Behavioral Genetics of the Fly

(*Drosophila melanogaster*)

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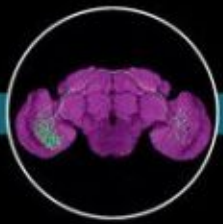

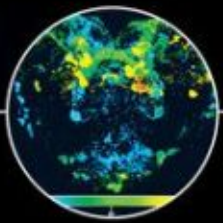

Edited by Josh Dubnau

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The common fruit fly – *Drosophila melanogaster* – has been the subject of genetics research since the early twentieth century. The complete genomic sequence of *Drosophila* was published in 2000 and it is still the model organism *par excellence* for the experimental study of biological phenomena and processes. It is also by far the best model for studying gene function in mammals, including humans.

Presenting state-of-the-art studies on the behavior of *Drosophila*, this volume discusses normal and pathological models of neurobehavioral disorders and encompasses the specialized methods that have been used, from anatomical, histological, immunohistological, and neurophysiological to genomic, genetic, and behavioral assays.

A comprehensive and thorough reference, this volume is a valuable resource for students and researchers alike across several disciplines of the life sciences, including behavioral genetics, neurogenetics, behavioral neuroscience, molecular biology, evolutionary, and population biology.

Josh Dubnau is Associate Professor at Cold Spring Harbor Laboratory. His research focuses on genetics and circuitry of memory and mechanisms of neurodegeneration. He received the Beckman Young Investigator Award and Human Frontiers Young Investigator Award. He is also the recipient of a Transformative R01 from the NIH. Former President of the International Behavioral and Neural Genetics Society, he has been twice selected by graduate students for the Winship Herr Teaching Award.

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Behavioral genetics is the study of the role of genetics in animal (including human) behavior. The genetic dissection of behavior in simple animals can provide insight into the mechanisms that regulate human behavioral traits. Once behaviors are understood at the genetic level, it allows the development of animal models of human pathological behaviors, such as stress, anxiety, depression, and addiction, which in turn furthers our understanding of these behaviours and opens windows of opportunity for the discovery of treatments. The Cambridge Handbooks in Behavioural Genetics series covers the behavioral genetics of different animal species, some because of their usefulness in medical research and others for the simplicity of their nervous systems. The series is of interest to researchers across several disciplines of the life sciences, primarily those with an interest in the behavioral sciences. The books are advanced texts, from graduate student upwards, and are useful resources for advanced genetics courses, such as genetics of model organisms, mammalian molecular genetics, developmental genetics, and animal models of human disease.

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Preface

Chapter 1

The anatomical organization of the compound eye's visual system

Ian A. Meinertzhagen

Introduction

The visual system of the fly's compound eye is noted both for its modular composition and for crystalline regularity. In the compound eye, each module or ommatidium has a fixed complement of 26 cells that includes eight uniquely identified photoreceptor neurons (Ready et al., 1976). An outer ring of six cells, R1–R6, surrounds two central cells R7 and R8 in each ommatidium. Backed by extensive genetic analysis of their development and function, R1–R6 constitute by far the best-understood sensory neurons in any invertebrate visual system, and among the best-known neurons of any nervous system. During its development from the eye imaginal disk, the pattern of ommatidia in the compound eye is impressed upon neurogenesis in the primordia of the underlying optic lobe (Meinertzhagen and Hanson, 1993) and, as a result, the optic lobe neuropiles are likewise modular in their composition, comprising a clear array of cartridges in the first neuropile, the lamina (Braitenberg, 1967) and a less obvious array of columns in the second neuropile, the medulla (Campos-Ortega and Strausfeld, 1972; Strausfeld and Campos-Ortega, 1972). The lamina and medulla are some of the most orderly and well-characterized neuropiles of the entire fly's brain, and models for the brains of all animal species, invertebrate or otherwise.

Often overlooked or simply not acknowledged, most essential details of the neuroanatomy of the optic lobe were established not in *Drosophila*, but in larger fly species – mostly the housefly *Musca domestica*, before observations on *Drosophila* became ascendant. Anatomical studies on the optic lobe in *Drosophila* are, in fact, undergoing an intense renaissance at the time of writing this review, yielding to new genetic and imaging technologies that support a sense of promise that many long-outstanding questions will soon be resolved. Particular issues include the number of individual cell types, their synaptic circuits, and neurotransmitter systems, and whether each type is discrete, distinguishable from all other types. The groundwork for these questions in *Drosophila* was laid by a commendably accurate report of the cell types derived from Golgi impregnation (Fischbach and Dittrich, 1989), which is still current. Anticipating the topic of this chapter, Meinertzhagen and Hanson (1993) provide summary diagrams of the adult optic lobe that occasional readers have found useful.

The compound eye

The compound eyes are the most obvious of the fly's seven visual systems (Hofbauer and Buchner, 1989), and their regular array of corneal lenslets has been a favorite object for microscopists since the time of Hooke (1665). Each corneal facet is a regular hexagon with two horizontal sides, that forms part of an array with horizontal z rows aligned parallel to an equator and two oblique rows (x,y). At its greatest vertical height, each eye contains about 30 such rows, divided equally between dorsal and ventral ommatidia on either side of the equator, with a similar number of x and y rows (Ready et al., 1976). The hexagonal shape of the ommatidium, and the ommatidial lattice that results, is refined during development, when excess pigment and bristle cells are removed (Cagan and Ready, 1989). It is therefore the loss of these cells that confers the regularity of the ommatidial photoreceptor array that is critical for the eyes' isotropic spatial resolution.

The ommatidium and its pattern of axonal projection

The organization and structure of photoreceptor neurons (Ready et al., 1976), and their rhodopsin expression patterns (e.g., Mikeladze-Dvali et al., 2005) in *Drosophila* have all been extensively reviewed elsewhere. Each photoreceptor neuron has a single light-absorbing rhabdomere that is separate from that of its neighbors, with the two rhabdomeres of R7 and R8 situated axially in tandem, that of R7 sitting on top of R8 (Ready et al., 1976). Starting with R8, R1–R6 assemble in a developmental sequence as three pairs of neurons, in which R2 and R5 are recruited first, followed by R3/R4 and then by R1/R6; R7 is added last (Tomlinson and Ready, 1987). Thus, R1–R6, which in many ways are matched in their anatomy and function, with each expressing a single rhodopsin Rh1 (O'Tousa et al., 1985), are not in fact a single class but are actually paired,

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as in other insect ommatidia. When viewed in cross-section, the six outer rhabdomeres of R1–R6 form an asymmetrical trapezoidal pattern that is reflected at an equator, a line of pattern mirror-image symmetry between ommatidia in the dorsal and ventral regions of the eye field (Dietrich, 1909). A consequence of that pattern is that the optical axis of each photoreceptor diverges slightly from those of other photoreceptors in the same ommatidium, each photoreceptor as a result viewing a slightly different point in visual space (Kirschfeld, 1967; Franceschini and Kirschfeld, 1971). Congruence between the angle of their divergence and the angular divergence between the optical axes of neighbouring ommatidia, imparted by the curvature of the retina, means that the axis of each R1–R6 photoreceptor exactly aligns with another R1–R6 photoreceptor sitting behind a neighboring facet, so that one photoreceptor each beneath seven such facets then view the same point in visual space. The axons of R1–R6 innervate the lamina, and while these enter the lamina as ommatidial bundles, the individual axons of each single bundle diverge at the distal face of the lamina neuropile. During that divergence, the axons of exactly those photoreceptors that view the same point in space then converge upon a single cartridge in the lamina (Braitenberg, 1967; Trujillo-Cenóz, 1965), in a pattern of so-called neural superposition (Kirschfeld, 1967). The axon sorting zone distal to the lamina is a complex layer of interweaving, a miracle of morphogenesis within which axon trajectories are established with great accuracy (Horridge and Meinertzhagen, 1970). Dorsal and ventral ommatidia have mirror symmetrical patterns of interweaving, and because more axons extend in a direction towards the equator than away from it, a zone of hyperinnervation is formed by cartridge rows on either side of the equator with a reciprocal zone of hypoinnervation at the lamina's rim (Meinertzhagen, 1972; Fröhlich and Meinertzhagen, 1987). These details were all firmly established from studies on large fly species, chiefly on *Musca* and the blowfly *Calliphora*.

The three main systems of photoreceptor input to the visual system are thus R1–R6, R7 and R8. R1–R6 provide input to motion-sensing pathways (Heisenberg and Buchner, 1977; Joesch et al., 2011; Rister et al., 2007), while R7 and R8 provide independent spectral inputs to the medulla (e.g., Heisenberg and Buchner, 1977; Gao et al., 2008). R7 and R8 with respective peak sensitivities in the UV (R7) and blue (R8) each express one of two rhodopsins (Morante and Desplan, 2004). Each cell type thereby comprises in turn two subtypes, and all four subtypes have distinct spectral sensitivities (Hardie and Kirschfeld, 1983). Pairs of R7 and R8 cells coordinately express a particular rhodopsin to construct one of two types of ommatidial rhodopsin partnerships, pale or yellow (Franceschini et al., 1981; Mazzoni et al., 2008). The R1–R6 and R7/R8 systems, previously considered independent (Yamaguchi et al., 2008), have recently been shown to converge, R7 and R8 also contributing to the motion pathway (Wardill et al., 2012). A proposed structural basis for that convergence is provided by gap junctions that form in a shallow zone of the lamina within which the axons of R7 and R8 make glancing contact with that of R6, and less frequently with R1 (Shaw et al., 1989). The opportunity for that contact arises, in turn, from the sorting zone of photoreceptor axons that enables neural superposition, which requires the axon of R6 to pass between those of R7 and R8 to reach its correct cartridge.

The terminals of R1–R6

The synaptic terminals of R1–R6 in the lamina are God's gift to neuroanatomy. They are aligned like drinking straws, so that a single section samples many profiles, thus allowing rigorous quantification of synaptic organelles. Exploiting these features, and the opportunity to generate whole-eye mosaics of mutant neural genes that would be lethal elsewhere in the nervous system (Stowers and Schwarz, 1999; Newsome et al., 2000) the synaptic terminals of R1–R6 have provided a test bed for the diagnosis of mutant synaptic gene action. Selected examples include genes that regulate: mitochondrial transport (Stowers et al., 2003); vesicle endocytosis (Fabian-Fine et al., 2003; Dickman et al., 2005); or the role of activity on axon sorting and photoreceptor synapses (Hiesinger et al., 2006). R1–R6 terminals form tetrad synapses that release histamine (Hardie, 1989; Sarthy, 1991). Capitate projections are synaptic organelles formed where neighboring epithelial glia (below) invaginate into a R1–R6 terminal to form a stalked organelle with a spherical ~200-nm head, single or multiple, borne on a ~80 nm diameter stalk (Stark and Carlson, 1986). The base of the stalk is a site of endocytotic membrane retrieval (Fabian-Fine et al., 2003), and the head a postulated site of histamine recycling (Fabian-Fine et al., 2003) expressing the AMPylation protein Fic (Rahman et al., 2012), the whole functioning as a proposed integrated recycling organelle.

The optic lobe

The fly's visual world maps upon four separate, successive neuropiles of the optic lobe, which sits beneath the compound eye. These comprise (Fig. 1.1): first, the distal curved lamina; beneath it the large, concentric second neuropile, the medulla; and, beneath this, two face-to-face neuropiles, the lobula and posterior lobula plate, that lie orthogonal to the medulla's internal face (Strausfeld, 1976). These neuropiles are all modular, with an array of columns – called cartridges in the lamina – that exactly matches that of the overlying ommatidia (Braitenberg, 1967). Each ommatidium projects as an axon bundle, which then undergoes the pattern of divergence required for neural superposition, as described above. Each cartridge projects, in turn, as a bundle of 11 axons that connects it to the medulla by way of the external chiasma (Strausfeld, 1971a; Meinertzhagen, 1976). Each horizontal row of cartridge axon bundles inverts its anteroposterior sequence as a coherent sheet, each sheet folding over on itself in parallel with its neighbors, either by a clockwise twist in the dorsal half of the right eye, above the equator, or by a counterclockwise twist in the ventral half, to map onto a

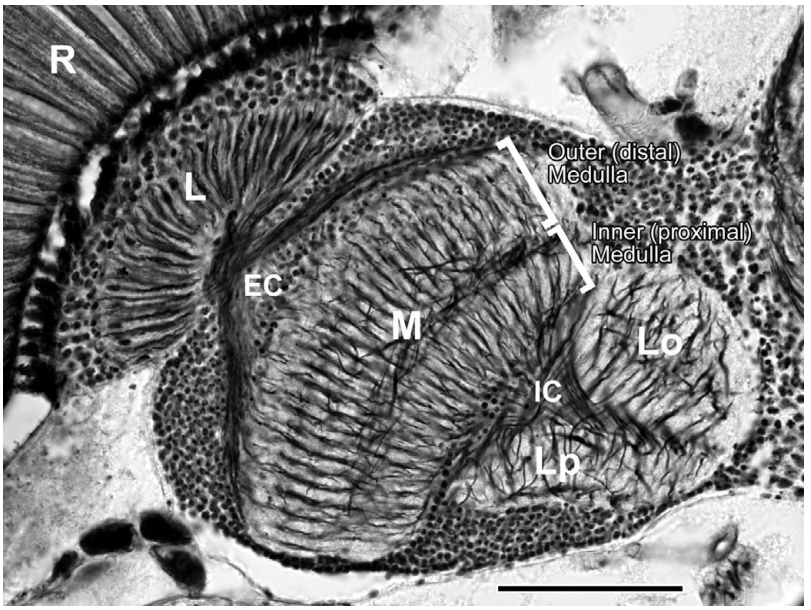

Fig. 1.1. The *Drosophila* visual system in horizontal section, showing rows of cartridges parallel to the equator in the lamina (L) connecting with rows of columns in the medulla (M) via the external chiasma (EC). Outer (distal strata M1–M6) and inner (proximal strata M7–M10) halves of the medulla are separated by a middle stratum connecting to Cuccatti's bundle, that contains many of the medulla's tangential neurons. Axons extend between the medulla's proximal face and the lobula and lobula plate neuropiles via the inner chiasm. R, retina; IC, internal chiasma; Lo, lobula; Lp, lobula plate. Scale bar: 50 μm. (Image of Bodian preparation reproduced from Takemura et al., 2008.)

horizontal row of medulla columns (Braitenberg, 1970). A corresponding inner chiasma with a more complex composition connects the medulla with the neuropiles of the lobula complex (see below).

These tracts are constituted by axons of columnar relay neurons, having their axon running the length of a column. Accounts especially by Strausfeld and others using Golgi impregnation and other classical light microscopic methods, established a library of cell types in different fly species (e.g., Strausfeld, 1979, 1971b, 1976; Strausfeld and Lee, 1992). Major studies on *Drosophila* came only after these earlier accounts and, at least initially, were mainly confirmatory.

Definition of morphological cell types

The landmark Golgi study of Fischbach and Dittrich (1989) provided what is still the most comprehensive single published account of cell types in *Drosophila*, assigning neurons to classes based on the direction of axon outgrowth – whether at right angles to the neuropile, as for columnar neurons, so as to project a retinotopic map onto the lamina, medulla, and lobula – or across the neuropile, as for tangential neurons. Further distinctions among these are based on the extent and stratum of each cell's dendrite arborizations Figs. 1.2, 1.3). The third class of intrinsic neurons are distinct from both columnar and tangential neurons insofar as they arborize only in a single neuropile, and are thus the substrate for local circuit interactions. While having much to commend it, a parallel nomenclature of "columnar neurons", which contact photoreceptors from a single ommatidium only, and "non-columnar neurons" that integrate information from broader receptor fields (Morante and Desplan, 2008), will not be adopted in this account.

From the evidence of Golgi impregnation alone, the optic lobe in *Drosophila* has a total of 113 morphological cell types, although even this large number appears to be a considerable underestimate, perhaps by about a third (Drs. A. Nern and G.M. Rubin, personal communication). Of these, the lamina has 12 types of neurons (Tuthill et al., 2013; Fig. 1.2), while the medulla has a reported minimum of 59, thus at least half the optic lobe's total. The cells are arranged in columns, one per ommatidium, and strata, ten in the medulla, six identified in the lobula and four in the lobula plate (Fischbach and Dittrich, 1989). The numbers of strata are thus in some proportion to the numbers of types of co-stratifying neurons they segregate (see below).

In addition to cells that relay within or between the optic neuropiles, visual projection neurons connect the optic lobe with the central brain. Among the 44 types identified in a screen of *Gal4* lines, 24 are associated with the lobula, of which 14 arborize specifically in the lobula and the remaining 10 contribute not only to the lobula, but also the medulla and/or lobula plate (Otsuna and Ito, 2006).

Some particular types of neurons and their numbers of subtypes

Including the lamina cells identified below, which all appear to be definitively identified, Golgi impregnation also reveals the main classes of columnar relay neurons (Fischbach and Dittrich, 1989). For the medulla, these are: the single class of five lamina L-cell types, which terminate in the distal medulla (Fig. 1.2); transmedulla cells (Tm, 30 including all reported subtypes – see Fig. 1.3), which penetrate the medulla and terminate in the lobula; and similar to these, TmY cells (14 including all subtypes), which have an axon that splits to terminate in both the lobula and lobula plate. Medulla intrinsic cells (Mi,

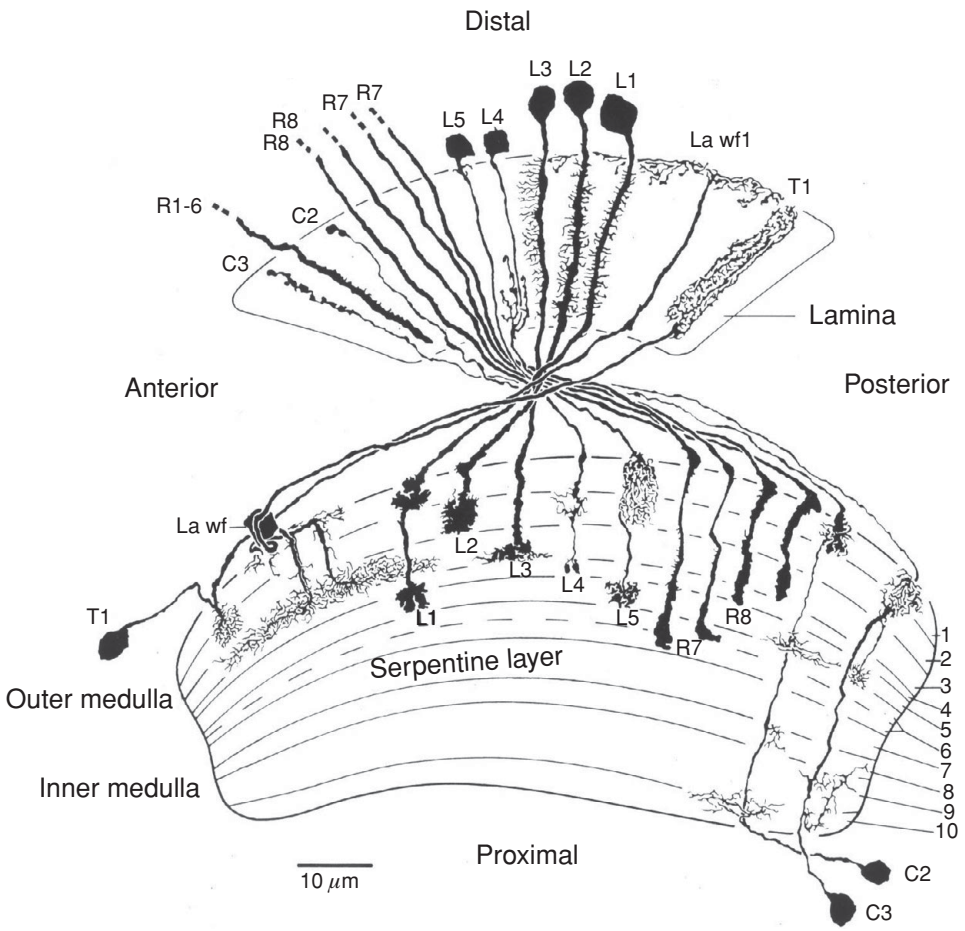

Fig. 1.2. The lamina's cell types impregnated by the Golgi method, shown in the same plane as in Fig. 1.1. Photoreceptor neurons R1–R6 innervate the lamina; lamina columnar cells L1–L5 relay to the medulla; photoreceptor neurons R7 and R8 innervate the distal medulla; T1 and a lamina wide-field cell (La wf1), both with somata in the medulla cortex, and C2 and C3, with somata between the posterior edge of the medulla and the lobula plate cortex, all innervate the lamina from a centrifugal direction. Lamina tangential and intrinsic (amacrine) neurons are omitted. (Reproduced from Meinertzhagen and Hanson, 1993, after Fischbach and Dittrich, 1989.)

12 reported subtypes) do not project to the lobula, but instead connect distal with proximal medulla strata, between strata 1 and 6 and strata 8 and 10, typically with dendritic arbor(s) in the former and a terminal in the latter and so relaying signals centripetally. T1 and two C cells (C2 and C3) are additional classes that project centrifugally from the medulla to the lamina (Fig. 1.2). The lobula and lobula plate neuropiles have four additional classes of columnar neuron, three with cell bodies in the lobula plate cortex: Tlp, Y and T cells. Translobula plate neurons (Tlp, seven reported subtypes) connect different layers of the lobula plate with lobula stratum Lo4. Y cells (five subtypes) have an axon that penetrates the lobula plate to bifurcate in the inner chiasma and project to both the lobula and proximal medulla, although no clear morphological distinction between dendrites and terminals is obvious. T cells (11 reported subtypes) also have their cell body in the lobula plate cortex, but form two major types depending on whether they arborize in the medulla or not. T2 and T3, for example, do, and, like the medulla centrifugal neurons, C2 and C3, with cell bodies nearby, both arborise in the proximal medulla (like Y cells), with T2 also arborizing in the distal medulla; in addition, both T2 and T3 project to the lobula. Similar to these, T4 also has an axon that divides in the inner chiasma, doubles back, and then innervates the lobula. T5 does the same but does not arborize in the medulla. T4 and T5 are numerous, with apparently up to four representatives per column (see below). In addition to these medulla neurons, lobula columnar neurons (Lcn, 6 reported Golgi subtypes) have cell bodies outside the optic lobe, arborizing in the deep lobula and projecting to the central brain. They form one of the many classes of visual projection neuron that project between optic lobe and brain, of which *Gal4* lines identify 14 more associated with the lobula (Otsuna and Ito, 2006), see above. Further illustrated details of cell types in *Drosophila* and associated nomenclatural issues are to be found in both these publications (Fischbach and Dittrich, 1989; Otsuna and Ito, 2006).

In addition to these columnar neurons, tangential neurons have an axon that spreads across the visual field, in many cases with an exuberant arbor, ten reported subtypes in the medulla, six in the lobula, and two in the lobula plate. Those of the lobula plate (Fischbach and Dittrich, 1989), the lobula plate giant tangential cells (LGTCs), have received particular attention because they signal wide-field information on motion, either horizontal (HS cells) or vertical (VS cells). Finally, additional cell types include the optic lobe intrinsic, or amacrine, neurons, those of the lamina (Lai), the medulla's distal (Dm, nine subtypes) or proximal (Pm, three subtypes) strata, or the lobula (Li, two subtypes).

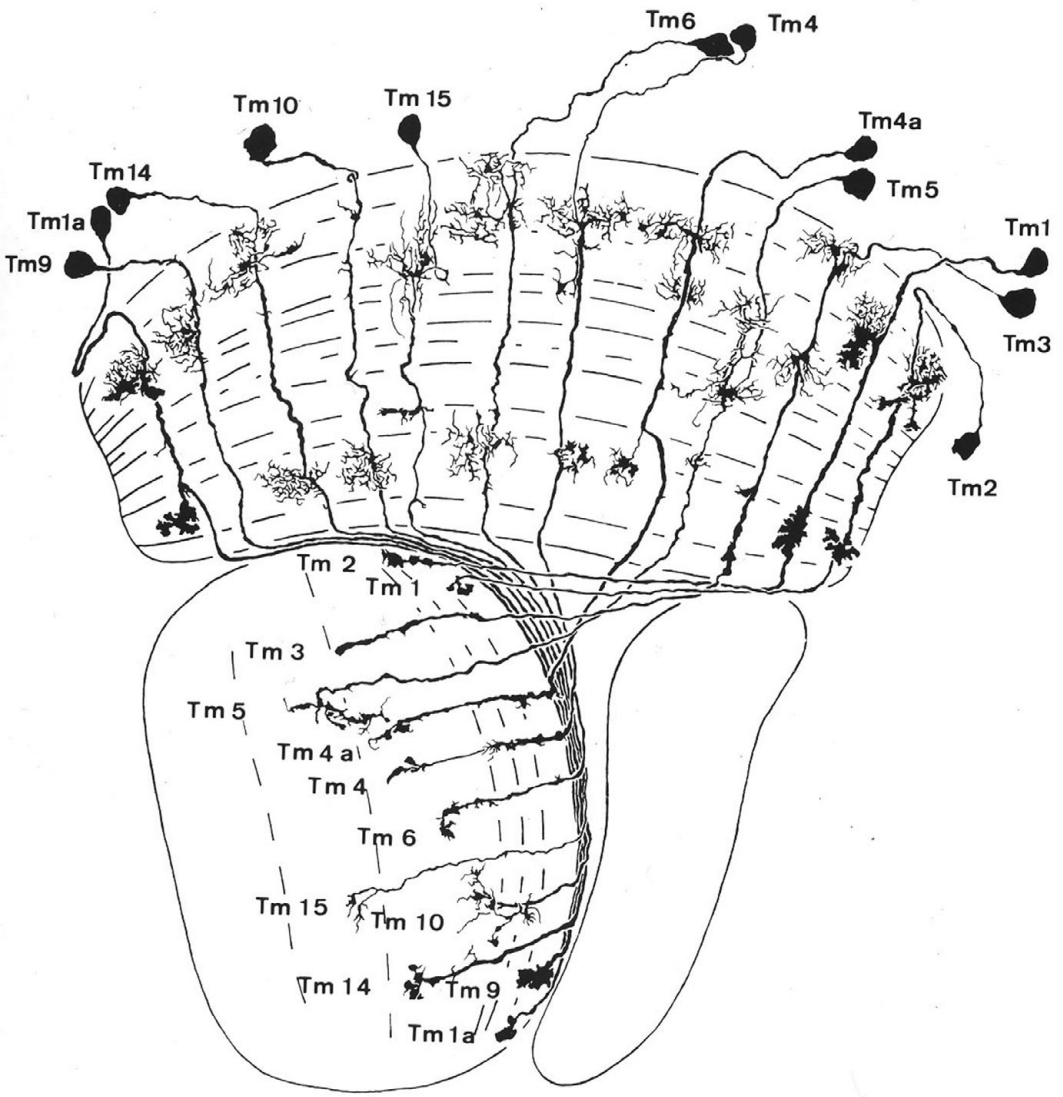

Fig. 1.3. Selected transmedulla (Tm) cells with somata in the medulla cortex, having axons that penetrate the medulla and terminate in the lobula. Tm1 and Tm2 are L2's chief targets (Takemura et al., 2011). Shown with the same orientation as in Fig. 1.2; scale bar 20 μm. (Reproduced from Meinertzhagen and Hanson, 1993, after Fischbach and Dittrich, 1989.)

In most cases these neurons have exquisite morphological phenotypes, and the careful assignment of a cell to a particular class has relied on accurate human observation and judgment that is particularly critical in the relay pathways for the many types of columnar neuron that connect successive strata and neuropiles. These judgments support an elaborate taxonomy based on several features: the direction of the axon, the site of its termination, the stratum of arborizations (from the ten in the medulla), and the width of the arbor (whether confined to a single column or extending across multiple columns). In parallel, screens of two major *Gal4* driver collections (Jenett et al., 2012; Hayashi et al., 2002) reveal the 12 cell types of the lamina (Tuthill et al., 2013), and many other classes of neuron, especially for the medulla (Drs. A. Nern and G.M. Rubin, personal communication) and lobula (Otsuna and Ito, 2006), some not previously reported from Golgi impregnation. In a more limited way, a *Gal4* line for the histamine channel protein gene *ort* (Gengs et al., 2002) expresses in neurons that are candidate targets for photoreceptor histamine release. It identifies L1–L3 (Rister et al., 2007) and several medulla cells, including a medulla amacrine cell Dm8 and four transmedulla cells, Tm2, Tm5, Tm9, and Tm20 (Gao et al., 2008).

Insofar as the taxonomy of cell types is based on human judgments, it is to some extent subjective. The close agreement between the forms of these cells seen from Golgi impregnation (Fischbach and Dittrich, 1989) and those seen in single *ort*-expressing neurons (Gao et al., 2008) rather gratifyingly implies that the human arbitration of different cell classes actually mirrors developmental decisions made by the fly. The latter must ultimately reflect the genetic steps that specify each neuron type. For example, Brain-specific homeobox protein is expressed in lamina cells L4 and L5 and in medulla cell Mi1, and is required to specify the fate of all three (Hasegawa et al., 2013).

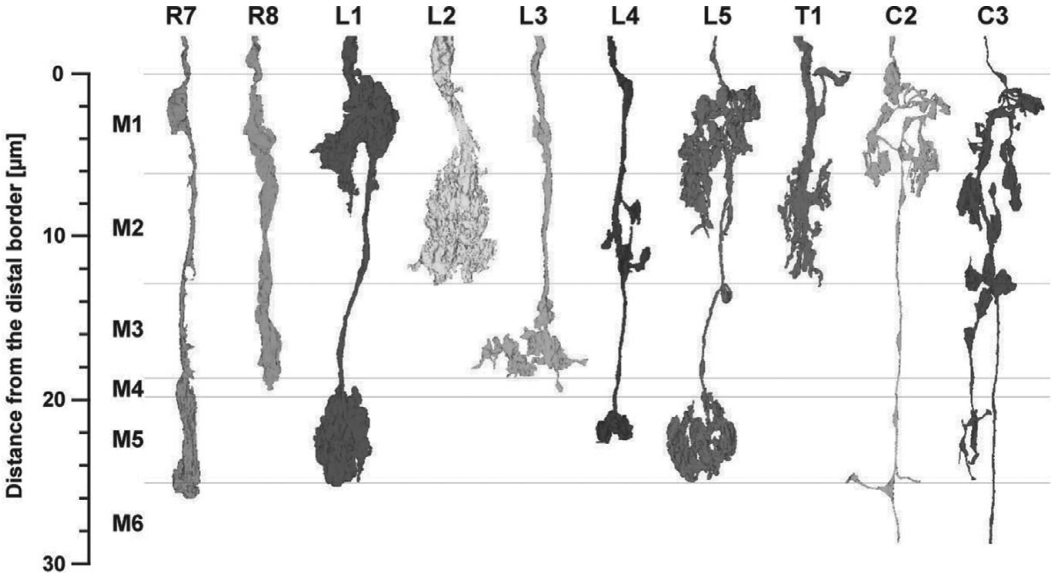

Fig. 1.4. Terminals of ten neurons reconstructed from serial-section EM with input terminals in strata M1–M6 of the distal medulla. The neurons are: R7 and R8, L1–L5, C2 and C3, and T1. Viewed from anterior looking posterior, in the plane of the chiasma. (Reproduced from Takemura et al., 2008.)

In addition to congruence between Golgi and genetic evidence, some neurons – such as L2 and Tm2 (Meinertzhagen et al., 2009) – have also been studied from serial-section EM, from which technically demanding three-dimensional reconstructions reveal yet a third means to view the same cells (Fig. 1.4), one that exerts no bias upon the choice of particular cells, but in which it may not be possible to reconstruct all tiny neurites.

With this spirit of conquest over some of the technically difficult approaches, and a groundswell of opinion to support the view that each type is discrete, morphologically determinate, and discriminable from all other types, it is still difficult to assess the exact extent of variation among the arbors of the same cell type, and to assert the absence of yet more subtle subtypes. Thus Tm5 identified from Golgi impregnation is now seen from inspection of repeated examples in a reporter line to comprise three subtypes, each with a minute difference in its arborisation (Gao et al., 2008). We may anticipate other such subtleties, although close inspection of 379 cells reconstructed from serial-EM, most as multiple representatives of 56 classes of medulla neurons (Takemura et al., 2013), does not reveal widespread cases.

Finally, the neurotransmitter phenotype of the optic lobe's cell types contributes another layer of evidence, although this is often conflicting. Inconsistencies, especially between transmitter immunolabelings and genetic reporter lines are even obvious in the simple lamina (e.g., Kolodziejczyk et al., 2008). These become more obvious in the deeper neuropiles, among the cells identified by reporter lines for acetylcholine (Cha-positive: Raghu et al., 2011), glutamate (dvGlut-positive: Raghu and Borst, 2011), and GABA (dVGAT-positive: Raghu et al., 2013). Used to drive green fluorescent protein (GFP) these lines provide clear evidence of cell morphology, sometimes identifying hitherto unreported cell types, but sometimes supporting neurotransmitter phenotypes that are at variance with other evidence. To give but one example, L4 is ChAT-immunoreactive (Kolodziejczyk et al., 2008) and expresses *Cha* transcripts (Takemura et al., 2011), both implying its cholinergic nature, but expresses with a *dVGAT-Gal4* reporter, consistent with a GABA phenotype (Raghu et al., 2013).

The lamina: a tiny constituency of identified neurons

The lamina's distinctive array of cartridges, one per ommatidium (Braitenberg, 1967) – thus numbering more than 750 (Ready et al., 1976), is a particular feature of this neuropile in flies. All the optic neuropiles are, in fact, modular but the appearance of that modularity in the lamina of flies arises from the principle of neural superposition, because each cartridge is surrounded by the terminals of R1–R6 that converge upon it from neighboring ommatidia, and because these are wrapped in turn by isolating glia. More than this, each cartridge has an identical cellular composition. Present in every cartridge are five lamina monopolar cells L1–L5, two medulla centrifugal cells C2 and C3, and a third medulla cell T1. T1 is a mystery: morphologically it appears to be centrifugal but in *Drosophila* it lacks presynaptic sites in either lamina or medulla (Takemura et al., 2008). C2 and C3 have cell bodies that arise from deep in the optic lobe, in the cortex of the lobula plate. They have a GABA phenotype (Kolodziejczyk et al., 2008) and thus qualify as a substrate for inhibitory centrifugal feedback between medulla and lamina.

To these five are added contributions from four other less well-characterized cell types that are infraperiodic, having fewer cells than there are cartridges. (a) Two are wide-field neurons (Lawf1, Lawf2) having processes that spread into neighboring cartridges. Lawf2 is labelled by a *Gal4* line for the transcription factor Homothorax *hth-Gal4* (Hasegawa et al., 2011) and has recently been independently confirmed

(Tuthill et al., 2013). Lawf1 and Lawf2 arborize in different medulla strata, Lawf1 in M1 and M4, Lawf2 in M1 and M8–M10 (Hasegawa et al., 2011). Both were considered tangential cells (Kolodziejczyk et al., 2008) although the direction of their axons, orthogonal to the face of the medulla, is in fact columnar. Lawf1 is probably a cell that expresses GFP driven by a *Gal4* line for choline acetyltransferase (*Cha-Gal4*) and was redesignated *Cha*-Tan, while Lawf2 expresses a *Gal4* for the ionotropic GABAA receptor subunit RDL, *rdl-Gal4*, and was redesignated *rdl*-Tan (Kolodziejczyk et al., 2008). The complete forms of these cells have now been confirmed as Lawf1 and Lawf2 (Tuthill et al., 2013), and partial EM reconstructions and their synapses reported (Rivera-Alba et al., 2011). There are many such cells, but not one each per cartridge. (b) A third cell type is the highly synaptic lamina amacrine (Lai) neuron. These have cell bodies beneath the lamina with ascending axons that spread synaptic processes into a number of cartridges, those of a single cartridge probably deriving from a single Lai cell. The amacrine processes partner the basket arborizations from T1 cells, both cells contributing one of a pair of neurites that lies between neighboring R1–R6 terminals. (c) Except for Lawf2, all the above cells were reported from Golgi impregnation (Fischbach and Dittrich, 1989), along with a fourth, final cell type. (d) The latter is a lamina tangential neuron partly reported by Fischbach and Dittrich (1989) as Lat, now reported to correspond to about four cells per optic lobe (Tuthill et al., 2013) that innervate a distal plexus of the lamina. These cells arborize in the anterior, so-called accessory medulla involved in circadian regulation (Helfrich-Förster et al., 2007). A second contender for the Lat cell arises from a pair of somata in the posterior protocerebrum with bilateral axons that bypass the medulla of both sides, traverse the chiasma and posterior margin of the lamina, to give rise to upwardly directed varicose neurites that penetrate the lamina cortex. These cells are called LBO5HT in large fly species and are 5-HT immunoreactive (Nässel, 1991). They lack synaptic release sites and are thought to be sources of 5-HT acting as a neuromodulator, for example mediating circadian changes in the visual system (Pyza and Meinertzhagen, 1996). Resolving the candidacy of these two cells must await further evidence.

The lamina's synapses

The cartridge is like a wooden interlocking burr puzzle with tightly packed space-filling cells. These are predominantly cylindrical in shape, and their mutual packing is mostly the problem of how to fit all cell profiles optimally into the cartridge cross-section. This fit reflects a compromise between two complex demands: first, wiring economy – to minimize the distance between connections; and second, volume exclusion – the displacement of large neurites from regions that are rich in synaptic connections (Rivera-Alba et al., 2011).

A complete matrix of synaptic connections between the cells in a single wild-type cartridge has been reported (Meinertzhagen and O'Neil, 1991), as have estimates of pathway strength derived from the numbers of such connections (Meinertzhagen and Sorra, 2001), reports that have recently been amplified (Rivera-Alba et al., 2011). Thus, taking 20 synapses as the threshold, the strongest pathways are from R1–R6 to L1–L3 and amacrine Lai cell processes, each R1–R6 terminal forming about such 50 input synapses. Each synapse is a tetrad that releases histamine; the terminals of R7 and R8 in the medulla also contain histamine (Pollack and Hofbauer, 1991). Other strong pathways include the synaptic connections from amacrine cell neurites, which feed back to R1–R6 or provide input to L3, T1 or epithelial glia (see below), for which the neurotransmitter may be glutamate (Sinakevitch and Strausfeld, 2004; Kolodziejczyk et al., 2008). The amacrine feedback synapses onto T1 beg to be better characterized. These occur at so-called gnarl contacts, where a thin sheet from surrounding epithelial glia is interposed so as to occlude a direct contact between the amacrine and T1 cells. The same is variably true for feedback synapses to R1–R6, where a thin sheet of epithelial glia intrudes at some but not all sites of amacrine synaptic contact, possibly nullifying the presence of a synapse between these two neurons (Meinertzhagen and O'Neil, 1991). Taking a lower threshold of eight synapses brings in additional pathways from amacrine to L2, as well as inputs to L2 from C2 and C3, and the collaterals of L4 that invade from the two anterior neighbouring cartridges (Meinertzhagen and Sorra, 2001). L5 lacks clear or significant synaptic engagements in the lamina.

The medulla, a plenitude of cell types

As summarized above, the medulla has an entire army of morphological cell types, at least half of all those reported for the optic lobe (Fischbach and Dittrich, 1989). Most are columnar, and of the medulla's 59 or so cell types reported from Golgi impregnation, possibly 30 are in turn Tm cells. The dendrites of these neurons can be restricted to a single column, or spread widely. Thus, dendrites of the same cell class can either extend outside column borders, often intermingling with those of others of the same cell type, so as to shingle the retinal field, or abut the neighboring column borders so as to tile the field. A number of genes are now identified that mediate the tiling of neurite arbors, for example through homophilic interactions between immunoglobulin family members Turtle, that mediate repulsion between R7 terminals (Ferguson et al., 2009), and Dscam2, for the terminals of L1 (Millard et al., 2007). In addition to columnar neurons, tangential neurons are fewer in number (ten reported for the medulla, but likely a considerable underestimate), and spread laterally, usually within just a single stratum, and often across the entire medulla field.

As first realized long ago for *Musca* (Campos-Ortega and Strausfeld, 1972), the medulla's array of columns is home to two patterns of columnar cell types. From counts of both the cells in the medulla cortex and the number of columns these populate, it is clear that on average only about 35 of the >60 cell types occupy each medulla column (calculated in Meinertzhagen and

Sorra, 2001), with a corresponding number of 13.5 for lobula columns, which contain 26 reported cell types. Clearly therefore, not all cell types have an axon in all columns. Some – such as Tm1 and Tm2 (Takemura et al., 2011) – are in all, while many others are not. Campos-Ortega and Strausfeld (1972) refer to the former as synperiodic (1 cell : 1 column). and perhaps only 25 types, including input terminals, are synperiodic, present as one cell in every column (Drs. A. Nern and G.M. Rubin, personal communication; Takemura et al., 2013) and the axons of all other types scattered less frequently. Two classes, Tm3 and Tm4 – along with T4 and T5, are ultraperiodic, having multiple representatives in each column; together with the 25 cells above, these are all considered to be modular, because they are found in each and every column (Takemura et al., 2013). Essentially, nothing is known about how other medulla cell types might populate the array of columns, however. They include those that arborize within one column but are infraperiodic, having fewer cells than medulla columns (1 cell : *n* columns). Many may have arborizations in every column and thus can be predicted to pool information from multiple columns. Defining their spacing relative to synperiodic cells depends on identifying the position of the axon relative to the borders of neighboring columns, but in neither case are these well defined. Moreover, the lateral spread of dendrites may ensure an even representation in neighboring columns, for example by tiling the medulla's array of columns (Millard et al., 2007; Ferguson et al., 2009), without close reference to the position of the axon that generates the dendrites. In practice, it may therefore be difficult to distinguish between infraperiod cells and those that are aperiodic, lacking a fixed distribution among columns. In addition to columnar cells, each column contains the neurites of tangential and local amacrine-like cells with wide-field arborizations not easily reconstructed by means of EM (Takemura et al., 2013).

Given the variable composition of infra- and aperiodic cell types, relative to the defined contributions from modular neurons, there can be no clear unit structure of the medulla neuropile. Unlike the lamina, this is anyway unlikely to contain a fixed blend of cell types, and insofar as the distribution patterns of medulla cells may be random, there may be no minimal structural unit, or medullon (Campos-Ortega and Strausfeld, 1972), containing all representative cell types. Two types of column may correspond to the pale and yellow subtypes of R7 and R8 pairs in the ommatidia, and the pattern of these across the eye is random (Bell et al., 2007). Some cell types may be very few in number, too, which will hinder the final search for their connections, while six other types seen from EM reconstructions (Takemura et al., 2013) are simply not reported from Golgi impregnation.

Single-cell clones from *Gal4* lines that report the expression of different transcription factors have already been used to identify a large number of medulla cell types and their likely contribution to spectral pathways (Morante and Desplan, 2008). Further analyses from reporter lines can be relied upon to confirm and add many other details.

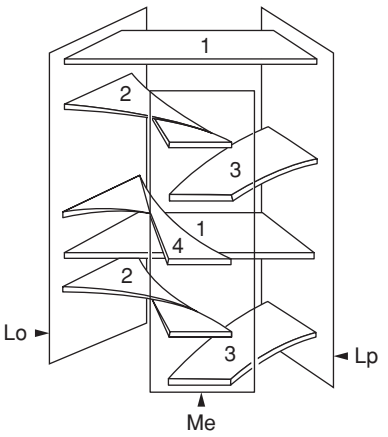

Fig. 1.5. Sheets of axons in the internal chiasma of *Musca*, with alternating direct and twisted strata between medulla (Me), lobula (Lo), and lobula plate (Lp) neuropiles. (Reproduced from Meinertzhagen and Hanson, 1993.)

Finally, the medulla's busy marketplace of neurites is, like any social network, highly stratified. Each stratum can be viewed as delimiting the network's combinatorial complexity, the range and number of contacts formed between synaptic partners, and thus as a corollary of packing so many different cell types into a single neuropile. Inputs arriving from the lamina establish the six strata of the distal medulla by terminating at specific strata, which they accomplish in a sequence of steps during which afferent input axons respond to specific cues in target layers (Ting et al., 2005). First, in the late third-instar larva and early pupa, axons from R7 and R8 grow to temporary layers in the medulla, R8 arriving before R7 and terminating more superficially. The axons of L1–L5 then follow, insinuating themselves between the temporary layers formed by R7 and R8. In the mid pupa, R8 axons then extend down to the R7 temporary layer, to form their final recipient stratum, M3. R7 axons then descend yet deeper to their final recipient stratum, M6. Interactions between classes of afferent axons are not needed for each class to locate its specific stratum, which it does instead presumably through afferent–target interactions (Ting et al., 2005). These steps require the actions of a range of identified cell adhesion molecules, as recently reviewed (Schwabe and Clandinin, 2012).

Going down: the neuropiles of the lobula complex

The lobula complex comprises two neuropiles, the lobula and its thinner, flatter, posterior partner, the lobula plate (Strausfeld, 1976). At the proximal surface of the medulla, the axonal composition of column bundles is not clear and awaits resolution. The axons that connect the medulla with lobula and lobula plate neuropiles through the internal chiasma are, like those of the external chiasma, are also arranged as a succession of coherent, horizontal sheets of axon bundles. The arrangement of these is much more complex than in the external chiasma, however. In *Musca* (Braitenberg, 1970), each layer of axons in the inner chiasma is reported to contain four sheets (Fig. 1.5): (1) an unfolded sheet between lobula and lobula plate; (2) a folded sheet generating the inverted projection of a row of medulla

columns upon a row of lobula columns, with a counterclockwise twist; (3) an unfolded sheet of a row of medulla columns upon a row of lobula plate columns; and (4) a folded sheet generating the inverted projection of a row of medulla columns upon a row of lobula columns, like (2) but with a clockwise rotation (Braitenberg, 1970). In the *Musca* lobula some large terminals form a hexagonal array that occupies every second column in every second row, i.e., one in six columns. The regularity of this array suggests that some infraperiodic cells at least must have a fixed distribution. These inputs have yet to be identified in *Drosophila*, however, although the lobula receives columnar input predominantly from medulla Tm and TmY cells.

The medulla interneurons of the lamina's two major cell types, L1 and L2, overlap the arbors of bushy T cells that have cell bodies in the lobula plate cortex (Strausfeld, 1984), of which T4 and T5 in large fly species both have up to four cells per column (Strausfeld and Lee, 1991) and *Drosophila* has four subtypes, a–d, overall (Fischbach and Dittrich, 1989). Each subtype segregates into one of the four strata of the lobula plate, a specific stratum for each subtype (Fischbach and Dittrich, 1989). These strata also segregate the dendrites of HS and VS cells, to which the terminals of T cells provide proposed anatomical synaptic input, albeit identified only for T4 input to an HS cell (Strausfeld and Lee, 1991; Takemura et al., 2013). Information on the lobula plate's HS and VS cells, either HS cells that signal horizontal motion (Hengstenberg et al., 1982) during rotation about the fly's vertical axis, or VS cells that signal rotation around vertical axes within the fly's equatorial plane (Krapp and Hengstenberg, 1997), is mostly derived from studies on large fly species, although recordings have been reported from dye-filled *Drosophila* HS cells (Schnell et al., 2010), and T cell inputs to giant LPTCs of the lobula plate have also recently been shown to be functional in *Drosophila*. Thus genetic interruption of that input by T4/T5-Gal 4 driven expression of two effector lines, UAS-*shi* or UAS- *Kir2.1*, procures conditional blockade of motion-sensitive responses in the LPTCs, but not responses to flicker (Schnell et al., 2012). Dendrites of both T4 and T5 cells express both Rdl-type GABA receptors, and are thus presumed to receive inhibitory input (Raghu et al., 2007), as well as Dα7-type nicotinic cholinoceptor subunits specifically on higher-order dendritic branches (Raghu et al., 2009). These expression patterns suggest that directional selectivity of the LPTCs is achieved by dendritic integration among excitatory cholinergic inputs and inhibitory GABA-ergic inputs from local motion detectors having opposite preferred directions.

In *Drosophila* three HS and six VS cells are reported (Scott et al., 2002; Rajashekhar and Shamprasad, 2004). In addition, three classes of neuron on each side of the brain that express the transcription factor *Odd-skipped* project into the lobula plate as tangential neurons; one has a contralateral and two have both ipsi- and contralateral projections (Levy and Larsen, 2013).

The lobula plate's four strata are thus defined in *Drosophila* by two criteria: first, the presence of dendrites from the HS and VS cells; and second, the segregation of terminals from T4 and T5's four subtypes, a, b, c and d (Fischbach and Dittrich, 1989; albeit subtype T4b is missing from their account). To these two criteria should be added a third, the stimulus-specific uptake of ^{3}H-2-deoxyglucose (2-DOG) when the fly is exposed to large-field gratings moving in a preferred direction and with a specific orientation (Buchner and Buchner, 1984; Buchner et al., 1984; Bausenwein and Fischbach, 1992). In sequence, the four strata are: An inner stratum Lopl (or HS layer), next to the inner chiasma, containing most of the dendrites of the HS cells and the terminals of T4a and T5a, which 2-DOG labels by front-to-back motion; next, stratum Lop2, which contains the terminals of T5b and probably T4b and which 2-DOG labels by back-to-front motion; next, stratum Lop3, which contains the terminals of T4c and T5c and which 2-DOG labels by upward motion; and last, the most posterior stratum Lop4 (or VS layer), which contains most dendrites of the VS neurons and the terminals of T4d and T5d, and which 2-DOG labels by downward motion. The lobula plate's outputs from these tangential cells relay information about directional motion in anti-parallel preferred directions to descending pathways which then project to the circuits of the thoracic nervous system that mediate flight, as identified in large fly species (e.g., Strausfeld, 1989; Strausfeld and Lee, 1991).

The axons of columnar neurons in the lobula segregate and project next to a group of discrete optic glomeruli in the lateral protocerebrum (Otsuna and Ito, 2006; Strausfeld and Okamura, 2007). These have been compared with those of the olfactory system (Mu et al., 2012). Eleven glomeruli in the posterior ventral, and seven in the posterior region of the lateral procerebrum each receive exclusive and often monolithic input from a single class of lobula columnar neuron (Lcn), while the optic tubercle is an additional glomerulus that receives non-Lcn input as well (K. Shinomiya, personal communication). Fourteen types of visual projection neuron have been identified extending between the lobula and protocerebrum (Otsuna and Ito, 2006) and although little is known about their function, the lobula as a whole is predicted to be involved in detecting object features (Douglass and Strausfeld, 2003a) but also exhibits motion sensing elements (Douglass and Strausfeld, 2003b). Two such neurons are tangential cells, LT10 and LT11, which a recent report implicates in the detection of second-order motion (Zhang et al., 2013).

The optic lobe's synaptic circuits

Since the time of Ramón y Cajal (Cajal and Sánchez, 1915), synaptic circuits in the optic lobe have been constructed from contacts between neurons, terminal to dendrite, with the specificity of those contacts dictated by the co-stratification of both. Such constructions rely upon three basic assumptions: the correct identification of the axon's terminal and dendrites for each optic lobe neuron; the assignment of an exclusively presynaptic role to the former, and a postsynaptic role to the latter; and the assignment of neither role to the axon itself. While true in general, each assumption is often violated (Takemura et al., 2008), and sites of synaptic contact can, in fact, only be confirmed

at present from electron microscopy (EM). More than this, EM studies often reveal synapses between unexpected synaptic partners, and thus reveal the importance of local circuit as well as relay neurons. Moreover, EM accounts alone reveal the numbers of synaptic contacts, and thus the likely pathway strength, between partner neurons. The existence and strength of connections predicted by terminal-to-dendrite overlaps, and those seen in EM reconstructions, have received recent quantitative comparison in a column of the medulla, where their correlation is seen in fact to be highly variable (Takemura et al., 2013).

Violated though these assumptions may be, it seems most likely that strata are one way to limit synaptic interactions to those between neurons that co-arborize in the same stratum. Using this line of reasoning, Bausenwein et al. (1992) superimposed the density profiles of Golgi impregnated columnar cell types to analyze the connectivity between the medulla strata. This approach assumes that the density of arborizations reflects accurately the density of synaptic contacts, as opposed to their dispersion, but clearly identified at least three main visual pathways.

Pathway 1 has input pathways in strata M1 and M5 and connects stratum M10 to the lobula plate, with its HS and VS LPTCs (Borst et al., 2010). Pathway 2 has input in stratum M2 and connects stratum M9 to superficial layers in the lobula, which in turn connect to the lobula plate. These pathways are proposed to receive input from R1–R6, either via L1 (terminating in M1 and M5) or L2 (terminating in M2), and their neurons have narrow-field dendritic arbors. The pathways were originally suggested to play a major role in motion detection, a conclusion supported by the stimulus-specific 2-DOG labeled bands seen after wide-field visual stimulation (Bausenwein and Fischbach, 1992). That suggestion was later validated by genetic dissection approaches for L1 (pathway 1) and L2 (pathway 2), that suggested, in turn, that these lamina neurons provide inputs to two motion-sensing channels (Rister et al., 2007).

Pathway 3 has input in M8 either from stratum M3 (pathway 3a) or from M4 and M6 (pathway 3b), layers that get their major input from L3 and R8 or L4 and R7, respectively. This pathway then connects M8 to deep layers of the lobula. Some neurons of pathway 3 have wide-field dendrites that must pool inputs over multiple columns that have been suggested to be involved in computing form and spectral information. One such pathway for the latter comes from pooled R7 inputs to an amacrine neuron, Dm8, and subserves UV phototaxis (Gao et al., 2008).

Overall, we see that divergence at the first synapse, the R1–R6 tetrads (Meinertzhagen and O'Neil, 1991), establishes input to pathways 1, 2, and 3a, whereas R8 and R7 are thought to provide input to pathways 3a and 3b, respectively. The synaptic contacts observed from serial-section EM largely bear out these suggestions but add a multitude of new details.

The motivation of motion

Interest in the organization of insect visual systems rests in large measure on a cornerstone computational model of motion detection, the Reichardt elementary motion detector (EMD). This computes correlations between input signals that are separated in time and space to predict motion-sensing outputs (for review see Borst and Egelhaaf, 1989; Borst et al., 2010). The attraction of the EMD detector lies both in its computational simplicity and in its robustness. No less, for decades it has offered vision scientists a simple solution to a compelling problem in neurobiology. But knowledge of the EMD's biological implementation as actual connections between specific neurons has always remained tantalisingly incomplete. Certain cell types have been implicated from terminal-to-dendrite overlap criteria and electrophysiological recordings, notably in the medulla (for review, see Douglass and Strausfeld, 2003a), but only recent EM evidence of the actual connections made by identified neurons reveals those anatomically qualified to act as circuits underlying this detector (Takemura et al., 2013).

Past accounts from all fly species have given particular attention to pathways 1 and 2, above, for L1 and L2. Following earlier suggestions both are now known to provide the substrate for motion sensing. Thus, interrupting synaptic function in L1 and L2 together suppresses optomotor (Rister et al., 2007; Clark et al., 2011) and electrophysiological (Joesch et al., 2010) responses to wide-field motion stimuli. By virtue of its proposed electrical coupling to the other by means of gap junctions, either neuron alone may, however, produce a wild-type motion response (Joesch et al., 2010). Differential effects have been reported after separately inactivating either cell, leaving the other intact. Thus, separately L1 may signal posterior-to-anterior motion across the retina and L2 anterior-to-posterior motion (Rister et al., 2007), or light and dark moving edges (Clark et al., 2011) respectively; or a yet wider range of even more subtle behavioral deficits that reveal the roles of these two cells in basic motion detection (Tuthill et al., 2013).

L1's and L2's pathways in the medulla, and the cells that constitute these, are now known. For the L2 pathway the chief targets are Tm1 and Tm2, representing a binary split that generates two parallel pathways (Takemura et al., 2011), rather as upstream L1 and L2 receive matched inputs from R1–R6 tetrads in the lamina (Meinertzhagen and Sorra, 2001). Compared with the input to L1/L2 pairs at lamina tetrads, however, these inputs are not matched exactly and only two thirds of L2's synapses provide input to both Tm1 and Tm2 (Takemura et al., 2013). L2 also provides input to Tm4 from the same column and the Tm4 cells of neighbouring columns. For the L1 pathway, each L1 terminal has two major targets: Mi1, which receives input almost exclusively within a single column, and a group of Tm3 cells which, like L2's Tm4 targets, have dendrites spreading in from neighbouring columns. Together these two cell types contribute 85% of the identified inputs to T4 and are therefore T4's sole major pathways from L1 (Takemura et al., 2013).

What of the medulla inputs from three other L-cell types? L5, long considered a synaptic orphan (Takemura et al., 2008), forms only a few casual synapses in the lamina, but is highly synaptic in the medulla, where it receives massive input from

the terminal of L1 and a smaller input from the terminal of L2. L3 acts combinatorially with the L1 and L2 pathways to provide input to circuits for detecting moving light and dark edges (Silies et al., 2013). It provides input to Tm9, among other Mi and Tm cells (Takemura et al., 2013).

The medulla pathway of L4 is associated with that of L2. In addition to receiving input from L2 in medulla stratum M2, Tm2 also receives inputs from two retinotopically posterior neighboring columns via the trifid terminals of L4. These provide input to the descending, so-called walking-leg dendrites of Tm2 in medulla stratum M4 (Takemura et al., 2011). Thus a combined L2/L4 pathway to Tm2 exists in the medulla, and this resembles the one seen in the lamina, where L4 provides reciprocal input directly to L2 (Takemura et al., 2011; see Fig. 1.6). Single-cell transcript profiles for neurotransmitter genes of individual identified neurons indicate that, whereas L1 expresses a glutamate-positive profile, all three cells of the L2 pathway (L2, L4, and Tm2) express an acetylcholine one, including nicotinic acetylcholine receptors that are presumed to mediate fast transmission (Takemura et al., 2011). L2 lacks a transcript for vesicular glutamate transporter, and this fails to confirm the expression both of *Gal4* under the control of a *dvGlut* promoter fragment (Raghu and Borst, 2011) and immunoreactivity to glutamate itself (Sinakevitch and Strausfeld, 2004; Kolodziejczyk et al., 2008), contradictions that await clarification.

The pathways of L1 and L2 eventually provide input to T cells that in turn provide input to the giant LPTCs. For L1's pathway, as we saw above, those inputs come via L1's chief medulla target neuron Mi1, and a neighboring group of Tm3 cells. Both terminate on the dendrites of T4 in proximal medulla stratum M10 (Takemura et al., 2013). L2's major medulla targets, Tm1 and Tm2 (Takemura et al., 2011, 2013), both terminate in superficial strata of the lobula, on the dendrites of T5 (K. Shinomiya and I. A. Meinertzhagen, unpublished observations). Thus T4 provides a direct pathway from the proximal medulla to the lobula plate, and T5 provides an indirect pathway, via its targets in the lobula (Douglass and Strausfeld, 1996). Dendrites of both T4 and T5 cells express DNA coding for the Rdl-type GABA receptor fused to an epitope tag, and are thus interpreted to receive inhibitory input (Raghu et al., 2007), as well as immunoreactivity to a Dα7-type nicotinic cholinoceptor subunit specifically on higher-order dendritic branches (Raghu et al., 2009). These expression patterns suggest that directional selectivity of the LPTCs may be achieved by dendritic integration among excitatory cholinergic inputs and inhibitory GABAergic inputs from local motion detectors having opposite preferred directions.

The receptive field organization of inputs to T4 and T5 is also critical in establishing directional selectivity in the target LPTCs. The Mi1 terminals and Tm3 dendrites converging upon T4 cells in M10 are separate components of T4's receptive field that overlap substantially, but are slightly displaced from each other, by less than one inter-ommatidial distance. As a result, they are anatomically qualified to constitute the two arms of a

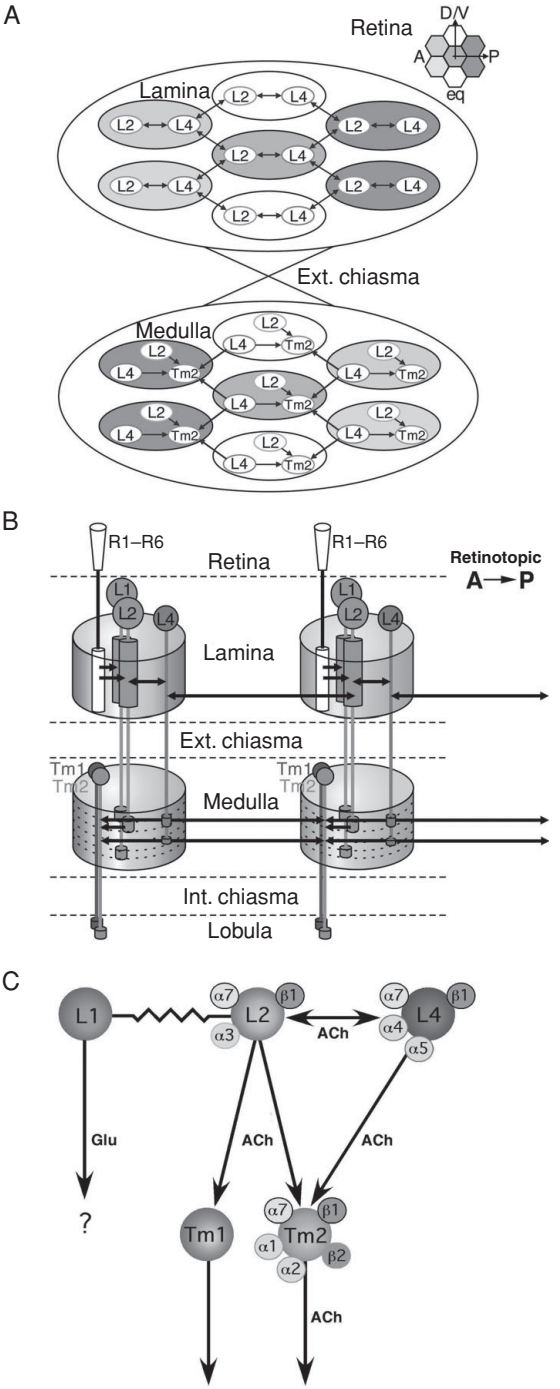

Fig. 1.6. Inputs from lamina cells L2 and L4 converge upon medulla cell Tm2. A, L1 and L2 receive matched inputs from R1–R6 at the lamina's tetrad synapses; and B, are electrically coupled (resistor symbol: Joesch et al., 2010), but express different neurotransmitter phenotypes, for glutamate (L1) or acetylcholine (L2). L4, which also expresses a cholinergic phenotype (B), is reciprocally connected to the L2 cells of two anterior lamina cartridges (A). In the medulla, L2 provides input to two target neurons, Tm1 and Tm2. Tm2, in turn, expresses a cholinergic phenotype and also receives input from L4, to constitute a three-element pathway with Tm2 outputs (B). To reveal the congruence of the direction of connections between columns the chiasma between lamina and medulla has been uninverted. B: Each candidate cholinergic neuron expresses a shared pair of fast nicotinic receptor subunits (nAcRα7/β1) as well as type-specific nAcR subunits. (Reproduced from Takemura et al., 2011.)

correlation-based motion detector, one that via its terminal provides input to a particular stratum (Lo1–Lo4) within the lobula plate (Takemura et al., 2013). As we have seen above, each lobula plate stratum has a preferred direction defined by the pattern of its stimulus-specific 2-DOG incorporation (Bausenwein and Fischbach, 1992). Critically, the direction of displacement between the Tm3 and Mi1 receptive field components for a particular T4 is consistent with that T4 cell's directional preference, as defined by the depth of its terminal in the lobula plate and the stratum (Lo1–Lo4) to which this corresponds (Takemura et al., 2013). Reported in parallel, optical recordings from T4 (and T5) cells reveal directionally tuned responses (Maisak et al., 2013) that are thus the congruent functional outcome of the anatomical receptive field organization. Details for the medulla's Tm inputs to the lobula have yet to be reported. Preliminary evidence indicates only that those inputs to the branched dendrites of T5 cells come from Tm9, which forms large terminals in lobula stratum Lo1, together with the smaller terminals of Tm1 and Tm2, and the deeper terminals of Tm4, all of which are present in each column (Shinomiya and Meinertzhagen, unpublished observations). Unclear so far is how the two inputs from the L2 pathway, Tm1 and Tm2, might integrate at the T5 dendrite, especially if these might be antagonistic, whereas Tm9 seems to be anatomically qualified as the pathway by which L3 acts combinatorially with the L2 pathway (Silies et al., 2013).

Anatomical inputs to the medulla feed a complex synaptic matrix

Seven main input pathways to the medulla are provided by: the terminals of R7 and R8; the terminals of L1–L3, which receive R1–R6 input in the lamina; and two centrifugal cells, C2 and C3, that ascend in the medulla, cross in the external chiasma, and innervate the lamina. In the lamina C2 provides input to L1–L3 and C3 provides it to L2, in both cases presumably inhibitory, while in the medulla L1 provides input to both C2 and C3, which is reciprocated for C2. In addition, a third medulla cell T1 receives strong amacrine cell input in the lamina, but lacks presynaptic sites either there or in the medulla and is thus of indeterminate status, but could possibly form gap junctions and so be electrically coupled with cells in either neuropile.

Details of these cells' synaptic connections in *Drosophila* are derived for the ~480 synapses of a lamina cartridge (Meinertzhagen and O'Neil, 1991; Meinertzhagen and Sorra, 2001; Rivera-Alba et al., 2011) and the ~2500 synapses of a medulla column (Takemura et al., 2008; Takemura et al., 2013), the latter annotated only for those of the 27 cells – including input terminals – that are considered modular. First, to summarize what has previously been said, R1–R6 provide input to L1 and L2 at tetrad synapses at which these two lamina cells are invariable partners (Meinertzhagen and O'Neil, 1991; Meinertzhagen and Sorra, 2001). Then L1 synapses on to Mi1, while L2's pathway is marked by a binary split and provides input at medulla synapses to Tm1 and Tm2, many sites at L2's mostly tetrad synapses incorporating these as joint postsynaptic partners, along with other neurons. The numerically strongest medulla pathways, such as those of L1 onto Mi1 and of L2 to Tm1 and Tm2 have up to about 150 synapses (Takemura et al., 2013), five times more than the tetrad pathway from a single R1–R6 terminal to L1 and L2. Both L2 and Tm2 synapses are genotypically cholinergic and use nicotinic receptor subunits (Takemura et al., 2011).

The anatomical definition of these and all other medulla synapses, is currently the subject of further intense investigation. The task is not entirely simple. The complexity of the medulla, the largest single neuropile of the fly's brain, with its host of reported cell types (Fischbach and Dittrich, 1989) in roughly a million cubic microns (Rein et al., 2002) with an estimated packing density of about one synapse per 0.77 μm^3 (S. Takemura and I. A. Meinertzhagen, unpublished observations), compiled with the density in the lamina, with one synapse per 0.67 μm^3 (Rivera-Alba et al., 2011), were of course all reasons that progress in identifying synaptic circuits in the fly's visual system had always been stalled. The chief issues are the numbers of medulla cell types and the fineness and complexity of their arborisations. Recent developments using automated reconstruction approaches from serial-section EM (Chklovskii et al., 2010) and other improved techniques, have now opened this neuropile to active analysis. Even so, cataloging synaptic contacts by current serial-section EM methods proceeds at a snail's pace that makes it difficult to determine whether an identified connection occurs repeatedly in different columns, without supplementary light microscopic analysis of overlaps between single labeled neurons (Takemura et al., 2011). For this reason, successful adoption of the GRASP (GFP Reconstitution Across Synaptic Partners) method, developed in *C. elegans* (Feinberg et al., 2008) and now successfully applied in *Drosophila* (Gordon and Scott, 2009; Gong et al., 2010), would seem to offer a valuable ancillary.

Synapses are divergent polyads

Unlike the neuromuscular junction (Atwood et al., 1993), but like the synapses of all neuropiles of the brain reported to date, the optic lobe's chemical synaptic contacts are almost invariably multiple-contact polyads, typically with an average of four postsynaptic sites at each presynaptic active zone. The latter is identified in flies by a presynaptic dense body or synaptic ribbon, T-shaped in cross-section, but actually a tiny table comprising a platform surmounting an ossiform or cruciform pedestal (Fröhlich, 1985; Prokop and Meinertzhagen, 2006) that designates the site of vesicle exocytosis during neurotransmitter release (Saint Marie and Carlson, 1982). Most structural information comes from the R1–R6 tetrads in the lamina. These have a quadripartite organization contributed by contacts from L1 and L2, as two median postsynaptic elements, and two polar postsynaptic contacts from different combinations of three cells, L3, epithelial glia, and a lamina amacrine

neuron (Nicol and Meinertzhagen, 1982; Fröhlich and Meinertzhagen, 1983). The arrangement has a high level of geometrical and dimensional stereotypy (Fröhlich, 1985), each synapse forming a tiny multiplex connector between an R-cell site and its four postsynaptic elements. Synapses elsewhere in the visual neuropiles may have less stereotypy, but follow the same general plan of construction.

The reason for the predominance of multiple-contact synapses is not known. Three reasons that have been advanced are: the economy of cost-sharing the energetic requirements of presynaptic release; as a means to enrich circuit interactions between limited numbers of neurons; and to satisfy a need to match inputs to two or more postsynaptic cells (Meinertzhagen and Sorra, 2001). The first need may be more pressing at tonic synapses with high rates of neurotransmitter release, such as the tetrads (Stuart et al., 2007). The particular need to match inputs, for example to L1's and L2 at tetrads, is perhaps special, and flies employ the redundant expression of two members of an immunoglobulin family of cell adhesion molecules, Dscam1 and Dscam 2, to ensure that L1 and L2 are obligate partners at all tetrads and that homotypic L1/L1 or L2/L2 pairings are excluded (Millard et al., 2010). As a result, all conforming tetrads receive matched inputs from R1–R6 (Meinertzhagen and Sorra, 2001). L1's and L2's partnership has been highly conserved during the evolution of ancestral fly groups (Shaw and Meinertzhagen, 1986), suggesting it may play an essential computational function in fly motion vision. Despite the marked similarity in their lamina inputs, however, the terminals of L1 and L2 form quite different output circuits and have few common targets (Takemura et al., 2008, 2013).

So far, it is clear then that synapses are divergent, and that synaptic contacts upon particular targets are shared amongst a population of multiple-contact synapses. Wholly unclear at this stage is whether interactions might exist between the postsynaptic elements that cohabit a single synaptic site, and thus what significance, if any, to attach to the combination of those postsynaptic elements.

Microcircuits and network motifs

Simple as it is, the lamina incorporates a richness of synaptic contacts (e.g., Meinertzhagen and O'Neil, 1991) that was hard to imagine from light microscopy alone. Thus only about 60% of the lamina's synapses, which total 480 in a single completely analyzed cartridge, are afferent tetrads that relay R1–R6 input to L1–L3 (Meinertzhagen and Sorra, 2001). Reciprocal connections abound, as do motifs, such as serial synapses, that contain three elements. Higher-order network motifs of four elements, particularly bi-parallel and bi-fan motifs, also exist but their frequency has not formally been reported. The release of the medulla connectome (Takemura et al., 2013) now provides a far more extensive database from which to draw such further analyses.

In general, the sizes of different synaptic contacts are rather similar, about 0.1 μm^2. Some idea of the strength of transmission can therefore be gained from the number of synaptic contacts connecting any two elements. It is clear that many "strong" pathways comprise large numbers of contacts, about 50 presynaptic sites for each R1–R6 terminal and up to about 100 sites for the terminals of L1 and L2 (Takemura et al., 2008). Many other pathways are numerically much weaker, some may have only one or two synapses and thus be functionally questionable, or possibly even misidentified. For example, the semi-automated procedures used to generate the medulla connectome are estimated to identify all connections with more than five synapses with a confidence level >95% (Takemura et al., 2013) so that in practice numerically weak connections are ignored. The relationship between the number of synaptic contacts between two neurons and the gain of synaptic transmission, the polarity of that transmission, or the kinetics of identified postsynaptic receptors all remain unknown; information on all of these will be needed to interpret structural maps of connections, as part of the field of functional connectomics (Meinertzhagen and Lee, 2012).

Gap junctions may offer surprises

So far, synaptic circuits have only been considered as groups of neurons connected by chemical synaptic contacts defined by ultrastructural criteria. Electrical coupling by means of gap junctions has been reported only at three major sites in the visual system. The first lies between the lamina terminals of R1–R6 (Ribi, 1978; Shaw and Stowe, 1982) some of which, viewed as membrane appositions, are lacking in the proximal lamina of the innexin mutant *shakB2* (Shimohigashi and Meinertzhagen, 1989). The second lies between L1 and L2, which have recently been shown to exhibit dye coupling (Joesch et al., 2011). The site of these unexpected gap junctions has still to be confirmed. In the lobula plate, three wide-field HS LPTCs that signal horizontal motion show ipsilateral coupling in *Drosophila* (Schnell et al., 2010). Extensive coupling, either electrical (Cuntz et al., 2007; Haag and Borst 2004) or dye coupling (Haag and Borst 2005), has also previously been shown between both the VS and HS systems of LPTCs in *Calliphora*. T1 is also a further candidate site for coupling between neurons in the medulla, as identified above, but many other such sites must augment the current analysis of chemical synaptic circuits in that neuropile. In addition to neurons, various sites of gap junction contact exist between classes of glial cell, known mostly from these cells in the lamina of *Musca* (Saint Marie and Carlson, 1983a).

Except for R1–R6, anatomical identification of gap junction contacts at all these sites has yet to be shown at EM level. The brightest prospect for that analysis must first await refinement of molecular reagents for the eight innexin genes in *Drosophila* (*inx2*, *inx3*, *inx5-inx7*, *shakB, ogre* and *zpg*: Phelan and Starich, 2001), of which four (*shakB, ogre*, *inx2* and *inx3*) are reported to express in the retina or optic lobe (Crompton et al., 1995; Zhang

et al., 1999; Stebbings et al., 2002). The developmental functions of *ogre* and *shakB*(*neural*) affect transmission at the R1–R6 tetrad synapses in the lamina, *ogre* being required in R1–R6 and *shakB*(*neural*) at least in their target lamina cells (Curtin et al., 2002a), presumably L1 and L2. The action of neither mutant can be rescued by alternative innexin genes (Curtin et al., 2002b), indicating not only that the junctions are heterotypic but also the combinatorial specificity of innexin partner proteins in coupled cells, suggesting that many junctions elsewhere in the optic lobe may exhibit similar specificity.

Optic lobe glia

In the lamina, there are six layers of glia that show a diversity scarcely less than the neurons they delineate and embrace. They are arranged in three pairs of layers comprising, from the basement membrane and extending in succession centrally: the surface fenestrated and pseudocartridge glia; the cortex distal and proximal satellite glia; and the neuropile epithelial and marginal glia.

Two major sources of confusion have existed over glial identities, both of them nomenclatural and causing recurrent vexation, at least to the author. First, glia have received the same names in the larva as in the adult without evidence that one transforms into the other. This was true for subretinal glia, which refer both to glia in the larval eye disk and optic anlage and to glia in the adult optic lobe, even though these are, in fact, unrelated (Edwards and Meinertzhagen, 2010). Second, genetic markers have failed to distinguish glial subtypes that clearly differ by structural criteria. Thus subretinal glia of the adult visual system were first designated by enhancer trap line 3–109 (Winberg et al., 1992; Perez and Steller, 1996) which expresses in both fenestration and pseudocartridge glia, and the identities of these two cells subsequently became merged in the *Drosophila* literature, even though they were recognized long ago as separate in the housefly (Saint Marie and Carlson, 1983a,b); the literature on *Musca* was simply ignored. A recent review now advocates abandoning this subretinal terminology (Edwards and Meinertzhagen, 2010). The developmental origins of the adult glia from the larval stage have now been traced, at least for the glia of the lamina (Edwards and Meinertzhagen, 2011).

These glia in the lamina have many functions that will not be considered further here, except to note that most seem to play some role in recycling photoreceptor histamine (Borycz et al., 2002; Richardt et al., 2003; Stuart et al., 2007). Epithelial glia play a direct role in converting histamine to its β-alanyl metabolite, carcinine, as part of a photoreceptor–glial shuttle pathway (Stuart et al., 2007) and other glia (marginal, proximal satellite, and fenestrated) play additional storage roles, acting in concert with a vertical recycling pathway for β-alanine that involves the pigment and cone cells of the overlying ommatidia (Borycz et al., 2011).

Little is known about how *Drosophila* specifies glia from neurons. In the lamina, proximal satellite glia may share developmental mechanisms with the fifth monopolar cell, L5. L5 specification requires Bsh (above) and in the absence of Bsh L5 transforms into proximal satellite glia. The transformation has been taken to suggest that the developmental mechanisms of these two cell types are coupled (Hasegawa et al., 2013).

Functional analysis by genetic dissection

This chapter considers properly only the anatomical organization of the optic lobes, but most would consider that knowledge to be of insufficient purpose, except as a basis to consider the fly's ultimate visual behavior and how the fly processes visual information. *Drosophila*, of course, offers the opportunity to undertake such analyses causally, using genetic reagents. Various studies now stand as models for how fly vision may be analysed, using genetic dissection approaches either to disrupt synaptic circuits, or to reinstate circuit function in mutant flies in which such function is suppressed. The following are models: (1) the pathways for motion provided by L1 and L2 (Rister et al., 2007; Clark et al., 2011; Tuthill et al., 2013); and (2) the pathways for UV phototaxis arising from R7 (Gao et al., 2008). The significance of such studies lies in the fact that they establish a causal relationship between identified neurons and circuits and the visual behavior these support. The analysis requires detailed information of the synaptic circuits that are being interrupted, as was the case in those studies. The approach proceeds cell by cell, and depends on the availability of drivers that can express the effector construct exclusively in one specific cell type, as well as of specific quantitative tests of vision that reveal the behavioural deficit or rescue in genetically transformed fly. *Drosophila* has outstanding qualifications for all three requirements: genetics, anatomy, and behavior, and such approaches are possible in large part because vision is not essential, so that vision-impaired flies are nevertheless viable.

Relationships to other fly species

Strictly this topic lies even further beyond the bounds of the present chapter, but becomes important when findings from other species are imported uncritically into the *Drosophila* field. Thus, while it is a culpably blinkered outlook not to consider data from larger fly species, it is perilously uncritical to invoke such findings without confirmation in *Drosophila*. The large body of comparative data on cell types derived originally from Golgi impregnation and whole-cell fills (e.g., Strausfeld, 1970, 1971b, 1976; Strausfeld and Lee, 1991; Buschbeck and Strausfeld, 1996) but also from immunocytochemical analysis (e.g., Sinakevitch et al., 2003) bears testament to many detailed similarities and conserved features of optic lobe neurons. Those occurring in the L1 and L2 pathways that underlie motion sensing have been the subjects of specific morphological study (Buschbeck and Strausfeld, 1996). It is likewise clear that many differences at the synaptic level may also exist, even at the first synapse formed by R1–R6 (Shaw and Meinertzhagen, 1986) but more extensively among other neurons of the lamina (Shaw and Moore, 1989). These are just the accessible tip of what is likely to be a large number of such evolutionary changes, even though

not all neuron classes need have changed as a result of such evolutionary progressions.

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Chapter 2

Neuropeptides regulating *Drosophila* behavior

Dick R. Nässel

Introduction

Neuropeptides act at various levels in the nervous system both as primary messengers, as neuromodulators and as circulating hormones. There are numerous molecular forms of neuropeptides and their functional roles are extremely diverse. Peptide functions encompass regulatory roles in development, reproduction, metabolism, ion and water homeostasis, and also in various behaviors, including locomotion, feeding, aggression, and reproductive behavior as well as in learning and memory (for review see Bendena et al., 2012; Johnson, 2006; Nässel and Winther, 2010; Taghert and Veenstra, 2003). Many neuropeptides also modulate contractions of heart, visceral and skeletal muscle. Some well-studied functions of peptides are in triggering and orchestrating ecdysis motor behavior, foraging, and feeding behaviors and in roles as output factors and modulators of the biological clock circuits. At the circuit level neuropeptides act as neuromodulators and have been analyzed as such in the olfactory system and in the central complex.

In *Drosophila* about 45 genes have been identified that code for precursors of neuropeptides and peptide hormones. Several of these precursors encode multiple neuropeptides and a total of at least 80 peptides may exist in the fly (Nässel and Winther, 2010; Yew et al., 2009). Surprisingly, some novel neuropeptides, were detected in *Drosophila* quite recently (Colombani et al., 2012; Garelli et al., 2012; Ida et al., 2011a; Ida et al., 2011b; Sellami et al., 2011). The targets of neuropeptides and peptide hormones in *Drosophila* are approximately 45 G-protein-coupled peptide receptors (GPCRs), a few tyrosine kinase type receptors, and at least one membrane receptor guanylate cyclase (Hauser et al., 2006b; Nässel and Winther, 2010). Several of these peptides and receptors have been investigated for their roles in behavior. It is, however, not an understatement to say that, even for the best-investigated neuropeptides in *Drosophila*, rather little is known compared to mammalian neuropeptides. This gap in knowledge is gradually decreasing with the availability of more powerful molecular and genetic techniques to study peptide signaling also in the tiny *Drosophila* (Jones, 2009; Olsen and Wilson, 2008; Simpson, 2009). Therefore, we are still in a rather exploratory phase of neuropeptide research in the fruitfly.

One of the difficulties in studying neuropeptides is the diversity in functions of a given neuropeptide. Most neuropeptides are functionally pleiotropic and can be released from a huge variety of neuron or cell types in the CNS, periphery, intestine, endocrine cells, glia, and so on. The functions may also change with the development of the organism. Here I summarize some key facts about roles of *Drosophila* neuropeptides in regulation of behavior (and associated physiology). For more general aspects of insect or *Drosophila* neuropeptides, especially for peptide roles in development, reproduction, metabolism, and other homeostatic regulations, the reader is referred to comprehensive reviews (Antonova et al., 2012; Bendena et al., 2012; Coast et al., 2002; Ewer, 2005; Giannakou and Partridge, 2007; Johnson, 2006; Nässel, 2002; Nässel and Winther, 2010; Taghert and Veenstra, 2003).

Some features of *Drosophila* neuropeptides

Of the 45 known *Drosophila* genes that encode precursors of neuropeptides, peptide hormones, and protein hormones, eight encode precursors of insulin-like peptides (DILPs), five encode protein hormones, and the rest shorter or longer peptides (Garelli et al., 2012; Hewes and Taghert, 2001; Nässel and Winther, 2010; Vanden Broeck, 2001). There are in addition secretory peptides/proteins, like sex peptide, produced by the male accessory glands that display hormone-like activities after transfer to the female fly (Kubli, 2003). Mass spectrometry identified 38 peptides in extract of the larval nervous system and 42 in adults (Baggerman et al., 2002; Predel et al., 2004; Yew et al., 2009), but one can predict more than 80 neuropeptides from the precursor sequences in *Drosophila*. It remains to be determined whether all of these are actually produced and available for release.

The majority of the neuropeptides, and peptide- and protein hormones activate one or two of the 45 known GPCRs (Hauser et al., 2006a; Hewes and Taghert, 2001). Most, if not all, of the eight *Drosophila* insulin-like peptides (DILP1–8) are likely to activate a single tyrosine kinase receptor, the *Drosophila* insulin receptor, dInR (Brogiolo et al., 2001; Fernandez et al., 1995; Grönke et al., 2010). Prothoracicotropic hormone (PTTH) acts on a receptor tyrosine kinase (Torso) that stimulates

Behavioral Genetics of the Fly (Drosophila melanogaster), ed. J. Dubnau. Published by Cambridge University Press.

extracellular signal-regulated kinase (ERK) phosphorylation (Rewitz et al., 2009). The peptide eclosion hormone acts on a membrane bound receptor guanylate cyclase in endocrine so-called Inka cells (Chang et al., 2009). For some peptide ligands, more than one GPCR has been identified, but the majority seem to activate only one. Commonly, the multiple related peptide products of a given precursor gene activate the same GPCR; one exception is the products of the *Dtk* (tachykinin gene) where one receptor (DTKR) can be activated by all 6 *Dtk* products (DTK-1–6), whereas the other (NKD) only by DTK6 (Birse et al., 2006; Poels et al., 2009).

In *Drosophila*, as in other insects, neuropeptides are distributed in stereotypic patterns of neurons and neurosecretory cells in the CNS and other sites (Park et al., 2008; Santos et al., 2007). Most neuropeptides are found in small numbers of neurons/neurosecretory cells, many of which can be individually identified (Fig. 2.1). Thus, *Drosophila* is a convenient organism for studying peptidergic signaling at the single neuron level or at the level of small systems of neurons. The *Drosophila* brain consists of about 100 000 neurons (Chiang et al., 2011; Simpson, 2009), and only a small fraction of these are peptidergic. Peptidergic neuron numbers in the entire CNS range from 2 (eclosion hormone) or 4 (SIFamide), over 10–60 for most neuropeptides, to some exceptional peptides (proctolin and short neuropeptide F) that are found in 400 to several thousands (see (Nässel and Winther, 2010; Park et al., 2008)).

Neuropeptides are produced by a huge variety of neuron types and secretory cells. In *Drosophila* various neuropeptides can be found in olfactory sensory neurons (OSNs), interneurons of many different types, neurosecretory cells, motor neurons, efferent neurons supplying various muscles and body wall, and endocrine cells of midgut and at peripheral locations, for example in the large Inka cells. Surprisingly few of the neuropeptides have been mapped in any detail in the CNS of adult *Drosophila*, although most have been localized to neurons/neurosecretory cells in the larval CNS (Park et al., 2008; Santos et al., 2007). Even fewer peptide receptors have been mapped to neurons. Here, the peptide and receptor distributions will be discussed, when known, in relation to behavioral analysis that is described next.

Neuropeptides in *Drosophila* behavior

Analysis of neuropeptide function in *Drosophila* has a relatively short history and is linked to the fairly recent possibility of using molecular genetics to perform targeted interference with expression of peptides and their receptors. Obviously, the small size of the fly has been a limiting factor, precluding classical endocrinological or pharmacological approaches that have been successfully used in larger insects. Thus, many neuropeptides have not yet been analyzed experimentally in *Drosophila* and others only superficially. In the following, I will highlight some aspects of physiology and behavior where we know that peptides play important regulatory roles.

Peptidergic modulation of feeding-related behaviors

In *Drosophila* several neuropeptides have been implicated in the regulation of feeding and food search (foraging). These act at different levels and in different neuronal or neuroendocrine systems. Some have been studied in larvae, others in adults, a few in both stages. The peptides investigated in relation to feeding in *Drosophila* are (details later): Allatostatin A (AstA), hugin-pyrokinin (hugPK), neuropeptide F (NPF), short NPF (sNPF), sex peptide, DILPs, leucokinin (LK), drosulfakinin (DSK) and adipokinetic hormone (AKH). Other neuropeptides act in neuronal circuits driving motor neurons that control the feeding apparatus or intestine (see Audsley and Weaver, 2009; Spit et al., 2012) and they are not dealt with here.

HugPK is derived from the precursor encoded by the *hugin* (*hug*) gene that is expressed by about 20 neurons in the subesophageal ganglion of larvae and adults (Melcher and Pankratz, 2005; Meng et al., 2002) (Fig. 2.1A). The importance of *hug* in feeding was indicated by its up-regulation in the feeding mutants *klumpfuss* and *pumpless* (Melcher and Pankratz, 2005). These authors showed that *hug* producing neurons and hugPK inhibit feeding. Thus ectopic expression of *hug* results in reduced food intake, decreased growth and larval lethality. Furthermore, it was shown that *hug* is down-regulated in starved or amino acid deprived larvae. In adult flies inactivation of the *hug* neurons affected the response to a new food source (Melcher and Pankratz, 2005). Wild-type flies display an evaluation phase when encountering a new food source, and thus a delay before feeding, whereas flies with inactivated *hug* neurons start feeding immediately. It was therefore concluded that the *hug* neurons might be part of a circuit evaluating a new food source. This makes sense since the *hug* neurons appear to have inputs from gustatory receptors in the subesophageal ganglion and outputs in several regions associated with control of feeding: the ventral nerve cord, dorsal protocerebrum, the pharynx and the neurohemal organ corpora cardiaca (Bader et al., 2007; Melcher et al., 2007; Melcher and Pankratz, 2005). Interestingly, hugPK and its two GPCRs (CG8784 and CG8795) appear to be orthologs of mammalian neuromedin U and its receptors, also known to decrease food intake and feeding (Melcher et al., 2006).

NPF and its receptor NPFR are considered orthologs of vertebrate neuropeptide Y (NPY) and its receptors (see Brown et al., 1999; Garczynski et al., 2002; Nässel and Wegener, 2011; Wu et al., 2003). NPF signaling has been explored in control of feeding in larval and adult *Drosophila*, especially in relation to nutritional status and food quality. The peptide is expressed in a small number of neurons in the brain (Fig. 2.1A), as well as in the midgut (Brown et al., 1999; Wu et al., 2003) and its expression is developmentally regulated in some of the neurons of the subesophageal ganglion (Wu et al., 2003). At the transition between feeding and non-feeding wandering larval stages the NPF expression is down-regulated, emphasizing its role in feeding. These NPF cells also respond to gustatory stimulation with sucrose by increase in NPF expression (Shen and Cai,

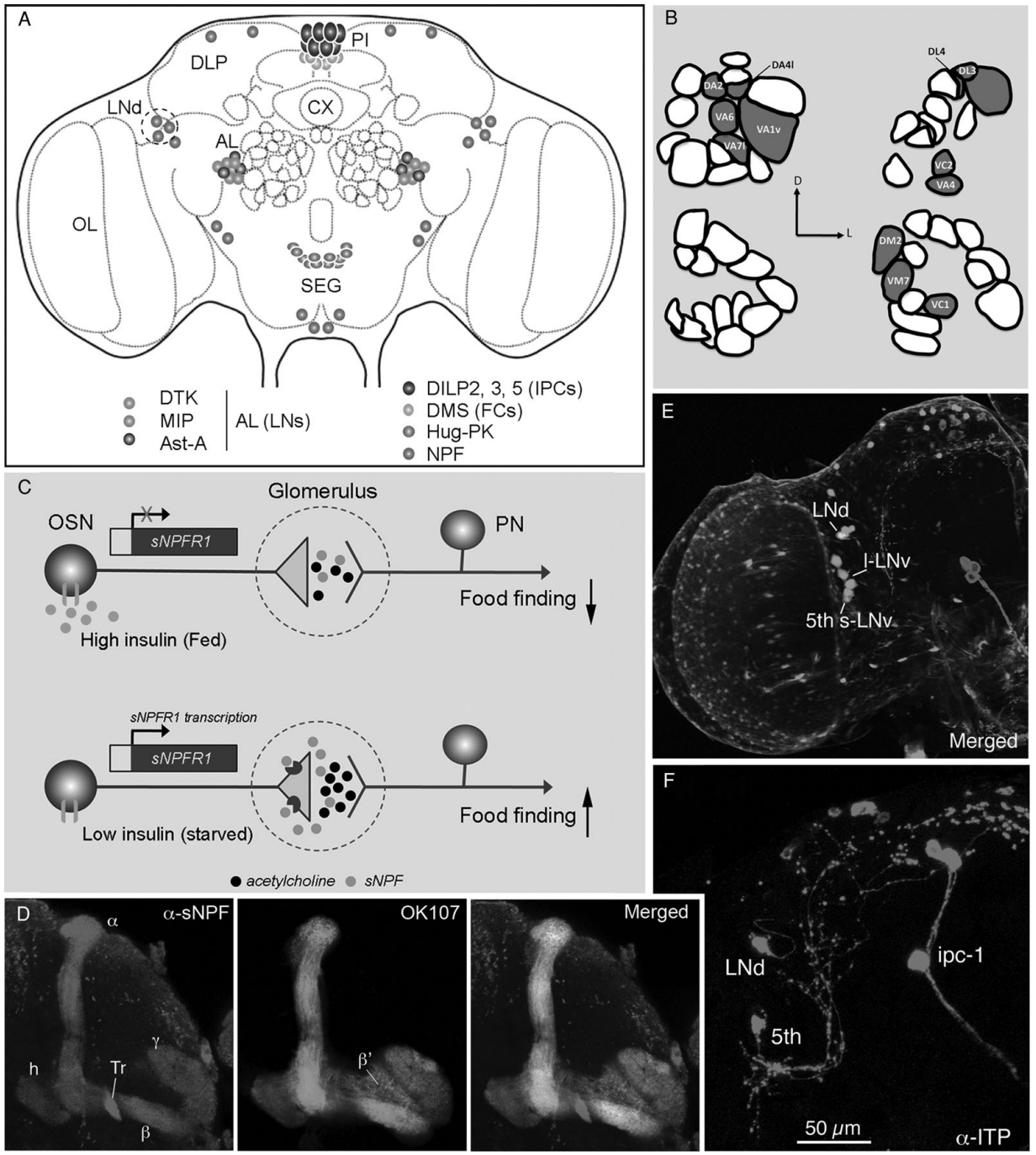

Fig. 2.1. Neuropeptide distribution in the *Drosophila* brain. A Schematic depiction of the distribution of a selection of neuropeptides in neuronal cell bodies in the brain. Note that the correct numbers of neuronal cell bodies are not displayed for clarity of the figure (but are given below). However, the ones shown are in their correct positions. The antennal lobes (AL) are supplied by local neurons (LNs) expressing tachykinin (DTK, 20 neurons per hemisphere), myoinhibitory peptide (MIP, 10–15 neurons) and allatostatin A (Ast-A, 3 neurons). In the pars intercerebralis (PI) there are 10–14 insulin-producing cells (IPCs; dark blue) and about 10 feminizing cells (FCs; orange), possibly expressing myosuppressin (DMS). Spread in different parts of the protocerebrum and subesophageal ganglion of the male brain are the 26 NPF expressing cells (red), three pairs of which are LNd clock neurons (circled). In the subesophageal ganglion (SEG) there are about 20 neurons (purple) expressing hugin-pyrokinin (hug-PK). Other acronyms: CX, central complex; DLP dorso-lateral protocerebrum; OL, optic lobe. B Distribution of sNPF in axon terminations of olfactory sensory neurons (OSNs) in a subset of the antennal lobe glomeruli (marked red) shown in exploded view. Redrawn from (Carlsson et al., 2010) with permission. C The olfactory sensory neurons (OSNs) are regulated by insulin signaling. This panel shows an OSN synapsing on a projection neuron (PN) in a fed fly (top) and a hungry one (bottom). The synapse is located within an antennal lobe glomerulus. In the fed fly, the level of circulating insulin (DILPs) is high and the activated insulin receptor (dInR) on the OSN inhibits transcription of the sNPFR1. Thus, there is low expression of the receptor presynaptically on the OSN axon termination and signal transfer at the synapse is weak. As a result, food finding is low. In the hungry (starved) fly, insulin levels are low and the transcription of sNPFR1 in the OSNs is activated. Consequently, presynaptic sNPFR1 expression increases and released sNPF activates the presynapse leading to enhanced release of acetylcholine and thus increased signaling in the synapse: food finding increases. From (Nässel, 2012) with permission (the figure is redrawn and compiled from original data in (Root et al., 2011) and (Wang, 2012)). D Distribution of sNPF in intrinsic neurons (Kenyon cells) of the mushroom body (magenta). The enhancer trap Gal4 line OK107 was used to drive GFP in most Kenyon cells (green). In the merged image it is clear that sNPF is present in axons in alpha, beta and gamma lobes, but not in those in alpha and beta lobes. From (Johard et al., 2008) with permission. E and F A subset of the clock neurons express ion transport peptide (ITP). In E *tim*-Gal4 expressing clock neurons are seen with IPT immunolabeling. One of the LNd clock neurons and the fifth small LNv (s-LNv) coexpresses ITP. In F these neurons are seen labeled with anti-ITP together with a set of large neurosecretory cells (ipc-1). (E) and (F) from (Johard et al., 2009) with permission.

2001). Overexpression of NPF extends the larval feeding stage and causes delayed pupariation, whereas knockdown/silencing leads to an abbreviated feeding phase and premature food aversion (Wu et al., 2003).

NPF signaling is also regulating food choice in feeding larvae. A wild type larva will stop feeding if encountering food of low quality or with bad taste. However, if they have been starved for some time they will eat even low quality (e.g., more solid) or noxious food. Experiments have shown that this deprivation-motivated feeding is regulated by NPF. Silencing of NPF signaling leads to larvae that are even more aversive to noxious or solid food, whereas overexpression of the NPFR leads to increased consumption of non-palatable food (Wu et al., 2003; Wu et al., 2005a; Wu et al., 2005b). NPF signaling is also required for feeding at low temperature, a behavior that only starved larvae display (Lingo et al., 2007). When NPFR1 is overexpressed in fed larvae, it triggered cold-resistant feeding activity.

An interesting aspect of NPF function is in regulation of ethanol intake in flies. Activation of the NPF system decreases alcohol preference, whereas inactivation increases it (Shohat-Ophir et al., 2012). This appears linked to a reward system that also includes sex behavior, and will be dealt with in a later section.

A link between NPF and insulin (DILP) signaling has been found. The NPFR is negatively regulated by DILP signaling and interference with the dInR in NPFR-expressing neurons produced behavioral effects related to the NPF-induced ones (Wu et al., 2005a; Wu et al., 2005b). Thus down-regulation of DILP signaling in NPFR neurons led to fed larvae feeding on non-palatable food that is normally rejected and up-regulated DILP signaling induced food aversion in starved larvae. It was furthermore shown that DILP signaling negatively regulated cold-resistant food acquisition in larvae (Lingo et al., 2007). DILP signaling has been implicated in the transduction of hunger signals to the CNS, including the olfactory system, and will be dealt with separately below.

Two systems of DILP producing neurons have been investigated for their roles in feeding. A set of DILP7 expressing neurons in the abdominal ganglia innervate the intestine and two send axons to the subesophageal ganglion (Miguel-Aliaga et al., 2008). Silencing these neurons does not affect normal feeding, but when flies are kept on poor nutritional conditions, they eat faster than control flies (Cognigni et al., 2011). Silencing the brain IPCs (Fig. 2.1A; see also Fig. 2.3) that produce DILP2, 3 and 5, on the other hand, leads to flies decreasing feeding in response to poor nutritional conditions (Cognigni et al., 2011). These authors propose that the two DILP systems regulate feeding in response to scarce nutrients.

As noted above, increased levels of certain DILPs suppresses starvation-dependent larval feeding (Wu et al., 2005b) and so does global activation of the dInR (Britton et al., 2002). One target of DILP signaling that regulates feeding in *Drosophila* larvae is the mushroom body Kenyon cells (Zhao and Campos, 2012). Knockdown of dInR or PI3K activity with mushroom body-specific Gal4 drivers reduces food intake in larvae, but also proliferation of the Kenyon cells. It is therefore not clear whether the DILP signaling to the Kenyon cells affects only development or also acute function of the mushroom bodies. Furthermore, these findings are not consistent with DILPs acting as satiety signals to the mushroom bodies, as would have been anticipated from earlier studies. Thus, the regulation of feeding by DILP signaling appears complex and requires further study.

The four peptides encoded by the sNPF precursor are widespread in the CNS and are likely to be multifunctional (Nässel et al., 2008; Nässel and Wegener, 2011). sNPF and its receptor sNPFR1 play prominent roles in feeding (Hong et al., 2012; Lee et al., 2008; Lee et al., 2004). It seems that the peptides regulate food intake in larvae and thus growth, and in contrast to NPF there seems to be no role for sNPF (or change in expression) at the transition from feeding to wandering stages. It was postulated that sNPF regulates the IPCs and stimulates DILP signaling (Lee et al., 2008), see also (Kapan et al., 2012). Thus overexpression of the sNPFR1 in IPCs increased food intake and body size, and decreased signaling led to smaller flies. A more recent paper identifies a signaling pathway activated by sNPF that includes protein kinase A and CREB modulation of the *minibrain* (*mnb*) product and FOXO regulation, and subsequent promotion of feeding (Hong et al., 2012). Interestingly, sNPF and the sNPFR1 are expressed in the olfactory sensory neurons of the antennae and the receptor expression is under negative control by DILP signaling (Root et al., 2011). Thus olfaction-based food search is regulated by hunger-driven up-regulation of the sNPFR1 via decreased DILP signaling (see Fig. 2.1C).

Leucokinin (LK) is known as a diuretic hormone in *Drosophila* (Coast et al., 2002), but was recently also found to regulate meal size in the adult fly (Al-Anzi et al., 2010). Knockdown of LK or its receptor (LKR) or genetic ablation of the neurons expressing these, both lead to an increase in meal size, but a decrease in meal frequency. It was suggested that leukokinin signals to LKR-expressing neurons in the brain that innervate the foregut and therefore regulate food intake neuronally and not hormonally (Al-Anzi et al., 2010). Also, the authors propose that the LK signaling may mediate information on gut distention to regulate meal size. In this context it was also tested whether ablation of the *hugin* and NPF neurons, discussed above, affected meal size, and they did not (Al-Anzi et al., 2010). Another peptide type proposed as satiety factors in insects is the sulfakinins (DSK in *Drosophila*), known to be related to mammalian cholecystokinin (Downer et al., 2007; Spit et al., 2012). The role of DSK as a satiety factor has recently been demonstrated in *Drosophila* where a small set of brain neurons express the peptide, including a subset of the brain IPCs (Söderberg et al., 2012). Inactivation of the DSK producing neurons or diminishment of DSK peptide leads to flies with deregulated food intake and food choice. In fact, it was sufficient to diminish DSK levels in the IPCs to obtain an effect on feeding.

Adipokinetic hormone (AKH) is only produced in the endocrine cells of the corpora cardiaca (see Fig. 2.3D) and has long been known to regulate carbohydrate and lipid metabolism in the fat body (see Gäde et al., 1997). In *Drosophila* it was shown that, in addition to the metabolic roles, AKH may trigger food search behavior in hungry flies (Isabel et al., 2005; Lee and Park, 2004). Ablation of the AKH producing neurons led to hypoactive flies that live longer at starvation than hungry hyperactive controls (thus displaying increased starvation resistance). The AKH receptor is primarily expressed in the fat body and this expression is responsible for metabolic phenotypes (e.g., starvation resistance) seen in receptor mutants (Bharucha et al., 2008; Buch et al., 2008; Katewa et al., 2012). Bharucha and coworkers did, however, not observe an effect of reduced AKH signaling on locomotor activity. Overexpression of AKH increases fat metabolism, locomotor activity and extends lifespan of flies (Katewa et al., 2012). These authors also suggest that AKH signaling, enhanced lipid metabolism, and increased exercise are crucial in the protective effects of life-extending dietary restriction. The AKH receptor is also expressed on a subset of the gustatory neurons that are mediating attractive taste (Bharucha et al., 2008), but the functional role in this system has not been explored.

Recently the Allatostatin A (AstA) type peptides have been shown to be important in foraging and feeding behavior in *Drosophila* (Hergarden et al., 2012; Wang et al., 2012). Knockdown of AstA or one of its receptors, DAR-1, results in a reduced foraging in larvae in the presence of food, but not in food absence (Wang et al., 2012). Another study showed that activation of AstA expressing neurons inhibits starvation-induced feeding behavior in adult flies (Hergarden et al., 2012). These flies increase their food intake and also display enhanced responsiveness to sugar. The effects on feeding behavior are not accompanied by effects on metabolism or energy storage and the authors suggest that the AstA activation is a consequence of metabolic satiety signals. The AstA activation thus induces food aversion and inhibits motivation to feed (Hergarden et al., 2012).

Sex peptide is transferred to the females at copulation via semen from the male accessory glands (Kubli, 2003). In the female fly sex peptide induces a change in behavior that lasts for about a week: the females become refractory to male courtship, they alter their locomotor activity rhythm and they increase feeding (Carvalho et al., 2006; Isaac et al., 2010; Kim et al., 2010; Kubli, 2003; Rezaval et al., 2012; Yapici et al., 2008). The increased feeding appears to be an indirect effect of sex peptide, since mated sterile females do not increase their food intake (Barnes et al., 2008). This suggests that the increased nutritional demands at egg production induced by the mating and sex peptide is the cause of increased feeding.

Associated with feeding there is a homeostatic regulation of water and ion balance. Several peptide hormones have been implicated in this regulation: diuretic hormones DH31 and DH44, leucokinin, *capability* gene derived peptides and ion transport peptide (Coast et al., 2002). Recently, pigment-dispersing factor (PDF), previously known for its central role in certain clock neurons (Renn et al., 1999), was shown to induce contractions in the renal tubules after peripheral release (Talsma et al., 2012).

Neuropeptides in reproductive behavior

In this section only behavioral aspects of reproduction will be highlighted. Direct or indirect peptidergic regulation of sexual maturation and fecundity will be ignored here. For roles of DILPs, corazonin and other peptides in these functions the reader is referred to reviews (Antonova et al., 2012; Bergland et al., 2012; De Loof et al., 2001; Soller et al., 1997; Toivonen and Partridge, 2009). A few peptides have been implicated in regulating mating behavior in *Drosophila*: SIFamide, sex peptide, NPF, and DILP7.

SIFamide is expressed in four neurons with cell bodies in the pars intercerebralis and densely arborizing processes in most neuropil regions of the brain and ventral nerve cord (Terhzaz et al., 2007; Verleyen et al., 2004). A notable exception is the mushroom body lobes that seem totally devoid of SIFamide innervation. Ablation of the SIFamide expressing neurons or knockdown of SIFamide led to flies with increased courtship activity, even promiscuity: males increased courtship to both males and females, whereas females became more sexually receptive (Terhzaz et al., 2007). Thus, SIFamide seems to inhibit the vigor of sexual activity in both sexes. It is not clear what circuitry is affected by the peptide and the relation to, for instance, *Fruitless* (*Fru*) splice form expressing neurons is not known. Many of the neurons expressing the male form of *Fru* (Fru^M) have been implicated in male-specific behaviors, including courtship (see Dickson, 2008; Kahsai et al., 2012; Yamamoto, 2008).

Sex peptide has already been mentioned in its role in inducing a post mating response in females which includes down-regulation of female mating behavior: females become refractive to male courtship for about a week (Kubli, 2003). The sex peptide receptor is localized to 6–8 sensory neurons with cell bodies and "dendrites" in the uterus and axons terminating in abdominal and subesophageal ganglia (Häsemeyer et al., 2009; Rezaval et al., 2012; Yang et al., 2009).

NPF was claimed to display a sex-specific expression in brain neurons: in the male brain 26 neurons were detected and in females 20 (Lee et al., 2006) (Fig. 2.1A). The six extra NPF neurons in males are bilateral sets of clock neurons (LN_ds) (Fig. 2.1A). When these neurons are genetically ablated or feminized by expression of the $transformer^F$ gene, the male files display reduced courtship activity (Lee et al., 2006). The authors propose that the male-specific NPF neurons modulate *Fru*-regulated courtship behavior, but also that NPF may play a role in a clock-controlled sexual dimorphism in locomotor activity. It should be noted that a later study suggests that the six NPF expressing clock neurons are present in both sexes (Hermann et al., 2012), but possibly their function is still sex specific (see further details in section on clock system). A recent

paper showed that sexual deprivation in male flies leads to an increased consumption of ethanol and that this was linked to reduced levels of NPF (Shohat-Ophir et al., 2012). These authors found that activation of NPF neurons produces a reward signal and that both mating and ethanol consumption are rewarding (and associated with high levels of NPF). The specific NPF neurons involved have not ben identified, but it was proposed that they are different from the ones mediating the NPF effect in sugar reward memory (Shohat-Ophir et al., 2012), discussed later in this chapter.

The oviposition motor program and choice of substrate for egg laying seems to be regulated by one of the seven DILPs, the relaxin-like DILP7 (Yang et al., 2008). DILP 7 is expressed in a small set of neurons in abdominal ganglia, and in females the reproductive tract is innervated by axons from some of these neurons (Miguel-Aliaga et al., 2008; Yang et al., 2008). Some of these DILP7 neurons also have arborizations in the subesophageal ganglion where gustatory sensory neurons terminate (see Vosshall and Stocker, 2007). Inactivation of the DILP7 neurons leds to loss of ovipositor motor programs and render Bd the females sterile (Yang et al., 2008). Flies were tested in a behavioral assay for choice of substrate for egg laying where it was found that the females avoided sucrose-containing medium. Overexpression of DILP7 in DILP7 neurons, or globally, rendered flies more prone to lay eggs on sucrose-based medium (Yang et al., 2008). The authors proposed that the DILP7 neurons are important for the decision-making process during egg laying site selection. In the light of the effect of other DILPs on sensitivity of olfactory sensory neurons (see section on olfaction), it is possible that the DILP7 neurons regulate the sensitivity of gustatory receptors or circuits in the gustatory pathway necessary for probing the egg-laying substrate. Both the abdominal and subesophageal ganglion are supplied by branches of DILP7 neurons and these regions receive inputs from gustatory receptors of the proboscis and ovipositor (Vosshall and Stocker, 2007).

Neuropeptides in aggression

Aggression is more prominent in male flies that defend territories and fight over females (Dierick and Greenspan, 2007; Vrontou et al., 2006). As mentioned in the previous section, NPF was found to display a male-specific expression in six brain neurons (but see previous section) and it is the only peptide so far studied in relation to aggressive behavior in *Drosophila* (Dierick and Greenspan, 2007). Silencing of the NPF neurons in males leads to increased fighting frequencies. Also feminizing the NPF neurons with Tra^F produces more aggressive males. These findings suggest that NPF decreases aggression. However, the inhibitory NPF action is presumed to be on a male-specific neuronal circuit required for aggressive behavior (Dierick and Greenspan, 2007). Interestingly, the effect of NPF to decrease aggression levels may be linked to the effect on courtship behavior: knockdown of NPF leads to suppressed courtship behavior (Lee et al., 2006) and increased aggressive behavior (Dierick and Greenspan, 2007). Males defending a territory rapidly switch between aggression and courtship depending on whether the invader is a male or a female and thus NPF in the male-specific neurons may provide a switch between opposite social behaviors (Dierick and Greenspan, 2007).

Neuropeptides in olfaction

The functional organization of olfactory system and olfactory behavior of *Drosophila* has been extensively investigated (see Vosshall and Stocker, 2007). However, the roles of neurotransmitters and neuropeptides in olfactory processing are less known and most studies so far have concerned GABA and its receptors in olfaction (Masse et al., 2009; Root et al., 2008; Wang, 2012; Wilson, 2011; Wilson and Laurent, 2005). One study has identified the neuropeptides present in the *Drosophila* antennal lobe by a combination of mass spectrometry and immunocytochemistry (Carlsson et al., 2010). It was found that not less than seven different neuropeptides are expressed in neurons with processes in the antennal lobe: allatostatin A (AstA), *Drosophila* myosuppressin (DMS), *Drosophila* tachykinin (DTK), IPNamide, myoinhibitory peptide (MIP), SIFamide and short neuropeptide F (sNPF). The distribution of some of these is shown in Fig. 2.1A. The major types of neurons in the antennal lobe are: axon terminations of OSNs, local neurons (LNs), projection neurons (PNs) and extrinsic (or centrifugal) neurons. Neuropeptides are so far found in all types except PNs. It is noteworthy that sNPF was detected in a subset of the OSNs (Fig. 2.1B); this was the first neuropeptide to be clearly identified in sensory neurons of an insect (Carlsson et al., 2010; Nässel et al., 2008). In fed wild-type flies sNPF positive OSN axon terminations are seen in 13 of the 50 glomeruli, suggesting odor-specific functions of the peptide (Carlsson et al., 2010).

Two peptide receptors have been identified in antennal lobe structures: the sNPF receptor sNPFR1 in OSNs and the DTK receptor DTKR also in OSNs and probably in LNs (Ignell et al., 2009; Kahsai et al., 2010b; Root et al., 2011; Winther and Ignell, 2010). Both these receptors have presynaptic functions in regulation of sensitivity of OSNs. Interestingly, the sNPFR1 in the OSNs is regulated by insulin signaling (Root et al., 2011). When the fly is hungry, insulin signaling decreases and this leads to diminished activation of the insulin receptor (dInR) expressed on OSNs (Fig. 2.1C). Since the activated dInR down-regulates sNPFR1 expression on OSNs, the diminished insulin signaling leads to increased sNPFR1 expression and this increases odor sensitivity in certain odor channels (Root et al., 2011). These authors showed that sNPFR1-mediated presynaptic odor facilitation of Or42b expressing neurons is necessary for starvation-induced food-search behavior, and that insulin signaling regulates this facilitation. Curiously, the OSNs express both sNPF and sNPFR1, and no other antennal lobe neurons produce sNPF. This suggests that the OSNs utilize sNPF as a cotransmitter with acetylcholine and that it acts predominantly presynaptically to regulate neurotransmitter release in an autocrine loop. The other peptide DTK is produced by LNs and acts on OSNs to

inhibit synaptic activity (Ignell et al., 2009). This changes sensitivity to odors in specific ways, but the behavioral relevance is not yet clear. There is no data so far on the functional role of the other five neuropeptides.

Neuropeptides in mushroom bodies and learning

The mushroom bodies are prominent paired protocerebral neuropils of the insect brain known to play important roles in olfactory learning and memory (for review see Davis, 2005; Heisenberg, 2003). Curiously, the neurotransmitter of the numerous endogenous neurons of the mushroom body, the Kenyon cells, is not known in any insect. In *Drosophila* sNPF was, however, detected in most of the Kenyon cells (Fig. 2.1D) (Johard et al., 2008), but the functional role of the peptide has not yet been determined.

The only peptide so far to be implicated in mushroom body circuits and learning is NPF. This peptide is expressed in 20–26 brain neurons in *Drosophila*, and a subset of these appear to be presynaptic to dopaminergic neurons that invade the mushroom body lobes (Krashes et al., 2009). These authors showed that the NPF expression is in circuits important for motivational activation in output of appetite-related memory in *Drosophila*. Starvation increases performance in olfactory reward learning and well-fed flies do not learn well. It was shown that stimulation of activity in the NPF neurons mimics food deprivation and promotes appetitive memory performance in fed flies (Krashes et al., 2009). This memory requires expression of the NPF receptor on a set of six dopaminergic neurons that innervate the mushroom body. Inactivation of these dopaminergic neurons increases memory performance in fed flies, whereas stimulating them suppresses memory in hungry flies. It thus appears that the NPF neurons and the NPF receptor expressing dopaminergic neurons serve as a motivational switch in the mushroom body circuits and control appetitive memory output (Krashes et al., 2009).

The *Drosophila* insulin receptor substrate CHICO is expressed in the Kenyon cells even in adult flies (Naganos et al., 2012) suggesting that these cells are targeted by insulin signaling. These authors showed that *Chico* mutants display defects in olfactory learning and that memory formation could be restored after *Chico* rescue specifically in mushroom bodies. Like in the study on mushroom bodies and feeding (Zhao and Campos, 2012), the studied effects of *Chico* impairment are developmental and influence growth and proliferation. Conditional knockdown of *Chico* or *dInR* in adult Kenyon cells is required to determine acute effects of insulin signaling to the mushroom bodies in learning and feeding.

Peptidergic modulation of locomotor activity

Locomotor activity can be regulated at multiple levels in the CNS. Local motor circuits in the ventral nerve cord are controlled by higher centers in the brain and subesophageal ganglion (Ritzmann and Büschges, 2007). In the brain the central complex and mushroom bodies are known to regulate and coordinate locomotor behavior (Serway et al., 2009; Strausfeld, 1999; Strauss, 2002). The central complex controls velocity of motion, maintenance of activity, symmetry of locomotion and orientation (Strauss, 2002) and the mushroom bodies regulate aspects of walking, and suppress locomotion over longer periods (Martin et al., 1998; Serway et al., 2009). Also other brain systems, like AKH and DILP-producing cells, control aspects of locomotor activity and in the following the roles of neuropeptides in sexually dimorphic activity patterns, foraging, and short-term actions in locomotor control are summarized. Roles of neuropeptides in circadian locomotor activity are discussed in the next section.

Sexually dimorphic locomotor activity has been observed in *Drosophila*. Locomotor activity in flies is clustered in bouts of motion, followed by periods of inactivity and the organization of these bouts is sexually dimorphic. Female flies stop and start with a higher frequency than males (Martin et al., 1999). The control of this sexually dimorphic behavior resides in two distinct populations of neurons in the pars intercerebralis of the brain, the IPCs (Belgacem and Martin, 2006; 2007), and in about ten neurons called the feminizing cells, FCs (Belgacem and Martin, 2002) (Fig. 2.1A). Ablation of the IPCs results in male flies with a feminized locomotor profile, suggesting that DILP signaling may control sex-specific behaviors. It was found that the DILP receptor dInR is expressed in endocrine cells of the corpora allata (CA), and knockdown of dInR in these cells abolishes the sexual dimorphism in locomotor activity (Belgacem and Martin, 2002). The CA cells secrete juvenile hormone (JH), probably under direct or indirect control of brain DILPs, and this seems to regulate the sexually dimorphic locomotor activity (Belgacem and Martin, 2006; 2007). Other studies have implicated the IPCs in regulation of locomotor activity as well as sleep–wakefulness (Crocker et al., 2010; Mattaliano et al., 2007) and we will return to this in the section on insulin signaling.

The FCs also regulate the sexual dimorphic locomotor activity. It was shown that genetically feminizing these neurons (but not the IPCs) in male flies eliminated the sexual dimorphism and that this feminization can be mimicked by feeding males with a JH inhibitor (Belgacem and Martin, 2002). It is not clear what the relation is between signaling from the FCs and the IPCs with respect to control of locomotor activity or how JH influences the circuits regulating locomotion.

Starved flies become hyperactive as a reflection of increased search for food. Ablation of the cells producing the metabolic hormone AKH leads to a loss of this hyperactivity at food deprivation (Isabel et al., 2005; Lee and Park, 2004). At starvation, AKH therefore seems to regulate both a mobilization of stored carbohydrate and lipids and induces locomotion to find new sources of nutrition. In the cockroach the AKH signaling was proposed to act via octopamine-expressing neurons in the ventral nerve cord to increase locomotor activity (Wicher et al., 2006).

Drosulfakinin (DSK) and its receptor CCKLR regulate larval locomotion and the DSK signaling is necessary for the

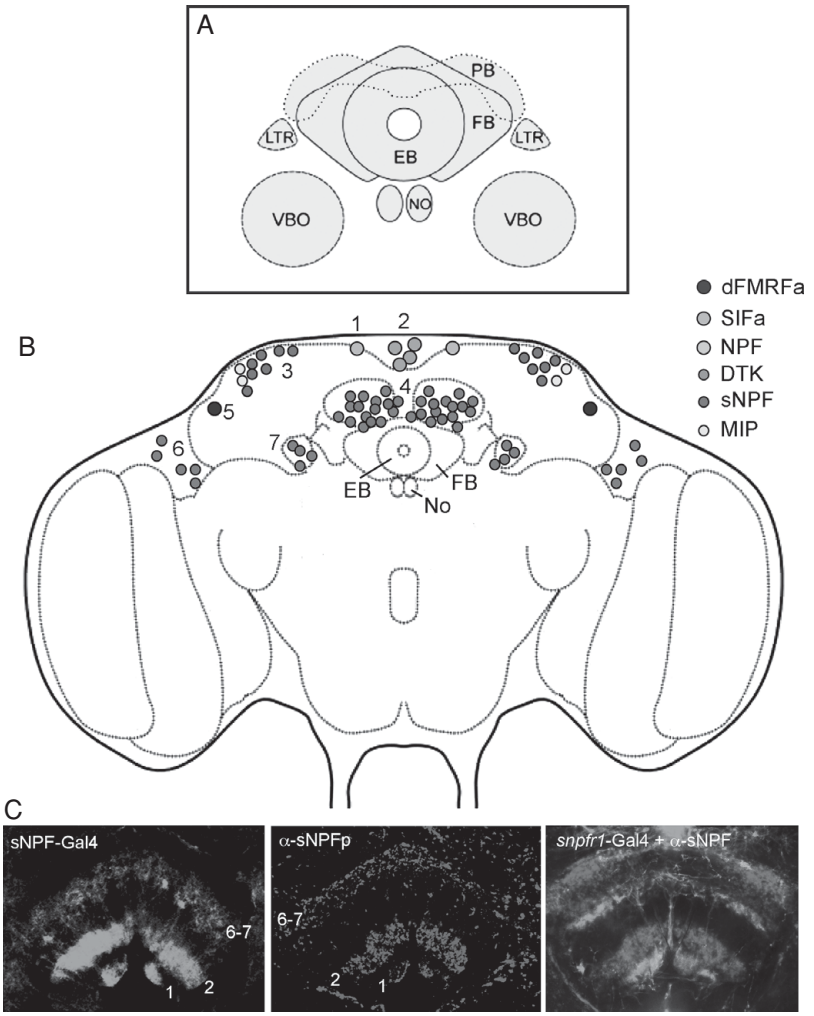

Fig. 2.2. Peptidergic neurons innervating the central complex (CX). A Schematic depiction of the CX in frontal view (dorsal is up). B There are seven clusters of peptidergic cell bodies (1–7) in the protocerebrum sending processes to the CX. These express tachykinin (DTK) in three clusters labeled 4, 6, and 7, dFMRFamide in two neurons (5) myoinhibitory peptide (MIP) in cluster 3, short neuropeptide F (sNPF) in two clusters (3 and 4), neuropeptide F (NPF) in two large neurons (1) and SIF amide in 4 neurons in the pars intercerebralis (2). These neuropeptides display different distributions inlayers of the fan-shaped or ellipsoid bodies of the CX (see (Kahsai and Winther, 2011)). This figure is redrawn from (Kahsai and Winther, 2011). C Expression of sNPF producing neurons in specific layers (layers 1, 2, 6, and 7) of the fan-shaped body of the CX. The left panel shows *snpf*-Gal4 driven GFP and the middle one immunolabeling with antiserum to the sNPF precursor. The right panel shows expression of the sNPF receptor (sNPFR1) using *snpfr1*-Gal4-driven GFP, together with sNPF immunolabeling. These images are slightly altered from (Nässel et al., 2008) and (Kahsai et al., 2012) with permission.

stress-induced escape response in larvae (Chen et al., 2012). A previous study by the same authors indicated an important role of DSK signaling for the normal development of the neuromuscular junction (Chen and Ganetzky, 2012).

Different neuron types of the central complex have been shown to express peptide products of eight neuropeptide encoding genes: DTK, sNPF, myoinhibitory peptide (MIP), allatostatin A (AstA), proctolin, SIFamide, NPF, and dFMRFamide (Kahsai and Winther, 2011). These are distributed in different sets of neurons innervating various neuropil regions of the central complex (Fig. 2.2): the fan-shaped body, the ellipsoid body, the nodules or the protocerebral bridge. All eight peptides were detected in different layers of the fan-shaped body. Peptide GPCRs have been detected in the fan-shaped body for sNPF (Fig. 2.2C), DTK, and proctolin (Birse et al., 2006; Johnson et al., 2003; Kahsai et al., 2012; Poels et al., 2009). Two of the peptides, DTK and sNPF, were investigated for roles in control of locomotor activity (Kahsai et al., 2010b). By using various enhancer trap-Gal4 lines combined with immunolabeling, neuron sets were identified that express DTK or sNPF; these were subsequently targeted by Gal4-UAS mediated RNAi to knock down either of the peptides in specific neuron types in the central complex. It was found that DTK knockdown in certain neurons resulted in flies with increased center zone avoidance, in other neurons knockdown resulted in flies with an increase in activity–rest bouts (Kahsai et al., 2010b). Knockdown of sNPF in specific neurons indicated a role in fine-tuning of locomotor activity levels. Thus the two peptides seem to be important for spatial orientation, activity levels, and temporal organization of spontaneous walking. The data for these two peptides suggest a circuit-specific contribution to locomotor control in the central complex (Kahsai et al., 2010b; Kahsai and Winther, 2011). In the study only two neuropeptides of the eight were investigated and only in a subset of the neurons expressing them in the fan-shaped body; still it is apparent that neuropeptides may play very distinct roles in fine tuning of locomotor control and that this control is specific to subsets of central complex neurons. Furthermore, the central complex (ellipsoid body) seems to be important in visually guided behaviors and visual learning, as well as courtship behavior (Becnel et al., 2011; Joiner and Griffith, 2000; Liu et al., 2006; Pan et al., 2009; Sakai and Kitamoto, 2006) and future analysis of neuron-specific

neuropeptide signaling in all these functions is a daunting task.

Neuropeptides in the clock system

In *Drosophila* sets of about 150 clock neurons form circuits that drive circadian behavior and physiology and are synchronized with the environmental daily rhythms of light and temperature (for review see Peschel and Helfrich-Förster, 2011). These clock neurons are distributed in seven bilateral groups, each of which has distinct functions within the network (Helfrich-Förster et al., 2007; Kaneko et al., 1997; Taghert and Shafer, 2006). Some of these clock neurons are shown in Fig. 2.1E. The first neuropeptide to be identified in a clock neuron was pigment-dispersing factor (PDF) (Helfrich-Förster, 1995). PDF is expressed in two types of lateral ventral neurons (s-LN_vs and l-LN_vs) and serve both as an output factor of the clock and as substance synchronizing activity within parts of the clock network (Im and Taghert, 2010; Peschel and Helfrich-Förster, 2011; Renn et al., 1999; Yoshii et al., 2009). A few other peptides have later been detected in other subsets of clock neurons: IPNamide, neuropeptide F (NPF), short neuropeptide F (sNPF), and ion transport peptide (ITP) (Johard et al., 2009; Lee et al., 2006; Shafer et al., 2006). As an example ITP-expressing clock neurons are shown in Fig. 2.1E and 2.1F. Except for NPF, the functional roles of these peptides in the clock are not yet known. It has been proposed that the NPF expressing clock neurons play a role as oscillators in evening activity of the flies (Hamasaka et al., 2010; Hermann et al., 2012; Lee et al., 2006). It was recently shown that NPF is expressed in clock neurons of both males and females and that it is not only produced in LN_d type neurons, but also in the fifth s-LN_v and in a subset of the l-LN_vs. Furthermore, it was found that the clock driven rhythm in courtship motivation depends on the evening oscillator, but not on the NPF expressing LN_ds of this oscillator (Hamasaka et al., 2010).

Insulin signaling in behavior and stress

In *Drosophila* DILPs play multiple roles in behavior in addition to the traditional roles in development, metabolism, reproduction, and lifespan (for review see Antonova et al., 2012; Baker and Thummel, 2007; Géminard et al., 2006; Giannakou and Partridge, 2007; Teleman, 2010). These behaviors include specific locomotor activity, sleep–wakefulness, stress-related behavior, ethanol sensitivity, feeding and olfactory behavior (for review see Nässel, 2012). The role of DILP7 produced by neurons in abdominal ganglia was already dealt with in relation to reproductive behavior and feeding (Cognigni et al., 2011; Yang et al., 2008). In the following the focus will be on the DILPs that are produced by median neurosecretory cells (MNCs) of the brain. A set of 10–14 MNCs, are known to produce DILP2, 3, and 5 (Brogiolo et al., 2001; Cao and Brown, 2001; Ikeya et al., 2002; Rulifson et al., 2002), and are referred to as insulin producing cells, IPCs (Fig. 2.1A, 2.3). These IPCs have their presumed dendrites in the pars intercerebralis (Fig. 2.3A, B) and release sites in the corpora cardiaca, anterior aorta and proventriculus of the intestine (Fig. 2.3C). Analysis of actions of the IPCs, and the three DILPs, have been performed in various ways: genetic ablation of the brain IPCs, inactivation of signaling in the IPCs, or by *Dilp2, 3, 5* mutant analysis. Each of these approaches has some caveats, apart from the fact that they mostly were not performed conditionally in adult flies. For instance, the two first approaches could produce phenotypes caused by other factors released from the IPCs; additional peptides (DSKs) are produced in a subset of the IPCs in the larva (Park et al., 2008) and adults (Söderberg et al., 2012). Thus, it is not always clear whether interference with the IPCs, that do not specifically eliminate the three DILPs, causes behavioral effects that are only DILP related. Another possibility is that release of DILPs targets not only the fat body and the canonical signaling pathway downstream to the dInR, but also other neurons and cells where other signaling pathways may be recruited (Fig. 2.3A). A few of these DILP targets have already been discussed in previous sections: DILP signaling (non-specified) to NPFR expressing neurons in the brain that regulate feeding and to OSNs in the antennae that lead to regulation of the sNPFR1 and changes in olfactory sensitivity and food search (Fig. 2.1C) (Root et al., 2011; Wu et al., 2005a; Wu et al., 2005b). Also, the roles of the IPCs in sex-specific locomotion, probably via JH, and food ingestion have been mentioned (Belgacem and Martin, 2007; Cognigni et al., 2011).

Other behaviors that may be under control of DILP signaling from IPCs, or at least depend on IPC activity, are (Fig. 2.3A): sleep–wakefulness, and ethanol sensitivity (Corl et al., 2005; Crocker et al., 2010). It was shown that the IPCs express the octopamine receptor OAMB and that manipulations of octopamine signaling to the IPCs affect sleep–wakefulness (Crocker et al., 2010). Octopamine activates the IPCs via increases in cAMP and activation of protein kinase A (PKA) and thereby promote wakefulness. However, this study did not provide evidence for a role of bona fide insulin signaling from the IPCs in the sleep–wakefulness regulation. In contrast, the role of IPCs in the flies' sensitivity to alcohol intoxication appear to depend on insulin signaling (Corl et al., 2005). When inhibiting PKA signaling specifically in IPCs or testing flies with mutated dInR or its response element CHICO, the effect was increased ethanol sensitivity. The authors showed that the expression of dInR in neurons of the CNS was required for the response.

The brain IPCs seem to be central as an interface between nutritional status of the fly and control of physiology and behavior. However, the regulation of the activity of the IPCs and their production and release of DILPs has been little studied. Cell autonomous nutrient sensing has been suggested in IPCs of adult *Drosophila* (Fridell et al., 2009). However, in the larva, the fat body expresses an amino acid transporter (slimfast) that acts in systemic nutritional sensing, and at feeding an unidentified humural signal molecule is released by the fat body to activate the IPCs in the brain (Geminard et al., 2009). Additionally, neuronal controls of IPC activity have been demonstrated.

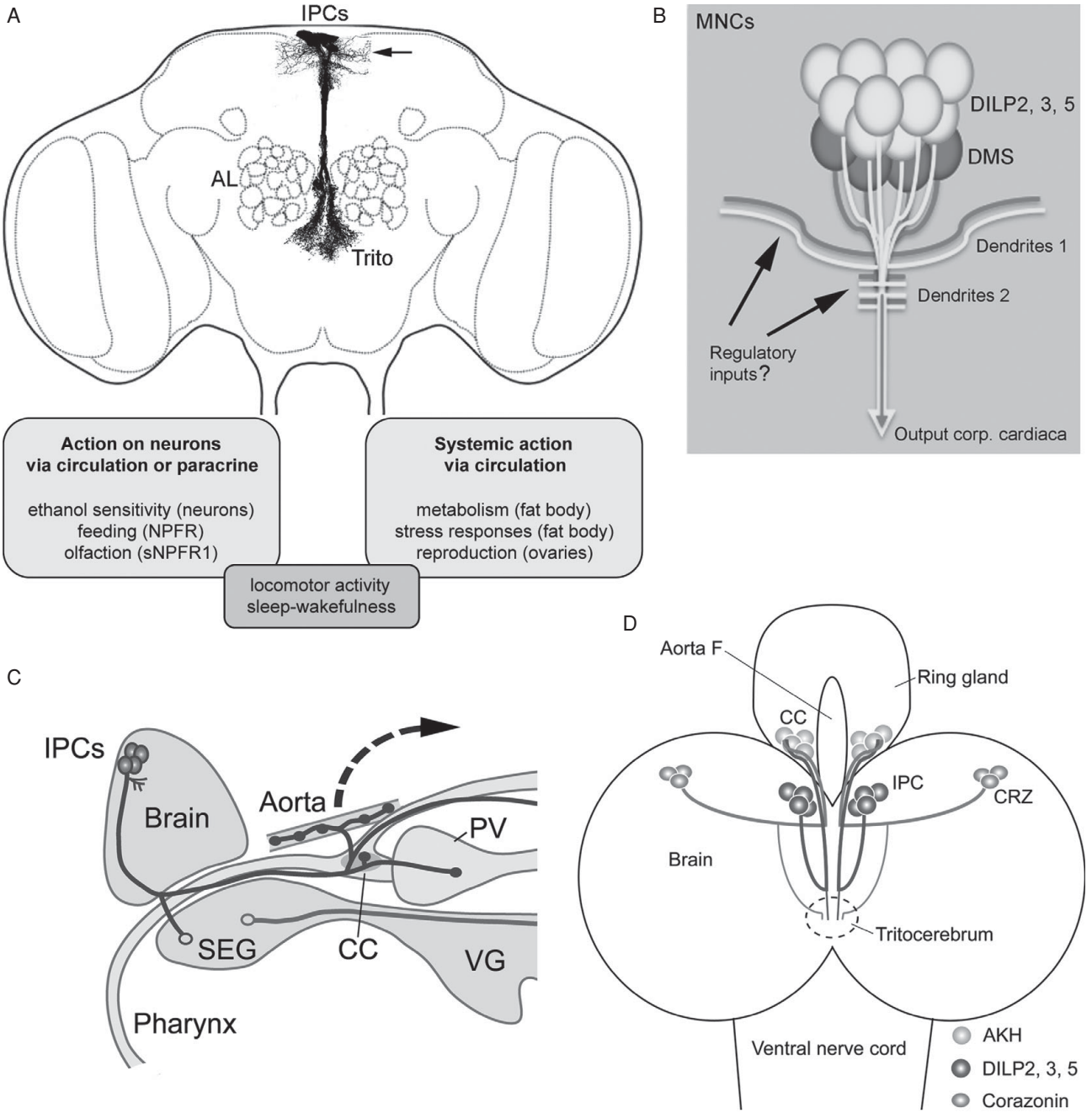

Fig. 2.3. Insulin producing cell in the *Drosophila* brain. A The insulin producing cells (IPCs) in the pars intercerebralis (PI) have pleiotropic functions. In the adult brain 10–14 IPCs have branches in the PI and tritocerebrum (Tritoc) and send axons that terminate in the corpora cardiaca, aorta and anterior midgut (shown in Fig. 2.3C). The branches in the PI and tritocerebrum may be dendritic, but they may also serve as peptide release sites. All the IPCs produce DILP2, 3, and 5, and many of them express drosulfakinin peptide (DSK). As described in the text, the IPCs release DILPs into the circulation (and maybe within the brain; paracrine signaling). In adult flies the systemic actions of DILPs (via circulation) are in regulation of carbohydrate and fat metabolism/storage, stress responses and reproduction and fecundity (in females). DILP functions that are known to be mediated by activation of neurons are regulation of ethanol sensitivity (unspecified neurons), feeding (brain neurons expressing the NPF receptor, NPFR) and olfactory sensitivity (OSNs in antennae expressing the sNPF receptor sNPFR1). Additionally, locomotor activity and sleep–wakefulness are regulated by the IPCs, by not yet defined pathways (although locomotor activity might be indirect via activation of juvenile hormone signaling). Possibly feeding is also directly or indirectly regulated by the IPCs, probably via the co-released DSK, which was shown to be a satiety factor (Söderberg et al., 2012). B The median neurosecretory cells (MNCs) include IPCs and a set of cells expressing myosuppressin (DMS). The IPCs express DILP2, 3, and 5, as well as DSK. C A sagittal view of the brain and anterior intestine with associated aorta and corpora cardiaca (CC). The IPCs send axons to the aorta, CC, proventriculus (PV) and crop (not shown here); these areas are likely release sites for DILPs. SEG, subesophageal ganglion; VG, ventral ganglion. D Schematic depiction of larval brain and ring gland and three sets of peptidergic cells/neurons. These relations are shown in the larva for clarity; the adult neurons/cells display similar relations, but are more complicated to depict. The DILP producing IPCs and corazonin producing neurons (CRZ) send axons to the corpora cardiaca (CC) of the ring gland and to tritocerebrum. Cells producing adipokinetic hormone (AKH) are located in the CC. The functional relations between these cells are discussed in the text. A – C are altered from (Nässel, 2012) and D from (Nässel and Winther, 2010), all with permission.

Experiments suggest that sNPF stimulates insulin signaling in the brain IPCs (Lee et al., 2008), octopamine activates IPCs via the OAMB receptor (Crocker et al., 2010), GABA and the metabotropic $GABA_B$ receptor, as well as DTKs and the receptor DTKR, inhibit IPCs (Birse et al., 2011; Enell et al., 2010), whereas serotonin via 5-HT1A regulates IPCs in a more complex fashion (Luo et al., 2012). It is, however, not clear what neuronal pathways utilize these neurotransmitters to regulate IPCs or what their inputs are; what are the sensory inputs that regulate IPCs via the five transmitters mentioned? Recently, however, a bilateral set of dorsal lateral peptidergic neurons (DLPs) was identified as the source of sNPF and corazonin that target the IPCs (Kapan et al., 2012). These DLPs stimulate the IPCs and affect metabolism, stress resistance and *Dilp* transcript levels in the brain. The DLPs were previously shown to express receptors for the diuretic peptide hormones DH31 and DH44 as well as the allatostatin A receptor DAR2 (Johnson et al., 2005; Veenstra, 2009); three peptides known to be produced by enteroendocrine cells in the midgut (Veenstra et al., 2008). Thus, it is possible that peptidergic signals from the gut, that report nutritional status, target the DLPs which in turn activate the IPCs (Kapan et al., 2012).

In addition to the neurotransmitters systems, it is possible that the AKH producing cells and IPCs communicate and regulate each other's activity (Kim and Rulifson, 2004) (see Fig. 2.3D). In fact, in larval *Drosophila* the AKH producing cells in the corpora cardiaca express ATP-activated potassium channels (with sulphonylurea receptor-like protein) and thus display cell autonomous carbohydrate sensing and could actually signal nutritional status to the IPCs (Kim and Rulifson, 2004). In adult flies this may be slightly different since IPCs seem to be autonomous nutrient sensors (Fridell et al., 2009), but reciprocal feedbacks with AKH cells may exist.

Finally, a few other neuropeptides/peptide hormones have been implicated in regulation of stress responses: corazonin, and CRF-like and CGRP-like diuretic hormones (Boerjan et al., 2010; Veenstra, 2009; Zhao et al., 2010), as well as sNPF and DTK (Kahsai et al., 2010a). It was shown that genetic ablation or silencing of the activity of the corazonin-producing neurons (DLPs) in the *Drosophila* brain (Fig. 2.3D) led to extended lifespan during starvation, osmotic, and oxidative stress, whereas activation by expression of an active Na-channel produced the opposite phenotype (Zhao et al., 2010). Furthermore, activation of corazonin-producing DLP neurons led to male flies with decreased locomotor activity and silencing the neurons produced hyperactive flies. Manipulations of the DLPs also impacted triglyceride and dopamine levels. Taken together, the findings suggest that the corazonin signaling is important in shaping the stress responses in flies and, interestingly, it was found that the role of DLP and corazonin in stress regulation is sexually dimorphic (Zhao et al., 2010). Corazonin and its receptor display similarities to vertebrate gonadotropin-releasing hormone (GnRH) and its receptor (Hauser et al., 2006b) and as mentioned the corazonin producing DLP neurons express receptors for CRF- and CGRP-related diuretic hormones (Johnson, 2006; Johnson et al., 2004). Thus, the *Drosophila* circuit displays similarities to a mammalian hypothalamic stress-regulating system where GnRH cells express receptors for CRF and CGRP and suppress GnRH release at stress (Zhao et al., 2010). The recent finding that the DLPs produce both corazonin and sNPF and target the IPCs to modulate insulin signaling from these cells (Kapan et al., 2012) partly explains the role of corazonin in stress responses.

