Advances in Experimental Medicine and Biology 800

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Cellular and Molecular Control of Neuronal Migration

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Cellular and Molecular Control of Neuronal Migration

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ISSN 0065-2598 ISSN 2214-8019 (electronic)
ISBN 978-94-007-7686-9 ISBN 978-94-007-7687-6 (eBook)
DOI 10.1007/978-94-007-7687-6
Springer Dordrecht Heidelberg New York London

Library of Congress Control Number: 2013954377

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

The brain is the most intricate and fascinating organ of our body and its development requires the rigorous control of a multitude of molecular signaling and cellular events. Cell migration is one of these events and represents a fundamental cornerstone of brain development, and homeostasis in the mature brain. The project of writing this book was intended to extend the science and lively discussions beyond a symposium devoted to neuronal migration that we organized at the 8th Federation of European Neuroscience Societies (FENS) meeting held in Barcelona, on July 15, 2012. We decided to set up a collection of individual chapters written by leaders in the field of neuronal migration and covering various areas and structures of the nervous system, ranging from the cerebral cortex to the spinal cord, the cerebellum, and the postnatal rostral migratory stream and olfactory bulb. The prime motivation for writing and editing this work was to provide a clear overview of the distinct and overlapping molecular signaling pathways and cellular principles of neuronal migration in several brain structures during development and after birth. With this consolidated illustration and detailed discussion of the mechanisms controlling neuronal migration, we hope to provide a useful and focally up to date resource to the community. Besides, each chapter raises novel important questions and provides perspectives for future research on neuronal migration. Despite tremendous efforts, many outstanding questions still remain and need to be addressed since numerous diseases are caused by disruption of neuronal migration during early development of the brain. Although a substantial catalogue of signaling pathways and susceptibility genes has been compiled and implicated in the pathology of neuronal migration disorders, the precise function of most of these genes in the sequential steps of neuronal migration remains elusive. Thus, we also strive to stimulate future research which should have the goal to provide a clear conceptual framework at the molecular and cellular level in order to understand the underlying basis of neurodevelopmental migration disorders. In all the chapters, we intended to govern most recent questions and novel methodological approaches used in the field. We are very grateful to each and every author for their scientific contribution to this book and wish to thank Martijn Roelandse at Springer-Verlag for his patience and support of this project.

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Molecular Pathways Controlling the Sequential Steps of Cortical Projection Neuron Migration

1

Simon Hippenmeyer

Abstract

Coordinated migration of newly-born neurons to their target territories is essential for correct neuronal circuit assembly in the developing brain. Although a cohort of signaling pathways has been implicated in the regulation of cortical projection neuron migration, the precise molecular mechanisms and how a balanced interplay of cell-autonomous and non-autonomous functions of candidate signaling molecules controls the discrete steps in the migration process, are just being revealed. In this chapter, I will focally review recent advances that improved our understanding of the cell-autonomous and possible cell-nonautonomous functions of the evolutionarily conserved LIS1/NDEL1-complex in regulating the sequential steps of cortical projection neuron migration. I will then elaborate on the emerging concept that the Reelin signaling pathway, acts exactly at precise stages in the course of cortical projection neuron migration. Lastly, I will discuss how finely tuned transcriptional programs and downstream effectors govern particular aspects in driving radial migration at discrete stages and how they regulate the precise positioning of cortical projection neurons in the developing cerebral cortex.

Keywords

Neuronal migration • Cortex development • *Lis1* • *Ndel1* • Reelin • MADM • Transcriptional regulation

1 Introduction

The cerebral cortex is the largest structure of the human brain, composed of a sophisticated network of billions of excitatory projection neurons and inhibitory interneurons. The assembly of functional cortical circuits requires the synchronized segregation and interconnection of the

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L. Nguyen and S. Hippenmeyer (eds.), *Cellular and Molecular Control of Neuronal Migration*, Advances in Experimental Medicine and Biology 800, DOI 10.1007/978-94-007-7687-6_1,

distinct classes of cortical neurons. The most prevalent installation theme of cortical neurons is their coalescence into stratified laminae (Cajal 1911). The mature cortical cytoarchitecture consists of six distinct layers with different cellular composition and function as a result of eminent developmental processes. As such, precisely coordinated migration of both projection and interneurons from their respective birth places to their final target areas in the developing embryo, is essential to achieve appropriate lamination and subsequent circuit formation in the cerebral cortex. While cortical interneurons are born in the ventrally located ganglionic eminences and migrate along distant tangential routes (Batista-Brito and Fishell 2009; Faux et al. 2012; Marin 2013; Wonders and Anderson 2006), cortical projection neurons are generated within the ventricular (VZ) and subventricular (SVZ) zones in the dorsal telencephalon (Fietz and Huttner 2011; Franco and Muller 2013; LaMonica et al. 2012; Lehtinen et al. 2011; Lui et al. 2011) and migrate along radial trajectories to reach their final settling position (Ayala et al. 2007; Marin et al. 2010). The laminar positioning of cortical projection neurons is one of the best described modes of radial neuronal migration in the mammalian brain. Cortical layering occurs in an 'inside-out' fashion whereby earlier born neurons populate deep layers and later born neurons occupy progressively upper layers (Fig. 1.1) (Angevine and Sidman 1961; McConnell 1995; Polleux et al. 1997; Rakic 1974, 2007). Thus, as part of a holistic developmental program, the sequential generation of faithful cell fates and concerted migration to correct laminae is critical for the assembly of the cortex. Sophisticated live-imaging approaches have revealed that radial projection neuron migration occurs in discrete phases (Kriegstein and Noctor 2004; Nadarajah et al. 2003; Tabata and Nakajima 2003). Moreover, the cortical projection neurons need to migrate across several distinct compartments from their birthplace in the ventrally located VZ/SVZ through the relatively less cell dense intermediate zone (IZ) and into their target zone, the developing cortical plate (CP). How this journey through different cellular environments is orchestrated, and the regulatory cues coordinating the execution of the *specific sequential steps* of radial migration from the VZ/SVZ through the IZ to the CP *in vivo*, are still mostly unclear. Nonetheless, in the last decades an impressive catalog of signaling pathways has been compiled, and described to play fundamental roles in cortical neuron migration (Ayala et al. 2007; Heng et al. 2010; LoTurco and Bai 2006; Marin et al. 2010), but the functional relationships between these molecular cues and the discrete steps of cortical neuron migration are still mostly enigmatic. Thus, the precise cellular and molecular mechanisms regulating each and every step during radial migration remain inexplicit although it is conceivable that many extrinsic cues and intrinsic signaling pathways impinge on the cytoskeleton [comprehensively discussed in the literature (Govek et al. 2011; Heng et al. 2010; Kawauchi and Hoshino 2008)] to orchestrate the cellular and subcellular events required in migrating neurons travelling through the distinct compartments in the developing cortical wall. I will focus in this chapter on recent advances that led to fundamental new insights into how a balanced interplay of cell-autonomous and non-autonomous functions of candidate signaling molecules precisely regulates the sequential stages of radial migration. The role of the LIS1/NDEL1-complex, the function of the Reelin signaling pathway and how specific transcriptional programs govern discrete steps in cortical projection neuron migration will be discussed in detail.

2 The Sequential Steps of Cortical Projection Neuron Migration

Nascent cortical projection neurons migrate in a step-wise fashion from their birth place in the VZ/SVZ through the intermediate (IZ) zone in order to reach the cortical plate (CP) and settle at appropriate positions to build up the six cortical layers (Ayala et al. 2007; Marin et al. 2010). While in the rodent the migration paths are still in the range of a few hundred microns, in the developing human cerebral cortex, radially

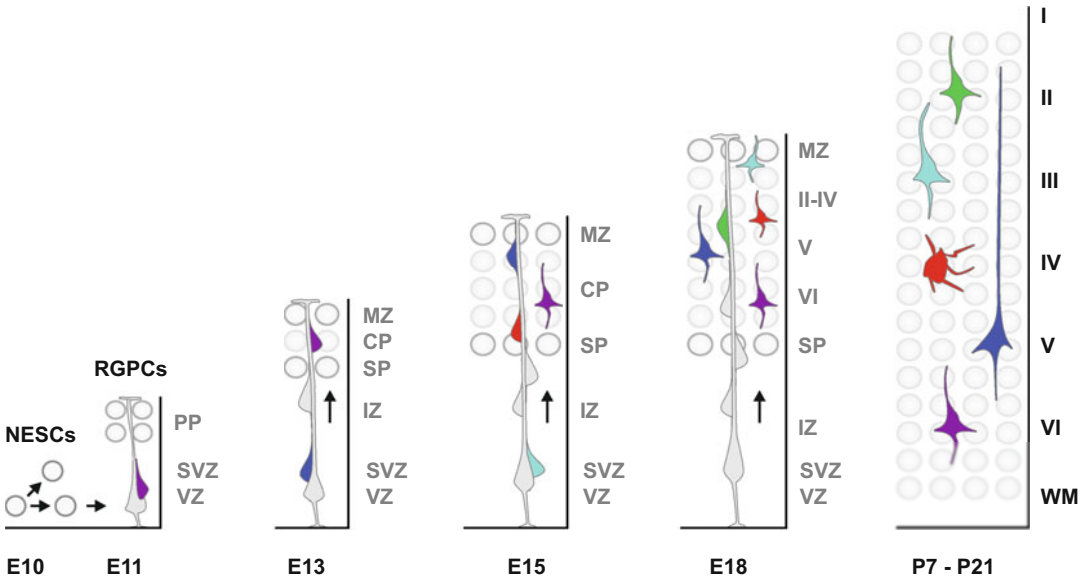

Fig. 1.1 Developmental sequence of inside-out cortical layer assembly. During cortical layer formation, earlier born neurons occupy lower layers and later born neurons progressively occupy upper layers. In the developing mouse cortex, NESCs initially divide mostly symmetrically in the VZ to expand the stem cell pool. The first asymmetric divisions produce cells that occupy the PP. Subsequently, the earliest neurons (*purple*, future layer VI) forming the CP, split the PP into the SP and MZ, and settle at appropriate positions. The next wave of neurons (*dark blue*, future layer V), migrate from the VZ/SVZ through the IZ and settle in the CP above the previously generated neurons (*purple*, layer VI). This developmental sequence is repeated for the *red* (layer IV), *cyan* (layer III) and *green* (layer II) neurons until all cortical layers have been established. During the first three postnatal weeks, a phase of consolidation takes place where cortical neurons finish axon and dendrite genesis, form synaptic connections and assemble into microcircuits. Abbreviations: *VZ* ventricular zone, *SVZ* subventricular zone, *PP* preplate, *SP* subplate, *MZ* marginal zone, *IZ* intermediate zone, *CP* cortical plate, *WM* white matter, *I-VI* cortical layers 1–6, *NESCs* neuroepithelial stem cells, *RGPCs* radial glia progenitor cells

migrating neurons travel approximately 2 cm (Bystron et al. 2008; Meyer 2007; Molnar et al. 2006). Migration routes across the developing cortical wall can be complex (Kriegstein and Noctor 2004; Nadarajah et al. 2003; Tabata and Nakajima 2003) and it has become increasingly clear that radial migration of cortical projection neurons occurs in a tightly regulated manner. Time-lapse and videomicroscopy approaches (Noctor 2011; Tabata and Nakajima 2008; Tsai and Vallee 2011) with the goal to trace the migration paths of individual cortical projection neurons have revealed that (1) radially migrating neurons proceed though several distinct migratory phases; (2) change their morphology along the way and (3) adjust their mode of migration while transiting through the different zones along the radial migratory path (Nadarajah et al. 2001; Noctor et al. 2004; Sekine et al. 2011; Tabata and Nakajima 2003; Tsai et al. 2005).

At early stages of corticogenesis, the migration distances for newly-born neurons are still short but as development continuously progresses, nascent neurons migrate along progressively longer distances. Therefore the patterns of neuronal migration are somewhat different during early versus late corticogenesis (Nadarajah and Parnavelas 2002). The earliest postmitotic neurons delaminate and migrate away from the ventricular surface primarily by somal translocation – by pulling up the soma in the vertical direction with a process stably attached to the pial surface – and form the transient preplate (PP) structure (Allendoerfer and Shatz 1994; Nadarajah et al. 2001; Price et al. 1997; Super et al. 1998). The next wave of postmitotic neurons moves toward the pial surface and

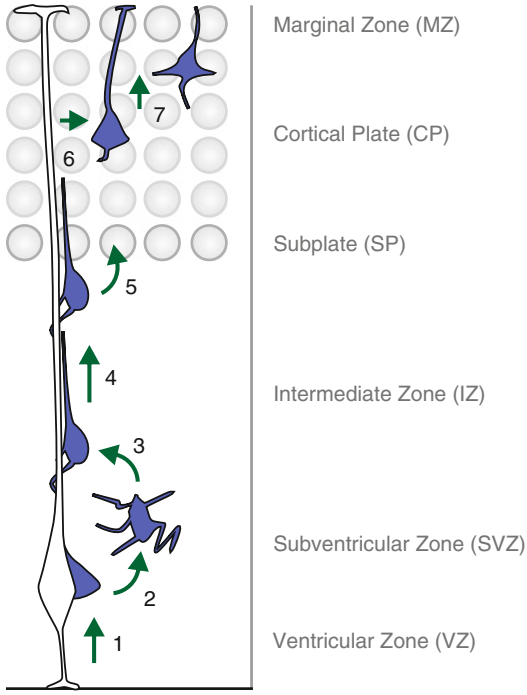

Fig. 1.2 The sequential steps of cortical projection neuron migration. Recent live-imaging experiments (see main text for details) have traced the path of migrating cortical projection neurons and revealed multiple discrete steps and phases along the entire migration journey: (*1*) nascent neurons delaminate from the ventricular surface in the VZ and move to the SVZ where they adopt a multipolar configuration (*2*). Upon multi-to-bipolar transition (*3*), neurons engage in locomotion along the radial glia fiber (*4*) until they reach the SP, enter the CP target area (*5*) and continue to migrate towards the MZ. Once the cortical projection neurons have reached the uppermost area of the CP, they detach from the radial glia fiber (*6*) and execute terminal somal translocation (*7*) to conclude the radial migration and settle in their appropriate position

splits the PP into the marginal zone (MZ) and the subplate (SP), thus establishing the first neuronal alignment as cortical plate (CP). The CP expands in the vertical direction as consecutive waves of neurons contribute successively to all the layers VI-II in an inside out fashion (Fig. 1.1) (Angevine and Sidman 1961; McConnell 1995; Polleux et al. 1997; Rakic 1974).

During the stages of progressive CP aggregation, nascent cortical projection neurons generated in the proliferative VZ undergo a series of sequential migration steps until they reach their final destination in the CP (Fig. 1.2). First, neurally committed cells delaminate/detach from the neuroepithelium at the ventricular surface (Itoh et al. 2013) and move radially away to the SVZ. Within the SVZ neurons 'sojourn' for about 24 h or longer and most adopt a multipolar morphology, extending and retracting processes in all directions (Noctor et al. 2004; Tabata and Nakajima 2003). During this phase, multipolar neurons tend to migrate tangentially in an apparent random fashion (Jossin and Cooper 2011; Noctor et al. 2004) while critical signaling cues induce polarization to predetermine the future axon of the neuron (Barnes and Polleux 2009) (see also Chap. 6). A substantial fraction but not all neurons move retrogradely back towards the VZ before they transform their shape, take on their bipolar morphology and enter the next phase in their migration journey (Noctor et al. 2004). Subsequently, migrating neurons with bipolar morphology locomote along radial glial fibers (Nadarajah et al. 2001; Noctor et al. 2004; Rakic 1972). During this stage of cortical projection neuron migration, the radial movement proceeds in a saltatory fashion by leaps rather than gradual transitions. Cortical projection neurons in the locomotion mode repeat several basic events which underlie their progressive advancement: (1) rapid extension and retraction of the leading neurite which protrudes several dozens of microns from the soma; (2) formation of one or more unsteady swellings/dilatations of the plasma membrane in the leading process; (3) advancement of the centrosome towards and/or into the swelling; (4) forward displacement of the nucleus and soma, a process also known as nucleokinesis; and (5) retraction of the trailing cytoplasmic region and basal process which usually occurs concurrently with nuclear translocation in step 4 (Schaar and McConnell 2005; Tsai and Gleeson 2005; Vallee et al. 2009) (see also Chaps. 2, 4 and 7). Neurons in locomotion mode travel through the IZ until they reach their appropriate target area, the CP. Below the CP, radially migrating neurons have to first pass the SP and then need to invade the earlier generated neurons that occupy the deepest cortical layers. Since the environment of the CP differs significantly from the one in the IZ, especially regarding the density

and arrangement of neurons, it is conceivable that the transition from the IZ into the CP must be tightly regulated. The regulatory mechanisms that control the passage of locomoting neurons from the IZ into the CP are however mostly unknown but may involve instructive or simply permissive guidance cues that could act as a gatekeeper for cortical projection neurons to enter their prospective CP target zone. In a next step, neurons then move within the CP further towards the outermost MZ. Once the leading edge of the apical neurite of the locomoting neuron reaches the MZ, the soma with the nucleus rapidly moves up to the top of the CP while the tip of the apical process remains stably attached to the MZ (Nadarajah et al. 2001; Sekine et al. 2011). This last step – terminal somal translocation – is critical for migrating cortical projection neurons to move past all their predecessors in order to ensure appropriate establishment of the inside out lamination within the CP. Recent observations indicate that the uppermost part of the CP comprises of distinct features than the lower CP and was termed primitive cortical zone (PCZ) (Sekine et al. 2011). Interestingly, time-lapse analyses revealed that locomoting neurons seem to transiently pause just below the PCZ and switch at this stage into the terminal translocation mode (Sekine et al. 2011). Once cortical projection neurons have reached their final destination they complete their global differentiation program, finish axon and dendrite genesis, commence synaptogenesis and assemble into microcircuits.

3 Role of the LIS1/NDEL1-Complex in the Regulation of Discrete Steps During Cortical Neuron Migration

The importance for appropriate execution of the migration program in cortical projection neurons during brain development is highlighted in patients that suffer from neuronal migration disorders, such as isolated lissencephaly sequence (ILS) or Miller-Diecker Syndrome (MDS) (Dobyns and Truwit 1995; Gleeson and Walsh 2000; Guerrini and Parrini 2010; Pilz and Quarrell 1996; Ross and Walsh 2001). Lissencephaly is characterized by smooth brain surface, abnormal brain morphology and function; and lissencephaly patients suffer from mental retardation and epilepsy. About 40 % of ILS and virtually 100 % of MDS cases occur due to the loss of one copy of the gene called *Lissencephaly-1* (*LIS1, also known as PAFAH1B1*) on human chromosome 17 (Cardoso et al. 2003; Lo Nigro et al. 1997; Pilz et al. 1998; Reiner et al. 1993). Lissencephaly is thus autosomal dominant but the concrete underlying basis of the clinical symptoms, and the mechanisms by which a reduction of LIS1 protein results in human lissencephaly are not well understood. It is however evident that reduced levels of LIS1 negatively affect radial neuron migration (Wynshaw-Boris 2007). Interestingly, it has been documented that increased expression of *LIS1* in the developing brain also leads to brain abnormalities in mice and human (Bi et al. 2009). Below, I will first describe the LIS1 protein-interactome and then elaborate on its function in the sequential steps of cortical projection neuron migration *in vivo*.

3.1 The LIS1/NDEL1-Complex and Its Interacting Partners

The LIS1 protein is evolutionarily conserved from yeast to man and was first identified as one of the nuclear distribution mutants (*NudF – for nuclear distribution F*) in the filamentous fungus *Aspergillus nidulans* (Morris 2000; Xiang et al. 1995) amongst other mutants displaying also defective nuclear distribution including *NudA*, *NudC* and *NudE* (Efimov and Morris 2000; Xiang et al. 1999). Orthologues of *Lis1* have been identified and cloned in many organisms including *Drosophila* (Liu et al. 2000) and mice (Hirotsune et al. 1997) whereby the murine LIS1 protein differs in only one aminoacid from the human version (Hirotsune et al. 1998; Reiner et al. 1993). In higher eukaryotes, LIS1 interacts with cytoplasmic dynein (*NudA* orthologue in *A. nidulans*) (Faulkner et al. 2000; Sasaki et al. 2000; Smith et al. 2000), which is critically involved in subcellular transport and directed

cell movement (Kardon and Vale 2009). *Lis1* interacts also with the two *Nde1* and *Ndel1* – formerly known a *NudE* and *NudEL* – paralogues that are homologous to *A. nidulans NudE* (Efimov and Morris 2000; Feng et al. 2000; Kitagawa et al. 2000; Niethammer et al. 2000; Sasaki et al. 2000; Smith et al. 2000). Structural analysis have revealed that both LIS1 and NDEL1 form homodimers (NDEL1 also tetramers) and that dimeric forms of NDEL1 are the principal configurations that interact with LIS1 (Bradshaw et al. 2009; Derewenda et al. 2007; Soares et al. 2012; Tarricone et al. 2004; Zylkiewicz et al. 2011). The interaction of LIS1 with NDE1 and NDEL1 critically influences the activity of cytoplasmic dynein (McKenney et al. 2010; Mesngon et al. 2006; Shmueli et al. 2010; Vallee et al. 2012; Yamada et al. 2008). In migrating cortical neurons, cytoplasmic dynein in concert with LIS1 act along microtubules to create forces, that eventually promote the saltatory nuclear movement toward the centrosome (Tsai and Gleeson 2005; Vallee et al. 2009). In fact, a recent model proposes that the Syne-1/2-SUN1/2 nuclear membrane proteins critically function in nucleokinesis by bridging the nucleus to the cytoskeleton via LIS1 and cytoplasmic dynein whereby the minus-end-directed microtubule motor dynein-dynactin complex then promotes nucleokinesis toward the centrosome (Zhang et al. 2009). This nucleus-centrosome coupling is the driving force and underlying molecular basis for the "two-stroke" model of neuronal migration (see also Chaps. 2, 4 and 7).

The regulation of the LIS1/NDEL1-complex and its functional interaction with dynein is controlled at multiple levels and it has been demonstrated that NDEL1 contains specific phosphorylation sites that are targeted by CDK5 (Niethammer et al. 2000) and Aurora A (Mori et al. 2007, 2009). NDEL1 is also regulated by palmitoylation on a conserved cysteine residue and it has been shown that palmitoylation of NDEL1 is essential for the function of cytoplasmic dynein and its downstream activities, including the control of cortical neuron migration (Shmueli et al. 2010). Besides posttranslational modifications regulating the LIS1/NDEL1-complex and thus dynein activity, NDEL1 (much more than LIS1) also serves as a structural and signaling platform for a large amount of interacting proteins [reviewed in (Chansard et al. 2011; Moon and Wynshaw-Boris 2013)].

3.2 Functional Analysis of the *Lis1*/*Ndel1* in Cortical Neuron Migration In Vivo

The biochemical and biophysical studies described above resulted in a relatively detailed molecular model of how the LIS1/NDEL1-complex regulates dynein-mediated nuclear/somal migration (Tsai and Gleeson 2005; Vallee et al. 2009). In contrast, very little is known how the LIS1/NDEL1-complex orchestrates neuronal migration during cortical development *in vivo*. *Lis1* and *Ndel1* are essential genes and complete ablation of *Lis1* and *Ndel1* in knockout mice results in early embryonic lethality at E5.5 preimplantation stages (Cahana et al. 2001; Hirotsune et al. 1998; Sasaki et al. 2005), and thus complicating the functional analysis of *Lis1*/*Ndel1* function *in vivo*. Besides controlling neuronal migration, *Lis1* has also essential functions in cell proliferation and neurogenesis which further compromises loss-of-function analysis of LIS1 during cortical neuron migration (Faulkner et al. 2000; Hippenmeyer et al. 2010; Tsai et al. 2005; Yingling et al. 2008). However, the dose of *Lis1* is clearly very important and reduced levels of LIS1 in mice results in defects in the radial migration of several different types of neurons including cortical projection neurons (Cahana et al. 2001; Gambello et al. 2003; Hippenmeyer et al. 2010; Hirotsune et al. 1998). Systematic examination of the consequences of dosage reduction of LIS1 on cortical development was achieved by comparing wild-type ($Lis1^{+/+}$), null heterozygotes ($Lis1^{ko/+}$), compound heterozygote (null/hypomorph, $Lis1^{ko/hc}$), and conditional null ($Lis1^{ko/hc}$;Cre) and it was found that *Lis1* likely controls the efficiency of neuronal migration in a dose-dependent manner (Cahana et al. 2001; Gambello et al. 2003; Hippenmeyer et al. 2010; Hirotsune et al. 1998; Youn et al. 2009). It remains

unclear from these genetic *in vivo* studies whether *Lis1* has specific functions at discrete steps in the radial migration process. In contrast, *in utero* electroporation of *Lis1* small interference RNA and short hairpin dominant negative *Lis1* in wild-type background in rat and mouse brains caused a dramatic accumulation of multipolar cells in the SVZ (Shu et al. 2004; Tsai et al. 2005). In addition, the above *Lis1* knockdown experiments indicate that even bipolar neurons in the IZ critically depend on *Lis1* function for locomotion, presumably by regulating nuclear translocation through centrosome-nucleus coupling (Shu et al. 2004; Tsai et al. 2005; Tsai and Gleeson 2005; Vallee et al. 2009). These findings are also in line with the total inhibition of migration phenotype observed in cortical slice assays upon complete loss of *Lis1* in conditional null ($Lis1^{ko/hc}$;Cre) (Youn et al. 2009). We have recently applied the MADM (Mosaic Analysis with Double Markers) strategy (Zong et al. 2005) to ablate *Lis1* in sparse subpopulations of cortical projection neurons (Hippenmeyer et al. 2010). In the experimental MADM paradigm, homozygous mutant cells are labeled in one color (e.g. green by GFP), wild-type cells in another color (e.g. red by tdTomato) and heterozygous cells in yellow (i.e. green and red together) in an otherwise unlabeled background (Hippenmeyer et al. 2010; Zong et al. 2005). MADM-based functional gene analysis has the advantage that essential genes can be studied at any stage during development since the sparseness of gene knockout in single cells allows the bypass of embryonic lethality. Consequently, we have quantified the distribution of $Lis1^{+/+}$, $Lis1^{+/-}$ and $Lis1^{-/-}$ cortical projection neurons at adult stages and noticed a significant reduction of $Lis1^{+/-}$ and $Lis1^{-/-}$ neurons in the uppermost layers II/III but concomitant increase in lower layers. Interestingly, there was no significant difference between $Lis1^{+/-}$ and $Lis1^{-/-}$ in their laminar distribution although the migration of $Lis1^{-/-}$ neurons is (at least initially) significantly delayed at birth when compared to $Lis1^{+/-}$. In contrast, $Lis1^{+/+}$ neurons displayed a significant cell-autonomous migration advantage when compared to $Lis1^{+/-}$ and $Lis1^{-/-}$ neurons, respectively. The presence of MADM-labeled $Lis1^{-/-}$ neurons in all cortical layers indicates that non-autonomous and/or community effects could strongly influence the positioning of these $Lis1^{-/-}$ mutant (and heterozygous $Lis1^{+/-}$) neurons (see also below), given the critical function of *Lis1* in promoting nuclear/somal translocation. Future detailed MADM-based analysis of *Lis1* at embryonic stages may promise further insight into the cell-autonomous and possibly cell-nonautonomous functions of *Lis1* in regulating the sequential steps of cortical neuron migration. In summary, *Lis1* acts in a dose-sensitive manner to regulate the efficiency of neuronal migration, likely by promoting somal translocation via nucleus-centrosome coupling in migratory neurons and catalyzes the exit from the multipolar conformation to the bipolar state of nascent neurons in the embryonic SVZ (Fig. 1.3a). Experiments involving mosaic ablation of *Lis1* by MADM in overall heterozygote $Lis1^{+/-}$ mice also indicate a significant degree of intersecting community effects (upon reduction of *Lis1*) and/or cell-nonautonomous function(s) for *Lis1* in regulating neuronal migration (Hippenmeyer et al. 2010; Youn et al. 2009).

Like for *Lis1*, homozygous complete knockout of *Ndel1* in mice results in embryonic lethality (Sasaki et al. 2005) and although heterozygote $Ndel1^{+/-}$ mice do not show obvious phenotypes, further reduction of *Ndel1* in $Ndel1^{ko/hc}$ (null/hypomorph) animals results in mild neuronal migration phenotypes (Sasaki et al. 2005; Youn et al. 2009). *Ndel1* RNAi knockdown experiments by *in utero* electroporation independently revealed an essential function for *Ndel1* for appropriate cortical projection neuron migration (Shu et al. 2004). The function of *Ndel1* involves proper coupling of the centrosome to the nucleus, presumably in a *Lis1* dependent manner since the requirement for *Ndel1* in cortical neuron migration could be partially compensated by overexpression of *Lis1* (Shu et al. 2004). Complete loss of *Ndel1* in conditional knockout mice ($Ndel1^{ko/hc}$;Cre) led to total inhibition of neuronal migration in organotypic cortical brain slices as revealed by high resolution time-lapse two-photon videomicroscopy (Youn et al. 2009). However, by using the same migration assay, reduction of NDEL1

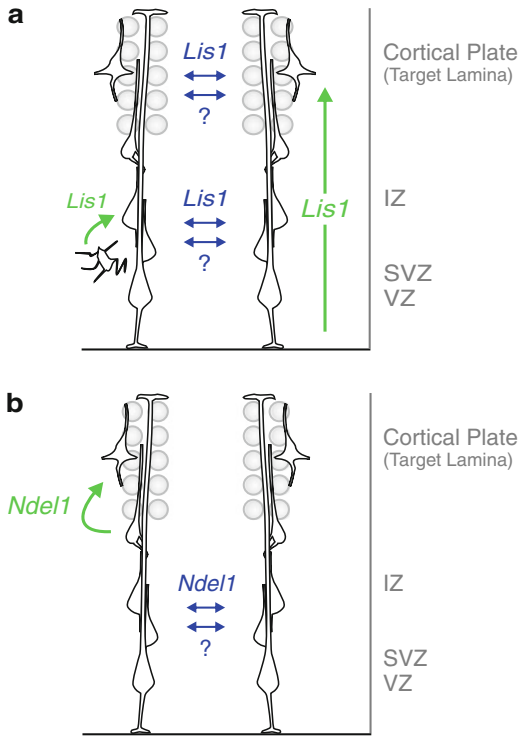

Fig. 1.3 Cell-autonomous and non-autonomous functions of *Lis1/Ndel1* at specific steps during radial migration. Models of cell-autonomous (*green*) and non-autonomous (*blue*) *in vivo* functions of LIS1 (**a**) and NDEL1 (**b**) in the developing cortex. LIS1 cell-autonomously regulates the efficiency of neuronal migration in a dose-dependent manner and has a role in the multi-to-bipolar transition of cortical projection neurons. NDEL1 cell-autonomously controls invasion and/or migration within the developing CP target laminae. Extensive interactions among migrating neurons, either mediated by specific cell-nonautonomous effects of LIS1/NDEL1 or through a general community effect, promote migration of *Ndel1*$^{-/-}$ cells before reaching the target laminae and *Lis1*$^{-/-}$ cells along the entire path under sparse knockout conditions (Adapted from (Hippenmeyer et al. 2010) with permission)

protein levels to about 35 % in *Ndel1*$^{ko/hc}$ allowed these neurons to migrate, albeit at reduced speeds. The slower migrating neurons in *Ndel1*$^{ko/hc}$ displayed multiple branches, like multipolar cells, and exhibited a branched migration pattern (Youn et al. 2009). Such branched migration is very similar to the movements of neurons lacking p35 (Chae et al. 1997; Gupta et al. 2003), which is the activator of CDK5 (Tsai et al. 1994). In *p35*$^{-/-}$ mutant mice (Chae et al. 1997) the vast majority of radially migrating cortical projection neurons displayed branched migration behavior (Gupta et al. 2003). Importantly, p35/CDK5 phosphorylates NDEL1 at specific sites (S198, T219 and S231) and this phosphorylation is essential for NDEL1 function (Niethammer et al. 2000; Sasaki et al. 2000). Therefore it is likely that the similar neuronal migration phenotypes of neurons lacking p35 or having reduced levels of NDEL1 could be causally related. Whether p35/CDK5-mediated NDEL1 phosphorylation is critically required during the entire radial neuron migration process or just at discrete steps is an important open question. In fact, loss of CDK5 function results in the accumulation migrating cortical projection neurons in the IZ and many of these *Cdk5*$^{-/-}$ mutant neurons remain in a multipolar configuration (Gilmore et al. 1998; Ohshima et al. 1996, 2007). Thus, p35/CDK5-induced phosphorylation of NDEL1 could in principle, besides promoting migration, trigger the multi-to-bipolar transition although distinct pathways including other CDK5 target molecules likely act in parallel in these sequential radial migration steps.

In order to get further insights and identify the critical step(s) in cortical neuron migration controlled by NDEL1 function *in vivo*, we pursued functional MADM analysis of *Ndel1* (Hippenmeyer et al. 2010). By using developmental time course and single clone analyses, the phenotype of mutant *Ndel1*$^{-/-}$ MADM-labeled cells revealed that *Ndel1* cell-autonomously regulates a very specific step in cortical neuron migration: the entry into the CP (Fig. 1.3b). Live-imaging experiments of MADM-labeled neurons in organotypic slice preparations further indicated that the speed of migration for *Ndel1*$^{+/+}$ and *Ndel1*$^{-/-}$ neurons is not significantly different as long as the neurons migrate within the IZ. In contrast, the majority of *Ndel1*$^{-/-}$ neurons failed to cross the IZ-CP border (Fig. 1.4). Altogether, the MADM experiments showed that *Ndel1*$^{-/-}$ neurons in a mosaic, mostly heterozygous, environment can migrate through the VZ/SVZ and the entire IZ. However, in the MADM setting, *Ndel1*$^{-/-}$ neurons are unable to enter and/or migrate into their CP target lamina. The analysis of several different types of MADM-labeled *Ndel1*$^{-/-}$ mutant neurons, besides cortical projection

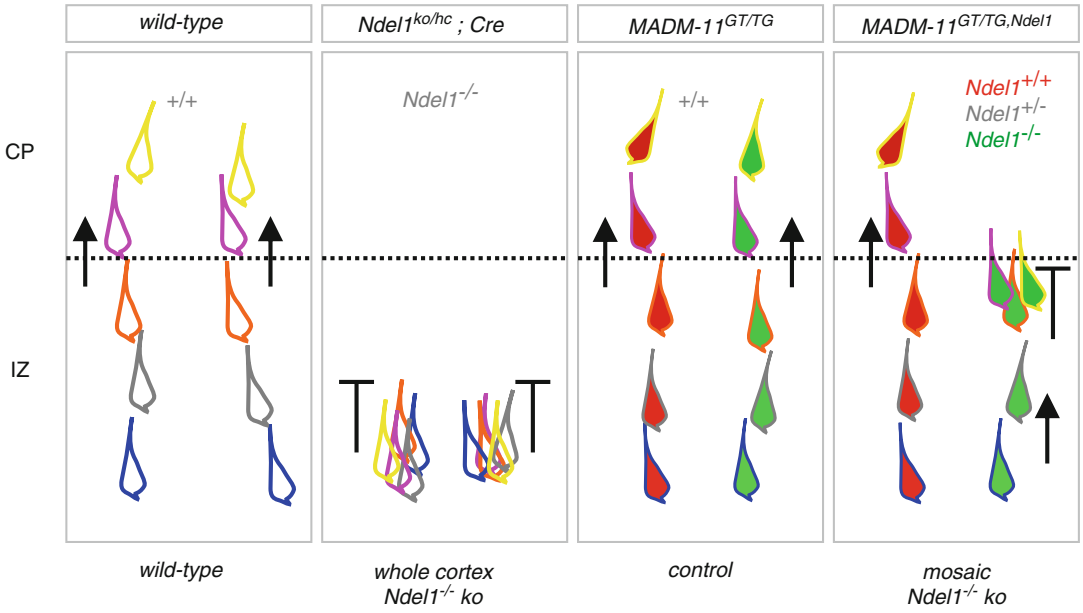

Fig. 1.4 Migration phenotypes of *Ndel1*$^{-/-}$ cortical projection neurons in whole and sparse knockout experimental paradigms. Distinct migration phenotypes in the IZ and CP in wild-type, *Ndel1*$^{ko/hc}$;Cre (whole cortex *Ndel1*$^{-/-}$ knockout), *MADM-11*$^{GT/TG}$ (control, all cells *Ndel1*$^{+/+}$) and *MADM-11*$^{GT/TG,Ndel1}$ (mosaic *Ndel1*$^{-/-}$ knockout). The different colored outlines of the migrating neurons shall indicate sequential positions at distinct times during progressive radial migration. This summary is based on the experimental time-lapse imaging data from (Hippenmeyer et al. 2010; Youn et al. 2009)

neurons, indicates that NDEL1 is generally required for the invasion of the target area in the course of radial neuronal migration (Hippenmeyer et al. 2010). The mosaic analyses afforded by MADM not only revealed cell-autonomous functions of *Ndel1* (and *Lis1*, see above) but indicates a significant amount of cell-nonautonomous effects which critically contribute to the regulation of discrete steps in radial neuron migration. Most indicative for such cell-nonautonomous effects is the fact that the phenotypes resulting from sparse MADM-based gene knockout are distinct from those observed in whole cortex knockout (Hippenmeyer et al. 2010; Youn et al. 2009) (Fig. 1.4). For instance, *Ndel1*$^{-/-}$ mutant neurons were unable to move in cultured organotypic brain slices from *Ndel1*$^{ko/hc}$;Cre mice (complete loss of NDEL1 in cortex) but MADM-labeled *Ndel1*$^{-/-}$ neurons (in genetic mosaic, mostly normal environment) could migrate at regular speeds in IZ and only stalled once they reached their CP target area. Thus, it is conceivable that the inability of *Ndel1*$^{-/-}$ mutant neurons to migrate in *Ndel1*$^{ko/hc}$;Cre mice is potentially a consequence of adversive cell-nonautonomous effects. What could be the molecular nature of such 'negative' effects? Neuronal migration requires the dynamic adjustment of cell adhesion molecules such as N-cadherin (see also below) and possibly axon guidance molecules (see also Chap. 9). Thus, one could speculate that the overall molecular landscape at the cell membrane might be non-permissive for migration and/or even inhibiting when *Ndel1*$^{-/-}$ neurons contact each other. Conversely, in a mosaic MADM environment, most *Ndel1*$^{-/-}$ mutant neurons only get in contact with *Ndel1*$^{+/-}$ or *Ndel1*$^{+/+}$ neurons and cross-inhibiting *Ndel1*$^{-/-}$ – *Ndel1*$^{-/-}$ cell contacts are minimized. On the other hand, migrating MADM-labeled *Ndel1*$^{-/-}$ neurons, as long as progressing through the VZ/SVZ and the IZ, could even be positively influenced by the neighboring *Ndel1*$^{+/-}$ or *Ndel1*$^{+/+}$ neurons. Such 'positive' effects might also account for the positioning of MADM-labeled *Lis1*$^{-/-}$ in upper cortical layers

(see above). The precise nature of the 'positive' cell-nonautonomous effects and how they account for positioning and/or migration of mutant *Ndel1*$^{-/-}$ and *Lis1*$^{-/-}$ cortical projection neurons is currently unclear. In principle two scenarios could be possible: (1) community effects whereby isolated *Ndel1*$^{-/-}$ or *Lis1*$^{-/-}$ neurons, despite defective in the intrinsic cytoskeletal migration machinery (e.g. nucleus-centrosome coupling), may still "piggy-back" on normally migrating abutting neurons; and (2) neurons migrating in a crowd could actively signal, in a *Lis1*/*Ndel1*-dependent manner, to each other in order to boost the intrinsic migration machinery. Molecularly, such a mechanism poses however a significant challenge since intracellular cytoplasmic proteins would need to signal inside-out and trans-cellular to neighboring migrating cells. Such a signaling pathway likely would need to involve transmembrane receptors and possibly extracellular matrix components, and some sort of a sensor connecting to cytoskeletal force generators in the 'signal-receiving' cell. Interestingly, cell-on-cell migration of *Drosophila* invasive border cells, which also depends on cell-autonomous requirement for *dLis1*, has been proposed to involve "piggy-backing" (Yang et al. 2012). More generally, community effects have been observed in a variety of other cell types moving collectively (Friedl and Gilmour 2009; Tada and Heisenberg 2012; Theveneau and Mayor 2013).