A set of 8–10 large lateral neurosecretory cells (ipc-1) in the protocerebrum of adult *Drosophila,* and axon terminations in neurohemal release sites, was shown to express products of three neuropeptide genes: sNPFs, DTKs and ion transport peptide (ITP; see Fig. 2.1F) (Kahsai et al., 2010a). Targeted knockdown of either sNPF or DTK expression in these cells led to increased sensitivity to starvation and desiccation, but seemed to have no effect on starvation-induced hyperactivity. Although the peripheral targets of this hormonal signaling have not been identified it is suggestive that intact sNPF and DTK signaling from the ipc-1 cells is required for regulation of homeostasis at nutritional and osmotic stress (Kahsai et al., 2010a). Also, the role of ITP in these cells has not yet been investigated.

Summary and conclusions

It is apparent that only a small number of *Drosophila* neuropeptides and peptide hormones have been analyzed with respect to roles in regulation of behavior and related physiology. Many otherwise well-studied peptides were not discussed here because only their roles in regulation of homeostasis or developmental processes have been investigated (see Coast et al., 2002; Johnson, 2006; Nässel and Winther, 2010; Taghert and Veenstra, 2003). A few of the peptidergic systems discussed in this review bear structural and functional similarities to ones also present in mammals. For instance the insulin-like peptides, insulin signaling pathway, and many of the insulin functions are well conserved over evolution (Baker and Thummel, 2007; Brogiolo et al., 2001; Giannakou and Partridge, 2007; Grönke et al., 2010). Other examples of peptides that are partially functionally conserved from flies to humans are components of the NPF/NPY and hugin/Neuromedin U signaling in feeding, or corazonin/GnRH in stress regulation (de Bono and Bargmann, 1998; Garczynski et al., 2002; Melcher et al., 2006; Nässel and Wegener, 2011; Wu et al., 2003; Wu et al., 2005b; Zhao et al., 2010).

It is quite common that insect neuropeptides have pleiotropic functions, but of course exceptions might exist. Peptides that are produced by very few neurons or neurosecretory cells (SIFa, eclosion hormone) may have more dedicated functions than those expressed in numerous cells. On the other hand, there are only 10–14 brain IPCs producing three DILPs that each might be multifunctional. Clear examples of neuropeptides that have pleiotropic functions are sNPFs, proctolin and DTKs. These peptides are produced by large numbers of diverse types of neurons and specific peptide functions may depend on the circuits where they are released. Thus,

for instance, DTKs act in presynaptic regulation of olfactory sensory neurons, in modulation of central complex neurons subserving locomotor control, in regulation of insulin signaling in renal tubules and brain, modulation of gut contractions, but also seem to have roles in the visual system and other brain regions (Birse et al., 2011; Ignell et al., 2009; Kahsai et al., 2010b; Nässel, 2002; Siviter et al., 2000; Söderberg et al., 2011; Winther et al., 2006; Winther et al., 2003). These distributed functions are likely to be uncorrelated (non-orchestrating) and probably DTKs are neuromediators that act at targets without a built-in functional message. In contrast, neuropeptides, such as individual DILPs, HugPKs, and NPF, might each serve in orchestrating functions. Hence, a DILP may signal to multiple targets to orchestrate a response to feeding and changed nutritional status, NPF could be a motivational or rewarding signal optimizing CNS circuits for a changed environmental situation and HugPK act as an interface between taste inputs and feeding.

Another role of neuropeptides, mostly neglected here, is as neuromodulators or cotransmitters in neurons. It is well known from crustaceans and mollusks that colocalized neuropeptides and classical neurotransmitters cooperate in neuronal circuits or at muscles to increase the plasticity of rhythm generating circuits (or networks) and muscle dynamics (Brezina and Weiss, 1997; Nusbaum and Blitz, 2012; Nusbaum et al., 2001). Possibly the multiple neuropeptides in the *Drosophila* antennal lobes and central complex act both as neuromodulators and cotransmitters; and indeed several cases of colocalized neuropeptides and GABA and acetylcholine have been detected in these and other brain regions (Carlsson et al., 2010; Ignell et al., 2009; Johard et al., 2009; Kahsai and Winther, 2011; Nässel et al., 2008).

It would be interesting to see how neuropeptides also contribute to circuit regulation in *Drosophila*, although the classical electrophysiological/pharmacological approach, used successfully in mollusks and crustaceans, may seem less feasible in flies. Probably the antennal lobe with its fairly well-described functional circuitry can serve for activity imaging in relation to peptide application or interference with peptide signaling (see Ignell et al., 2009; Root et al., 2011). Actually even the anatomical analysis of peptidergic circuitry in the CNS is still in its infancy for *Drosophila* and for most other insects. One way to go might be to combine mapping of functional circuits by genetic markers, like for example the courtship behavior circuits, with analysis of neuropeptide distribution. With the huge arsenal of GAL4 and UAS lines, mutants, and other molecular genetics tools available, we shall certainly see a rapid increase in knowledge in neuropeptide signaling in *Drosophila*. However, the complexity in signaling mechanisms in peptidergic and other neurotransmitter systems and the combinatorial possibilities of synaptic connections in the multiple circuits of the fly brain are likely to keep scientists busy for quite a while.

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Chapter 3

Vision

Nina Vogt and Claude Desplan

Introduction

When a fly moves through space in its environment, the animal processes information from optic flow to stay on course while in flight, or it fixates objects during its search for food. When startled, flies move towards the light. This chapter attempts to describe the anatomy of the *Drosophila* retina and the processing of visual information in the photoreceptors, or how a visual stimulus is transformed into a neuronal correlate. We also focus on a behavior dependent on all photoreceptors, the innate spectral preference.

Some of the properties of a visual stimulus that can be detected by the visual system of *Drosophila* are intensity, contrast, motion, wavelength, and polarization (Borst, 2009; Heisenberg and Wolf, 1984). The efficiency of a light stimulus depends on the sensitivity curves of the photoreceptors involved in the visual task. For *Drosophila*, the wavelengths of visible light range from around 250 nm to 650 nm, and thus from UV to orange (Hardie, 1985). Depending on the visual task, different types of photoreceptors might be involved, thus the effective range of light might vary.

Generally, light properties can be measured in two ways, radiometrically or photometrically. Radiometry deals with the physical properties of light, while photometry is concerned with the perception of light by the human eye. Especially when describing visual stimuli for color-related behaviors, it is important to consider these differences. A useful radiometric unit is irradiance, measuring power per area (in W/m^2). The corresponding photometric unit is illuminance (in lux). The term "intensity" is often used in an imprecise way, but is defined as a photometric unit measuring power per unit solid angle (in cd). Two stimuli of equal irradiance but different spectral composition may have very different illuminance values, due to the different sensitivity of the human eye to various wavelengths. For example, orange light of a certain irradiance will be perceived as less bright than a green light of the same irradiance. More importantly, two stimuli that appear equally bright to the human eye might not be perceived as isoluminant by *Drosophila*, due to the different sensitivities of *Drosophila* and human photoreceptors.

The light levels that *Drosophila* encounters vary considerably in the course of a day. For example, in bright sunlight, up to 1000 W/m^2 can be reached. These values decrease to around 100 W/m^2 on an overcast day and to 1 $\mu W/m^2$ in moonlight. *Drosophila* is mostly active during dusk and dawn and is therefore exposed to intermediate light levels. Still, the dynamic range of light detection by the fly visual system is extremely large.

Anatomy of the retina

Drosophila has several light-sensing organs with different sensitivities and functions. Non-image-forming structures such as the Bolwig's organ (the larval eye), the eyelet (located beneath the retina) and the ocelli are not considered here, as this discussion is focused on the adult compound eyes (Green et al., 1993; Helfrich-Förster et al., 2002; Hu et al., 1978). They allow the fly to see 85% of its environment, with only a narrow blind spot at the back of the fly head (Heisenberg and Wolf, 1984). There is minimal overlap between the eyes in the frontal visual field. This means that *Drosophila* cannot perceive depth the way humans do by triangulating the distance of an object using the different projections on both retinas (stereopsis).

The *Drosophila* compound eye consists of a hexagonal lattice of approximately 800 unit eyes or ommatidia (Hardie, 1985). Each ommatidium has its own small lens that is secreted by cone cells and focuses incoming light onto the photoreceptors (Fig. 3.1A). The ommatidia are optically isolated from each other by several layers of pigment cells (Johannsen, 1924).

Within each ommatidium, the light-sensitive structures, the rhabdomeres, of six photoreceptors (R1–R6), are organized in a trapezoidal fashion while the rhabdomeres of two photoreceptors (R7 and R8) are located in the center (Fig. 3.1B) (Wolken et al., 1957). The outer photoreceptors R1–R6 span the whole thickness of the ommatidium, while the inner photoreceptors R7 and R8 are arranged on top of each other, with R7 more distally located than R8. R1–R6 project to the lamina, the first optic neuropil within the optic lobe, while R7 and R8 do not arborize in the lamina, but instead continue to send their axons to the underlying medulla (Fischbach and Dittrich, 1989; Meinertzhagen and O'Neil, 1991).

Behavioral Genetics of the Fly (Drosophila melanogaster), ed. J. Dubnau. Published by Cambridge University Press.

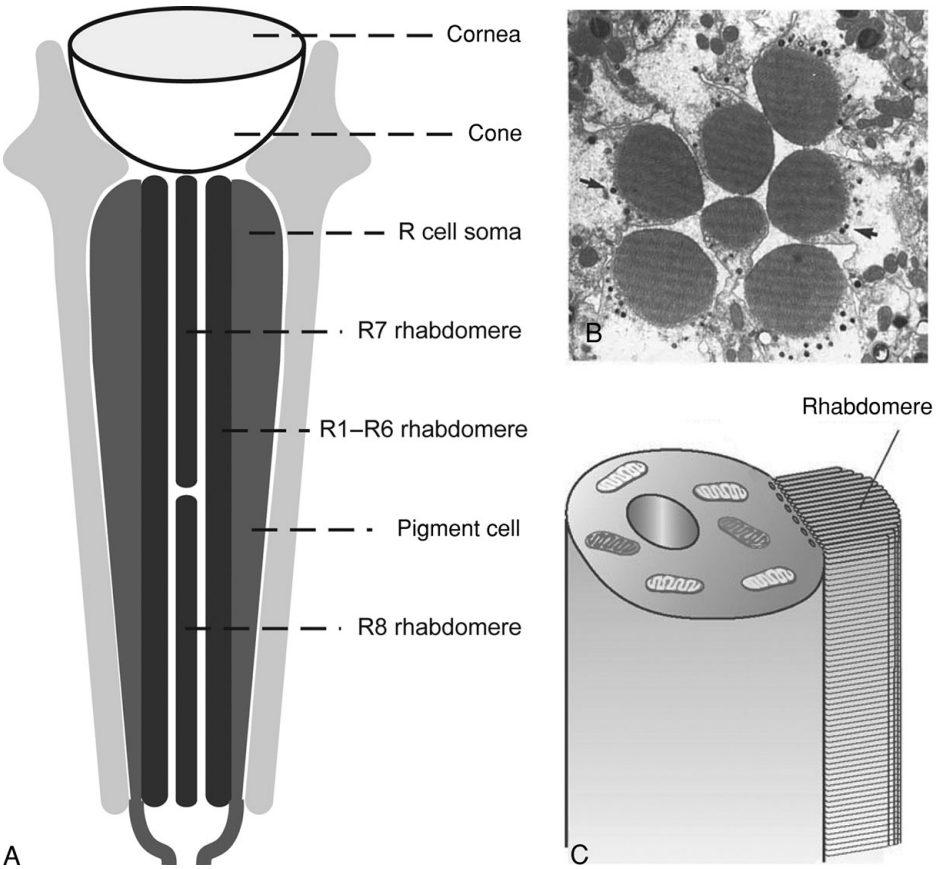
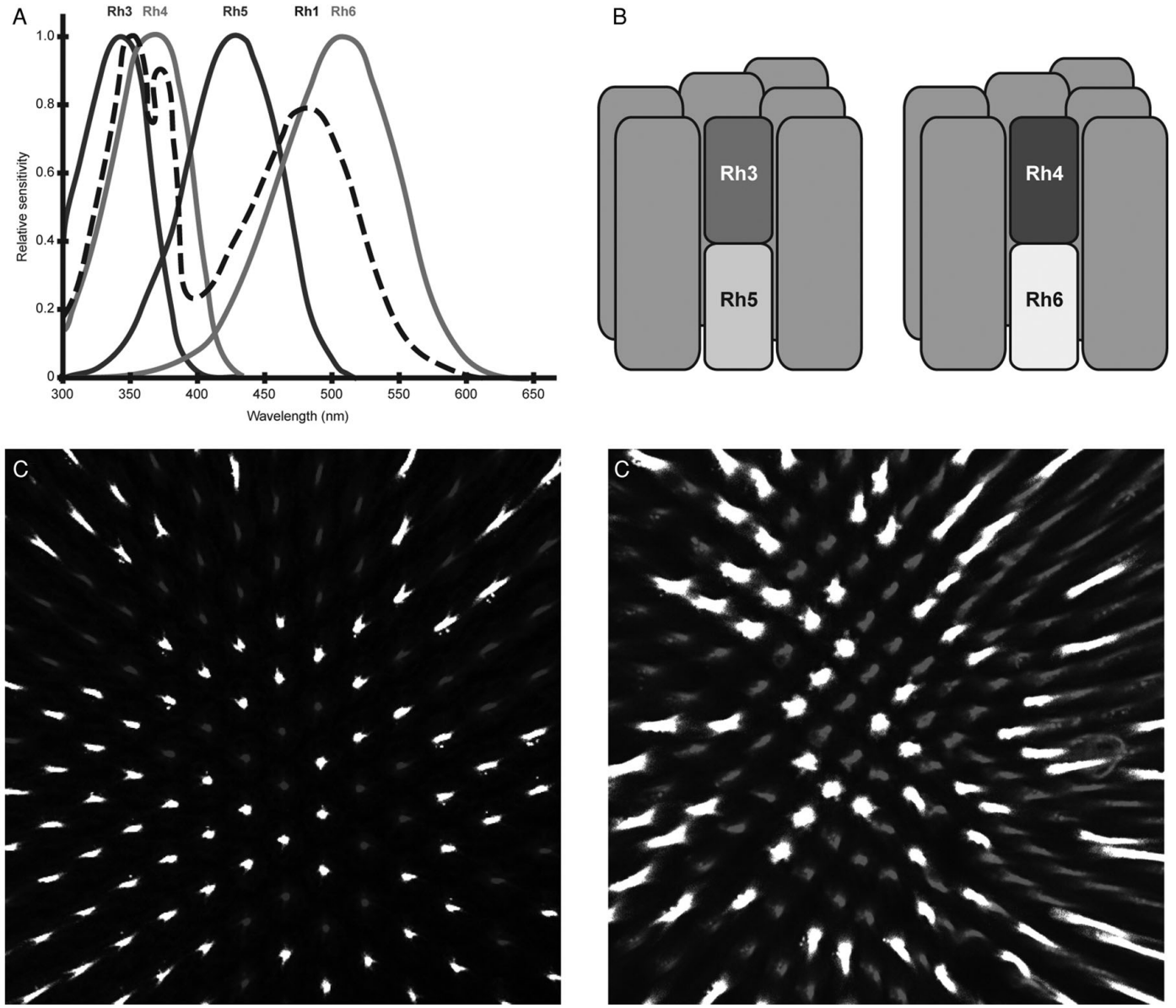


Fig. 3.1. Ommatidial and photoreceptor structure. A Schematic overview of an ommatidium. B Electron-microscopic cross-section through an ommatidium (from Leonard et al., 1992). C Cartoon of a photoreceptor (modified from Hardie and Raghu, 2001).

The phototransduction cascade occurs in rhabdomeres, a specialized apical compartment of the photoreceptor cell (Fig. 3.1C). In the rhabdomeres, the visual pigments are concentrated in tightly packed microvilli, which have a diameter of about 60 nm and are 1–2 µm long (Hardie and Raghu, 2001; Wolken et al., 1957). In contrast to vertebrate rods, where the phototransduction machinery is located in intracellular discs, *Drosophila* photosensation therefore takes place at the plasma membrane. Each of the approximately 30 000 microvilli acts as a semiautonomous compartment in phototransduction (Suzuki et al., 1993). Most insects have fused rhabdoms, where all photoreceptors within one ommatidium share the same light path. In *Drosophila* and other dipterans, the rhabdomeres for individual photoreceptors are separate, and the light paths for each of the photoreceptors R1–R6 within one ommatidium are different, whereas R7 and R8 are located on top of each other and point in the same direction in space (Wolken et al., 1957). Thus, light is first filtered in the more distal R7 before it reaches R8. Groups of six outer photoreceptors from neighboring ommatidia share a common, parallel optical axis with the two inner photoreceptors from the central ommatidium, and their axons are grouped together into a single retinotopic (visual sampling) unit or cartridge in the lamina. This organization is called neural superposition (Braitenberg, 1967; Kirschfeld, 1967; Kirschfeld, 1973). With this arrangement, information about light from a single point in space is transmitted to the lamina through six photoreceptor axons present in six different ommatidia, which increases sensitivity at low light intensities and reduces noise (Land, 1997; Scholes, 1969).

Properties of the visual system

The spatial resolution and sensitivity of the *Drosophila* eye is determined by the angular spacing of ommatidia, the optical quality of the lenses and the rhabdomere diameter (Land, 1997). Smaller lens size allows for a higher pixel density, but this comes at the cost of lower acuity as smaller lenses lead to increased blur due to diffraction. Also, less light can pass through smaller lenses, resulting in lower signal-to-noise ratios. Furthermore, pixel size is determined by the focal length of the lens and the rhabdomere diameter. In *Drosophila*, the lens diameter is 17 µm, while the interommatidial angle is 4.5°, which is larger than in *Calliphora* (1°) or *Musca* (2.5°) and is an adaptation to the smaller body size of *Drosophila* (Franceschini and Kirschfeld, 1971; Gonzalez-Bellido et al., 2011; Land, 1997). However, visual performance is determined by the acceptance angle of the photoreceptors, which is defined by both the interommatidial angle as well as the characteristics of the lens and the rhabdomere diameter. In *Drosophila*, this parameter has initially been estimated to be between 5 and 6°, but recent measurements suggest a value of 8.2° for R1–R6 (Buchner, 1976; Gonzalez-Bellido et al., 2011; Heisenberg and Wolf,



(Grayscale) Fig. 3.2. Rhodopsin diversity. A Spectral sensitivity of *Drosophila* rhodopsins (modified from Yamaguchi et al., 2010). B Ommatidial subtypes. 30% of ommatidia are of the *pale* subtype and express Rh3 and Rh5. The remaining 70% are of the *yellow* subtype and express Rh4 and Rh6. C Cross-section through the R7 layer, stained for Rh3 (in cyan) and Rh4 (in red). Courtesy of R. Johnston. D Cross-section through the R8 layer, stained for Rh5 (in blue) and Rh6 (in yellow). Courtesy of J. Rister.

1984). The acceptance angle for the inner photoreceptors R7 and R8 is probably smaller due to the smaller diameter of their rhabdomeres (R7: 1.75 μm vs. R1–R6: 2.34 μm,), making them less sensitive to light (Kirschfeld and Franceschini, 1968).

Another important feature of the *Drosophila* visual system is its temporal resolution. This property depends on the speed and noisiness of the phototransduction cascade in the photoreceptors. Initial measurements in *Drosophila hydei* resulted in flicker fusion rates of 60–100 Hz, while in recent measurements *Drosophila melanogaster* photoreceptors responded accurately to naturalistic stimuli only up to 43 Hz (Gonzalez-Bellido et al., 2011; Miall, 1978). This discrepancy may be due to different adaptation states or temperatures (Cosens and Spatz, 1978).

Photoreceptor diversity

The outer photoreceptors R1–R6 appear to be homogeneous in morphology and function. They express the broadband rhodopsin 1 (Rh1), which is encoded by *ninaE* and has two sensitivity peaks at 360 nm and 486 nm (Fig. 3.2A) (Feiler et al., 1988; O'Tousa et al., 1985; Zuker et al., 1985). The UV sensitivity is due to the presence of a second chromophore or accessory sensitizing pigment, which is presumably a vitamin

A derivative (Kirschfeld and Franceschini, 1977). Upon activation, rhodopsin (R form) is converted to metarhodopsin (M form) with an absorbance maximum at 566 nm (Ostroy et al., 1974). The M form is stable and only reverts back to the R form upon absorption of another photon. In *Drosophila*, the red pigmentation of the eye is permeable to long wavelengths, thus facilitating the conversion of the M form to the R form. R1–R6 are sensitive to a wide range of light intensities and have been shown to function in motion detection (Heisenberg and Buchner, 1977).

The rhodopsin expression profiles of R7 and R8 are more complex than those of R1–R6. The photoreceptors R7 and R8 express different rhodopsins, and therefore are ideal candidates for color processing. Two different types of ommatidia, *pale* and *yellow*, are distributed throughout most of the *Drosophila* retina in a stochastic fashion (Fig. 3.2B) (Bell et al., 2007; Franceschini et al., 1981; Kirschfeld et al., 1978). The two types of ommatidia can be distinguished by a technique called water immersion microscopy, and the expression of different rhodopsins (Rh) in R7 and R8 (Franceschini and Kirschfeld, 1971; Pichaud and Desplan, 2001; Rister and Desplan, 2011). Under water immersion, *pale* ommatidia do not autofluoresce while *yellow* ommatidia show yellowish autofluorescence. *Pale* ommatidia represent about 30% of the total population. They express Rh3 in R7 and Rh5 in R8 photoreceptors (Fig. 3.2C, D) (Chou et al., 1996; Chou et al., 1999; Mazzoni et al., 2008; Papatsenko et al., 1997). Rh3 is a UV-sensitive rhodopsin with absorption peaks at 331 nm for the R form and 468 nm for the M form (Feiler et al., 1992; Fryxell and Meyerowitz, 1987; Zuker et al., 1985). Conversely, the blue-sensitive Rh5 absorbs best at 442 nm (R) and 494 nm (M) (Salcedo et al., 1999). *Yellow* ommatidia comprise the remaining 70% of ommatidia (Chou et al., 1996; Chou et al., 1999; Papatsenko et al., 1997). These ommatidia harbor UV-sensitive Rh4 in R7 and green-sensitive Rh6 in R8 (Fig. 3.2C,D). The R form of Rh4 absorbs best at 355 nm, while the absorption peak of the M form lies at 470 nm (Feiler et al., 1992; Montell et al., 1987). Rh6 is unusual, as the R form absorbs most efficiently at longer wavelengths (peak at 515 nm) than its corresponding M form (468 nm) (Salcedo et al., 1999). Peak absorbances of the rhodopsins normally expressed in R7 and R8 have been measured in R1–R6 expressing those rhodopsins in mutants lacking Rh1 expression. *Yellow* R7 photoreceptors in the dorsal third of the retina co-express UV-sensitive Rh3 and Rh4 (Mazzoni et al., 2008). The behavioral significance of this phenomenon is unclear, but one hypothesis is that this co-expression could broaden the wavelength sensitivity for navigation in sunlight (Stavenga and Arikawa, 2008).

One to two rows of ommatidia at the dorsal margin (DRA) of the eye display a specialized architecture and are involved in polarization sensitivity (Fortini and Rubin, 1991; Labhart and Meyer, 1999; Wernet et al., 2003; Wernet et al., 2012). Their photoreceptors R7 and R8 have shorter rhabdomeres with enlarged diameter and both express Rh3 (Fortini and Rubin, 1990; Wada, 1974). The microvilli of R7 and R8 are organized orthogonal to each other. However, in the DRA, the rhabdomeres are not twisted in contrast to those in the rest of the eye, making them ideally suited to detect the orientation or e-vector of light polarization in the sky (Hardie, 1984; Labhart and Meyer, 1999; Wernet et al., 2012; Wunderer and Smola, 1982).

The phototransduction cascade

Phototransduction is the process of converting light energy into an electrical response in the photoreceptors (Hardie, 1985; Montell, 2012). In contrast to phototransduction in vertebrates, light exposure of invertebrate photoreceptors results in the opening rather than closure of cation channels and thus depolarization of the photoreceptor membrane. Na^+ and Ca^{2+} influx occurs within 20 ms after photoreceptor illumination (Ranganathan et al., 1991). The process involves a G protein-mediated amplification cascade starting with the G protein-coupled rhodopsin and its chromophore 3-hydroxy-retinal (Vogt and Kirschfeld, 1984). Due to amplification, the photoreceptors can respond to even a single photon with a change in membrane potential, generating quantum bumps that appear to correspond to the activity of the phototransduction cascade within a single microvillus in the rhabdomere (Hardie, 1985; Henderson et al., 2000; Wu and Pak, 1975). Individual quantum bumps are then added up to give the total response. Phototransduction has been studied in detail in photoreceptors R1–R6, but is probably similar in R7 and R8.

Upon light absorption, the chromophore 3-hydroxy-retinal associated with rhodopsin photoisomerizes from its *11-cis* to an *all-trans* form, resulting in metarhodopsin (Fig. 3.3). This activated form induces the dissociation of the heterotrimeric G protein complex (Scott et al., 1995). The $G_q\alpha$ subunit then activates phospholipase C (PLCβ, encoded by *norpA*) (Bloomquist et al., 1988). The substrate for PLC is phosphatidylinositol-4,5-bisphosphate (PIP_2), which is converted into the second messengers diacylglycerol (DAG) and inositol-1,4,5,triphosphate (IP_3). DAG remains membrane-bound while IP_3 is soluble. This hydrolysis leads to the opening of TRP channels in the microvillar membrane resulting in Ca^{2+} influx into the cytosol (Hardie and Minke, 1992; Montell and Rubin, 1989; Niemeyer et al., 1996; Phillips et al., 1992). TRP most likely forms homomultimeric channels, but also heteromultimerizes with TRP-like (TRPL) (Xu et al., 1997). A further TRP relative, TRPγ, is also expressed in photoreceptors and heteromultimerizes with TRPL (Xu et al., 2000). TRP-only channels are responsible for about half of the light induced current, while TRPγ-TRPL channels appear to carry the remainder of the current (Reuss et al., 1997).

The exact mechanism leading from the generation of second messengers to the opening of Ca^{2+} channels has long been elusive. Mutations in the IP_3 receptor have no effect on light-induced electrical responses of photoreceptors, precluding a direct function for IP_3 in the phototransduction cascade (Acharya et al., 1997; Raghu et al., 2000a). The second product of PIP_2 hydrolysis, DAG, is another candidate for gating the TRP channels. DAG activates protein kinase C (PKC), but

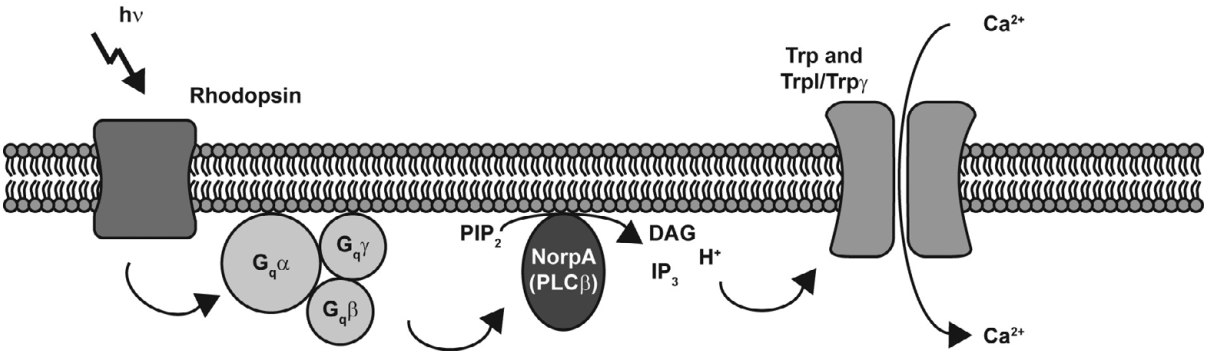


Fig. 3.3. Model of the phototransduction cascade.

a mutation of the eye-specific PKC gene *inaC* does not impair the generation of quantum bumps (Smith et al., 1991). However, PKC is required for response termination and adaptation (Hardie et al., 1993; Smith et al., 1991). DAG might also be converted to polyunsaturated fatty acids (PUFAs) by DAG lipase (*inaE*), generating other potential effector molecules. Indeed, PUFAs can activate TRP and TRPL channels, but exogenous DAG application (which would be a substrate for DAG lipase) does not appear to have a major effect on TRP channels, making it unlikely that DAG and PUFAs are major players in opening the TRP channels (Chyb et al., 1999; Estacion et al., 2001).

Another consequence of the generation of IP_3 and DAG is the depletion of their precursor PIP_2. In vertebrates, PIP_2 modifies the activity of ion channels of the Kir and TRP families (Chuang et al., 2001; Huang et al., 1998). Indeed, PIP_2 depletion has been implicated in TRP and TRPL channel activation in *Drosophila* phototransduction (Hardie et al., 2001). More importantly, the hydrolysis of PIP_2 not only generates the second messengers DAG and IP_3, but also leads to the release of a proton (Huang et al., 2010). This can be directly measured as a pH decrease in light-activated photoreceptors. Interestingly, PIP_2 depletion without acidification does not lead to significant TRP and TRPL channel opening, while a pH decrease in PIP_2 depleted cells results in major current flow through TRP and TRPL channels. Thus, the gating of TRP and TRPL channels in phototransduction might be mediated by both PIP_2 depletion and by concurrent acidification.

Response termination

The phototransduction cascade has to be quickly inactivated in order to allow a new cycle of activation and therefore to provide high temporal resolution. In fact, 100 ms after a light stimulus is terminated, the phototransduction cascade is completely deactivated (Ranganathan et al., 1991). This requires the efficient shutdown of each individual step in the cascade. The trigger of the phototransduction cascade, metarhodopsin, is disabled by binding to arrestin (Byk et al., 1993; Dolph et al., 1993). Two arrestins are expressed in *Drosophila* photoreceptors. Arrestin 2 is the major player, while arrestin 1 is much less abundant. Arrestin-bound metarhodopsin is converted back to rhodopsin by exposure to orange light, followed by the dissociation of rhodopsin and arrestin (Byk et al., 1993; Ostroy et al., 1974). If the photoreceptors are exposed to prolonged stimulation with short-wavelength light (such as blue light), the pool of available arrestin is depleted and activated metarhodopsin can continue to trigger the phototransduction cascade, resulting in a prolonged depolarizing afterpotential (PDA) (Cosens and Briscoe, 1972; Minke et al., 1975). Without exposure to long-wavelength light, photoreceptors can stay depolarized for several hours, even in the dark.

Inactivation of the next step of the phototransduction cascade requires the hydrolysis of G protein-bound GTP. The intrinsic GTPase activity of $G_q\alpha$ is too low for efficient response termination. However, the downstream effector PLC acts as a GTPase-activating protein, which conveniently ensures that PLC gets activated before $G_q\alpha$ is inactivated (Minke et al., 2000). PLC in turn is inhibited by high Ca^{2+} levels, which occur upon TRP channel opening, providing a feedback mechanism for response termination (Hardie et al., 2001). As described above, PLC generates two second messengers from PIP_2: IP_3 and DAG. While IP_3 does not appear to function in phototransduction, DAG action has to be efficiently terminated for proper response kinetics. This is achieved through DAG phosphorylation by DAG kinase, encoded by the gene *rdgA* (Masai et al., 1993; Raghu et al., 2000b).

Finally, Ca^{2+} influx through TRP and TRPL channels results in strongly elevated Ca^{2+} levels up to 200 μM, which result in inactivation of several components of the transduction cascade (Hardie, 1996; Oberwinkler and Stavenga, 2000; Postma et al., 1999). Foremost, the channels are rapidly inactivated in response to rising Ca^{2+} levels, possibly through phosphorylation by the DAG- and Ca^{2+}-responsive protein kinase C (*inaC*) (Hardie et al., 1993). Rising Ca^{2+} levels can also contribute more directly to response termination. TRPL harbors two calmodulin binding sites, which are involved in Ca^{2+}-dependent channel inactivation (Scott et al., 1997). TRP channels are blocked in a voltage-dependent manner by divalent ions such as Ca^{2+} (Hardie and Mojet, 1995).

Photoreceptor adaptation

The *Drosophila* visual system can respond to light intensities ranging over more than six orders of magnitude. To achieve this without response saturation, photoreceptors undergo light adaptation. Multiple mechanisms operate on different timescales. Ca^{2+}-dependent adaptation is fast (less than 1 min). Upon adaptation to high light levels, the quantum bumps generated by the absorption of single photons become smaller, due to elevated Ca^{2+} levels (Juusola and Hardie, 2001). Indeed, manipulating extracellular Ca^{2+} affects bump shape, confirming a major role for Ca^{2+} in fast adaptation (Henderson et al., 2000). In the fully adapted state, photoreceptors can resolve up to 3 x 10^5 photons per second (Juusola and Hardie, 2001). In addition, at elevated Ca^{2+} levels, pigment granules within the photoreceptors move close to the rhabdomere where they absorb light, thus providing a further level of adaption (Kirschfeld and Vogt, 1980).

Long-term adaptation involves several mechanisms. Prolonged exposure to light results in translocation of arrestin 2 molecules from the cell body into the rhabdomere, a process that is dependent on the concentration of metarhodopsin, and thus the level of photoreceptor activation (Lee et al., 2003; Satoh et al., 2010). Thus, more arrestin 2 is available to inactivate metarhodopsin. Furthermore, TRPL can be translocated from the rhabdomere membrane into an intracellular compartment in the cell body, thereby reducing sensitivity (Bähner et al., 2002). Finally, $G_q\alpha$ can also translocate away from the rhabdomere membrane into the cytosol in order to adapt to high light levels (Frechter et al., 2007). In general, long-term adaptation reduces the efficiency and thus the frequency of bump generation.

Photoreceptor output

Photoreceptors encode light intensity with graded potentials and do not generate action potentials. Their depolarization leads to the graded release of the neurotransmitter histamine from the photoreceptor terminals (Buchner, 1991; Hardie, 1987; Pollack and Hofbauer, 1991; Sarthy, 1991). Two histamine-gated ion channels are expressed in the *Drosophila* visual system, Ort/HclA and HclB (Gengs et al., 2002; Gisselmann et al., 2002; Pantazis et al., 2008; Witte et al., 2002; Zheng et al., 2002). Both are chloride channels. HclB is restricted to glia and helps shape the response of the postsynaptic targets of R1–R6 in the lamina, the lamina monopolar cells (Pantazis et al., 2008). Ort/HclA is required for postsynaptic responses in both the lamina (downstream of R1–R6) and the medulla (downstream of R7 and R8) (Gao et al., 2008; Gengs et al., 2002; Pantazis et al., 2008). Mutations in the *ort* gene lead to defects in motion detection, a behavior that is dependent on the photoreceptors R1–R6, and also to impaired spectral preference behavior, which can be driven by all photoreceptors (Gao et al., 2008; Heisenberg and Buchner, 1977; Rister et al., 2007; Yamaguchi et al., 2010). Upon light exposure, photoreceptors depolarize and release histamine that leads to hyperpolarization of lamina monopolar cells (in the case of outer photoreceptor activation) due to chloride influx, resulting in a sign inversion at the first synapse in the visual system (Stuart, 1999). Therefore, although *Drosophila* photoreceptors depolarize in response to light while vertebrate photoreceptors hyperpolarize, the net output is basically the same.

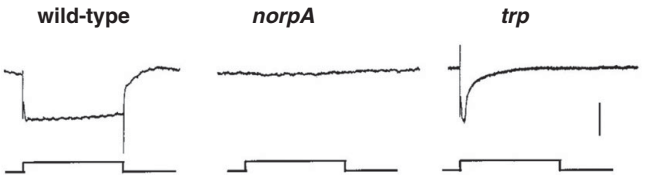


Fig. 3.4. Electroretinogram of wild-type, *norpA* and *trp* mutant flies (from Montell, 1999). Flies were dark-adapted and then subjected to a 5 s pulse of white light as indicated below the recordings. Scale bar represents 5 mV.

After release, histamine has to be rapidly removed from the synaptic cleft. This is most likely achieved by finger-like protrusions of glial cells (capitates projections) that reach the photoreceptor terminals (Stark and Carlson, 1986). The glial cells rapidly take up histamine and convert it into carcinine through the action of the *ebony* gene product (Borycz et al., 2002; Richardt et al., 2003). The inactive carcinine is released back into the synaptic cleft and can then be taken up by photoreceptors through the neurotransmitter transporter inebriated (Gavin et al., 2007). The enzyme encoded by *tan* reconverts carcinine into histamine (Borycz et al., 2002; True et al., 2005).

Electroretinogram

Measuring an electroretinogram (ERG) has been a successful method to isolate and characterize mutants in the visual system of *Drosophila* (Heisenberg, 1971; Mehta et al., 2005; Pak et al., 1970). It is a robust and simple technique, which is especially useful for high-throughput applications. To measure ERGs, an electrode is placed onto, or slightly beneath, the cornea and compared to a reference electrode inserted into the abdomen of the fly. Usually, measurements are performed on white-eyed flies because the lack of optical isolation between ommatidia allows for a more uniform illumination of photoreceptors in the retina. The absence of the red pigment in these flies does not change the ERG properties (Hengstenberg and Götz, 1967).

The ERG measures the electrical responses of photoreceptors and cells in the lamina. The photoreceptor contribution is dominated by photoreceptors R1–R6, due to their number and size. The characteristic waveform of the ERG consists of several components (Fig. 3.4). The photoreceptors contribute a negative tonic potential, also called the receptor potential (Heisenberg, 1971). At the onset of a light stimulus, lamina cells respond with a positive phasic "ON" response, while a negative phasic "OFF" response is detected upon cessation of the light stimulus.

Mutations in different parts of the visual transduction pathway can have very different effects on the ERG. For example, *ninaE* mutants (*rh1*) are characterized by the absence or strong reduction of the ERG response upon light exposure (Johnson

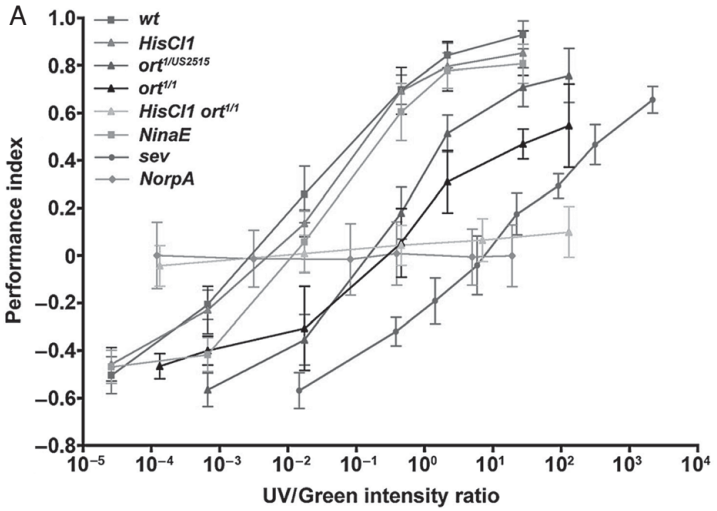


Fig. 3.5. Spectral preference behavior of mutants affecting the visual system of *Drosophila* (modified from Gao et al., 2008). Flies were given a choice between UV light at various intensities and a constant green light. A Intensity-response curves. The response is calculated as a performance index (number of flies in UV minus number of flies in green, divided by the total number of flies). B Attractiveness of UV over green light (defined as the UV/green ratio where the performance index is zero).

and Pak, 1986). In *trp* mutants, the receptor potential decays during prolonged light exposure, while the ON transient generated by the lamina is normal and the OFF transient is absent (Lo and Pak, 1981; Montell et al., 1985). In contrast, mutations in the histamine receptor *ort* have normal receptor potentials, but the ON and OFF transients are absent (Gengs et al., 2002).

By varying the conditions of the light stimulus, the contributions of different cells to the ERG can be isolated. The lamina potential can be extracted by either using low light intensities or by sinusoidally modulating the stimulus at high frequencies (Heisenberg, 1971). Under these conditions, the ERG has been shown to consist predominantly of the lamina potential (Heisenberg, 1971). This is due to the properties of lamina neurons which can strongly amplify small changes in the receptor potential (Autrum et al., 1970). In order to isolate the response of the photoreceptors R7 and R8, R1–R6 have to be saturated by prolonged exposure to blue light, thus inducing a prolonged depolarizing afterpotential (PDA) (Minke et al., 1975). In this case, further light stimulation results in the appearance of an additional receptor potential on top of the afterpotential. This potential lacks the ON and OFF transients as expected for a response generated by the inner photoreceptors, which bypass the lamina. Alternatively, the ERG can be recorded in *ninaE* mutants, however R1–R6 degenerate in the absence of Rh1, which might also impair R7 and R8 function (Leonard et al., 1992).

Spectral preference behavior

Phototaxis, the movement towards a light source, is an innate behavior involving all photoreceptors (Fischbach, 1979; Heisenberg and Buchner, 1977; Yamaguchi et al., 2010). This behavior can be easily studied in the lab using a T-shaped maze or similar devices. Often, "fast" phototaxis is distinguished from "slow" phototaxis, the former being considered an escape response of startled flies, the latter a choice behavior of undisturbed flies (Benzer, 1967; Heisenberg and Götz, 1975; Rockwell and Seiger, 1973). Phototaxis experiments can be carried out either as a choice between light and dark sides or as "differential" phototaxis or spectral preference behavior, in which the flies choose between two different light sources (Bertholf, 1932; Schümperli, 1973). In contrast to true color vision, which is independent of intensity, spectral preference behavior is strongly dependent on the intensity of the visual stimuli. Genetic screens for mutants with defects in phototactic behavior have identified a number of genes involved in phototransduction, retinal degeneration, photoreceptor development or targeting (Benzer, 1967; Hotta and Benzer, 1969; Lee et al., 2001; Pak et al., 1969).

The phototactic response spectrum was determined to be in the range from 250 nm to 650 nm as early as 1932, coinciding well with the sensitivity spectrum of all photoreceptors (Bertholf, 1932). However, UV light is much more efficient in eliciting a phototactic response compared to light of longer wavelengths (Fig. 3.5) (Gao et al., 2008; Schümperli, 1973; Yamaguchi et al., 2010). For example, the phototaxis threshold for UV light is around 10^{-7} W/m^2 for light-adapted flies, while the threshold for green light is ten times higher (Gao et al., 2008).

Interestingly, pure UV light is more attractive to *Drosophila* than a mixture of the same UV light and additional green light (Fischbach, 1979; Heisenberg and Buchner, 1977). Furthermore, the attractiveness of green light decreases when higher intensities are used (Gao et al., 2008). This suggests that photoreceptor input is not simply added up, but non-linear interactions between different photoreceptors or downstream processing pathways may exist.

In order to understand the contribution of the different photoreceptor subtypes to spectral preference behavior, mutants affecting the function or development of these subtypes have been analyzed (Fischbach, 1979; Heisenberg and Buchner, 1977; Hu and Stark, 1977; Yamaguchi et al., 2010). Initially, in the absence of specific rhodopsin mutations, photoreceptors were manipulated using *sev*, *rdgB* and *ora* mutations (Fischbach,

1979; Heisenberg and Buchner, 1977; Hu and Stark, 1977). *sev* mutants lack the UV-sensitive R7 photoreceptors and mainly express Rh6 in R8 (Chou et al., 1999; Harris et al., 1976), while *ora* mutants are actually *ninaE, ort* double mutants and therefore defective in both Rh1 as well as the histamine receptor Ort (Gengs et al., 2002; O'Tousa et al., 1989; Zheng et al., 2002). *rdgB* mutations lead mostly to the degeneration of R1–R6 (Harris and Stark, 1977). Fast phototaxis appears to be mainly mediated by the inner photoreceptors R7 and R8, as UV preference in a UV vs. green choice experiment is abolished in *sev* mutants, while *rdgB* mutants are not impaired (Hu and Stark, 1977). On the other hand, all photoreceptor types are required for slow phototaxis, as both *sev* and *ora* mutants show a strong reduction in UV attraction when given a choice between UV and green lights (Fischbach, 1979).

More recently, the contribution of different photoreceptor subtypes to differential phototaxis has been analyzed in even more detail (Yamaguchi et al., 2010). Two sets of experiments were conducted: a UV vs. blue choice and a blue vs. green choice. Manipulations in photoreceptors R1–R6, R7, *pale* R8 or *yellow* R8 each resulted in impairments in at least one of the two choice experiments, confirming that all photoreceptors are necessary for proper phototaxis behavior. On the other hand, all photoreceptor subsystems (R1–R6, R7 and R8) are sufficient for this behavior as they can independently mediate light attraction.

Conclusions

As described above, the anatomy of the retina and the cell biology of photoreceptors are understood in great detail. Furthermore, much progress has been made in understanding innate behaviors such as motion detection and phototaxis. The next challenges in the field of *Drosophila* vision will be to decipher the complete neural networks downstream of the photoreceptors and to understand more complex behaviors such as true color vision. While the lamina as the first target of R1–R6 has been studied extensively, both on the anatomical and functional level, comparatively little is known about the connectivity and function of medulla neurons (Gao et al., 2008; Meinertzhagen and O'Neil, 1991; Meinertzhagen and Sorra, 2001; Rister et al., 2007; Takemura et al., 2008). Furthermore, initial attempts to study true color vision in *Drosophila* have been made, but little progress has been achieved since then (Hernandez de Salomon and Spatz, 1983; Menne and Spatz, 1977; Tang and Guo, 2001). With recent genetic and technical advances, these challenges are now being addressed.

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Chapter 4

Sensory coding of olfaction and taste

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Introduction

Insect chemosensory systems are tasked with the challenge of detecting and discriminating thousands of chemicals in the environment. Chemical stimulus quality and intensity impart key information to drive essential behaviors including location and selection of food, mates, and oviposition sites. The olfactory and gustatory systems harbor the capacity to encode properties of chemical stimuli by way of large, highly divergent chemoreceptor gene families. The identification of *Drosophila* chemoreceptor genes hailed a new era of molecular and neurophysiological research in this model organism. The past decade or so has been witness to remarkable progress in our understanding of the principles by which odorants are encoded by the olfactory system: the manner in which olfactory sensory neurons (OSNs) are molecularly and functionally organized, and the anatomical and physiological mechanisms governing the transmission of their activity to higher brain centers. The same period has seen key progress in elucidating peripheral taste coding mechanisms, although much remains to be discovered about higher-order processing of taste information.

Here we review the organization and function of peripheral chemosensory neurons in the fly, and summarize the current understanding of chemosensory processing in the central nervous system. We include a synopsis of recent advances that have brought new perspectives to the idea of plasticity in olfactory and gustatory circuits, pointing to sophisticated mechanisms by which chemosensory input can be modified to reflect the context in which a chemical stimulus is perceived. We conclude with some intriguing examples of functional interactions between olfactory and gustatory systems, a new area of study that has the potential to uncover principles of convergence in the fly central nervous system. The field is now poised to unravel the mechanisms by which responses of olfactory and gustatory systems are integrated to result in specific behavioral routines, and how the changing needs of the animal are encoded via modulation of sensory input.

Chemosensory neurons and receptors

The peripheral olfactory system

Olfactory sensory neurons (OSNs) are housed in stereotypical combinations in porous cuticular structures called sensilla that cover the surfaces of the third antennal segments and the maxillary palps on the *Drosophila* head (Stocker, 1994). Antennal sensilla are sub-divided into three morphological types – basiconics, coeloconics, and trichoids – that are distributed in distinct, overlapping zones (Venkatesh and Naresh Singh, 1984). Only basiconic sensilla are present on the maxillary palps (Naresh Singh and Nayak, 1985). OSNs are also located in other sub-structures of the antennae – the three-chambered sacculus compartment, and a bristle-like projection called the arista (Stocker, 1994).

Olfactory sensilla, which can house up to four OSNs, are further sub-divided into 23 functional classes – 12 antennal basiconics (ab1–ab12), 4 coeloconics (ac1–ac4), 4 trichoids (at1–at4), and 3 maxillary palp basiconics (pb1–pb3) – based on their unique response profiles to large panels of volatile odorants and their molecular identities (Table 4.1). At least three different features of odorant responses can be used to distinguish individual OSNs: the level of spontaneous activity, the excitatory or inhibitory response to individual odorants, and the temporal dynamics of the response. Although not absolute, there appears to be some degree of functional specialization among the three morphological types – basiconic OSNs are tuned to general fruit and plant volatiles, trichoid OSNs to pheromones, and coeloconic OSNs to volatile products of microbial degradation and fermentation. Furthermore, OSNs are grouped in an invariant manner within sensilla. A recent study describing inhibitory effects between neurons within the same sensillum (Su et al., 2012) suggests that the stereotypical grouping of OSNs carries functional significance.

The functional identity of each OSN is determined by the membrane-bound receptor(s) it expresses (Dobritsa et al., 2003). Those mapped to OSNs belong to one of three large

Behavioral Genetics of the Fly (Drosophila melanogaster), ed. J. Dubnau. Published by Cambridge University Press.

Table 4.1. Organization of the *Drosophila* olfactory system

Neuron	Receptor(s)	Glomerulus	PN Class	Strongest ligand(s)	Behavior valence
Antennal large basiconic sensilla					
ab1A	Or42b	DM1	l	propyl acetate	attractive
ab1B	Or92a	VA2	ad	2,3-butanedione	attractive
ab1C	Gr21a/Gr63a	V		carbon dioxide	aversive
ab1D	Or10a	DL1	ad	methyl salicylate	aversive
ab2A	Or59b	DM4		methyl acetate	attractive
ab2B	Or85a	DM5	l	ethyl-3-hydroxybutyrate	attractive
ab3A	Or22a/b	DM2	l	ethyl hexanoate	attractive
ab3B	Or85b	VM5d		2-heptanone	
Antennal small basiconic sensilla					
ab4A	Or7a	DL5	ad	E2-hexenal	aversive
ab4B	Or56d				
ab5A	Or82a	VA6	ad	geranyl acetate	
ab5B	Or47a	DM3	ad	pentyl acetate	
ab6A	Or98b	VM5d			
ab6B	Or49b	VA5		2-methylphenol	
ab7A	Or98a	VM5v		ethyl benzoate	
ab7B	Or67c	VC4		ethyl lactate	
ab8A	Or43b	VM2	ad	ethyl butyrate	
ab8B	Or9a	VM3	ad	2-pentanol	
ab9A	Or69aA/B	D	ad		aversive
ab9B	Or67b	VA3	ad	acetophenone	
ab10A	Or49a/Or85f	DL4			aversive at high conc.
ab10B	Or67a	DM6	ad	ethyl benzoate	
ab11A				citronellal	
ab11B					
ab12A				citronellal	
ab12B				benzaldehyde	
Antennal coeloconic sensilla					
ac1A	Ir31a/Ir8a	VL2p		2-oxopentanoic acid	
ac1B	Ir75d/Ir25a	VL1		pyrrolidine	
ac1C	Ir92a/Ir76b	VM1		ammonia	
ac2A	Ir75a/Ir8a	DP1l		Propionic acid, acetic acid	
ac2B	Ir75d/Ir25a	VL1		pyrrolidine	
ac2C	Ir76b				
ac3A	Ir75a/b/c/Ir8a	DL2?		butyric acid	
ac3B	Or35a/Ir76b	VC3			
ac4A	Ir76a/b/Ir25a	VM4	ad	phenylethylamine	
ac4B	Ir75d/Ir25a	VL1		pyrrolidine	
ac4C	Ir84a/Ir8a	VL2a		phenylacetaldehyde	
Antennal trichoid sensilla					
at1A	Or67d	DA1	l,v	11-*cis*-vaccenyl acetate	
at2A	Or23a	DA3	ad		
at2B	Or83c	DC3	ad		aversive at high conc.

Table 4.1. (*cont.*)

Neuron	Receptor(s)	Glomerulus	PN Class	Strongest ligand(s)	Behavior valence
at3A	Or2a	DA4m			
at3B	Or19a/b	DC1	ad	1-octen-3-ol	
at3C	Or43a	DA4l		1-hexanol	
at4A	Or47b	VA1lm	ad,v	male extract	
at4B	Or65a/b/c	DL3	l		
at4C	Or88a	VA1d	ad	male and female extract	
sacculus I					
	Ir25a				
	Ir40a	VP1?			
	Ir93a	VP1?			
sacculus II					
	Ir25a				
	Ir40a	VP1?			
	Ir93a	VP1?			
sacculus III					
	Ir8a				
	Ir21a?				
	Ir64a	DP1m	ad	strong acids	
arista					
	Ir21a	VP3?			
	Ir25a				
Palp basiconic sensilla					
pb1A	Or42a	VM7	ad	propyl acetate	
pb1B	Or71a	VC2	l	4-methylphenol	
pb2A	Or33c/Or85e	VC1		(-) fenchone	
pb2B	Or46a	VA7l	ad	4-methylphenol	
pb3A	Or59c	l	ad		
pb3B	Or85d	VA4			

families of chemoreceptor genes: *Odor receptor* (*Or*), *Ionotropic receptor* (*Ir*), or *Gustatory receptor* (*Gr*) genes.

Odor receptors

Or genes belong to an insect-specific superfamily that encodes proteins unrelated in sequence or membrane topology to olfactory receptors in other organisms, and were discovered independently by bioinformatic and differential expression screens (Clyne et al., 1999, Vosshall et al., 1999, Gao and Chess, 1999). OSNs of basiconic and trichoid sensilla typically express a single *Or* gene along with an obligate co-receptor, *Or83b* or *Orco*, which is required for proper dendritic localization and function of the odor receptor protein (Larsson et al., 2004, Vosshall et al., 1999, Vosshall et al., 2000, Neuhaus et al., 2005). Epitope-tagging studies of Or and Orco proteins revealed a membrane topology with an intracellular N-terminal domain, which is inverted to that of canonical G-protein coupled receptors (Benton et al., 2006, Wistrand et al., 2006, Lundin et al., 2007). In keeping with the structural dissimilarity, Or/Orco complexes were found to be novel ligand-gated ionotropic receptors capable of rapid signal transduction in the absence of G-protein second messenger signaling when expressed in human cell lines and *Xenopus* oocytes (Sato et al., 2008, Wicher et al., 2008). However, one study reported an additional, slower metabotropic response that is also ligand dependent (Wicher et al., 2008). Disruption of key genes involved in metabotropic signaling pathways reduced odor sensitivity, but did not abolish it completely, consistent with some, although not exclusive, role for G proteins (Smart et al., 2008, Kain et al., 2008, Chatterjee et al., 2009). Detailed studies concerning the topology and signaling of Or/Orco complexes in endogenous neurons are now needed to fully understand the mechanistic properties of this receptor complex.

In each OSN, the specific or "tuning" Or is the determinant of its odor coding features. Expression of any Or in an "empty" basiconic OSN lacking its endogenous tuning receptors, but

retaining Orco, resulted in the host neuron adopting the odorant response properties of the OSN that the exogenous Or was derived from (Dobritsa et al., 2003). This so-called "empty neuron" strategy became instrumental in decoding individual tuning Ors (Hallem et al., 2004, Hallem and Carlson, 2006), which in concert with comprehensive molecular and transgenic expression analyses (Couto et al., 2005, Fishilevich and Vosshall, 2005) led to a detailed Or-to-OSN-to-function map of the peripheral olfactory system. The molecular organization of fly OSNs revealed remarkable parallels to olfactory systems of vertebrate animals, in which an individual OSN selects only one from among ~1000 odorant receptor genes to express (Vassar et al., 1993).

The interaction of an odorant with a select Or/Orco receptor, and thereby its corresponding OSN, is typified by a response of a characteristic type (excitatory or inhibitory), strength, and temporal decay. Or/Orco receptors are unique not only with respect to which odorants they respond to but also in their breadth of tuning. Some Or receptors such as Or35a are broadly tuned and respond to several structurally diverse odorants; by comparison, others such as Or85a are far more selective in their responses (Hallem and Carlson, 2006). Similarly, odorants themselves vary in the number and degree to which they activate various receptors. For example, 1-hexanol activates a number of different Ors from several different sensillar classes across both olfactory organs (Hallem and Carlson, 2006); by contrast only *Or67d*- and *Or65a*-expressing OSNs in trichoid sensilla are activated by the sex pheromone 11-*cis*-vaccenyl acetate (cVA) (Ha and Smith, 2006, van der Goes van Naters and Carlson, 2007). The identity and intensity for most general odors is thus largely represented at the periphery via differential activity across ensembles of OSNs.

Ionotropic receptors

Neurons that express *Or*/*Orco* genes accounted for ~70% of the OSNs in the antennae, positing that the remaining OSNs, mainly housed in coeloconic sensilla, are likely to express other classes of receptors (Couto et al., 2005, Yao and Carlson, 2010, Yao et al., 2005). The recent identification of variant ionotropic glutamate receptor genes (*Irs*) that represent an ancient family shared throughout protostomes, revealed exclusive expression of *Irs* in all but one of the coeloconic OSNs (Benton et al., 2009), ac3B, which expresses *Or35a*/*Orco* in addition to *Ir76b*. Reporter analysis suggests that *Ir* genes are also expressed in olfactory neurons of the sacculus and the arista (Benton et al., 2009, Ai et al., 2010, Silbering et al., 2011). Two members of the *Ir* gene family, *Ir8a* and *Ir25a,* are broadly expressed in multiple OSN classes and are thought to function as co-receptors. Similar to *Orco,* co-expression of *Ir8a* or *Ir25a* with ligand specific *Ir(*s) is required for proper shuttling to the dendritic membrane and function of Ir complexes (Abuin et al., 2011).

Unlike the general one-receptor-per-neuron rule for *Or* genes, OSNs express combinations of up to 4 *Ir*s in addition to either *Ir8a* or *Ir25a* (Abuin et al., 2011). Co-expression of *Ir8a* and *Ir84a* was sufficient to generate a response to phenalacetaldehyde in *Xenopus* oocytes, suggesting a tuning subunit/co-receptor complex reminiscent of Or/Orco receptors (Abuin et al., 2011). In the *in vivo* "empty neuron" system, however, at least three different Irs, *Ir25a, Ir76a,* and *Ir76b*, were required to reconstitute the phenylethyl amine response of the *Ir*-expressing coeloconic OSN of their origin (Abuin et al., 2011). Although other combinations of Irs have not been matched with ligands in this manner, systematic analysis of coeloconic responses to a variety of odorants revealed that several *Ir*-expressing OSNs are more narrowly tuned than their *Or*-expressing counterparts, with $Ir8a^{+}$ OSNs responding to a variety of acids and $Ir25^{+}$ OSNs responding to amines (Silbering et al., 2011).

Canonical ionotropic glutamate receptors (iGluRs) in mammalian nervous systems are ion channels gated by the neurotransmitter glutamate, which is recognized by an extracellular ligand-binding domain (Mayer, 2006, Sobolevsky et al., 2009). This ligand- binding domain is conserved in many classes of iGluRs described thus far: AMPA, kainite, and NMDA receptors. The divergent *Ir* family found in *Drosophila* functions instead to detect odorants and bears significant differences in the ligand-binding region (Mayer, 2006, Benton et al., 2009), leading to the model that Irs also serve as ion channels, gated by various odorants instead of glutamate.

Further investigations are required to understand the exact mechanisms of heteromeric Ir complex function, including the role of individual Irs within such complexes and their means of ligand-activated signal transduction.

Gustatory receptors

A family of 60 *Gr* genes encoding 68 divergent receptor proteins was identified soon after the *Or* gene family (Clyne et al., 2000, Scott et al., 2001). Although *Gr* genes are primarily expressed in taste neurons and are discussed in more detail below, two prominent members that are highly conserved between flies and mosquitoes, Gr21a and Gr63a, were mapped to a single basiconic OSN that is tuned to carbon dioxide (Jones et al., 2007, Kwon et al., 2007). Co-expression of the two receptors in the empty neuron system conferred a response to CO_2, providing evidence for a heteromeric Gr receptor (Jones et al., 2007, Kwon et al., 2007). The strength of the CO_2 response was significantly enhanced by the inclusion of the Gq protein (Yao and Carlson, 2010), suggesting a role for second messenger signaling mechanisms in Gr function. Correspondingly, a knockdown of Gq affects the level of CO_2 response, but not general odorant responses of other OSNs (Yao and Carlson, 2010). Gr21a/Gr63a remains the sole illustration of Gr function in OSNs; at least one other *Gr*, *Gr10a*, has been mapped to an OSN (Scott et al., 2001, Fishilevich and Vosshall, 2005), but its functional relevance is not yet clear.

Odorant binding proteins

Insect odorant-binding proteins (OBPs) are a large, conserved family of proteins, many of which are concentrated in the sensillar lymph of olfactory and gustatory sensilla and are thought to

facilitate interactions of odorants with membrane-bound olfactory receptors (Pelosi and Maida, 1995). In *Drosophila* there are as many as 51 predicted members of the OBP family, several of which are expressed in specific regions of the antenna (Hekmat-Scafe et al., 2002, McKenna et al., 1994, Galindo and Smith, 2001, Pikielny et al., 1994). The protein LUSH is expressed in trichoid sensilla and evidence suggests that it binds the male aggression and anti-aphrodisiac pheromone cVA (Kim et al., 1998, Shanbhag et al., 2001a, Xu et al., 2005). LUSH was shown to be required for odor-evoked responses in trichoid sensilla and behavioral responses to cVA (Xu et al., 2005). One current model for *Drosophila* pheromone detection invokes cVA-bound LUSH protein, which is conformationally different from free LUSH, as the ligand that activates Or67d/Orco complexes (Laughlin 2008). A recent study, however, challenges this model directly and instead proposes a more supportive rather than direct role for the OBP LUSH in pheromone detection (Gomez-Diaz et al., 2013).

The roles for many of the additional OBPs in olfactory coding are largely uncharacterized. Some evidence suggests that a family of 12–14 sensory neuron membrane proteins (SNMPs) may also be involved in odor detection (Benton et al., 2007, Rothenfluh et al., 2006). One member, encoded by *Snmp*, is necessary for activation of Or67d/Orco by cVA (Benton et al., 2007, Gomez-Diaz et al., 2013). However, the precise role of Snmp, as well as other members of this family, is not yet known.

The peripheral taste system

Taste neurons in *Drosophila* are instead distributed in sensilla on the surface of the labellum, distal segments of the legs (tarsi), anterior wing margins and the ovipositor (Stocker, 1994). Although the general organization of taste sensilla is similar to that of the olfactory trichoids, they are distinguished by two defining features: a single pore at the tip of the sensillar shaft, and the presence of a mechanosensory neuron in each sensillum. Taste neurons or gustatory receptor neurons (GRNs) are also found paired with mechanosensory neurons in smaller papillae, which dot the oral surface of the labellum (taste pegs) and internal pharyngeal organs.

The positional organization of external taste sensilla displays a remarkable stereotypy as compared to the olfactory system. On the labellum, which is the best-characterized taste organ thus far, each half is decorated by ~30 sensilla in overlapping rows running along the anterior-posterior axis. The morphology and location of each labellar sensillum allows its unambiguous identification as a unique member of one of three sensillar types (Shanbhag et al., 2001b, Hiroi et al., 2002), a feature that has greatly aided functional analysis of individually identified taste neurons (Table 4.2). Although comparable analyses have not yet been performed for taste sensilla in other organs, anatomical studies indicate that such stereotypy exists for those as well.

Taste neurons appear to be dedicated for compounds of distinct taste categories associated with stereotyped acceptance or rejection behaviors, revealing an organizational logic for the fly taste system that is similar to the one found in mammals (Yarmolinsky et al., 2009). Chemicals of a single taste category typically excite distinct GRNs in labellar sensilla, defining them as sweet-, salt-, water-, or bitter-sensing neurons (Fujishiro et al., 1984, Meunier et al., 2003). The former three are present in all of the ~20 labellar taste bristles containing four gustatory neurons, although there are some quantitative differences in sugar responses across different sensilla (Hiroi et al., 2002). On the other hand, the tuning properties of the "fourth" neuron were found to be more heterogeneous. In the small S-type medial sensilla and intermediate I-type lateral sensilla of the labellum, this neuron responds to alkaloids and other classes of bitter compounds (Weiss et al., 2011). By contrast, strong activators have not been found for this neuron's counterpart in L-type sensilla (Weiss et al., 2011), although it has also been implicated as a detector of noxious compounds by virtue of its activation by high concentrations of salt (Fujishiro et al., 1984), which are behaviorally aversive (Tompkins et al., 1979). Recently, a subset of the bitter-sensing neurons was also shown to be responsive to low pH or "sour" stimuli (Charlu et al., 2013), providing further evidence of functional heterogeneity within deterrent neurons. Emerging evidence suggests that some degree of peripheral integration can occur in the gustatory system as well. Aversive compounds such as alkaloids or carboxylic acids can influence the activities of sweet- and salt-sensing neurons (Meunier et al., 2003, Lee et al., 2010, Charlu et al., 2013) and are thus likely to dictate behavioral responses by their combined action on two or more categories of taste neurons.

Sensilla in other taste organs have not been studied to the same extent, although the functional organization of at least a subset of tarsal sensilla displays similarities to ones found in the labellum (Meunier et al., 2000, Meunier et al., 2003). Of interest are a number of tarsal sensilla that are sexually dimorphic and do not carry the canonical sweet-, water-, and bitter-sensing neurons. Recently these sensilla were found to respond to pheromones (Starostina et al., 2012, Liu et al., 2012, Thistle et al., 2012, Toda et al., 2012, Lu et al., 2012).

Gustatory receptors

Gr expression was analyzed via the *GAL4/UAS* binary expression system to understand the molecular organization of the taste system (Thorne et al., 2004, Wang et al., 2004, Weiss et al., 2011). Generally, individual sweet- or bitter-sensing neurons were found to express multiple *Gr* genes, such that distinct neuronal classes emerged with unique combinations of receptors (Weiss et al., 2011). Although verification of this Gr-to-neuron map by direct *in situ* hybridization or immunolocalization remains a challenge, the presence of numerous Grs in each GRN parallels the expression patterns of T1R and T2R families of mammalian taste receptors (Yarmolinsky et al., 2009) and is in stark contrast with the one receptor-per-neuron organization of *Or*-expressing OSNs. A Gr-to-neuron map for the labellum showed that sweet neurons express Gr5a along with subsets of seven related receptors (Weiss et al., 2011). Expression

Table 4.2. Organization of labellar taste sensilla

Sensillum class	GRN	Receptor(s)	Response
L (L1 – L9)	Sweet	Gr5a, Gr61a, Gr64e, Gr64f	Sugars, glycerol
	Salt	None identified	Inorganic salts
	Water	Ppk28	Water
	Aversive	None identified	High salt
	Sweet	Gr5a, Gr64e, Gr64f	Sugars, glycerol
	?	Ppk23, Ppk29?	Pheromones?
S-a (S0-S2, S6, S7, S10)	Water	Ppk28	Water
	Bitter	Gr8a, Gr22b, Gr22d, Gr22e, Gr28a, Gr28b.a/e, Gr32a, Gr33a, Gr36b. Gr36c, Gr39a.a/b/d, Gr39b, Gr57a, Gr58b, Gr59a, Gr59b, Gr59c, Gr59d, Gr66a, Gr89a, Gr92a, Gr93a, Gr93b, Gr98b, Gr98c, Gr98d Painless, TrpA1	High salt, bitter compounds, allelochemicals
	Sweet	Gr5a, Gr64e, Gr64f	Sugars, glycerol
	?	Ppk23, Ppk29?	Pheromones?
S-b (S3, S5, S9)	Water	Ppk28	Water
	Bitter	Gr8a, Gr22e, Gr22f, Gr28a, Gr28b.a/d/e, Gr32a, Gr33a, Gr36a, Gr38c, Gr39a.a, Gr39b, Gr59a, Gr88a, Gr89a Painless, TrpA1	High salt, bitter compounds, allelochemicals, acidic pH
	Sweet	Gr5a, Gr64e, Gr64f	Sugars, glycerol
	?	Ppk23, Ppk29?	Pheromones?
I-a (I0 – I6)	Water	Ppk28	Water
	Bitter	Gr32a, Gr33a, Gr39a.a, Gr59c, Gr66a, Gr89a Painless, TrpA1	High salt, bitter compounds, allelochemicals
	Sweet	Gr5a, Gr64e, Gr64f	Sugars, glycerol
	?	Ppk23, Ppk29?	Pheromones?
I-b (I8 – I10)	Water	Ppk28	Water
	Bitter	Gr22b, Gr28a, Gr28b.a/e, Gr32a, Gr33a, Gr39a.a, Gr47a, Gr66a, Gr89a Painless, TrpA1	High salt, bitter compounds, allelochemicals, acidic pH
Oral taste pegs		Labeled by *E409-GAL4*	Carbonated water

of Gr5a and the other "sweet" receptors is excluded from bitter-sensing neurons (Jiao et al., 2007), which themselves can express as many as 30 of the remaining Grs (Weiss et al., 2011). Other analyses with more limited sets of lines suggest that some receptors may be exclusive to one taste organ whereas others may be shared by more than one (Thorne et al., 2004, Wang et al., 2004).

Interestingly, a number of *Gr-GAL4* drivers showed expression outside known chemosensory neurons. Some drivers labeled sensory neurons in propiosensory chordotonal organs and in the auditory Johnston's organ (Thorne and Amrein, 2008) suggesting roles for this family in sensing other classes of stimuli, a possibility that was recently substantiated by the observation that Gr28b is expressed in light-sensing dendritic neurons of the larval body wall and mutants lacking this receptor are defective in phototransduction and light avoidance behavior (Xiang et al., 2010). Various *Gr-GAL4* drivers were also found to label neuroendocrine cells of the gut (Park and Kwon, 2011), which allows for the possibility that Grs may be involved in sensing nutrients in the gastrointestinal tract of the fly similar to what has been found for some T1R and T2R receptors in vertebrates (Sternini et al., 2008). Interestingly, analysis of *Gr43a-GAL4* revealed expression in few neurons in the lateral protocerebrum of the brain in addition to some expression in peripheral taste organs (Miyamoto et al., 2012). *Gr43a* encodes a fructose receptor (Sato et al., 2011) that is thought to be activated by post-prandial rises in levels of hemolymph fructose (Miyamoto et al., 2012).

Gr proteins are likely to have the same inverted membrane topology as Or proteins based on epitope-tagging analysis of the Gr9 receptor of *Bombyx mori* (Zhang et al., 2011). Although second messenger signaling has been implicated to some extent in taste neuron sensitivity to sugars (Ishimoto et al., 2005, Ueno et al., 2006, Kain et al., 2010, Bredendiek et al., 2011), the rules of Gr protein assembly and ligand-dependent activation remain open questions. Given their complex combinatorial expression patterns, investigating Gr function in ligand recognition has proven to be difficult using mutation analysis alone. Two Grs, Gr5a and Gr64a, are broadly required for the detection of complimentary sets of sugars (Dahanukar et al., 2001, Dahanukar et al., 2007, Jiao et al., 2007, Slone et al., 2007). Similarly, five Grs are expressed in all bitter-sensing labellar neurons and have been proposed as "core" receptors involved in detecting bitter tastants (Weiss et al., 2011). Mutant analyses have suggested that Grs are likely to work in combinations to detect both sweet and

bitter ligands: the requirement for Gr64f partially overlaps with that of Gr5a as well as Gr64a (Jiao et al., 2008); similarly, at least three different Grs, Gr33a, Gr66a, and Gr93a are necessary for caffeine response (Moon et al., 2006, Moon et al., 2009, Lee et al., 2009). In each case, however, it is unclear whether the Gr serves a general co-receptor function or whether it directly confers ligand specificity.

In contrast to the empty neuron system for deorphanizing Or receptors, there has been limited success with heterologous expression of Gr receptors. Expression in *Drosophila* S2 cells (Gr5a) or COS-7 cells (Gr43a) showed that a single Gr protein could confer ligand-specific responses (Chyb et al., 2003, Sato et al., 2011), and at least in the case of Gr43a that ligand-dependent activity was independent of G protein signaling mechanisms (Sato et al., 2011). In another study, Gr64e was ectopically expressed in the CO_2 -sensitive neuron of the olfactory system, which also expresses *Gr21a* and *Gr63a,* and was found to be sufficient for glycerol sensitivity (Wisotsky et al., 2011). More recently, calcium imaging of selected classes of taste neurons using *Gr-GAL4* drivers and *UAS-GCaMP3.0* was used to identify a role for *Gr61a* in response to glucose (Miyamoto et al., 2013). However, elucidating the composition of a functional taste receptor and the precise contributions of individual subunits awaits a more comprehensive analysis.

Trp channels

Trp (Transient receptor potential) channels are highly conserved ion channels that have broad sensory roles in phototransduction, olfaction, taste, hearing, and thermosensation (Venkatachalam and Montell, 2007). Two *Drosophila* TRPA proteins, dTrpA1 and painless, are expressed in bitter taste cells and are required for responses to various allelochemicals that have pungent or irritant qualities (Al-Anzi et al., 2006, Kang et al., 2010). In mammals, the thermosensitive TRPM5 and capsaicin-activated TRPV1 channels also function in taste cells (Caterina et al., 1997, Zhang et al., 2003, Talavera et al., 2005, Riera et al., 2009). However, there is no evidence that *Drosophila* TRPM or the TRPV proteins, Nanchung and Inactive, are expressed in taste neurons.

Pickpocket channels

The *Drosophila* genome includes a family of ~30 *pickpocket* genes that encode Degenerin/ENaC proteins, many of which are expressed in taste neurons. Deg/ENaC channels are involved in detecting a variety of stimuli (Ben-Shahar, 2011), and recent studies suggest that members of this family also have diverse gustatory functions in the fly. One member, *ppk28*, is specifically expressed in water-sensing taste neurons and is activated by low osmolarity (Cameron et al., 2010). Two others, *ppk23* and *ppk29*, have been linked to populations of taste neurons in the labellum and tarsi that are largely distinct from Gr-expressing sweet and bitter neurons (Lu et al., 2012, Thistle et al., 2012, Toda et al., 2012). In the labellum, *ppk23-GAL4* labeled neurons that were largely distinct from water, sweet or bitter neurons, possibly coinciding with those previously identified as salt-responsive cells. In tarsi, *ppk23-GAL4* is co-expressed with *fruitless*, but not *Gr32a* (bitter), *Gr64f* (sweet), or *ppk28* (water), in paired GRNs that have sexually-dimorphic axonal projections, suggesting a role for these neurons in detecting cues that drive courtship behaviors (Lu et al., 2012, Thistle et al., 2012, Toda et al., 2012). Indeed, *ppk23*$^+$ neurons were found to be responsive to female-enriched hydrocarbons, 7,11-heptacosadiene and 7,11-nonacosadiene (Thistle et al., 2012, Toda et al., 2012). Further functional imaging experiments revealed that the two neighboring *ppk23*$^+$ neurons are specialized sex-specific pheromones sensors – one is selectively activated by female-enriched hydrocarbons and the second by male-enriched cuticular hydrocarbons, 7-tricosene and 7-pentacosene (Thistle et al., 2012). Behavioral analyses support a role for ppk23 in both male–female and male–male recognition: males lacking functional *ppk23* neurons showed reduced initiation and completion of courtship towards females (Lu et al., 2012, Toda et al., 2012, Thistle et al., 2012), as well as increased courtship towards males (Thistle et al., 2012). Two other DEG/ENaC genes, *ppk25* and *nope*, are also expressed in sexually dimorphic male gustatory neurons in tarsi and mutants lacking either one of these genes had similar impairments in male–female courtship (Liu et al., 2012, Starostina et al., 2012). However, the anatomical and functional overlap between *ppk23*$^+$ and *ppk25*$^+$ neurons remains to be determined. *ppk11* and *ppk19* channels are also implicated in salt detection in larvae (Liu et al., 2003), but the rest of this family remains uncharacterized.

Ionotropic receptors

Members of the newly identified *Ir* family are not restricted to OSNs and several are reported to be expressed in gustatory neurons as well (Croset et al., 2010). Although our current understanding about their expression or function in the taste system is limited, future studies will undoubtedly reveal the extent to which this ancient family is involved in contact-mediated chemosensation.

Central representation of chemosensory activity

The olfactory system

Glomerular maps of odor responses

The axons of both antennal and maxillary palp OSNs terminate in an ordered fashion in a pair of antennal lobes (AL) in the fly brain (Table 4.1), which is the site where olfactory processing begins. Each antennal lobe is comprised of approximately 50 discrete spheroidal units or glomeruli (Stocker et al., 1990, Laissue et al., 1999). The axons of all OSNs of the same functional class fasciculate and converge on one, or in few instances two, glomeruli (Couto et al., 2005, Fishilevich and Vosshall, 2005, Gao et al., 2000). In the AL, invariant synaptic connections are made between the axon termini of OSNs and the dendrites of

projection neurons (PNs) (Stocker et al., 1990). Most classes of OSNs send bilateral projections to glomeruli in each of the two ALs (Stocker et al., 1990, Couto et al., 2005). Despite this redundancy, the fly can determine the originating direction of an olfactory cue via higher levels of neurotransmitter release in the ipsilateral AL as compared to the contralateral AL (Gaudry et al., 2013).

Patterns of odor-evoked activity were monitored across the whole system by assaying changes in calcium in the AL, either from axons of OSNs or dendrites of PNs (Wang et al., 2003). Each odor recognized at the periphery elicits a stereotypic pattern of glomerular activity reflecting the specificity of Or/Orco responses of the corresponding OSNs. Furthermore, low odorant concentrations evoked sparse activation of glomeruli, which was more dispersed at higher odorant concentrations suggesting one possible mechanism by which odor intensity is encoded (Wang et al., 2003). From the glomerular activity map it also became clear that neurons responding to similar classes of chemicals converge onto glomeruli that are scattered throughout the AL. This suggests that rather than a chemotopic map in the central nervous system, it is more likely that the topographic map created at the periphery is maintained at the AL.

Interglomerular integration of olfactory input

The one-to-one connectivity between OSNs and PNs suggested the existence of a discrete, parallel channel for processing information from each OSN class. However, this idea was brought into question by two observations. First, the AL contains a complex network of interglomerular connections via lateral interneurons (LNs) (Stocker et al., 1990). Second, a systematic comparison of OSN and PN responses found that odor-receptive fields of PNs are generally stronger and broader than those of their cognate OSNs (Bhandawat et al., 2007). The latter observation suggested that PNs could receive excitatory input from LNs making presynaptic connections with other glomeruli, which was corroborated by measurements of "silent" PN activity from glomeruli that lacked their own functional presynaptic OSNs (Olsen et al., 2007, Shang et al., 2007). Interestingly, the tuning of each "silent" PN varied across PN classes, indicating a role for ensemble activity patterns of OSNs as signatures of odor identity (Olsen et al., 2007). Although initial studies failed to find evidence for inhibitory interactions between glomeruli, surgical and genetic manipulations to removal lateral input to PNs led to an increase in the tuning breadth of some individual PNs, suggesting a role for lateral inhibition in olfactory coding (Olsen and Wilson, 2008). The inhibition was shown to occur via GABAergic interneurons that directly blocked OSN to PN transmission (Root et al., 2008, Olsen and Wilson, 2008). Overall, the current view is that the various channels influencing OSN-to-PN transmission allow for superior division of odorant representation across PN activity by boosting the signal-to-noise ratios of glomerular activity patterns.

Propagation of olfactory input to higher brain centers

PNs in the AL relay olfactory information gathered at the periphery to higher processing centers in the *Drosophila* brain. The organization of the PN network is similar to that of the OSNs in that the dendrite of a single PN typically innervates a single glomerulus, effectively maintaining the peripheral one-to-one topographic map. On average, a single glomerulus is innervated by three PNs that make synaptic connections with approximately 30 OSN axons (Wong et al., 2002, Marin et al., 2002). PNs belong to one of three broad classes named on the basis of the relative positions of their cell bodies in the AL: anterodorsal, lateral, and ventral PNs (Marin et al., 2002). PNs within a given class are all derived from a single progenitor and make stereotypic connections in the AL (Jefferis et al., 2001). Thus, the architecture of this second-order signaling network is also genetically prespecified. Activity in PNs is relayed to two olfactory processing centers in the *Drosophila* protocerebrum: the mushroom body (MB) and the lateral horn (LH). The MB is involved in olfactory learning and memory (de Belle and Heisenberg, 1994, Davis, 2005). The role of the LH is less clear, but it has been implicated in innate behaviors (Heimbeck et al., 2001, Kido and Ito, 2002) as well as in bilateral and multimodal integration of sensory information (Gupta and Stopfer, 2012).

Single-cell labeling experiments allowed for identification of PN glomerular targets as well as characterization of their axon branching and terminal arborization patterns. Interestingly, PNs that innervate the same glomerulus have stereotypic projection patterns in the LH (Marin et al., 2002). Although there is some overlap, the cognate OSN class of a PN can be reliably predicted on the basis of the pattern of axon branching and arborization in the LH alone. Thus, the spatial map of olfactory activity appears to be conveyed to the LH, with some degree of overlap that may allow for convergence of olfactory input from multiple OSN classes in third order neurons. The PN axons that extend to the MB are simpler in terms of numbers of arborizations, and initial studies were unable to demonstrate clear topographic stereotypy as seen in the LH (Marin et al., 2002).

Subsequent high-resolution mapping of PN processes confirmed class-specific stereotypic arborizations in the LH and demonstrated a previously unreported degree of stereotypy in the MB. In-depth analysis of PN projection patterns, overlaid with the Kenyon cell (KC) dendritic map, established five groups in the LH and four in the MB (Jefferis et al., 2007, Lin et al., 2007). Superimposition of this spatial organization of the higher olfactory centers with the established Or-OSN-PN map and Or/OSN responses at the periphery exposed a spatial separation of PN classes that respond to general fruit odors from those that respond to specific pheromones, offering the first evidence that anatomical segregation in the LH is linked to biologically distinct functions (Jefferis et al., 2007).

In contrast to the stereotypy of the topographic connectivity between PNs and KCs as defined by such mapping, recent functional tracing experiments to define PN–KC relationships suggest that PNs converge on to the dendrites of KCs in a

seemingly random manner, and thereby disparate between individuals (Caron et al., 2013). Although there is no apparent organization of glomerular inputs to individual KC cells, KC axons make connections with spatially segregated extrinsic output neurons in the various lobes of the MB, which are involved in different forms of learned behavior (Tanaka et al., 2008, Sejourne et al., 2011). These findings invoke mechanisms that would allow an individual fly to adapt its olfactory circuitry and acquire behavioral valence through prior experience.

Glomerular activity and behavioral output

Studies of individual receptor function and glomerular activation patterns have given insight into how odorant identity and intensity are represented in the AL. How the complex glomerular activity patterns are translated to behavioral output is less clear. A behavioral screen with 110 single odorants to determine innate positive or negative valence for each found that a majority were classified as either attractive or neutral whereas only 6 of the tested compounds bore repellent properties (Knaden et al., 2012). Surprisingly, there was no obvious correlation between odorant valence and its chemical category, or its activation pattern in the peripheral olfactory system. However, the patterns of PN activation were separated by valence. In particular, six glomeruli that are clustered in the lateral region of the AL were strongly activated by the aversive odorants, raising the possibility that they may be components of hard-wired repellent circuits (Knaden et al., 2012).

Results of another study support the idea that one or more "aversive" glomeruli recruited at higher concentrations can be responsible for concentration-dependent switches in valence that are observed for many odorants (Semmelhack and Wang, 2009). The study examined behavior of *Drosophila* to apple cider vinegar, a low concentration of which activated six glomeruli in the AL and was behaviorally attractive. Selective silencing and activation of individual OSN classes, and thus individual glomeruli, revealed that two of the engaged glomeruli, DM1 (Or42b) and VA2 (Or92a), mediated the attraction. On the other hand, wild-type flies showed a robust aversion to apple cider vinegar at high concentrations. Analysis of the glomerular activity map revealed that an additional glomerulus, DM5 (Or85a), was activated at the increased concentration (Semmelhack and Wang, 2009). Genetic manipulation of glomerular activity showed that this single glomerulus could account for the valence reversal at high concentrations of apple cider vinegar, suggesting that the DM5 glomerulus is hard-wired to generate avoidance behavior.

While most general odorants are represented in combinatorial glomerular activity, the peripheral and AL representations of carbon dioxide and strong acids are distinct exceptions (de Bruyne et al., 2001, Suh et al., 2004, Ai et al., 2010). Each of these compounds activates one or two OSN/glomeruli and is perceived as aversive, suggesting a "labeled-line" avoidance circuit for its detection. Carbon dioxide, for example, activates the Gr21a/Gr63a receptor in ab1C OSNs (de Bruyne et al., 2001, Jones et al., 2007, Kwon et al., 2007), the axons of which terminate in the V glomerulus in the AL (Suh et al., 2004). Similarly, strong acids activate *Ir64a*$^{+}$ OSNs in the sacculus that target the DC4 glomerulus (Ai et al., 2010).

To date, the most significant advances in linking olfactory system wiring to behavior have come through studies of pheromone detection and courtship behavior. Courtship by the *Drosophila* male comprises a complex set of innate behavioral sequences (Hall, 1994) set in place by the male-specific isoform of the *fruitless* gene, Fru^{M} (Manoli et al., 2005, Stockinger et al., 2005). Courtship is influenced, in part, by the detection of the male-emitted pheromone cVA. In both sexes, one of two OSN classes that detect cVA expresses *Or67d* and projects to the DA1 glomerulus in the AL (Kurtovic et al., 2007). Despite identical first order projections, cVA elicits disparate behavioral responses in males and females. In males, cVA promotes aggression towards other males (Wang and Anderson, 2010) and suppresses courtship towards both males and females (Ejima et al., 2007). In contrast, cVA detection in females stimulates an increase in receptivity to courting males (Kurtovic et al., 2007). Exposure to cVA generates similar responses in OSNs and PNs in both sexes (Datta et al., 2008), suggesting that the differences in behavior are generated in higher brain centers. Tracing the axons of PNs that innervate the Or67d/DA1 glomerulus revealed a high density of arborizations in the ventral region of the LH in males, but not in females (Datta et al., 2008). Given that the narrowly tuned *Or67d* olfactory channel expresses *fru* in the OSN and the cognate PN, the sexually dimorphic arborizations in the LH were examined in *fru* mutant males. The arborizations in the ventral region of the LH were significantly reduced in *fru* mutant males, showing instead arborization resembling that present in wild-type females (Datta et al., 2008). *fru* mutant males court other males with an increased frequency, which suggests that the *fru*-regulated axon topography in the LH contributes to the sexually dimorphic behavioral responses to cVA.

Sexual dimorphism of the specialized cVA pathway continued in higher order neurons (Ruta et al., 2010). Four clusters of cell bodies are in close proximity to the DA1 PN terminal arborizations. Among these putative third order neurons, one dorsal cluster (DC1), which showed responses following stimulation of the DA1 glomerulus but not other glomeruli, was specific to males. DC1 axons were traced to the lateral triangle and the superior medial protocerebrum (SMP tract), neuropil structures that are only present in males. Further tracing of this circuit revealed male-specific DN1 neurons, which extend dendrites into the lateral triangle and the SMP tract, and send long axons down to the ventral nerve cord. These DN1 axons terminate in the thoracic and abdominal ganglia and intermingle with motor neurons. DN1 neurons receive excitatory signals in response to cVA and DA1 activation, and this excitation requires input from the third order DC1 neurons (Ruta et al., 2010). The specificity of the cVA circuit has been key in

following a neural pathway from olfactory detection to motor output.

The taste system

Presynaptic termini of GRNs are located in the subesophageal ganglion (SOG) in the central nervous system (Stocker, 1994). The functional separation of sweet- and bitter-sensing GRNs at the periphery is reflected in non-overlapping axonal projections in the medial region of the SOG (Thorne et al., 2004, Wang et al., 2004, Marella et al., 2006). Axons of labellar *Gr5a*$^+$ sweet neurons terminate ipsilaterally in a region that abuts but does not overlap with an area innervated by projections of labellar *Gr66a*$^+$ bitter neurons (Wang et al., 2004). Imaging tastant-evoked activity in the SOG using a genetically encoded calcium sensor confirmed unique representations for sweet and bitter tastants by showing that the two identified regions of the SOG were activated either by one or the other class of tastants but not both (Marella et al., 2006). Axons of other classes of GRNs, include those specialized for detection of water and carbonation have also been traced to areas within the SOG (Inoshita and Tanimura, 2006, Fischler et al., 2007, Cameron et al., 2010). Additionally, GRNs from different organs terminate in distinct areas of the SOG, even when they are labeled by the same Gr receptor (Stocker, 1994, Wang et al., 2004, Thorne et al., 2004).

Although sub-regions of the SOG are not morphologically demarcated like the glomeruli in the AL, a recent study used transgenic strategies to label neurons of gustatory sensilla and identified 11 separate zones within a region named the "primary gustatory center" of this ganglion, which receives input from GRNs and mechanosensory neurons of the labellum and pharynx (Miyazaki and Ito, 2010). This high-resolution neuroanatomical analysis confirms the idea that mechanosensory and chemosensory inputs are relayed to distinct areas, which was first put forth by classical dye tracing studies (Murphey et al., 1989). Eight of the identified zones received input from GRNs in a manner distinguishing oral taste pegs, labellar bitter neurons, labellar sweet neurons and pharyngeal neurons from each other. The distinctions were not always perfect – there was some overlap between bitter neurons of the labellum and pharynx, and no discernible differences were found in the distribution of termini of sweet- and water-sensing taste neurons, suggesting that gustatory input may converge at least in part at the very first level of taste processing. Overall, GRN representation in the SOG appears to separate both the quality and positional information about the taste stimulus, although behavioral evidence to support the functional significance of the latter is lacking.

A cluster of ~20 neurons in the SOG that express the neuropeptide precursor *hugin* are candidate gustatory interneurons, since blocking their activity resulted in a drastic reduction in food intake (Melcher and Pankratz, 2005). Subsets of *hugin*$^+$ neurons were found to project to one of various targets including the protocerebrum, the ring gland neuroendocrine center, and pharyngeal muscles (Melcher and Pankratz, 2005, Bader et al., 2007). Although these and other neurons that display arborizations in the SOG have been described, none have been demonstrated to be direct synaptic targets of GRNs. Thus, higher order gustatory neurons remain to be identified, leaving many open questions about how information is processed and integrated along gustatory circuits.

Sophisticated processing by chemosensory neurons

Encoding chemical blends in sensory neurons

Olfactory coding of odor blends

Thus far, properties of OSNs have been described in terms of response profiles to monomolecular odorants. In nature, however, many odors encountered are mixtures and can be perceived, at least by humans, as unique fragrances (Laing and Francis, 1989). This unique perception has been thought to be the product of sophisticated central processing. There is mounting evidence, however, that OSN activity itself can reflect information about the context in which an odorant is received. For example, the presence of odorants that inhibit the Gr21a/Gr63a CO_2 receptor can disrupt the innate avoidance behavior to CO_2 in *Drosophila* (Turner and Ray, 2009). Other work that examined mixtures containing both excitatory and inhibitory components for a given Or/Orco receptor demonstrated that individual OSNs have the capacity to generate responses to mixtures that differ from the mere sum of its components. Recordings to such binary mixtures showed a change not only in the firing frequency of the OSN but also in the timing of the response (Su et al., 2011). Thus, odorant mixtures can generate unique signatures in the periphery that afford the freedom to discriminate blends from individual components alone, even across a range of concentrations.

Each component of an odor mixture has unique physicochemical properties that likely affect the rates in which their vapors reach the fly's olfactory organs. In a mixture of a "fast" excitatory odorant with a "slow" inhibitory odorant, the presence of the inhibitory odorant sharpened the activation response (Su et al., 2011). The reverse experiment ("slow" activator paired with a "fast" inhibitor) was carried out in a *Drosophila* OSN that ectopically expressed a mosquito olfactory receptor (Su et al., 2011). In this case, a biphasic response was observed where spontaneous activity was reduced upon initial exposure to the binary mixture and activation was marked by a slower response profile. Taken together, these experiments suggest that a given OSN has the power to generate unique responses to blends of odorants due to varying response dynamics of the constituents.

Gustatory coding of tastant blends

The separation of GRN responses at the periphery in a manner that correlates with tastant acceptance or rejection suggested

that GRN circuits operate as labeled lines that are hard-wired to convey unambiguous information about whether or not to feed. Molecular genetic analyses have provided ample support for this model by showing that specific GRNs are necessary and sufficient to execute stereotypical food selections (Thorne et al., 2004, Marella et al., 2006, Wang et al., 2004). Because of this, behavioral decisions are thought to arise from higher order summation of positive and negative inputs.

Like complex odor blends, tastants are often found in the context of mixtures, and responses to mixed stimuli are not always predictable from responses to the individual components. While there is no evidence of communication between GRNs of the same sensillum, there are reports of aversive stimuli acting to inhibit responses of taste acceptance neurons to their phagostimulatory agonists. Work in the blowfly has shown that a range of alkaloids can inhibit responses to sugars (Dethier and Bowdan, 1989), an observation that has been confirmed, albeit with a limited panel, in *Drosophila* (Meunier et al., 2003). Likewise, carboxylic acids have been shown to modify response of the salt neuron in blowflies, enhancing it when present at low concentrations and inhibiting it at higher concentrations (Murata et al., 2002). Electrophysiological analyses in other insects has revealed instances in which responses to mixtures are simply additive effects, or lower than expected, from responses to individual components (Chapman, 1995). Examples of synergistic effects have also been reported – both when two stimuli act upon the same neuron and when they act upon different neurons within the same sensillum. Although such interactions have not yet been investigated in great detail in *Drosophila*, these studies suggest that information about tastants can be integrated, at least to some degree, in the sensory neurons of the insect taste system.

Modulation of sensory neuron by starvation

Starvation-induced changes in olfactory neurons

A change in an organism's internal physiological state often leads to distinct changes in behavior. The stereotypic nature of chemoreceptor expression and first-order connectivity would suggest that such plasticity relies largely on modulation of central processing. However, circadian changes in responses and spontaneous spike amplitudes of OSNs (Krishnan et al., 1999, Krishnan et al., 2008) suggested that OSN responses are not rigid. Also, a recent study demonstrated that flies show a robust increase in food-search behavior that is largely dependent on modulation of olfactory processing at the periphery. Food odor-evoked changes in calcium-influx in PNs showed that some neurons in the AL were subject to modulation in response to starvation (Root et al., 2011). Specifically, three glomeruli (DM1, DM4, and DM2) showed enhanced odor-evoked responses and two (VM2 and VA3) showed decreased responses following starvation (Root et al., 2011). This modulation was specific to the glomerulus and not to the odor tested. Thus, a change in internal state appears to cause specific changes in olfactory representation in the brain along with changes in behavior.

Analysis of food odor-evoked activity in OSNs and PNs revealed that this glomerular-specific change in olfactory representation occurred at the level of transmission of OSN signal to PNs (Root et al., 2011). The *Drosophila* neuropeptide sNPF, known to promote feeding behavior (Lee et al., 2004), is expressed in a subset of OSNs along with its receptor sNPFR1 (Carlsson et al., 2010). Knockdown of sNPF in OSNs, using *Orco-Gal4* and *UAS-RNAi* transgenes, abolished the starvation-mediated increase in OSN signaling and the corresponding enhancement in food-search behavior. This loss of starvation-induced modulation was absent if either sNPF or sNPFR were knocked down in PNs. Further knockdown experiments with OSN-specific drivers refined the starvation-dependent requirement of sNPF/sNPFR to Or42b OSNs that project to the DM1 glomerulus. Moreover, overexpression of sNPFR1, but not sNPF, in Or42b OSNs in fed flies was sufficient to induce a starved phenotype (Root et al., 2011). Together, the results suggest that a starvation-regulated increase in sNPFR expression in Or42b neurons brings about changes in DM1 activity, leading to an increase in food-search behavior.

There is also conclusive evidence to link the regulation of sNPFR1 expression in OSNs to insulin signaling. A combination of genetic and pharmacological manipulation was used to show that insulin receptor-mediated signalling was both necessary and sufficient for the up-regulation of sNPFR1 and the subsequent enhancement of odor-evoked activity in Or42b OSNs (Root et al., 2011). The study thus uncovered a simple yet credible mechanism for how a change in internal state is translated to a change in sensory input via insulin signaling, and in so doing brings about an appropriate behavioral modification to meet the physiological needs of the fly.

Starvation-induced changes in gustatory circuits

It's long been known that hunger and satiety regulate feeding behavior. Studies in blowflies have shown that feeding behaviors are modulated by biogenic amines (Brookhart et al., 1987, Long and Murdock, 1983, Long et al., 1986), although their neural substrates within gustatory circuits are still not well understood. A recent study used an innovative transgenic system to report dopamine (DA) receptor activity and found high levels of reporter labeling in sweet taste neurons of starved flies (Inagaki et al., 2012). Pharmacological manipulation of dopamine levels resulted in changes in behavioral sensitivity to sucrose, as did knocking down the level of DopEcR, one of four *Drosophila* DA receptors, specifically in sweet GRNs. A proposed mechanism of GRN modulation from this work is that starvation, at least during the early stages, triggers release of dopamine on to sweet GRNs, which increases the value of their input in feeding circuits. The site of dopamine release, however, is not yet clear. One candidate is a single dopaminergic neuron, named TH-VUM, which has extensive

bilateral projections in the SOG and was shown to be necessary for starvation-induced increase in sucrose sensitivity (Marella et al., 2012). TH-VUM, which was not responsive to sugars, was relatively silent in fed flies and far more active in starved flies consistent with a role in driving hunger-induced behavioral modifications. However, TH-VUM-dependent behavioral modulation relied on the D2R receptor, whose function is dispensable in sweet neurons, indicating that much remains to be learnt about the molecular and cellular mechanisms of dopamine action on feeding circuits. Nevertheless, these results offer a first glimpse of how such modulatory changes may occur at the level of peripheral taste neurons. Future studies will likely focus on other neuromodulators as well, since changes in behavioral sensitivity upon longer periods of starvation appear to be independent of dopamine (Inagaki et al., 2012).

Other lines of evidence suggest that internal nutrient-sensing pathways convey information about nutrient deprivation or mating status to feeding circuits via the target-of-rapamycin/Ribosomal S6 kinase (TOR/S6k) and insulin signaling pathways, which result in compensatory feeding behaviors (Ribeiro and Dickson, 2010, Vargas et al., 2010). For example, flies deprived of protein in their diet develop a heightened preference for protein over time. To what extent such modulation occurs at the level of GRNs is not clear. However, insects such as locusts have been shown to alter their peripheral sensitivity to tastants that are excluded from their diet (Simpson et al., 1991), and it will be intriguing to determine whether this occurs in *Drosophila* as well. Collectively, these examples highlight the fascinating capacity of peripheral chemosensory neurons to relay meaningful environmental information to the central nervous system in a variety of contexts.

Interactions between smell and taste

There are a few examples of environmental cues that can be detected by both olfactory and gustatory systems. The common insect repellant DEET (N, N-diethyl-*m*-toluamide) is detected by olfactory and gustatory neurons, and leads to aversive behaviors in both cases. Volatile DEET evokes repellency, observed in flies where contact with DEET is prohibited. The mechanisms of volatile DEET repellency are controversial and two different models have been put forth for its action on OSNs: one in which DEET activates repellent OSNs in an Orco-dependent fashion (Syed and Leal, 2008, Ditzen et al., 2008), and a second in which DEET modifies responses of some OSNs to their cognate ligands thereby acting to confuse normal olfactory coding (Pellegrino et al., 2011). DEET also acts as a contact repellent, relying on bitter taste neuron-mediated rejection of DEET-laced substrates, which occurs even with severely reduced olfactory input (Lee et al., 2010). Taste neuron responses to DEET are dependent on *Gr33a*, *Gr66a,* and *Gr32a*, confirming the idea that it is directly detected by bitter GRNs. Studies of behavioral responses to DEET highlight an example of a compound with the same valence for olfactory and gustatory behaviors, perhaps ensuring a strong avoidance response to a toxic chemical.

On the other hand, acetic acid is an example of a single cue that can drive olfactory and gustatory behaviors with opposite valence (Joseph et al., 2009). Flies avoid lingering on substrates that contain acetic acid at an environmentally relevant concentration. This behavior is dependent on signaling from antennal OSNs, and is exaggerated in *white rabbit (whir)* mutants that are suspected to have an enhanced sense of smell (Joseph et al., 2009, Rothenfluh et al., 2006). Despite this olfactory-mediated avoidance of acetic acid, mated female flies preferentially lay their eggs in media containing acetic acid at the same concentration for which they exhibit "positional" avoidance. Oviposition preference to acetic acid was dependent upon a functional gustatory system, as selective disruption of gustatory neurons led to a deficit in this behavior, while removal of the antennae had no effect (Joseph et al., 2009). It will be interesting to determine where the competing olfactory and gustatory inputs converge, whether in the brain or at the level of motor output that generates behavior.

Although behavioral studies in olfaction and taste have generally been isolated, it is increasingly apparent that chemosensory behaviors can be influenced by both olfactory and gustatory input. The exact nature of interactions between the two systems remains unknown, but at least in one case the input from the gustatory system has been shown to have a dominant influence on olfactory-mediated behavior. The Or67d/cVA circuit is known to promote male–male aggression and suppress male–male courtship (Ronderos and Smith, 2010, Kurtovic et al., 2007, Wang and Anderson, 2010). Recent studies have found that similar opposing effects on courtship and aggression behavior are driven by *Gr32a*-expressing bitter GRNs via recognition of the male-specific hydrocarbon (z)-7-tricosene (Miyamoto and Amrein, 2008, Wang et al., 2011, Koganezawa et al., 2010). Investigation of the manner in which the effects of cVA and 7-tricosene are related revealed a requirement for *Gr32a* in taste neurons for cVA-mediated enhancement of aggression (Wang et al., 2011). Conversely, *Or67d* was dispensable for 7-tricosene-mediated aggression. Thus, the olfactory effect of cVA on aggression was found to be dependent on gustatory input but not visa versa, suggesting that gustatory signaling is epistatic to olfactory input in stimulating male–male aggression. Similar hierarchical interactions between the two systems are also implicated in suppression of male–male courtship. Currently, the regions in the brain where the two systems converge are poorly understood. Identification of such regions would be instrumental toward understanding how information encoded in various classes of sensory neurons is integrated to influence behavior.

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Chapter 5

Nociception

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Introduction

Pain is a universally experienced but poorly understood process. 116 million people in the USA alone live with chronic pain (Council, 2011). Nociception is the neural process of detecting and responding to noxious stimuli, and serves a protective function in avoiding potentially tissue damaging sensory stimuli. Nociception is extremely important for survival as it allows animals to avoid and react to potentially harmful conditions. Animals use specialized sensory neurons called nociceptors to sense noxious stimuli (Woolf and Ma, 2007). Appropriate control of nociceptor function is critical to maintaining overall health, since failures in controlling the nociception system often cause pathological states, such as neuropathic pain (Campbell and Meyer, 2006).

Pathological pain can be a consequence of an environmental event, such as a traumatic accident or a surgical procedure, or the pain can be idiopathic and of unknown etiology. In the case of chronic pain syndromes that are triggered by a causal insult that causes chronic pain in the area of the original injury, such as post-thoracotomy pain, it is largely unknown why some individuals develop severe and long-lasting chronic pain, while others do not. An important goal of pain research is to explain the mechanisms that lead to the development of pain syndromes. An especially powerful way to achieve this goal is through the identification of genes that play a functionally important role in pain responses, which is the approach that we describe here.

Historically, approaches to the genetic analysis of pain have been primarily applied in humans and mice (LaCroix-Fralish et al., 2007). In human studies pedigrees with extreme insensitivity or hypersensitivity to pain have led to the identification of several important pain signaling molecules (Kremeyer et al., 2010; Raouf et al., 2010; Momin and Wood, 2008; Cox et al., 2006; Choi et al., 2006). In addition, candidate gene studies have identified gene variants that contribute to pain states in mice and humans (LaCroix-Fralish et al., 2007). More recently, genome-wide association studies (GWAS) in humans have the potential to ascribe variability in pain sensitivity to genetic variants in the population of interest (Kim et al., 2009). The strength of the GWAS approach lies in the large number of single nucleotide polymorphisms (SNPs) that have been identified by the human genome project. However, this strength imposes limitations because the many SNP variants make it difficult to achieve the statistical significance needed to detect associations that are above the noise of genetic variation (Goldstein, 2009). A statistically more powerful approach can be taken through the study of inbred mouse strains that have been found to show extreme differences in pain responses among lines (Mogil et al., 1999a,b). Greater statistical power can be achieved because the breeding of the mice is controlled by the investigator. The genetic pedigrees can then be used to find the loci that vary among the strains and which also segregate with the pain trait.

Although these approaches to pain genetics used in mice and in humans have led to many important discoveries (LaCroix-Fralish et al., 2007) they have also met with certain formidable obstacles. These studies clearly show that natural genetic variation contributing to pain phenotypes does exist, and the important variants can be identified. Yet, in both the human and the mouse models, in many cases, even if a polymorphism can be found, it may be very difficult to assign the polymorphism to a specific gene function. In cases when variation is found in non-coding DNA, then finding the affected gene itself is a challenge. If the gene can be found, does the polymorphism increase the activity of a nearby gene or does it decrease it? Does the polymorphism affect gene expression in the nociceptor neurons, in microglial cells, dorsal horn interneurons or in the brain? Many years of research by many laboratories may be needed to move beyond the initial identification of the variant in order to understand the mechanisms that lead to the association with pain.

In addition, the association studies can never identify the complete set of molecular elements that define the pain system because they rely on natural variation. The genes that do vary may not be the best therapeutic targets for pharmacological intervention. And most importantly, many of the critical genes involved in pain signaling may not have variants that lead to disease. This is because natural selection against deleterious variants will likely act to eliminate variation in key nociception genes. This means that many genes can never be identified

Behavioral Genetics of the Fly (Drosophila melanogaster), ed. J. Dubnau. Published by Cambridge University Press.

through the examination of population variability or may only be identified as extremely rare variants.

In *Drosophila*, these problems can be overcome through the use of forward genetics. We have developed a system that uses *Drosophila* as a platform for discovering genes required for the function of nociceptive sensory neurons (Caldwell and Tracey, 2010; Hwang et al., 2007, 2012; Tracey et al., 2003; Zhong et al., 2010, 2012). Understanding nociceptors is important to understanding pain because sensory drive in nociceptors contributes to the development of central sensitization and subsequent chronic pain (Seltzer et al., 1991). Furthermore, congenital pain syndromes are caused by mutations in genes that affect the function of nociceptive sensory afferents in humans (Kremeyer et al., 2010; Raouf et al., 2010; Momin and Wood, 2008; Cox et al., 2006; Choi et al., 2006). Thus, a fundamental and basic understanding of the molecular mechanisms of nociceptor neurons is important for an understanding of chronic, neuropathic, and inflammatory pain.

Drosophila is an invertebrate. This may be seen as a limitation to this approach because of the phylogenetic distance between flies and mammals. However, our research and that of others has shown that *Drosophila* nociception pathways are evolutionarily conserved. The dTRPA1 channel, the TRPA channel Painless, the degenerin/epithelial sodium channel (DEG/ENaC) Pickpocket, the calcium channel subunit α2δ3 and the Piezo channel play critical roles in *Drosophila* nociception (Tracey et al., 2003; Zhong et al., 2012; Neely et al., 2010, 2011; Coste et al., 2012; Kim et al., 2012). Importantly, TRPA channels, DEG/ENaCs and the calcium channel subunit α2δ3 are also important for mammalian nociception. In fact, familial episodic pain syndrome (FEPS) results from a missense mutation that changes a single amino acid of the hTRPA1 gene (Kremeyer et al., 2010), mammalian homologues of DEG/ENaCs called acid-sensing ion channels (ASICs) have been implicated in many types of nociceptive signaling including mechanical nociception (Deval et al., 2010), and rare polymorphisms in the calcium channel subunit α2δ3 gene are linked to reduced pain phenotypes in humans (Neely et al., 2010). In addition, it has been shown that a TNF-alpha-like pathway triggers thermal allodynia in *Drosophila* larvae (Babcock et al., 2009). Larvae that are exposed to strong ultraviolet (UV) irradiation, release the Eiger (TNF-alpha) ligand from the epidermis. This in turn triggers allodynia through the TNF receptor Wengen, which is expressed in nociceptor neurons (Babcock et al., 2009). Thus, given the conserved functions of these genes in mammalian pain signaling, mechanistic insights that have been uncovered in *Drosophila* have a high probability of illuminating our understanding of mammalian nociception.

Nociception behaviors and circuits in *Drosophila*

Drosophila larvae show a stereotypic escape locomotion in response to noxious thermal, mechanical or chemical stimuli (Tracey et al., 2003). When performing escape locomotion, the larva rotates around its long body axis in a corkscrew like manner. This nocifensive escape locomotion (NEL) is specifically triggered by noxious stimuli and is unambiguously separable from other forms of larval locomotion such as reverse or forward peristalsis. Two nociception assays, thermal and mechanical, have been established (Tracey et al., 2003) and are used to test nociceptive sensory function in larval *Drosophila*. Thermal nociception can be tested by stimulating larvae with the gentle touch of a probe heated to a noxious temperature. Interestingly, the temperature threshold to induce robust larval NEL is around 39 °C, which is similar to the temperature threshold for nociceptor firing in mammals, including primates (Tillman et al., 1995). Mechanical nociception can be tested by using Von Frey fibers which deliver a designated maximum mechanical force. Larval NEL is triggered by harsh mechanical stimuli of greater than 30 mN and innocuous tactile stimuli do not evoke the stereotypic rolling responses (Tracey et al., 2003; Kernan et al., 1994). Detailed materials and methods for thermal and mechanical nociception assays are provided below.

The Class IV multidendritic (md) neurons have been identified as polymodal nociceptors in larval *Drosophila* (Hwang et al., 2007). The md neurons are sensory neurons located just beneath the larval epidermis, and tile the larval body wall with their dendritic arbors (Grueber et al., 2002). Based on the complexity of the dendritic structures, md neurons are classified into Class I to Class IV in the order from least to most complex dendritic arbors (Grueber et al., 2002). Hwang et al. (2007) demonstrated that silencing the activity of the Class IV md neurons significantly disrupts NEL responses to noxious heat and mechanical stimulation. Silencing of Class II and III md neurons impaired mechanical nociception but had little effect on thermal nociception. Additionally, optical activation of the Class IV md neurons using the algae-derived blue light-gated cation channel Channelrhodopsin-2 elicits NEL, but optical activation of the Class I, II, or III md neurons fails to evoke larval NEL (Hwang et al., 2007). Collectively, these results show that larval Class IV md neurons are necessary for sensing thermal and mechanical noxious stimuli and sufficient to trigger NEL, thus fulfilling the criteria of a polymodal nociceptor. Recently it was also shown that the Class IV md neurons mediate light avoidance behaviors in response to high intensity ultraviolet or blue light (Xiang et al., 2010). The Class IV md neurons send their axons centrally to the larval ventral nerve cord (Grueber et al., 2007), but the secondary neurons and downstream nociceptive circuits are unknown.

Although the Class IV md neurons are clearly nociceptive, it is still possible that there are additional nociceptive pathways in *Drosophila* larvae. Two recent papers report an assay that involves immersing larvae in a water droplet resting on a petri dish (Chattopadhyay et al., 2012; Oswald et al., 2011). The petri dish with the water droplet was then placed on a hotplate heated to 70 °C or 95 °C. Interestingly, this technique elicited an NEL like behavior in the larvae. However, the temperature measured in the water droplet was only around 29 °C when the

NEL like behavior was triggered. This temperature is far below the nociceptive threshold that we have observed. Oswald et al. also reported that blocking the Class IV md neurons results in an elevated temperature threshold for the rolling-like behavior observed in the water droplet (Oswald et al., 2011). Since a thermal probe heated to 29 °C never elicits larval NEL in wild-type larvae (Tracey et al., 2003; Zhong et al., 2012; Babcock et al., 2009) and recordings from the Class IV neurons show that they respond to temperatures above 39 °C (Xiang et al., 2010), these observations raised the possibility that there might be an unknown nociception processing pathway that triggers larval NEL in response to the low heat stimuli delivered systemically in the water droplet. However, there are important caveats in the interpretation of these experiments. For instance, because larvae can make contact with the surface of a petri dish the temperature of the petri dish surface must be taken into account. This caveat was not considered in either study. Experiments in our laboratory have found that the temperature of the petri dish surface indeed rises much more rapidly than the water droplet. In addition, NEL behavior is only rarely observed when larvae are placed in a water droplet equilibrated to 31.5 °C on a plate heated to 32.5 °C (J. Robertson and W.D. Tracey, Jr unpublished observations).

Adult flies have also been used in thermal nociception assays. A wide variety of stimuli, including an infrared laser beam, hot plate, heated ring barrier and heated chamber have been used to stimulate adult flies (Neely et al., 2010; Aldrich et al., 2010; Xu et al., 2006). Among these assays, the heated ring barrier and heated chamber assay can test a group of animals at once, and thus have the potential to provide a relatively high-throughput experimental system. One potential weakness of adult thermal nociception assays is that innocuous thermosensation pathways mediated by antennal (Sayeed and Benzer, 1996) and internal (Hamada et al., 2008) sensory neurons will also repel flies from temperatures between 25 °C and 32 °C. In addition, the jump reflex or avoidance behaviors which are used as indicative responses for nociception in adult flies are generally elicited by various other non-noxious stimuli such as airflow, light, sound, and odors. Therefore, experimental conditions need to be carefully controlled to exclude non-specifically activated responses. The neurons responsible for nociception in adult flies have not been identified. Although the larval Class IV md neurons persist through metamorphosis to the adult fly (Shimono et al., 2009), their function in the adult stage remains to be demonstrated.

Nociception-related genes in *Drosophila*

Since the field of *Drosophila* nociception has emerged nearly a decade ago, only a handful of genes have been found to be involved in *Drosophila* nociception. However, these few examples present convincing evidence that flies can be used to uncover novel evolutionally-conserved molecules responsible for nociception.

Transient receptor potential A (TRPA) channels

The TRP channels are a large family of cation channels known to function in mediating a wide range of sensory modalities, including taste, hearing, thermosensation, hygrosensation and nociception (Gallio et al., 2011; Kim et al., 2003; Liedtke et al., 2003; Liu et al., 2007; McKemy et al., 2002; Montell and Rubin, 1989; Niemeyer et al., 1996; Peier et al., 2002; Sidi et al., 2003; Smith et al., 2002; Walker et al., 2000; Xu et al., 2002). TRP channels have four subunits, and can be either homomeric or heteromeric (Hoenderop et al., 2003). The TRPA channel family plays key roles in mechanical, chemical and perhaps cold-induced nociception in mammals, although the role for TRPA1 in cold nociception is controversial (Brierley et al., 2009; Bautista et al., 2006; Story et al., 2003; Knowlton et al., 2010; McMahon and Wood, 2006; Kwan et al., 2006; Macpherson et al., 2005; Bandell et al., 2004; Jordt et al., 2004). The TRPA channel is also the first TRP channel subfamily that has been associated with a human pain-related syndrome. A gain-of-function mutation in the *TRPA1* gene is linked with a heritable familial episodic pain syndrome (FEPS) in a Colombian family (Kremeyer et al., 2010; Waxman, 2010). Two TRPA channels in *Drosophila* have been found to play an important role in nociception, which strongly argues that the molecular mechanisms for noxious stimuli sensation are evolutionarily conserved.

painless (*pain*) was the first reported nociception gene in *Drosophila*. *pain* was identified from an insertional mutagenesis-based forward genetic screen for mutations that cause insensitivity to noxious heat (Tracey et al., 2003). *pain* mutants show severe insensitivity to both noxious thermal and mechanical stimulation, but respond normally to innocuous gentle touch. The nociception phenotype of *pain* is not likely to be due to developmental defects in the motor circuit because *pain* mutants are coordinated and capable of responding to higher noxious temperatures (≥52 °C). The gene *pain* encodes an ion channel that belongs to the TRPA channel family. Consistent with the multimodal sensory function of TRPA channels, the *pain* mutant is also defective in avoiding allyl isothiocyanate (AITC), which is an irritant compound found in wasabi (Al-Anzi et al., 2006).

Patch-clamp recordings from the heterologously expressed Painless channel in HEK293 cells demonstrated that Painless is directly gated by heat above 41 °C (Sokabe et al., 2008), supporting its role in sensory transduction for noxious heat. However, direct activation of Pain by AITC or osmotic pressure has not yet been observed (Sokabe et al., 2008). Recently, a non-canonical isoform of Pain that lacks ankyrin repeats was identified (Hwang et al., 2012). Expression of this non-canonical Pain isoform in larval nociceptors is capable of rescuing mechanical nociception, but not thermal nociception in a *pain* mutant (Hwang et al., 2012). This suggests that the ankyrin repeats domain of Pain is important for its thermoceptive function. A mechanosensory role for the Pain channel has been implied in mechanical stress responses in the heart (Senatore et al., 2010)

and gravity sensing (Sun et al., 2009), but whether Pain functions as a mechanotransducer or downstream as a signal amplifier in these contexts has yet to be determined.

Another TRPA channel involved in fly nociception is *dTRPA1*, the *Drosophila* orthologue of mammalian *TRPA1*. The function of *dTRPA1* was first described in thermotaxis (Rosenzweig et al., 2010), and it has been subsequently revealed that the canonical dTRPA1 isoform (known as dTRPA1-A) is a thermosensitive channel directly gated by innocuous warming (>27 °C) (Hamada et al., 2008). The role of *dTRPA1* in sensing various noxious or repellent chemicals has also been shown (Kang et al., 2010; Kwon et al., 2010; Kim et al., 2010), supporting the hypothesis that the mechanisms of chemical nociception are evolutionally conserved.

Recently, *dTRPA1* was shown to be involved in thermal and mechanical nociception (Zhong et al., 2012; Neely et al., 2011; Babcock et al., 2011). Paradoxically, *dTRPA1* involvement in thermal nociception was not consistent with the activation threshold of dTRPA1-A, which is around 27 °C, much lower than the temperature threshold of larval thermal nociception (39 °C). Furthermore, the known dTRPA1-GAL4 strains did not drive reporter expression in the larval nociceptors (Hamada et al., 2008; Rosenzweig et al., 2005). This puzzle was resolved by the discovery of a novel non-thermosensitive isoform of dTRPA1, which has an alternative transcriptional start site located upstream of the canonical isoform (Zhong et al., 2012). Enhancer elements from the DNA region upstream of the non-thermosensitive transcriptional start site drives expression of GAL4 reporters almost exclusively in the larval nociceptors. In addition, when expressed in larval nociceptors, the non-thermosensitive dTRPA1 isoform rescues the thermal nociception phenotype in the dTRPA1 null mutant. Conversely, the expression of the canonical dTRPA1-A thermosensitive isoform results in a gain-of-function phenotype, and lowers the temperature threshold for NEL to 30 °C. These results suggest that the non-thermosensitive isoform functions in larval thermal nociception, and that dTRPA1 is not a direct thermosensor for noxious heat in larval nociception. Structurally, the non-thermosensory dTRPA1 is distinct from the canonical thermosensitive isoform only in the alternative exons flanking the ankyrin repeats domain, which indicates that these regions are important in determining the thermodynamics of the channel. Similar to the canonical isoform, the non-thermosensitive dTRPA1 isoform is responsive to irritant chemicals such as AITC (Zhong et al., 2012; Kang et al., 2012). In the adult fly, the non-thermosensory isoform, but not the thermosensory isoform, is found to be important for chemical nociception against nuclear electrophiles mediated through gustatory neurons (Kang et al., 2012).

straightjacket (*stj*)

stj, an α2δ3 voltage-gated calcium channel subunit, was identified as a conserved nociception gene in a study by Neely and colleagues that applied findings from the fly to mice and humans (Neely et al., 2010). *stj* was first isolated as a candidate nociception gene in a genome-wide RNAi screen based on a high-throughput noxious heat avoidance assay in adult flies. Flies with neuronal specific knockdown of *stj* fail to avoid noxious heat in a heated chamber and are consequently paralyzed, implicating a possible defect in thermal nociception. The thermal nociception phenotype seen in *stj* mutants has been confirmed using the thermal nociception assay in larvae, and the phenotype is rescued by a genomic fragment containing the *stj* locus. Based on these results, the authors generated knockout mice for the mammalian *stj* orthologue *CACNA2D*, and found that the knockout mice are deficient in thermal and inflammatory nociception, which is consistent with the prediction from *Drosophila* phenotypes. Furthermore, identification of the *stj* phenotype has subsequently led to the discovery that rare polymorphisms in the human orthologue of the *CACNA2D3* gene are associated with reduced acute and chronic pain. In mice, *stj* is not detectably expressed in the nociceptors or DRG neurons. Furthermore, the knockout mice exhibit impaired activation of higher-order pain centers and abnormal sensory cross-activation in the brain by thermal and tactile stimuli, implicating that *stj* might be required for higher-order processing of nociceptive signals in the brain. Although the localization of *stj* in the nociception pathway is unclear in *Drosophila*, its relatively simple nervous system and powerful tools to dissect circuitry functions will be useful to uncover the site of action of the gene in nociceptive circuits.

pickpocket (*ppk*)

pickpocket genes constitute a large family of DEG/ENaC sodium channels in the *Drosophila* genome (Adams et al., 1998). DEG/ENaC channels are thought to form sodium-selective trimers (Firsov et al., 1998; Kosari et al., 1998; Mano and Driscoll, 1999; Gessmann et al., 2010). Several lines of evidence have suggested that channels from this family play a role in mechanosensation and nociception. Mec-4 and Mec-10, DEG/ENaC channel subunits in *C. elegans*, have been shown to comprise a mechanosensitive channel complex required for gentle touch sensation (O'Hagan et al., 2005; Suzuki et al., 2003; Ernstrom and Chalfie, 2002). The ASIC3 DEG/ENaC channel in mice is thought to be required for sensing a noxious pinch (Price et al., 2001). Interestingly, snake venom proteins elicit robust nociception behavior in mice through the activation of the ASIC1 DEG/ENaC channel in nociceptors (Bohlen et al., 2011).

A role for the canonical *ppk* gene (*ppk1*) in larval nociception was first suggested by its expression pattern. Indeed, until the discovery of the non-thermoceptive dTRPA1, *ppk* was the only gene known to be specifically expressed in the nociceptors (Ainsley et al., 2003). Consistent with its specific expression pattern, *ppk* mutant larvae are defective in mechanical nociception, but normal for both thermal nociception and gentle touch sensation (Zhong et al., 2010). In addition, RNAi knockdown of *ppk* severely impairs mechanical nociception, but does not affect optogenetically induced NEL. Thus, a potential role for

the PPK channel subunit in mechanotransduction of noxious mechanical force has been proposed. Although patch-clamp recordings from Class IV neurons that were grown in culture identified a PPK dependent transient current that was gated by acidity (Boiko et al., 2012) the role of PPK as a direct mechanotransducer has yet to be demonstrated with electrophysiological approaches.

Dmpiezo

The *piezo1* and *piezo2* genes have been found to be capable of conferring robust mechanically activated currents when heterologously expressed in mammalian cultured cells (Coste et al., 2010). The *piezo* genes are well conserved, but the structure is unlike any previously described ion channels. Depending on the species, Piezo has 24–36 transmembrane domains, and does not have an identifiable pore structure. Two recent studies on *Drosophila piezo* (*Dmpiezo*) have further elucidated the role of Piezo in mechanical nociception *in vivo*. Coste et al. (2012) performed electrophysiological analyses of heterologously expressed Piezo channels and found that DmPiezo forms mechanically-activated, homo-multimeric, non-selective cation channels (Coste et al., 2012). Utilizing the genetic tools of *Drosophila,* Kim et al. (2012) provided *in vivo* evidence that DmPiezo functions as an important component of larval mechanical nociception. Behavioral analyses demonstrated that larvae with a Dm*piezo* deletion are insensitive to mechanical nociception, but responded normally to thermal nociception and innocuous gentle touch (Kim et al., 2012). A *Dmpiezo* GAL4 line drives UAS reporter gene expression in a variety of sensory neurons including the larval Class IV nociceptors. Finally, recordings from dissociated Class IV nociceptors isolated from the *Dmpiezo* deletion mutants showed no mechanically activated currents (Kim et al., 2012). Interestingly, Piezo and PPK1 seem to function in parallel pathways in detecting mechanical nociception, while Pain and Piezo may function in the same pathway.

amnesiac (*amn*)

amn was originally isolated as an associative olfactory memory mutant (Quinn et al., 1979). The *amn* gene is predicted to encode a putative neuropeptide precursor, which exhibits weak sequence similarity to mammalian pituitary adenylyl cyclase activating peptide (PACAP) (Feany and Quinn, 1995). When tested in thermal nociception assays, *amn* genetic mutants or ubiquitous knockdown of *amn* with RNAi show significantly reduced or delayed nociceptive responses to noxious heat in both the adult and larvae (Aldrich et al., 2010). These mutant phenotypes are rescued by ubiquitous overexpression of wild type *amn*, but the site of action of the putative neuropeptide in nociception circuits is unknown.

NPFR1

NPFR1 is a member of the Neuropeptide tyrosine (NPY)-like receptors (Feng et al., 2003). NPY-like receptors and their ligand NPY have been implicated in modulation of a number of physiological processes in vertebrates such as sleep, food intake, and nociception (Held et al., 2006; Heilig et al., 1991; Brumovsky et al., 2007). In nociception, NPY and NPY like-receptors are thought to have an anti-nociceptive role. Intrathecal injection of NPY in rats has been reported to cause reduced responses to noxious heat (Hua et al., 1991), and knockout mice for the NPY1 receptor display hyperalgesia (Naveilhan et al., 2001). In *Drosophila*, overexpression of *NPFR1* with painless-GAL4 has been reported to lower sensitivity to noxious heat, implicating its conserved anti-nociceptive role (Xu et al., 2010).

Nociceptive sensitization and mediators in *Drosophila*

The nociceptors can be sensitized due to tissue-damage and/or inflammation. In vertebrates, numerous extracellular signaling molecules secreted from injured or inflamed tissue have been identified as modulators of nociceptor sensitivity (Hucho and Levine, 2007). These inflammatory mediators activate diverse intracellular signaling cascades through the activation of a variety of receptor types and ultimately lead to plastic change of nociceptor sensitivity. Sensitized nociception can occur due to hyperalgesia and/or allodynia (Sandkuhler, 2009). Hyperalgesia is indicated when there is an exaggerated response to a normally noxious stimulus such that a normally painful stimulus is even more painful. Allodynia occurs when a normally innocuous stimulus is perceived as painful.

An experimental paradigm to probe nociceptive sensitization has been developed for *Drosophila* larvae (Babcock et al., 2009). After exposure to UV-C radiation, epidermal tissue damage is seen and larvae show sensitized nociception behavior. This UV-induced nociceptive sensitization includes both hyperalgesia and allodynia, since larvae develop faster responses to noxious heat stimulation as well as NEL in response to normally innocuous temperatures (Babcock et al., 2009). In this UV-induced nociceptive sensitization model, thermal hyperalgesia and allodynia are developed in different time courses. Hyperalgesia is observed from 8 to 16 hours after UV irradiation but diminished after 24 hours, while allodynia persists from 8 to 24 hours after UV treatment.

The tumor necrosis factor (TNF) signaling pathway is activated through apoptosis of damaged epidermal cells, and mediates thermal allodynia in *Drosophila* (Babcock et al., 2009). TNF-α has been extensively studied as an inflammatory cytokine which has a role in modulating immune and nociceptive neuronal responses (Ware, 2011; Leung and Cahill, 2010). Babcock and colleagues examined the role of *Drosophila* TNF and its receptor, named *eiger* and *wengen* respectively, using the UV-induced nociceptive sensitization model. They found that either epidermis-specific knockdown of *eiger* or nociceptor-specific knockdown of *wengen* by RNAi abolishes UV-induced thermal allodynia without affecting basal nociception (Babcock et al., 2009). Additionally, RNAi-mediated blocking of apoptotic caspase-3 (*dronc*) activity in damaged epidermal cells

prevents the development of allodynia. The proposed model involves the Eiger TNF ligand produced and secreted from apoptotic epidermal tissue which activates the Wengen receptor expressed in the nociceptors. Interestingly, knockdown of *eiger*, *wengen*, and *dronc* caspase does not affect the development of hyperalgesia, suggesting that allodynia and hyperalgesia are mediated by genetically separable signaling cascades. Because TNF signaling has been suggested to be involved in nociceptive sensitization in mammals (Sandkuhler, 2009), the identification of TNF as a mediator of fly allodynia implies that the molecular machinery mediating nociceptive sensitization is also conserved between flies and mammals.

Recently, the Hedgehog (HH) signaling pathway has been reported to function in nociceptive sensitization in *Drosophila*. Babcock et al. (2011) have demonstrated that nociceptor-specific functional disruption of HH signaling components by either expressing RNAi or a dominant negative form of HH signaling components blocks thermal allodynia and hyperalgesia after UV-induced tissue damage, while the number and gross morphology of the nociceptors and basal nociception behavior are not affected (Babcock et al., 2011). Epistasis analyses showed that the HH signaling pathway functions in parallel with the TNF signaling pathway in allodynia development. Additional epistasis analysis uncovered interactions between the HH and TNF pathways and TRPA channels. In the case of allodynia, both Pain and dTRPA1 are necessary, and Pain interacts with both the TNF and HH pathways. In the case of hyperalgesia, however, only dTRPA1 is necessary, and it interacts with the HH signaling pathway. Interestingly, the authors have also provided evidence implying that the HH signaling pathway plays a role in modulating nociceptive sensitization in mammals. Pharmacological inhibition of the HH receptor Smoothened results in sustained or enhanced morphine-mediated analgesia in rodent inflammatory or neuropathic pain models.

Conclusions

In summary, *Drosophila* provides robust behavioral assays and powerful forward genetic screening systems to study nociception. Nociception studies in *Drosophila* have shown that a number of nociception genes are functionally conserved in flies and mammals, and even led to discoveries of novel signaling molecules in mammalian nociception such as$\alpha 2\delta 3$ voltage-gated calcium channel subunit and *hh*. The fact that flies have played a key role to find the unexpected nociception signaling pathways further emphasizes the potential of the fly nociception model.

Thermal and mechanical nociception assays in *Drosophila* larvae

Materials

Common

- Deionized water
- Standard cornmeal molasses fly food vials
- Dry yeast
- 60 × 15 mm petri dishes
- Digital video camera (SONY DCR-DVD610)
- Leica MZ6 stereomicroscope
- MM99 Adaptor S/N: 1685 (Martin Microscope Company)
- Halogen dual fiber optic light source (Schott)
- Forceps
- Transferring pipette

Thermal nociception assay

- Variable auto transformer, single phase input, 0–120 VAC Output, 12A (Variac)
- BAT-12 thermometer (Physitemp)
- IT-23 MLT1402 T-type ultra fast thermocouple probe (Physitemp)
- Soldering irons (Two soldering irons are required; one is to be remodeled to a custom-made thermal probe, another is to be used for soldering.)
- Solder
- Whetstone

Mechanical nociception assay

- Pasteur pipette
- Nylon monofilament fishing line (Shakespeare Omniflex 6 lb test, diameter 0.009 inch [0.23 mm])
- Weighing scale

Equipment setup

Stereo microscope and digital video camera

A conventional stereomicroscope is set up with a halogen light source. A digital video camera is mounted on the stereomicroscope through the MM99 adaptor (C-mount). The optical zoom setting of the digital video camera is adjusted to minimize vignetting of the viewing field.

Custom-made thermal probe

For the thermal nociception assay, a custom thermal probe can be made from a soldering iron. Using a whetstone, the tip of the soldering iron is filed down to a pointy chisel shape, approximately 0.6 mm wide at the tip (Fig. 5.1). The tip of the IT-23 thermocouple is attached on the back of the chisel-shaped tip using solder. It is critical to place the thermocouple on the very tip of the thermal probe. A dollop of solder is added onto the thermal probe in order to entirely cover the IT-23 thermocouple and insulate it from the exterior. The solder on the thermal probe also confers greater heat capacity to the probe, which prevents temperature fluctuation during experiments. After cooling for several hours the soldered probe is ready to use. Connect the IT-23 thermocouple to the BAT-12 thermometer. The power cord of the thermal probe should

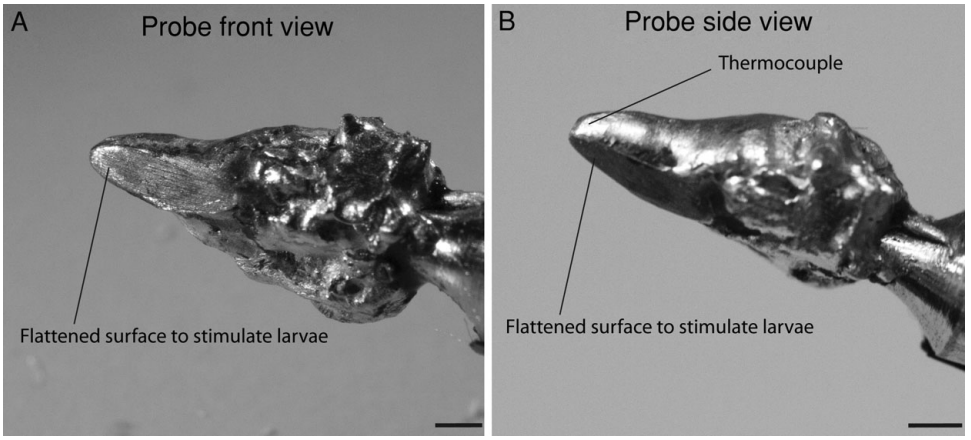

Fig. 5.1. The thermal probe.The frontal view A and the side view B of the tip of the thermal probe. An IT-23 thermocouple (Physitemp) is attached on the backside of the flattened tip and the temperature is measured with a model BAT-12 microprobe thermometer (not shown, Physitemp). To achieve consistent delivery of heat stimuli, larvae are touched by the flattened side of the chisel shaped tip. Scale bars = 1 mm.

be plugged into the 12 A variac transformer so that the temperature of the thermal probe can be controlled by adjusting the voltage on the thermal probe. Typically 20–25 volts are needed to drive the temperature of the probe into the noxious range.

Von Frey fibers

Von Frey fibers are used to deliver mechanical stimuli in the mechanical nociception assay. Von Frey fibers can be made by attaching nylon monofilament fishing line to a Pasteur pipette whose tip has been truncated and bent to 90 ° angle with a Bunsen burner. The mechanical forces produced by Von Frey fibers can be adjusted by varying the length of fishing line hanging over from the end of the Pasteur pipette. A Von Frey fiber with a longer protruding fishing line generally produces weaker mechanical force. The maximum mechanical force delivered by a particular Von Frey fiber can be measured by depressing a weighing balance using the Von Frey fiber. The mechanical force produced by a Von Frey fiber reaches its maximum at the instant when the fiber begins bending. Thus, the reading of a weighing balance when the fishing line of a Von Frey fiber bends represents its maximum force. The force in mN can be calculated by multiplying the reading of the balance in grams by 9.8 (the value of the acceleration of gravity).

Methods

Fly crosses and husbandry

Wandering third instar larvae should be used in both thermal and mechanical nociception assays. To obtain experimental larvae, six virgin females are crossed to three males in a cornmeal molasses fly food vial and the cross is kept for five-six days at 25 °C with 75% relative humidity and a 12-hour light/dark cycle. Multiple crosses should be set up to increase the number of experimental larvae. Since day-to-day variations cannot be eliminated in behavioral experiments, control crosses should be tested side-by-side with experimental genotypes.

General experimental conditions

All behavioral experiments are performed at room temperature (21∼23 °C) under the stereomicroscope.

Thermal nociception assay

1. The variac transformer, BAT-12 thermometer, halogen light source and digital video camera are turned on. The voltage on the variac transformer is adjusted to heat the custom-made thermal probe up to the intended temperature, which can be monitored through the BAT-12 thermometer. The focus, exposure and white balance settings of the digital video camera are adjusted properly to achieve fine video recordings.
2. Wandering third instar larvae are gently washed from the food vials to petri dishes using deionized water.
3. Larvae need to be tested in a shallow aqueous environment. Excess water is carefully removed from the petri dishes using the transferring pipette to leave water shallow enough for larvae to crawl along the bottom of the dish, but not so deep that the larvae are suspended. A few particles of dry yeast can be added to the dish and dissolved so that the surface tension of the water is lowered and the bottom of the dish is evenly covered by the aqueous solution.
4. The video camera is started to record the experiments. A larva is gently touched with heated thermal probe on the lateral side of abdominal segments 4 to 6 until the animal achieves a 360° roll along its long body axis or 10 seconds have passed. The tested larva is discarded using forceps to avoid repeated testing on the same larva. For statistical comparison, it is desirable to test > 90 larvae in total for each genotype. To ensure the reproducibility of results, it is also recommended to repeat the same experiment on multiple days using larvae from independent parental flies.
5. Larval nociception responses are analyzed on the recorded movies. The latencies from heat stimulation to roll are measured for each tested animal with a digital stopwatch (for example http://tools.arantius.com/stopwatch).

6. Average latency is calculated for each genotype. Appropriate statistical tests (for example Wilcoxon rank sum test) are used to compare a control strain and a given experimental genotype. A histogram with 11 bins (< 1 sec, < 2 sec, …,< 10 sec and > 10 sec) can be made to show the distribution of latencies. In wild-type animals, larval nocifensive rolling is typically seen within 2 seconds using a 46 °C thermal probe.

Mechanical nociception assay

1. Wandering third instar larvae are washed into petri dishes with a shallow aqueous environment as described in step 3 of the thermal nociception assay.
2. Videotaping is started. A noxious mechanical force is delivered to a larva by depressing the Von Frey fiber down perpendicularly on the dorsal midline of abdominal segments 4 to 6. The Von Frey fiber should be rapidly pressed down until the fishing line begins bending and immediately released to deliver only instantaneous maximum mechanical force. Each animal is poked up to 3 times until the larva shows NEL to mechanical stimulation. The tested larva is discarded from the dish using forceps. In our lab, approximately 70–80% of wild type larvae typically show NEL after the first stimulation with a 50 mN Von Frey fiber. Some practice may be required to obtain reproducible results for beginners. To minimize potential bias, it is recommended to perform the assay blind to experimental genotypes. In addition, the experiments should be repeated on multiple days using animals from independent crosses. It is desirable to test > 90 animals per genotype in total.
3. For statistical comparison, larval responses can be binomially scored ("roll at the first poke" or "no roll") and a percentage of larvae which show nocifensive rolling behavior in response to a particular noxious mechanical force can be calculated. A cumulative percentage of larvae which show the nocifensive rolling behavior at each poke (roll at the first, second, third poke and no roll) can also be calculated. Appropriate statistical tests for proportional data (for example, Fisher's exact test) can be used to compare a control strain and experimental genotype.

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Chapter 6

Flight behavior: Degradation of flight muscle power and locomotor capacity in transgenic *Drosophila*

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Introduction

Drosophila displays an impressive diversity of flight techniques and vision-guided behaviors. The limits of these behaviors and thus aerial capacity depend on several key factors including the ability of the underlying neuromuscular system to control wing movements and the maximum mechanical power output delivered by the asynchronous indirect flight muscle (A-IFM). What we experience as flight behavior thus reflects the output of a complex high-speed feedback cascade that turns sensory information into aerodynamic forces. This chapter summarizes the recent progress related to the link between A-IFM function and flight behavior in *Drosophila* from a behavioral genetics perspective, presenting behavioral data on A-IFM mutants that have been tested in free and tethered flight essays. These mutants cover a wide range of flight muscle modifications such as alterations in the number of muscle fibers, the phosphorylation capacity of proteins essential for muscle stiffness, actin–myosin cross-bridge cycling, and flight muscle oxygen supply.

Aerial performance of flies

Flight is an essential component of most insect behaviors: wing flapping is required to escape from predators, for the localization of food and mating partners and allows responses to various forms of stress such as thermal treatment and desiccation. Aerial locomotion is thus a major component of fitness in fruit flies (Gilchrist et al., 1997; Jordon et al., 2006).

Drosophila displays an impressive diversity of sophisticated aerobatic behaviors such as obstacle avoidance reactions, escape responses and elaborate starting and landing programs. These maneuvers result from the interplay between genetically predetermined behavioral programs, feedback coming from the insect's sensory structures and mechanical forces acting on body and wings. The interplay between sensory stimuli and the formation of muscle commands for motor control during vision-guided behaviors such as object fixation behavior and optomotor responses has been thoroughly analyzed on different levels of investigation (Duistermars et al., 2007; Götz et al., 1979; Heisenberg and Wolf, 1984; Kern and Egelhaaf, 2000; Mronz and Lehmann, 2008). Using elaborate sensory input, *Drosophila* steers and maneuvers by changing many aspects of wing kinematics such as the amplitude and frequency of the wing stroke, and the timing and speed of wing rotation at the stroke reversals (Dickinson et al., 1999; Lehmann and Dickinson, 1998; Fig. 6.1A). The limits of these kinematic alterations, and thus the constraints on the fly's aerial maneuverability, depend on several key factors including mechanical constraints set by the thoracic exoskeleton, the ability of the underlying neuromuscular system to precisely control wing movements, and the maximum power output of the flight muscles. What we experience as flight behavior in *Drosophila* thus reflects the output of a complex feedback cascade that turns sensory information into muscle mechanical and, subsequently, locomotor forces by the activation of flight muscles.

Although the maximum mechanical power output and the efficiency of the locomotor musculature for flight can be estimated from *in vitro* biophysical experiments, the values determined from such experiments in *Drosophila* are substantially lower than the maxima that occur in the flying animal (Lehmann and Dickinson, 1997; Tohtong et al., 1995). As a consequence, a systems-level perspective on power production is a necessary bridge in any attempt to link the function and performance of flight musculature with its specific role for wing motion and flight force control in the behaving animal. Moreover, the cost of locomotion in flying insects is rarely constant but varies as the animal changes speed and direction. Ultimately, the muscles of the insect must compensate for these changing requirements by varying the amount of muscle power that they produce. An important key to understand *Drosophila* flight behavior is thus to unravel the function of the A-IFM and its significance for mechanical power output during various forms of aerial maneuvers.

Bioamines and their significance for flight

Besides walking, flight is probably the most complex behavior, and understanding its underlying genetic architecture requires a broad perspective on the various structures of the flight motor including their physiological functions. Although to date no complex trait associated to flight has been genetically dissected in all detail, behavioral genetic analyses offer beneficial tools to determine the significance of particular genes for the function of the flight feedback cascade. The *Drosophila* flight system

Behavioral Genetics of the Fly (Drosophila melanogaster), ed. J. Dubnau. Published by Cambridge University Press.

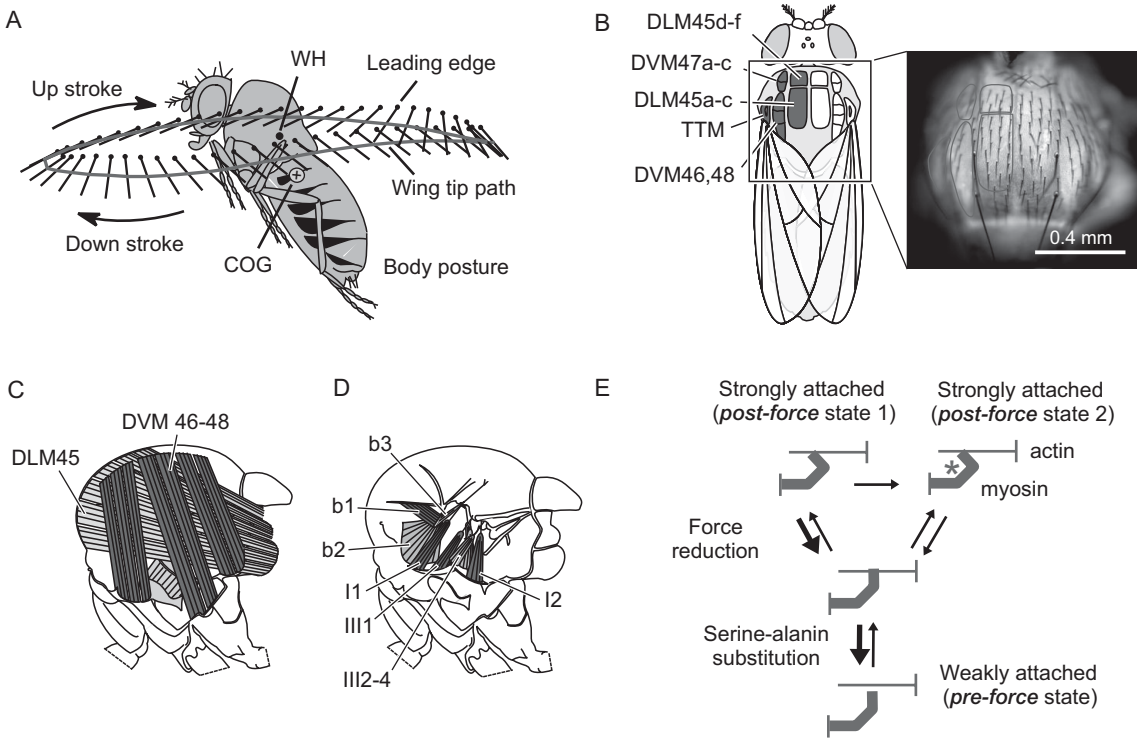

Fig. 6.1. Wing motion and flight muscles in *Drosophila*. A Body posture and wing motion during hovering flight. The black lines show snapshots of the wing blade at the center of force production, sampled every 100 μs throughout the stroke cycle, where black dots indicate the wing's leading edge. WH, wing hinge; COG, center of gravity. B Dorsal attachment sites of the indirect flight muscle (A-IFM) inside the thorax. Fluorescence image on the right shows the A-IFM during flight in a mutant expressing the calcium indicator Cameleon. Red lines in the hemisoma circle attachment sites of the muscles shown on the left. DLM, dorso-longitudinal muscle; DVM, dorso-ventral muscle; TTM, tergo-trochanter muscle. The TTM connects the scutum with the coxa of the middle leg. It is termed "jump muscle" because of its activation during take-off behavior. C Side view on DLM and DVM fibers and their insertion points inside the thorax. D Insertion points of the synchronous flight control muscles. b1–3, muscles of the basalare; I1–2 and III1–4, muscles of the pterale. E "Three-state cross-bridge model" of actin–myosin interaction for A-IFM contraction in *Drosophila*. Site-directed mutagenesis of serine to alanine in the myosin light-chain kinase shifts the balance between the contraction states from *post-force* state 1 to the non-binding *pre-force* state.

thus serves as a model system for basic processes related to complex body control. Since some of these processes are conserved between flies and humans (Adams et al., 2000), genes that affect development and function of the *Drosophila* locomotor system may be also relevant in humans. A prominent example of this link are several human degenerative diseases related to motor control such as Parkinson's disorder which is caused by a degeneration of dopaminergic neurons in the nigrostriatal area and an accumulation of Lewy bodies (Olanow and Tatton, 1999; Scholtissen et al., 2006). It has been shown that, in *Drosophila*, mutations with reduced levels of dopamine are associated with variable changes in locomotor activity, where higher dopamine concentrations typically cause an increase in locomotor activity (Connolly et al., 1971).

Other biogenic amines such as octopamine (tyramine-β-hydroxylase, TβH), the invertebrate homolog of noradrenalin, cause more complex behavioral changes in flight, such as alterations in flight maintenance and flight initiation, rather than in the dendritic structure of flight motoneurons, the structure of the flight muscles or kinematic parameters such as stroke frequency (Brembs et al., 2007). Flight deficits of TβH null mutants can be fully rescued by octopamine substitution, but also by blocking tyramine receptors that are enriched in the TβH null mutant. Microinjection of octopamine into the isolated ventral nerve cord of locust produces similar effects, initiating walking and flight motor patterns (Sombati and Hoyle, 1984). Besides octopamine, the neurotransmitters serotonin and γ-aminobutyric acid (GABA) enhance and inhibit locomotor activity in *Drosophila*, respectively. Treatments with the GABA antagonist bicuculline (BIC), for example, can restore previously suppressed locomotor activity by applications of GABA reuptake transport inhibitors, which is similar to the role of GABA for the control of locomotion in vertebrates (Leal and Neckameyer, 2002). Since locomotion is a complex trait, it was further investigated by mapping quantitative trait loci (QTL) for locomotor behavior. In one study, fine mapping of four QTL to 12 chromosomal regions produced 13 positional candidate genes affecting locomotor activity, including the enzyme *Dopa decarboxylase* (*Ddc*) that catalyzes the final steps in the serotonin and dopamine syntheses. According to the data available so far, it seems likely that a large part of the natural variation in locomotor flight activity in *Drosophila* results from the variations in the synthesis and availability of these two bioamines (Jordon et al., 2006).

Muscular requirements and muscle function

The production and control of aerodynamic forces during flight results from the interplay in neural activation between two functionally, physiologically and anatomically distinct classes of flight muscles: the asynchronous, indirect flight muscle (A-IFM) composed of the dorso-longitudinal (DLM) and the antagonistic dorso-ventral (DVM) muscle, and the synchronous flight control muscles (Fig. 6.1B–D). In *Drosophila* there are 16 flight control muscles on each body side, where 14 muscles insert at the wing hinge and two muscles, the pleurosternal muscles 1 and 2, insert inside the thorax shell to allow control of thoracic stiffness and thus stroke frequency (Dickinson and Tu, 1997; Tu and Dickinson, 1996). In contrast, stroke amplitude is modulated by at least the first and second basalare muscle (b1 and b2) and the first control muscle of the pterale (I1). While the A-IFM mainly delivers the mechanical power for wing motion by deforming the thoracic exoskeleton, flight control muscles reconfigure the wing hinge in order to allow changes in both wing movements and power transmission from the A-IFM to the flapping wings.

Since insect flight requires higher levels of mechanical power than any other form of animal locomotion, the A-IFM of *Drosophila* offers morphological and physiological specializations such as stretch activation and shortening deactivation at an oscillation frequency of more than 200 Hz. The A-IFM oscillates at nearly full actin–myosin filament overlap. In the prevailing model for muscle function, stretch activation describes the transition from a non-force-producing (weekly bound state) cross-bridge state to a force-producing (strongly bound state) state ('three-state cross-bridge model'; Tawada and Kawai, 1990; Zhao and Kawai, 1993; Fig. 6.1E). It has been suggested that the transmission of strain to the myosin filament (thick filament) affects strain-sensitive rate constants of the cross-bridge cycle and thus the distribution of cross-bridge states in the A-IFM myofibrils (Granzier and Wang, 1993a; 1993b). In contrast to vertebrate synchronous muscles, asynchronous nerve impulses at 5–10 Hz repetition rate maintain an elevated level of A-IFM intracellular calcium. This calcium primes the A-IFM for stretch activation, converting the detached actin and myosin filaments (detached state) into a weakly bound state, instead of directly controlling muscle tension by calcium waves (Vigoreaux, 2006). Thus, the asynchronous A-IFM of insects produces only small mechanical forces when stimulated electrically (Josephson, 2006). The function of the A-IFM in *Drosophila* is similar to the vertebrate cardiac muscle, namely to generate power during oscillatory contractions, and it is beneficial for understanding muscle function in a larger context. This includes, for example, studies on the functional significance of structural alterations of muscle myosin for muscle tension and power (Moore et al., 2000; Ramanath et al., 2011; Swank et al., 2006; Yang et al., 2010, 2008), on the muscle proteins obscurin (Katzemich et al., 2012), troponin (Kržič et al., 2010), and fligthin (Barton et al., 2005), on aging effects such as age-dependent degradation of muscle ultrastructure and mitochondrial damage (Miller et al., 2008) and muscle mechanical properties of the A-IFM (Swank, 2012).

The A-IFM of myosin regulatory light chain (Mlc2) transgenic *Drosophila*

Modulation of mechanical power output and the control stretch activation in the indirect flight muscle is essential for *Drosophila* to cope with the changing power requirements during maneuvering flight. Besides changes in intra-muscular calcium concentration (Gordon and Dickinson, 2006), A-IFM-specific proteins and a unique extension of the myosin regulatory light chain (MLC2) may also play a key role in A-IFM power control (Bullard et al., 1985; Bullard et al., 1988; Tohtong et al., 1995; Vigoreaux et al., 1993). *Drosophila* MLC2 exhibits two conserved serines at the positions 66 and 67 that are phosphorylated during cross-bridge cycling by the myosin light chain kinase (MLCK). A replacement of these amino acids by non-phosphoryable alanines changes the chain of events with actin and myosin during cross-bridge cycling. A change in the constitutive level of MLC2 phosphorylation is thought to be correlated with changes in stretch activation and net A-IFM power, by reducing the number of attached cross-bridges rather than by changing the kinetics of the power-producing step during cross-bridge cycling. An alternative explanation of A-IFM power loss owing to MLC2-phosphorylation refers to the human heart and skeletal muscle (Poetter et al., 1996). Mutation of the human regulatory light chain supposedly modifies the regional elasticity of the myosin neck, which includes regulatory and essential light chains. Assuming that the strain sensitivity of *Drosophila* myosin originates in the neck region of the myosin molecule, a removal of the MLCK-dependent phosphorylation side on the MLC2 diminishes oscillatory power output by altering neck compliance (Dickinson et al., 1997).

Changes in viscoelastic properties of A-IFM in Mlc2 mutants

On the level of muscle structure, the replacement of each of the two MLC2 serines into alanines using site-directed mutagenesis has only little effect in *Drosophila*. The sarcomeric structure of the serine double mutant ($Mlc2^{S66A,S66A}$) appears to be normal by optical and low magnification electron microscopy. This indicates that phosphorylation of the myosin regulatory light chain is not essential for muscle structure and development, although power output is severely attenuated (Table 6.1). The power reduction is probably caused by the complete detachment of myosin heads from actin filament in the "three-state cross-bridge model". The model incorporates MgATP binding, MgATP hydrolysis, or release of phosphate and MgADP during the pre-force, post-force, and recovery state (Maughan et al., 1994). In the recovery state, the myosin head remains weakly attached to the actin filament. The MLCK-phosphorylation dependent recruitment of power-generating

Table 6.1. Mean flight parameters of tethered wild type and transgenic *Drosophila*, flying at maximum locomotor capacity in a virtual reality flight simulator. Amplitude, stroke amplitude of the flapping wings; frequency, stroke frequency of a complete flapping cycle; force, aerodynamic lift production of both flapping wings opposing gravity and normalized to *Drosophila* body weight (100%); power, muscle mechanical power output of the indirect flight muscle (A-IFM) during wing flapping and normalized to flight muscle weight; η_M, muscle efficiency, i.e., the efficiency with which the A-IFM converts chemical energy into mechanical forces for wing motion; η_M, aerodynamic efficiency, i.e., the ratio between the minimum power requirements for flight (Rankine–Froude power) and the power actually produced by A-IFM; η_T, total efficiency of the flight apparatus, i.e. the product between muscle and aerodynamic efficiency and a measure for the overall efficacy of the flight apparatus; activity, a relative measure for locomotor activity and flight motivation compared to wild type behavior (+, increase; −, decrease); *, estimated value

Strain	Amplitude (degrees)	Frequency (Hz)	Force (%)	Power (WKg^{-1})	η_M (%)	η_A (%)	η_T (%)	Activity
wild type ♂	162	226	116	77	11.4	25.4	2.9	0
wild type ♀	170	207	135	80	9.7	26.9	2.6	0
$Mlc2^{S66A,S67A}$ ♀	172	168	103	55	9.9	25.3*	2.5*	–
fln^0/fln^+ ♀	171	198	115	70	9.4	25.7	2.4	–
fln^+/fln^+ ♀	163	212	103	65	8.9	25.7	2.3	–
sply ♀	178	215	96	67	12.0	23.9	2.9	–
drd ♂	116	179	29	12	2.6	23.9	0.6	+

cross-bridges changes the equilibrium between the recovery and the relaxation state, in which the myosin is fully detached from the actin filament.

The viscoelastic properties of wild type A-IFM that are the frequency-dependent changes in composite stiffness of isolated, skinned A-IFM fibers, significantly differ from fibers of $Mlc2^{S66A}$, $Mlc2^{S67A}$, and the double mutant $Mlc2^{S66A,S67A}$. Deconvoluted Nyquist plots of contraction and relaxation processes, however, suggest that the net oscillatory power delivered from the DLM fibers to the experimental apparatus is not significantly different between $Mlc2^{S67A}$ and wild-type controls, whereas $Mlc2^{S66A}$ and the double mutant exhibit a 33% and 31% reduction in peak *in vitro* power output of the isolated fibers, respectively, as shown in Table 6.1 (Dickinson et al., 1997).

Flight tests of *mlc*2 mutants

Despite the normal muscle ultrastructure, MLC2 mutation of the indirect flight muscle produces severe impairments of flight behavior in tethered flies during visually-invoked optomotor lift stimulation in a virtual reality flight simulator (Lehmann and Dickinson, 1997). Myosin phosphorylation-site mutant strains cannot achieve sustained flight, although they could generate at least a few wing strokes when stimulated with visual patterns. They exhibit a stereotyped flight initiation reflex upon removal of their tarsal substrate. Although the values of maximum flight force and maximum A-IFM mechanical power are lower in MLC2 mutants, most of these flies generate enough power and lift to support body weight. In $Mlc2^{S66A}$ and $Mlc2^{S66A,S67A}$, the stroke frequency during hovering flight is significantly below wild-type (Table 6.1). These mutants obviously compensate for a reduced number of cross-bridge cycles and wing stroke frequency by elevating the stroke amplitude of their wings and thus muscle strain. In $Mlc2^{S67A}$ little compensation is required, because the number of recruited cross-bridges is similar to wild type. The changes in MLCK of *Drosophila* in all substitution lines result in a decrease in A-IFM metabolic activity of up to 17% in the double mutation line $Mlc2^{S66A,S67A}$. Muscle efficiency, i.e., the efficiency of the ATP-mechanical conversion process in the A-IFM, is similar in MLCK mutants compared to wild type and varies between 9.7 and 9.9% according to Table 6.1.

Flightin

Flightin is a novel 20 kDa, multiply phosphorylated, myosin binding protein found in indirect flight muscles of *Drosophila* and other insects with asynchronous flight muscles. A null mutation in the flightin gene (fln^0) severely compromises thick filaments of the myofibril assembly and muscle integrity resulting in muscle degeneration and loss of flight ability. The myofibril is a multiprotein structure designed to produce and transmit contractile forces through the interaction of myosin containing thick filaments and actin containing thin filaments. In insect indirect flight muscles, these filaments are organized in a double hexagonal lattice and, as in other striated muscles, are stabilized laterally by structures at the M-line and Z-band. In particular, thick filaments are anchored at the center of the sarcomere through their association with unknown M-line proteins, and connected to the Z-band through projectin and kettin. In addition to myosin heavy chain (MHC) and its two associated (regulatory and essential) light chains, paramyosin/mini-paramyosin and flightin have been shown to be essential for normal thick filament development and elevated stiffness of the asynchronous muscle fiber (Arredondo et al., 2001; Liu et al., 2003; Reedy et al., 2000). A single amino acid substitution in the myosin rod (glutamate 1554 to lysin, the Mhc^{13} allele) prevents the accumulation of flightin *in vivo* and its binding to MHC *in vitro* (Kronert et al., 1995). Thus, similar to the flightin null mutation, Mhc^{13} flies are near flightless and their A-IFM undergoes a time-dependent hypercontraction that is characterized by myosin proteolysis, thick filament instability, and sarcomere degeneration.

Muscle structure of flightin "resuced" and "tetraploid" lines

To better understand the role of flightin in A-IFM function and flight behavior of *Drosophila*, previous studies generated transgenic animals that express a chimeric Actin88F promoter-flightin gene construct in wild-type (fln^{+}/fln^{+}) and flightin null (fln^{0}/fln^{0}) genetic backgrounds. Transgenic P-element transformed P[fln^{+}] fln^{0} "rescued" flies have a smaller number of thick filaments per myofibril than wild-type flies (782 vs 945) but have normal A-IFM, while transgenic P[fln^{+}] fln^{+} "tetraploid" flies have normal number of thick filaments. Flightin expression levels in both transgenic strains are similar to wild type. In contrast, flightin expression levels are reduced in a myosin heavy chain "tetraploid" strain that produces excess myosin and excess thick filaments. It has thus been suggested that regulation of flightin expression is independent of gene copy number and that the number of thick filaments assembled per myofibril is influenced independently by myosin and flightin expression (Barton et al., 2005).

Flight essays and flightin phenotypes

The functional significance of flightin for flight behavior and power output in *Drosophila* may be estimated *in vitro* by sinusoidally stretching and relaxing A-IFM skinned fibers in a force rack and measuring mechanical force production while the fibers undergo a full stretching–relaxation cycle (*work loop technique*). The applied length changes are similar to those that occur during flight and typically amount to 2%–3% the muscle's resting length (Barton et al., 2005). An alternative method to estimate A-IFM power output *in vivo* is to determine the power requirements for flight using aerodynamic theory (Casey et al., 1981; Ellington, 1984). Estimates of power requirements result from biomechanical and aerodynamic considerations such as energetic cost to overcome wing inertia (inertial power) during flapping motion, aerodynamic drag on wings (profile power), the energetic costs to generate lift (induced power) and drag on the animal body during forward motion (parasite power). The latter method has been applied to tethered animals flying in a virtual reality flight simulator (Lehmann and Dickinson, 1997), and flight force estimates were derived from unrestrained animals flying in a cylindrical free-flight arena under optomotor stimulation (Mronz and Lehmann, 2008; Figs 6.2A, 6.3C–D).

The mechanical properties of A-IFM skinned fibers during the sinusoidal stretching show no significant differences in active viscoelastic properties (dynamic stiffness) in flightin "rescued" and "tetraploid" transgenic flies vs. wild type (Barton et al., 2005). By contrast, mechanical analyses of skinned fibers from newly eclosed fln^{0} and Mhc^{13} flies show similar deficits in passive and dynamic stiffness, and a loss of the stretch activation response resulting in no net positive work output, compared to wild type controls. This effect is most likely due to an internal absorption of much of the actomyosin generated force (Henkin et al., 2004). Dynamic stiffness is composed of the elastic and the viscous modulus, where the elastic modulus is a measure of the fiber compliance. In "rescued" flies it statistically corresponds to the wild type at the frequency of maximum power generation. The "rescued" fibers produce normal, triphasic responses, indicative of restoration of wild-type function. The viscous modulus is a measure of the work produced (negative values) and work absorbed (positive values) by the fiber during stretching. In the flightin "rescued" fibers it is nearly identical to wild type and the value at the frequency of maximum power is also not statistically different from wild type.

Tethered and free flight capacity of flightin mutants

While flying in a virtual reality flight simulator, flight performance of transgenic "rescued" and multi-gene copy "tetraploid" lines of tethered *Drosophila* is significantly reduced during maximum locomotor capacity compared to wild type flies. Although transgenic flies generate enough flight force to sustain hovering flight, their reduced capability to produce flight force in excess of hovering flight force is due to a reduction in stroke amplitude ("tetraploid" line) and frequency ("rescue" and "tetraploid"). Muscle (~10%) and aerodynamic (~26%) efficiency appear to be similar in transgenic and wild-type lines. Thus, the reduced myofibrillar diameter in "rescued" A-IFM does not appear to have a deleterious effect on flight parameters and dynamic stiffness (Barton et al., 2005). The muscle mechanical power output from "rescued" fibers is more similar to wild-type than the corresponding values from "tetraploid", although the latter has the normal number of thick filaments per sarcomere. Likewise, normalized force and mechanical power measured in the flight simulator for "rescued" flies are more similar to wild-type values than to "tetraploid" values, as are muscle and aerodynamic efficiency. The observation that all values follow a similar trend suggest that the presence of extra copies of the flightin gene, while restoring the quota of thick filaments, has a moderately unfavorable effect on flight muscle function in tethered flight of *Drosophila*.

The reduction in maximum flight force production in "rescued" and "tetraploid" flightin lines has a pronounced effect on locomotor behavior and aerial capacity during free flight. Compared with tethered flight, freely flying *Drosophila* must keep its balance, cope with parasitic drag to overcome the drag on the moving body, and compensate for centrifugal forces to keep on track during turning behavior (Fig. 6.2B). During fast yaw turning, centripetal forces opposing centrifugal forces are up to 70% of the total locomotor reserve that is available for maneuvering flight and pay load in *Drosophila* (Mronz and Lehmann, 2008). Consequently, besides maximum flight endurance and maximum forward velocity, the reduced ability to boost aerodynamic force production during directional turning is considered a major factor that attenuates free flight behavior in transgenic lines.

Maximum locomotor performance of a freely cruising fly can be elicited by optomotor stimulation via rotation of the fly's visual environment. While trying to compensate for the

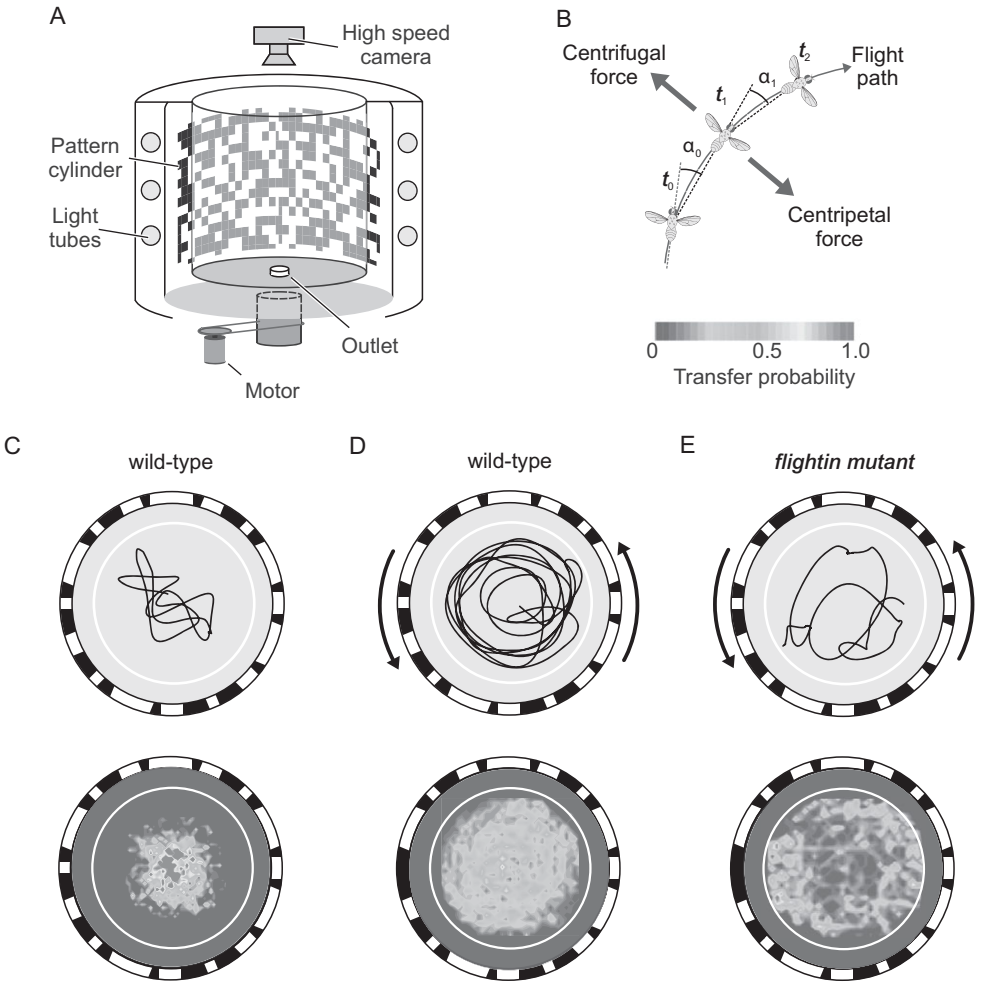

Fig. 6.2. Free flight essay and free flight behavior in *Drosophila*. A Flight arena used for scoring maximum flight capacity in freely cruising flies in a stationary visual environment and during optomotor stimulation by rotation of the surrounding random dot pattern. B Yaw turning in *Drosophila* produces centrifugal forces that need to be compensated by the production of centripetal flight forces. If locomotor reserves of the fly are small, elevated centrifugal forces may cause unwanted side-slipping movements of the animal. C–E Top view on flight paths of single *Drosophila* (upper row) and mean transfer probability of several flies (lower row) during flight in a stationary visual environment in *c* and during optomotor stimulation with a 500° s^{-1} rotating environment in *d* and *e*. The traces in *c* and *d* are measured in wild type, the data in *e* are collected from an A-IFM mutant (flightin fln^0/fln^+ 'rescued' mutant) with reduced flight muscle mechanical power output. Mutants with limited A-IFM power output cannot cope with elevated centrifugal forces during yaw turning while flying forward at fast speed, and are thus pulled away from the arena center.

visually induced retinal slip, *Drosophila* reaches its maximum motor capacity at a maximum forward velocity of 1.2 m s^{-1} and 500° s^{-1} directional turning rate (Mronz and Lehmann, 2008). Since "rescued" and "tetraploid" flightin lines exhibit a 29% and 34% reduction in peak A-IFM power output, respectively, compared to wild type, they are unable to fully compensate centrifugal forces during directional turning. In a cylindrical free flight arena, this causes unwanted side-slipping movements, pulling the animal towards the surrounding walls of the experimental setup. Transfer probability plots indicate that wild-type flies are able to stay closer to the center of the arena while turning, owing to their ability to produce elevated centripetal flight forces (F.-O. Lehmann, unpublished results, Fig. 6.2D–E).

Effect of sphingosine-1-P lyase on A-IFM function and flight behavior

Sphingolipids are important cell complex lipids. As components of membrane lipid rafts or as second intra-extracellular messengers, they ubiquitously participate in determining cell fate under stress conditions, apoptosis, and in *Drosophila* also in egg-laying behavior. Sphingosine-1-phosphate lyase mainly catalyzes the conversion of sphingosine-1-phosphate to fatty aldehyde and ethanolamine phosphate (Hannun et al., 2001; Merrill jr *et al.*, 2001; Prieschl and Baumruker, 2000; Pyne and Pyne, 2000). Bioactive sphingolipid metabolites, ceramide, sphingosine and sphingosine-1-P generate opposite effects, where sphingosine and ceramide action upon cellular functions or fate are opposed by sphingosine-1-P action. This balanced functional activity between bioactive sphingolipids has also been referred to as the sphingolipid rheostat (Herr et al., 2003).

At the level of flight muscle tissue, sphingolipids seem to play a functional role in the regulation of physiological adaptations to fatigue and activity of the plasma membrane, the sarcoplasmic reticulum calcium channels in vertebrates and A-IFM calcium channels in fruit flies. Adult fruit flies carrying a null mutation in the *sphingosine-1-P lyase (sply)* gene, encoding for a terminal key enzyme in the sphingolipid metabolic pathway, accumulate upstream metabolites such as sphingosine-1-P, sphingosine, and ceramide. This accumulation leads to near flightless phenotypes associated with an asymmetric degeneration of single muscle fibers, while the remaining A-IFM fibers

apparently remain completely intact (Herr et al., 2003; Sonnenmoser et al., 2011). Ultrastructural analysis of degenerating fibers shows the presence of mitochondrial swelling.

A-IFM structure and function in *sply*

S*phingosine-1-P lyase* null mutants are characterized by a loss of one or two of the 12 dorso-longitudinal (DLM) muscle fibers of the thorax. The hemisoma of $sply^{05091}$ has on average only 4.14 fibers and $sply^{14a}$ 5.97 DLM fibers, compared to the six fibers of the intact muscle. Despite the reduction in number of DLM fibers, the total cross sectional area of the DLM is similar in *sply* and wild type of approximately 11 000 μm^2 (Sonnenmoser et al., 2011). The intact A-IFM fibers in *sply* thus slightly increase in diameter, apparently compensating for the loss of muscle tissue. According to low magnification electron microscopy, the intact structure of *sply* muscle fibers suggest only minor changes in flight behavior. This assumption is further fueled by electrophysiological experiments, showing similar resting potentials of the DLM membrane (~-82 mV) and similar mean latency between an electrical brain stimulus and the onset of muscle action potential by muscle activation via the giant-fiber-pathway (~1.6 ms) in *sply* and wild-type *Drosophila* (Sonnenmoser et al., 2011). However, behavioral flight tests indicate severe attenuations in locomotor capacity of this mutant during tethered flight conditions.

Tethered flight capacity of *sply* transgenic *Drosophila*

The virtual reality flight simulator allows us to estimate maximum locomotor capacity in tethered flying *Drosophila* by means of visual stimulation. In the attempt to stabilize the motion of a visual stimulus on its retina via the optomotor feedback, *Drosophila* maximizes its locomotor output by increasing stroke amplitude in response to vertically, upward moving horizontal stripes (Lehmann and Dickinson, 1997; Fig. 6.3E). Under these flight conditions, aerodynamic force production of *sply* transgenic lines is reduced up to ~29% compared to wild-type controls (Table 6.1). Morphometric analyses suggest that this loss in peak force production is due to a significant reduction in wing length and wing area, while maximum stroke amplitude and frequency are not significantly different between *sply* and wild type. Despite their attenuation in maximum locomotor capacity, tethered *sply* mutants are able to generate flight forces close to hovering force, owing to a reduction in body mass. However, since total DLM cross-sectional area differs only little between transgenic line and wild type, while aerodynamic force production is significantly reduced, *sply* causes a 34% reduction in muscle tension per muscle cross-sectional area. The ultimate reason for this loss in contraction strength is not known yet, because the electrophysiological properties of *sply* A-IFM fibers appear to be normal.

Considering the role of *sply* for the function of muscle calcium channels, however, it appears possible that *sply* attenuates A-IFM calcium activation due to a reduction of calcium influx from the extracellular space. A reduction in intramuscular calcium level lowers the ability of the A-IFM to maximize mechanical power output, which was demonstrated by calcium imaging of the *Drosophila* A-IFM using the trangene calcium indicator Cameleon (Gordon and Dickinson, 2006). This assumption is in agreement with the finding that muscle efficiency of S*phingosine-1-P lyase* null mutants is similar to that of wild-type controls (~12%). It is also in agreement with the muscle efficiency measures of *Mlc*2 trangenic *Drosophila* lines because both mutations are thought to reduce the probability of A-IFM cross-bridge cycling, which lowers ATP cleavage and thus metabolic costs.

Drop-dead mutation and A-IFM oxygen shortage

While wild-type *Drosophila* live several weeks after eclosion, *drop-dead* mutants have shortened live-spans and typically die within several days after hatching. *Drop-dead* carries an X-chromosomal recessive mutation that causes brain degeneration, due to a loss in glia function. Thus, in *drop-dead* (drd^1, drd^{x1}) most neurons lack their complete glia sheaths (Buchanan and Benzer, 1993). In *drd* there is an acceleration of temporal pattern expression in some age-related markers (lacZ-marked wg, en, and 206) whose expression pattern of β-gal is correlated with aging (Bier et al., 1989; Freeman, 1991; Helfand et al., 1995; Helfand and Naprta, 1996; Kassis et al., 1991; O'Kane and Gehring, 1987). It has thus been concluded that the normal *drd* gene product prevents brain degeneration by establishing glia function (Buchanan and Benzer, 1993).

The role of the *drd* gene product is not only limited to brain function. A recent study on gut function in *Drosophila* has shown that mutants carrying the strong allele drd^{lwf} have reduced defecation rates and increased volumes of crop contents, including an abnormal spontaneous motility of the crop (Peller et al., 2009). The *drd* gene product facilitates the transfer of food from the crop to the midgut of the animal. Drd^{lwf} mutants also have abnormal triglyceride and glycogen stores within the first four days after eclosion. Unpublished results on drd^{x1} associate glia cell degeneration with a lack of tracheole cell development. X-ray phase-contrast imaging in a synchrotron shows that drd^1 males yield pronounced changes in tracheal geometry compared to wild type (F.-O. Lehmann, unpublished observations). Major tracheal air sacs in the thorax and head of drd^1 males are consistently reduced or completely collapsed, suggesting a severe change in tracheolar diffusivity for respiratory gases and thus oxygen supply to flight muscles and the nervous system (Fig. 6.3A). The latter findings link neurodegeneration with the function and development of the tracheal system in insects. Assuming that an abnormal tracheal development with subsequent degradation of both oxygen supply (oxygen shortage, hypoxia) to the mitochondria and elimination of carbon dioxide from the nervous and muscle tissue provokes the *drd* phenotype, demanding respiratory processes such as locomotor behaviors and in particular flight should be severely impaired in this mutant. Behavioral tests on walking flies demonstrate that *drd* null mutants are less motivated to

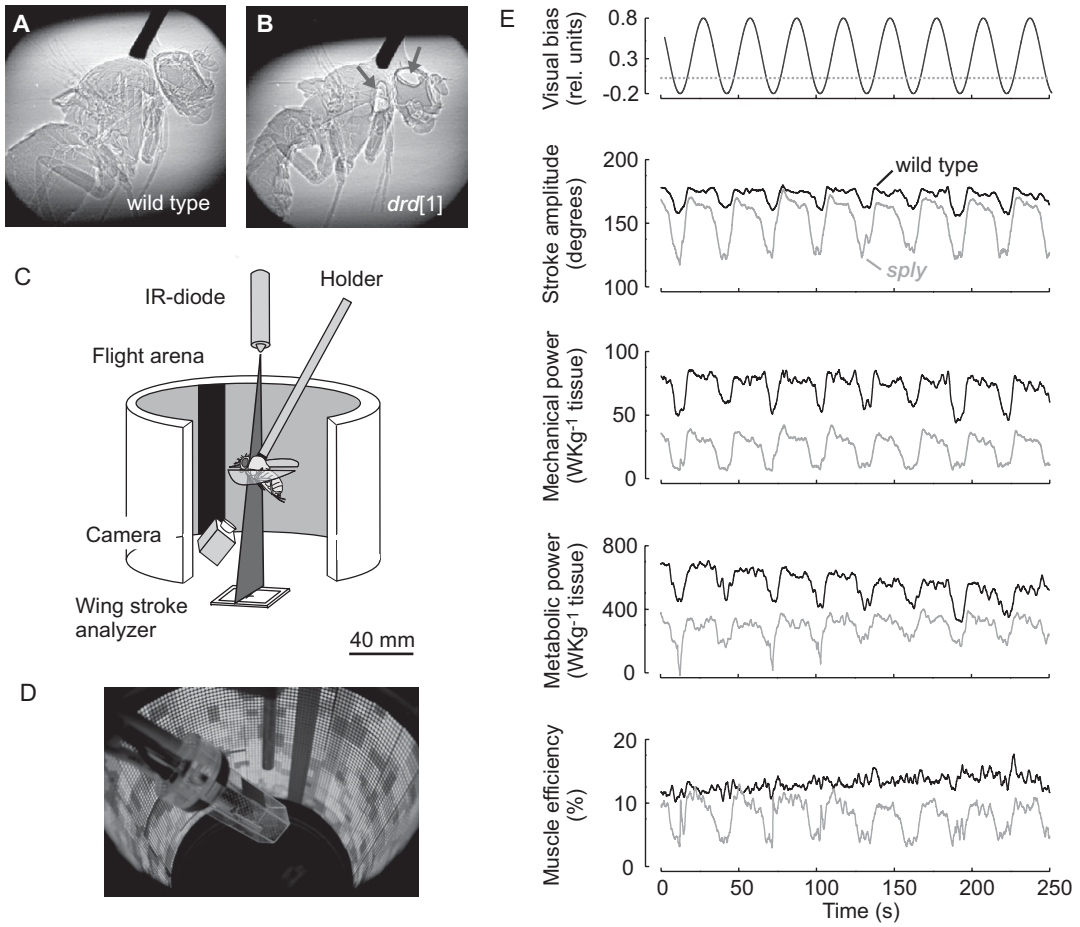

Fig. 6.3. Behavioral essay for scoring wing motion and flight performance in tethered flight of *Drosophila*. A X-ray phase-contrast images of wild-type male *Drosophila* and B the mutant *drop-dead* during tethered flight in a synchrotron at Argonne APS (Illinois, USA). Red arrows indicate collapsed air sacs of the fly's tracheal system inside the thorax and head of the *Drosophila* mutant which supposedly cause hypoxia under high respiratory demands in flight. C Schematic drawing of a closed-loop visual feedback flight simulator for tethered flying fruit flies. The animal is tethered to a holder and wing motion is measured via an infra-red light path (red). The shadows of the beating wings are tracked by an optical wing stroke analyzer. Visual patterns are computer-controlled and displayed inside the cylindrical simulator. The pattern moves according to the fly's actions. D Image of the cylindrical flight simulator in *c* showing a random-dot visual pattern and a respiratory chamber for measuring in-flight carbon-dioxide release in single flies. E Simultaneous changes in wing motion, flight muscle mass-specific A-IFM power output, mass-specific metabolic power, and muscle efficiency in wild-type and *sply* mutants, responding to a vertically oscillating random-dot open-loop visual pattern and while yaw-heading in closed-loop towards a vertical black stripe (see pattern in *D*). Minimum and maximum locomotor capacity of *Drosophila* can be retrieved from the changes in optomotor response due to the motion of the random-dot background pattern. Stroke amplitude and muscle power are minimum when the visual pattern moves downward and maximum when the pattern moves upward inside the flight simulator.

run, while speed and path trajectories are similar to wild type (Lehmann and Cierotzki, 2010).

Staggering behavior and free flight essay

A usual and prominent behavior in *drd*^{1} is the recurrent complete loss in body posture control termed "staggering" (Buchanan and Benzer 1993). Stagger behavior in *drd* prevents the fly from regular leg coordination and can clearly be distinguished from other types of locomotor behaviors such as grooming and walking. Stagger bouts are initiated randomly and their rate of occurrence increases within 5 days after eclosion until the mutant dies. The decrease in body stability with increasing age is, though less pronounced, also measurable in wild type (Lehmann and Cierotzki, 2010). This supports the assumption that the *drd*^{1} phenotype is related to an ageing process, rather than implying a dedicated behavioral deficit for locomotion.

The two findings, oxygen shortage and stagger behavior, have recently fueled an experimental evaluation of the *drd* mutation in flight (F.-O. Lehmann, unpublished observations). Owing to the changes in tracheal development and oxygen supply, *drd*^{1} males suffer from a significant loss in their flight motivation and capacity during free flight, despite a 28% reduction in body weight. Flight behavior and carbon dioxide release patterns of *drd*^{1} are more variable compared to wild type. When tested in free-flight on their ability to take-off and to sustain hovering flight force, *drd*^{1} males are weak fliers on the first day after eclosion, when only 25% of the animals generate sufficient lift to support their body mass (wild type, ~90%). With increasing age, *drd*^{1} increasingly looses its ability to initiate and maintain flight, whereas wild-type flight capacity changes only

slightly. Approximately 75% of *drd*^{1} mutants are flightless before they die on day five. Free-flight capacity in *drd*^{1}, however, transiently recovers on the second day after eclosion. The reason for this transient recovery is unknown until now.

Flight of drop-dead transgenic flies in a flight simulator

The response of tethered flying *drd*^{1} animals to optomotor lift stimuli presented inside a virtual reality flight simulator confirms the results recorded in free flight essays (Fig. 6.3C,D). On average, 2 days old *drd*^{1} males are not capable to achieve active flight, because their mean lift production amounts to only 30% of the force required to compensate for their body weights. Stroke amplitude and stroke frequency is reduced compared to wild type *Drosophila* (Table 6.1). The low wing flapping speed in *drd*^{1} lines reduces the A-IFM power requirements for wing flapping by 84% mainly owing to a reduction in aerodynamic drag. Flight metabolic activity in *drd*^{1} decreases accordingly, but out of proportion. Thus, unlike A-IFM efficiency in mutants with altered A-IFM development and ultrastructure (*sply*), muscle stiffness (*fln*) or cross-bridge cycling (*Mlc*2), muscle efficiency in *drd* is significantly reduced (~2.5%) compared to wild type controls (~11%). Assuming that the electrophysiological properties of the muscle membrane and calcium activation capacity of *drd* A-IFM are normal, reduced A-IFM oxygen supply apparently produces a *Drosophila* phenotype in flight that is different from muscle mutants with alterations in flight muscle stiffness and phosphorylation-dependent cross-bridge cycling.

Concluding remarks

Genetic dissections of traits associated with *Drosophila* flight are challenging because flight results from a multi-step, mulit-level locomotor feedback cascade. Current research on the role of biogenic amines largely highlights flight control on the level of motor activity and motivation, while research on the significance of the flight muscular system opens a window towards an understanding of power delivery and kinematic control of wing motion. All A-IFM mutants tested on their maximum muscle mechanical power output, muscle efficiency and aerial performance in free and tethered flight paradigms, show pronounced changes in flight behavior due to a reduction in muscle power. The energetic costs for flight typically change in proportion with this changing power production, thus, muscle efficiency of the A-IFM does not differ between transgenic and wild type flies, except in mutants facing an A-IFM oxygen shortage. Since flight in *Drosophila* is inherently unstable with respect to body posture control, the sensory and neuromuscular requirements for flight are much higher than those for walking or other motor behaviors. It thus appears reasonable to assume that posture control during aerial maneuvers reflects a behavioral output close to the animal's maximum locomotor capacity, with respect to power, speed and accuracy. Consequently, genetically induced alterations of the locomotor cascade should impair flight more strongly than any other type of locomotor behavior. This is of relevance in particular with respect to research on human motor disorders, since some genes that affect development and function of the *Drosophila* locomotor system are also relevant in humans. The goal of this book chapter was to highlight this issue, presenting results on flight behavior and locomotor performance in transgene *Drosophila*. Ultimately, this research contributes to our understanding of how aerial maneuverability is achieved and determined in an animal that employs the energetically most demanding tissue for locomotion – the asynchronous indirect insect flight muscle.

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Chapter 7

Behavioral genetics of *Drosophila* female post-mating responses

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Introduction

Drosophila melanogaster spend most of their larval lives gathering resources, and their pre-mating adult lives maturing sexually and gaining access to mates. However, once a sexually mature female fly has mated, there is a critical transition in her allocation of time and resources: the female's "priorities" shift towards producing progeny, facilitated by the postmating storage of sperm that prolongs her reproductive capability for weeks after mating. The female's reproductive tract also undergoes its final maturation only after mating (Kapelnikov et al., 2008a).

Part of this post-mating transition includes behavioral changes that are long lasting, cut across a wide array of behaviors, and resculpt the way that many behavioral programs are executed. In other words, mating acts as a "social modulator" of behavior, triggering a broad, concerted suite of behavioral effects through stimuli such as seminal fluid proteins, pheromones, and sperm. As will be discussed in this chapter, mating results in dramatic changes in an array of behaviors, including egg-laying behavior, mating receptivity, appetitive behavior, and locomotor activity.

It is noteworthy that in many cases[1] mating acts to increase, decrease, or modify behaviors that the female is already performing, rather than activating entirely new behavioral programs. After mating, females become much less receptive to – and more likely to actively reject – male courtship advances. Mated females lay large numbers of fertilized eggs. While virgin females are capable of ovulating and ovipositing eggs, the stimulus of mating causes these processes to occur at much higher rates. Mating also modulates female feeding behavior, increasing the amount of feeding and changing dietary preferences. Females change their diurnal activity patterns in response to mating, increasing their daytime locomotion. Finally, in addition to producing more eggs, mated females actively store and utilize sperm.

A substantial effort towards investigating *Drosophila* social behavior has been applied to understanding innate social behaviors (esp. courtship and aggression), and these model systems have proven to be very informative. Unlike preprogrammed innate behaviors, post-mating changes reflect a facultative behavioral switch[2]. Post-mating behavioral changes seemingly require neural plasticity in response to mating stimuli, although many of these plasticity mechanisms await elucidation.

Insect seminal fluid proteins (Sfps) induce major post-mating effects, including effects on female post-mating behaviors (Sirot et al., 2009; Avila et al., 2011). Some of these effects occur only for a short time: for example, the *D. melanogaster* Sfp ovulin stimulates ovulation only on the first day after mating (Heifetz et al., 2000). Other effects persist for much longer after mating, contributing to the "long-term response" in *D. melanogaster*: for example, the Sfp Sex Peptide (SP) stimulates egg production and decreases receptivity by females for several days after mating (Chapman et al., 2003; Liu and Kubli, 2003; Peng et al., 2005).

Female flies exhibit more dramatic and well-characterized changes in behavior after mating than males, so in this chapter we focus only on female postmating behaviors. Specifically, we will discuss how mating causes changes in females' mating receptivity, egg-laying behavior, feeding, locomotor activity, egg production, and sperm management. We will argue that the study of postmating behavior is a powerful model system for learning how even a single social experience can dramatically alter diverse behavioral programs.

Courtship receptivity

A courting male displays a stereotyped behavior, tapping the female's abdomen, extending and vibrating a wing, and attempting copulation (Villella and Hall, 2008). Sexually mature virgin females are receptive to male courtship, but after mating the female's likelihood of remating plummets by approximately 90%, as assessed in 1-hour assays, for several days, and females

[1] The exception here is sperm storage, which is discussed later.

[2] To be sure, innate behaviors and socially responsive behaviors are not mutually exclusive. Social experiences affect innate behaviors in the short term by eliciting them and in the long term, for example, through courtship conditioning and aggressive experience. Also, the ability to respond to social cues is innate.

Behavioral Genetics of the Fly (Drosophila melanogaster), ed. J. Dubnau. Published by Cambridge University Press.

that do mate exhibit longer latencies to mating (Manning, 1962; Kalb et al., 1993; Liu and Kubli, 2003). An unreceptive female will flee courting males or actively reject courtship attempts by kicking the male or by extruding her ovipositor (Connolly and Cook, 1973).

The postmating decrease in female receptivity occurs in two phases (Tram and Wolfner, 1998), each controlled by different male Sfps: PEBII acting in the first several hours after mating (Bretman et al., 2010), and SP maintaining low receptivity for the next several days (Chen et al., 1988; Chapman et al., 2003; Liu and Kubli, 2003). PEBII is a major component of the posterior mating plug, a solid mass that fills the posterior of the mated female uterus. PEBII suppresses remating for approximately 4 hours after the initial mating. This timing corresponds well with the period during which the mating plug remains in place: the mating plug is pushed out before oviposition of the first egg (approximately 3 hours after mating). We do not know how PEBII inhibits remating: it could act chemically by affecting receptor-signaling or physically by maintaining the integrity of the sperm plug, which may, for example, stretch the uterus and stimulate sensory neurons (Clark and Lange, 2001; Häsemeyer et al., 2009; Yang et al., 2009).

While PEBII's short window of activity dovetails with the ephemerality of the sperm plug, SP's longer-term effect is due to the long-term storage of sperm (Manning, 1962; Chapman et al., 2003; Liu and Kubli, 2003). SP is a 36-amino acid peptide that exerts multiple effects on mated females (see below), including nearly eliminating female receptivity to mating. SP binds the sperm inside the female, so when females store sperm, they store SP as well. The gradual proteolytic cleavage and release of SP from sperm tails maintains an active titer of "free" SP activity (Peng et al., 2005).

Using autoradiography, labeled SP was seen to bind targets in the female brain, ventral ganglia, and oviduct (Ottiger et al., 2000; Ding et al., 2003) (See Fig. 7.1 for a diagram of the structures referred to in this chapter). The SP receptor (SPR) was identified in an RNAi screen for neural genes that are necessary in females for postmating receptivity and egg-laying behaviors (Yapici et al., 2008). SPR is a G-protein coupled receptor that likely reduces PKA signaling (Chapman et al., 1996; Yapici et al., 2008; Yang et al., 2009; Poels et al., 2010). *SPR* is expressed in most of the regions in which SP binding has been observed, including the suboesophageal ganglion, the cervical connectives, the ventral ganglia, and the oviduct (Yapici et al., 2008).

SPR expression in a remarkably limited set of neurons, as few as three neurons per paired cluster of multidendritic sensory neurons along the uterus of the female reproductive tract (Fig. 7.1), is necessary and sufficient for SP's effect on receptivity (and egg-laying, see below) (Häsemeyer et al., 2009; Yang et al., 2009; Rezával et al., 2012). The location of these SP effector neurons is consistent with the identification of SP binding sites at the oviduct (Ottiger et al., 2000; Ding et al., 2003). The subset of sensory neurons capable of inducing the SP response coexpress *pickpocket* (*ppk*), a marker for peripheral, proprioceptive sensory neurons (Grueber et al., 2007), with *fruitless* (*fru*), (Ryner et al., 1996; Lee et al., 2000; Usui-Aoki et al., 2000; Kvitsiani and Dickson, 2006; Villella and Hall, 2008; Häsemeyer et al., 2009; Yang et al., 2009) and *doublesex* (*dsx*) (Billeter et al., 2006; Kimura et al., 2008; Rideout and Goodwin, 2008; Villella and Hall, 2008; Rideout et al., 2010; Rezával et al., 2012), sex determination genes with sex-specific splicing (note that sex-specific *fru* protein products are only found in males).

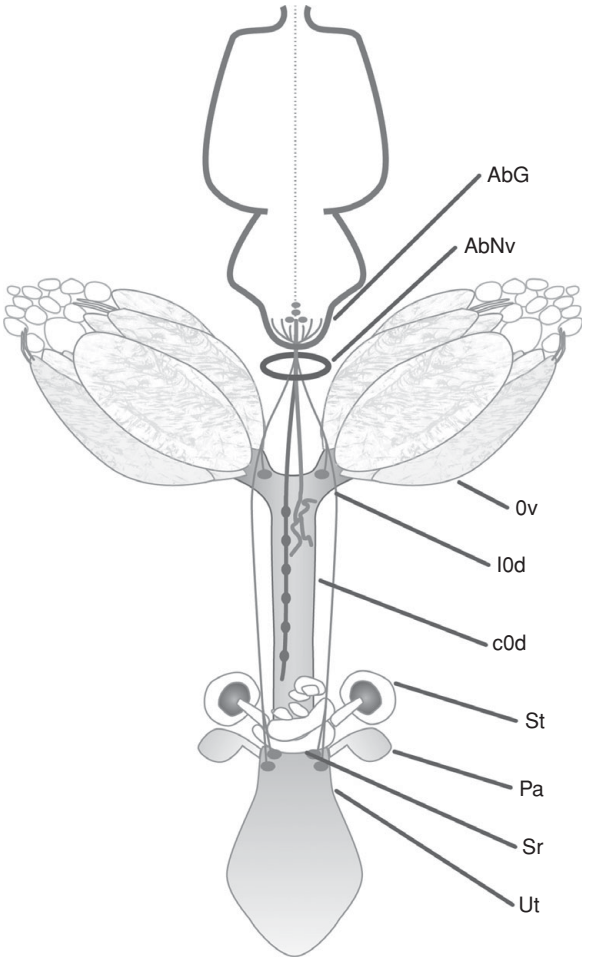

Fig. 7.1. The female reproductive tract and its innervation by the nervous system. OA and glutamate (Glu, green) neurons project their axons through the abdominal nerve (AbNv), innervating many compartments of the reproductive tract (Monastirioti, 2003; Middleton et al., 2006; Rodríguez-Valentín et al., 2006; Kapelnikov et al., 2008a). Innervation from OA neurons (pink) in the abdominal ganglia (AbG) can be observed throughout the lateral and common oviducts (lOd and cOd, respectively) and the ovaries (Ov), but is limited here for clarity. Only unilateral depictions of bilaterally symmetric OA and Glu axons are shown. Neurons releasing Glu and OA are depicted as distinct, but both factors might in fact be co-released (Rodríguez-Valentín et al., 2006). Multidendritic fru^+/ppk^+ sensory neurons critical for SP's postmating behavioral changes (blue) are found on the lOds and the anterior uterus (Ut), and project to the central nervous system, ramifying in the AbG and perhaps in the brain (Häsemeyer et al., 2009; Yang et al., 2009). Spermatheca (St), parovaria (Pa; also known as female accessory glands), and seminal receptacle (Sr).

Specifically, post-mating responses can be affected by silencing either fru^+ neurons (resulting in increased rejection in virgins) (Yang et al., 2009) or dsx^+ neurons (increased remating in mated females) (Rideout et al., 2010; Rezával et al., 2012). Further, either ectopically expressing membrane-bound

SP (mSP) or restoring SPR expression in an SPR mutant background in *fru*$^+$ or *dsx*$^+$ neurons induces a post-mating response in virgin females (Häsemeyer et al., 2009; Yang et al., 2009; Rezával et al., 2012). However, these inductions of post-mating behavior are suppressed when SPR expression is suppressed in *ppk*$^+$ neurons (Häsemeyer et al., 2009; Yang et al., 2009; Rezával et al., 2012). The most parsimonious explanation is that an overlapping subset of neurons expressing all three *ppk*$^+$/*fru*$^+$/*dsx*$^+$ markers is sufficient to induce a post-mating response. It is noteworthy that, while ectopically expressing mSP in *ppk*$^+$neurons does induce a post-mating response, it does not induce the full postmating response (Yang et al., 2009). Additionally, ectopically expressing mSP in *dsx*$^+$ cells while suppressing mSP expression in *ppk*$^+$ neurons does not fully eliminate the postmating response (Rezával et al., 2012). This could indicate that SP requires *fru*$^+$/*dsx*$^+$ neuronal populations other than *ppk*$^+$ neurons for the full postmating effect, suggesting that the complete postmating response circuit may not be contained only in the paired cluster containing three *ppk*$^+$/*fru*$^+$/*dsx*$^+$ neurons. In addition, using different expression paradigms, egg-laying behavior can be separated from receptivity, suggesting that subsets of the *ppk*$^+$/*fru*$^+$/*dsx*$^+$neurons may be contributing differentially to distinct post-mating responses (Rezával et al., 2012). Further, these experiments utilize ectopically expressed mSP, while endogenous, male-derived SP has been shown to bind to several places throughout the female (Ottiger et al., 2000; Ding et al., 2003). SP's post-mating behavioral effect also requires *apterous*-expressing interneurons of the ventral nerve cord that ascend to the brain (Ringo et al., 1991; Soller et al., 2006), although it is not clear where these neurons lie in the circuit controlling SP's effect on post-mating behavior. Nonetheless, since knocking-down SPR expression in *fru*$^+$ (Yapici et al., 2008; Yang et al., 2009) and *dsx*$^+$ (Rezával et al., 2012) neurons virtually eliminates the post-mating response, additional neurons beyond the small uterine cluster that are required for the full complement of the post-mating response are likely *fru*$^+$ or *dsx*$^+$ or both.

Curiously, SPR is not predominantly localized along the somata or dendrites of the critical *fru*$^+$/*ppk*$^+$ sensory neurons, although these regions are adjacent to the reproductive tract, where SP is most highly concentrated. Instead, SPR is most abundant along the axons that project to the central nervous system (Yang et al., 2009), suggesting the possibility that SP may need to enter the hemolymph (Peng et al., 2005; Pilpel et al., 2008) or be actively localized to exert its behavioral function.

The *fru*$^+$/*ppk*$^+$ and *dsx*$^+$/*ppk*$^+$ sensory neurons project to the central nervous system and ramify extensively in the abdominal ganglia and in the suboesophageal ganglion (Häsemeyer et al., 2009; Yang et al., 2009; Rezával et al., 2012). It has been noted (Häsemeyer et al., 2009; Rezával et al., 2012) that abdominal ganglia neurons control egg-laying behavior (Monastirioti, 2003; Cole et al., 2005; Rodríguez-Valentín et al., 2006), another behavior that is also increased by SP (see below). Additionally, the suboesophageal ganglia's proximity to the auditory center (Kamikouchi et al., 2006) may allow SP to regulate female responses to male courtship stimuli, such as courtship song. Since *fru*$^+$ and *dsx*$^+$ neurons are found in both of these regions in females (Billeter and Goodwin, 2004; Manoli et al., 2005; Stockinger et al., 2005; Rideout et al., 2010), SP receptor *fru*$^+$ neurons may in turn relay their information to *fru*$^+$, *dsx*$^+$, or *fru*$^+$/*dsx*$^+$ interneurons, which could propagate the signal throughout a *fru*$^+$-or *dsx*$^+$-labeled circuit controlling aspects of post-mating behavior (Kvitsiani and Dickson, 2006; Häsemeyer et al., 2009). Early support for such a *dsx*$^+$ network has been found by reducing neuronal activity in a small group of *dsx*$^+$ neurons in the abdominal ganglia, and observing that these females fail to decrease remating receptivity after mating (Rezával et al., 2012). These neurons send descending projections widely throughout the reproductive tract and ascending projections to the suboesophageal ganglion.

Interestingly, while *fru* and *dsx* mark neurons important for female post-mating receptivity (Kvitsiani and Dickson, 2006; Häsemeyer et al., 2009; Yang et al., 2009), *fru*$^+$ and *dsx*$^+$ circuits are critical for controlling other sex-specific behaviors, such as male aggression (Vrontou et al., 2006; Chan and Kravitz, 2007) and courtship (Ito et al., 1996; Ryner et al., 1996; Anand et al., 2001; Demir and Dickson, 2005; Manoli et al., 2005; Stockinger et al., 2005; Billeter et al., 2006; Datta et al., 2008; Kimura et al., 2008; Rideout et al., 2010; Yu et al., 2010; Meissner et al., 2011; Ito et al., 2012), which has been extensively reviewed (for review, Manoli et al., 2006; Yamamoto, 2007; Dauwalder, 2008; Villella and Hall, 2008; Siwicki and Kravitz, 2009).

SP is not the sole ligand for SPR: SPR is also activated by myoinhibitory peptides (MIPs) in *D. melanogaster* (Kim et al., 2010; Poels et al., 2010) and a *Bombyx mori* SPR ortholog is activated by a *B. mori* MIP ortholog (Yamanaka et al., 2010). Although MIPs bind to SPR with higher affinity than SP *in vitro*, MIPs do not induce an SP-like post-mating switch in behavior (Kim et al., 2010). Phylogenetic analyses of *SP*, *SPR* and *MIP* suggest that MIPs, rather than SP, may be the ancestral ligand of SPR: *SPR* and *MIP* orthologs can be unambiguously detected across insects (Kim et al., 2010; Poels et al., 2010; Yamanaka et al., 2010), whereas SP, to date, has only been detected in some *Drosophila* lineages and one mosquito lineage (Cirera and Aguade, 1998; Wagstaff and Begun, 2005; Dottorini et al., 2007). Two lines of evidence suggest that any ancestrally retained SPR functions regulated by MIPs are distinct from the SP/SPR control of post-mating behavior. First, MIPs do not induce SPR-regulated post-mating behaviors (Kim et al., 2010; Poels et al., 2010). Second, *SPR* and *MIP* are expressed in preadult stages and in both adult sexes, whereas *SP* is only expressed in adult males (Kim et al., 2010; Poels et al., 2010). Although SPR is sensitive to ligands other than SP, and SPR is expressed more widely than in *ppk*$^+$/*fru*$^+$/*dsx*$^+$ neurons along the reproductive tract, the post-mating switch seems nonetheless to be unique to SP and inducible at *ppk*$^+$/*fru*$^+$/*dsx*$^+$ sensory neurons.

Female pheromonal profiles change after mating (i.e., *cis*-vaccenyl acetate and 7-tricosene levels increase), and these changes can alter sexual attractiveness to suppress subsequent male courtship. These pheromonal changes have been

attributed to direct contributions from the male during mating as well as female changes in pheromone production after mating (for example, see Tompkins and Hall, 1981; Scott, 1986; Friberg, 2006; Yew et al., 2008; Billeter et al., 2009; Everaerts et al., 2010). These post-mating changes in pheromonal profiles strongly influence sexual behavior after mating, and the reader is referred to the above citations for further discussion of the topic.

Virgin females do reject courting males at low levels, including upon influence of social context (Billeter et al., 2012), so the neural circuitry required for courtship rejection is intact in virgin females (Connolly and Cook, 1973); however, mating greatly increases the likelihood of its activation. Several studies have identified mutants that exhibit constitutively high levels of rejection of courting males, independent of mating status (for review, see Yamamoto, 2007). For example, *icebox* mutations in the *neuroglian* gene decrease female mating and increase rejection of courting males; other behavioral traits, sperm storage, and lifespan appear normal in *icebox* mutant females (Kerr et al., 1997; Carhan et al., 2005). In another example, the *muscleblind* allele, *chaste*, decreases virgin females' mating probability, affecting female receptivity by increasing the rate of decampment from male courtship (Juni and Yamamoto, 2009). Females mutant in *dissatisfaction* show high levels of rejection as well as defects at neuromuscular junctions, which are discussed further below (Finley et al., 1997). Virgin females mutant in *spinster* also show high levels of rejection and exhibit synaptic overgrowth at larval neuromuscular junctions (Suzuki et al., 1997; Nakano et al., 2001; Sweeney and Davis, 2002; Sakurai et al., 2010). It is possible that the genes whose mutations suppress mating receptivity in virgin females might influence the postmating decrease in receptivity. In this scenario, the constitutive rejection behavior of *icebox*, *chaste*, *dissatisfaction*, and *spinster* mutant females might reflect a constitutive activation of the receptivity suppression circuit that is upregulated after mating. A number of these genes are important for neural development and might affect female behavior in response to mating stimuli (e.g., Sfps) by affecting neuronal plasticity. A prediction of this model is that the neural substrates affected by these mutations are also modulated by mating to reduce receptivity.

Egg-laying behavior

Once mature oocytes are produced in the ovary, the eggs must be released into the lateral oviduct (Fig. 7.1); this release is defined as ovulation. The eggs must then pass through the lateral oviduct, the common oviduct, and enter the uterus (also referred to as the genital chamber or bursa copulatrix) where they are fertilized and held. Once the female has found a suitable site for oviposition, the egg is extruded from the uterus and oviposited. Here, we refer to this entire process, from ovulation to oviposition, as egg-laying behavior. After mating, females dramatically increase their egg-laying behavior; this increase is most easily observed as an increased number of oviposited eggs. Virgin females may lay several unfertilized eggs each day, whereas a mated female will typically lay approximately 35–85 eggs within 24 hours after mating, then laying fewer each day for several days (Kalb et al., 1993; Chapman et al., 2003; Liu and Kubli, 2003). While the increase in oviposited eggs is the most obvious to casual observation, all aspects of egg-laying behavior are upregulated by mating.

Ovulation and egg progression through tract

Several male – and female – derived signaling molecules affecting ovulation have been identified. The nervous system sends multiple projections to the female reproductive tract that are instrumental for egg-laying behavior. Female signaling systems alone (i.e., before mating) allow low levels of ovulation. Males increase ovulation rates through compounds transferred during mating. For example, the male-derived Sfp ovulin induces a post-mating increase in ovulation rate.

The best-studied of the female signaling systems that regulate ovulation are neurons that release octopamine (OA). OA acts as a neuromodulator in the nervous system affecting a wide range of behaviors, including locomotion, aggression, flight and release of energy stores. The OA signaling system is often compared to the vertebrate "fight-or-flight" adrenergic system (e.g., see Roeder, 2005 for review and discussion). OA is synthesized from tyrosine, which is first metabolized to tyramine (TA) by tyramine decarboxylase 2 (TDC2). The enzyme tyrosine β-hydroxylase (TβH) then converts TA to OA in the nervous system (Barker et al., 1972; Monastirioti et al., 1996; Cole et al., 2005). Mutational analysis has permitted the dissection of processes that require OA function. For example, the null mutation $T\beta H^{M18}$, which eliminates OA but increases accumulated TA levels, abolishes ovulation: eggs are only observed in the ovary in these mutant females (Monastirioti et al., 1996). The mutant phenotype is rescued by feeding females OA, showing that the phenotype is caused by the loss of OA, not by an increase in TA. The mutant's defect is also rescued by driving $T\beta H$ expression in a group of abdominal ganglia neurons in $T\beta H^{M18}$ mutant females, indicating that OA signaling from abdominal neurons is critical for ovulation (Monastirioti, 2003). After mating, type II boutons at the oviduct neuromuscular junction (NMJ), which release OA (Middleton et al., 2006; Rodríguez-Valentín et al., 2006), increase in number, suggesting that OA signaling is increased by mating (Kapelnikov et al., 2008a).

OA controls ovulation by affecting muscle contractions in the reproductive tract. Pharmacologically applied OA enhances spontaneous contractions of the muscle sheath surrounding the ovary, and suppresses evoked contractions of the oviduct in isolated reproductive tracts (Cole et al., 2005; Middleton et al., 2006; Rodríguez-Valentín et al., 2006). These opposing effects on muscle contractions on different reproductive tract compartments led to the model that increasing signaling from this single neuromodulator may act on the ovarian muscle sheath to induce contractions and push the egg into the oviduct, while inducing the oviduct to relax and accept the egg (Middleton et al., 2006).

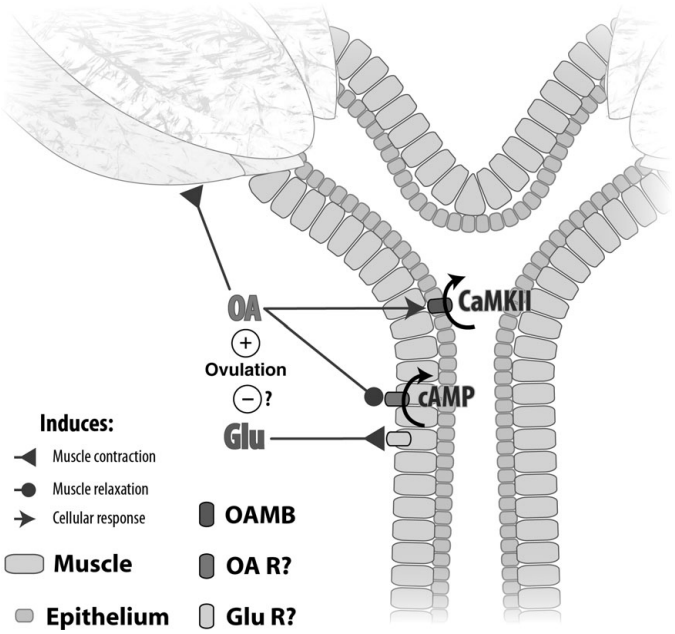

Fig. 7.2. Ovulation is increased by OA at the ovary and oviducts, acting on muscle and epithelium (Monastirioti et al., 1996; Middleton et al., 2006; Rodríguez-Valentín et al., 2006; Lee et al., 2009). OA activates contractions of the ovarian muscle (triangle synapse), but blocks contractions of the oviduct (circle synapse) via cAMP signaling. Glutamate (Glu) induces oviduct contractions (triangle synapse), perhaps decreasing egg-laying behavior. OA interacts with epithelium through the OAMB receptor and CaMKII signaling. The OA and Glu receptors for muscle (OA R? and Glu R?) await identification. OA might block oviduct contractions by acting in parallel with Glu on oviduct muscle or perhaps by presynaptically blocking Glu signaling (Nishikawa and Kidokoro, 1999).

While OA is clearly important for ovulation, a role for tyramine in this process in *Drosophila* remains to be clarified (but, for locust, see Lange, 2009). Pharmacological application of TA did not lead to detectable changes in spontaneous ovary contractions. The $tdc2^{RO54}$ mutant allele for the enzyme that converts tyrosine to tyramine also causes problems with egg-laying behavior. But, unlike $T\beta H^{M18}$ mutant females, $tdc2^{RO54}$ mutants do ovulate eggs. However, those eggs never proceed to the uterus to be oviposited (Cole et al., 2005). Females mutant for *tdc2* have neither TA nor OA, whereas *TβH* females have no OA, but accumulate elevated levels of TA. The difference between $tdc2^{RO54}$ and $T\beta H^{M18}$ ovulation phenotypes may stem from their differences in TA levels, since both mutants lack OA. These results suggest that TA may play another, unidentified role in moving ovulated eggs to the uterus (see Cole et al., 2005 for further discussion of relative roles of OA and TA in egg laying behavior). It may also be important to consider that tyramine may have distinct actions depending on its concentration, as suggested by evidence in the locust (Donini and Lange, 2004). The recent identification of TA-specific receptors in *D. melanogaster* may help clarify TA's role in ovulation (Cazzamali et al., 2005).

OA works in concert with glutamate (Fig. 7.2), the primary excitatory neurotransmitter at invertebrate NMJs, to affect contractions of the oviduct (Rodríguez-Valentín et al., 2006). Pharmacologically applied glutamate induces oviduct contractions, and OA acts antagonistically to block glutamate-induced oviduct contractions. OA and glutamate may act independently upon muscle, but it is also possible that OA presynaptically blocks glutamate neurotransmission, as has been observed at the larval NMJ (Nishikawa and Kidokoro, 1999).

Although much of the evidence points towards a role for OA in NMJ signaling, *oamb*, an OA receptor, is required in the oviduct epithelium for normal passage of the egg through the reproductive tract (Lee et al., 2003; Lee et al., 2009). Thus the epithelium also appears to play an important role in OA signaling in egg-laying behavior. Thus far, two different cell-signaling mechanisms have been identified for oviduct muscle contractions and OAMB signaling: OA's effect on evoked muscle contractions acts through cAMP (Rodríguez-Valentín et al., 2006), but the OAMB receptor in the oviduct epithelium works though CaMKII (Lee et al., 2009) (Fig. 7.2). These pathways need not be mutually exclusive, and cross-talk among the muscular and epithelial mechanisms is possible to facilitate egg movement through the tract.

In addition to female factors that control ovulation, male factors transferred during mating also affect egg-laying behavior. The Sfp ovulin rapidly increases the rate of ovulation, acting only within the first 24 hours after mating (Herndon and Wolfner, 1995; Heifetz et al., 2000). The *ovulin* gene encodes a 264 amino acid polypeptide that is cleaved upon entering the female reproductive tract (Monsma and Wolfner, 1988; Monsma et al., 1990; Park and Wolfner, 1995; Heifetz et al., 2005; Ravi Ram et al., 2006). Because some ovulin enters the hemolymph, it remains unclear whether ovulin acts on targets within the reproductive tract or on more distant targets, such as the central nervous system (Monsma et al., 1990; Lung and Wolfner, 1999). Interestingly, the *ovulin* gene is under remarkably strong positive selection: it can be identified in only 8 of the 12 sequenced drosophilids (Wagstaff and Begun, 2005) and it shows extremely rapid sequence evolution across these species (Aguadé et al., 1992; Tsaur and Wu, 1997; Wagstaff and Begun, 2005). While explanations for *ovulin*'s rapid evolution remain speculative, these observations suggest that *ovulin* has important fitness consequences for males, females, or both, either through its effect on ovulation or through an unidentified pleiotropy.

Virtually nothing is known about how ovulated eggs are moved into the uterus. One could speculate that peristaltic contractions of the common oviduct push the egg into the uterus. Alternatively, it has been hypothesized that OAMB mediates ciliary movements along the apical membrane of the common oviduct epithelium to facilitate movement of the egg (Lee et al., 2009).

Oviposition

To oviposit, or deposit an egg upon a substrate, the female must first find a suitable site to lay her eggs. She does this by probing the substrate with her proboscis and ovipositor. Once an

acceptable site has been found, the female bends her abdomen ventrally, contacts the substrate with her ovipositor, and rocks anteriorly–posteriorly until the egg has been deposited upon the substrate (Yang et al., 2008).

Mating induces an increase in oviposition rates. A major factor in this oviposition increase is the transfer of SP, acting on fru^{+}/ppk^{+} sensory neurons through SPR along the female reproductive tract, identical to the sensory neurons involved in SP's effect on mating receptivity discussed above (Kvitsiani and Dickson, 2006; Häsemeyer et al., 2009; Yang et al., 2009). In fact, much of what it is known about SP's role in receptivity holds for its role in oviposition. It remains a feasible hypothesis that SP's pathway to control both receptivity and egg-laying behavior is largely shared, particularly at its upstream end.

Oviposition site selection

Finding a suitable site for oviposition is crucial, as larval diet will be largely determined by the egg's oviposition site, and larval feeding efficiency is important for survival to adulthood (Ohnishi, 1979). Carson (1971) proposed that the "major specificity of the ecology of *Drosophila* relates to the niche in which the female of the species deposits her eggs." The process of site selection for oviposition utilizes a sensory processing network distinct from general female chemotaxis (see below) and requires integration of environmental and social stimuli (Del Solar and Palomino, 1966; Markow and O'Grady, 2008). Considering the importance of oviposition site selection, it may not be surprising that this process demonstrates higher-order characteristics of social learning and decision-making.

Evidence suggests that oviposition site preference is distinct from – and not merely a consequence of – a general preference to reside upon a particular substrate (place preference). For example, Joseph et al. (2009) showed that while female oviposition preference for acetic acid is positive, females avoid residing on substrates with acetic acid. Similarly, longer chain acids elicited a positive oviposition preference and a negative place preference, but mutations in *OBP57d* or *OBP57e* increased the oviposition preference, while not affecting the place or feeding preference (Harada et al., 2008). Thus, oviposition site preference and place preference appear to utilize separate sensory integration processes, yielding distinct behavioral outputs.

Preferable oviposition sites are rife with nutrients, free of toxins, and conducive to development. Females looking to oviposit will avoid non-optimal nutritional substrates, such as high sucrose (Yang et al., 2008), noxious chemicals, such as quinine (Mery and Kawecki, 2002), smooth exposed surfaces (Atkinson, 1983), and unfavorable growth conditions, such as cool temperatures (Fogleman, 1979; Schnebel and Grossfield, 1986) and dry conditions (Spencer, 1937; for review, Markow and O'Grady, 2008). In spite of these general preferences, site choice is not uniform across different strains of *D. melanogaster*, and natural variation exists for site preferences (Miller et al., 2011).

Females also utilize social information when selecting oviposition sites (Del Solar and Palomino, 1966; Mainardi, 1968; but see Atkinson, 1983). The presence of the pheromone *cis*-vaccenyl acetate (cVA) on an oviposition substrate enhances a female's preference for that site (Bartelt et al., 1985). Additionally, cVA acts as a general aggregation signal to males and females, recruiting them to sites of copulation and oviposition (Mainardi, 1968; Bartelt et al., 1985). Females primarily receive cVA from males during mating, although they also produce small amounts themselves (Butterworth, 1969; Jallon et al., 1981; Guiraudie-Capraz et al., 2007; but see Yew et al., 2009). Evidence suggests females deposit male-derived cVA on a substrate while they are ovipositing, which may allow one female to use cVA detection to eavesdrop on another female's prior identification of an acceptable egg-laying substrate (Bartelt et al., 1985). Although a mated female receives cVA as a result of one social interaction, mating, this molecule affects another social interaction between ovipositing females.

Interestingly, cVA function is context dependent: it serves as an oviposition signal for gravid females and as a courtship and aggression cue in males and females (Butterworth, 1969; Jallon et al., 1981; Zawistowski and Richmond, 1986; Wang and Anderson, 2010). This behavioral context-dependence may be explained by the sexual dimorphism of a neural circuit downstream of cVA reception (Datta et al., 2008; Ruta et al., 2010).

Gravid females are equipped with higher-order neural processes to aid them in oviposition site selection: social-learning and decision making. Observing other females' oviposition on a substrate can cause a female to prefer that substrate in later test trials (Sarin and Dukas, 2009; Battesti et al., 2012). This social learning (Leadbeater and Chittka, 2007) cannot be attributed to an exposure to cVA itself, as exposure to cVA-treated food in a conditioning trial did not affect oviposition site preference in later test trials (Sarin and Dukas, 2009). Additionally, the conditioned preference is not detectable in later test trials if only the media with oviposited eggs are presented instead of females ovipositing their eggs, indicating that conditioned preference requires direct observation of the training females, rather than the presence of eggs or other substrate chemical cues (Battesti et al., 2012). The sensory cue conditioning females' site preferences may be either another non-cVA chemical cue or the visual cue of observing oviposition behavior itself.

Recently, oviposition site selection has been proposed to be a model system for simple decision-making (Yang et al., 2008; Joseph et al., 2009). In an unforced choice egg-laying assay, females were presented with two substrates, each of which they actively assessed. Their decision was easily scored as the presence of eggs on a given substrate (Yang et al., 2008). These experiments showed that oviposition site preference is context dependent, as an egg-laying site may be repulsive when presented with a more appealing site, but the same site may become acceptable when presented alone. Furthermore, silencing the neurons expressing *insulin-like-peptide 7* resulted in the elimination of oviposition substrate selectivity, suggesting that these neurons might be important for integrating stimuli to decide on an oviposition substrate (Yang et al., 2008).

Genetic analyses of oviposition

Once a suitable oviposition site has been selected, oviposition behavior can be initiated. Current understanding of *Drosophila* oviposition is largely based on genetic investigations, with little known about the physiology (however, extensive physiological studies of oviposition have been conducted in locust; for review Lange, 2009).

Studies of gynandromorph females demonstrated that thoracic tissue must be female for proper oviposition (Szabad and Fajszi, 1982). Virgin females with chemically ablated mushroom bodies increase oviposition, but this increase disappears upon injection of SP, suggesting that mating could overcome mushroom body inhibition of oviposition (Fleischmann et al., 2001). Further, *dissatisfaction* (*dsf*) appears to be critical for oviposition, as *dsf* mutants lack uterine musculature innervation and do not deposit eggs, even though eggs are present in the uterus (Finley et al., 1997). Genetically silencing neurons projecting to the reproductive tract causes a decreased ability to move eggs from the uterus to the substrate (Rodríguez-Valentín et al., 2006), providing further evidence that central nervous system input is important for ovipositing.

Cellular trafficking in neurons is also important for oviposition: vesicular trafficking proteins in the p24 family, *logjam* (*loj*), *eclair* (*eca*), and *baiser*, are required for oviposition (Carney and Taylor, 2003; Bartoszewski et al., 2004; Saleem et al., 2012). For example, mutant *loj* females are able to move eggs through the reproductive tract into the uterus, but are unable to oviposit. Consequently, multiple eggs accumulate throughout the reproductive tract, including in the uterus and oviducts (Carney and Taylor, 2003). Restoration of *loj* or *eca* expression in the nervous system rescues this oviposition defect (Bartoszewski et al., 2004; Boltz et al., 2007; Saleem et al., 2012), suggesting a neural requirement of *loj* and *eca* for oviposition. Expressing *loj* in olfactory neurons and olfactory processing centers (Boltz et al., 2007) or in neuromodulatory neurons, specifically, peptidergic and octopaminergic neurons (Saleem et al., 2012) was sufficient to rescue the oviposition defect, suggesting that initiation of the oviposition behavior may be gated by sensory cues and reinforces the role neuromodulators play in egg-laying behavior.

Products of the secretory cells of the spermathecae are also important for oviposition: genetic ablation of these cells leads to retention of eggs in the uterus to the striking extent that sometimes late-stage embryos and young larvae can be found in the uterus of females lacking spermathecal secretory cells (Schnakenberg et al., 2011). Although many putative secreted proteins have been identified (Allen and Spradling, 2008; Prokupek et al., 2008), it is not clear to what extent spermathecal secretions interact with the female nervous system and male proteins to influence oviposition.

It has been demonstrated in several insect groups (e.g., *Aedes*, *Locusta*, *Tribolium*, *Rhodnius*) that neuropeptides play important roles in female reproductive physiology (Taghert and Veenstra, 2003; Lange, 2009; Altstein and Nässel, 2010; Van Wielendaele et al., 2013). For example, mutants of several insulin-like peptides reduce egg-laying in *Drosophila* (Grönke et al., 2010). Other neuropeptides have been shown to exhibit a direct effect of reproductive tract muscle. For example, in the locust, a FMRFamide-like peptide and proctolin have been shown to affect oviduct muscle contraction (see reviews for more examples, Lange, 2009; Van Wielendaele et al., 2013). Presumably, numerous neuropeptides are similarly important in *Drosophila* reproductive physiology, however, a detailed role for this class of signaling molecule has yet to be established for *Drosophila*. Developing *Drosophila* as a model for neuropeptide signaling in reproductive physiology would provide a strong genetic model system to screen for neuropeptides controlling reproductive physiology and to understand physiological circuits in vivo.

Appetitive behavior

Once a female has mated, her dietary needs change, both quantitatively and qualitatively. *Drosophila* females increase feeding after mating (Carvalho et al., 2006). This effect is caused, at least in large part, by male-derived SP: mates of *SP* mutant males do not increase feeding, and ectopic expression of *SP* in virgin females increases feeding. Silencing fru^+ neurons of virgin females increases feeding behavior similarly to that seen in the SP-mediated post-mating response (Barnes et al., 2008), which is consistent with SPR reducing synaptic output of fru^+/ppk^+ neurons (Häsemeyer et al., 2009; Yang et al., 2009). It is likely that increased feeding after mating serves the need for additional resources required for increased egg production (see below). Mutant females that do not produce mature oocytes do not show this increase in feeding behavior (Barnes et al., 2008). In accordance with the mating-dependent change in feeding amount, excretion changes after mating as well (Cognigni et al., 2011). Notably, a highly concentrated form of excreta is unique to mated females. Production of this type of excreta depends upon the female's receipt of SP. It is possible that SP triggers changes in the activity of enteric neurons innervating the intestinal tract, such that in which case the post-mating excretion changes may not be entirely a consequence of post-mating feeding changes, but likely involve in addition a separate neuronal circuit.

In addition to increasing the amount of food that they eat, mated females shift their dietary preferences. Mated females are more likely than virgin females to prefer yeast-rich media to sucrose-rich media, perhaps because protein is critical for increased egg production (see below) (Kubli, 2010; Ribeiro and Dickson, 2010; Vargas et al., 2010). Females mated to *SP* mutant males exhibited a weaker postmating dietary switch than normally mated females. Also, *SPR* mutant females do not exhibit the switch in post-mating dietary preference, and restoring *SPR* expression in all multidendritic ppk^+ sensory neurons rescues the switch, suggesting that SP signaling through ppk^+ neurons, possibly fru^+/ppk^+ neurons, contributes to the postmating

change in dietary preference (Ribeiro and Dickson, 2010). Although mutant females unable to produce mature eggs do not increase their feeding behavior (Barnes et al., 2008), they still exhibited the dietary switch towards a protein-rich source (Ribeiro and Dickson, 2010). This suggests that the switch in overall feeding rates may be a response to nutrient depletion, whereas the switch in dietary preference may be a direct effect of SP and SPR (Barnes et al., 2008; Ribeiro and Dickson, 2010).

The neuronal population that responds to mating by controlling these feeding changes is still unknown. However, there are interesting observations that neurons in the mushroom body are important, on the one hand, for integrating multiple signals for appetitive learning (Schwaerzel et al., 2003; Margulies et al., 2005; Kim et al., 2007; Zhao and Campos, 2012; Kim et al., 2013), and on the other hand that neurons in the mushroom body modulate oviposition rates (Fleischmann et al., 2001). Moreover, the octopamine receptor, whose presence in the oviduct epithelium is essential for egg movement through the female reproductive tract (Lee et al., 2003; Lee et al., 2009), is also expressed in neurons in the mushroom body. It is possible that these similarities are coincidental, but they do raise the intriguing possibility that these two post-mating processes might share some common neuronal and/or regulatory mechanisms.

Diurnal activity

Diurnal patterns of activity are also subject to change after mating. Daytime intervals of locomotor inactivity, used as a proxy for sleep, are sexually dimorphic, with female flies spending less time than males sleeping during the day (Andretic and Shaw, 2005). This sexual dimorphism has been traced to mating status: virgin females exhibit daytime sleep patterns like those of males, whereas mated females exhibit reduced daytime sleep (Isaac et al., 2010). It has been proposed that this post-mating increase in locomotion contributes to the previously mentioned post-mating increase in feeding behavior by raising activity levels overall (Isaac et al., 2010).

The postmating change in diurnal activity pattern is related to receipt of SP: females mated to *SP* null males retain the activity patterns of virgins. Further, females that do not receive sperm during mating exhibited this postmating change for only a single day, compared to a 10 day long effect upon normal matings (Isaac et al., 2010). The sperm-dependent persistence of this effect is consistent with gradual release of sperm-bound SP in mated females (Peng et al., 2005), although this has not yet been directly tested.

Gamete maintenance

Egg production

Metabolic demands for a newly mated female shift to support oogenesis and other aspects of egg-laying behavior. Although virgin females exhibit low levels of oogenesis, the rate of oogenesis increases greatly after mating, at least in part because mating causes oocyte development to proceed more efficiently past a developmental checkpoint (Soller et al., 1997). Males exert their influence on the female's oogenesis via SP, which may, at least initially, work through juvenile hormone (JH) signaling to influence oogenesis rate (Moshitzky et al., 1996; Soller et al., 1997, 1999). Since JH does not affect other aspects of the postmating behavior, SP's action through JH signaling may involve a pathway separate from that controlling egg-laying and remating behaviors (Soller et al., 1999). Consistent with this, the region of SP that affects a females' JH levels after mating (SP's N-terminus) differs from the region that regulates egg-laying and remating behaviors (SP's C-terminus), suggesting that two different mechanisms of action might control these two groups of postmating responses (Schmidt et al., 1993; Moshitzky et al., 1996; Fan et al., 2000; Ding et al., 2003; Peng et al., 2005).

Sperm management behavior: storage and usage of sperm

The female's long-term storage of sperm upon mating is critical for her subsequent reproductive success, as it allows her a long window of reproductive capacity even after a single mating. After sperm are transferred to the female, they enter one of two types of specialized storage organs: the seminal receptacle and the paired spermathecae (Bloch Qazi et al., 2003; Schnakenberg et al., 2012) (Fig. 7.1), from where they will be retrieved to fertilize her eggs over a 2-week period. In addition to providing a reservoir of sperm for progeny production, sperm storage indirectly affects female post-mating behaviors because gradual release of the active portion of SP from stored sperm (Peng et al., 2005), extends many aspects of the female's postmating behavior for days after mating, as described above (Chapman et al., 2003; Liu and Kubli, 2003; Isaac et al., 2010).

Sperm storage requires active contributions from the female. Females with masculinized nervous systems exhibited a severe defect in the quantity of stored sperm. In fact, female sperm storage organs with masculinized neural input were as defective in sperm storage as sperm storage organs that were removed along with the abdomen from the rest of the female after mating (Arthur et al., 1998a). The sperm storage defect was particularly strong for the spermatheca, suggesting that sex-specific components of the nervous system are particularly critical for spermathecal sperm storage (Arthur et al., 1998a). In fact, sperm storage organ function from many insect species integrates neuronal input (Lange and Dasilva, 2007, and references within). Further, a genome-wide association study has implicated several neural genes in the selective use of sperm received from multiple previous mates (Chow et al., 2013). Sperm storage requires the transformation of the uterus from a tightly constricted organ with a closed lumen towards a more open, turgid organ shortly after mating begins (Adams and Wolfner, 2007; Avila and Wolfner, 2009). These uterine

changes require male-derived Sfps, including the glycoprotein Acp36DE, suggesting that sperm storage requires an interaction between male and female components (Avila and Wolfner, 2009; Sirot et al., 2009; Avila et al., 2011).

Stored sperm must be released efficiently from storage at a rate that will lead to optimal fertilization rates (Bloch Qazi and Wolfner, 2006). Sperm release from storage requires SP (Avila et al., 2011) as well as the prior action of Sfps that bind SP to sperm (Ravi Ram and Wolfner, 2007; Ravi Ram and Wolfner, 2009). Mutant SP that cannot be released from sperm does not promote release of sperm, so SP release is necessary for normal sperm release (Avila et al., 2010). It is currently unknown whether SP regulates sperm release through the same neurons through which it regulates other behavioral aspects of the postmating response. The neuromodulators tyramine and octopamine in the female have also been shown to regulate rates of sperm release (Avila et al., 2012). Mutations in the enzyme metabolically upstream of both tyramine and octopamine exhibited higher sperm retention (and presumably, lower rates of sperm release) in the spermathecae and seminal receptacle. However, females mutant for the enzyme metabolically upstream of only octopamine only showed evidence of lower sperm release rates only in the seminal receptacle. These data indicate that females can control sperm maintenance using endogenous neuromodulators, but also that these neuromodulators can be use to differentially regulate sperm maintenance across distinct sperm storage organs (Avila et al., 2012).

It is worth noting that, of all the post-mating behaviors discussed so far, sperm storage is unique in that this behavior is exclusive to mated females. All of the other postmating changes discussed thus far reflect wide-ranging, concerted quantitive changes in behavior. Virgin females, however, do not show the uterine shape changes important for sperm storage. Nonetheless, results of Arthur et al. (1998a) indicate that the network is already in place in unmated females, and may simply be activated by mating stimuli.

Transcriptomic post-mating response

Given that mating causes dramatic changes in the behavior of female flies, it is of interest to determine whether gene expression changes underlie (or at least correlate with) these behaviors. Several groups have compared the transcriptomes of whole females, female brains and heads, or female reproductive tracts before and after mating (Lawniczak and Begun, 2004; McGraw et al., 2004; Mack et al., 2006; Kapelnikov et al., 2008b; McGraw et al., 2008; Innocenti and Morrow, 2009; McGraw et al., 2009; Dalton et al., 2010; Gioti et al., 2012). Consistent with the view we have presented here – that most postmating behavioral changes appear to be either quantitative changes of pre-existing behaviors or are behaviors that arise due to modulations of pre-existing circuitry – the onset of postmating behaviors does not correlate with dramatic changes in the transcriptome. Rather, almost all of the transcriptome changes seen in the first few hours after mating are small in magnitude (mostly less than two-fold; McGraw et al., 2004). (Of course not all of the modulated transcripts need be related to post-mating behavioral changes; for example the genes of largest response are ones that appear to have roles in immunity or metabolism.) That small changes correlate with, and can potentially underlie, behavioral changes is not unprecedented; in honeybees, only small-magnitude changes in transcript levels are seen in the brains of workers responding to queen mandibular pheromone, despite their dramatic changes in behavior (Grozinger et al., 2003). It is unknown if large-magnitude changes in transcript abundance that are highly tissue- or cell-specific go undetected due to the sensitivity of the assay. To date it has not been possible to assign any of the *Drosophila* postmating transcriptome changes to modulation or onset of particular behaviors. There are some tantalizing candidates, such as genes with known neural or muscle functions or genes expressed in the fat body (Dalton et al., 2010), a tissue with important metabolic functions that also expresses genes that have been associated with mating behaviors in males (Lazareva et al., 2007; Dauwalder, 2008). Future study will be needed to determine the roles of any of those genes, and others, in post-mating behaviors.

How are post-mating behavior changes induced?

Much of the research involving sex-specific behaviors in *D. melanogaster* has focused on the role of sex-determination genes in developmentally specifying circuits. Circuits are irreversibly sex-specified through mechanisms including changes in sexually dimorphic projection patterns of neurons or programmed cell death of neurons (Ditch et al., 2005; Kimura et al., 2005; Datta et al., 2008; Kimura et al., 2008; Mellert et al., 2010; Rideout et al., 2010; Ruta et al., 2010; Yu et al., 2010; Ito et al., 2012). Some aspects of post-mating behavior circuits are also sex-specific. Ectopically expressing the male-specific FRU^M protein in virgin females nearly abolishes mating receptivity and egg-laying behavior to levels even lower than in normal virgin females (Kvitsiani and Dickson, 2006). Females with masculinized nervous systems also show severe defects in sperm storage (Arthur et al., 1998a).

It is likely that the sex-specification of post-mating behavior circuits occurs before – not in response to – mating. The sex determination of the nervous system and resultant innate courtship behavior is likely complete by adulthood (Belote and Baker, 1987; Arthur et al., 1998b; Kimura et al., 2005). Thus, the fully mature adult female nervous system may require two distinct stages of development: the first stage may generate a nervous system capable of responding to mating stimuli, while the second stage may release the full complement of post-mating behaviors triggered by mating (a similar model

was proposed for female reproductive maturation in general; Kapelnikov et al., 2008a).

How might the female nervous system respond to mating stimuli to produce dynamic post-mating behavioral changes? Mating dramatically changes the amounts of behaviors performed (e.g., egg-laying behavior, courtship receptivity), shifts decision making processes (e.g., oviposition site selection, dietary switch, receptivity), and releases other inactive behaviors (e.g., sperm storage). Therefore, mating-induced neuronal changes may not involve gross anatomical reorganization of neurons. Instead, mating could modulate post-mating behavior circuits by altering neuronal activity or changing synaptic strengths of pre-existing synapses. For example, suppressing activity of fru^+ or dsx^+ neurons causes changes in female postmating responses, such as receptivity inhibition, increased egg-laying, and increased feeding behavior (Kvitsiani and Dickson, 2006; Barnes et al., 2008; Rideout et al., 2010; Rezával et al., 2012). Further, suppressing activity of fru^+/ppk^+ sensory neurons mimicks the effects of the receipt of SP on receptivity and oviposition. This suggests that changes in *fru*/*dsx* neuronal circuit activity may control at least some postmating behavioral changes (Häsemeyer et al., 2009; Yang et al., 2009). Mating has also been shown to induce synaptic changes at the reproductive tract NMJ: it causes an increase in the number of putatively-OA releasing boutons on the lateral and common oviduct musculature, suggesting an increase in synaptic strength (Kapelnikov et al., 2008a). Post-mating responses might thus represent a distinct second stage of neuronal development composed of modulation of neuronal membrane or synaptic characteristics, separate from a first stage of hard-wired specification. Post-mating behavior provides a strong model system for understanding how modulations of neurons can cause diverse and long-lasting changes in behavior.

A model of two distinct stages of female behavioral development forms the testable prediction that essential functions of *fru* and *dsx* for post-mating behavior are conferred prior to mating, rather than during the post-mating transition. Further, some nervous system regions may be good candidates to look for modulatory post-mating responses. The motor neurons that project to the reproductive tract have already been shown to exhibit synaptic plasticity in response to mating (Kapelnikov et al., 2008a). Might these motor neurons exhibit increased excitability after mating? Might auditory sensory neurons that transduce male courtship song become less excitable after mating, as females are less accepting of male's courtship stimuli? Or, might the increased preference for yeast reflect an increase in the excitability of gustatory neurons sensing protein content? Since many insects exhibit a decrease in flight ability after mating (Jones et al., 1978; Collatz and Wilps, 1985), might flight muscle NMJs weaken in response to mating? The identification of several markers for neurons controlling post-mating behaviors (e.g., *fru*, *dsx*, *ppk*, *tdc2*) will be helpful in manipulating the activity or measuring the responses (e.g., calcium imaging, synaptic morphology) of subsets of neurons.

Conclusions

Females exhibit a diverse array of behavioral changes upon mating, including decreased mating receptivity, increased egg-laying behavior, increased feeding, altered locomotion patterns, increased egg production, and differences in the way that sperm are stored and utilized. These changes reflect higher-order effects such as social (e.g., receptivity) and decision-making behavior (e.g., oviposition site selection, dietary switch, receptivity) to metabolic processes (e.g., locomotion and feeding) to physiological and morphological changes (e.g., ovulation and uterine confirmation changes). In contrast to innate, hard-wired aspects of sex-specific behavior, postmating behavioral shifts reflect a plastic response that is both long-lasting, and reversible over time.

As mechanisms for post-mating behaviors become better described, it will be interesting to discover how mating stimuli modulate neural networks to modify behaviors. Are several seemingly unrelated behaviors governed by a common set of mechanisms, or even a singular post-mating switch? Or is each behavior induced by its own distinct mechanism? That a single signaling molecule, SP, affects receptivity, locomotor activity, and feeding behaviors could suggest that they are all induced through a common mechanism. However, it is as yet unclear whether all of these effects involve the same mechanism (i.e., SPR signal transduction in multidendritic sensory neurons along the female reproductive tract). Other data favor the idea that different postmating processes are induced by different mechanisms. For example, one of SP's effects (increased oogenesis) requires a different portion of SP than do the others (receptivity and egg-laying), and for some postmating behaviors, Sfps in addition to SP are essential, for example, ovulin for ovulation (Heifetz et al., 2000) and Acp36DE for uterine contractions (Avila and Wolfner, 2009).

At least some post-mating changes likely can be attributed to changes in neuronal activity or synaptic strength, rather than to the specification of new circuitry. Thus, post-mating behavior provides a powerful model system to understand how modulations of intact circuits can lead to dramatic behavioral shifts. Applying powerful new techniques such as conditional neuronal activation (Schroll et al., 2006; Parisky et al., 2008; Shang et al., 2008; Pulver et al., 2009) (CHAPTER 7.7 GRIFFITH), *in vivo* imaging of neural responses (Tian et al., 2009), and MARCM analysis (Lee and Luo, 2001; Kimura et al., 2008; Yu et al., 2009; Yu et al., 2010) to dissect the genesis of post-mating behaviors will provide a tractable understanding of how a wide variety of behaviors can be released by a specific social input.

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Chapter 8

Circadian rhythms

Nara I. Muraro and M. Fernanda Ceriani

Introduction

Every time one wakes up minutes before the alarm clock is set to go off, it seems logical to imagine that there must be a time-keeping mechanism inside us. And that is indeed the case. Circadian rhythms (*circa*: around, *diem*: day) are biological rhythms with a period of approximately 24 h and have been described from cyanobacteria to humans. Their presence clearly confers an immense adaptive value to organisms, allowing them to anticipate the daily changes in light and temperature generated by the rotation of our planet, and to adjust their behaviors and physiology accordingly. Circadian rhythms have the ability to persist in constant conditions and are not affected by moderate temperature changes.

Circadian clocks have been traditionally modeled as a three-part system composed of a molecular clock (the oscillator) that is synchronized by environmental clues (the inputs) and produces daily variations in downstream parameters (the outputs) (Fig. 8.1A). The more important inputs or *zeitgebers* (time giver, in German) are the daily light–dark cycle and the variation in ambient temperature; however, other regular events such as food availability (Xu et al., 2008) and social interaction (Levine et al., 2002) can also function as synchronizing inputs. A vast amount of circadian research has been focused on understanding the mechanism of the oscillator itself and the relation to its synchronizing inputs and, although clock outputs can be easily identified, unraveling the mechanisms by which the oscillator controls them has proven more challenging. Nowadays, thanks to the great deal of information that has been gained about the molecular oscillator, together with high throughput analysis, we are a step forward in defining the mechanisms of circadian output regulation. Indeed, a great proportion of genes have been found to cycle in a circadian manner in different tissues and conditions (Claridge-Chang et al., 2001; McDonald and Rosbash, 2001; Ceriani et al., 2002; Ueda et al., 2002; Keegan et al., 2007; Wijnen et al., 2006; Kula-Eversole et al., 2010; Nagoshi et al., 2010; Hughes et al., 2012; Rodriguez et al., 2012).

There is also evidence of direct effects of the inputs over the outputs, effects that bypass the oscillator (an effect known as masking); one example is the arousal effect of light on locomotor activity, which is independent of the lights-on anticipation that depends on a working oscillator (Wheeler et al., 1993). The oscillator can also affect the perception of the inputs; for instance, *Drosophila* L1 and L2 monopolar cells axon size shows daily oscillations, providing a way of adapting the visual system sensitivity to the daily changes in light conditions (Pyza and Meinertzhagen, 1999; Emery et al., 1998 and see below). Therefore, the traditional inputs→oscillator→outputs way of depicting circadian clocks should be viewed with an open mind.

Circadian rhythms are possibly the most studied of all *Drosophila* behaviors. They gained that leading role thanks to pioneer work from the Benzer lab, which successfully attempted to find a link between individual genes and behavior. Back in 1971, Konopka and Benzer performed a mutagenesis screen and searched for mutants with altered eclosion behavior (Konopka and Benzer, 1971). They identified different mutants with long and short eclosion periods, as well as a mutant that showed no eclosion rhythms at all; amazingly, the three mutations were found to reside on the same X-chromosome gene designated *period* (*per*); and were named per^{LONG}, per^{SHORT} and per^{01} respectively. This fortuitous event had foundational effects both on the study of *Drosophila* behavioral genetics and in the field of Chronobiology.

More than two decades elapsed from the discovery of *Drosophila per* in the 1970s to the explosion of circadian-related research that took off in the 1990s. Indeed, the development of molecular biology techniques allowed the identification not only of many other clock molecules, but also the initial unraveling of the oscillator molecular mechanism. Genetic screens in *Drosophila* (Sehgal et al., 1994; Rutila et al., 1998; Kloss et al., 1998; Rutila et al., 1998; Allada et al., 1998; Stanewsky et al., 1998; Martinek et al., 2001; Akten et al., 2003; Lin et al., 2002) and mouse (Vitaterna et al., 1994) revealed the molecular players necessary for behavioral rhythmicity. With the *Drosophila* genome at hand and thanks to a reasonably conserved homology of sequence, mammalian clock genes were identified. The pursuit of the molecular mechanism of the circadian oscillator had begun.

Behavioral Genetics of the Fly (Drosophila melanogaster), ed. J. Dubnau. Published by Cambridge University Press.

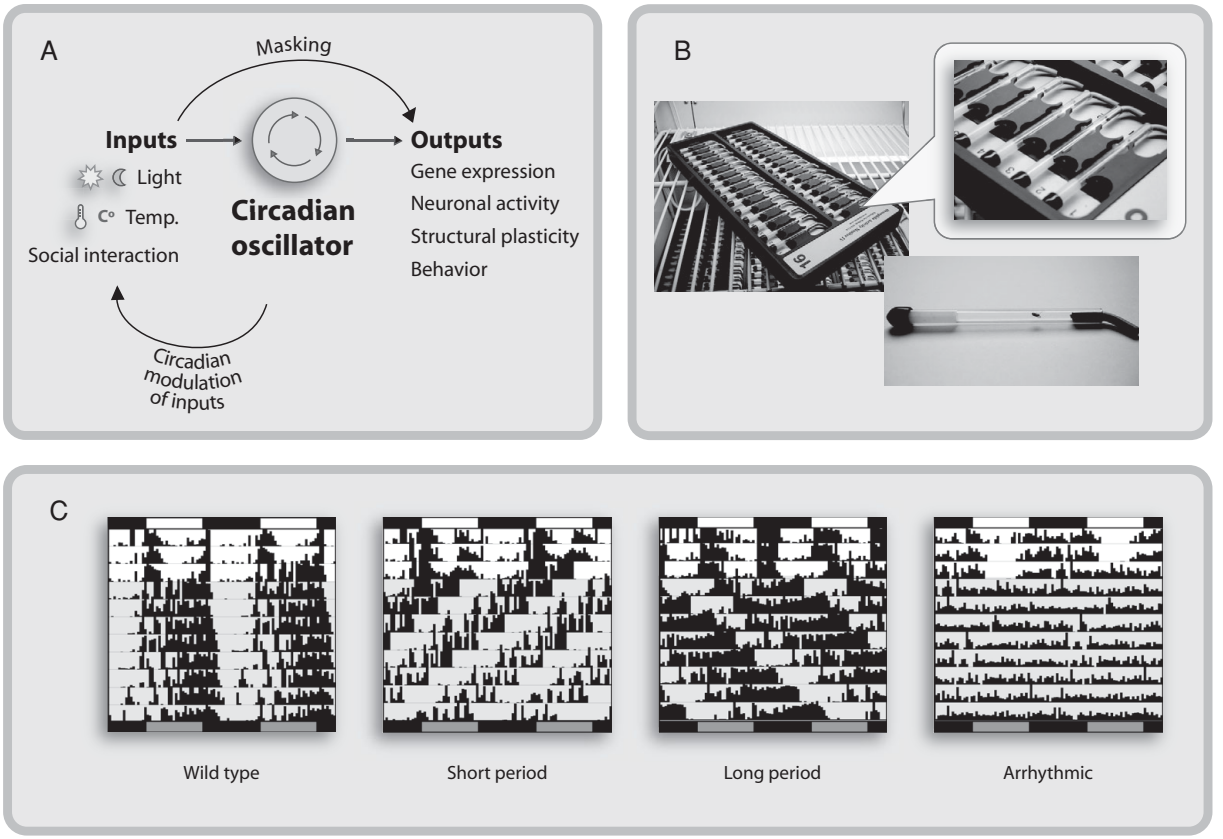

Fig. 8.1. *Drosophila* circadian basics. A Circadian oscillator scheme. B The image displays a *Drosophila* locomotor activity monitor inside an incubator along with magnifications of the monitor and an individual tube housing an adult fly on freshly prepared fly food (on the left of the tube). C Examples of circadian locomotor activity actograms; from left to right: examples of a wild-type, short period, long period and arrhythmic flies are shown. After 3 days of light–dark entrainment, flies were kept in constant darkness, represented with a shadowed background.

Circadian genes and proteins

Although eclosion was the first observed clock output in *Drosophila*, it is the rhythm in locomotor activity the more commonly examined circadian behavior. *Drosophila* adult males in isolation present a stereotypical crepuscular activity pattern with two peaks of activity; one early in the morning that anticipates dawn and another peak in the evening which anticipates dusk; around midday they exhibit a reduced activity period or "siesta." To monitor activity in the laboratory, flies are housed individually in thin glass tubes that are placed in a device containing an infrared light beam (which cannot be seen by flies) and a detector halfway along the length of each tube (Fig. 8.1B). A computerized system detects the number of times a fly crosses the light beam per time unit and creates an activity profile for each individual fly. The activity monitors are placed in an incubator with adjustable temperature and light conditions. Usually, flies are entrained with one circadian parameter, for example a 12 h light–12 h dark (LD) cycle. After a few days, termed the entrainment phase, the incubator settings are changed to constant conditions (usually constant dark, DD). If the circadian clock is working properly, the activity pattern would be preserved under free running conditions, perhaps with a slightly altered period but still close to 24 h (the period observed in the absence of synchronizing inputs is the real oscillator period, and it varies in different species). However, if the clock has been accelerated, delayed or damaged, the free running activity patterns would present a short period, long period or show arrhythmicity, respectively (Fig. 8.1C). The activity pattern is double plotted (2 days in each line) for easy appreciation of the period and is referred to as an actogram. This kind of strategy has been repeatedly employed to identify the clock molecules that will be described in this section.

Within clock neurons, the *Drosophila* circadian oscillator is established by self-sustaining, cell-autonomous, interlocking transcriptional-translational negative feedback loops of several clock genes and proteins. The main players in the core feedback loop are the products of the originally described *per* gene (Konopka and Benzer, 1971) together with *timeless* (*tim*) (Sehgal et al., 1994) (Myers et al., 1995) and two transcription factors of the bHLH (basic Helix Loop Helix) family named *clock* (*Clk*) (Allada et al., 1998; Darlington et al., 1998) and *cycle* (*cyc*) (Rutila et al., 1998). At midday, CLK and CYC heterodimerize and drive the transcription of target genes containing the circadian E-box (CACGTG) (Hao et al., 1997) sequence in their promoter regions, such as *per* and *tim*. This causes the accumulation of *per* and *tim* mRNA first, and PER and TIM

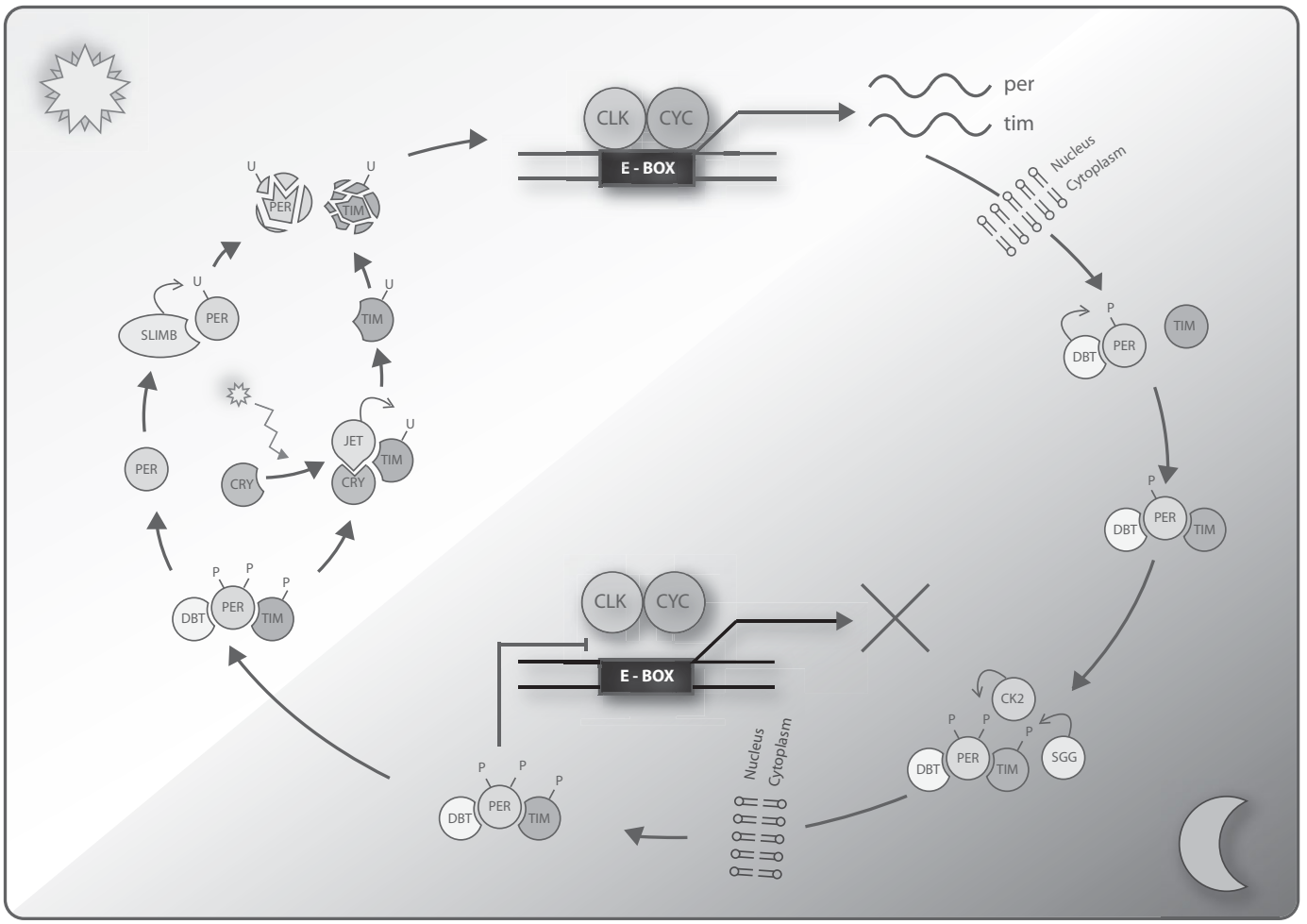

Fig. 8.2. Schematic diagram of the molecular clock. During the day, the absence of PER/TIM allows CLK/CYC mediated transcription of E-box containing genes such as *per/tim* (top). PER/TIM proteins accumulate during the night thanks to the stabilization of PER by some DBT phosphorylation (right). When the PER/TIM/DBT complex has achieved a given concentration it is translocated to the nucleus, a phenomenon associated to phosphorylation of both PER (by CK2) and TIM (by SGG) (bottom right). PER/TIM presence in the nucleus inhibits transcription of E-box containing genes (bottom). During the day, light activates the photoreceptor CRY and mediates the sequestration and ubiquitination of TIM by the JET complex (left). Without TIM, PER is ubiquitinated by SLIMB and degraded, derepressing CLK/CYC mediated transcription of E-box containing genes, and starting a new cycle (top). See details and references of these processes in main text.

proteins a few hours later, when night falls. PER and TIM are only able to accumulate in the dark because TIM is degraded in the presence of light (see below). Moreover, TIM stabilizes PER by protecting it from a kinase named doubletime (DBT) (Kloss et al., 1998; Price et al., 1998), without TIM, DBT phosphorylates PER and targets it for proteasomal degradation (Grima et al., 2002). Phosphorylation also plays a key role in nuclear translocation, which is facilitated by two kinases Casein kinase 2 (CK2) (Akten et al., 2003; Lin et al., 2002) and Shaggy (SGG, the ortholog of the mammalian glycogen synthase kinase 3) (Martinek et al., 2001), which phosphorylate PER and TIM respectively. The phosphorylated PER–TIM–DBT complex, now in the nucleus, binds to CLK, phosphorylates it and inhibits transcription of E-box containing genes (Bae et al., 2000) (Fig. 8.2).

Wild-type *Drosophila* become arrhythmic when kept under constant light (LL) conditions (Konopka et al., 1989). The persistence of rhythmicity in LL in mutants for the blue light photoreceptor *cryptochrome* (*cry*) (Stanewsky et al., 1998) unraveled the mystery of TIM light sensitivity. In the morning, light is sensed cell-autonomously by CRY and, by a still not completely understood mechanism, light-activated CRY is able to interact with TIM, making it labile to ubiquitination by the Jetlag (JET) protein complex (Ceriani et al., 1999; Rosato et al., 2001; Busza et al., 2004; Koh et al., 2006; Peschel et al., 2006). As a consequence of TIM degradation, PER is also destabilized, a step which involves PER ubiquitination by the E3 ubiquitin ligase Supernumerary limbs (SLIMB) (Chiu et al., 2008) followed by proteolysis. Without PER, CLK repression comes to an end, allowing the CLK–CYC heterodimer to restart transcription of *per* and *tim*, closing the loop and beginning a new cycle.

Additional E-box containing genes, also transcriptionally controlled by the CLK–CYC heterodimer provide further regulation to the core oscillator. These interlocked secondary feedback loops involve the genes *vrille* (*vri*) (Blau and Young,

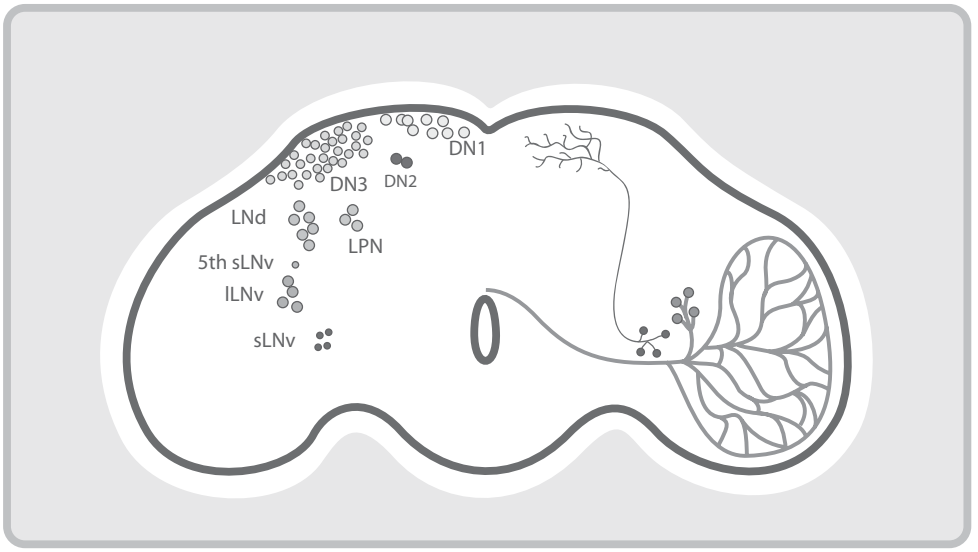

Fig. 8.3. *Drosophila* clock neurons scheme. An adult *Drosophila* brain with the traditional clock neuron clusters on the left side. On the right side, PDF positive sLNvs and lLNvs with their axonal arborizations are shown; the sLNvs axons projecting towards the dorsal protocerebrum and the lLNvs towards the optic lobe on the ipsi and the contralateral side.

1999; Cyran et al., 2003), *PAR domain protein 1ε* (*pdp1ε)* (Benito et al., 2007) and *clockwork-orange* (*cwo*) (Kadener et al., 2007; Lim et al., 2007; Matsumoto et al., 2007). A great deal of information has already been gained on the molecular mechanisms of the biological clock and it is beyond the scope of this chapter to describe them in detail. Further reading on this matter can be found on recent review articles (Peschel and Helfrich-Forster, 2011; Hardin, 2011; Glossop, 2011). The phosphorylation/dephosphorylation balance of defined residues in specific proteins is, as it has been already mentioned, crucial for setting the pace of the molecular clock. However, other post-transcriptional regulatory mechanisms play important roles such as alternative splicing of clock genes, mRNA stability and regulation by microRNAs. This subject has been reviewed by Green and colleagues (Kojima et al., 2011).

At the molecular level, the conservation of *Drosophila* and mammalian circadian oscillators is remarkable. The main differences between them are: (1) the complexity of the protein networks involved, as mammals possess more than one clock gene of each category (possibly due to genome duplication (Kasahara, 2007)); (2) orthologs do not necessarily play the same role within the loop (CRY being the most striking example of this (Stanewsky et al., 1998; Kume et al., 1999)); (3) the lack of a cell-autonomous photoreceptor protein in mammalian clock cells (a role that CRY plays in fly clock neurons; in contrast, in mammals light is exclusively sensed by retinal photoreceptors and the information relayed to the suprachiasmatic nucleus, the mammalian master circadian regulator); (4) in mammals there are extra players in the interlocked-loop that involve retinoid-related orphan receptor genes such as *Rev-erbα* and *Rora* (for review see Jetten, 2009).

Finally, it is necessary to take into account that the molecular oscillator mechanism described here is likely to present variations in different clock cells, for instance there is a subset of *Drosophila* clock neurons that do not express CRY and, therefore, other mechanisms may be in place to regulate TIM stability under light dark conditions. The next section examines the available information on different clock cell clusters and their putative connectivity in the *Drosophila* brain.

Circadian cells and circuits

Circadian rhythms in animals are typically coordinated by a central circadian pacemaker that lies within the brain (or retinal ganglion in some mollusk (Jacklet, 1969) and insect (Page, 1982) species). In mammals this corresponds to a small hypothalamic region named the suprachiasmatic nuclei (SCN) (Ralph et al., 1990). In *Drosophila*, clock genes are expressed in approximately 150 neurons that have been classified in seven groups according to their anatomical location (Fig. 8.3). In each brain hemisphere there are three dorsal neuron clusters (DNs) corresponding to 16 DN1s, 2 DN2s and around 40 DN3s; and four lateral neuron (LN) groups comprising five small ventral lateral neurons (sLNvs), four large ventral lateral neurons (lLNvs), six dorsal lateral neurons (LNds) and three lateral posterior neurons (LPNs). Although the complete circadian network is necessary for a coherent and plastic circadian control of behavior, several lines of evidence point to the LNvs as the location of the core pacemaker in *Drosophila*. For instance, using mosaic analysis in per^{01} mutants it was found that *per* expression only in LNs was sufficient for rescuing circadian behavior (Ewer et al., 1992). In addition, a careful analysis of *disconnected* (*disco*) mutants, which lack LNs and are arrhythmic under free running conditions, showed that in the rare occasion where rhythmic flies were observed, it correlated with the presence of at least one LNv (Helfrich-Forster, 1998).

The seven groups of clock neurons that were originally defined on their anatomical position and size are nowadays being subdivided and re-defined according to the expression

of additional markers. For instance, the DN1s have been subdivided into posterior and anterior groups, according to their developmental time and the expression of the GLASS transcription factor only in the posterior subset (Shafer et al., 2006). Moreover, the photoreceptor CRY has been found in LNs and only a subset of DN1s (Yoshii et al., 2008), including two anterior DN1s that are distinguished by the additional expression of the neuropeptide IPN-amide (Shafer et al., 2006). The LNvs case is interesting since all lLNvs and four out of the five sLNvs express the neuropeptide Pigment Dispersing Factor (PDF) (Kaneko et al., 1997); the fifth sLNv lacks PDF, but expresses ion transport peptide (ITP) and choline acetyltransferase (Johard et al., 2009). The information gained on this subject is extremely useful when it comes to design genetic tools. Indeed, the promoters of many clock neuron-specific transcripts have been used to generate GAL4 and GAL80 constructs that provide, using different combinations, a way of expressing a gene of interest in a specific clock neuron subset (Dubruille and Emery, 2008).

Is there a hierarchy among clock neurons? Early on, Pittendrigh and Daan proposed the existence of two distinct oscillators to account for the morning and evening activity peaks observed in diurnal animals, implying that each of the two daily activity peaks would depend on the action of specific subsets of neurons (Pittendrigh and Daan, 1974). Using genetic tools to rescue *per* expression in a per^{01} background or to ablate specific subsets of clock neurons using pro-apoptotic genes, it was proposed that the morning activity peak was commanded by the sLNvs (the M oscillator) and the evening oscillator (the E oscillator) by the LNds, DNs and the fifth PDF-negative sLNv (Stoleru et al., 2004; Grima et al., 2004; Rieger et al., 2006). This simplified model lost consistency when locomotor activity was analyzed using different constant light conditions like LL or dim LL (Picot et al., 2007; Murad et al., 2007; Rieger et al., 2009). Nowadays, the more accepted model considers the sLNvs as the main pacemaker in DD conditions, but regards the two oscillators as plastic entities, composed of subsets of clock neurons that change their predominance according to the photoperiod (Rieger et al., 2006; Stoleru et al., 2007; Dubruille and Emery, 2008).

Although the location of clock neuron subsets in the *Drosophila* brain is well described, their connectivity is still highly unexplored. A thorough analysis of axonal projections of the different clusters predicts a possible connection of the sLNvs with at least some DNs (Helfrich-Forster et al., 2007). Moreover, several clock neuronal clusters, like the lLNvs, LNds and DN1s, extend their axons contralaterally, suggestive of a role in coordination of the clocks in each brain hemisphere (Helfrich-Forster et al., 2007). The lLNv projection trees arborise massively in the optic lobes, which lead to analysis of their role as light arousal neurons (Shang et al., 2008). However, all this anatomical data does not necessarily imply real connectivity. The lack of a connectivity map can be mainly attributable to the absence, for decades, of an electrophysiologically accessible preparation of the clock circuit in flies. This issue has been solved in the mid to late 2000s (Park and Griffith, 2006; Sheeba et al., 2008; Cao and Nitabach, 2008; Fogle et al., 2011; McCarthy et al., 2011). A refinement of this preparation might soon provide real connectivity information of the circadian network in *Drosophila*.

Light, the main circadian *zeitgeber*, has several input routes into the circadian system in addition to the previously mentioned cell-autonomous photoreceptor CRY. Photic information is also sensed by the retinal photoreceptors of the compound eye, the Hofbauer–Buchner eyelets and the ocelli. All these inputs contribute in some way to circadian synchronization; however, some have a predominant role under specific light conditions associated, in nature, to different photoperiods (Rieger et al., 2003). Finally, important regulators of any neuronal process, the glial cells, have taken a protagonist role in the circadian field as well. Indeed, glial expression of a molecular oscillator of similar characteristics to the neuronal one has been reported, and its role in modulation of circadian outputs recently reviewed (Jackson, 2011).

In animals, self-sustained oscillation of clock genes has been found not only in the master circadian regulator but also in several other tissues, named peripheral clocks. At the molecular level, peripheral clocks are built slightly differently from the central clocks, but conserve the main molecular players (Hardin et al., 2003; Glossop and Hardin, 2002). Peripheral clocks control defined outputs and, in *Drosophila*, are synchronized by cell-autonomous CRY-mediated detection of light (Plautz et al., 1997), and thus work with a high degree of independence from the central pacemaker. For instance, in a per^{01} background, *per* rescue restricted to the LNs was able to restore circadian locomotor activity, but not circadian olfactory electroantennogramm responses (Krishnan et al., 1999). Along this line, circadian rhythms in olfactory behavior depend on *per* rescue in the antenna, but not in the LNvs (Zhou et al., 2005). In *Drosophila*, peripheral clocks have been found in the eyes (Zerr et al., 1990; Cheng and Hardin, 1998), gustatory sensillae (Chatterjee et al., 2010), fat bodies (Xu et al., 2008) and Malpighian tubules (Giebultowicz et al., 2000) among others.

As we have reviewed in this section, *Drosophila* as a model organism has not only taken the lead in the discovery of the molecular aspects of circadian oscillation, but also the cellular basis of it. Although the mammalian SCN has been subjected to many studies, and advances in defining neuronal sub-populations expressing different neuropeptides and neurotransmitters has been achieved, the creation of genetic tools in mammalian systems has been slow. On the contrary, the identity of clock neurons in *Drosophila* is more defined and genetic tools for dissecting their roles are constantly being created and refined. Further analysis of the *Drosophila* circadian circuit is likely to provide an immense amount of information about the role of individual clock neurons within the networks that regulate clock outputs. The big challenge is now to find the outputs of each sub-cluster of clock neurons and the connectivity between them and with other, non-circadian, neurons.

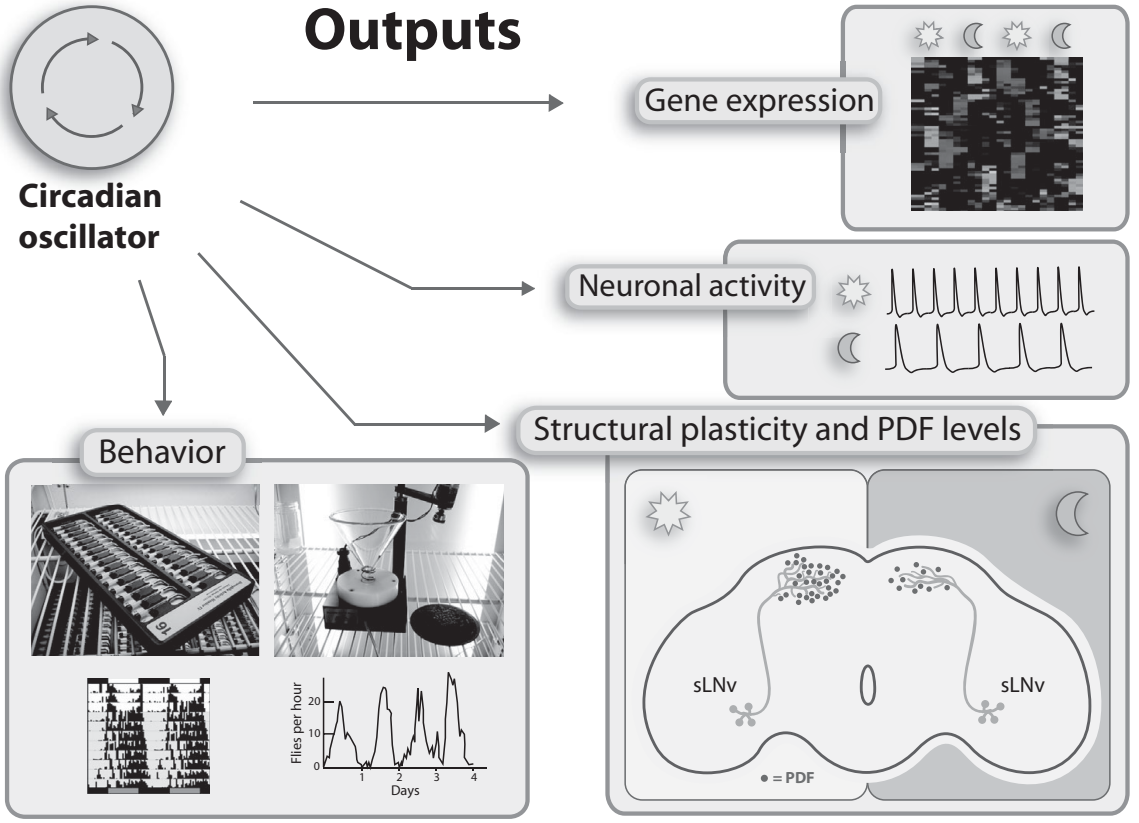

Fig. 8.4. Circadian oscillator outputs. The cartoon depicts some of the reported outputs of the *Drosophila* circadian clock: daily variation of gene expression detected by microarray analysis; circadian variation of clock neuron activity; circadian plasticity of sLNv dorsal projections and PDF levels; and circadian behaviors such as locomotor activity and adult eclosion (locomotor activity and eclosion monitors are photographed).

Circadian outputs

An output of the circadian clock can be defined as any parameter that is regulated by the molecular clock but is not an intrinsic part of it. Circadian outputs are seen at many levels; they range from the cyclic expression of specific molecules and the regulation of membrane excitability within clock neurons, to the structural remodeling of specific neuronal projections and the regulation of complex behaviors (Fig. 8.4). This subject has been comprehensively reviewed recently (Frenkel and Ceriani, 2011); this section examines some of the most significant clock outputs described so far in *Drosophila*.

Within clock neurons, circadian variation in mRNA expression could be part of the molecular oscillator mechanism (if the cycling mRNA corresponds to a core clock gene); however, a vast number of the cycling mRNAs found through high throughput microarray and sequencing experiments have turned out to be non-core clock related, and thus are likely to be associated to a clock output (Claridge-Chang et al., 2001; McDonald and Rosbash, 2001; Ceriani et al., 2002; Ueda et al., 2002; Keegan et al., 2007; Wijnen et al., 2006; Nagoshi et al., 2010; Kula-Eversole et al., 2010; Hughes et al., 2012; Rodriguez et al., 2012). The molecular clock could regulate output gene expression directly (for example, via an E-box motif and CLK/CYC-mediated transcriptional regulation) or indirectly, through additional transcription factors such as PDP1/VRI (Cyran et al., 2003).

It is the electrical properties of neurons that dictate their role within a circuit and these properties depend on the type and quantity of ion channels. Therefore, ion channels represent a particularly interesting candidate group of genes predicted to cycle in clock neurons. Indeed, several ion channel mRNAs, such as *Ir* (*inwardly rectifying potassium channel*) and *SK* (*small conductance calcium-activated potassium channel*) have been found to be enriched at certain times of day in LNs (Kula-Eversole et al., 2010). Moreover, mutations or down-regulation of ion channel genes that lead to behavioral alteration of circadian outputs have been reported (Cirelli et al., 2005; Lear et al., 2005a; Fernandez et al., 2007; Hodge and Stanewsky, 2008; Ruben et al., 2012).

One of the earlier pieces of evidence for the self-sustainability of circadian systems was the observation of the persistence of cyclic electrical activity under free running conditions in tissue islands containing rat SCN, which showed increased electrical activity during the subjective day and reduced activity during the subjective night (Inouye and Kawamura, 1979). Moreover, thanks to the development of SCN slice

preparations, mammalian models have been extremely useful in determining the electrophysiological characteristics of clock neurons (Kuhlman and McMahon, 2006; Ko et al., 2009; Colwell, 2011). In that aspect *Drosophila* has lagged way behind; however, the development of a preparation to perform whole cell patch-clamp recordings from *Drosophila* clock neurons (Park and Griffith, 2006; Sheeba et al., 2008; Cao and Nitabach, 2008) has provided an opportunity to start filling this gap. By exploiting this preparation, some information about the lLNvs electrophysiological characteristics has been gained: (1) lLNvs, as SCN clock neurons, show circadian variations in electrical activity and resting membrane potential (Sheeba et al., 2008; Cao and Nitabach, 2008); (2) lLNvs present two firing modes, bursting and tonic; interestingly, the same neuron can change from one pattern to the other and the proportion of bursting neurons is higher during the early morning (Sheeba et al., 2008) and (3) lLNvs respond to light with an increase in firing rate and resting membrane potential (Sheeba et al., 2008), which is in agreement with their proposed role as arousal neurons and was found to be a CRY dependent phenomenon (Fogle et al., 2011). The sLNvs have clearly proven to be less accessible for electrophysiological recordings and, although daily variations in resting membrane potential have been reported (Cao and Nitabach, 2008), no additional information is available yet. The electrical properties of other *Drosophila* clock neurons remain unexplored.

Electrical activity often results in the release of neurotransmitter molecules, so, what are the neurotransmitters employed by clock neurons? Cross-reactivity of a LNvs epitope with crustacean pigment dispersing hormone (PDH) anti-sera gave the first hint of the importance of a related molecule in these cells. Notably, the antisera revealed not only the cell nuclei (as PER immunoreactivity did) but also the dorsally projecting sLNvs axons and the optic lobe lLNv projecting axons (Helfrich-Forster, 1995). Afterwards, it was found that pdf^{01} mutants, that lacked expression of the PDH orthologue PDF, had a progressive loss of rhythmicity under free-running conditions (Renn et al., 1999). The progressiveness and lack of complete penetrance of the phenotype suggested that the mutation in pdf^{01} was affecting a clock output rather than the core oscillator itself. Analysis of PER cycling in different pacemaker clusters suggested that PDF was acting as a crucial signal for synchronization between them (Lin et al., 2004), a theory supported by the finding of a PDF receptor (PDFR) molecule expressed in different clock neuron subsets (Hyun et al., 2005; Lear et al., 2005b; Mertens et al., 2005; Im and Taghert, 2010). Although no daily change in *pdf* mRNA was detected (Park and Hall, 1998), PDF immunoreactivity was found to cycle in the dorsal projections of sLNvs, a property that was lost in clock mutants such as per^{01} and tim^{01} (Park et al., 2000). There is still controversy about the way the clock regulates PDF; however, the fact that ion channel mutations affect PDF levels clearly indicates a role for membrane excitability in PDF release (Lear et al., 2005a; Fernandez et al., 2007; Hodge and Stanewsky, 2008; Depetris-Chauvin et al., 2011). In addition to PDF, the sLNvs are predicted to use another, still unknown, classical chemical neurotransmitter. This is suggested by the fact that sLNvs axonal termini contain not only dense core PDF-filled vesicles, but also small clear vesicles that would provide fast neurotransmission (Miskiewicz et al., 2004; Yasuyama and Meinertzhagen, 2010) and are predicted to be released by a VAMP-dependent mechanism (Umezaki et al., 2011). The identity of this additional neurotransmitter remains elusive. The involvement of other neurotransmitters in different clock neuron clusters has been postulated, including: neuropeptide F, short neuropeptide F, ITP, acetylcholine (Johard et al., 2009), glutamate (Hamasaka et al., 2007), dopamine, serotonin (Hamasaka and Nassel, 2006) and histamine (Hong et al., 2006).

Another clock-controlled output is the daily remodeling of neuronal structures, a phenomenon termed circadian plasticity (Mehnert and Cantera, 2011). This was first described in the first optic neuropil in the housefly (Pyza and Meinertzhagen, 1995) and *Drosophila* (Pyza and Meinertzhagen, 1999). They showed a circadian variation on axon caliber of L1 and L2 monopolar cells, with swelling during the early day and early night. This correlated with the two main periods of crepuscular activity when visual processing is most necessary. This phenomenon was dependent on clock genes, persisted in DD and was abolished in LL. However, rescue experiments revealed a complex regulation, with some aspects of monopolar cell structural plasticity dependent on the central pacemaker and others dependent on glial cells (Pyza and Gorska-Andrzejak, 2004).

Another structure found to undergo circadian plasticity corresponds to the terminals of the dorsally projecting sLNv axons (Fernandez et al., 2008). Complexity of these terminals shows circadian variation with an "open", more complex, structure in the early morning and a "closed" structure during the early night, correlating with daily changes in PDF levels in the same protocerebral region. This kind of circadian plasticity persists in constant conditions and is abolished in core clock mutants (Fernandez et al., 2008). Other examples of circadian plasticity include the changes in synaptic vesicles evidenced at the ultrastructural level in terminals of MN5 motoneurons (Ruiz et al., 2010) and photoreceptors (Barth et al., 2010). It would not be surprising if, in the future, additional structures are found to experience circadian structural plasticity.

The coordinated clock controlled changes in gene expression, neuronal activity and neuronal structure may end up impacting on the daily changes of behavior. Behavior is clearly affected by the time of day, therefore, anyone willing to assay any behavior in the laboratory should take this into account. In addition to the already mentioned locomotor activity and adult eclosion behaviors, others have been found to exhibit circadian modulation, namely: courtship (Hamasaka et al., 2010), short term memory (Lyons and Roman, 2009), optomotor response (Barth et al., 2010) and gustatory behaviors (Chatterjee et al., 2010) among others.

Concluding remarks

These are exciting times for *Drosophila* Chronobiology, a time to revise what decades of research really mean and start to dissect the mechanisms of circadian networks in light of the new information and the novel techniques available. We now know that the clock is not just "a clock," but a complicated clockwork mechanism composed of neuronal and non-neuronal oscillators.

What is missing in the field? The vast amount of genetic tools available today for the *Drosophila* neurobiologist has recently been reviewed (Venken et al., 2011). Still, to keep dissecting the roles of specific subsets of clock neurons, even more cell type specific expression drivers are necessary. Intersectional genetics could provide a useful alternative (Potter et al., 2010). In addition, promoter analysis of specific genes could offer another clean means of addressing the relevance of specific neuron subtypes in the context of a wild-type circadian network. Notwithstanding, a more precise definition of the connectivity between the circadian network including mapping synapses between clock neurons and other neurons (circadian or not), and finding out which neurotransmitters are involved, open up a window of opportunity. Needless to say, as the *Drosophila* adult brain becomes a less intractable preparation for electrophysiological recordings many of these unsolved mysteries will come to light.