The adaptor protein 14-3-3ε is encoded by *YWHAE* in human and deleted in all MDS patients (Cardoso et al. 2003; Toyo-oka et al. 2003; Wynshaw-Boris 2007). The precise function of 14-3-3ε during brain development is unclear but it has been shown that *Ywhae* genetically interacts with *Lis1* in mice and that the 14-3-3ε protein binds to p35/CDK5 phosphorylated NDEL1 (Toyo-oka et al. 2003). The 14-3-3ε/NDEL1 interaction is important for binding to LIS1 and the dynein motor. Thus, the tripartite LIS1/NDEL1/14-3-3ε complex appears as a key regulator of neuronal migration. Interestingly, sparse MADM-mediated conditional knockout of *Ywhae* in cortical projection neurons did not significantly affect their radial migration although *Ywhae*$^{-/-}$ pyramidal CA1 cells in hippocampus showed slight defects in migration (Hippenmeyer et al. 2010). Since multiple isoforms of 14-3-3 are expressed in the brain (Takahashi 2003), distinct 14-3-3 isoforms could compensate for the loss of 14-3-3ε function. Along the same lines, genetic redundancy could also, at least in part, explain some of the divergent phenotypes that were observed when comparing the individual MADM-induced *Lis1*$^{-/-}$, *Ndel1*$^{-/-}$ and *14-3-3ε*$^{-/-}$ mutant neurons to each other. For instance *Nde1*, the second homologue of *A. nidulans NudE* and paralogue of murine *Ndel1*, shares 55 % sequence homology with *Ndel1* (Feng et al. 2000; Sweeney et al. 2001) and could possibly compensate for some *Ndel1* functions, especially during the early stages of neuronal migration in VZ/SVZ and IZ. Although the overall phenotype of *Nde1*$^{-/-}$ mutant mice is distinct from *Ndel1*$^{-/-}$ mutants, NDE1 can interact with LIS1 and this interaction is critical to control cortical lamination and thus cortical neuron migration (Feng et al. 2000; Feng and Walsh 2004; Pawlisz et al. 2008). Alternatively, LIS1, NDEL1 and 14-3-3ε may also each act in a 'complex'-independent manner to execute distinct component-specific functions, at discrete steps and transitions during radial migration of cortical projection neurons. Recent biochemical experiments support such a model whereby for example both LIS1 and NDEL1 proteins may act independently in certain contexts and that their interaction can amplify and/or modulate those activities (Zylkiewicz et al. 2011).

4 The Reelin Pathway Controls Sequential Phases in Cortical Neuron Migration

Cortical layering occurs in an 'inside-out' fashion whereby earlier born neurons populate deep layers and later born neurons occupy progressively upper layers (Angevine and Sidman 1961; McConnell 1995; Polleux et al. 1997; Rakic 1974, 2007). The Reelin signaling pathway plays fundamental roles in cortical neuron migration and inside-out lamination, and has attracted unmatched attention, by now for over half a decade. In human, mutations in the *RELN* gene

(encoding RELN also known as Reelin) are associated with autosomal recessive lissencephaly and cerebellar hypoplasia (Hong et al. 2000) but the precise function and mode of action of the Reelin signaling cascade at the cellular and molecular level *in vivo* remains unclear.

Historically, Falconer initially described the *Reeler* mouse mutant phenotype which includes ataxia, tremors and a reeling gait (Falconer 1951), hence the name *Reeler*. A multitude of anatomical studies has been carried out but the most striking feature of the *Reeler* mouse is that laminated brain structures including the neocortex are disorganized with misplaced projection neurons (Caviness and Rakic 1978; Hamburgh 1963; Tissir and Goffinet 2003). Upon cloning of the Reelin gene *Reln* in mouse it became evident that Reelin encodes a large (3,461 amino acids) secreted glycoprotein (D'Arcangelo et al. 1995). Reelin is mostly expressed and secreted from the earliest born cortical 'pioneer' Cajal-Retzius cells which form the outermost layer of the PP and later the MZ (D'Arcangelo et al. 1995; Ogawa et al. 1995). Reelin binds to a receptor complex – VLDLR (very low-density lipoprotein receptor)/LRP8 (low-density lipoprotein related receptor 8, formerly known as APOER2) (D'Arcangelo et al. 1999; Hiesberger et al. 1999) and strikingly, the phenotype of *Vldlr/Lrp8* double knockout mice is basically indistinguishable from the *Reeler* phenotype (Trommsdorff et al. 1999). The Reelin signal is transmitted via the intracellular signaling adaptor DAB1 which is phosphorylated by Src-family kinases upon Reelin binding (Arnaud et al. 2003; Bock and Herz 2003; Howell et al. 1999), and *Dab1* mutant mice (Howell et al. 1997; Sheldon et al. 1997; Ware et al. 1997) exhibit neurological and anatomical phenotypes the are indistinguishable from the ones in *Reeler* mutant mice (Trommsdorff et al. 1999). Both VLDLR and LRP8 do not possess intrinsic kinase activity and it has been shown that the axon guidance molecule ephrin-B can act as a co-receptor for Reelin (Senturk et al. 2011). Reelin binds to the extracellular domain of ephrin-Bs and thereby associating with the VLDLR/LRP8 complex. This leads to clustering of ephrin-Bs and Src kinase-mediated DAB1 phosphorylation and activation (Senturk et al. 2011). Finally, Notch signaling, executing most important functions during neurogenesis (Kageyama et al. 2009) has recently been shown to play a critical role in transducing the Reelin signal during neuronal migration (Hashimoto-Torii et al. 2008) although the precise mechanisms how Notch functions as a Reelin downstream mediator still remain enigmatic.

Despite tremendous efforts to decipher the biological Reelin function(s) in cortical neuron migration, important aspects are still unclear and several somewhat conflicting hypotheses have been put forth. It has been proposed that Reelin might act (1) as a repellent cue (Ogawa et al. 1995; Schiffmann et al. 1997); (2) as a stop signal (Frotscher 1997; Sheppard and Pearlman 1997); (3) to stimulate detachment of migrating cortical neurons from the radial glia process (Dulabon et al. 2000; Sanada et al. 2004) or (4) specifically could regulate radial glia-independent somal translocation (Franco et al. 2011; Jossin and Cooper 2011; Sekine et al. 2012) (see also below).

One reason for the apparent pleiotropy of the Reelin deficiency phenotypes could be that besides cell-autonomous functions of Reelin signaling in migrating neurons, environmental factors, community and other cell-nonautonomous effects might critically influence Reelin-sensitive neurons (Franco et al. 2011; Sanada et al. 2004). In any case, several recent experiments convincingly suggest that Reelin may play not only one but instead several important cell-autonomous roles and likely acts at multiple discrete steps during radial migration of cortical projection neurons. First, Reelin-dependent LRP8 downregulation could be important for nascent neurons to delaminate and start their migration by uncoupling them from neural progenitor cells (Perez-Martinez et al. 2012). It is however not clear if this proposed early function acts more or less as a permissive signal rather than acting as an instructive cue. Second, Reelin plays an essential role in the multipolar-to-bipolar transition while neurons migrate within the IZ (Jossin 2011; Jossin and Cooper 2011). In fact, migrating neurons in the IZ show the highest level of "functional Reelin receptors" and the Reelin

signal could diffuse from the MZ to the IZ (Jossin et al. 2007; Uchida et al. 2009). Jossin and Cooper propose a three step model how Reelin regulates the multipolar-to-bipolar transition of cortical neurons in the IZ. In a first step, multipolar neurons migrate in an apparently stochastic mode in the IZ where they encounter Reelin, which leads to activation of the small GTPase RAP1, presumably via pDAB1-CRK/CRKL-C3G signaling (Ballif et al. 2004; Voss et al. 2008). RAP1 is a Ras-related GTPase which plays important roles in the regulation of the actin cytoskeleton, membrane trafficking and cell adhesion (Gloerich and Bos 2011). In the second step, active RAP1 increases the surface level of N-Cadherin in the multipolar neurons. The increased levels of surface N-Cadherin could then allow the multipolar neurons to sense environmental cues allowing the proper polarization and exit of the multipolar state to adopt bipolar morphology (Jossin 2011). While the precise intracellular signaling mechanisms controlling RAP1-mediated N-Cadherin regulation during multi-to-bipolar transition and migration towards the upper IZ remain to be elucidated, Reelin also regulates later aspects in the radial migration process. Upon transition to the bipolar morphology, cortical neurons migrate along the radial glia processes towards the cortical plate. This glia-dependent migration (locomotion) mode appears to be independent of Reelin signaling. In contrast, the very last step – terminal somal translocation – which seems to occur in a glia-independent manner requires DAB1-mediated Reelin signaling (Franco et al. 2011; Jossin and Cooper 2011; Sekine et al. 2012). Interestingly, such glia-independent somal translocation is the predominant mode of migration of early born neurons occupying the future lower cortical layers (Nadarajah and Parnavelas 2002). Thus DAB1-mediated Reelin signaling likely promotes cell-autonomously the splitting of the PP by deep layer neurons.

Because early and late born neurons travel different distances and through varying cellular compartments in their individual migration journeys, it is likely that the molecular machineries driving their migration could be different for future deep versus upper layer neurons, respectively. Therefore, neurons destined for different layers most likely have additional distinct requirements for functional Reelin signaling. Nevertheless, for the very last step in the migration process – terminal somal translocation – which is conserved in early and late born neurons, Reelin is equally important in both early and late born neurons (Franco et al. 2011; Jossin and Cooper 2011; Sekine et al. 2012). Mechanistically, Reelin signaling via RAP1 and subsequent control of N-cadherin function seems to promote terminal somal translocation (Franco et al. 2011; Jossin and Cooper 2011). The functions of N-cadherin in the control of radial neuron migration appear however pleiotropic since also glia-dependent locomotion depends on N-cadherin function (Kawauchi et al. 2010). How N-cadherin signaling – triggered either by Reelin-RAP1-mediated intracellular redistribution (multi-to-bipolar transition and glia-independent terminal somal translocation) or via Reelin-independent pathways (glia-dependent locomotion) – regulates the distinct sequential steps of neuronal migration is currently not well understood. Precise regulation of N-cadherin levels through endocytic and/or coordinated intracellular trafficking pathways could be an effective way for appropriately tuning of N-cadherin levels/activity (Kawauchi et al. 2010). As such, during the locomotion process of migrating neurons, N-cadherin undergoes RAB5-dependent endocytosis at the trailing end and is efficiently shuffled to the plasma membrane at the forward end via a RAB11-dependent recycling pathway. Conversely, RAB7-dependent lysosomal degradation pathways with increased degradation of N-cadherin and possibly other cell adhesion molecules seem also important for the final terminal somal translocation step (Kawauchi et al. 2010). Although the Reelin-DAB1-CRK/CRKL-C3G-RAP1 pathway dynamically regulates N-cadherin activity and terminal somal translocation, overexpression of N-cadherin alone was not sufficient to rescue loss of Reelin-mediated DAB1 signaling (Franco et al. 2011). Therefore, distinct signaling molecules and/or cell adhesion molecules have additional roles in the transmission of the Reelin signal for the promotion of terminal somal translocation. Consequently, it has been demonstrated that

Reelin triggers C3G-RAP1-mediated integrin activation in an inside-out signaling fashion specifically during somal translocation (Sekine et al. 2012). In this pathway, Reelin acts as input signal through VLDLR/LRP8-DAB1-CRK/CRKL-C3G to activate RAP1 which recruits effectors to promote conformational change and thus activation of α5β1-integrin, which then triggers adhesion to fibronectin in the ECM of the MZ to mediate terminal somal translocation (Sekine et al. 2012).

Altogether and despite tremendous efforts and progress in deciphering the biological functions of the Reelin signaling pathway, the precise mechanism of the highly complex Reelin signaling cue and how it regulates the proper positioning of neurons in the CP remains unclear and somewhat controversial. There is an emerging picture whereby Reelin controls radial neuronal migration at several distinct steps (Fig. 1.5). Reelin acts (1) during delamination and separation of nascent neurons from neuronal progenitors; (2) to regulate the multi-to-bipolar transition; and (3) most importantly during somal translocation of early born lower layer neurons and terminal somal translocation of late born upper layer neurons. Interestingly, the Reelin and LIS1 pathways described above are likely to intersect at certain stages during cortical neuron migration (Assadi et al. 2003). However, the cytoarchitectural phenotypes of the cortex in mice with absent Reelin or LIS1 are distinct (Caviness and Rakic 1978; D'Arcangelo et al. 1995; Hirotsune et al. 1998; Yingling et al. 2008) although loss of function of either pathway cause lissencephaly in human (Hong et al. 2000; Reiner et al. 1993). At which steps during radial migration the Reelin and LIS1 signaling pathways crosstalk to exert their critical function(s) in driving migrating cortical neurons awaits further investigation.

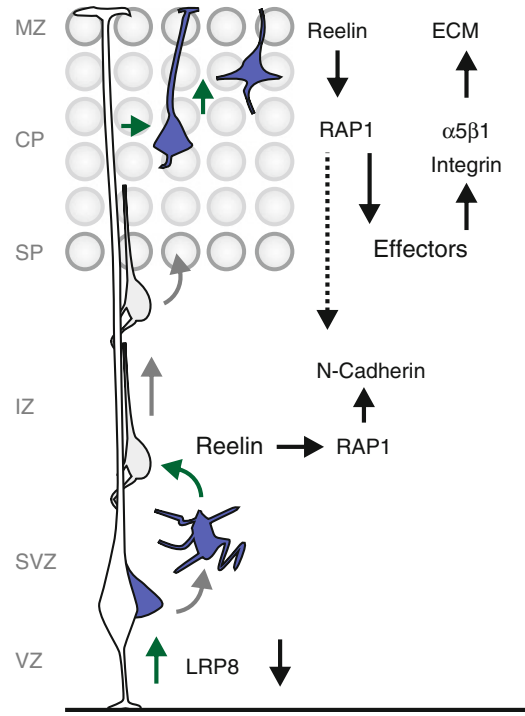

Fig. 1.5 Reelin signaling requirements at discrete stages of radial migration. Nascent cortical projection neurons downregulate LRP8 in a Reelin-dependent manner while delaminating from the ventricular surface. Reelin signaling, via RAP1 and N-Cadherin, regulates the multi-to-bipolar transition. During the last stages of cortical neuron migration, Reelin signaling controls terminal somal translocation via N-Cadherin and inside-out activation of α5β1-Integrin signaling

5 Transcriptional Programs in the Regulation of Specific Transitions During Radial Neuron Migration

The assembly of functional cortical microcircuits depends on a global and holistic developmental program including specific transcriptional regulatory schedules. Transcription factors have been shown to play crucial roles in many phases of cortical development including patterning in neuroepithelia, neurogenesis, arealization and neuronal specification (Martynoga et al. 2012; O'Leary et al. 2007; Rash and Grove 2006). Critical transcriptional programs control in particular the acquisition of cell-type and laminar identity in the neocortex (Leone et al. 2008; Molyneaux et al. 2007). It becomes now increasingly clear that finely tuned transcriptional programs do also control other aspects, besides instructing cell identity in the course of neurogenesis, such as driving migration and precise positioning of cortical projection neurons. Interestingly, neurons migrating tangentially along extended pathways and crossing many

intermediate targets before reaching their final positions require several levels of transcriptional regulations. As such, specific classes of cortical interneurons, motor and pontine neurons in hindbrain for instance deploy transcriptional control over their long distance tangential migrations (Chedotal and Rijli 2009; Di Meglio et al. 2013; Marin 2013). Recent studies implicate critical transcriptional changes as well in the regulation of discrete steps of the radial migration journey of cortical neurons. Such transcriptional changes are likely crucial for adjusting the molecular landscape of migrating neurons. For instance, the transition through distinct compartments (e.g. from VZ/SVZ through the IZ into the CP target area) could require the adjustment of cell surface receptors, intracellular signaling or regulation of cell adhesion in response to changes in the extracellular matrix. The precise regulation of transcriptional programs and their impact on sequential stages of cortical projection neuron migration at the mechanistic level is however poorly understood and just about to be deciphered.

5.1 Transcriptional Migration Control of Early and Late Born Cortical Projection Neurons

Certain transcription factors play a role in the development of some but not other classes of cortical projection neurons. As such it appears that particular genetic programs seem to regulate specifically the fate and positioning of early born lower layer neurons and, that other programs are important for migration and molecular differentiation of later born upper layer neurons. The transcription factor SOX5 (Kwan et al. 2008; Lai et al. 2008) is specifically expressed in postmitotic early born SP, layer VI and in a subset of layer V projection neurons but not in VZ/SVZ progenitors and nascent projection neurons migrating in the IZ. Ablation of *Sox5* in mice showed that *Sox5* controls the molecular differentiation and development of axonal projections but also regulates critical steps in the migration of the early born deep layer projection neurons (Kwan et al. 2008; Lai et al. 2008). In particular, the splitting of the PP is not complete and some populations of deep layer neurons failed to migrate past earlier born neurons in $Sox5^{-/-}$ mutant mice. Thus, *Sox5* appears to play a crucial role in the final step of neuronal migration (somal translocation) and *Sox5* deficient neurons show a somewhat similar phenotype like neurons in *Reeler* mutant mice (see above). How *Sox5* regulates the terminal steps of radial migration of early born deep layer neurons is currently unknown but the answer likely lies within the group of SOX5-regultated downstream target genes acting at the final stage of deep layer neuron positioning. Interestingly, however, late born upper layer $Sox5^{-/-}$ neurons migrate normally and past earlier born projection neurons (Kwan et al. 2008; Lai et al. 2008). These studies indicate that the molecular mechanisms, controlling the migration of certain classes of late born projection neurons past earlier born neurons, might not be equal for all cortical layers. More likely, the regulation of the molecular mechanisms promoting the passage of neurons past their predecessors may involve adjustment according to the respective layer landscape. Since the overall cortical architecture is substantially different for earlier born neurons than for later born neurons, appropriate readjustment of the migration machinery may be necessary. For instance, layer V neurons 'only' need to migrate past the SP and one layer (layer VI), whereas layer III neurons need to migrate past the SP, layers VI, V and IV in order to reach their final settling area. Thus the migration path is not only longer for layer III neurons but requires the passage of multiple and distinct classes of earlier born neurons.

The identity and migration of cortical upper layer neuron is critically regulated by two related POU domain transcription factors POU3F2 (BRN-1) and POU3F3 (BRN-2) acting to some extent redundantly (McEvilly et al. 2002; Sugitani et al. 2002). *Pou3f2/3* are co-expressed in progenitors in the VZ and in the CP in most layer II-V cortical projection neurons. Analysis of *Pou3f2/3* double knockout mice revealed that the generation of late born neurons was affected but that the cortical projection neurons that were successfully generated, could migrate away from

the VZ and expressed some markers characteristic for differentiating neurons (McEvilly et al. 2002; Sugitani et al. 2002). However, the *Pou3f2/3* double mutant neurons failed to pass the SP and layer VI and accumulated below the SP in an apparently cell-autonomous manner. The migration phenotype of *Pou3f2/3* double mutant neurons resembles to some extent the phenotype of neurons lacking p35/CDK5 activity [see also above; (Gilmore et al. 1998; Ko et al. 2001)]. Interestingly, POU3F2/3 directly bind to the promoters of both *p35* and *p39* genes, redundantly regulate the cell-autonomous expression of the p35/39 regulatory subunits for CDK5 (McEvilly et al. 2002) and control expression of DAB1 (Sugitani et al. 2002). It is thus likely that *Pou3f2/3* control neuronal positioning through direct regulation of the expression of components of the Reelin and p35/CDK5 signaling pathways although the precise role of these pathways in early versus late born neurons needs to be analyzed more detail. In addition to the above described, apparently cell-population specific transcriptional programs, another series of transcriptional programs fine-tunes key downstream intracellular pathways that drive neuronal migration at defined sequential steps in radially migrating projection neurons.

5.2 Transcriptional Programs Regulating Discrete Steps in Radial Cortical Neuron Migration

The findings illustrated above indicate that distinct classes of neurons have different requirements for adjusting their migration efficiency through the VZ/SVZ, IZ and growing CP compartments, and thus exploit specialized cell-type specific transcriptional programs to tune their migration machinery. Besides the important layer-specific transcriptional regulation, finely-tuned transcriptional programs, their downstream target genes and effectors also play critical roles in the discrete steps and transitions during the migration process of cortical projection neurons.

Members of the bHLH (basic helix-loop-helix) family of proneural transcription factors such as Neurogenin1/2 and ASCL1, which exert key roles in neurogenesis (Martynoga et al. 2012), encoded by *Neurog1/2* and *Ascl1* (achaete-scute complex homolog 1), also promote the migration of nascent cortical projection neurons (Ge et al. 2006; Hand et al. 2005; Heng et al. 2008). Two members of the Snail superfamily of transcription factors, Scratch1 and Scratch2, appear to be expressed under the control of the above *Neurog1/2* and *Ascl1* proneural genes in the developing cortex and control the very first step of the neuronal migration process – detachment or delamination from the apical surface in the ventricular zone (Itoh et al. 2013). On a mechanistic level, Scratch proteins mediate transcriptional repression and/or downregulation of E-cadherin based adhesion in order to promote the detachment of neurons from the ventricular surface (Itoh et al. 2013) (Fig. 1.6a).

Apart from promoting Scratch-mediated delamination during the very first step of neuronal migration, proneural genes also regulate later but discrete steps during the migration journey of nascent cortical projection neurons. In particular, *Neurog1/2* has been initially shown to be involved in controlling the expression of genes with prominent functions in neuronal migration such as *RhoA*, *Dcx* and *p35* (Ge et al. 2006) but other target genes such as *Rnd2* encoding a small GTP-binding protein appear to play even more critical roles in translating the *Neurog2* requirement in neuronal migration (Heng et al. 2008). On the other hand, *Ascl1* promotes migration of newly-born neurons by direct regulation of another Rnd family member: *Rnd3* (Pacary et al. 2011). Both RND2 and RND3 inhibit RhoA signaling but lack intrinsic GTPase activity, are thus constitutively bound to GTP (Chardin 2006) and are thought to be regulated at the level of their expression, post-translational modification and intracellular localization (Madigan et al. 2009; Riento et al. 2005). Importantly, *Rnd2* and *Rnd3* control distinct key steps in the migratory process (Heng et al. 2008; Pacary et al. 2011) (Fig. 1.6b). While *Rnd2* regulates the transition from the multipolar state to a bipolar arrangement in the IZ, *Rnd3* controls the

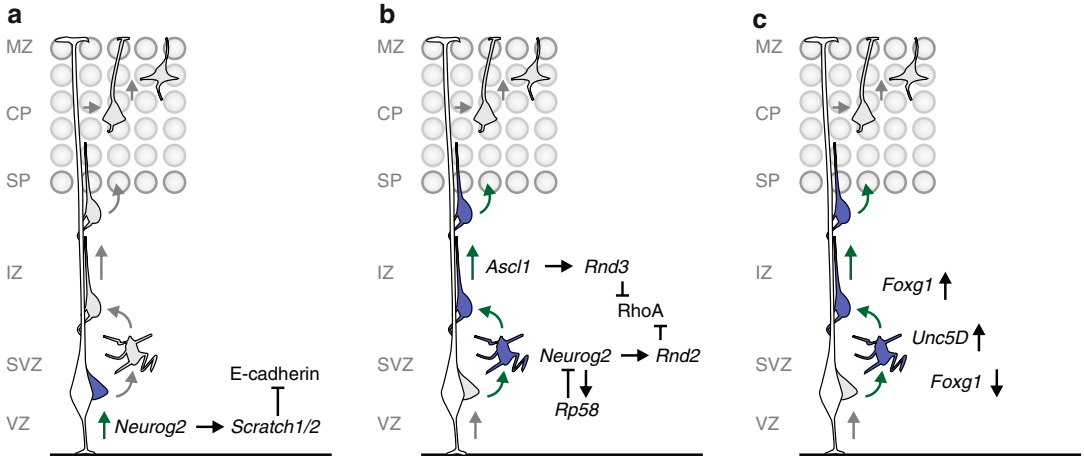

Fig. 1.6 Transcriptional regulation of cortical projection neuron migration. (**a**) The transcriptional regulators *Scratch1/2* (downstream of *Neurog2*) mediate the very first step in cortical neuron migration – delamination form the ventricular surface – via transcriptional repression of the cell adhesion molecule E-Cadherin. (**b**) *Rnd2* (downstream of *Neurog2*) and *Rnd3* (downstream of *Ascl1*) both inhibit RhoA signaling but promote distinct steps in the migratory process of cortical projection neurons. *Rnd2* controls the multi-to-bipolar transition and *Rnd3* is required for glia-dependent locomotion. *Neurog2* expression is tightly regulated via RP58-mediated transcriptional negative feedback. (**c**) Dynamic regulation of *Foxg1* is critical for several steps during cortical neuron migration. Downregulation of *Foxg1* in the early multipolar phase is necessary for initiation of *Unc5D* expression and to proceed from the early to the late multipolar phase. Reiteration of *Foxg1* expression in migrating cortical projection neurons is required at later stages in order to enter the CP

locomotion of migratory neurons in the CP. Consequently, *Rnd2* and *Rnd3* cannot replace each other although the mechanism of action for both involves the inhibition of RhoA activity (Heng et al. 2008; Pacary et al. 2011). Interestingly, RND2 and RND3 seem to execute their functions via RhoA inhibition in distinct cellular compartments of the migrating neurons (Pacary et al. 2011). Since RND2 is primarily associated with early endosomes it may be involved in the selective intracellular trafficking while neurons are multipolar and thus contribute to the proper polarization and transition from multi-to-bipolar morphology. In contrast, RND3 is localized also at the plasma membrane (besides early and recycling endosomes) and appears to drive locomotion of migratory cortical projection neurons by inhibiting RhoA-mediated actin polymerization (Pacary et al. 2011). Altogether, the proneural *Neurog2* and *Ascl1* regulate consecutive phases of cortical neuron migration via induction of *Rnd2*/*Rnd3* which then inhibit RhoA activity in distinct cellular compartments catalyzing multi-to-bipolar transition and driving locomotion, respectively (Fig. 1.6b).

Since the function of *Neurog2* via induction of *Rnd2* is highly specific for a particular transient step in the migration process, it is essential to regulate and tune that *Neurog2*/*Rnd2* function appropriately. Negative feedback regulation could be an ideal mechanism for dosing the *Neurog2*/*Rnd2* action. Indeed, the transcriptional regulator RP58, a zinc-finger transcriptional repressor from the BTB/POZ-domain family (Aoki et al. 1998), is a downstream target of *Neurog2* and appears to control neuronal migration via the regulation of the multipolar-to-bipolar transition (Ohtaka-Maruyama et al. 2013). Moreover, RP58 has been shown to directly repress *Neurog2* expression by binding to regulatory elements in the region near the *Neurog2* genomic locus. Thus, while RP58 itself is a target gene of *Neurog2* (Ohtaka-Maruyama et al. 2012; Seo et al. 2007), it ensures transient and to the point action of the *Neurog2*/*Rnd2* pathway for controlling multipolar-to-bipolar transition during

neuronal migration via negative feedback regulation (Ohtaka-Maruyama et al. 2013) (Fig. 1.6b).

As illustrated above, the progression of the multi-to-bipolar transition, of cortical projection neurons in the course of their migration journey, requires extensive regulation. The forkhead transcription factor FOXG1, acting as a critical regulator of early telencephalic development (Hanashima et al. 2004; Kumamoto et al. 2013; Martynoga et al. 2005; Muzio and Mallamaci 2005), is dynamically expressed while migrating pyramidal neurons progress through the multi-to-bipolar transition (Miyoshi and Fishell 2012) (Fig. 1.6c). The dynamic *Foxg1* expression is critical, because at the beginning of the multipolar phase, *Foxg1* needs to be downregulated in order to allow expression of *Unc5D* (Sasaki et al. 2008) which facilitates the transition from early to late multipolar phase and thus migration through the IZ (Miyoshi and Fishell 2012). However, reiteration of *Foxg1* expression is required for neurons to switch from the multipolar state to the bipolar morphology and for entering into the CP (Miyoshi and Fishell 2012) (Fig. 1.6c). Gene expression experiments along within the above study strongly indicate that *Foxg1* could control the expression of cell adhesion molecules (including the above mentioned putative Netrin receptor UNC5D) in cortical projection neurons during the multi-to-bipolar transition. In summary, cortical projection neurons need to migrate through highly distinct environments (VZ/SVZ, IZ, and CP) in the course of their radial migration journey and transcriptional programs mediated by *Foxg1* and other transcription factors fine tune the complement of receptors on the extracellular surface of migration cells. This can allow the optimal sensing of the extracellular cue repertoire which then can serve as a guide through the different zones across the developing cortical wall.

6 Perspectives

Newly-born cortical projection neurons need to migrate from their birthplace to their final position to fulfill their appropriate function. It has become well established that nascent cortical neurons migrate in a step-wise fashion and progress through sequential migration phases, from the VZ/SVZ through the IZ in order to reach their final positions in the developing CP, and build up the six cortical layers. The importance of neuronal migration for correct brain development is highlighted in patients that suffer from certain brain malformations which include the 'migration' disorder Lissencephaly. Although tremendous efforts have revealed a variety of signaling pathways regulating neuronal migration, our understanding of the precise cellular and molecular mechanisms, controlling specifically the *sequential steps and transitions* during cortical neuron migration, remains incomplete. Thus, future studies are required in order to clarify the relationship between particular signaling pathways and the discrete steps of cortical neuron migration. The precise nature of cell-autonomous and non-autonomous effects of gene function(s) and/or community effects in the control of neuronal migration are also not well understood. Such knowledge is however relevant for our molecular and mechanistic understanding of the precise nature of devastating neurodevelopmental disorders, including Lissencephaly, where non-autonomous and/or community effects might influence the severity of the condition in human patients. More generally, investigations are necessary along these lines to decipher both the nature and exact interplay of the cell-autonomous and cell-nonautonomous functions of candidate genes, and their effectors, in regulating the precise stages during the radial cortical neuron migration process.

Acknowledgements I apologize to the all the authors whose relevant work could not be included in this chapter due to space constraints and thank Carl-Philipp Heisenberg, Michael Sixt and Mariapia Postiglione for helpful comments on the manuscript. This work was supported by IST Austria (Institute of Science and Technology Austria) institutional funds.

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The Dynamics of Neuronal Migration

2

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Abstract

Proper lamination of the cerebral cortex is precisely orchestrated, especially when neurons migrate from their place of birth to their final destination. The consequences of failure or delay in neuronal migration cause a wide range of disorders, such as lissencephaly, schizophrenia, autism and mental retardation. Neuronal migration is a dynamic process, which requires dynamic remodeling of the cytoskeleton. In this context microtubules and microtubule-related proteins have been suggested to play important roles in the regulation of neuronal migration. Here, we will review the dynamic aspects of neuronal migration and brain development, describe the molecular and cellular mechanisms of neuronal migration and elaborate on neuronal migration diseases.

Keywords

Neuronal migration • Neural stem cells • Radial Glia • Microtubules • Neuronal migration disorders • Lissencephaly

1 Neurons Originate from Two Proliferative Zones of the Cerebral Cortical Wall

Neuronal migration is a critical step in the formation of the nervous system. During development, neurons migrate from their birthplace to their final destination, where they form neuronal architectures, such as laminated structures and nuclei that are necessary for information processing in the nervous system. The majority of neurons in the cerebral cortex, the pyramidal excitatory neurons, are born either within the ventricular zone

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L. Nguyen and S. Hippenmeyer (eds.), *Cellular and Molecular Control of Neuronal Migration*, Advances in Experimental Medicine and Biology 800, DOI 10.1007/978-94-007-7687-6_2,

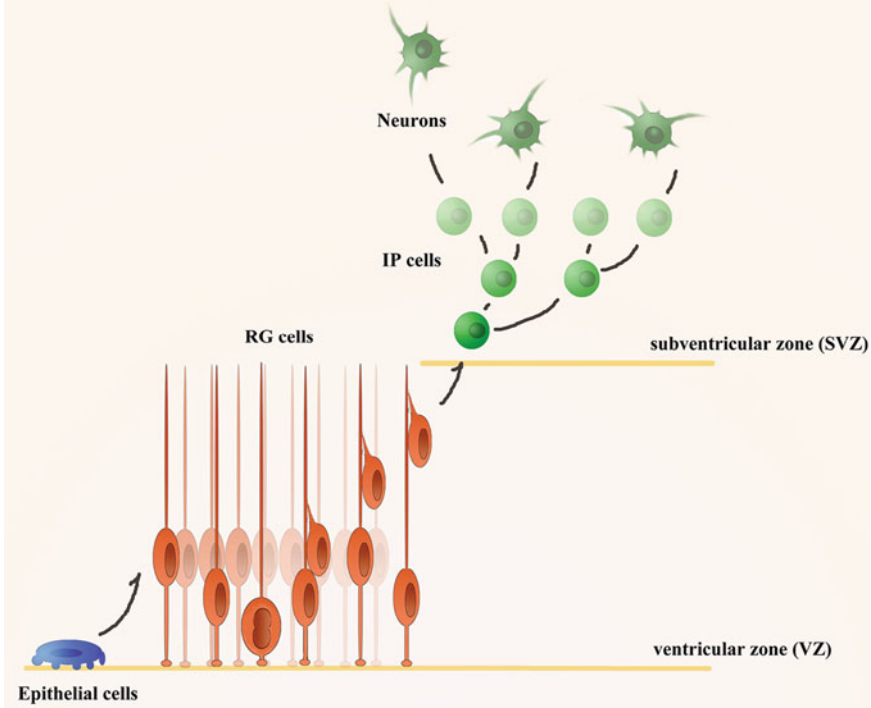

Fig. 2.1 Proliferation of neuronal stem cells and interkinetic nuclear migration

(VZ) or the subventricular zone (SVZ) (Kriegstein and Noctor 2004; Noctor et al. 2001; Tamamaki et al. 2001; Wu and Wang 2012) (Fig. 2.1). Neuronal progenitors in the VZ are the radial glial (RG) cells that span the entire neocortical wall and maintain contact both at the ventricular and pial surfaces throughout their mitotic division cycles. The RG cells are the major population of neural progenitor cells occupying the proliferative VZ in the developing mammalian neocortex (Malatesta et al. 2000; Miyata et al. 2001; Noctor et al. 2001). Radial glia cells undergo interkinetic nuclear migration, whereby the nucleus moves within the cytoplasm of elongated neuroepithelial progenitor cells in synchronization with the cell cycle phase (Gotz and Huttner 2005; Miyata et al. 2004; Noctor et al. 2004). The nucleus ascends to the upper region of the VZ during S phase, and later descends to the apical part of the VZ (Fig. 2.1).