This chapter has reviewed the main discoveries that aided the unraveling of the mechanisms governing a complex innate behavior, the circadian rhythms. To do this, researchers have subjected a variety of gene mutations and genetic manipulations to unnatural environmental conditions such as constant dark, constant light of different intensities or unusual photoperiods, in order to make the system trip and obtain information on the roles of the particular molecules/cells on a specific aspect of circadian regulation. Although extremely informative, this approach could be, at times, excessively reductionist. Therefore, one must not loose sight of the system as a whole, keeping in mind that circadian rhythms have developed in organisms in the wild, to guide their behaviors and physiology under light–dark cycles of naturally changing photoperiods and unforeseeable weather conditions (Kyriacou et al., 2008; Schiesari et al., 2011).

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Chapter 9

Courtship learning

Leslie C. Griffith

Introduction

The courtship rituals of insects are rich and varied. Almost every species has signature behaviors that serve to enhance the reproductive success of the individual performing them (Eisner, 2003). These behaviors are believed to be important for demonstrating both the appropriateness (sex, species, mating status) and the fitness of the animal to potential mates. Both males and females necessarily play a part in courtship behavior, minimally providing cues to the opposite sex for initiation and culmination of the process but usually engaging in a more complex exchange of information (Villella and Hall, 2008). In *Drosophila melanogaster*, the most obvious observable mating behavior is the courtship display of the male; females mating behavior is robust, but more subtle (Ferveur, 2010). Male courtship is now widely used as a model of a complex, genetically specified, innate behavior (i.e., one that can occur without learning).This stereotyped behavior is one that probably reflects a dependent action pattern, since the component behaviors occur in a fixed order once the behavior is initiated (Hall, 1994).

While the structure of the behavior itself may be innate, the specific conditions that lead a male to make the decision to engage in and to maintain courtship are not hardwired. Even as species-specific courtship plays an important adaptive role, the ability to inhibit the behavior under particular circumstance is also adaptive. Courtship behaviors are not without cost. An individual can risk exposure to predators when preoccupied with a mating ritual. Some mating rituals are flamboyant, drawn out and energy intensive. Time spent in mating is time that cannot be spent finding food. There is also an opportunity cost, particularly for elaborate behaviors, since during the period that an animal is focused on performing for a single other individual it cannot engage other potential mates. These costs mean that animals need to have mechanisms by which they selectively deploy their courtship behavior. The costs of courtship also suggest that, if a particular sensory cue predicts that a target is not receptive, then it would be advantageous for the animal to learn to suppress courtship toward all individuals displaying that cue. In fact, male courtship behavior is very plastic and is strongly modulated by a male's previous experience. In this chapter I will focus on the plasticity of *Drosophila melanogaster* male courtship behaviors and how males learn to modulate them using a wide variety of situational cues which likely engage a wide variety of neuronal circuits.

How a male decides to court

Plasticity of courtship occurs at the level of the decision to vigorously engage in the behavior. An understanding of how the decision to initially court, and to continue to court, is made, i.e., what information a male considers and how he processes that information, is critical to understanding how he modifies his behavior. Each insect species relies on a particular set of sensory cues to determine if a potential target is likely to be appropriate and receptive. The cues can involve any or all sensory modalities: vision, olfaction, gustation, hearing or somatosensation.

Drosophila melanogaster males are fairly indiscriminate about what they will consider courting. Pretty much any other fly: male, female, young or old is a potential target. Decapitated flies, dead flies, oenocyte-deleted flies which have almost no cuticular hydrocarbons, solvent extracted "dummy" flies and flies of other species (Gailey et al., 1986, Billeter et al., 2009, Ejima et al., 2005, Ishimoto et al., 2009) will even engender some level of initial courtship. This low initial threshold means that males do not lose opportunities for potential matings. Attentional processes may act to trigger the courtship program, since even white noise can increase the probability that a male will initiate courtship (Ejima and Griffith, 2008). Vision may serve a similar role; initiation of courtship is lower in the dark, visually defective males court less, and females that do not provide a moving visual stimulus are courted less (Pan et al., 2011, Joiner and Griffith, 1997, Ejima and Griffith, 2008). Whether these attentional processes and being in a heightened arousal state are simply permissive, or whether they are directly stimulatory for courtship, is unknown.

Once a target has been acquired, the initiation, continuation and vigor of the courtship ritual is governed largely by chemosensory (olfactory and gustatory) information. At a distance, olfaction is most likely the dominant specific sensory modality (c.f. Heimbeck et al., 2001), but once the male is in close proximity to the female, he acquires gustatory information by tapping and licking the female. Gustatory receptors in

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the foreleg, palps and proboscis are important for maintenance and progression of courtship (Greenspan and Ferveur, 2000, Ebbs and Amrein, 2007). The majority of the relevant molecules are likely cuticular hydrocarbons (Ferveur, 2005, Siwicki et al., 2005, Tompkins et al., 1980), but only in some cases have specific ligand/receptor relationships been established. Recent advances in mass spectrometry have allowed a more detailed analysis of the compounds present on flies of different ages and sexes (Yew et al., 2008, Everaerts et al., 2010, Arienti et al., 2010), and this is expanding the number of compounds that could act as social and learning signals. Even more interestingly, it is now becoming clear that there is transfer of many compounds between individuals during social interactions and that this transfer is likely to have an influence on behavior (Everaerts et al., 2010). One such compound, the lipid *cis*-vaccenyl acetate (cVA), has been studied and can affect how males modify courtship in response to mated females in terms of learning, as opposed to simply acting as an anti-pheromone as has been shown for several other compounds.

Both positive and negative chemosensory cues are believed to be important contributors to olfactory regulation of courtship. Electrophysiological evidence suggests that only four olfactory receptors respond specifically to fly odors (van der Goes van Naters and Carlson, 2007). Two of these, Or67d and Or65a, appear to be specific for cVA, a lipid made in the male ejaculatory bulb (Butterworth, 1969, Guiraudie-Capraz et al., 2007) that is transferred to the female vaginal tract during mating (Ejima et al., 2007). This compound has multiple roles; in the context of food odor it can act as an attractant (Schlief and Wilson, 2007, Bartelt et al., 1985), while in the context of a social interaction with another fly, it is an important signal of "inappropriateness." When males come in contact with other males, cVA signaling via Or67d increases aggression (Wang and Anderson, 2010). cVA signaling via Or65a can attenuate this aggression, presumably by inhibiting Or67d-mediated signaling (Liu et al., 2011). cVA can also inhibit male-male courtship (Kurtovic et al., 2007, Zawistowski and Richmond, 1986) and act as an associative cue to cause a generalized decrease in courtship of females (Ejima et al., 2007).

The nature of the stimulatory pheromones sensed by the olfactory system is still obscure. The Or47b and Or88a receptors respond to both male and female odors (van der Goes van Naters and Carlson, 2007). GABAergic signaling to Or47b-positive neurons has been shown to have a role in localization of females (Root et al., 2008), although elimination of Or47b does not affect latency to copulate with virgin females (Wang et al., 2011). The natural roles of Or47b and Or88a have been difficult to tease out; one reason for this may be that there are likely to be complex interactions between different olfactory neuron pathways (Liu et al., 2011) and between olfactory and gustatory pathways (Wang et al., 2011, Inoshita et al., 2011) which hierarchically regulate multiple aspects of social behavior.

The gustatory system also has both stimulatory and inhibitory roles in courtship. The Gr68a receptor appears to sense an unknown compound on females (Bray and Amrein, 2003) that can enhance the vigor of male courtship. Inhibitory pheromones are sensed by gustatory receptor neurons that are associated with bitter taste, such as those expressing Gr66a, Gr32a and Gr33a (Lacaille et al., 2007, Miyamoto and Amrein, 2008, Moon et al., 2009). While the full range of inhibitory ligands is unknown, one substance that has a role is Z-7-tricosene which requires the Gr32a gene product to be sensed (Miyamoto and Amrein, 2008) and acts via the Gr66a-expressing bitter receptors (Lacaille et al., 2007, Inoshita et al., 2011). The situation is complex due to the fact that many of the gustatory receptor neurons express multiple Gr genes and these proteins likely function as multimers (Montell, 2009).

Measuring and dissecting mechanisms of modification of courtship

The suppression of courtship toward a specific type of target is usually manifested by both a decrease in the initiation of courtship and a decrease in the vigor of courtship (Ejima et al., 2005), so may involve the response to any or all of these sensory cues. To engage plasticity mechanisms in a specific way, males need to find some unique identifier for the inappropriate target type that will not lead them to exclude potentially suitable mates. In some cases the male can habituate to the identifier, e.g., in short-term learning to suppress courtship to young males. But for the male to engage associative learning mechanisms that could lead to long-term memory formation, this identifier (which for most purposes can be thought of as a conditioned stimulus, or CS in analogy to classical conditioning) needs to be associated with some aversive stimulus or event (which would be analogous to the unconditioned stimulus, or US). In the case of courtship learning, it also appears that the association must occur in the context of active courtship. This lends an operant-like aspect to courtship learning. In cases where chemical cues for a specific type of associative learning have been defined, providing these chemicals to a male fly in the absence of a courtship object does not generate behavioral changes (Tompkins et al., 1983, Ackerman and Siegel, 1986, Ejima et al., 2007), unlike habituation, where the chemical stimulus alone is sufficient to cause modification of courtship (Ejima et al., 2005, Gailey et al., 1982). This linkage makes the association context-dependent and may function to increase the perceived importance of the associated cues.

There are many potential but unsuitable mates, and where it has been examined, males are easily able to learn to avoid entire classes of fly. Interestingly, however, the details of what sensory modalities are used to define CS cues and the nature of the US appear to vary widely, dependent on both the courtship object in question and the exact conditions under which the male encounters that courtship object. This variability highlights the robustness of learning in this context; males can and will use many types of cues to learn to avoid inappropriate targets. It also means that small changes in the learning environment can shift what the male learns in surprising ways, perhaps

by alterations in salience, a much discussed but poorly understood parameter. Experimental design therefore becomes very important if one is interested in the mechanisms of learning.

In general, assessment of plasticity of courtship behavior involves a two part assay (for protocols, see Ejima and Griffith, 2011, Ejima and Griffith, 2007). In the first part, the male is exposed to a trainer fly. The conditions under which the male encounters the trainer provide the cues he will use, so must be considered carefully. The size of the chamber, the lighting, the presence of additional odors, the mobility of the trainer and the length of time the male is exposed to the trainer, will all have an influence on memory formation. Some time after training, the experimental male is placed in a clean chamber with a tester fly and his level of courtship toward a tester target is measured. The amount of time allowed to elapse between training and testing provides a way to discriminate between short-term memory (STM) and long-term memory (LTM) processes. The nature of the tester, i.e., if it is the same type of fly as the trainer (same sex, age, mating status), provides a way of assessing the specificity of the memory formed.

The level of courtship (or some proxy for courtship, e.g., song or wing extension) is assessed at the beginning and the end of the training period, usually by measuring a "courtship index," the percent of a 10 min observation period spent in courtship activity. This is done to ensure that the male performs an adequate amount of courtship, since learning requires exposure to the cues in the context of the behavior. In wild-type animals, there is also a decrement in the courtship of the trainer over this period; the final courtship index is significantly less than the initial. Memory is assessed by measurement of another courtship index after the training period with a tester female. This value is usually compared to the courtship index of a sham-trained male who spent his training period in an empty chamber. If training has produced a memory, the courtship index of the trained male will be significantly less than that of a sham-trained male of the same genotype. One of the nice features of this behavior is that a failure to form memory is reflected in an increase in courtship behavior, i.e., sick flies are less likely to give false positives.

Types of courtship learning: Courtship suppression

The existence of plasticity mechanisms for learning can help fine tune the search for a mate. If a male is exposed to a particular type of unsuitable target enough, he can learn to ignore that subset of flies or terminate his efforts more quickly. In the following sections I will discuss the major types of courtship learning, organized by the type of fly used as a trainer during the behavior modification. As will be noted, in some cases the behavioral suppression extends to additional types of flies; this is determined by varying the tester fly. I will also discuss what we know about the sensory and signaling pathways used for each type of learning, and what, if anything, we know about where plasticity is occurring in the courtship circuitry.

Mated females

The first example of learning related to courtship was the observation that mature males exposed to a previously mated female in a small arena (0.4 cm^2) would subsequently show less courtship toward virgin females (Siegel and Hall, 1979). This suppression was a form of STM which lasted for 2–3 h and was sensitive to mutation in the *amnesiac* locus, a previously known mutant that disrupts olfactory memory consolidation (Keene et al., 2006). This last finding, that the suppression of courtship was disrupted by a known plasticity gene, was strongly suggestive that this phenomenon was mediated by learning as opposed to the actions of some anti-pheromone present on mated females. The subsequent finding that suppression could not be generated simply by extracts of mated females (Ackerman and Siegel, 1986, Tompkins et al., 1983), further strengthened the case that the change in courtship vigor was a measure of memory formation.

This paradigm has been used in many forms by many researchers. In a form close to the original, using a small training chamber in which the male receives a constant exposure to the mated female trainer, only STM is formed. This type of training is likely analogous to "massed" training, which in classical conditioning does not engage LTM mechanisms (Margulies et al., 2005). This means that the formation of memory is unlikely to require new transcription or translation, but will rely on extant signaling proteins to modify neuronal function (McGaugh, 2000).

The STM formed by mated female training generalizes to all females (Ejima et al., 2005), suggesting that in this case the CS the male learns is one that is found on all female flies, regardless of age or mating status. Training with mated females did not affect the level of courtship toward immature males, indicating that the memory was specific to a female CS. Training with virgin females does not produced a generalized suppression (Ejima et al., 2005, Siegel and Hall, 1979) although it can induce age-specific plasticity (see below). This generalized form of STM can be mimicked by training males with virgin females in the presence of cVA, which probably contributes to the US (Ejima et al., 2007). The other chemical cues that are involved as the CS are likely to be stimulatory cuticular hydrocarbons (Siwicki et al., 2005, Tompkins et al., 1980).

The STM produced with mated female training is sensitive to alterations in many signal transduction pathways. These include reducing signaling in PKA (Ackerman and Siegel, 1986, O'Dell et al., 1999, Gailey et al., 1984), CaMKII (Griffith et al., 1993) and PKC (Kane et al., 1997) cascades. While these kinase pathways are well known to affect synaptic plasticity, disruption of normal regulation of excitability by mutating potassium or sodium channel genes can also block memory formation, implying that plasticity of intrinsic properties is also important (Cowan and Siegel, 1984, Tompkins et al., 1983). Many additional neuronal signaling proteins have also been implicated in this type of courtship learning (for review see Griffith and Ejima, 2009).

The cellular circuits underlying this memory have been probed in several ways. The enrichment of expression of many plasticity-related genes in the mushroom bodies (Davis, 1993) strongly suggested that this structure would be involved in courtship memory, as it is in odor-shock conditioning. Accordingly, ablation of mushroom body formation using hydroxyurea feeding during development blocks STM (McBride et al., 1999). Other brain regions were probed using a transgene for a CaMKII inhibitor (Griffith et al., 1993) expressed under control of the UAS/GAL4 system, which allowed cell-specific manipulation of kinase activity (Brand and Dormand, 1995). The formation of memory was found to be reduced by expression of the inhibitor in the mushroom body gamma lobe and areas of the lateral protocerebrum as well as parts of the central complex (Joiner and Griffith, 1999). The recent identification of putative "decision centers" for courtship song in the lateral protocerebrum (von Philipsborn et al., 2011, Kohatsu et al., 2011) suggest that this relatively underexplored area could be very relevant to courtship learning and memory.

Joiner and Griffith, 1999 also looked at the behavior during the training period and provided evidence that exposure to mated females can engender at least two types of plasticity that depend on separate neuronal circuits. Inhibition of CaMKII in subsets of antennal lobe neurons and a different set of lateral brain cells could decrease the ability of males to modulate their courtship levels during the training period without affecting their ability to form memory as assessed by subsequent, temporally dissociated, testing. This type of effect, memory without decrement during the training period, was also seen with inhibition of PKC (Kane et al., 1997). The converse, a pure effect on memory and mushroom body structure, but no effect on courtship reduction during training, is found in *dfmr1*, a mutant in the *Drosophila* homolog of the Fragile X gene (McBride et al., 2005). These data suggest that the circuit mediating the decrease in courtship during the training period is anatomically distinct from, and not required for, memory formation. Whether this early plasticity is associative has not been determined.

Manipulating the training conditions to give the male an "inter-trial interval" by either increasing the size of the chamber so he can disengage from the female or removing him from the chamber periodically allows the male to form LTM, which can be measured over a week later (McBride et al., 1999). The LTM assay with mated females has been used to show that genes in the classical cAMP pathway (Sakai et al., 2004), *period,* a circadian gene (Sakai et al., 2004), and several other neuronal signaling genes including *Notch, orb2, Ecdysone receptor* and *blistered* (Presente et al., 2004, Keleman et al., 2007, Donlea et al., 2009, Ishimoto et al., 2009), are all required for LTM in this paradigm. The circuitry for this type of memory has not been extensively explored in an unbiased way, but mushroom bodies are required (McBride et al., 1999).

The specificity of the LTM that is formed, i.e., whether it is generalized to all females or whether males specifically can learn to avoid only mated females, has recently begun to be addressed. Early studies were consistent with findings for STM in that training with a mated female was shown to reduce subsequent courtship of virgin females (McBride et al., 1999, Presente et al., 2004, Sakai et al., 2004, Ishimoto et al., 2009). Recently, however, it has been found that some forms of training can produce LTM that is specific to mated female testers (Keleman et al., 2012).This type of LTM is dependent on activation of dopaminergic neurons and is mediated by an increase in sensitivity to cVA, a compound not present on virgin females. The exact nature of the training difference that produces this specific memory have not been explored, but it is likely that female rejection behavior during training is a key component for producing this type of plasticity.

Virgin females

For many years it was thought that only mated females could catalyze changes in male courtship behavior toward other females. This supposition was based on control experiments carried out by Siegel and Hall (1979) in which they trained males with mature virgin females and showed that there was no change in behavior toward mature female testers. In these control experiments, however, 20/24 of the males mated during the training period. Subsequent investigation of the conditions required for memory formation has shown that copulation during training prevents males from learning to suppress courtship, possibly by either removing of an important component of the US aversive signal (failure to copulate) or by providing a positive reinforcing cue (sexual gratification) which overrides the US (Ejima et al., 2005). These data suggested that the control experiments in the literature did not adequately address the specificity issue for training.

To look more thoroughly at the issue of whether males could learn to suppress courtship with virgin trainers, Ejima et al. (2005) trained males with virgin females of different ages under conditions in which males could not copulate with the trainer. What they found is that training with virgins could produce an associative memory that allowed males to discriminate between females of different ages. Males trained with an immature female (less than 1 day old) suppress courtship toward immature females, but not mature (mated or virgin) testers. Males trained with mature virgins showed memory with mature mated or virgin females, but not with immature virgins. The CS in these cases was shown to be an olfactory component of the cuticular hydrocarbons. The US is unknown, but may be associated with performance of the courtship behavior without completion of copulation.

Immature males

Inexperienced mature male flies will vigorously court young (less than 1 day old) males (Cook and Cook, 1975, Jallon and Hotta, 1979, Tompkins et al., 1980). Like courtship of mated females, there is a decrease in intensity over time, to approximately half the initial level in 30 min (Gailey et al., 1982). After such exposure, a male fly will show markedly reduced

courtship of a new immature male, but will show no decrement in courtship of females (Gailey et al., 1982). The suppression of young male courtship produced by previous experience with a young male lasts for several hours.

This reduction of courtship is thought to be due to habituation to aphrodisiac compounds made by very young males. The cuticular hydrocarbon profile of immature males is very different from that of a mature male. They make compounds that can stimulate courtship (Tompkins et al., 1980, Vaias et al., 1993) and have very low levels of cVA (Butterworth, 1969). Exposure of a mature male to an arena that has housed immature males (Gailey et al., 1982), hexane extracts of immature male cuticle (Ejima et al., 2005) or synthetic immature male pheromones (Vaias et al., 1993), in the absence of an actual fly, are each sufficient to reduce courtship of a subsequently presented immature male. Like other forms of habituation, a strong sensitizing stimulus, e.g., vortexing, can cause dishabituation (Ejima et al., 2005).

In simple systems, the mechanism of habituation to a substance often involves a change in sensitivity of the peripheral receptor. In the case of young male habituation, however, it is clear that the process is central, since ablation of higher centers (mushroom bodies) or depletion of central neuromodulators (dopamine or octopamine) block plasticity (Neckameyer, 1998, O'Dell, 1994). Mutants in the classical plasticity related genes *dunce, amnesiac* and *rutabaga* also block habituation (Gailey et al., 1982), as does normal aging (Neckameyer et al., 2000). While the cellular circuits for this particular nonassociative plasticity have not been fully explored, other examples of centrally mediated habituation have been documented in *Drosophila* (McCann et al., 2011, Das et al., 2011).

Training with artificial cues

While most examples of associative courtship learning use another animal as a trainer to provide both the CS and US cues, there are a number of examples in the literature where investigators have added additional, more defined, cues, to try to more precisely understand the nature of this type of plasticity. The first example of this was the use of a bitter compound, quinine, paired with an immature virgin female, to induce courtship suppression of a mature virgin female (Ackerman and Siegel, 1986). The quinine was interpreted to act as an US, and this experiment provided early evidence that courtship suppression could be generated by engaging associative learning mechanisms. Benzaldehyde has also been used as a cue to cause generalized courtship suppression (Ejima et al., 2005).

Types of courtship learning: Courtship enhancement

The ability of males to learn to suppress courtship of unreceptive or inappropriate targets has been the main focus of research in courtship learning, and there are obvious adaptive aspects to this type of learning. It would also be advantageous, however, to be able to enhance courtship response to classes of targets that are receptive or situations that are associated with potential mating success. Female sexual receptivity has been shown to be increased by the male courtship song (Kyriacou and Hall, 1984). This sensitization has been termed "acoustic priming" and is dependent on CaMKII (Griffith et al., 1993). The existence of similar mechanisms for males is suggested by several studies, which are described below. These behaviors represent potential new avenues for the study of courtship learning.

Courtship venue and sensitization

The size of the training chamber can have a significant impact on the level of courtship during training and the ability of a fly to learn to avoid mated females (Zawistowski and Richmond, 1987, Ewing and Ewing, 1984). Small chambers promote high levels of courtship, presumably by forcing the male into close proximity of the female and thus enhancing the exposure of the male to proximity-dependent cues like low volatility chemicals. Larger chambers can also be used for training, e.g., the use of food tubes (*c.* 5 mL volume) for training for LTM (McBride et al., 1999), but the amount of time the male must be exposed to the female to make memory is longer. This is likely due to requirement for both performance of courtship (which only occurs at close range) and the role of cVA, a lipid of relatively low volatility.

When the training chamber gets very large, e.g., 35–40 mL food vials, the ability to form generalized memory with a mated female is lost; males do not modify their subsequent courtship of virgin females (Zawistowski and Richmond, 1987, Dukas, 2005). This may be due to the very low amounts of total courtship or the short exposure time during training. In the case of Zawistowski and Richmond, they report courtship indices on the order of 25%, but they only allowed the male to be exposed to the female for 30 min. Dukas' protocol produced courtship indices that were even lower, 7% and the training period lasted only 1 h. In neither study was there an effect of mated female training on subsequent courtship of a virgin.

While the generalized memory of mated female training was gone in the large chamber, Dukas (Dukas, 2005) found that there was a specific memory formed for mated females. Males experienced with mated females had a longer latency to court them. This is analogous to what was seen by Keleman (Keleman et al., 2012). In examining virgin courtship after mated female training, this study found not only no decrease in courtship, but rather the experience with the mated female caused a small but significant decrease in the latency to courtship of a subsequently presented mature virgin female. Examining this effect further, it was found that experience with an immature virgin female, which is also an unreceptive target, produced a similar potentiation of mature virgin and even mated female courtship.

The author's interpretation was that the courtship experience allowed a behavioral refinement. It is worth noting that the flies tested were progeny of a wild population and not a standard laboratory resident strain. It also possible that there was some

sort of sensitization occurring, perhaps via a volatile chemical cue or a compound deposited on the vial by the trainer female. In the absence of a clear negative signal from cVA, this putative positive cue may act as a courtship enhancer. A similar type of phenomenon, an increase in courtship-like wing display lasting 30–60 min after a brief exposure to a virgin, has been documented (Medioni and Manning, 1988). This prolonged state of sexual arousal may be due to peripheral sensitization, since it is not affected by mutation of the *dunce* gene. These types of sensitization are interesting and mechanistically underexplored forms of plasticity.

Training with food odors

Associative learning mechanisms can also be employed to enhance courtship in particular environments. The Greenspan group found conditions that would allow a male to learn to increase courtship of a normally unattractive target (Broughton et al., 2003). In their paradigm, a mature male was exposed for 30 min to an immature virgin female in the presence of a food-related odor (grape juice, apricot juice, or a mixture of isoamyl alcohol and ethyl acetate). Males were then tested, after a 1 h rest in a clean chamber, for courtship of a decapitated mature male in the presence or absence of odorant. All odorants were able to produce a pairing-dependent and extinguishable associative memory. Formation of this memory was blocked by expression of a CaMKII inhibitor peptide under control of the heat shock promoter.

This is a particularly interesting form of plasticity and may actually represent a suppression of courtship inhibition. Mature males normally do not court other mature males, but rather engage in aggressive behavior (Chen et al., 2002). This aggressive response is tied into the sensing of cVA on the other male (Wang and Anderson, 2010), but can be modified by social experience (Liu et al., 2011). It would be interesting to determine if the food odor associative learning is mechanistically similar to social amelioration of aggression.

Social learning

Social interactions can have significant effects on many behaviors, including aggression and courtship. While not yet well studied in terms of classical plasticity mechanisms, there have been many observations that suggest that flies can actually learn from group and individual social experiences. One interesting example is the ability of females to discriminate in favor of unfamiliar conspecific males (Odeen and Moray, 2008). This set of experiments indicates that flies can remember individuals, even if they have not copulated with that individual. Group interactions can also modulate mating (Krupp et al., 2008). Wild-type flies in mixed genotype groups which contain circadian mutants, mate more than wild type males in homogenous groups of the same size. These effects may be traceable to changes in cuticular hydrocarbons in the mixed genotype groups, implicating changes in the chemosensory milieu. These are but two examples of the type of complex interactions flies engage in. The cellular and molecular underpinnings of these behaviors are likely to involve plasticity mechanisms.

Conclusions

Is courtship plasticity really "learning"? The answer is unequivocally positive. While courtship behaviors are probably hardwired into the fly nervous system, the ability of a male to access that behavioral circuit is clearly gated by his experience. The behavioral data show that courtship can be affected by plastic processes that are largely consistent with the principles that have been derived by psychologists to understand human learning. In some cases the ability to manipulate the presentation of the cues has allowed courtship learning to be clearly shown to be associative (Ackerman and Siegel, 1986, Broughton et al., 2003, Ejima et al., 2005). In others, the learning clearly reflects habituation in that it requires just one cue and can be dishabituated by a strong stimulus (Gailey et al., 1984, Ejima et al., 2005). Both long-term and short-term plasticity have been demonstrated (McBride et al., 1999), with a differentiation between the two in terms of training protocol that supports the idea that these represent true forms of STM and LTM. At the molecular level, all forms of courtship learning that have been examined appear to be dependent on signal transduction pathways that, while largely identified first in *Drosophila* learning screens, are known to be critical for learning and memory in mammals.

Reproductive behaviors are critical to species survival and are therefore robust and usually redundantly driven. Plasticity of reproductive behaviors is also robust since learning when and where to engage in courtship has adaptive value, allowing a male to maximize his chance of success and minimize his output effort. The number of ways a male can learn to modify his behavior is fairly astonishing and our understanding of this type of learning is growing even as the number of reports on different ways a fly can learn increases. While these behaviors can be complex since they involve multiple sensory pathways and can be triggered by redundant mechanisms, they provide a beautiful window into how learning provides a critical adjunct to natural, ethologically relevant, behavior.

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Chapter

10

Place memory

Daniela Ostrowski and Troy Zars

Introduction

Most of the time people can easily orient themselves in their daily life, e.g., moving through a familiar room in the dark. Sometimes, however, orienting in a new and/or complex environment can be a challenge: as in it can be a challenge to remember where you parked the car in a large garage. Indeed, it is when a task like this is difficult that it is most obvious that we use memories to orient in space. The ability to encode information about the environment and one's spatial orientation within the environment is called spatial learning / memory (Bitterman, 1996). Besides humans, animals ranging in nervous system complexity from primates and rodents to simpler organisms like the fly *Drosophila* and the nematode *C. elegans* are capable of at least some sorts of spatial learning (Kahsai and Zars, 2011, Zars, 2009, Qin and Wheeler, 2007, Wang and Spelke, 2002, Burgess et al., 2002, Morris, 1984).

How do animals form spatial memory? Insects provide a great opportunity to understand mechanisms of spatial memory formation. Desert ants (*Cataglyphis bicolor*) can travel up to 200 m from their nest when foraging and are capable of integrating their path to find the shortest route home (Collett, 2010, Wehner, 2003, Collett and Collett, 2000). Honeybees (*Apis mellifera*) can even communicate with each other the location of a food source within an environment (Menzel et al., 2011, Esch et al., 2001). How do these small brains accomplish this task? Despite numerous behavioral studies of spatial orientation and memory in insects, and invertebrates in general, not much is known about the cellular and circuit mechanisms of place memory.

The model organism *Drosophila melanogaster* has been used for decades to highlight the processes supporting various types of learning and memory (Zars, 2011, Zars, 2010, Keene and Waddell, 2007, Davis, 2005). More recently, spatial learning and memory has become a focus of investigation in the fly. New genetic tools and behavioral assays have been used to identify neuronal and molecular mechanisms of place memory. Here we review (1) the behavioral assays used to study place memory, (2) what brain structures have been identified to play an important role in place memory, and (3) what genes interfere with spatial memory.

Behavioral assays

At the moment two different types of behavioral assays have been developed to analyze underlying mechanisms of place memory in *Drosophila*. First, variations on the heat-box apparatus, in which individual flies are trained in a dark, narrow chamber to avoid one chamber half with high temperatures (up to 45 °C) as a negative reinforcer have been used (Sitaraman and Zars, 2010, Zars et al., 2000, Wustmann et al., 1996). Second, a heat-maze apparatus, conceptually based on the Morris water maze, tests spatial memory with obvious visual landmarks (Ofstad et al., 2011, Foucaud et al., 2010). In this second apparatus, walking flies orient with respect to visual cues to find a cool tile (20 °C) within an otherwise hot floor.

Place memory in the heat-box paradigm

The heat-box apparatus consists of multiple rectangular chambers (each 40 × 4 × 2.5 mm) in which single flies are allowed to walk freely back and forth (Zars, 2009, Zars et al., 2000, Wustmann et al., 1996) (Fig. 10.1A). The position of a single fly within each chamber is recorded at 10 Hz and 0.32 mm resolution throughout an experiment. Fast temperature changes within the chambers are provided by Peltier-elements on top and bottom. Before each training session, the flies are provided a pre-test phase, typically 30 seconds. During this time, flies can freely run from end to end in the chamber with no danger of increasing temperatures. On average, flies show very little or no spontaneous side, preference within the chamber (i.e., the front half or the back half of the chamber) (Diegelmann et al., 2006, Zars et al., 2000). During conditioning (the training phases) one chamber half is defined as the side associated with punishment and the other as not. Every time the fly enters the punishment-associated side, the whole chamber heats up to an aversive temperature (33–45 °C). The return of the fly to the other side quickly cools down the chamber to a non-aversive temperature (24 °C) (Zars, 2001, Sayeed and Benzer, 1996). The following post-test measure of place preference is performed, and the chamber is kept at the same non-aversive temperature (24 °C). Wild-type flies still avoid the chamber-half associated with the high temperature punishment and spend more time

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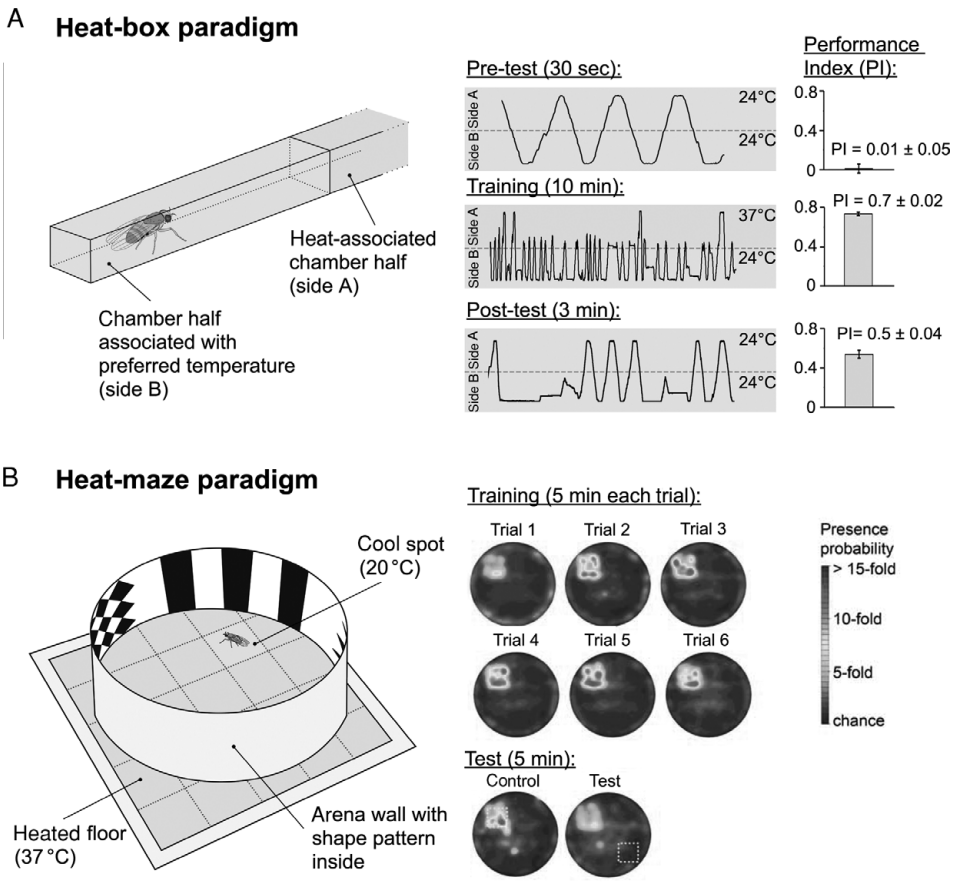

Fig. 10.1. Behavioral paradigms for testing place memory. A The heat-box paradigm is used to train single flies to avoid one half of a long narrow chamber. One half of the chamber is associated with a high temperature, the other half not. A conditioning experiment is composed of Pre-test, Training, and Post-test phases. In the Pre-test phase any spontaneous side preferences are measured, which are typically close to zero. Training for several minutes associates one half of the chamber with a high temperature, like 37 °C. Flies typically avoid the chamber half associated with high temperature (a PI of 0.7 is shown). A Post-test measures persistent avoidance of the chamber-half previously associated with high temperature. B The heat-maze paradigm has a platform with a largely uniform warm temperature with one cool tile. Conspicuous patterns on the wall allow flies to orient in the arena. The probability of locating to the cool tile increases with trial number, and persists in the test phase (adapted from Foucaud et al., 2010).

within the unpunished side. A performance index (PI) for this type of place memory is calculated by the difference in time a fly spent in either chamber half (unpunished side vs. punished side) divided by the total time within a session. The PI can vary from 1.0 to −1.0. Zero indicates that, on average, the flies spent equal time on both sides of the chamber, whereas 1.0 shows a perfect side preference of the fly for the unpunished chamber half. Since all experiments are performed in the dark, flies aren't able to use visual cues for orientation. In fact, by ruling out other possibilities, flies are thought to use tactile and/or ideothetic cues for orientation (Putz and Heisenberg, 2002, Wustmann and Heisenberg, 1997).

Conditioning in the heat-box has an operant component. The natural explorative behavior of a fly, walking back and forth in the chamber, causes a high temperature punishment when it walks into the dangerous part of the chamber. With the correlation of spatial position and punishment, flies change their behavior by reducing locomotor activity and avoiding the chamber half associated with high temperatures (Putz and Heisenberg, 2002). Yoking experiments showed that locomotor changes and spatial memory formation strongly depend on the control of the behavior/punishment contingency. In these experiments a master fly is conditioned as described above. The yoked fly's chamber, on the other hand, is linked to the master fly and heats up whenever the master fly is located within the punishment-associated half of the chamber. That means yoked flies have no control of the chamber temperature. Yoked flies show a smaller reduction in locomotor activity and no spatial preference in a subsequent post-test (Sitaraman and Zars, 2010, Putz and Heisenberg, 2002).

Many properties of *Drosophila* place conditioning are similar to other associative memory tests across species, suggesting evolutionary conserved mechanisms. Memory strength strongly depends on the intensity of reinforcing stimuli, characterized by an asymptotic curve (Herrnstein, 1997). For heat-box place memory in *Drosophila* temperatures from 33 °C to 45 °C were used as aversive stimuli to induce place memories. The higher the temperature, the stronger the fly's avoidance of the chamber side associated with punishment. In addition, increasing training duration (ranging from 2 min to 20 min) also increases place memory levels (Diegelmann et al., 2006, Putz and Heisenberg, 2002). Flies also avoid temperatures below 24 °C (24 °C is the preferred temperature of *Drosophila* given free choice on a gradient (Hamada et al., 2008, Sayeed and Benzer, 1996)). Interestingly, low temperatures do not have the same reinforcing properties as high temperatures. For example, a 20 min training period using 15 °C as an aversive temperature induces a PI of about 0.2. In contrast, using the same training duration and 30 °C as reinforcer, which is even a smaller difference compared to 24 °C, induces much stronger memory (PI of

about 0.4) (Zars and Zars, 2006). The differences in the magnitude of memory with warm and cold temperatures could be due to different thermosensors (hot vs. cold thermosensors) and/or different sets of neurons that processes temperature information (Zars, 2001).

In nature *Drosophila* are challenged by a variable temperature environment. *Drosophila* are capable of modulating conditioned behavior and spatial memory performance with dynamic reinforcement intensities (Zars and Zars, 2009). Matching experiments showed that a switch from either lower temperatures to higher (24 °C to 33 °C) or higher temperatures to lower (41 °C to 33 °C) change both the side preference during training and the memory performance in the post-test. Within 2 minutes of training, corresponding to one or two experiences with a new reinforcer intensity, flies match their conditioning behavior to the new reinforcement intensity.

In many organisms memory strength of associative learning can also be modulated by the type of training. Using the same duration of training, but inserting short periods of rest (spaced training) enhances later memory levels. For *Drosophila*, improvement of memory formation with spaced training is documented for both classical conditioning with odor and operant conditioning using the heat-box paradigm (Putz and Heisenberg, 2002, Tully et al., 1994). The place memory PI increases about 0.2, from 0.55 to 0.75, when using spaced training of 6 cycles (1 cycle: 2 min training, 1 min rest) compared with training sessions without any interruption (12 min total training time).

Another common feature of associative memory for both vertebrates and invertebrates is that pre-exposure to either aversive or appetitive stimuli can influence later associative learning using the same stimulus. Interestingly, the pre-exposure effect can either enhance or inhibit later memory formation. For place memory in *Drosophila*, it has been shown that a pre-exposure to aversive temperatures (41 °C) enhances later spatial memory if using training conditions that usually result in low memory levels (low temperature reinforcer, short training session) (Sitaraman et al., 2007). This place memory enhancement lasts at least 20 minutes (longer periods have not been tested). Furthermore, yoking experiments showed that the lack of prediction for high temperatures, and not exposure to high temperatures itself, causes the increased memory performance (Sitaraman and Zars, 2010). In these experiments flies were trained for one, two, four, or seven sessions (each 6 min) with 41 °C reinforcement. Master flies had control of the temperature environment through place preference and yoked flies received the same temperature changes as their master fly, but without any behavioral control. Subsequent conditioning with low temperature reinforcement (30 °C) showed an enhancement of spatial memory in the yoked fly group already after two training sessions (about 1 min of high temperature exposure in total). By contrast, with multiple training sessions the master fly group showed lower spatial memory performance, indicating matching conditioning behavior, consistent with the results from more straightforward matching tests (Zars and Zars, 2009).

Changes in spatial preference in *Drosophila* usually decrease to negligible levels within minutes in the absence of the reinforcing contingency, indicating the extinction of fly's place memory. However, if flies were allowed to rest in a food vial, then placed back in the heat-box and given a short reminder (which alone induces no spatial preference), flies can retrieve spatial memory (Putz and Heisenberg, 2002). Longer rest intervals before the memory tests revealed that spatial preference lasts up to 2 hours when using a 4-min spaced training (40 °C reinforcement) and a 30-second reminder prior to the post-test. Although not studied in detail, retention of place memory can last longer if using longer training periods and memory decay is slightly slower in spaced training compared to massed training conditions (Putz and Heisenberg, 2002).

Place memory in the heat-maze paradigm

The heat-maze (Foucaud et al., 2010) consists of a large circular arena (19 cm diameter), enclosed by a heated ring to prevent escape by a fly (Fig. 10.1B). The floor of the arena is heated by Peltier elements to an aversive temperature (37 °C or higher), except for a so called safe zone whose temperature is set to 20 °C. Individual flies with clipped wings are put in the heat-maze and constantly recorded with a camera; walking traces are used in the analysis of place preference. Visual cues, either proximal (colored dots on the floor) or distal (shape-patterns on a wall surrounding the arena) help the flies to orient and navigate within the maze. Naïve flies initially use thigmotaxis and random search to explore the new environment. Wild-type *Drosophila* already show a high preference for the "safe zone" within 3 minutes of the first training session (about 20-fold higher chance that a fly resides within the safe zone). Throughout several training trials (on the order of 30 min) flies improve their performance of locating the safe zone by decreasing the latency and decreasing the distance traveled in escaping the hot parts of the floor. In addition, flies increase the total time spent in the safe zone and decrease the distance moved within the heated arena. Both proximal and distal cues result in improvement of locating the safe zone. Furthermore, a slight gender difference was found using distal cues. Compared with females, male flies showed a higher locomotor activity, but performed worse in locating the safe zone.

Through the training phase, flies change their search strategies from more general (random search, thigmotaxis) to more accurate (e.g., scanning or direct search). An abrupt change of the environment after six training periods impairs the flies' performance to find the safe zone. For example, linking the safe zone to a different wall pattern causes an increase of latency to the first contact to the preferred area. The flies initially still search for the safe zone in the quadrant with the previous represented wall pattern. These results show that *Drosophila* indeed are able to acquire and remember a representation of its environment and can both improve its orientation and navigation to a preferred area by using visual cues. Interestingly, flies also improve their performance to find the safe zone if

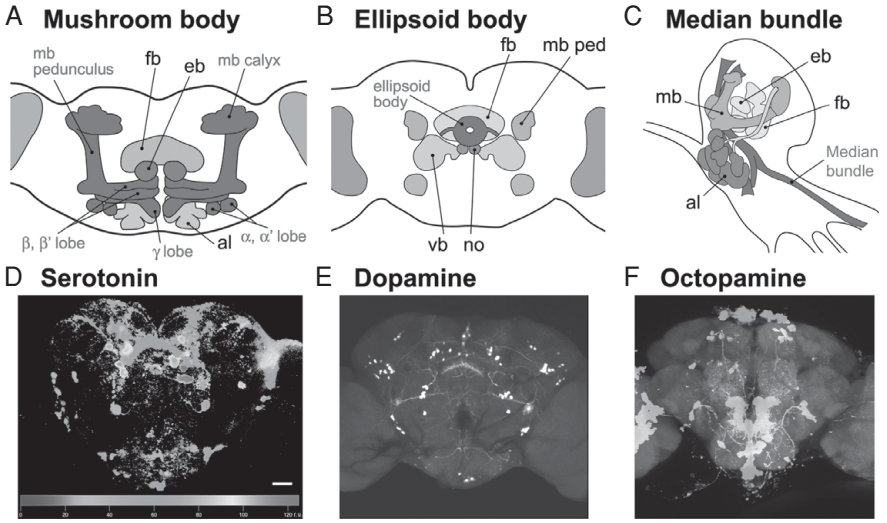

Fig. 10.2. Organization of the fly brain. Parts of the fly brain examined for roles in place memory. A–C Major neuropil structures like the mushroom bodies (mb, oblique horizontal view), ellipsoid body (eb, anterior view), and median bundle (sagittal view) have been implicated in place memory (labeled in red). Other major neuropil structures are labeled for position references: fan-shaped body (fb), antennal lobe (al), ventral body (vb), nodulli (no), and peduncle of the mushroom bodies (mb ped). Furthermore, three aminergic modulatory systems have been examined for roles in place memory. D The serotonergic system has been implicated in establishing normal place memory. Depth encoded image shows cell bodies and innervation sites of serotonin neurons labeled with anti-serotonin (blue is anterior, red is posterior). E–F Although the dopaminergic (TH-GAL4) and octopaminergic (TDC-GAL4) systems have been implicated in several forms of aversive and appetitive memories, manipulation of these neurons does not alter place memory. Scale bar = 50 μm.

wall patterns are presented randomly relative to the safe zone throughout training sessions. Analyses of the flies' search strategies reveal differences compared to flies in a constant environment. Flies of the random group fail to develop accurate search strategies, but still decrease their thigmotaxis. This indicates that flies may also use other orientation cues than visual landmarks and/or have the ability to adapt easily to changes in the environment.

A similar experimental set-up also tests the ability of *Drosophila* for visually guided place memory (Ofstad et al., 2011). Instead of four different wall patterns, LED panels present either spaced horizontal, vertical or diagonal bars. Furthermore, in this experimental set-up flies were trained and tested individually or in groups. After each training cycle (10 cycles in total, each 5 min) flies remain within the heat-maze arena, but the orientation of wall patterns and the cool spot is rotated by 90°. Similar to the results of Foucaud et al. (2010), almost all flies (94%) locate the cool spot in the heated arena within the first training session and improve their ability to find the safe zone during training. With every rotation of the wall pattern and the safe zone, they needed less time to find the new target's position by using shorter paths and more directed search. While flies trained with uncoupled wall pattern presentation, or in the dark, did not show improvement in some parameters used to quantify efficiency for finding the cool tile, flies were still able to find the cool tile and spent significantly more time in the cool tile quadrant than the other quadrants. Thus, visual landmarks aid the localization of the cool tile, but they are not the sole cue that can be used to solve the task.

In a probe trial of 60 seconds (11th trial) the wall pattern is again rotated but the cool spot is not re-established. The calculated probe memory index displays the time flies spend searching for the cool spot in the quadrant where they were trained to expect the cool area minus the time they spend in the quadrant that has the same distance from the starting quadrant but in the wrong direction. After ten training sessions, the place memory performance index is about 0.5. A memory retention curve revealed significant preference for the quadrant with the expected cool spot up to 2 hours after training. Flies tested 4 and 6 hours after training still showed a memory that was significantly different from zero, but not different from flies trained with wall patterns unlinked to the cool spot.

Neural systems involved in place memory

The neural systems underlying place memory in *Drosophila* are still not fully understood. Nevertheless, some ideas of which parts of the adult brain are important for place memory have been described. The following section will discuss what role the mushroom body, ellipsoid body, and median bundle neurons may have in place memory. The modulatory neurotransmitters serotonin, dopamine and octopamine will also be examined.

Mushroom bodies

The *Drosophila's* mushroom bodies (MBs, also sometimes called the *corpora pedunculata*) are two mirror symmetrical paired neuropil structures formed by ~2000 densely packed neurons called Kenyon cells (Fig. 10.2). Dendritic arborizations of Kenyon cells form a cup-shaped structure called the calyx. Kenyon cell axons run strictly parallel, forming the peduncle and finally either bifurcate into vertical (α, α′) and horizontal lobes (β, β′) or shape a third type of lobe – the γ-lobe. Several studies revealed a quadripartite structure of the Kenyon cells organization (Tanaka et al., 2008, Heisenberg, 2003, Lee et al., 1999, Ito et al., 1997, Yang et al., 1995). The dendritic arborizations within the calyx can either be clustered in four gusset-like domains or diffusely organized across the matrix of the calyx. The axons of Kenyon cells that have their dendrites in gusset like domains project to α and β-lobes and cells with dendritic arborizations in the calyx matrix supply the α′ and β′-lobes. Clawed Kenyon cells that project into the γ-lobe have dendrites within the entire calyx. Axonal projections of the Kenyon cells are furthermore organized into concentric layers in the peduncle and lobes, in which younger neurons project

first into the core to shift to the surrounding layers as they differentiate (Kurusu et al., 2002). During development of the fly the Kenyon cells are born in a specific temporal order. Neurons that project into the γ-lobe of the adult MB are born first (prior to the mid-3rd instar larval stage). Neurons projecting into α′/β′ lobes are born between the mid-3rd instar larval stage and puparium formation and finally, neurons that form α/β lobes are born after puparium formation. Beside Kenyon cells other "intrinsic" neurons were described whose cell bodies are located in the protocerebrum distant to the Kenyon cell bodies. Their axonal branches bifurcate and form synaptic connections exclusively within various lobes (Tanaka et al., 2008, Ito et al., 1998).

Various neurons with extrinsic properties have been described. The projection neurons of the olfactory pathway are one example of neurons that have connectivity with the MB. Projection neurons have their dendritic region within the antennal lobe glomeruli, the main output region for olfactory sensory neurons. The projection neurons run through the inner and medial antenna-cerebral tract to the calyx of the MB as well as to the lateral horn (Caron et al., 2013, Jefferis et al., 2007, Tanaka et al., 2004, Wong et al., 2002, Marin et al., 2002). Other extrinsic neurons, that have their dendritic regions in different parts of the protocerebrum, have been described to innervate all of the lobes of the MB, and connect to most regions of the central brain, including connecting different regions of the mushroom bodies and the superior lateral protocerebrum (Tanaka et al., 2008, Ito et al., 1998).

The complex anatomical structure of the *Drosophila* MB and the numerous extrinsic neurons suggest a key role for this structure in many behaviors. Neuroanatomy suggests that the MBs are involved in olfaction. Olfaction *per se* is not severely affected in MB altered flies, but associative memory of olfactory information is strongly impaired (Kahsai and Zars, 2011, McGuire et al., 2005, Heisenberg, 2003, Schwaerzel et al., 2002, McGuire et al., 2001, Dubnau et al., 2001, Connolly et al., 1996, de Belle and Heisenberg, 1994). In other associative learning tasks, independent of the olfactory pathway, the MBs also play an important role. Although straightforward visual pattern memory requires normal central complex function (see below), retrieval of visual pattern memory after context changes and courtship memory strongly depend on MB function (Zars, 2010, Liu et al., 2006, Joiner and Griffith, 2000, McBride et al., 1999, Liu et al., 1999, Joiner and Griffith, 1999). The MBs are also necessary for some non-learning tasks. For example, flies lacking most of the MBs as a result of a mutation fail to decrease walking activity measured over several hours (Martin et al., 1998). In addition, an open field study revealed that MB altered flies show diminished avoidance behavior of the central part of an open arena (Besson and Martin, 2005).

In ants, bees, and cockroaches the MBs have been implicated in visually guided place memory (Mizunami et al., 1998, Withers et al., 1995, Bernstein and Bernstein, 1969). Ofstad et al. (2011) analyzed the function of MBs in visually guided spatial memory of *Drosophila* in the heat-maze apparatus. Flies with impaired MB function using several GAL4 driver lines, or hydroxyurea-treatment (a pharmacological procedure that eliminates dividing cells) to selectively remove a large majority of the Kenyon cells, leaves flies with normal place memory. This likely indicates that visually guided place memory formation is independent of MB function in *Drosophila*. Whether or not the MBs may be important in spatial memory tasks with more complex conditioning, e.g., context changes or multiple stimuli for classical and operant components (as it is shown for visual learning in general) needs to be investigated further.

Spatial memory formation in *Drosophila* can also be formed independent of obvious visual cues. This type of memory has been studied using the heat-box apparatus. Flies with chemically ablated MBs, caused by hydroxyurea-treatment, still have the ability to form spatial memory in the heat-box (Wolf et al., 1998, de Belle and Heisenberg, 1994). In addition, more complex place conditioning with context changes result in the same phenotype compare to flies with intact MBs, indicating no important role for MBs in place memory (Putz and Heisenberg, 2002). But, hydroxyurea-treated flies still have 10% of MB cells left, which may be the important MB neurons that participate in the formation of spatial memory. On the other hand, using the same protocol to impair MB function was successfully used to completely abolish the fly's ability for associative memory with odors (de Belle and Heisenberg, 1994).

An important set of experiments used ectopic expression of a wild-type *rutabaga* cDNA (rut^+ cDNA) to rescue the impaired spatial memory phenotype of *rutabaga* mutant flies with expression in different subsets of the brain. The *rutabaga* gene encodes a type 1 adenylyl cyclase, mutation of which leads to a severe deficit for flies in several associative learning paradigms, including place memory (Zars et al., 2000, Wustmann et al., 1996, Levin et al., 1992). The four different Gal4 enhancer trap lines that rescue the *rutabaga* mutant phenotype show expressions in at least one of the MB lobe systems. Lines c772 and c115 highly express in all lobe systems (α/β, α′/β′ and γ-lobe). The line c522 showed expression only in the α′/β′-lobes and c271 only in the α/β-lobes. One conclusion is that MBs do not play a significant role in place memory since the expression patterns in the MBS of the four rescue lines differ (Zars et al., 2000). Another conclusion could be that the expression of rut^+ cDNA in any one of the lobe systems is sufficient to rescue the *rutabaga* phenotype. In cockroaches, it has been shown that lesions in MB β-lobes (but not α-lobes or calyces) impair place memory (Mizunami et al., 1998), which indicates that some structures of the MB may be necessary for accurate place memory.

Ellipsoid body

The general organization of the central complex (CC) structures is conserved in several insect species and the neuroanatomical organization suggests integration of neuronal information from both brain hemispheres (Strausfeld and Hirth, 2013, Hanesch et al., 1989) (Fig. 10.2). The ellipsoid body (EB) of *Drosophila,* a core part of the CC, appears as a circular neuropil structure

located in the center of the CC. Besides the EB three other brain neuropils (protocerebral bridge (PB), fan-shaped body (FB) and paired noduli (NO)) and two accessory areas (paired ventral bodies (VBO) and paired lateral triangles (LTR)) form the CC. All CC units are somehow linked via various neurons, where one neuron can connect up to three different substructures. About 30 different neuron types were identified based upon morphology and topography and mainly classified as either large-field or small-field neurons (Strausfeld and Hirth, 2013, Young and Armstrong, 2010, Renn et al., 1999, Hanesch et al., 1989). Large-field neurons are mostly extrinsic neurons that arborize in only one single substructure and link it to accessory structures or other central brain regions. The majority of small-field neurons are intrinsic neurons that interconnect small subdivisions of either one or more CC substructures.

The EB is organized in a concentric pattern of 12–16 glomeruli around its circumference. Furthermore, it is divided into two principal disks along the anterior–posterior axis. The main input into the EB is provided by large field neurons called ring (R)-neurons. The primary neurite of R neurons run through a tract and bifurcates into dendrites that connect to the paired lateral triangles and an axon that forms a ring-like connectivity within the EB. Four different types (R1–R4) have been described, based on the position their axon enters the EB neuropil and based on the arborization patterns within the EB (Hanesch et al., 1989). R1–R3 neurons project to the center of the EB via the EB canal and arborize outwardly, while R4 neurons enter the EB neuropil from the periphery and project inwardly within the outer ring. R1–R3 neurons are further classified regarding their arborization zones within the EB. R1 neurons project exclusively to the inner ring, R2 neurons exclusively to the outer ring (like R4 neurons) and terminals of R3 neurons are formed within both the inner and mid-rings. In addition, R4 neurons can be distinguished into R4d neurons whose axons arborize in the distal zone and R4m neurons whose axons project to the medial domain of the outer ring. With respect to the anterior–posterior axis, axons of R2, R3 and R4 neurons project to the anterior disk, while R1 neurons project to the posterior disk. The described arborization patterns of R1–R4 neurons reveal a topographic segregation, whereby most of the EB neuropil is innervated. A topographic segregation was also found for the dendritic regions of R neurons within the lateral triangles that in turn receive afferent input from different parts of the brain (e.g., optic foci). This indicates a morphological and functional division of R neurons (Renn et al., 1999). Only a few extrinsic R neurons (ExR1 and ExR2) are described so far. These neurons receive input from brain regions outside the CC and have arborizations in the dorsal fan-shaped body, the distal EB and the paired ventral bodies. Postsynaptic structures of ExR1 within the EB have also been shown (Young and Armstrong, 2010). Various intrinsic small field neurons (6 different types) with arborizations within the EB and other structures of the CC have been described (Young and Armstrong, 2010, Hanesch et al., 1989). Thus, connectivity to all CC substructures has been described.

Two sets of neurobehavioral experiments that alter the EB impact the understanding of spatial memory mechanisms. A novel version of the Buridan paradigm was used to test a seconds-long orientation memory (Neuser et al., 2008). In the Buridan paradigm, individual flies normally walk back and forth between two landmarks (Strauss and Pichler, 1998). A test for an orientation memory comes from the presence of a distracting landmark, which a fly will orient towards, then removal of all landmarks. A fly will accurately re-orient toward the initial landmark target up to four seconds after distraction, showing that flies can maintain an orientation goal for several seconds. Flies with a defective ellipsoid body (ebo^{KS263} mutants) show no orientation memory, while mushroom body affected flies (e.g., HU ablation) still have orientation memory. Furthermore, blocking synaptic activity of R neurons (R1, R3, or R4 neurons) prevented the formation of an orientation memory completely. Finally, rescue experiments of S6 kinase II (S6K2) mutants within R neurons restored functional orientation memory (more on this gene below), indicating an importance of this kinase signal for an orientation memory. The function of R neurons of the EB in visually guided spatial memory using the heat-maze apparatus has also been recently examined (Ofstad et al., 2011). Blocking neuronal activity in R4/R1 neurons or R1 neurons alone impair spatial memory formation, whereas blocking the activity of R4 neurons alone do not interfere with spatial memory formation. It could be that the EB function in supporting a seconds-long orientation memory is critical in establishing a longer lasting visually guided place memory.

Median bundle

The median bundle consists of two large bundles that run through the center line (anterior / posterior) of the brain (Fig. 10.2). Numerous neurons project axons through the bundles in both directions (ascending or descending). Neurons with ascending axons project to the roof of the protocerebrum where they form an extensive system of terminals. The median bundle also contains axons of neurons that originate in the protocerebrum from cell bodies in the *pars intercerebralis*. These neurons have dendrites that extend over the dorsal medial protocerebrum and axons that descend through the medial bundle to terminate in the tritocerebral ganglion (Armstrong et al., 1995).

Neurons of the median bundle have been described to function in several behaviors, including place memory. In *Drosophila* courtship (Manoli and Baker, 2004, Joiner and Griffith, 1999), genes that are critical for various behavioral phases are expressed in neurons of the median bundle, and altering activity of these neurons alters courtship behavior (Stockinger et al., 2005, Manoli and Baker, 2004). Neurons of the median bundle have also been discussed for place memory in *Drosophila* (Zars et al., 2000). The *Drosophila* type 1 AC mutant (*rutabaga*) flies perform poorly in the heat-box paradigm. Rescue experiments were performed to restore the

rutabaga mutant phenotype using four GAL4 enhancer trap lines to drive the expression of a wild-type *rutabaga* cDNA in subsets of CNS neurons (Zars et al., 2000). A comparison of the expression pattern of the four different lines identified neurons of the antennal lobes, median bundle and the ventral ganglion as candidates for *rutabaga*-dependent place memory.

Biogenic amines

Biogenic monoamines are important neuroactive molecules in both vertebrate and invertebrate animals. Physiologically, they can act as neurotransmitters, neuromodulators, or neurohormones. In the *Drosophila* CNS dopamine (DA), 5-hydroxytryptamine (5HT or serotonin), histamine (HA), and octopamine (OA) are the major amines observed (Fig. 10.2). They are expressed in distinct cell populations of both the larval and adult nervous system and modulate multiple behaviors, including memory formation (Sitaraman et al., 2008, Hoyer et al., 2008, Schroll et al., 2006, Kume et al., 2005, Saraswati et al., 2004, Schwaerzel et al., 2003, Baier et al., 2002, Monastirioti et al., 1996). In classical olfactory conditioning a positive or negative stimulus can be associated with the presence of a specific odor. An odor that is associated with a negative stimulus (e.g., electric shock) can become more repulsive to a fly when compared to a second odor not previously associated with shock. On the other hand, an odor associated with a reward (e.g., sugar) can become more attractive. With this approach, it has been shown that octopamine and dopamine can strongly influence aversive and rewarded olfactory memories (Liu et al., 2012, Aso et al., 2012, Waddell, 2010, Claridge-Chang et al., 2009, Schroll et al., 2006, Schwaerzel et al., 2003).

The role of serotonin, dopamine and octopamine has also been studied in place memory (Sitaraman et al., 2010, Zars, 2009, Sitaraman et al., 2008, Diegelmann et al., 2006). Mutation of the *white* gene, an X-linked ATP-binding cassette (ABC) transporter, causes the typical white-eyed phenotype in flies. The *white* gene has been extensively used as a genetic and transgenic marker in manipulating gene functions. Importantly, overexpression or loss-of-function of the ABC-transporter can also change the fly's behavior (e.g., courtship behavior and aggression) (Hoyer et al., 2008, Zhang and Odenwald, 1995). *white* mutant flies were tested for place memory using the heat-box (Sitaraman et al., 2008, Diegelmann et al., 2006). Compared with red-eyed wild-type flies, *white* mutant flies have about a 60% decrease in place memory levels. Measurements of biogenic amines in fly heads revealed a strong reduction in serotonin and dopamine, indicating that the ABC-transporter modulates biogenic amine levels. This correlation suggested that reduction in serotonin and/or dopamine caused defects in place memory. Further analysis of flies with solely altered serotonin (DDC-GAL4; TH-GAL80 line) or dopamine (TH-GAL4 line) activity revealed the importance of serotonin but not dopamine in place memory. The role of octopamine was also analyzed. Octopamine levels of *white* mutant flies were not altered and flies with genetically altered octopamine levels (TβH mutant flies or via a TDC2-GAL4 line) perform as well as wild-type flies in the heat-box (Sitaraman et al., 2010, Sitaraman et al., 2008). Thus, octopamine does not play an obvious role in place memory.

Genetics of place memory

Several of the earliest genes identified with a role in memory arose from unbiased behavioral screens. These mutants ultimately linked gene products that regulate cAMP signaling and other cascades with olfactory memory formation. The *rutabaga* and *dunce* genes encode a type 1 adenylyl cyclase and cAMP-phosphodiesterase, respectively (Levin et al., 1992, Han et al., 1992, Nighorn et al., 1991). Because the *rutabaga* gene product has the properties predicted for a coincidence detector (Dudai et al., 1984, Lechner and Byrne, 1998), that is requiring both G-protein and Ca^{2+} / calmodulin input to increase cAMP levels, this gene has received much attention in memory investigations. The *dunce* product has the enzymatic activity that is expected to complement the *rutabaga* function. The *dunce* product is also necessary for normal olfactory memory formation. Neuropeptides have also been implicated in olfactory memory formation. The *amnesiac* gene encodes putative neuropeptides that may influence the cAMP / PKA cascade via mushroom body extrinsic neurons. The *amnesiac* gene is predicted to encode three neuropeptides, one of which has some homology to the PACAP peptide (Moore et al., 1998, Feany and Quinn, 1995). The S6K2, a cGMP-dependent protein kinase (PKG), and *tribbles* kinases implicate new kinase cascades in memory formation (LaFerriere et al., 2008, Mery et al., 2007, Putz et al., 2004). Despite the long history for the role of the *radish* (*rsh*) gene in olfactory memory, this gene falls into a class for which it is not clear how it acts in a cell or neural circuit (Krashes and Waddell, 2008, Folkers et al., 2006).

Although there are relatively few genes that have been investigated in *Drosophila* place memory, some general conclusions can be supported. Most of the genes that have been identified as having a role in place memory are also necessary for other forms of memory. Those genes with a more common role in memory formation include genes implicating the cAMP signaling pathway and several additional kinases. Mutation of the *rutabaga* and *dunce* genes, as well as the *amnesiac* gene, shows that this pathway is important for place memory (Zars, 2010, Diegelmann et al., 2006, Putz, 2002, Zars et al., 2000, Wustmann et al., 1996). Furthermore, the S6K2 was identified in a screen for mutant flies in the heat-box place memory paradigm. Semidominant alleles have an effect on place memory (Zars, 2009, Putz et al., 2004). Interestingly, loss of function alleles of S6K2 have an impact on the seconds-long orientation memory (Neuser et al., 2008). Furthermore, mutation of a *tribbles* encoded kinase has been shown to decrease place memory and alter olfactory memory (LaFerriere et al., 2008). Moreover, the *radish* gene, usually implicated in consolidating olfactory memory several hours after training, is required within minutes of training for a place memory (LaFerriere et al., 2011b). The

radish gene may, therefore, be critical for a memory phase with different temporal properties, depending on the learning situation. Finally, the *arouser* EPS8L3, implicated in actin remodeling in the cell, is necessary for both place memory and olfactory memory (Laferriere et al., 2011a). There are, however, some genes that have a restricted role in memory formation. Examination of the *foraging* PKG gene shows that it is critical for olfactory memory, but flies with the same alleles do not have altered place memory (Chen et al., 2011, Gioia and Zars, 2009, Mery et al., 2007). Although not investigated at the single gene level, the wild-type levels of place memory with manipulation of the dopaminergic and octopaminergic system suggests that a broad group of genes that are important for olfactory memory will not be critical for spatial memory (Sitaraman et al., 2010, Sitaraman et al., 2008, Schroll et al., 2006, Schwaerzel et al., 2003).

Conclusions

Although a wide range of animals can use spatial information to form memories, it is only relatively recently that investigation of the behavioral, circuit, and molecular mechanisms that support this type of memory in the insects has been initiated. *Drosophila melanogaster* can alter place preference using high temperature as an aversive cue. This can be accomplished in both simple linear chambers as well as in more open environments with conspicuous visual landmarks. Depending on the training type, memories can be induced that last from minutes to hours. Different parts of the fly brain have been investigated for roles in place memory. So far, the mushroom bodies have not been shown to have a significant role in place memory. Parts of the central complex and the median bundle have been implicated in this type of memory. While the number of genes that have been found to have a role in *Drosophila* place memory is still rather small, the cAMP / PKA pathway, some specific kinases, and a few other genes have been implicated in this form of memory. It is clear, however, that the genes involved in place memory only partially overlap with the genes involved in *Drosophila* olfactory memory. Thus, the genes responsible for memory formation have some common core elements while others are more specific to the learning context.

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Chapter

11

Neurodegeneration models in *Drosophila*

Bidisha Roy and George R. Jackson

Introduction

Neurodegenerative diseases in humans are clinical conditions characterized by the selective and progressive loss of neuronal structure and function, leading to behavioral, cognitive, and motor deficits that eventually can result in death (Skovronsky et al., 2006). Neurodegenerative diseases can be classified under various categories. The subdivisions can be based on their clinical manifestations: movement disorders such as Parkinson's disease (PD) and Huntington's disease (HD), motor neuron diseases such as amyotrophic lateral sclerosis (ALS), and dementias, including Alzheimer's disease (AD) and frontotemporal lobar degeneration (FTLD). These disorders can also be classified based on the kind of neurons affected, e.g., dopaminergic neurons in PD, GABAergic neurons in HD and motor neurons in ALS, or on their origin (familial disorders) or dependence on external factors like environment (sporadic diseases).

Complex patterns of inheritance, population-based genetic heterogeneity, and insufficient family pedigree data have led to difficulties in analysis of genes and pathways in human genetic studies. Researchers have generated models to study functional aspects of genes and their proteins identified in neurodegenerative diseases. These include yeast (Winderickx et al., 2008), *C. elegans* (Teschendorf and Link, 2009), cell lines, and mammals (rodent models, Gama Sosa et al.). However, most of these models have fulfilled only some of the criteria expected to apply in human neurodegenerative studies. An ideal model is expected to encompass a wide range of features, such as behavioral dysfunction, neuronal death, pathophysiological hallmarks such as aggregate formation, and population-based genetic homogeneity. In addition, the model should have a reasonable lifespan to allow study of age-dependent neurodegeneration. These properties will, in turn, help in deciphering the underlying pathophysiological basis of the disease and its progression, and they may help in the design of therapeutic interventions. Yet another desirable attribute is the ability to generate large numbers of genetically homogeneous individuals that can enable us to decipher mechanisms underlying neurodegeneration in a quantitative and statistically significant manner. One organism that meets most of these criteria is the vinegar fly, *Drosophila melanogaster*.

Drosophila

The word *Drosophila* is derived from two Greek words: **Dros** (dew) and **phila** (lover). It belongs to the order of the Diptera and the family of Drosophilidae. The species is commonly referred to as the fruit fly or the vinegar fly. *Drosophila melanogaster* was among the first organisms used for genetic analysis, and today it is one of the most widely used and genetically best known of all eukaryotic organisms.

Although flies have substantial divergence from the humans in the evolutionary tree, there are many similarities between the two in their fundamental cellular processes. These include regulation of gene expression, subcellular trafficking, synaptogenesis, synaptic transmission, and cell death. Many genes and signaling pathways are conserved between flies and humans. Many of the signaling pathways such as Wnt, Ras/ERK (extracellular regulated kinase) and Toll-like pathways were first identified in flies, paving the way for discovery of their mammalian homologs. About 75% of known human disease genes have a recognizable match in the genome of fruit flies and 50% of fly proteins have mammalian homologs. The identity at the nucleotide level or protein sequence between fly and mammal is usually approximately 40% between homologs; and 80%–90% in the conserved functional domains. The fly is being used to study mechanisms underlying aging and oxidative stress, immunity, diabetes, cancer, and drug abuse. *Drosophila* is also being used as a genetic model for several human diseases including neurodegenerative disorders such as PD, HD, AD, and spinocerebellar ataxias.

There are multiple reasons for using *Drosophila* as a model system to study neurodegeneration: they are cheap, incur low maintenance costs and can give rise to a large number of genetically identical progeny. *Drosophila* has a short lifespan, ranging from 40 to 120 days, depending on diet and stress. Flies have simpler genetics (the entire *Drosophila* genome is encoded by roughly 13 600 genes as compared to 27 000 human genes, located on only four pairs of chromosomes as compared to 23 pairs in human) and simpler nervous systems (~100 000 neurons as compared to ~100 billion neurons in humans). These 100 000 neurons organize to form discreet circuits in compartmental neuropil structures that

Behavioral Genetics of the Fly (Drosophila melanogaster), ed. J. Dubnau. Published by Cambridge University Press.

mediate complex behaviors like walking, climbing, flight navigation, learning and memory, circadian rhythms, courtship, feeding, aggression, and grooming. Owing to very well-described anatomy and developmental biology, as well as the availability of diverse molecular and genetic tools, *Drosophila* is one of the most extensively used genetic model organisms to study complex biological processes. As compared to other organisms such as *C. elegans* and the mouse, the fly provides a very powerful genetic model system for the analysis of neurodegenerative disorders related to human disease: it is complex enough (as compared to*C. elegans*) for studies to draw parallels with humans, but still simple enough (as compared to mouse) to permit in-depth structural and functional analysis for deciphering the molecular mechanisms underlying disease.

Genetic tools available in the fly

One of the major aims underlying the study of neurodegeneration in animal models is to decipher molecular mechanisms leading to pathogenesis. Successful accomplishment of this goal would enable design of therapeutic strategies to ameliorate disease. *Drosophila melanogaster* serves as an excellent system with a wide range of genetic capabilities that can help in addressing disease pathogenesis.

Widely used genetic strategies in flies to address questions pertaining to neurodegenerative disorders are:

- Misexpression of a human disease gene, in its wild type or mutant form.
- Loss and gain of function of the *Drosophila* homologue of a human disease gene.
- Genetic screens to identify enhancers and suppressors that are able to modify a phenotype caused by the above.

Therapeutic strategies used in flies pertaining to neurodegenerative disorders:

- Compound screening to identify compounds (metabolites, chemical drugs or plant extracts) that could ameliorate disease phenotypes.
- Genetically to express conformation specific antibodies against the toxic pathophysiological aggregates (oligomers).

Misexpression of a gene refers to the introduction of a foreign gene in the fly to investigate its functional properties and elucidate its interactions with other *Drosophila* proteins. The GAL4 / UAS [Upstream Activating Sequence system] (Brand and Perrimon, 1993) allows misexpression of any gene of interest in *Drosophila* in a spatially and temporally specific pattern. Human neurodegenerative disease conditions have been mimicked by using nervous system-specific GAL4 drivers. Robust phenotypic read-outs in the brain including inclusion bodies or other aggregates, neuronal cell loss and behavioral abnormalities have made *Drosophila* a successful model system for studying neurodegeneration. Several other tissues also have been used as a phenotypic read-out system, including the compound eye, wing (Greeve et al., 2004) and bristles (Yeh et al., 2010), because a degenerative phenotype can be generated without affecting the animal's survival. The compound eye is used because it yields phenotypes that can be easily scored under the dissection microscope. The eye model has been widely utilized for genetic enhancer / suppressor screens. (Sang and Jackson, 2005; Ambegaokar and Jackson, 2011).

The GAL4/UAS system of expression (Brand and Perrimon, 1993) uses yeast-derived GAL4, a transcriptional activator that binds to the UAS enhancer element, driving expression of the gene immediately downstream of the UAS. Two transgenes are required: (1) a "driver" transgene expressing GAL4 under control of tissue-specific enhancers; and (2) a UAS "reporter" transgene encoding the gene of interest. There are several advantages of the binary expression system. It allows deleterious genes to be expressed in a conditional manner. Precursor lines can be crossed as needed to carry out the experiments to avoid accumulation of toxic effects of the disease specific proteins. Second, a wide variety of tissue specific enhancer lines can be used to drive the protein of interest using a single UAS transgenic line. This feature helps in generating models of neurodegeneration, as different tissue types or even different cell types within the same tissue may manifest different phenotypes caused by the same misexpressed protein. The GAL4–UAS system can also be used to express the desired toxic protein in different neurons and validate the susceptibility of certain types of neurons against the others. Finally, the GAL4–UAS system often yields much higher expression of the gene of interest than direct promoter-fused transgenes, and expression can be amplified further by adding more copies of either the driver or the UAS construct. However, there are some potential pitfalls with this approach: (i) degeneration can occur due to excess GAL4 protein itself (Kramer and Staveley, 2003); (ii) the apparent neurodegenerative phenotype can arise due to degeneration of accessory cells unrelated to neurodegeneration; (iii) the severity of a degenerative phenotype may relate not to the amount of protein expressed but rather to the manner in which the UAS construct has been integrated into the fly genome (position effect).

The second approach focuses on techniques available to study the function of a *Drosophila* gene/protein. These include generation of mutants for loss-of-function analysis, GAL4/UAS-mediated RNA interference for knockdown and over-expression for gain-of-function analyses (Leung and Waddell, 2004; Nichols, 2006) (Dietzl et al., 2007; Venken and Bellen, 2007). The fundamental aim of this approach is to gain insights into the role and function of a *Drosophila* homologue of a human disease gene. Existence of a satisfactory homology between the fly and human protein enables one to make hypotheses and predictions about the potential function of the protein and the associated pathogenic pathway(s). Such an approach has shown that mitochondrial dysfunction can result from defective PINK1/Parkin signaling (Dodson and Guo, 2007; Park et al., 2009; Van Laar and Berman, 2009).

The third strategy is to perform unbiased genetic screens to identify enhancers and suppressors on a genome-wide scale. The compound eye of the fruit fly has been the favorite of researchers to carry out large scale modifier screens. A "rough" eye phenotype acts as a scoring index for genes that either enhance (enhancers) or suppress it (suppressors). This strategy (or smaller scale candidate-based approaches/ biased screens) have aided in identifying several key interacting partners of Tau, Aβ and α-synuclein (Scherzer et al., 2003; Shulman and Feany, 2003; Whitworth et al., 2005; Cooper et al., 2006; Karsten et al., 2006; Blard et al., 2007; Tan et al., 2008; Ambegaokar and Jackson, 2011). Loss of thoracic bristles or abnormalities in wing shape and size are other scoring parameters that can be used to identify potential interactors with a disease causing protein.

Flies also can be used for compound screens. A *Drosophila* compound screen is designed by feeding flies with their usual food to which a defined concentration of the compound has been added. This strategy has resulted in improvements relevant to human disease, including extended lifespan in models of AD (Rajendran et al., 2008) and prolonged survival of dopaminergic neurons in models of PD (Auluck et al., 2005; Faust et al., 2009) as well as the complete rescue of disease-related phenotypes (Chang et al., 2008).

There are two approaches by which flies can be used to screen for therapeutics. Positive hits from traditional *in vitro* mammalian cell culture HTS (high throughput screen / compound screening) can be fed to the transgenic flies expressing the toxic proteins to rapidly identify the effective compounds which have an ameliorating effect on the disease phenotypes. Drugs with efficacy in *D. melanogaster* models, however, will still need to be validated in mammalian whole-animal disease models. Alternatively, neurodegenerative models created in the fly can be directly screened for small molecules that rescue the phenotype or conduct genetic screens to identify modifiers of the phenotype, which could be putative therapeutic targets for the given disease. After initial primary screening, positive hits can be validated by testing in additional fly models of the disease.

Neuronal toxicity in the disease conditions is mediated by dysfunction of multiple cellular processes. One curative strategy could be administration of combinatorial drug therapy, in which multiple drugs restore the various basic cell functions. The other would be a more direct approach in which therapeutic is designed against the toxic species. On this line, intrabodies serve as an excellent therapeutic strategy. Intrabodies are recombinant antibodies usually derived from a monoclonal antibody of interest by cDNA cloning of the antigen binding domain. The variable heavy and light chains (V_H and V_L) from the monoclonal antibody are then joined together by a synthetic cDNA encoding a flexible polypeptide linker. Alternatively, intrabody libraries can be constructed and cloned in phage or displayed on yeast for selection and binding to specific antigens. A variant form of the intrabody, referred to as single domain antibodies have also been developed. They are functional single-domain (VH or VL but not both) intrabodies which have been selected for specific targets. These single-domain intrabodies have the ability to block protein–protein interaction, are more stabilized and display better folding properties. An anti-HD C4 single chain (sc) Fv [C4-scFv] has shown shown dramatic phenotypic rescue in a *Drosophila* HD model. The flies displayed higher survival rates, increase in life span and decrease in aggregate formation (Wolfgang et al., 2005).

Parkinson's disease (PD)

PD is the second most common neurodegenerative disorder of the central nervous system, affecting an estimated 1% of people over the age of 65 years (Lees, 2010). PD belongs to a group of conditions called movement disorders and is the result of loss of a majority of dopaminergic neurons in the substantia nigra of the midbrain. PD is a debilitating neurodegenerative disease associated with tremor, rigidity, bradykinesia, and postural instability. The pathological hallmark of PD is the Lewy body, a cytoplasmic inclusion comprised primarily of the protein α-synuclein (Forno et al., 1996; Engelender, 2008) in surviving dopaminergic neurons. PD is predominantly sporadic, but there are familial cases, as well (10%–15%).

Familial PD is linked to mutations in genes including *SNCA [synuclein, alpha (non-A4 component of amyloid precursor*, Polymeropoulos et al., 1997], *LRRK2* [leucine-rich repeat kinase 2, Paisan-Ruiz et al., 2004], *PARK2* [parkinson protein 2, E3 ubiquitin protein ligase (parkin), Kitada et al., 1998], *PINK1* [(PTEN)-induced kinase 1,Valente et al., 2004)], *DJ-1* (Bonifati et al., 2003) and *ATP13A2*. Additionally, mutations in the vacuolar protein-sorting-associated protein 35 (VPS35) gene (Vilarino-Guell et al., 2011; Zimprich et al., 2011) and the translational initiator, EIF4G1 (Chartier-Harlin et al., 2011) recently have also been linked to familial PD. The PARK9 locus is associated with Kufor-Rakeb disease, an autosomal recessive, juvenile onset form of parkinsonism (Williams et al., 2005). Mutations in the gene for ATP13A2, a P5-type ATPase, were subsequently identified in these cases as well as in some young onset forms of PD (Ramirez et al., 2006; Di Fonzo et al., 2007). P-type ATPases maintain an ion gradient across the cell membrane and employ membrane potential to manufacture ATP. It is unclear how loss of function mutations to ATP13A2 lead to parkinsonism, although interference with localization and lysosomal function has been reported (Gupta et al., 2008). ATP13A2 protects against α-synuclein toxicity, suggesting a link between these two genetic pathways (Gitler et al., 2009). The Kufor–Rakeb syndrome is slightly distinct from PD: patients with this syndrome do not exhibit tremor; furthermore, disease features include spasticity (resulting from corticospinal tract degeneration) and dementia (which is absent in idiopathic PD).

On the other hand, sporadic PD has been attributed to complex interactions between genetic and environmental factors (e.g., pesticides) (Olanow and Tatton, 1999; Dauer and Przedborski, 2003; Abeliovich and Beal, 2006). Understanding of the

genes responsible for familial forms of PD and their interplay with environmental factors would aid in developing therapeutics for both familial and sporadic forms of PD. The use of classical genetic analysis in *Drosophila melanogaster* has proved to be beneficial in solving this problem to considerable extent. *Drosophila* has a complex nervous system, including a subset of around 200 neurons that contain the neurotransmitter dopamine. Although the anatomy of the fly brain and the distribution of dopaminergic neurons in the central nervous system of *Drosophila* differ from those of vertebrate brains, many fundamental cellular and molecular biological features of neuronal development and function are conserved between vertebrates and invertebrates. In particular, the *Drosophila* genome encodes homologs of five of the eight PD-related genes that have been identified (Whitworth et al., 2006). These include the Uch (ubiquitin hydrolase/ligase), parkin (E3 ubiquitin – protein ligase), DJ-1a and DJ-1b (oxidative stress sensors), a PINK1 homolog, and dardarin /LRRK2 homolog kinase.

In order to investigate pathophysiological mechanisms underlying familial and sporadic PD, various animal models have been generated. *Drosophila* has been extensively exploited to conduct some of these studies and over time has proved to be a reliable model capable of recapitulating parkinsonian phenotypes (Bilen and Bonini, 2005; Whitworth et al., 2006). Some of the clinical hallmarks of PD, e.g., selective degeneration of subsets of dopaminergic neurons in the brain, as well as motor impairment, can be readily mimicked in the flies. Various PD models have been generated in flies: these include the PINK1, parkin, LRRK2, DJ-1, α-synuclein and pesticide (environmental toxicant) models.

PINK1 models

Mitochondrial dysfunction has been implicated in PD. Exposure to environmental mitochondrial toxins leads to PD pathology. Of the various genes linked to familial PD (West et al., 2005), three (parkin, DJ-1, and PINK1) are associated with early onset autosomal recessive PD, in which a loss of function (most likely) of a single gene product results in the clinical manifestations of parkinsonism. All the three autosomal recessive PD genes are involved in mitochondrial function. Sporadic PD also is linked to mitochondrial dysfunction; environmental factors implicated in PD cause reduced mitochondrial complex I activity in animal models (Abou-Sleiman et al., 2006). Furthermore, markers of oxidative stress and decreased mitochondrial complex I levels are observed in brains of patients with sporadic PD. Thus, accumulating evidence suggests a role for mitochondrial dysfunction in PD pathogenesis.

One of the candidates underlying mitochondrial dysfunction in PD is PINK1, a putative serine/ threonine kinase with a mitochondrial targeting sequence (Valente et al., 2004). The *Drosophila* PINK1 gene encodes a polypeptide of 721 amino acids with a molecular mass of about 80 kDa. The kinase domain exhibits 60% similarity (42% identity) to that of human PINK1. Consistent with the localization of human PINK1 (Valente et al., 2004), *Drosophila* PINK1 also is localized to mitochondria (Park et al., 2006).

Two different approaches were used to generate *Drosophila* PINK1 models: transposon-mediated mutagenesis and the interference RNA (RNAi) technique. Guo and colleagues (Clark et al., 2006) observed male sterility, apoptotic muscle degeneration, increased sensitivity to oxidative stress, and fragmentation of mitochondrial cristae in *dPINK1* mutants. In addition to these phenotypes, another group found that *dPINK1* mutants exhibit dopaminergic neuronal degeneration accompanied by defects in locomotion (Park et al., 2006). Furthermore, ultrastructural analysis and a rescue experiment with *Drosophila* Bcl-2 demonstrated that mitochondrial dysfunction underlies the degenerative phenotypes of *dPINK1* mutants (Park et al., 2006). Using transgenic RNAi, Lu and colleagues demonstrated that knockdown of dPINK1 function results in energy depletion, shortened life span and degeneration of both indirect flight muscles and selected dopaminergic neurons (Yang et al., 2006). Muscle pathology was preceded by mitochondrial enlargement and disintegration. These phenotypes were rescued by the wild type but not the pathogenic C-terminal deletion form of human PINK1, indicating functional conservation between fly and human PINK1 (Yang et al., 2006). Zhang and coworkers (Wang et al., 2006), using RNAi-mediated knockdown of dPINK1, produced progressive loss of dopaminergic and photoreceptor neurons, both of which were rescued by expression of human PINK1. These investigators also showed that expression of human SOD1 and treatment with the antioxidants SOD and vitamin E ameliorated photoreceptor degeneration in *dPINK1* RNAi flies (Wang et al., 2006). Taken together, these studies strongly implicate mitochondrial dysfunction and oxidative stress in PINK1 pathogenesis.

Similarities between the phenotypes of dPINK1 and dparkin mutants encouraged investigation of the epistatic relationship between PINK1 and parkin. Overexpression of parkin rescues male sterility and mitochondrial morphology defects of dPINK1 mutants. Double mutants lacking both PINK1 and parkin function show phenotypes identical to those observed for either mutant alone. Overexpression of PINK1 has no effect on *dparkin* mutant phenotypes. These observations suggested that PINK1 and parkin function in the same pathway, with PINK1 acting upstream of parkin (Clark et al., 2006) (Park et al., 2006; Yang et al., 2006). This idea is further supported by the observation that Parkin protein level was found to be reduced in dPINK1 RNAi animals (Yang et al., 2006).

Prominent mitochondrial morphological defects in *dPINK1* and *dparkin* mutants prompted investigation of the mitochondrial fission/fusion pathway, a conserved mitochondrial remodeling process that controls the dynamic distribution and morphology of mitochondria in all eukaryotes (Chan, 2006). Mitochondrial fission and fusion have also been shown to be important for regulating synaptic structure and plasticity (Li et al., 2004), and imbalance of mitochondrial fission/fusion can lead to neurodegeneration (Bossy-Wetzel et al., 2003). Pallanck and colleagues (Poole et al., 2008) suggested

that the PINK1/parkin pathway promotes mitochondrial fission and that the loss of mitochondrial and tissue integrity in *PINK1* and *parkin* mutants result from reduced mitochondrial fission. Heterozygous loss of function mutations of *drp1*, which encodes dynamin related protein I, a key mitochondrial fission-promoting component (Frank et al., 2001;Hoppins et al., 2007), are largely lethal in a *PINK1* or *parkin* mutant background. Conversely, the flight muscle degeneration and mitochondrial morphological alterations that result from mutations in *PINK1* and *parkin* are strongly suppressed by increased *drp1* gene dosage and by heterozygous loss of function mutations affecting the mitochondrial fusion-promoting factors OPA1 (optic atrophy 1, a GTPase) and Mfn2 (mitofusin 2). Finally, these investigators found that retinal phenotypes associated with increased PINK1/parkin pathway activity are suppressed by perturbations that reduce mitochondrial fission and enhanced by those that reduce mitochondrial fusion.

Additional efforts of de Strooper and coworkers (Morais et al., 2009) found that, in *Drosophila* neurons, PINK1 deficiency affects synaptic function, as the reserve pool of synaptic vesicles is not mobilized during rapid stimulation. They also showed that this deficit could be rescued by adding ATP to the synapse, thus suggesting a possible role of PINK1 in energy maintainence under conditions of heavy demand. Using a mouse model, this group also reported that wild-type human PINK1, but not PINK1 containing PD-associated mutations, can rescue complex I deficiency. Thus, PD-related mutations in PINK1 affect complex I activity and synaptic function, providing yet another putative mechanism for PINK1-mediated mitochondrial dysfunction in PD.

PINK1 has been proposed to play yet another role in PD: protection against α-synuclein-mediated neurotoxicity. α-synuclein pathology has been reported to be mediated by mitochondrial dysfunction (Hsu et al., 2000; Martin et al., 2006). Haywood and Staveley (Haywood and Staveley, 2004) reported that parkin overexpression using the GMR-GAL4 driver rescued the α-synuclein retinal neurodegeneration phenotype in *Drosophila*, presumably through targeting α-synuclein for degradation. As discussed previously, PINK1 and parkin seem to function in the same pathway, and this pathway is necessary for proper mitochondrial function and morphology (Clark et al., 2006; Park et al., 2006; Poole et al., 2008). PINK1 has been shown to protect neurons from the dopaminergic neurotoxin MPTP (Haque et al., 2008), and MPTP pathology has been attributed to the toxicity of α-synuclein (Dauer et al., 2002; Klivenyi et al., 2006). Todd and Staveley (2008) demonstrated that overexpression of PINK rescues α-synuclein-induced impairment of climbing and retinal degeneration.

Parkin models

Mutations in the parkin gene are thought to be the second most common genetic cause of sporadic PD, after LRRK2/dardarin (Kitada et al., 1998; Foroud et al., 2003) (Klein et al., 2003; Lincoln et al., 2003; Hedrich et al., 2004; Gilks et al., 2005; Hernandez et al., 2005). Parkin mutations originally were identified in families with autosomal recessive juvenile parkinsonism (AR-JP) (Kitada et al., 1998). *In vitro* studies have indicated that parkin functions as an E3 ligase (Giasson and Lee, 2001; Hattori and Mizuno, 2004; Moore et al., 2005), although additional roles in microtubule-based transport (Ren et al., 2003) and regulation of dopamine transporter activity have been suggested (Jiang et al., 2004). It is generally thought that the loss of E3 ligase activity contributes to the pathogenesis of parkin-linked PD.

In order to understand the function of parkin, mutations in the endogenous gene were generated by several groups (Greene et al., 2003; Pesah et al., 2004; Cha et al., 2005). Characteristic features of these mutant flies include mitochondrial pathology and apoptotic muscle degeneration. In addition, these mutant flies also display reduced lifespan, sterility, reduced cell number and size, and hypersensitivity to oxidative stress (Pesah et al., 2004). Initial analysis failed to report any significant loss of dopaminergic neurons in the dorsomedial clusters (DMC, the populations previously reported to be selectively affected in α-synuclein transgenic flies); however, these neurons appear to be smaller in *parkin* mutant flies. On the other hand, subsequent work by Pallanck and colleagues used whole mount confocal analysis and reported significant dopaminergic neuron loss in the PPL1 cluster (Whitworth et al., 2005). Work reported by Lu and colleagues (Yang et al., 2003), used a different approach to create the parkin loss of function phenotype. They created transgenic RNAi against the parkin gene, which also enabled them to attain tissue and cell specific gene knockdown. Targeted expression of *parkin* double-stranded RNA in *Drosophila* fails to elicit loss of dopaminergic neurons in the DMC, suggesting that fly parkin is not essential for the maintenance of dopaminergic neurons in this cluster under normal physiological conditions.

Lu and coworkers found that targeted overexpression of human parkin-associated endothelin like receptor (Pael-R), a parkin substrate protein (Imai et al., 2001), does result in a reduction of tyrosine hydroxyase positive neurons in the DMC, and that this phenotype is exacerbated in the *parkin* RNAi background (Yang et al., 2003). Conversely, overexpression of human parkin suppresses Pael-R-induced toxicity. Together, these *in vivo* genetic interaction studies confirm the biochemical relationship between parkin and Pael-R and indicate that accumulation of abnormal Pael-R protein in parkin-deficient dopaminergic neurons might be one of the causes of neuronal death. These results validate work in mice showing that Pael-R induces degeneration of dopaminergic neurons in the substantia nigra via endoplasmic reticulum stress and dopamine toxicity. Furthermore, Pael-R toxicity is enhanced under parkin inactivation conditions (Kitao et al., 2007). These data confirm that the *Drosophila* model recapitulates some features of the vertebrate system and hence serves as a reliable platform for understanding molecular mechanisms underlying both familial and sporadic PD cases.

Taking another approach, Sang and coworkers (Sang et al., 2007) demonstrated that expression of mutant but not wild-type human parkin in *Drosophila* causes age-dependent, selective degeneration of dopaminergic neurons accompanied by progressive motor impairment. These investigators generated transgenic lines expressing two mutant forms of human parkin derived from familial AR-JP, Gln311Stop (Q311X) and Thr240Arg (T240R) (Shimura et al., 2000). Both these mutant forms produce age-dependent neurodegeneration and neuronal dysfunction in younger flies. These data suggest a possible dominant mechanism underlying the pathological phenotypes caused by mutant parkin in *Drosophila*. Additionally, Sang and coworkers demonstrated that overexpression or knockdown of the *Drosophila* vesicular monoamine transporter, which regulates cytosolic dopamine homeostasis, partially rescues or exacerbates, respectively, the degenerative phenotypes caused by mutant human parkin. These results support a model in which the vulnerability of dopaminergic neurons to parkin-induced neurotoxicity results from the interaction of mutant parkin with cytoplasmic dopamine.

Yet another function has been attributed to parkin in fly PD models: parkin and α-synuclein may interact functionally in the disease process. Parkin colocalizes with α-synuclein in Lewy bodies, and an O-glycosylated form of α-synuclein has been reported to be a substrate of parkin (Shimura et al., 2001). In cell culture systems, overexpression of mutant α-synuclein decreases proteasome function; this can be counteracted by overexpression of parkin (Petrucelli et al., 2002). In a rat lentiviral model of PD, overexpression of wild-type parkin protects against the toxicity of α-synuclein A30P. Animals overexpressing parkin show significant reductions in α-synuclein-induced neuropathology, leading to preservation of tyrosine hydroxylase-positive cell bodies in the substantia nigra and to sparing of tyrosine hydroxylase-immunoreactive nerve terminals in the striatum. Additionally, parkin-mediated neuroprotection has been associated with an increase in hyperphosphorylated α-synuclein inclusions, supporting a role for parkin in the genesis of Lewy bodies and suggesting an intriguing neuroprotective role for α-synuclein inclusions (Lo Bianco et al., 2004). In *Drosophila*, overexpression of parkin suppresses α-synuclein-induced degeneration of dopaminergic neurons (Yang et al., 2003). This suppression is not associated with changes in total cellular α-synuclein levels. The authors speculated that toxic species of α-synuclein, if targeted by parkin, may represent only a small portion of the total α-synuclein pool. Alternatively, parkin suppression of α-synuclein toxicity may be mediated through a mechanism unrelated to α-synuclein degradation by the ubiquitin-proteasome pathway. Another group (Haywood and Staveley, 2004) reported protective effects of parkin against α-synuclein toxicity in *Drosophila* using alternative assays of α-synuclein-induced toxicity, i.e., decline of climbing activity and retinal degeneration using the dopamine decarboxylase- and *glass* multimer reporter-GAL4 drivers, respectively.

LRKK2 models

LRRK2 mutations are one of the most common genetic causes of PD (Paisan-Ruiz et al., 2004; Zimprich et al., 2004). Among the large number of *LRRK2* mutations that have been identified, the G2019S (Lesage et al., 2006) and the G2385R (Tan and Schapira, 2008) variants appear to be most prevalent. LRRK2 is a large protein (2527 amino acid residues) composed of multiple domains, including a GTPase domain and a kinase domain capable of exhibiting GTP-dependent phosphorylation activity (West et al., 2005). In general, disease-associated mutations of LRRK2 increase its kinase activity and thereby its toxicity (Smith et al., 2006; West et al., 2007). However, significant variations have been observed in many cases; hence, the precise mechanisms by which LRRK2 mutations give rise to disease is not entirely clear. As an example, the I2012T LRRK2 mutant appears to have reduced kinase activity (West et al., 2007), and several LRRK2 mutants also aggregate when expressed in cultured cells (Greggio et al., 2006).

To understand the role of LRRK2 mutations *in vivo*, a number of groups generated *Drosophila* models of mutant LRRK2-associated PD. Smith and colleagues (Liu et al., 2008) reported that misexpression of human wild-type LRRK2 or the G2019S mutant in flies triggers photoreceptor and dopaminergic degeneration. Similarly, Lu and colleagues (Imai et al., 2008) observed neurodegeneration in flies expressing *Drosophila* orthologs of human LRRK2 Y1699C or I2020T mutants, although the degeneration in these cases case was confined to certain dopaminergic neuronal clusters and failed to affect the eye. In contrast, Chung and coworkers (Lee et al., 2007) reported that transgenic flies over-expressing human wild type or R1441C mutant LRRK2 fail to show any defects in tissues examined, including dopaminergic neurons and muscle. Lim and coinvestigators (Ng et al., 2009) reported that transgenic flies expressing G2019S, Y1699C or G2385R LRRK2 mutants, but not wild type, exhibit late onset loss of dopaminergic neurons in selected clusters that is accompanied by locomotion defects. Furthermore, LRRK2 mutant flies displayed reduced lifespan and increased sensitivity to rotenone, a mitochondrial complex I inhibitor. They also found that coexpression of human parkin in LRRK2 G2019S-expressing flies protected against age or rotenone-dependent dopaminergic neurodegeneration. Taken together, these data suggest a link between LRRK2, parkin and mitochondria in the pathogenesis of LRRK2-related parkinsonism.

DJ-1 models

Mutations in the *DJ-1* gene are associated with the *PARK7* locus and are an extremely rare cause of inherited and sporadic parkinsonism (Bonifati et al., 2003; Moore et al., 2003). Studies in PD patients have suggested that DJ-1 loss of function accounts for disease in these individuals. Based on these findings, investigators have tried to develop model organisms that recapitulate DJ-1-linked parkinsonism by generating homozygous null alleles for *DJ-1*. Most of the initial models focused on

vertebrates, but *Drosophila* models also have been developed to further investigate the contribution of DJ-1 dysfunction to the pathogenesis of PD.

There are two DJ-1 homologs in *Drosophila*, DJ-1A and DJ-1B. DJ-1A is a closer homolog of human DJ-1 than DJ-1B. Bonini and colleagues engineered DJ-1A and DJ-1B double-knockout flies, which had normal viability and lifespan; however, they displayed a selective sensitivity to environmental toxins such as paraquat and rotenone. This sensitivity was speculated to result primarily from loss of DJ-1B protein, which becomes modified upon oxidative stress (Meulener et al., 2005). Min and coworkers, on the other hand, found that DJ-1B loss of function mutants have an extended survival of dopaminergic neurons and resistance to paraquat, as well as an acute sensitivity to hydrogen peroxide treatment. There is also a compensatory upregulation of DJ-1A expression in the brain of the DJ-1B mutant, suggesting that overexpression of DJ-1A in dopaminergic neurons may be sufficient to confer protection against paraquat (Menzies et al., 2005). The differential sensitivity of the DJ-1B-mutant to paraquat in these two studies was speculated to be due to different genomic deletions or genetic backgrounds.

Using the RNAi technique, Lu and colleagues showed that knocking down DJ-1A in a cell type-specific manner results in the accumulation of reactive oxygen species, hypersensitivity to oxidative stress, and dysfunction and degeneration of dopaminergic and photoreceptor neurons (Yang et al., 2005). These investigators also reported that DJ-1A RNAi flies exhibit stronger phenotypes than the DJ-1A or DJ-1B genetic mutants: the RNAi-treated flies suffer dopaminergic and photoreceptor neuron loss, as well as shortened lifespan. The authors explained these findings as possible off-target effects associated with the RNAi approach or the existence of certain compensatory mechanisms associated with genomic deletions. In order to decipher genes and pathways that could act as modifiers of DJ1-associated neurodegeneration, this group used a DJ-1A RNAi-induced retinal phenotype. This search led to the isolation of components of the PI3K/Akt signaling pathway as specific genetic modifiers. Reduction of PI3K/Akt signaling enhanced DJ-1A RNAi phenotypes, whereas activation of PI3K/Akt signaling significantly rescued DJ-1A RNAi phenotypes. The modifying effects of PI3K/Akt signaling on DJ-1A RNAi phenotypes were also shown to manifest in dopaminergic neurons.

Mak and colleagues (Kim et al., 2005) reported DJ-1A to be a genetic modifier of a PTEN-induced small eye phenotype in *Drosophila*. Additionally, the authors performed biochemical studies in mammalian cells and found that DJ-1 RNAi results in decreased phosphorylation of PKB/Akt, whereas DJ-1 overexpression leads to hyperphosphorylation of PKB/Akt and increased cell survival. Futhermore, they have shown that DJ-1 expression correlates negatively with PTEN immunoreactivity and positively with Akt hyperphosphorylation in breast cancer tissues. These studies hint at the existence of common biochemical pathways shared between seemingly disparate diseases such as PD and cancer.

Synuclein models

The most common histopathological hallmark of PD is the Lewy body, a ubiquitinated proteinaceous inclusion containing α-synuclein in the neuronal perikaryon. Similar inclusions also have been observed in neuronal processes and are referred to as Lewy neurites. α-synuclein has been implicated as one of the major proteins in the pathogenesis of the PD (Cookson, 2005a).

Synucleins are a group of small soluble proteins, predominantly found in neural tissues in addition to certain forms of tumors. The group is made up of α-, β-, and γ-synuclein; among these, α-synuclein has the best characterized role in neuropathogenesis. Missense mutations (A53T mutation: alanine being replaced by threonine at amino acid position 53 (Polymeropoulos et al., 1997), the A30P mutation: substitution of alanine with phenylalanine at position 30 (Kruger et al., 1998), the E46K mutation: substitution of glutamic acid with lysine at amino acid position 46 (Zarranz et al., 2004) and genomic duplication and triplication of the α-synuclein gene (Ibanez et al., 2004; Singleton et al., 2003) cause autosomal dominant familial PD. Knockout of α-synuclein in mice results in functional deficits of the nigrostriatal dopamine system (Abeliovich et al., 2000). Furthermore, *in vitro* studies indicate that α-synuclein can self aggregate and form fibrils.

The first PD model in the fly system was reported by Feany and Bender (Feany and Bender, 2000). These authors expressed a human α-synuclein transgene in all *Drosophila* neurons. This resulted in an age-dependent loss of dopaminergic neurons; other neurons including serotoninergic neurons remained largely unaffected. Death of dopaminergic neurons was observed upon the expression of both the wild type α-synuclein and the two mutants (A53T and A30P) identified at that time. Feany and Bender also reported that some of the dopaminergic neurons accumulated intracellular aggregates resembling Lewy bodies. The inclusions contained α-synuclein filaments 7–10 nm in diameter, comparable to those in human Lewy bodies. These authors used a climbing test to determine whether expression of human α-synuclein produced motor behavioral deficits characteristic of PD; this assay originally was designed to follow age-related changes in *Drosophila*. When tapped down to the bottom of a tube, flies rapidly climb up to the top in what is known as a negative geotactic response (Hirsch, 1959). Young flies expressing α-synuclein performed well in this test, but aged transgenic flies frequently fell back to the bottom of the tube. Flies expressing wild-type α-synuclein or the A53T mutant performed similarly to one other, but climbing defects were more severe in flies expressing the A30P mutant protein. Thus, this fly model replicated many of the characteristic features of the PD. However, data reported by Mardon and colleagues (Pesah et al., 2005) called into question some of the findings of this PD model after observing no change in the number of dopaminergic neurons of the dorsomedial cluster in various α-synuclein transgenics. One possible explanation for this contradictory result could be related to use of whole mount immunofluorescence analysis of fly brain with

an antibody against tyrosine hydroxylase (TH), a marker for dopaminergic neurons, whereas earlier studies used peroxidase immunohistochemistry in paraffin sections. Whole mount immunohistochemistry coupled with confocal microscopy may yield more reproducible results than paraffin sections, since all dopaminergic neurons in the targeted cluster can be viewed simultaneously in a single preparation, reducing the chance of missing cells as can occur in serial paraffin sections. An alternative explanation is that α-synuclein overexpression does not lead to neuronal death, but rather to dopaminergic dysfunction and thus reduced expression of TH, rendering them undetectable in paraffin sections. Mardon and coworkers also failed to show any defects in the negative geotactic assay in animals misexpressing α-synuclein (Pesah et al., 2005). These findings suggest that dopaminergic cell loss or climbing defects observed with the misexpression of α-synuclein are not fully penetrant under all conditions.

Work reported by Pallanck and colleagues, however (Trinh et al., 2008), showed that increasing expression of α-synuclein elicited an age-dependent loss of dopaminergic neurons in the PPL1 (protocerebral posterior lateral 1) cluster of the *Drosophila* brain. Based on the observation that heritable forms of PD result from increased gene dosage of α-synuclein (Chartier-Harlin et al., 2004; Singleton et al., 2003), this group engineered an expression construct bearing sequence alterations designed to improve the translational efficiency of this cDNA in *Drosophila*. Furthermore, they maximized α-synuclein expression by making use of flies bearing two copies each of the UAS-synuclein transgenes and GAL4 drivers, which approximately doubled the abundance of α-synuclein protein relative to flies bearing a single copy of each of these transgenes. This work seems to have succeeded at generating a more robust *Drosophila* model for studying synucleinopathies.

Mechanisms underlying synuclein-mediated neurotoxicity

The neuropathological effects of α-synuclein have been attributed to a number of factors, including post-translational modifications or mutations that lead to formation of toxic aggregates, induction of oxidative stress, effects on dopamine homeostasis, and indirect effects via interaction with various other molecules. Fly models have been beneficial in deciphering and validating some of these basic molecular mechanisms.

Formation of toxic aggregates

Abnormalities of protein aggregation have been implicated as a key feature underlying pathogenesis of many neurodegenerative diseases, including PD (Cookson, 2005b; Trojanowski and Lee, 2000). Since α-synuclein aggregates are a prominent component of Lewy bodies, considerable effort has been expended toward investigating the mechanisms that regulate aggregate formation. Feany and coworkers (Periquet et al., 2007) reported that the central hydrophobic region of α-synuclein (known as the non-β-amyloid component (NAC) of AD senile plaques) is essential for aggregation of the protein. On the other hand, the C-terminal region acts as an inhibitor or negative regulator of α-synuclein aggregation. These investigators found that misexpression of a truncated form of α-synuclein lacking amino acid residues 71–82 did not lead to the formation of large aggregates, oligomeric species, or loss of tyrosine hydroxylase-immunoreactive neurons. However, when the C-terminal truncated form of the α-synuclein was misexpressed, increased aggregation into large inclusion bodies and accumulation of high molecular weight α-synuclein species, as well as enhancement of neurotoxicity, were observed. The authors speculated that oligomeric synuclein aggregates are potentially toxic and do not confer neuroprotection, unlike larger conformers.

This kind of internal posttranslational modification of synuclein has been attributed to candidates including calpain I, a calcium-dependent protease that has been implicated in numerous diseases including AD and stroke (Glading et al., 2002; Vanderklish and Bahr, 2000). α-synuclein is a substrate for calpain cleavage (Mishizen-Eberz et al., 2003), and calpain-cleaved α-synuclein species are similar in molecular weight to truncated α-synuclein fragments that promote aggregation and enhance cellular toxicity (Li et al., 2005; Murray et al., 2003; Serpell et al., 2000) Murray et al., 2003). Rohn and coworkers (Dufty et al., 2007) have shown the presence of calpain cleaved α-synuclein in the fly brain.

Phosphorylation

Several other post-translational modifications of α-synuclein have been described, including phosphorylation at Ser129 (Fujiwara et al., 2002) and nitration at tyrosine residues (Giasson et al., 2000). These post-translational modifications have been speculated to regulate the aggregation and toxicity of α-synuclein. To assess the role of Ser129 phosphorylation in α-synuclein toxicity and inclusion formation, Chen and Feany (Chen and Feany, 2005) performed mutagenesis studies in the *Drosophila* α-synuclein model of PD. Substitution of Ser129 to non-phosphorylatable alanine completely suppressed dopaminergic neuronal loss caused by human α-synuclein, whereas changing Ser129 to the phosphomimetic aspartate enhanced α-synuclein toxicity. These investigators also showed that G protein-coupled receptor kinase 2 (Gprk2) phosphorylated Ser129 and enhanced α-synuclein toxicity *in vivo*. Furthermore, blocking phosphorylation at Ser129 substantially increased aggregate formation. In subsequent work, Feany and colleagues (Chen et al., 2009) reported that α-synuclein is also phosphorylated at tyrosine 125 in transgenic *Drosophila* expressing wild type human α-synuclein, and that this tyrosine phosphorylation protects from α-synuclein neurotoxicity. They showed that levels of soluble oligomeric species of α-synuclein were increased by phosphorylation at serine 129 and decreased by tyrosine 125 phosphorylation. Tyrosine 125 phosphorylation was seen to be diminished during the normal aging process in both humans and flies. Furthermore, these investigators

observed that cortical tissue from patients with the PD-related synucleinopathy, dementia with Lewy bodies, showed less phosphorylation at tyrosine 125. Their work seems to suggest that α-synuclein neurotoxicity in PD may result from an imbalance between the detrimental, oligomer-promoting effect of serine 129 phosphorylation and a neuroprotective action of tyrosine 125 phosphorylation that inhibits toxic oligomer formation.

Induction of oxidative stress

Oxidative stress has been suggested to play an important role in the pathology of PD (Dawson and Dawson, 2003; Jenner, 2003; Dauer and Przedborski, 2003). Oxidative stress is increased in affected brain areas of PD patients, but whether the production of reactive oxygen species (ROS) is a cause or a consequence of PD is unknown. Dopamine is a highly reactive molecule that can oxidize to form reactive quinones, which in turn make dopaminergic neurons more susceptible to oxidative insults. Certain environmental toxins such as rotenone inhibit mitochondrial complex I, releasing oxygen free radicals, and are toxic to dopaminergic neurons. Rotenone administration also increases α-synuclein aggregation and formation of Lewy bodies in an animal model of PD (Betarbet et al., 2000), perhaps as a consequence of an unbalanced redox status in dopaminergic neurons.

Studies in the fruit fly have provided important insights into mechanisms underlying oxidative stress-mediated PD neuropathology. Overexpression of glutathione S-transferase rescues dopaminergic neuronal loss caused by mutation of *Drosophila* parkin, a gene whose function is compromised in autosomal recessive juvenile parkinsonism (AR-JP), also linking oxidative stress to familial PD (Menzies et al., 2005; Whitworth et al., 2005). Botella and colleagues have demonstrated that dopaminergic neurons are sensitive to hyperoxia-induced oxidative stress and that mutant forms of α-synuclein promote toxicity under these conditions (Botella et al., 2008). Furthermore, co-expression of Cu/Zn superoxide dismutase protects against dopaminergic neuronal loss induced by mutant α-synuclein, thus identifying oxidative stress as an important factor in the pathology of autosomal dominant PD.

Interaction between α-synuclein and other PD genes

LRRK2 and α-synuclein

Despite a lack of reports in *Drosophila,* a few observations in cell and animal models have hinted toward a possible role of LRRK2 in enhancing α-synuclein mediated neurotoxicity. LRRK2 has been speculated to be the kinase that mediates α-synuclein phosphorylation and leads to enhanced neurotoxicity. One report has shown that recombinant α-synuclein can be directly phosphorylated by cell lysates from HEK293 cells overexpressing LRRK2 (Qing et al., 2009). However, there is no evidence that LRRK2 causes increased α-synuclein phosphorylation in animal systems. Another set of observations linking LRRK2 and α-synuclein may be the role of LRRK2 in modulating microtubule dynamics and axonal transport. Experimental evidence indicates that LRRK2 causes tau hyperphosphorylation, which induces destabilization of microtubules (Melrose et al., 2010; Lin et al., 2010). Altered microtubule stability could lead to improper transport of vesicle-bound α-synuclein with consequent protein accumulation and, in turn, cell death. Another potential mechanism by which LRRK2 could promote α-synuclein aggregation is through impairment of autophagy. There are few studies highlighting a role of LRRK2 in the autophagic pathway. LRRK2 null mice display impaired autophagy, accumulation of α-synuclein in the kidneys and consequent cell death, suggesting that LRRK2 is implicated in the autophagic pathway (Tong et al., 2010). Moreover, chaperone-mediated autophagy inhibition leads to an accumulation of soluble high molecular weight and detergent-insoluble species of α-synuclein, suggesting that chaperone-mediated autophagy dysfunction may play a role in the generation of such aberrant species in PD (Vogiatzi et al., 2008). Thus, these studies suggest that autophagy impairment caused by mutant LRRK2 may result in accumulation of misfolded α-synuclein similar to the effect of α-synuclein mutations, thereby enhancing cellular toxicity.

DJ1 and α-synuclein

Even though there have been no *Drosophila* models to validate interaction between DJ1 and α-synuclein, DJ-1 has been shown to modulate α-synuclein aggregation state in a cellular model of oxidative stress (Batelli et al., 2008). In this model, inactivation of DJ-1 led to increased aggregation of α-synuclein in human neuroblastoma cells(SK-N-BE). In a separate cellular model study, overexpression of wild-type DJ-1 inhibited protein aggregation and cytotoxicity, caused by A53T human α-synuclein (Zhou et al., 2005). In conclusion, DJ-1 has a neuroprotective role in ameliorating α-synuclein mediated toxicity.

PINK1/ parkin and α-synuclein

Although a direct molecular interaction between parkin and α-synuclein remains controversial, several studies have shown that coexpression of parkin rescues α-synuclein induced dopaminergic neurodegeneration, motor dysfunction and retinal degeneration in α-synuclein flies. These studies suggest that up-regulation of parkin expression may provide a novel therapy for PD (Yang et al., 2003; Haywood and Staveley, 2004). On the other hand, overexpression of PINK1 has been shown to rescue loss of climbing ability and neurodegeneration induced by α-synuclein expression in *Drosophila* (Todd and Staveley, 2008). Furthermore, it has been suggested that parkin and PINK1 function in a common pathway in maintaining mitochondrial integrity and morphology, as demonstrated using *Drosophila* models (Clark et al., 2006; Park et al., 2006; Poole et al., 2008). In

conclusion, PINK1 and parkin ameliorate α-synuclein phenotypes in flies and PINK1/α-synuclein and parkin / α-synuclein interactions in fly models have validated results obtained in cell and animal models.

Pesticide models

The vast majority of late onset idiopathic PD cases are sporadic and of largely unknown etiology. Epidemiological studies have implicated gene–environment interactions in the pathogenesis of sporadic PD. Pesticides have been identified as potential environmental toxins, and epidemiological studies have supported their role in sporadic PD cases. Hence, pesticide models have become popular in the investigation of PD. Post mortem and epidemiological studies (Di Monte, 2003; Di Monte et al., 2002) have suggested that impairment of mitochondrial function by environmental toxins contributes to PD. Rotenone, a commonly used natural pesticide prepared from the roots of certain tropical plants, has emerged as a key tool in this arena. This lipophilic compound freely crosses cell membranes, accesses cytoplasm and mitochondria, and inhibits mitochondrial complex I. Chronic exposure to rotenone has been used to model PD in the rat, where it induces dopaminergic neurodegeneration, parkinsonian behavior and formation of cytoplasmic inclusions in nigral neurons similar to Lewy bodies (Betarbet et al., 2000). However, owing to variability in observations derived from rats of the same strain, this model has been considered somewhat problematic and thus suboptimal for testing therapeutics. Various explanations have been put forward for the mixed results obtained, including the fact that rotenone leads to nonselective neuronal death in this model (Hoglinger et al., 2003) and differences in the mode of administration of the pesticide (Sherer et al., 2003).

Several groups have adopted the invertebrate system to examine mechanisms underlying pesticide-mediated neurotoxicity. The pesticide model for PD has been investigated in fruit flies and data obtained have confirmed the reliability of the fly for further studies. Coulom and Birman (Coulom and Birman, 2004) showed that chronic exposure to rotenone in *Drosophila* results in PD-like neurodegeneration and behavioral defects. Rotenone-treated flies display characteristic locomotor impairments as measured by negative geotaxis. At the cellular level, dopaminergic neurons are selectively lost in all clusters. Both the locomotor impairments and dopaminergic cell loss increase with the concentration of rotenone. Addition of L-DOPA (3, 4-dihydroxy-L-phenylalanine) into the feeding medium rescues the behavioral deficits but not neuronal death, as is the case with human PD patients. In contrast, the antioxidant melatonin (*N*-acetyl-5-methoxytryptamine) alleviates both symptoms and neuronal loss, supporting the use of this agent in preventing oxidative stress in PD.

It has been speculated that certain genetic backgrounds increase susceptibility to pesticides. *Drosophila* provides an advantageous platform for the identification of genetic factors that promote susceptibility to environmental toxicants; hence the fly paves roads toward design of strategies to combat sporadic PD. Investigations reported by O'Donnell and coworkers (Chaudhuri et al., 2007) have shown that paraquat, a commonly used herbicide, can produce a wide array of parkinsonian symptoms in *Drosophila* that are associated with loss of specific subsets of dopaminergic neurons. Flies fed with paraquat display a shorter lifespan, defects in negative geotaxis and loss of neurons in specific dopaminergic clusters of the fly brain. Male flies exhibited paraquat-related signs earlier than their female counterparts, supporting results of epidemiological studies showing an increased incidence of PD in the male population. These investigators confirmed that paraquat-mediated neurotoxicity is due to the generation of oxidative stress by measuring changes in catalase activity. Paraquat-fed males exhibited increased catalase activity as compared to control male flies. In order to test the hypothesis that variation in dopamine regulating genes, e.g., those that regulate tetrahydrobiopterin (a requisite cofactor in dopamine synthesis) can alter susceptibility to paraquat-induced oxidative damage, these investigators checked the susceptibility to paraquat of *Drosophila* mutant strains that have increased or decreased dopamine and tetrahydrobiopterin production. Surprisingly, protection against neurotoxicity of paraquat is conferred by mutations that elevate dopamine function, whereas mutations that diminish dopamine pools increase susceptibility. This result was intriguing and surprising in the background of extensive research suggesting that dopamine contributes to oxidative load (Stokes et al., 1999). The authors explained these results by suggesting a model in which paraquat, which is similar in structure to MPP^+, has unique access to dopaminergic neurons that can be modulated via competition with extracellular dopamine. They also showed that loss of function mutations in a negative regulator of dopamine production, *Catecholamines-up*, delay the onset of neurological symptoms, dopaminergic neuron death and morbidity during paraquat exposure, while simultaneously conferring sensitivity to hydrogen peroxide (Chaudhuri et al., 2007).

In summary, work in *Drosophila* has provided key insights in understanding PD and has made significant contributions to our understanding of the role of some genes involved in heritable forms of PD. Flies provided the first *in vivo* evidence that PINK1 and parkin regulate mitochondrial integrity by acting in the same serial pathway. This finding hinted at the existence of a common pathogenic pathway in some recessive familial forms of PD. In *Drosophila*, mutations in α-synuclein, DJ-1, parkin, PINK1, and LRRK2, as well as mitochondrial toxins implicated in sporadic PD, render animals more susceptible to oxidative stress, lending support to the hypothesis that some PD cases are caused by gene–environment interactions and that oxidative damage might be a point of convergence in the pathology of PD. Although α-synuclein PD models in flies have been controversial, continued efforts to improve expression levels (e.g., codon optimization) can make the phenotypes stronger and robust. *Drosophila* models have opened up tremendous opportunities to explore the role of genetic and environmental

factors in PD and the pathways in which they might be involved. Both genetic and environmental toxin-induced *Drosophila* PD models provide a system for therapeutic compound identification. Even though the effects of several compounds have been analyzed with regard to behavioral, neurodegenerative, or biochemical phenotypes of such models, leading to the identification of potentially therapeutic compounds in the last 15 years, efforts need to be invested in generating high throughput compound screening along with highly penetrant and robust phenotypes to find new drugs for PD.

Alzheimer's disease (AD)

AD is the most common form of dementia, affecting approximately 26 million people worldwide, and is currently the sixth leading cause of death in the USA. Pathological hallmarks include formation of amyloid plaques and neurofibrillary tangles (NFT), leading to progressive memory loss. The NFT primarily consist of tau, a microtubule-binding protein that, when hyperphosphorylated, leads to the formation of insoluble fibrillar deposits. Amyloid plaques, on the other hand, are composed mainly of amyloid Aβ, small peptides composed of 40 or 42 amino acids (Selkoe, 2000). These peptides are cleaved from the larger amyloid precursor protein (APP) by β-secretase and γ-secretase (De Strooper and Annaert, 2000; Turner et al., 2003).The β-secretase activity is mediated by a single protein called the beta site APP cleaving enzyme or BACE, whereas γ-secretase is a protein complex consisting of presenilin (Psn), nicastrin, aph1, and pen2 (Turner et al., 2003).

Tauopathies

Pathological tau inclusions are found in several other neurodegenerative diseases apart from AD, including frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), corticobasal degeneration, Pick's disease, and progressive supranuclear palsy. These diseases are classified as tauopathies (Gendron and Petrucelli, 2009).

Tau is a microtubule binding protein and is known to regulate microtubule stability and organization. Six tau isoforms exist in human brain tissue, and they are distinguished by their number of microtubule binding repeats. Three isoforms have three binding domains and the other three have four binding domains. The binding domains are located in the carboxy terminus of the protein and are positively charged, allowing it to bind to the negatively charged microtubule. Those isoforms with four binding domains are better at stabilizing microtubules than those with three. The isoforms arise from alternative splicing of exons 2, 3, and 10. Neurotoxicity of tau is thought to be due to two major mechanisms. First, hyperphosphorylation of tau hampers its interaction with tubulin and thereby destabilizes the microtubule; this, in turn, affects axonal transport and leads to neuronal death. Second, neurotoxicity is attributed to the formation of hyperphosporylated tau aggregates and NFT that in turn sequester the normal tau and disrupt its normal function (Alonso et al., 2008).

The first evidence that *Drosophila* could be exploited as a model for tau toxicity came from experiments in which bovine tau was used as an axonal marker (Murray et al., 1998). Subsequently, Shepherd and coworkers found that expressing this construct in sensory neurons resulted in significant degeneration of axonal projections within the thoracic ganglia (Williams et al., 2000). Pan-neuronal induction of this construct also resulted in disrupted axonal transport (Torroja et al., 1999). Further, Feany and colleagues expressed both wild-type human tau and variants containing mutations associated with FTDP-17 (Wittmann et al., 2001) in the CNS, which led to abnormally phosphorylated tau, progressive degeneration, and early death; these effects were stronger for the mutant forms. All these results established that the fly could serve as a reasonable model for tauopathies. However, none of the tau forms induced NFT formation, which suggested that apart from NFTs, there exists some other non filamentous toxic species of tau. These observations in flies validated results obtained in conditional mouse model expressing mutant human tau, where NFT formation do not correlate with neuropathological phenotypes. (Santacruz et al., 2005).

Mechanisms underlying Tau mediated neurotoxicity

Tau phosphorylation and neurotoxicity

A number of kinases, including glycogen synthase-3β (GSK-3β), cdk-5, protein kinase A (PKA), and microtubule-affinity regulating kinase (MARK2), have been shown to regulate tau phosphorylation in biochemical studies (Lee et al., 2001). Fly models also have established that phosphorylation of tau modulates its pathogenicity. Expressing human tau pseudophosphorylated at 14 sites causes a more severe degenerative phenotype as compared to wild-type tau (Khurana et al., 2006). On the other hand, expressing human tau with mutations in sites that render tau phosphorylation resistant ameliorate neurodegenerative effects in the eye (Steinhilb et al., 2007). One of the kinases implicated in the hyperphosphorylation of tau is GSK-3β. The *Drosophila* homolog of GSK-3β is known as Shaggy (Sgg). Pan-neuronal expression of wild-type human tau in flies induces degeneration but does not cause NFT (Wittmann et al., 2001). However, when tau is coexpressed with Sgg, this not only increases the phosphorylation of tau and exacerbates the degenerative phenotype in the eye, but also results in the formation of pretangle-like structures (Jackson et al., 2002). Chan and coworkers have showed that co-expression of either Sgg or Cdk-5 enhances both tau phosphorylation and its degenerative effect (Chau et al., 2006). The interaction of Sgg with human tau has also been confirmed in studies of axonal transport defects in motorneurons. In this case, co-expression of constitutively active Sgg enhances the transport defects, while treatment with GSK-3β inhibitors suppresses the phenotype (Mudher et al., 2004). Apart from these findings, the role of MARK2 in tau phosphorylation has also been investigated in flies.

Overexpression of PAR-1, the fly ortholog of MARK, increases tau phosphorylation and enhances its toxic effects, whereas mutating the PAR-1 phosphorylation sites in tau abolishes its toxicity (Nishimura et al., 2004). This group also asserted that phosphorylation of tau by PAR-1 is a prerequisite for downstream phosphorylation events, most likely including tau phosphorylation by Sgg and cdk-5. In contrast, Chatterjee and colleagues showed that, while mutating the PAR-1 sites in tau did decrease photoreceptor degeneration, it did not prevent phosphorylation of tau by Sgg. In addition, a mutant form of tau resistant to Sgg phosphorylation retains its deleterious effects (Chatterjee et al., 2009). Interestingly, mutations in the Sgg sites of tau increase its affinity for microtubules. This suggests that either decreased or increased binding to microtubules can have a toxic effect, thereby highlighting the need for a precisely regulated interaction between tau and microtubules in normal function of the neuron.

Other mechanisms underlying tau related neurotoxicity

Hyperphosphorylation of tau is widely believed to be a key mechanism underlying its neurotoxicity. However, a few studies in *Drosophila* have suggested that this mechanism may be less important than generally accepted. In a functional genetic screen to identify modifiers of tau-induced neurotoxicity using the 2N/4R (full-length) isoform of wild-type human tau, loss-of-function and gain-of-function alleles showed poor correlation with tau phosphorylation (Ambegaokar et al., 2011). Parmentier and coworkers (Talmat-Amar et al., 2011), using axonal transport and neurohormone release defects as phenotypic readouts, demonstrated the existence of stronger toxicity of hypophosphorylated tau for neuronal function, when compared to normal or pseudophosphorylated tau.

Studies have shown that tau can also affect the actin cytoskeleton. Phosphorylated tau induces the accumulation of filamentous actin resembling the Hirano bodies found in patients with AD or Pick's disease, while co-expression of tau with actin in the eye increases the severity of its degenerative phenotype (Fulga et al., 2007). It is not yet known whether this interaction is due to a direct effect of tau on actin or an indirect one, possibly due to an interaction between the microtubule and actin cytoskeleton (Sider et al., 1999).

Other post-translational modifications of tau have also been implicated in potentiating its neurotoxic properties. Steinhilb and coworkers (Reinecke et al., 2011) found that mutations that disrupted endogenous calpainA or calpainB activity in transgenic flies suppressed tau toxicity. Expression of a calpain-resistant form of tau in *Drosophila* revealed that mutating the putative calpain cleavage sites that produce the 17 kD fragment was sufficient to abrogate tau toxicity *in vivo*. Furthermore, they found toxicity in the fly retina associated with expression of only the 17 kD tau fragment. Thus they established a fly tauopathy model to show the importance of calpain-mediated tau proteolysis in contributing to neurotoxicity.

Tau levels can be modulated by the ubiquitin-proteasome or autophagic clearance pathways. A fly model demonstrated that the induction of autophagy by rapamycin reduced the degenerative phenotype of wild type or mutant (R406W) human tau when expressed in the eye (Berger et al., 2006). The role of the proteasome pathway in tau degradation was investigated by Lecourtois and coworkers (Blard et al., 2007); they expressed a dominant negative form of the 20S proteasome β6 subunit along with wild-type human tau and showed that this resulted in increased tau accumulation, including tau that was hyperphosphorylated by Sgg/GSK-3β (Blard et al., 2007). Surprisingly, a hyperphosphorylated variant of tau that was resistant to proteasome degradation accumulated when dominant negative Sgg was co-expressed, suggesting that phosphorylation by another kinase produces a degradation resistant variant of tau. Tau-induced neurodegeneration also has been linked to aberrant cell cycle regulation and oxidative stress responses. Abnormal activation of the cell cycle accompanies Tau^{R406W} or Tau^{V337M}-induced retinal degeneration, and co-expression of genes that promote the cell cycle (cyclin A, B, or D) enhances this phenotype (Khurana et al., 2006). In contrast, blocking cell cycle progression by co-expressing the cdk2 inhibitor Dacapo (the fly homolog of p21/p27) or the E2F1 inhibitor Rbf1 (retinoblastoma factor 1) reduces the neurodegenerative effects of tau. Feany and coworkers have suggested that these effects are mediated through the TOR (target of rapamycin) kinase pathway, which activates cell cycle progression in both flies and mammalian cells. Enhanced cell cycle activation also appears to be the mechanism underlying oxidative stress-induced neurodegeneration. Modulating the antioxidant defense mechanisms by removing one copy of either superoxide dismutase or thioredoxin reductase aggravated the degenerative phenotype induced by tau, although it did not affect its pattern of phosphorylation (Dias-Santagata et al., 2007).

Functional genetic screen using loss-of-function and gain-of-function alleles performed using the 2N/4R (full-length) isoform of wild-type human tau expressed in the fly retina identified several modifiers of tau mediated neurotoxicity included kinases (shaggy/GSK-3beta, par-1/MARK, CamKI and Mekk1), genes related to autophagy, the cell cycle, RNA-associated proteins and chromatin-binding proteins constituted a large fraction of identified modifiers. Other functional categories identified included mitochondrial proteins, lipid trafficking and Golgi proteins, kinesins, dynein, and the Hsp70/Hsp90-organizing protein (Hop) (Ambegaokar et al., 2011). An unbiased genetic modifier screen performed by Shulman and Feany (Shulman and Feany, 2003) identified phosphatases, kinases, transcription factors, cation transporters, and several unknown proteins as modifiers of tau.

Puromycin sensitive aminopeptidase (PSA) was picked up as a candidate of tau degradation in the studies conducted by Geschwind, Jackson and coworkers, where microarray experiments identified differentially expressed genes in wild-type mice vs. mice expressing mutant tau, and then their interactions were confirmed with tau in the fly model (Karsten

et al., 2006). Thus, this paved way for PSA as a potential therapeutic candidate for ameliorating neurotoxic effects of Tau. Another therapeutic candidate to be modeled in the flies against neurotoxic effects of Tau is nicotinamide mononucleotide (NAD) adenylyl transferase (NMNAT), a protein that has both NAD synthase and chaperone function. Zhai and coworkers (Ali et al., 2012) showed that overexpression of NMNAT significantly suppresses both behavioral and morphological deficits associated with tauopathy by means of reducing the levels of hyperphosphorylated tau oligomers. Recently, Mudher and coworkers (Quraishe et al., 2013) showed that microtubule-stabilizing drug, NAPVSIPQ (NAP) or davunetide, prevents as well as reverses human tau-mediated neuronal dysfunction phenotypes (characterized by microtubule destabilization, axonal transport disruption, synaptic defects, and behavioral impairments) in the flies. The drug did not alter phosphorylated tau levels, indicating that it bypassed toxic tau altogether. They established microtubule stabilisation as a disease-modifying therapeutic strategy for conferring protection against tau-mediated neuronal dysfunction.

Amyloid-related neurotoxicity

One of the hallmarks of AD is the accumulation of Aβ in senile plaques (Hardy and Selkoe, 2002). Mutations leading to increased production of Aβ cause familial AD (Chartier-Harlin et al., 1991; Goate et al., 1991; Sherrington et al., 1996). In order to investigate the toxic function of Aβ peptides in *Drosophila*, transgenic fly models have been created that specifically express either Aβ40 or Aβ42 (Finelli et al., 2004; Crowther et al., 2005; Iijima et al., 2004). Expression of Aβ42 results in amyloid deposits and degeneration in the fly eye (Finelli et al., 2004) and brain (Crowther et al., 2005; Iijima et al., 2004). However, both peptides induce defects in an olfactory associative learning assay when expressed pan-neuronally (Iijima et al., 2004). The performance of flies expressing either protein decline in an age-dependent manner. These experiments indicate that Aβ peptides alone are sufficient to induce AD-like phenotypes. In addition, they also suggest that learning defects do not require visible plaque formation, similar to results obtained by Crowther and colleagues using negative geotaxis (Crowther et al., 2005). In this study, the deficits also were observed before the appearance of large extracellular deposits and instead correlates with the intracellular accumulation of Aβ (in this case Aβ42 and the Arctic mutation found in patients with early onset familial AD).

Iijima et al. investigated the effects of different aggregation rates by expressing Aβ42 containing the Arctic mutation, which increases aggregation, or an artificial mutation shown to decrease aggregation (Iijima et al., 2008). Expressing the Arctic mutation results in higher levels of Aβ42 oligomers as compared to the normal Aβ42, while the artificial mutation reduced the formation of oligomers. These differences in aggregation tendency correlate with detrimental effects on lifespan and locomotion. However, both mutations increase short-term memory deficits in comparison to flies expressing normal Aβ42, with the artificial mutation causing an even earlier onset than the Arctic mutation. These findings support the earlier results by this group, which suggested that the aggregation propensity does not determine the severity of memory deficits. They further showed that each form of Aβ42 has distinct effects on neuronal degeneration, with the Arctic mutation causing mostly vacuoles in the cortex (where all neuronal cell bodies are located, similar to the gray matter in vertebrates). In contrast, the artificial mutation induced vacuolization in the neuropil. The specificity of these pathologies correlates with the localization of aggregates, because Aβ42-Arctic showed large deposits in the cell bodies, whereas the artificial mutation primarily resulted in deposits in neurites. This finding suggests that although aggregation levels can affect some phenotypes, differences in aggregation rates alone do not determine pathogenicity.

Amyloid precursor protein (APP) is an integral membrane protein expressed in many tissues and concentrated in the synapses of neurons. Its primary function is not known, though it has been implicated as a regulator of synapse formation, neural plasticity and iron export. APP is best known as the precursor molecule whose proteolysis generates beta amyloid (Aβ). In order to investigate the genetic factors that affect the processing of APP protein to form the cleaved toxic species, it is important to express the full-length form. This would, in turn, aid in designing therapeutic strategies to target APP processing. Fly lines that express full-length human APP695 were generated by Paro and colleagues (Fossgreen et al., 1998) To ensure β-cleavage of APP in this model, a human BACE construct was co-expressed with APP695, which together with endogenous fly γ-secretase produced toxic Aβ fragments (Greeve et al., 2004). Histological analysis revealed the formation of amyloid deposits and age-dependent degeneration in these flies, in addition to a decreased lifespan. Surprisingly, the same phenotypes were induced after expression of APP695 alone, suggesting that flies possess an endogenous BACE-like enzyme. Western blot analysis confirmed the production of an Aβ fragment in these flies, although it was slightly larger than the Aβ produced by coexpression of human BACE. Flies expressing full-length APP also were used to investigate the effects of altering the processing pattern of APP by either genetic or pharmacological means (Greeve et al., 2004). Increasing the levels of endogenous presenilin (dPsn) or a variant of dPsn that contains mutations linked to familial AD enhanced the phenotypes in this model, whereas removing one copy of the presenilin gene had the opposite effect. Similarly, treating these flies with BACE or γ-secretase inhibitors ameliorated the phenotypes, suggesting that toxic effects are indeed due to the production of Aβ peptides from the full-length protein.

A link between cholesterol homeostasis and APPL processing has been reported by Kretzschmar and coworkers (Tschape et al., 2002). This report showed that the *Drosophila* mutant *löchrig* (*loe*) exhibits an age-dependent degeneration of the

CNS, which is enhanced by knockout of APPL. The *loe* mutation affects a specific isoform of the γ-subunit of AMP-activated protein kinase (AMPK), a negative regulator of hydroxymethylglutaryl (HMG)-CoA reductase and cholesterol synthesis in vertebrates. Western blot analysis revealed that the *loe* mutation reduces APPL processing, whereas overexpression of Loe increases it. These results describe a novel function of AMPK in neurodegeneration and APPL/APP processing, which could be mediated through HMG-CoA reductase and cholesterol ester.

Drosophila models also have been used to investigate the role of ubiquilin in late onset AD. Genetic variants of human ubiquilin-1 have been associated with a higher risk for late onset AD, and ubiquilin-1 can bind to presenilin in cell culture (Bertram et al., 2005; Kamboh et al., 2006). Tanzi and coworkers demonstrated that an RNAi knock-down of *Drosophila* ubiquilin (dUbqln) in the brain results in neurodegeneration and shortened life span. When dUbqln is reduced in the eye, it enhances the eye degeneration caused by expression of dPsn, whereas overexpression suppresses the small eye phenotype (Li et al., 2007). In addition, this group reported that co-expression of dUbqln reduces full-length APP and the amyloid intracellular domain. On the other hand, Guo and colleagues reported that loss of dUbqln suppresses dPsn-induced eye degeneration and that overexpression of dUbqln causes degeneration (Ganguly et al., 2008). These investigators also showed that misexpression of human ubiquilin variants associated with increased risk for late onset AD induce a more severe degeneration as compared to wild-type human ubiquilin. They further reported the binding of dUbqln to dPsn. The results from both groups suggest that ubiquilin is involved in the regulation of Psn. However, clear cut mechanistic roles underlying the ubiquilin and presenilin interaction remain to be elucidated.

In order to identify novel modifiers of AD phenotypes, genetic interaction screens have been performed using both APP/presenilin (van de Hoef et al., 2009) and Aβ42 expressing flies (Cao et al., 2008). Roughly 200 genes were identified from these screens; these include genes involved in vesicular transport, protein degradation, stress response, chromatin structure and the γ-subunit of AMP-activated protein kinase (AMPK), a protein complex involved in energy metabolism and cholesterol homeostasis.

Further, Patridge and coworkers (Sofola et al., 2010) showed that expression of Aβ42 in adult neurons increased GSK-3 activity, and inhibition of GSK-3 (either genetically or pharmacologically by lithium treatment) rescued Aβ42 toxicity. Aβ42 toxicity was also reduced by removal of endogenous fly tau and within the limits of detection of available methods; tau phosphorylation did not appear to be altered in flies expressing Aβ42. They concluded that the GSK-3-mediated effects on Aβ42 toxicity occurred by tau-independent mechanisms, because the protective effect of lithium alone was greater than that of the removal of tau alone. Finally, they showed that Aβ42 levels were reduced upon GSK-3 inhibition, pointing to a direct role of GSK-3 in the regulation of Aβ42 peptide level, lending support to the potential therapeutic use of GSK-3 inhibitors in ameliorating Aβ toxicity. Fernandez-Funez and coworkers (Casas-Tinto et al., 2011) showed up-regulation ER stress response factor X-box binding protein 1 (XBP1) and down-regulation of ryanodine receptor (RyR) suppressed Aβ toxicity, thereby uncovering them as targets for AD therapeutics.

A high throughput screen for inhibitors of Aβ42 aggregation on a collection of 65000 small molecules nailed a potential compound D737, which was most effective in inhibiting Aβ42 oligomerization and reducing Aβ42-induced toxicity in cell culture. Treatment with D737 increased the lifespan and locomotive ability of flies in a *Drosophila melanogaster* model of Alzheimer disease (McKoy et al., 2012).

Effects of synergistic interaction between tau and APP proteins

The hypothesis that tau and APP/Aβ might act synergistically in potentiating neurotoxicity was investigated in *Drosophila* by White and colleagues (Torroja et al., 1999). They described an interaction between the endogenous APPL protein and bovine tau. Expression of either tau or APPL alone resulted in the accumulation of vesicles in larval motoneurons (an effect also seen after expression of human APP (Gunawardena and Goldstein, 2001); this phenotype became significantly more severe when both are co-expressed. These flies showed defects in cuticle hardening and wing expansion in 99% of the eclosing adult flies, while these phenotypes were only occasionally observed when only one construct is expressed (approximately 7% for APPL, and 0.5% for tau). Other studies have showed that co-expression of Aβ42 with tau in the eye enhances the degenerative phenotype as compared to tau alone, accompanied by a substantially greater accumulation of filamentous tau (Fulga et al., 2007). Studies in flies have provided further evidence for the hypothesis that this interaction is mediated by the effects of APP on tau phosphorylation. Induction of Aβ42 and tau in motorneurons not only enhances the synaptic bouton phenotypes and larval crawling defects caused by tau alone, but also increases the phosphorylation of tau (Folwell et al., 2010). These histological and behavioral defects are suppressed by treatment with LiCl, a common inhibitor of GSK-3β, indicating that Aβ may regulate tau phosphorylation via GSK-3β/Sgg. However, similar experiments by Wang et al. suggest that the effects of APP on tau phosphorylation can be mediated by PAR-1 (Wang et al., 2007). Specifically, they showed that PAR-1 is phosphorylated by the tumor suppressor protein LKB-1, which in turn promotes PAR-1-dependent phosphorylation of tau. Expression of full-length APP increases the phosphorylation of both PAR-1 and tau, an effect dependent on the presence of LKB-1.They also showed that LKB-1 affects the toxic function of tau; knocking-down LKB-1 expression suppresses the eye degeneration caused by PAR-1 or APP / tau expression, whereas overexpression of LKB-1 enhances the toxic effects induced by PAR-1 and tau. Together with the observation that expression of APP enhances the PAR-1 phenotype in an LKB-1 dependent manner,

these results suggest a pathway in which APP activates LKB-1, which in turn phosphorylates PAR-1, leading to hyperphosphorylation of tau.

In summary, hallmarks of AD include amyloid plaques, comprised mainly of Aβ, and NFT, which primarily consists of tau. Many aspects of fly models have supported that phosphorylation of tau modulates its pathogenicity. These models have focused on GSK-3β, cdk-5 and PAR1 kinases as mediators of tau phosphorylation. However, some studies in *Drosophila* suggest that this link is highly complex and possibly less important than generally accepted. Experiments using flies also have suggested that either decreased or increased binding to microtubules can have a toxic effect. Tau-induced toxicity can be mediated by effects on other cytoskeletal proteins, e.g., actin. Tau levels can also be modulated by the proteasome or autophagic clearance pathways. Tau-induced neurodegeneration has been linked to aberrant cell cycle regulation and oxidative stress responses. The most innovative results using *Drosophila* have, and will continue to, come from unbiased forward genetic screens that identify modifiers of tau neurotoxicity, as well as functional validation of candidates derived from mammalian genome-wide high throughput experiments (e.g., genome wide association studies and functional transcriptomics). Amyloid-related neurotoxicity has been also modeled in the fly. Expression of Aβ42 (amyloid beta peptide) produces amyloid deposits and degeneration in the fly eye. Genetic factors that affect the processing of APP protein to form the cleaved toxic species have been, and will continue to be, studied in the fly. Additionally, synergistic interaction between tau and APP proteins have been established.

Huntington's disease

Huntington's disease is the prototypic member of a family of autosomal dominant, late-onset diseases that are caused by expanded CAG triplet repeat sequences that encode expanded polyglutamine (polyQ) repeats in the affected protein (Q is the single letter code for glutamine). The polyQ diseases belong to a larger family of protein conformation diseases, many of which also cause dominant, late-onset neurodegeneration. These disorders are caused by mutations or cellular events that lead to accumulation of abnormal structural forms of a particular protein. HD and other polyQ diseases are associated with the formation of abnormal protein aggregates thereby leading to motor dysfunction, early death, movement disorders, or dementia. HD is caused by a polyQ repeat expansion toward the amino terminus of huntingtin, a large (~350 kDa) protein of as yet unknown biochemical function, ubiquitously expressed, with expression, beginning in the embryo (Duyao et al., 1995; Group, 1993; Li et al., 1993; Strong et al., 1993). Expansions above ~39 repeats invariably lead to disease, whereas individuals with ≤35 are disease free (Gusella and MacDonald, 1995). The number of polyQ repeats determines the age of symptom onset. Some of the early fly models of polyQ diseases were established by Zipursky and coworkers (Jackson et al., 1998) and by Bonini and colleagues (Warrick et al., 1998). The first invertebrate model of HD (Jackson et al., 1998) was generated by expressing truncated wild type and mutant forms of huntingtin, whereas Warrick et al. (1998) reported a model of SCA 3 (spinocerebellar ataxia Type 3) or Machado–Joseph disease (SCA3/MJD) expressing truncated ataxin 3 (also referred to as MJD). Both papers demonstrated that increased polyQ expansion leads to more severe age-dependent degeneration and repeat length-dependent nuclear aggregation. Neurotoxicity due to interactions between phosphatidylinositol 3-kinase/AKT signaling and 14–3–3/ataxin1 protein have been shown in a fly model of SCA1. (Chen et al., 2003)

Changes in genes that regulate degradation of misfolded proteins (e.g., chaperones and ubiquitin ligases) have been shown to modify degenerative effects of polyQ proteins (Al-Ramahi et al., 2006; Bilen and Bonini, 2007; Fernandez-Funez et al., 2000; Warrick et al., 1999). One of the E3 ligases that interacts with polyQ is CHIP (C-terminus of Hsc-70 interacting protein) (Al-Ramahi et al., 2006). CHIP also targets the microtubule-binding protein tau for ubiquitination and degradation (Dickey et al., 2006; Petrucelli et al., 2004). Genes related to apoptosis (Bae et al., 2005; Sang et al., 2005; Warrick et al., 1998) and some signaling pathways (Chen et al., 2003; Scappini et al., 2007) also have been reported to modulate polyQ degeneration. Another screen has identified Drosophila VCP, an AAA+ ATPase superfamily member, as a dominant suppressor of polyQ pathology (Higashiyama et al., 2002).Artificially engineered proteins containing only polyQ tracts expressed outside the context of a disease-associated gene are toxic (Kazemi-Esfarjani and Benzer, 2000; Marsh et al., 2000) and can form nuclear and axonal inclusions in neurons (Gunawardena et al., 2003). However, the toxic effects of these polyQ tracts can be diminished if additional non-glutamine amino acids are added, even by as few as 26 amino acids that comprise an epitope tag (Marsh et al., 2000). In addition, expansion of polyQ tracts in non-disease-associated genes that contain functional polyQ tracts, such as *Dishevelled* (*dsh*) in *Drosophila* produces a mild phenotype as compared to expression of the polyQ tract alone; instead, they appear to disrupt the normal function of the host protein, yielding phenotypes similar to *dsh* loss of function mutations (Marsh et al., 2000). The presence of other proteins or peptides with polyQ tracts also appears to modulate polyQ degeneration, depending on the type of proteins/peptides expressed. Expression of exon 1 of human huntingtin with a normal polyQ tract of 20 glutamines (Q20) has no toxic effect on its own, whereas coexpression of huntingtin-Q20 strongly accelerates degeneration and increases nuclear inclusions induced by expression of huntingtin-Q93, perhaps by enhancing seeding for aggregation (Slepko et al., 2006).

Increased expression of wild-type ataxin-2, a polyQ-containing protein associated with spinocerebellar ataxia 2 (SCA2), enhanced both ataxin-1- (Al-Ramahi et al., 2007) and ataxin-3-induced toxicity (Lessing and Bonini, 2008) through a direct interaction with both proteins. The normal ataxin-2 appears to be recruited to the nucleus by the other mutant

ataxin proteins, where it can produce toxic effects, even with a normal polyQ length (Al-Ramahi et al., 2007). On the other hand, coexpression of wild-type ataxin-3, a protein involved in ubiquitin binding and the ubiquitin–proteasome cycle, can suppress the toxicity of several polyQ proteins, including pathogenic forms of ataxin-3, huntingtin, and ataxin-1, likely by targeting these harmful proteins for degradation (Warrick et al., 2005).

Polyglutamine proteins such as huntingtin have been shown to bind and inhibit the acetyltransferase function of histone acetylases. This observation led to the design of genetic and pharmacological strategies for maintaining acetylation levels by reducing histone deacetylase (HDAC) activities. These strategies have been effective in suppressing polyQ-induced toxicity in cellular and fly models (Agrawal et al., 2005; Pallos et al., 2008; Steffan et al., 2001). HDAC Rpd3 was found as a common modifier by two different groups (Fernandez-Funez et al., 2000; Pallos et al., 2008), and the HDAC coactivator Sin3a was also found as a common modifier by the Marsh and Botas groups (Steffan et al., 2001, Fernandez-Funez et al., 2000), as well as independently by a third group (Bilen and Bonini, 2007). HDAC6 has also been shown to promote autophagy, which can specifically degrade polyQ proteins (Pandey et al., 2007). Other regulators of autophagy, like phosphoinositide-dependent kinase-1 (PDK1), p70 ribosomal S6 kinase (S6K) (Nelson et al., 2005), and endosomal sorting complex required for transport (ESCRT) complexes (Rusten et al., 2007) have also been shown to modify polyQ toxicity, suggesting that endosomal/lysosomal/autophagy pathways, in addition to the ubiquitin–proteasome system, have a role in degrading toxic proteins and aggregates.

Some *Drosophila* and mammalian models (Klement et al., 1998; Saudou et al., 1998) have suggested that translocation to the nucleus of the mutant polyQ protein is necessary to induce neurodegeneration. Additionally, *Drosophila* models showed that toxicity can be alleviated if the mutant protein is trapped outside of the nucleus (Takeyama et al., 2002). Furthermore, transcription factors and other nuclear proteins have been found to modify polyQ toxicity (Bilen and Bonini, 2007; Branco et al., 2008; Fernandez-Funez et al., 2000; Steffan et al., 2001; Taylor et al., 2003). These results suggest that dysregulation of transcription is an important event in polyQ-mediated toxicity and addresses the presence of inclusions localized to the nucleus. However, the role of these nuclear inclusions is still unclear, as several studies have shown that toxicity can persist in the absence of inclusions and, conversely, that toxicity can be mitigated with little effect on inclusion formation or stability (Bilen and Bonini, 2007; Klement et al., 1998; Saudou et al., 1998). Additionally, some investigations have shown that CUG or CAG codon expansions in mRNA can lead to neurotoxicity without the need for translation and that increased repeat length correlates with increased neurodegeneration (Le Mee et al., 2008; Li et al., 2008; Mutsuddi et al., 2004). These observations provided a platform in understanding the reasons, that RNA binding proteins are strong modifiers of polyQ toxicity (Bilen and Bonini, 2007; Bilen et al., 2006; Fernandez-Funez et al., 2000; Le Mee et al., 2008; Li et al., 2008; Murata et al., 2008; Mutsuddi et al., 2004; Satterfield and Pallanck, 2006) and microRNAs modify polyQ-mediated neurodegeneration (Bilen et al., 2006). In yet another avenue, Rab11 (involved in endosomal recycling) rescued synaptic dysfunction and behavioral deficits in a *Drosphila* model of HD. This work highlighted a potential novel HD therapeutic strategy for early involvement, prior to neuronal loss and clinical manifestation of the disease. Giorgini and coworkers (Steinert et al., 2012) found that expression of mutant htt in the larval neuromuscular junction decreased the presynaptic vesicle size, reduced quantal amplitudes and evoked synaptic transmission and altered larval crawling behaviour. Overexpression of Rab11 reversed all the synaptic dysfunction phenotypes.

Using these *Drosophila* models of polyglutamine disease, a number of pharmacological interventions have been tested. Many chemical compounds aimed to reduce polyQ protein aggregation have been identified that also lessen neurodegenerative phenotypes in *Drosophila* (Kazantsev et al., 2002; Nagai et al., 2003; Pollitt et al., 2003; Zhang et al., 2005). HDAC inhibitors, either alone or in combination with aggregation inhibitors, have shown great potential (Agrawal et al., 2005; Ehrnhoefer et al., 2006; Pallos et al., 2008; Steffan et al., 2001) in reducing neurotoxicity in fly HD models. The use of intracellular single chain antibodies ("intrabodies") that target protein aggregates have been shown to strongly ameliorate toxicity and significantly lengthen lifespan in a *Drosophila* HD model (Wolfgang et al., 2005).Thompson and coworkers (Sontag et al., 2012) nailed on methylene blue (MB) as a potential therapeutic agent for HD. MB inhibited recombinant Huntingtin protein aggregation *in vitro*, decreased oligomer number and size, and accumulation of insoluble mutant Htt in cells. In functional assays, MB increased survival of primary cortical neurons transduced with mutant Htt, reduced neurodegeneration and aggregation in a *Drosophila* model of HD, and reduced disease phenotypes in R6/2 HD modeled mice. Meclizine, a clinically used drug and known to cross the blood brain barrier suppressed apoptotic cell death in a murine cellular model of polyglutamine (polyQ) toxicity and displayed protective effect against neuronal dystrophy and cell death in *C. elegans* and *Drosophila* models of polyQ toxicity (Gohil et al., 2011). Meclizine's mechanism of action strongly correlated with its ability to suppress mitochondrial respiration. Thus, meclizine holds strong therapeutic potential in the treatment of polyQ toxicity disorders.

Additionally, by performing high-content small molecule and RNAi suppressor screens, by tracking subcellular distribution of mRFP-tagged pathogenic Huntingtin and assaying neurite branch morphology via live-imaging, in a *Drosophila* primary neural culture Huntingtin model, Littleton and coworkers (Schulte et al., 2011) identified suppressors that could reduce Huntingtin aggregation and/or prevent the formation of dystrophic neurites. They identified lkb1, an upstream kinase in the mTOR/Insulin pathway, and four novel drugs, Camptothecin, OH-Camptothecin, 18β-Glycyrrhetinic

acid, and Carbenoxolone, that were strong suppressors of mutant Huntingtin-induced neurotoxicity.

In summary, flies have been beneficial in deciphering the modifiers of polyQ mediated toxicity. Genes that regulate degradation of misfolded proteins, e.g., chaperone proteins and ubiquitin ligases anas well as those d those related to apoptosis modify degenerative effects of polyQ proteins. The presence of other proteins or peptides with polyQ tracts also appears to modulate polyQ dependent degeneration, depending on the type of proteins/peptides expressed. The toxicity of several polyQ proteins, including pathogenic forms of ataxin-3, huntingtin, and ataxin-1 have been modeled in the fly. Regulators of autophagy, including HDAC6, PDK1, p70 ribosomal S6 kinase (S6K) and ESCRT complexes also have been shown to modify polyQ toxicity. Transcription factors, nuclear proteins and microRNAs also modify polyQ mediated neurodegeneration. *Drosophila* models of polyglutamine diseases have been used to test a number of pharmacological interventions (FDA approved drugs and natural products) to reduce polyQ protein aggregation, as well as HDAC inhibitors. Intracellular single chain antibodies ("intrabodies") that target protein aggregates have been tested in *Drosophila* and can serve as potential therapeutic interventions for the future.

Amyotrophic lateral sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS) is a disease of motorneurons in the brain and spinal cord that control voluntary muscle movement. ALS is also known in the USA as Lou Gehrig's disease. In about 10% of cases, ALS is caused by a genetic defect. Mutations in genes including copper or zinc superoxide dismutase (SOD), TAR DNA binding protein (TDP43) and vesicle associated membrane protein / synaptobrevin-associated membrane protein B (VAPB) have been implicated in familial ALS and studied in fly models. Neuronal inclusions of SOD proteins, leading to oxidative stress and axonal transport blockages have been found in familial ALS cases (Wood et al., 2003). Null alleles of the *Drosophila* homolog of SOD showed reduced longevity and increased susceptibility to oxidative stress (Phillips et al., 1989). Studies expressing either mutant or wild-type *Drosophila* or human SOD also showed neurotoxicity and increased aggregation (Phillips et al., 1989; Watson et al., 2008). Sequestration of wild-type *Drosophila* SOD by their mutant counterparts and functionally orthologous human SOD proteins were pointed out as the key pathophysiological mechanism underlying neuronal toxicity in both these studies.

The TDP43 (TAR DNA binding protein-43) gene has been linked to both familial and sporadic ALS. This gene encodes a heterogeneous nuclear ribonuclear protein (hnRNP) transactive response-DNA binding protein with a molecular weight of approximately 43 kDa, also known as "TDP-43" (Gitcho et al., 2008; Kabashi et al., 2008; Sreedharan et al., 2008; Van Deerlin et al., 2008). Ubiquitin-positive and tau-negative inclusions in neurons from patients with frontotemporal lobar degeneration (FTLD) are TDP-43-immunoreactive in both familial and sporadic cases (Neumann et al., 2006). Post-translational modifications of the TDP43 protein (for example, its cleavage, phosphorylation, and aggregation) and its translocation from nucleus to cytosol have been identified as pathophysiological mechanisms underlying TDP43-mediated neuronal toxicity (Gendron et al., 2010; Kwong et al., 2007). TDP-43 is highly conserved between humans, mice, *Drosophila* and *Caenorhabditis elegans*, and functions in DNA binding and regulation of splicing (Ayala et al., 2005; Wang et al., 2004).

Endogenous *Drosophila* TDP43 (dTDP / TBPH) and human TDP43 (hTDP-43) both have two RNA recognition motifs (RRMs) and a glycine rich region (GRR) toward the C-terminus, but the carboxy terminal domain is longer in dTDP than in hTDP-43. dTDP has 531 amino acids in contrast to 414 amino acids for hTDP-43. dTDP runs at a higher molecular band (around 55kDa) in comparison to the human counterpart (Feiguin et al., 2009; Lu et al., 2009). Some of the reagents that have served as useful tools to understand the function of endogenous dTDP-43 are: (a) dTDP null alleles (genomic deletions created by imprecise P element excisions) created by Feiguin and coworkers et al. (2009); (b) A point mutation introducing a stop codon – Q367X – used by Gao and colleagues (Lu et al., 2009); and, (c) RNAi lines used by all groups (Feiguin et al., 2009; Li et al., 2010; Lu et al., 2009) to down-regulate dTDP. Flies bearing homozygous null dTDP mutations, or depletion by RNAi expression, show larval lethality. Flies heterozygous for the mutation appear functionally similar to controls. Thus, dTDP is indispensable for development to adulthood and survival. Expression of wild-type hTDP43 in a dTDP mutant background is sufficient to restore survival to adulthood, as well as rescue locomotor functions, and hTDP-43 and dTDP overexpression yield similar phenotypes, demonstrating functional conservation between human and *Drosophila* TDP-43. Amino acid sequence analysis has revealed the existence of 59% sequence identity in the RNA recognition motifs of the human and the *Drosophila* protein (Ayala et al., 2005). Overexpression of hTDP-43 in a dTDP wild-type background leads to larval lethality and motor impairments, similar to mutant dTDP null phenotypes. Loss-of-function alleles of dTDP and hTDP-43 overexpression yield similar phenotypes with regard to reduced number of axonal branches, number of synaptic boutons and neuritic processes. However, overexpression of dTDP or hTDP-43 promote dendritic branching in sensory neurons (Lu et al., 2009), indicating differences in TDP-43 function in axons and dendrites.

In an independent study, Hirth and coworkers (Diaper et al., 2013) have shown that both loss and gain of dTDP / TBPH severely affect development and result in premature lethality. TBPH dysfunction causes impaired synaptic transmission at the larval neuromuscular junction (NMJ) and in the adult. Electrophysiological recordings at the larval NMJ along with tissue-specific knockdown revealed that alterations of TBPH function predominantly affect pre-synaptic efficacy, suggesting that impaired pre-synaptic transmission is one of the earliest events in TDP-43-related pathogenesis.

Work reported by Tibbetts and colleagues (Hanson et al., 2010) showed that TDP43 is mainly localized to the nucleus of motor neurons, with minor levels of aggregated or insoluble forms distributed in the cytoplasm. On the other hand, Wu and coworkers (Li et al., 2010) reported axonal inclusions in motor neurons with coincident axonal swelling, as well as the appearance of TDP-43 in sarkosyl-insoluble material, although most TDP-43 appears to be soluble and localized to the nucleus. However, cells expressing TDP-43 have abnormal amounts of highly condensed chromatin within the nucleus (Hanson et al., 2010). Clearance of cytosolic and insoluble TDP-43 by the proteosome or autophagy (by coexpression of ubiquilin) fail to rescue degenerative effects and, in fact, worsen the degenerative phenotype (Hanson et al., 2010).These data hint at a novel mechanism of early toxicity in the cell that possibly involves chromatin assembly, regulation of transcription and/or splicing. This observation provides a possible explanation for the nuclear exclusion of TDP-43 seen in the neurons of patients with ALS: the cell's security mechanism to exclude TDP-43 from its toxic locus of action in the nucleus.

A high-content, genome-wide RNAi screen to identify pathways controlling TDP-43 nucleocytoplasmic shuttling shortlisted 60 genes, whose silencing increased the cytosolic localization of TDP-43 (Kim et al., 2012). These genes included nuclear pore complex components and regulators of the G2/M cell cycle transition. In addition, the type 1 inositol-1,4,5-trisphosphate (IP3) receptor (ITPR1), an IP3-gated, endoplasmic reticulum (ER)-resident calcium channel, was also identified as a strong modulator of TDP-43 nuclear-cytoplasmic shuttling. Knockdown or chemical inhibition of ITPR1 induced TDP-43 nuclear export in primary neurons and cells and aided the recruitment of TDP-43 to ubiquilin-positive autophagosomes. This suggested that diminished ITPR1 function leads to autophagosomal clearance of TDP-43. The functional significance of the TDP-43-ITPR1 genetic interaction was tested in *Drosophila*, where mutant alleles of ITPR1 were found to significantly extend lifespan and mobility of flies expressing TDP-43 under a motor neuron driver.

Studies reported by both Wu and Gao (Li et al., 2010; Lu et al., 2009) showed that the RNA recognition motifs of TDP-43 is important for its neurotoxicity, in contrast to reports that the cleaved carboxy terminal fragments are abundant in TDP-43 inclusions. Lecourtois and coworkers reported biochemical data showing that human TDP-43 proteins expressed in adult fly neurons are abnormally phosphorylated on the disease-specific Ser409/Ser410 site and processed (Miguel et al. 2011).

Recently, the Pandey and McCabe groups independently demonstrated interaction between FUS/TLS (fused in sarcoma / translated in liposarcoma) and TDP-43 (Lanson et al. 2011; Wang et al., 2011). FUS is another DNA/RNA-binding protein found to be mutated in sporadic and familial forms of ALS. Lanson et al., 2011, demonstrated that ALS-associated mutations in FUS/TLS cause adult-onset neurodegeneration via a gain-of-toxicity mechanism that involves redistribution of the protein from the nucleus to the cytoplasm and is likely to interact with TDP-43. They showed that ectopic expression of human ALS-causing FUS/TLS mutations in *Drosophila* causes an accumulation of ubiquitinated proteins, neurodegeneration, larval crawling defects, and early lethality. Mutant FUS/TLS localizes to both the cytoplasm and nucleus, whereas wild type FUS/TLS localizes only to the nucleus, suggesting that the cytoplasmic localization of FUS/TLS is required for toxicity. Furthermore, they found that deletion of the nuclear export signal strongly suppresses toxicity, suggesting that cytoplasmic localization is necessary for neurodegeneration. Interestingly, they also observed that FUS/TLS genetically interacts with TDP-43 in a mutation-dependent fashion to cause neurodegeneration *in vivo*.

McCabe and colleagues (Wang et al., 2011) reported that *Drosophila* mutants in which the homolog of FUS is disrupted exhibit decreased adult viability, diminished locomotor speed, and reduced lifespan as compared with controls. These phenotypes are fully rescued by wild-type human FUS, but not by ALS-associated mutant FUS proteins. Further, they found that mutants of dTDP had similar, but more severe, deficits. Through cross-rescue analysis, they demonstrated that FUS acted together with and downstream of TDP-43 in a common genetic pathway in neurons. They also found that these proteins associated with each other in an RNA-dependent complex.

A mutation linked to ALS was identified in the locus ALS8, which encoded vesicle-associated membrane protein / synaptobrevin- associated membrane protein B (VAPB). Three independent groups (Chai et al., 2007; Ratnaparkhi et al., 2008; Tsuda et al., 2008) established VAPB-mediated neurodegeneration models in *Drosophila*. All three studies employed wild-type and disease-associated mutant VAPB and demonstrated an increased propensity of the mutant VAPB to aggregate or form inclusions. The mutant VAPB recruited wild-type protein. Bellen and colleagues (Tsuda et al., 2008) reported that VAPB is cleaved and secreted; however, mutant VAPB fails to be secreted and induces endoplasmic reticulum stress, initiating the unfolded protein response. Synaptic bouton morphology defects (Chai et al., 2007; Ratnaparkhi et al., 2008), impairment of transmission (Chai et al., 2007), involvement of signaling pathways like BMP (Ratnaparkhi et al., 2008) and ephrin (Tsuda et al., 2008) have been implicated in pathological phenotypes in these models.

These studies provided novel insights into VAPB function and possible mechanisms that can lead to motorneuron degeneration. Studies reported by Ratnaparkhi and colleagues (Ratnaparkhi et al., 2008) showed that increased expression of wild-type VAPB in sensory neurons leads to loss of notal bristles on the dorsal posterior thorax and that bristles could be restored when wild-type VAPB was inhibited.

In conclusion, VAPB, TDP43 and FUS proteins have been successfully shown as players in ALS fly models. The propensity to aggregate, nuclear to cytoplasmic translocation, and regulation of signaling pathways have been highlighted as mechanisms underlying ALS. Presently, it remains unclear

whether TDP43-mediated neurotoxicity is caused by the aggregation of TDP-43 into inclusions [formation of aggregates], caused by cytoplasmic accumulation of TDP-43 or by loss of TDP-43 from the nucleus. Studies in flies have pointed toward each of these mechanisms underlying pathogenesis. Since TDP-43 is known to have roles in RNA metabolism, pre-mRNA splicing and repression of transcription, using *Drosophila melanogaster* as a model, Morton and coworker (Hazelett et al., 2012) generated loss-of-function and overexpression genotypes of TAR-DNA binding protein homolog (TBPH) to study their effect on the transcriptome of the central nervous system (CNS). Interestingly, comparison of parallel high-throughput RNA sequencing between knockout of TDP-43 and its overexpression revealed primarily nonreciprocal and non-overlapping gene expression changes in the central nervous system of *Drosophila*. This study was pivotal in proving that cytoplasmic mislocalization of TDP-43 in an overexpression experiment does not have the same consequence as loss-of-function in the nucleus. At the functional level, *Drosophila melanogaster* may prove to be useful in validating RNA targets of TDP43 screened using UV cross-linking and immunoprecipitation (UV-CLIP) in cells (Xiao et al., 2011) and TDP43 RNA library generated from the rat cortical neurons using RIP-seq [RNA Immunoprecipitation followed by deep sequencing] (Sephton et al., 2011).

Concluding remarks

Drosophila models for various human neurodegenerative diseases have appeared on a reasonable scale in the last 15 years. *Drosophila* has helped to mimick several important neurodegenerative diseases, including HD and the polyQ diseases, ALS and other late-onset neurodegenerative diseases such as PD and Tauopathies. These models have nailed several cellular processes like axonal transport, synaptogenesis, autophagy, and apoptosis underlying neuronal degeneration. Various post-translational modifications (like phosphorylation, truncation / cleavage) of the disease causing protein have been shown to enhance neurotoxicity. Aggregation, nuclear to cytoplasmic translocation, transcriptional and translational regulation, modulation of signaling pathways, cytoskeletal disorganization have been nailed as some of the basic mechanisms underlying pathogenesis in these disease models.

The various genetic tools available in the fruit fly allows one to investigate the basic fundamental mechanisms underlying disease and to design screens to look for putative modifiers of the disease phenotype. Unbiased high-throughput forward genetic screens have proved to be useful in pointing to cellular pathways that influence the severity of a particular disease. Yet another avenue where the fruit fly can prove to be useful is in the validation of targets found in the genome-wide association studies (GWAS). GWAS and transcriptomics have emerged during the last few years as powerful strategies to identify novel biological disease pathways. However, given the vast amount of novel data generated in these experiments, and the fact that the function of many of the identified genes or loci are not known, the biological interpretation of these studies is difficult. A couple of research reports have been published using interesting strategies tackling this problem. The first study combines transcriptome analysis of mice overexpressing tau with functional validation in *Drosophila* (Karsten et al., 2006). The second study starts with target identification in an AD GWAS study, followed by validation in the fly (Shulman et al., 2011). In both studies, *Drosophila* has played a central role in the functional validation of targets found in mice or patients.

However, the true potential of these models will only be realized if the pathways identified in genetic screens and the efficacy of therapeutic strategies in different models allow one to identify the overlapping and the unique features of the different diseases. Another area of venture will be the effort to make *Drosophila* models more accessible to high-throughput and automated screening for therapeutics.

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Chapter 12

Drosophila model of cognitive disorders: Focus on memory abnormality

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Memory abnormality and cognitive disorders

Cognitive disorders are one of the most puzzling problems plaguing human beings. Along with the human cost, cognitive disorders cause tremendous socio-economic burdens. From the pathogenic origin, cognitive disorders can be divided into the inherited cases led by mutations of specific disease-related genes, such as fragile X syndrome (FXS), Down syndrome (DS), tauopathy, and familial Alzheimer's disease (FAD), and the sporadic cases triggered by the combination of both genetic-susceptibility and environmental factors. These more complex disorders include sporadic AD, posttraumatic stress disorder (PTSD) and schizophrenia (Sz). Human genetic studies of these diseases have identified a plethora of candidate genes that may contribute to their inception and evolution (Bertram et al., 2010; Lubs et al., 2012; Sturgeon et al., 2012; Sullivan et al., 2012).

Worth noting, although cognitive disorders have heterogeneous origins and affect various aspects of cognitive functions, the majority exhibit a primary disruption in learning and memory capacity. Memory is the process of information encoding, storage, and retrieval. It is one of the most fundamental cognitive functions and is closely related to other critical cognitive functions, including attention, the organization and understanding of languages, problem-solving, as well as decision-making. Indispensable as memory is, it is greatly vulnerable to all kinds of internal and external factors, including heredity, disease, aging, drugs, and environment. At present, memory abnormality is considered as a core endophenotype in the research of cognitive diseases, and is studied extensively. Studies on the functions of disease-related genes under normal and pathological conditions are not only important in revealing the pathogenesis and mechanisms of diseases, but also instrumental in understanding the way our brain works, and to bridge the gap between genes and behaviors in neurological research.

To this end, various animal models have been established to emulate the pathophysiological and clinical phenotypes of the cognitive disorders through genetic manipulation, brain lesion, or pharmacological approaches. Studies have been performed to uncover the molecular and cellular mechanisms underlying these phenotypes, and to screen for candidate drugs that could rescue the clinical-related manifestations.

As a model system that has made great contributions to the research of genetics and development, *Drosophila* likewise proves to be an excellent model on disease research. Here, we will elaborate on the *Drosophila* model of cognitive disorders, particularly the ones with memory abnormalities, and review the progress in this field.

Why flies? *Drosophila* as a model for the study of cognitive disorders with memory abnormality

The fruit fly *Drosophila melanogaster* is well known for its advantages as a research animal model. In addition to the short lifespan, inexpensive rearing cost, genetic tractability, and available tools for spatial–temporal regulation of gene expression, *Drosophila* has several inherent properties that are especially favorable for studying cognitive disorders.

First is the high degree of evolutionary conservation and the relatively lower level of genetic redundancy in the fly genome. Genome analysis has revealed that 74% of human disease-related genes have their counterpart in *Drosophila*, and 10% of these genes are involved in neurological diseases (Reiter et al., 2001; Chien et al., 2002). The high degree of conservation in the genes and signaling pathways is the foremost prerequisite of studying the functions of disease genes endogenously in *Drosophila*. Another advantage relevant to evolution is the minimal genetic redundancy in *Drosophila* compared with mammals (Mendonca et al., 2011). Generating null and hypomorphic mutant alleles of a particular gene is feasible in *Drosophila* and the relatively small number of paralogs reduces the chance that compensatory mechanisms obscure the functional consequences of perturbation to signaling pathways or cellular processes.

In addition to the conservation of gene function, there is a high degree of homology at the level of behavioral features. Recent evidence has shown convincingly that several behaviors in human and fly, including sleep (Sehgal and Mignot, 2011), circadian rhythm (Allada and Chung, 2010), addiction (Kaun

Behavioral Genetics of the Fly (Drosophila melanogaster), ed. J. Dubnau. Published by Cambridge University Press.

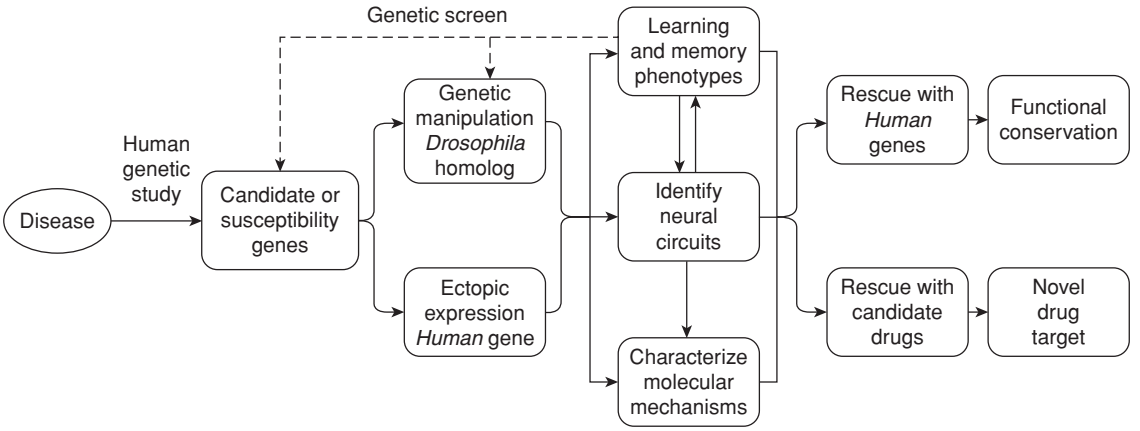

Fig. 12.1. The research strategy for the study of cognitive disorders with memory abnormality using *Drosophila* as a model system.

et al., 2012), as well as learning and memory (Dubnau, 2003), not only exhibit phenotypic similarity, but also share molecular and cellular mechanisms. Thus, it is rational to believe that the discoveries made on the functions and interactions of the genes required in particular behaviors in fruit flies will provide insights to the neurological roles of the cognate genes in human.

A third favorable characteristic of flies as a model is that the nervous system exhibits a moderate level of complexity. The human brain is the most complex and elaborate organ in the living world, with innumerable neurons and connections. It is a huge challenge to study the genetic regulation of physiological properties and behavioral phenotypes in such an elaborate system, let alone the inevitable ethical problems. On the other hand, it may be problematic to model behavioral disorders including the molecular, cellular, circuit and behavioral levels, using an oversimplified model. The brain of *Drosophila* seems ideal in that it is considerably simpler than that of a mammal, but far more complex than that of worms, which also are an excellent genetic model. The fly brain has well-organized centers for distinctive functions, including olfactory, visionual, and gustatory processes as well as learning and memory (Chang et al., 2011). And, the fly brain appears to operate on the same fundamental principles as its mammalian counterpart, and therefore, is an efficient and ideal system for the study of cognitive diseases.

Last but not least, large quantities of genetically homogeneous progeny are easily obtained in flies. Practically speaking, hundreds of *Drosophila* offspring with identical genetic background can be produced by a single cross in a fly laboratory. Coupled with the short life cycle, *Drosophila* is no doubt a preferred system for inheritance pattern identification and high throughput drug screening.

How to study cognitive disorders with memory abnormalities in *Drosophila*

Besides all these inherent advantages, well-characterized and highly effective research strategies in *Drosophila* also make it a unique model for the research of cognitive diseases. Common research strategies in *Drosophila* disease model are summarized and detailed in Fig. 12.1 and below.

In the reverse genetic approach, the disease-related gene is selected according to the research on human genetic or other animal models, and then a *Drosophila* model is established to duplicate aspects of the corresponding disease, such as memory deficits and neurodegeneration. Under the condition that the *Drosophila* homolog of the disease-gene is available, we can manipulate the endogenous homolog gene by taking advantage of the genetic tractability in *Drosophila*. The most commonly used are fly mutants accumulated during the past century that were produced by means of chemical, radiation and transposon mutagenesis. In addition, the fruit fly has a variety of genetic toolkits that provide the means to regulate gene expression in specific tissues and cell types and during a particular time-window. These include the Gal4-UAS binary expression system, and the TARGET as well as Geneswitch systems, that were developed subsequently (for details about these tools, see review by Venken et al., 2011, and chapter by Zhang *et al.*). Almost every *Drosophila* homolog of a human disease gene can be knocked down in a spatially–temporally controlled manner by these tools coupled with the *Drosophila* RNAi library. In the case that the *Drosophila* homolog of the disease gene is not available, the wild-type or pathogenic-related form of human disease gene can be misexpressed in the disease affected brain structures or neuron subtypes in *Drosophila*. The genetically modified flies are then subjected to the inspection of different paradigms, ranging from molecular and biochemical levels to the physiological level and ultimately the behavioral level. The phenotypes exhibited under such scrutiny provide information about how the genes are involved in the etiology and progression of the disease. Next, genetic and pharmacological rescue experiments can be performed to confirm the specificity of the preceding genetic manipulations and the functional conservation between genes from human and fly.

There are two classes of high throughput screens that fall under the forward genetic approach. One is unbiased blind

screening, in which a sizable cohort of fruit fly mutants is tested under specific pathogenic-related paradigm. Follow-up genetic analysis is subsequently performed on the mutants that exhibit clinically relevant manifestations to determine the genes and pathways that are involved in the phenotypes. Novel, and even unexpected, genes and signaling cascades may be identified in such screens. An alternative approach is to screen for genetic or pharmacological modifiers of a particular disease gene based on the aforementioned reverse genetic disease model. Genes or drugs that exacerbate or ameliorate the disease phenotypes can be discovered in such screens, and can be further studied as novel therapeutic targets.

Many fly cognitive disorder models have been established via the above-mentioned research strategy. The fly models of cognitive disorders with memory abnormality have been summarized in Table 12.2. As the memory abnormality component for these models in *Drosophila* is the focus of our discussion, learning and memory paradigms used in such studies are briefly introduced in Box 12.1. These models have profoundly improved our understanding of the mechanisms of both clinically devastating diseases and the basic mechanisms of learning and memory. Next, we will elaborate the lessons learnt about the properties and functions of disease-related proteins, potential therapeutic targets and candidate treatment, as well as the molecular underpinnings of learning and memory.

Box 12.1. Paradigms for the study of learning and memory in *Drosophila* disease models

The classic *Drosophila* olfactory associative conditioning paradigm

In the training phase of this paradigm, two odors are presented to the flies sequentially. One of the odors is coupled with an electric shock (aversive stimulus) or sugar (reward), while the other is not accompanied with the reinforcing stimulus. During the test phase, flies are allowed to choose between the two odors that are presented simultaneously. Flies can be trained to avoid or prefer the odor previously paired with shock or sugar. The memory performance can be tested at different time points after training to probe immediate, intermediate or long-term memory (Tully and Quinn, 1985; Tully et al., 1994; Davis, 2005).

Courtship conditioning

Courting male fruit flies will perform a characteristic ritual to the females. Virgin females are receptive to these behaviors of the male. However, the mated females will reject the advances of males. Experiences of unsuccessful mating suppress subsequent courtship activities of the rejected male, so that the rejected male will not even court other virgin females for a period. An associative learning occurs in this process by coupling the failure courtship experience with the aversive pheromones emitted by the mated female (Siegel and Hall, 1979; Hall, 1994).

Olfactory and visual associative learning in larvae

In the olfactory conditioning procedure, *Drosophila* larvae are trained to associate odorants with positive (fructose) or negative (quinine or soldium chloride) gustatory reinforcers. In the visual conditioning procedure, larvae are trained to associate light or dark with positive (fructose) or negative (quinine or soldium chloride) gustatory reinforcers. During testing, individual larva are allowed to choose between odors or lights that previously were paired with reinforcers (Scherer et al., 2003; Gerber et al., 2004).

Aversive phototaxis suppression (APS) assay

Flies in the training session of APS learn to associate light with aversitve stimuli such as quinine and humidity. The test session is carried out subsequently in a T-maze, in which flies will avoid light (Seugnet et al., 2009).

Revealing the functions of disease genes in evolutionarily conserved memory phenotypes

The cognitive diseases discussed here exhibit a spectrum of clinical manifestations, such as neurodegeneration, developmental defects, emotional aberrations, etc. But all share overlapping dysfunction in learning and memory capacity, and for each case there are established *Drosophila* models that emulate these abnormalities. These diseases include Alzheimer's disease (AD), tauopathy, fragile X syndrome (FXS), Down syndrome (DD), schizophrenia (SZ), Neurofibromatosis type I (NF1), and Noonan syndrome (NS). The major clinical features and their specific memory phenotypes of these cognitive diseases are summarized in Table 12.1, and the related disease genes and corresponding fly models are summarized in Table 12.2.

Alzheimer's disease

Alzheimer's disease (AD) is the most common dementia. Although familial AD (FAD) constitutes less than 5% of the total cases, it exhibits identical clinical manifestations with the sporadic cases, suggesting that common pathogenic mechanisms are shared in different forms of AD. Mutations in β-amyloid precursor protein (APP), presenilins 1 and 2 and tau account for the FAD, and are respectively relevant to the main cellular hallmarks of AD including the extra-neuronal senile plaques, composed of Aβ peptides, and intra-neuronal neurofibrillary tangles (NFT), composed of hyper-phosphorylated microtubule-associated tau protein (MAPtau) (Goedert and Spillantini, 2006).

Aβ peptides are produced by sequential proteolytic cleavage of the APP at the β and γ sites with the β-site APP-cleaving enzyme (BACE) and the γ-secretase presenilins (PS1 and PS2), respectively. The cleavage of APP results in both Aβ40 and Aβ42. Aβ42 is more liable to form oligomers and fibrils than Aβ40, and is the predominant form of amyloid peptide found in senile plaques. What's more, the mutations of APP, PS1 and PS2 in FAD promote the production, aggregation, and stability against clearance of Aβ42. Thus, the Aβ hypothesis has been proposed, in which Aβ42 is considered as the culprit of AD pathogenesis (Hardy and Selkoe, 2002; Hardy, 2009).

Table 12.1. Summary of the clinical features and memory phenotypes of cognitive diseases with memory abnormality

Disease	Clinical features	Memory phenotypes
Alzheimer's disease	Fatally and irreversibly progressive memory loss and subsequent neurodegeneration	Early stage: Impairment in learning and immediate memory; Late stage: Long-term memory loss
Tauopathy	Subtype-dependent; parkinsonism, dementia, motor neuron disorder	Various degrees of frontotemporal dementia
Fragile X syndrome	Mental retardation and autistic behaviors including social withdrawal and repetitive behaviors	Severe working memory defect
Down syndrome	Mental retardation, learning and memory deficits, delayed physical growth, congenital heart disease, facial dismorphology, and early development of Alzheimer's disease	Deficits in prefrontal working memory, spatial associative memory, declarative and explicit memory
Schizophrenia	Positive symptoms including hallucination, delusion; negative symptoms including flat emotion, alogia, asociality, avolition; cognitive deficits including memory impairment, disorganized speech and thinking	Severe impairment in working memory and immediate and delayed recall in episodic memory
Neurofibromatosis type I	Multiple benign and malignant nervous system tumors, white matter lesions in the brain, and high incidence of complex cognitive symptoms	Both verbal and non-verbal learning disabilities
Noonan syndrome	Dysmorphic facial features and other developmental problems, including learning and memory abnormalities and mental retardation	Severe learning and memory disabilities

Based on the Aβ hypothesis, three kinds of *Drosophila* model were established. Progress in the studies of other pathological-related phenotypes, including neurodegeneration, premature death, and motor dysfunction have been extensively reviewed elsewhere (Iijima-Ando and Iijima, 2010; Moloney et al., 2010). Here, we will only discuss the fly models with learning and memory phenotypes. The first kind is fly models related to APP. The *Drosophila* homolog of APP is amyloid precursor protein-like (Appl) gene. Acutely knocking-down Appl or overexpressing human APP in mushroom bodies led to specific impairment in protein synthesis dependent long-term memory, but left the learning ability intact (Goguel et al., 2011). The evidence that Appl is required in long-term memory suggests that normal function of APP may contribute to the cognitive defect in AD pathology.

The second class includes fly models that directly overexpress Aβ peptides. Besides neurodegeneration and premature death, a study by Iijima et al., also found severe behavioral phenotypes resembling AD in Aβ transgenic flies, including locomotor disability and especially memory loss (Iijima et al., 2004). Notably, the memory loss induced by pan-neuronal overexpression of Aβ40/42 peptides was age-dependent and occurred much earlier than the neurodegeneration in the brain, which is similar to the observations in mouse models and AD patients (Terry et al., 1991; Hsiao et al., 1996; Chen et al., 2000; Selkoe, 2002). Later studies by the same group found that the Aβ-induced AD-like phenotypes including the severe age-dependent memory loss were related to the augmentation of phosphatidylinositol 3-kinase (PI3K) pathway, and inhibition of PI3 kinase per se or its upstream receptor EGFR suppressed the Aβ-induced manifestations (Chiang et al., 2010; Wang et al., 2012).

Other groups turned their attention to the *Drosophila* PS (dPS) gene. In dPS null mutants, synaptic transmission as well as the olfactory and visual associative learning in larvae was severely impaired (Knight et al., 2007). Besides supporting the evolutionarily conserved role of PS in synaptic transmission and learning, this study also indicates that the role of PS in these functions may be independent of Aβ, for dPS is not involved in Aβ peptide production in fly due to the lack of Aβ region in *Drosophila* Appl. Intriguingly, the endogenous expression level of dPS is essential to *Drosophila* cognitive functions. Even a 50% reduction in the dPS dosage resulted in age-dependent defective courtship learning and memory, which could be prevented by either pharmacological inhibition or genetic reduction of *Drosophila* metabotropic glutamate receptor (DmGluRA), the inositol trisphosphate receptor (InsP3R), or inositol polyphosphate 1-phosphatase (McBride et al., 2010). This study not only confirms the involvement of PS in AD pathology, but also suggests PS may exert its function through enhanced mGluR signaling and calcium release regulated by InsP3R.

Tauopathy

Pathological aggregation of the hyper-phosphorylated microtubule-associated tau protein (MAPtau) in neurons and/or glia is a characteristic feature of a group of neurodegenerative diseases collectively called tauopathies, including Alzheimer's disease, corticobasal degeneration, Pick's disease, progressive supranuclear palsy, and frontotemporal dementia (Lee et al., 2001). Various degrees of frontotemporal dementia are a dominant feature of tauopathy.

The *Drosophila* homolog of human tau, lacks the N-terminal repeats in the human isoforms (Heidary and Fortini, 2001). Therefore, most *Drosophila* models of tauopathy were established by overexpressing the wild-type or mutant forms of human tau protein. A spectrum of neurotoxicity and dysfunction was found with overexpression of wild-type or mutant forms of human tau. The pathologies in these models

Table 12.2. Fly models for cognitive disorders with memory abnormality

Disease	Related Genes	Encoded Protein	Protein Function	Fly Homolog	Fly Model	Sources
	APP	Amyloid precursor protein	Pre-synaptic protein	Appl (CG7727)	Knocking-down endogenous Appl: *UAS-Appl-42673RNAi, UAS-Appl-G3RNAi*; Overexpression of human APP: *UAS-hAPP*	(Goguel et al., 2011)
Alzheimer's Disease	APP	Aβ	Pre-synaptic protein	None	Overexpression of Aβ peptides: *UAS-Aβ42, UAS-Aβ40*	(Iijima et al., 2004; Chiang et al., 2010; Wang et al., 2012)
	PSN-1/2	Precenilin	Gamma-secretase activity	dPs (CG18803)	Null mutant: psn^{W6}, psn^{C4},psn^{B3}, psn^{I2}, psn^{K2}, psn^{S3}, psn^{C1}, $psn^{C2.1}$, psn^{EMS46}, $psn^{l(3)48015}$, $psn^{l(3)49314}$ Overexpression of *Drosophila* wild-type dPs: *UAS-psn*	(Knight et al., 2007; McBride et al., 2010)
Tauopathy	Tau	Tau	Microtubule stabilization	Tau (CG31057)	Overexpression of vertebrate and *Drosophila* tau: *UAS-bTau, UAS-dTau, UAS-hTau*^{WT}, *UAS-hTau*^{R406W}, *UAS-hTau*^{0N3R}, *UAS-hTau*$^{0N4R\text{-}V377M}$, *UAS-hTau*^{R406WS2A}, *UAS-hTau*^{2N4R}, *UAS-hTau*$^{2N4R\text{-}STA}$	(Morales et al., 2002; Selkoe, 2002; Mershin et al., 2004; Ali et al., 2012)
Fragile X Syndrome	FMR1	FMRP	Protein and RNA binding protein	dfmr1 (CG6203)	Null mutant: $dfmr1^{3}$, $dfmr1^{B55}$, Knocking-down of endogenous fmr1: *UAS-fmrRNAi(1–7), UAS-fmrRNAi(1–10), UAS-fmrRNAi(2–1),* Overexpression of *Drosophila* fmr1: UAS-fmr1,	(McBride et al., 2005; Bolduc et al., 2008; Bolduc et al., 2010a; Kanellopoulos et al., 2012)
Down Syndrome	Dyrk1A	Dyrk1A	Serine-threonine protein kinase activity	Minibrain (CG42273)	Hypomorph mutants: mnb^{1}, mnb^{2}, mnb^{3}, mnb^{4}	(Heisenberg et al., 1985; Tejedor et al., 1995)
	RCAN1	RCAN1/ Calcipressin1	Protein binding	Nebula/ Sarah (CG6072)	Hypomorph mutants: nla^{1}, nla^{2}; Transposon-excision mutant: nla^{PJ}; Overexpression of Drosophila nebula: nla^{t1}, nla^{t2}	(Chang et al., 2003)
Schizophrenia	DTNBP1	Dysbindin	Synaptic transmission and homeostasis	Ddysb (CG6856)	Hypomorph mutant: $dysb^{1}$; Overexpression of *Drosophila* and human dysbindin: *UAS-Ddysb, UAS-Hdysb, UAS-dysb, UAS-dysb-venus*; Knocking-down of endogenous dysbindin: *UAS-DdysbIR1/2*	(Dickman and Davis, 2009; Shao et al., 2011)
Neurofibromatosis Type 1 (NF1)	Neurofibromin1 (NF-1)	NF-1	Ras GTPase activator activity	Nf1 (CG8318)	Null mutants: $NF1^{P1}$ and $NF1^{P2}$; Hypomorph mutant: $NF1^{c00617}$; Heat-shock inducible transgenic fly: *hsNF1*; Overexpression of *Drosophila* wild-type NF1: *UAS-dNF1*; Overexpression of human wild-type and mutatant of NF1: *UAS-hNF1*, *UAS-hNF1*^{L847P}, *UAS-hNF1*^{R1276P}, *UAS-hNF1*^{K1423E}, *UAS-hNF1*^{GRD1}, *UAS-hNF1*^{GRD2}, *UAS-hNF1*$^{\Delta GRD2}$, *UAS-hNF1*^{Cterm}, *UAS-hNF1*^{Nterm}	(Guo et al., 1997; The et al., 1997; Guo et al., 2000; Tong et al., 2002; Hannan et al., 2006; Ho et al., 2007; Buchanan and Davis, 2010)
Noonan Syndrome	PTPN11	SHP2	Protein tyrosine phosphatase activity; protein binding	csw (CG3954)	Overexpression of wild-type or GOF mutants of *Drosophila* csw: *UAS-csw*^{WT}, *UAS-csw*^{D61Y}, *UAS-csw*^{A72S}, *UAS-csw*^{T73I}, *UAS-csw*^{E76K}, *UAS-csw*^{J282V}, *UAS-csw*^{N308D}; Spontaneous occurring hypomorph mutant: csw^{Jf}	(Oishi et al., 2006; Pagani et al., 2009)

include abnormal tau accumulation, premature death, cell-type selective neurodegeneration, defective synaptic transmission, axonal transport disruption, locomotor dysfunction, and cognitive defects. The molecular mechanisms underlying these functions are extensively discussed in other reviews (Iijima-Ando and Iijima, 2010; Gistelinck et al., 2012).

The fly models of tauopathy also cause learning and memory defects. Pan-neuronal overexpression of vertebrate and *Drosophila* tau results in severe defects in olfactory associative learning and selective aberration in mushroom body integrity (Mershin et al., 2004; Kosmidis et al., 2010). Notably, the severity of memory defect and brain structure disruption were related to the properties of mutations in tau protein, consistent with the notion that abnormal phosphorylation is responsible for the cell-type specific toxicity and dysfunction (Mershin et al., 2004; Steinhilb et al., 2007; Kosmidis et al., 2010; Papanikolopoulou et al., 2010). The overexpression of human tau induced similar deficits in APS conditioning as in the olfactory conditioning (Seugnet et al., 2009; Ali et al., 2012; Box 12.1).

Fragile X syndrome

Fragile X syndrome (FXS) is a common heritable mental retardation featured with cognitive impairment and autistic behaviors, including social withdrawal and repetitive behaviors. A trinucleotide (CGG) repeat expansion in the 5′ untranslated region of the FMR1 gene, leading to no or little functional expression of the FMRP protein due to the hypermethylation of the FMR1 gene, is responsible for almost all of the cases of fragile X syndrome (Hagerman and Hagerman, 2002). The *Drosophila* homolog dfmr1 is highly conserved with human FMR1 (Wan et al., 2000), which prompted the development of fly model of the FXS based on loss-of-function of the dfmr1.

These fly models successfully duplicated key pathological features of FXS by exhibiting abnormalities in neuronal morphology (Morales et al., 2002), synaptic structure and function (Zhang et al., 2001; McBride et al., 2005), courtship behavior (Dockendorff et al., 2002; McBride et al., 2005), circadian rhythm (Dockendorff et al., 2002; Morales et al., 2002), social interaction (Bolduc et al., 2010b), as well as learning and memory. Much has also been learned from these models about the underlying molecular and cellular bases of these behaviors (for intensive reviews, see McBride et al., 2012; Tessier and Broadie, 2012). The fly FMR1 gene is an excellent example.

Immediate recall and short-term memory in the courtship conditioning procedure were found to be defective due to developmental defects in mushroom body with loss-of-function of dfmr1 (McBride et al., 2005). In addition to the effects on immediate recall and short-term memory, dfmr1 and its interaction with argonaute1 and staufen are also required for long-term memory in the classic olfactory conditioning procedure. Interestingly, this long-term memory defect was found to involve excessive baseline protein synthesis in the dfmr1 mutant, and thus could be rescued by the protein synthesis inhibitors (Bolduc et al., 2008). This is consistent with the known function of FMRP in RNA trafficking, metabolism, and suppression of unregulated synaptic translation (Antar and Bassell, 2003). A further genetic interaction study demonstrated a synergistic effect on long-term memory of interactions between dFMR1 and cheerio, the *Drosophila* homolog of Filamin A responsible for periventricular nodular heterotopia. The heterozygous mutants of either dfmr1 or cheerio exhibit intact long-term memory, whereas the double heterozygous mutant animals exhibit disrupted long-term memory (Bolduc et al., 2010a). Consistent with the studies in mouse model, the learning and memory deficits induced by dfmr1 deficiency in *Drosophila* also involve metabotropic glutamate receptor (mGluR)-mediated inhibition of cAMP signaling pathway (Kanellopoulos et al., 2012).

Down syndrome

Down syndrome (DS), also known as Trisomy 21, is the most common chromosomal abnormality in human, caused by full or partial trisomy of chromosome 21 (Reeves et al., 2001). The overexpression or gain-of-function of the genes located at the "Down syndrome critical region (DSCR)" in human chromosome 21 is a likely culprit contributing to the pathogenesis of the disease.

Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (DYRK1A) is one of the 33 genes located at human DSCR and is closely associated with DS manifestations (Park et al., 2009). The first study on Dyrk1A was performed on its *Drosophila* homolog minibrain (mnb). Dyrk1A was found to be involved in the regulation of neural development through its serine–threonine protein kinase activity (Tejedor et al., 1995). Furthermore, the mnb^{1} mutant also showed several abnormal behaviors, including effects on walking speed, olfactory learning, visual pattern fixation, and negative geotaxis (Heisenberg et al., 1985; Tejedor et al., 1995).

The expression level of regulators of calcineurin 1 (RCAN1, also known as DSCR1), lying inside the DSCR, is increased approximately two-fold in brains of DS patients (Fuentes et al., 2000; Ermak et al., 2001). The protein encoded by RCAN1, known as calcipressin 1, belongs to a protein family called calcipressins that are named, based on their function as inhibitors of calcineurin (Fuentes et al., 2000). Calcineurin plays critical roles in the synaptic plasticity as well as learning and memory (Mansuy, 2003; Baumgartel and Mansuy, 2012). However, whether RCAN1 is also involved in learning and memory and whether the mental retardation in DS is related to RCAN1 overexpression remain unknown. The *Drosophila* homolog of RCAN1, nebula, is significantly up-regulated in the brain during development (Strippoli et al., 2000). Chang et al. found that both overexpression and down-regulation of nebula resulted in severe learning and memory deficits. Further study suggested that such learning impairment was not caused by developmental defects, and was instead attributed to biochemical disturbances in the activities of calcineurin and PKA, the phosphorylation of CREB, and the transcription level of d-jun. What's more, similar biochemical perturbations were also found in the tissue of DS patients, indicating that the mental retardation in DS may involve overexpression of RCAN1 (Chang et al., 2003). Subsequent studies further uncovered the fact that nebula is indispensable for the maintenance of normal mitochondria function (Chang and Min, 2005) and sleep homeostasis (Nakai et al., 2011) in *Drosophila*. Recently, nebula and other two genes located on human chromosome 21 and overexpressed in DS, intersectin (dap160 in fly) and synaptojanin (snyj in fly), were found to act synergistically in the regulation of synaptic morphology and endocytosis in *Drosophila* (Chang and Min, 2009), which gives an excellent example of the power of systematical overexpression of multiple DS related genes in the fly model.

Schizophrenia

Schizophrenia is a debilitating and severe mental disorder characterized by hallucination, delusion, and cognitive deficits. The reduction of memory capacity, occurring prior to other symptoms, is a core component of the cognitive deficits in schizophrenia (Tandon et al., 2009). As genetic factors are

strongly associated with the etiology of schizophrenia, a long list of susceptibility genes has emerged through linkage and association studies (Owen et al., 2005).

Dysbindin (also known as *dystrobrevin binding protein 1*, *DTNBP1*), one of the most promising susceptibility genes, is found to associate with schizophrenia in a series of genome-wide association studies (Guo et al., 2009; Talbot et al., 2009). The protein and mRNA levels of dysbindin are found dramatically decreased in brain regions involved in schizophrenia pathology (Talbot et al., 2004; Tang et al., 2009). In studies of the sandy mouse, a null mutant of dysbindin (Li et al., 2003), mounting evidence suggests that dysbindin is required in glutamatergic transmission and dopamine receptor trafficking. *dysbindin* mutants also cause pathological behavioral anomolies, including abnormal activity, social withdrawal, and memory deficits (Cox et al., 2009; Talbot, 2009).

However, it is unclear how dysbindin regulates multiple neurotransmitter systems simultaneously, and how the pathophysiological phenotypes resulting from dysbindin down-regulation relate to the behavioral manifestations. Shao *et al.* studied the function of the *Drosophila* dysbindin homolog (Ddysb), and found that down-regulation of Ddysb protein level in glutamatergic neurons resulted in glutamatergic transmission deficits and subsequent learning and memory impairment (Shao et al., 2011). The learning defect was induced by knocking-down dysbindin specifically in the glutamatergic neurons and was restored by glutamate receptor agonist (Shao et al., 2011). This provided direct evidence for the notion that the learning and memory aberration associated with dysbindin deficiency is due to abnormal glutamatergic transmission. The mechanism underlying the function of dysbindin in neurotransmission and learning may be attributable to its regulation of synaptic homeostasis in *Drosophila* (Dickman and Davis, 2009). At the same time, decreased dysbindin protein levels in glial cells led to disrupted dopamine metabolism and hyperactivity as well as mating abnormalities. These studies in the fly model provide new insight into the pathological effects of dysbindin disruption (Shao et al., 2011).

Revealing the underlying mechanisms of learning and memory

Besides revealing the functions of disease genes *per se*, studies with *Drosophila* disease models also contribute to the understanding of mechanisms of learning and memory.

NF1 and genetic dissection of biochemical pathways of learning and memory

Neurofibromatosis type I (NF1) is a dominant single-gene neurocutaneous disorder featuring multiple benign and malignant nervous system tumors, white matter lesions in the brain, and high incidence of complex cognitive symptoms. Up to 65% of individuals diagnosed with NF1 exhibit learning disabilities (Rosser and Packer, 2003). Mutations in the human NF1 gene, encoding neurofibromin, are the principal culprits for inception of the disease. The neurofibromin protein, containing a central GAP-related domain (GRD), was initially identified as a guanosine triphosphatase (GTPase)-activating protein for Ras (Ras-Gap), which acts to inhibit the intrinsic activation of Ras (Ballester et al., 1990). Although deficiency in NF1 had been associated with learning and memory deficits in human (North et al., 1994; Ferner et al., 1996) and mice (Silva et al., 1997), the underlying mechanism was largely unknown.

Great advances in our understanding of the involvement of NF1 in learning and memory, especially the functions other than its Ras-Gap activity, originate mainly from the studies of NF1 in the *Drosophila* model. *Drosophila* NF1 shares 60% amino acids sequence identity with the human homolog, and is highly conserved in the GRD region (The et al., 1997). The body and wing sizes of homologous *Drosophila* NF1 mutants were smaller than that of wild-type flies, but counterintuitively, instead of being ameliorated by manipulating the cell growth-related Ras signaling pathway, the reduced body size was restored by expressing a constitutively active PKA transgene (The et al., 1997). A back-to-back study found for the first time that NF1 was required in the cellular response to pituitary adenylyl cyclase-activation polypeptide (PACAP38) by the regulation of rutabaga-encoded adenylyl cyclase (AC). The Ras-Gap property, however, was indispensable in this process (Guo et al., 1997). Data from both of these studies indicated that NF1 might possess functions other than Ras-Gap.

Shortly after the initial connection of NF1 to cAMP pathway (Guo et al., 1997; The et al., 1997), Guo et al. found that the G protein-stimulated AC activity consists of NF1-dependent and NF1-independent components, and that NF1 was involved in learning and short-term memory via the rutabaga-AC/cAMP pathway independent of its role in development (Guo et al., 2000). A later biochemical study further trisected AC pathway into the classical $G\alpha_s$-dependent AC pathway stimulated by Phe-Met-Arg-Phe-amide (FMRFamide) and dopamine, the NF1- and $G\alpha_s$-dependent AC pathway stimulated by serotonin, histamine and PACAP (see also Guo et al., 1997), and the EGFR, NF1, and Ras/MAPK involved AC pathway stimulated by growth factors (Hannan et al., 2006). Moreover, the authors found that the C-terminal region of NF1 was closely related to its function in the second AC pathway (Hannan et al., 2006). The regulation of G protein-activated AC and cAMP pathway by NF1 was subsequently confirmed in mammals by the observations that cAMP-related defects in NF1 mutant fly was rescued by human NF1 transgene as well as AC and cAMP activities were compromised in NF1 knockout mice (Tong et al., 2002; Dasgupta et al., 2003; Brown et al., 2010).

In concert with the functional division of NF1 in Ras/MAPK and cAMP signaling cascades attributable to the GRD and C-terminal domains, the effects of NF1 on protein synthesis, independent short-term memory, and protein synthesis dependent LTM were also bifurcated. Ho et al., found that the C-terminal domain of NF1, which is required for cAMP signaling, was critical for immediate memory. In contrast, the GRD region of NF1,

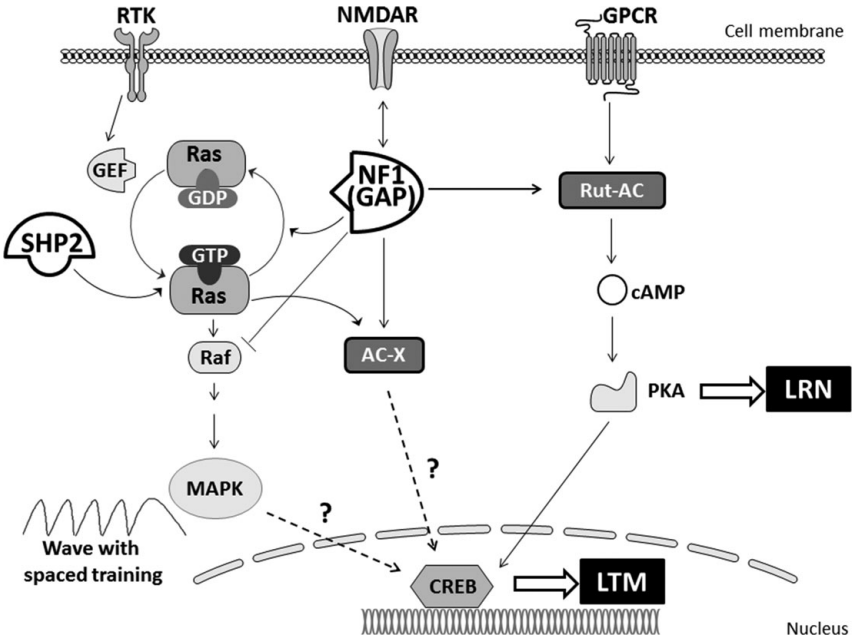

Fig. 12.2. Illustration of the signaling pathways involved in NF1 and Noonan syndrome.

which signals via the Ras/MAPK pathway, was necessary and sufficient for the formation of LTM (Ho et al., 2007). In addition, this functional dissection of the domains of NF1 for different signaling pathways and memory phases is important in that it settled a discrepancy between the studies of mouse and fly. As is discussed above, in fly cAMP pathway was associated with defects of learning or immediate memory led by NF1 deficiency (Guo et al., 2000), whereas in mouse Ras/MAPK pathway was considered accountable for the defects of spacial learning in the Morris water maze paradigm (Costa et al., 2001; Costa et al., 2002; Li et al., 2005). Authors in this study reasoned that the spatial learning derived from training in the Morris water maze is a protein synthesis-dependent memory that required the NF1-mediated Ras pathway and thus is, in essence, consistent with the observations in fly (Ho et al., 2007).

In contrast to rut-AC, which functions in both the acquisition and stabilization phases of memory, NF1 was found to be required specifically in memory acquisition. In addition, its function in memory acquisition was refined to a subset of mushroom body neurons (Buchanan and Davis, 2010). Recently, a new receptor tyrosine kinase (RTK) Alk was identified as an upstream regulator of NF1 in both growth and learning in *Drosophila*. However, the learning defect of NF1 mutants could only be rescued outside mushroom body neurons (Gouzi et al., 2011). The contradiction may due to the fact that the Gal4 line (c739-Gal4) used in the first study included neurons extrinsic to mushroom body, and to the fact that the Gal80 line (MB-Gal80) used in the second study failed to label all the neurons in the mushroom body. Therefore, further investigation is necessary to settle this discrepancy. Collectively, NF1 acts downstream of GPCR and RTKs in mediating immediate and long-lasting memories, respectively, via the regulation of cAMP and Ras pathways by different functional domains (Fig. 12.2). Research on *Drosophila* NF1 model shed light, not only on the mechanism of learning and memory symptoms in NF1 disorder, but also on the delineation of signaling cascades involved in distinct memory phases.

Noonan syndrome and spacing effect

The sustainability of memory highly depends on the training patterns. Spaced training, in which repetitive training trials are intervened by resting intervals, produces a more stable and longer lasting form of memory than does the massed training, where equal number of training trials is consecutively repeated without rest intervals. This so-called "spacing effect" on memory formation, is highly conserved across species ranging from aplysia, fruit fly, to human (Ebbinghaus, 1885; Carew et al., 1972; Tully et al., 1994; Cepeda et al., 2006; Philips et al., 2007). The spacing effect has attracted considerable experimental and theoretical attention because of its close relevance to psychology, education, therapy, and marketing (for review, see Naqib et al., 2012). Despite these endeavors, however, several critical questions about the spacing effect remain to be unraveled. What are the molecular and physiological underpinnings of the rest requirement? What are the mechanistic effects of repeated training? How does the brain time and count the rests and repetitions? Work in the *Drosophila* model of the Noonan syndrome has advanced our understanding of these questions.

Noonan syndrome (NS) is an autosomal dominant congenital disorder characterized by dysmorphic facial features and other developmental problems, including learning and memory abnormalities and mental retardation (Noonan, 1968; Tartaglia and Gelb, 2005). Pathogenically, over 60% of NS cases are attributable to mutations in the genes of Ras/MAPK signaling pathway, including *PTPN11*, *SOS1*, *RAF1*, *BRAF*, and *KRAS*

(Gelb and Tartaglia, 2006). The gain-of-function (GOF) mutations in *PTPN11*, encoding the non-receptor protein tyrosine phosphatase SHP-2, account for approximately 50% of clinically diagnosed NS patients (Tartaglia et al., 2001; Tartaglia et al., 2003). The positions of amino acids mutated in clinical NS cases are highly conserved between human *PTPN11* and the *Drosophila* ortholog *corkscrew* (*csw*).

Pagani and colleagues started their study from the inquiry of the learning and memory phenotypes of flies overexpressing various *csw* transgenes harboring clinically relevant GOF mutations (Pagani et al., 2009). They found that overexpression of the GOF *csw* transgene in the mushroom body specifically impaired the protein synthesis-dependent long-term memory (LTM) produced by spaced training. Although *csw* is essential to development, its role in LTM formation involves an acute physiological effect after development because similar LTM impairment resulted from swiftly inducing expression of the GOF *csw* transgene during adulthood. Interestingly, rather than enhance the LTM *per se*, overexpression of wild-type (WT) *csw* shortened the inter-trial intervals in spaced training, thereby eliciting LTM with massed training rather than with spaced training. This phenomenon prompted the authors to hypothesize that *csw* regulates the duration of resting period needed to produce LTM and provided a means to investigate the underlying mechanism. As *PTPN11* is an integral component of the Ras/MAPK signaling cascade, and the activity of MAPK was reported to be involved in the regulation of dendritic morphology (Wu et al., 2001), long-term plasticity of synaptic transmission (Michael et al., 1998), as well as memory formation (Kelleher et al., 2004; Mayford, 2007; Philips et al., 2007), the authors assayed the activity of MAPK at different time points during or after spaced training. They found that, during spaced training, a transient MAPK activity was induced shortly after a training trial, and was reset immediately by the training of the next trial, which forms a wave of MAPK activity correlated with each trial during spaced training. In the massed training procedure, however, the resting period is too short to induce an increment of MAPK activity until the end of the last trial. Moreover, the alteration of the MAPK activity wave also ideally explained the LTM phenotypes resulting from overexpression of WT and GOF *csw*. The overexpression of WT *csw* reduced the latency of the rise of MAPK activity and thus shortened the optimal rest interval between trials, whereas the overexpression of GOF *csw* resulted in high and prolonged MAPK activity, but was devoid of the reset of MAPK activity by the following trial, and thus cannot form sufficient MAPK activity waves during a routine spaced training. In concert with the above evidence, both pharmacologically inhibiting SHP-2 phosphatase activity and prolonging the inter-trial intervals rescued the LTM deficits in flies overexpressing GOF csw (Pagani et al., 2009).

The intriguing MAPK activity wave hypothesis (as illustrated in Fig. 12.2) proposed in this study shed light on the mechanism of the spacing effect and LTM formation in several ways. CREB activity has been identified as the switch to form LTM from spaced training nearly two decades ago, and a number of critical proteins were found to act upstream of CREB in response to spaced training. These include eukaryotic translation initiation factor 2 (EIF2α) (Bartsch et al., 1995; Chen et al., 2003) and protein phosphatase I (PP1) (Hagiwara et al., 1992; Genoux et al., 2002). However, the question of how the timing of spaced training is determined remained elusive. This study provided insight into the mechanism of timing in underlying the spacing effect by identification of MAPK activity waves as defining the inter-trial interval in spaced training (Pagani et al., 2009). Despite this advance, the upstream modulators of the MAPK activity waves and the molecular machinery of "counting" the number of MAPK activity waves still need to be elucidated. Pursuing the connection between Ras/MAPK and cAMP/CREB cascades in learning and memory, a recent study found that simultaneous activation of both MAPK and PKA increased the induction of CREB as well as long-term synaptic facilitation and LTM (Zhang et al., 2012). Therefore, it will also be of interest to see whether MAPK and CREB function in series or in parallel during the spacing effect.

Fragile X syndrome and translational control of LTM

The formation of LTM is dependent on new protein synthesis. Although most efforts to reveal the mechanism of gene expression in the context of LTM formation focus on transcription, especially transcription factors like CREB, accumulating evidence suggest that translational control is another critical step in the regulation of LTM formation (for review, see Costa-Mattioli et al., 2009).

RNA-binding proteins (RBPs) contribute to the translational control underlying LTM formation by sequestering mRNAs and repressing translation during their transport to dendrites, while releasing mRNAs and reactivating translation when they arrive at the right locations. Thus, the regulation of mRNA translation by RBPs might act as a rate-limiting step in the translational control of LTM. Research in *Drosophila* has found that RBPs including staufen and GLD-2 are specifically involved in the regulation of LTM formation (Dubnau et al., 2003; Kwak et al., 2008) and provide circumstantial evidence of the notion that local translational activation is essential to memory formation and consolidation. The direct evidence came from a study on the *Drosophila* model of FXS.

Lack of FMRP has been reported to lead to an increase in general protein synthesis (Qin et al., 2005). In this study excessive protein synthesis induced by the deficiency of the RBP FMRP was demonstrated to cause the LTM impairment in the dfmr1 null and knockdown mutant (Bolduc et al., 2008). Acute induction of dfmr1 RNAi in the mushroom body before training specifically impaired 1 day memory after spaced training, but had no effect on 1 day memory after massed training. It is noteworthy that three independent protein synthesis inhibitors at moderate concentration successfully rescued the memory defect in the dfmr1 mutant (Bolduc et al., 2008). Notably, this study in the fly model of FXS was echoed in a recent report that direct disruption of the translational machinery in

the fmr1/S6K1 double knockout mice rescued the abnormalities in dendritic morphology, synaptic plasticity, and cognitive function present in fmr1 knockout mice (Bhattacharya et al., 2012).

At first glance, it is counterintuitive that a process requiring protein synthesis such as LTM is compromised by increases in overall protein synthesis. According to the evidence from above studies, however, it is likely that the protein synthesis required by LTM formation is not indiscriminate, but takes place with specific timing such as after spaced-training and specific subcellular locations such as particular dendrites. This conjecture is consistent with the fact that the morphological difference between stubby/mushroom- and filopodia-shaped dendritic spines became blurred in fmr1 knockout mice, but was restored in fmr1/S6K1 double knockout mice (Bhattacharya et al., 2012), suggesting indiscriminate general protein synthesis resulted in the loss of identity of synapses related to memory formation.

Implications for development of drug targets

Accessibility to medium- or high-throughput genetic or pharmacological screens is the predominant advantage of *Drosophila* for translational research. Two complementary approaches have been adopted in screens for therapeutic compounds: hypothesis-driven tests of candidate compounds and unbiased screens of random chemical libraries for novel drug candidates. In the former approach, candidate compounds from particular disease-related pathways are tested in disease models for their effects on the pathogenic- or clinically relevant phenotypes. This approach is theory-oriented and therefore is fairly efficient. However, drugs from pathways not implicated from previous studies will be missed. To compensate for the shortcomings of this approach, the alternative approach is employed to generate a more comprehensive list of drug candidates. What's more, completely novel and even unexpected disease relevant pathways can be discovered to underlie the diseases initiation or progression in such screens, which in turn deepens and extends our understanding of the disease pathogenesis. The prerequisite for a feasible pharmacological screen, however, is the availability of easy, clear, and reliable readouts, such as synaptic transmission at the NMJ, neurodegeneration of the compound eye, premature lethality, locomotor dysfunction, as well as learning and memory. The strategies and examples of using this approach for *Drosophila* disease models have been nicely reviewed (Shulman et al., 2003; Giacomotto and Segalat, 2010; Newman et al., 2011), so only a few successful translational studies in *Drosophila* will be discussed in this section.

The most prevalent targets in hypothesis-based pharmacological screens are either genes underlying neurotoxicity and inducing the neural dysfunction, or genetic factors involved in the metabolism, aggregation, and clearance of the disease characteristic proteins, although dysbindin mRNA and protein levels were found to be significantly decreased in hippocampus and prefrontal cortex of patients with schizophrenia (Talbot et al., 2004; Tang et al., 2009), and dysbindin risk variants in both patients with schizophrenia and normal population is associated with cognitive decline and memory loss (Hashimoto et al., 2009a; Hashimoto et al., 2009b; Luciano et al., 2009; Markov et al., 2009; Wolf et al., 2009), the relation between dysbindin deficiency and cognitive defects. Shao et al., found in their fly model that the learning performance of the Ddysb mutant flies was fully restored by glycine, an NMDA receptor agonist, and subsequent genetic rescue was obtained by expressing fly Ddysb specifically in glutamatergic neurons. This further strengthened the hypothesis that dysbindin regulates cognitive functions by the modulation of glutamatergic transmission (Shao et al., 2011).

As mentioned above, the *Drosophila* model of FXS based on loss-of-function mutants of dfmr1 exhibited abnormal courtship behavior, defective immediate recall of short-term courtship memory and developmental defects of mushroom body neurons (McBride et al., 2005). It has been hypothesized that the misregulation of mGluR accounts for many clinically relevant phenotypes in the fmr1 knockout mice (Huber et al., 2002; for review, see Bear et al., 2004). Thus, the authors of the fly study went on to test this hypothesis and to validate the fly model of FXS by feeding flies with four independent mGluR antagonists or with lithium. They found that these agents restored all the above-mentioned phenotypes in dfmr1 mutant flies, and interestingly, the restoration of naive courtship behavior and the cognitive capacity was independent of the rescue of mushroom body dismorphology (McBride et al., 2005), suggesting that mGluR may be a potential target for the treatment of cognitive symptoms in FXS.

Markers of oxidative injury were found in the brain tissues of postmortem patients of age-related neurodegeneration including AD and oxidative stress is implicated as a mediator of Aβ induced neurotoxicity (Andersen, 2004; Zhu et al., 2004). Pharmacological manipulation of the antioxidant pathways, such as feeding the flies with antioxidant vitamin E (Dias-Santagata et al., 2007) and iron-binding agent clioquinol (Rival et al., 2009) was found to suppress the tau- and Aβ-induced neurotoxicity respectively in fly, indicating that oxidative stress may be a general pathogenic mechanism in neurodegenerative diseases. Although AD pathology is characterized by extracellular amyloid aggregations, it remains elusive whether the extracellular senile plaques are the cause or the result of AD. Crowther et al., found that the intracellular non-amyloid Aβ alone may result in AD-related phenotypes ranging from non-amyloid aggregates, brain vacuolus, locomotor dysfunction, and premature death. Interestingly, these phenotypes were rescued by feeding flies with Congo Red, which could reduce Aβ aggregation *in vitro* (Crowther et al., 2005). A similar study on tauopathy found that rapamycin alleviated tau-induced toxicity by reduction of insoluble tau (Berger et al., 2006). These studies suggest the possibility to treat these neurodegenerative diseases by interfering with the abnormal aggregation and deposition of corresponding hallmark

proteins. As is discussed in previous sections, it is presumed that tau-induced neurotoxicity is correlated with its phosphorylation. GSK-3β inhibitors, lithium (Mudher et al., 2004; Cowan et al., 2010), and AR-A014418 (Mudher et al., 2004) blocked tau phosphorylation and thus significantly restored the pathology-related phenotypes, including cytoskeletal integrity, axonal transport, and locomotor activity, demonstrating that GSK-3β is a potential target for the treatment of tauopathies.

The above provide examples of hypothesis-driven drug testing. A good example of a high-throughput screen of random chemical libraries is the discovery of the involvement of EGFR in the AD pathogenesis. Wang et al., screened from 2000 kinase modulators with the olfactory learning paradigm in Aβ42 transgenic flies. Forty-five of the total 2000 compounds could effectively rescue the Aβ-induced memory loss in flies, and the therapeutic effects of four out of nine effective modulators were further confirmed in APP/PS1 double transgenic mouse model. Surprisingly, three of the four compounds significantly suppressed the Aβ42-induced EGFR phosphorylation. The discovery was further validated with the hypothesis-driven approach in the same paper, in which the authors found that clinical EGFR inhibitors effectively restored the memory capacity of both the fly and mouse AD models (Wang et al., 2012). This study highlighted a novel potential target for the treatment of AD. Another example comes from a medium-throughput screen based on an automated locomotor paradigm carried out by Mahoney et al., The authors developed several AD models that rely on overexpressing the derivatives and mutations of human APP and human tau, and found histone deacetylase (HDAC) inhibitors, gamma-secretase inhibitors, aggregation inhibitors, and cholinesterase inhibitors were effective in restoring the locomotor behaviors of their fly models (Mahoney et al., 2010). Although no novel targets were discovered in this screen, these agents were independently validated in mouse models and *in vitro* cell-culture AD studies.

Future directions

Numerous genes are discovered to be associative to diseases every year in human genetic studies. Closely following this literature should provide abundant resources for model system research in *Drosophila*. However, most of the cognitive disorders and their endophenotypes, such as abnormalities in learning and memory, are likely to result from altered genetic interactions rather than from mutations in single genes (Hsu et al., 2008; Raj et al., 2012). Therefore, knockdown of one gene at a time is not conceptually sufficient to provide an understanding of the genetic bases of neurological disease. *Drosophila* is an ideal model with inherent advantages for studying the synergistic effects of multiple genes simultaneously (van Swinderen and Greenspan, 2005). Classic epistasis experiments in *Drosophila* (Yamamoto et al., 2009) can highlight functional gene networks involved in learning and memory and ultimately the evolution of disease.

Besides genetic complexity, another characteristic of cognitive disorders is that their inception is usually attributable to both genetic and environmental risk factors. The cellular effectors that respond to various environmental stresses such as nuclear factor-κB, p38 MAPK, stress-activated protein kinases (SAPK)/Jun amino-terminal kinases (JNK) pathways and heat-shock proteins are highly conserved between human and *Drosophila* (Minowada and Welch, 1995). *Drosophila* is highly sensitive to environmental changes and stresses such as starvation, heat, and drugs that induce oxidative stress. And flies adapt swiftly both in terms of physiological and behavioral responses compared with mammals. Thus it will be more tractable to study the gene–environment interactions that impact cognitive disorders in *Drosophila*. Such studies already have contributed and will continue to contribute to understanding mechanisms that explain "nature and nurture" interactions underlying learning and memory.

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Chapter

13

Age-related memory impairment in *Drosophila*

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Introduction

Age-related memory impairment (AMI) is a debilitating phenotype of brain aging. AMI has been generally considered to be an overall and non-specific decay of memory processes that results from dysfunction of neural networks. However, behavioral genetic study with *Drosophila* has demonstrated that AMI of olfactory aversive memory after single cycle training results from the decay of one memory component, middle-term memory (MTM), and not from other components. Significantly, suppression of cAMP/PKA signaling activity in the mushroom bodies (MBs) delays AMI. Involvement of cAMP/PKA signaling pathway is also shown in AMI of prefrontal cortex-dependent working memory in mammals. Although many advances have been made in the study of pathways involved in aging, much remains to be elucidated on how these pathways affect memory formation to cause AMI. Due to its short lifespan, powerful genetics, and well-characterized and conserved pathways involved in memory and lifespan, *Drosophila* will be a useful model system for studying the molecular mechanisms underlying AMI. In this review we overview how increased cAMP/PKA signaling causes AMI.

Although many research groups have reported anatomical and physiological changes associated with AMI (Foster, 1999, Shimada, 1999), neither its underlying molecular mechanisms nor its genetic relationship with aging are clearly understood. A major obstacle in performing genetic and behavioral analyses of AMI has been the long lifespan of animal models. That is, while recent genomic analyses identify a number of genes with altered expression upon aging, it is difficult to carry out behavioral genetics to test whether mutations in these genes affect AMI in mammalian system. *Drosophila* has numerous advantages in studying AMI, including a relatively short lifespan (30 to 40 days of age after eclosion), powerful genetics, and a quantifiable and well-characterized assay for memory. Hence, *Drosophila* offers a unique opportunity for understanding the molecular mechanisms of AMI.

AMI in *Drosophila*

Using a courtship conditioning paradigm, Savvateeva et al. showed a decay in memory upon aging for mutants in the kynurenine pathway (Sauvage et al., 2000, Savvateeva et al., 1999). However, they did not find significant memory impairment in aged wild-type flies. In contrast, AMI in wild-type flies can clearly be observed using an aversive Pavlovian olfactory conditioning paradigm.

When memory is observed in 10-day old flies, a mild but significant reduction is seen in performance measured 3 min after a single olfactory aversive conditioning trial (3-min memory is operationally defined as learning, LRN). However, further reduction in LRN does not occur upon further aging. In contrast, 20-day-old flies show drastic reduction in memory measured 1 h after training and this reduction becomes more dramatic at later age (Fig. 13.1A). Memory retention of these aged flies is reminiscent of memory retention of *amnesiac* (*amn*) mutants, which are defective for middle-term memory (MTM). That is, similar to *amn* flies, old flies display remarkable reduction in memory at 1 h after a single cycle training session, while initial learning and memory at 7 h after training are not much different from young flies. Furthermore, *amn* flies do not show further reduction in memory upon aging, while mutants that disrupt other memory phases such as acquisition and short-term memory (STM) show significant decay in memory upon aging (Fig. 13.1B). These occlusion results strongly suggest that AMI is a preferential disruption of *amn*-dependent MTM.

The *amn* gene encodes a neuropeptide precursor that is thought to be cleaved into three neuropeptides, two of which have homologies to mammalian pituitary adenylyl cyclase activating peptide (PACAP) and growth hormone releasing hormone (GHRH) (Feany & Quinn, 1995). *amn* gene products are preferentially expressed in dorsal-paired medial (DPM) neurons that project their terminals to the lobes of the MBs (Waddell et al., 2000).

AMI mutants and cAMP/PKA signaling

Since AMI consists of a decrease in *amn*-dependent MTM, it is possible that aging results in downregulation of *amn*. However, *amn* expression does not decrease upon aging and overexpression of an *amn* transgene does not suppress AMI (Tamura et al., 2003). Furthermore, there are no apparent morphological changes in DPM cells in aged flies (Tamura et al., 2003).

Behavioral Genetics of the Fly (Drosophila melanogaster), ed. J. Dubnau. Published by Cambridge University Press.

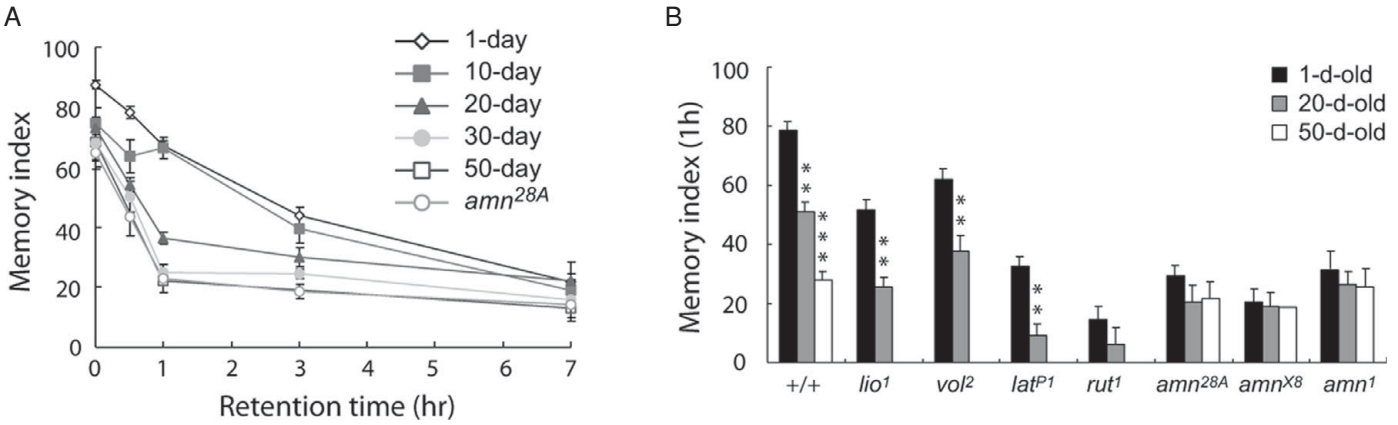

Fig. 13.1. AMI caused by preferential disruption in *amnesiac*-dependent MTM.
A Significant AMI is observed in 20-day and older flies as a severe impairment in memory one hour after training. Memory retention characteristics of aged flies are highly reminiscent of that of the middle-term memory mutant *amnesiac*. While there are minor differences in 0 h and 7 h memory, there is a prominent reduction in memory between these timepoints (especially in 1 h memory). B Age-related changes in 1 h memory in lio^1, vol^2, lat^{P1}, rut^1, amn^{28A}, amn^{x8} and amn^1 mutants. In contrast to other memory mutants, 1 h memory is not reduced upon aging in *amn* mutants, even at 50 days of age. Modified from (Tamura et al., 2003).

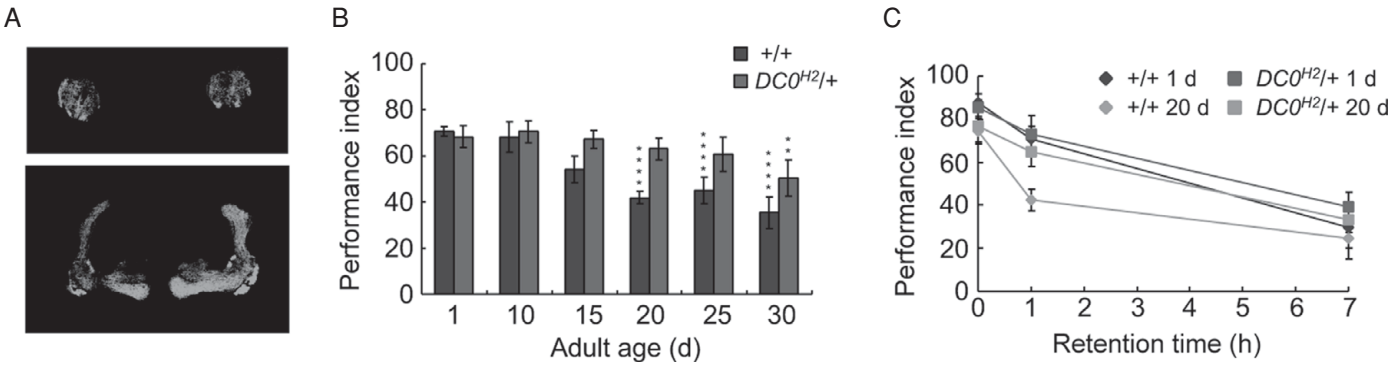

Fig. 13.2. Suppression of AMI by *DC0* mutations.
A Expression of DC0 gene product, Pka-C1. Pka-C1 is highly expressed in the lobes and calyx of MB. B The $DC0^{H2}$/+ heterozygous mutation delays the onset of AMI. AMI does not occur in $DC0^{H2}$/+ flies until 30 days of age C Comparison of memory retention curves in DC0/+ mutants and wild-type controls at 1-day and 20-days of age. In contrast to wild-type flies, there are no significant changes in memory retention upon aging at any timepoint tested in $DC0^{H2}$/+ flies. Modified from (Yamazaki et al., 2007).

Given that DPM neurons innervate the lobes of the MBs, downstream signaling of *amn* likely resides in the MBs. From screening based on this hypothesis, heterozygous mutations in *DC0* were identified as strong AMI suppressors. The *DC0* gene encodes the catalytic subunit of PKA (Pka-C1). Pka-C1 are highly expressed in the MBs (Fig. 13.2A), and activity of PKA in *DC0*/+ flies is reduced to approximately 60% of that in wild-type control animals (Yamazaki et al., 2007, Skoulakis et al., 1993). Rescue experiments confirm that PKA function in the MBs is involved in AMI. Interestingly, both the memory and AMI defects of *amn* mutants are restored in *amn;DC0*/+ double mutants. These results are consistent with the idea that Amn peptides released from DPM neurons act to inhibit PKA in the MB (Fig. 13.3), although both PACAP and GHRH stimulate PKA activity in mammalian systems. Recent studies demonstrate that AMI can be ameliorated by stimulating DPM neurons (Tonoki & Davis, 2012), implicating that aging may disrupt releasing machineries of DPM neurons.

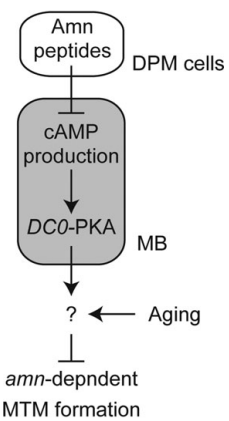

Fig. 13.3. cAMP/PKA signaling underlying AMI. While neither cAMP production nor PKA activity is increased, downstream undiscovered molecule is increased upon aging thereby impairs formation of *amn*-dependent MTM.

Remarkably, *DC0*/+ flies do not show significant AMI until 30 days of age (Fig. 13.2B), which corresponds to normal lifespan of control wild-type flies. Increasing lifespan could, in principle, delay onset of AMI. However, the lifespan of *DC0*/+

flies are not increased. Memory retention curve of *DC0*/+ flies is not altered upon aging (Fig. 13.2C). This excludes the possibility that AMI still occurs in *DC0*/+ flies, but is obscured by a shift in retention kinetics of MTM relative to wild-type flies.

AMI has often been proposed to result from a gradual, irreversible accumulation of deleterious byproducts over time. However, AMI can be reversed by acutely reducing PKA activity in the aged MBs (Yamazaki et al., 2010). This indicates that AMI in *Drosophila* is caused by an age-dependent increase in PKA-dependent signaling that can be reversed by acute interventions at old age. In addition to MTM, long-term memory (LTM), which is produced by multiple training sessions with rest interval between each and sensitive to feeding protein synthesis and transcription inhibitors, is also disrupted in aged flies (Mery, 2007). However, it is still not clear whether this type of AMI is also suppressed by reducing PKA activity.

cAMP/PKA signaling has been shown to play a critical role in learning and memory. That is, a large fraction of memory mutants can be linked to cAMP/PKA signaling. As demonstrated by genetic manipulations of *dnc*, which encodes a cAMP-specific phosphodiesterase, not only reduction of cAMP/PKA activity, but also increases in its activity disrupts memory formation. Overexpessing Pka-C1 in the MB also impairs MTM without affecting LRN (D. Yamazaki unpublished observations). Since *DC0* mutation suppresses AMI, one might infer that age-related increase in cAMP/PKA activity may cause AMI. However, neither cAMP level nor PKA activity is increased upon aging, indicating that age-related increase in downstream components of the cAMP/PKA pathway may be responsible for AMI (Fig. 13.3).

Involvement of cAMP/PKA signaling in AMI is also suggested in mammalian systems. AMI of prefrontal cortex (PFC)-dependent working memory in aged rats can be ameliorated by reducing PKA activity (Ramos et al., 2003). Significantly, the improvement of working memory is greater in aged rats with more severe cognitive deficits, while memory enhancements are not observed in young adult rats. These data also suggest that age-related increase in activity of cAMP/PKA signaling leads to a decline in PFC-dependent working memory. However, basal levels of adenylyl cyclase isoforms, AC2 and AC3, and phosphodiesterase isoforms, PDE4a, PDE4b, and PDE4d do not show age-related changes. Instead, CRE binding activity, which is likely to be a downstream effect of cAMP/PKA (Coven et al., 1998), significantly increases in the PFC and higher number of cells show immunostaining with anti-phospho-CREB antibody in aged rats (Ramos et al., 2003). However, it has not been determined whether age-related increase in transcriptional activity of CREB is responsible downstream of cAMP/PKA for the decline in PFC-dependent working memory in aged rats.

In contrast to PFC-dependent working memory, it has been widely reported that increases in the cAMP/PKA pathway activity can ameliorate AMI. Analogs of cAMP or agonists of dopamine D1/D5 receptors, which are positively coupled with adenylate cyclase increase both the protein synthesis-dependent phase of hippocampal LTP (L-LTP) and hippocampus-dependent LTM in aged mice (Bach et al., 1999). Also, concentrations of rolipram, a cAMP phosphodiesterase inhibitor, that increase stimulated levels of cAMP without affecting basal levels, improves hippocampus-dependent LTM in aged mice (Barad et al., 1998). These results suggest that aging may lead to a reduction of cAMP/PKA activity in the brain resulting in AMI. However, there is a caveat to this model. It is likely that the improvement of memory and LTP by stimulation of PKA is not specific to aged animals. In many cases, memory is also improved in young animals, raising the possibility that decreasing PKA activity is not the cause of AMI (Wang et al., 2004). Rather, increasing PKA activity improves memory in general.

How cAMP/PKA signaling leads to AMI?

In contrast to age-related neurological disease such as Alzheimer's, Parkinson's and Huntington's, mammalian AMI does not necessarily accompany morphological changes in neurons. There are reports showing that overall numbers of hippocampal neurons and synaptic connections are not altered in aged, learning impaired rats (Geinisman et al., 2004, Rapp & Gallagher, 1996). Similarly, no significant neural degeneration is observed in *Drosophila* at the age of AMI onset (Iijima et al., 2004, White et al., 2010). In contrast, AMI always accompanies physiological changes in neuronal cells. A well-known physiological consequence associated to AMI is a dysregulation of intracellular Ca^{2+} level in neurons where larger Ca^{2+} influx occurs via L-type voltage-gated Ca^{2+} channel. Ca^{2+} plays a key role in expression of synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD).

In the hippocampus of aged rats, the threshold for LTP induction is increased, while the threshold for LTD induction is reduced (Fig. 13.4A) (Foster, 1999). Increased Ca^{2+} influx through LVGCCs enhances Ca^{2+}-dependent K^+ channel activity which, in turn, increases afterhyperpolarization (AHP) amplitude and duration (Norris et al., 1998). An increase in AHP, the hyperpolarization following action potentials (Fig. 13.4B), should decrease the probability of LTP induction requiring higher frequency stimuli (increase in threshold for LTP induction). Evidence that an increased Ca^{2+}-dependent K^+ channel activity suppresses LTP induction comes from a study demonstrating that the Ca^{2+}-dependent K^+ channel blocker apamin reduces the threshold for LTP in aged rats (Norris et al., 1998). Moreover, there is an elevated expression of small-conductance Ca^{2+}-activated K^+ channels (SK channels) in the hippocampus of aged mice, and the injection of antisense oligomer for SK channels both ameliorates AMI of a hippocampus-dependent memory task and increases the probability of LTP induction (Blank et al., 2003). Significantly, the LVGCC blocker, nimodipine, ameliorates AMI of eye blink conditioning in rabbits (Deyo et al., 1989, Straube et al., 1990). Also, the LVGCC blocker nifedipine facilitates LTP and reverses the increased probability of LTD induction in aged hippocampal slices (Norris et al., 1998). Notably, LVGCC expression is highly

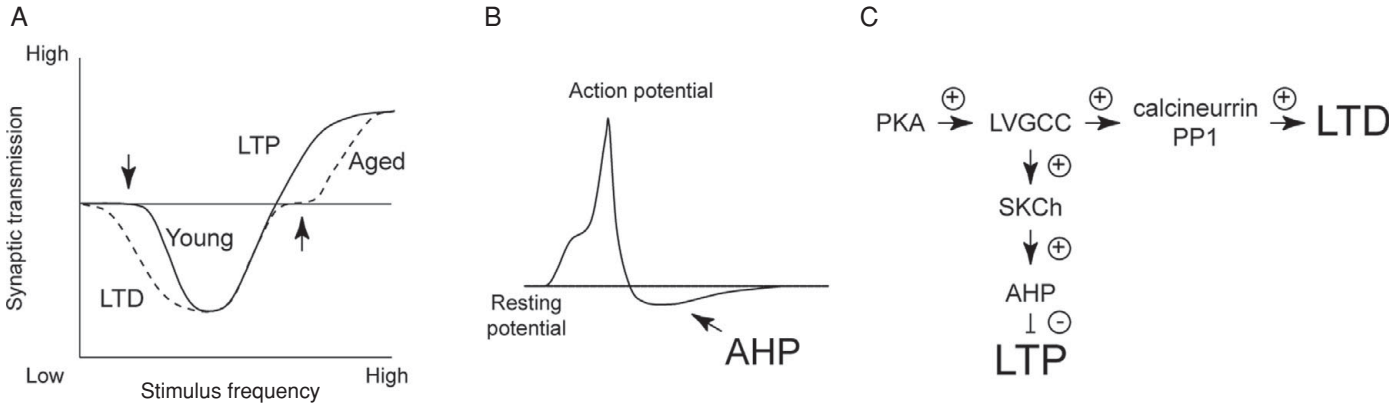

Fig. 13.4. Possible role of PKA for Ca^{2+} dysregulation in aged brain.
A Age-related change in metaplasticity. As compared with young hippocampus, aged hippocampus requires higher frequency of stimuli for induction of LTP, while lower frequency of stimuli-induced LTD. B Afterhyperpolarization (AHP) appears after induction of action potential. C Hypothetical role of PKA activity for altered plasticity.

up-regulated upon aging (Thibault et al., 2001, Thibault & Landfield, 1996) and channel activity is strongly enhanced by PKA phosphorylation (Davare & Hell, 2003), suggesting that LVGCC may be a candidate substrate that can cause AMI upon aging (Fig. 13.4C). In support of this hypothesis, LVGCC phosphorylation by PKA has been shown to be increased in the hippocampus of aged rats (Davare & Hell, 2003).

Besides Ca^{2+} dysregulation, in aged PFC, increased cAMP/PKA signaling is suggested to increase open state of HCN (hyperpolarization-activated cyclic nucleotide-gated) and KCNQ potassium channels in pyramidal neurons producing recurrent firing during the delay period of working memory task (Wang et al., 2011). Given that such recurrent firing during the delay period is required for working memory, age-related increase in cAMP/PKA signaling may cause AMI of PFC-dependent working memory by increasing activity of HCN and KCNQ channels. PKA activity may also be involved in formation of neurofibrillary tangles (NFT). Accumulation of NFT shows strong correlation with age-related memory loss (Morrison & Hof, 1997), and phosphorylated tau protein is a major component of NFT. PKA is one of several kinases that phosphorylate tau protein facilitating NFT formation (Liu et al., 2004, Morrison & Hof, 1997).

The linkage between lifespan and AMI

Given that AMI occurs as a function of biological aging, there must be crosstalk between lifespan regulation and AMI, and identifying common signaling pathway for these processes is essential for understanding AMI.

Calorie or dietary restriction (CR), which reduces metabolic activity, is perhaps the best known non-genetic intervention for extending lifespan and works in organisms as diverse as yeast, *Drosophila*, *C. elegans*, and mammals. Reducing metabolic rate is thought to extend lifespan via decreasing production of reactive oxygen species (ROS), the by-products of normal metabolism. In this model, ROS cause molecular damage to DNA, proteins, and lipids that accumulates over time, eventually causing aging and death (Kirkwood & Austad, 2000). Supporting this idea, mutations that extend lifespan tend to have increased tolerance to dietary paraquat, a toxic free radical generator (Johnson et al., 1999). Furthermore, overexpression of antioxidant genes such as SOD1 and catalase, as well as feeding SOD/catalase mimetics, have been shown to increase lifespan in *Drosophila* and *C. elegans* (Melov, 2000, Orr & Sohal, 1994, Parkes et al., 1998). It has been reported that a diet rich in antioxidants improves AMI in canines. In rats, feeding mitochondrial metabolites decreases oxidative damage of nucleic acids in the brain and improves performance in memory tasks in aged animals (Liu et al., 2002). Also, infusion of catalytic scavengers of ROS into the brains of rats both ameliorates oxidative damage and reverses cognitive defects associated with age (Liu et al., 2003). Thus it seems that oxidative damage may be a cause of both aging and AMI. However, recent studies in *Drosophila* demonstrate that onset of AMI is not associated with an increase in ROS production upon aging (Hirano et al., 2012).

Lifespan extension by CR occurs through a number of converging or interrelated pathways, including the insulin-like growth factor (IGF) pathway, the target of rapamycin (TOR) kinase pathway, and pathways involving the histone deacetylases SIR2 and RPD3 (Blander & Guarente, 2004, Raught et al., 2001, Saltiel & Kahn, 2001). Both the IGF and TOR pathways function by sensing nutrient availability and regulating cell and organismal growth. IGF stimulation activates kinase cascades, which ultimately result in phosphorylation of the transcription factor, FOXO (Brunet et al., 1999, Hwangbo et al., 2004, Libina et al., 2003, Ogg et al., 1997). Unphosphorylated FOXO activates transcription of factors that retard cell growth and proliferation, while phosphorylated FOXO is sequestered in the cytoplasm. In yeast, TOR signaling is similar to IGF signaling, resulting in phosphorylation and cytoplasmic localization of transcription factors involved in responses to various stresses (Beck & Hall, 1999). In mammals, TOR kinase activity affects

the activity of ribosomal protein S6 kinase and binding of the translation repressor 4EBP1 to initiation factor eIF4E to regulate translation in response to nutrient sensing. The TOR and IGF pathways are evolutionarily well conserved, and reduction in their activities extends lifespan in several species (Clancy et al., 2001, Hwangbo et al., 2004, Kapahi et al., 2004, Murphy et al., 2003, Vellai et al., 2003). It is likely that longevity by CR occurs through IGF and TOR signaling, since the effects of CR and reduction in these pathways are not additive (Clancy et al., 2002). Furthermore, data from *C. elegans* indicates that the IGF and TOR pathways themselves are at least partially overlapping, since the increased lifespans seen upon inhibiting the TOR pathway are not additive with increases due to mutations in *daf-2*, the *C. elegans* homolog to the IGF receptor (Vellai et al., 2003).

While signaling pathways involved in lifespan regulation has been extensively identified, it is still largely unknown how they associate to AMI. Although CR extends lifespan and ameliorates AMI in some cases, there are some reports that CR can extend lifespan without ameliorating cognitive aging in rats as well as in flies (Burger et al., 2010, Markowska, 1999, Yanai et al., 2004). Similarly, while reducing insulin/IGF signaling extends lifespan and improves AMI (Kauffman et al., 2010), some studies show that activation of the IGF signaling pathway in the brain ameliorate AMI (Markowska et al., 1998, Sonntag et al., 2000). These results indicate that optimal conditions for lifespan extension and for amelioration of AMI may not be the same. Hence, further identification of AMI-specific mutants and behavioral analyses of lifespan mutants are required to explore the linkage between lifespan regulation and AMI. As a novel animal model, *Drosophila* will be useful in reconciling these conflicts and elucidating the molecular mechanisms of AMI.

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Chapter

14

Functional analysis of natural clock gene variation

Charalambos P. Kyriacou

Introduction

More than 40 years ago, Ronald Konopka, working in Seymour Benzer's laboratory in Pasadena isolated the 24-hour *period* mutants by using chemical mutagenesis (Konopka and Benzer, 1971). This classic work, performed in *Drosophila melanogaster*, heralded the advent of "neurogenetics," a field that has been consolidated and expanded over more than four decades to include genetic model and non-model organisms. The behavioral phenotypes that are now studied with the neurogenetic approach in *Drosophila* extend far beyond circadian rhythms. Indeed, the list is almost endless and includes learning and memory, olfaction, courtship, food searching, locomotion, pain and thermal sensing, drug addiction, sleep, etc., etc. In fact, there is barely a behavioral phenotype that we can define in a human that cannot be studied in some way in the fly. This extends to even complex disorders such as mental illness. While most neurogenetic analysis is focused on the genes, cells, and neuronal networks that generate a phenotype, not much attention is devoted to natural genetic variation. Perhaps this is not seen to be as important as the mutagenesis used to make the severe variants that allow us to dissect behavioral pathways. Yet, using *Drosophila* to study the more subtle behavioral genetic variation that is available in populations, or between species, has provided an adaptive evolutionary perspective to neurogenetics that has also led to functional insights into how some of these "neuro" genes underlie their behavioral phenotypes (Kyriacou et al., 2008).

Basic circadiana

As this chapter will focus on circadian behaviour, some minimal introduction into the field is required, particularly in the features of the clock that will be genetically dissected in subsequent sections. It is hardly surprising that the rotation of the planet around its own axis drives a 24-hour oscillation in many environmental factors (i.e., light, temperature, humidity, etc.) that exert a strong selection pressure on life. The ability to organize biological resources to anticipate these daily oscillations would be expected to be adaptive. Thus the behavior and/or metabolism of most, if not all, living species evolved to follow a 24-hour schedule. When isolated from environmental time cues (e.g., constant darkness, DD), organisms are still able to maintain biological rhythms with a period of approximately 24 hours, indicating the existence of an endogenous oscillator pacemaker, generally referred to as the circadian clock (Latin: *circa* = about, *dies* = day). Without the presence of environmental cycles, the circadian clock is placed into *free-running* conditions allowing its internal periodicity to cycle. For most species, the *free-running* period in DD ranges from 23 to 25 hours (Dunlap et al., 2004). Interestingly, very little change in the period is observed when an organism is submitted to different temperatures, indicating the presence of compensatory regulation in the pacemaker mechanism (Zimmerman et al., 1968). This fundamental aspect allows the circadian clock to adapt to daily and seasonal variations of temperature. But, despite its self-sustainable nature, the oscillator needs to be synchronized to environmental cycles on a daily basis to maintain an exact 24-hour rhythm. These environmental cycles or *zeitgebers* (German: "time giver"), act as input factors to the pacemaker mechanism, regulating the turnover dynamics of some of its components, in a process called "entrainment" (Dunlap et al., 2004). For many species, the strongest and most important *zeitgeber* is the daily light–dark (LD) cycle, although other non-photic cues (e.g., temperature cycles, humidity cycles, social interactions, food availability) have also been attributed to play an important role (Dunlap et al., 2004). Indeed, recently, quasi-natural observations of fly behavioral rhythms revealed the surprising result that temperature was a more important environmental zeitgeber than light (Vanin et al., 2012).

period, timeless and seasonal behavior

We have briefly touched on the *per* gene, which is so important in the development of the field. Within *per* is found a repetitive Thr–Gly encoding repeat located in the middle portion of the coding sequence (Yu et al., 1987). This unusual sequence was observed to have natural molecular variation, which was distributed as a latitudinal cline in Europe (Costa et al., 1992). To cut a long story short (that has been reviewed many times in the past (Kyriacou et al., 2008; Costa and Kyriacou, 1998), balancing selection was maintaining the *per* polymorphism and not drift (according to statistical analysis of the sequences) (Rosato

Behavioral Genetics of the Fly (Drosophila melanogaster), ed. J. Dubnau. Published by Cambridge University Press.

et al., 1997a). Given this result, one felt particularly justified in attempting to find out why these molecular variants were being maintained at such frequencies. It turns out that the clock of the variant found at high frequencies in the south of Europe was more adapted to warmer temperature, whereas that in the north was more adapted to colder temperatures (Sawyer et al., 1997). But, imagine that genetic drift was responsible for these spatially distributed *per* allelic frequencies and not balancing selection. Would it have been worth the considerable effort to find out whether the variants had altered phenotypes if they were not of any differential adaptive value? The answer is that further investigation would probably not have been pursued, because embedding the study of natural variation within the context of adaptation provides an enhanced biological perspective of the phenotype.

The *per* example highlights the use of a cloned gene as a candidate, not necessarily to look for correlations between sequence variation and a phenotype, but more to look for sequence variation in the first instance, and then to see whether this looks interesting from an evolutionary perspective. The final fillip to the *per* story is that variation of the Thr–Gly sequence motif does account for a large fraction of the circadian clock's natural ability for "temperature compensation," by which it maintains a 24 h cycle at different temperatures, and is a cardinal characteristic of all circadian timers (Sawyer et al., 1997). Imagine one had performed this experiment in reverse and used a popular modern approach and obtained lines from various locations, inbred them, and then performed the QTL-type analysis for temperature compensation (a lot of work). One would have found a significant QTL on the *X*-chromosome (*per* is sex-linked) and one might have then tested *per* as a candidate locus, but one would always be worried about assigning a phenotype to the locus itself, rather than to sequences in linkage disequilibrium. The neurogenetic approach solves this problem because the different *per* variants can be placed in identical genetic backgrounds via transgenesis, and indeed when this was done, it was clear that the thermal phenotype was caused by the Thr–Gly polymorphism (Sawyer et al., 1997). I present this story as a nice example of how an evolutionary approach to neurogenetics can enhance the functional analysis of behavior as a by-product of studying the population genetics of the gene.

A more recent set of studies of circadian clock gene that has undergone the population genetics treatment is *timeless (tim),* which encodes the partner of *per*. Briefly, the PER and TIM proteins are negative autoregulators, and their mRNA and protein products cycle with 24 h periods. They interact with the positive transcriptional regulators CLOCK and CYCLE to generate a negative feedback loop, which forms one of the several feedback loops that define the intracellular oscillator. This is discussed in detail in the chapter by Ceriani. For the moment, all the reader requires to know is that TIM represents the light-sensitive negative autoregulator, and through its physical interactions with the dedicated circadian blue light photoreceptor (Rosato et al., 2001), Cryptochrome (CRY), which is itself activated by light, leads to a resetting of the clock at dawn (see Ceriani).

The *tim* gene has two natural variants which differ by a 5′ deletion/insertion of a single *G* nucleotide (Rosato et al., 1997b). In the case of *ls-tim,* the insertion, which occurs 5′ of an *ATG* start codon, recruits an additional N-terminal 23 amino acids because it brings into frame a further upstream *ATG* codon. Thus *ls-tim* can (and indeed does) generate two length isoforms of TIM, L-TIM (long-TIM) from the upstream *ATG* and S-TIM (short-TIM) from the downstream *ATG* (Fig. 14.1A) (Tauber et al., 2007). A second allele, *s-tim,* found initially in laboratory stocks, has this *G* nucleotide deleted so that, after 19 residues from the upstream *ATG,* translation of L-TIM grinds to a halt because of a premature stop (Fig. 14.1A) (Rosato et al., 1997b). However, the main product of *s-tim* is now the shorter S-TIM isoform that initiates at the downstream *ATG*. So, here we have an interesting polymorphism involving translational starts, whereby one allele, *ls-tim,* generates both L-TIM^{1421} and S-TIM^{1398} isoforms, whereas *s-tim* generates a very short 19 residue variant (at least on paper) but its main product is S-TIM^{1398}. This minor length difference of 23 residues between L- and S-TIM might not be expected to make much difference to how TIM functions, yet as we shall see, it has major implications for how the clock responds to light.

This polymorphism extends into natural populations and is distributed as a latitudinal cline, in that from southern Italy traveling northwards to Scandinavia, the frequency of *ls-tim* goes from ~80% to about 20%, a very dramatic change in frequencies (Fig. 14.1B) (Tauber et al., 2007). Using statistical tests that have been designed to distinguish between various types of selection and drift, the results suggested that directional selection was at work, i.e., one of the two alleles was being favored (Tauber et al., 2007). This was itself a complete surprise, as one would have imagined *a priori,* a balancing selection scenario, just as in the case of the *per* polymorphism, so that it might be adaptive to have high *ls-tim* frequencies in the south of Europe and vice versa in the north. However, this was not the case and begged the question of which allele was being favored by selection. Phylogenetic analysis revealed that *ls-tim* was the new variant and had been derived in Europe a few thousand years ago, after flies colonized Europe from sub-Saharan Africa (Tauber et al., 2007). In Africa, *ls-tim* was not found in over 100 different sequences from various regions of central Africa, supporting the view that *ls-tim* was a European allele and that the ancestral state was *s-tim*. Indeed, the sibling species *D. simulans* has the *s-tim* variant only in Europe. Consequently, if *ls-tim* was the new variant, and was only a few thousand years old, it must logically be the variant under directional selection in order to establish itself in such high frequencies.

Having shown that the polymorphism was under selection, what was the selective agent? As TIM encodes the light-sensitive component of the clock, and because *ls-tim* and *s-tim* did not show any differential temperature compensation, perhaps some kind of photoperiodic selection was at work that could explain the geographic distribution? Indeed, it was observed that *ls-tim* flies, irrespective of which population they come from, are less light sensitive in terms of the way their clock is reset by

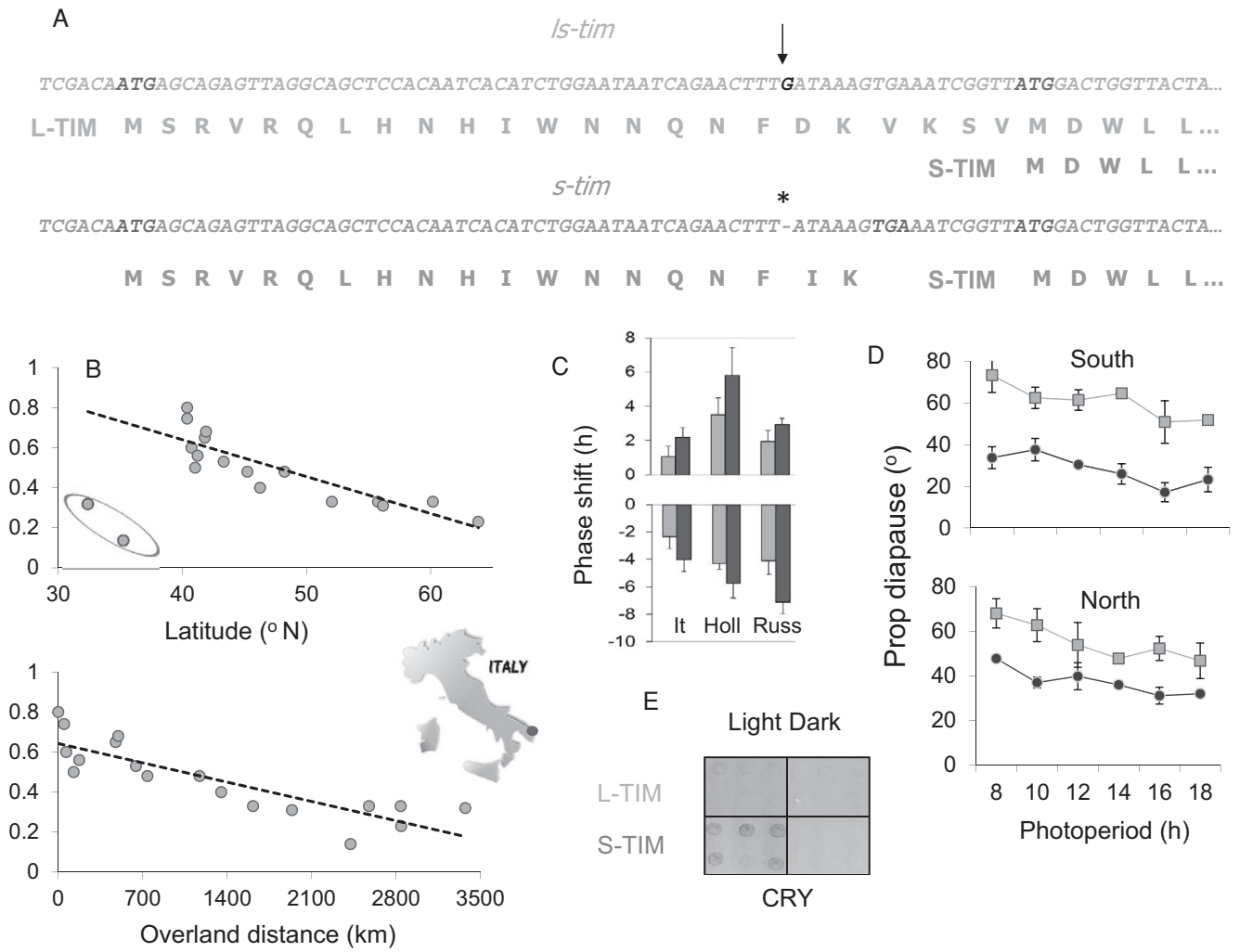

Fig. 14.1. Functional implications of the *timeless* polymorphism.
A The sequence of *ls-tim* (green) and *s-tim* (blue). Note the *ATG* start and *TGA* stop codons (red) and the *G* nucleotide insertion (arrowed) in *ls-tim* which allows the upstream *ATG* to be recruited to generate L-TIM. B Frequency of *ls-tim* at different European latitudes. Top panel. The regression line is drawn through points that represent populations sampled from southern Italy, traveling generally northwards to Scandinavia. The two points at lower latitudes represent population from Crete and Israel that are clearly outliers (ringed). Bottom panel. Replotting the frequencies as overland distance from Novoli (red point on map of Italy), brings all points into a straight line. C *ls-tim* (green) flies from natural Italian (It), Dutch (Holl) and Russian (Russ) populations show reduced circadian locomotor phase shifts in response to short light pulses delivered both late during subjecting night, that lead to advances (top, +ve) and early in subjective night that lead to delays (bottom –ve). *s-tim* in blue. D *ls-tim* flies from northern and southern European populations show enhanced diapause responses. E Yeast two-hybrid experiment showing robust S-TIM-CRY interaction in the light compared to L-TIM (redrawn from Kyriacou et al., 2008; Sandrelli et al., 2007).

light compared to *s-tim* (Sandrelli et al., 2007). Thus, a pulse of light might reset the clock of an *ls-tim* fly by 2 hours, but that might be 4 hours in *s-tim* depending on the genetic background (Fig. 14.1C). Also, consider that the further north one travels in Europe, the more extreme is the photoperiod, particularly in mid-summer when flies are up and about (in winter they diapause). Constant light makes the fly clock arrhythmic (indeed, it does this to all clocks) and such arrhythmicity is metabolically stressful (think about insomnia!). Thus flies in the UK, for example, where the mid summer photoperiod is LD20:4 in Leicester (indeed, it never really gets truly dark at this time), will be struggling to remain rhythmic. One evolutionary resolution to this problem was identified by Pittendrigh many years ago in his studies of the clocks of *Drosophila* species. He suggested and demonstrated that the higher the latitude, the less responsive is the clock to light. He suggested that this could be managed by having an underlying oscillator with a larger amplitude, which would be more resistant to the effects of light stimuli (Pittendrigh and Takamura, 1989; Pittendrigh et al., 1991). One can think of this as a pendulum that has a larger swing…its swing will be less affected if I put my hand on it momentarily than a pendulum with a small swing. When the molecular oscillations of *tim* were examined at the mRNA level, no evidence for a difference in the amplitude of the rhythm was observed between the two *tim* variants (Sandrelli et al., 2007). Another way of reducing circadian light responsiveness might be that the

light is somehow "filtered" before it gets into the clock mechanism, so might this be the mechanism by which the *ls-tim* light response is modulated?

ls-tim has another phenotype, which may reflect a decreased sensitivity to light and that is reflected in the fly's seasonal response. *D. melanogaster* have a shallow diapause, which can be easily scored as a reproductive arrest in the female. At 12 °C and in long winter nights, females stop producing eggs in their ovaries, whereas at the same temperature, but with short nights and long days to mimic summer photoperiods, they maintain their egg production. This is a photoperiodic response that can be easily scored. Interestingly, irrespective of whether the population comes from the north or south of Europe, *ls-tim* females will go into diapause at much higher frequencies than *s-tim* (Fig. 14.1D) (Tauber et al., 2007). So, even though they are seeing long days, *ls-tim* females interpret them as short days, and move into diapause prematurely, again representing a reduction in the response of the diapause mechanism to light and suggesting some kind of photo-filtering.

One could be lured into a sense of security here in that here we have a polymorphism with quite dramatic and similar effects on two light-related phenotypes. One involves changing the photosensitivity of the clock and the other alters diapause levels, possible by measuring day-length with some kind of TIM related timer. Not only does this make a genetic and functional connection between these two phenotypes (something that has vexed the entomology community for some time (Emerson et al., 2009), but also, it may perhaps illuminate the spatial distribution of *ls-* and *s-tim* in Europe. Alas, the reader may have noted that *ls-tim* shows phenotypes (reduced circadian light sensitivity and premature diapause) that might be expected to be more adaptive in northern rather than southern Europe, yet its highest frequencies are found in southern Italy! The solution to this puzzle relates to the age of the new allele. If the *ls-tim* mutation occurred in southern Italy, it would be expected to spread out from that site in every direction, because we assume that seasonal selection would be a factor throughout all of Europe. Indeed, when samples were taken further south from Italy, in Crete and Israel, the frequency of *ls-tim* fell dramatically, completely upsetting the original latitudinal cline (Fig. 14.1B) (Tauber et al., 2007). When, instead of latitude, the frequency of *ls-tim* was plotted as direct distance from the site of highest *ls-tim* frequency (near the town of Novoli in Puglia), a linear relationship was restored, except for the population in Crete, that stubbornly remained an outlier. However, as flies are human commensals, and in the past would have largely migrated with humans over land, all frequencies including the recalcitrant one from Crete fell into a simple linear relationship with overland distance from Novoli (from Novoli to Crete go northwards to Croatia, across the Balkans, down to southern Greece then a short hop overseas to Crete) (Fig. 14.1B). This result suggested that the spread of *ls-tim* was generated by selection in every direction, because all of Europe has a seasonal environment, thermally and photoperiodically (Tauber et al., 2007). Naturally, we might expect that seasonal selection in the northern part of Europe might be even more intense than in the south and new results support this view (V. Zonato and C. P. Kyriacou, unpublished observations).

The results of the analyses described above therefore point to a new mutation arising in southern Italy, then spreading slowly by directional selection because of the adaptive seasonal phenotypes it conveys. In addition, demographic processes such as human migration from Italy (for example, during the Roman expansion) might have helped the spread of *ls-tim*. Another alternative that cannot be definitively excluded is that perhaps there is directional selection for high *ls-tim* frequency in a narrow range of latitudes around southern Italy, but that balancing selection takes over outside these geographical ranges. Demographic and more subtle balancing selection scenarios can both be tested with appropriate experimentation.

Finally, at the molecular level, what is it that makes *ls-tim* flies less circadian and seasonally light-responsive? TIM physically interacts with CRY when the lights come on at dawn and this leads to TIM degradation. In the absence of TIM, PER is then vulnerable to phosphorylation by Doubletime kinase (DBT) which then leads to PER degradation (see Chapter 8). When PER and TIM are taken out in this way after dawn, their repressive influence on the positive transcription factors CLOCK and CYCLE, which activate *per* and *tim* transcription is lifted, and the two positive factors re-initiate the molecular cycle, which eventually leads to PER and TIM repressing their own genes late at night via their interactions with CLOCK-CYCLE (Ceriani chapter). Thus could *ls-tim,* by generating the L-TIM isoform, be altering the dimerization of TIM with CRY? In a yeast two-hybrid assay, this is exactly what happens, with L-TIM showing a reduced level of interaction with CRY than S-TIM (Fig. 14.1E). This would be expected to reduce the circadian light response, but also to reduce the degradation of TIM. When head TIM levels are examined over the circadian cycle, *ls-tim* flies show much higher levels of TIM than do *s-tim,* at all phases, just as expected from the yeast results[1417].

Jetlag mediates TIM and CRY degradation

In LL, wild-type flies become arrhythmic, because first TIM then PER are degraded, and the clock is placed into a phaseless state. However, some laboratory strains were observed to remain rhythmic in LL (Koh et al., 2006), rather like the cry^b mutant (which is almost a *cry*-null, hence light cannot 'enter' the clock so it free-runs as if in DD (Emery et al., 2000). Meiotic mapping and deletion analysis of this phenotype from these strains suggested that a locus encoding an F-box protein that may be a component of the Skp1/Cullin/F-box (SCF) E3 ubiquitin ligase complex could be the culprit. Sequencing of this gene (termed *jetlag, jet)* revealed two mutations in leucine-rich repeats (LRRs), a common one, jet^c, found in all but one line, and a rare one, jet^r. In DD, the two *jet* mutants showed normal circadian behaviour, but showed reduced photic responses to brief light pulses, which correlated with a reduction in light-dependent degradation of TIM in clock neurons. Furthermore,

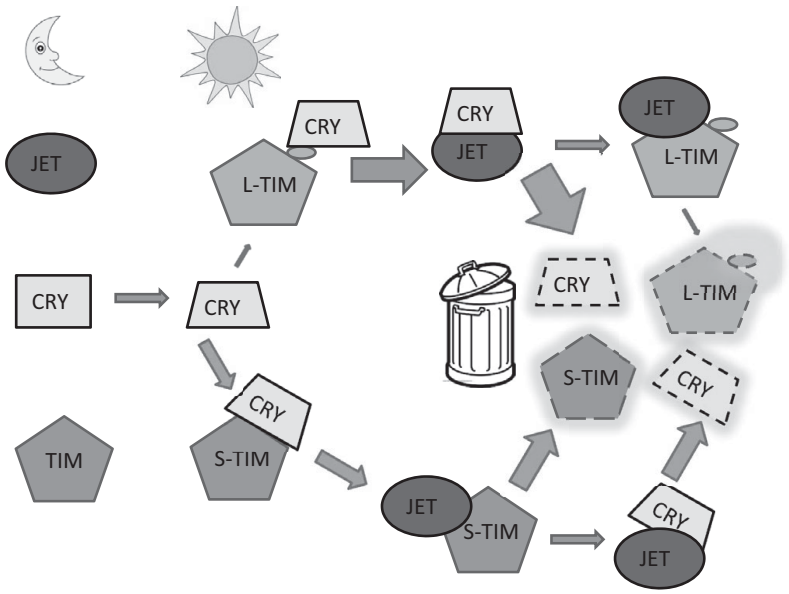

Fig. 14.2. Model for L-TIM, S-TIM and CRY degradation by JET. At night, CRY, TIM and JET do not interact, but at dawn, light activates a conformational change in CRY, which can now bind TIM and JET. In *ls-tim* flies that express L-TIM, the additional N-terminal sequence prevents efficient binding to CRY, so CRY now binds to JET and is degraded, leaving even less CRY to interact with L-TIM. The interaction between L-TIM and CRY creates a post-translational change in TIM so that it becomes a target for JET, but the weak L-TIM-CRY binding slows this process down, so that it takes much longer for L-TIM to be degraded, so it appears more stable than S-TIM. S-TIM on the other hand binds strongly to TIM and this protects CRY from JET mediated degradation, while at the same time priming S-TIM for interaction with JET and efficient degradation. JET then binds CRY and leads to its degradation. Consequently in S-TIM expressing flies, TIM degradation occurs earlier than CRY degradation. The resulting stability of L-TIM in *ls-tim jet* mutant flies means that flies continue to be rhythmic and free-run in LL (redrawn and modified from Peschel et al., 2009).

addition of JET to *Drosophila* cell lines expressing CRY and TIM, resulted in a light-dependent degradation of TIM, which was significantly reduced with JET^r rather than JET^c. JET physically interacts with TIM, strongly in the light, but also with SKpA in these cell lines (Koh et al., 2006).

All of these results suggest that JET is an important factor in the CRY-dependent degradation pathway of TIM. However, an interesting subtlety exists in that another research group similarly identified a laboratory strain called *Veela*, which only expresses rhythmicity in LL when jet^C is in linkage disequilibrium with *ls-tim* (Peschel et al., 2006). Neither single *ls-tim* nor the *jet* mutants, nor *s-tim* jet^C double mutants show this LL effect. Indeed the proximity of *tim* to *jet* on chromosome 2 initially led to much confusion about the robustness of the *jet* mutant LL phenotype. However, TIM cycling in LL was observed in clock neurons, correlating with the unusual LL rhythmic behavioural phenotypes of *ls-tim* jet^C. The physical interaction among TIM, CRY and JET have led to a sophisticated model for the light resetting complex, where light activates CRY, which binds strongly to S-TIM and generates efficient S-TIM degradation, but, counter-intuitively, this also protects and stabilizes CRY from the effects of JET. The weaker L-TIM-CRY interaction and less efficient L-TIM degradation leads to less stable CRY because it is now susceptible to JET and degradation, and even less CRY reduces further the L-TIM circadian photosensitivity (Peschel et al., 2009) (Fig. 14.2). Thus, the *jet* polymorphisms led to a much deeper understanding of the light-resetting mechanism, yet disappointingly, it appears that jet^C and jet^r were mutants induced in laboratory genetic backgrounds and are not (at least yet) found in nature. However, it is possible that other natural polymorphisms in *jet* may be present in the sequence that could modulate the light response, and, like *ls-tim* and *s-tim,* become substrates for natural selection.

Seasonal *per* splicing and the siesta

We have touched on seasonal aspects of clock biology, particularly with the discussion of light sensitivity and *tim* polymorphisms, and its implications for circadian phenotypes as well as overwintering strategies such ovarian arrest. There are other mechanisms that may adapt circadian behaviour to the seasons and one that has received particular attention is the thermally regulated splicing of *per*. Under colder temperatures splicing of a 3′ intron (with no coding sequence implications) leads to an earlier upswing in the cycle of abundance of the *per* transcript and the protein in the fly's head. This correlates with an earlier phase in the locomotor behaviour of the fly, so that the large evening component (E) also moves to an earlier phase during the light portion of the day (Fig. 14.3) (Majercak et al., 1999). This would appear to provide an adaptive response, in that conversely, at hotter temperatures, the E component moves to the latter parts of the day and into the night, thereby avoiding potentially dessicating conditions of the middle of the day. There is also a morning (M) locomotor component which also moves in the opposite direction (although not as dramatically) with hotter temperatures, so that the M episode comes earlier in the day, again avoiding the hotter parts of the day (Vanin et al., 2012; Majercak et al., 1999). This stretching of the time between M and E behavior under hotter temperatures has been termed the "siesta."

This model has been complicated recently by the finding that, under natural environmental conditions, flies actually get very active in the middle of the day when the temperature is >30 °C as often occurs during southern European (and American) summers. Flies show an anti-siesta, rather than a siesta, and this may be some kind of escape response, even though the flies are kept in shaded habitats during these experiments (Vanin et al., 2012). However, it is interesting that this response is clock

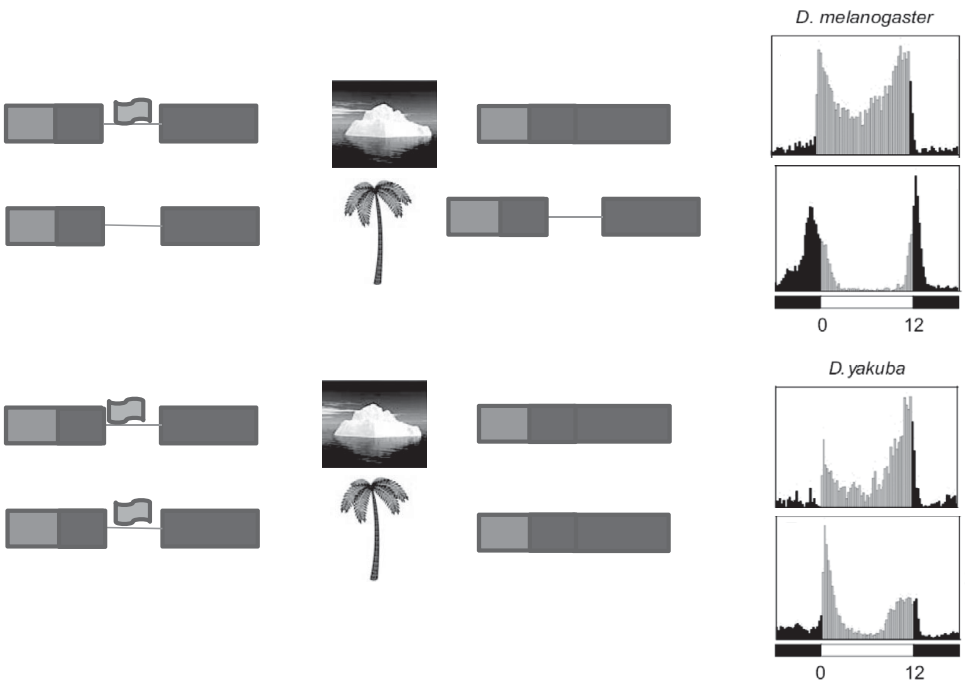

Fig. 14.3. Interspecific *per* splicing of 3′UTR and the siesta.
In *D. melanogaster,* a weak splice site on a *per* 3′ intron that divides the 3′ UTR (red, blue represent coding sequences in that 3′ exon) may be bound by the splicing apparatus (green flag) only under colder conditions (iceberg), leading to splicing out of the intron. Splicing leads to an earlier upswing of *per* mRNA and PER protein, leading to an advanced evening onset of locomotor activity and consequent reduction of the siesta, which is the quiet period between the morning and evening locomotor components that is prevalent at warmer temperatures. In *D. yakuba,* a strong splice site means that efficient *per* 3′ splicing occurs at both cold and warm temperatures, and consequently the behavioural profile does not change for this tropical species. These experiments are carried out at constant temperature and rectangular light-dark cycles (locomotor data reproduced from Low et al., 2008).

modulated, because per^s mutants which have a fast endogenous oscillator (period of 19 h) start their anti-siesta some hours earlier than per^L (which has a 29 h period). In addition, the splicing of the *per* intron has a linear relationship with average daily temperature from 5 °C to 30 °C in the wild, yet the length of the siesta is largely constant until average daily temperatures reach 22 °C (a hot day). Above average temperatures of 22 °C, if we ignore the anti-siesta which kicks in at around average temperatures of 25 °C, the E component of activity does move later and later, consistent with the splicing/siesta model (Vanin et al., 2012) (Fig. 14.3).

per splicing is modulated by both heat and light, and mutations in various genes that affect both light and heat sensitivity, show changes in the splicing pattern (Majercak et al., 2004; Collins et al., 2004). Locking the splice mechanism into constitutive splice or non-splice modes has the same behavioral effect, in that flies are locked into a summer phenotype, with an E component that is delayed into the early night (Majercak et al., 1999). Thus it is not the different transcripts that convey the behavioral phenotype, but the act of splicing itself, suggesting that enhancing 3′ end formation of the pre-mRNA might be the causal factor for the thermal phenotype. Another curious and interesting feature of this post-transcriptional mechanism is the variation that is observed among different *Drosophila* species of the *melanogaster* subgroup. The tropical species *D. yakuba*, which is found in Africa, also splices *per,* but the splicing does not change with temperature, and coincidentally or causally, nor does the siesta (Low et al., 2008). Bioinformatic analysis reveals *D. yakuba* to have an optimal splice site compared to *D. melanogaster*'s rather weak site and molecularly this was confirmed by a clever use of *Drosophila* S2 cells. In addition, *D. simulans,* a sympatric cosmopolitan sibling species of *D. melanogaster* also showed splicing and behavioral changes with temperature, whereas another tropical species, *D. santomea*, behaved as their close cousins *D. yakuba* for both molecular and behavioral phenotypes (Low et al., 2008). Thus, a species-specific pattern emerges suggesting that tropical species' circadian behaviour need not be thermally modulated and this can be ensured by having an optimal *per* splice site that is available for interacting with the spliceosome at all temperatures. The weak *D. melanogaster/simulans per* splice sites are only activated at colder temperatures, which may be required for a more stable assembly of spliceosomal proteins, or for the protein-RNA interactions that are required for splicing. Whatever the mechanism, this elegant work has implications beyond *Drosophila*, in that such a mechanism based on optimal/suboptimal splice sites may convey not only species-specific characteristics for the circadian phenotypes, but also might represent a more general mechanism for thermal adaptations in poikilotherms (Low et al., 2008).