Intermediate progenitor (IP) cells reside within the VZ and often divide at ventricular surface at early stages of neurogenesis (Franco and Muller 2013; Noctor et al. 2004). However, as neurogenesis proceeds, the IP cells migrate to a distinct proliferation layer adjacent to the VZ, the SVZ. Retroviral labeling and time-lapse imaging in embryonic rodent cortical slice cultures as well as staining for neuron markers was used to demonstrate that IP cells most often undergo one round of symmetric division to produce two neurons (Attardo et al. 2008; Haubensak et al. 2004; Kriegstein and Noctor 2004; Noctor et al. 2008). In contrast to RG cells, IP cells seem to lack apical-basal polarity (Attardo et al. 2008; Kriegstein and Noctor 2004; Miyata et al. 2004; Noctor et al. 2004). The 'two-step pattern' of neurogenesis, involving RG cells and IP cells, appears to be the predominant principle for cortical neurogenesis in rodents (Haubensak et al. 2004; Kriegstein and Noctor 2004; Miyata et al. 2004). It has been suggested that the emergence of the SVZ and its constituent IP cells may have been responsible for the evolutionary increase in cortical thickness and layering that presumably occurred in the interval between a

reptile-like mammalian ancestor and early mammals (Cheung et al. 2007). As mentioned above, neurons are usually born in a position, which differs from their terminal destination. Thus, neurons need to migrate from their place of birth to their final position using several types of cellular mechanisms.

2 The Cellular Mechanisms of Neuronal Migration

The formation of the central nervous system (CNS) depends on two crucial early development events: (1) the proliferation and differentiation of neural stem cells as discussed above, leading to generation of a variety of different types and numbers of neurons; (2) the migration of postmitotic neurons from the VZ and SVZ to appropriate areas and specific locations within the central nervous system where these neurons establish functional neural circuits with each other (Marin and Rubenstein 2003). During central nervous system development, neurons utilize three modes of migration: radial migration, tangential migration, and chain migration (Marin and Rubenstein 2003). In the developing cortex, later-born projection neurons migrate radially along the elongated fiber of radial glia (RG) cells to reach their final destinations at the interface between cortical plate and marginal zone (Hatten 2002; Kriegstein and Noctor 2004; Noctor et al. 2001; Rakic 2007; Tamamaki et al. 2001). Meanwhile, GABAergic cortical interneurons born in the ganglionic eminences migrate tangentially into the developing cortical wall (Anderson et al. 1997; Ang et al. 2003; de Carlos et al. 1996; Marin and Rubenstein 2003). In the postnatal brain, the radial migration mode still remains the primary way for laminar positioning of newly generated granule neurons of the dentate gyrus of the hippocampus (Nowakowski and Rakic 1979). Interestingly, olfactory interneurons migrate from the SVZ of the lateral ventricles in the adult brain to the olfactory bulbs via the rostral migratory stream (RMS), by a specific mode of migration named chain migration (Lois and Alvarez-Buylla 1994; Lois et al. 1996) (see also Chap. 9).

Within the CNS, despite differences in migratory pathways and migration modes among the distinct neuronal subtypes, it is believed that most migratory processes are driven by similar cell-intrinsic mechanisms, and determined by extracellular cues to a large extent. Neurons contain a heterogeneous network of filamentous structures known collectively as the cytoskeleton consisting of the actin microfilaments, the neurofilaments (called intermediate filaments in non-neuronal cells), and the microtubules. Actin is prominent in axons, and is particularly abundant in growing tips of axons, the growth cones. It plays a critical role in orchestrating dynamic changes of cellular morphology. Microtubules form long scaffolds that extend the full length of the neuron, and they also take part in cell movement and cell division. Neurofilaments are the bones of the cytoskeleton and the most abundant fibrillar components of the axon. They are long filaments of approximately 10 nm in diameter, intermediate in size between actin filaments (about 5 nm) and microtubules (about 20 nm). Unlike microtubules, neurofilaments are very stable and remain mostly polymerized within neurons.

Additionally, the extracellular matrix (ECM) acts as a major extracellular signaling mechanism influencing the development of the central nervous system (see also Chap. 9). The ECM is composed of five classes of macromolecules – collagens, elastin, proteoglycans, hyaluronan, and adhesive glycoproteins, such as laminins, reelin, tenascins, etc. The ECM plays important roles during CNS development by acting as a mechanical support, by providing essential survival signals and by regulating neuronal migration, which will be further discussed.

Neuronal migration is a cyclical multi-step process that consists of collectively interrelated but independent discrete events, including four major cell biological stages: polarization, protrusion, adhesion, and retraction (Lauffenburger and Horwitz 1996; Pollard and Borisy 2003; Ridley et al. 2003). Each individual neuron responds to certain extracellular signaling stimuli and the cellular migration process is rapidly initiated. The migration process is coordinated by internal and external signaling mechanisms allowing the cell to form transient specialized structures that

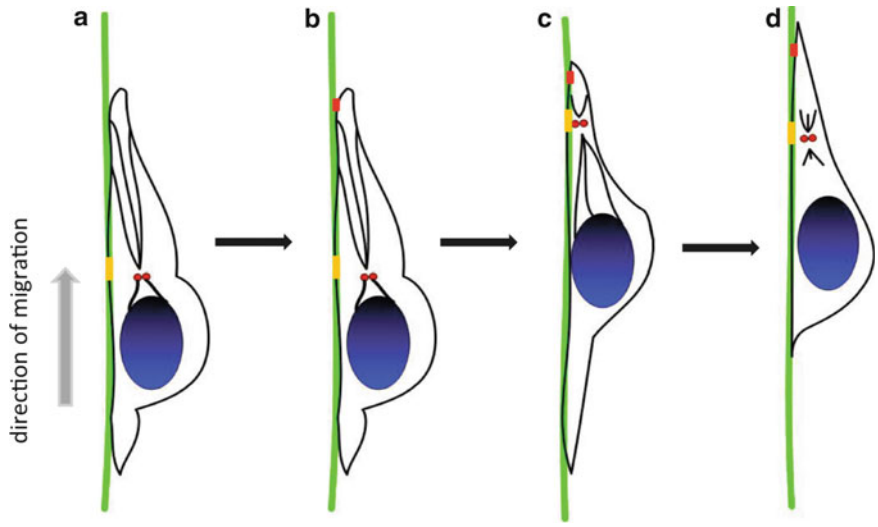

Fig. 2.2 Multi-step process of neuronal migration. (**a**) Cell movement in a certain direction and formation of the leading edge, (**b**) Attachment of the leading edge to surrounding substrates, (**c**) Remodeling of microtubules and nuclear translocation, (**d**) Retraction of the trailing edge

permit each neuron to complete the entire cell migration process. Specifically, the neuron first needs to polarize in a certain direction, to form an active and extensive leading edge that allows the dynamic protrusions of neurite to move forward and initiate the cell migration cycle. Subsequently, somal translocation, the most characteristic feature of neuronal migration, involves two consecutive steps. The first step in somal translocation is the formation of a cytoplasmic swelling in the leading edge, immediately ahead of the nucleus. This cytoplasmic dilatation has been observed in both tangentially and radially migrating neurons (Bellion et al. 2005; Konno et al. 2005; Schaar and McConnell 2005). The second step is nucleokinesis, which repositions the nucleus forward into the cytoplasmic dilatation following the centrosome by coordination of many cytoskeletal and signaling molecules (Bellion et al. 2005; Godin et al. 2012; Solecki et al. 2009; Tsai and Gleeson 2005) (Fig. 2.2). The leading edge of migrating neuron displays diverse morphologies in different neuronal types, for instance, the cortical tangentially migrating interneurons dynamically integrate their two leading edge branches into the migratory cycle (Martini et al. 2009; Okada et al. 2007). In contrast, radially migrating neurons seem to have a single leading edge migrating along radial glia fibers (Gupta et al. 2003; Rakic 1972). Parallel to these events, rearrangements in adhesive complexes that link the ECM to the cytoskeleton can lead to attachment and stabilization of neurons to the surrounding substrates, which is very helpful to move forward. Cells modulate adhesion by controlling the surface density, and state of activation of their adhesion receptors. A variety of extracellular stimuli activate intercellular signaling pathways and cytoskeleton components in neurons, which enhance or inhibit the ligand-binding activity of the adhesion receptors to influence cellular adhesion function. As the cell moves forward, the trailing edge must remain in the rear and retract to enable the cell to advance. During neuronal migration, cytoplasmic rearrangements and organelle repositioning also participate in this process. The nucleus is the most remarkable organelle during the forward movement of the soma, and this process is commonly referred to as nucleokinesis (Bellion et al. 2005; Godin et al. 2012; Solecki et al. 2009; Tsai and Gleeson 2005).

The cytoskeleton is the major intrinsic determinant of the shape and migration mode of a

neuron. Actin filaments play a central role in the leading process formation, intracellular rearrangement events and all of the mechanical steps during the migration cycle, since the actin meshwork can provide the major driving force for cell movement. In addition, other cytoskeletal systems are also required for cell migration. As such, a mechanical role for microtubules is also important during cell movement. For example, microtubules also grow during the elongation of the leading edge, and nuclear movement seems to involve the participation of microtubules (Tanaka et al. 2004). Additionally, microtubules also associate with some important signaling proteins to control neuronal migration. CDK5, a serine/threonine cyclin-dependent kinase, modulates nucleokinesis through phosphorylation of many microtubule-associated proteins, including Lissencephaly (LIS1) and Doublecortin (DCX), which play well-established roles in nucleokinesis (see also Chaps. 1, 5 and 6).

The ECM signals define the timing, the direction, and the final destination for the migrating neurons. The coordination of ECM and cytoskeleton can initiate cell polarization and provide grounds for neuronal migration and lamination by affecting different modes of cellular migration, such as radial, tangential and chain migration, in distinct ways and controlling specific aspects of neuronal migration. For example, lamінins is an ECM glycoprotein that have active roles in promoting neuronal migration through binding to cell surface receptors such as integrin and then transducing information to the cytoskeleton (Belvindrah et al. 2007; Chen et al. 2009; Mobley et al. 2009; Stanco et al. 2009). Reelin is an extracellular molecule secreted by Cajal-Retzius cells, and its binding to its receptors induces a series of phosphorylated signaling cascade that regulates microtubule dynamics and triggers neurons to migrate into their proper destination in the cortex (Beffert et al. 2004; D'Arcangelo et al. 1999; Gonzalez-Billault et al. 2005; Hiesberger et al. 1999; Howell et al. 2000). The Reelin signaling pathway is one of the most well-known signaling mechanisms involved in the assembly of the cortical cytoarchitecture (see also Chaps. 1 and 9). Reelin, the product of the *Reln* gene, is secreted by Cajal-Retzius (CR) cells in the marginal zone (MZ) and binds to the transmembrane lipoprotein receptors APOER2 and VLDL, which are expressed by migrating neurons and RG cells. The promotion of neuronal detachment from RG fibers is a major function of Reelin (Franco et al. 2011). Disruptions in the Reelin signaling pathway in mice cause disorganized lamination while in human causing lissencephaly and cerebellar hypoplasia (Hong et al. 2000). A multistep mechanism has been proposed based on recent research, in which Reelin orients multipolar neurons to polarize their migration by activating RAP1/DAB1, then controlling N-cadherin function to regulate somal translocation (Franco et al. 2011; Jossin and Cooper 2011).

3 The Molecular Mechanisms of Neuronal Migration

Neurons within the developing neocortex find their final destination specifically through a rearrangement of their cytoskeleton in response to extracellular cues mediated by various intracellular signaling pathways (Ayala et al. 2007). In this part, we will discuss the extracellular and intracellular signal mechanisms that regulate the behavior of the cytoskeletal components (Fig. 2.3). Extracellular molecules and surrounding cellular architecture, which function corporately as a microenvironment, play a critical role in neural migration in an inside-out fashion to their respective lamina of neocortex. In the CNS, ECM glycoproteins display a dynamic expression pattern in developing and adult brain (Franco and Muller 2011). The interactions between neuroblasts and ECM molecules are dynamic and mediated via cellular receptors and molecules. In detail, cell surface receptors for ECM are integrins while ECM molecules include Reelin, laminin, proteoglycans and tenascin, which take profound effect in neural migration and lamination in the developing neocortex (Barros et al. 2011). In addition to the glycoproteins mentioned above, the secreted netrins and slits function in axonal outgrowth and guidance,

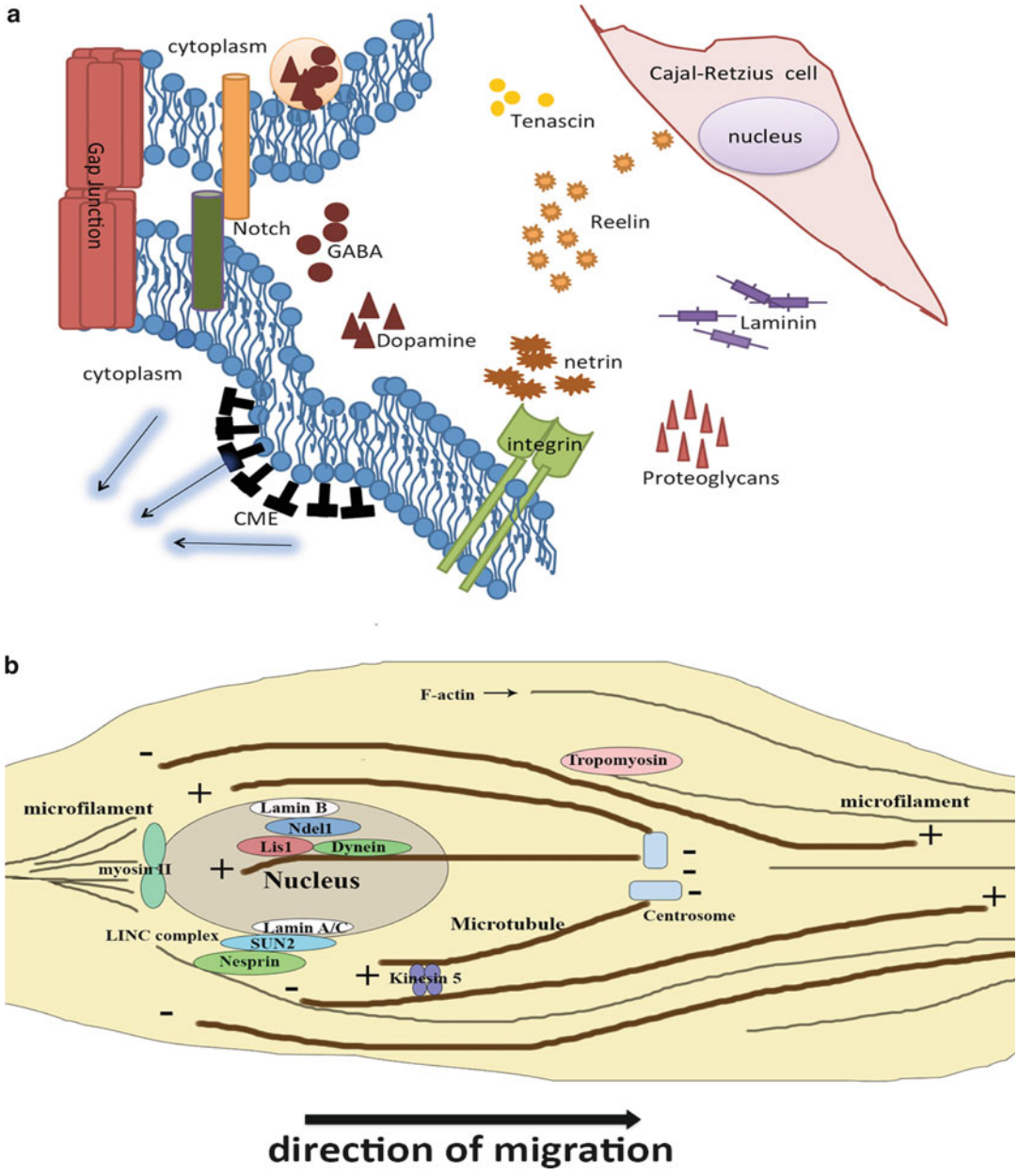

Fig. 2.3 The molecular mechanism of neuronal migration. (**a**) Molecules in ECM secreted by surrounding cells regulate neuronal migration. Notch signaling pathway and endocytosis are involved in some locomotion regulation. (**b**) Cytoskeletons, including microfilament and microtubule, and attached molecules play a role in pushing nucleus and cytoplasm to move forward

controlling cell adhesion, neuronal migration and polarity (Bradford et al. 2009; Ypsilanti et al. 2010).

Cellular communication can influence neuronal migration in the neocortex by regulating neuroblast behavior. Cell communication can occur in a cell-cell contact fashion. It has been suggested that cell-cell communication and/or adhesion through gap-junction-mediated interaction by connexin 43 plays a crucial mechanistic

role in radial migration of cortical projection neurons (Elias et al. 2007); and in switching cell migration from tangential to radial manner to allow interneurons to move to their correct laminar position (Elias et al. 2010). Furthermore, cell communication can also capitalize on paracrine signaling in neocortical migration. Chemokines, a family of secreted cytokines, function in neuronal migration during developing brain in addition to their roles in pathological states. To give an example, stromal cell-derived factor 1 (SCD1) reinforces the motility of neuroblasts, migrating from the SVZ towards the olfactory bulbs by upregulating epidermal growth factor receptor (EGFR) and α6 integrin in nearby cells, resulting in enhancement of their ability to bind to laminin in the vascular niche (Asensio and Campbell 1999). Moreover, neurotransmitters are implicated in functioning in modulating the migration of cortical neurons as well (Heng et al. 2007). Dopamine can influence tangential migration of cortical GABAergic neurons by redistributing cytoskeletal elements (Bhide 2009; McCarthy et al. 2007). GABA transiently released near target destinations for migrating neurons, acts as a chemoattractant during corticogenesis by modulating cortical neuronal movement via multiple classes of receptors (Owens and Kriegstein 2002). Blockage of $GABA_B$ receptors with a specific antagonist, results in altered tangential migration of cortical interneurons (Lopez-Bendito et al. 2003). TorsinA plays a role in GABAergic neuron migration in the embryonic brain by tethering the nucleus to the cytoskeleton (McCarthy et al. 2012).

The intracellular mechanisms initiated by the extracellular cues are in critical operation during neocortical migration. In addition to the signaling pathways mentioned above, there are additional critical intracellular signal pathways. Notch signaling, which is widely known as a vital regulator of neural stem cells and neural development, affects neuronal migration by altering the morphology of migrating neurons. Increased Notch signaling leads to a bipolar morphology that favors migration, while decreased Notch signaling leads to a more multipolar morphology that stalls migration (Hashimoto-Torii et al. 2008). Notch signal regulates neural migration in different patterns. One scenario involves a Reelin signaling related mechanism, and another possible mechanism involves the regulation of microtubule dynamics (Ables et al. 2011). A recent study further indicates that a Reelin-Notch crosstalk is required during cortical neuron migration (Hashimoto-Torii et al. 2008). In addition, endocytosis is also involved in neocortical migration. Clathrin–mediated endocytosis (CME) takes effect in regulating substrate detachment to enable soma translocation in migrating neurons by modulating the subcellular distribution of cell adhesion proteins at the neuroblast surface (Shieh et al. 2011).

Actin filaments, one of the major components of cytoskeleton, are the structural element of the lamellipodia and filopodia as mentioned above. Thus, actin can modulate the leading process of migrating neurons. Molecules such as tropomyosin could regulate the polymerization and depolymerization of actin and thus influence neuronal migration. In addition, actin also affects nuclear movement in many kinds of cells including the developing neurons (Luxton et al. 2011). But how these molecules mediate the contact between actin and the nucleus remains still unclear. A very important motor protein which could bind to F-actin named myosin II has been shown to influence neuronal migration (Vallee et al. 2009). Researchers who used blebbistatin, the inhibitor of myosin II, have found that actomyosin filaments are accumulated at the rear of nucleus (Bellion et al. 2005; Vallee et al. 2009). These results showed that myosin II may push nucleus to move forward and control the nuclear movement. Also, diabetes mellitus condition influences the brain function by modifying expression of myosin II (Calabria et al. 2011). Recent studies further indicate that a protein complex named linker of nucleoskeleton and cytoskeleton (LINC) complex, which is composed by nesprin proteins and SUN proteins, could connect with both actin and nucleus (Luxton et al. 2011). Maybe this complex constitutes the key that could explain how actin could drive nuclear movement.

Microtubules could affect nuclear movement by mutual antagonistic motors. Cytoplasmic

dynein, a motor protein, moves toward the minus-end of microtubules (Vallee et al. 2009) and can be regulated by LIS1 (see also Chap. 1). LIS1 is encoded by the *LIS1* gene and can cause Lissencephaly when mutated in human. Both LIS1 and cytoplasmic dynein can interact with the evolutionary conserved protein NDEL1 (Chansard et al. 2011). NDEL1 can recruit LIS1 and dynein to the nuclear envelope (Chansard et al. 2011; Vallee et al. 2009). This microtubule-LIS1-cytoplasmic dynein complex could link to SUN-nesprin complex and accelerate nuclear movement. In certain instances, neurons need to retard this movement. A slow motor molecule called kinesin-5 is thought to be the brake of nuclear movement (Falnikar et al. 2011). Thus, several motor proteins modulate the nuclear movement.

4 Clinical Perspectives – Neuronal Migration Diseases

Neuronal migration is a critical process in cortical development and the defects in neuronal migration can lead to devastating brain diseases. For example, patients suffering from Lissencephaly harbor cortical malformations resulting from defective radial neuron migration and failure of cortical fold formation (see also Chaps. 1 and 5). As discussed earlier, the loss function of *LIS1* is a major cause of lissencephaly, leading to abnormal nuclear translocation during neuronal migration and hence impairing brain gyrus formation (LaMonica et al. 2012; Wu and Wang 2012). Mutation of Reelin, a molecule that plays a critical role in neuronal migration regulation during cortical development as discussed above, also lead to lissencephaly (Hong et al. 2000). In addition, various mutation of Reelin have also been reported in neuropsychiatric disorders, including schizophrenia, bipolar disorder and autism, and neurodegenerative disease, like Alzheimer's disease (AD) (Botella-Lopez et al. 2006; Chin et al. 2007; Kelemenova et al. 2010; Nahin et al. 1991; Persico et al. 2001; Rogers et al. 2011).

5 Neuronal Migration and Therapeutic Strategies for Brain Disorders

Although not many neurons migrate in the adult brain, cellular migration research is still important with respect to stem cell therapies for some brain diseases. Neural stem cells have been considered as potential and effective methods for neurodegenerative diseases and CNS injury due to their great abilities to proliferate and differentiate into specific terminal cell types. There are numerous studies using animal models, such as rodents and primates, showing that transplantation of neural stem cells could be effective to some extent in the treatments of Parkinson's disease (PD), Amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD) and stroke (Blurton-Jones et al. 2009; Darsalia et al. 2007; Kelly et al. 2004, 2005; Kim et al. 2006; Redmond et al. 2007; Takagi et al. 2005). Defining the experimental conditions to promote stem cells migration to the desired regions is one of the most important challenges that need to be overcome for the success of neurological diseases therapies. One can expect that efficient migration to the desired destinations would favor appropriate neural stem cell differentiation, and integration into neuronal circuits. Indeed, the regulation of stem cell migration involves the contribution of ECM components and intracellular signals, including some members of the cytokine family. Stem cell factor (SCF), originally was characterized as a molecule playing an important role in the regulation of hematopoiesis, spermatogenesis and melanogenesis during development. Recent further studies have revealed that both *SCF* messengers and proteins are highly expressed in neurons at injured sites in the brain. SCF signals promote neural stem cell migration to lesions *in vitro* and *in vivo* via the activation of its receptor c-kit (Jin et al. 2002; Sun et al. 2004). In a rodent model of Huntington's disease, the activation of SCF and c-kit signaling pathways are required for transplanted NSCs to migrate to the diseased striatum (Bantubungi et al. 2008).

In a rat model of stroke, human neural stem cells are able to migrate to the ischemic lesions and differentiate into neurons after transplantation (Darsalia et al. 2007; Kelly et al. 2004). Recently, further studies in stem cell therapy for stroke have demonstrated that pre-differentiated brain-derived adult human progenitor cells migrate more efficiently to the stroke damaged area after transplantation *in vivo*, due to the signaling of chemokine receptor 4 (CXCR4) and its ligand, stromal cell-derived factor-1α (SDF-1α), which are both highly expressed in pre-differentiated cells and ischemic regions respectively (Olstorn et al. 2011).

With the rapid emergence of sophisticated molecular biological cell-label techniques and powerful cell-tracing imaging systems, future studies on the regulation of neural migration in embryonic (developing) and adult (mature) CNS will not only reveal the fundamental underlying basis of pathological neurological disorders, but also open new avenues to find prospective candidate drugs and therapeutic strategies for CNS diseases and injury.

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The Impact of JNK on Neuronal Migration

3

Justyna Zdrojewska and Eleanor T. Coffey

Abstract

Incorrect placement of nerve cells during brain development leaves us at risk of diseases and conditions ranging from epilepsy and mental retardation to schizophrenia and dyslexia. The developing brain produces cells at an impressive rate, with up to 250,000 new cells generated every minute. These newborn cells migrate long distances in sequential waves to settle in the layers that make up the cerebral cortex. If a nerve cell moves too fast or too slow during this journey, it may not take the correct route or reach its appropriate destination. Much knowledge has been accumulated on molecular cues and transcriptional programs regulating cortical development. More recently, components of the c-Jun N-terminal signaling cascade have been brought to light as important intracellular regulators of nerve cell motility. In this chapter, we focus on this family of protein kinases, their upstream activators and downstream targets in the context of neuronal migration. We first present basic information on these molecules, much of which derives from studies outside the nervous system. We then highlight key findings on JNK signaling in brain where it phosphorylates brain-specific proteins that influence microtubule homeostasis. Finally, we summarize recent findings from transgenic mice on the regulation of neuronal migration by JNK cascade components and by JNK substrates.

Keywords

Radial migration • JNK • Stathmin • Microtubule-associated Proteins (MAPs) • Mitogen-activated Protein Kinase (MAPKs)

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L. Nguyen and S. Hippenmeyer (eds.), *Cellular and Molecular Control of Neuronal Migration*,
Advances in Experimental Medicine and Biology 800, DOI 10.1007/978-94-007-7687-6_3,

1 The JNK Family Protein Kinases – Basic Pathway Components

The JNK family of protein kinases consists of proline-directed kinases that transfer phosphate to serine or threonine residues that immediately precede a proline. JNKs belong to the large group of mitogen activated protein kinases (MAPKs) (Fig. 3.1). Although JNKs are phylogenetically more distant from ERKs than are the p38s, there is approximately 40 % sequence identity between JNK and ERK MAPKs (Kyriakis et al. 1994). The MAPKs in turn belong to the CMGC group (containing Cyclin dependent kinases (CDKs), MAPKs, Glycogen synthase kinase-3 (GSK-3) and CDK-Like kinases) (Manning and Davis 2003). JNK was originally discovered as a stress-induced protein kinase activity isolated from liver of cycloheximide-challenged rats (Kyriakis and Avruch 1990). Its name was later changed to JNK when it was revealed that it specifically phosphorylated the transcription factor c-Jun on serine 63 and serine 73, sites that induced AP1 transcriptional activity (Hibi et al. 1993; Dérijard et al. 1994). Indeed the stress-associated function of JNK was studied intensively for some years in the context of cell stress and apoptosis, and much basic information on JNK cascade components as well as information on substrate recognition derived from these studies. We now know that the mammalian genome encodes three *Jnk* genes, *Jnk1 (Mapk8)*, *Jnk2 (Mapk9)* and *Jnk3 (Mapk10)* that are alternatively spliced within the catalytic domain between subdomains IX and X (producing α and β splice variants), and at the C-terminus producing long (54 kDa) and short (46 kDa) forms (Kyriakis and Avruch 2012). In humans the *JNK* genes are located on chromosomes 10, 5 and 4 for *JNK1*, *JNK2* and *JNK3* respectively. In mouse however they localise to chromosomes 14, 11 and

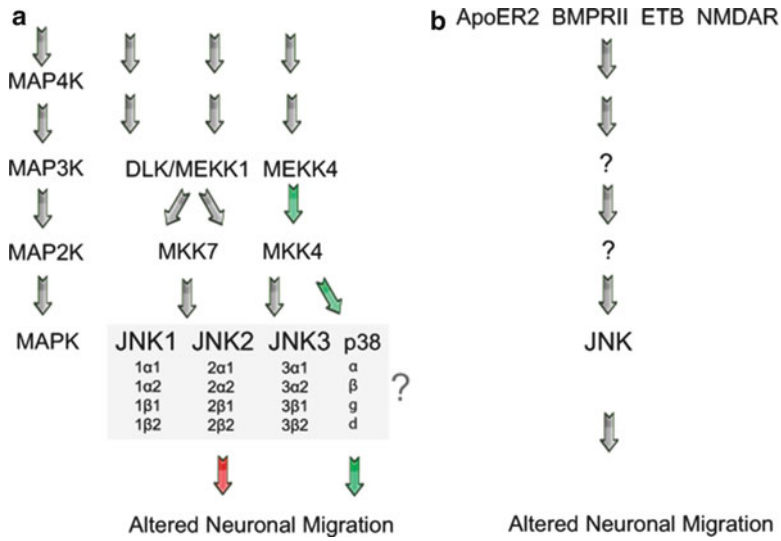

Fig. 3.1 Scheme of the JNK cascade and the upstream components that regulate cell migration during cortical development. (**a**) JNKs are the effector kinases that reside at the end of a classical MAPK signalling cascade. Studies in transgenic mice have indicated a role for DLK and MEKK4 in the regulation of neuronal migration, and for MEKK1 in the regulation of epithelial cell migration during eye development. MEKK4 signals via p38 to positively regulate neuronal migration (*green arrow*), in contrast JNK1 slows (*red arrow*) the rate of multipolar and bipolar cell migration. The MAPK splice variants involved in migration regulation are not known, though multiple forms are expressed in brain. (**b**) Depicted in the scheme are those receptors that activate JNK in neuronal systems in various contexts and may contribute to JNK regulation during neuronal migration. ApoER2 is the Reelin receptor that couples to JNK (Trommsdorff et al. 1999), BMP7 binding to BMPRII and activates JNK (Podkowa et al. 2010), endothelin-1 activates JNK via the ETB receptor in developing brain (Mizuno et al. 2005). NMDA receptor activation also induces JNK activity in neurons (Mukherjee et al. 1999)

5 and in rat to chromosomes 16, 10, 14 (Haeusgen et al. 2011). JNKs act as the final effector kinases within a classical protein kinase cascade consisting of MAPKKKs (MAP3Ks), MAPKKs (MAP2Ks) and MAPKs (Johnson and Nakamura 2007; Kyriakis and Avruch 2001). Like other MAPKs, JNKs are activated by phosphorylation of two residues within the activation loop of the kinase. For JNKs the conserved activation sites are T183 and Y185. Phosphorylation of the TPY motif is catalyzed only by MKK4 and MKK7. MKK4 is a dual specificity kinase that shows preference for phosphorylation of Y185 of JNK, while MKK7 phosphorylates T183 (Wada et al. 2001; Kishimoto et al. 2003). It is important to note that MKK4 can also phosphorylate and activate p38. Further signalling diversity is conferred by the existence of several splice variants each, two variants of MKK4 and six variants of MKK7. While MKK4 and MKK7 splice forms show different properties in terms of JNK TPY phosphorylation and subsequent kinase activation in cell lines (Wada et al. 2001; Kishimoto et al. 2003), we do not yet understand how they are coordinated to regulate JNK in the nervous system. Activation of MKK4 and MKK7 is achieved through phosphorylation by a number of MAP3Ks that are expressed in brain and can be divided into several families such as MEKKs, mixed lineage kinases (MLKs), dual leucine zipper kinase (DLK), apoptosis signal-regulating kinases (ASKs), leucine zipper bearing kinase (LZK), transforming growth factor β-activated kinase (TAK), and these are in turn activated by MAP4Ks including hematopoietic progenitor kinase (HPK) and germinal center kinase (GCK/MINK1) (Bogoyevitch and Kobe 2006; Kyriakis and Avruch 2012).

2 JNKs Regulate Cell Death in Developing and Ageing Brain

In non-neuronal cells JNK activity is low and triggered in response to stressful stimuli. However, in primary neuron cultures and in brain tissue JNK activity is anomalously high even in the absence of stress (Coffey et al. 2000; Coffey and Courtney 1997; Hu et al. 1997). This elevated activity in brain derives mainly from JNK1 isoforms as 50 % of resting JNK activity is lost in the cortex of *Jnk1*$^{-/-}$ mice, while JNK2 and JNK3 isoforms contribute less (Tararuk et al. 2006). Precise measurements of stress responsive JNK in neuronal systems are for this reason compounded by contaminating constitutive kinase activity. However, JNK2/3 isoforms have been isolated using isoform-specific antibodies and shown to be activated by stress in the *trophic factor deprivation* model of developmental death in neurons (Coffey et al. 2002). JNK3 has been implicated in excitotoxic damage in brain and *Jnk3*$^{-/-}$ adult mice show reduced sensitivity to kainate-induced seizures (Yang et al. 1997b; Kuan et al. 2003; Pirianov et al. 2007) while *Jnk3*$^{-/-}$ neonates are less susceptible to hypoxia-ischemia induced damage (Pirianov et al. 2007). Furthermore, cortical and hippocampal neurons derived from *Jnk3*$^{-/-}$ mice are protected from beta-amyloid-induced death (Morishima et al. 2001). In the peripheral nervous system, *Jnk3*$^{-/-}$ neurons are protected from axotomy-induced death and JNK pathway inhibitors protect in models of Parkinson's disease (Keramaris et al. 2005; Maroney et al. 1999; Brecht et al. 2005). Studies evaluating neuronal death during development, have shown that all JNKs (1, 2 and 3) contribute to toxicity, and significant protection requires silencing of all three genes (Björkblom et al. 2008). Moreover, the JNK substrates mediating neuronal death are not yet formally identified. While increased phosphorylation of JNK substrates c-Jun and ATF2 accompanies JNK activation in a range of neuronal death models, efficient knockdown of *c-Jun* or *Atf2* in neurons does not protect from death (Björkblom et al. 2008), and c-Jun phosphorylation was unchanged in *Jnk1/2*$^{-/-}$ double mutants suggesting that c-Jun phosphorylation by JNK is not essential for brain morphogenesis and the accompanying apoptosis changes (Behrens et al. 1999; Sabapathy et al. 1999). Although it is not easy to formulate a simple model to explain how JNKs contribute to both pathological and physiological functions, for example based on isoform dependence alone, the signaling complexity does not take away from the wealth of evidence that indicates that these kinases are major effectors of cell death in

the nervous system. Indeed, inhibition of JNK activity is elevated in post-mortem brain from patients with Parkinson's Disease and Dementia with Lewy bodies and in Alzheimer's patients (Ferrer et al. 2001, 2002), and inhibition of JNK provides neuroprotection in an extensive range of neuronal death models (Waetzig et al. 2006).

3 Developmental Expression in the Nervous System

Jnk1 and *Jnk2* are expressed ubiquitously throughout the body including brain, while *Jnk3* expression is almost exclusively expressed in brain with low levels found in heart and testis (Kuan et al. 1999). *Jnk1*, *2* and *3* mRNAs are high in embryonic brain (Kuan et al. 1999) and in adult brain JNK1 protein expression is elevated in the cortex, hippocampus and striatum while JNK3 expression is high in the hippocampus, lower levels are detected in the cortex, striatum and cerebellum (Brecht et al. 2005). Directly upstream from JNK, *Mkk4* and *Mkk7* mRNAs are ubiquitously expressed in adult mouse brain, whereas in the developing nervous system, *Mkk4* appears before E10 after which it is also expressed in liver and thymus (Wang et al. 2007c). As development proceeds MKK4 protein levels increase in brain, reaching a stable level in adulthood, while MKK4 simultaneously decreases in the liver and thymus (Lee et al. 1999). In contrast, MKK7 shows wide tissue expression both in during development and in adult. However at later stages of embryogenesis MKK7 levels in brain, skin and hair follicle increase (Yao et al. 1997).

4 Lessons Learned from Genetic Disruption of JNKs and Upstream Kinases in Mice

4.1 *Jnk1* and *Jnk2* Regulate Early Brain Morphogenesis

Genetic ablation of *Jnk1* and *Jnk2* in mice results in embryonic death at around E11.5 with defective neural tube closure (Kuan et al. 1999). These mice show regional specific apoptosis with decreased cell death in the hindbrain neuroepithelium just prior to neural tube closure, and increased apoptosis and caspase-3 activation in the forebrain (Sabapathy et al. 1999; Kuan et al. 1999). Neural tube closure is a process that requires cell migration, cell death and cell proliferation. However, major defects in neural crest cell migration were not described in *Jnk1/2*$^{-/-}$ double mutants (Kuan et al. 1999; Sabapathy et al. 1999). Similarly the number of BrdU positive cells (reflecting the proliferating population), were unchanged and the exencephaly phenotype (where the brain is completely exposed or protrudes outside of the skull) was therefore attributed to deregulated apoptosis. The open neural tube phenotype showed a gene dosage effect since *Jnk1*$^{-/-}$*;Jnk2*$^{+/-}$ mice displayed exencephaly, while *Jnk1*$^{+/-}$*;Jnk2*$^{-/-}$ mice did not (Sabapathy et al. 1999), indicating that JNK1 plays a dominant role in regulating the events underlying neural tube closure (Sabapathy et al. 1999). These studies marked a turning point in the field of JNK research as they established an important role for JNK function in the developing nervous system and demonstrated a critical role in regulation of cranial morphogenesis. Subsequent work has shown that JNKs are fundamentally important for migration of several cell types including epithelial cells (Huang et al. 2004; Yamasaki et al. 2012). Therefore, the possibility that migration defects contribute to the exencephaly phenotype of *Jnk1/2*$^{-/-}$ double mutant mice cannot be excluded. Indeed these studies focused on quantitative measures of apoptosis and qualitative measures of cell proliferation. A more detailed analysis of cell migration in *Jnk1/2*$^{-/-}$ double mutants during early embryogenesis may therefore be worth a re-visit.

It is notable that the classic phenotypic neural tube defect observed in *Jnk1/2*$^{-/-}$ double mutant mice is not replicated by genetic ablation of either of the upstream regulators *Mkk4* or *Mkk7* (Table 3.1) (Wang et al. 2007b, c; Wada et al. 2004), while double knockout mice die at E8.5 prior to neural tube closure. Thus the activation of JNK, in the context of neural tube closure, may

Table 3.1 Summary of the major phenotypes in *Jnk* and *Map2k* transgenic mice

	Jnk1$^{-/-}$	*Jnk2*$^{-/-}$	*Jnk3*$^{-/-}$	*Jnk1*$^{-/-}$;*Jnk2*$^{-/-}$	*Mkk4*$^{-/-}$	*Mkk4*$^{flox/flox}$; *Nestin-Cre*	*Mkk7*$^{-/-}$	*Mkk7*$^{flox/flox}$; *Nestin-Cre*
Phenotype	Viable. Disorganized neuron positioning in cortex, increased multipolar and bipolar motility rate[a]. Decreased duration of multipolar stage[a]. Anterior commissure lost by 3 months[b] Altered dendrite morphology[b,c]	Viable. No overt brain malformations	Viable. No overt brain malformations	E11.5 lethal Impaired neural tube closure[d]	Die E11.5-E13.5 from anemia and abnormal hepatogenesis[g]	Irregular Purkinje cell positioning and delayed radial migration[i] Degeneration of the anterior commissure in adult mouse[i]	E 11.5-E12.5 lethal[j]	Enlarged ventricles by E18.5. Diminished striatum, disturbed development of axonal tracts[l] Reduced neurite length at E15.5[l]. Altered radial migration l (Yamasaki et al. 2011)
Cell death proliferation	A small decrease in cell cycle exit in embryonic brain[a]	Resistance to MPTP toxicity[e]	Resistance to MPTP and kainate induced neurotoxicity and seizures[e,f]	Increased death in hindbrain, decreased death in forebrain at E11.5[d]	Increased hepatocyte apoptosis and liver degeneration[g]		Decreased cell proliferation and defective liver formation with reduced parenchymal hepatocyte number[j]	Increased presence of autophagic vacuoles[l]
Tissue expression profile of respective MAPK/MAP2K	*Jnk1* from E7 onwards in different tissues including brain, heart, liver, lung[d]	*Jnk2* E7 onwards in brain, heart, lung, liver[d]	*Jnk3* E11 onwards in brain and lower levels in heart and testis[d]	*Jnk1/Jnk2* E7 onwards in various tissues including brain, heart, liver, lung[d]	*Mkk4* expressed by E10 in CNS. E12 onwards, transcripts in liver, heart, immune system[h]		*Mkk7* is present in embryonic brain in epithelial tissues[k]	

[a]Westerlund et al. (2011), [b]Chang et al. (2003), [c]Björkblom et al. (2005), [d]Kuan et al. (1999), [e]Hunot et al. (2004), [f]Yang et al. (1997b), [g]Ganiatsas et al. (1998), [h]Lee et al. (1999), [i]Wang et al. (2007c), [j]Wada et al. (2004), [k]Yao et al. (1997), [l]Yamasaki et al. (2011)

require a coordinated regulation by both MKK4 and MKK7, or alternatively may involve an unknown mechanism.

The JNK substrates contributing to cranial morphogenesis have not been clarified though transcription factor targets of JNK could be central. Mice null for the JNK substrates *c-Jun* or *Atf2* do not display defects in cranial morphogenesis indicating that they either play redundant roles in this process or none at all. Interestingly, *c-Jun*$^{-/-}$ mice do share the *Mkk4* and *Mkk7* phenotype of deregulated hepatocyte proliferation, while *Atf2*$^{-/-}$ mice die at birth with respiratory defects (Eferl et al. 1999; Maekawa et al. 1999). More recently, a screen for novel JNK substrates in brain revealed an actin regulatory protein (required for neural tube formation) which is myristoylated Alanine Rich C-Kinase Substrate Like protein-1 (MARCKSL1), also known as F52, Mac MARCKS and MRP (Björkblom et al. 2012). MARCKSL1 is phosphorylated on three sites by JNK and genetic deletion of MARCKSL1 results in exencephaly (Wu et al. 1996; Björkblom et al. 2012). MARCKSL1 is the only known JNK substrate to date that phenocopies *Jnk1/2*$^{-/-}$ double mutant mice.

4.2 JNK1 Regulates Multipolar Stage Exit and the Rate of Movement of Multipolar and Bipolar Cells in Developing Cortex

Movement of cells from their place of birth to their final destination is a fundamental feature of brain morphogenesis. The speed at which neurons move to form the layers of the cortex could affect their final placement. The role of JNK in regulating the rate of nerve cell movement has been examined in the context of multipolar cell migration and bipolar cell locomotion and is discussed in the following paragraph. The multipolar phase occurs when the precursors of pyramidal neurons in the intermediate (IZ) and subventricular (SVZ) zones take on a multipolar shape and display a movement that is characterized by frequent changes in direction and a slower speed compared to locomoting cells moving on radial glia processes (Tabata and Nakajima 2003). The molecular mechanisms of multipolar migration and multipolar-to-bipolar transition have only recently received attention, as described in more detail below (Westerlund et al. 2011; Tabata et al. 2009; Pacary et al. 2011). At the level of gene regulation, transcription factors determining glutamatergic neuron differentiation are known as gene regulators of multipolar transition. Recently a role for the transcriptional repressor RP58 governing multipolar-to-bipolar conversion has been described. RP58 represses *Ngn2* transcription and thereby regulates the *Ngn2-Rnd2* pathway (Geisen et al. 2008; Nóbrega-Pereira et al. 2008; Ohtaka-Maruyama et al. 2013) (see also Chap. 1).

JNK1 is directly implicated in regulation of multipolar phase exit and neuronal migration during cortical development (Westerlund et al. 2011). This study used *in utero* electroporation to label neuroepithelial progenitors at E15.5 and demonstrated that GFP-expressing neurons in *Jnk1*$^{-/-}$ mice migrated faster than those in wild-type mice. *Ex vivo* imaging in organotypic slices demonstrated a bipolar cell movement rate of 5–10 μm per hour in wild-type compared to 15–20 μm per hour in *Jnk1*$^{-/-}$ cortex. The multipolar cells also moved at higher speeds and transit more quickly to the bipolar phase with subsequent pial-directed migration (Westerlund et al. 2011). This was illustrated by a relatively large ratio of bipolar to multipolar cells in the IZ and reduced duration of the multipolar phase. Furthermore, the JNK binding domain (JBD) of JIP [JIP1a(1–277)], a peptide inhibitor of JNK that binds tightly to JNK and prevents substrate binding (Dickens et al. 1997), was used in cortical neurons. Neurons expressing GFP-JBD *in vivo* reached the cortical plate (CP) faster than in control mice (Mizuno et al. 2005; Westerlund et al. 2011). Despite the altered migration speed, no overt lamination defect was detected in *Jnk1*$^{-/-}$ mice, in contrast to *Cdk5*$^{-/-}$ or *p35*$^{-/-}$ mice that exhibit disturbed layering (Gilmore et al. 1998; Gupta et al. 2003). However, in *Jnk1*$^{-/-}$ mice, the final cell positioning was quite markedly disorganized and crowded (Westerlund et al. 2011).

This mal-positioning may be a consequence of the unusually rapid transit through the multipolar phase, when it is most probable that choices are made to influence the final cellular placement. This could include selection of the appropriate radial glial scaffold for instance. The findings from *Jnk1*$^{-/-}$ mice compare strikingly well to those obtained with *Reeler* mice (Britto et al. 2011), and are discussed in more detail later in this chapter. These results thus suggest that JNK1 may be a conveyor of Reelin's directive on cell positioning in the developing cortex.

While it is not clear whether JNK2 or JNK3 regulate neuronal migration, it is worth noting that opposing results were obtained using the cytosolic or nuclear-targeted JBD inhibitor. Inhibition of JNK in the cytoplasm using compartment specific inhibitors (Björkblom et al. 2005; Tararuk et al. 2006) led to an accelerated migration phenotype, thereby mimicking *Jnk1*$^{-/-}$ mice, while inhibition of nuclear JNK had the opposite effect (Westerlund et al. 2011). These results suggest that in addition to cytosolic targets, JNK controls a gene transcription program that is required for migrating neurons, while in the cytoplasm JNK phosphorylates proteins that slow down migration. Thus the subcellular location of activated JNK is consequential in terms of functional outcome.

4.3 Other Evidence for JNK Regulation of Radial Migration

Interestingly, while *Jnk1*$^{-/-}$ mice present with accelerated multipolar and radial migration (Westerlund et al. 2011), Mizuno and colleagues obtained similar results from neuronal progenitor cells *in vitro* and in developing brain (Mizuno et al. 2005). They found that the vasoactive peptide endothelin-1 induced JNK activity via the Gq heterotrimeric G protein, which activates phospholipase C. They further showed that endothelin-evoked inhibition of radial migration was reversed when the JNK inhibitor JBD was expressed.

4.4 Upstream of JNKs, MAP2Ks Regulate Liver Development and Cortical Migration

Upstream regulators of JNK, MKK4 and MKK7 play essential roles in liver morphogenesis. In contrast to *Jnk* knockout mice, genetic disruption of *Mkk4* or *Mkk7* results in defective liver development and poor survival as embryos die between E11.5 and E12.5, displaying defects in hepatocyte proliferation (Table 3.1) (Ganiatsas et al. 1998; Nishina et al. 1999; Yang et al. 1997a; Wada et al. 2004). Strikingly, the *Mkk4*$^{-/-}$ and *Mkk7*$^{-/-}$ phenotypes match closely that of *c-Jun*$^{-/-}$ mice (Eferl et al. 1999). However, they diverge noticeably from *Jnk1/2*$^{-/-}$ double mutant mice which do not show liver morphogenesis defects, but rather impairment of cranial morphogenesis (Kuan et al. 1999). Early embryonic lethality has prevented more detailed study of the nervous system from *Mkk4*$^{-/-}$ mice and targeted deletion strategies have therefore been applied. Targeted disruption of *Mkk4* and *Mkk7* was accomplished in the nervous system using transgenic *Nestin–cre* mice. In the case of *Mkk4*, this resulted in substantial but incomplete loss of MKK4 expression in brain (Wang et al. 2007c). Constitutive JNK activity was reduced postnatally by 80 % and p38 activity by 25 %, indicating a significant impact on downstream signaling events in the postnatal brain. Newborn *Mkk4*$^{flox/flox}$;*Nestin–Cre* mice did not exhibit overt differences to their wild-type littermates, however they stopped growing a few days after birth and died within 3 weeks showing defects in growth, balance and righting reflex (Wang et al. 2007c). Substrate phosphorylation was correspondingly reduced in these mice. MAP1B phosphorylation decreased from postnatal day 5 onwards and neurofilament light chain phosphorylation decreased from postnatal day 10 onwards. By adulthood, the mice display axonal tract defects. Specifically, fasciculation of the anterior commissure and corpus callosum is substantially disrupted. While the telencephalon was not studied in great detail, defective positioning of Purkinje cells in the cerebellum was noted.

To further examine MKK7 function in the nervous system, *Mkk7*$^{flox/flox}$; *Nestin–Cre* mice were generated with specific deletion of *Mkk7* in neural stem cells (Yamasaki et al. 2011). MKK7 expression was significantly reduced from E16.5 onwards, while there was a compensatory gain in MKK4 expression. *Mkk7*$^{flox/flox}$; *Nestin–Cre* mice were comparable to wild-type mice up to E18.5 but as with *Jip3*$^{-/-}$ mice, they died due to failed respiration (Kelkar et al. 2003). Examination of embryonic brains revealed serious defects in axonal tract formation (reduced size of the corpus callosum, anterior commissures and internal capsule). Electron microscopy examination of axons in *Mkk7*$^{flox/flox}$; *Nestin–Cre* mice revealed defects in neurofilaments and the presence of autophagic vacuoles and swollen mitochondria. Consistent with the neurofilament defects, neurons isolated from *Mkk7*$^{flox/flox}$; *Nestin–Cre* showed reduced axon length (Yamasaki et al. 2011). In line with this observation, inhibition of JNK in cultured cortical and hippocampal neurons retards axodendritic growth (Tararuk et al. 2006; Oliva et al. 2006) and *Jnk1*$^{-/-}$ mice show disrupted axon tracts (Chang et al. 2003). Detailed inspection of the brain tissue revealed enlarged ventricles, reduced striatum, and severe defects in axon formation. The features were not observed in the *Mkk4*$^{flox/flox}$; *Nestin–Cre*, thereby implying that MKK4 and MKK7 can exert functionally distinct outcomes. Moreover genetic disruption of *Mkk7* in the nervous system moderately altered radial migration.

In conclusion, disruption of either *Mkk4* or *Mkk7* in the CNS, leads to a reduction in JNK activity in the brains of these mice and reduced phosphorylation of the JNK substrates c-Jun, MAP1B and NFL. However, the temporal influence of these kinases on substrate phosphorylation differs, MKK7 playing a critical phosphorylation role at earlier developmental time points. These studies also show that MKK7 and MKK4 can play differential roles in directing JNK substrate phosphorylation, MKK7 is a critical upstream component in DCX phosphorylation while MKK4 seems to play no role in targeting JNK towards this substrate (Wang et al. 2007c; Yamasaki et al. 2011).

4.5 MEKK1 and DLK Regulate Migration Upstream of MAP2K

Several upstream regulators of JNK signalling were identified, most of which have not been systematically studied in the nervous system. Among those studied however, MEKK4 and DLK stand out because genetic deletion of these genes results in defective cell migration during cortical formation. MEKK1 (MEK kinase 1) is a MAP3K that directly phosphorylates and activates MKK4 and MKK7, though it has a preference for MKK4. MEKK1 therefore activates p38 and JNK but also ERK (Kyriakis and Avruch 2012). MEKK1 is essential for embryonic stem cell migration, keratinocyte migration. Mice lacking MEKK1 display impaired eyelid closure due to disturbed epithelial cell movement, these mice are born with open eyes (Xia et al. 2000; Xia and Kao 2004). JNK activity is reduced by 50 % in *Mekk1*$^{-/-}$ mice and JNK is thought to contribute to the eyelid closure phenotype (Takatori et al. 2008). MEKK1 co-localizes with α-actinin along actin stress fibers (Christerson et al. 1999) suggesting an involvement of the actin cytoskeleton in MEKK1-regulated cell movement. On the other hand, MEKK1 activates JNK in cells treated with microtubule toxins and targeted disruption of *Mekk1* in embryonic cells results in loss of JNK function and increased apoptosis, suggesting that MEKK1 can protect cells from apoptotic death (Yujiri et al. 2000), possibly as a consequence of disturbed microtubule integrity. While MEKK1 can regulate migration in non-neuronal cells via JNK (Xia and Kao 2004; Xia et al. 2000), there is no evidence that MEKK1 regulates migration in neurons.

MEKK4, like MEKK1, is a MAP3K that can regulate JNK and p38 activity (Takekawa et al. 2005). Mice lacking *Mekk4* develop neural tubes defects including cranial exencephaly, spina bifida and curly tail. They also present with severe periventricular heteropia (Chi et al. 2005). The activity of MKK4 was decreased in these embryos, as was p38, however JNK activity remained unchanged. *Mekk4*$^{-/-}$ embryos showed massively elevated apoptosis before and during

neural tube closure. Rakic and colleagues subsequently showed that siRNA knockdown of *Mekk4* using siRNA retarded neuronal migration and BrdU-labelling of *Mekk4*$^{-/-}$ embryos at E14.5 reveals impaired exit of BrdU positive cells from the ventricular zone (VZ) (Sarkisian et al. 2006). Importantly however, while p38 was strongly inhibited, the JNK pathway activity was unaltered in *Mekk4*$^{-/-}$ brain, and phosphorylation of the JNK substrate DCX was unaffected (Sarkisian et al. 2006; Abell et al. 2005). This data indicate that MEKK4 is required for neuroepithelial cell survival early and radial migration later during cortical development. Impaired migration in *Mekk4*$^{-/-}$ embryos correlates with upregulated Filamin A expression and elevated neuroepithelial cell apoptosis may be due to loss of GADD45-induced p38 activation (Takekawa et al. 2005). Thus MEKK4 is a critical regulator of radial migration during corticogenesis. Its mechanism of action most likely involves p38 as JNK activity is not altered in *Mekk4*$^{-/-}$ brain (Chi et al. 2005).

DLK is a MAP3K that is highly expressed in developing brain and spinal cord (Hirai et al. 2006). In *C. elegans* DLK regulates MKK4 and p38 activity and is required for nerve regeneration (Nakata et al. 2005; Hammarlund et al. 2009). In fly and mouse, DLK activation promotes stress responses and Wallerian degeneration (Miller et al. 2009; Ghosh et al. 2011). DLK has also been associated with radial migration regulation in the developing neocortex. At E16, DLK expression is concentrated in the IZ of the telencephalon while by E18, it accumulates in the SVZ (Hirai et al. 2002). Ectopic expression of DLK using adenoviral gene transfer to E13 mice activates JNK and arrests cell migration in the SVZ, while transfer of a kinase dead DLK had no effect. This data implied that DLK negatively regulated radial migration. However, in contrast, it was later reported that radial migration was arrested in *Dlk1*$^{-/-}$ mice or upon treatment with SP600125, an ATP-competitive kinase inhibitor of JNK (Hirai et al. 2006). A second study also showed that SP600125 retarded radial migration in cortical slices (Kawauchi et al. 2003). These results were attributed to JNK inhibition, however results obtained using SP600125 are difficult to interpret, as this molecule is not specific for JNK. A kinome screen has shown that SP600125 inhibits 74 kinases (of 353 tested) at a concentration of only 10 μM. Among those kinases inhibited are several MAPK pathway upstream regulators e.g. MEK1, MEK2, MKK3, MKK4 and MKK6 [results from KINOMEscan Library of Integrated Network based Cellular Systems (LINCS) data base]. Nonetheless, these studies highlighted an important role for DLK in regulating radial migration (Hirai et al. 2002; Kawauchi et al. 2003).

5 JNK and Cell Migration – A Closer Look

Several lines of evidence have shown that JNK is required for migration in a variety of cell types including epithelial cells, fibroblasts, endothelial cells, various cancer cell lines and in aortic vascular smooth muscle cells (Huang et al. 2003; Malchinkhuu et al. 2005; Björkblom et al. 2012; Kavurma and Khachigian 2003). This large and concurrent data set derives largely from wound healing assays and the use of *Jnk*$^{-/-}$ cells, or pharmacological inhibition (Javelaud et al. 2003; Huang et al. 2003). The situation in neurons has been less clear however. Studies of mouse genetics have revealed that components of the JNK cascade namely JNK1, MEKK4 and DLK influence neuronal migration in the cortex, albeit in different directions (inhibitory versus facilitatory). In freshly isolated cerebellar granule neurons and in cortical neurons, JNK1 activity retards migration as demonstrated using *Jnk*$^{-/-}$ cells or upon expression of the JNK inhibitor JBD (Westerlund et al. 2011; Björkblom et al. 2012). These authors also find that in non-neuronal cells JNK facilitates migration, thus inhibitors of JNK retard motility, in agreement with a large literature. Interestingly, ectopic expression of a neuron-enriched JNK substrate (MARCKSL1) in fibroblasts reverses this migration phenotype to one that mimics JNK action in neuronal cells, where JNK retards migration (Björkblom et al. 2012). Therefore the conflicting reports as to whether JNK activates or inhibits migration in

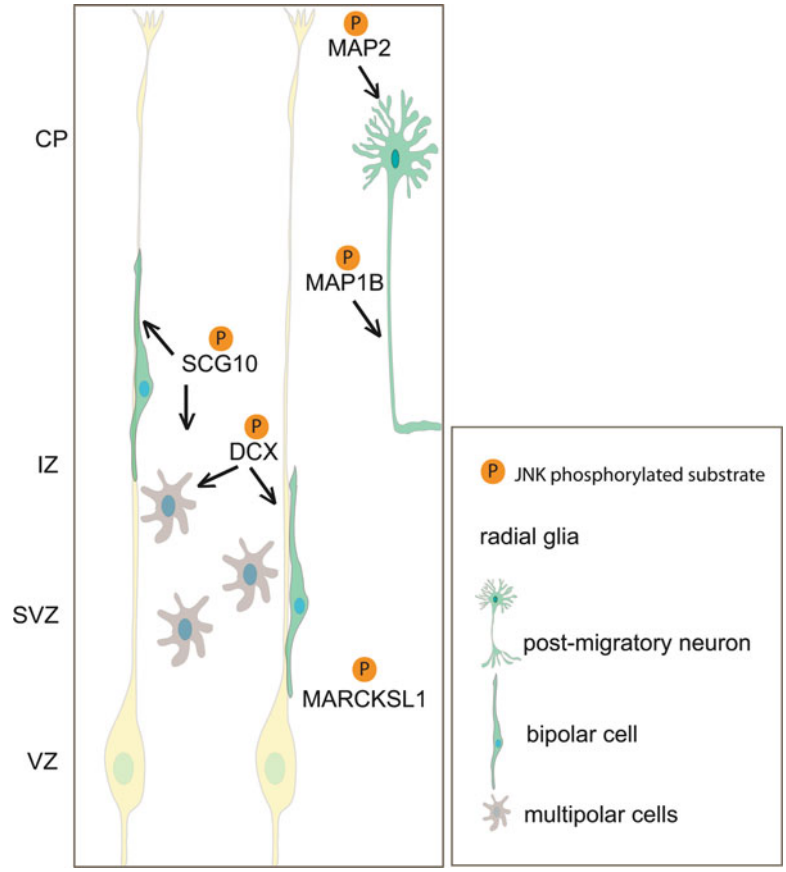

Fig. 3.2 Model depicting neuronal JNK substrates and their respective roles in regulating neuronal migration. JNK phosphorylates SCG10 and DCX, microtubule regulatory proteins that have a major influence on multipolar transition and bipolar cell migration. When neurons have reached their final position, JNK phosphorylation of MAP1b and MAP2 regulates axonal extension and dendritic arbor development. MARCKSL1 is also phosphorylated by JNK and regulates neuronal migration *in vitro* (Björkblom et al. 2012). Whether MARCKSL1 regulates radial migration remains to be seen. *CP* cortical plate, *IZ* intermediate zone, *SVZ* subventricular zone, *VZ* ventricular zone

neuronal and non-neuronal cells may depend on JNK target expression. Perhaps the functional multiplicity displayed by JNK in controlling cell migration is not altogether surprising given the signaling diversity at the MAP3K and MAP4K level of the JNK cascade, the components of which least well defined in neurons and brain tissue. The striking differences in phenotypes, while comparing even MAP2K (*Mkk4* and *Mkk7*) single knockouts with MAPK (*Jnk*) knockout mice, indicates that cooperative activation of JNKs by MKK4 and MKK7 may be critical for JNK's management of migration in neuronal cells.

5.1 JNK's Association with Controllers and Executors of Neuronal Migration

DCX: It is generally accepted that MAPK cascades function linearly, the MAPK is the effector kinase and the upstream kinases do not signal orthogonally. The substrate of the effector kinase therefore reveals information on mechanism. Among the well-characterised substrates for JNKs are several microtubule-modifying proteins, including the classical neuronal migration protein doublecortin, also known as DCX (Fig. 3.2). DCX is a developmentally regulated,

brain-specific microtubule-associated protein that was first identified in patients with missense mutations in DCX and lissencephaly syndrome (characterized by smooth brain), subventricular heteropia and cortical dysgenesis (des Portes et al. 1998; Francis et al. 1999). DCX protein is phosphorylated by JNK on T331, S334 and T321 (sites from human DCX isoform 2) (Gdalyahu et al. 2004) and phosphorylation of these sites is important for neurite length regulation and neuronal migration (Gdalyahu et al. 2004; Bai et al. 2003). DCX binds to microtubules and enhances polymer formation, it may do this by regulating microtubule nucleation (Fourniol et al. 2010). More recently, DCX has been shown to regulate F-actin through its C-terminal region and this is thought to involve its interaction with spinophillin (the F-actin-binding, regulatory subunit of protein phosphatase 1). Crosslinking of actin and microtubules by DCX is proposed to be important in growth cone guidance as *DCX*$^{-/y}$; *Dclk1*$^{-/-}$ mutant neurons are unresponsive to netrin guidance cues (Fu et al. 2013). Furthermore, DCX is required for multipolar transit and bipolar cell locomotion (LoTurco and Bai 2006). DCX phosphorylation by JNK2 may dissociate it from microtubules (Jin et al. 2010) thereby increasing microtubule plasticity and influencing migration. Whether JNK1 or JNK3 isoforms phosphorylate DCX in developing brain and alter migration rate remains to be seen.

Reelin: Reelin is an extracellular glycoprotein that negatively regulates migration in the developing cortex, hippocampus and cerebellum giving rise to the *Reeler* mouse phenotype (Tissir and Goffinet 2003). An important link between Reelin and JNK signalling has been established. Mutations in Reelin or in two of its multiple receptors APOER2 and VLDL (Trommsdorff et al. 1999), or in the DAB1 protein that serves as intracellular adaptor protein leads to a lissencephaly in humans (Trommsdorff et al. 1999; Hong et al. 2000). A proline rich region in the cytoplasmic tail of APOER2 binds to two of the JNK scaffold proteins, JIP1 and JIP2. The function of the JIP scaffolds is to co-assemble JNK and distinct MAP2Ks and MAP3Ks thereby facilitating the activation of JNK by these upstream regulators (Manning and Davis 2003; Whitmarsh 2006). Importantly binding of JIP to APOER2 does not disturb the assembly of a complete JNK signalling module consisting of its upstream activators MLK3 and MKK7 (Stockinger et al. 2000). Therefore APOER2 is capable of organizing a scaffold signalling complex that activates JNK, and it could be expected that Reelin binding to its receptor will regulate JNK activation, though this still requires experimental validation. Reelin also controls neuroblast migration to the olfactory bulb via the receptor APOER2 (Hellwig et al. 2012). Whether or not JNK regulates cell motility in the rostral migratory stream is not known.

Notably, *Reeler* mice mimic *Jnk1*$^{-/-}$ mice in several significant ways. Firstly, multipolar and bipolar cells in *Reeler* mice move faster than in wild-type mice and transit more quickly through the multipolar phase (Britto et al. 2011). This mimics the accelerated migration of multipolar and bipolar cells in *Jnk1*$^{-/-}$ mice with reduced duration of the multipolar phase (Westerlund et al. 2011). Another feature of migration in *Reeler* mice was the disturbed trajectories of migrating neurons that exhibited increased meandering (Britto et al. 2011). Whether neuronal trajectories are altered during cortical development in *Jnk1*$^{-/-}$ mice has not been reported. Finally, *Reeler* mice are characterized by abnormal reorganisation of preplate (PP) neurons, exemplified by delayed and incomplete PP splitting (Sheppard and Pearlman 1997). Similarly, *Jnk1*$^{-/-}$ mice display more prominent chondroitin sulphate proteoglycan staining in the superplate that may reflect differences in PP splitting (Westerlund et al. 2011). Together these findings point to JNK as a likely intracellular purveyor of Reelin's *stop signal* during formation of the cortex.

5.2 JNK1 Regulation of Microtubules

The microtubule cytoskeleton and its posttranslational modifications are critical determinants of neuronal migration and there are several examples of mutant mice, where disruption of genes

that directly or indirectly affect microtubule homeostasis impose migration defects. For example, missense mutations in DCX (des Portes et al. 1998; Francis et al. 1999), TUBA1A (Kumar et al. 2010), TUBG1 (Poirier et al. 2013) and Elongator (Creppe et al. 2009) result in significantly altered migration patterns in the developing cortex. In this context, JNK1 has emerged as a prominent regulator of microtubule integrity in brain. Microtubule length is decreased in brains from adult mice lacking *Jnk1* (Chang et al. 2003), microtubule dynamics is altered in cortical neurons upon expression of the JBD inhibitor of JNK (Tararuk et al. 2006), and there is a significant increase in tyrosinated tubulin in *Jnk1*$^{-/-}$ neonates, suggesting increased microtubule plasticity (Westerlund et al. 2011). Consistent with this function in maintaining brain microtubule stability, axon formation is also disturbed in *Jnk1*$^{-/-}$ mice, for example, the anterior commissure develops normally but is disrupted by postnatal day 12 and is absent in adult brain (Table 3.1) (Chang et al. 2003).

We now know that JNK displays a preference for phosphorylating microtubule regulatory proteins. DCX is one example (Gdalyahu et al. 2004), phosphorylation of its S322 by JNK disrupts microtubule binding (Jin et al. 2010). The dendrite specific high molecular weight MAP2 is another (Björkblom et al. 2005), as is MAP1b (Chang et al. 2003; Kawauchi et al. 2005). It is not therefore surprising that JNK is associated with regulation of dendrite architecture (Björkblom et al. 2005; Rosso et al. 2005) and the autism spectrum disorder susceptibility gene TAOK2, acting via JNK, is essential for basal dendrite formation (de Anda et al. 2012).

SCG10: Aside from DCX, the tubulin interacting protein SCG10 has been validated as a JNK substrate *in vitro* (Neidhart et al. 2001) and in brain (Neidhart et al. 2001; Tararuk et al. 2006). SCG10 plays a critical role in regulating neuronal migration during cortical development as shRNA against SCG10 increases radial migration rate (Fig. 3.2) (Westerlund et al. 2011). JNK phosphorylates SCG10 on S62 and S73 and phosphorylation of S73 is significantly reduced in *Jnk1*$^{-/-}$ brain (Tararuk et al. 2006). SCG10, also known as stathmin-2, is a member of the stathmin family of microtubule destabilizing proteins, the other members being stathmin, SCLIP, and RB3 (Riederer et al. 1997). Tissue specific expression of SCG10 in brain is under the control of the neuron restrictive silencing factor (NRSF) (Sone et al. 2011). Expression peaks in embryonic brain and decreases thereafter (Stein et al. 1988). Knockdown of SCG10 significantly altered radial migration, thereby highlighting SCG10 as a new player in the regulation of migration during cortical morphogenesis (Westerlund et al. 2011). SCG10 may act downstream of JNK1 in slowing the pace of radially migrating neurons as expression of exogenous SCG10 rescues the migration phenotype observed upon inhibitor JBD expression and in *Jnk1*$^{-/-}$ mice (Westerlund et al. 2011). Consistent with this, in *Drosophila melanogaster* which encodes only one stathmin, RNAi-mediated knockdown of *D-stathmin* disrupts nerve cell placement, with disturbed axonal organisation in both central and peripheral nervous system (Ozon et al. 2002). Interestingly, neurite extension is one of the first hallmarks of migrating neurons (Rakic 1971), and SCG10 phosphorylation is associated with neurite growth (Grenningloh et al. 2004; Tararuk et al. 2006). SCG10 is also present at the Golgi apparatus and in growth cones, where it concentrates alongside loose microtubules (Stein et al. 1988). SCG10, like the other stathmin family members (stathmin, SCLIP and RB3) is a microtubule destabilizing protein. This function is controlled by phosphorylation on sites that include the JNK phosphorylation sites S62 and S73 (Antonsson et al. 1998; Neidhart et al. 2001). Cells expressing pseudo-phosphorylated GFP-SCG10S62DS73D display more stable microtubules than cells expressing GFP-SCG10^{S62AS73A} (Westerlund et al. 2011). While it is not known precisely how SCG10 regulates migration, the model proposed in this study suggests that SCG10 acting locally on microtubules in the growth cone may contribute to protrusion force and forward movement of neurons.

Rnd2 and *Rnd3* encode atypical Rho GTPases that facilitate radial migration by inhibition of RhoA signalling (Pacary et al. 2011).

Like SCG10, these proteins control multipolar to bipolar transition (*Rnd2*) and locomotion (*Rnd3*) to the CP (see also Chap. 1). Defects in migration observed upon *Rnd3* knockdown were rescued by F-actin depolymerisation indicating a central role for the actin cytoskeleton. Importantly, gene knockdown of *Rnd2* confined cells at the multipolar stage in the IZ and prevented migration to the CP. This suggested a critical role for *Rnd2* in exit from the multipolar phase. Interestingly, SCG10 was found in a yeast two hybrid assay to interact with another member of the Rnd family, RND1 (Li et al. 2009). This interaction was confirmed by immunoprecipitation. *Rnd1* is highly expressed in brain though unlike RND2 and RND3, it lacks GTPase activity (Nobes et al. 1998). RND1 binds to the central domain of SCG10 regardless of its phosphorylation state, and regulation of axon extension by RND1 requires SCG10 (Li et al. 2009). RND2/3 and SCG10 have been independently shown to regulate migration and mutipolar stage exit, but whether they cooperate to exert this function or act exclusively remains to be seen.

5.3 JNK Scaffolds and Migration

The JNK signaling cascade is shaped by interaction with scaffolding proteins. Organization of JNK and other MAPK kinases into signaling cascades provides a high level of organization and specific stimulation in response to external stimuli (Davis 2000). The JNK scaffolds consist of JIP1, JIP2, JIP3 (or JSAP-1) JIP4, JNK-interacting leucine zipper proteins, plenty of SH3 (POSH), beta-arrestin 2, CRK3 and IKAP (Morrison and Davis 2003). For the purpose of this review we will discuss only those scaffolds playing a role in migration, for others we refer the reader to the literature (Davis 2000; Morrison and Davis 2003).

JIP3 like JIP1 is a JNK scaffold that is highly expressed in brain (Koushika 2008). Disruption results in lung failure, perinatal death and the absence of the telencephalic commissure. Neuronal positioning was also altered in these mice (Chang et al. 2003; Kelkar et al. 2003). The JNK scaffold POSH interacts with JIPs to scaffold JNK cascade components and promote activation of JNK (Kukekov et al. 2006). In *Drosophila* POSH is required for epidermal dorsal closure, as is DJNK (Sluss et al. 1996; Zhang et al. 2010). In mice, POSH is strongly expressed in the VZ and IZ of developing cortex and knockdown of *Posh* impairs migration to the CP, while overexpression of *Posh* advances migration (Yang et al. 2012). POSH is however also considered a strong regulator of neuronal apoptosis and POSH expression promotes death in a Parkinson's model (Wilhelm et al. 2007). Most of the analysis of JNK scaffolds and cell migration are carried out in the context of cancer, where JIP3 has also received attention. For example high levels of *Jip3* mRNA correlate with advanced brain tumor malignancy (Takino et al. 2002) and the mechanism of JIP3 regulation of cancer cell motility may involve focal adhesion kinase (Takino et al. 2002, 2005; Wang et al. 2007a). JIP4 on the other hand regulates the migration of HeLa cells (Gantulga et al. 2008) and SH2-containing inositol polyphosphate 5-phosphatase 2 (SHIP2), a new interacting partner for JIP1, regulates a variety of cellular processes including cytoskeletal organization, adhesion and cell migration (Xie et al. 2008).

5.4 *Caenorhabditis elegans* and *Drosophila melanogaster*

Fundamental insights leading to new understanding of cortical development, draws from studies in model organisms. It is therefore worth remarking on the JNK signalling cascade in this context. The JNK pathway in *C. elegans* is less complex than in mammals, and one JNK homolog, *Jnk-1* is expressed in worm with two possible splice variants (Villanueva et al. 2001). Upstream of JNK-1, two MAP2Ks, *Jkk-1* and *Mek-1* (*Mkk7* homolog), are expressed that can activate JNK-1 in response to distinct stimuli (Kurz and Ewbank 2003). Mutant worms null for *Jnk-1* show defective body movement and coordination, associated with D-type motor neurons as do *Jkk-1* null

worms (Villanueva et al. 2001). JNK signalling was also identified as being important for vesicular cargo localisation in worms, and *Jkk-1* mutants show mislocalized synaptic vesicle markers (Byrd et al. 2001). In *Drosophila melanogaster* however, the situation is different. *Drosophila* expresses one JNK homolog *dJnk*. Flies lacking *dJnk* (encoded by *basket),* show defects in dorsal closure, a mid-embryogenesis process involving morphogenic movement of epithelial cells (Sluss et al. 1996; MacKrell et al. 1988). This phenotype was rescued when *basket* was reintroduced to the developing embryo indicating that DJNK plays an important role in cell spreading. The actin nucleating protein p150-Spir has been identified as a DJNK substrate in *Drosophila melanogaster*. It is a member of the Wiscott-Aldrich syndrome protein (WASP) homology domain 2 (WH2) family of proteins that play a role in actin reorganization (Ramesh et al. 1999). It was identified in a yeast 2-hybrid system as an interaction partner for DJNK (Otto et al. 2000). While mammalian homologs of p150-Spir (formin-1 and formin-2) are highly expressed in developing brain (Schumacher et al. 2004) and regulate actin nucleation and elongation, it remains to be seen whether these proteins are substrates for JNK in brain.