Further species-specific circadian phenotypes

In the previous section we have also moved from discussing intra-species polymorphism to investigating the implications of inter-species genetic variation. The differences in *per* gene coding sequences among Drosophilids is quite dramatic, and early studies of *Drosophila* species PER noted the patchwork nature of conserved and non-conserved regions (Colot et al., 1988; Thackeray and Kyriacou, 1990). Inter-specific transformation of the *pseudoobscura per* coding sequences under the control of the *melanogaster per* promoter into per^0 hosts gave

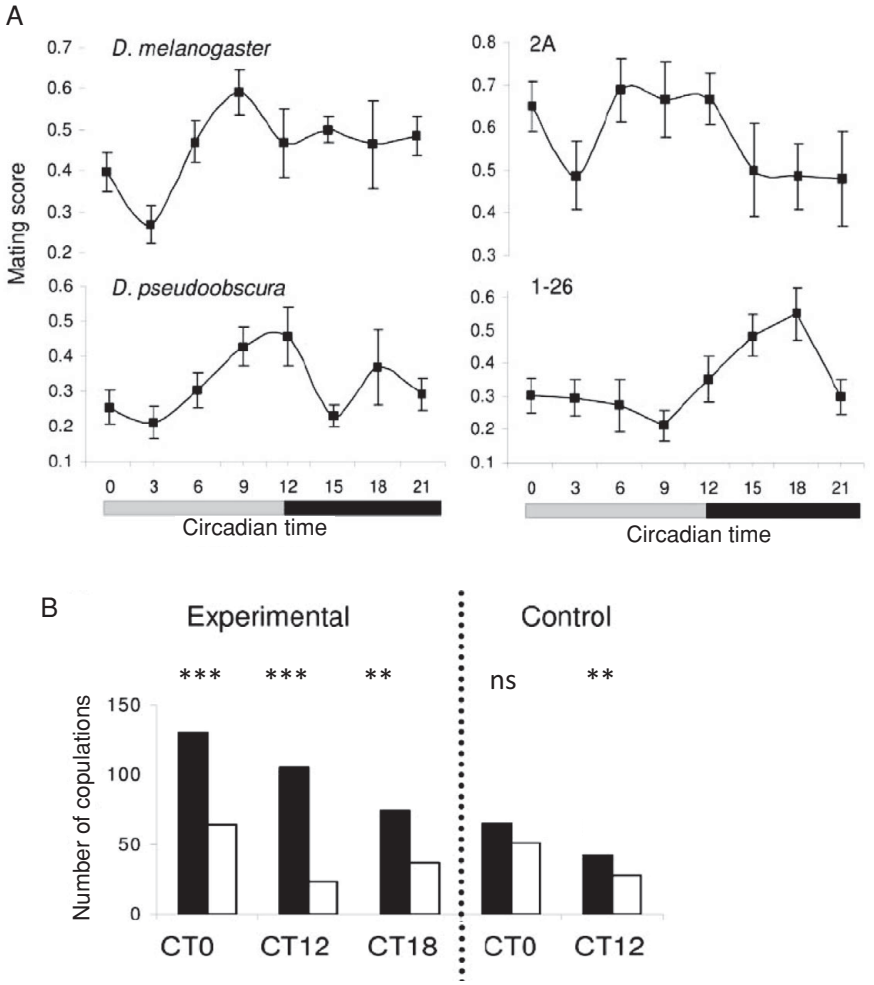

Fig. 14.4. *period* is a species-specific reservoir for mating timing.
A The mating times of a population of *D. melanogaster* peak a few hours earlier than *D. pseudoobscura* in constant darkness. This effect is amplified when *D. melanogaster per*^{0} transformants carrying the conspecific *per* transgene (2A) are compared with transformants carrying the *D. pseudoobscura per* transgene (1–26). B When males and females of each transformant class are mixed together the number of homogamic compared to heterogamic matings are significantly elevated at different phases of the circadian cycle (CT0 = subjective lights on, CT12 subjective lights off). Two different control strains of *per*^{0} mutants transformed with the *melanogaster period* gene do not show such high levels of assortative mating (*** $P < 001$ ** $P < 0.02$) (Figure redrawn from Tauber et al., 2003 with permission from Elsevier).

a modest rescue of rhythmicity with longer periods, but more importantly, the different phase of locomotor rhythms of *pseudoobscura* was transferred to the *D. melanogaster* transformants (Petersen et al., 1988; Tauber et al., 2003). Incidentally, chimeric transgenes in which the coding region was divided up between the two species around the Thr–Gly region were also very informative in terms of detecting changes in temperature compensation, and revealing a role in the clock's thermal response due to dynamic selective changes in this region (Peixoto et al., 1998).

In addition to species-specific phases of circadian locomotor behavior, fruit fly species also prefer to mate at different times of the day, for example, the sympatric *D. melanogaster* and *D. simulans* sibling species (Sakai and Ishida, 2001), and this could contribute to the sexual isolation between them, and support their pheromonal differences, which also play a key role in their mating behaviors (Billeter et al., 2009). Not surprisingly, these mating rhythms can be disrupted by clock gene mutations (Sakai and Ishida, 2001). The *D.pseudoobscura per* transformants mentioned above also show a temporal mating pattern that is different from that of *D. melanogaster* and these mating patterns correlate with the locomotor differences between the species (Fig. 14.4) (Tauber et al., 2003). The *D. melanogaster per*^{0} transformants carrying *D. pseudoobscura per* sequences also show the parental species preference in mating rhythms, indeed, it is somewhat exaggerated compared to control *D. melanogaster per* transformants. So, what happens when we put together the two sets of transformants, males and females of each type? A very strong assortative mating is observed, so that like mates with like, and the pairings reflect the mating rhythms of the two species (Fig. 14.4) (Tauber et al., 2003). In other words, in a laboratory environment we can set up a sexual isolation that could represent the first steps in the speciation process, simply by manipulating a single gene!

These remarkable results were the first to show that complex species-specific adaptive phenotypes could be determined by natural variation in a single gene. Previous to this, and for many decades, the dominant view on the genetic basis of species-specific adaptations, the so-called "infinitesimal model," was that these adaptations were the end-point of selection at many loci, each with a tiny effect, which when added up generated the phenotypic difference (Coyne, 1992). While this may be true for some adaptations, inter-specific transformations using *period* as described above, demonstrated that this need not be the case. Nor is this an unique example of single gene control

of complex species-specific features. In studies of *Drosophila* courtship songs, species-specific rhythmic song features that cycle every 40–70 s, or song characters that distinguish the sound pulse patterns of different species, also map to *per* or to another song/clock gene, *nonA (no-on-transient-A)* (Wheeler et al., 1991; Campesan et al., 2001). Furthermore, behavioral studies in this "geno-evo-behavioral" area are buttressed by evolutionary analyses of development ("evo-devo"), in which changes in species-specific body plans can be caused by coding or *cis* regulatory region changes in single homeobox genes or in those of their downstream targets (Galant and Carroll, 2002; Ronshaugen et al., 2002).

Conclusions and perspectives

The evolutionary analysis of "behavioral" genes is still very much in its infancy. I have focused here on clock genes as they represented the first example in which population genetic analysis was applied to genes that were identified by the neurogenetics approach. Another well-known example is the *foraging (for)* locus, which is described in the chapter by Sokolowski. Here, a different approach was taken in that initially, a larval behavioral food searching phenotype was discovered, which appeared to be polymorphic within natural *D. melanogaster* populations, and this was used to map and then molecularly identify the locus responsible. Again, what appeared at first to represent a quantitative character, "roving" or "sitting," was surprisingly found to be due to changes in a single gene, *for* (see Sokolowski chapter).

There are probably lots more surprises to come. Many behavioral phenotypes are now given the "genomics" treatment, in which quantitative trait loci (QTLs) are established for a phenotype which can contribute varying amounts to the phenotypic variation, from quite substantial to tiny. Mapping the responsible loci within the QTL (which usually includes many genes) can also be performed by using deletion analysis. However, a variation on the theme is to use transcriptome information to map natural variation in behavior. The first time this was done was by selecting for positive and negative geotaxis and then profiling the change in gene expression of the transcriptome, which led to a number of candidate genes, which were then tested by using mutants (Toma et al., 2002). More recently, fly aggression has been given the genome treatment, with the result that >1500 loci were reported to contribute to the variation in levels of this complex phenotype that had also been bidirectionally selected (Edwards et al., 2006). One wonders which of these loci carry genetic variation in natural populations that might be meaningful in an evolutionary context. Obviously, one cannot study the variation of all genes at once, or can one?

Next generation sequencing methods mean that whole fly genomes can be sequenced rapidly and so population genetic tests could conceivably be performed systematically on adjacent sliding windows of a defined size sequentially through the genome, indicating regions where there was evidence for selection. This has been performed recently by comparing patterns of variation between sequences from flies collected in the USA and in the ancestral African homeland (Langley et al., 2012). Very interestingly, one of the many analyses reveals a region of putative selection very close (~2kb) to the site of the *tim* polymorphism supporting the earlier analysis on European populations (Tauber et al., 2007). Such genome analyses will be able to highlight areas within the genome which show the signatures of selection and worthy of further study if a known behavioral gene is lurking in the vicinity. This would provide a rational starting point for examining behavioral genes that have not yet undergone the evolutionary treatment.

This type of genomics approach represents the future of behavioral genetics, yet the painstaking analysis of natural variation on candidate single genes may still have an enormous amount of biological insight to offer.

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Chapter

15

Natural variation in foraging behavior

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Introduction

Organisms are said to be foraging when they search for, and exploit, food resources (Stephens et al., 2007). Foraging behavior varies both within and between species. When food is limited, foraging behavior can significantly affect survivorship and reproduction. Both genetic variation and phenotypic plasticity in foraging-related traits enable animals to cope with fluctuating abiotic and biotic factors that affect food availability (Kaun and Sokolowski, 2008; Meuller et al., 2005.) Foraging behavior also serves to restore stability to an animal's internal environment in spite of changes in the external world. In *Drosophila melanogaster*, metabolic homeostasis is dependent upon phenotypic traits that regulate the intake, storage, and mobilization of nutrient substrates (Leopold and Perrimon, 2007).

Gene by environment interactions using natural genetic variants provide an inroad to questions about both the adaptive and functional character of naturally occurring genetic variation (Sokolowski, 2001). Our task here was to focus on a gene–environment perspective in the study of natural variation in *D. melanogaster* foraging-related behaviors. We only briefly touch on the expanding literature that uses mutational analyses to investigate mechanisms underlying feeding and metabolism. In the latter part of this chapter the rover and sitter genetic variants of the *foraging* gene are used to illustrate this perspective.

The single gene mutant analysis for behavior-genetics pioneered by Seymour Benzer (Benzer, 1973) has been aptly used to investigate the molecular and neurobiological mechanisms underlying food related behaviors in *D. melanogaster* (Douglas et al., 2005; Melcher et al., 2007). This method has revealed the role of numerous genes involved in feeding such as *malvolio*, a transmembrane transporter found to play a role in taste (Rodrigues et al., 1995), *takeout,* a mutant that exhibited aberrant circaddian locomotory behavior during starvation (Sarov-Blat et al., 2000), mutations in the leucokinin pathway caused flies to consume larger meals (Al-Anzi et al., 2010), and *hugin* which modulated feeding behavior in response to nutrient signals (Bader et al., 2007), to name but a few examples.

Genes, environments and natural variation in food-related traits

Behavior genetics also has a history in artificial selection (Hirsh, 1959) and more recently evolutionary biologists have used natural selection experiments in the laboratory to investigate food-related traits. In natural selection experiments flies are reared under certain conditions for many generations and the responses to these environmental conditions are measured as a change in phenotype between the various environments. For example, flies reared at high population densities for many generations fare well in these conditions, but do poorly in low density conditions (Mueller et al., 1991). In addition, feeding rate is increased in the high density selection lines due to larval competition for limited food, where high density conditions reduce both the quality and quantity of food (Guo et al., 1991). Flies have also been selected for their ability to endure nutrient stressed environments and bouts of starvation (see Rion and Kawecki, 2007 and references therein). Relevant findings from these studies indicate there is widespread genetic variation and plasticity for these food-related traits. It would appear that any trait that you artificially select for will exhibit a selection response. So why is there so much variation for food-related traits? To begin to answer this question, we need to identify genes in natural populations that affect food-related traits and then ask whether or not these genes and behaviors are responsive to the environment.

Identifying the genes involved in food-related traits is an important initial step to studying the behaviors associated with food. Instances of natural variation in food-related traits have been genetically mapped using genetic markers such as single nucleotide polymorphisms (SNPs). This process of associating variation in a quantitative trait with genetic variation is known as quantitative trait locus (QTL) mapping (Mackay, 2001). This method has successfully identified numerous loci associated with many behaviors, including those involved in food search, consumption and metabolic homeostasis. For instance, multiple QTLs associated with starvation resistance have been detected in two wild-type *D. melanogaster* populations (Harbison et al., 2004). Starvation resistance is the ability for an

Behavioral Genetics of the Fly (Drosophila melanogaster), ed. J. Dubnau. Published by Cambridge University Press.

organism to successfully function during bouts of food deprivation. This study identified 12 genes affecting starvation resistance; among these genes were some that mediated metabolic and other feeding-related traits.

Searching for food requires that flies detect the appropriate chemical cues that signify a potential meal. Olfactory responses, important in the search for food, vary due to allelic variation in a set of odorant binding proteins (Obp) (Wang et al., 2007, 2010). Adult flies respond to odorants commonly found in fruits like benzaldehyde, acetephenone, hexanol, and hexanal with different degrees of avoidance and attraction. These differences were statistically associated with single nucleotide polymorphisms (Wang et al., 2007, 2010), an indication that within these populations, there was widespread variation in the ability to detect and approach different food odors.

Once food is located, the next step is tasting it. A naturally occurring dimorphism in adult gustatory behavior was identified in two commonly used laboratory *D. melanogaster* strains, Canton-S (CS) and Oregon-R (OR) (Tanimura et al., 1982). By employing a two-choice feeding assay, they showed that CS flies ingested trehalose-containing food preferentially compared with OR flies. Importantly, no difference in sensitivity to other sugars was found in these lines. This difference in behavior mapped to the *Tre* (*trehalose sensitivity*) locus that resides on the X chromosome between cytological regions 5A10 and 5B1–3 (Tanimura et al., 1988).

The *Tre* gene was later identified by Ishimoto et al., 2000 as encoding a seven-transmembrane domain G protein-coupled receptor protein (GPCR), a gustatory receptor protein-encoding gene *Gr5a* (Ueno et al., 2001). Sequence analysis of over a dozen wild-caught and laboratory isofemale fly lines with either high (Tre^{+} allele) or low (Tre^{01} allele) trehalose sensitivity demonstrated that a single-nucleotide polymorphism (Ala218Thr) in *Gr5a* cosegregates with the trehalose gustatory phenotype. While other genetic factors are recognized to make a contribution to the observed behavioral dimorphism, the Ala218Thr SNP is the only naturally occurring polymorphism in *Gr5a* known to influence trehalose sensitivity (Inomata et al., 2004).

The behavioral dimorphism generated by this naturally occurring allelic variation in *Gr5a* was hypothesized to be a product of differential transmission of trehalose-related chemosensory signals in sugar gustatory receptor neurons (Isono et al., 2005). Electrophysiological readings obtained from labellar l-type sensilla using tip recording techniques revealed very low spike generation in OR (Tre^{01}) flies in response to high concentrations of trehalose. In contrast, CS (Tre^{+}) flies displayed prolific spike generation in response to even relatively low concentrations of trehalose, as compared to OR (Tre^{01}) flies (Isono et al., 2005). The neurophysiological phenotypes revealed by this study were hypothesized to be a product of disrupted ligand–receptor binding, or of altered G-protein activity.

The question of why natural variation in gustatory perception of a nutritious sugar exists, as well as the mechanistic basis

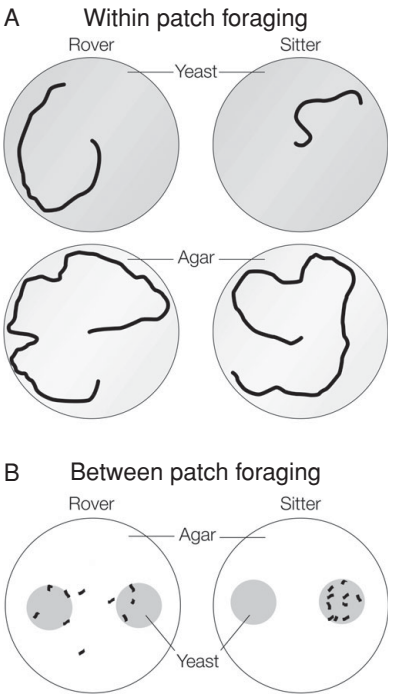

Fig. 15.1. A Typical foraging path lengths of third instar rover and sitter larvae on a yeast substrate and on a non-nutritive substrate (agar). B Rover and sitter patterns of between patch foraging. (Reproduced from Sokolowski, 2001. With permission from MacMillan).

of this dimorphism, is still under investigation. Analyses of wild populations of *D. melanogaster* identified a roughly 25:75 allelic ratio of Tre^{+}:Tre^{01} (Ueno et al., 2001). Sequence analysis of *Gr5a* in *D. simulans* revealed Thr in position 218, suggesting that low trehalose sensitivity is the ancestral functional form of this gustatory receptor (Inomata et al., 2004).

Intimately associated with the search for food is the decision to eat a food source once located. There are a number of techniques available to measure food consumption in larval (Kaun et al., 2007) and adult (Ja et al., 2007; Wong et al., 2009) flies (see Fig. 15.1). Food is a mixture of different types and ratios of macronutrients, as well as potentially hazardous contaminants, and measuring nutritional targets can be ascertained (Simpson and Raubenheimer, 1993). Whether a larval or an adult fly, the decision to eat is based on the central integration within a fly's brain of external feeding stimuli with internal physiological signals that convey information regarding the fly's metabolic homeostatic condition. Often, feeding decisions reflect the stage of development the fly is in. The optimal ratio of protein to carbohydrate intake (P:C) for longevity in flies is 1:16 while egg-laying rate was optimized at 1:2 and lifetime egg production at 1:4 (Lee et al., 2008). Thus the macronutrient ratios are largely dependent on the life history phenotype being measured and the life stage the fly. Another important factor is the condition of the fly in prior developmental stages; this is highlighted in the larva to adult transition discussed below.

Larval life history and feeding, consequences for adult life

Although a clear difference in morphology and behavior can be seen between pre-pupation and post-pupation flies, what

happens in the larval stages, especially with regard to feeding, can have major implications for adult life. Larvae feed by extending the anterior end of their body through peristaltic action, gripping the feeding medium with mouth hooks, and ingesting food as they move through the medium, or remain in place and repeatedly "bite" into the food with their mouth hooks. Larvae spend most of their time searching for food and feeding to build up enough mass to pupate and metamorphose into adult flies (Reaume and Sokolowski, 2006).

Early third instar larvae are able to attain the critical weight necessary to pupate and successfully emerge as an adult fly (Bakker, 1961). Food ingested after reaching this minimun weight allows larvae to pupate at a higher weight resulting in larger flies. By mid-third instar, larvae treat food and non-food (agar) substrates the same (Sokolowski et al., 1984) and wander in search of a place to pupate (Riedl et al., 2007). Larvae reared in nutrient-deficient or crowded feeding environments either pupate early or delay pupation depending on their strain and the environmental conditions.

Like adult flies, larvae are exposed to heterogeneity in their feeding environments. Larval feeding history impacted both larval and adult fitness (Foley and Luckinbill, 2001; Kolss et al., 2009). Larvae reared in nutritionally poor environments produced smaller and lighter adult flies than larvae reared in a nutritionally rich larval environment (Kolss et al., 2009). Larger flies had more stored lipids (Baldal et al., 2006; Chippindale et al., 1996). This is significant because a positive correlation has been observed between adult starvation resistance and lipid storage (Chippindale et al., 1996; Djawden et al., 1998; Vermeulen et al., 2006). An adult that is resistant to starvation stress can allocate more time to non-foraging related activities such as mating and oviposition. Larval nutrient history and traits that confer greater foraging success in the larvae are expected to have beneficial consequences that extend beyond pupation and eclosion into adulthood.

Kolss et al. (2009) demonstrated the impact of larval nutrient history on adult nutrient stress tolerance, and also identified an interesting trade-off between larval and adult foraging-related traits. Lines selected for tolerance to chronic larval nutritional stress over many generations exhibited greater egg-to-adult viability and faster larval development on both poor and normal food. However, these selected lines had decreased tolerance to adult malnutrition and starvation resistance relative to unselected lines. This effect was independent of whether the assayed populations had been reared on poor or normal food (Kolss et al., 2009). Selection for tolerance to poor foraging conditions at one point in the life history of *D. melanogaster* was associated with a trade-off with adult metabolic phenotypes. Such an effect can be hypothesized to directly impact, not only foraging-related traits in adults, but also other fitness components related to mate searching and fecundity (Prout and McChesney, 1985).

D. melanogaster larvae encounter significant heterogeneity in their feeding environment. The decision to remain within, or leave, a food patch is therefore one of the most crucial faced by larvae, and the role played by genes in mediating this process is thus expected to differentially impart lasting consequences on the survival and fitness of adult rovers and sitters. The role of the *foraging* gene in food patch leaving is discussed later in this chapter.

Larval crowding is a major factor that impacts the quality of the larval feeding environment. In low density conditions, food is abundant and relatively uncontaminated by waste products. High larval density leads to deterioration of food quality and quantity, accumulation of wastes and increased interactions between individuals. Increased larval feeding rate and decreased nutrient assimilation into fat body stores were found under high density conditions (Guo et al., 1991; Mueller, 1990). Conditions of high larval density resulted in tolerance towards the effects of larval crowding (Borash and Ho, 2001). Adults selected under high density evolved higher lipid content and greater starvation resistance than those reared under normal densities. In high larval density, nutrient-poor and competitive environments, larvae that move into new feeding patches (albeit of unknown quality) may stand a better chance of survival.

The *foraging* gene and its effect on food-related behaviors

A prominent example of genetic variation underlying a naturally occurring behavioral polymorphism arose from studies of *D. melanogaster* larval foraging behavior. The remainder of this chapter will focus on the *foraging* gene and its naturally occurring rover and sitter variants. This model will illustrate an example of gene–environment interplay on food-related behaviors. Roles for *foraging* in other taxa will also be discussed near the end of this chapter.

We begin this discussion with a historical overview of the *foraging* gene story. Significant variation in the distance (path length) larvae travelled while feeding was found in *D. melanogaster* larvae from an orchard in Toronto, Canada (Sokolowski, 1980). Analysis of the population revealed a dimorphism in larval foraging strategies, which was termed "rover" and "sitter." Rovers exhibited significantly longer foraging path lengths on nutritive yeast media than sitters (Fig. 15.2A and B). These differences in behavior exhibited gene by environment interactions; they were conditional on the presence of a food (yeast paste) and absent on non-nutritive agar (Sokolowski, 1980). In a Toronto orchard population the ratio of larvae exhibiting the rover vs. sitter foraging behaviors was 70:30 (Sokolowski, 1980).

Genetic analysis of the rover–sitter larval foraging path length trait began with its localization to the second pair of chromosomes using chromosome substitution analysis. Quantitative genetic analysis confirmed that larval path length differences in the rover and sitter strains had a predominantly autosomal basis and fit a single gene model of inheritance with rover genetically dominant to sitter (de Belle and Sokolowski, 1987). A single gene basis for this trait was confirmed by subsequent mapping of the rover–sitter trait to the *foraging* (*for*) gene

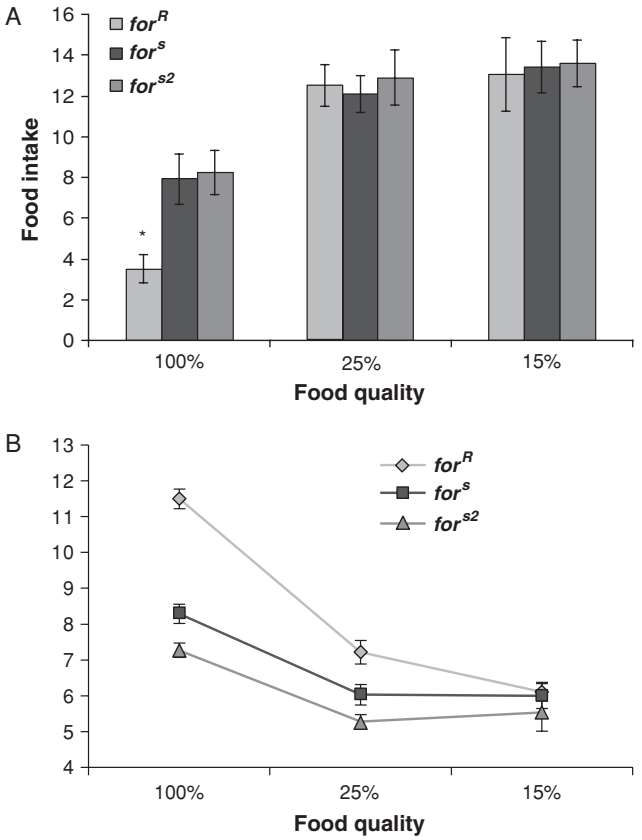

Fig. 15.2. A Food intake measures of rover (for^R), sitter (for^S), and sitter mutant (for^{S2}) larvae on 100%, 25% and 15% food quality. B PKG enzyme activity for rover (for^R), sitter (for^S), and sitter mutant (for^{S2}) larvae at 100%, 25%, and 15% food quality. (Reproduced from Kaun et al., 2007. With permission of the Company of Biologists.)

(de Belle et al., 1989a) and later cloning of this gene (Osborne et al., 1997).

In the 1980s it was difficult to localize a quantitative trait such as *foraging* because the fly genome had not been sequenced. As a result analytical techniques based on mutagenesis screening called lethal tagging were used (de Belle et al., 1989a). This approach helped with later cloning of the gene because mutants in the *foraging* gene were generated as part of this approach (de Belle et al., 1989a, 1993). The use of morphological mutant phenotypic markers in recombination mapping was also avoided because the pleiotropic effect of these markers on development and behavior were likely to confound behavioral analysis of rover–sitter differences. Lethal tagging was developed as a method to map genes affecting quantitative behavioral variations that were susceptible to genetic background effects (de Belle et al., 1989a).

Mutagenesis was used to tag the gene responsible for the rover–sitter behavioral difference with a discrete easily quantifiable mutation; lethality was chosen for this purpose (de Belle et al., 1989a). To accomplish lethal tagging, rover male flies were mutagenized and crossed to sitter, and the resultant F1 were screened both for a change from rover to sitter larval foraging behavior and for the induction of a lethal phenotype. Five independent lines arose from this mutagenesis screen. All lines behaved as sitter, all were lethal and three lines had pupal lethal mutations that did not complement with each other. The chances of altering the behavioral phenotype *and* of generating a lethal mutant that didn't complement in three lines was very unlikely given approximately 2000 lethal complementation groups on the second pair of chromosomes. Thus, the lethal mutation generated in the three non-complementing lines was either within the *foraging* gene or had tagged it. The non-lethal lines had second site mutations that were crossed off two mutant sitter *foraging* alleles called (*for*^{s2}) and (*for*^{s1}), these were generated in addition to the established rover (*for*^{R}) and sitter (*for*^{s}) natural variants. Complementation analysis of lines exhibiting sitter mutant behavior as well as deficiency mapping showed that the *foraging* locus resided within cytological region 24A3-C5. Another series of mutagenesis screening studies using ethyl methanesulphonate (EMS), which generated point mutations in a single locus, further narrowed the location of *foraging* to region 24A3–5 on the polythene chromosome map (de Belle et al., 1993). This study also first suggested that in addition to its behavioral role, *for* might function as a vital gene.

This set the stage for the molecular identification of *for* (Osborne et al., 1997). The *dg2* gene which encodes a cGMP-dependent protein kinase (PKG) lies within the *for* region 24A3–5. The candidacy of *dg2* as *for* was demonstrated in a number of ways. Mutations in *for* were mapped to within *dg2*. Sitter to rover phenotypes were reverted by excision of a P-element thought to be inserted into *dg2* (but see Wang et al., 2008). Characterization of rover–sitter PKG expression and activity differences showed that whole adult *for*^{R} heads had small, yet significantly higher, levels of PKG enzyme activity than *for*^{s} and sitter mutant heads (Osborne et al., 1997). The expression of *dg2* cDNA behind a leaky heat shock promoter in a *for*^{s} genetic background generated larvae that exhibited rover-like larval foraging path lengths that did not significantly differ from *for*^{R}. Additionally, dissected nervous systems from these transgenic larvae had significantly higher PKG enzyme activity than *for*^{s} larvae. Together, these data suggested that *for* was synonymous with *dg2*.

foraging and larval behavior

Differences in foraging behavior between rovers and sitters have the potential to place the two behavioral morphs on different developmental trajectories. For instance, rovers leave a food patch more frequently than sitters, and this trait can expose rovers to increased risk of parasitism by wasps (Sokolowski and Turlings, 1987), desiccation (Sokolowski, 1985), and a much greater degree of uncertainty about where their next meal is coming from. The use of a larval foraging assay consisting of discrete food patches distributed across a non-nutritive surface allowed Sokolowski et al. (1983) to demonstrate artificial selection for one foraging strategy over another in populations of *D. melanogaster*. Using starting populations consisting of a mixture of rovers and sitters, artificial selection for larvae

that remained on a food patch yielded a population of animals exhibiting predominantly sitter-like foraging behavior within only six generations.

Graf et al. (1989) investigated the role of *for* in mediating foraging behavior in a variety of different food quality environments as well as in response to a short 4-hour period of food deprivation. When foraging on media containing different amounts of yeast (ranging from no-yeast to all-yeast), rover larvae exhibited a highly variable response in path length to the different feeding media compared to sitters. Rovers also consistently displayed longer path lengths on a given substrate than did sitters and these scores were highest on the all-yeast substrate. Interestingly, following a 4 h food deprivation period, the path lengths of early third larval instar rovers and sitters both decreased significantly as compared with well-fed larvae; however, food-deprived rover path length scores remained significantly longer than sitter scores. Thus, while an environmental treatment can impact both strains with similar magnitude, the inherent strain differences remain. These data reveal an aspect of phenotypic plasticity inherent to both the rover and sitter genetic variants, and raise questions regarding the molecular and physiological events that underlie these patterns of gene-environment interaction (Graf and Sokolowski, 1989).

Density- and frequency-dependent selection

Environmental factors are ever important elements to the success or failure of an individual searching for food. In some cases, the most important environmental factor can be one's conspecifics – the social environment. How dense and crowded a location is and what type of individuals make up this location are highly influential factors that impact larval development and adult life. Sokolowski et al. (1997) demonstrated density-dependent selection on *for*. An equal proportion of rovers and sitters were introduced into replicate populations that consisted of either high or low density populations. Thus natural selection occurred under these different conditions in the laboratory. The frequency of sitter and rover individuals was measured after 74 generations. They found that the frequency of sitter individuals increased in low density conditions. Complementation and deficiency analysis showed that the observed low density-dependent selection of sitters resulted from changes in the frequency of for^s alleles (Sokolowski et al., 1997).

Negative frequency-dependent selection on alternative alleles is one way that genetic polymorphisms can be maintained in populations. In this case, the fitness of each type decreases as its frequency increases. Fitzpatrick et al. (2007) grew rover and sitter larvae together at different frequencies in vials where food was limited and larval competition is severe. Fitness, measured as larval survivorship to pupation, was higher in the type (rover or sitter) that was less frequent. This was only true when food was limited. The actual mechanism by which negative frequency-dependent selection occurs is not yet understood.

Evolutionary trade-offs can also affect the frequency of natural genetic behavioral variants. Donlea et al. (2012) showed that natural variation in *for* provides allele specific resiliency in one environment and vulnerability in another, an example of gene–environment interaction. This may exist as a trade-off between the effects of sleep deprivation and starvation on memory. The high PKG levels of rovers confer protection from sleep deprivation; sleep-deprived rovers exhibit high short-term and long-term memory. In contrast under these conditions, the low PKG levels of sitters confer a reduction in short and long term memory. This reduction in memory can be restored by expression of *for* in the α and β lobes of the mushroom body. On the other hand, rovers exhibit a reduction in STM and poorer survival from starvation than do sitters. The reduction in STM as a consequence of starvation can be restored to a sitter level by expressing for-RNAi in rover mushroom bodies. Thus, differences in the expression of *for* may cause resiliency in one environment but vulnerability in another. This suggests that *for* is a modulator of behavior in response to different environmental contexts.

foraging and larval metabolic strategies

Natural variation in *for* affects larval food acquisition in an environmentally dependent manner (Kaun and Sokolowski, 2008). In addition to mediating locomotor behavior while feeding, *for* plays a role in food intake, absorption and allocation (Kaun et al., 2007; Kaun and Sokolowski, 2008). Even though rover larvae move more than sitters, well-fed rovers have lower food intake than sitters, but rovers also absorb more of the glucose that they ingest. When larvae are chronically food-deprived throughout their larval life and then provided with good-quality food, rovers and sitters increase their food intake to a common level. PKG activity is correlated with food intake across different food deprivation treatments (Fig. 15.3). Thus, rovers exhibit plastic responses to chronic food deprivation and PKG activity levels track the food intake measures. Interestingly, glucose absorption by rover larvae reared in a low food environment remained elevated (Kaun et al., 2007). This may explain why rover larvae exhibit greater survivorship to eclosion and decreased developmental delay relative to sitters when reared in a nutritionally deficient environment.

The differences in nutrient absorption observed between rover and sitter larvae are reflected in the levels of different macronutrient reserves contained within the larvae. In rovers, ingested glucose is allocated preferentially to lipid stores, whereas in sitters it is biased towards carbohydrate stores (Kaun et al., 2008). Carbohydrate levels in the fat body and in whole body measures are significantly higher in well-fed sitters. Kent et al. (2009) also found rover–sitter differences in nutrient storage in adult heads. Indeed, the characteristics of rover larvae in comparison to sitter larvae are largely mirrored in their adult counterparts.

Early life experience and adult behavior

Burns et al. (2012) showed that larval but not adult nutritional deprivation influences adult exploratory behavior and

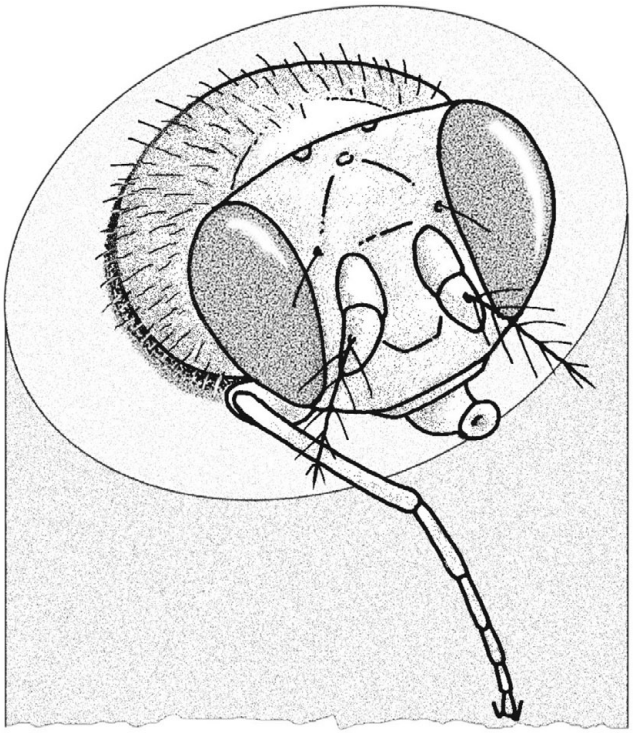

Fig. 15.3. Proboscis extension response (PER) assay. Mounted fly is presented a solution to its tarsi. Fly will extend proboscis upon tasting desired nutrients. (Reproduced from Scheiner et al., 2004. With permission from Cold Spring Harbor Laboratory Press).

fecundity. Larvae fed on an 85% reduction in yeast and sugar throughout their larval lives produced adult flies that showed higher levels of darting exploration in an open field assay. This larval diet-induced change in darting exploration was affected by allelic variation in the *Drosophila foraging* gene. Sitters showed increased darting behavior following dietary treatment; in contrast, rovers were unaffected. This *foraging* gene by early larval nutritional environment interaction also influenced fecundity. In both cases, sitters were more affected by larval nutritional deprivation. Transgenic expression of *foraging* in the mushroom bodies was sufficient to increase adult darting exploration when larvae were reared under standard conditions, but not under conditions of nutritional adversity.

foraging and adult behavior

A useful measure for adult feeding and tasting is the proboscis extension response (PER), where a fly is mounted and presented a solution containing some concentration of sugar. If the fly is able to detect these sugars, they extend their proboscis (Fig. 15.4). This assay has been used to demonstrate that *for* also affects gustatory behavior of adult flies (Scheiner et al., 2004). for^R adult food deprived flies displayed a higher sucrose response than for^s sitter flies and sucrose responsiveness became increasingly rover-like as the duration of food deprivation increased. Additionally, rovers took longer to habituate to a feeding stimulus than sitters. A subsequent study that used

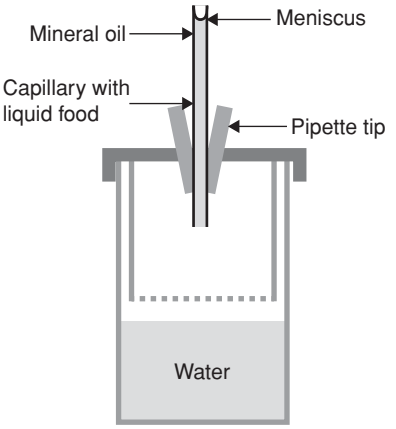

Fig. 15.4. The capillary feeder (CAFE) assay delivers liquid food to the fly in a quantifiable manner. Mineral oil prevents rapid evaporation, and the chamber provides humidity. (Modified from Ja et al., 2007. With permission from the National Academy of Sciences).

the GAL4-UAS (Brand and Perrimon, 1993) to transgenically increase the expression of *for* pan-neuronally in a sitter genetic background and showed a significant increase in SR (Belay et al., 2007).

Flies must land on and walk across a substrate to locate food. Nagle and Bell (1987) examined the responses of individual food-deprived adult flies following ingestion of a sucrose drop and reported significant rover–sitter differences in their patterns of post-feeding locomotion. Specifically, sitters remained close to the drop exhibiting what is called intensive search behavior, whereas rovers walk away from the drop in what is called ranging. Pereira and Sokolowski (1993) reported very similar post-ingestion locomotory differences between wild-type for^R rovers and for^s sitters and for^{s2} sitter mutants. As with the larvae, there were no strain differences in adult locomotor behavior in the absence of feeding stimuli. These findings demonstrate that the known polymorphism in larval foraging behavior mediated by naturally occurring variation at *for* persists into adulthood, where it influences feeding-related phenotypes.

Tortorici and Bell (1988) compared the relative foraging success of rovers and sitters in different feeding environments. A foraging assay consisted of either single or multiple feeding patches. Rovers showed a greater tendency to leave a food patch and were less likely to return to that patch. In multi-patch feeding assays, sitters never left their initial patch, whereas rovers commonly left their starting patch and found other patches. This pronounced difference between the two behavioral morphs supports the hypothesis that the rover foraging strategy in adults, as in larvae, promotes the discovery of novel feeding environments. As with larvae, rover and sitter adults exhibited phenotypic plasticity in response to food deprivation and food quality (Bell and Tortorici, 1988). The consistency with which the behavioral differences across strains persisted through two different life stages has prompted the question of whether or not function conservation of *for* persists across species.

for in other taxa

Gene function can be conserved across species. Knowledge of gene function in one species can be used to understand its

function in other species – this is known as the candidate gene approach (Fitzpatrick et al., 2005). Using the candidate gene approach, the *foraging* gene of *D. melanogaster* has been identified as a gene influencing foraging and food-related behaviors in a number of other species. Below, we describe the role for *foraging* orthologs in *C. elegans,* honey bees, and ants.

The nematode worm, *C. elegans*, feeds on bacterial lawns. As they feed, the worms dwell on their food and exhibit low locomotion. A loss of function mutation in *egl-4*, a gene that encodes a cGMP-dependent protein kinase (PKG), resulted in an increased prevalence of roaming behavior, where the worms moved faster and for longer periods of time (Fujiwara et al., 2002). This is opposite to the gene expression pattern differences in *Drosophila*, where the higher levels of PKG led to more rover-like behavior. Another mutation in *egl-4,* resulted in a novel dominant allele that exhibited increased gene expression and influenced a number of phenotypes including body size and intestinal fat storage (Raizen et al., 2006). Like *for* in *D. melanogaster*, *egl-4* is highly pleiotropic, influencing chemosensory behavior, synaptic transmission, dauer formation, olfactory responses, body size, and lifespan (Daniels et al., 2000, L'Etoile et al., 2002, and Hirose et al., 2003).

Honey bee (*Apis mellifera*) workers undergo an age-based transition from nursing duties, while young towards foraging for the hive at later ages. Ben-Shahar et al. (2002) found that this nurse to forager transition, which occurs at around 3 weeks of life, is associated with changes in the expression of *Amfor* – the honey bee ortholog of *for*. Forager heads from three different colonies contained higher amounts of *for* mRNA compared with nurse heads. Although the transition from nurse to forager occurs typically with age, it is possible for nurses to become foragers precociously if the needs of the hive demand it. When colonies with only young workers (1 day old) were established, within 1 week many of these workers took on the role of foragers precociously. These precocious foragers also had higher *Amfor* mRNA in their heads relative to their nurse cohorts. To further investigate the causal role of *Amfor* in the foraging behaviors of honey bees, pharmacological treatments were utilized. 8-Br-cGMP, a PKG activator was fed to nurse bees, which subsequently led to a cumulative increase in foragers in the colony. This demonstrated that, although thousands of genes (Whitfield et al., 2003) are known to differ between nurse and forager bees, it was sufficient to change the level of the *foraging* gene enzyme to induce a change from nurse to forager (Ben Shahar et al., 2002).

Like honey bees, ants also exhibit social structure with behavioral castes. Red harvester ants, *Pogonomyrmex barbatus*, are divided into two distinct worker castes. Young workers remain in the nest while older workers forage outside. This is similar to the honey bees with the exception that the *foraging* expression is reversed, workers were shown to have higher *for* mRNA compared to foragers (Ingram et al., 2005), but see also Ingram et al. (2011). Another ant species, *Pheidole pallidula* has two morphologically distinct castes, majors and minors. Majors are larger and are primarily used for colony defence, while minors are typically foragers. In unmanipulated colonies PKG activity in the heads of majors was found to be higher than that of minors (Lucas and Sokolowski, 2009). The duties of each caste were not exclusive and one caste could be opted to assist the other when the situation required. For instance, minors recruited majors to assist in foraging when the prey was too large for the minors to handle on their own. This prey stimulus, resulted in a significant decrease in PKG activity in majors and minors relative to control situations. Stimulating foraging behaviors was also possible by food deprivation and PKG activity was decreased in majors during this treatment.

This behavioral flexibility was present in minors as well. When intruder ants were introduced to the colony, brain PKG was significantly higher in both majors and minors relative to controls (i.e., introduction of a nest mate). If differences in PKG activity levels is related to foraging and defense behaviors, then these trends suggest that increased PKG activity in both castes would both reduce foraging and increase defensive behaviors. Indeed, application of 8-Br-cGMP reduced the number of workers interacting with a prey stimulus (Lucas and Sokolowski, 2009). PKG activation increased defensive behavior in major workers only. However, it is also possible that the minor workers' response to a defence stimulus was increased, but that the responsive behavior was not characterized in the assay (e.g., egg removal from brood chambers).

That the *foraging* gene is conserved and plays a role in distinct behavioral phenotypes across various taxa is apparent. How *for* operates to produce the varying behaviors in each species may be unique to each, but the answers are bound to be discovered by looking at where, when and under what environmental circumstances *for* is expressed (Reaume and Sokolowski, 2011). One common location is neuronal. In bees, the expression differences were localized to the optic lobes and Kenyon cells of the mushroom bodies; In ants, there was differential expression in the mushroom bodies and suboesophageal ganglion between majors and minors.

Future questions

Normal individual differences in food-related behaviors are found in natural populations of *D. melanogaster*. These differences arise from interplay between genes and the environment. Although allelic variation can predispose an individual to behave in a certain manner, the environmental context affects the expression of these genes. Individuals carrying one allele may be more plastic or sensitive to the environment than those with another allele.

Foraging-related behaviors are particularly sensitive to an individual's internal and external environments, as it is expected that organisms with different metabolic phenotypes will behave differently in the same environment. We have discussed how food composition, availability, distribution, nutritional balance, experience, and early nutritional adversity all contribute to the generation of foraging-related behaviors. Between-species comparisons from a candidate gene approach (Fitzpatrick et al.,

2005) and the use of a nutritional geometric framework (Simpson et al., 2010) may contribute to an understanding of gene–environment interactions in foraging-related traits. Epigenetic phenomena may be of particular interest to the question of how organisms cope with environmental change (Kramer et al., 2011).

Although we did not review the mechanistic literature on feeding, it is worth pointing out that there are many questions currently being investigated in this domain that are important for our understanding of foraging behavior. For example, how is the environment perceived, how is the information encoded, and how do decisions about foraging change pre- and post-ingestion? Additionally, how is nutritional information communicated to the brain, what tissues are involved, how does the environment influence communication between these tissues? Gut, fat, endocrine organs, and the immune system all contribute to this conversation with the brain. Which signaling pathways and neural circuits are involved in this communication and are they conserved across species and life stages? Finally, are there critical periods in the development of these circuits? These questions provide thought-provoking fodder for future studies.

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Chapter 16

Using neuron specific RNAi in *Drosophila* for understanding the molecular and neuronal basis of behavior

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Introduction

Research into the genetic basis of behavior encompasses a large variety of scopes. While genes are being found to code for an expanding number of functions (ranging from coding to non-coding) (Brent, 2008; Negre et al., 2011; Graveley et al., 2011) their possible implications in functional neuroscience continues to grow. There are multiple reasons why studying genes in the context of the nervous system has been and continues to be highly relevant for understanding its function. Genes code for proteins and small regulatory RNAs which control the transcriptional and post transcriptional signaling that guides the patterning, differentiation and wiring of the nervous system. Understanding how specific neurons are interconnected and how they obtain a specific identity is not only a highly important endeavor by itself but it also gives us a genetic access to these populations, allowing us to manipulate them at the molecular level (Manoli et al., 2005; Stockinger et al., 2005; Scott et al., 2005). More recently, however, the field has focused on identifying gene products which confer specific properties to neuronal ensembles allowing them to fulfill behaviorally relevant roles in the animal. Examples for these are genes coding for molecular machineries controlling plasticity of neurons (Margulies et al., 2005; Abel and Lattal, 2001), sensory receptors enabling neurons to detect physical properties of the external environment (Yarmolinsky et al., 2009; Kaupp, 2010), receptors allowing neurons to react differentially to neuromodulators and neuropeptides (Blenau and Baumann, 2001; Piomelli, 2003) and molecular machineries allowing neurons to count the passage of time (Yu and Hardin, 2006). Ultimately, the power of genetics in the context of the neuronal basis of behavior, which is what we will focus on in this chapter, is the ability to non-invasively, manipulate highly complex molecular processes in specific neuronal populations, be it in a hypothesis-based or hypothesis-free approach.

The use of *Drosophila melanogaster* genetics to dissect the biological basis of behavior was largely pioneered by Seymour Benzer (Konopka and Benzer, 1971; Vosshall, 2007). His ground-breaking work on circadian rhythms, courtship, and memory exposed both the promises and the challenges posed by classic chemical mutagenesis screens for genes governing behavioral responses. The past three decades of *Drosophila* neurogenetics have witnessed a dramatic expansion of the genetic toolset available for the analysis of the molecular underpinnings of behavior (Venken et al., 2011), which together with new and improved behavioral paradigms and assays like the gap-climbing assay (Pick and Strauss, 2005), the aggression assay (Chen et al., 2002) or the nutrient choice assay (Ribeiro and Dickson, 2010) has led to a real gold rush in the field (Vosshall, 2007; Sokolowski, 2001). One of the most promising tools is tissue-specific RNAi (TSRi). It allows for neuron-specific inactivation of defined gene functions enabling the experimenters to efficiently identify new gene functions and probe their involvement within specific neuronal subsets. This approach has allowed the field to follow in the steps of the Benzer lab and expand our understanding to such diverse behaviors as sleep (Luo and Sehgal, 2012; Tomita et al., 2011; Chung et al., 2009), nociception (Neely et al., 2010a) and post-mating behaviors (Yapici et al., 2008; Chapman et al., 2003).

In this chapter we will describe the use of TSRi in *Drosophila* to study the molecular and neuronal basis of behavior. We will start by describing this technology, discuss its advantages when compared to other gene disruption methods, review the history of this approach, present the currently available transgenic RNAi resources, discuss the different types of uses of the method focusing on large-scale screens, target verification and neuronal mapping, and finish by elaborating on opportunities and open questions on the use of this new gene analysis method.

TSRi as a novel gene discovery and analysis tool

The challenges posed by a classic chemical mutagenesis screen for a behavioral response are obvious. Additionally to the difficult and time-consuming primary screen, the fact that the identification of the affected gene relies on the statistical scoring of every single recombinant chromosome using a behavioral assay makes it extremely difficult to map the locus of interest. Behavior can also only be studied in the developed *Drosophila* larva or imago and it is therefore very difficult to analyze genes involved in development which can lead to lethality.

Behavioral Genetics of the Fly (Drosophila melanogaster), ed. J. Dubnau. Published by Cambridge University Press.

Furthermore, despite recent progress (Bellen et al., 2011), transposon mediated gene disruption is biased (Bellen et al., 2004) making it difficult to efficiently generate mutants in all genes.

The sequencing of the complete genomes of higher organisms, including *Drosophila melanogaster* (Adams et al., 2000) asks for an approach which allows a systematic, non-random probing of gene functions in complex processes. In the last decade RNA interference (RNAi) has emerged as a novel approach for performing such functional genomic analyses. This method is based on the seminal discovery of endogenous gene silencing mediated by double-stranded RNA (dsRNA) in *Caenorhabditis elegans* (*C. elegans*) (Fire et al., 1998; Montgomery et al., 1998). In *Drosophila,* as in all metazoans, RNAi can be triggered via distinguishable mechanisms: the small interfering RNA (siRNA), the microRNA (miRNA), and the piwi-interacting RNA (piRNA) pathways (extensively reviewed in (Kim et al., 2009; Carthew and Sontheimer, 2009; Czech and Hannon, 2011; Ghildiyal and Zamore, 2009). Only the siRNA and miRNA pathways require activity of Dicer (Lee et al., 2004; Vagin et al., 2006), a dsRNA-specific ribonuclease (Bernstein et al., 2001), and are the pathways used for experimentally inducing gene silencing. While in worms and mammals, one Dicer protein feeds the siRNA and miRNA pathways (Hutvagner et al., 2001; Grishok et al., 2001; Ketting et al., 2001; Knight and Bass, 2001), in *Drosophila* two Dicer proteins co-exist: Dicer-1 (Dcr-1) and Dicer-2 (Dcr-2) (Lee et al., 2004). In the siRNA pathway, long dsRNA molecules are cleaved by Dcr-2 in the cytoplasm, yielding siRNA duplexes (~21 nt). siRNAs are then loaded into Argonaute-2 (AGO2) to form the RNA-induced silencing complex (RISC) that mediates endonucleolytic degradation of complementary mRNA targets, ultimately leading to silencing of the corresponding gene. In the miRNA pathway, a 60–70 nt pre-miRNA molecule containing mismatches, as well as specific secondary stem–loop structures is processed by Dcr-1 in the cytoplasm, generating a ~22–23 nt microRNA/microRNA* duplex. This duplex is then loaded into Argonaute-1 (AGO1) to form the RISC, leading in this case primarily to translation repression and destabilization of complementary mRNA targets. The association of siRNAs with AGO2, and of miRNAs with AGO1 is a general rule, but exceptions have been discussed (Czech and Hannon, 2011). Less common mechanisms for target regulation by siRNAs and miRNAs have also been highlighted (Kim et al., 2009; Carthew and Sontheimer, 2009; Czech and Hannon, 2011; Ghildiyal and Zamore, 2009). In general, gene loss-of-function can be achieved either by "knock-out" or "knock-down" strategies. While gene deletion ("knock-out") always leads to a complete loss-of-function, siRNAs and miRNAs trigger post-transcriptional gene silencing (sometimes incomplete), commonly referred to as "knock-down."

dsRNAs can be produced either *in vitro* and injected into the organism, or endogenously by the expression of a "hairpin-loop RNA," which upon transcription folds back on itself and thereby produces the desired dsRNA. This approach is termed transgenic RNAi. In contrast to other organisms such as *C. elegans* in *Drosophila* RNAi is cell autonomous. Therefore, transgenic RNAi combined with the Gal4-UAS system for controlled gene induction in *Drosophila* (Brand and Perrimon, 1993) allows the silencing of genes of interest in almost any tissue in a time-specific manner (Kennerdell and Carthew, 2000). Although long dsRNA and therefore the Dcr-2 pathway is the most commonly used silencing approach, both long dsRNAs and artificial miRNAs (shRNAs) can nowadays be transgenically delivered in *Drosophila* using UAS based vectors (Kennerdell and Carthew, 2000; Haley et al., 2008; Ni et al., 2011). It is these techniques called tissue-specific RNAi (TSRi) that this chapter focuses on.

Advantages of TSRi over classic mutant approaches

Compared with classic genetic loss-of-function approaches, TSRi offers many advantages for neurogenetic studies. In the case of behavior, two are most important: its tissue-specific nature and that the identity of the manipulated gene is inherently known.

Especially relevant for behavioral analyses, the tissue-specificity of the tool allows probing the function of genes exclusively within the nervous system. This allows overcoming pleiotropic effects like early lethality, as well as non-specific phenotypes due to the requirement of the gene in other tissues, which are not directly involved in the behavior of interest (Chung et al., 2009; Copf et al., 2011; Liu et al., 2007; Stavropoulos and Young, 2011; Melicharek et al., 2010; Kain et al., 2010). Furthermore, given the inherent cellular diversity and parallel nature of the nervous system, it is very important to be able to probe gene functions in defined and small neuronal subsets. Importantly, this allows researchers to identify neuronal subsets in which specific molecular functions are required for the generation of the studied behavior. The challenge is to achieve a loss-of-function condition in the smallest possible set of neurons (ideally only the ones of interest), while disrupting the gene function in all neurons of interest. Clonal approaches like MARCM (Lee et al., 2000) have been successfully used for identifying and characterizing molecules involved in neurodevelopmental processes (del Valle Rodriguez et al., 2012). Unfortunately their use for behavioral studies is limited. The challenges faced can easily be exemplified in the case of the olfactory system. As there are many neurons expressing the same olfactory receptor, removing the receptor from only a subset of neurons should still allow the remaining neurons to detect and convey the olfactory information to the brain, obscuring the loss-of-function effect in the targeted neurons. It is this requirement which makes it very difficult, or almost impossible, to use classic clonal approaches like MARCM for systematically studying gene functions in behavior. They normally produce multiple clones, and being stochastic in nature requires correlating the observed behavioral phenotypes with concomitant large number of histological analyses. Additionally, if the recombinase would be driven by a specific Gal4 line, the mosaic nature

of mitotic clonal approaches would always produce non mutant sister clones (Golic, 1991) making it very difficult to recover a homogenous mutant population. Methods in which the sister clones are genetically ablated (for review see Blair, 2003) are also poorly suited, as it would be very challenging to assign the cause of the obtained behavioral phenotype to either the loss of the molecular function or to the function of the ablated neurons.

The second big advantage of TSRi is the capacity to immediately determine the identity of the gene eliciting the desired phenotype without the need for time-consuming and sometimes challenging positional cloning steps. Knowing the behavioral phenotype (or absence of it) associated to specific gene functions also allows for systems biology-type analyses; within screens and among screens.

Furthermore, knowing the gene identity also allows the experimenter to choose to directly test specific genes of interest, groups of genes, or all genes in the genome. When working with very large numbers of TSRi lines, one can gain valuable time for the laborious detailed follow-up analysis by first prioritizing groups that are more likely to generate insightful phenotypes.

We will focus on two main caveats of the method, off-targeting and the partial nature of the knock-down as well as how to circumvent them in a later section.

History of TSRi

In 2000, three independent laboratories reported the first successful cases where inheritable transgenes delivering dsRNA, could trigger RNAi in *Drosophila* (Kennerdell and Carthew, 2000; Lam and Thummel, 2000; Fortier and Belote, 2000). In these seminal pieces of work transgene induction was achieved either by heat or by Gal4-inducible promotors. In the same year, circadian behavior in *Drosophila* was shown to be disturbed by silencing *period* in TIMELESS neurons, using the Gal4-UAS system (Martinek and Young, 2000). This was the earliest report using TSRi within *Drosophila* behavioral neuroscience, illustrating how it can aid characterizing the loss-of-function effect of specific genes identified by reverse genetics using nervous system or even neuron specific loss-of-function situations. Since then, TSRi has been widely used to address the molecular basis of many different behaviors. These include learning and memory (Liu et al., 2007; Wu et al., 2007; Lee et al., 2011), circadian rhythms (Luo and Sehgal, 2012; Chung et al., 2009; Nagoshi et al., 2010), and feeding behavior (Lee et al., 2004; Krashes et al., 2009; Wu et al., 2005).

Given that about 45% of the fly genes share an ortholog with humans, and ~53% of human disease genes are conserved in *Drosophila* (Forslund et al., 2011), this organism has emerged as a model organism for human disorders, including neurological (Lessing and Bonini, 2009). As studying human genetic disorders in *Drosophila* requires knowing the molecular nature of the gene of interest, neuronal specific TSRi has been the method of choice for innumerous behavioral analyses of such disorders. These include Fragile X syndrome (Bushey et al., 2009; Bolduc et al., 2008), Alcoholism (Schumann et al., 2011) and many others (Melicharek et al., 2010; McQuibban et al., 2010; Gonzales and Yin, 2010; Pak et al., 2011; Gouzi et al., 2011; Nedelsky et al., 2010; Orso et al., 2005; Faust et al., 2009; Feiguin et al., 2009; Lin et al., 2011; Singh et al., 2010).

Despite being highly informative, early uses of TSRi relied on the prior identification of specific candidate genes to be tested (reverse genetics) and therefore missed one of the biggest advantages of using the *Drosophila* system: the possibility to perform unbiased forward genetic screens. Pioneering work in the nematode *C. elegans* using dsRNA injection, soaking or feeding had proven that RNAi can be used to systematically probe large numbers of genes including covering the whole genome (Carpenter and Sabafini, 2004; Boutros and Ahringer, 2008). In *Drosophila*, large-scale cell-based screens based on exogenously synthesized dsRNA libraries allowed the investigation of innumerous aspects of cell biology (Mohr et al., 2010), including axonal outgrowth (Sepp et al., 2008). Given that TSRi relies on the generation of transgenic lines for every gene of interest, adapting this technique to whole genome wide screens is a gigantic infrastructural and technical challenge. It requires designing, cloning, generating, and maintaining transgenic fly lines, stably carrying RNAi inducing transgenes for all genes in the genome. Indeed, three independent initiatives motivated by the work of Ryu Ueda, Barry Dickson, and Norbert Perrimon originated the three public collections of RNAi flies available nowadays. In 2005, the National Institute of Genomics-FLY (NIG-FLY) initiative released the first 1000 transgenic lines of a collection that today comprises 11 726 RNAi lines. The first full-genome RNAi library comprising 22,270 transgenic lines covering 88% of the genome was released in 2007 by the Vienna *Drosophila* Research Center (VDRC) (Dietzl et al., 2007), and was updated with 10 740 additional new generation lines in 2009 (Keleman et al., 2009). The latest published library, the Transgenic RNAi Project (TRIP) (Ni et al., 2009), currently distributes around 7000 lines with good perspectives of the full-genome coverage in the near future (Ni et al., 2011). The onset of publicly available RNAi collections fostered the first neuronal genome-wide RNAi screens tackling behavior in *Drosophila*. These screens allowed the identification of key neuronal molecules governing behavior. In 2008, the *Sex Peptide Receptor* (*SPR*), mediating a post-mating switch in reproductive behavior was identified in a pan-neuronal RNAi screen (Yapici et al., 2008). More recently, a full-genome pan-neuronal RNAi screen for genes involved in thermal nociception allowed the identification of *straightjacket* (*stj*), which culminated with uncovering a pain sensitivity gene in humans (Neely et al., 2010a). Both screens were performed using the VDRC libraries.

Available RNAi collections and targeting tools

RNAi collections

Since the first public collections of transgenic RNAi lines were made available to the *Drosophila* community, neuroscience

research requiring RNAi-based tools saw immense progress, not only by enriching the targeted gene set, but also by allowing a constant technical improvement of RNAi based experiments. Three collections are publicly available nowadays: the VDRC (www.stockcenter.vdrc.at), the NIG-FLY (www.shigen.nig.ac.jp/fly/nigfly/), and the TRIP (www.flyrnai.org/TRiP-HOME.html) collections. These collections together publicly released more than 50 000 independent UAS-RNAi lines, allowing a virtual coverage of almost all the *Drosophila* genome using different targeting sequences of which the VDRC collection is the most complete one. Beyond distributing the RNAi lines on a per cost basis the centers also provide control background lines as well as lines for enhancing RNAi efficiency.

Targeting tools

Initially, fly transgenesis in these collections relied on random P-element transformation of RNAi delivering vectors (Vienna GD series, NIG-FLY). This could yield significant variability of gene silencing efficiency among independent RNAi lines due to insertion site effects (Dietzl et al., 2007). In order to circumvent this limitation, phiC31-mediated site-specific integration (Groth et al., 2004) of RNAi delivering vectors has already been adopted in more recent collection releases (Vienna KK series, TRIP Valium 1/10/20 series). In addition, the incorporation of *gypsy* insulator sequences in some RNAi delivering vectors has significantly enhanced the knock-down efficiency (TRIP Valium 10/20 series) (Ni et al., 2009). Furthermore, the improvement of bioinformatics tools for the design of the targeting constructs, e.g., Next-RNAi (Horn et al., 2010) and DSIR (Vert et al., 2006), used in the new generation libraries is thought to have reduced non-specific targeting events, while also strongly enhancing the specific knock-down efficiency.

Until very recently, all the RNAi delivering vectors employed to generate these collections promoted the generation of long (300-600 bp) dsRNAs as gene silencing initiators. Long dsRNAs were proven effective in generating loss-of-function phenotypes in somatic tissues, but were proposed to be very inefficient at silencing genes in the *Drosophila* female germline (Ni et al., 2011), a limitation which recently has been proposed to be circumventable (Wang and Elgin, 2011; Handler et al., 2011). Furthermore, the TRIP initiative released a new set of transgenic lines based on shRNAs generating vectors that allow potent gene silencing in the germline (Valium 22) and simultaneous potentially high efficiency knock-down in the soma (Valium 20) (Ni et al., 2011). It is expected that in the next years, a full-genome transgenic library with these new generation vectors will be available (Ni et al., 2011).

The large number of transgenic RNAi lines offered by the currently available collections increases the chance of easily testing distinct RNA hairpins targeting a single gene, which confirms phenotype specificity. Even for cases where multiple TSRi lines are not available for a given gene, the pGD264 (pMF3) (Dietzl et al., 2007), the pKC26 and pKC43 [85], the pUAST-R57 (Pili-Floury et al., 2004), and the VALIUM (Ni et al., 2009, 2011; Keleman et al., 2009) series vectors used in the VDRC, NIG-FLY, and TRIP initiatives, respectively, can be readily obtained and independent transgenic lines generated. Additionally, alternative vectors to generate *Drosophila* transgenic RNAi lines have been developed by various research groups (Haley et al., 2008, 2010; Lee and Carthew, 2003; Kondo et al., 2006, 2009; Kalidas and smith, 2002; Giordano et al., 2002; Piccin et al., 2001; Reichhart et al., 2002; Chen et al., 2007), and some are available at DGRC (https://dgrc.cgb.indiana.edu/vectors/).

From gene tailored, to gene cluster to genome wide studies

The implementation of TSRi in *Drosophila* neuroscience laboratories allowed gaining fast insights on individual gene functions within the nervous system. Indeed, the gene and neuronal specific manipulations permitted by TSRi allow aiming at the identification and study of genes governing behavior in *Drosophila*. With this aim three distinct approaches using TSRi can be envisaged: (i) gene tailored, (ii) gene cluster, and (iii) genome-wide studies. Following any of these depends largely on a particular laboratory/ research project objective.

Gene-tailored studies

Gene-tailored studies using TSRi are useful for pinpointing the relevance of a single gene for a behavior of interest. Furthermore, by confining gene knock-down to the fly nervous system these studies are particularly valuable in confirming the neuronal cell-autonomous dependency of a behavioral phenotype (Stavropoulos and Young, 2011; Bolduc et al., 2008; Pak et al., 2011; Kang et al., 2011; Lim et al., 2007, 2011; Ishimoto et al., 2009; Hamada et al., 2008; Eddison et al., 2011; King et al., 2011; Chen et al., 2011). In the context of neuronal populations, combining TSRi with Gal4 lines that drive expression in specific neurons has proven useful in mapping gene function onto neuronal networks (Martinek and Young, 2000; Krashes et al., 2009; Kang et al., 2011; Lim et al., 2007; Hamada et al., 2008; King et al., 2011; Chen et al., 2011; Hasemeyer et al., 2009; Li et al., 2011). For negative controls the driver can simply be combined with the isogenic host strain in which the tested RNAi line has been generated. These strains can be obtained from the corresponding stock centers. The success of gene tailored studies relies on the existence/ generation of robust transgenic RNAi reagents whose knock-down efficiency and specificity must be experimentally assured beforehand. This is not much different than working with classic gene lesions where the nature of the studied allele has to be ensured with tests for loss-of-function efficiency using different alleles, and the specificity of the observed phenotype has to be confirmed via rescue experiments. We will discuss specific strategies to ensure efficiency and specificity of the observed phenotypes later in this chapter.

Gene cluster studies

A neglected but very interesting approach is to select groups of genes to be tested in form of a small focused screen. The groups of genes can be chosen to reflect many different criteria, but normally are enriched for molecular, functional or genomic features, which make them more likely to give an informative insight into the studied process. These groups can for example compromise genes encoding kinases (Yu et al., 2011) or miRNA targets (Luo and Sehgal, 2012), genes which have been shown to be involved in synaptic plasticity or chemosensation or finally genes which show a high homology to vertebrate genes (Neely et al., 2010) or have been identified in functional genomics approaches to be expressed in neurons of interest (Nagoshi et al., 2010). Obviously, most of these classifications rely on high-quality annotations as attempted by functional genomics initiatives such as the *Kyoto Encyclopedia of Genes and Genomes* (KEGG) (Kanehisa et al., 2012), the Gene Ontology consortium (GO) (Ashburner et al., 2000), or modENCODE (Celniker et al., 2009). While the improvement of these annotations will strongly benefit the design and analysis of such focused small scale RNAi screens, TSRi can also be an essential tool for improving gene annotation (Groth et al., 2008; Mummery-Widmer et al., 2009; Schnorrer et al., 2010).

Being able to select the genes to be studied is a special advantage of TSRi and highlights one of its biggest strengths. The interest of this approach is the reduced time and effort required to screen a small subset of the genome (e.g., 100–200 genes) while dramatically reducing the costs and infrastructure associated with a full-genome approach. Furthermore, having a starting hypothesis allows one to generate a subgroup of candidates simplifying the incorporation of the identified molecular components into a meaningful mechanistic framework. As an example, starting from a gene set retrieved from a whole-genome microarray (Ueda et al., 2002), 133 genes were screened with TSRi and 5 novel circadian genes could be identified (Matsumoto et al., 2007). Similarly, the identification of *nemo* as a component of the circadian oscillator was achieved by screening a CMGC kinase subset for short-period rhythms (Yu et al., 2011; Chiu et al., 2011). Another advantage of this strategy is that focusing on smaller set of genes will allow one to screen multiple RNAi lines, possibly even from different libraries decreasing the risk of false negatives. Also, the possibility to obtain more replicas of the same genotype will lead to a reduction in false positives, which is especially relevant for behavioral approaches where the assays are inherently noisier than cellular readouts. Finally, an interesting possibility offered by concentrating on a smaller subset of genes is to focus on gene functions and test these in a panel of several behavioral assays uncovering the differential requirement of genes in multiple behaviors.

Genome-wide studies

The possibility to perform unbiased forward genetic screens has been one of the main reasons for the success of *Drosophila* (St Johnston, 1967). When the laboratory or the field has a special interest in a specific behavior, performing a whole genome screen is an interesting possibility to consider. This specially applies to novel questions, which make it very difficult and sometimes misleading to propose any specific molecular or functional hypotheses on which genes could be underlying the behavior of choice. The advantages of this approach are its comprehensive scale and the fact that it is non-biased. Being comprehensive allows systems biology type of analyses while being non-biased allows for truly novel and unexpected insights beyond solutions fitting a predefined mechanistic framework. Despite being an approach with unmatched potential, the scale of an approach such as a full-genome screen requires compromises to be made at the level of data collection, so that a reasonable throughput is achieved. The effort required for testing ~13 000 genes makes behavioral assays requiring detailed observations or time consuming manual scoring very difficult to screen. Ultimately, the screeners have to design a strategy that will allow them to test all the genes in the genome in a realistic time frame, while allowing for a reasonable false positive to false negative ratio. These parameters are set using control genes as well as pilot screens which allow for a reasonable calibration of the full-genome screen. Realistically, one has to commit a minimum of 1 year for a whole genome screen requiring a steady stream of around 250 tested genotype per week.

Designing and performing large scale screens

The scale of full-genome or other large-scale screens (e.g., all vertebrate conserved genes) requires a special type of experimental design and strategy. In general, screens can be divided into four different stages: the preparatory, the primary screen, the validation and secondary assay, and the screen analysis stages. We will discuss the main points to be considered when designing and performing the screen. However, as for all experiments, the strategy to be employed depends very much on the question asked, and devising a successful and smart screen is as much a technical skill as a creative enterprise.

Preparatory stage

Assay development

The preparatory stage is the most decisive one in any screen. Most importantly, the experimenters have to develop a behavioral assay, which will allow them to score for a specific phenotype while allowing for a reasonably high throughput. General wisdom has it that the more specific the scored phenotype is, the more successful the screen will be. Ideal assays are very robust, therefore requiring few replicas, being easy and fast to score, and producing easily classifiable and recordable phenotypes. The phenotypes should be either *all or nothing* qualitative ones (e.g. copulation or no copulation) (Yapici et al., 2008), or alternatively quantitative ones using a predetermined scalable scoring system going for example from 1–10 (e.g., rate of impairment in flying) (Dietzl et al., 2007). It is important to note

that assays can also be devised in which one does not directly score for the behavior of interest, but for the consequence of such a behavior (e.g., egg laying). Despite indirect measurements being more prone to misinterpretations and non-specific results, the possibility to screen large numbers of flies in a short time can justify such approaches (Yapici et al., 2008). The probabilistic nature of behavior, the sensitivity of behavior to changes in environmental variables, as well as the complexity of behavioral descriptions make devising a screenable behavioral assay a special challenge. The classic example of the countercurrent setup developed by Seymour Benzer for screening for phototaxis mutants (Benzer, 1967), however, shows that these limitations can be overcome with ingenuity. Furthermore, the development of high throughput video tracking and automated behavioral analysis is opening the possibility to perform large-scale screens for more complex behaviors such as those relying on social interactions (Dankert et al., 2009; Branson et al., 2009).

Choosing the Gal4 driver

One of the most important decisions when designing an *in vivo* RNAi screen is the choice of the appropriate Gal4 line. The line has to be temporally and spatially specific enough to exclude non-specific phenotypes such as lethality or unwanted developmental effects, and strong enough to allow for a robust knock-down (Mummery-Widmer et al., 2009). Depending on the available knowledge of the neuronal basis of the examined behavior, the choice can range from pan-neuronal Gal4 lines to drivers for specific neuronal subpopulations. Given the goal to uncover unexpected mechanisms, and the fact that full-genome screens are often performed for poorly understood biological phenomena, most *in vivo* neuronal RNAi screens have been performed using the *elav-Gal4* pan-neuronal driver (Neely et al., 2010a; Yapici et al., 2008). Because it is expressed at high levels (Bushey et al., 2009), the pan-neuronal *nSyb-Gal4* driver (generated by Julie Simpson) (Pauli et al., 2008) is a good alternative for performing neuronal RNAi experiments (Pospisilik et al., 2010). If the screen is successful, the identified RNAi lines can be used to identify Gal4 lines marking the neurons in which the gene function is implemented as done in the case of *SPR* (Hasemeyer et al., 2009), and these Gal4 lines can then be used to rescreen the lines which produced non-specific phenotypes. Furthermore, as described later, in the case of neuronal RNAi experiments it is advisable to increase the efficiency of the RNAi knockdown by concomitantly overexpressing *Dcr-2* (Neely et al., 2010a; Yapici et al., 2008; Dietzl et al., 2007). This can be easily done by generating a double balanced line which contains the Gal4 driver and the *UAS-Dcr-2* transgene.

Choosing the conditions of the screen and the pilot screen

The critical conditions of the screen include which Gal4 line to use, the screening temperature, the number of replicas, and the sex and age at which the flies are tested, use of selected positive and negative control genes, and calibrating the conditions to obtain a reproducible and strong behavioral phenotype with the positive controls, while observing no effects with the negative control genes (Neely et al., 2010a; Mummery-Widmer et al., 2009). In cases for which no control genes are known, the focus has to be on ensuring that the control background (the Gal4 line together with *UAS-Dcr-2* crossed to the library background) does not produce a scorable phenotype while using screening conditions that were successful in other similar screens.

The final step of the screen preparation is performing a pilot screen. In this screen a set of a few hundred randomly selected RNAi lines, together with the control genes, are tested under the same conditions as the planned full-genome screen. The goal is to assess the feasibility of the screen and to ensure that the positive controls are picked, while few of the randomly selected lines score as positive. It is also at this stage that the strategy for defining mutant phenotypes is normally determined. This is not a trivial task as behaviors are often scored quantitatively, and RNAi knock-downs are often partial, sometimes leading to weak phenotypes of even key genes. Statistical approaches such as the z-score are often used (Birmingham et al., 2009), but the screeners can also decide to use an arbitrary but informed cut-off based on experience or reasonable workload.

Primary screen

The fact that behavioral assays are highly labor intensive makes it difficult to use strategies employing a high number of replicas to ensure a low variability in the wild-type distribution. It is therefore important to carefully decide how to reduce the number of false positives. One can either perform many replicas in the first round of the screen or allow for more false positives in the first round and eliminate them in subsequent post-screen repetitions. If one has, for example, to reduce the noise of the primary screen by performing twice as many assays on all the genes in the genome, that will double the amount of time the primary screen will take. If one performs a lower number of assays in a first round of the screen and then increases the number of assays by retesting the 10% of genes with the best score, this will only increase the screening time by one fifth. For this reason, it is often advisable for a primary screen to use a somewhat lower threshold for defining mutant phenotypes (allowing for more false positives but also for the recovery of weak phenotypes) than would be advisable for tissue culture screens. This has then to be combined with a follow-up retest strategy.

It has to be considered that, in contrast to classic mutagenesis approaches, knowing the identity of the tested gene and having the lines available in stock centers, allows the screeners to retest lines even after changes in the screen analysis strategy without having to maintain the original fly stock.

The primary screen is the longest and normally most labor-intensive part of the screen demanding a long-term focused commitment from the screeners. It consists of systematically crossing all the selected RNAi lines to the Gal4 driver and testing the offspring for behavioral phenotypes using the predetermined assay. Given the number of required assays, it is extremely important to generate a streamlined screening

protocol, schedule, and infrastructure that will support the screen. The fly husbandry part should not be underestimated, and combining the Gal4 line with a Y chromosomal *hs-hid* transgene (Starz-Gaiano et al., 2001) for the easy and efficient generation of virgins is, for example, highly advisable. Great care should also be given to the recording of the behavioral phenotype. The development of a database to keep track of the genotype, phenotype, and screening parameters such as screening day, cross-batch, screener, lethality stage (if observed) as well as general observations is a big advantage. Barcodes for tracking the cross and the assays have proven to be an efficient solution for minimizing typing and tracking mistakes. Furthermore, negative and if available positive control genes should be part of every screening batch. They can be used to ensure the confidence in the assays performed in each batch and can then be combined at the end of the screen to determine the statistical distribution of the phenotypes.

One of the big advantages of RNAi is that it is non-random and therefore, as described before, it is possible to enrich for genes of interest at the beginning of the screen. The availability of stock centers makes it possible to have the lines shipped in a way that allows the experimenters to maintain a steady number of tested lines without having to generate them and having to maintain the negative lines. Depending on the strategy of the screen and the costs of stock maintenance, it is even possible to envisage strategies in which all stocks get discarded after testing and the positive ones get re-ordered at the end of the primary screen.

As discussed above, one of the most widely used screening strategies consists in retesting all lines that achieved a specific phenotype threshold. These lines are then randomly and blindly re-introduced into the primary screen and are kept for further analysis if scored again as positives (Neely et al., 2010a, b; Dietzl et al., 2007; Mummery-Widmer et al., 2009; Schnorrer et al., 2010). Importantly, this enrichment strategy has to be validated by showing that the rate of positives in the group of retested genes is higher when compared to the whole genome set. If this is not the case, the screening parameters or strategy need to be reassessed.

The final result of the primary screen consists in a collection of retested RNAi lines having a high probability of eliciting a behavioral phenotype when combined with a neuronal Gal4 line.

Validation and secondary assays

The lines from the primary screen need to undergo a post-screen validation to ensure the reproducibility and the specificity of the observed phenotype. This can be done at the end of the screen or in parallel to it. Given the large scale of assays performed in the primary screen, it is advisable to test the reproducibility of the identified phenotype using the direct statistical comparison of the knock-down with a control background. Often, instead of using the assay developed for the screen, which has been improved for efficiency, a classic higher quality version is used. If reproducible, the nature of the gene knockdown should be validated using the strategies described below. Alternatively, the gene validation step can be prioritized, and lines with different targeting constructs can be ordered from other libraries and tested as part of the primary screen.

It is also at this stage that the temporal requirement for the identified gene function can be probed using conditional approaches such as TARGET (Temporal and Regional Gene Expression Targeting – a method based on a temperature sensitive allele of the Gal4 inhibitor Gal80) (Tomita et al., 2011; Liu et al., 2007; Wu et al., 2007; Lee et al., 2011; Bolduc et al., 2008; Gouzi et al., 2011; Lim et al., 2007; Eddison et al., 2011; McGuire et al., 2003; Xia and Tully, 2007; Sitaraman et al, 2008) and GeneSwitch (a method based on a hormone-dependent Gal4) (Copf et al., 2011; Ishimoto et al., 2009; Roman et al., 2001; Osterwalder et al., 2001; Roman and Davis, 2002; Han et al., 2000; Jepson and Reeman, 2009). These methods allow for example to test the requirement of gene functions in the adult nervous system.

Given the complexity of the biological processes underlying the generation of behavior, the specific involvement of the identified gene function in the behavior of interest has to be ensured. Here, secondary assays can use related, but orthogonal behaviors to exclude the general behavioral impairment in the mutant animal (Neely et al., 2010a; Kang et al., 2011). For example, in a screen for heat nociception genes (Neely et al., 2010a), secondary defects at the level of phototaxis (Benzer, 1967), geotaxis (Strauss and Heisenberg, 1993) and thermal sensitivity (Siddiqi and Benzer, 1976; Grigliatti et al., 1973) have been excluded from screen hits. Alternatively, known mechanisms interfering with the behavior of interest, but which are not of interest to the screeners, can be excluded with secondary assays, as testing for circadian rhythm perturbations when studying sleep mutants (Chung et al., 2009; Stavropoulos and Young, 2011; Shimizu et al., 2008). Secondary assays can also focus on probing the function of interest using a different assay relying on the same molecular machinery. For example, in the screen for post-mating behaviors, the specificity of *SPR* was tested by assaying female receptivity in addition to egg laying which had been the screening assay (Yapici et al., 2008).

At the end of the post-screen validation and the secondary assays, the experimenters will be rewarded with a unique dataset of genes involved in his behavior of interest.

Screen analysis

Unique for RNAi screens is the possibility to use systems biology analysis tools for mining the full-genome screening results. Comprehensively knowing the identity of the perturbed genes and being able to associate them with a phenotype score is a distinctive feature of RNAi screens. Furthermore, *in vivo* RNAi screens normally produce a large number of positives (e.g., between 580 (Neely et al., 2010a) and 847 (Schnorrer et al., 2010) genes), making it important to analyze them in a systematic and coherent way. The idea is to go beyond the one gene one phenotype paradigm and treat the behavior of interest as a system controlled by networks of interacting gene

products. This approach is widely used in tissue culture screens and has also been applied for analyzing gene sets obtained from *in vivo* whole genome RNAi screens (Neely et al., 2010a; 2010b; Schnorrer et al., 2010). Annotations of gene function are available in public databases such as KEGG (www.kegg.jp) (Kanehisa et al., 2012) or GO (www.geneontology.org) (Ashburner et al., 2000), and in *Drosophila* dedicated databases such as FlyMine (www.flymine.org) (Lyne et al., 2007) or ModENCODE (www.modencode.org) (Celniker et al., 2009). In the last years, innumerous user-friendly web based bioinformatics tools to improve post-screen analysis have become available, from which we highlight several. RNAiCut (groups.csail.mit.edu/cb/RNAiCut) (Kaplow et al., 2009) introduces information from protein–protein interaction networks to aid determining significance thresholds for *Drosophila* functional genomic data. GeneMANIA (genemania.org) (Warde-Farley et al., 2010) allows gene prioritizing based on genomics and proteomics data, and currently integrates 174 *Drosophila* sets of co-expression, protein–protein interaction, or genetic interaction data. COMPLEAT (www.flyrnai.org/compleat) (Vinayagam et al., 2013) estimates the enrichment for literature annotated and predicted protein complexes in a given gene dataset. Two independent databases, FlyRNAi (www.flyrnai.org) (Flockhart et al., 2012), and GenomeRNAi (genomernai.de/GenomeRNAi) (Horn et al., 2007) provide constantly updated results from tissue culture and in vivo *Drosophila* RNAi screens, allowing the easy contextualization of data from novel screens. As a whole, these tools can be used for prioritizing genes to be subsequently studied. This approach is the one that is most commonly used for analyzing *in vivo* RNAi screens, and good examples of its success are the identification of the importance of the COP9 signalosome for Notch signaling (Mummery-Widmer et al., 2009), as well as the identification of NOT3 as a conserved regulator of heart function (Neely et al., 2010b).

A tantalizing possibility is the use of these tools in combination with mathematical models of systems such as network theory for extracting new types of functional information from the screens (Geschwind and Konopka 2009; Przytycka et al., 2010; Alon, 2007). For example, one could attempt to identify groups of proteins working together either in complexes or as information relay systems to generate specific aspects of behavior. Given the complexity of the nervous system and behavior, the possibility to use these sophisticated analysis systems seems highly appropriate. Realistically, however, the novelty of the mathematical approaches, the complexity of *in vivo* systems as well as the technical drawbacks of RNAi such as its variable knock-down efficiency make this a challenging and poorly explored possibility.

Verification of RNAi phenotypes

As with other methods, TSRi has also its drawbacks and requires the same verification care, as with any gene disruption approach. The main caveats to its use are the targeting specificity (false positives) and the variation in the degree of knock-down (false negatives). Even if both issues have been effectively tackled in the design of the new generation of vectors, constructs and librarie, they still require special attention on the side of the experimenter.

Off-targeting

The problem of targeting specificity originates from the worry that the observed phenotype is not due to the loss-of-function of the predicted gene. The main source for such artifacts is the non-specific knock-down of other genes (off-targeting) (Kulkarni et al., 2006; Moffat et al., 2007; Fedorov et al., 2006; Ma et al., 2006; Jackson et al., 2006) or to a less extent, in the case of the libraries based on random transgenesis, due to the overexpression of genes adjacent to the insertion site of the RNAi delivering vector (Dietzl et al., 2007). Off-targeting can be caused by the generation of siRNAs that are able to target non-predicted genes or the presence of so-called CAN (CA[AGTC]) repeats within the expressed constructs (Perrimon et al., 2010). Even if this problem is more prevalent in tissue culture experiments (Perrimon et al., 2010), and is thought to only affect few constructs of the publicly available libraries (less than 2% (Dietzl et al., 2007)), it still requires some consideration for *in vivo* approaches (Ni et al., 2008, 2009). Obviously, the use of genomic mutants, i.e., physical lesion at gene DNA level, to verify the knock-down phenotype is ideal (Yapici et al., 2008; Kain et al., 2010; Ishimoto et al., 2009; Takahama et al., 2012), but sometimes it is not a feasible solution due to the lack of mutants or of a viable allele. These caveats can be circumvented by homologous recombination, which has made exquisitely targeted genomic manipulations a realistic endeavor (Venken et al., 2011). Furthermore, the recent development of TALEN, and CRISPR/Cas-based methods for genome engineering, promises to significantly reduce the effort required for engineering loss-of-function alleles (Gaj et al., 2013). In the future, these new methods might enable the verification of RNAi phenotypes of larger sets of candidate genes using null mutants.

Other experimental strategies to confirm the causal link between gene loss-of-function and phenotype are currently preferred. One strategy relies on the correlation of visible knock-down effects between experiments using different targeting constructs (Tomita et al., 2011; Neely et al., 2010a; Yapici et al., 2008; Copf et al., 2011; Bolduc et al., 2008; Eddison et al., 2011; Yu et al., 2011; Chiu et al., 2011). By analogy to testing multiple alleles in classic mutagenesis approaches, the logic is that, if different non-overlapping RNAi constructs designed to target specifically the same gene lead to the same phenotype, the likelihood that these effects are all due to off-targeting is very low. This approach is especially attractive, as with the availability of different libraries, multiple independent knock-down constructs can be simply ordered and do not need to be designed and transgenic flies generated. The problem with this approach, however, is that, due to differences in knock-down efficiency,

some constructs fail to generate phenotypic effects, leading to a false verification failure. This problem can be overcome by correlating the phenotype strength with knock-down efficiency using qRT-PCR. The identification of multiple genes within a signaling cascade or a molecular complex is also a strong indication for the specificity of each knock-down (Neely et al., 2010b).

An alternative and complementary approach is the use of rescue constructs that encode a functional protein, but with enough sequence divergence to escape targeting by RNAi. They can either evade targeting through the use of an exogenous UTR (Stielow et al., 2008), silent mismatches in the *Drosophila melanogaster* coding sequence (Schulz et al., 2009) or the use of the gene sequence of a closely related species (Faust et al., 2009; Kondo et al., 2009; Ejsmont et al., 2009; Langer et al., 2010). Under certain circumstances, simple overexpression of the unmodified gene has also been observed to allow for the rescue of the knock-down phenotype presumably by diluting out the available siRNAs (Stavropoulos and Young, 2011; Lim et al., 2007).

Knock-down efficiency

A major issue when using RNAi is the onset of false negatives, due to hypomorphic nature of the knock-down phenotype. For the first generation random insertion libraries this problem was estimated to affect up to 40% of genes (Dietzl et al., 2007). Multiple variables can influence knock-down efficiency, some of which have been improved in the subsequent generations of libraries and some which can be influenced by the experimenter. It is, however, clear that ultimately the efficiency of knock-down is gene, tissue, and developmental stage specific, making it very difficult to systematically ensure a complete knock-down for all genes in all tissues. To reduce the false-negative rate, two strategies can be used. First, the screening temperature can be increased from 25 (Neely et al., 2010a) to 27 (Schnorrer et al., 2010) or 29 (Neely et al., 2010b) degrees Celsius, enhancing Gal4-mediated RNAi transgene expression levels and therefore knock-down. Furthermore, if possible, males should be used for RNAi experiments as they have been shown to display stronger RNAi phenotypes than females (Ni et al., 2008). Finally and most relevant for behavioral experiments is the fact that knock-down in post-mitotic neurons seems to be especially inefficient (Dietzl et al., 2007). It is for this reason that when performing neuronal RNAi experiments as a general strategy the use of *Dcr-2* overexpression is advised as it has been shown to double the recovery of RNAi phenotypes (Dietzl et al., 2007). Despite the fact that neuronal *Dcr-2* overexpression has neither been reported to elicit major non-specific phenotypes nor to specifically induce off-targeting events, one needs to be aware of potential *Drc-2* side effects, as for any overexpression situation.

Finally, an advisable validation step can be the verification of knock-down efficiency using qRT-PCR (Tomita et al., 2011; Dietzl et al., 2007; Chen et al., 2011) or antibody-based methods such as Western Blot (Eddison et al., 2011; King et al., 2011; Chen et al., 2011) or immunohistology (Liu et al., 2007; Wu et al., 2007; Hamada et al., 2008). By correlating the level of knock-down with the strength of the phenotype, one can consolidate the confidence in the causal connection between the targeted gene function and the studied process. Given its universality and quantitative nature, qRT–PCR has been the method of choice for the verification of tissue culture RNAi experiments (Mohr et al., 2010). However, the heterogeneous cellular nature of the nervous system, together with the differences in knockdown efficiency among tissues and between Gal4 lines for specific subpopulations of neurons, might pose obstacles in determining knock-down levels with *in vivo* RNAi experiments. The gene of interest could, for example, be expressed in a large set of cells masking the knock-down induced in the targeted small set of neurons. Alternatively, no visible behavioral phenotype upon strong knock-down could be due to the perdurance of a protein or the sufficiency of few molecules to sustain the process of interest, leading to the erroneous dismissal of the requirement of the studied gene. Therefore, despite knock-down quantification methods being advisable and useful, genetic verification methods based on behavioral phenotype reproduction and rescue are the method of choice.

Using TSRi to map neurons

Classically, the goal of behavioral genetics was mostly focused on identifying molecular mechanisms underlying the generation of behavior. While this is still an important goal, the scope of neurogenetic manipulations has expanded over the last decade. Instead of being used for loss-of-function genetics to characterize gene function *per se*, TSRi has been used as a tool to ask new questions beyond gene function. By silencing specific genes, encoding key structural, functional, or fate-determining proteins in different parts of the nervous system, neuronal ensembles underlying specific behaviors could be uncovered using TSRi. Examples of this approach are the use of TSRi against *paralytic,* a gene encoding a voltage-gated sodium channel to silence neurons (Zhong et al., 2010); the silencing of the *white*-ABC transporter to perturb serotonergic and dopaminergic neurons [134]; the use of knockdown of the sex determination gene *transformer* (Fernandez et al., 2010) or the courtship gene *fruitless* (Manoli et al., 2005; Meissner et al., 2011) to probe for neurons underlying sexually dimorphic behaviors like courtship and aggression, and finally the manipulation of genes encoding receptors such as *SPR* (Hasemeyer et al., 2009) or the *neuropeptide F receptor 1* (Krashes et al., 2009) for mapping neurons underlying post-mating behavior and learning and memory.

The identification of *SPR* and the neurons in which it acts in the female to control behavioral changes that occur upon mating illustrates the usefulness of TSRi. *SPR* was originally identified in the first genome-wide pan-neuronal RNAi screen. It was focused on identifying genes important for post-mating behaviors (Yapici et al., 2008). To define the neuronal requirement for SPR function, Häsemeyer and colleagues inverted the logic of

the initial screen, crossing the *SPR* RNAi line to a collection of 998 Gal4 lines and scoring the female progeny for post-mating phenotypes (Hasemeyer et al., 2009). Using this strategy the authors were able to pinpoint the action of SPR to a small subset of internal sensory neurons that innervate the female uterus and oviduct, identifying a novel and unexpected mode of action of the Sex peptide on the female nervous system. *SPR* highlights the power of combining molecular mechanisms identified using genome-wide inducible RNAi screens with collections of Gal4 lines to assign gene functions to specific neuronal subsets.

Future opportunities

Nowadays, TSRi has become a standard technique to characterize molecular functions at the basis of behavior in *Drosophila*. Its use is, however, still mainly restricted to the analysis of specific gene functions and not so much for identifying novel genes using unbiased large-scale screens. Given the fact that the generation of full-genome transgenic RNAi libraries is a rather new development, we expect that large-scale TSRi screens for molecular components controlling specific behaviors will become more widely used. This development will be further spurred on by advances in behavioral assay development as well as by an increased knowledge in the neuronal networks underlying the generation of specific behaviors, and the availability of Gal4 lines targeting these restricted neuronal subsets (Jenett et al., 2012).

Technically, TSRi has undergone a dramatic development over the last years, as witnessed by the implementation of new targeting strategies and by the generation of next generation libraries. While the specificity and the potency of the silencing tools have improved, the validation of the lines is still left to the single experimenter. Therefore, it is foreseeable that the next step in the development of TSRi libraries is not so much to continue improving the technique itself, but to improve the validation and annotation of the specific lines available in the publicly existing libraries. We are aware that this is a considerable challenge, given the fact that the efficiency and specificity of the knock-down effect is dependent on many variables, such as the targeted tissue or the used Gal4. Also, the scale of such verification, for example, by qRT–PCR requires a large amount of resources. Therefore, two strategies can be envisaged to verify the available RNAi lines: one relying on a centralized effort by the organizations maintaining the libraries or dedicate consortia; and an other in which a centralized database is used to collect information gathered by users of the libraries. The result would be a curated database of information on each line available to the community, which would include not only the published observed phenotypes, but also lines which lead to an efficient knock-down without eliciting a phenotype. This will be very valuable when planning experiments and also for the systematic mining of phenotypic data using systems biology approaches.

The available RNAi libraries make it easier and easier to target annotated coding genes. While this fulfills the need of most experimenters, there is still a window of opportunity for approaches in which non-coding as well as products of poorly annotated open reading frames (Ladoukakis et al., 2011) are targeted and tested for their implication in cellular and neuronal processes. This might require the development of new targeting strategies, such as the recently developed shRNAs as well as the generation of new libraries. Such new targeting strategies could be derived from novel endogenous post-transcriptional regulators such as the recently described circular RNAs (Hansen et al., 2013; Memczak et al., 2013).

One of the main hurdles for systematic and comprehensive behavioral genome-wide screens is the large infrastructural and time effort required for performing such big screens. In tissue culture as well as in *C. elegans* this hurdle has been overcome using sophisticated automated and robotics approaches (Boutros and Ahringer, 2008). Adapting these methods to *in vivo* behavioral set-ups is a big challenge, but one which automated behavioral tracking and analysis is making possible, and if complemented with automated fly handling could allow for more ambitious systematic large scale approaches.

In yeast, *C. elegans* as well as in tissue culture, genetic interaction screens have been highly successful in uncovering unexpected mechanistic interactions (Krastev et al., 2011; Horn et al., 2011; Dixon et al., 2009; Lehner et al., 2006; Tong et al., 2004). Expanding the throughput of *Drosophila* behavioral screens would unlock the potential to easily perform genetic interaction TSRi screens.

As mentioned throughout this chapter, one of the main advantages of RNAi approaches is the possibility to do systems biology type of analyses of the data, a possibility researchers have just started exploring. Applying network theory and modeling to large datasets of comprehensive gene - behavioral phenotype relationships will be important for uncovering new non-linear mechanistic relationships underlying the generation of behavior; especially if these analyses rely on data generated with systematically validated tools and data. Furthermore, in analogy to the microarray gene expression field 10 years ago, there is a need for a common ontology and standard for maintaining and sharing *in vivo* RNAi phenotype data. A central and standardized repository for *in vivo* RNAi data would allow the comparative mining of these data across behavioral paradigms with the promise of elucidating general molecular mechanisms at work in the brain to generate different behaviors. Ultimately, the goal is to systematically map gene functions onto different identified neuronal sets, allowing us to generate a model of how different molecular mechanisms act in neuronal networks to generate complex behaviors.

Current behavioral neuroscience research is strongly focused on identifying neuronal networks as well as characterizing how their structure and activity within their neurons correlates with, and affects, behavior. While for these questions the use of rodent, fish and nematode models often has clear advantages, it is difficult to envisage that any of these systems will be able to match the ease with which it is possible to manipulate and investigate the behavioral function of a large

set of molecular mechanisms in specific neuronal subsets in *Drosophila*: an advantage the field largely owes to the possibilities offered by TSRi. We are only at the beginning of systematically exploring the molecular mechanisms acting within specific neurons to generate behavior. A fascinating and rewarding path lies ahead of us.

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Chapter 17

Systems genetics of behavior in *Drosophila*

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Behaviors as quantitative traits: Complex genotype–phenotype relationships

The expression of behaviors is determined by the interplay of coordinated ensembles of genes and environmental factors. Behaviors are unique among complex traits, such as morphological or life history traits, in two respects. First, behaviors are an expression of the nervous system. Thus, understanding behaviors requires not only characterization of their genetic underpinnings, but also understanding of how genetic architecture relates to the function of specific neuronal circuits. Second, most behaviors reflect an interaction between the organism and its environment. Thus, the genetic networks that drive behaviors are likely to show substantial environmental plasticity. Finally, it should be noted that polymorphisms that give rise to phenotypic variation in behavior provide the substrate for natural selection and the stage on which adaptive evolution occurs.

Unlike Mendelian traits, where the genotype allows a direct quantitative prediction of the phenotypic value, predictions of phenotypic values based on genotype are not straightforward for complex polygenic traits (Mackay et al., 2009). Here, the phenotype manifests itself as an emergent property from complex genetic networks. Understanding the relationship between genotype and phenotype requires a detailed characterization of genome-wide spatial and temporal regulation of transcription and intermediate endophenotypes, e.g., the dynamics of the transcriptome, the proteome, and – in some instances – the metabolome, establishing causal relationships between DNA sequence variants, between transcripts within co-regulated and /or interacting networks, and between gene products that mediate cellular and physiological processes that give rise to the organismal phenotype. This complex relationship between genotype and phenotype is not static, but is expected to change dynamically as a function of circadian or seasonal time, changes in physiological state, and constantly evolving changes in social and physical environmental conditions. Whereas until recently, the challenge of such a broad integrative analysis might have been perceived as insurmountable, recent advances in next generation sequencing technology, computational biology, proteomics, and neural imaging, now enable systems genetics approaches to study the complex relationship between genotype and organismal phenotype.

Drosophila melanogaster provides an ideal model organism for systems genetics analyses of behaviors, because large numbers of genetically identical individuals can be reared rapidly and economically under controlled environmental conditions. Flies are amenable not only to extensive genetic manipulations, but also to neuroanatomical and electrophysiological studies, and they display a wide range of behaviors that can be quantified using simple behavioral paradigms, including olfactory behavior (Anholt et al., 1996), courtship and mating (Hall, 1994; Villella and Hall, 2008), gustation (Tanimura et al., 1982; Montell, 2009), foraging (Osborne et al., 1997), phototaxis (Benzer, 1967), negative geotaxis (Hirsch and Erlenmeyer-Kimling, 1962), startle behavior (Jordan et al., 2007; Yamamoto et al., 2008 and 2009), open field locomotion (Valente et al., 2007), aggression (Chen et al., 2002; Edwards et al., 2006), sensitivity to alcohol (Weber, 1988; Guarnieri and Heberlein, 2003), flight (Heisenberg et al., 2001; Frye and Dickinson, 2004), circadian behavior (Konopka and Benzer, 1971), learning and memory (Quinn et al., 1974; Tully and Quinn, 1985), and sleep (Harbison et al., 2009a).

Genes that contribute to behavior can be classified as genes that contribute to the manifestation of the phenotype and a subset of those genes that harbor alleles that contribute to variation in the phenotype. The former can be identified through mutational analysis, the latter through quantitative genetic approaches and analysis of naturally occurring variation.

Genetic dissection of behavior

Induced mutations

In contrast to developmental genetics, where chemically induced null mutations or mutations of large effects that result in extreme phenotypes are desired, studies on the genetics of behavior require the development of a viable individual and thus depend on the analysis of hypomorphic mutations. The two most common approaches to induce such mutations are the use of *P*-element insertions (transposon tagging) and RNAi-mediated targeted suppression of gene expression. A

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variety of transposable element constructs are available and, although different constructs have been developed to enable different experimental manipulations, their common attribute is that they tend to insert in regulatory regions of genes, which results in interference with or, sometimes, enhancement of gene expression (Spradling et al., 1995; Bellen et al., 2004). Depending on the insertion site, effects on adult behavior may result from gene disruption at earlier developmental stages (Sambandan et al., 2006). The distribution of transposon insertion sites is not random. Hot spots and cold spots are evident (Bellen et al., 2004). The latter comprises gene clusters, such as members of odorant receptor, gustatory receptor, and odorant binding protein gene families, which are refractory to *P*-element insertions. When the insertion of a transposable element affects the expression of a gene that contributes to the manifestation of a behavioral phenotype it will give rise to a statistically significant aberration of the behavior as measured by quantitative bioassays (Anholt, 2004).

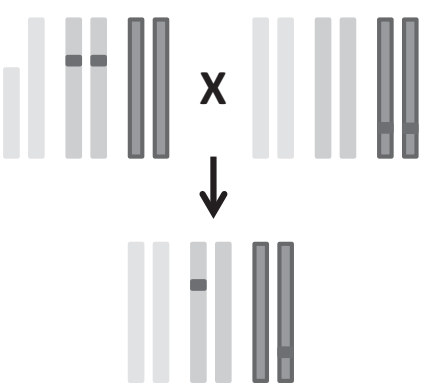
The conclusion that the candidate gene indeed contributes to the phenotype requires further evidence, as movement of *P*-elements in the genome can result in mutations unrelated to the final insertion site, affect chromatin structure that results in effects of genes located at a distance from the insertion site, affect multiple genes if the *P*-element is inserted in an intergenic region, or could affect a gene nested within the sequence of the candidate gene. Mobilization of the transposon that results in excision from its insertion site should restore the aberrant phenotype to control values to provide proof that the *P*-element is indeed responsible for the observed mutation. The gold standard of evidence, however, is demonstration that a wild-type transgene introduced into the mutant background can rescue the phenotype.

It is important to control the genetic background when conducting *P*-element insertion screens, since effects of *P*-elements are often subtle and can be masked or modulated by genetic background variation. Studies that employed co-isogenic backgrounds in which *P*-element insertion lines were genetically identical, except for the *P*-element insertion site, revealed a large mutational target for all behavioral phenotypes analyzed to date. Approximately 29% of *P*-element mutations tested for alcohol sensitivity and resistance differed significantly from co-isogenic *P*-element free controls (Morozova et al., 2011). About 37% of *P*-element insertion lines tested for startle-induced locomotion (Yamamoto et al., 2008), about 6% of *P*-element insertion lines tested for olfactory avoidance behavior (Sambandan et al., 2006), and 35% of *P*-element insertion lines tested for aggression (Zwarts et al., 2011) differed significantly from the co-isogenic control. In addition, 15% of *P*-element insertion lines with known effects on physiology, development, and behavior affect 24-h sleep time (Harbison and Sehgal, 2008). These large mutational targets show that a significant proportion of the genome contributes to the manifestation of each phenotype, thus implying pervasive pleiotropy.

Targeted suppression of gene expression by RNAi can be used as an independent screen or to confirm the effects of *P*-element induced mutations on behavior. Here, the binary GAL4/UAS expression system (Brand and Perrimon, 1993) is used to drive expression of RNAi by crossing transgenic flies that express the yeast transcription factor GAL4 under a tissue-specific promoter with flies that contain an RNAi construct inserted behind the GAL4-specific UAS promoter. A large library of RNAi constructs has been generated as a community resource, in which the UAS–RNAi constructs are inserted in a defined PhiC31 docking site that does not give rise to positional effects and allows effective expression of the RNAi construct (Groth et al., 2004; Dietz et al., 2007). The effectiveness of RNAi constructs on levels of the targeted transcript or protein is variable. RNAi effectiveness can be enhanced by introducing an *UAS–dcr2* construct that promotes further degradation of the targeted endogenous mRNA (Pham et al., 2004). It is clear, however, that there is no linear relationship between the magnitude of reduction in the expression of a target gene by either a transposon insertion or RNAi and the effect on the phenotype. Thus, small effects on the expression of a particular gene product can have dramatic consequences on a behavioral phenotype and, vice versa, large effects on transcript abundance can exert only small phenotypic effects. This is not surprising when one considers the complexity of additive and epistatic interactions among ensembles of genes that underlie the manifestation of a behavioral phenotype.

Natural variation

Naturally segregating polymorphisms provide the substrate for adaptive evolution and a treasure trove of naturally occurring mutations that underlie phenotypic variation. Analyses of different clines of *D. melanogaster* in Australia, Europe, and Argentina have documented behavioral differences between populations in aggression (Hoffmann, 1989), circadian regulation (Kyriacou et al., 2008), and olfactory behavior (Lavagnino et al., 2008), respectively. Artificial selection studies, together with whole genome transcript profiling have identified genes that harbor alleles that contribute to negative geotaxis (Toma et al., 2002), aggression (Edwards et al., 2006; Dierinck and Greenspan, 2006), and alcohol sensitivity (Morozova et al., 2007). When properly conducted (that is bidirectional selection in replicates starting from a heterogeneous base population), changes in hundreds to thousands of transcripts are apparent between the selected populations, again bearing testimony to the complex genetic architecture of behavioral traits (Edwards et al., 2006; Morozova et al., 2007). A complete understanding of the genetic architecture of behavior requires not only understanding how transcriptional variation relates to variation in organismal phenotype, but also how variation at the level of the DNA itself impacts variation in transcript abundance. This requires genome-wide association (GWA), studies which previously were not possible in *Drosophila*. Recently, however, a panel of wild-derived inbred lines with fully sequenced genomes has become available as a community resource to enable GWA studies (Mackay et al., 2012). This panel, referred



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2	-	-	*2,3*	*2,4*	*2,5*
3	-	-	-	*3,4*	*3,5*
4	-	-	-	-	*4,5*
5	-	-	-	-	-

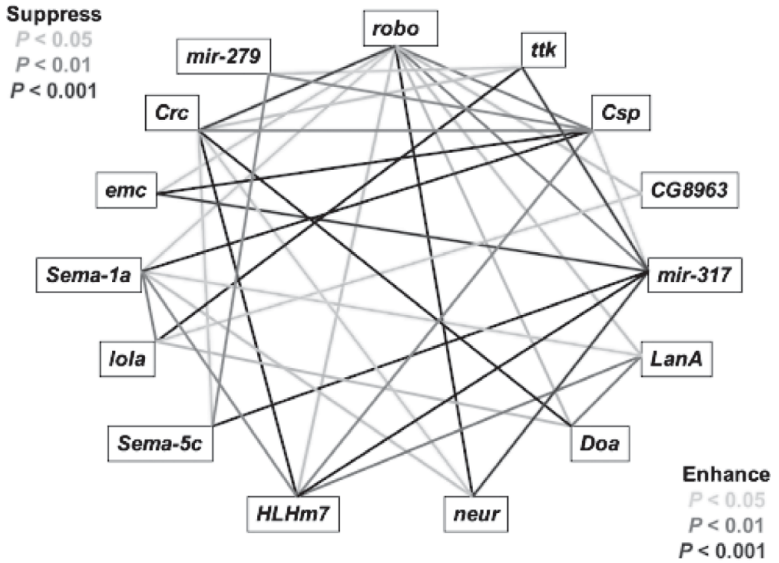

Fig. 17.1. Building epistatic networks among co-isogenic *P*-element insertion lines. Parental strains homozygous for different *P*-element insertions affecting the same behavioral phenotype are crossed to form double heterozygotes. The three major Drosophila chromosomes are represented as yellow, pink and blue bars, and the *P*-element insertions are indicated as horizontal red bars. In a half-diallel crossing design each *P*-element insertion line is crossed in a non-reciprocal manner to all other *P*-element insertion lines, as indicated in the upper right diagram. Analyses of deviations of observed from predicted phenotypic values allows inferences about enhancer and suppressor effects, which can be represented diagrammatically as illustrated for epistatic interactions among 15 co-isogenic *P*-element insertions that affect startle behavior (modified from Yamamoto et al., 2008). Solid arrows indicate genes in which *P*-element insertions in the same genetic background also affect olfactory behavior (Sambandan et al., 2006) and dotted arrows indicate *P*-element insertions in the same genetic background that also affect aggression (Zwarts et al., 2011), providing examples of pleiotropy.

to as the *Drosophila melanogaster* Genetic Reference Panel (DGRP), has provided a critical link in enabling systems genetics studies of behavior in *Drosophila* and will be described in greater detail below.

Fundamental principles of the genetics of behavior

Genetic context: epistasis

A classic approach of examining interactions between two loci that affect a trait is to assess whether mutants at these loci in combination have an additive effect on the phenotype or whether the effect is non-additive. The latter case is indicative of enhancer or suppresser effects, which are defined as "epistasis" (Phillips, 2008). Note that this definition of epistasis is statistical, and does not necessarily imply direct molecular interaction or even a relationship within the same cellular pathway of the interacting loci. Thus, epistatic interactions can be confounding factors in linkage mapping and GWA studies, when the genetic background is not appropriately controlled.

Epistatic effects are often underestimated, since they include both enhancer and suppresser effects, which may compensate when examined globally across the genome. One method for assessing epistatic interactions among multiple genes, which can be readily deployed in Drosophila, is to generate all possible double heterozygotes among a series of co-isogenic *P*-element insertion lines that affect the behavioral phenotype of interest in a classic half-diallel crossing design (Fig. 17.1; Fedorowicz et al., 1998). This design allows an estimate of the average

heterozygous effect contributed by each *P*-element insertion mutant in combination with all others, referred to as the "General Combining Ability (GCA)." Knowledge of the GCA values of both homozygous single *P*-element insertion parents will enable an estimate of the phenotypic value of double heterozygous offspring (the "Specific Combining Ability, SCA") under the null hypothesis of complete additivity (Griffing, 1956). When the predicted SCA is statistically significantly different from the observed SCA, epistasis is inferred between the two *P*-element tagged loci, either in the direction of the mutant phenotype (enhancer effects) or the wild-type phenotype (suppresser effects). This analysis is only possible when all *P*-element insertions are in the same genetic background and is limited in practice, as the addition of more lines to the half-diallel crossing design exponentially increases the workload. Nonetheless, such analyses have shown surprisingly extensive epistasis among independently isolated *P*-element mutations affecting olfactory behavior (Fedorowicz et al., 1998; Sambandan et al., 2006), startle behavior (Yamamoto et al., 2008), negative geotaxis (van Swinderen and Greenspan, 2005) and aggression (Zwarts et al., 2011), and indicate that the genetic architecture of behavior is determined by epistatic networks of pleiotropic genes (Fig. 17.1). Moreover, the composition of these epistatic networks is dynamic and sensitive to environmental conditions, i.e., enhancer/suppresser effects among mutations affecting the trait within the same genetic background are flexible, even though their main mutational effects are stable (Sambandan et al., 2006).

Examination of the effects on the transcriptome of single *P*-element insertions affecting olfactory behavior has shown widespread effects on gene regulation (Anholt et al., 2003). This is an important observation as it implies that the effects of newly arising mutations can affect the composition of transcriptional networks and thus give rise to indirect pleiotropic phenotypic effects. Transcriptional profiling of double heterozygous *P*-element insertion lines associated with aggression showed widespread effects on genome-wide transcript abundance levels that exceeded effects of the homozygous parents, again illustrating the prevalence and complexity of epistatic interactions both at the level of the transcriptome and the organismal phenotype (Zwarts et al., 2011).

Pleiotropy

Pleiotropy is defined as the ability of a gene to influence multiple phenotypes. Pleiotropy is an inherent feature of complex traits, as implied by the large mutational target of behavioral phenotypes mentioned above. A careful analysis of pleiotropic effects, however, shows that different alleles may affect different phenotypes differentially. For example, different *P*-element insertion alleles of *neuralized*, in which the transposon insertion sites are only a few base pairs apart, have different effects on olfactory behavior, aggression and startle-induced locomotion (Fig. 17.1; Rollmann et al., 2008). Similarly, different polymorphisms in *Catsup*, which encodes a negative regulator of tyrosine hydroxylase, the rate limiting enzyme in the dopamine biosynthetic pathway, are associated with variation in longevity, locomotor behavior, and sensory bristle number (Carbone et al., 2006). Most of these polymorphisms were in coding regions, had large effects, and were present at relatively low allele frequencies. The realization that pleiotropic effects are properties of "alleles" rather than "genes" adds a dynamic dimension to the manifestation of pleiotropy. Changes in allele frequencies as a result of natural or artificial selection can alter the pleiotropic landscape among gene ensembles. Whereas pleiotropy can exert constraints on selection, such constraints may depend on the pleiotropic nature of the segregating alleles and, conceivably, in heterozygotes may discriminate between different alleles. Allelic effects on pleiotropy may in turn influence the dynamics of epistatic interactions and generate variation in the structure of epistatic networks within a natural population, which in turn may contribute to variation in organismal phenotypes.

Environmental context: Phenotypic plasticity and genotype-by-environment interactions (GEI)

The relationship between genotype and phenotype is further confounded by environmental effects. Among complex traits, behaviors are expected to be especially sensitive to environmental factors, since they represent an organism's responses to and interactions with the environment. The ability of a genotype to express different phenotypes under different conditions is referred to as "phenotypic plasticity." When different genotypes respond differently to different environments, GEI is inferred, which is evident from non-parallel reaction norms. Phenotypic plasticity is the counterpoint of phenotypic robustness. In 1942, Waddington developed the concept of "canalization" to explain the observed robustness of developmental processes under variable conditions (Waddington, 1942). Whereas phenotypic plasticity enables organisms to adapt rapidly to changing conditions, excessive phenotypic plasticity that may compromise optimal development will cause a reduction in fitness. Canalization results in phenotypic robustness, and the balance between plasticity and robustness in the most frequently encountered environments determines optimal adaptation. It has been postulated that rapid evolution of the human genome combined with dramatic environmental and cultural perturbations in the past two generations might cause decanalization that results in the manifestation of common genetic diseases (Gibson, 2009). Gibson and Wagner (2000) have postulated the notion of cryptic genetic variation in which most of the genetic variation is suppressed by canalization (which likely involves suppressing epistasis), and is released during major environmental perturbations.

Phenotypic plasticity is a common phenomenon during the life cycle. The sleep/wake cycle and fluctuations in circadian activity are examples of different behavioral phenotypes that are contingent on physiological state and the daily light–dark cycle. The honey bee, *Apis mellifera*, provides a striking example of behavioral phenotypic plasticity during its life cycle when bees

progress from nurses to foragers. The temporal progression of this process can be environmentally modulated (Robinson et al., 2005). One of the most striking examples of phenotypic plasticity comes from the water flea, *Daphnia*, which develops a helmet and spiny tail as defensive traits when grown in the presence of predators (Dodson, 1989).

What is the genetic basis of canalization and phenotypic plasticity? The Hsp83/Hsp90 chaperone protein has been implicated as a "capacitor" of genetic canalization (Queitsch et al., 2002). *Hsp90* mutants give rise to a wide range of morphological aberrations both in *Drosophila* and *Arabidopsis thaliana* under various conditions (Rutherford and Lindquist, 1998; Sangster et al., 2008). However, Hsp90 is not the only capacitor that may mediate genetic canalization. miRNAs may also function as canalization capacitors. Of special interest are the Piwi interacting RNAs (piRNAs). This class of RNAs suppresses the activity of transposable elements, and compromised piRNA function may result in mutations, due to mobilization of transposons (Salathia and Queitsch, 2007; Specchia et al. 2010). Furthermore, the demonstration of extensive suppressing epistasis that modulates the effects of mutations that affect startle behavior (Yamamoto et al., 2009), sleep and olfactory behavior (Swarup et al., 2012) suggests that canalization may also be attributable to the buffering capacity of epistatic genetic networks and that the dynamics of these networks may regulate phenotypic plasticity.

Several studies have shown that only a small segment of the transcriptome responds to environmental changes. When *Drosophila mojavensis* larvae are grown on different species of cactus, only 7% of the genome was differentially expressed (Matzkin et al., 2006). Furthermore, when a synthetic outbred population of *D. melanogaster* was exposed to 20 different rearing conditions, only 15% of expressed transcripts were environmentally variable (Zhou et al., 2012). These transcripts comprised two categories. One category (Class I) consisted of transcripts that were genetically and environmentally variable and comprised gene ontology categories associated with detoxification, metabolism, proteolysis, heat shock proteins, and transcription regulation. The second class of transcripts (Class II) showed low genetic variance, but greater environmental variation. These transcripts exhibited sexually dimorphic expression and were enriched for reproductive functions. These Class II transcripts may have low genetic variance under the standard rearing condition due to canalization, and their extensive environmental variation under different rearing conditions may reflect the release of cryptic genetic variation. Both classes of transcripts (especially Class II) evolve more rapidly than the robust segment of the transcriptome (Zhou et al., 2012). Thus, the environmentally responsive segment of the transcriptome may serve as a homeostatic buffer for overall robustness.

A subset of transcripts that account for phenotypic plasticity is expected to show GEI. When larvae from 41 wild-derived inbred lines were raised on different nutrient media, subsequent analysis of adult olfactory behavior showed that approximately 50% of phenotypic variation was attributable to GEI. Transcriptional analysis revealed that only 20 genes showed GEI at the level of gene expression, some of which were associated with physiological responses to environmental chemicals (Sambandan et al., 2008).

Constructing transcriptional networks

Modulated modularity clustering

If transcripts are interacting in a network, it is reasonable to expect that their abundance will co-vary in response to a common perturbation. Such perturbations may be environmental, genetic, chronological, or otherwise; regardless of its nature, the signal of interest is a correlated response. For years, grouping transcripts on the basis of correlated expression patterns has been an important step in microarray studies (Verducci et al., 2006; Lee and Saeed, 2007). Hierarchical clustering has been, and remains, popular for this purpose, but other more sophisticated methods exist. In recent years, graph clustering techniques have risen in prominence as an alternative to more traditional agglomerative approaches. One such method is Modulated Modularity Clustering (MMC) (Stone and Ayroles, 2009). The goal of MMC is to group transcripts on the basis of correlated responses to a perturbation. MMC attempts to optimally partition transcripts into intercorrelated modules, so that a specific global objective function (i.e., modulated modularity) is maximized. The transcripts comprising each module can be viewed as participants in a hypothesized network. Independent support can then be sought for each module-directed network hypothesis. In simple terms, MMC organizes transcripts in clusters in such a way that expression of each transcript shows stronger correlations with other members of the same cluster than with those outside the cluster. The procedure is unbiased in that the number of covariant modules is not determined *a priori*.

Application of MMC to the analysis of transcriptional profiles of 40 wild-derived inbred lines showed that the transcriptome, as measured at a single time point under standard growth conditions, is highly genetically intercorrelated and organized as 241 modules (Ayroles et al., 2009). Analyses of these modules show that they comprise transcripts associated with similar biological processes. Furthermore, values of organismal phenotypes could be regressed on transcript abundance values and the residuals of such regressions could be organized by MMC to reveal transcriptional modules associated with phenotypic variation of life history traits and responses to physical stress (Ayroles et al., 2009; Zhou et al., 2012), as well as behavioral phenotypes, including startle behavior (Ayroles et al., 2009), aggression (Edwards et al., 2009; Fig. 17.2), alcohol sensitivity (Morozova et al., 2009), and sleep (Harbison et al., 2009b).

Interpreting transcriptional networks

Annotation, when available, provides a powerful means of corroborating and interpreting transcriptional networks. Gene Ontology (GO) enrichment, for example, can implicate

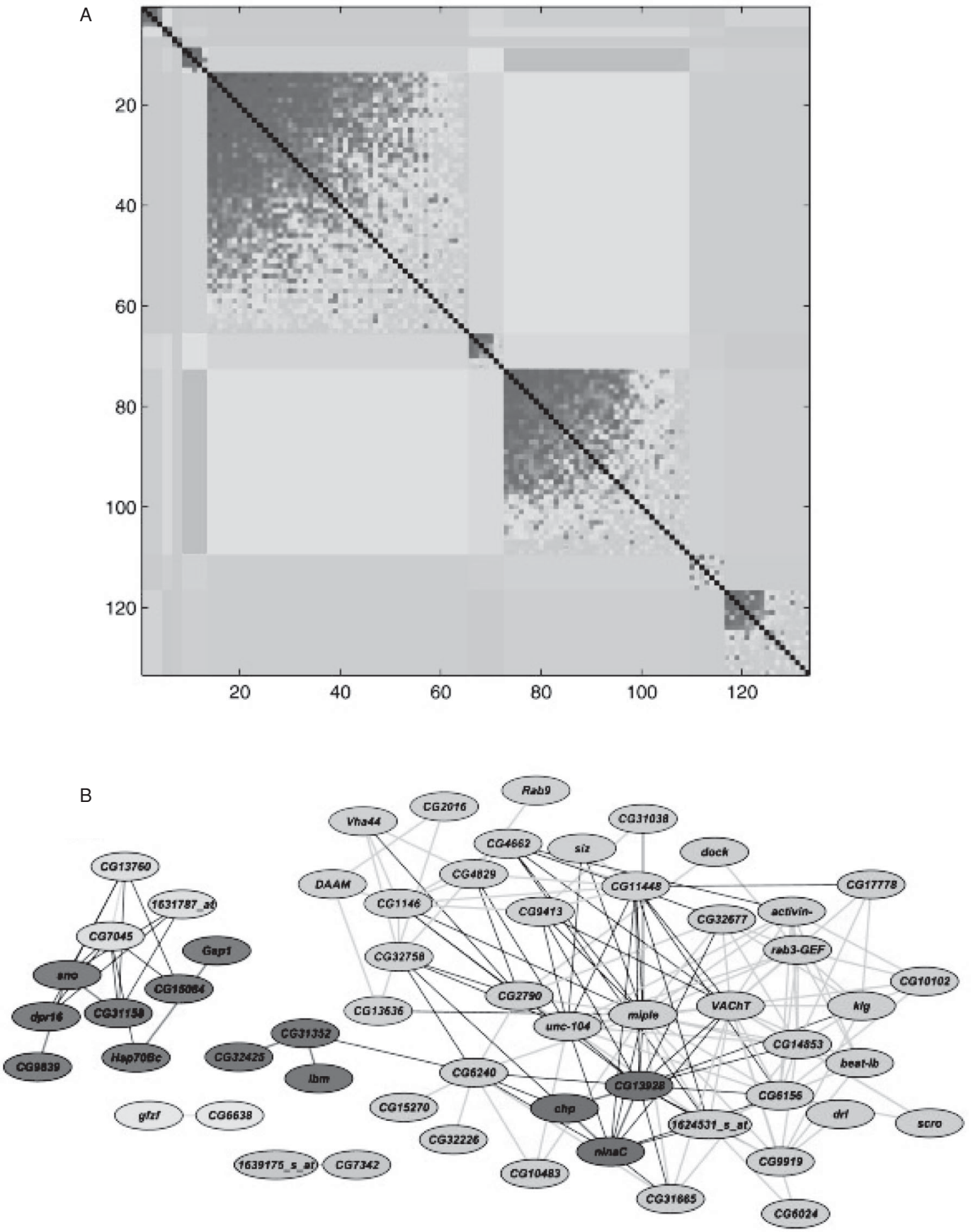

Fig. 17.2. Modules of correlated transcripts associated with variation in aggressive behavior. A Heat map of correlated probe sets generated by MMC. Modules are represented along the diagonal with the strongest correlated modules in the upper corner. The strength of correlations within the modules decreases down the diagonal. B Network view of the most highly correlated ($r \geq 0.7$) probe sets where the edges represent correlated transcripts and the color-coding of nodes represents the different modules depicted in A. (Modified from Edwards et al., 2009).

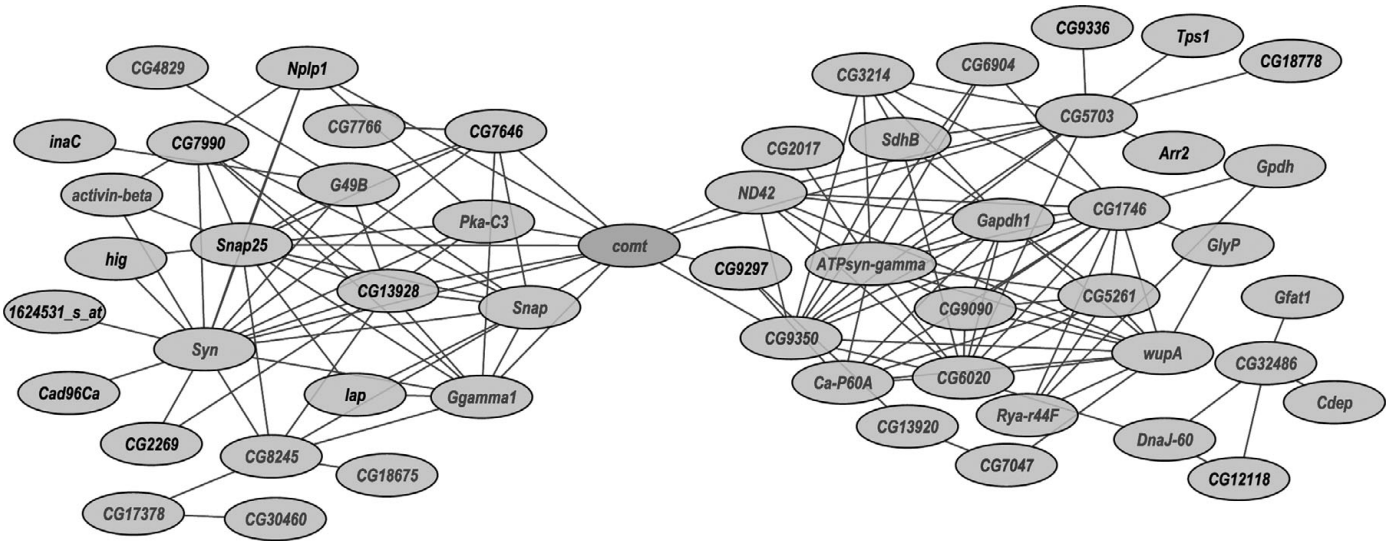

Fig. 17.3. Highly connected gene clusters within a module associated with development of alcohol tolerance in *Drosophila*, identified by MMC. Gene ontology analyses show that transcripts in the cluster to the left are associated with synaptic function, whereas transcripts associated with oxidative phosphorylation are enriched in the cluster depicted on the right. Both clusters are connected via *comt (comatose),* which encodes a protein implicated in ATP-dependent synaptic vesicle release. Both clusters contain unannotated transcripts. Such transcripts can be hypothesized to function in synaptic transmission (left cluster) or energy metabolism (right cluster) based on the "guilt by association" principle. Drosophila genes with human orthologues (68.5%) are indicated in blue font. All genes depicted have transcriptional genetic correlations ≥ 0.7. (From Morozova et al., 2009).

networks as performing a particular function or localizing to a particular cellular location (Huang et al., 2009). This implication, in turn, can be used to hypothesize functions for unannotated transcripts within the module based on the "guilt by association" principle (e.g., Morozova et al., 2009; Fig. 17.3). In this way, the statistical groupings from expression data and the biological groupings in the GO hierarchy are complementary.

Constructing co-variant networks around focal genes

The availability of whole genome transcript profiles of 40 wild-derived inbred lines makes it possible to computationally mine the modular structure of the transcriptome by identifying co-regulated genes for any given focal transcript and to identify polymorphisms in the focal gene associated with transcript abundance of the same gene (*cis* effects) or other genes (*trans* effects) (Mackay et al., 2009). Here, each transcript can be considered a trait, and hence is referred to as an expression QTL (eQTL). An example of this type of application is a study that investigated how functional diversification of the family of *Odorant binding protein* (*Obp*) genes affects the organization of the transcriptome (Fig. 17.4; Arya et al., 2010). First, all six *Obp* genes located on the *X* chromosome were sequenced in 219 inbred wild-derived lines. Polymorphisms in *Obp8a*, *Obp19a*, *Obp19b*, and *Obp19c* were associated with variation in olfactory responses, and polymorphisms in *Obp19d* were associated with variation in lifespan. Next, the transcriptional context of each gene was characterized by identifying expression polymorphisms where genetic variation in these *Obp* genes was associated with variation in expression of transcripts genetically correlated to each *Obp* gene. Gene ontology analyses of these computational modules centered on each focal *Obp* gene showed that diversification of the *Obp* family has organized distinct transcriptional niches that reflect their acquisition of additional functions (Fig. 17.4; Arya et al., 2010).

A variation on this theme used a combination of *P*-element insertional mutagenesis and computational analysis of natural variation based on whole genome transcript profiles to build co-regulated networks associated with alcohol sensitivity (Morozova et al., 2011). Here, *P*-element insertional mutagenesis identified genes that impact alcohol sensitivity, and these genes served as focal genes for the construction of co-regulated modules. RNAi-mediated suppression of the expression of correlated genes could then be used to validate their effects on the phenotype and those could then, in turn, be used as focal genes to expand the network through an iterative approach accompanied by functional validation (Fig. 17.5; Morozova et al., 2011). Although co-regulated transcriptional networks do not provide information about causal relationships among their constituent transcripts, they provide a framework for subsequent functional studies on the genetic basis of the behavioral phenotype of interest.

Association analyses

The *Drosophila melanogaster* Genetic Reference Panel (DGRP)

Systems genetic analyses of behaviors require linking DNA sequence variants to variation in transcript abundance and variation in organismal phenotypes. Because *Drosophila* is highly

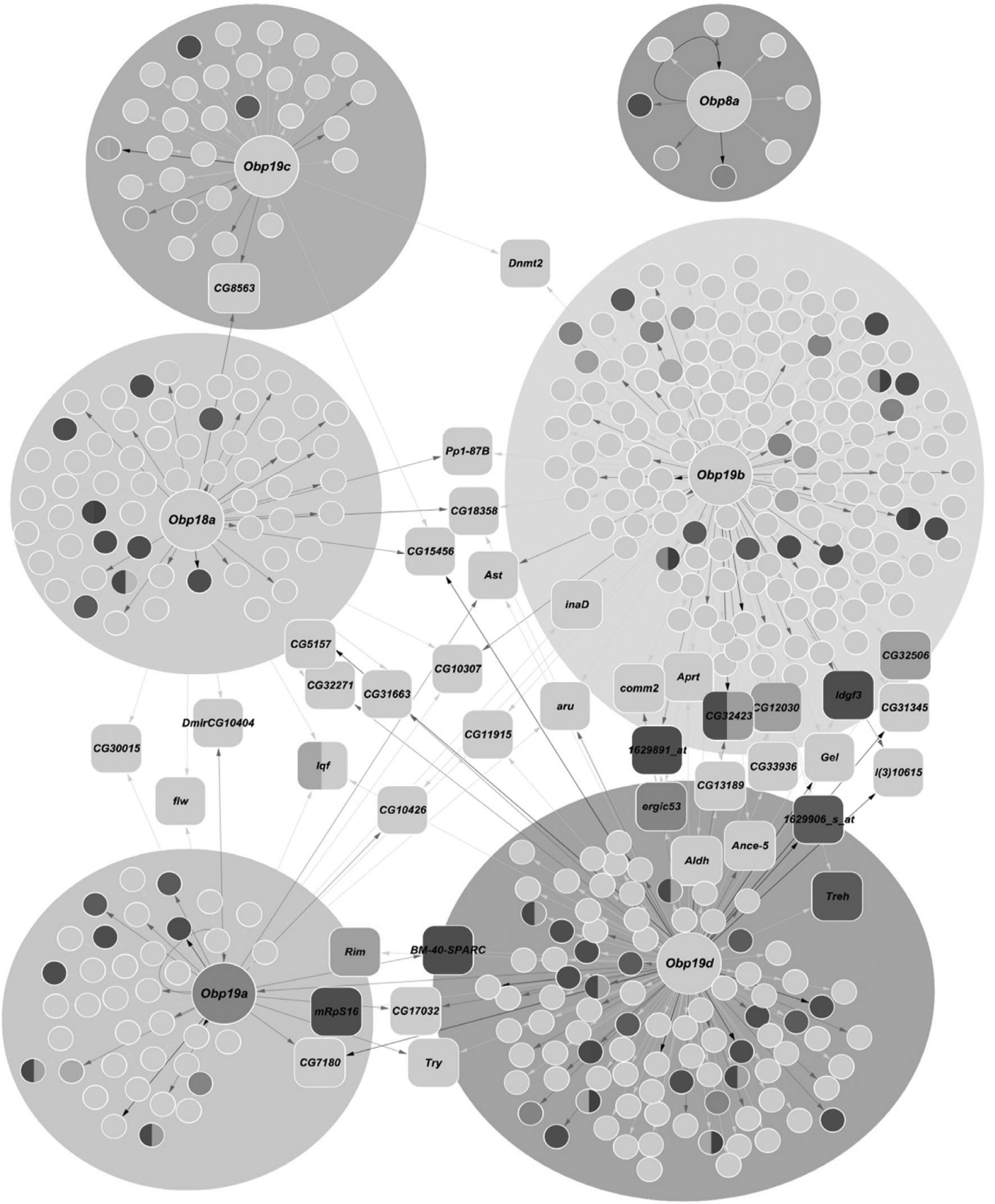

Fig. 17.4. Diagrammatic representation of computationally derived transcriptional niches of six *Obp* genes located on the *X*-chromosome. Transcripts associated with more than one *Obp* network are indicated in rectangles. Transcripts that are significantly associated with a phenotype after regression analysis ($P < 0.01$) are in colors other than light gray (dark blue, starvation resistance; maroon, copulation latency; green, longevity; purple, olfactory behavioral response to hexanol; turquoise, olfactory behavioral response to hexanal; orange, olfactory behavioral response to benzaldehyde; light blue, olfactory behavioral response to acetophenone). *Obp8a* contains a SNP associated with its own transcript abundance, indicated by the curved arrow, which is an example of a *cis*-eQTL. Gene ontology analyses of these transcriptional networks illustrate functional diversification of these *Obp* genes following gene duplication with different niches showing enrichment of gene ontology categories related to olfactory behavior, synaptic transmission, detection of signals regulating tissue development and apoptosis, postmating behavior and oviposition, and nutrient sensing (From Arya et al., 2010).

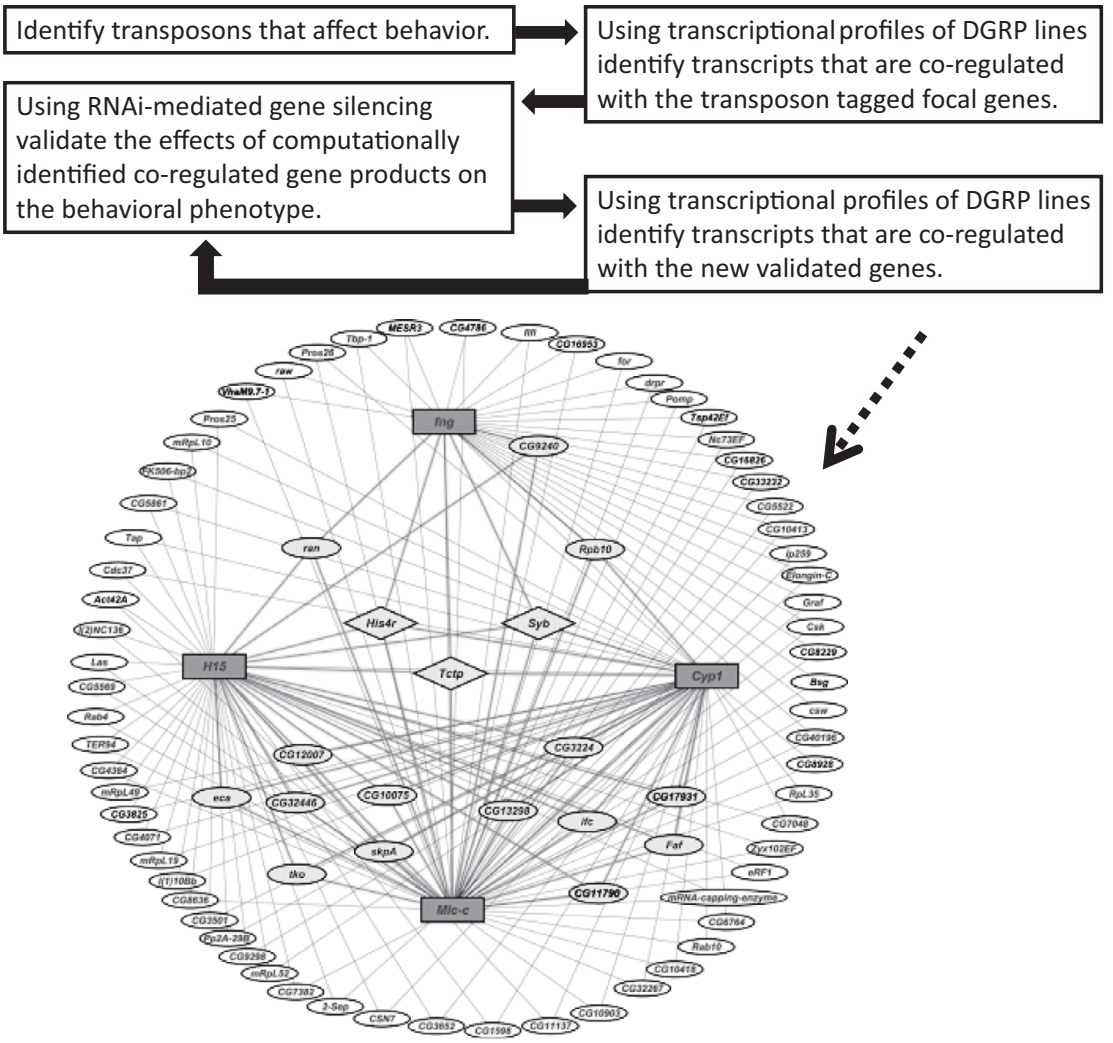

Fig. 17.5. Combining *P*-element insertional mutagenesis and analysis of natural variation to build transcriptional networks of co-regulated transcripts. The flowchart illustrates an iterative process in which identification of transposon-tagged genes can be used as focal genes for the identification of co-regulated transcripts among 40 DGRP lines (Ayroles et al., 2009), followed by verification of their effects on the phenotype through RNAi-mediated gene silencing. This method can be successful despite differences in genetic backgrounds between *P*-element insertion lines, wild-derived lines and GAL4-UAS lines expressing RNAi, as illustrated by the transcriptional network for alcohol sensitivity that has resulted from this analysis, shown in the figure. Four focal genes, initially implicated in alcohol sensitivity, are shown in red squares. Only genes connected to more than two of the focal genes are indicated. Genes connected to two focal genes are shown at the periphery of the circle in white ovals and connected by grey lines. Genes interconnected by three and more networks are indicated on a yellow background and connected by orange lines. Diamond shapes indicate genes connected to all four focal genes. *Drosophila* genes with annotated human orthologues are indicated in blue font (Modified from Morozova et al., 2011).

polymorphic and linkage disequilibrium decays on average within 200 bp, complete sequence information is necessary to identify SNPs that may be causally associated with phenotypic variation. Thus, previous association analyses have been limited to studies on single or few genes at a time. Genome-wide association analyses have become possible in *Drosophila* only with the advent of the DGRP (Mackay et al., 2012). Construction of this resource began in 2002 with the collection of gravid *Drosophila* females from peaches at the Raleigh, North Carolina, farmer's market. Offspring from isofemale lines was subjected to 20 generations of full sib inbreeding and this resulted in 345 wild derived inbred lines. Genetic drift results in minimal genetic variation among individuals within each line, while at the same time genetic variation among lines is preserved and reflects the variation found in the original population. The DGRP consists of a subset of 205 of these lines with fully sequenced genomes that are publicly available for genome-wide association studies.