5.5 Migration in Cancer, Ectopic Expression of Neuronal JNK Substrates

In cancer, the regulation of cell motility by activation of the JNK pathway may be significant in terms of tumor metastasis. In approximately 5 % of cancers, there is a loss of function mutation in the *Mkk4* gene and a variety of studies have implicated MKK4 in cancer cell metastasis (Whitmarsh and Davis 2007). However, while many studies suggest a role for JNK signalling in the regulation of cancer, JNK activity is also associated with suppression of tumour development (Kennedy and Davis 2003). In the context of this chapter, it is worth noting that some neuron-specific JNK substrates, including SCG10 and MARCKSL1 are ectopically expressed in cancers (Wang et al. 2007a; Björkblom et al. 2012; Lee et al. 2006). SCG10 is indeed up-regulated in hepatomas while *MARCKSL1* is up-regulated in a broad range of cancers. MARCKSL1 protein was strongly increased in prostate carcinomas and in metastatic breast cancer (Wang et al. 2007a; Björkblom et al. 2012). Moreover, MARCKSL1 enhances prostate cancer cell migration in a JNK-dependent manner while in neurons MARCKSL1 also regulates migration in a JNK-regulated manner (Björkblom et al. 2012). MARCKSL1 is phosphorylated by JNK on C-terminal residues (S120, T148 and T183). A phosphomimicry mutant of MARCKSL1 where the JNK sites are mutated to aspartate, acts to bundle F-actin in a reconstituted system and this stabilization of actin filaments correlates with inhibited migration of cortical neurons and of the PC3 prostate cancer cell line (Björkblom et al. 2012). Interestingly both MARCKSL1 and JNK are critical for brain morphogenesis as disruption of either leads neural tube closure defects (Chen et al. 1996; Kuan et al. 1999; Sabapathy et al. 1999).

6 Concluding Remarks

The last decade of research on JNKs has revealed that in addition to transcription factors, JNKs phosphorylate cytosolic proteins, a number of which are neuron-specific proteins that regulate the cytoskeleton. It is not surprising therefore that JNKs are pivotal players in neuronal migration and brain development. It has also emerged that JNK regulation of migration in neurons is complex and some apparently conflicting findings have been described. For example knockout of *Jnk*1 produces one migration phenotype and knockout of an upstream kinase evokes another response (Table 3.1). This is perhaps not surprising given that individual MAPKs from yeast to mammals share certain MAP2K and most MAP3K upstream regulators. These activator kinases are recruited via scaffold proteins to activate select MAPKs and direct distinct functional outcomes. In addition, functional outcome can be controlled at the effector kinase level itself. There, four splice variants exist for JNKs 1, 2 and 3. These variants may behave differently in terms

of substrate recognition and cellular response thereby further increasing the signalling diversity. Finally, the effector proteins phosphorylated by JNK in brain have not been fully characterized and we rely on substrate screens to identify these. Finding answers to these questions will ultimately yield improved insight on the cellular mechanism of neuronal migration as well as other aspects of brain development.

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Novel Functions of Core Cell Cycle Regulators in Neuronal Migration

4

Juliette D. Godin and Laurent Nguyen

Abstract

The cerebral cortex is one of the most intricate regions of the brain, which required elaborated cell migration patterns for its development. Experimental observations show that projection neurons migrate radially within the cortical wall, whereas interneurons migrate along multiple tangential paths to reach the developing cortex. Tight regulation of the cell migration processes ensures proper positioning and functional integration of neurons to specific cerebral cortical circuits. Disruption of neuronal migration often lead to cortical dysfunction and/or malformation associated with neurological disorders. Unveiling the molecular control of neuronal migration is thus fundamental to understand the physiological or pathological development of the cerebral cortex. Generation of functional cortical neurons is a complex and stratified process that relies on decision of neural progenitors to leave the cell cycle and generate neurons that migrate and differentiate to reach their final position in the cortical wall. Although accumulating work shed some light on the molecular control of neuronal migration, we currently do not have a comprehensive understanding of how cell cycle exit and migration/differentiation are coordinated at the molecular level. The current chapter tends to lift the veil on

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L. Nguyen and S. Hippenmeyer (eds.), *Cellular and Molecular Control of Neuronal Migration*, Advances in Experimental Medicine and Biology 800, DOI 10.1007/978-94-007-7687-6_4,

this issue by discussing how core cell cycle regulators, and in particular $p27^{Kip1}$ acts as a multifunctional protein to control critical steps of neuronal migration through activities that go far beyond cell cycle regulation.

Keywords
Radial migration • Cerebral cortex development • p27 • Myosin II • Microtubules

1 Introduction

The cerebral cortex is a complex and highly evolved brain structure that emerges from the dorsal telencephalon as a result of birth and migration of multiple neuron waves that settle "inside-out" as adjacent layers in the cortical plate (CP). Six neuronal layers are sequentially produced between E11 and E18 in mouse and each of them contains a characteristic set of neuron subtypes that connect with specific cortical and subcortical regions (Gupta et al. 2002; Hevner et al. 2003) (Fig. 4.1). In addition, cortical neurons are regionally organized into specialized areas that underlie elaborated motor, cognitive and perceptual abilities (Rash and Grove 2006). The cortex is mainly composed of glutamatergic projection neurons that are born in ventricular and subventricular zones of the dorsal telencephalon (VZ and SVZ, respectively), while the population of GABAergic interneurons arises from ventral progenitors located in medial and caudal ganglionic eminences (MGE and CGE, respectively) (Fig. 4.2a). The extraordinary degree of organization of the cerebral cortex reflects the complexity of the migratory movements required to generate it. One of the most remarkable features of the cerebral cortex is the wide-range of distinct migration patterns undertaken by neurons to integrate into functional neural circuitry. In contrast to projection neurons that show rather simple morphology and engage locally in directed migration along radial glia fibers (Gupta et al. 2002), interneurons undergo dynamic branching and reach the cortical wall by travelling along tangential paths (Anderson et al. 1997; Tamamaki et al. 1997) that run across various substrates (Yokota et al. 2007) (Fig. 4.2b). Neuron migration is orchestrated by both extracellular and intracellular cues (Ayala et al. 2007; Marin and Rubenstein 2001, 2003; Bielas et al. 2004), and computation of these signals ultimately drives cytoskeleton remodelling to support cell motility. Accumulating knowledge of the molecular control of neuronal migration [reviewed in (Metin et al. 2006; Tsai and Gleeson 2005)] revealed a significant role for proteins associated with or regulating the actin and microtubule (MT) cytoskeletons. In cortical neurons, some critical MT regulators are dynein and its cofactor LIS1, CDK5, and Doublecortin (Kappeler et al. 2006; Kawauchi et al. 2006; Koizumi et al. 2006; Rakic et al. 2009; Tanaka et al. 2004; Tsai et al. 2007). Moreover, some small GTPases (Kholmanskikh et al. 2003, 2006) and selected F-actin regulators (Bellenchi et al. 2007; Nagano et al. 2002) promote actin microfilament modification during neuronal migration in the cortex. Not surprisingly, most cortical malformations associated with neurological disorders arise as a consequence of mutation in genes that encode cytoskeletal proteins or their modifiers (Breuss et al. 2012; Cushion et al. 2013; Gleeson et al. 1998; Jaglin et al. 2009; Pilz et al. 1998; Reiner et al. 1993). Recent works performed by us and others shed some new light on novel cytoskeleton-related mechanisms that regulate specific steps of neuronal migration in the developing cerebral cortex.

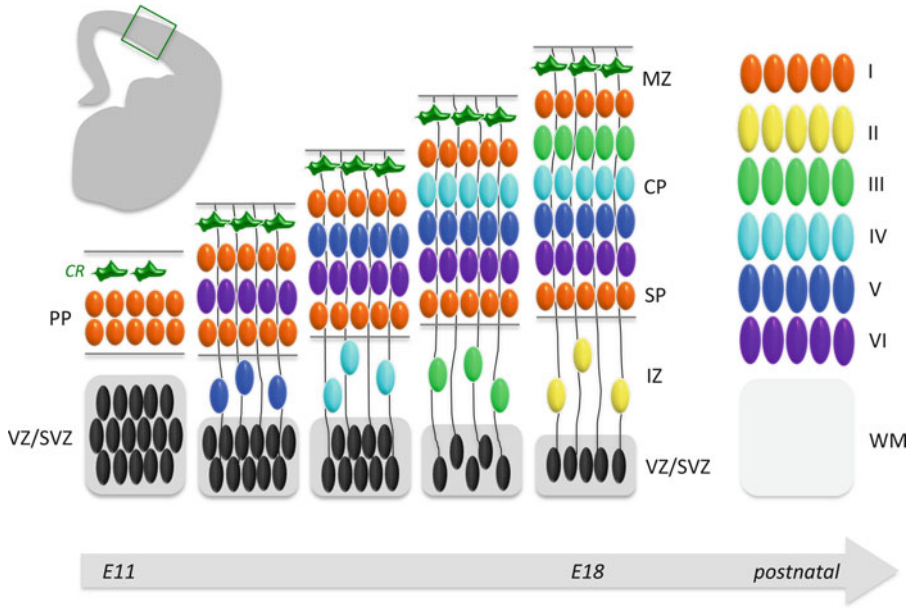


Fig. 4.1 Cortical layering process in mouse embryos. Half coronal section through the forebrain of an E14.5 mouse embryo (*upper left scheme*). Successive waves of post-mitotic projection neurons are generated between E11 and E18 in the dorsal telencephalon (cortical interneurons are not represented). A magnified area of the neocortex (*boxed with a green square* on the coronal section) shows the sequential establishment of the adjacent cortical layers (see timeline). The first wave of projection neurons splits the preplate (*PP*) into a subplate (*SP*) and a marginal zone (*MZ*), which also includes migrating Cajal-Retzius cells (*CR*). These cells release the glycoprotein Reelin that serves as migration signal. Projection neurons arise from committed progenitors located in the ventricular (*VZ*) and subventricular (*SVZ*) zones and migrate radially towards the cortical plate (*CP*) by locomotion on radial glia processes (*black lines*). At birth, the neocortex is composed of six molecularly distinct (*different colours*) layers (*I–IV*) established in an inside-out fashion above the white matter (*WM*)

First identified as cell cycle inhibitors, mediating the growth inhibitory cues of upstream signalling pathways, the cyclin-CDK inhibitors of the Cip/Kip family composed of $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$ have emerged as multifunctional proteins with roles extending beyond cell cycle regulation. Cip/Kip proteins in general, and $p27^{Kip1}$ (renamed p27 further in the text) in particular regulate cell migration in various tissues in physiological or pathological conditions (Baldassarre et al. 2005; Besson et al. 2004b; Itoh et al. 2007; Kawauchi et al. 2006; McAllister et al. 2003; Nguyen et al. 2006). In this chapter, we discuss how p27 acts as a modular protein to coordinate critical steps of neuronal migration in the developing cerebral cortex.

2 Old Players, New Functions – Revisiting the Role of Core Cell Cycle Regulators in Cerebral Cortical Neurogenesis

The generation of cortical neurons by progenitor cells is a finely tuned process that requires a tight coordination of multiple cellular activities, including cell specification, cell cycle exit, cell migration and neuronal differentiation. Achievement of these multiple biological steps relies on implementation of specific genetic inputs as well as on molecular signaling pathways triggered by specific extracellular cues, including growth factors and neurotransmitters (Heng et al. 2007; Nguyen et al. 2001). However,

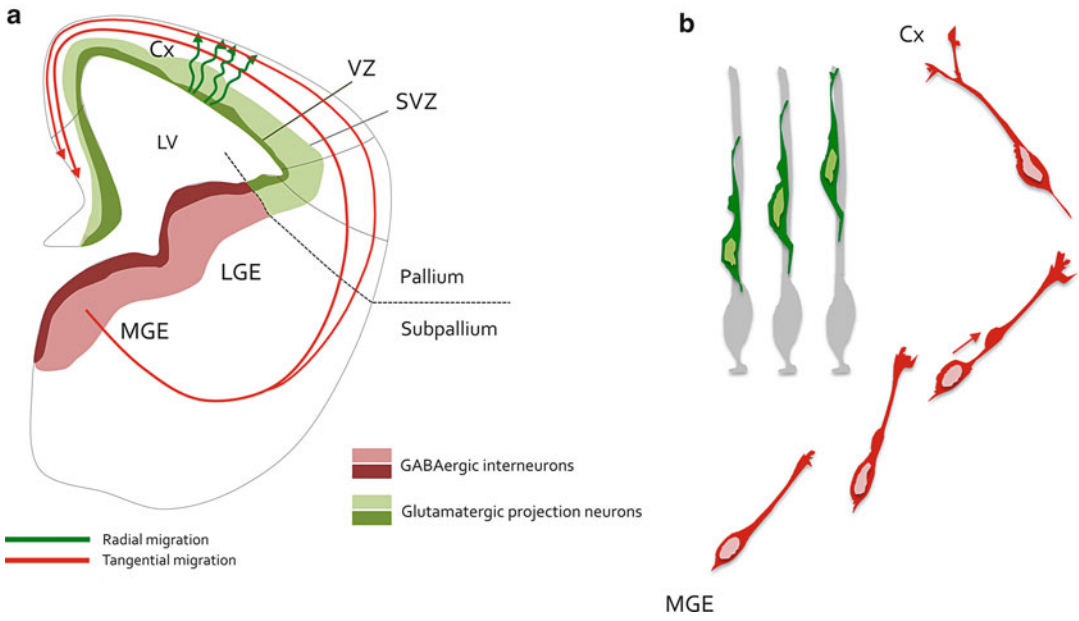


Fig. 4.2 Neuronal migration in the developing cerebral cortex. (**a**) Scheme representing a hemisection of an E14.5 mouse embryo forebrain. Glutamatergic projection neurons are born around the lateral ventricle (*LV*) from stem and cortical progenitors residing in the pallial ventricular (*VZ*, *dark green*) and subventricular (*SVZ*, *light green*) zones. Projection neurons migrate radially (*green tortuous arrows*) to integrate the cortical plate of the cortex (*Cx*). Cortical interneurons are GABAergic and arise from the subpallial medial (*MGE*) and caudal (*CGE*) ganglionic eminences. They are born from stem and progenitor cells located in the VZ (*dark red*) and SVZ (*light red*). These neurons undergo radial migration along various paths (*red*) to integrate the developing cortical wall. (**b**) Morphological remodelling of cortical neurons during migration. Radial migration of projection neurons (*green*) is a directed movement characterized by locomotion on radial glia guides (*light grey*). These neurons are bipolar during locomotion and undergo short-distance nucleokinesis and limited remodelling of the leading process. On the other hand, cortical interneurons (*red*) navigate in the forebrain parenchyma through a migration process characterized by an extensive nucleokinesis (*red arrow*) that comes together with a highly dynamic growth cone, which give rise to temporary branches that are both requires for proper migration

the mechanisms that integrate these cellular processes into appropriate developmental programs remain poorly understood. Accumulating evidence supports the existence of a complex relationship between cell cycle components and factors promoting embryonic development in various animal models (Godin et al. 2012; Nguyen et al. 2006; Ohnuma et al. 1999; Reynaud et al. 2000; Vernon et al. 2003; Joseph et al. 2009). More specifically, Cip/Kip proteins are expressed in both, cycling progenitors and postmitotic neurons of the mouse developing cerebral cortex where they modulate neural specification and migration in addition to their "first-identified" function as cell cycle regulators (Itoh et al. 2007; Kawauchi et al. 2006; Nguyen et al. 2006; Tury et al. 2011). Our work revealed that the most abundant Cip/Kip in the embryonic telencephalon is p27, which promotes projection neurons specification through stabilization and thus progressive accumulation of the basic helix-loop-helix (bHLH) proneural transcription factor neurogenin 2 (Ngn2) in dorsal telencephalic progenitors (Nguyen et al. 2006). In addition, p27 acts as key cellular cytoskeleton modulator, thus promoting specific migratory steps of both cortical projection neurons and interneurons (Godin et al. 2012; Nguyen et al. 2006). Later during adulthood, the brain retains stem/progenitor cells in the subventricular zone (SVZ) of the lateral

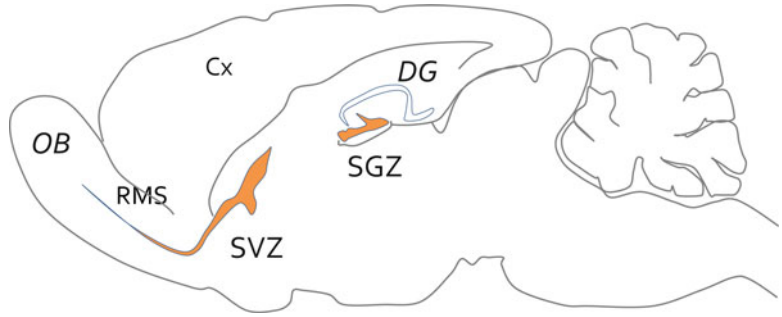


Fig. 4.3 Neurogenic niches of the adult brain. Scheme of a parasagittal section made through the brain of an adult mouse. While no more neurogenesis takes place in the cortex (*Cx*), two focal brain neurogenic areas (*orange*) have been clearly identified after birth. The more productive one is the subventricular zone (*SVZ*) of the lateral ventricle. Adult stem cells are quiescent and upon activation give birth to transient amplifying progenitors that further differentiate into neuroblasts that migrate along the rostral migratory stream (*RMS*) towards the olfactory bulbs (*OB*). They differentiate into granular or periglomerular neurons. The second neurogenic niche is the subgranular zone (*SGZ*) of the dentate gyrus (*DG*), which give rise to limited number of excitatory granule neurons that settle into the granular layer of the DG

ventricles and in the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus that support continuous generation of new neurons (Fig. 4.3). All Cip/Kip proteins are expressed in the postnatal brain where they play central function for the regulation of neurogenesis. Indeed, they are detected in adult neurogenic regions where they control proliferation kinetics (Doetsch et al. 2002; Li et al. 2009) and promote cell cycle exit (Pechnick et al. 2008) of specific progenitor subtypes. In addition, p21 and p57 control the pool of brain neural stem cell (NSC) by contributing to the regulation of their quiescence (Furutachi et al. 2013; Kippin et al. 2005). Interestingly, the number of stem cells increases in selected adult tissues (including the retina and the lung) of a knockin mouse line characterized by expression of a cell cycle dead mutated version of p27 [$p27^{CK-}$, a mutant that cannot interact with cyclins and CDKs (Besson et al. 2004b); see also Box 4.2]. This suggests that p27 controls stem cell amplification in selected adult tissues (Besson et al. 2007). Although classical function as cell cycle regulators have been attributed to Cip/Kip proteins in the adult brain, there is currently no demonstration of any additional cell cycle unrelated activities in this specific context. This issue should be further explored.

3 Kip Movin' in the Brain – p27 Promotes Neuron Migration by Regulating Cell Cytoskeleton Dynamics

During migration, neurons undergo major morphological changes (Box 4.1) that are accompanied by dramatic shifts in cytoskeleton structure and centrosome positioning. Cell migration is initiated by the protrusion of a leading neurite ending with a structure similar to a growth cone (Tsai and Gleeson 2005). Extension of the highly polarized leading process requires protrusion forces exerted by actin polymerization, microtubule growth and establishment of adhesion complexes that link the extracellular substrate to the actin cytoskeleton (Marin et al. 2006). The centrosome, which comprises the microtubule organisation centre (MTOC), is located ahead of the nucleus in the direction of migration. This elongation step is followed by formation of a swelling, characterized by a cytoplasmic dilatation, that encompass the centrosome, the golgi apparatus, the mitochondria and the endoplasmic reticulum. The nucleus and the centrosome are attached together, through a fork-like structure that enwraps the nucleus in a cage like fashion (Xie et al. 2003). Then, the nucleus relocates into

Box 4.1 Cortical Neurons: Two Distinct Modes of Migration

The two major classes of cortical neurons adopt distinct modes of migration to reach their final position within the cortical plate. Projection neurons undergo directed migration along straight radial glia guides (Gupta et al. 2002). The migration process is divided into four phases (see also Chap. 1): bipolar progenitors migrate through the subventricular zone (SVZ) independently of the radial glia scaffold. When they reach the intermediate zone (IZ), projections neurons stop their migration, sprout multiple neurites and become multipolar (Noctor et al. 2004). Neurons further convert to a bipolar shape, attach to radial glia and move to the cortical plate by glia-guided locomotion. Once connecting to the pia, neurons undergo a final nuclear translocation to settle at appropriate position in the cortical plate (Noctor et al. 2004). Cortical interneurons extend multiple branches and move along various tangential paths that run across different substrates in the telencephalon (Anderson et al. 1997; Tamamaki et al. 1997), including progenitor cells, post-mitotic neurons as well as radial glia fibers (Yokota et al. 2007). Migration of interneurons results from successive cycles of morphological changes that couple saltatory progression of their cell body with dynamic remodelling of their leading process. The nucleus alternates between resting phases that correlate with elongation of the leading process and movement phases associated with splitting of growth cone-like structures that give rise to new branches (Bellion et al. 2005). This stepwise behavior relies on cytoskeletal transformations that promote rostral translocation of a cytoplasmic dilatation encompassing the centrosome and the Golgi apparatus into the extending leading process. This is followed by forward migration of the nucleus, and its perinuclear cytoplasm, a process named nucleokinesis (Marin et al. 2010). Finally, interneurons undergo retraction of the trailing process and branching of the leading process (Bellion et al. 2005).

the swelling through nucleokinesis (Schaar and McConnell 2005). Nuclear movement varies depending on the cell type with short and high amplitude for projections neurons and interneurons, respectively. Discrepancies about the contribution of actin and microtubule networks to the forward migration of the nucleus argue for either pulling or pushing forces to generate somal motion (see also Chap. 7).

3.1 p27, an Unexpected Regulator of Cell Migration

The contribution of p27 to cell migration was first reported in hepatocellular carcinoma cells: transduction of a TAT-p27 protein promoted their migration in vitro (Nagahara et al. 1998). Further studies confirmed that p27 acts as a regulator of cell migration in a variety of cell types, including fibroblasts, vascular smooth muscle cells, endothelial cells, and cortical neurons (Diez-Juan and Andres 2003; McAllister et al. 2003; Sun et al. 2001; Godin et al. 2012; Nguyen et al. 2006) (Fig. 4.4). Besson and collaborators showed that p27 null mouse embryonic fibroblasts (MEFs) have reduced motility compared to wild type MEFs and re-expression of p27 rescued motility defect of p27 null MEFs (Besson et al. 2004b). Furthermore, re-expression of a p27 mutant that cannot bind cyclins and CDKs ($p27^{CK-}$; see Box 4.2) also restored migration, suggesting that the effect of p27 on cell motility was independent of its cell cycle activity (Besson et al. 2004b). The first demonstration of an *in vivo* cell cycle-unrelated activity of p27 came from the analysis of neuronal migration during embryonic development. Overexpression of wild-type or $p27^{CK-}$

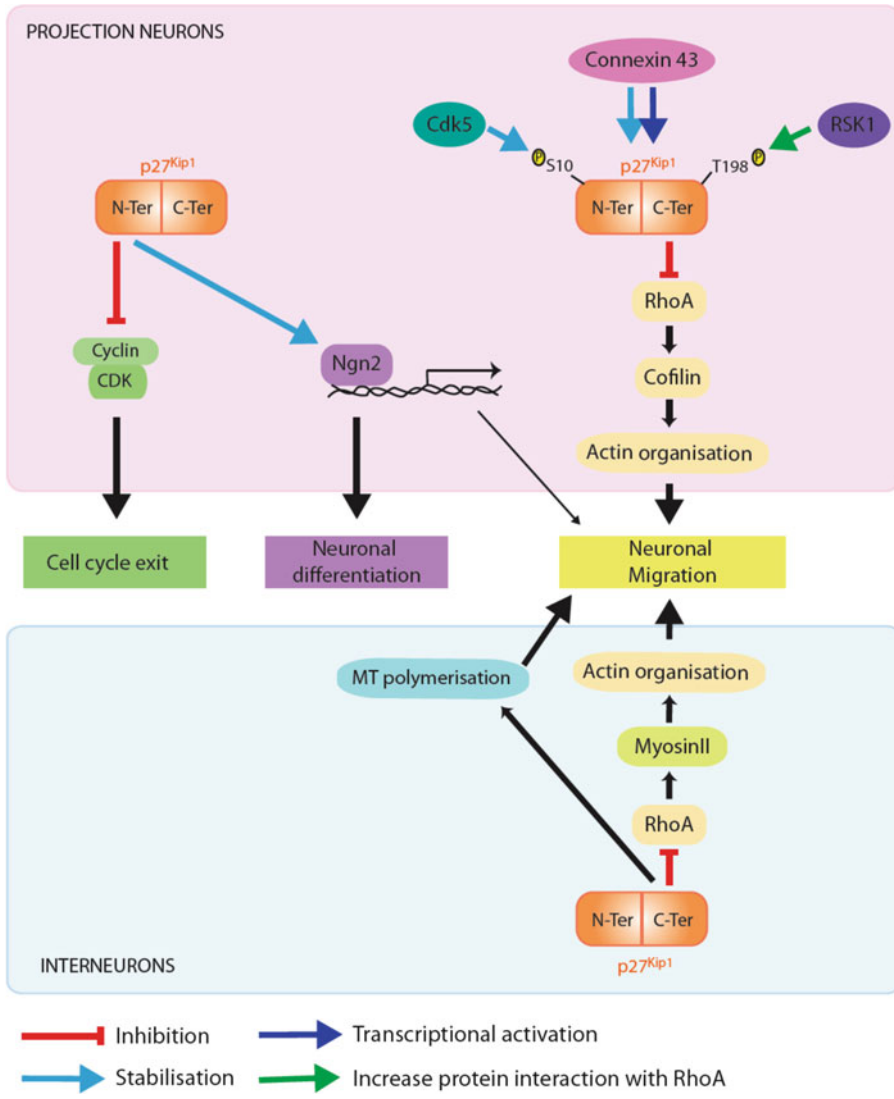
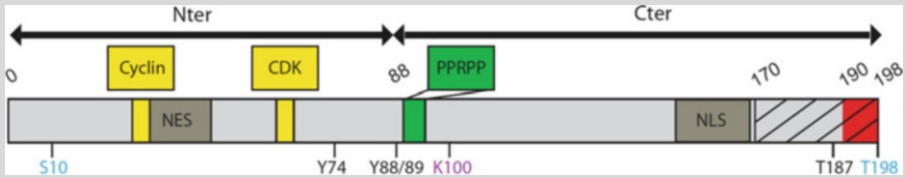


Fig. 4.4 Different roles of p27 during corticogenesis. p27 promotes cell cycle exit by associating with specific Cdk/cylins complexes through a N-terminal binding domain and hence blocking their catalytic activity and preventing G1-S phase transition (molecular pathway in *green*). By stabilising Ngn2 in the nucleus of cortical progenitors, p27 regulates neuronal differentiation, an activity that resides in its N-terminal half (molecular pathway in *purple*). p27 promotes the migration of both types of cortical neurons (projection neurons, *upper panel*; cortical interneurons, *lower panel*) by blocking RhoA signalling pathway, an activity residing in its C-terminal domain (molecular pathway in *yellow*). While p27 regulates actin cytoskeleton through activation of cofilin, an actin depolymerizing factor in projection neurons, it controls actomyosin-contractions by fine-tuning myosin II activity in cortical interneurons. In cortical interneurons, p27 additionally controls migration through regulation of MT dynamics (pathway in *blue*). p27 could also indirectly promote migration through Ngn2 inducing the transcription of target genes which regulate radial migration. Phosphorylation of p27 at Serine 10 (Ser10) by cdk5 regulates its stability and cytoplasmic localization. Phosphorylation at threonine 198 enhances p27 interaction with RhoA (*green arrow*). Finally connexin 43 acts as an upstream regulator of p27 by controlling both synthesis (*dark blue arrow*) and stability (*light blue arrow*)

Box 4.2 Major Domains and Post-translational Sites of p27 Protein

A detailed examination of the p27 protein identifies two major regions, the N-terminal, which according to homology with p21 and p57 showed the capability of inhibiting the kinase activity of cyclin-CDK complexes, and a C-terminal region that interacts with several proteins involved in processes not correlated to the cell cycle control. p27 interacts with cyclins and CDK through two distinct domains (30/32 and 62/64; Box 4.2). N-terminal half, but not Cyclin-CDK interactions domains, is required for p27-dependent stabilisation of Neurogenin2 (Ngn2) protein in cortical progenitors, probably through interaction domains with specific ubiquitin ligases that may target Ngn2 to the proteasome for degradation (Nguyen et al. 2006). When localized at cytosolic cellular compartment, p27 interacts with various proteins including RhoA (Red box, 190–198) (Godin et al. 2012), Rac, stathmin (dashed box, 170–198) (Baldassarre et al. 2005), Grb2 (90–96) (Kardinal et al. 2006) and 14-3-3 (Fujita et al. 2002). While both halves of p27 protein binds to microtubule *in vitro*, proline-enriched domain located in the C-terminal region (green box) is crucial for its microtubule-associated function (see further in the text) (Godin et al. 2012). Shuttling between nucleus and cytoplasm is crucial for regulation of p27 functions. A bipartite nuclear localization signal (NLS, 152/153-166/168), which is recognized by the alpha/beta importins, allowing p27 transport into the nucleus (Sekimoto et al. 2004; Zeng et al. 2000) and a putative nuclear export signal (NES, 32–45) have been identified. Finally, p27 post-translational modifications may also control its function, by regulating subcellular localisation (pS10, pY74, pY88, pY89) (Viglietto et al. 2002; Sekimoto et al. 2004; Liang et al. 2002; Kardinal et al. 2006; Ishida et al. 2002; Fujita et al. 2002, 2003), stability (pS10, pT187, AcK100) (Tsvetkov et al. 1999; Perez-Luna et al. 2012; Ishida et al. 2000) or protein-protein interaction (pT198) (Larrea et al. 2008; Fujita et al. 2003). Phosphorylation sites that may regulate p27 migratory activities are highlighted in blue.



efficiently promoted migration of projection neurons to the cortical plate, while overexpression of other members of the cip/kip family did not affect radial migration (Nguyen et al. 2006). Indeed lack of p27 impaired: **(1)** Radial migration of projection neurons: p27 knockdown projections neurons remained in subventricular and intermediate zones (Nguyen et al. 2006; Kawauchi et al. 2006) and they failed to acquire multipolar morphology in the intermediate zone (Kawauchi et al. 2006); **(2)** Tangential migration of interneurons: conditional removal of p27 in interneurons led to decreased migration velocity that arose from both nucleokinesis and branching defects (Godin et al. 2012). Lack of p27 expression in cortical interneurons delayed the course of tangential migration rather than permanently blocking it. Radial and tangential migrations in p27CK mice were not affected suggesting that cortical migration properties of p27 are independent of its cell cycle activity (Godin et al. 2012; Nguyen et al. 2006). We will further discuss the mechanisms by which p27 regulates migration with an emphasis on cortical neurons.

3.2 p27 and Actomyosin-Based Cytoskeleton

The first understanding of the mechanisms by which p27 regulates cell migration came from studies performed with *p27*$^{-/-}$ mouse embryonic fibroblasts. They revealed that p27 promotes migration by inhibiting the Rho-kinase pathway (Box 4.3) (Besson et al. 2004b). Motility of *p27*$^{-/-}$ mouse fibroblasts was impaired as a result of increased RhoA activity. Basal migration level could further be restored by inhibiting the Rho-kinase ROCK (Besson et al. 2004b). Indeed, *p27*$^{-/-}$ cells had increased numbers of stress fibers and focal adhesions as well as elevated levels of Rho-GTP (Besson et al. 2004b). Overexpression experiments showed that p27 interacts with RhoA, thereby preventing RhoA activation by interfering with RhoA binding to its GEFs, but not to its effectors (Besson et al. 2004b). It's worth noting that modulation of p27 expression level can lead to distinct migratory responses depending on the cellular context (Besson et al. 2004a).

The regulation of RhoA by p27 is critical for proper migration of both projection neurons and interneurons in the cortex of developing mice (Godin et al. 2012; Nguyen et al. 2006; Kawauchi et al. 2006; Itoh et al. 2007) (Fig. 4.4). Although, inhibition of RhoA signalling by p27 resulted in changes in actin cytoskeleton in neurons, discrepancy between downstream mechanisms have been described in these two neuronal classes. Recent work performed with projection neurons suggested that p27-mediated block of Rho-kinase pathway promoted actin reorganisation by activating cofilin, an actin-severing enzyme. Indeed, knockdown of p27 in cortical projection neurons lead to increased phosphorylation of cofilin through ROCK but not PAK1 signalling pathways (Gungabissoon and Bamburg 2003), suggesting that defect of migration arose from excessive stabilisation of the actin network in p27-deprived projection neurons (Kawauchi et al. 2006). These data were supported by some work performed with fibroblasts showing that p27 suppresses the activity of the RhoA-ROCK (Rho-kinase) pathway, thereby decreasing Ser 3 phosphorylation of cofilin (Besson et al. 2004b). However, the extent to which cofilin mediates p27 activity in neuronal actin cytoskeleton remodelling remains unclear since overexpression of a constitutively active form of cofilin prevents radial migration rather than promoting it

Box 4.3 RhoA, a Master Regulator of Actin Dynamics

The Rho family of GTPases, which includes Rho, Rac and Cdc42, regulate cell morphology, cytokinesis and cell motility through reorganization of actin filaments (Bar-Sagi and Hall 2000). RhoA activation promotes actin stress fiber formation, focal-adhesion assembly, as well as actin-myosin contractility (Etienne-Manneville and Hall 2002). The RhoA pathway leads to a decrease in stress fibers and focal adhesions, which increases cell motility. RhoA exists in a GDP-bound inactive state and a GTP-bound active state. Guanine nucleotide exchange factors (GEFs) catalyse the release of GDP, allowing GTP to bind RhoA, leading to activation of its downstream effectors; the Rho-kinases, ROCK1 and ROCK2. Stress fiber formation and focal-adhesion assembly and stability are mediated by the activation of LIM domain-containing protein kinase (LIMK) by ROCK, which in turn phosphorylates and inhibits cofilin, an actin depolymerization factor. ROCK also controls actomyosin contractility, by directly phosphorylating the light chain of myosin II (MLC-II) (Amano et al. 1997) or indirectly via inhibition of the MLC phosphatase MLCP (Kimura et al. 1996) or activation of the MLC kinase MLCK (Chrzanowska-Wodnicka and Burridge 1996). Importantly, myosin activation promotes cell migration by driving translocation of the cell body and retraction of the rear of the cell during migration (Webb et al. 2002).

(Kawauchi et al. 2006). In addition, the lack of p27 expression in cortical interneurons correlated with partial inactivation of cofilin but without an accumulation of actin microfilaments. This could be explained by the concomitant expression of gelsolin, another actin-severing protein, as detected in cortical interneuron *in vivo* (Godin et al. 2012). Together, these results suggest that the severing of actin is driven by multiple and redundant mechanisms, as G/F actin ratio remained unchanged in $p27^{-/-}$ interneurons (Godin et al. 2012) (Fig. 4.4). Indeed the main candidate target of the Rho-kinase pathway in migrating interneurons was myosin II (Fig. 4.4). This protein complex is involved in actomyosin-based contractions and p27 fine-tuned myosin II activity at the rear of the nucleus and within the growth cone. The lack of p27 led to a hyperactivation of myosin II that came together with increased frequency of nucleokinesis as well as excessive branching activity. These cellular defects were rescued by treatment with pharmacological inhibitors of the Rho effector ROCK or of the myosin light chain kinase (MLCK) (Godin et al. 2012). Although nucleokinesis and leading process branching activities were rescued, the elongation of neurites remained affected, suggesting the existence of additional substrate of p27 in migrating interneurons. In addition, targeting The RhoA/myosinII pathway was not sufficient to rescue basal level of velocity (Fig. 4.5).

Altogether, these data demonstrate that p27 controls Rho-kinase/myosin II activity to fine-tune actomyosin-based contractions that take place behind the nucleus during nucleokinesis and the leading process growth cone. However, there is an apparent discrepancy between the wide cytoplasmic distribution of p27 and the discrete location of actomyosin activity in migrating interneurons. Several works showed that p27 undergo multiple and dynamic post-translational modifications among which some are important to regulate its nucleo-cytoplasmic shuttling (Viglietto et al. 2002; Sekimoto et al. 2004; Liang et al. 2002; Kardinal et al. 2006; Ishida et al. 2002; Fujita et al. 2002, 2003; Zhang et al. 2013; Connor et al. 2003). Such modifications may indeed control the specific and dynamic accumulation of a pool of p27 at the rear of the nucleus and in the growth cone of actively migrating interneuron. This hypothesis should be further investigated.