Polymorphisms in the DGRP and statistical considerations for accurate SNP calling

The DGRP design redistributes the genetic variation segregating in a wild population as fixed differences between inbred

lines. Indeed, most single nucleotide polymorphisms in the DGRP fall into this category, though in some cases residual heterozygosity within an inbred line is observed. There are about 5.4 million SNPs among the DGRP lines. The identification of SNPs in the DGRP is predicated upon obtaining accurate genotypes, but one must be careful when defining the term "genotype" for an inbred line. Recall that each inbred line of the DGRP is itself a population, albeit a population with very low heterozygosity. At sites where the inbreeding process has led to homozygosity, the population is monomorphic and the meaning of a line genotype is clear. When there is residual heterozygosity, however, "genotype" most properly refers to the multiple alleles that are present *and* the frequencies at which they are segregating. Residual heterozygosity is complicating for two reasons: first, methodology designed for genotyping individuals does not apply; and, second, rare alleles within a line are difficult to distinguish from sequencing error. Given sequencing reads obtained from a DNA pool of flies for each of the DGRP lines, the challenge of SNP calling is to identify between line variation in the face of low levels of within line variation and an unknown degree of sequencing and genome assembly error. These concerns can be mitigated by exploiting the experimental design. Because the DGRP lines are derived from a common population, it is possible to estimate ancestral allele frequencies from the collective sequence data. Given the ancestral allele frequencies at any one site, the probability that residual heterozygosity remains can be calculated. Thus, the DGRP lines could be genotyped by estimating both ancestral allele frequencies and sequencing/assembly error probabilities so that heterozygosity and error could be probabilistically disentangled (Mackay et al., 2012). Recently, complete information on copy number variants in the DGRP has also become available, which shows 751 177 segregating insertion/deletions, among which 3191 biallelic indels affecting 2418 genes are naturally segregating alleles, with an especially large number of indels segregating among the rapidly evolving chemoreceptor gene families. Chromosomal inversions have also been characterized for all the lines, making the DGRP the best characterized genetic reference panel for any eukaryotic organism available to date.

GWA studies in the DGRP reveal extensive epistasis

Huang et al. (2012) conducted GWA studies for starvation stress resistance, startle behavior, and chill coma recovery in the DGRP and identified SNPs associated with genetic variation in each trait. This study compared GWA on line means with GWA on line variants (vGWA) as well as extreme QTL mapping. The latter consists of genotyping differentially segregating alleles in individuals with phenotypic values at the tails of distributions in an advanced intercross population derived from a base population generated by a round robin cross-design of 40 DGRP lines. Surprisingly, the three different analyses identified different SNPs. Differences between GWA and vGWA results can be expected, since GWA is designed to detect mean shifts, whereas vGWA contrasts variances of the two genotypes at each SNP, as expected with epistatic interactions in which the effect of an undetected interacting locus has a much greater effect in one genetic background than the other at the tested locus. Discordance between SNPs identified by GWA of differentially segregating alleles in the AIL populations could be attributed to context-dependent effects and to variants that are not common in the DGRP but at intermediate frequencies in the AIL population (Huang et al., 2012).

Similar observations were obtained for variation in olfactory behavior, where GWA of line means, vGWA, and extreme QTL mapping of differentially segregating alleles in an advanced intercross population derived from DGRP lines with extreme phenotypic values for olfactory behavior showed non-overlapping SNPs. If GWA results depend on genetic context, one can predict that combined analyses of results obtained under different conditions of genetic background would converge on a common underlying cellular framework. Indeed, when candidate genes harboring SNPs associated with phenotypic variation in olfactory behavior were pooled from GWA, vGWA, and extreme QTL mapping analyses and subjected to network enrichment analysis, a significant cellular network emerged, comprosed of genes associated with axon guidance, inositol triphosphate signaling, cyclic GMP metabolism, cell adhesion, and neural development, implying that polymorphisms may contribute to natural variation in olfactory perception through subtle variations in neuronal signaling and connectivity of the nervous system (Swarup et al., 2013; Fig. 17.6).

These results demonstrate that epistasis dominates the genetic architecture of complex traits, including behaviors, in *Drosophila* and that differences in allele frequencies in outbred populations can give rise to different SNPs reaching statistical significance in GWA studies. These observations are highly relevant to studies in human populations, as discordant findings in different populations could arise from epistatic interactions that are sensitive to differences in allele frequencies.

eQTL: inferring *cis* and *trans* regulation of transcripts within networks

Under the assumption that co-regulated transcripts will show correlated expression levels across lines, methods such as MMC can be used to hypothesize groups of transcripts within networks. The manner in which these transcripts interact can then be resolved, with error, in a second step, using statistical approaches and knowledge of the underlying biology. Statistical methods for this task often rely on conditional dependencies in the data; for example, if in a network transcript Y is the sole intermediary between transcripts X and Z, then the abundances of X and Z depend on each other only through that of Y (Fig. 17.7). That is to say, conditional on Y, X, and Z are independent, and equipped with expression measurements

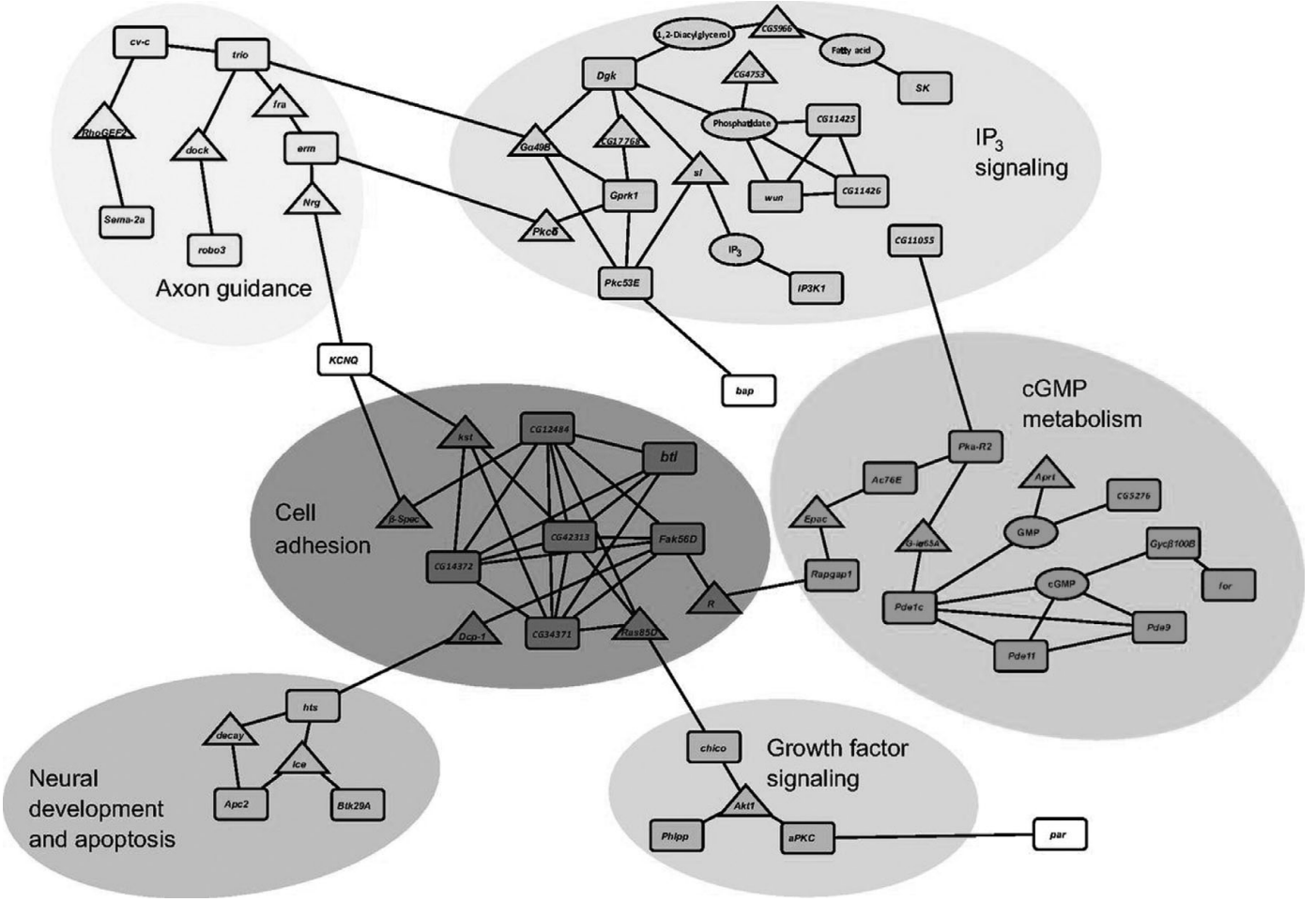

Fig. 17.6. A cellular network identified by combined GWA, vGWA, and extreme QTL mapping analyses. Analysis using the R-Spider algorithm (Antonov et al., 2010) reveals an enriched cellular network among candidate genes ($P < 0.005$). This algorithm tests the significance of the network by Monte Carlo simulations, in which the same number of randomly selected genes is used to form the null distribution of the size of the network. Candidate genes are indicated by rectangles, missing genes (i.e., genes without significant associations) by triangles, and metabolites by circles. Distinct interconnected cellular processes are highlighted by colored backgrounds (From Swarup et al., 2013).

of X, Y, Z across lines one expects the correlation between X and Z to vanish given Y (i.e., zero partial correlation). These statistical arguments can be supplemented with biological reasoning. If, for example, there is a genetic variant at the X locus that strongly associates with the transcript abundance of each of X, Y, Z, then it is plausible that X lies upstream of Y and Z in the network topology. In this case, the genetic variant is an eQTL for all three transcripts; it is *cis* to X and *trans* to Y and Z (Fig. 17.7).

Validation and follow-up experiments

Statistical associations, though powerful hypothesis generators, are insufficient to establish causality. Sometimes, the signal can be replicated in a second association mapping population, thereby providing an additional independent line of evidence. Alternatively, a statistical association may gain support from an overlapping peak in a linkage study, for example through an F2 design. Accumulating statistical evidence in this way can refine the set of loci and variants to be tested, but true validation requires direct experimental evidence. In *Drosophila* this is best achieved through the use of mutations or RNAi-mediated gene silencing. If mutations in a gene that harbors SNPs statistically associated with a behavioral phenotype, indeed, result in aberrant behavior, one can reasonably assume that the natural variation in this gene associated with phenotypic variation is likely to be causal. Final proof for causality could be obtained by allelic replacement of one candidate allele by another under conditions that preserve the genetic background. However, development of this technology remains a challenge for the future.

Considerations for future endeavors

Whereas the 205 sequenced DGRP lines have been instrumental in enabling systems genetics studies in *Drosophila*, a

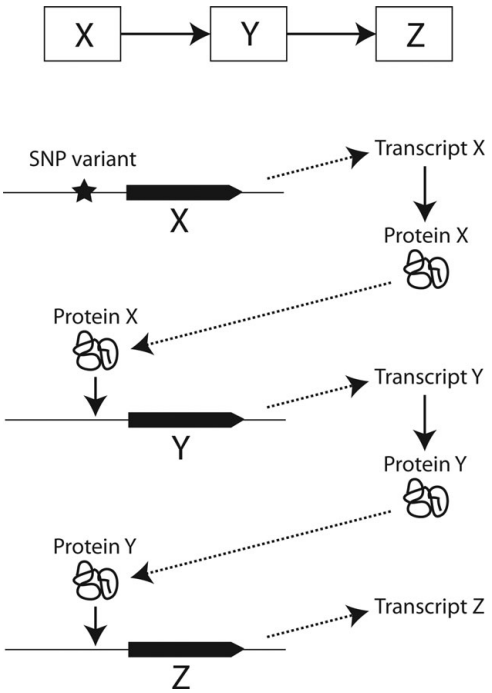

Fig. 17.7. Resolving dependencies between transcripts in a network. A transcriptional cascade involving three loci (X, Y, Z) in which the protein products of X and Y are enhancers of Y and Z, respectively. Expression at X influences expression at Z but only though expression of Y; therefore, conditional on Y, X, and Z are independent. The same would be true if the cascade were reversed so that Z enhanced Y and Y enhanced X, as here again Y is along the path between X and Z. Knowledge of an associated SNP variant in the regulatory region of the X locus suggests the presence of a *cis*-eQTL. This "genetic perturbation" drives the correlated expression between transcripts X, Y, Z by directly mediating the abundance of transcript X and indirectly mediating that of Y and Z.

larger collection of fully sequenced wild-derived inbred lines from the same population would provide greater statistical power for association analyses and allow the detection of SNPs with smaller effect sizes. It is likely that rapid developments in parallel sequencing technologies will make expansion of this resource in the future economically feasible.

Whereas the transcriptome is the direct read-out of gene expression, the proteome is the ultimate endophenotype that directly mediates the expression of the organismal trait. Thus, understanding the relationship between DNA sequence variants and variation in organismal phenotypes will require a comprehensive quantitative analysis of the relationship between the transcriptome and the proteome. Effects of epigenetic factors on the genotype-phenotype relationship will also have to be taken into account.

Gene expression patterns, interactions among transcripts, and composition of the proteome will change dynamically in response to changes in the environment, a consideration that is especially relevant for behavioral traits. Development of quantitative models that can predict behavioral phenotypes in the face of the dynamics of their underlying genetic architecture is a daunting challenge for the future. Finally, determining the relationship between genetic networks and neural circuits will be critical for an integrative systems genetics understanding of the manifestation of behaviors.

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Chapter

18

Olfactory learning and memory assays

Christopher J. Tabone and J. Steven de Belle

Introduction

Olfactory learning and memory are widely investigated in *Drosophila* neuroscience. Assays can serve as simple measurements of behavioral plasticity in whole animals by harnessing the innate importance of olfactory cues for the fly (Lebreton et al., 2012). A successful fly learning assay needs to do only one thing well: Ask a simple question that has a restricted set of possible answers. *Drosophila* tend to perform rather poorly on the insect-equivalent of an essay question, in contrast to honeybees for example (Menzel and Giurfa, 2006; Giurfa and Sandoz, 2012). However, we can gather a great deal of useful information from a fly by administering carefully considered true-or-false exams. Indeed, binary options form the basis of many successful experiments. The first robust olfactory-based assay to offer reproducible measurement of learning and memory in *Drosophila* is not yet 40 year old (Quinn et al., 1974). Currently, many laboratories conduct a breadth of olfactory learning experiments involving an ever-expanding list of devices and protocols. In this chapter we outline experimental techniques used in some of the more widespread assays and explain how they can be implemented effectively to measure olfactory learning and memory in *Drosophila*.

Olfactory learning and memory experiments are particularly challenging for a variety of reasons. Unlike visual or auditory stimuli that can be switched on and off with temporal precision, odor delivery and elimination are not trivial binary operations. Once presented, odors tend to disperse in a chaotic fashion and linger in the environment. We ask the relatively simple fly to tell us about experiences with these odors through our observations of behavior. Moreover, their experiences are context dependent and preceding events have profound impacts on the outcomes. Thus, a successful experiment relies on proper rearing and precise control of physical parameters – which is where we begin. Next, we classify behavioral assays in a matrix of categories that include (1) developmental stage (adult or larva), (2) unit of measurement (population or individual), (3) type of learning (non-associative, associative or complex), and (4) in cases of associative learning, the nature of reinforcement (aversive or appetitive). This matrix is incomplete. We have selected assays for discussion based on their historical precedent, instrumental contributions to our field, and if we find them compelling in their potential to demonstrate learning principles in a classroom setting (i.e., inexpensive, low-tech, minimal gadgetry). Many protocols retain similar features such as a common use of electric shock or sucrose reward for negative and positive reinforcement, respectively. All assays deliver stimuli in spatial and temporal arrangements established experimentally to generate different types of learning and consolidated memory.

The term "paradigm" has gathered multiple meanings in several disciplines, but remains broadly accepted to represent a set of (1) practices that define a field of research or (2) universally recognized scientific achievements that, for a time, provide model problems and solutions for a community of researchers (Kuhn, 1970). From these definitions we suggest that "paradigm" represents more than a device, a protocol, or outcomes generated from them, and have refrained from using it in this review.

Experiments

As a rule, practically any aspect of environmental variation at any time in development or during an experiment is a candidate influence on behavior. Care must be taken to minimize these extraneous variables to maximize signal-to-noise in comparisons of experimental groups. This can be accomplished in a number of ways, most notably by attentive fly rearing and tightly-regulated environmental conditions during experiments. It should include proper control of the genetic background among all strains of flies (e.g., de Belle and Heisenberg, 1996). To the conscientious and observant fly-pusher, many of the standard procedures used in rearing flies for behavior assays are already in practice or similar to those implemented in general stock maintenance and other types of experiments.

Flies and behavior

Drosophila used for behavior should be kept in incubators or climate chambers, typically held at 24 °C or 25 °C with humidity between 40% and 60%, and cycles of either 16:8 or 12:12 L:D (see Greenspan, 1997). Identical rearing conditions

Behavioral Genetics of the Fly (Drosophila melanogaster), ed. J. Dubnau. Published by Cambridge University Press.

for all experimental stocks are important. Overcrowding must be avoided since the density of larvae and adults can directly affect the development of the nervous system, particularly brain structures important for learning (Heisenberg et al., 1995). Maintaining no greater than ~100 flies per bottle (on 40 mL food), and transferring adults every 2 or 3 days typically prevents overcrowding. Food composition is also critical for behavioral experiments, and lack of sufficient nutrients can negatively affect learning performance (e.g., Guo et al., 1996). We have tried many recipes and found the one served up by the kind folks at the Bloomington stock center to be both convenient and sufficient for behavioral tests (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/bloomfood.htm).

Flies of different genetic backgrounds should be outcrossed to a common stock for at least six generations (e.g., Rosato and Kyriacou, 2006). This can remove accumulated modifiers of behavior (polymorphisms, mutations etc.), that may exist between laboratory fly strains. If time is critical, even one round of outcrossing is better than none as it should exchange and equilibrate on average half of these alleles. Different genetic backgrounds may influence the expression of genes required for learning and memory so care should be taken to check for the continued expression of a mutant phenotype after outcrossing (de Belle and Heisenberg, 1996). This concept has gained wide acceptance and different laboratories tend to favor one of two approaches. Common co-isogenic backgrounds selected for optimal or reliable performance will minimize genetic variability in the experiment and possibly enable detection of important behavioral differences among groups (e.g., de Belle et al., 1989). However, choose the wrong background lacking alleles important for expression of a particular trait and your phenotypes may vanish (M. Boyle and A. Whittaker, Dart Neuroscience, personal communication). Alternatively, outcrossing all strains to a common but variable wild-type background affords assessment of genetic or physiological manipulations across many possible genomes (e.g., de Belle and Heisenberg, 1996). This method is more reflective of flies in their natural setting and should be generally applicable except for behavioral differences only revealed in specified backgrounds (e.g., Helfrich-Förster et al., 2002; Serway et al., 2009).

For larval experiments, aged animals are most commonly collected at the third instar stage (96–120 hr), either by careful extraction with a brush or through the use of a sucrose solution (e.g., Aceves-Piña and Quinn, 1979). Larvae should be gently washed with tap water immediately before experiments to remove residual media. In adult experiments, flies aged 2- to 6-days-old are preferred for behavior studies (e.g., Simon et al., 2006) and should be transferred to fresh media where they can groom for at least 30–60 min, but preferably overnight. In the absence of a specific plan to retrain or retest, flies should be discarded after each experiment. This avoids complications due to behavioral changes from experience, possible contamination of parent stocks due to reintroduction of flies and ensures that only naive responses are recorded for all experiments.

The use of anesthetic agents (such as CO_2 and chilling) has negative effects on fly behavior (Baron, 2000) and should be avoided within 24 hr of an experiment. Where anesthesia is absolutely necessary, chilling is the method of choice (Baron, 2000). Ether as an anesthetic agent is known to have long-term impacts on behavior and should be avoided (Kaplan and Trout, 1969, van Dijken et al., 1977).

Environment

The significant challenge in measuring any behavior, especially experience-dependent behavior is to minimize unintended contributions of external (environmental) and internal (physiological) influences. Because flies have poor vision above 600 nm (Heisenberg and Wolf, 1984), we recommend that all experiments are conducted in a controlled environmental chamber under dim red light to minimize the impact of visual stimulation on olfactory behavior. For wild-type flies, a temperature of 24 °C or 25 °C, or rearing temperature is usually optimal. Humidity levels are best held constant between experiments if possible, and higher levels (>65%) are required for aversive experiments with electric shock reinforcement to help conduct electrical charge through the cuticle of adult flies (see below). Without consistently high humidity, stimulus salience is reduced, leading to a significant drop in learning performance (Tully and Quinn, 1985). Unintended chemical, light, sound, and mechanical stimulation should be kept to a minimum. For example, avoid opening and closing the chamber door during an experiment. Especially for olfactory assays we recommend abstinence from strong or aromatic deodorants, perfumes, soaps, cologne, and even toothpaste (especially if you plan to aspirate flies). Any variables that cannot be controlled should be documented (e.g., time, experimenter, etc.). Care should be taken to observe consistency of all physical parameters, both between each run of an experiment and between experiments. Experimental and control groups should receive identical regimes of stimulus presentation so that significant behavioral differences can be attributed to intended manipulations (e.g., mutations). As in all behavioral experiments, genotypes or other distinctions should be concealed from the experimenter until data is collected to avoid unintended biases.

Devices used for olfactory behavior should be cleaned of debris on a daily basis (we use test-tube brushes) and all odors and dilutions prepared new for each set of experiments. If the training apparatus becomes excessively dirty, we recommend distilled water and a light detergent for cleaning. Some laboratories use water only. Flies demonstrate better and more reproducible learning in a device after several rounds of training and testing have occurred (Tully and Quinn, 1985), indicating that perhaps lingering chemical cues from other flies serve to "condition" the apparatus. For this reason, we recommend cleaning only sparingly and never during an experiment. If detergent is used, be sure to rinse the device thoroughly with distilled water.

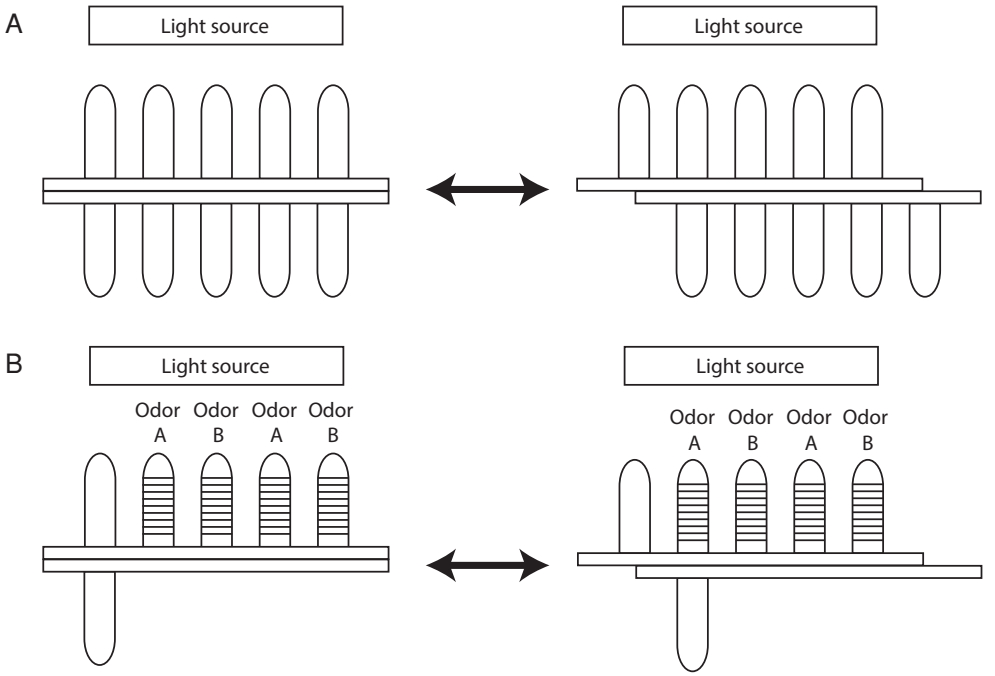

Fig. 18.1. Habituation assays. A Jump response. Individual flies are loaded into an inverted tube enclosed by a wire-mesh. Both air and odor enter through a small tube at the top of the device and are exhausted from through the bottom. The arrow indicates the direction of airflow. Individual flies placed inside the tube receive 3 s presentations of odor to elicit a startle response. Repeated exposure to odor reduces the frequency of startle, providing a measure of habituation. The assay can be run manually (McKenna et al., 1989) or automatically with computer-controlled solenoids and tracking software (Asztalos et al., 2007a, Sharma et al., 2009). B Y-maze. This is used for short- and long-term habituation (Das et al., 2011). Groups of ~15–30 flies are introduced into the bottom tube of the maze and allowed to climb into the top arms. The arrow indicates the direction of airflow and the gray circle indicates the exhaust port. One tube arm is connected to an odor source and the other to a blank airflow. Naive odor avoidance is assessed by counting the number of flies that avoid the Y-maze arm containing odor. After exposure to odor in a separate bottle (habituation), flies are re-assessed for their avoidance of the odor. An increase in the number of flies entering the odor-containing arm is indicative of habituation.

Habituation of adults

Habituation is a simple, non-associative form of learning that measures the decline in response to repeated presentations of a single stimulus (Rankin et al., 2009). Several of the more common olfactory habituation assays used to interrogate flies are described in this section.

Chemosensory jump and startle response assays

The chemosensory jump assay is easy to use and generates robust measures of behavioral plasticity (Fig. 18.1A; McKenna et al., 1989). Individual flies are placed into an inverted plastic culture tube connected to an odor source (top) and a vacuum exhaust (bottom). After allowing the fly to climb about a third the height of the culture tube, a brief pulse of odorant is administered through the top-connected odor tube. A positive response is counted if the fly reacts by jumping off the side wall of the tube and landing on a thin mesh screen below. This response is not traumatic, as the fly resumes normal walking and climbing behavior shortly after the odorant pulse is administered.

Automation combined with the basic principles of this assay have led to a more robust and higher throughput assay (Asztalos et al., 2007a, Sharma et al., 2009). Computer-controlled solenoids are used to precisely administer 4 s odor pulses to flies isolated in chambers similar to those used in earlier jump response studies. Timing for both the inter-stimulus-interval (ISI) and inter-trial-intervals (ITI) can be modified through adjustments in the controller software. Habituation is observed as the declining tendency of flies to jump after repeated odor presentation over time. This change in behavior can be recorded and analyzed with image recognition software. An important control used to distinguish habituation from sensory fatigue or other non-specific causes of lowered olfactory response is to demonstrate dishabituation. This can be easily induced by vortexing the test chamber. Upon subsequent odor presentations, flies that previously showed habituation should demonstrate elevated responses similar to those of naive flies, whereas continued odor presentation in the case of fatigue should elicit a further decline in response.

In one of the only documented measurements of olfactory sensitization Asztalos et al. (2007b) modify their assay used for measuring dishabituation (above) by vortexing flies prior to testing jump responses. In their report they use genetic dissection to distinguish sensitization from dishabituation.

The startle response assay is another observable olfactory-based habituation test for small populations of flies that is similar in several respects to the chemosensory jump assay (Cho et al., 2004). Upon presentation of an aversive odor, flies will exhibit a brief increase in locomotion (i.e., "startle") that

can be quantified and distinguished from normal movement. Groups of 20 flies are placed in an acrylic observation chamber and their responses to repeated pulses of ethanol vapor are monitored with visual tracking software. As in the automated chemosensory jump assay, declining startle responses over time can be assessed with adjustments of both the ISI and ITI.

Y-maze assay

In addition to these automated procedures (above), recent work by Das et al. (2011) has demonstrated simple olfactory habituation through the use of the Y-Maze assay (Fig. 18.1B). A similar concept was first adopted for olfactory studies in *Drosophila* by Thorpe to examine habituation (1939) and later to screen for mutants with olfactory defects by Rodrigues and Siddiqi (1978). The Das et al. (2011) Y-Maze consists of a removable glass test tube (bottom) connected to an acrylic collar (middle) containing an exhaust port attached to a vacuum. At the top of the collar sits a branching "Y" glass piece onto which two vertical glass tubes are connected. Air bubbled through odorant in water (e.g., 10^{-3} dilution of ethyl buterate, EB) is drawn downward to the maze through one tube and blank air bubbled through water alone is drawn through the other at rates of from 60 to 65 ml min^{-1}. To assess odor avoidance, groups of 20–30 flies are starved overnight on moist filter paper. The next day they are introduced to the bottom of the maze and allowed to walk up towards the choice point of the "Y" for 15 to 30 s, while a vacuum draws odor or air downward from the top glass tubes into the middle exhaust. The number of flies in each arm is then counted. The maze is then gently tapped to knock the animals back into the bottom tube and rotated 180° to exchange the locations of the odor- and air-containing arms. At the choice point, flies are typically less inclined to avoid the odor when they are habituated to it. A response index (RI) represents the average odor avoidance of four such tests ($n = 1$). RIs are calculated as the mean of the normalized proportion of flies that avoid the odor and range from 1 (complete avoidance) to −1 (complete preference). A score of 0 reflects no preference.

To measure short-term habituation (STH), flies are first assessed for their naive odor avoidance as described above. They are then exposed to odor for 30 min and immediately re-assessed for their avoidance response in the Y-Maze. Flies are habituated by passive exposure to odors in a small glass jar sealed with a cotton plug. Odorant (e.g., 5% EB diluted in paraffin oil) is held in an Eppendorf tube covered with perforated cling-wrap to allow diffusion into the jar. RIs of flies avoiding the odor both pre- and post-habituation are then compared as a metric of habituation. For example, naive flies will avoid a mildly noxious odor about 80% of the time, whereas after habituation avoidance is closer to 20%.

Long-term habituation (LTH) can also be studied in the Y-Maze. In these experiments, LTH is induced by exposing newly eclosed (<12 hr) adult flies to an odor (e.g., 20% EB) suspended in food for 4 days with the odorant suspended in a normal culture bottle containing food. The control group is similarly exposed to paraffin oil. The RIs calculated for the exposed and control group are then compared and used as a measure of LTH. This form of habituation typically lasts from 4 to 7 days and recovers spontaneously over time.

Aversive classical conditioning of adults

Associative learning is based on temporal relationships of two or more stimuli (Walker, 1987). In classical conditioning, one stimulus is conditioned (CS) when paired with another (punishment or reward; US) and acquires the ability to evoke a conditioned response (CR) comparable to that of the US (Pavlov, 1927). In operant conditioning aversive or appetitive stimuli are experienced and learned as consequences of a subject's own behavior (Brembs, 2009). In this section we focus on olfactory classical conditioning. While operant visual learning assays are well established in flies (e.g., Wolf and Heisenberg, 1991) and some assays discussed below have some operant features (i.e., training is, in part, contingent on the flies' behavior), a purely operant olfactory assay has not been established in *Drosophila*. Krashes and Waddell (2010) provide additional useful information about olfactory conditioning assays for flies.

Quinn harris benzer assay

The first reliable olfactory conditioning assay was designed for populations of adults and used electric shock as an aversive unconditioned stimulus (US). It was developed by Quinn et al. (1974) and based on a device originally employed for counter-current fractionation of fly populations to measure innate responses to light (phototaxis) or gravity (negative geotaxis) (Benzer, 1967). It consisted of two rows of tubes, one moveable and aligned, so that flies could walk freely from tubes in the bottom row to those in the top. At the beginning of an experiment, groups of ~35 to 50 flies were placed into the first lower tube and allowed to walk into the first upper tube for a specified amount of time. The upper tubes were then shifted to the right so that the first tube registered with the second on the bottom. After gently knocking the flies back to the bottom, the tubes were returned to their original alignment and flies again were permitted to walk upward. These steps were repeated five times. A measure of the population's innate response to either gravity or light was calculated as the sum of the percentages of flies that completed each number of possible transitions (0–5).

Through a modification of this counter-current device, Quinn et al. (1974) conditioned flies to avoid an odor (conditioned stimulus; CS) that had been paired with electric shock (US). This became known as the Quinn Harris Benzer (QHB) assay (Fig. 18.2). Electrified copper grids painted with an odorant were rolled and placed in the tubes to present paired stimuli. Shock-associated odor (CS^+) was present only in the reinforced tube. Flies were moved by gentle tapping and shifted to the next conditioning tube, where a second unreinforced odor (CS^-) was presented. They were then moved through the final two tubes of the device containing either CS^+ or CS^- odors. With successful conditioning, more flies entered the

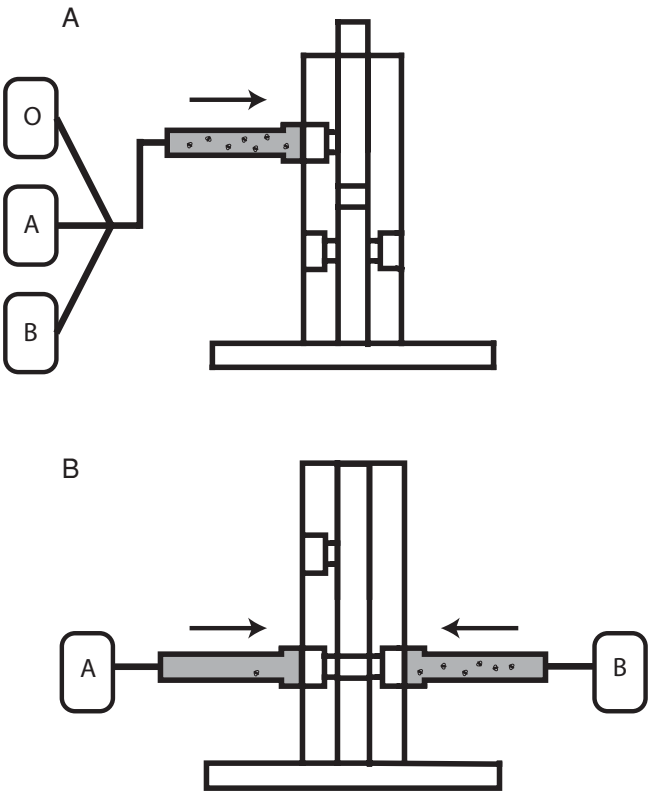

Fig. 18.2. Quinn, Harris, Benzer (QHB) Assay. Flies are introduced to the single bottom tube used to transit flies in training and testing. The first upper tube is a blank rest tube. The remaining four upper tubes contain rolled electrifiable grids alternatively painted with two different odorants (CS^+, CS^-). Electric shock (US) is applied to tube 2 (or 3 in reciprocal experiments) during conditioning. Flies are positioned beneath different tubes and permitted to walk upward in response to gravity and light. They are gently tapped into the lower tube after each transition. Conditioning is three cycles of 60 s rest (tube 1), 15 s CS^+ + US (tube 2), 60 s rest (tube 1), 15 s CS^- (tube 3). In the test, flies entering tubes 4 (CS^+) and 5 (CS^-) are counted. A learning index (λ) is calculated from alternate CS/US pairings in reciprocal experiments. Redrawn from Quinn et al., 1974.

test tube containing the CS^- odor than the one with the CS^+. Control for innate odor bias was achieved by training and testing a second group of flies with reinforcement switched from the first odor to the second. A learning index (λ) was calculated as the mean of the normalized proportion of flies avoiding the conditioned odors from each test respectively. In theory, λ could range between 1 and −1. A score of 1 indicated perfect learning and 0 indicated no learning. In practice, λ for wild-type flies rarely exceeded 0.4.

While the assay offered consistent aversive conditioning, several drawbacks were apparent from its design and application. Most importantly, training was reliant on flies climbing up from a blank tube into a copper-grid lined tube and remaining there during conditioning. After only a single round of training, animals became "hesitant" to enter the conditioning tube – an operant response to the US – and often did not receive sufficient training (Quinn et al., 1974). The ability of flies to walk into the different tubes was also reliant to some degree on normal motor function; mutant stocks deficient in locomotion could not be properly conditioned. Furthermore, odor delivery depended on painting the shock grids between experiments, a process that resulted in poor control over precise levels of odor exposure. These confounds, in addition to the narrow range of scores that could be obtained even immediately after training, prompted workers to abandon the QHB assay in favor of more innovative devices developed by the emergent fly "mnemogenetics" community of the 1980s (Heisenberg, 1989).

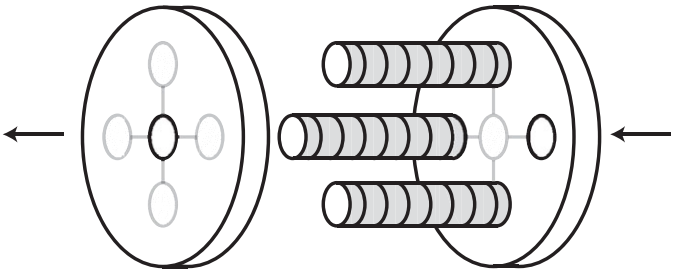

Fig. 18.3. T-maze assay. Arrows show direction of air flow carrying odors drawn from a port at the rear of the machine. A Training. For aversive conditioning an electrifiable training tube has either a rolled printed copper grid (Tully and Quinn, 1985) or an embedded double-wound copper coil (Wang et al., 2007). For appetitive conditioning it has rolled filter paper with either a sucrose solution or water (Schwaerzel et al., 2003). Odors are CS^+, CS^- (A, B) or blank (0). B Testing. Flies are transferred in an elevator to a choice point between converging air currents carrying A or B odors in collection tubes connected to the lower ports of the machine. They are trapped in either tube by raising the elevator at the end of the test and counted. In this example, flies were conditioned to avoid the odor A (CS^+). A performance index (PI) is calculated from reciprocal tests as in the QHB assay. Redrawn from Tully and Quinn, 1985.

T-maze assay

In an effort to improve on the QHB assay, Tully and Quinn (1985) established a more robust method of assessing aversive olfactory learning and memory in populations of flies using the now well known T-Maze (Fig. 18.3). This device is a mainstay in odor learning studies, because it offers absolute control over stimulus presentation essential for conditioning (Pavlov, 1927) and simple binary fractionation of animals. Based on these benefits, it has also been adapted for other types of conditioning and behavior experiments (some are described below). It consists of an inverted "T" shape, usually constructed of acrylic plastic, to which tubes used for both training and testing can be connected. It also has a sliding center piece that provides ports to draw air carrying odors through the device as well as an "elevator" to transport flies from their training tube to a choice point between CS odors in collection tubes. CS odors were delivered in the original T-maze assay by passing air over the surfaces of odorants held in small cups at the ends of training and testing tubes (Tully and Quinn, 1985). A simple method of equilibrating innate response differences to odors was to vary surface area (de Belle and Heisenberg, 1994b). Currently, more precise control of odor delivery is achieved by bubbling humidified room air through small quantities of odorants diluted in paraffin or mineral oil held in individual glass chambers (Tully et al., 1994b; Tabone and de Belle, 2011). This also provides a simple means

to adjust odor concentration in different experiments. Dilutions are commonly effective in the range of 10^{-3} or 10^{-4}. The optimal voltage of electric shock reported in the literature varies. Generally it ranges from 60 to 90 V DC but can be as high as 120 V without detriment (de Belle and Heisenberg, 1996). Tully and Quinn (1985) suggested that significant increases in learning scores are not normally observed at voltages greater than 60 V DC. However, salience of voltage as a US is dependent on humidity (as noted above), which is likely variable and accounts for much of the discrepancy.

Flies are trained in the T-maze in groups of approximately 100. They are either gently tapped or aspirated into an acrylic training tube lined with either an electrifiable copper grid (as above) or an embedded double-wound copper coil (Schwaerzel et al., 2003; Presente et al., 2004). The training tube is then plugged into the upper port of the T-maze (Fig. 18.3A). Flies are first acclimated to a consistent airflow of a "blank" odor (room air) drawn through the training tube at a rate of 650 to 750 mL min^{-1} for 60 to 90 s. Following acclimation, a single odor (CS^+) is passed through the tube for 5 s. Immediately after this initial presentation, electric shock (US) is delivered in dc pulses of 1.25 or 1.5 s duration, typically 0.2 Hz for 60 s. During this time the odor continues to flow uninterrupted at a rate identical to the blank odor presentation. In classical conditioning it is imperative to deliver the US *after* the initial CS presentation. Presenting the US first can lead to inhibition of conditioning (Tully and Quinn, 1985). After 60 s of odor and pulsed shock conditioning, the chamber is cleared with the blank odor for 45 to 60 s. A second unreinforced odor (CS^-) is then introduced for 60 s (in an identical manner to CS^+ presentation). It is important that flies remain completely undisturbed to prevent aversive memory formation with the CS^-. A second presentation of blank odor follows the CS^- for an additional 45 to 60 s to clear the training tube of residual odor and complete the training cycle. Flies are then gently tapped into the elevator where they remain for 60 to 90 s while collection tubes are prepared and attached to the bottom ports of the T-maze. Next, they are lowered to the choice point where they are free to move between CS^+ and CS^- odors delivered from opposite ends of both collection tubes (Fig. 18.3B). After 120 s the elevator is raised, confining flies to either tube. The tubes are then removed, flies anesthetized and counted. A performance index (PI) is calculated as the normalized percent of flies avoiding the shock-conditioned odor (CS^+) (Tully and Quinn, 1985):

$$PI = [(\#\,\text{avoiding}\,CS^+ - \#\,\text{avoiding}\,CS^-) / \Sigma] \times 100$$

As with the QHB assay, control for innate odor bias and other non-associative factors is achieved by training and testing a second group of flies with US pairing switched to the previously unpaired odor. A single PI ($n = 1$) is then the average of PI^{CS1} and PI^{CS2}. PIs can range from 100 (perfect learning) to −100, with 0 indicating no learning. Typically, wild-type flies are strongly repelled by shock-punished odors when tested immediately after training and should score from 80 to 90 or even higher. A negative score indicates a preference for adversely conditioned odors (CS^+), which to our knowledge has not been demonstrated.

PIs calculated from flies tested 1 min after training only approximate the learning that occurs when there is no direct metric available. They actually measure something quite different – the recall of very short term memory. Operationally, we extrapolate backward 1 min and assume that decay over this short time is minimal. For assessment of memory, a strength of Pavlovian conditioning is that subjects receive known intensity, duration, and number of presentations of the CS and US. Longer-lasting memory can be examined by carefully removing flies from the training device, usually storing them on fresh food and testing them at later time points. Different phases of memory can be distinguished by single, massed, or spaced training protocols and by the length of time between training and testing (Margulies et al., 2005; Davis, 2011). With a single training session, short-term memory (STM) typically lasts around 1 hr. Intermediate or middle-term memory (ITM/MTM) forms during the first hr post-training and lasts about 5 hr. Memory from a single cycle of training will decay completely within 24 hr. Flies trained with multiple training sessions (see Automation and Throughput section below) exhibit different forms of memory depending on whether the training sessions contain rest intervals (spaced) or not (massed). Importantly, flies exposed to either spaced or massed training receive equivalent amounts of paired stimulus exposure. Anesthesia-resistant memory (ARM) is formed by both spaced and massed training, and has been reported to last for 24–72 hr, while not requiring *de novo* protein synthesis (Tully et al., 1994). Long-term memory (LTM) is induced by spaced training only, can last for upward of 1 week, and is reliant on the synthesis of new protein (Tully et al., 1994b).

The T-maze assay addressed all of the challenges plaguing its predecessor. Pairing of shock and odor is guaranteed as flies are trapped in a training tube with >95% of its inner surface, electrifiable. They do not find the small unpunished surface, as flies are positively amenotactic and attracted toward the air flow source at the opposite end of the tube. Moreover, odor delivery is quantifiable and can be carefully regulated. Pure odorants are generally preferred as they are more consistent than fruit, beer or other compound odorants. Among many odor combinations tested (Dudai, 1977), 3-octanol (OCT) and 4-methylcyclohexanol (MCH) tend to give consistently reliable and high learning scores with wild-type flies and remain the most commonly used for conditioning. Benzaldehyde (BAL) is occasionally substituted in some studies (e.g., de Belle and Heisenberg, 1994; Xia and Tully, 2007).

Sensory acuity tests are a critical part of assessing the performance of *Drosophila* in any olfactory-based assay. Genotypically distinct flies may differ in odor perception and/or innate odor avoidance, shock reactivity or general mobility. The source could be pleiotropy of a mutation primarily influencing behavioral plasticity or variation in alleles elsewhere in the genome (in theory, they can be eliminated by outcrossing unless tightly linked; de Belle and Heisenberg, 1996). Either way, these deficits

impair performance in the assay and confound comparisons of learning or memory among different groups of flies. Controls to detect them are similar in most respects to the tests themselves. Another advantage of the T-maze is that it offers convenient task-relevant measures for these controls.

To assess naive odor avoidance, flies are loaded into the upper arm of the T-maze and allowed to acclimate to airflow for 90 s, as in conditioning. The control is identical to the test except that flies are presented with a choice between a single odor and blank airflow for 120 s. Naive responses to odors are very sensitive to concentration and highly reproducible (e.g., Wang et al., 2007; Masek and Heisenberg, 2008; Tabone and de Belle, 2011). Ideally, avoidance PI scores between 60 and 90 are desired. Odors that are too strongly repellant at a particular concentration may negatively affect conditioning, particularly in their role as a CS^-. Odors that elicit very low avoidance may result in weak salience and lack of association with the US during training. Naive odor avoidance for experimental and control groups of flies should not be different in the same experiment (Boynton and Tully, 1992). In situations where differences are found, the options are to adjust odorant concentration (e.g., Wang et al., 2007) or consider selecting alternative odorant pairs (e.g., de Belle and Heisenberg, 1994).

Electric shock reactivity controls are conducted in a similar manner, except that electrifiable training tubes are substituted for collection tubes at the lower ports of the T-maze. After 90 s of acclimatization, electric current identical to that used in conditioning is applied to one of them for 120 s, while the other remains unpowered. Ideally, close to 100% of the flies will avoid the electrical shock by moving to the unpowered shock tube. Shock application should be alternated between left and right tubes with each subsequent test, so that the tubes are "conditioned" equally by flies throughout the experiment (see below). Assuming that sensory acuity controls are normal, motor behavior is deemed to be sufficient for demonstrating conditioned behavior. This can also be validated with independent assessments of motor behavior (e.g., Benzer, 1967; de Belle and Heisenberg, 1996).

A number of useful optimizations and tests can help to achieve reliably high performance with the T-maze assay. Perhaps the most critical of these is to ensure consistent and balanced air flow. This should be carefully metered and measured. While O-rings are placed at several points in the T-maze to minimize leaks, they nonetheless often manage to bias experiments by altering air flow and odor concentration. A simple solution adopted by several groups is to seal all connections with Parafilm®, thereby alleviating the problem – whether or not it exists in your system. In addition to demonstrating comparable naive avoidance of single odors among experimental groups (above), an equal distribution of flies between both the CS^+ and CS^- odors without aversive conditioning (i.e., odor balance) is also an important consideration. Ideally, naive flies should distribute themselves equally in both collection tubes, giving a PI = 0. This balance may skew greatly in instances of high odor concentrations or differences in odor preference. Extreme unbalanced responses may be detrimental, with the repulsive nature of one or both odors overriding conditioning. In these cases, flies will tend to choose collection tubes containing the least repellent odor, regardless of prior conditioning. Adjustments involving lowering or raising concentrations of both odors are necessary to find an optimal CS balance and salience for training.

Salience of the US in training and control experiments is dependent on voltage, and pulse duration and frequency (Tully and Quinn, 1985). As noted above, voltage can be adjusted along with humidity for one type of US salience optimization. Shock duration and frequency are commonly set from 1.25 to 1.5 s and 0.2 Hz. These values were found to be in excess of those needed for optimal training of wild-type flies but are also sufficient for optimal training of genetic or other variants that can be mildly resistant to the US (Tully and Quinn, 1985). At a minimum, these parameter settings should serve as a benchmark for those establishing this aversive conditioning assay. Adjustment to sub-threshold values has utility when attempting to identify variants with enhanced learning or memory capacities (see below). Reliable US delivery requires that training tubes have their inner surfaces fully electrifiable. In addition to gentle cleaning to remove possible debris that can hinder shock delivery, some laboratories occasionally use light abrasives to remove oxidation and maintain optimal electrical contact with the fly. The integrity and (usually) soldered connections of printed grids are particularly delicate and should be regularly tested with a multimeter. Any disruption of the circuit will interrupt the US.

Variants and developments

Punishment

While electric shock is the most common aversive US used in associative conditioning experiments with adult *Drosophila*, other mechanisms of reinforcement have been demonstrated. For example, Mery and Kawecki (2005) have shown that a mechanical shaking US with a test tube shaker can be used to induce both forms of persistent olfactory memory (ARM and LTM). Delivering 2000 rpm vibrations for 1 s every 5 s for 30 s during odor exposure is sufficient to form an aversive memory to an odor.

Throughput and automation

Building upon the T-maze, several groups have made developments in throughput and automation of the aversive conditioning assay through the use of computer controlled relay systems (Tully et al., 1994b; Murakami et al., 2010; Tabone and de Belle, 2011; Chen et al., 2012). These have contributed greatly to the efficiency, accuracy, and flexibility of training that can now be used to study plasticity of olfactory behavior in flies. The CS^+, CS^- odors, and US electric shock can be administered through the use of controllers and computer programs such as National Instruments LabView® or other custom software. An automated approach provides better consistency between

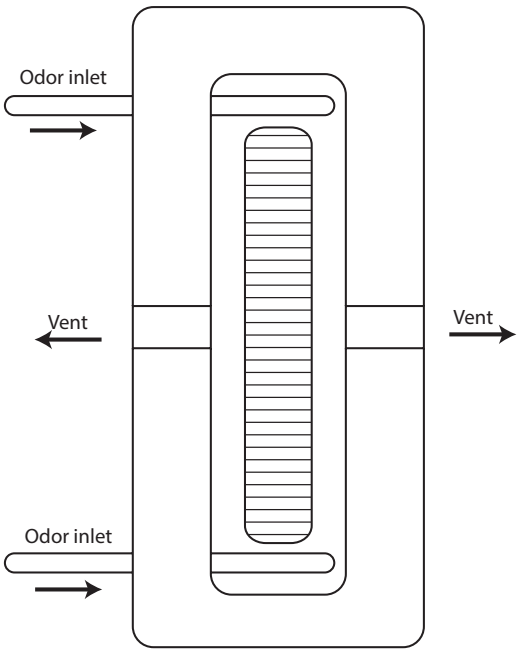

Fig. 18.4. Barrel assay. This multi-tube device provides simultaneous conditioning of four separate groups of flies with a single odor source (other variants include systems with six or eight tubes). Arrows indicate the direction of airflow through a unified intake and exhaust. Groups of 35–50 flies are introduced into the electrifiable grid-lined tubes (gray) and conditioned as in the T-maze (Pascual and Préat, 2001). The device is also modified for appetitive conditioning with sugar (Colomb et al., 2009). After training, flies are removed for testing in a T-maze.

experiments by precisely controlling delivery of the odor and shock in addition to minimizing any undesired disturbance of the flies from handling of the training device. The first example of such a system was by Tully et al. (1994b), demonstrating persistent forms of memory in flies. Their "robo-trainers" consist of multiple self-contained modules, each with an electrified tube connected to a manifold providing three separate 750 mL min^{-1} airflows delivering blank air and both odors to the flies under the control of an external computer. Multiple groups of flies can be trained automatically without a need for researcher intervention. This allows for implementation of both massed and spaced training for the study of ARM and LTM. Modifications in training can include varying the number of trials and the length of inter-trial-interval (ITI), for example. Although training is automated, it is important to note that testing is not. Flies must be removed from the robo-trainers and retained on fresh food until testing in a regular T-maze as above. Some groups have found that holding trained flies at a lower temperature (e.g., 18 °C; Li et al., 2013) leads to more consistently reproducible memory scores (J. Dubnau and S. Xia, personal communication).

Another extension of the T-maze for aversive training is the so-called "barrel" assay (Pascual and Préat, 2001; or "Gatling gun," M. Heisenberg, personal communication; Fig. 18.4). This type of system utilizes similar shock tubes, but allows for simultaneous conditioning of multiple groups of flies. After loading several electrifiable tubes with 35 to 50 flies, circular manifolds are fastened on both ends and serve as common odor intakes and exhausts. Using this method, odors are drawn from a single vacuum source and distributed among multiple tubes. A training regimen identical to conditioning in the T-maze can be used. Following training, flies are carefully removed from the tubes and introduced to separate T-mazes for testing.

Single fly assay

Recent experiments by Claridge-Chang et al. (2009) demonstrate the viability of a fully automatic olfactory-based training system for individual flies. Each fly is introduced into a rectangular training block with opposing electrifiable surfaces (Fig. 18.5). Odors are passed from either end of the chamber

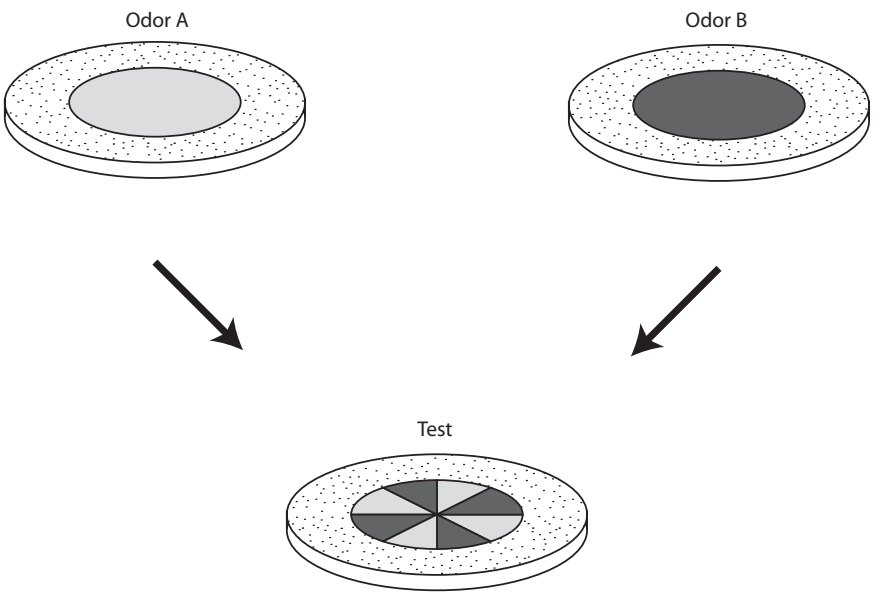

Fig. 18.5. Single fly assay for aversive conditioning. Single flies are introduced to this fully automated assay through a side port of a chamber lined above and below with an electrifiable grid. Flies are conditioned to avoid odor (CS^+) presented from both ends of the chamber paired with shock, followed by an unpaired odor (CS^-), and tested in the same chamber. CS^+ and CS^- odors are presented from opposite ends of the chamber in the test. Fly movement is tracked with a CCD camera through a transparent window on the side of the chamber. The assay can be run in both classical and operant modes. The time a fly spends in either end of the chamber in the presence of each odor is used to calculate a learning score. Redrawn from Claridge-Chang et al., 2009.

and flow towards a vacuum exhaust located in the middle of the device, while electric shock can be delivered to both the top and bottom grids. Flies are free to move throughout the chamber and their position is tracked by an infrared-sensing camera. The assay can be run in classical conditioning mode, with experimenter-determined stimulus presentation mirroring closely the training parameters employed for populations in the T-maze. Learning is assessed by simultaneously presenting both the CS^+ and CS^- from opposite ends of the chamber and tracking the amount of time spent by the fly at either end of the chamber. This entire procedure allows for training and testing of *Drosophila* with no intervention on the part of the researcher, thus minimizing potential complications due to human error. Dimly backlit chambers are stacked in groups of 20, imaged by a remote camera that captures the position of each fly over time. Data and analysis suggest that it generates metrics with a range and dynamics similar to the T-maze (Claridge-Chang et al., 2009; S. Xia, Dart Neuroscience, personal communication).

Because this assay is fully automated and the actions of single flies can be used in closed loop mode to drive stimulus presentation, it is (so far) uniquely able to investigate aspects of operant olfactory learning, while retaining some Pavlovian characteristics (Brembs and Heisenberg, 2000; Colomb and Brembs, 2010). This novel aspect of the automated single fly assay represents an important addition to the tools available for studying olfactory learning in flies.

One facet of the single fly assay is very different from discriminative olfactory assays for fly populations. While two odors are presented (OCT and MCH in the original report), only one fly is exposed to only one reinforced odor. The average of training to both odors is not used. Instead, odor preferences assessed prior to training represent baseline responses that can be modified by pairing with electric shock US.

Appetitive classical conditioning of adults

Appetitive variants of aversive assays

Apart from aversive conditioning, adult *Drosophila* have been trained to form appetitive memories in both the QHB and T-Maze devices in experiments that closely resemble those described above (e.g., Tempel et al., 1983; Schwaerzel et al., 2003). We describe the T-maze variant of this appetitive assay here. The electrifiable copper grids normally used for conducting shock are replaced with rolled filter paper spanning the length of the training tube. One training tube has paper soaked in a 2 M sucrose solution, while the other has paper soaked in water only. Whereas electric shock US is always unpleasant, the salience of sugar as a US is dependent on hunger and satiated flies are generally poorer learners. Consequently it is important in these experiments to starve adults overnight (up to 20 hr) beforehand on damp filter paper (Krashes et al., 2009). During training, odor is channeled through the tube for 30 s (CS^+). Following this paired presentation, flies are transferred to a new tube containing filter paper soaked in water alone and a second odor is then passed through this tube for another 30 s (CS^-). Testing is conducted as above when training involves an aversive US. Calculations of λ and PI are the inverse, however, reflecting positive changes in response to training due to the appetitive US and giving negative avoidance scores (Schwaerzel et al., 2003).

The barrel device (Pascual and Préat, 2001) has been adapted by Colomb et al., (2009) for appetitive conditioning. In this assay flies are loaded into barrels through which odor can pass, but flies are restricted from access to sugar by rotating lock mechanism. Upon administration of the odor, the device is rotated in such a fashion that the flies are immediately allowed access to the sucrose US. The authors note that around 90% of the flies extend their proboscis within 5 s of rotation and sucrose presentation (Colomb et al., 2009). Testing is still performed outside of the barrel system in the T-maze and PI calculation is the same as in Schwaerzel et al. (2003).

Sliding box assay

While appetitive conditioning has been successfully adapted from aversive conditioning assays (above), these conversions are not without some technical challenges. Most significantly, since positive reinforcement (US) is usually administered as sucrose absorbed onto filter paper, the experimenter must remove the US or transfer the flies to a new chamber between presentations of the reinforced CS^+ and unreinforced CS^-. This is time consuming and usually introduces a disturbance that can add noise to the experiment. A more recent take on the appetitive assay developed by Kim et al. (2007) addresses this issue with a clever but simple device that is designed from the beginning as an appetitive assay. The "sliding box" assay (Fig. 18.6) has a movable "box-on-rails" for gently transporting flies between any of six positions, where they can be conditioned to odors paired with or without sucrose US. Flies are loaded into a rectangular sliding box, which sits between a set of rails. Water- or sucrose-soaked filter papers can be inserted in the different sections along each rail. The top and bottom of the sliding box are open, exposing flies to different sections of filter paper as the box is moved. Generally, all compartments but the second one have filter paper soaked with water and the second one has paper with a sucrose solution. Odors are drawn into the sliding box through an opening on one side.

The training trials in this assay are modeled after those used for the appetitive T-maze assay. Flies are exposed to blank air over the first section of water-soaked filter paper. The box is then moved to the second compartment, where flies are presented with odor (CS^+) paired with sucrose (US) for 60 s. They are then moved to the third compartment for a rest period with blank air and water only for 45 s. Movement to the fourth compartment offers a presentation of a second odor (CS^-) with water for 60 s. For testing, flies are transferred from the box to the choice point in a T-maze as described above. Reciprocal training to account for odor bias and PI calculations are both

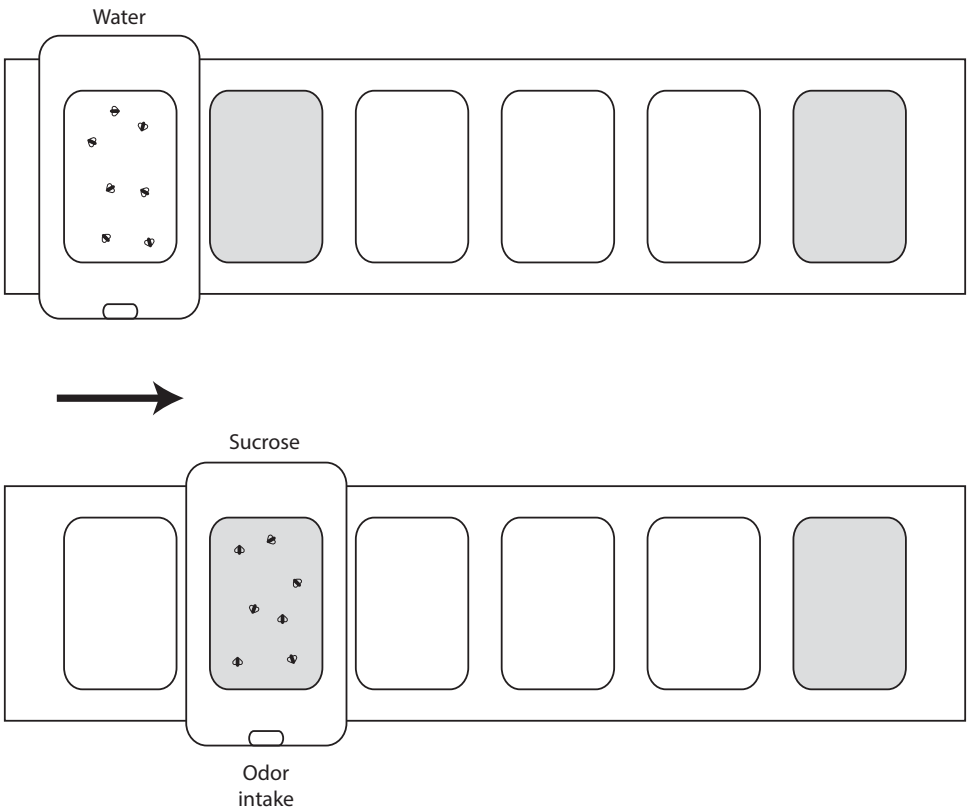

Fig. 18.6. Sliding box assay for appetitive conditioning. The device consists of a sliding chamber between two acrylic rails. Flies placed in the chamber are moved between positions where they are exposed to filter paper with sucrose or water. Odors are drawn into the box through an opening on one side. For conditioning, animals are loaded into the sliding box and moved to a rest location with water only for 30 s (top). The box is then carefully shifted to a panel where they are presented with sucrose (US) and an odor (CS^+) for 1 min (bottom). Flies are next shifted to water for 45 s (position 3), followed by the CS^- odor presentation with water for 1 min (position 4). After conditioning, flies are moved to a T-maze to for testing. A second set of flies are trained in a reciprocal manner and tested. Redrawn from Kim et al., 2007.

conducted in the same manner as for appetitive conditioning. Scores obtained are higher (about 40) than those from other appetitive assays. In a comparison Kim et al. (2007) noted that they achieved a mean score of less than 20 using the T-maze. Multiple (2–4) training trials also improve learning over a single trial and appear to give optimal scores with a 1 min ITI and sucrose concentration of 2 M. With this combination of parameters, the assay generates memory that can be observed 12 hr post-training.

Proboscis extension reflex assay

Experience-dependent change in the proboscis extension reflex (PER) has been widely used to examine learning and memory in the honey bee *Apis mellifera* (Sandoz et al., 1995). In this procedure, individual animals are carefully restrained by placement into small brass tubes, leaving only their heads exposed. In one training regimen, an odor CS is presented for 6 s and during the last 3 s a sucrose solution (20%–40%) is delivered via microsyringe, first to the antennae and then subsequently to the proboscis (US) (Bitterman et al., 1983). After multiple spaced trials with 10 min intervals, honey bees respond with a proboscis extension on the presentation of the CS alone. Unpairing the two stimuli during training for a separate group of individuals serves as the associative control (Bitterman et al., 1983).

Chabaud et al. (2006) have adapted the honeybee PER associative conditioning assay for *Drosophila*. Female flies, grown at 18 °C, aged 2 days, and starved for 24 hours are immobilized in the end of 1 mL micropipette tips while leaving their head and forelimbs exposed. Airflow is delivered to the fly at a rate of 140 mL min^{-1} through either a pipette containing odorant-soaked filter paper or a control blank pipette. The authors use banana odor delivered for 10 s (CS) with presentation of a 0.5 M sucrose solution (US) to the labellum with a filter paper tip during the last 5 s. This procedure is repeated five times with a 15 min ITI. An explicitly unpaired control group experiences the US 7.5 min after exposure to the CS. Memory is assayed after 10 min by presenting the CS odor for 10 s and observing whether the fly exhibits PER. Successfully conditioned flies demonstrate PER increases of about 30% that persist up to 1 hr post-training, on average. A benefit of this assay is that conditioning of restrained individual flies may also permit interventions and/or observations of neural and cellular processes underlying plasticity in behavior (e.g., Yu et al., 2004; Wang et al., 2008; see imaging below).

Arena assay

The "Arena" assay was developed for appetitive conditioning of single flies in the early 1980s (Fig. 18.7; Heisenberg et al., 1985; Heisenberg, 1989). A training arena consists of a dish (110 mm diameter) containing a small quantity of odorant in paraffin oil covered with tissue paper that has absorbed 1 M sucrose. The arena is surrounded by a water mote to ensure that the fly remains on task rather than wandering off. Individual starved

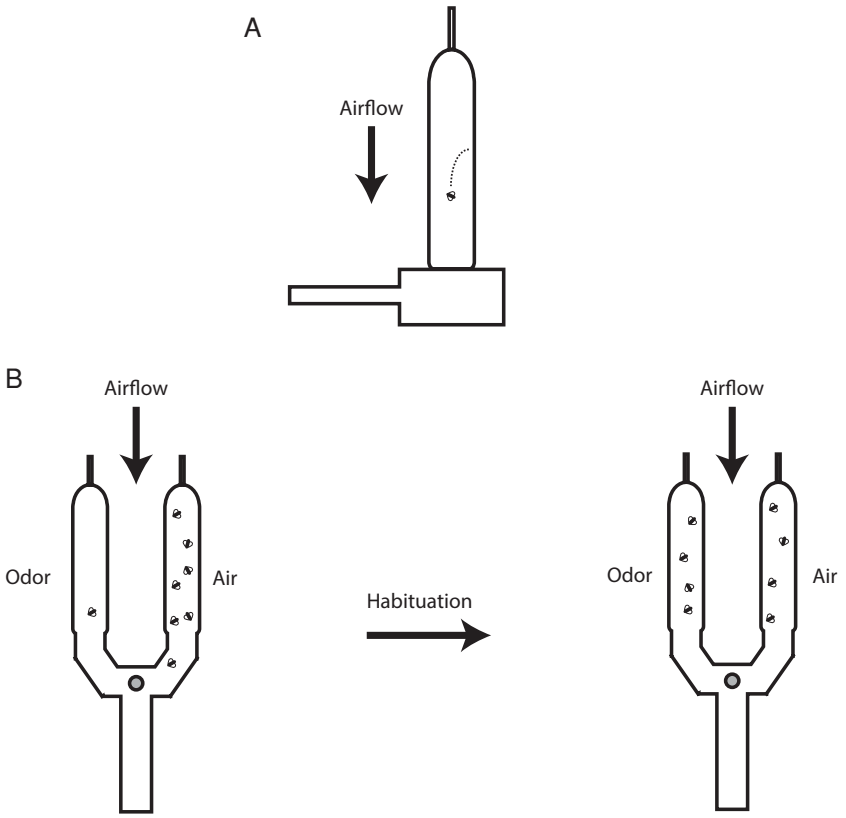

Fig. 18.7. Arena assay for appetitive conditioning. Single starved flies with clipped wings are conditioned for 10 s bouts on a circular platform containing 50 μl of odorant (CS^+) covered with tissue paper soaked in 1M sucrose (US) (top). A water mote prevents flies from escaping. Flies are conditioned with 2–3 bouts and carefully placed in vials after each. The test arena (bottom) has eight radial compartments, each containing a previously paired (CS^+) or novel (CS^-) odorant (20 μl) in alternating pattern. The amount of time a fly spends over the CS^+ odorant is a measure of learning for that odor. The mean scores calculated from two flies trained in a reciprocal manner are used to generate a learning index (λ). Redrawn from Heisenberg et al., 1985.

flies aged 3 to 6 days old with clipped wings are conditioned in 10 s bouts on the training arena where the odor (CS) is paired with unrestricted access to sucrose reinforcement (US). This procedure is repeated two or three times for each fly, after which they are carefully removed and tested. In the test, a fly is placed at the center of a similar round arena with tissue paper covering eight radial compartments. These alternately contain two different odorants, one previously reinforced with sucrose while the other is novel for the fly. Over a period of 2 min, the proportion of time spent in each compartment is used to calculate a learning index (λ). Learning is demonstrated when flies spend more time in a compartment containing a previously reinforced odor. It is noteworthy that this is not a balanced discriminative olfactory assay as training does not involve two odors (one odorant was previously reinforced with sucrose while the second is not presented during conditioning). To balance differences in odor preference within and between genotypes (or other groupings of flies), the final λ value is calculated as the mean score of two flies trained to different odors (Heisenberg, 1989). The arena assay is elegant in its simplicity but limited in that it generates comparably low learning indices (λ usually < 0.04, similar to Tempel et al., 1983) with high variability (Heisenberg et al., 1985). However, it is low-tech, requiring only a few constructed plastic parts. A teaching laboratory version of this conditioning experiment can be easily rigged with Petri dishes that will capture the essential features of the experiment for small numbers of patient students with steady hands.

Elemental and complex conditioning of adults

In a majority of olfactory conditioning experiments, one odor (CS) is paired temporally with one reinforcement (US). This reduced presentation of stimuli is usually sufficient for reproducible demonstrations of behavioral plasticity in the laboratory. However, it bears little resemblance to learning in a natural environment. To gain an appreciation for the capacity of a fly to extract meaningful signals from noise, we can examine conditioning with multiple and compound odors in a variety of different combinations. This section highlights several olfactory learning experiments involving multiple odors that should provide a starting point for those interested in complex conditioning.

Odor discrimination

The ability of *Drosophila* to discriminate between different odors and between different concentrations of a single odor has been investigated using a variety of experimental approaches. Borst (1983) used appetitive conditioning to show that flies are proficient in both of these tasks. He paired odors with sucrose in the tubes of an adapted counter current distribution device (Benzer, 1967). Flies were tested by transferring them to the choice point of a T-maze and exposed in converging air currents to two unique odors, two identical odors with varying

concentrations, or combinations of multiple odors. His results revealed that flies are quite capable of discriminating between different concentrations of the same odor. They are also capable of discerning individual components of compound odors.

More recent studies by Eschbach et al. (2011b) have examined the ability of *Drosophila* to discriminate a variety of aversively conditioned compound odors in the T-maze. Binary odor blends were created by combining odors streams from individual odorant sources into a single airflow during training and testing. Flies were aversively trained with both single and binary compound odors. They were subsequently tested for their capacity to both extract information from a compound (AB) about a previously punished component (A^+), or to recognize a component (A) of previously punished compound (AB^+). These experiments showed that *Drosophila* partially generalize mixtures and their components. The elements of a mixture AB are equivalent to the mixture itself in conditioning and testing. The reciprocal is also true: Flies conditioned to avoid either A or B will also avoid an AB mixture during the testing phase of aversive conditioning. The authors also note that odor mixture "learnability" is influenced by their physiochemical properties. For example, flies can more easily generalize an aversively conditioned odor (A^+) to a binary mixture (AB) during a test when A and B are chemically similar.

Young et al. (2011) further investigated complex conditioning with an extensive list of experimental challenges for the fly. The authors examined learning with binary mixtures (AB^+ CD^-), overlapping mixtures (AB^+ BC^-), positive patterning (AB^+ A^- B^-), negative patterning (A^+ B^+ AB^-), and biconditional discrimination (AB^+ CD^+ AC^- BD^-). Flies were able to learn many of these more complicated sets of odor and punishment pairings, except for negative patterning and biconditional discrimination. In agreement with earlier studies of complex aversive conditioning (Tabone and de Belle, 2011; Eschbach et al., 2011b) there is strong evidence for "elemental" learning wherein flies are capable of recognizing the individual components of a mixture as unique elements. All of these findings are important considerations when designing experiments involving odor mixtures.

Odor quality and intensity

The manner in which flies learn and remember odor quality, identity, and concentration was investigated by Xia and Tully (2007) and Masek and Heisenberg (2008). Both groups took advantage of aversive conditioning protocols in the T-maze. Xia and Tully (2007) demonstrated that odor identity, but not intensity, is discriminated by a mushroom body-dependent mechanism. However, following aversive conditioning, both odor identity and intensity discrimination require mushroom body activity.

Masek and Heisenberg (2008) highlighted several characteristics of odor processing in their experiments. In contrast with Tully and Quinn (1985), the authors demonstrate that differential conditioning with the CS^- does not optimize avoidance of the CS^+ in the test. They show that aversive conditioning to an odor at a particular concentration will be avoided during a test if the concentration is within an order of magnitude. Beyond this range, odors are processed as distinct entities. Flies also respond differently to conditioning trials with multiple presentations of the same odor at different concentrations compared to conditioning with multiple odors of unique chemical composition. For identical odors, flies recall the most recent concentration in instances of sequential training, while memories for two unique odors are stored simultaneously. Finally, chemically similar odors at low concentrations (e.g., isoamylacetate and amylacetate) are often generalized and difficult for to distinguish.

Temporal and second-order conditioning

Apart from elemental and compound learning, olfactory-based assays have also been used to investigate learning in episodes of competing temporal memories (Yin et al., 2009). Flies trained to avoid two different conditioned stimuli at different times (A^+ and B^+) can be tested for their choice between these two odors. Yin et al. (2009) found that stronger US (in this case, electric shock voltage) lead to stronger odor avoidance, independent of order. The same outcome was found for 1 hr memory. Flies conditioned with three different odors, each at a different voltage (30 V, 45 V, 60 V), demonstrated "flexibility of choice" and again avoided the higher voltage-associated odor when given varying pairs of odors during the testing phase (30 V vs. 45 V or 45 V vs. 60 V). If the time between two conditioning bouts is extended to more than 30 min, flies demonstrate a "recent-takes-all" approach, where only the most recent conditioning episode, regardless of the shock-associated conditioning value, takes priority over any older conditioning episode. However, memory of earlier conditioning with higher voltage tended to reduce scores of later training with weaker US (Yin et al., 2009).

Complex olfactory learning by means of second-order conditioning can also be demonstrated with flies. Tabone and de Belle (2011) used a previously negatively reinforced odor to train flies to avoid a novel odor. Three conditioning trails of one odor (CS1) paired with shock (US) followed by three pairings of a novel odor (CS2) with the first odor ($CS1^+$ in the absence of shock) was sufficient to generate a conditioned avoidance of the $CS2^+$ *vs* $CS3^-$. Second-order learning in this experiment depends on proper temporal pairing of both the CS1 and US during the first training session and CS2 and $CS1^+$ during the second training session. The $CS2^+$ odor was presented in a pulsed pattern during the second round of training similar to the manner in which the US was presented during conditioning of CS1 in the first round of training. Unpairing either the first or second training session results in an abolishment of learning.

Learning in larvae

The *Drosophila* larva is a comparatively simple invertebrate model for olfactory learning. Most experiments use mid-third

instar larva for convenience, as they are large and their behavioral repertoire is rather limited in its focus on feeding prior to pupariation (Sokolowski, 1985). Reflective of their limited behavioral needs, larvae have a relatively few olfactory receptor neurons, olfactory glomeruli, and projection neurons compared with the adult fly yet they retain a capacity to form olfactory-based memories (Gerber and Stocker, 2007). Furthermore, adults and larvae are physiologically different animals sharing a common genome. Thus, the same diverse set of genetic and transgenic tools can be used for dissecting and comparing the mechanisms underlying olfactory learning at both stages of development.

Habituation

Habituation in *Drosophila* larvae using an olfactory-based assay is described by (Larkin et al., 2010). These authors use two different approaches to induce a decline in attraction to repeated presentations of a single odor as a measure of habituation. In one, between 20 and 55 feeding mid-third instar larvae are isolated from fly food and placed into a Petri dish (35 mm diameter) with 600 μL of Ringer's solution. A small filter paper rectangle (20 mm × 15 mm) containing 100 μL of odorant (10^{-2} dilution of ethyl acetate, EA) or water (control) is placed on the inside of the lid and closed over the dish. Following 5 min of exposure, larvae are carefully removed and transferred to a separate dish (9 cm diameter) for assessment of habituation. The testing dish contains ~10 ml of 2% agar with two plastic cups on opposite ends. Within each cup is a 5 mm filter paper disk soaked in 25 μL of either odorant (EA at 10^{-3} dilution) or distilled water (control). After placing the larvae in a marked 2 mm circle at the center of the dish, the lid is shut and the animals are allowed to freely wander for 5 min. The larvae at either side of the plate are counted after a specified period of time (those remaining at the center are not included). A response index (RI) is calculated as above for conditioning in the T-maze. An RI of 1 indicates complete attraction, −1 is complete repulsion and 0 represents no preference. EA and most odorants and concentrations chosen for habituation experiments normally elicit a positive response in naive larvae, so reduced RI scores indicate habituation. In the second method of inducing habituation, Larkin et al. (2010) place larvae in a 500 mL flask on 1% agar and expose them to air bubbled through an odorant for 5 min. Testing is identical to the first method. This form of habituation lasts for about 1 hr, is odor-specific and recovers spontaneously.

Aversive conditioning

Larvae can be conditioned by negative association of odors (CS) with electric shock (Aceves-Piña and Quinn, 1979; Tully et al., 1994a; Khurana et al., 2008; Pauls et al., 2010), quinine, or NaCl (Scherer et al., 2003). Two approaches have been used for electric shock training, one including the addition of a layer of lithium chloride (LiCl) to the top of the conditioning agar (Khurana et al., 2008) and one without (Aceves-Piña and Quinn, 1979, Tully et al., 1994a, Pauls et al., 2010). However, both approaches include introducing a small amount of LiCl into the agar itself.

The first report of aversive conditioning in *Drosophila* larvae was by Aceves-Piña and Quinn (1979). The authors created an electrically conductive gel capable of delivering an aversive shock to larvae by mixing 1.4% agarose with 0.15M LiCl. Approximately 80–100 third-instar larvae are isolated, cleaned and placed onto gel in a Petri dish (150 mm diameter). They receive 30 s exposures to amyl acetate (AA) through a humidified current of air (300 ml min^{-1}) originating from a bubbler containing pure odorant (CS^+). For the duration of this exposure, 90 V electric shock US is delivered at 60 Hz from two brass electrodes inserted into opposite sides of the gel. After a 90 s rest period with blank air, larvae receive a second odor (OCT, CS^-) for an additional 30 seconds without the US. Testing is performed after a second 150 s rest exposure to blank air. Around 30–40 larvae are then moved from the training plate to a Petri dish (90 mm diameter) containing two capillary tubes placed at opposite ends of the dish, each containing either the CS^+ or CS^- odorant (Fig. 18.8). The plate is covered and larvae are allowed to move freely for 3 min. After this time, their location on either side of the midline on the plate is tallied. A larva is scored as avoiding the odor when on the opposite side of the plate, across the midline. The assay is performed in reciprocal fashion with a different group of larvae. The mean of both scores is calculated as above for the T-maze to generate a single learning index (λ).

Khurana et al. (2008) describe one modification of the procedure (above) to train and test olfactory conditioning in larvae. This variant uses about 400 larvae (96–102 hr post-hatch) in Petri dishes containing 1.5% agarose with 10 mL of 20 mM LiCl. In an attempt to conduct electric current more effectively and elevate US salience the authors spread 0.5 ml of LiCl over the surface of the plate. Rather than piping in odor for training as above, these authors spread odorant (20 μL) along the lid of the plate and place it over the dish. The larvae are exposed to the CS^+ odor for 60 s with shock administered during the last 30 s only. Electric current (AC) is passed through the dish with a gradient of 14 V/cm across two electrodes embedded in the agar at opposite sides of the plate (US). A test plate of agar has paper filter discs at opposite sides that have each absorbed one of two odorants (CS^+, CS^-). Larvae are placed at the center of the plate, their position after 2 min is recorded and a learning index is calculated as above. The authors note that multiple cycles of this training protocol can be repeated to generate robust longer forms of memory.

Another variant of the aversive conditioning assays for larvae is reported by Pauls et al., 2010. Groups of about 30 larvae are placed on a 2.5% agar plate pre-mixed with 0.01 M LiCl and containing an odorant, with two electrodes spaced on opposing ends of the plate. After a 30 s exposure to the odor, a 100 V AC current is applied to the plate for an additional 30 s with odor (CS^+). The larvae are then moved to a neutral "resting" plate for 5 min followed by exposure to a second odor on a new plate for 60 s (CS^-). Following this second exposure to an unreinforced odor, larvae are moved to a test

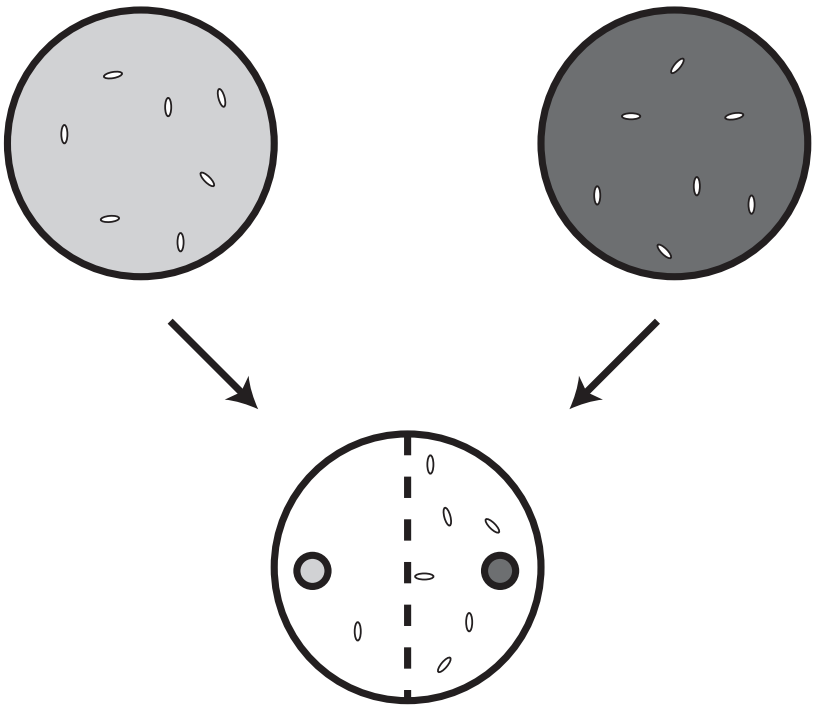

Fig. 18.8. Larval olfactory conditioning. Larvae are exposed to different odors paired with either aversive (Aceves-Piña and Quinn, 1979; Khurana et al., 2008; Pauls et al., 2010) or appetitive (Gerber et al., 2007) reinforcement in Petri dishes (top). They are moved to the center of a test dish with both odorants placed at opposite sides of the dish (bottom). Larval movement and position on either side of the midpoint (dashed line) in response to the conditioned odors is recorded at the end of a test. The hypothetical result shown in this illustration could reflect aversive reinforcement in the light gray dish (left), appetitive reinforcement in the dark gray dish (right) or both.

plate and allowed to choose between the two odors as described above.

Drosophila are holometabolous insects that undergo near complete reorganization of their nervous systems. Nonetheless, there are several accounts of persistent memories in adults that were established from experiences in larvae (e.g., Borsellino et al., 1970). One report in flies supports this idea, suggesting that memories may be retained in structures or circuits that avoid rewiring and are retained to adulthood (Tully et al., 1994a). In these experiments, approximately 80–100 third-instar larvae are introduced into a square training chamber containing 1.5% agarose with 2 mM LiCl (3 mm thickness) and embedded copper electrodes. Odors, EA, or isoamyl acetate (IA) are presented with an airflow rate of 35 ml min^{-1} from opposite sides of the chamber and exhausted from above. The CS^+ odor is trained for 60 s with 90 V AC shock delivered during the last 30 s of exposure. This is followed by a 90 s rest period during which larvae are carefully moved to a second chamber used solely for the second odor. In this way, each odor is presented in a separate device to avoid complications from lingering odor traces. The CS^- odor is presented for 60 s followed by another 90 s rest period. Larvae are returned to the first chamber and the cycle is repeated a total of eight times for a single training session. Larval learning can be tested or animals can be allowed to develop for testing as adults. In the larval test, animals are placed in the center of an identical chamber with CS^+ and CS^- odors delivered from either side, and performance assessed as above. The adult tests are made in the T-maze as described above.

Mechanosensory punishment, as a clever alternative to electric shock reinforcement, has recently been demonstrated to condition larvae to odor stimuli (Eschbach et al., 2011a). Similar to assays described above, third-instar larvae are placed in a Petri dish containing 1% agarose solution and exposed to either 1-octanol or n-amyl acetate (CS^+). The authors then used a loudspeaker mounted under the dish to punish larvae in the presence of the odor with a frequency of 100 Hz in 200 ms pulses every 1 s for 5 min (US). Conditioned larvae are then moved to another dish without reinforcement for 5 min (CS^-). A performance index (PI) is calculated from the number of larvae that move to avoid the CS^+ odor on test plates in reciprocal experiments as above.

Appetitive conditioning

Several laboratories have provided methods for simple appetitive conditioning of larvae. This form of learning is fairly straightforward as it does not require electrodes or the use of LiCl in the agar media. Appetitive conditioning is typically performed with reciprocal training of two odors (e.g., Neuser et al., 2005, Gerber and Stocker, 2007) or training of a single odor (e.g., Honjo and Furukubo-Tokunga, 2005) with a sucrose or fructose US reward. As with the Arena assay for adult flies, appetitive learning assays for larvae are particularly well suited for demonstrating the principles of associative conditioning in the classroom or teaching laboratory, as they are simple to run and have minimal equipment requirements.

In experiments conducted by Neuser et al. (2005), a small group (8) of third-instar larvae is placed on a Petri dish (85 mm diameter) containing 1% agar, 2 M fructose, and the CS^+ odorant. Following exposure for 1 min, the larvae are carefully moved to a completely empty plate for 1 min rest. They are then

transfer to a third plate agar and the with the CS^- odorant for 1 min. Within 2 min after training, animals are tested on a new plate containing agar and both odorants in small containers on opposite sides. Movement is recorded for approximately 5 min and a score is generated as described for tests of aversive conditioning in larvae. A second group of larvae are conditioned reciprocally to avoid the opposite odor with all other perimeters being consistent. The final PI is calculated as the average of the two tests. The authors note that three repeated training trials are sufficient to generate the strongest possible appetitive behavior.

Honjo and Furukubo-Tokunga (2005) report a slightly different approach for appetitive conditioning. In their version of the assay, several hundred 72 to 76 hr-old larvae were removed from food media and made buoyant for ease of collection in 15% glucose (a common method for collecting larvae in large numbers; e.g., Aceves-Piña and Quinn, 1979). Petri dishes (85 mm diameter) are prepared with 2.5% agar and 10 µL of undiluted odorant absorbed in a filter paper disk (55 mm diameter) secured to the underside of the dish lid (CS). For reinforcement, 1 mL of 1 M sucrose is spread across the surface of the plate (US). Larvae are introduced to the plate and conditioned for 30 min. They are then carefully removed, washed in distilled water and transferred to a test plate. This has 2.5% agar and two filter disks (10 mm diameter) placed on opposite sides (Eppendorf tube lids prevent the disks from touching the agar). One disk receives 2.5 µL of odorant while the other is blank. Between 50 and 100 larvae are placed onto the plate and allowed to move for 3 min. The proportion of animals found within a 3 cm diameter space around the odorant filter paper are counted as the response index (RI). A separate control group experiences the same training minus sucrose US and tested similarly. The two RI scores are compared as a measurement of appetitive learning.

Dual conditioning

Most conditioning experiments discussed to this point present one odor (CS^+) with reinforcement (US) and a second odor is unpaired (CS^-). Scherer et al. (2003) describe a differential conditioning assay for single larvae in which both odors are paired, but with opposing reinforcement. One odor (A^- or B^-) is paired with an aversive US (0.2% quinine hemisulfate or 2 M NaCl) and the other (A^+ or B^+) is paired with an appetitive US (1 M fructose). Separate Petri plates (85 mm diameter) are prepared with 1% agar containing one or the other US. Odorants used in this experiment are 10 µL of either 1-octanol (OCT) or amylacetate (AM), placed into the Petri dishes in small Teflon cups. Two treatment conditions were used for the experiments, one in which the animals are negatively conditioned to AM and positively conditioned to OCT (A^-/B^+) and the second in which reciprocal conditioning was used (A^+/B^-). Larvae were placed onto a dish containing both the odor and positive or negative reinforcement for 1 min and then carefully moved to a second dish with the second odor and reciprocal reinforcement. This procedure was repeated a total of ten times for a single training session. Individual larvae are tested by placing them onto a new plate with two Teflon cups with odorant on opposite sides of the dish. The position of the larva is observed for a total of 5 min, after which time a score was calculated based on the position of the larva in an identical manner to previous larval assays. These scores were then pooled for all animals trained for a particular odor for the final performance index.

Imaging olfactory learning

Observing *in vivo* neural activity with targeted transgenic reporters while a fly is learning and establishing or recalling a memory sounds fantastic – because it is. It has been made possible by the synthesis of fluorescent proteins and bioluminescent reporters throughout the past decade (Fiala et al., 2002, Yu et al., 2004; Martin et al., 2007; Davis, 2011). Three major approaches have been adopted for visualization of learning in the *Drosophila* brain: (1) calcium-sensitive fluorescent indicators such as GCaMP (Wang et al., 2008) and Cameleon (Fiala et al., 2002; Riemensperger et al., 2005), (2) GFP-aequorin, a calcium-sensitive bioluminescent reporter (Martin et al., 2007), and (3) synapto-pHluorin, a pH indicator (Yu et al., 2004). All of these indicators can be expressed in restricted subsets of neurons with the GAL4/UAS binary expression system. Also critical to the success of these experiments is an established method of conditioning single flies to odors paired with electric shock reinforcement.

Fly preparation for experiments involving GCaMP, Cameleon and synapto-pHLourin follow similar procedures for securing and exposing the brain of the fruit fly. Most preparations involve the careful insertion of the fly into the end of a micropipette tip, securing the limbs and wings against the inside of the tip and leaving the antennae exposed at the smaller open end (Davis, 2011). The fly's head is secured in place with wax against a coverslip with a hole cut to allow unobstructed imaging of the brain. The top cuticle of the head is then carefully removed and Ringer solution is applied either by hand or from a gravity-fed stream to maintain homeostasis during imaging. Copper coils or a copper grid can be inserted from the large opening of the pipette tip and pressed against the abdomen of the fly to provide a means for delivering the electric shock US. Odor presentation is carefully controlled by computer-driven solenoids with an exhaust located upstream of the specimen's antennae. Yu et al. (2004) employ a Leica TCS confocal with 20× objective and 488 nm excitation. Differential conditioning is with CS^+ and CS^- odors each presented for 60 s in a 100 mL min^{-1} stream of pressurized air. The electric shock US (90V DC in 1.25 s pulses at 0.2 Hz) is paired with the CS^+. Using this experimental setup, distinct changes in antennal lobe activity can be visualized after aversive conditioning. Working with the bioluminescent reporter GFP-aequorin requires a slightly different preparation due in part to the nature of the photon detector. After the head capsule and neural sheath are removed, flies are immobilized in an acrylic block which is subsequently mounted in a slice chamber to hold the position

of the fly. Similar to fluorescent imaging techniques, Ringer solution is applied to the excised area of the head capsule. Imaging is performed through the use of a highly sensitive photon detector (IPD 3, Photek Ltd.) capable of observing biolumenescence (Martin et al., 2007).

Conclusions

In this chapter we have reviewed many of the prevalent and robust assays used to measure diverse forms of odor learning in *Drosophila*, along with a few of their more common variants. Some approaches have undoubtedly been overlooked. It is perhaps useful here to point out where significant "assay gaps" exist and what we expect may lead to important developments in olfactory assays in the foreseeable future.

A majority of all publications that include any *Drosophila* olfactory learning and memory report about Pavlovian conditioning experiments. Combined with fly genetics and neurobiology, this method has been rewarding in the advancement of our knowledge about behavioral plasticity. But obviously other important classes of experiments are under-represented among the list of assays available to the fly behaviorist. One of these is olfactory sensitization. A small number of papers describe elevated sensitization responses to odors following a strong but temporally unrelated stimulus (e.g., Asztalos et al., 2007b). It may be particularly worthwhile to develop olfactory sensitization for investigating its cellular and physiological basis (as in Aplysia; Braha et al., 1990), since these phenomena have been mostly ignored in *Drosophila* as far as we can tell. Another surprisingly understudied form of behavioral plasticity is operant olfactory conditioning. With the development of automated and precise mass-flow odor delivery to single flies (e.g., Claridge-Chang et al., 2009), it is now possible to examine operant olfactory behavior and compare its fundamental principles with those of classical conditioning in a manner similar to plasticity of visual learning (e.g., Heisenberg et al., 2001; Colomb and Brembs, 2010).

Several common features emerge from the experimental procedures discussed in this review. The most important of these is the notion of timing. Windows of stimulus presentation, coincidence and rest should be determined empirically when developing or optimizing an assay. It is notable that the timing critical for establishing change in behavior is conserved across many of the assays described here. For example, the window of CS odor presentation is consistently about 60 s and the onset of presentation must always precede pairing with the US in classical conditioning. Inter-trial intervals in spaced training to generate LTM are generally in the order of 15 min for flies. These apparent "rules" are clues that will lead to an understanding of the mechanistic basis of behavioral plasticity. Another common aspect of olfactory experiments is the effective range of odor concentration. While this varies somewhat between experiments, many laboratories have determined that less is more: Most odors tend to be learned best at concentrations of around 10 or even lower. Early experiments using undiluted odors were successful in demonstrating olfactory learning, but optimizing responses are far easier when working with lower concentrations. We encourage an investment in tweaking the parameters of olfactory assays to attain optimal and reliable performance.

As with many technical fields of research, advances in automation, throughput, imagery, and combinations thereof are rapidly moving our field in new directions. All else being equal, simple and elegant assays are preferable to high-tech gadgetry. However, more often the answers to some important questions are dependent on novel developments. Eventually, automation in olfactory assays is usually desirable, leading to experiments that are more reproducible by the exclusion of capricious human influences (e.g., bias, habits, etc.). Multiple researchers may run the same experiment without including their own differences as a variable in the analysis. When introduced correctly, automation can free up time for the investigator and enhance throughput of an experiment. Nonetheless, establishing a behavioral experiment begins with observation. Automation and other developments can follow once you have watched animals with your own eyes, counted their numbers, measured their movements, recorded the timing of events, and entered the data in your lab book.

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Chapter

19

Intersectional strategies for cell type specific expression and transsynaptic labeling

Taylor R. Fore and Bing Zhang

Introduction

The brain continues to puzzle mankind with its unparalleled complexity and awesome power of creativity. To unlock the mystery of the brain, neuroscientists have used various methods to explore its structure and function. Observing the behavioral consequence of brain lesion(s) caused by stroke, viral infection, or surgery has proven an effective approach for studying the brain. Very few students of introductory neuroscience are not impressed by the textbook story of the brain injury of Mr. Phineas Gage in a mining accident. From a metal rod-pieced brain, neurologists learned the importance of the frontal lobe in personality and decision-making. Equally unforgettable is the story of Mr. Henry Molaison (better known by his initials, H.M.), who lost the ability to form long-term memory after a brain surgery. From H.M. and similar "memory" patients, neuroscientists have identified the hippocampus as a key site for formation of long-term declarative memories. These classical examples underscore the importance of correlating specific brain structures with behaviors.

Drosophila is a major model organism for studying neural basis of behavior. Unlike the uncontrolled lesions experienced by Mr. Gage, *Drosophila* neurobiologists undertake powerful, controlled, and often precise genetic approaches to study brain and behavior. Seymour Benzer pioneered the genetic dissection of behavior in *Drosophila* (Benzer, 1967). The discovery of *period* (*per*) represents one of the best examples of forward genetic approach (from "gene mutations" to "behaviors") in dissecting genes and behaviors (Konopka and Benzer, 1971). Studies of *per* in flies have led to important discoveries of conserved circadian rhythms in mammals (Hall, 2003; Reddy et al., 1984; Rosbash, 2009; Zehring et al., 1984). Besides this classical mutational approach, *Drosophila* biologists have also developed some of the most sophisticated and versatile transgenetic 'tools' to manipulate genes, subset neurons, and behaviors (Venken et al., 2011; Zhang et al., 2010). For neural circuit mapping specifically and for fly genetics in general, there are a number of notable landmark "big bang" developments that led the way for today's "cosmic explosion" in transgenetic tools. The first one is the advent of the P-element-mediated germ line transformation technique by Gerry Rubin and Allan Spradling (Rubin and Spradling, 1982; Spradling and Rubin, 1982). This is followed by the enhancer-trap technique by Cahir O'Kane, Hugo Bellen, and Walter Gehring (Bellen et al., 1989; O'Kane and Gehring, 1987). The third is the establishment of Gal4-UAS binary expression system by Andrea Brand and Norbert Perrimon (Brand and Perrimon, 1993). The fourth landmark technique is the introduction of FLP-FRT and chromosomal engineering in flies (Golic and Lindquist, 1989; Struhl and Basler, 1993; Xu and Rubin, 1993). The fifth technique that has transformed *Drosophila* biology is the MARCM (mosaic analysis with repressible markers) method developed by Tzumin Lee and Liqun Luo (Lee and Luo, 1999).

In this chapter, we aim to highlight the recent techniques for cell type specific expression and for transsynaptic labeling. An important premise behind the intersectional approach is to uncover the minimal number of neurons (or glia) and the simplest circuit underlying a specific behavior. To know the whole, one must first know the parts. Only with the knowledge of neural circuits underlying each behavior can we begin to understand the logic and operation of the nervous system in an organism. Although our studies use fruit flies, the techniques and findings from these studies also have impacts on other animals, including humans. The techniques such as Gal4-UAS, FLP-FRT, and MARCM have all been used in mammalian genetics (review by (Luo et al., 2008)). Beyond basic biology, the drive to dissecting brain circuits also has a significant impact on understanding the dysfunction of the brain. Most neurological disorders in humans such as fragile-X syndrome and the Rett syndrome result from developmental or functional changes of brain circuits, even though it may be caused by mutations in a single gene (for review see Krueger and Bear, 2011; Zoghbi and Warren, 2010). Other mental disorders, such as schizophrenia, likely having more complex genetic and environmental factors, are also found to be associated with altered brain circuits (Akil et al., 2010).

Binary systems

To better manipulate gene expression in either whole flies or select tissues, fly biologists have developed a number of elegant ways in which they import the transactivation systems

Behavioral Genetics of the Fly (Drosophila melanogaster), ed. J. Dubnau. Published by Cambridge University Press.

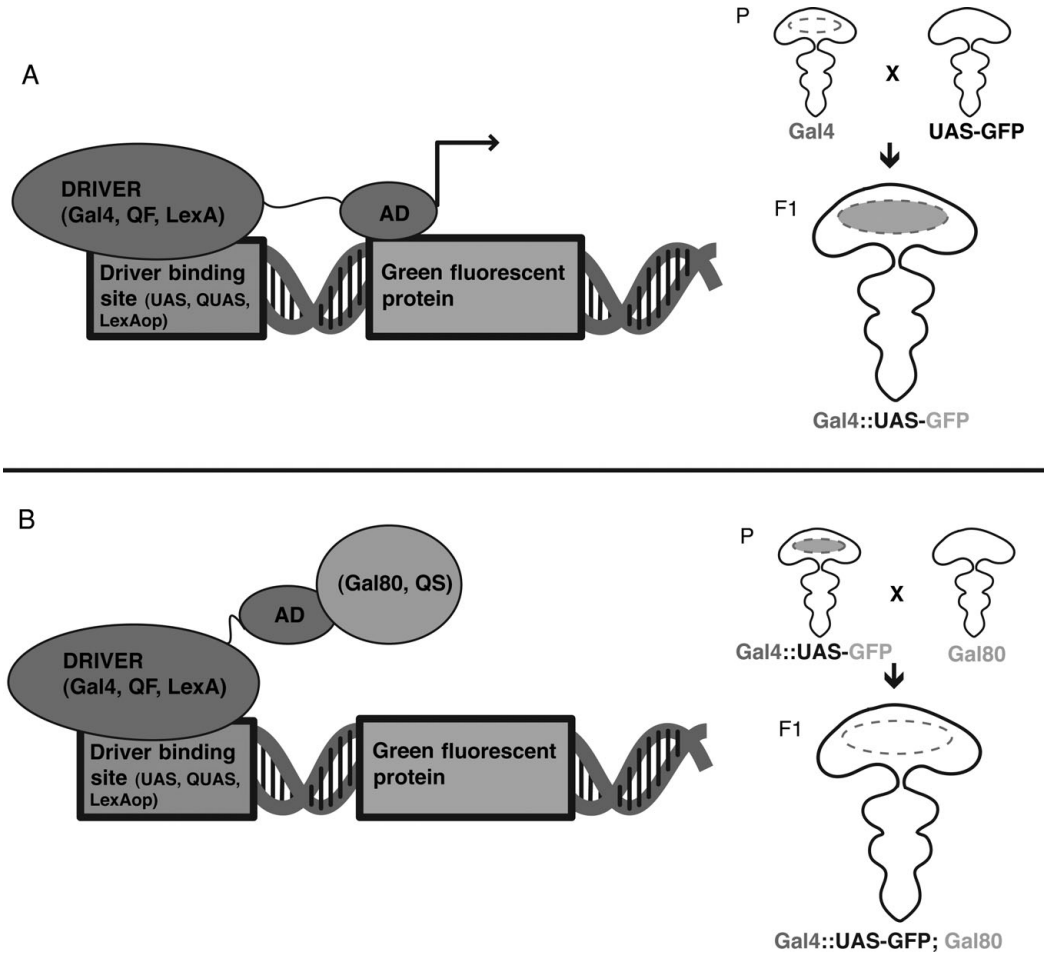

Fig. 19.1. Binary expression systems commonly used in *Drosophila*. A Gal4-UAS, LexA-LexAop, and the QF-QUAS pairs are binary expression systems, imported and modified from yeast, bacteria, and fungus, for gene expression in *Drosophila*. They share similar features in that they have two separate modules, one of which is the driver (Gal4, QF, or LexA) and another is the drivee (UAS, QUAS, or LexAop). The driver contains a DNA-binding domain (DBD) and a DNA-activation domain (AD) whereas the drivee contains binding sites for the driver and can induce transcription of the fused transgene (GFP as an example) upon activation by driver binding. The spatial expression of the driver is usually controlled by endogenous promoters or enhancers.

In the example shown on the right side, Gal4 is expressed in the oval-shaped area of the fly brain. Alone UAS-GFP will not produce any product, but the cells expressing Gal4 will also be producing GFP when the binary system is combined in the F1 generation. For temporal control, heatshock promoter-induced Gal4 and hormonally induced GeneSwitch have been used to induce UAS-gene expression for defined periods at different developmental stages. Similar temporal versatility can be incorporated into LexA and QF.

B The Gal4-UAS and the Q systems can also be negatively regulated by the transcriptional repressor Gal80 and QS, respectively. Upon binding to the C-terminal domain of the AD domain, Gal80 represses the activity of Gal4 and prevents it from activating UAS-gene expression. QS works in a similar fashion as Gal80 to block the transcription of QUAS-gene by repressing QF activity. In the example shown on the right side, ubiquitously expressed Gal80 effectively blocks the expression of GFP driven by the hypothetical Gal4 active in the subset brain cells. $Gal80^{ts}$ provides additional flexibility in the Gal4-UAS system to temporally control gene expression. Readers should be aware that some of the non-traditional Gal4s such as Split Gal4 may not be repressible by Gal80 as their AD domain does not contain the binding sites for Gal80. Quinic acid, which can be fed to flies, relieves QS repression of QF, and thus also allowing one to gain temporal control of the Q system.

from other organisms (yeast, bacterium, or fungus) to flies. The Gal4–UAS binary system was the first to be developed (Brand and Perrimon, 1993; Fischer et al., 1988), whereas the LexA-LexAop system (Lai and Lee, 2006) and the Q system (Potter et al., 2010) are examples of recent additions. These binary systems differ in many aspects, but they are similar in that one module contains the DNA binding and activation domains and serves as a tissue-specific "driver" (such as neuron-Gal4). Another module "drivee" contains binding sites for the driver and it is fused with a transgene of interest (such as UAS–GFP). Only when these two modules are together in the same cell does the transgene (e.g., GFP) begin to be transcribed and expressed (Fig. 19.1). Each of these binary systems will be briefly discussed below, as they serve the foundation for intersectional restrictions.

Gal4-UAS

The UAS transgene contains the upstream activating sequence (UAS) from a yeast *gal* promoter, which can be used to control any target genes of interest (such as UAS–GFP). The Gal4 transgene uses a promoter to direct expression of the Gal4

transcription factor. Where Gal4 is expressed, the target gene under the control of UAS is also expressed ((Brand and Perrimon, 1993; Fischer et al., 1988); for review see Duffy, 2002). This binary system allows any number of target genes to be expressed in GAL4-delineated cells (Fig. 19.1A).

The tissue-specificity of Gal4 expression is achieved via enhancer-traps or by fusing with specific promoters (including enhancer-bashing by the Rubin group (Pfeiffer et al., 2008)). To date, fly biologists have created thousands of Gal4 driver lines that produce relatively tissue-specific and reproducible expression patterns. The Gal4–UAS method has enabled numerous developmental and morphological studies and a number of brain-behavioral studies (e.g., (Broughton et al., 2004; Dubnau et al., 2001; Gong et al., 2010; Gordon and Scott, 2009; Keene et al., 2004; Kitamoto, 2001; Rezával et al., 2012)). The impact of the Gal4–UAS system has been tremendous such that it is not an overstatement that the Gal4–UAS system has revolutionized *Drosophila* biology.

While most enhancer-trap Gal4 lines provide reasonably good spatial expression patterns, they cannot be controlled temporally. Heatshock-induced Gal4 (hs-Gal4) can and has been used to provide the temporal solution. However, heatshock lacks the spatial resolution and makes it particularly challenging for behavioral studies. To overcome this hurdle, the laboratories of Haig Keshishian and Ron Davis developed the GeneSwitch system to allow researchers to turn on UAS–gene expression by feeding flies with the antiprogestin mifespristone (RU486) (Osterwalder et al., 2001; Roman et al., 2001). GeneSwitch, originally developed by Bert O'Malley's group in mammalian cells, is a chimeric transactivator containing the Gal4 DNA-binding domain, part of the DNA activation domain of the human p65 protein fused with a mutant progesterone receptor-binding domain (Wang et al., 1994). The chimeric protein can be driven by a tissue-specific promoter; once produced it accumulates inside nuclei, but remains inactive. Upon RU486 binding, GeneSwitch becomes active, binds to the UAS-sites, and thereby turns on gene transcription. These temporal and inducible capabilities significantly boost the versatility of the Gal4–UAS system (Nicholson et al., 2008; Osterwalder et al., 2001; Roman et al., 2001).

Gal80: Repression of Gal4 and temperature-sensitive Gal80

Gal80 is a yeast translational repressor that represses Gal4 activity by binding to the carboxyl terminal 30 amino acids of Gal4 (Ma and Ptashne, 1987) (Fig. 19.1B). In the mosaic analysis with a repressible cell marker (MARCM) method, Gal80 is used to repress Gal4-driven GFP expression in non-mutant cells such that the mutant clone can be readily marked by GFP (Lee and Luo, 1999). Added to classical balancer chromosomes (such as CyO-tub::Gal80), ubiquitously expressed Gal80 is used to "keep" stocks where a specific Gal4–UAS-effector pairing is lethal or deteriorating. Increasingly, Gal80 is used to restrict Gal4 expression in subsets of cells or tissues in intersectional approaches (see below). Enhancer-trap Gal80 lines with various tissue-specific expression can be produced for such a purpose (Suster et al., 2004). For example, Scott Waddell's group used a mushroom body (MB)-specific Gal80 line to demonstrate the specificity of MB–Gal4-mediated gene manipulation in learning and memory (Keene et al., 2004). Other alternative usage of Gal80 for intersection will be discussed further in the next section.

Gal80 repression of Gal4 was empowered with a temporal advantage when Ron Davis' laboratory introduced a temperature-sensitive version of Gal80, Gal80^{ts} (McGuire et al., 2003). At lower temperatures, e.g., 19 °C, Gal80^{ts} is functional in repressing Gal4 activity, but it loses this ability at temperatures above 30 °C (McGuire et al., 2003). At 30 °C Gal80^{ts} can be turned off quickly and the UAS–gene transcript can be detected by RT–PCR in as early as 30 min. This relief of Gal80 is reversible if the flies are returned to 19 °C, in which a significant reduction in UAS–gene transcript is detectable after a 12–18 h recovery at 19 °C (McGuire et al., 2003). This reversibility is another strength of Gal80^{ts}. Both Gal80^{ts} and GeneSwitch are powerful spatiotemporal methods called temporal and regional gene expression targeting (TARGET) (McGuire et al., 2003; 2004). A major utility of TARGET is to control UAS-effector expression at different developmental stages to examine the spatial and temporal requirement of the gene product for a specific developmental or behavioral process. Using the TARGET method, McGuire and colleagues showed that transient expression of the *rutabaga* (*rut*)-encoded adenylyl cyclase in adult MBs is sufficient to rescue learning and memory defects in *rut* mutants.

UAS-effectors and UAS-reporters

Among the thousands of UAS-lines produced by fly colleagues, some have emerged as favorites for fly neurobiologists interested in neural circuits and behaviors. These UAS-lines can be divided into two categories: reporters and effectors. UAS-reporters including UAS–XFP and UAS–photoconvertible FPs are used to label cells of interest. UAS-effectors are used to manipulate neuronal excitability, synaptic transmission, or simply to kill or degenerate cells (also see a review by (Venken et al., 2011); see Table 19.1). To silence neuronal excitability, UAS–Kir2.1 (inward-rectifying K channel; (Baines et al., 2001; Paradis et al., 2001)), UAS–EKO (Shaker K channel; (White et al., 2001)), and UAS–dORK (an open K channel; (Nitabach et al., 2002)) are used to express potassium channels, which hyperpolarize the cell membrane, so that the probability for firing action potentials will be significantly reduced or abolished. For example, Nitabach et al. demonstrated an essential role of pacemaker neurons in free-running circadian rhythm using UAS–dORK or Kir2.1 to silence the pacemaker neurons (Nitabach et al., 2002). To enhance neuronal excitability, UAS–NaChBac (a bacterial Na channel; Nitabach et al., 2006) or UAS–TrpM8 (cold-activated cation channel; Peabody et al., 2009) or UAS–TrpA1 (heat-activated cation channel;

Table 19.1. List of select UAS-effector and reporter lines

UAS-line	Effect	Reference
Labeling (Fluorescent)		
UAS-mCDX::XFP	Membrane bound fluorophores	(GFP) Lee and Luo, 1999; (mRFP) Pfieffer et al., 2010
UAS-NLS::XFP	Fluorophores with a nuclear localization signal	(GFP) Neufeld et al., 1998; (mRFP) Wen et al., 2008
UAS-DenMark	mCherry dendritic marker	Nicolai et al., 2010
UAS-PA-GFP	Photoactivatable GFP	Patterson et al., 2002; Datta et al., 2008
UAS-Dendra2	Photoconvertible fluorophore, irreversibly transitions from GFP to RFP	(Vacuolar ATPase) Williamson and Hiesinger, 2010; (Dpp) Zhou et al., 2012
Silence Neuronal Activity		
UAS-Kir2.1	Inward rectifying K+ channel	Baines et al., 2001
UAS-EKO	Shaker K+ Channel	White et al., 2001
UAS-dORK	Open rectifier K+ channel	Nitabach et al., 2002
Excite Neuronal Activity		
UAS-NaBac	Increases sodium conductance	Nitabach et al., 2006
UAS-TrpM8	Cold-activated cation channel	Peabody et al., 2009
UAS-dTrpA1	Heat-activated cation channel	Rosenzweig et al., 2005
UAS-ChR2	Photogated ion channel	Schroll et al., 2006
UAS-P2X2	ATP-gated ionotropic purinoceptor, increases cation conductance	Zemelman et al., 2003; Lima and Miesenböck, 2005
Ablate Cells		
UAS-Reaper	Proapoptotic gene reaper	Zhou et al., 1997
UAS-Ricin A	Catalytic subunit of Ricin Toxin	Hidalgo et al., 1995
UAS-DTI	Diphtheria toxin gene subunit A	Han et al., 2000
Silence Synaptic Activity		
UAS-Shibire^{ts1}	Dynamin activity is blocked at restrictive temperatures (>29 degrees)	Kitamoto, 2001
UAS-TNT	Tetanus Toxin, cleaves Synaptobrevin thus silencing SV exocytosis	Sweeney et al., 1995
Excite Synaptic Activity		
UAS-Syx$^{3-69}$	A mutant Syntaxin 1A that enhances synaptic vesicle fusion	Lagow et al., 2007; Koles et al., 2012; Kottler et al., 2013

Rosenzweig, 2005) or UAS–ChR2 (a blue light-activated cation channel; Schroll et al., 2006), UAS–P2X2 (ATP-activated cation channel; Lima and Miesenbock, 2005; Zemelman et al., 2003) is used. To block synaptic transmission, UAS–TNT (a neurotoxin that cleaves the vesicle SNARE Synaptobrevin; Sweeney et al., 1995) or UAS–Shibire^{ts} (UAS–Shi^{ts}, a mutant dynamin that blocks synaptic vesicle recycling at restrictive temperatures; Kitamoto, 2001) can be used. To enhance synaptic transmission, UAS–Syx$^{3-69}$ is used to stimulate SNARE-mediated vesicle fusion (Koles et al., 2012; Kottler et al., 2013; Lagow et al., 2007). To ablate cells, UAS–reaper (Zhou et al., 1997), UAS–Ricin (Hidalgo et al., 1995), or UAS–DTI (Han et al., 2000) is effective. The temperature-sensitive feature in Shi^{ts1}, TrpA1, and TrpM8 is particularly powerful allowing one to add the temporal flexibility in any study. Dubnau and colleagues used UAS-Shi^{ts1} to dissect the role of MB in learning, memory consolidation and retrieval, which would be otherwise impossible to achieve with a constitutively active mutant shibire (Dubnau et al., 2001). Similarly, Peabody and colleagues used UAS-TrpM8 to test the developmental stage in which bursicon neuronal activity is involved in wing inflation in newly enclosed adult flies (Peabody et al., 2009).

As pointed out by Yoshihara and Ito (Yoshihara and Ito, 2012), one must keep in mind that each UAS-effector may have unexpected effects beyond its described "face value." Due to its critical role in endocytosis, Shi^{ts1} is expected to also affect all other endocytotic events in addition to synaptic vesicle recycling. Transmitters such as glutamate, ACh, GABA, and monoamines have different life cycles from neuropeptides. The former is reloaded into recycled synaptic vesicles whereas neuropeptides are packed into synaptic vesicles at the cell body. Hence, UAS-Shi^{ts1} should not be expected to have the same blocking effects on peptide release as on other transmitters.

LexA-LexAop

LexA/LexAop is a binary system that parallels the Gal4/UAS system (Lai and Lee, 2006). It features the bacterial transcriptional factor, LexA, which can bind the specific DNA bind site, LexAop, driving transcription of a downstream effector

(Fig. 19.1A,B). Lai and Lee introduced two transcriptional activation domains (TA) into the LexA/LexAop system, a Gal80-sensitive ("Gal80 suppressible") form of LexA, GAD, and a Gal80-insensitive form, VP16. These TAs allow for additional layer of control, in which the level of transcript and Gal80 sensitivity are adjustable, although the former is the unintentional nature of the TA itself. Unlike Gal4, LexA transcriptional level is not dependent on environmental temperatures, e.g., 18 °C and 29 °C (Pfeiffer et al., 2010). However, the strengths of the TAs do vary, Pfeiffer and colleagues demonstrated a decrease in transcript, measured via qRT–PCR and fluorescent intensity, in both the VP16 domain (1.0-fold) and GAD (0.6-fold) in comparison to a Gal4 control (2.3-fold) (Pfeiffer et al., 2010). In light of this, several new TAs have been engineered to improve the strength and Gal80 sensitivity (Pfeiffer et al., 2010; Yagi et al., 2010).

The LexA system can also be used to spatially control the mitotic recombination events in MARCM clones through the use of LexA-VP16 and LexAop-FLP (Shang et al., 2008).

The Q system

The Q system is a recent addition to the binary toolbox, which parallels Gal4/UAS (Potter and Luo, 2011; Potter et al., 2010). Borrowing from *Neurospora crassa's* regulatory elements, QF (QA-1F) is a transcriptional activator that recognizes and binds the *qa* gene cluster-binding site, QUAS, thus driving a downstream effector (Fig. 19.1A). The QS (QA-1S) element represses QF function (Fig. 19.1B). However, one key difference in QS repression, in comparison to Gal80, is that it can be relieved, in as quickly as 6 hours, via the feeding of quinic acid. Hence, quinic acid makes QS into an equivalent of $Gal80^{ts}$.

FRT-FLP, *cis* and *trans* recombination

Drosophila biologists also borrowed another great system from yeast, the FLP/FRT system (Golic and Lindquist, 1989; Struhl and Basler, 1993; Xu and Rubin, 1993). This system consists of flippase recombinase target sequence (FRT) and an FRT-specific recombinase flippase (FLP). FLP can induce recombination of chromosomes marked by FRTs either in *trans* or *cis* arrangements. In *trans* arrangements, where FRTs are located on each sister chromatid, FLP induces homologous chromosomal cross-over during mitosis. *Trans*-recombination has been extensively used for generating mutant clones, including MARCM analysis. FRT sites can also be arranged in *cis* on the same chromatid, and depending on the orientation of FRT sites in tandem FLP can have different effects. When two FRT sites are arranged in tandem in the same orientation, FLP excises the intervening sequence between the two FRT sites (such as in Tub^{P}stop>GFP, ">" denotes FRT sites) to allow GFP expression driven by the tubulin-promoter. In the *cis* excision design FLP can flip in (Tub^{P}stop>GFP) or flip out (Tub^{P}GFP>stop) gene expression (Struhl and Basler, 1993). When two tandem FRTs are arranged in opposite orientations, the intervening sequence can be inverted, which has been used to produce random labeling of cells with different XFPs such as the strategy used for BrainBow, dBrainBow, and Flybow (Hadjieconomou et al., 2011; Hampel et al., 2011; Livet et al., 2007).

Intersectional strategies

Despite the great power of the Gal4–UAS system, most Gal4 lines are not restrictive enough to be truly "tissue or cell-specific." For example, a "MB-specific" Gal4 line could also express elsewhere in the nervous system and even outside the nervous system. The lack of tissue-specificity may not be a major concern for some studies (such as morphological examination of mushroom bodies); however, it will complicate the interpretation of behavioral results, preventing one from assigning a specific neuronal structure to a specific behavior. Further, the expression pattern of a given "tissue-specific" Gal4 such as Elav-Gal4 or Repo-Gal4 is often too large to pinpoint the specific cells involved in a behavior. The great effort by Gerry Rubin and colleagues at Janelia Farm refines Gal4 expression through enhancer bashing (Jenett et al., 2012; Pfeiffer et al., 2008). This has produced, and will continue to produce, valuable refined Gal4 lines for the fly community. However, this approach does not fully resolve the tissue-or cell-specificity issue inherent to enhancers or promoters. Unfortunately, this tissue-or cell-specificity problem faced by Gal4 also remains for all other binary systems. Thus, behavior–brain circuitry studies can be significantly improved by employing new methodologies designed to refine the expression of Gal4, LexA, or QF. Because of the abundance of Gal4–UAS in the fly community, it is comforting to know that, in addition to refining each of these binary systems, LexA–LexAop and the Q offer additional flexibilities to intersect Gal4.

Split-Gal4

Ben White and colleagues developed the "Split Gal4" system to refine Gal4 expression (Luan et al., 2006) (Fig. 19.2). The Split Gal4 approach resembles the yeast "two-hybrid" system in that it splits Gal4 into two modules, a DNA-binding domain (Fig. 19.2A) and a transcriptional-activation domain (Fig. 19.2B), each driven by distinct promoters. Each module contains a heterodimerizing leucine zipper, thus the zipper will dimerize to form a functional Gal4 when both modules are expressed in the same cell (Fig. 19.2C). It achieves restricted UAS-transgene expression to cells that express both modules. Luan and colleagues (Luan et al., 2006) demonstrated the use of enhancer-trap lines to drive $VP16^{AD}$. Screening with a collection of ET–$VP16^{AD}$ lines in conjunction with a known promoter, such as CCAP–$Gal4^{DBD}$, they were able to discover novel overlapping promoters, thus identifying a subset of CCAP–Gal4 defined neurons in adult wing inflation.

In comparison to a traditional intact Gal4, the use of the $Gal4^{AD}$ or $VP16^{AD}$ results in a reduction of transcriptional activity to 52% and 84% (determined via β-galactosidase activity in SL2 cells). However, the authors demonstrated an increase in UAS–GFP activation when utilizing pan-neuronal Split

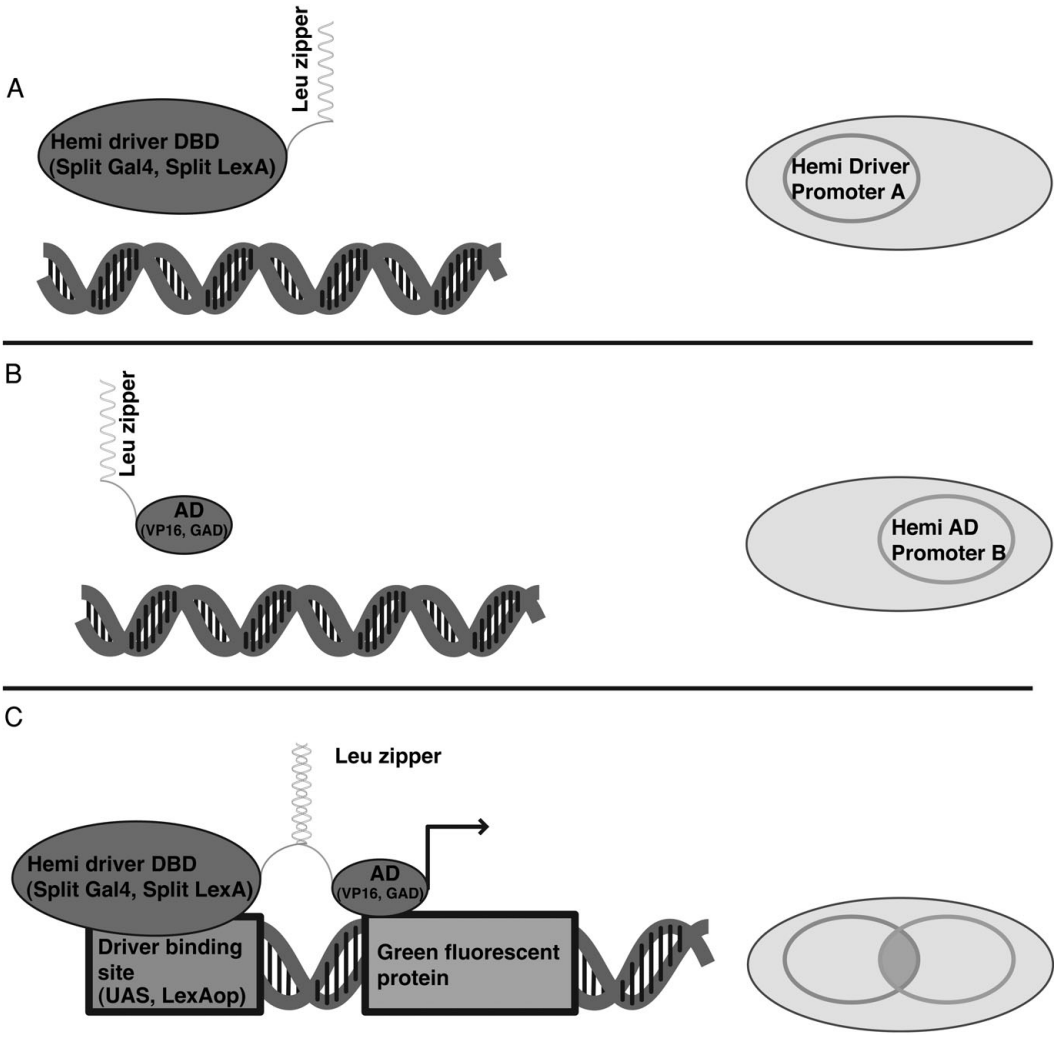

Fig. 19.2. The Split Gal4 system. Using the yeast "two-hybrid" concept, Gal4 can be split into two hemi-drivers, one containing the DBD fused with a Leucine zipper A and another containing the AD-leucine zipper B. Alone none of these hemi-drivers will be able to drive UAS–gene expression. When the two hemi-drivers are expressed in the same cell, a functional Gal4 will be reassembled and lead to UAS–gene transcription in a subset of cells C. If the AD used in the Split Gal4 is no longer Gal80 sensitive, this will lose the ability to further restrict Gal4-defined circuits through Gal80 intersection. Off-target activation of UAS–gene may complicate the interpretation of behavioral results. The same strategy can also be used to generate Split LexA and Split Q systems (a Split Q system has been developed in *C. elegans*).

Gal4 configuration featuring the $VP16^{AD}$ activation domain, in comparison to expression levels of comparable intact Gal4. The reduction in transcriptional activity of the Gal4 can be overcome by increasing UAS-effector copy number. This was demonstrated through the use of two copies of UAS-reaper, a proapoptotic gene, in CCAP positive neurons. Pfeiffer and colleagues improved efficacy of activation through the use of a p65 activation domain. However, the increased transcriptional efficiency can reduce viability due to cytotoxicity (Pfeiffer et al., 2010).

One downside is that the Split Gal4 system does not refine existing Gal4 lines, requiring the production of new DBD and AD transgenic lines. To overcome this limitation, White and colleagues are currently developing a method to convert Gal4 into a split Gal4DBD (B.H. White personal communication). Furthermore, the transcriptionally stronger AD, VP16, is not compatible with Gal80 repression due to the lack of Gal80-binding sites. Hence, "off-target" overlapping of Split Gal4 enhancer-trap lines could still complicate the interpretation of circuit-behavioral results. Currently, White and colleagues are developing and testing the Gal80 equivalent for the split Gal4 system. They are using a "killer zipper" to refine the expression pattern of the split Gal4 system. In this system, the killer zipper contains the same heterodimerizing leucine zipper as VP16, which enables it to bind to Gal4 DBD, thus competitively inhibiting VP16 binding (B.H. White, personal communication).

Split LexA

Another intersection method developed by Chi-Hon Lee's group is "Split LexA" system (Ting et al., 2011) (Fig. 19.3). Differing from the Split-Gal4 approach, Split LexA can be used to intersect pre-existing Gal4 lines because it uses Gal4 to drive the expression of UAS-LexA-DNA-binding domain (Fig. 19.3A) and then intersects with enhancer-trap LexA-DNA-activation domain $VP16^{AD}$ (Fig. 19.3B). When these two modules overlap in the same cell, a fully functional LexA reassembles and then drives the expression of LexAop-transgene (Fig. 19.3C). In this configuration, Gal4 can be first restricted by Gal80 and

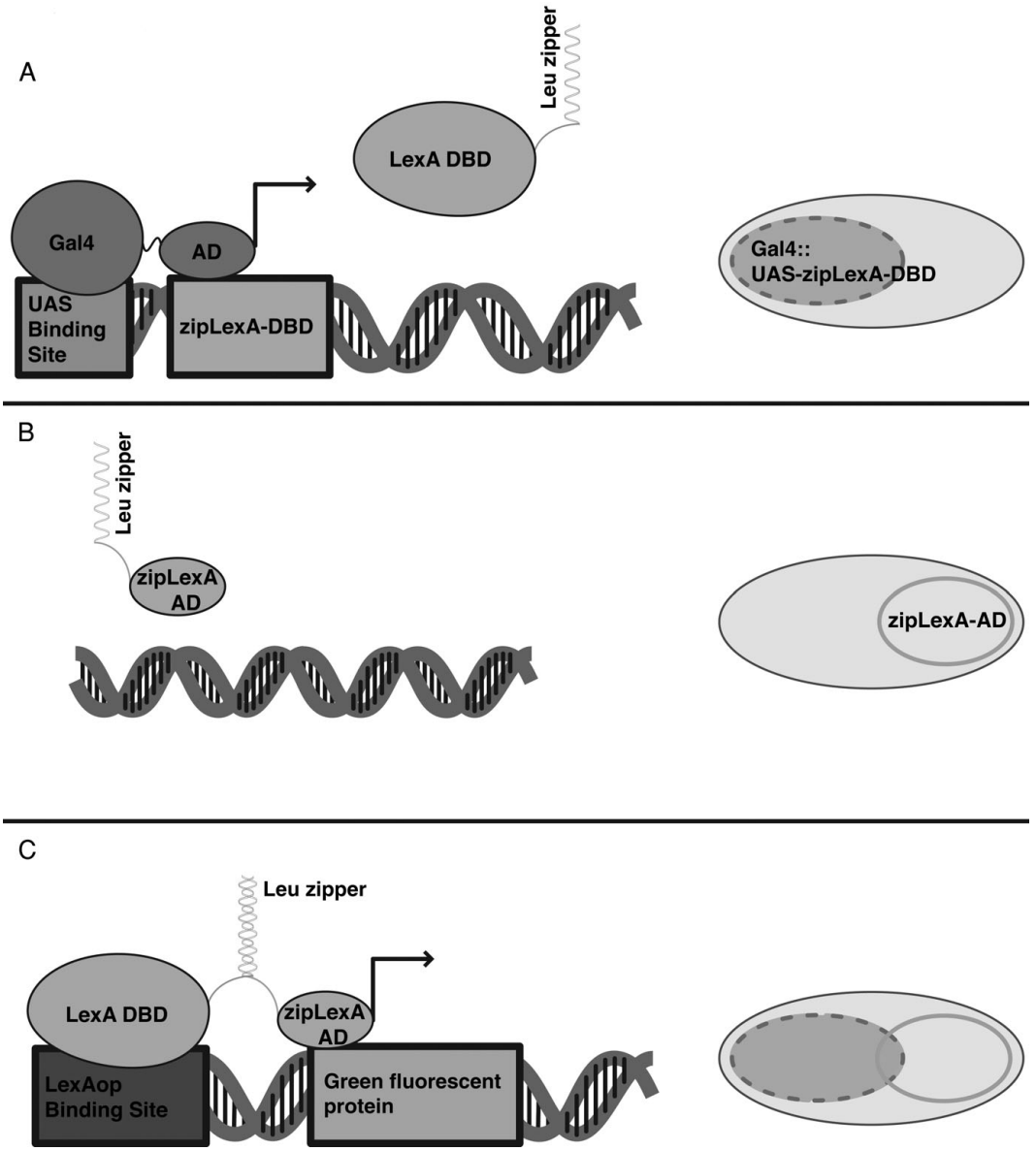

Fig. 19.3. The Split LexA system coupled to Gal4. Split LexA can be coupled to Gal4 to extend the power of both LexA-LexAop and Gal4–UAS systems. In this design, LexA-DBD is under the control of UAS A whereas LexA-AD is driven by specific promoters or enhancer-traps (e.g., ET-LexA-AD) B Only when the expression patterns of Gal4 and ET-LexA-AD overlap then is a functional LexA produced to restrict the Gal4-defined circuit C In addition, ET-LexA-AD can also be used to together with Split Gal4-DBD to restrict circuits.

then further refined through Split LexA. Although compatible with Gal4, this system does not work with existing UAS lines. It requires generation of the enhancer-trap LexA-DNA-activation domain.

The Split LexA method used the same heterodimerizing leucine zipper to generate hemi proteins as featured in Split Gal4 (Luan et al., 2006). This zipper module affords the Split LexA system further flexibility; it can be combined with current Split Gal4 activation domains, e.g., elav-VP16AD, thereby making it more versatile with traditional Gal4 and Split Gal4.

FINGR

The ET-FLP-induced intersectional Gal80/Gal4 repression (FINGR) method that we developed is built on the Gal4–UAS system with additional new components: (1) a set of two complementary FLP/FRT-mediated Gal80 converting tools, e.g., Tub^{P}Gal80>stop (Fig. 19.4A) and Tub^{P}stop>Gal80 (Fig. 19.4B). The FRT sites mediate *cis* recombination and excision of the FRT-flanked "stop" or "Gal80" sequence upon activation by FLP; and (2) enhancer-trap FLP (ET-FLPX2, with two copies of FLP) (Bohm et al., 2010; Fore et al., 2011). Gal4 is used to define the primary neural or glial circuitry of interest. Tissue-specific ET-FLPx2 can flip Gal80 in or out and effectively restrict Gal4 expression into a smaller unit (Fig. 19.4A,B).

The key reagent for the FINGR method is the ET-FLPx2 lines, which have significant advantages over the commonly used hs-FLP. Heatshock FLP is random in nature, making it difficult or nearly impossible to generate the same mosaics and thus less feasible for behavioral studies. We have mapped the FLP expression patterns in the central nervous systems of larvae and adult flies of approximately 500 ET-FLPx2 lines, which

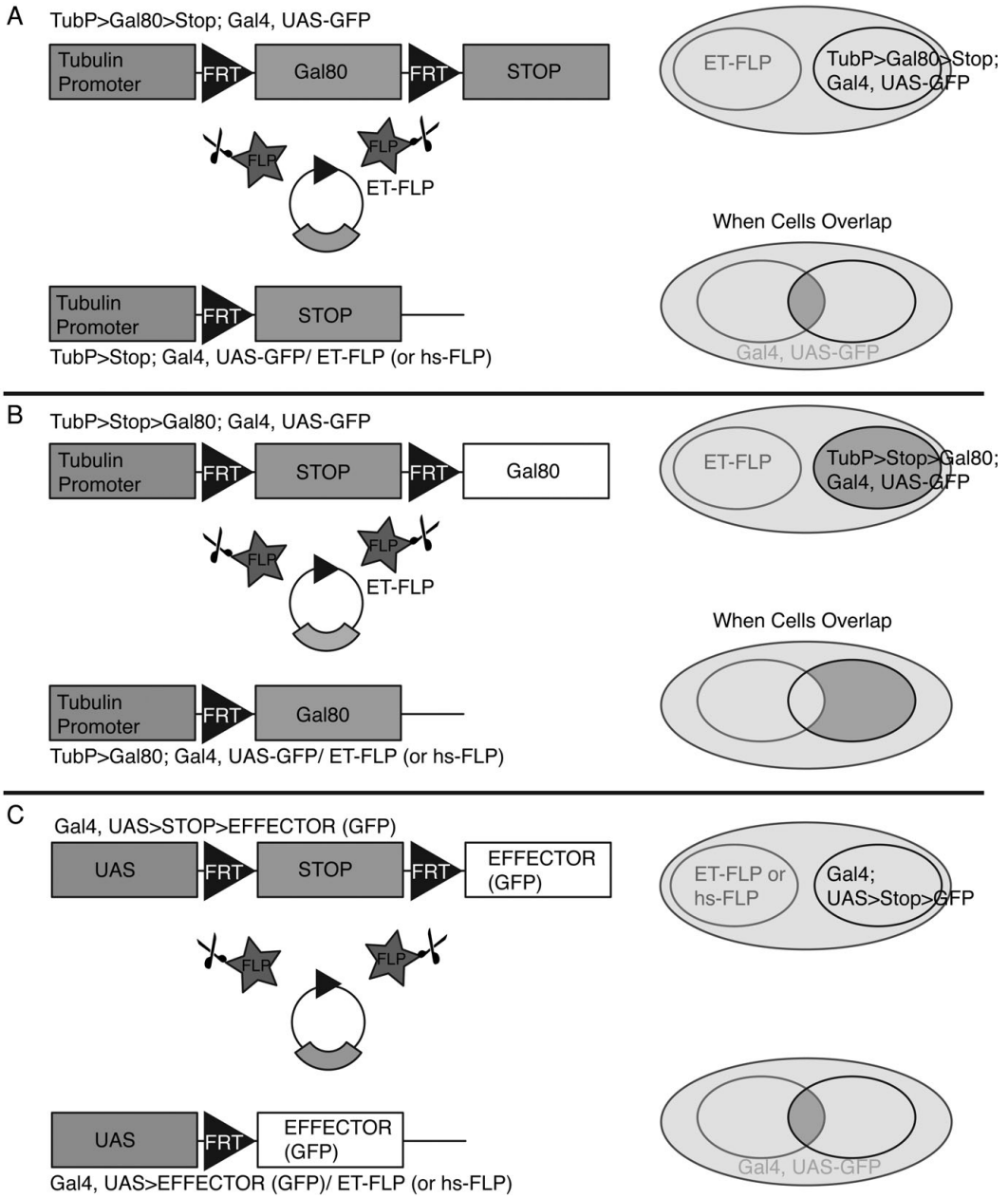

Fig. 19.4. Enhancer-trap Flippase-based Gal80/Gal4 intersectional methods. A Flip-out intersection: The cells in the black oval patch all contain Tub^{P}Gal80>stop; hence no GFP is expressed due to Gal80 repression of Gal4. However, Gal80 is flipped out only in a Gal4/ET-FLP (red oval) overlapping region allowing GFP expression in a more restricted patch (green oval). B Flip-in intersection: In the Tub^{P}stop>Gal80 configuration, the Gal4 oval patch expresses GFP constitutively. In cells with Gal4/ET-FLP overlap, ET-FLP flips out the ">stop" cassette, flips in Gal80, and turns off GFP expression to achieve circuit restriction in a fashion opposite to the flip-out intersectional method. (A and B) shows the FINGR (ET-FLP-induced intersectional Gal80/Gal4 repression) method, which is compatible with the vast collection of Gal4 and UAS reagents available in the fly community. ET-FLP lines with tissue-specific expression are key to the success of using this method for circuit mapping. C UAS>stop>effector flip-out intersection: Gal4 defines the patch of cells expressing UAS>stop>GFP, but there is no GFP expressed. When Gal4 overlaps with ET-FLP (or hs-FLP), "> stop" cassette is excised to active UAS-GFP expression to achieve restriction of the UAS-effector expression. Note that this method does not restrict Gal4 expression and it is not compatible with the traditional UAS lines.

are expected to facilitate neural circuit mapping (Fore et al., 2011; T.R. Fore et al., unpublished data). Unlike enhancer-trap Gal80, the use of Tub^{P}Gal80>stop and Tub^{P}stop>Gal80 "Gal80-converting tools" avoid potential developmental oscillations associated with certain promoters and allows permanent and strong expression or turning off of Gal80 once FLP-mediated recombination has taken place. As a proof of principle, we demonstrated the utility of the FINGR method in refining CCAP-Gal4 neurons in wing inflation. Notably, both Gal80 flip-in and flip-out worked for the same ET-FLPx2 lines in mapping the wing inflation circuit. Shang and colleagues (Shang et al., 2008) and Gordon and Scott (Gordon and Scott, 2009) have also applied the Gal80 flip-out version in neural circuit mapping using LexA-LexAop-FLP and hs-FLP, respectively.

The FINGR method has one major advantage of being compatible with the Gal4–UAS bipartite system, allowing one to tap into the vast collections of both reagents in the fly community. It also allows neural circuits to be further refined by using additional promoter-driven Gal80 and/or using multiple ET-FLP lines. However, the Tub^{P}stop>Gal80 configuration has one potential weakness in that it prevents the pairing of certain Gal4::UAS-effectors. For example, it will be impossible to generate Elav-Gal4::UAS-Kir2.1; Tub^{P}stop>Gal80 flies as this combination is lethal. Fortunately, this problem can be overcome with the use of balancers with ubiquitously expressed Gal80

(such as Elav-Gal4::UAS-Kir2.1; Tub^{P}stop>Gal80/TM6B, Tb, Tub::Gal80). Another potential weakness is that constitutive expression of a UAS-effector may affect the development of the nervous system or alter behavior in the Tub^{P}stop>Gal80 flip-in configuration. This weakness can be overcome by developing Tub^{P}stop>Gal80^{ts}. The Tub^{P}Gal80>stop flip-out configuration avoids the lethality and constitutive expression concerns faced by Tub^{P}stop>Gal80. However, one weakness is Gal80 perdurance in the flip-out configuration. As shown by Bohm and colleagues (Bohm et al., 2010), the same ET-FLPx2 line capable of achieving 100% wing inflation in CCAP-Gal4, UAS-Glued^{DN}, UAS-GFP, Tub^{P}stop>Gal80 flies was less effective in crumpling wings with CCAP-Gal4, UAS-Glued^{DN}, UAS-GFP, Tub^{P}Gal80>stop flies. As for all FLP/FRT-based methods, the FINGR method has to live with the stochastic nature of FLP, even with "tissue-specific" ET-FLP lines. To reduce potential variations, we produced ET-FLPx2 lines with two copies of FLP.

UAS>stop>effector Flip-in method

Another FLP-dependent intersection method was developed by Barry Dickson's lab, who used FLP to refine UAS-effector patterns via UAS>stop>effector (e.g., UAS>stop>shi^{ts} or UAS>stop>TNT)(Fig. 19.4C) (Stockinger et al., 2005). Each effector is only expressed when a FLP excises the >stop intervening sequence when the FLP overlaps with a Gal4 (Fig. 19.4C). Stockinger and colleagues showed that male flies of fru-Gal4, eyFLP, UAS>stop>Shi^{ts} (or UAS>stop>TNT) courted wild type females significantly less than wild type males did. Recently, Rezával and colleagues used this method to map the neurons within the *doublesex* circuits controlling behavioral changes of female flies following copulation (Rezával et al., 2012).

One should note that this method, in fact, *does not* restrict Gal4 patterns, but refines effector patterns defined by UAS>stop>effector in cells expressing both Gal4 and FLP. The stochastic nature of FLP remains a concern for this method, and it is not compatible with the traditional UAS lines.

Gal4-based Mosaic-inducible And Reporter-exchangeable Enhancer Trap (G-MARET)

One intersection method developed by Konrad Basler's group is similar to the Dickson method in that it does not restrict Gal4 pattern directly, but controls UAS>stop>effector expression (Yagi et al., 2010). In this G-MARET method, a constitutively active LexA (c-LexA), generated from tissue specific promoter using CONVERT (Yagi et al., 2010) or traditional enhancer trap methods, is used to activate LexAop-FLP, which in turn acts upon UAS>stop>effector. Only when c-LexA and Gal4 overlap, is UAS-effector expressed. To be effective, thousands c-LexA lines will need to be produced. This method is compatible with Gal4 but not with the traditional UAS lines.

Q System "Logic Gates"

While the Q system is an independent binary system and the number of Q drivers is limited at the present time, the "Logic Gate" strategy combines Q and Gal4 systems (Potter and Luo, 2011; Potter et al., 2010) (Fig. 19.5). Potter et al. demonstrated two "Logic Gate" intersectional strategies, which take advantage of the vast number of Gal4 drivers presently available and the FLP/FRT system.

In the FLP/FRT dependent "Logic Gate" strategy, two drivers (e.g. "Promoter A"-Gal4 and "Promoter B"-QF) are used to express FLP via UAS-FLP and FRT-flanked genes such as QUAS>stop>mCD8-GFP; or vice versa UAS>stop>mCD8-GFP and QUAS-FLP. Only in areas where the two drivers overlap will FLP-mediated recombination take place to excise the > stop sequence and allow for GFP expression (Fig. 19.5A).

Another "Logic Gate" strategy is based upon the subtraction of QF expression in regions of Gal4 and QF overlap. Through the use of UAS-QS, the QF repressor, Gal4 is able to suppress QF transcription. As a demonstration of this subtraction, Potter et al. used two fluorescent lines, UAS-GFP and QUAS-RFP, to create a triple labeled (green, red, and yellow) antenna lobe. After the addition of the UAS-QS, regions of overlapping expression resulted in the suppression of QF driven RFP (Fig. 19.5B). These strategies can also incorporate other QUAS or UAS effectors to modulate cellular dynamics. As a proof of this, Potter et al. utilized QUAS-Shi^{ts} in a FLP/FRT dependent "Logic Gate" to demonstrate which populations of PN neurons are responsible for the olfactory attractive response to ethyl acetate and CO_2.

These "logic gate" strategies could be applied to ET-FLPx2 lines utilized in the FINGR method to avoid the use of QUAS/UAS-FLP lines, thus allowing for the ability to modulate two overlapping circuits independently from one another. Another potential strategy involves expressing multiple transgenes without introducing binding competition from a UAS/QUAS-FLP. Alternatively, QS could be modified into a FINGR-QS system, Tub^{P}stop>QS or Tub^{P}QS>stop, making it compatible with the collection of ET-FLP currently available.

Recently, Stowers and colleagues demonstrated the efficiency of recombineering of Gal4 and QF cassettes into large BACs, in comparison to traditional restriction cloning, to generate new drivers which should aid in the generation of more Q drivers (Petersen and Stowers, 2011; Stowers, 2011).

Trans-synaptic match-making and activity-dependent trans-synaptic labeling

GRASP

GFP reconstitution across synaptic partners (GRASP) consists of two complementary fragments of GFP bound to an extracellular membrane protein, CD4 (Fig. 19.6). Alone, the fragments are unable to form a functional GFP; however, whenever each is individually expressed in opposing cells that from

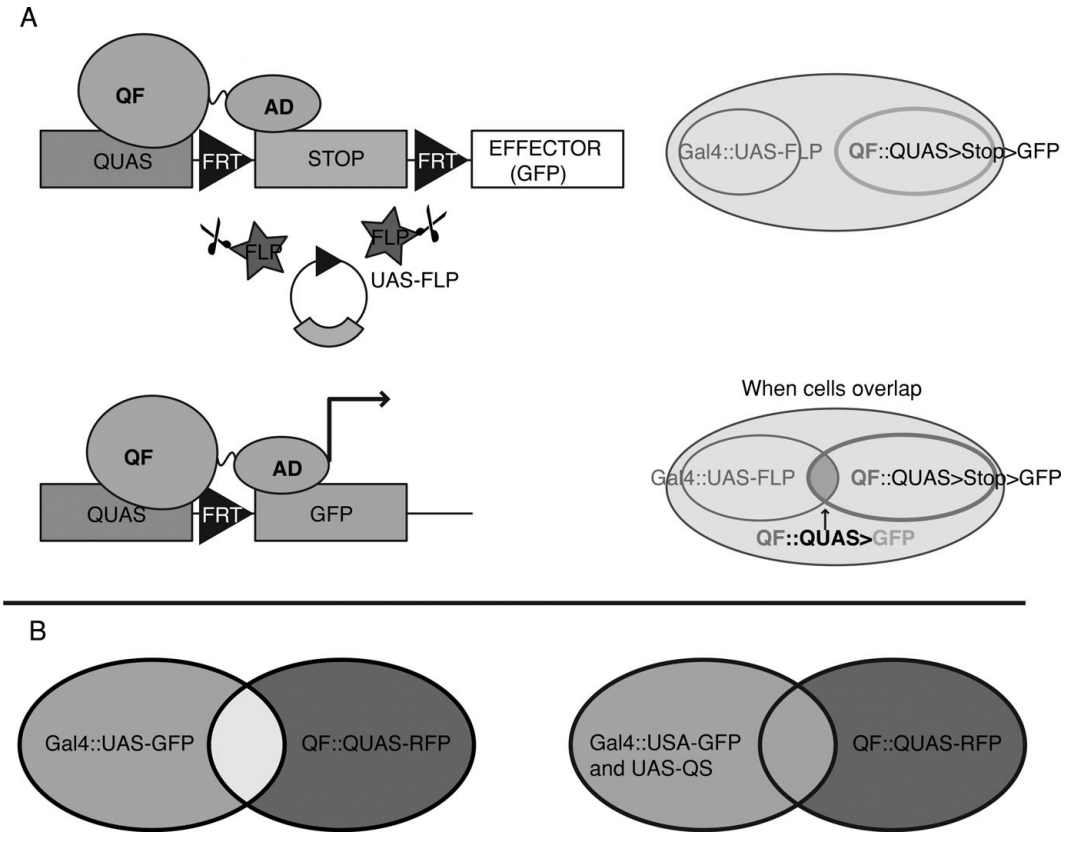

Fig. 19.5. Logic gates of the Q system. A An intersectional approach using both Gal4 and the Q system. Gal4 drives the expression of FLP through UAS-FLP. QF drives the expression of QUAS>stop>GFP. GFP is expressed only in cells in which Gal4 and QF overlaps. Gal4-driven FLP can be replaced with ET-FLP to intersect QF and thus restricting the number of cells that express UAS-GFP. B Promoter A-driven Gal4 and promoter B-driven QF can be used to map overlapping cells with the help of UAS-GFP and QUAS-RFP, respectively. Coupled with UAS-QS, the QF-defined circuit can be restricted by Gal4. If QUAS-RFP is replaced by a QUAS-effector (such as QUAS-Shi^{ts}), the neuronal activity of the refined QF circuit can be manipulated.

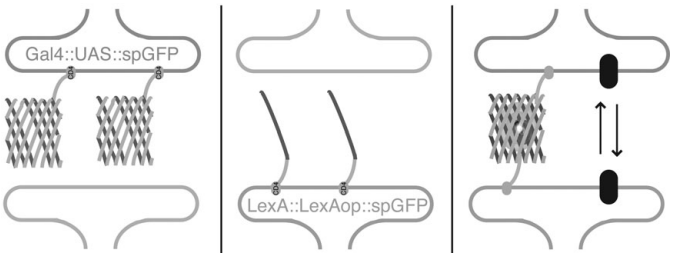

Fig. 19.6. GRASP the trans-synaptic matchmaker. GFP reconstitution across synaptic partners (GRASP) consists of two complementary fragments of GFP bound to an extracellular membrane protein, CD4. Alone, the fragments are unable to form a functional GFP; however, whenever each is individually expressed in opposing cells that from synaptic connections, a functional GFP is formed. The differential expression of the GFP fragments in presynaptic and postsynaptic membranes can be achieved using two binary systems, Gal4–UAS for the presynaptic cell (for example) and LexA-LexAop for the postsynaptic cell. The intersection of Gal4 and LexA expression patterns allows one to identify synaptic connections. Once identified morphologically, UAS-effectors such as UAS-NaChBac or UAS-TrPA1 can be used to excite the presynaptic cells and functionally test the postsynaptic response in a behavior or with imaging techniques (with the help of LexAop-GCamP or LexAop-cAMP reporters).

synaptic connections, a functional GFP is formed. GRASP was originally developed in *C. elegans* (Feinberg et al., 2008) and first adapted to *Drosophila* (Gordon and Scott, 2009), and soon to mammals (Kim et al., 2011). Gordon and Scott placed one fragment of the GFP under the control of Gal4, UAS-CD4::spGFP$^{1-10}$, and the other smaller fragment under LexA control, LexAop-CD4::spGFP^{11} (Gordon and Scott, 2009). As a proof of concept, Gordon and Scott expressed GRASP in known synaptic partners, olfactory receptor neurons (ORNs) and second-order projection neurons (PNs), demonstrating that the two individual halves were required and retained immunohistochemistry antigenicity in fixed tissue.

TANGO: trans-synaptic labeling

It takes two to tango! In the DSL-Notch signaling pathway, DSL-binding triggers cleavage of Notch and the release of the Notch intracellular domain, which in turn enters nucleus to activate gene transcription (Struhl and Adachi, 1998). Based on this concept, Barnea and colleagues developed the TANGO trans cell labeling method to convert transient ligand binding (presynaptic release) to a reporter gene expression and thus permanently mark the postsynaptic cell (Barnea et al., 2008) (Fig. 19.7A). In the TANGO design, the two key dancers are (1) a receptor fused with an intracellular transcriptional activator bridged by a protease cleavage site, and (2) a protease that only binds to the chimeric receptor and cleave free the transcriptional activator upon ligand activation of the receptor. Then the transcriptional activator enters the nucleus and turns on a reporter gene (such as GFP) expression. In mammalian culture cells Barnea tested three modules for G-protein-coupled receptors (GPCR), tyrosine kinase receptors, and steroids receptors. For each of these receptor types, they designed specific carrier proteins of the tobacco etch virus (TEV) protease, which

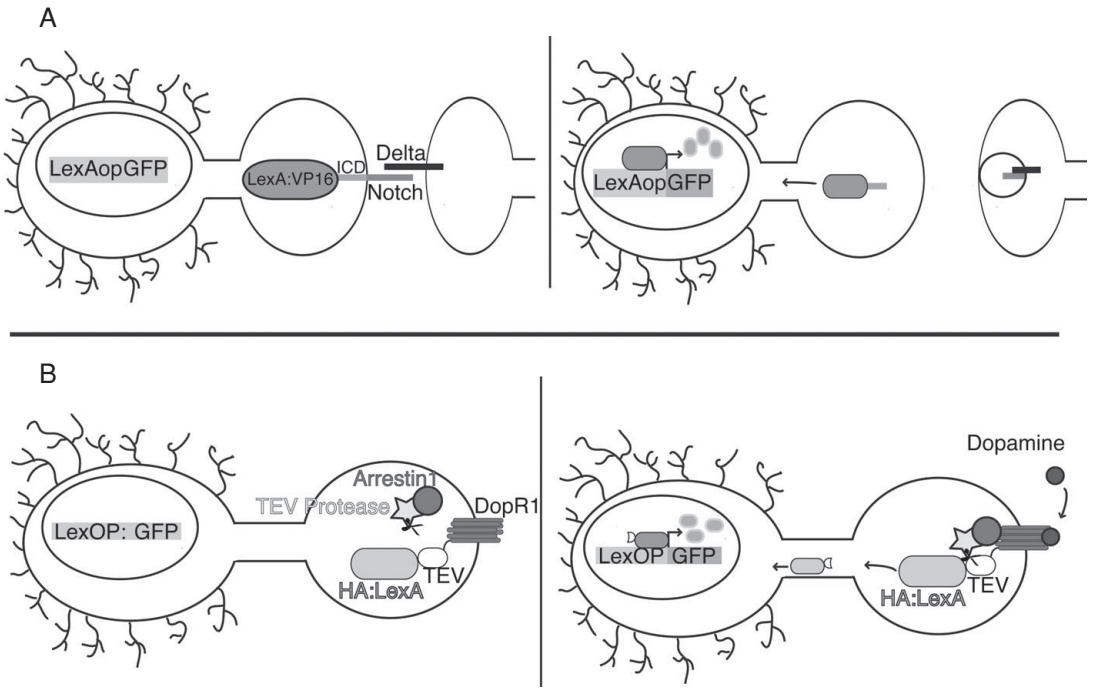

Fig. 19.7. TANGO trans-synaptic labeling. A The Delta/Notch-TANGO method takes advantage of the basic biology of Delta-Notch signaling mechanism in which DSL-binding triggers cleavage of Notch and the release of the Notch intracellular domain (NICD), which in turn enters nucleus to activate gene transcription. Fused to NICD, LexA-VP16 is able to enter the nucleus to turn on the LexAop-reporter gene expression (e.g., LexAop-mCD8GFP) to mark the postsynaptic cell. In *Drosophila* olfactory system, this TANGO labeling has been shown to be activity and Delta-dependent. It remains unclear whether presynaptic activity also regulates Delta at all synapses.B The DopR-TANGO method is used to label dopaminergic postsynaptic cells. In this design, arrestin1 serves as a specific carrier protein of the tobacco etch virus (TEV) protease, which normally does not bind to the receptor at rest but dimmerizes with receptors. This, in turn, allows TEV protease to cleave the transcriptional activator (LexA) from the receptor upon activation of the receptor. LexA then enters the nucleus to turn on the LexAop reporter gene (e.g., mCD2GFP) to mark the dopamine-receiving cell. Because Elav-GeneSwitch is used to express UAS-DoR1-TEV cleavage site-LexA-HA-2A-Arrestin1-TEV Protease transgene in all neurons, the current DopR-TANGO configuration can only be used to identify dopamine-responsive cells. False-positive postsynaptic cells may occur if dopamine levels are high in the hemolymph. The presynaptic dopaminergic cells can be marked with RFP using the TH-promoter Q system to further examine the morphological synaptic connections. Alternatively, presynaptic activity can be controlled by TH-QF-driven QUAS-TrpA1 to activate postsynaptic cells.

normally do not bind to the receptor at rest, but dimmerize with receptors and hence allow TEV protease to cleave the transcriptional activator from the receptor upon activation of the receptor.

Inagaki and colleagues from David Anderson's laboratory adopted the GPCR TANGO module and developed the first dopamine receptor TANGO transgenic flies (Inagaki et al., 2012) (Fig. 19.7B). Differing from the Barnea strategies, Inagaki and colleagues used endogenous GPCR in their TANGO design, thus allowing studies of neuromodulators native to flies. Using GeneSwitch they expressed UAS-DopR1-TEV cleavage site-LexA-HA-2A-Arrestin1-TEV Protease transgene in all neurons. Activation of DopR1 is then expected to recruit arrestin1-TEV protease to the chimeric receptor and free LexA to enter the nucleus and turn on LexAop-reporters (such as LexAop-CD2-GFP and LexAop-β-gal) (Fig. 19.7B).

One major concern is how effectively this method distinguishes background signal mediated by "resting" levels of dopamine from dopamine associated with a "potentiated" or "intensive" neuronal activities. The authors showed that perpetual expression with Gal4 could increase GFP levels and thus mask the sensitivity to detect dopamine response. By using a conditional Gal4 (GeneSwitch), the "resting" GFP levels were relatively low compared to experimental conditions. There are obvious limitations to GPCR-TANGO methods, as they are not applicable to classical fast transmitters via ionotropic receptors.

Lieber et al. from Gary Struhl's group utilized a version of DSL-Notch synaptic labeling consist of a chimeric Notch protein with either a Gal4-VP16 or LexA-VP16^{AD} cytosolic domain (Lieber et al., 2012). Upon Delta endocytosis and Notch cleavage, the transcriptional domain drives expression of a destabilized form of GFP, dGFP. While an NLS–GFP can be utilized to increase readout kinetics, using dGFP allows for the measurement of recent activity. Lieber et al. demonstrates this transient readout by assaying the olfactory response of olfactory receptor neurons to CO_2 and ethyl butrate. In comparison with other fluorescent activity indicators, e.g. GCaMP, this DSL–Notch indicator is slower; requiring a minimum of 6–12 hours, using NLS-GFP, to 3 days, using dGFP. DSL signaling is better appreciated for neurogenesis and early development (Bellen et al., 2010; Tien et al., 2009). The finding of DSL in TANGO transsynaptic labeling is important and suggests a role of DSL in postmitotic and mature nervous systems. It remains to be determined

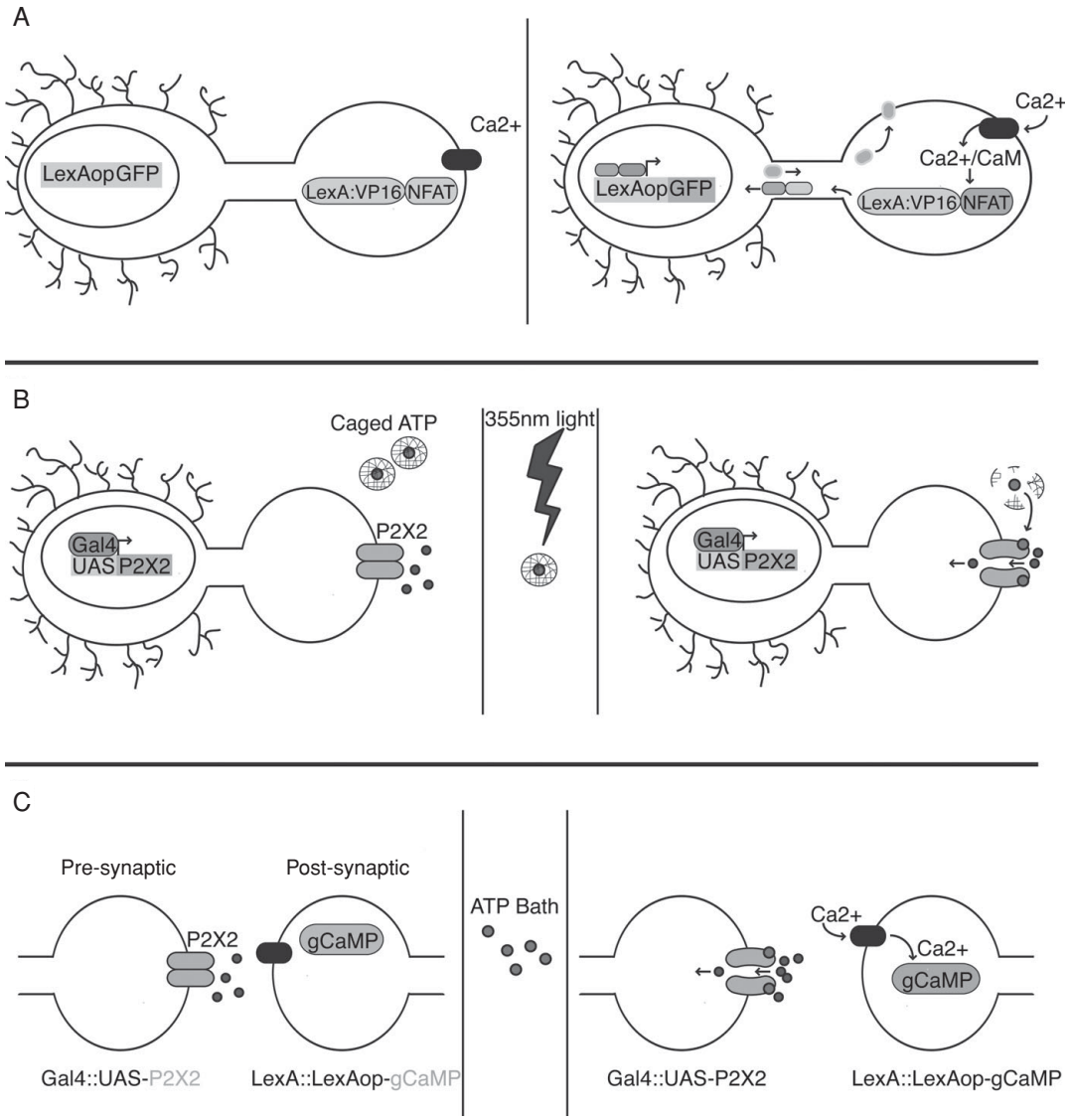

Fig. 19.8. Activity-dependent trans-synaptic labeling or imaging. A The CaLexA method uses Ca^{2+}-dependent translocation of the nuclear factor of activated T cells (NFAT) transcription factor to report neuronal activities. Calcium activates calcineurin, which in turn dephosphorylates NFAT and exposes the NLS on NFAT allowing NFAT to enter the nucleus. Coupled with NFAT is a mutant LexA without NLS (mLexA), which then turns on the expression of GFP reporter via LexAop-mCD8GFP. CaLexA reports all active neurons with rising Ca^{2+}, including postsynaptic cells downstream of a specific presynaptic input. B Uncaging ATP to activate postsynaptic cells expressing ATP receptor/channel P2X2. C Presynaptic cells expressing P2X2 receptors can be activated by uncaging ATP focally or by perfusion of ATP. The cells postsynaptic to the P2X2 cells can be identified by imaging intracellular (or synaptic) Ca^{2+} or second messenger cAMP for peptidergic or other cAMP-producing cells.

how neuronal activity regulates Delta and DSL signaling and whether DSL TANGO can be used for other synaptic partners.

In the present design, both DSL-TANGO and DopR-TANGO cannot label the presynaptic partners. For DopR-TANGO, presynaptic input seems defined as dopaminergic neurons, which can be marked with RFP via TH-QF::QUAS-RFP (when Gal4 is used to express TANGO).

CaLexA Calcium-dependent nuclear entry of LexA (CalexA): Activity-dependent transcription factors as reporters/circuit mapping

Nuclear factor of activated T cells (NFAT) is a transcription factor that enters the nucleus in response to the influx of calcium (Masuyama et al., 2012). Calcium activates calcineurin, which in turn dephosphorylates NFAT and exposes the NLS on NFAT, allowing NFAT to enter the nucleus. Because NFAT can be rapidly rephosphorylated inside the nucleus, it stays there only transiently and thus it has the potential to translate a relatively transient nerve activity into permanent XFP tags.

Taking advantage of these properties of NFAT, Jing Wang's group developed the CaLexA method in which they mutated LexA to delete its NLS, fuse the mutant LexA (mLexA) with NFAT, and thereby mLexA entry into nucleus will depend on both Ca (neuronal activity) and NFAT. Coupled with Gal4 and LexAop-linked GFP reporter (e.g., LexAop-mCD8GFP), CaLexA can be used to label active neurons in a circuit. One key reagent is UAS-mLexA-VP16-NFAT, which can be driven by any Gal4 and accumulates mLexA-VP16-NFAT in the cytosol. Upon calcium influx into the cell, mLexA-VP16-NFAT enters the nucleus to drive LexAop-mCD8GFP to transcribe, producing GFP as readout of neuronal activity levels (Fig. 19.8A).

This method can be combined with RFP to tag all circuits expressing Gal4 and use GFP to determine which cells within the Gal4 expression pattern are activated from stimuli. Fusing

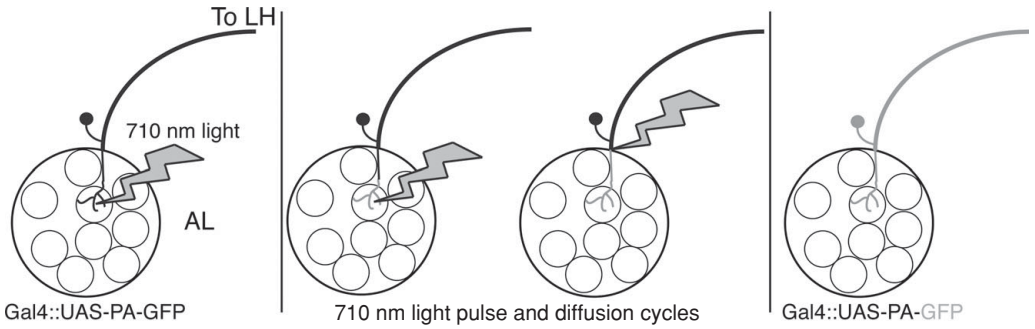

Fig. 19.9. Neuronal tracing using photoactivatable GFP (PA-GFP). A Prior to photoactivation, PA-GFP emits very little fluorescence. Exposure to either two-photon excitation (710 nm) or UV laser (390–415 nm) results in a 100-fold increase in GFP fluorescence. To label PNs innervating a single glomerulus, the glomerulus is exposed to 710 nm light pluses (30x for 30 sec) over a 15 minutes period, followed by a 15 minutes break to allow for the activated GFP to diffuse. Additional activation cycles are targeted at the glomerulus and the corresponding PN to enhance fluorescence levels for tracing.

with another effector (such as LexAop-mCD8GFP-Shi^{ts}) or adding another effector (LexAop-Shi^{ts}) separately, the CaLexA method will simultaneously mark and manipulate the downstream neurons selectively. The great advantage of the CaLexA system is that it is compatible with Gal4 drivers, allowing one to simply cross UAS-mLexA-VP16-NFAT with his or her favorite Gal4.

P2X2-Ca/cAMP imaging mapping synaptic connectivity

While TANGO and CaLexA methods offer activity-dependent readout of postsynaptic cells, the response is slow, taking hours or days to accumulate sufficiently high levels of GFP signaling for imaging or immunocytochemistry. Orie Shafer and colleagues have developed a 'physiogenetic' method using ATP to activate presynaptic neurons and then detecting postsynaptic calcium or cAMP levels in single neurons (Yao et al., 2012) (Fig. 19.8C). In this method, one population of neurons expresses the ATP-sensitive receptor/channel P2X2 (Lima and Miesenbock, 2005) (Fig. 19.8B) whereas another population of cells expresses GCaMP3.0 (Tian et al., 2009). This differential expression of P2X2 and GCaMP can be achieved using two different binary pairings, such promoter A-Gal4::UAS-P2X2 vs. promoter B LexA::LexAop-GCaMP3.0 or vice versa. In dissected brain preparations, bath perfusion of ATP (1–5 mM) will lead to GCaMP3.0 signal increase as rapidly as 30 sec if (a) the two cell populations or subset of them overlap or (b) when cell A synapses with cell B. The authors chose ATP perfusion instead of activating neurons with TrPA1 or TrPM8 because changing temperature can result in significant body movement of flies. Which in turn will impede live imaging of Ca^{2+} or cAMP in single neurons, although this works relatively well for imaging of larger brain areas such as neuropils (Suh et al., 2004; Wang et al., 2003; Yu et al., 2004). Focal perfusion of ATP or local ATP uncaging offers additional spatial resolution if a specific presynaptic Gal4 driver expresses too broadly. Channelrhodopsin-2 (ChR2) was not used for neuronal activation due to the overlap in wavelength of light for activation of ChR2 and GCaMP3.0. Newer versions of ChR2 activated by red light may offer additional ways to excite presynaptic cells (Tye and Deisseroth, 2012).

Tracing Connectivity using Photoactivatable GFP

Utilizing photoactivatable GFP (PA-GFP), it is now possible to trace nerve projections. PA-GFP originally generated and tested in cell culture by Patterson and Lippincott-Schwartz (Patterson, 2002), was enhanced and turned into a UAS line by Datta et al. (Datta et al., 2008). Driving expression of PA-GFP in projection neurons (PNs) using GH146-Gal4, Datta et al. was able to photoactivate the GFP in a single glomerulus, DA1, and then mark the specific PN or PNs that innervated the glomerulus (Fig. 19.9). Initially being activated at the synapse, GFP then diffused into the rest of the PN, enabling the investigators to characterize the projection pattern and morphology of the PN in male and female flies. Once the PNs were labeled with GFP, focal loose-patch was applied to monitor their action potential firing properties. PA-GFP has also been utilized to trace the dopaminergic neurons responsible for reinforcing odor aversion memory (Claridge-Chang et al., 2009) and to trace the connectivity between three areas of the auditory circuit (Lai et al., 2012). Theoretically, this technique could be used in any two-photon accessible *in vivo* preparation to map circuit connectivity.

Beyond *Drosophila*: Brain-behavior circuitry mapping in other genetic model organisms

Understanding the neural substrate of behavior is a common goal of all neuroscientists. Hence, most, if not all, of the techniques described here have also been employed by scientists working with other genetic model organisms. Lichtman and Denk pointed out that no matter if it is a big or a small brain the technical difficulties for dissecting brain structure and function are similar (Lichtman and Denk, 2011). The nervous system of *C. elegans* has approximately 300 neurons, but its behaviors are just as rich and complex as most other animals, including mammals (for review see Bargmann, 2012; Bendesky and Bargmann,

2011). Because of its well-defined cell lineage and transparent body, circuit-behavioral studies can be readily carried out by cell-specific laser ablation or cell-specific promoter-driven genes. The need for developing binary and intersectional methods is rather low such that most of the techniques described here are not in use in *C. elegans*. However, we note that Kang Shen's group recently adopted the Q system and developed Split Q intersection methods in *C. elegans* (Wei et al., 2012).

In zebrafish, Gal4–UAS-Gal80 are being perfected for manipulating gene expression and mapping neural circuits (Abe et al., 2011; Fujimoto et al., 2011; Kawakami et al., 2010; Scheer and Campos-Ortega, 1999). In mice, various strategies have been developed to map neural circuits. Cre-LoxP is more generally used than FLP-FRT for chromosomal engineering (Austin et al., 1981; Luo et al., 2008; Orban et al., 1992). MARCM, first developed in *Drosophila*, has been modified as mosaic analysis with double markers (MADM) for mice (Zong et al., 2005). The most basic but yet essential and heroic step is to map all neuronal connections in the brain ("connectomes") through BrainBow and other techniques (Livet et al., 2007); for review see Lichtman and Denk, 2011). The recent development of CLARITY, which effectively transforms the opaque brain into transparent, enabling whole brain imaging without the need to slice, will be a boost to connectomes (Chung et al., 2013). Besides transgenics, viruses are often used to deliver gene expression focally in subset of cells (reviewed by (Luo et al., 2008)). Focal release of transmitters through *uv* uncaging in combination with Ca or cAMP imaging is proven a powerful approach for mapping synaptic connectivity. Most early studies are limited to brain slices, however, and the findings may not be easily reconciled with behavioral context of intact animals. The application of photo-sensitive ion channels and related optogenetics offers the great opportunity to map brain circuits underlying behaviors in mice, other genetic model organisms, and primates (Boyden et al., 2005; Diester et al., 2011; Han, 2012; Lima and Miesenbock, 2005); for review see Bernstein et al., 2012; Tye and Deisseroth, 2012). The potential of optogenetics in deep-brain stimulation for treating Parkinson's disease, depression, substance addiction, and psychiatric disorders is exciting and potentially promising (Deisseroth, 2012). Whether it is basic research or clinical application, cell-specific expression systems and intersectional methods will be expected to play critical roles in the effort of understanding or treating brains.

Alternative to physical maps, one intriguing idea is to use DNA barcodes and high throughput sequencing to map the brain connectome (Zador et al., 2012). In this proposed method, each neuron can be barcoded with a unique short nucleotides using transgenic approach similarly employed for the BrainBow technique. If a neuron forms a synapse with a target neuron, then the barcodes will invade the synaptically connected cell through transsynaptic translocation (aided by viruses or other means). Finally, the two neurons' unique barcodes will be joined together. DNA sequencing of the joint barcode will reveal the specific pairing of these two neurons. Conceptually, this approach has the potential to reveal all synaptic connections in the brain. The authors further argued that this BOINC (barcoding of individual neuronal connections) method could be done at a much faster speed and at a much lower cost compared to microscopic approaches. At present, BOINC is a highly creative and attractive hypothesis; if successful it would significantly transform the study of brain circuits and synaptic plasticity.

Conclusions and future directions: Functionomics

As precise and informative as they are, neither genomics nor connectomics can predict the behavioral output of neurons or neural networks. Even for the best understood "simple" stomatogastric nervous system of crustaceans in which all neurons and their connections are known, the wiring diagram of the network will not tell us how the network will be operated (Harris-Warrick et al., 1992; Marder and Bucher, 2007). In the end, analysis of *structure* and *function* has to go hand in hand. Hence, "functionomics," defined as detailed maps of neurons or glia and neural circuits functioning for specific behaviors or cognition, will help neuroscientists achieve the ultimate goal of understanding the brain. This is also the goal of a recent initiative initially called Brain Activity Map (BAM) and later Brain Research through Advancing Innovative Neurotechnologies dubbed BRAIN (Chung et al., 2013). *Drosophila* will continue to play important roles in this endeavor. It is expected that new and clever intersectional methods will be developed, as there is need for refinement of each binary system and for different binary systems to intersect with Gal4. Temporal and inducible features can be added to the current intersectional methods. The methods described here have benefited from studies of non-nervous systems and should also be applicable to clonal (mosaic) analysis in these systems too.

A number of challenges remain facing current and future functionomics in *Drosophila*. The first challenge is the refinement of intersectional methods. At present, all of the intersectional methods described in this chapter are useful for neural circuit-behavior mapping. However, most of them have just passed the proof of principle stage and they are underdeveloped in terms of the need for a large collection of essential reagents (such as Split Ga4, Split LexA enhancer-trap lines). The FINGR method is relatively ready for behavior-brain mapping, as we have a collection of approximately 1,000 ET-FLPx2 lines. For the FINGR method to be truly useful for the broad fly community interested in different behaviors, more ET-FLPx2 lines will be needed, however. As with the evolution of Gal4–UAS resources, we expect that different labs will generate new lines and eventually have a large collection of needed reagents for all to share.

The second challenge is even greater, which is how to map the synaptic connections within a circuit. The TANGO methods will need to be tested and perfected for other synapses. New methods will be needed for transsynaptic labeling of

non-GPCR synapses such as glutamate, acetylcholine, and GABA. Dye injection has been effective for labeling electrically coupled neurons, but genetic methods will be needed to mark gap junction-coupled neurons. All of these transsynaptic labeling will ideally not depend on the use of viruses, but take advantage of transgenic techniques and the strength of fly genetics.

The third challenge is to dissect the contributions of the often-overlooked "other" brain cells, glia, to neural circuits and behaviors. Relative to neurons, glial cells are more abundant in the human brain (making up ~80% of brain cells although neurons out number glia in the fly brain), but they remain less well understood. However, the interest in glia is growing rapidly as we are learning important and surprising features of glial cells. Glial cells are now regarded as active partners of neurons, with broad functions in regulation of neuronal development and function. Not surprisingly, glia–neuron communication also has strong influences on behavior. By increasing the permeability of the blood-brain barrier, mutations in the G-protein-coupled receptors Moody make the flies more sensitive to cocaine and alcohol (Bainton et al., 2005; Schwabe et al., 2005). Glia-expressed Period and glia-specific Ebony both play important roles in circadian rhythm (Jackson, 2010; Suh and Jackson, 2007). Gliotransmission affects sleep homeostasis, cognition, and learning/memory in mammals (Halassa et al., 2009). Deletion of a glial solute carrier called Genderblind dramatically alters the courtship preference of male flies such that males court males instead of females (Grosjean et al., 2007). These examples highlight the value of genetic studies toward understanding glial roles in neuronal control of behavior.

The final challenge is how to factor in neuromodulators and hormones in the equation of understanding circuits and behavior. Neuromodulators and hormones may not be part of a wired circuit but they can have profound effects reshaping the activity of the neural circuit and behaviors. Neuromodulation has been studied in a number of animals, including *C. elegans*, *Drosophila*, and mammals (Bargmann, 2012). Neuromodulatory effects on circuit function are best exemplified in the stomatogastric nervous system of crustacea where a neuropeptide or a monoamine can completely reconfigure the synaptic strength of an anatomically defined circuit and dramatically alter the output of the circuit (Harris-Warrick et al., 1992). In humans, neuromodulators dopamine and serotonin (5-HT) have profound effects on the well being of individuals. For example, 5-HT reuptake blockers such as Prozac (Fluoxetine) are widely prescribed to treat depression in millions of people (although the mechanism by which 5-HT reliefs depression remains poorly understood). We strongly second the argument made by Cori Bargmann that an anatomically wired circuit is incomplete without weighing in the influence of neuromodulators and hormones (Bargmann, 2012).

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Chapter

20 Imaging *Drosophila* brain neurons for "FlyCircuit" analysis

Chih-Yung Lin and Ann-Shyn Chiang

Introduction

An open-access database, named "FlyCircuit" (http://www.flycircuit.tw/), has recently been established for online data archiving, mining, and 3D visualization of single neurons in a standardized *Drosophila* brain (Chiang et al., 2011). The adult *Drosophila* brain has approximately 100 000 neurons that can be classified into two categories: local neurons (LNs), whose neurites are contained within a restricted local brain region, and projection neurons (PNs), which connect with distant brain regions. FlyCircuit contains high-resolution 3D images of ~16 000 single cells from 9 different promoter-driven Gal4 lines covering most known neurotransmitter types. Although this is only around 10% of the total number of *Drosophila* brain cells, FlyCircuit likely contains the majority of anatomical cell types, because each cell type may be composed of multiple neurons that are identical in function (Kohl and Jefferis, 2011).

To track information flow in the brain, a comprehensive map of neural connections (i.e., a connectome) is urgently needed. Like Google Earth, a connectome spans multiple scales, ranging from macroscopic neural tracts linking brain regions, to intermediate mesoscopic connectivity between subregions, to microscopic synaptic connections. Thus far, two approaches have contributed to the reconstruction of the fly connectome. The first, used in several ongoing efforts, employs serial-section transmission electron microscopy for a semi-automated reconstruction of neural circuits at the level of the synapse (Briggman and Denk, 2006; Chklovshii et al., 2010). Although such an approach requires labor-intensive synapse identification and neurite tracing, a complete map of all synaptic connections within a single column in the medulla (for a volume of 90 μm × 90 μm × 80 μm) has been successfully reconstructed. The second approach, a parallel effort using confocal microscopy, has managed to bar code some 16 000 of the 100 000 neurons in the fly's brain, and reassembled those neurons into a complete brain containing 41 local processing units (LPUs) and 58 tracts (Chiang et al., 2011) (summarized in Fig. 20.1). The basis of this light microscopic approach is a technique that makes the *Drosophila* brain transparent (Liu and Chiang, 2003), and allows visualization of the 3D structure of individual neurons labeled by genetic mosaic analysis with a repressible cell marker (MARCM) (Lee and Luo, 1999) without physical sectioning. Although its limited resolution precludes detailed visualization of the synapse, the light microscopic approach offers several advantages: It enables the imaging of large tissue volumes, allows for rapid reconstruction of mesoscopic connections, and, perhaps most importantly, provides functional information such as neurotransmitter content and gene expression.

FlyCircuit allows researchers to add their own neurons of interest to the database for data mining and connectivity analysis (Fig. 20.2). Several tools have been implemented: (i) quantitative analysis of the spatial distribution of single neurons in relation to all LPUs, (ii) comparison of individual neurons, (iii) prediction of a neuron's neurotransmitter and receptor expression, (iv) identification of neural tracts connecting LPUs, (v) selection of Gal4 drivers labeling neurons of interest, and (vi) perhaps most importantly, predicting connections between individual neurons. Adding a neuron to the FlyCircuit database involves five steps: whole-mount immunostaining of all synapses, segmentation of the neuron of interest, alignment and transformation of the sample brain to the standard model brain, registration of the neuron of interest to the standard model brain, and proofreading and annotation. It is important to note that the accuracy of the FlyCircuit analysis depends largely on sample preparation and proper 3D imaging. Here, we present a step-by-step protocol for preparing the fly brain for high quality 3D imaging and FlyCircuit analysis.

Materials

- Fly stocks
- 1× phosphate buffer solution (PBS, 100 mM Na_2HPO_4/NaH_2PO_4, pH 7.2)
- Triton™ X-100 (Sigma-Aldrich, cat. no. T8787)
- Sodium azide (Sigma-Aldrich, cat. no. S2002)
- Paraformaldehyde (Electron Microscopy Sciences, catalog no. 15713-S)
- Normal goat serum (NGS, Lampire Biological Laboratories, catalog no. S2–0609)

Behavioral Genetics of the Fly (Drosophila melanogaster), ed. J. Dubnau. Published by Cambridge University Press.

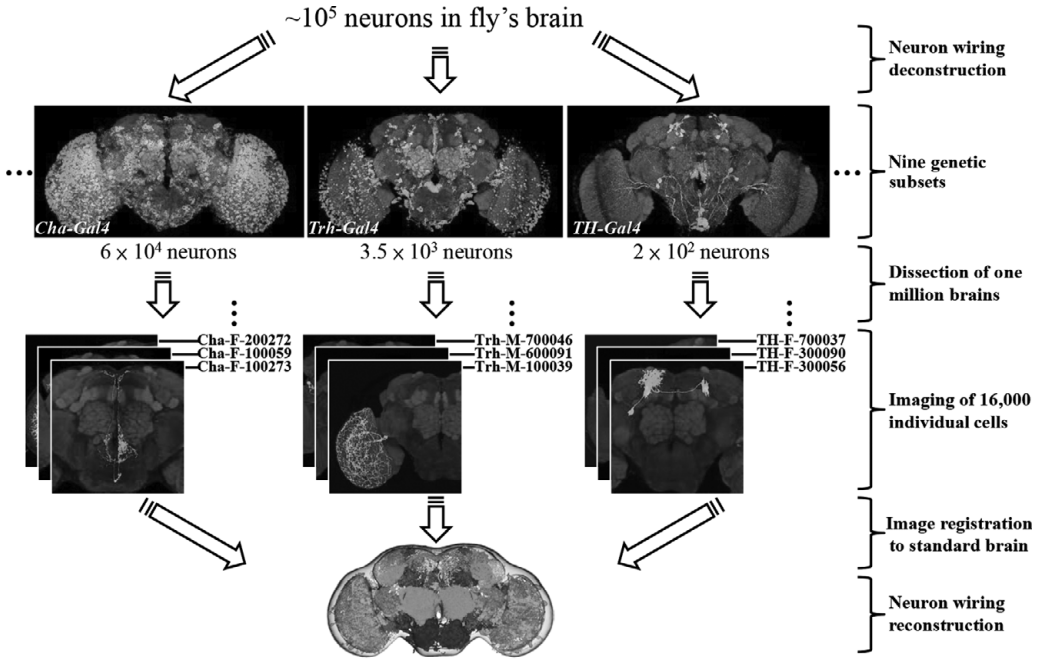

Fig. 20.1. Deconstruction and reconstruction of the fly brain. We selected nine different Gal4 lines, each containing a unique subset of neurons, for use in deconstructing the entire *Drosophila* brain, resulting in a sizeable collection of individual neurons. GFP-labeled images of 16 000 single cells derived from millions of dissected brains were segmented from confocal images, registered onto a common standardized brain template, and then reassembled into a complex brain network.

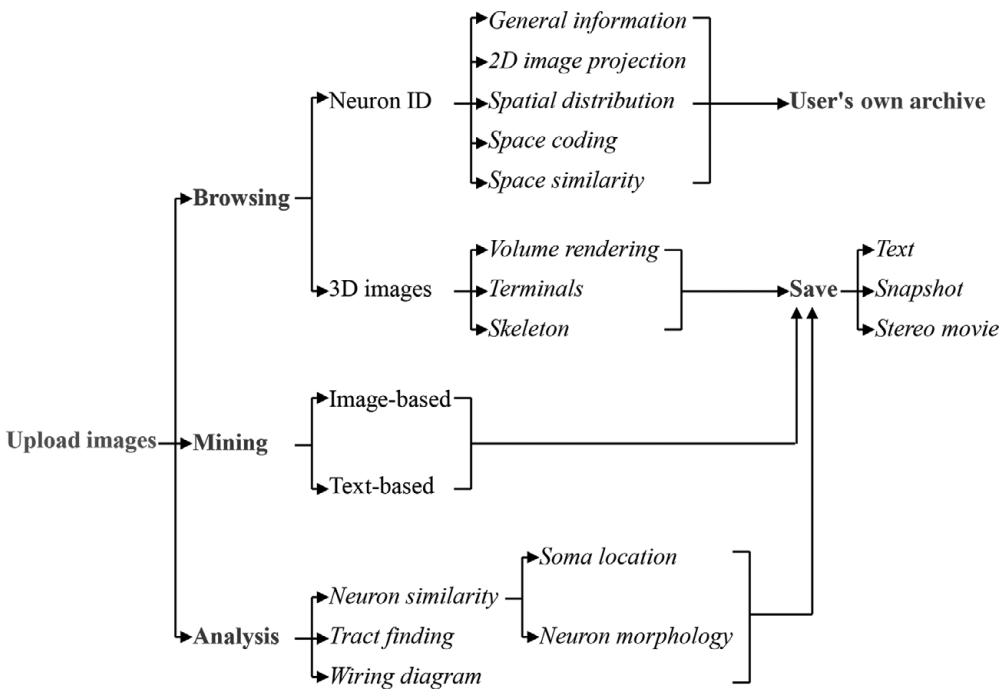

Fig. 20.2. A road map of FlyCircuit tools.

- Primary antibody: the synaptic marker mouse 4F3 anti-disks large (DLG) antibody (Developmental Studies Hybridoma Bank., Univ. of Iowa, USA; 1:50) is used to label all synapses in the *Drosophila* brain. Notably, the mushroom body and glomeruli of the antennal lobe are strongly labeled.
- Secondary antibodies and streptavidin-conjugated florescence dyes: these may include biotinylated goat anti-mouse IgG (Molecular Probes; 1:250) and Alexa Fluor 635 streptavidin (Molecular Probes; 1:500).
- FocusClear™ (CelExplorer, catalog no. FC-101), an aqueous sugar-based solution rendering biological tissue transparent.
- MountClear™ (CelExplorer, catalog no. MC-301), a mounting solution compatible with FocusClear™.

Equipment

- Standard fly culturing equipment and microscope
- 25 °C incubator to maintain fly strains

- Dissecting stereomicroscope (Carl Zeiss, Stemi 2000)
- Vacuum oven and pump (Risen Inc. RUD-30L)
- Microwave oven (2450 MHz, 1100 watts). The microwave energy required to heat 1 liter of water at room temperature was found to be 34.98 ± 1.60 kcal
- Orbital shaker (GenePure, OSR 201)
- 1.5-mL microcentrifuge tubes
- Two pairs of sharp forceps (Dumont, no. 55)
- Loop (~0.5 mm diameter)
- Dissection dishes
- 24-well culture plates
- Kimwipes
- No. 1 coverslips (Paul Marienfeld GmbH & Co. KG, catalog no. 01 010 50)
- Microscope slides (Paul Marienfeld GmbH & Co. KG, catalog no. 10 012 02)
- Clear nail polish
- Reinforcing rings (Wen Lung Printing Inc. catalog no. WL-8210)
- Zeiss LSM 510 confocal microscope equipped with an argon–krypton laser (458, 488, or 514 nm) and two HeNe lasers (543 and 633 nm)
- 20× objective lens (Zeiss, N.A. 0.75)
- 40× C-Apochromat water-immersion objective lens (Zeiss, N.A. 1.2; working distance, 280 μm)

Reagents

20% (vol/vol) PBT	Add 20 ml Triton-X 100 to 80 mL PBS (PBT) and store at 4 °C.
1% sodium azide	Add 0.5 g sodium azide to 50 mL PBT. Store at 20~25 °C. **Caution!** Toxic, handle as a potential carcinogen.
Fixation solution	Add 10 mL 16% w/v paraformaldehyde to 30 mL 0.25% (vol/vol) PBT in a 50-mL tube. Must be prepared fresh and placed at 4 °C. **Caution!** Toxic, handle as a potential carcinogen.
Washing buffer	Add 30 g NaCl and 5 mL 20% PBT to 995 mL PB. Store this nontoxic buffer at 40 °C.
Blocking buffer	10% (vol/vol) NGS containing 0.5 mL 20% PBT, 0.5 mL NGS, and 0.1 ml 1% sodium azide in 3.9 mL PBT. The solution may be stored for 24 hours at 4 °C.
Dilution buffer	Add 0.0625 mL 20% PBT, 0.05 mL NGS, and 0.1 mL 1% sodium azide to 4.7875 mL PBT. The solution may be stored for 24 hours at 4 °C.
Primary antibody	1:50 mouse 4F3 anti-disks large monoclonal antibody in dilution buffer.
Secondary antibody	1:250 biotinylated goat anti-mouse IgG in dilution buffer.
Fluorescent dye	1:500 Alexa Fluor 635 streptavidin in dilution buffer.

Procedures

Brain dissection

Careful dissection that retains the brain's original size and shape is a prerequisite for precise 3D alignment between the sample brain and the FlyCircuit model brain.

Timing

~3–5 min per brain

1. Anesthetize the adult fly on ice.
2. Place the fly in a dissection dish and immerse it in PBS.
3. Remove the brain from the head cuticle and gently clean the brain with forceps under a dissecting microscope.
4. Put 100 μL PBS in each well of a 24-well plate, and cool the plate on ice. Collect the dissected brain using a loop (i.e., without touching the brain) and place it in the cold PBS.

Fixation

Dissected brains should be fixed within 30 min.

Timing

~12–16 h

1. Place the brain in 4% paraformaldehyde in PBS on ice. Rapidly fix the brain by microwave irradiation for 90 s on a rotation plate, repeating three times. **Caution!** Seal the well with colored tape to prevent dehydration.
2. Keep the brain in blocking buffer at room temperature for 4 h. **Caution!** Mark the experiment date, strain name, gender, operator, heat-shock stage, and duration on the colored tape.
3. Expel the air trapped in the tracheal system by placing the sample, immersed in blocking buffer, inside of a vacuum chamber. Depressurize the chamber to −70 mmHg for 10 min and repeat for a total of four cycles.
4. Keep the brain in the blocking buffer at 4 °C overnight. **Caution!** Seal the well to avoid dehydration.

Immunohistochemistry

FlyCircuit uses anti-DLG-labeled neuropilar structures in the sample brain as landmarks for 3D alignment and transformation of MARCM-labeled neurons to the standard model brain. High-quality immunostaining is a prerequisite for precise structural registration. A standard protocol for sample preparation has been established, so that every brain maintains its original size and shape as much as possible.

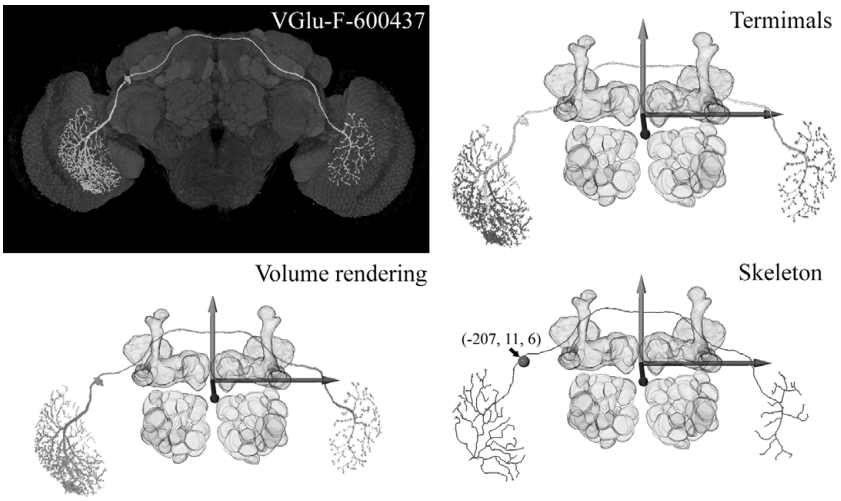

Fig. 20.3. Visualizing a neuron-of-interest uploaded to FlyCircuit. A Frontal view of an uploaded neuron with its neuron ID for cross-lab examination. B Volume rendering. C Spatial distribution of neuronal terminals. D Automated cell body identification and neuronal skeleton tracing.

Timing: 6 days

1. Wash the brain samples with washing buffer for 30 min at room temperature three times.
2. Incubate with anti-DLG antibody (120 μL/well) on an orbital shaker at 4 °C for 2 days. **Caution!** Seal the well to avoid dehydration.
3. Wash with washing buffer for 30 min at room temperature three times.
4. Keep the samples in washing buffer overnight on an orbital shaker at 4 °C. **Caution!** Seal the well to avoid dehydration.
5. Incubate the samples with biotinylated goat anti-mouse IgG (120 μL/well) for 2 days on an orbital shaker at 4 °C. **Caution!** Seal the well to avoid dehydration.
6. Wash with washing buffer for 30 min at room temperature three times.
7. Incubate with Alexa Fluor 635 streptavidin on an orbital shaker at 4 °C overnight. **Caution!** Seal the well to avoid dehydration.
8. Wash with washing buffer for 30 min at room temperature three times.
9. Clear the brains in FocusClear™ for 5 min, or until the brains become completely transparent at room temperature.
10. Mount the brains in a drop of MountClear™ under a coverslip separated by a spacer ring of ~ 200 μm thickness, so that the brain is not flattened.

Imaging

Timing: 60 min per brain

1. Brain samples containing a few well-separated single neurons labeled by MARCM were imaged. Samples with obvious deformities, weak GFP signal, or entangled neurons, were discarded.
2. For large neurons extending beyond the field of view under a 40× objective lens, we acquired two parallel stacks of confocal images covering the entire brain, with some overlap between the two hemispheres. We then stitched the two image stacks into a single large data set with a homemade 3D image-stitching algorithm, which used the overlapping region as a reference.
3. Each image stack contained ~120–140 optical sections with a 0.32 × 0.32 × 1.0 μm^3 voxel size, taken under a 40× objective lens. The following settings were used: scanning speed, 7; resolution, 1024 × 1024 voxels; zoom, 0.7; optical slice thickness, 1 μm; 25% overlap between adjacent slices, and line averaging of 4.
4. Two channels were simultaneously scanned: GFP-labeled neurons were excited using a 488-nm ArKr laser and Alexa Fluor 635-labeled neuropilar structures were excited using a 633-nm HeNe laser.

Image post-processing

Raw images were analyzed and processed with Avizo 6.0 (Visualization Sciences Group, Merignac Cedex, France).

1. For each stack of images, neurons are manually demarcated in several key slices using the *labelvoxel* module. We then apply the *interpolate* function to automatically outline the entire neuron. The precision of the demarcation is manually examined and adjusted.
2. The segmented single-neuron channel is then incorporated back into the original brain with the Zeiss LSM v.3 (Zeiss, Jena, Germany). This is used as the original image presented on the Neuron ID page in FlyCircuit.

Adding image data to FlyCircuit

1. Upload the stack of images of the DLG-immunostained brain.
2. Upload the stack of images of the GFP-labeled single neuron or expression pattern.
3. Write the annotation (optional).

4. Select the gender of the standard model brain.
5. Set the initial orientation and central position of the sample brain.
6. Using the DLG-immunostained channel as a reference, the program automatically aligns and warps the sample brain to the standard model brain. The resulting transformation matrix is then applied to merge the sample neurons into the standard model brain. For ease of further analysis and comparison, the voxel size for all the transformed images is re-sampled to x:y:z = 1:1:1. One can also curate manually if necessary.

Results and discussion

Once uploaded, FlyCircuit automatically generates a Neuron ID page that contains the cell body location and basic information about the neuron. Volume rendering and skeleton tracing of the uploaded neuron are automatically performed. Neuronal terminals can be visualized, counted, and analyzed (Fig. 20.3). More importantly, one can then use the data mining tools provided in FlyCircuit to search and compare the neuron of interest with the other 16 000 neurons stored in FlyCircuit (Chiang et al., 2011). For example, as a proof-of-concept exercise, the anterior cells (ACs), which act as an internal temperature sensor in the brain, have been added to FlyCircuit for connectivity analysis (Hamada et al., 2008). Spatial distribution analysis indicates that the axons of serotonergic ACs intersect with putative dendrites of a 5-HT1B-Gal4 neuron in the superior dorsofrontal protocerebrum (Shih and Chiang, 2011), which suggests that structural connections are present. This prediction requires functional confirmation, but remains invaluable, as most neurons of the central fly brain have unknown functions.

FlyCircuit provides a personal account for storage of a user's data and results. Users can decide whether they wish to make their data available for public examination. Combined with sophisticated genetic manipulations in *Drosophila*, FlyCircuit, with its cloud computing capability, provides a tour of the fly connectome, making it an important tool for planning perturbations of the neural circuits necessary for specific fly behaviors.

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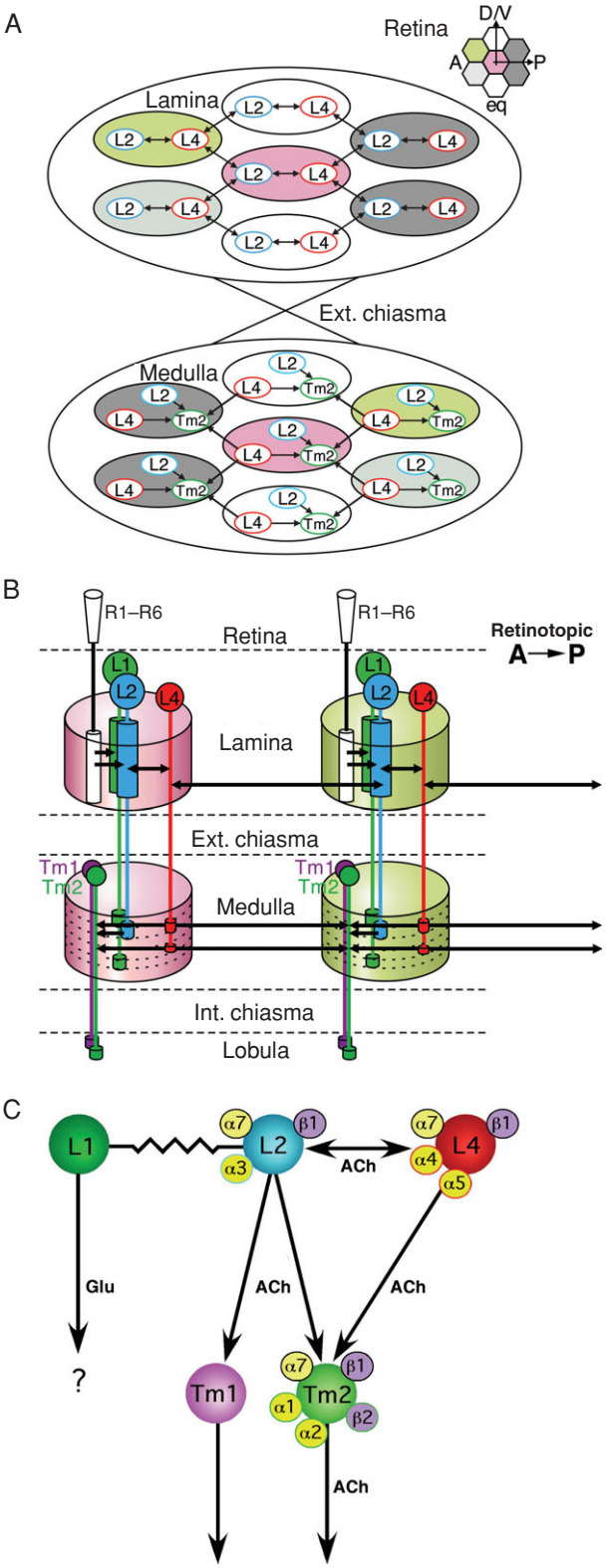

Plate 1.6 Inputs from lamina cells L2 and L4 converge upon medulla cell Tm2. For full caption text see page [11]

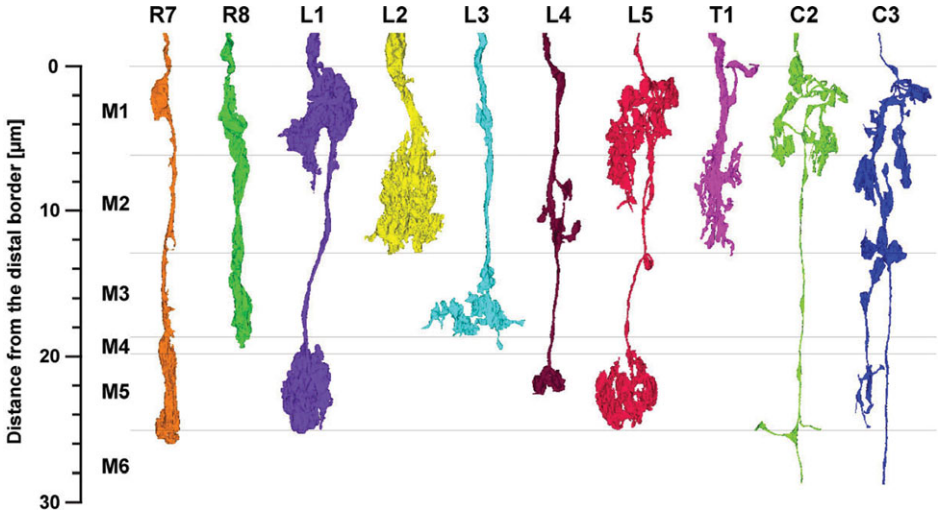

Plate 1.4 Terminals of ten neurons reconstructed from serial-section EM with input terminals in strata M1–M6 of the distal medulla. For full caption text see page [6]

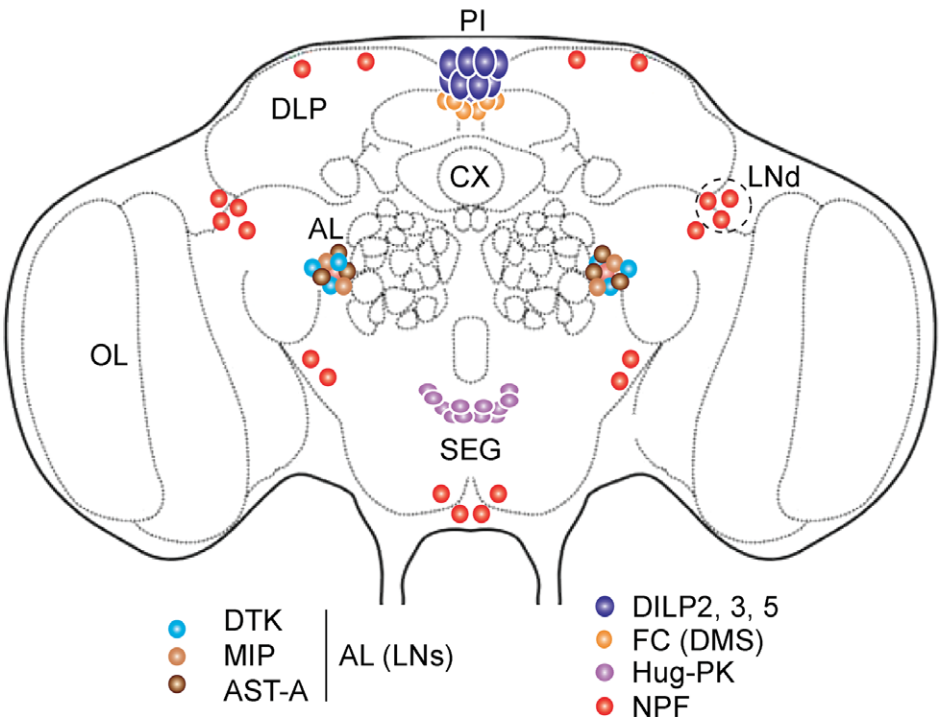

Plate 2.1 Neuropeptide distribution in the *Drosophila* brain. For full caption text see page [22]

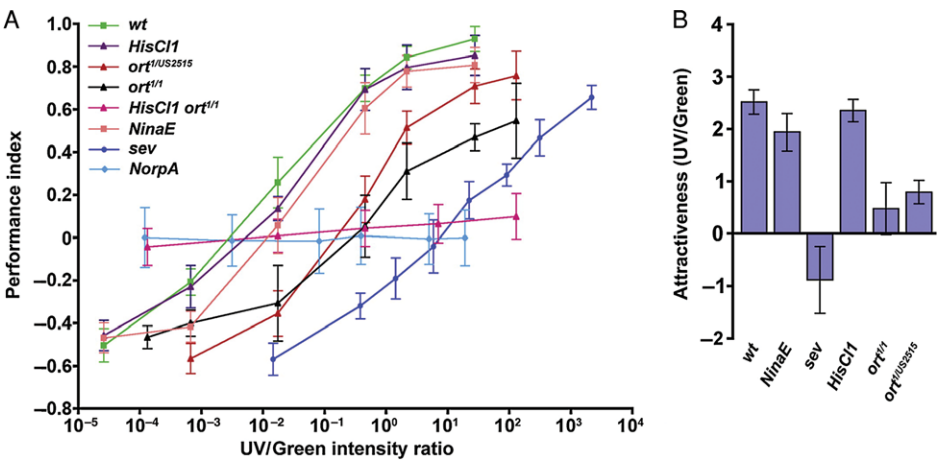

Plate 3.5 Spectral preference behavior of mutants affecting the visual system of *Drosophila* (modified from Gao et al., 2008). For full caption text see page [43]

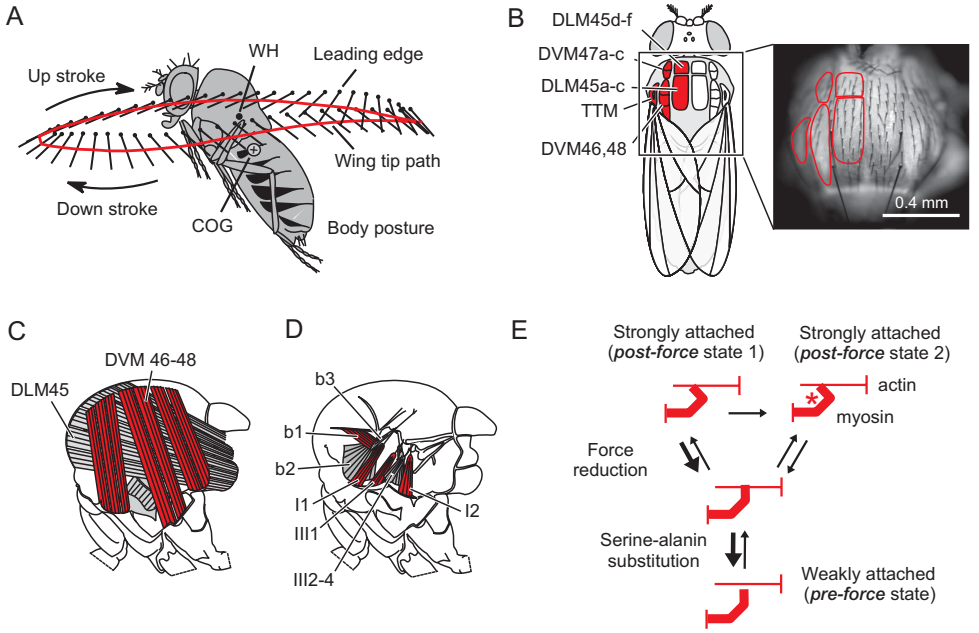

Plate 6.1 Wing motion and flight muscles in *Drosophila*. For full caption text see page [78]

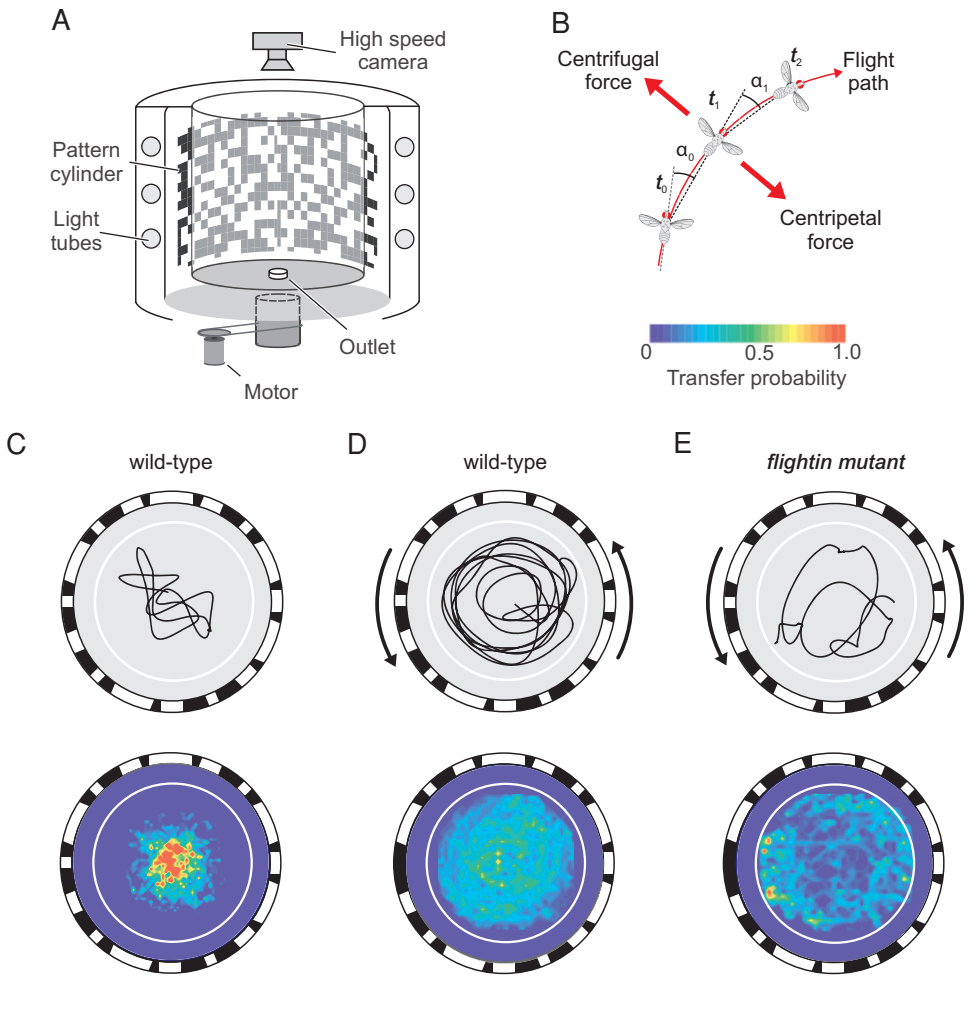

Plate 6.2 Free flight essay and free flight behavior in *Drosophila*. For full caption text see page [82]

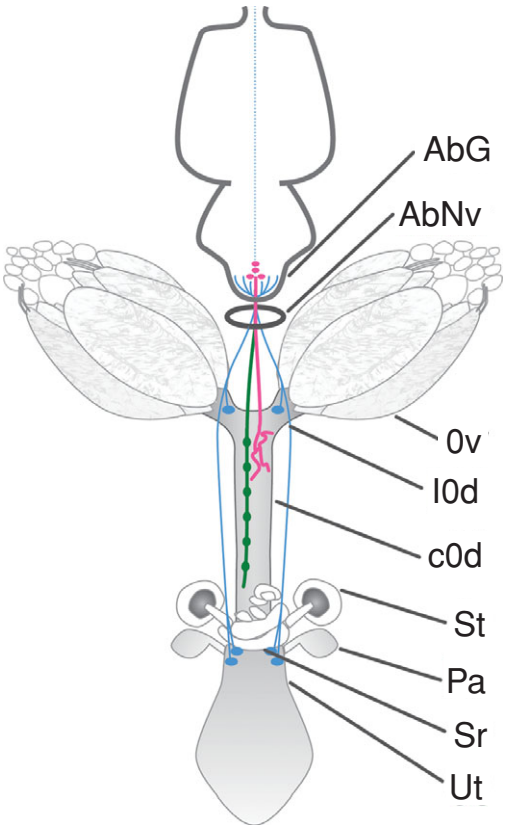

Plate 7.1 The female reproductive tract and its innervation by the nervous system. For full caption text see page [89]

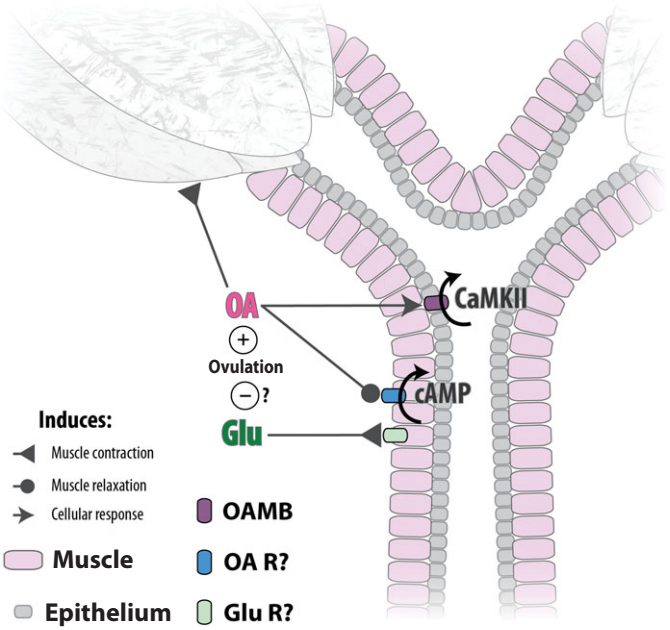

Plate 7.2 Ovulation is increased by OA at the ovary and oviducts, acting on muscle and epithelium (Monastirioti et al., 1996; Middleton et al., 2006; Rodríguez-Valentín et al., 2006; Lee et al., 2009). For full caption text see page [92]

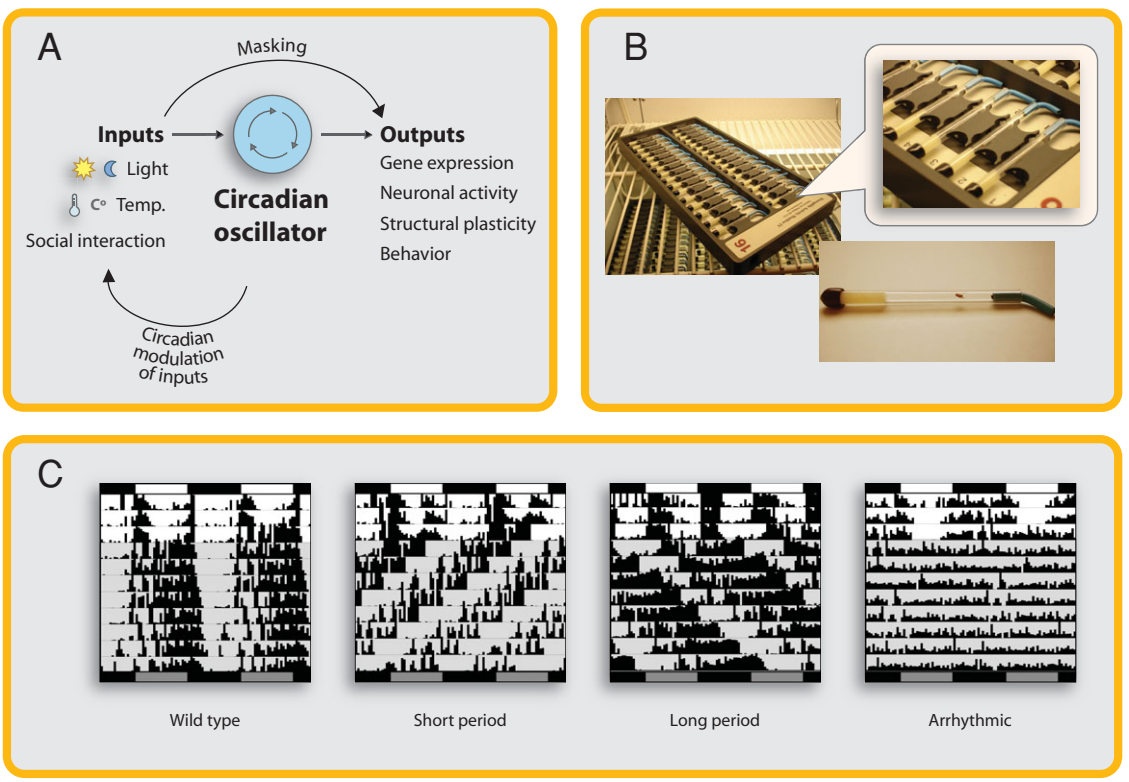

Plate 8.1 *Drosophila* circadian basics. For full caption text see page [105]

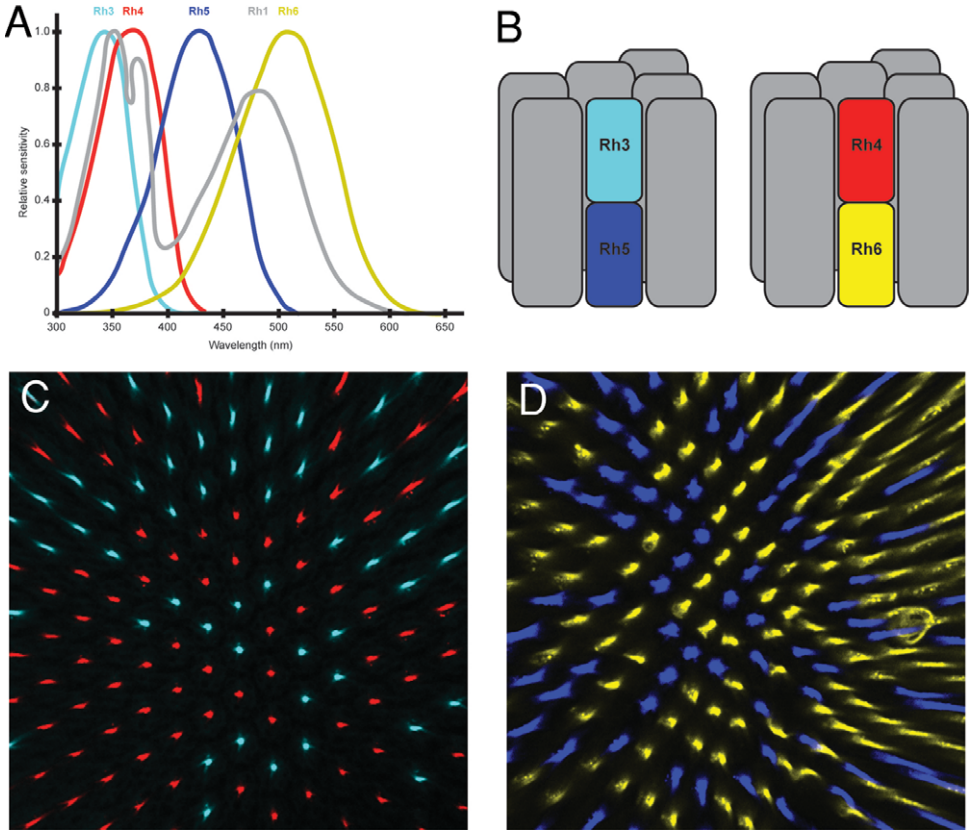

Plate 3.2 Rhodopsin diversity. (A) Spectral sensitivity of *Drosophila* rhodopsins (Modified from Yamaguchi et al., 2010). For full caption text see page [39]

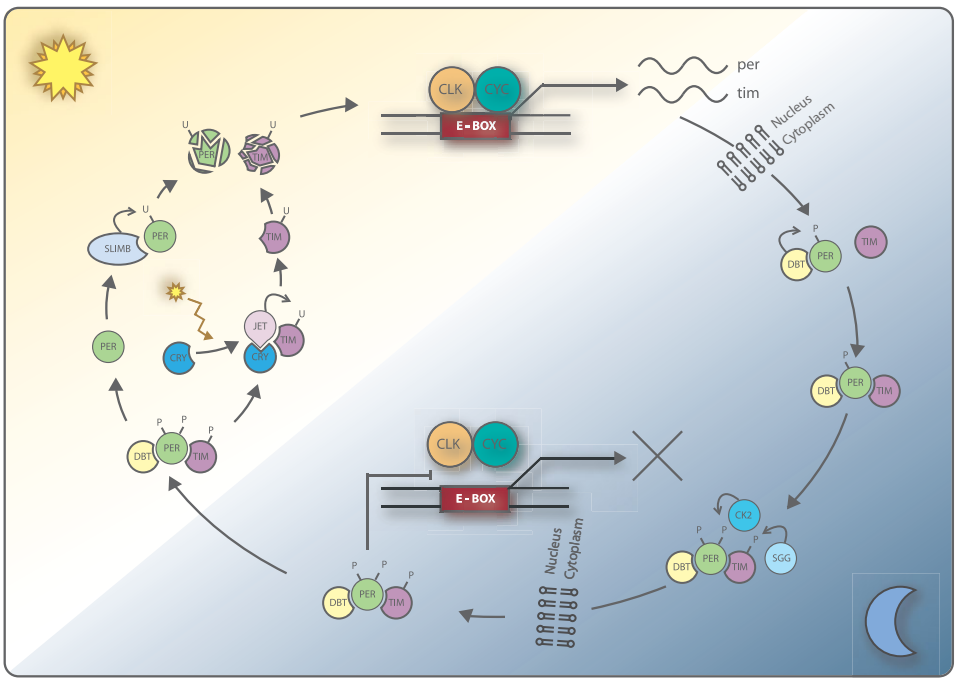

Plate 8.2 Schematic diagram of the molecular clock. For full caption text see page [106]

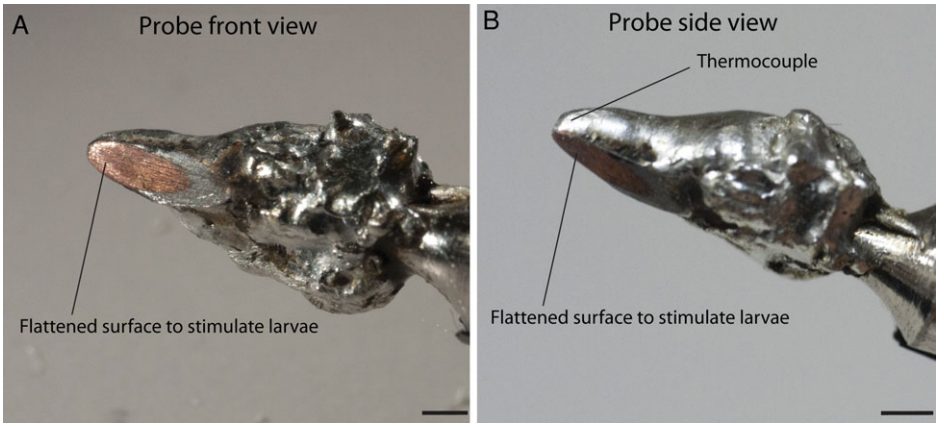

Plate 5.1 The thermal probe. For full caption text see page [72]

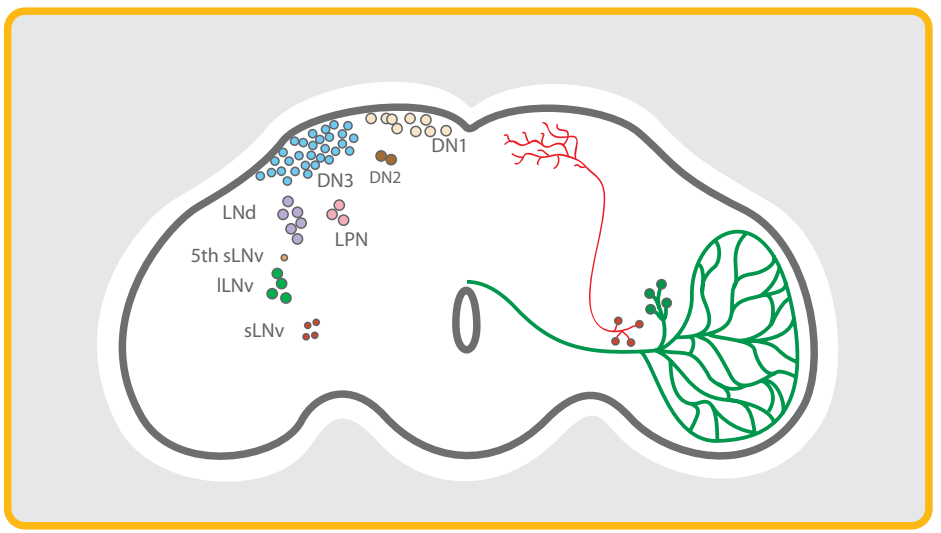

Plate 8.3 *Drosophila* clock neurons scheme. For full caption text see page [107]

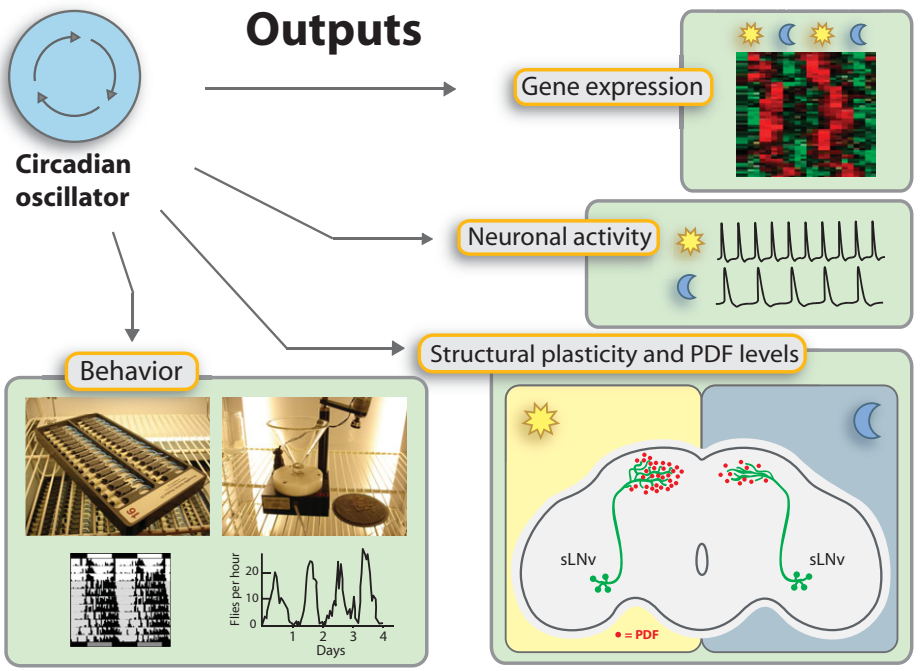

Plate 8.4 Circadian oscillator outputs. For full caption text see page [109]

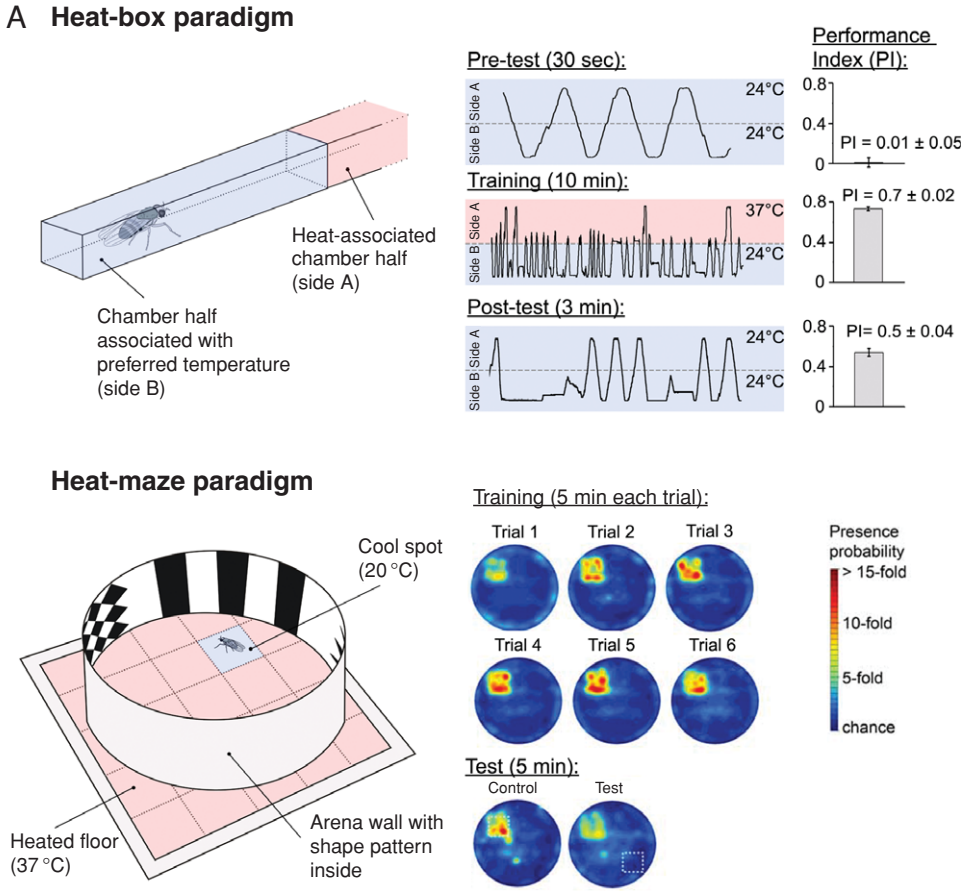

Plate 10.1 Behavioral paradigms for testing place memory. For full caption text see page [126]

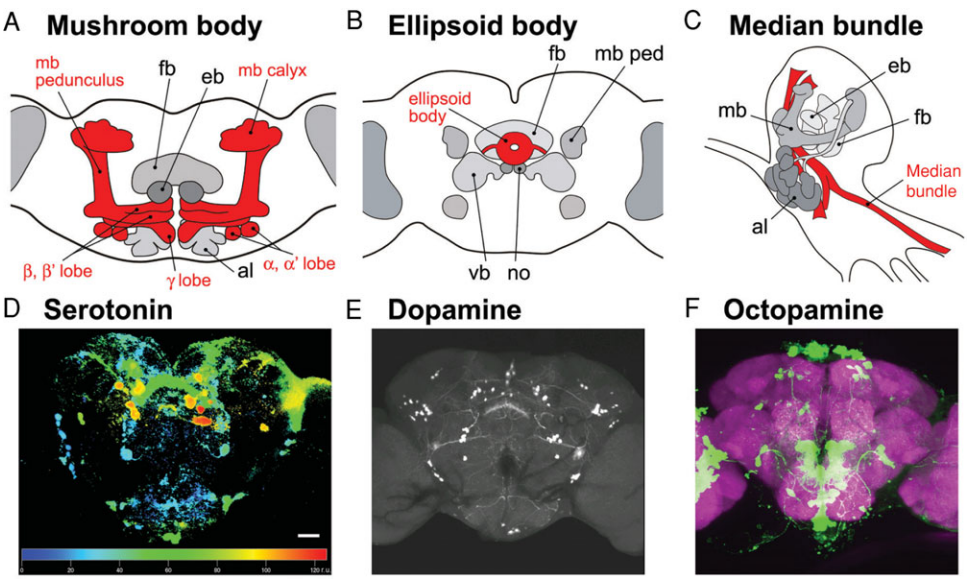

Plate 10.2 Organization of the fly brain. For full caption text see page [128]

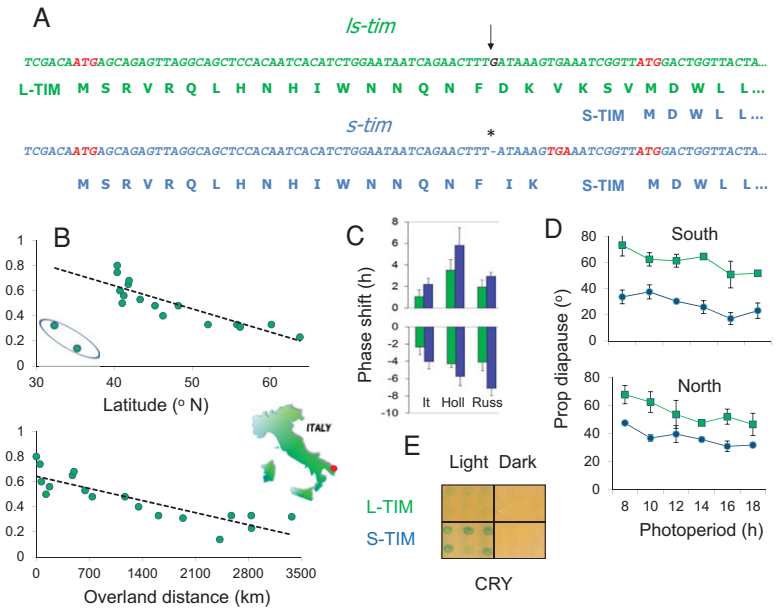

Fig 14.1 Functional implications of the timeless polymorphism. For full caption text see page [185]

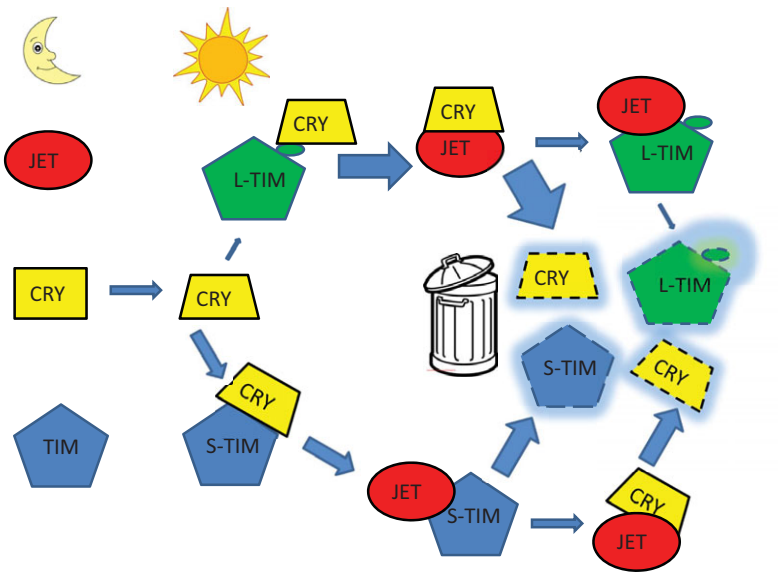

Plate 14.2 Model for L-TIM, S-TIM and CRY degradation by JET. For full caption text see page [187]

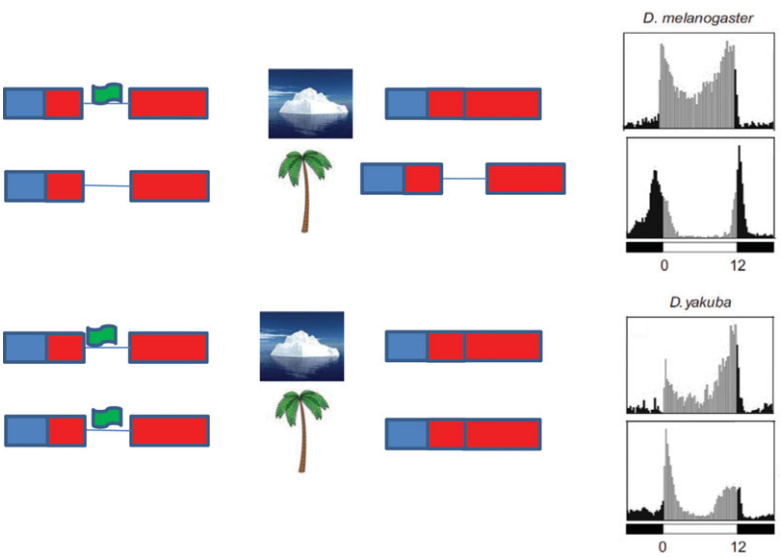

Fig 14.3 Interspecific per splicing of 3′UTR and the siesta. For full caption text see page [188]

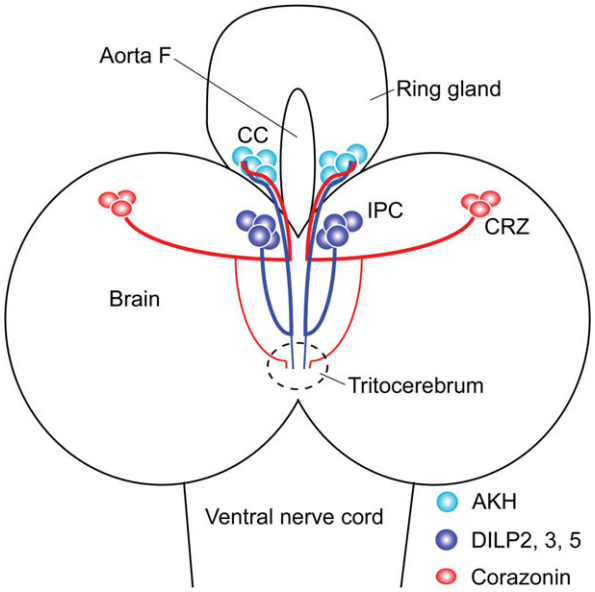

Plate 2.3 Insulin producing cell in the Drosophila brain. For full caption text see page [29]

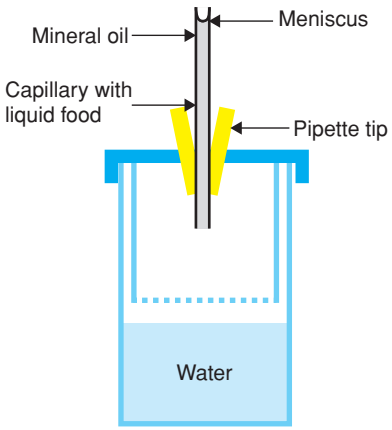

Plate 15.4 The capillary feeder (CAFE) assay delivers liquid food to the fly in a quantifiable manner. For full caption text see page [197]

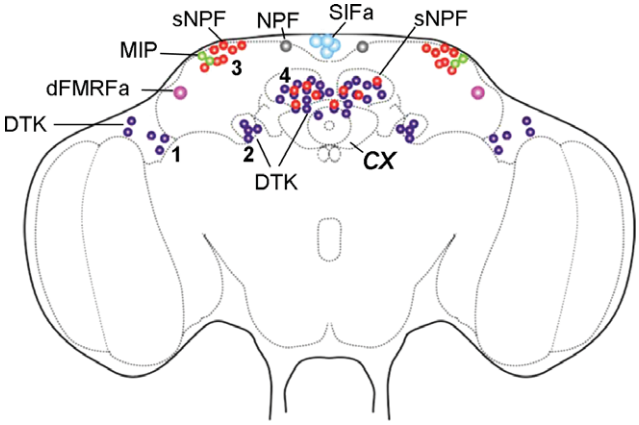

Plate 2.2 Peptidergic neurons innervating the central complex (CX). For full caption text see page [27]

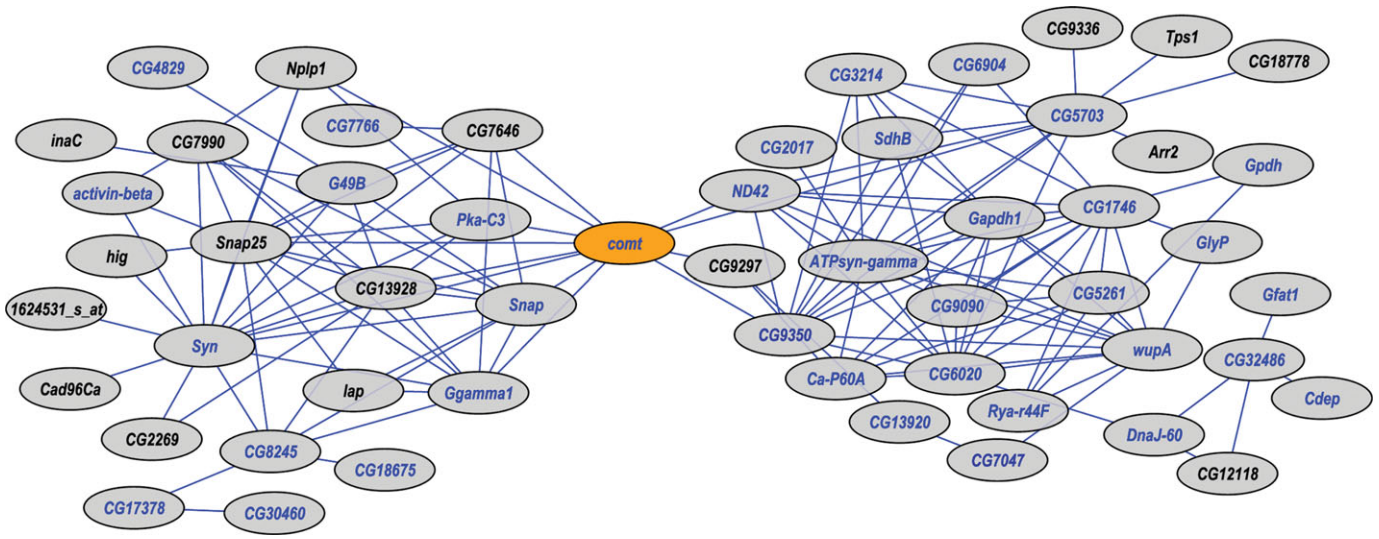

Plate 17.3 Highly connected gene clusters within a module associated with development of alcohol tolerance in *Drosophila*, identified by MMC. For full caption text see page [223]

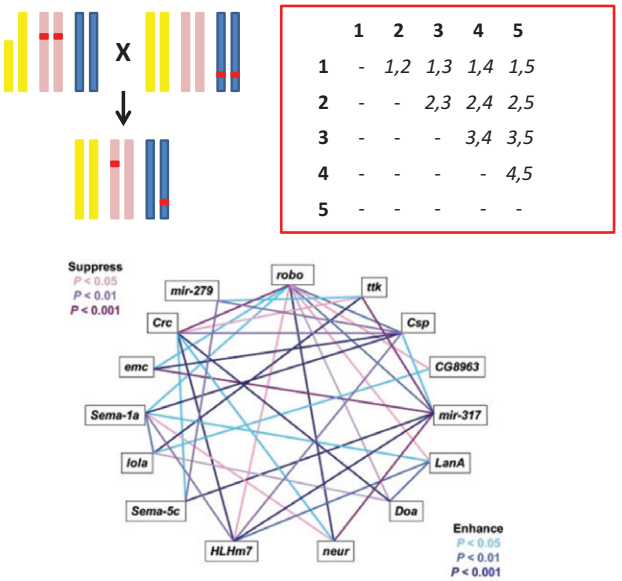

Plate 17.1 Building epistatic networks among co-isogenic P-element insertion lines. For full caption text see page [219]

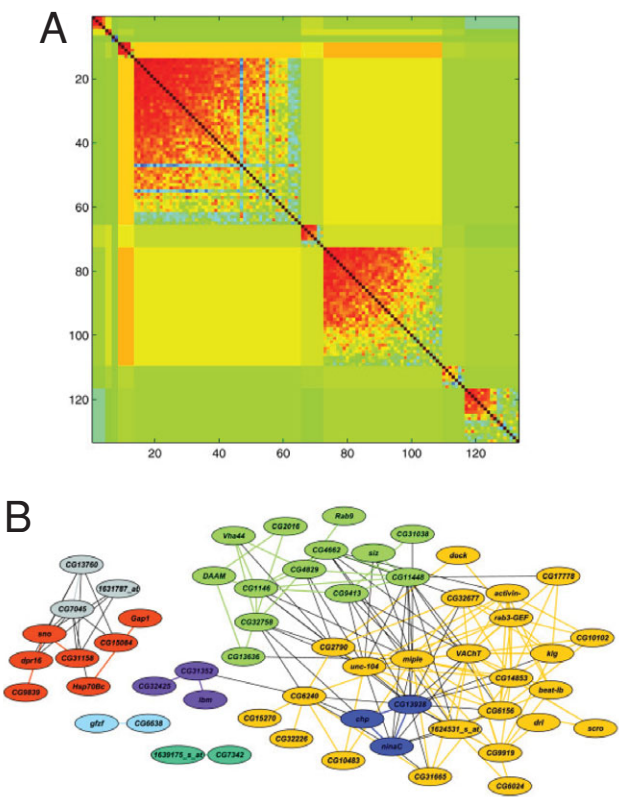

Plate 17.2 Modules of correlated transcripts associated with variation in aggressive behavior. For full caption text see page [222]

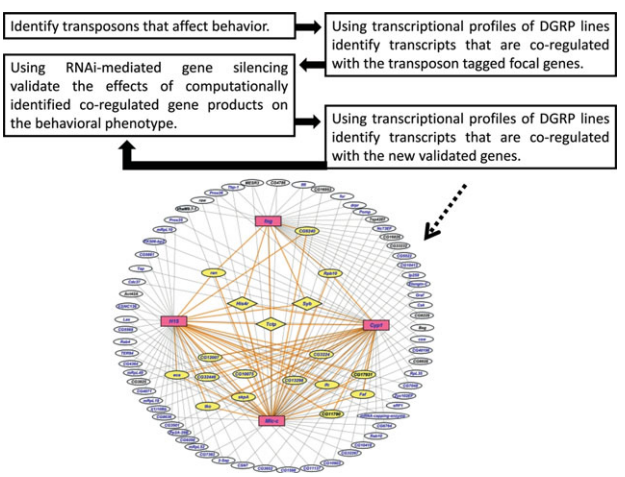

Plate 17.5 Combining P-element insertional mutagenesis and analysis of natural variation to build transcriptional networks of co-regulated transcripts. For full caption text see page [225]

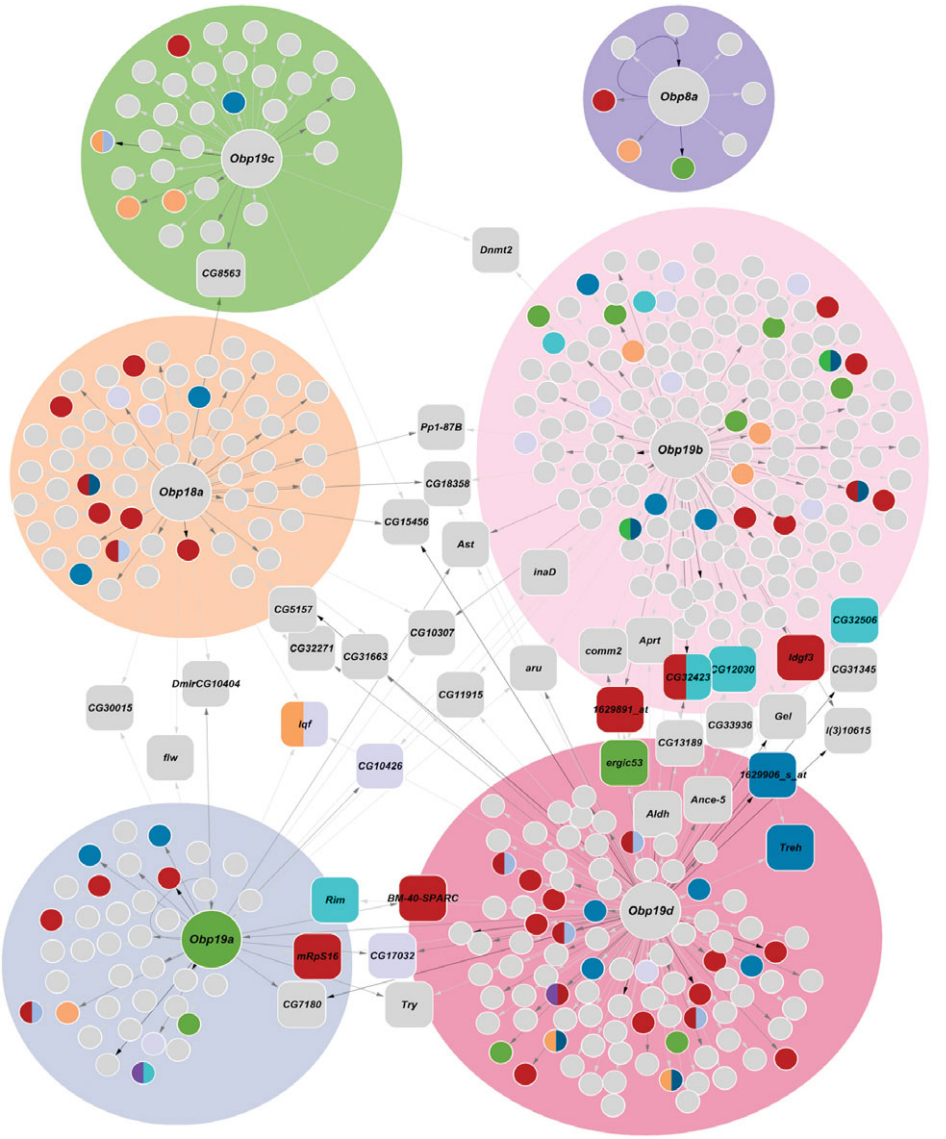

Plate 17.4 Diagrammatic representation of computationally derived transcriptional niches of six Obp genes located on the X-chromosome. For full caption text see page [224]

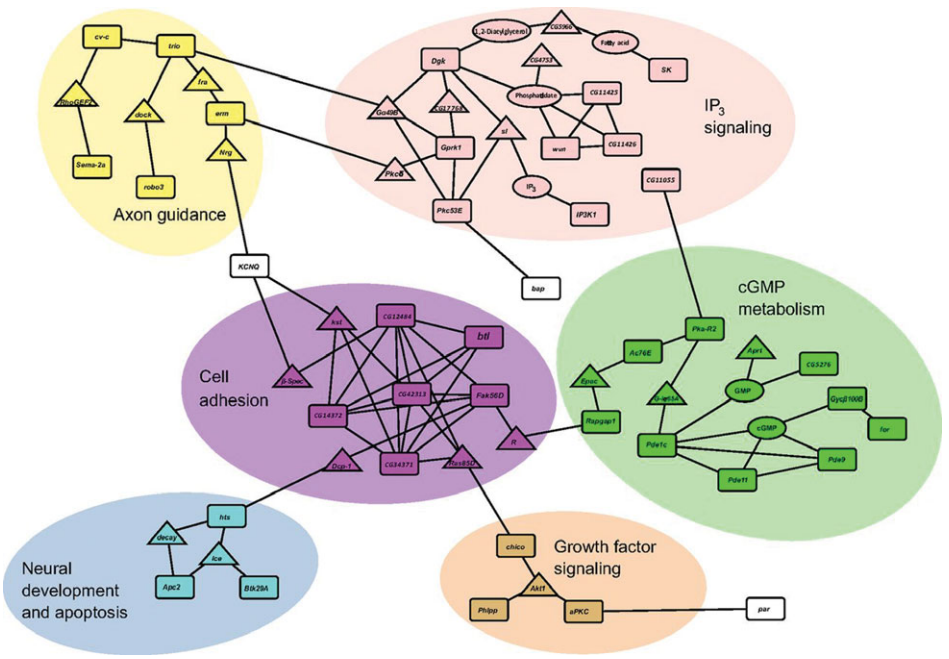

Plate 17.6 A cellular network identified by combined GWA, vGWA, and extreme QTL mapping analyses. For full caption text see page [227]

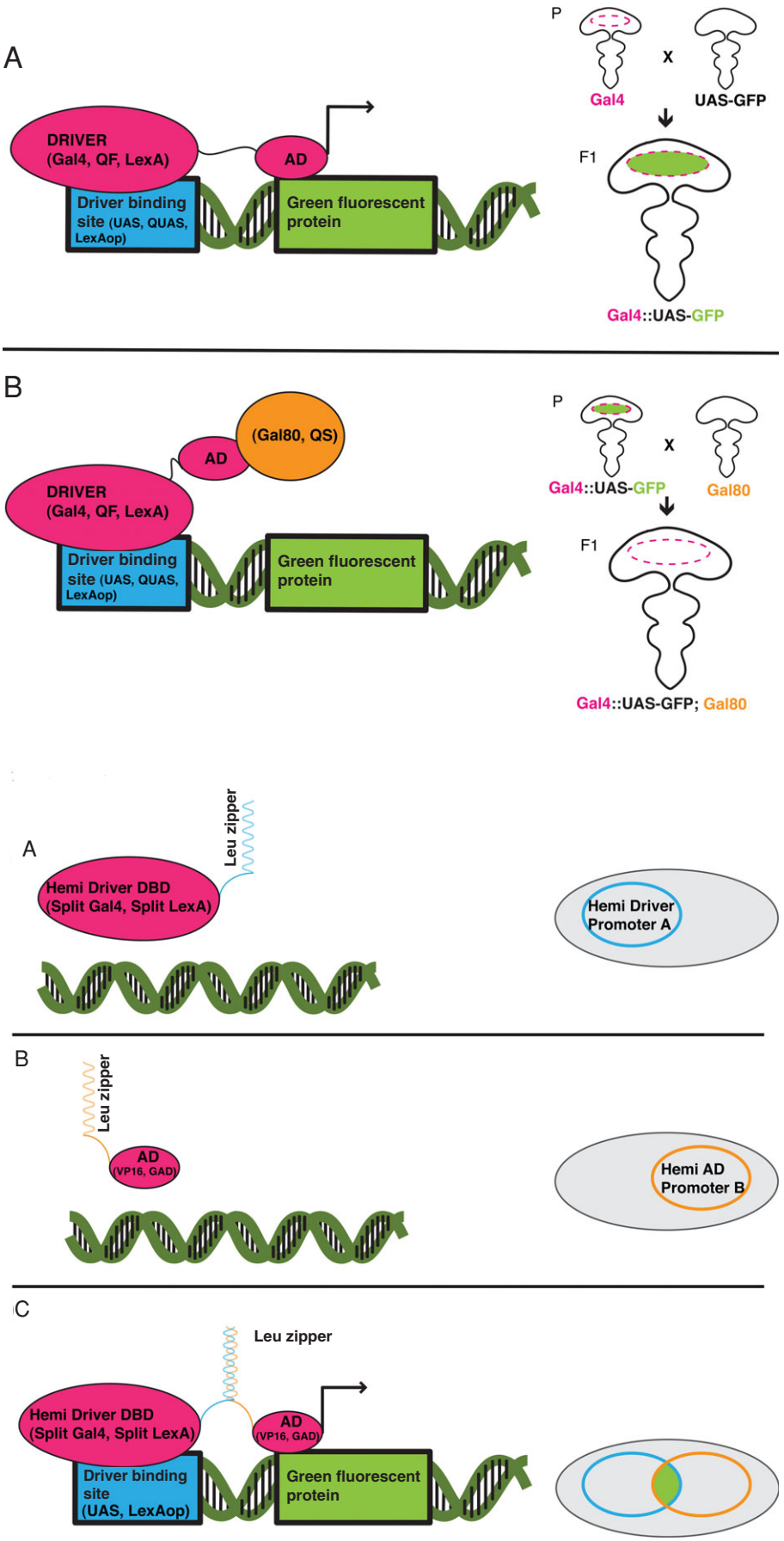

Plate 19.1 Binary expression systems commonly used in *Drosophila*. For full caption text see page [251]

Plate 19.2 The Split Gal4 system. For full caption text see page [255]

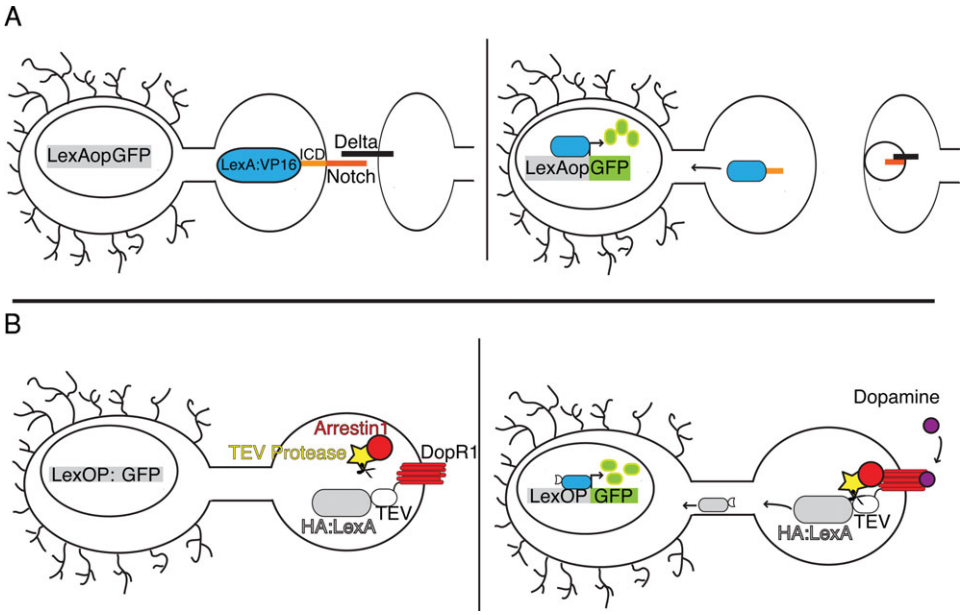

Plate 19.7 TANGO trans-synaptic labeling. For full caption text see page [260]

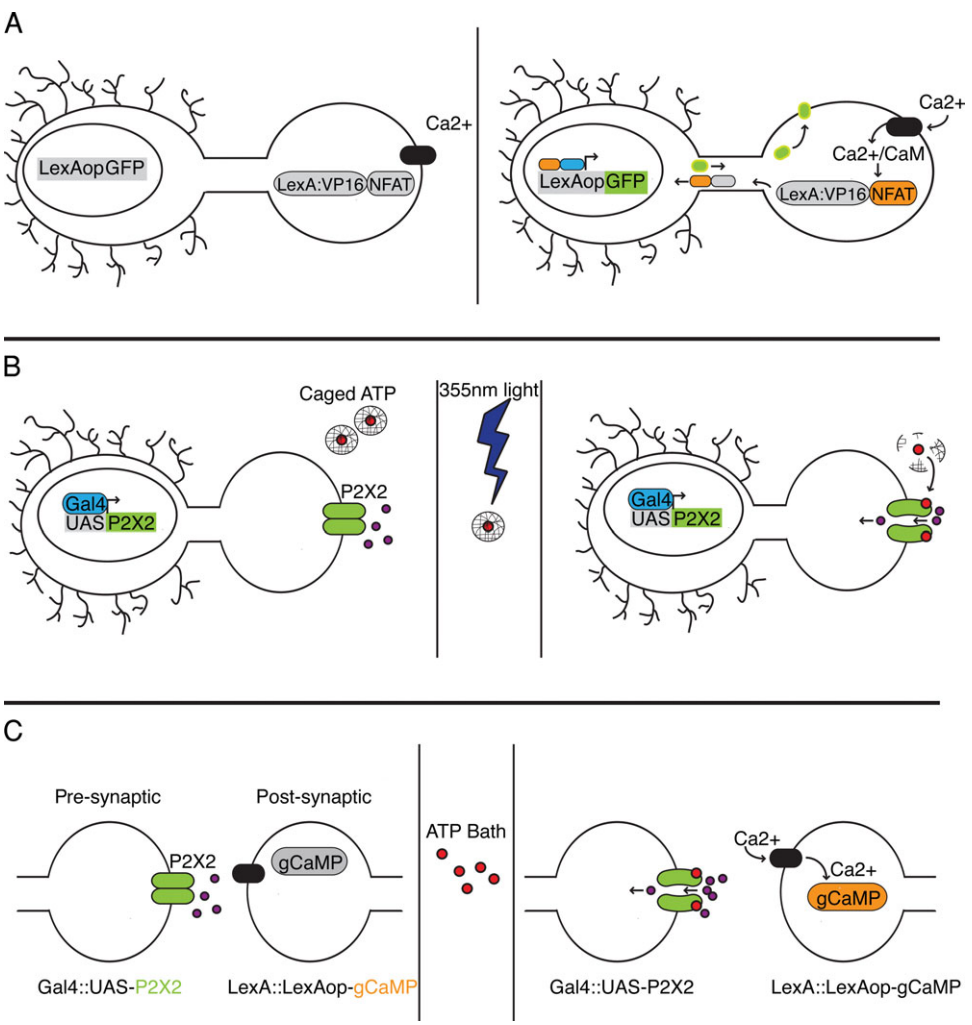

Plate 19.8 Activity-dependent trans-synaptic labeling or imaging. For full caption text see page [261]

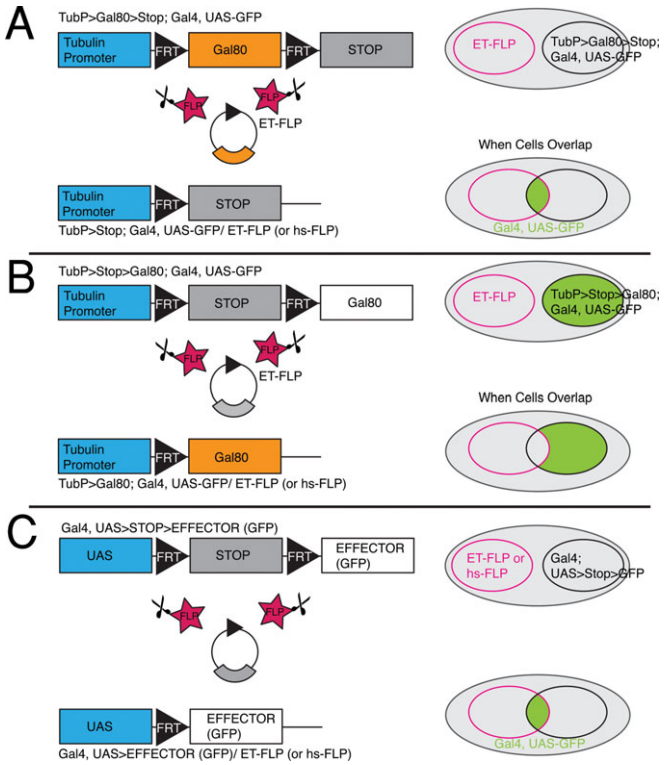

Plate 19.4 Enhancer-trap Flippase-based Gal80/Gal4 intersectional methods. For full caption text see page [257]

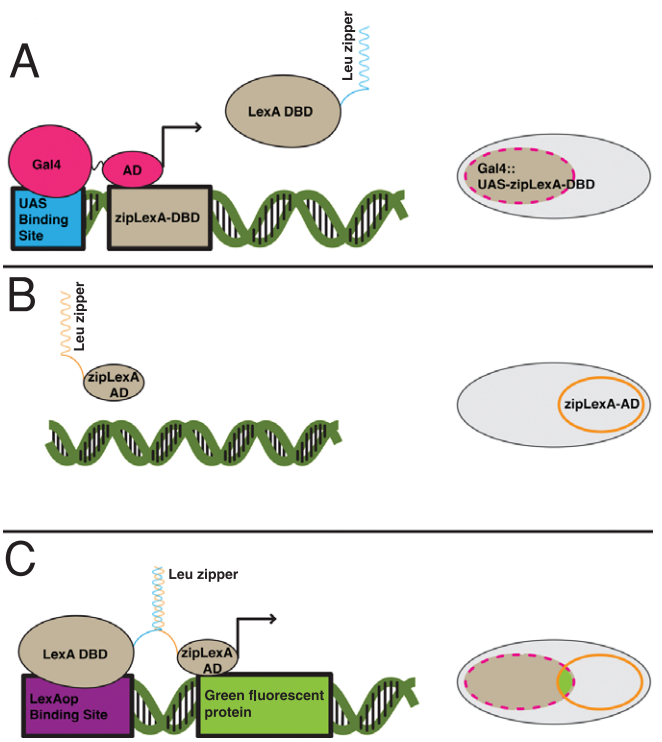

Plate 19.3 The Split LexA system coupled to Gal4. For full caption text see page [256]

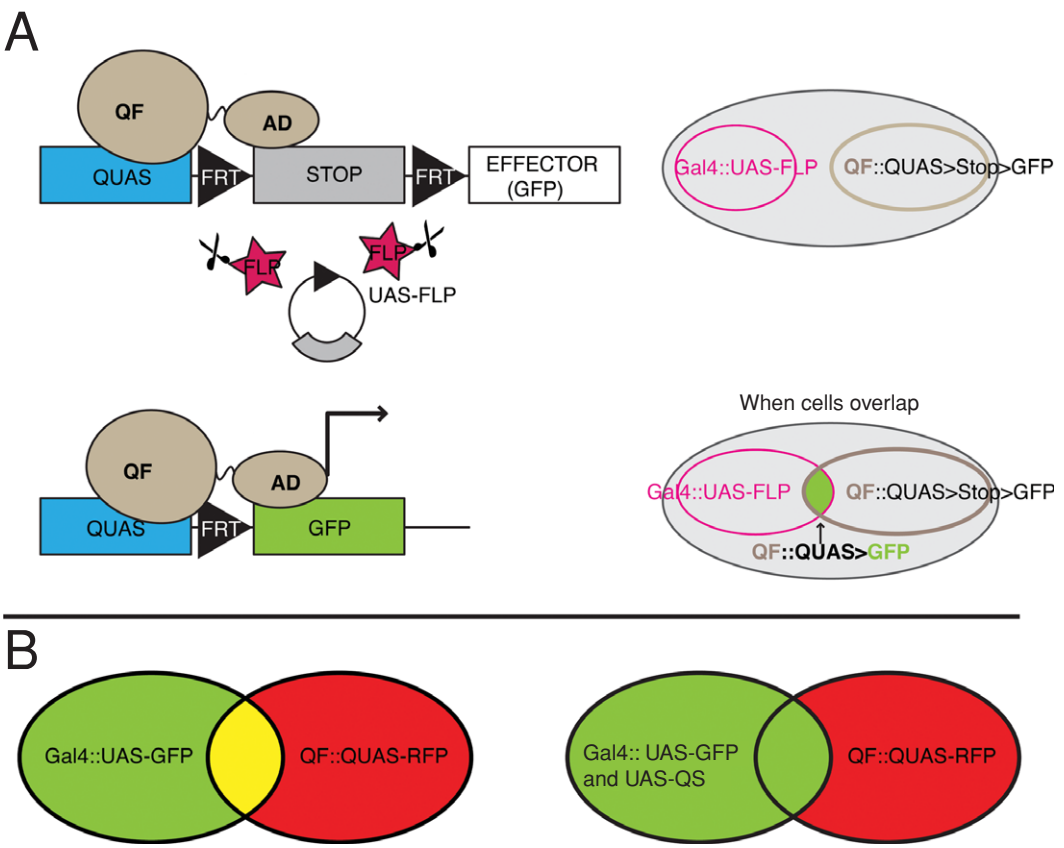

Plate 19.5 Logic gates of the Q system. For full caption text see page [259]

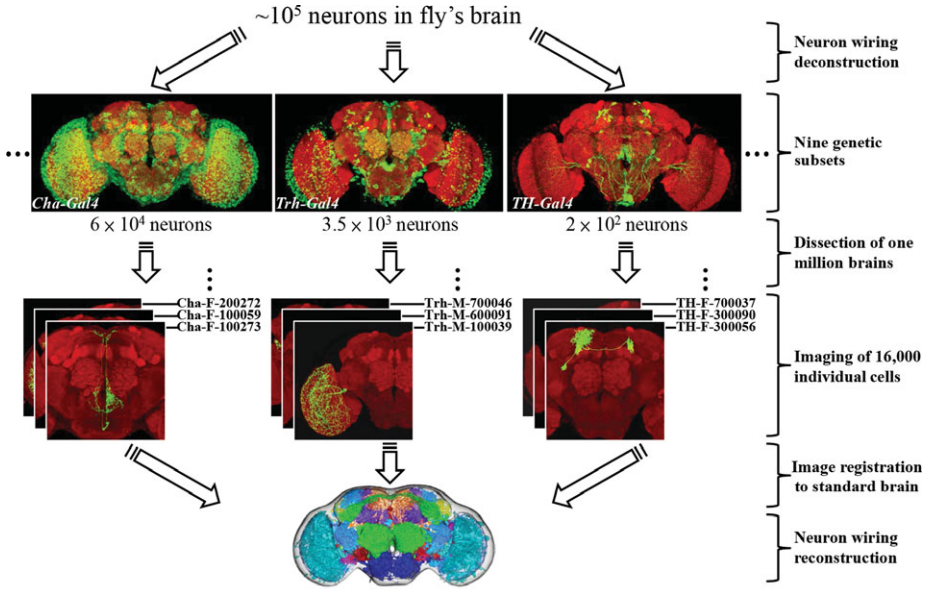

Plate 20.1 Deconstruction and reconstruction of the fly brain. For full caption text see page [269]

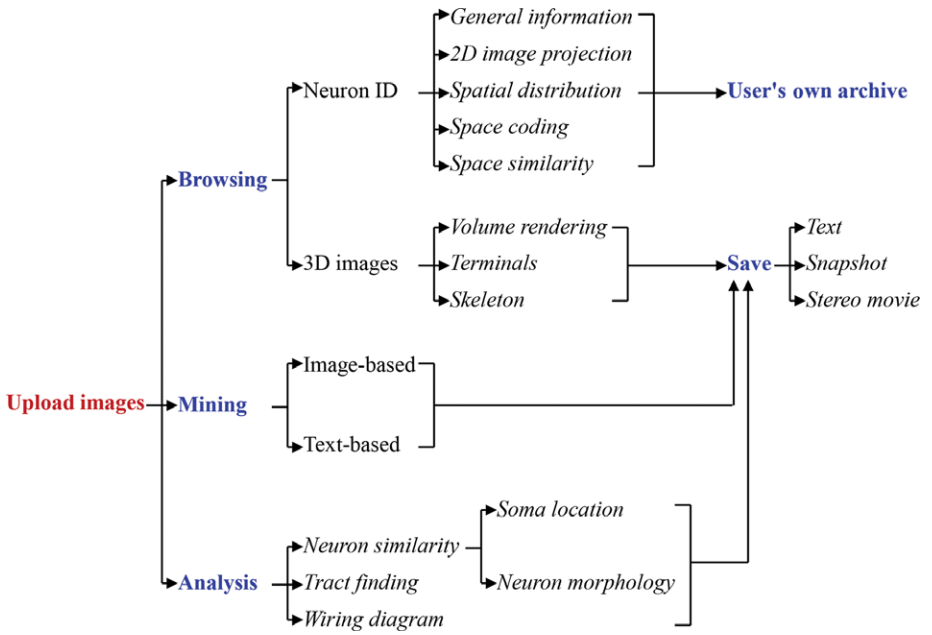

Plate 20.2 A road map of FlyCircuit tools.

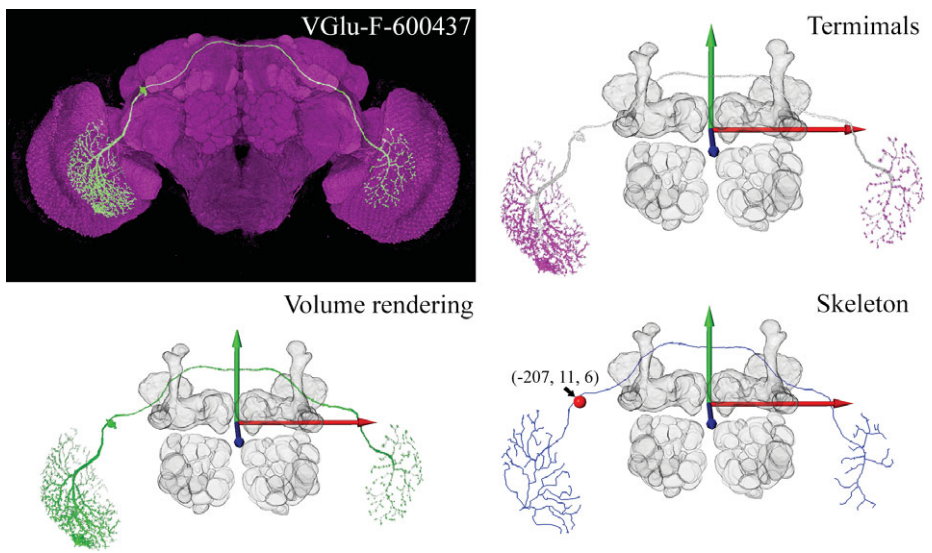

Plate 20.3 Visualizing a neuron-of-interest uploaded to FlyCircuit. For full caption text see page [271]

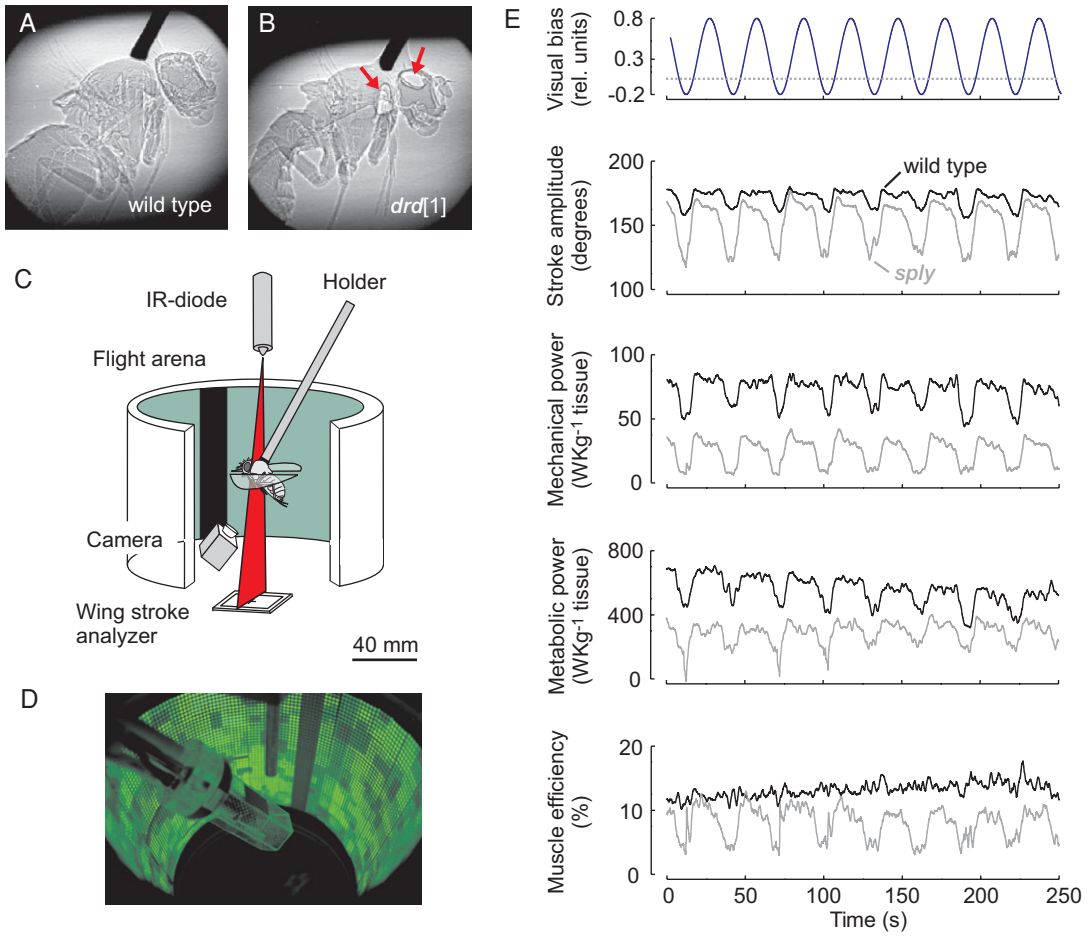

Plate 6.3 Behavioral essay for scoring wing motion and flight performance in tethered flight of *Drosophila*. For full caption text see page [84]

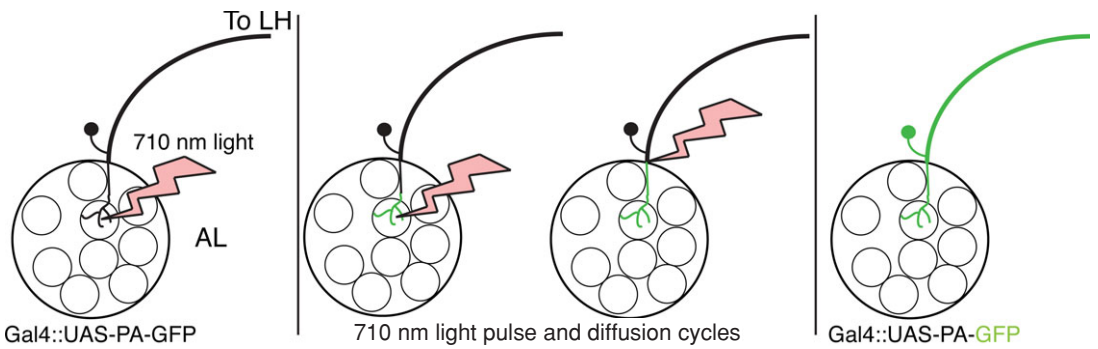

Plate 19.9 Neuronal Tracing using Photoactivatable GFP (PA-GFP). For full caption text see page [262]

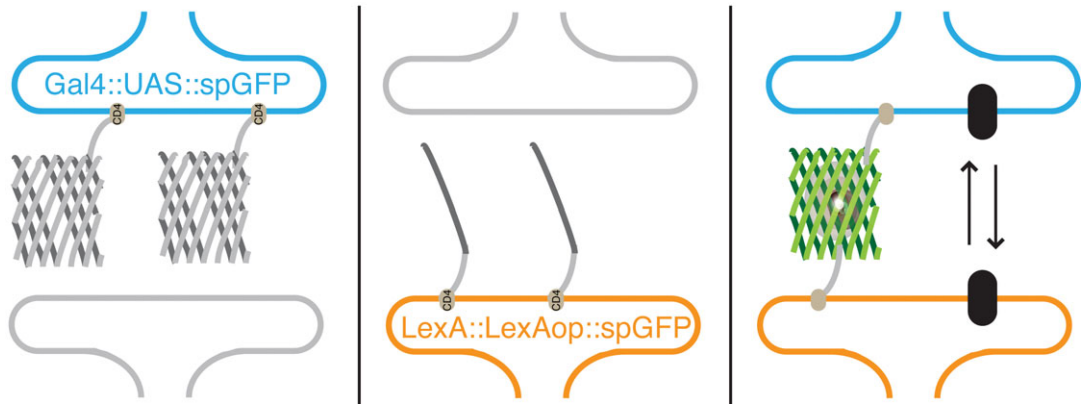

Plate 19.6 GRASP the trans-synaptic matchmaker. For full caption text see page [259]