3.3 p27 and Microtubule Dynamics

Further analyses of the role of p27 during migration of both fibroblast and cortical interneurons described p27 as a microtubule regulator. It has been demonstrated that cortical interneurons showed neurite elongation defects upon removal of p27 and that these defects were not rescued by tuning RhoA-dependent actin contractibility. This raised the possibility that p27 acts on both actin and microtubules cytoskeletons (Godin et al. 2012). This hypothesis has been recently validated by our laboratory that demonstrated that p27 associates with microtubule network in migrating interneurons and binds directly with both free and polymerized tubulin in vitro. While p27 had no effect on MT stability, it promoted MT polymerisation in vitro, a function that required the integrity of a proline-rich domain. Likewise, MT polymerisation rate was impaired in $p27^{-/-}$ MEFs, a defect that could be rescued by re-expressing p27 but not its proline mutant, p27 4A (see Fig. 4.5). Importantly, expression of the proline mutant could not rescue neurite extension in migrating p27-null interneurons, indicating that p27 controlled neurite extension by inducing MT polymerisation. While, Godin and collaborators identified p27 as a microtubule-associated protein (MAP) that regulates MT dynamics through direct interaction (Godin et al. 2012), other work performed with mouse fibroblasts (MEFs) and fibrosarcoma HT-180 cells showed that p27 also binds to stathmin and interfere with its ability to sequester free tubulin (Baldassarre et al. 2005). Interestingly a mutant form of p27 could not bind to stathmin promotes MT polymerisation as wild type p27, suggesting that stathmin-related activities of p27 are not required to regulate microtubule dynamics in cortical interneurons (Godin et al. 2012). It is worth noting that expression of the

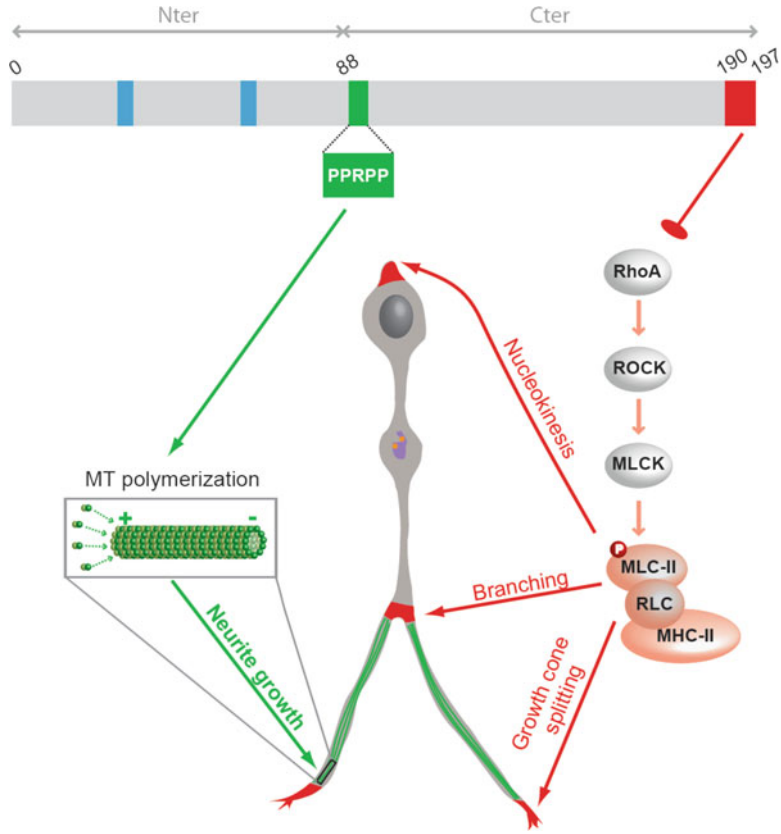


Fig. 4.5 Summary scheme illustrating the molecular pathways by which p27 regulates cortical interneurons tangential migration. p27 acts as a master regulator of cytoskeletal transformations to control cortical interneuron migration *in vivo*. p27 controls neurite extension and nucleokinesis through independent pathways. p27 controls nucleokinesis, branching and growth cone splitting through inhibition of RhoA and regulation of acto-myosin contractility (molecular pathway in *red*). p27 promotes neurites extension by regulation microtubule polymerisation (molecular pathway in *green*). p27 expression rescued tangential migration defects in brain slices from p27-null embryos, including numbers of interneurons that reached the cortex and mean interneuron velocity in knockout embryonic slices. However, a p27 mutant (p27190-4A) that lacks the ability to regulate both RhoA signalling (p27-190) and polymerisation of MTs (p274A) could not rescue interneuron migration, while single mutants only partially contributed to this process. This suggests that both novel cell cycle-unrelated activities are required to support proper tangential migration of interneurons in the cerebral cortex. Abbreviations, *MLC-II* for myosin light chain II, *RLC* for regulatory light chain, *MHC* for myosin heavy chain II. *Blue boxes*: cyclin and CDK interacting domain; *orange dots*: centrioles; *purple line*: golgi apparatus (Adapted from Godin et al. 2012)

proline mutant p27 4A efficiently rescued nuclear translocation frequency defects seen upon loss of p27 in cortical interneurons, suggesting that p27 regulates interneurons nucleokinesis in a MT polymerisation-independent manner. Likewise, neurite-extension defect was rescued by expressing a p27 mutant that fails to bind to RhoA. Altogether these data suggest that p27 controls neurite extension and nucleokinesis through two independent pathways, that both contribute to proper tangential migration of interneurons in the cerebral cortex (Godin et al. 2012) (Fig. 4.5). Neuritogenesis is a critical process that takes place in projection neurons undergoing bipolar-multipolar conversion. Thus, it would be interesting to assess whether p27 also control MT-dependent events during radial migration (Box 4.4).

Box 4.4 Microtubule Cytoskeleton and Migration

Microtubules are essential components of the cytoskeleton that play a major role in many cellular functions such as cell migration. Microtubules are hollow tubes composed of α and β tubulin dimers that are, in most cells, nucleated at the centrosome. Microtubule plus-ends are oriented towards the periphery of the cell and explore the cytoplasm in a very dynamic manner. Microtubules alternate between phases of growth (polymerisation) and shrinkage (catastrophe, depolymerisation). Their dynamics are highly regulated by tubulin post-translational modifications such as detyrosination or acetylation, and binding of microtubule-associated proteins (MAP). The microtubule network is even more dynamic and polarized in migrating neurons. Morphological changes that occur during neuron migration are accompanied by dramatic shifts in cytoskeleton structure and centrosome positioning (Higginbotham and Gleeson 2007). Microtubule growth and centrosomes play a key role in driving neurites extension and nuclear migration in neurons, respectively. The centrosome typically precedes and is coupled to the nucleus via perinuclear microtubules that envelop and capture the nucleus (Rivas and Hatten 1995). In one hand, models suggest that the centrosome first moves into the leading process and serves as a cue for forward displacement of the nucleus along the microtubules (Umeshima et al. 2007; Distel et al. 2010; Solecki et al. 2004). In another hand, additional studies reveal that the movement of nucleus and centrosome occur independently (Umeshima et al. 2007; Distel et al. 2010). The pulling effect of the centrosome is not likely to be the only driving force of nuclear translocation. Forward migration of the nucleus indeed could also depend on actin cytoskeleton.

3.4 Upstream Regulation of p27 Function During Migration

The mechanisms regulating the pro-motility role of p27 are not fully understood. However some regulatory mechanisms have been proposed (Fig. 4.4). p90 ribosomal S6 kinase (RSK1), an effector of both Ras/MEK/MAPK and PI3K/PDK1 pathways, drives phosphorylation of p27 at T198. This phosphorylation of p27 enhances RhoA-p27 binding, RhoA-ROCK inhibition and motility in melanoma cells (Larrea et al. 2008). In addition, a recent study indicates that connexin 43, a component of gap junction involved in both neural progenitor proliferation and neuronal migration (Elias and Kriegstein 2008), acts upstream of p27 to regulate the multipolar morphology of migrating neurons (Liu et al. 2012). The N terminal part of Connexin 43 increases the synthesis of p27 via intracellular cAMP mechanism, whereas C terminus reduces the degradation of p27 via inhibition of skp2 (S phase kinase-associated protein 2), the human F-box protein that regulates the ubiquitination of p27. Finally Cdk5 has been proposed as a master regulator of p27-dependent regulation of actin-base cytoskeleton. Cdk5 stabilizes p27 by phosphorylating the Ser10 site, eventually increasing the amount of p27 protein in the cytoplasm. It leads to an increase of non-phosphorylated, activated cofilin through the suppression of RhoA activity, which is required for actin cytoskeletal reorganization in the processes of migrating neurons (Kawauchi et al. 2006). Therefore, dynamic post-translational modification of p27 is a likely mechanism that drives its migratory functions in specific cellular context.

4 Conclusive Remarks

Although p27 has been discovered almost two decades ago, its contribution to cell migration has only been unveiled recently. Several intersecting

works showed its ability to control cell migration by connecting two distinct molecular pathways: the Rho kinase/actin-myosin II on one hand, and the microtubule cytoskeleton, either directly as a MAP or indirectly through inhibition of stathmin activity. Thus, p27 acts as a cytoskeleton modulator and its net activity on cell migration/motility also depends on the migration mode adopted by cells (directed versus ameboid-like migration). It is worth noting that p27 is intrinsically unstructured and as such, may indeed interact with various proteins found in specific subcellular context, including novel yet to be discovered partners for cell migration and beyond.

Acknowledgements We would like to apologize to all authors whose work could not be cited du to length restrictions. L.N. is Research Associate from the Belgian National Funds for Scientific Research (F.R.S- F.N.R.S.). His laboratory is funded by grants from the F.R.S.-F.N.R.S., the Fonds Léon Fredericq, the Fondation Médicale Reine Elisabeth, the Belgian Science Policy (IAP-VII network P7/20), ARC (ARC11/16-01), the Walloon excellence in lifesciences and biotechnology (WELBIO). J.G. has been awarded Marie-Curie and EMBO long-term fellowships.

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Microtubules and Neurodevelopmental Disease: The Movers and the Makers

5

Martin Breuss and David A. Keays

Abstract

The development of the mammalian cortex requires the generation, migration and differentiation of neurons. Each of these cellular events requires a dynamic microtubule cytoskeleton. Microtubules are required for interkinetic nuclear migration, the separation of chromatids in mitosis, nuclear translocation during migration and the outgrowth of neurites. Their importance is underlined by the finding that mutations in a host of microtubule associated proteins cause detrimental neurological disorders. More recently, the structural subunits of microtubules, the tubulin proteins, have been implicated in a spectrum of human diseases collectively known as the tubulinopathies. This chapter reviews the discovery of microtubules, the role they play in neurodevelopment, and catalogues the tubulin isoforms associated with neurodevelopmental disease. Our focus is on the molecular and cellular mechanisms that underlie the pathology of tubulin-associated diseases. Finally, we reflect on whether different tubulin genes have distinct intrinsic functions.

Keywords

Microtubule • Cytoskeleton • Neurodevelopment • Neuronal migration • Tubulinopathies

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L. Nguyen and S. Hippenmeyer (eds.), *Cellular and Molecular Control of Neuronal Migration*,
Advances in Experimental Medicine and Biology 800, DOI 10.1007/978-94-007-7687-6_5,

1 Introduction

Microtubules were discovered in the early 1950s by De Robertis and Franchi, who observed that axons of amphibian sciatic nerves were composed of "large bundle[s] of parallel, tightly packed fibrils" (De Robertis and Franchi 1953). They were similarly described by the neurocytologist Sanford Palay in 1956 as "numerous, long, tubular elements of the endoplasmic reticulum, about 180 Å wide and remarkably straight" (Palay 1956). Soon after Palay's description, various papers reported these structures in different cell types and organisms, such as avian and murine tumours or interstitial cells of hydra (De-The 1964; Slautterback 1963; Ledbetter and Porter 1964). Whereas Slautterback still assumed that they were membranous structures that could be involved in ion transport, De-Thê argued that the "protein nature of these microtubules is very probable" (De-The 1964; Slautterback 1963). In the same year, Ledbetter and Porter described a 13-fold radial symmetry of microtubules in plants and proposed an arrangement of longitudinal subunits that form hollow tubes (Ledbetter and Porter 1964). Shortly after this paper, the protein subunits that form microtubules were isolated, and finally, 1 year later, Mohri published the amino-acid composition of this protein and coined the expression tubulin (Mohri 1968; Shelanski and Taylor 1967). Stephens reported that there are at least two types of microtubule proteins, describing α- and β-tubulins and the tubulin-heterodimer (Stephens 1970). This work culminated in a conference in tubulin biology in 1975 (Taylor 1975) by which time a basic understanding of microtubule structure and function were established (see Fig. 5.1).

2 Tubulin Diversity and Genetics

Shortly after this conference, it became apparent that the α- and β- isoforms were not single entities, but rather existed in multiple flavours. N-terminal sequencing and biochemical separation experiments led Hayashi and colleagues to conclude that both types of tubulins include multiple isotypes consisting of slightly different amino acids (Bryan et al. 1978). The advent of molecular cloning further expanded the tubulin family. cDNA libraries constructed by Cowan and colleagues from chicken brain mRNA resulted in the identification of four α- and four β-tubulin genes; a list which has grown over the years (Krauhs et al. 1981; Lopata et al. 1983; Cleveland et al. 1978, 1980; Cowan et al. 1981; Wilde et al. 1982a, b; Cowan and Dudley 1983; Hall et al. 1983; Little et al. 1981; Ponstingl et al. 1981). With the completion of the human and mouse genome sequences, we now know that there are seven α- and eight β-tubulins in mice; and eight α- and nine β-tubulins in humans (Table 5.1).

With the exception of the carboxy terminus, the α- and β-tubulin isoforms exhibit a high degree of sequence homology; however, their expression pattern varies (Lewis et al. 1985; Villasante et al. 1986; Wang et al. 1986; Burgoyne et al. 1988). For instance, in humans the β-tubulin isoform TUBB1 is specifically found in platelets and megakaryocytes, whereas TUBB3 is expressed in post-mitotic neurons (Wang et al. 1986; Schulze et al. 2004; Liu et al. 2007). Similarly, in *Arabidopsis thaliana*, the *ArathTub9* isoform accumulates specifically in male reproductive tissue, the pollen, whereas *ArathTub1* is preferentially found in roots and leaves (Oakley et al. 2007; Snustad et al. 1992; Cheng et al. 2001).

3 The Multi-tubulin Hypothesis

The existence of this extended gene family with distinct expression patterns led investigators to speculate that the different tubulin isoforms possess unique functional properties, accounting for the extraordinary diverse role microtubules play in eukaryotic cells (Fulton and Simpson 1976). This concept, which is referred to as the multi-tubulin hypothesis, was advanced by Raff and co-workers who employed the

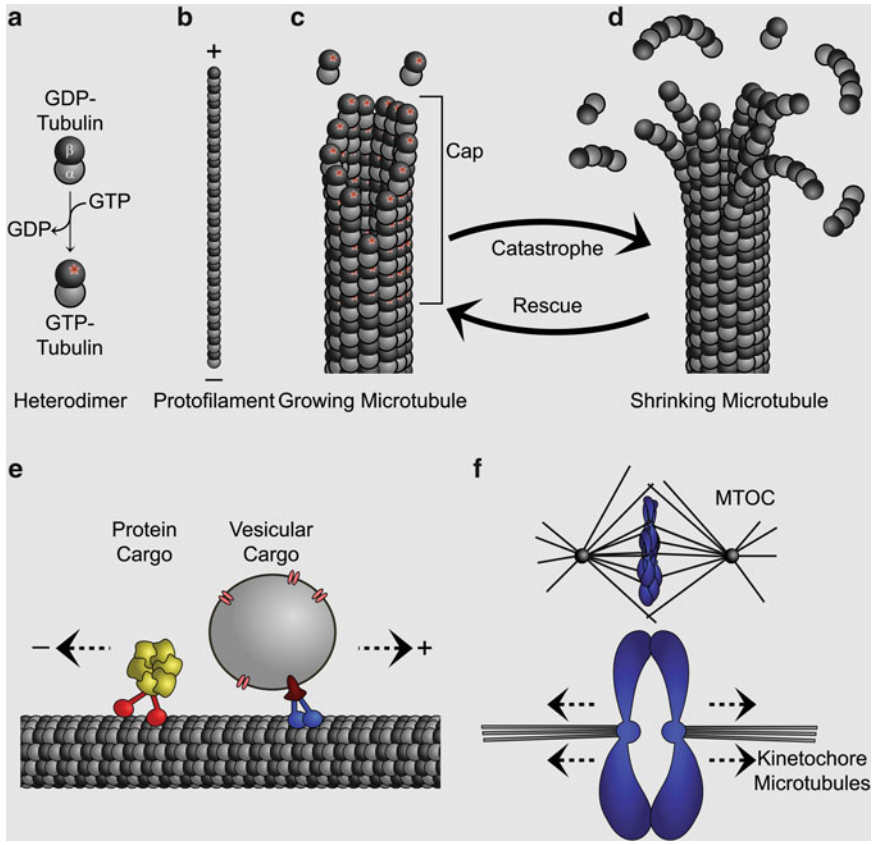


Fig. 5.1 Microtubule biology and cellular function. (**a**) Tubulin heterodimers are the basic building units of microtubules. Both isoforms can bind to a guanidine nucleotide. Whereas α-tubulin cannot hydrolyze and exchange GTP, **β**-tubulin can exist in two states: GTP- or GDP-bound. GTP-tubulin is the active state of the heterodimer with regards to polymerization capability (indicated with a *star*). (**b**) Tubulin heterodimers build up protofilaments by a juxtapositional head-to-tail arrangement. The β-tubulin is oriented towards the so called plus-end, the α-tubulin towards the minus-end. Protofilaments are arranged into a hollow microtubule and have lateral interactions with each other. Note that protofilaments are not formed per se, but are always part of the microtubule complex. (**c**) A polymerizing microtubule will add GTP-bound heterodimers, which will hydrolyze their nucleotide. This results in a GTP-tubulin cap at the plus-end of the microtubule. Due to low concentrations (*in vitro*) or the activity of certain accessory proteins the microtubule can change into a depolymerizing state; this transition is called catastrophe. (**d**) Depolymerizing microtubules are characterized by curved protofilaments (a structural trait of GDP-bound heterodimers) at the plus-tip end that leave the microtubule complex. The reversal of this state into a polymerizing microtubule is called rescue. (**e**) Rigid microtubules are used as transport highways for different cargo, such as macromolecular protein complexes or vesicles. Specialized motor proteins can attach to the microtubule lattice and show processive movement towards the plus- or the minus-end; these motors will bind to cargo and transport it along the microtubule. (**f**) Dynamic microtubules are needed for chromosome alignment in the metaphase plate (*upper panel*) and for sister chromatid separation during anaphase (*lower panel*). *MTOC* Microtubule Organizing Center, centrosome. Modelled on Akhmanova and Steinmetz (2008, 2010), Conde and Caceres (2009), Dogterom et al. (2005), Kuijpers and Hoogenraad (2011) and Schliwa and Woehlke (2003)

genetic tools available in *Drosophila* to replace the testes specific tubulin β-2 with the developmentally expressed β-3 tubulin (Kemphues et al. 1979). The β-3 isoform could support the assembly of a cytoskeletal array, but the substitution nevertheless resulted in defects of axoneme structure, meiosis, and nuclear shaping (Hoyle and Raff 1990; Raff et al. 1997). The Raff group was further able to show that the architecture of microtubules was influenced by the isoform

Table 5.1 List of all human and Murine Tubulin-Isotypes

	Symbol		Name	NCBI Gene ID	
	Mouse	Human	Mouse/Human	Mouse	Human
α-Tubulins	Tuba1a	TUBA1A	Tubulin, α 1A	22142	7846
	Tuba1b	TUBA1B	Tubulin, α 1B	7846	10376
	Tuba1c	TUBA1C	Tubulin, α 1C	22146	84790
	Tuba3a	–	Tubulin, α 3A	22144	–
	Tuba3b	–	Tubulin, α 3B	22147	–
	–	TUBA3C	Tubulin, α 3C	–	7278
	–	TUBA3D	Tubulin, α 3D	–	113457
	–	TUBA3E	Tubulin, α 3E	–	112714
	Tuba4a	TUBA4A	Tubulin, α 4A	22145	7277
	Tuba8	TUBA8	Tubulin, α 8	53857	51807
β-Tubulins	Tubb1	TUBB1	Tubulin, β 1 Class VI	545486	81027
	Tubb2a	TUBB2A	Tubulin, β 2A Class IIA	22151	7280
	Tubb2b	TUBB2B	Tubulin, β 2B Class IIB	73710	347733
	Tubb3	TUBB3	Tubulin, β 3 Class III	22152	10381
	Tubb4a	TUBB4A	Tubulin, β 1 Class VI	22153	10382
	Tubb4b	TUBB4B	Tubulin, β 4B Class IVB	227613	10383
	Tubb5	TUBB5	Tubulin, β 5 Class I	22154	203068
	Tubb6	TUBB6	Tubulin, β 6 Class V	67951	84617
	–	TUBB8	Tubulin, β 8 Class VIII	–	347688

For all isoforms the human and the murine gene symbols are given in addition to a full name. Note that a revised nomenclature for the β-tubulin isoforms is shown and based on the nomenclature of the α-tubulin isoforms (Khodiyar et al. 2007)

composition. Transgenic expression of the moth β-tubulin (Hvβt) alongside the β-2 isoform resulted in a Drosophila germline that was dominated by microtubules with 16 protofilaments, not the usual 13 (Hoyle and Raff 1990; Raff et al. 1997).

Parallel to these reports a mutagenesis screen performed in the group of Martin Chalfie identified a specific β-tubulin isoform (MEC7) that caused loss of touch receptivity in the nematode worm *C. elegans* (Savage et al. 1989, 1994). Similar to Raff and colleagues, they observed that the isoform composition of microtubules could affect the microtubule superstructure. MEC-7 mutants showed a shift from microtubules with 15 protofilaments to microtubules with just 11 protofilaments. This result was mirrored by another *C. elegans* strain harboring mutations in the α-tubulin MEC-12, which is also highly expressed in touch-sensitive neurons and is believed to co-assemble with MEC-7. Mutations in this tubulin again resulted in the loss of microtubules with 15 protofilaments (Fukushige et al. 1999).

These findings suggested that the protofilament number is a fixed inherent property of microtubules which is dependent on the tubulin composition. This is supported by the finding that the predominant configuration of mammalian microtubules in cells is 13 protofilaments (McIntosh et al. 2009; Tilney et al. 1973). However, *in vitro* experiments have shown that vertebrate tubulin-heterodimers by themselves assemble into microtubules ranging from 8 to 17 protofilaments (Chretien et al. 1992; Chretien and Wade 1991). Therefore, preference for a specific number of protofilaments for one isoform can only be determined by interaction with factors present *in vivo*.

So, what factors determine the protofilament number? Brouhard and colleagues have demonstrated that the microtubule associated protein

DCX (Doublecortin) stabilizes the 13 protofilament configuration *in vitro* and they argue that this might be one of the main mechanisms that ensure correct protofilament numbers in neurons (Bechstedt and Brouhard 2012). Likewise, the nucleation by a γ-tubulin ring complex contributes to a consistent width of cellular microtubules (Moritz et al. 2000). Posttranslational modifications may also play a role. Goodman and colleagues have shown that acetylation of the α-tubulin MEC-12 stabilizes the 15 protofilament configuration found in *C. elegans* touch receptive cells (Cueva et al. 2012). The deletion of the responsible acetylase, ATAT-2, results in highly variable protofilaments numbers. This observation has led Goodman and colleagues to the proposition that acetylation promotes the formation of salt bridges that mediate lateral interactions between protofilaments (Cueva et al. 2012). In their model an interaction between glutamate at position 55 and lysine 40 exists within the α-tubulin (αE55-αK40). This salt bridge is disrupted by acetylation of the K40 residue, favoring an interaction between adjacent heterodimers (αE55-α′H283), the angle of which is consistent with 15 protofilament microtubules (Cueva et al. 2012).

4 Posttranslational Modifications

Acetylation is but one of a myriad of different posttranslational modifications associated with the tubulins. Others include detyrosination, polyglutamylation, polyglycylation, palmitolyation and phosphorylation (Janke and Bulinski 2011; Westermann and Weber 2003). These modifications affect tubulin dynamics and stability, the interaction with motor proteins and also non-motor microtubule associated proteins. The amino acid sequence of individual tubulin isoforms influences their respective posttranslational modifications. For instance, in mice and humans TUBA8 lacks a lysine at the critical residue 40, and consequently cannot be acetylated. This contrasts with the remaining members of the α-tubulin family, all of which have this residue, and therefore can be subject to this modification (Fukushige et al. 1999; Stanchi et al. 2000). Likewise, some tubulin isoforms, such as the testis specific cα2 in chicken, lack a carboxy-terminal tyrosine residue and are therefore not subject to detyrosination (Pratt et al. 1987).

5 Tubulin proteins in Neurodevelopmental Disease – The Makers

Since their discovery in the 1950s it is has been clear that microtubule function is essential for the formation and function of the nervous system in a broad range of animal species, whether it be a nematode, a fruit fly, a frog or a rodent (Goldstein and Yang 2000; Gerson et al. 1976; Gray 1975, 1976; Ward et al. 1975; Poulain and Sobel 2010). It is no surprise that the same holds true for the development of the human brain. Microtubules facilitate neurogenic division, they drive neuronal migration, and they are required for neuronal differentiation and circuit formation (Ayala et al. 2007; Kuijpers and Hoogenraad 2011) (Fig. 5.2). Here we discuss the role of the different tubulins in these processes with a focus on human diseases caused by mutations in these genes (Fig. 5.3).

6 *TUBA1A* – The First

The tubulin gene family was first implicated in neurodevelopmental disease following the cloning of an N-ethyl-N-nitrosourea (ENU) induced *Tuba1a* mutation in the *Jenna* mouse mutant (Keays et al. 2007). It was identified in a screen for hyperactive behavior, but also showed defects in working memory and presented with an exaggerated acoustic startle response (Edwards et al. 2011; Keays et al. 2007, 2010). Histological examination revealed wave-like perturbations of the adult cortex, a fractured pyramidal layer of the hippocampus and structural abnormalities in the superior colliculus; defects which were

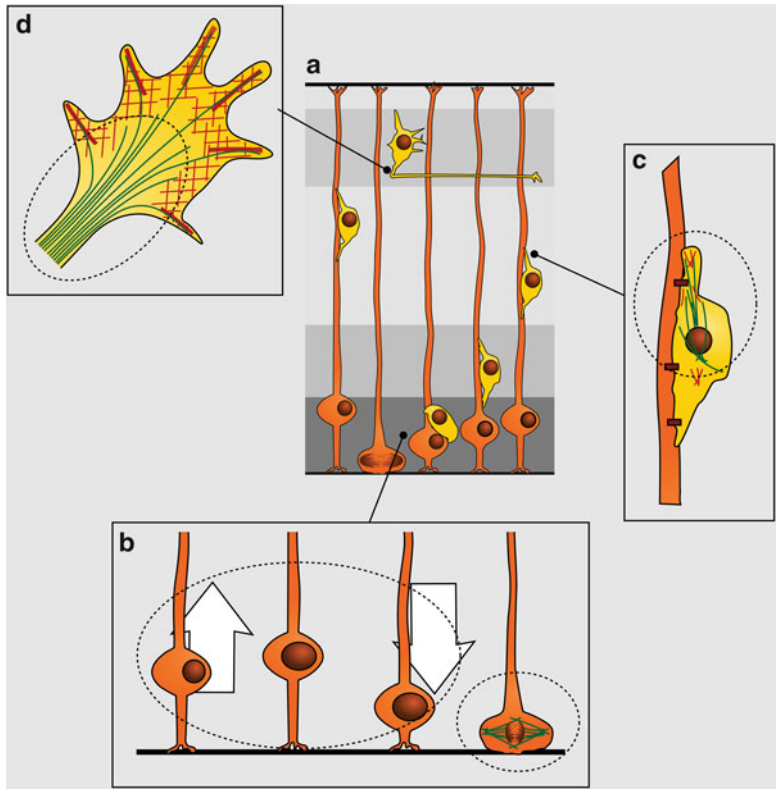


Fig. 5.2 Examples for microtubule functions in neurodevelopment. (**a**) Schematic of the developing cortex. Radial glial progenitors span the entirety of the cortex from the ventricular to the pial surface (shown in *orange*). They undergo mitosis to generate other types of progenitors (not shown) and neurons (shown in *yellow*). Postmitotic neurons migrate along their radial glial mother cell to their final destination in the cortical plate where they will differentiate and extend their axons. (**b**) Detailed view of the interkinetic nuclear migration of a radial glial cell. The cell nuclei migrate basally (*upwards*) during G1 phase, undergo S phase and migrate apically (*downwards*) during G2. Finally, cells will undergo mitosis at the ventricular surface. Microtubules are required for interkinetic nuclear migration and spindle formation (shown in *green*) which mediates sister chromatid separation in M phase. (**c**) Neuronal migration requires nuclear translocation. Nuclei are surrounded by a microtubule cage (nuclear cage; shown in *green*) that connects with the actomyosin network (shown in *red*) via the centrosome. (**d**) Differentiating neurons extend their neurites to form connections. Axonal projections have to cover large distances within the brain and the correct pathfinding requires the establishment of a growth cone. This specialized structure consists of microtubules (shown in *green*) that provide a rigid platform which interacts with the actin cytoskeleton (shown in *red*), facilitating the extension of the lamellipodia

attributed to impaired neuronal migration. These phenotypes were reminiscent of mouse models of lissencephaly (*Lis1*, *Dcx*, and the *Reeler* mouse), a disease which is characterized by a cortex with a smooth surface (Gleeson and Walsh 2000; Guerrini and Parrini 2010) (see also Chap. 1). Speculating that mutations in *TUBA1A* might cause neurodevelopmental disease in humans, a genetic screen identified two *de novo* mutations in this gene (R264C and R402H) in patients with lissencephaly (Keays et al. 2007).

The introduction of a *TUBA1A* genetic test into clinical practice has resulted in the identification of a host of disease causing mutations in this gene (Poirier et al. 2007, 2012; Fallet-Bianco et al. 2008; Bahi-Buisson et al. 2008; Morris-Rosendahl et al. 2008; Kumar et al. 2010; Lecourtois et al. 2010; Jansen et al. 2011; Mokanszki et al. 2012; Sohal et al. 2012; Hikita et al. 2013). Most patients identified have *de novo* mutations and present with a spectrum of phenotypes that extends from an absence (agyria), to a

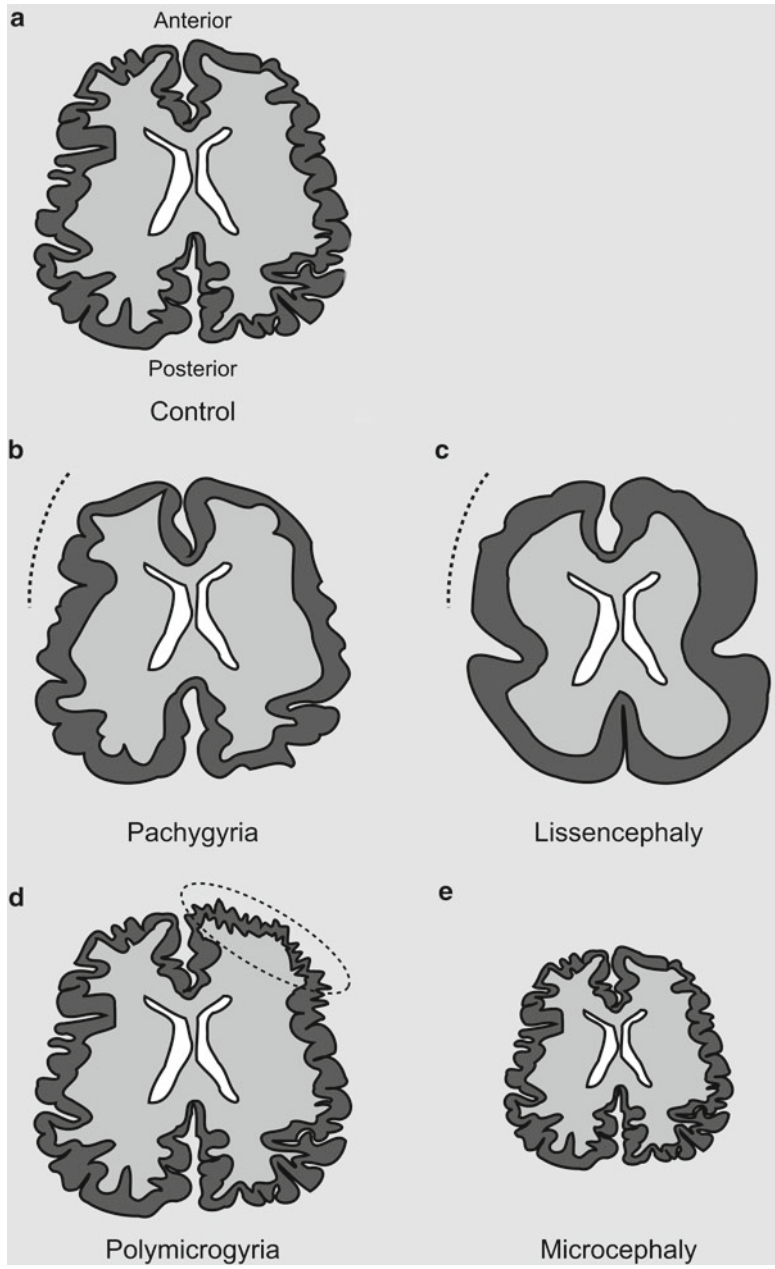


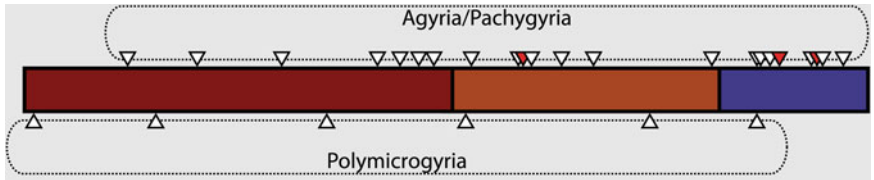
Fig. 5.3 Spectrum of tubulinopathies. (**a**) Depiction of an axial section of a control brain with regular distribution and number of sulci and gyri. (**b**) Pachygyric (meaning thick gyri) patients show a reduction in the number and increase in the size of their gyri (indicated with a *dotted line*). (**c**) Lissencephalic (meaning smooth brain) patients show a complete absence of sulci and gyri. Both pachygyria and lissencephaly can be present as a gradient from anterior to posterior. (**d**) Polymicrogyric (meaning many small gyri) patients show an increase in the number of gyri with a decreased size. This is often focally localized and asymmetric. (**e**) Microcephalic (meaning small head) patients show a reduction in overall brain size (−2SD from the mean). Microcephaly vera (or primary microcephaly) occurs in the absence of other cortical malformations

reduction (pachygyria) or even an increased number of gyri (polymicrogyria) (Poirier et al. 2007, 2012; Kumar et al. 2010). These cortical phenotypes are frequently accompanied by hypoplasia or agenesis of the corpus callosum, hypoplasia of the brain stem, dysgenesis of the basal ganglia, ventricular dilation, and hypoplasia of the cerebellum (Sohal et al. 2012; Kumar et al. 2010). In addition, almost all patients with *TUBA1A* mutations present with a reduction in brain size (−1 S.D. to −7 S.D. from mean), most classifying as microcephalic (less than −2 S.D. below mean; more than 90 %) (Poirier et al. 2007, 2012; Sohal et al. 2012; Kumar et al. 2010).

7 Molecular and Cellular Mechanisms of *TUBA1A* Mutations

What is the underlying molecular defect that results in the disease state in patients with *TUBA1A* mutations? TUBA1A, like all tubulins protein, has three major domains; an N-terminal domain (1–229), an intermediate domain (230–371), and a C-terminal domain (372–450). The N-terminal domain harbors a GTP binding pocket that, in the case of α-tubulins, is non-exchangeable and is thought to act as a structural co-factor (Nogales et al. 1997; Spiegelman et al. 1977). In the case of the *Jenna* mouse it was shown that the S140G mutation caused impairment in GTP binding, and, consequently, a dramatic reduction in heterodimer formation. The mutant heterodimers, however, were able to incorporate into the microtubule cytoskeleton, suggesting that the mutation acted by haploinsufficiency. Similarly, the human mutations V303G, L397P, and R402C all result in a reduction in heterodimer levels, which have been attributed to molecular defects in the tubulin folding pathway (Tian et al. 2008, 2010). It is apparent, however, that some disease causing tubulin mutations have no effect on the efficiency of chaperon mediated tubulin folding whatsoever. For instance, *in vitro* analysis of the P263T, L286F, R402H, and S419L mutations has shown that they do not cause impaired heterodimer folding. In the case of the P263T mutation the incorporation of mutant heterodimers into the microtubule lattice has a deleterious effect of microtubule dynamics and growth, lending itself to the conclusion that some tubulin mutations act by a dominant negative mechanism (Tian et al. 2010). Mutations that fall within this class may influence the binding of microtubule associated proteins such as DCX or the kinesins (Amos and Schlieper 2005). Tubulins might also interact with unknown microtubule associated proteins that are vital for the formation of the developing brain.

What are the underlying cellular mechanisms that give rise to *TUBA1A*-related disease? In addressing this question it is important to appreciate that *TUBA1A* is highly expressed in post-mitotic neurons, but not glia, in the human and mouse brain (Gloster et al. 1999; Bamji and Miller 1996). Murine expression studies have shown that TUBA1A is largely absent from the proliferative ventricular zone (VZ), and its expression peaks at embryonic day (E) 16.5 (Braun et al. 2010). The migration of neurons requires the extension of the leading process, the translocation of the nucleus and the retraction of the trailing process (Trivedi and Solecki 2011) (see also Chaps. 1, 2, 4 and 7). All of these processes are heavily reliant on a dynamic microtubule network, and could potentially be impaired by mutations in *TUBA1A*. Similarly, neurite outgrowth requires the stable support and dynamic force generated by microtubules (Dent and Gertler 2003). Defects in this process can cause inadequate crossing of the midline, resulting in an abnormal corpus callosum and neurological defects (Engle 2010). Disorders of axon guidance or migration, however, fail to account for the reduction in brain size that is observed in almost all patients with mutations in *TUBA1A*. This is particularly curious, given its post-mitotic expression. One explanation that might account for this phenotype is an increase in neuronal apoptosis, which has been observed in the adult superior colliculus in the *Jenna* mouse (Edwards et al. 2011). This explanation is consistent with the observation that *TUBA1A* associated microcephaly can increase in severity postnatally (Cushion et al. 2013).



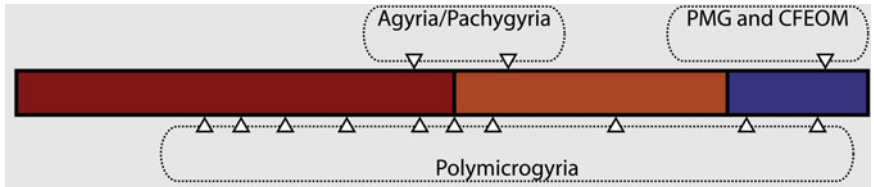
Gene Name:	*TUBA1A* (NCBI Gene ID 7846)
Genomic Location:	Chromosome 12
Size:	451 amino acids
Expression:	Predominantly post-mitotic neurons
Malformations:	Lissencephaly (Agyria) to Pachygyria; or Polymicrogyria; Microcephaly Hypoplasia or agenesis of the corpus callosum Hypoplasia of the brain stem Dysgenesis of the basal ganglia Ventricular dilation Hypoplasia of the cerebellum
Mutations	
Agyria/Pachygyria:	E55K; *L70S*; L92V; V137D; I188L; C200Y; Y210C; D218Y; I238V; P263T; R264C (Hotspot); A270T; L286F; V303G; G366R; R390C; N392S; L397P; R402H; R402C; R402L; S419L; R422C; R422H; M425K; G436R
Polymicrogyria:	I5L; Y161H; V235L; A333V; R390C

Fig. 5.4 Mutations associated with *TUBA1A*

Why do mutations in *TUBA1A* cause a spectrum of distinct neurological disorders? Initially this gene was strongly associated with lissencephaly/pachygyria, but it is now clear that *de novo* mutations can also cause polymicrogyria. For instance, a mutation in valine 235 (V235L) results in bilateral and asymmetric polymicrogyria, whereas mutations in arginine 402 (R402C, R402H) cause classic lissencephaly (Mokanszki et al. 2012; Kumar et al. 2010; Poirier et al. 2007). An analysis of the position of polymicrogyria and lissencephaly causing mutations reveals no obvious pattern (Fig. 5.4). It is conceivable that different diseases are a consequence of defects in different cellular processes associated with microtubule based neuronal migration. However, this would not account for the interesting case of the R390C mutation. This very same mutation has been reported to cause polymicrogyria in a 1-year-old boy and mild gyral simplification and total agenesis of the corpus callosum in another child (Poirier et al. 2012; Kumar et al. 2010). How does the same mutation cause two distinct migration phenotypes? One possibility could be the exposure to different environmental conditions *in utero*; or additional genetic factors that contribute to one or the other phenotype.

8 *TUBB2B* – Expanding the Spectrum

Given that mutations in *TUBA1A* cause neurodevelopmental disease, it was reasonable to speculate that mutations in the β-tubulins might also be pathogenic. Following a genetic screen of



Gene Name:	*TUBB2B* (NCBI Gene ID 347733)
Genomic Location:	Chromosome 6
Size:	445 amino acids
Expression:	Post-mitotic neurons, but also at lower levels in progenitors
Malformations	Polymicrogyria with or without CFEOM; or Agyria/Pachygyria Microcephaly Dysmorphic basal ganglia Cerebellar dys- or hypoplasia Abnormal corpus callosum Brain stem hypoplasia
Mutations	
Polymicrogyria:	G98R; L117P; G140A; S172P; I210T; L228P; A248V; F265L; T312M; R380L; R380S; R380C; D417N
Agyria/Pachygyria:	L207P; N256S
PMG and CFEOM:	E421K

Fig. 5.5 Mutations associated with *TUBB2B*

TUBB2A, *TUBB2B*, and *TUBB2C*, the Chelly group reported the identification of five cases of asymmetrical polymicrogyria (four patients, one aborted fetus) caused by mutations in *TUBB2B* (Jaglin et al. 2009). Besides asymmetrical polymicrogyria, each patient presented with additional features, such as microcephaly, dysmorphic basal ganglia, cerebellar dys- or hypoplasia, abnormal corpus callosum and brain stem hypoplasia. Similar to *TUBA1A*, the spectrum of *TUBB2B* related diseases has expanded rapidly. Engle and colleagues recently reported the occurrence of a mutation (E421K) that causes congenital fibrosis of the extraocular muscles (CFEOM), a specific defect of axon guidance, accompanied by polymicrogyria (Cederquist et al. 2012); axon guidance defects accompanied by polymicrogyria and schizenecephaly have also been reported for a G140A mutation (Romaniello et al. 2012). Pilz and colleagues have described a lissencephalic patient with a *TUBB2B* mutation (L207P), and Guerrini and colleagues have reported an individual with pachygyria and microcephaly with an N256S mutation (Cushion et al. 2013; Guerrini et al. 2012) (Fig. 5.5). To date biochemical analysis has been conducted on five *TUBB2B* mutations (F265L, I210T, L228P, S172P and T312M) and, similar to *TUBA1A* mutations, they influence tubulin heterodimer folding and their incorporation into microtubules in different ways. For instance, the S172P mutation results in arrested tubulin heterodimer folding, whereas the I210T is indistinguishable from the wild-type in biochemical and cellular assays.

As might be expected *TUBB2B* is highly expressed in post-mitotic neurons at key develop-

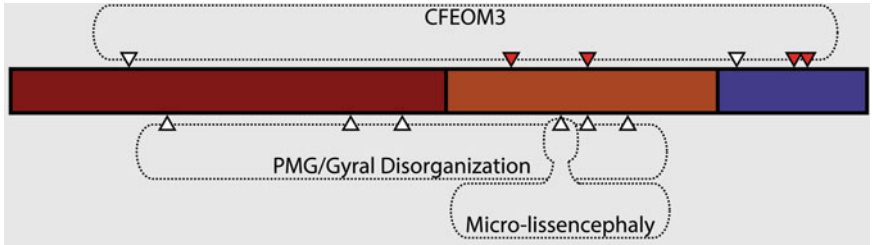
mental time-points, but is also found in progenitor cells at lower levels (Jaglin et al. 2009). *In vivo* knockdown experiments in the rat have shown that *Tubb2b* is required for radial migration. These data have led to the hypothesis that *TUBB2B*-related cortical malformations are due to a combination of impairment in neuronal migration and radial glial dysfunction (Jaglin et al. 2009).

9 *TUBB3* – The Janus Tubulin

The list of tubulinopathy causing genes expanded in 2010 with the addition of *TUBB3* by two independent studies. Engle and colleagues showed that six different heterozygous mutations in this gene caused congenital fibrosis of the extraocular muscles type 3 (CFEOM3), either in isolation or as a component of a syndrome (Tischfield et al. 2010). Interestingly, and in marked contrast to the previously described *TUBB2B* and *TUBA1A* mutations, no neuronal migration deficits or microcephaly could be observed in these patients. The pathogenicity of one mutation (R262C) was explored further by the creation of a transgenic mouse line that replicated various aspects of the human disease. The R262C mutation increased microtubule stability and impaired their interaction with the motor protein Kif21a. Ultimately, this resulted in defects of axon guidance and cranial nerve extension, but not cortical architecture (Tischfield et al. 2010). In the same year, the Chelly group reported six different *TUBB3* mutations (five heterozygous, one homozygous) in nine patients with malformations of cortical development associated with neuronal migration defects (Poirier et al. 2010). All patients suffered from polymicrogyria or gyral disorganization with microcephaly and cerebellar dysor hypoplasia. With the exception of one individual, these patients presented with brainstem hypoplasia, an abnormal corpus callosum and dysmorphic basal ganglia, but not the CFEOM3 phenotypes described by Engle and colleagues (Fig. 5.6). None of the mutations identified by the Chelly group were the same as those that cause CFEOM3; curiously, however, both studies described different mutations in the same residue, A302. Its substitution with a threonine caused CFEOM3, whereas the A302V mutation was pathogenic in a patient with gyral disorganization (Tischfield et al. 2010; Poirier et al. 2010). These data imply that the mutations probably act by a dominant mechanism and not by haploinsufficiency. This idea is supported by the observation that mutations that cause cortical malformations, in contrast to the ones causing CFEOM3, reduce microtubule stability (Tischfield et al. 2010; Poirier et al. 2010). Finally, it should be noted that the phenotypes associated with *TUBB3* have recently been expanded to include peripheral neuropathy, defective olfactory function, photophobia, cyclic vomiting and hypogonadotropic hypogonadism with analogies to Kallmann syndrome (Chew et al. 2013).

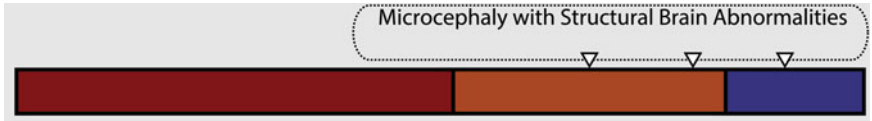
10 *TUBB5* – The Mitotic Tubulin

We have added another tubulin isoform to the list of disease-causing genes: *TUBB5* (Breuss et al. 2012). In contrast to the other tubulinopathies, the primary defect associated with *TUBB5* is microcephaly. We reported three unrelated individuals with *de novo TUBB5* mutations (M299V, V353I, E401K) with only one patient presenting with a notable migration phenotype (M299V) (Fig. 5.7). Similar to other tubulin isotype-dependent disorders, affected individuals presented with dysmorphic basal ganglia and corpus callosum abnormalities. Employing a transgenic mouse line that expresses GFP under the endogenous *Tubb5* promoter, we have shown that *TUBB5* is expressed in radial glial progenitors, intermediate progenitors, and post-mitotic neurons. Depletion of *TUBB5 in utero* by shRNA knockdown perturbed the cell cycle of progenitors and resulted in neuronal migration defects. Similarly, we have found that overexpression of two of the three *TUBB5* mutations (E401K and V353I) increased the percentage of progenitors in M-phase and altered neuronal positioning. Intriguingly, these two mutations affected the tubulin folding pathway in different ways. The behavior of the



Gene Name:	*TUBB3* (NCBI Gene ID 10381)
Genomic Location:	Chromosome 16
Size:	450 amino acids
Expression:	Exclusively post-mitotic neurons
Malformations:	CFEOM3; or PMG/Gyral disorganization Dysmorphic basal ganglia Cerebellar hypoplasia
Mutations	
CFEOM3:	R62Q; R262H; R262C; A302T; R380C; E410K; D417H; D417N
PMG/ Gyral disorganization:	G82R; T178M; E205K; A302V; M323V
Micro-lissencephaly:	M288V

Fig. 5.6 Mutations associated with *TUBB3*



Gene Name:	*TUBB5* (NCBI Gene ID 203068)
Genomic Location:	Chromosome 6
Size:	444 amino acids
Expression:	Progenitors and postmitotic neurons
Malformations:	Microcephaly Dysmorphic basal ganglia Abnormal corpus callosum Abnormal brain stem
Mutations	
Microcephaly with structural brain abnormalities:	M299V, V353I, E401K

Fig. 5.7 Mutations associated with *TUBB5*

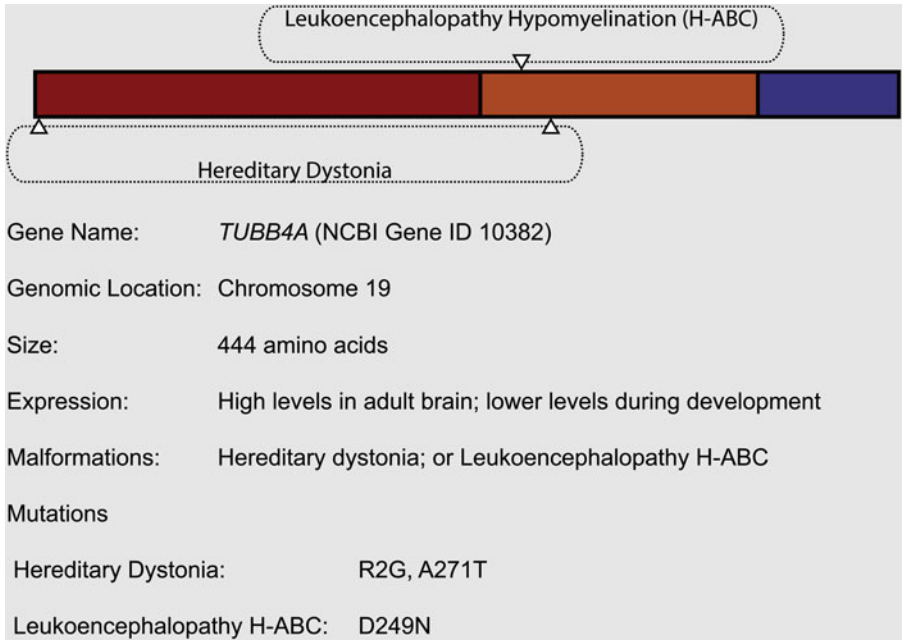


Fig. 5.8 Mutations associated with *TUBB4A*

V353I mutation was indistinguishable from wild-type tubulin, whereas the E401K mutation disrupted the chaperone-mediated folding with a consequent dearth of α/ß heterodimers that failed to incorporate into the cytoskeletal network. This result highlights that tubulin mutations that operate by different mechanisms can still result in similar phenotypes.

11 *TUBB4A* – Postnatal and Motor-Related

There is emerging evidence that the tubulinopathies are not limited to developmental phenotypes. In 2013 two independent groups reported the cloning of an R2G mutation in *TUBB4A* in a multigeneration Australian family that suffered from Whispering Dysphonia. Affected individuals in this family presented with a characteristic "hoppy horse" gait, laryngeal dysphonia, and a thin face (Hersheson et al. 2012; Lohmann et al. 2012). Klein and colleagues additionally described an A271T mutation in an unrelated familial case of segmental dystonia with spasmodic dysphonia (Lohmann et al. 2012). Complementing this finding, Vanderver and colleagues have reported that D249N mutations in *TUBB4A* cause a rare form of hereditary leukoencephalopathy, characterised by hypomyelination with atrophy of the basal ganglia and the cerebellum (H-ABC) (Simons et al. 2013) (Fig. 5.8). Most of the affected individuals, which originated from seven independent families, presented in infancy with motor dysfunction, but with normal cognitive and language development. While the underlying cellular and molecular mechanisms responsible for these phenotypes remain to be defined, it is known that the Asp249 residue forms a salt bridge with Arg2. This is important for the correct positioning of the T7 loop that interacts with the α-tubulin bound GTP. It is therefore a tenable hypothesis that disruption of this bridge impairs heterodimer stability or microtubule dynamics. Given the postnatal motor-deficits in those individuals with *TUBB4A* mutations, it is an unsurprising fact that this gene is expressed at low levels in the developing CNS, but is highly transcribed in adult cerebellum, brainstem and striatum (Breuss et al. 2012; Leandro-Garcia et al. 2010). It remains to be determined which cell types in the adult brain express this gene.

12 *TUBA8* – (Un)Related

TUBA8 was first cloned from a human adult skeletal muscle cDNA library, and was shown to be enriched in heart, skeletal muscle and testis (Stanchi et al. 2000). Sheridan and colleagues implicated this gene in polymicrogyria by undertaking genetic mapping of two consanguineous families (Abdollahi et al. 2009). They found linkage to a 7.42 Mb region that contained 230 genes, one of which was *TUBA8*. Candidate gene sequencing revealed a 14 base pair deletion in intron 1 of *TUBA8* that altered splicing. Despite assertions that this gene is widely expressed in developing neuronal structures, careful analysis in mice and humans has revealed that (unlike other disease-causing tubulins) *TUBA8* is expressed at extremely low levels in the developing brain (Braun et al. 2010). An alternative explanation for the reported polymicrogyria is that an unidentified mutation lies in another gene in the candidate interval. In the absence of additional unrelated patients with mutations in this gene the association of *TUBA8* with neurodevelopment disease should be considered tenuous at best. It may transpire that *TUBA8* is an innocent gene.

13 *TUBG1* – The Third Family Implicated

The tubulin superfamily is not limited to the α- and β-tubulins, but includes the γ-, δ-, ε-, ζ- and η-tubulins (McKean et al. 2001; Dutcher 2001; Oakley 2000; Oakley and Oakley 1989). Chelly and colleagues have recently shown that mutations in the γ-tubulin *TUBG1* cause complex cortical malformations (Poirier et al. 2013). The γ-tubulins are highly conserved in eukaryotes, forming a structural component of the centrosome known as the γ-tubulin ring complex (Oakley 2000; McKean et al. 2001). This complex is known to play a role in the nucleation of microtubules and regulation of the spindle during mitosis (Edgerton-Morgan and Oakley 2012). Chelly and colleagues reported three patients harboring missense *de novo* mutations in *TUBG1* (L387P, Y92C, T331P), one of the two isoforms in humans. Functional analysis revealed that the L387P mutation impairs chaperone mediated folding of TUBG1, whereas the W92C mutation results in decreased frequency of microtubule nucleation from the spindle body (Poirier et al. 2013).

TUBG1 is constitutively expressed throughout the body and its homozygous deletion results in an arrest of development at the morula/blastocysts stage due to mitotic spindle disorganization (Yuba-Kubo et al. 2005). Surprisingly, given the function of γ-tubulin in centrosome regulation, only two of these patients suffered from microcephaly. All patients showed agyria and/or pachygyria with abnormalities of the corpus callosum, highlighting the vanishing boundaries between disorders characterised by defects in proliferation, migration and differentiation. Consistent with this observation, *TUBG1* knockdown by *in utero* electroporation resulted in a drastic impairment in neuronal migration (Poirier et al. 2013). The coupling of the centrosome to the actin cytoskeleton is a critical requirement for the saltatory nuclear translocation in migrating neurons (Tsai and Gleeson 2005) (see also Chaps. 1, 2, 4 and 7).

14 Microtubule Associated Proteins – The Movers

Microtubules do not act alone, but rather in concert with an orchestra of microtubule associated proteins (MAPs) (Amos and Schlieper 2005) (see also Chaps. 4 and 6). The multitude of tubulin mutations that do not affect folding, and are able to incorporate into a functional cytoskeleton strongly suggest that they act by impairing the interaction with MAPs. There are a multitude of MAPs that could potentially be involved, including the microtubule stabilizer DCX (see also above) which is a key player in the pathogenesis of lissencephaly (Reiner 2013; Caspi et al. 2000; Gleeson et al. 1998). Here, we focus on the movers; dynein and kinesin.

These two classes of proteins are molecular motors that employ microtubules as intracellular

highways to delivery their molecular cargo (Vale and Milligan 2000). In addition they can also act as force generators or influence microtubule stability (Moore and Wordeman 2004; Mitchison and Mitchison 2010). While it is unclear whether isoform composition directly influences the interaction between microtubules and motor proteins, it has been shown that posttranslational modifications are important (Janke and Bulinski 2011). For instance, kinesin family motors increase their microtubule-binding upon detyrosination (Konishi and Setou 2009; Dunn et al. 2008). Similarly, for dynein motors, it has been shown that polyglutamylation directly regulates their interactions with microtubules (Suryavanshi et al. 2010; Kubo et al. 2010).

15 Dynein

Cytoplasmic dynein is a minus-end directed motor protein that consists of two heavy chains and a complex of associated light chains (Vallee et al. 2012; Rodriguez-Crespo 2011). The major cytoplasmic form, dynein 1 (DYNC1H1), is ubiquitously expressed and important for various functions ranging from vesicular transport to nuclear envelope breakdown (Vallee et al. 2012). The minor form, dynein 2 (DYNC2H1), is responsible for transport within cilia and flagella; their beating behavior in turn, is driven by the axonemal class of dyneins (Vallee et al. 2012). Chelly and colleagues reported *de novo* mutations in DYNC1H1 in nine independent cases of pachygryria and/or polymicrogyria (Poirier et al. 2013). Consistent with earlier findings that implicated dynein in peripheral neuropathy and an axonal (type 2) form of Charcot-Marie-Tooth disease, a subset of these patients also showed defects in the peripheral nervous system (Harms et al. 2012; Weedon et al. 2011; Poirier et al. 2013). Disease-causing missense mutations causing malformations of cortical development occurred throughout the protein; however, the mutations affecting the peripheral nervous system seem to cluster in the tail domain.

Although dynein has a multitude of cellular functions, the observed cortical malformations are most likely the result of deficient nuclear translocation in migrating neurons. The critical role dynein plays in this process has been revealed by experiments in the fungus *Aspergillus nidulans*, a eukaryotic model for nuclear migration (Willins et al. 1997). Morris and colleagues reported that mutations in the fungal homolog *NudA* block nuclear migration (Xiang et al. 1994). They further showed genetic interaction of this gene with the *LIS1* homolog, *NudF* (Willins et al. 1997). Subsequent functional characterization of this interaction revealed that dynein and Lis1 are acting in concert with Nde1/Nudel to couple the centrosome and the nucleus to the actin cytoskeleton (Tsai et al. 2007; Sasaki et al. 2000). The importance of this interaction is underlined by the finding that mutations in *LIS1* and *NDE1* cause neurodevelopmental disease (Reiner et al. 1993; Alkuraya et al. 2011; Bakircioglu et al. 2011) (see also Chap. 1).

16 Kinesins

The kinesin superfamily consists of 45 genes (also known as KIFs), classified into 15 families (Hirokawa et al. 2009). The progressive movement of most KIFs is directed toward the microtubule minus-end, although there are some family members that move toward the plus-end. Most are dimeric in structure, which enables them to "walk" along the surface of microtubules, driven by the hydrolysis of ATP. Their preferred substrates are 13 protofilament microtubules, underlining the importance of protofilaments number (Moores et al. 2006). Their main function is to transport of cellular cargo (Hirokawa et al. 2009), however, they also play an important role in the depolymerization of microtubules and force generation during mitosis (Moore and Wordeman 2004). These "movers" have also been implicated in neurological disease: Marchuk and colleagues identified a *KIF5A* mutation (N256S) as causative in hereditary spastic paraplegia, a neurodegenerative disorder (Reid et al. 2002); Engle and colleagues showed that a host of missense mutations in *KIF21A* cause the congenital axon guidance defects CFEOM1 and

CFEOM3 (Yamada et al. 2003, 2004); and Chelly and colleagues identified several mutations in both *KIF5C* and *KIF2A* that cause microcephaly with epilepsy and severe cortical phenotypes, such as polymicrogyria and agyria/pachygryria (Poirier et al. 2013).

17 Reflections and Directions

This review has catalogued those tubulin genes, the "makers", and those microtubule associated motors, "the movers", that cause neurodevelopmental disease. It is apparent that mutations in the neurodevelopmentally expressed "makers" (*TUBA1A*, *TUBB2B*, *TUBB3*, and *TUBB5*) cause a spectrum of diseases with overlapping phenotypes. At this juncture it is not possible to predict a disease phenotype given the residue or isoform mutated. This is because different tubulin mutations act by distinct mechanisms, some by haploinsuffiency, others by dominant means. Dominant mutations, in turn, have different effects on the stability and dynamic properties of microtubules, which is likely to be associated with the binding affinities of various MAPs. The question that arises is whether different tubulin proteins have intrinsic properties that make them distinct? Alternatively, could their unique expression patterns simply provide spatio-temporally critical concentrations? While the classic experiments in invertebrate systems strongly pointed towards tubulin specific function(s), the same cannot be said for the tubulinopathies, which have muddied the scientific waters. One way to address this issue would be to create a series of transgenic mouse models whereby the coding region of one gene of interest (e.g. *Tubb5*) is replaced by each of the seven other β-tubulin isoforms. Driven by the endogenous *Tubb5* promoter, this experiment would reveal, whether Tubb5 for instance, has a specific function in the developing telencephalon.

In the future, we expect that an understanding of tubulin gene function and the underlying molecular mechanisms that give rise to the tubulinopathies will play an important role in the development of novel therapeutics and diagnostic tools. There is growing evidence that neurodevelopmental disorders, once thought to be irreversible, may be treated effectively postnatally (Ehninger and Silva 2011). In the case of loss of function mutations in *TUBA1A* it is conceivable that a small molecule that increased the transcriptional activity at the *TUBA1A* genomic locus might be of utility (Kern et al. 2013). Finally, we expect that in the coming years the tubulinopathies will expand further, encompassing additional genes and disease states. To date genetic screening has primarily been biased by pre-conceived notions of the role of a particular isoform, and the availability of specific patient cohorts. There is already some evidence implicating *de novo TUBA1A* and *TUBB2B* mutations in autism spectrum disorders (Neale et al. 2012; Pinto et al. 2010). With the extension of exome, and eventually, whole genome sequencing into the clinic we expect that many more *de novo* mutations will be found.

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Mark/Par-1 Marking the Polarity of Migrating Neurons

6

Orly Reiner and Tamar Sapir

Abstract

Proper brain development requires the orchestrated migration of neurons from their place of birth to their final positioning, where they will form appropriate connections with their target cells. These events require coordinated activity of multiple elements of the cytoskeleton, in which the MARK/Par-1 polarity kinase plays an important role. Here, the various roles and modes of regulation of MARK/Par-1 are reviewed. MARK/Par-1 participates in axon formation in primary hippocampal neurons. Balanced levels of MARK/Par-1 are required for proper radial migration, as well as for migration in the rostral migratory stream. Normal neuronal migration requires at least two of MARK/Par-1 substrates, DCX and tau. Overall, the positioning of MARK/Par-1 at the crosstalk of regulating cytoskeletal dynamics allows its participation in neuronal polarity decisions.

Keywords

Microtubules • Neuronal migration • Neuronal polarity doublecortin • Tau

1 Introduction

The capacity of the mammalian brain to perform complex tasks depends on the activity of a large number of neurons spatially organized into regions with distinct functions. Neocortical neurons arise from dividing cells in the proliferative regions of the developing brain. In the cerebral cortex, the two main types of neurons include the excitatory glutamatergic neurons, which compose the majority of the neurons in the cerebral cortex, and the inhibitory GABAergic interneurons. These two types of neurons are born in physically distinct areas of the brain, therefore, they need to migrate, sometimes very long distances, to their final position using several types of cellular motility [reviewed in (Ayala et al. 2007; Marin and Rubenstein 2001, 2003; Kriegstein and Noctor 2004; Nadarajah and Parnavelas 2002; Tsai and Gleeson 2005; Reiner and Gerlitz 2013; Reiner and Sapir 2009; Reiner 2013)]. The six layers of the cerebral cortex are composed of

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L. Nguyen and S. Hippenmeyer (eds.), *Cellular and Molecular Control of Neuronal Migration*, Advances in Experimental Medicine and Biology 800, DOI 10.1007/978-94-007-7687-6_6,

neurons that are born in different areas but are subsequently organized according to their birthdates (Angevine and Sidman 1961; McConnell 1991). In addition, the precursors for olfactory bulb interneurons are generated from early postnatal ages and during adulthood in the subventricular zone (SVZ) (Luskin 1993), and migrate in the rostral migratory stream in a continuous way (Altman 1969; Lois et al. 1996; Lledo et al. 2006).

Genetic mutations, which affect polarity regulation and processes of neuronal migration in the developing brain, result in a wide array of human diseases [reviewed in (Lian and Sheen 2006; Reiner 2013)]. The most common disturbances of brain development affecting up to 4 % of children cause functional deficits, leading to epilepsy, mental retardation, behavioral disturbances and functional psychosis (Surveys 1989). Morphological abnormalities of the cortex also account for a substantial fraction (5–15 %) of epilepsy in adults (Hardiman et al. 1988; Brodtkorb et al. 1992; Hauser et al. 1993). In addition, a portion of cortical malformations in humans is genetic in origin [reviewed in (Walsh 1999; Gupta et al. 2002)].

Taking into consideration the vast importance of neuronal migration in health and disease, understanding of the basic molecular mechanisms underlying this phenomenon has been the focus of multiple studies [reviewed in (Reiner and Sapir 2009)]. A motile cell must have internal polarity in order to move in a specified direction. Locomotory polarity requires the coordinated polymerization of cytoskeletal elements such as microtubules and actin combined with regulated activities of the associated molecular motors. MARK/Par-1 appears to be at the crossroads of various biological functions including cell polarity and stability of microtubules [reviewed in (Tassan and Le Goff 2004; Timm et al. 2008b; Munro 2006; Hayashi et al. 2011; Cohen and Musch 2003; Hurov and Piwnica-Worms 2007; Bright et al. 2009; Marx et al. 2010; Reiner and Sapir 2009; Macara 2004; Matenia and Mandelkow 2009)]. MARK/Par-1 was initially identified as a protein involved in the regulation of cell polarity in the simple organism *C. elegans* (Par-1, partition defective gene 1) (Rose and Kemphues 1998; Betschinger and Knoblich 2004) and in parallel as a protein kinase involved in regulation of microtubule stability, MARK (microtubule affinity-regulating kinase) (Drewes et al. 1997) or also as EMK (ELKL motif kinase) (Espinosa and Navarro 1998). Therefore, we have further analyzed the roles of MARK/Par-1 in regulation of neuronal migration (Mejia-Gervacio et al. 2011; Sapir et al. 2008a, b).

2 MARK/Par-1 Polarity – Cytoskeletal Regulation and Disease

Par genes include six members (Par-1 to -6) and an additional member to this group was identified as atypical protein kinase C (aPKC) (Kemphues et al. 1988; Tabuse et al. 1998). Par proteins are evolutionary conserved with the exception of Par-2. Par-1, -4 and aPKC encode for serine/threonine kinases that exhibit interesting and functional enzyme-substrate interactions. Par-3 and Par-6 are well-studied scaffold proteins [reviewed in (Barnes et al. 2008; Munro 2006)]. They can form a ternary complex containing aPKC that can recruit the small-GTPase Cdc42 and thereby regulate the dynamics of actin and microtubule cytoskeleton, epithelial cell polarity, tight junction formation, mitotic spindle orientation, and cell migration. The mammalian orthologs of Par-1 are also known as MARK or EMK as stated above, and four gene family members exist in mammals (Tassan and Le Goff 2004; Marx et al. 2010). MARK/Par-1 is a central player in the localization of several cell polarity proteins. The importance of MARK/Par-1 to cell polarity is not limited to *C. elegans*. Par-1 is involved in polarization of the *Drosophila* oocyte (Doerflinger et al. 2006, 2010). Additional kinases share structural similarities with the MARKs, and they include several members of the AMPK (5′ AMP-activated protein kinase) subgroup of human protein kinases [reviewed in (Marx et al. 2010)]. All MARK isoforms contain six recognized segments: an N-terminal header (Fig. 6.1), catalytic kinase domain (KD), linker, ubiquitin-associated

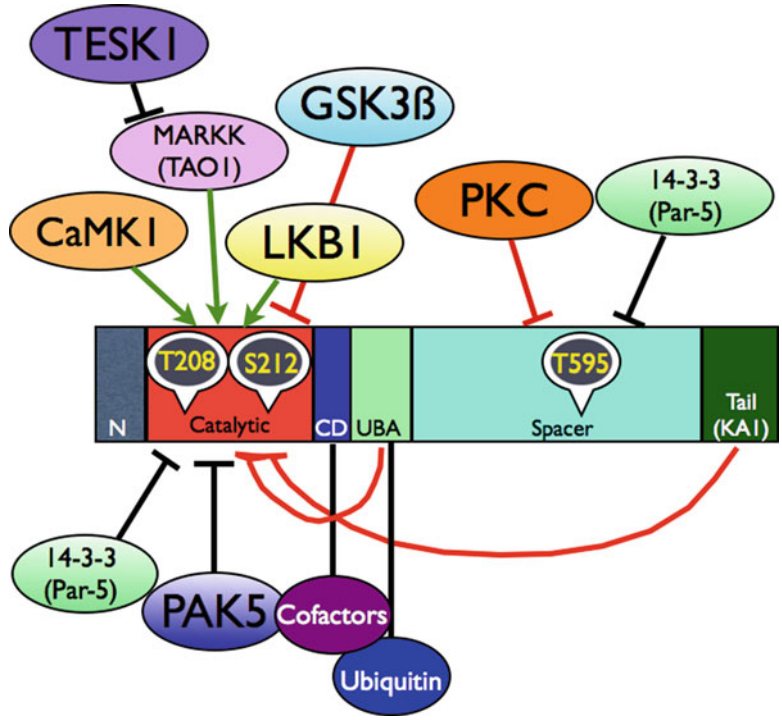

Fig. 6.1 MARK/Par-1 domains and major modes of regulation. MARK/Par-1 kinases consist of an N-terminal domain (N) a catalytic domain followed by a common docking domain-like CD-like motif, an auto-regulatory UBA domain, a spacer, and a tail domain (or kinase associated domain, KA1). MARK/Par-1 is subject to multiple means of regulations that occur at several sites along the protein. One requirement for MARK/Par-1 activity is the phosphorylation of a threonine at the activation loop (human MARK/Par-1 T208), which keeps the active site accessible to the substrate. The kinases MARKK (TAO1) and LKB1 activate MARK/Par-1 by phosphorylating it on T208 (*green arrows*). GSK3b inactivates the kinase by targeting a different residue in the same domain, Serine 212 (*red blocked lines*). CaMKI also targets the catalytic domain of MARK/Par-1. aPKC phosphorylation sites lies within the space domain and its phosphorylation allows the inhibitory binding of 14-3-3 to MARK/Par-1. This binding represents a second mode of regulation of MARK/Par-1, which involves protein-protein interaction (*Black blocked lines*). 14-3-3 binds to the spacer domain and relocalizes the kinase while PAK5 binds MARK/Par-1 at the catalytic domain itself. Intra-molecular inhibitory interaction (*red arched lines*) of the tail may block the catalytic domain. Alternatively, the tail may mediate binding of inhibitory partners. The regulatory role of the UBA domain is not fully recognized. Its homology to the UBA class of ubiquitin-associated proteins suggests that it may be able to interact with different forms of polyubiquitin. The common docking domain-like CD-like may exert its function by binding to yet uncharacterized cofactors (The scheme is adopted from Marx et al. 2010)

(UBA) domain, spacer, and tail domain (including the KA1 sequence motif). Other related kinases usually differ in the UBA domain. The isolated UBA domain exhibits conformational instability and very weak affinity of binding to ubiquitin, suggesting that this domain functions mainly in stabilizing of the adjacent kinase domains (Murphy et al. 2007). The MARKs were originally discovered because of the ability of MARK1 or MARK2 to regulate the stability of microtubules by virtue of phosphorylating microtubule associated proteins, such as tau and MAP-2, and reducing their affinity to microtubules (Drewes et al. 1997). Many MARK/Par-1 substrates were identified to contain conserved KXGS motifs, where the serine residue is phosphorylated (Drewes et al. 1997). The removal of microtubule-associated proteins from microtubules following MARK/Par-1 phosphorylation also affects microtubule-mediated transport especially in neurons. The tight association of microtubule-associated proteins to the microtubule 'tracks'

may impede the movements of motor proteins and their associated cargoes along microtubules (Mandelkow et al. 2004). Increased expression of MARK/Par-1 has been shown to correct abnormal transport resulting from overexpression of tau (Thies and Mandelkow 2007). Transport of mitochondria may be affected via the phosphorylation of a recently identified MARK/Par-1 interacting protein and substrate, which is the phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1) (Matenia et al. 2012). PINK1 affects the transport and autophagy of mitochondria through its interaction with Parkin, and this process is important for the survival of neurons (Clark et al. 2006; Park et al. 2006; Jones 2010; Vives-Bauza et al. 2010). Mutations in either Parkin or PINK1 are linked to familial Parkinson disease (PD) (see: http://www.thepi.org/parkinson-s-disease-mutation-database/). Thus, MARK/Par-1 is also linked with regulation of the transport and localization of mitochondria (Matenia et al. 2012).

MARK/Par-1 acts as a key organizer of microtubule arrays that govern polarized transport in both vertebrate (Cohen et al. 2004a, b; Suzuki et al. 2004) and invertebrate (Doerflinger et al. 2003) epithelia. It was identified as an important microtubule organizer in the leading edge of migrating cells downstream of Rac1 activity (Nishimura et al. 2012). The microtubule-associated proteins (MAPs) tau and MAP2 are well-known substrates of MARK/Par-1 (Drewes et al. 1997). MARK/Par-1 phosphorylates these MAPs at their corresponding KXGS motifs, which reside within their microtubule-binding domain, resulting in a dramatic reduction in their affinity to microtubules. The detachment of MAPs from the microtubules can lead to highly dynamic and unstable microtubules (Drewes et al. 1997, 1998). Tau, an axonal MAP, regulates not only microtubules stability but also axonal transport and has a pivotal role in neuronal function and survival (Matenia et al. 2012; Timm et al. 2011; Sydow et al. 2011; Li et al. 2011), as well as regulation of dendritic functions (Ittner et al. 2010). Tau is well known for its involvement in a group of neurodegenerative diseases collectively known as tauopathies [reviewed in (Hernandez and Avila 2007; Avila et al. 2004; Mandelkow and Mandelkow 1998; Rademakers et al. 2004)]. The most common tauopathy is Alzheimer's disease where hyperphosphorylated tau accumulates within paired helical filaments. During disease progression, hyperphosphorylated tau is missorted to the dendrites and cytoplasm and aggregates into neurofibrillary tangles (NFTs), a process that lead to neuron dysfunction and neurodegeneration (Thies and Mandelkow 2007). NFTs are a pathological hallmark of Alzheimer's disease (AD) as well as a collection of other neurodegenerative disorders that are referred to as tauopathies [reviewed in (Morris et al. 2011; Morfini et al. 2009; Hernandez and Avila 2007; Avila et al. 2004)]. Based on the recent findings of a dendritic function of tau and studies in mouse models, the role of tau in AD pathogenesis was revisited and it was placed in the amyloid-ß toxicity cascade (Morris et al. 2011). In addition, mutations within the human *MAPT* (tau) locus result in the neurodegenerative disease, frontotemporal dementia with Parkinsonism (Hutton et al. 1998; D'Souza et al. 1999). However, tau pathologies are not confined to neurodegenerative diseases. Microdeletions of a region encompassing the *MAPT* gene result in moderate intellectual disability with associated dysmorphic features (Shaw-Smith et al. 2006; Sharp et al. 2006; Koolen et al. 2006, 2008; Varela et al. 2006). The frequency of this microdeletion syndrome was estimated to be 1:13,000 to 1:20,000, thus suggesting it to be a common underlying cause for intellectual disability. When the same area on chromosome 17 is duplicated, patients exhibit behavioral problems and poor social interactions that are consistent with autism spectrum disorders (ASD) (Grisart et al. 2009; Kirchhoff et al. 2007). Interestingly, MARK1, one of the members of the MARK/Par-1 gene family has been suggested as a susceptibility gene for autism (Maussion et al. 2008).

3 MARK/Par-1 Regulation

MARK/Par-1 activity is regulated by several mechanisms [see Fig. 6.1 and review (Marx et al. 2010)]. The kinase can be activated by phosphorylation

on a conserved threonine in the activation loop, which can be executed by MARKK/TAO-1 (Timm et al. 2003) or LKB1 in association with STRAD and MO25 (Lizcano et al. 2004). Phosphorylation of an adjacent serine residue by GSK3β resulted in inhibition of MARK2/Par-1 activity in one study (Timm et al. 2008a) or activation in another study (Kosuga et al. 2005). Phosphorylation at other sites may affect MARK2/Par-1 activity or its interactions with additional proteins. For example, phosphorylation by aPKC induced binding of MARK/Par-1 to the scaffold protein 14-3-3/PAR-5, which resulted in relocation of the kinase from the cell membrane to the cytosol (Suzuki et al. 2004; Watkins et al. 2008; Hurov et al. 2004). In addition, several protein interactions regulate MARK/Par-1 activity independent of protein phosphorylation. For example, MARK/Par-1 interaction with PAK5 inhibited the activity of the kinase (Matenia et al. 2005). Furthermore, the C-terminal tail of MARK/PAR1 kinases (which includes the KA1 domain) is involved in binding to acidic phospholipids, which may be important for the interaction with subcellular fractions of the plasma and may also be involved in regulation of enzymatic activity (Moravcevic et al. 2010; Goransson et al. 2006; Hurov et al. 2004). Tight control of MARK/Par-1 may be achieved in part by its regulated degradation; it has been shown that active MARK/Par-1 phosphorylated by LKB1 is targeted for ubiquitination and degradation (Lee et al. 2012).

4 MARK/Par-1 in Primary Neurons

MARK/Par-1 is required for establishment of neuronal polarity in culture (Biernat et al. 2002) (Fig. 6.2). Within the axon, MARK/Par-1 mediated phosphorylation may interfere with the

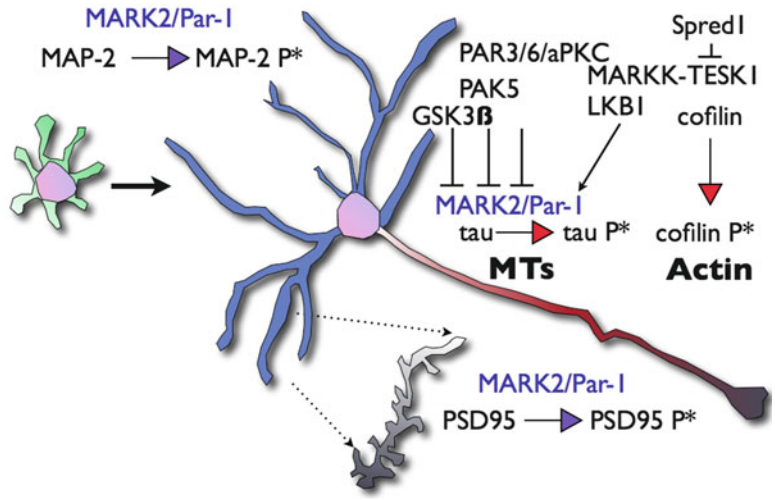

Fig. 6.2 Schematic representation of key substrates of MARK/Par-1 during neuronal polarization. Neuronal polarization of hippocampal neurons in vitro involves the transition from a non-polarized cell extending multiple undifferentiated neurites (*green* processes) to well-characterized cellular compartments; a single axon (*red*) and multiple dendrites (*blue*). Tau is the key microtubule-associated protein in axons and MAP2 is the key microtubule-associated protein in the dendrites. MARK/Par-1 phosphorylation alters their affinity the microtubule and affects microtubules dynamics. The Par3/Par6/aPKC complex counteracts excessive activity of MARK/Par-1 and relives possible inhibition of axonogenesis. In parallel, modulation of the actin cytoskeleton occurs upstream of MARK/Par-1. MARKK an activator of the kinase binds Sprouty-related protein with EVH-1 domain1 (Spred1) and the later interacts and inhibits specific protein kinase (TESK1) thus limiting the phosphorylation of Cofilin, which results in the stabilization F-actin. Phosphorylation of MAP2 by MARK/Par-1 shortens the length and reduces the branching of dendrites. At the mature dendrite, MARK/Par-1 regulates spine morphogenesis through phosphorylating of Postsynaptic Density Protein 95 (PSD-95)

maintenance of the barrier-mediated sorting in the initial axonal segment (Li et al. 2011). Knockdown of MARK/Par-1 induced formation of multiple axons in hippocampal neurons, whereas strong MARK/Par-1 expression inhibited axon formation. This inhibition was counteracted by the Par-3/Par-6/aPKC complex, which inhibits MARK/Par-1 (Chen et al. 2006). Recent research has demonstrated that Par-3 is also involved in regulation of microtubule stability. Furthermore, disruption of the microtubule regulatory activity of Par-3 impaired its function in axon specification (Chen et al. 2013). At least part of the functions of MARK/Par-1 in the axon may be attributed to the activities of one of its main substrates, tau [reviewed in (Matenia and Mandelkow 2009; Timm et al. 2006)]. Tau is mainly an axonal protein (Kempf et al. 1996; Mandell and Banker 1996) however, it is highly dynamic and its axonal sorting is regulated in part by active phosphorylation, for example by MARK/Par-1 (Li et al. 2011; Konzack et al. 2007). It should be noted that tau may be phosphorylated by additional kinases such as GSK-3β and CDK5 [reviewed in (Billingsley and Kincaid 1997; Drewes 2004)]. *In vitro* work also implicates GSK3β as one of the critical regulators of neuronal polarity [reviewed in (Polleux and Snider 2010)]. Experiments using several types of GSK-3 inhibitors indicate that GSK-3α/β act as negative regulators of axon formation because they lead to formation of multiple axons (Jiang et al. 2005; Yoshimura et al. 2005). The activity of MARK/Par-1 is not limited to axons, although high activity, which was visualized by a cellular biosensor, was noticed in the axon and growth cone of developing neurons (Timm et al. 2011). The active kinase was shown to inhibit the formation of dendrites via phosphorylation of MAP2 in hippocampal neurons (Terabayashi et al. 2007). A role for MARK/Par-1 has also been demonstrated in dendritic spines (Wu et al. 2012). This activity was mediated through MARK/Par-1 induced phosphorylation of the synaptic scaffolding protein PSD-95 (Fig. 6.2).

MARK/Par-1 is likely to be mediating an active crosstalk between the microtubules and actin microfilaments [reviewed in (Timm et al. 2006; Matenia and Mandelkow 2009)]. Neuronal polarization requires a continuous modulation of both actin microfilaments and microtubules [reviewed in (Witte and Bradke 2008; Arimura and Kaibuchi 2007)]. The regulatory interactions between MARK/Par-1 and several actin modulators are capable of mediating this crosstalk [reviewed in (Matenia and Mandelkow 2009)]. PAK5, a member of the Rac/Cdc42-associated Ser/Thr kinases inhibits MARK/Par-1 activity resulting in stable microtubules and in parallel induces actin dynamics (Matenia et al. 2005) (Fig. 6.2). A similar type of regulatory interactions occurs more upstream, one of the MARK/Par-1 activating kinases, MARKK has been found to interact with TESK1 and Spred-1 (Johne et al. 2008). TESK-1 belongs to the LIM kinase family and stabilizes actin organization by phosphorylating Cofilin. TESK1 can also bind and inactivate MARKK. Spred-1's interaction with TESK1, inhibits TESK1 kinase activity. Thus, the tripartite interaction of MARKK-TESK1 and Spred-1 can influence both the microtubules and the actin elements of the cytoskeleton.

In vivo, kinases belonging to the MARK family (SAD-A and SAD-B also known as BRSK1 and BRSK2) have been shown to regulate neuronal polarity (Kishi et al. 2005). Cortical neurons doubly knockout for SAD-A and SAD-B kinases were unable to specify a single neurite to become an axon *in vivo*. Additional studies demonstrated that the kinase LKB1 is the main upstream activator of SAD-A/B kinases in cortical neurons (Barnes et al. 2007).

5 MARK/Par-1 and Neuronal Migration

MARK2 is a member of a small family of proteins (Tassan and Le Goff 2004), thus, although it could be expected that knockout of the *Mark2* gene will result in a neuronal migration phenotype based on its cellular activities, developmental gene redundancy may explain why such a phenotype has not been described in the *Mark2* deficient mice. *In utero* electroporation has been proven to be an efficient way to circumvent gene

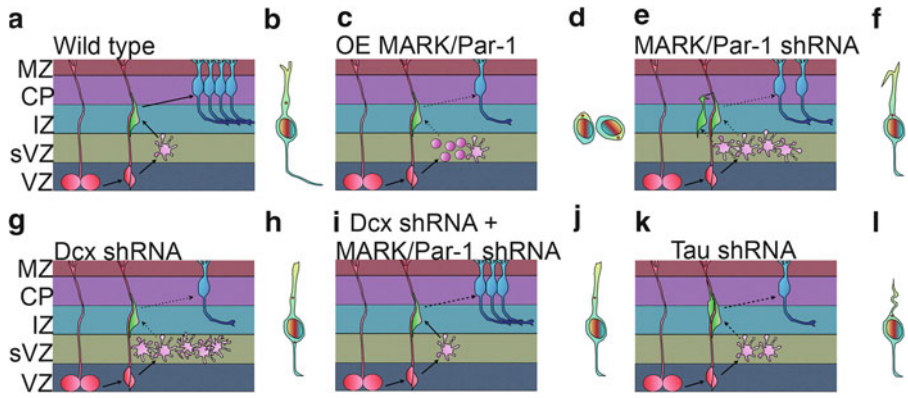

Fig. 6.3 MARK/Par-1 and neuronal migration. (**a**) Simplified scheme of the radial migratory path of excitatory neurons in the developing cerebral cortex. Cells born at the ventricular zone (*VZ*) from a radial progenitor (*red*) one of several types that occupy the subventricular zone (*sVZ*). The postmitotic cells lose their polarity and become multipolar (*pink*) before re-establishing a bipolar morphology and moving to the forming cortical plate (*CP*), sending connections to the marginal zone (*MZ*) and towards other brain areas. A migrating cell typically orients a leading edge towards the pial surface and the centrosome is located in front of the nucleus and serves as a microtubule organization center. (**b**–**j**) MARK/Par-1 affects neuronal migration via its dual role in regulating cellular polarity as well as microtubule dynamics. Over expression of MARK/Par-1 causes destabilization of the microtubule, complete loss of polarity cell rounding and inhibition in radial migration (**d**). Acute reduction in MARK2/Par-1 levels in the developing brain is also detrimental to normal migration. Neurons treated with MARK/Par-1 shRNA fail to migrate beyond the IZ and fail to repolarize. The leading edge of the migrating neurons is frequently hooked and distorted and the centrosomal motility is decreased (**f**). Knock down of DCX, a MARK/Par-1 substrate, cause cells to stall in a highly branched multipolar morphology (**g**) Bipolar cells occasionally display centrosome split and irregular centrosomal motility (**h**). (**i**) Dcx shRNA with additional reduction in MARK/Par-1 levels partially rescues this phenotype and restores centrosomal motility (**j**). (**k**) Introduction of tau shRNA in the developing brain inhibits neuronal migration. Less neurons reach the cortical plate. The leading edge of the migrating neurons is curved and thin (**l**)

redundancy, as previously demonstrated in case of the *Dcx* family of proteins (Bai et al. 2003; Koizumi et al. 2006) [reviewed in (Reiner et al. 2012)]. However, *Mark2* knockout mice do exhibit impairments in spatial learning and memory (Segu et al. 2008). Based on the tight correlation between abnormal neuronal migration and mental retardation, it may be hypothesized that these mice exhibit a subtle previously unappreciated phenotype in the developing brain.

During normal brain development, neuroblasts proliferate in the ventricular (VZ) and the subventricular (SVZ) zones (Fig. 6.3a). Following their postmitotic division, neurons adopt a multipolar morphology (Tabata and Nakajima 2003) from which they will transit to a bipolar morphology and migrate along radial glia (Fig. 6.3a, b). Once they reach the cortical plate (CP), they detach from the radial glia and form appropriate connections. Overexpression of MARK/Par-1 in the developing brain using *in utero* electroporation strongly inhibited neuronal migration; cells lost their polarity and adopted round rather than multipolar or bipolar morphologies (Fig. 6.3c, d). Reduction in the levels of polarity kinase MARK/Par-1 by shRNA resulted in a pronounced inhibition of radial neuronal migration (Sapir et al. 2008a) (Fig. 6.3e, f). Most of the shRNA treated neurons were stalled at the boundary between the intermediate zone (IZ) and CP (Fig. 6.3e, f). The inhibited neurons mainly exhibited a multipolar morphology. Some of the neurons that managed to migrate towards the CP, exhibited abnormal morphology with a curved or bifurcated leading edge pointing to the VZ (Sapir et al. 2008a) (Fig. 6.3f). The transient multipolar stage is sensitive to the levels of quite a few proteins; including DCX, LIS1, Filamin A and others (LoTurco and Bai 2006). Schematic presentation of brain sections treated with *Dcx* shRNA is shown in

Fig. 6.3g, h. The proteins mentioned above regulate cell polarity and motility in neocortical SVZ and IZ during radial migration. Interestingly, adding MARK/Par-1 kinase-dead on top of MARK/Par-1 shRNA allowed neurons to change their morphology from multipolar to bipolar. Nevertheless, this transition was not sufficient to allow successful migration to the CP. This result strongly suggests that there may be different modes of regulation for each phase. This was the first example where a transition to the bipolar morphology occurred without subsequent migration. These results strongly suggest for an unknown kinase-independent activity involved in morphology change. Reduction of kinase activity on its own also retarded neuronal migration and cells did not reach their expected position in the cortical plate. Reduction of kinase activity was achieved by expression of MARK/Par-1 kinase-dead which acts as a dominant negative and inhibits endogenous kinase activity, or via expression of PAK5, which binds to the catalytic domain of MARK2 and inhibits its activity (Matenia et al. 2005).

Taking into consideration the important role that MARK/Par-1 plays in migration of radially migrating neurons, we also examined its role in migration of postnatal neurons to the olfactory bulbs (Mejia-Gervacio et al. 2011). Rostral migratory stream (RMS) neuroblasts expressed MARK/Par-1 in the postnatal and adult brain. Knockdown of MARK/Par-1 revealed that it is required for correct positioning of the leading processes of the neuroblasts heading toward the olfactory bulb. Decreasing the expression levels of MARK/Par-1 in neuroblasts impaired the ability of cells to maintain a sustained forward direction of displacement in the RMS. As a consequence, the integration of newborn neurons into the olfactory bulb circuit was compromised.

It is likely that the observed phenotypes can be attributed at least in part to modulation of microtubule dynamics. MARK/Par-1 phosphorylates multiple substrates, some of them are MAPs, which change their affinity to microtubules following MARK/Par-1 phosphorylation. The substrates include tau, MAP2, MAP4 and DCX (Drewes et al. 1997; Biernat et al. 1993; Schaar et al. 2004). Overexpression of MARK/Par-1 in cells leads to hyperphosphorylation of MAPs on KXGS motifs and to disruption of the microtubule array (Drewes et al. 1997). As mentioned above, *in vivo* overexpression of MARK/Par-1 resulted in loss of neuronal polarity (Sapir et al. 2008a). Reduction in MARK/Par-1 resulted in more stable microtubules detected in primary neurons, and as a consequence *in vivo* neurons were stalled in the multipolar stage (Sapir et al. 2008a). Reduction in DCX, one of the substrates of MARK/Par-1, resulted in an opposite effect with more dynamic microtubules, yet *in vivo* neurons are stalled in the multipolar stage (Sapir et al. 2008b) as previously reported (Bai et al. 2003; Ramos et al. 2006) (Fig. 6.3g). One clear conclusion from the above-described experiments may be that proper neuronal migration requires very accurate control of microtubule dynamics. Tipping the balance in either direction inhibits neuronal migration. Based on this conclusion it was possible to postulate that simultaneous reduction of both DCX and MARK/Par-1 will allow for proper neuronal migration. Indeed, *in utero* electroporation of both DCX and MARK/Par-1 shRNA resulted in a partial rescue of neuronal migration. These results have practical implications following the clear demonstration that a reduction in the levels of one gene may ameliorate the phenotype observed in case of mutation of another gene in the same pathway.

Previous studies indicated that centrosomal motility and the coupling between the centrosome and nucleus through microtubules is important for neuronal migration [reviewed by (Solecki et al. 2006; Tsai and Gleeson 2005; Vallee and Tsai 2006)]. Centrosomal motility requires the activity of molecular motors and cytoskeletal integrity. Furthermore, this process is subject to a delicate balance of opposing activities thus suggesting that reversible post-translational modifications are likely to be involved in the regulation of dynamics of centrosomal motility and neuronal migration. When MARK/Par-1 levels were reduced, centrosomes moved very slowly (Sapir et al. 2008a) (Fig. 6.3f). The dynamics of observed centrosomal behaviour when DCX was reduced differed markedly (Sapir et al. 2008b). The centrosome separated into two centrioles,

which moved bi-directionally and fast (Fig. 6.3h). It has been proposed that the mammalian interphase centrosome consists of two independent units held together primarily as a result of the dynamic properties of the microtubule cytoskeleton (Jean et al. 1999). Furthermore, the balanced activities of kinases and phosphatases play an instructive role in centrosomal splitting (Meraldi and Nigg 2001). Therefore, it is possible to assume that reversible phosphorylation may also be involved in splitting of the centrosome in migrating neurons. DCX is a phosphoprotein and is dephosphorylated by phosphatases. The role of DCX kinases in neuronal migration has been well established, but phosphatases are likely to be as important. Of particular interest is the role of Protein Phosphatase 1 (PP1) in centrosomal splitting (Mi et al. 2007), since this phosphatase is capable of dephosphorylating DCX in a site-specific manner (Shmueli et al. 2006; Bielas et al. 2007). Nevertheless, the role of actin and the associated molecular motor myosin in the maintenance of centosomal integrity cannot be neglected. A basic role of the actin cytoskeleton in centrosomal splitting has been established (Euteneuer and Schliwa 1985; Thompson et al. 2004; Uzbekov et al. 2002). In migrating neurons the role of actin remodelling and the activity of myosin has proven to be essential for proper nuclear and cellular motility (Bellion et al. 2005; Schaar and McConnell 2005; Tsai et al. 2007; Ma et al. 2004). Both DCX and MARK/Par-1 are capable of mediating a crosstalk between the microtubule and actin cytoskeleton (Tsukada et al. 2003, 2005; Matenia et al. 2005; Johne et al. 2008). In summary, seamless motility of the polarized centrosome requires a tight balance of factors involved in regulation of the molecular motors and the cytoskeleton.

As mentioned above, tau is one of the key substrates of MARK/Par-1. Reduction of tau clearly inhibits neuronal migration in the developing cortex, thus revealing a clear developmental role for tau (Sapir et al. 2012) (Fig. 6.3k, l). Reduced tau levels affected the morphology of the leading edge in spite of normal morphology of radial glia (Fig. 6.3l). The leading edge of cells treated with shRNA to tau was crooked and thinner than in control cells. Furthermore, tau has a role in regulation of mitochondria in the migrating cells; both intracellular mitochondrial transport and morphology were severely affected following tau knockdown. Neurons that did reach the CP, were not entirely normal. They exhibited smaller cell somas, far less developed dendrites and a striking reduction in connectivity.

Collectively, these results demonstrate that balanced levels of MARK/Par-1, relative levels of at least two of its important substrates DCX and tau, as well as their phosphorylation status, are of importance in regulation of neuronal polarity, migration and later on connectivity in the developing brain.

6 Concluding Remarks

MARK/Par-1 belongs to a functionally conserved family of protein kinases. It plays key roles in several cellular processes including neuronal polarity and migration and it has major roles in more than one aspect of the neurodegenerative cascades in pathologies such as Alzheimer's disease. The protein is composed of functional domains, which serve as sites for many levels of regulatory modifications and interactions. Indeed the kinase activity is tightly controlled by means of post-translational modifications (phosphorylation), physical interactions, as well as intra- and inter-molecular regulations of the active kinase. MARK/Par-1 exerts its function by phosphorylating several effectors. The phosphorylation of MAPs such as DCX in the developing neuron, tau in the axon, MAP2 in the dendrite, and PSD-95 in the synapse, affect neuronal polarization and neuronal functioning. Its dual effects on polarization and on the dynamic properties of the microtubules make MARK/Par-1 regulated activity pivotal for proper advancement of radial migration in the developing cortex.

Acknowledgements O.R. is an Incumbent of the Berstein-Mason professorial chair of Neurochemistry. Our research has been supported in part by the Israel Science Foundation (grant no. 47/10), Minerva foundation with funding from the Federal German Ministry for Education and Research, a grant from the Chief Scientist

Office at the Israeli Ministry of Health, under the frame of ERA-Net NEURON (DISCover, IMOS 3-00000-6785), Fritz-Thyseen Foundation (grant Az. 10.11.2.161), the Benoziyo Center for Neurological diseases, the Helen and Martin Kimmel Stem Cell Research Institute, and the David and Fela Shapell Family Center for Genetic Disorders Research.

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The PAR Polarity Complex and Cerebellar Granule Neuron Migration

7

Joseph S. Ramahi and David J. Solecki

Abstract

Proper migration of neurons is one of the most important aspects of early brain development. After neuronal progenitors are born in their respective germinal niches, they must migrate to their final locations to form precise neural circuits. A majority of migrating neurons move by associating and disassociating with glial fibers, which serve as scaffolding for the developing brain. Cerebellar granule neurons provide a model system for examination of the mechanisms of neuronal migration in dissociated and slice culture systems; the ability to purify these cells allows migration assays to be paired with genetic, molecular, and biochemical findings. CGNs migrate in a highly polarized fashion along radial glial fibers, using a two-stroke nucleokinesis cycle. The PAR polarity complex of PARD3, PARD6, and an atypical protein kinase C (aPKC) regulate several aspects of neuronal migration. The PAR polarity complex regulates the coordinated movements of the centrosome and soma during nucleokinesis, and also the stability of the microtubule cytoskeleton during migration. PAR proteins coordinate actomyosin dynamics in the leading process of migrating neurons, which are required for migration. The PAR complex also controls the cell-cell adhesions made by migrating neurons along glial cells, and through this mechanism regulates germinal zone exit during prenatal brain development. These findings suggest that the PAR complex coordinates the movement of multiple cellular elements as neurons migrate and that further examination of PAR complex effectors will not only provide novel insights to address fundamental challenges to the field but also expand our understanding of how the PAR complex functions at the molecular level.

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L. Nguyen and S. Hippenmeyer (eds.), *Cellular and Molecular Control of Neuronal Migration*, Advances in Experimental Medicine and Biology 800, DOI 10.1007/978-94-007-7687-6_7,

Keywords
Cerebellum • Neuronal migration • Centrosome • Nucleokinesis • PAR complex

1 Introduction

In the developing brain, immature neurons must migrate from the proliferative germinal zones to their final destinations (Hatten and Heintz 1995; Marín et al. 2010; Manzini and Walsh 2011; Métin et al. 2008; Millen and Gleeson 2008; Vallee et al. 2009). They are guided along their way by association and disassociation with glial fibers that act as neuronal migration tracts (Hatten 1990; O'Rourke et al. 1992). Neurons throughout the brain migrate by saltatory motion, in which the highly dynamic forward extension of the leading process is followed by somal translocation (Edmondson and Hatten 1987). This two-step motion is a highly orchestrated process involving coordination of the actin and microtubule cytoskeletons and associated motor proteins (Trivedi and Solecki 2011; Bellion et al. 2005; Kawauchi and Hoshino 2007; Valiente and Marín 2010). The correct orientation and migration of these cells is fundamental to the proper formation of neural circuits. Errors in neuronal migration and germinal zone exit are implicated in developmental and cognitive disorders such as lissencephaly, mental retardation, epilepsy, and pediatric cancers (Métin et al. 2008; Kato and Dobyns 2003; Ross and Walsh 2001). The molecular mechanisms of neuronal migration provide insight into the progression and treatment of these diseases.

The cerebellar granule neuron (CGN), the most common cerebellar neuron, has been used as a model for studies of polarity and migration. CGNs are born prenatally in the rhombic lip of the developing brain and form a secondary germinal zone in the external granule layer (EGL) of the developing cerebellum (Fig. 7.1) (Gregory et al. 1988; Rakic 1971; Ryder and Cepko 1994). In the EGL, immature proliferative granule neuron progenitors (GNPs) follow tangential migration paths parallel to the surface of the developing brain. CGNs begin to terminally differentiate between postnatal days 6–8 (P6–8). This process comprises germinal zone exit, axon extension, transition from tangential to radial migration (perpendicular to the cerebellar surface) along Bergmann glia, and arrival at their final positions in the internal granule layer (IGL) (Rakic 1972; Komuro and Rakic 1998). As radial migration continues through to P15, the EGL disappears as all CGNs have evacuated this transient germinal zone and have migrated into the IGL.

Advances in microscopy have allowed ever more detailed views of the morphology of migrating neurons in both dissociated culture and slice imaging systems. CGNs have provided a prototypic model for examination of neuronal migration, progressing from studies of fixed cells to high temporal–resolution live imaging assays of migrating cells. First, electron microscopy of the developing cerebellum in Rhesus macaques showed the migration of individual CGNs perpendicular to the surface of the brain along radial fibers later identified as Bergmann glia, with leading and trailing processes extending from their elongated soma (Rakic 1971, 1972). At the junctions of migrating CGNs, electron microscopy identified interstitial densities, or regions of the cell in which submembranous cytoskeletal elements attach to microtubules, thereby anchoring the cytoskeleton to a point at which forward force can be generated from cell-cell contacts (Gregory et al. 1988). Subsequent time-lapse imaging revealed that CGNs are highly polarized, having dynamic leading and trailing processes, while the nucleus occupies most of the somal volume. This polarity facilitates nuclear movement as a crucial aspect of saltatory CGN migration (Edmondson and Hatten 1987; Rivas and Hatten 1995; Solecki et al. 2004).

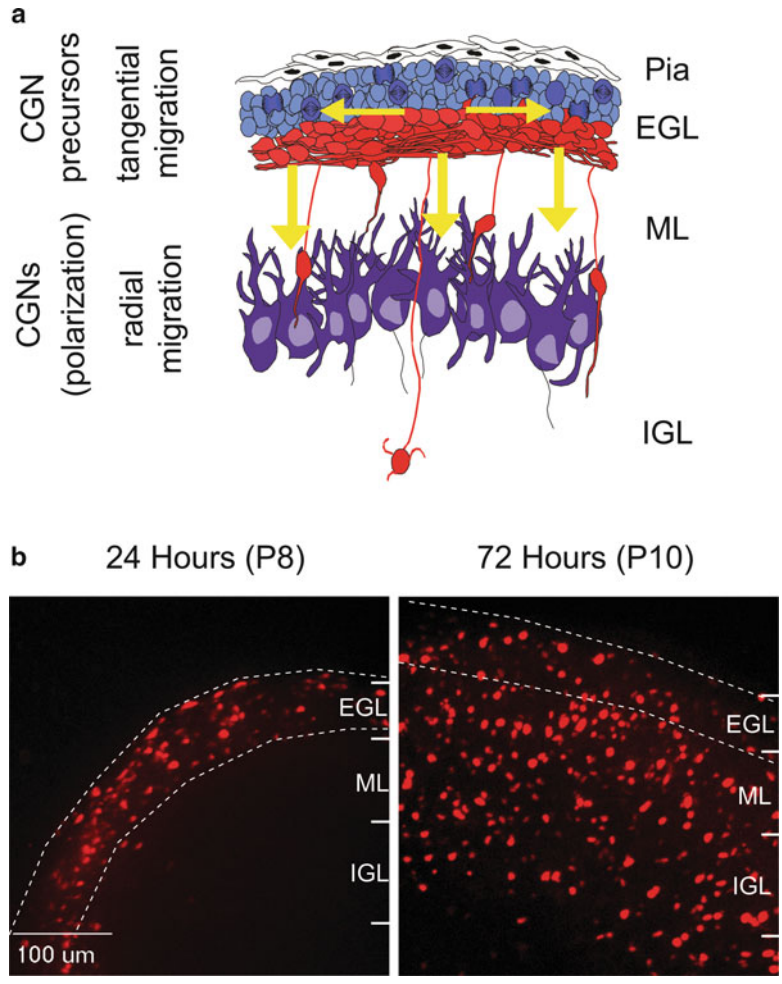

Fig. 7.1 Germinal zone migration in the developing cerebellum. (**a**) Cerebellar granule neuron precursors (*cGNPs*) migrate tangentially (*horizontal arrows*) within the External Granule Layer (*EGL*). They then transition to a radial migration mode (*vertical arrows*) and migrate along glial fibers through the Molecular Layer (*ML*) and into the Internal Granule Layer (*IGL*). (**b**) Cereballarslice cultures electroporated with CGN-specific H2B-mCherry nuclei to track neuronal migration. At postnatal day 8 (P8, 24 h post electroporation), H2B-mCherry labeled CGNs migrate tangentially through the EGL. By P10 (72 h post electroporation) most CGNs have evacuated the EGL and migrated radially into the ML and IGL

As with all neurons, the dynamic leading processes of CGNs are guided by extracellular cues but their movements are not synchronized with those of the neuronal soma (Edmondson and Hatten 1987). Polarized somal and organelle movement during CGN migration provided a foundation for understanding the basis of the saltatory movement cycle, in which the soma moves at an average rate of 33 ± 20 μm/h (Edmondson and Hatten 1987). Interestingly, forward movement of vesicles precedes somal movement, implying that specializations in cellular structures occur prior to somal movement. This concept was expanded with the observation that the centrosome enters the leading process prior to somal translocation, in what is termed the two-stroke motility cycle (Fig. 7.2) (Solecki et al. 2009). Strikingly, the saltatory timing first observed in early differential interference contrast (DIC) microscopy studies matches the two-stroke

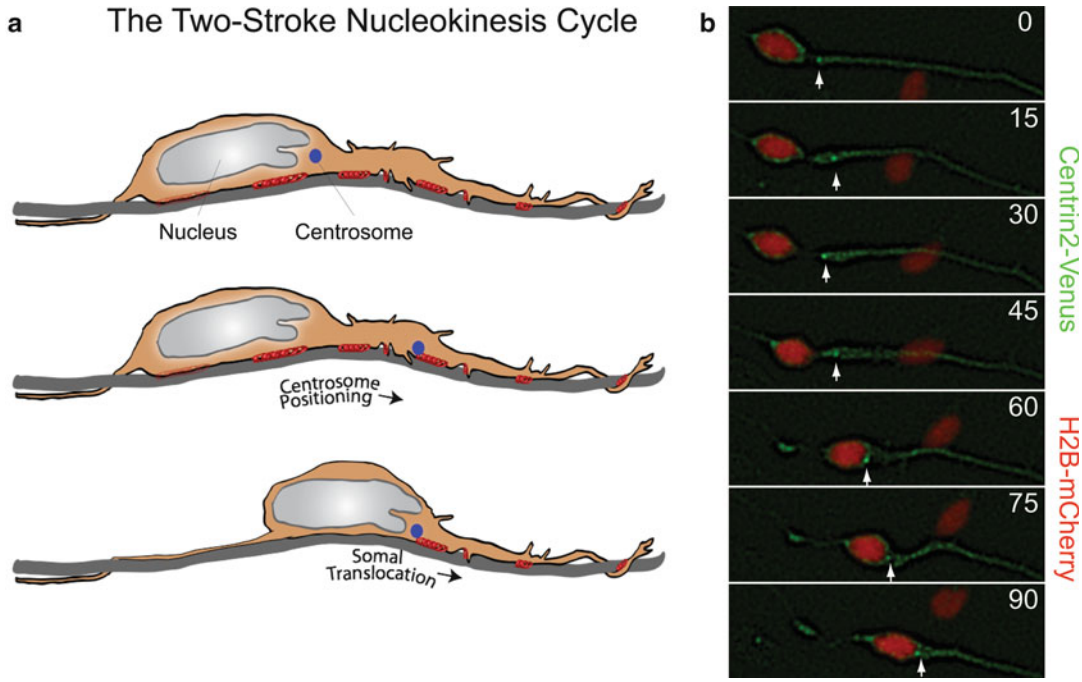

Fig. 7.2 The two-stroke nucleokinesis cycle of migrating neurons. (**a**) In the two stroke nucleokinesis cycle, the centrosome is positioned into the neuronal leading process before somal translocation. (**b**) Time-lapse imaging of a migrating CGN whose centrosome is labeled with Centrin2-Venus (*green*) (*white arrow*) and whose nucleus is labeled with H2B-mCherry (*red*). Centrosome positioning occurs 0 and 30 min, and somal translocation occurs between 45 and 90 min

motility cycle of centrosome and soma (Solecki et al. 2009). This original observation of the mechanisms of CGN migration was expanded to apply to several other neuronal subtypes (Bellion et al. 2005; Schaar and McConnell 2005; Tsai et al. 2007; Sakakibara et al. 2013; Yanagida et al. 2012; Yang et al. 2012; Shinohara et al. 2012). Recently it has been shown a cytoplasmic dilation develops within the leading processes of subventricular-zone neurons before nuclear translocation, similar to the morphologic change seen during actin and microtubule enrichment of the leading processes of CGNs (Schaar and McConnell 2005; Rivas and Hatten 1995). Thus *in vitro* studies of CGN morphology provide a cellular context for understanding the large-scale migration patterns within the developing brain.

Advances in *ex vivo* imaging have shown complex alterations in the morphology of migrating CGNs as they transit different environments and interact with different cell types. The shapes of radially migrating CGNs change as they pass through different layers of the developing cerebellum. The growth cone of the leading tip of migrating neurons has dynamic filopodia and lamellipodia, which are dynamic extensions that form and retract as the neuron samples its environment and moves forward (Gregory et al. 1988). In the molecular layer of the cerebellum, CGNs assume an extended shape as they move rapidly along Bergmann glia, while they assume a more rounded shape as they transiently and slowly migrate through the Purkinje cell layer. As CGNs enter the IGL, the cell body again assumes an extended shape for rapid movement independent of Bergmann glia (Komuro and Rakic 1998). Observation of tangentially migrating CGNs shows that in the EGL their velocity is dependent on their position. Their most rapid rate

of tangential migration occurs in the center of the EGL, where they maintain short leading and trailing processes. As they move to the bottom of the EGL, their tangential migration velocity slows and they extend longer leading and trailing processes. CGNs slowly migrate out of the EGL upon reaching its interface with the molecular layer and begin radial migration into the molecular layer (Komuro et al. 2001). Because the multiple modes of CGN migration involve region-specific rates and morphologies, the motor systems and cytoskeletal regulation mechanisms that regulate these different types of migration are of great interest.

The cytoskeletons of migrating neurons are dynamic, changing within the different migration environments. The leading processes of migrating neurons are enriched in microtubules and actin, which extend toward a tubulin cage surrounding the nucleus (Rivas and Hatten 1995). Regulation of the microtubule cytoskeleton is a driving factor in neuronal migration. The microtubule array of migrating neurons is highly polarized, as growing microtubule "plus" tips extend into the leading process and depolymerizing "minus" ends are oriented toward the nucleus (Rakic et al. 1996). The genetics of human neuronal migration disorders further highlight that microtubule cytoskeleton and its associated motor protein dynein are regulators of neuronal migration. The cytoplasmic dynein motor protein is a polypeptide of 12 subunits, comprising two identical heavy chains that contain the AAA ATPase domains required for activity, two intermediate chains involved in cargo anchoring, and additional intermediate and light chains whose functions remain unclear (Cho and Vale 2012; Dujardin and Vallee 2002; Feng et al. 2000). Genetic analysis of lissencephaly identified mutations in the dynein adaptor protein Lissencephaly 1 (LIS1) (Reiner et al. 1993; Dujardin et al. 2003; Faulkner et al. 2000; Hirotsune et al. 1998; Smith et al. 2000). Lissencephaly also results from mutations in the Doublecortin (*DCX*) gene, which encodes a microtubule bundling protein and is expressed in migrating neurons (Kato and Dobyns 2003; Francis et al. 1999; Gleeson et al. 1999; Allen et al. 1998). Both LIS1 and dynein play roles in radial neuronal migration (Tsai et al. 2007; Tanaka et al. 2004; Smith et al. 2000; Shu et al. 2004) (see also Chap. 1). As more genes that participate in the regulation of neuronal migration and brain development are identified, additional genetic causes of cognitive and developmental brain disorders will be recognized.

Active migration of CGNs requires coordination between the microtubule and actin cytoskeletons and their associated motor proteins (Ridley et al. 2003). The leading processes of migrating neurons are enriched in actin, and disruption of the actin cytoskeleton with cytochalasin B is sufficient to inhibit migration, implicating actin subunit assembly in migration (Rivas and Hatten 1995; Le Clainche and Carlier 2008). Actin-based motility is dependent on the myosin family of motor proteins. Myosin II contains two heavy chains that constitute the head and tail domains of the protein and four light chains that bind to the heavy chains (Vallee et al. 2009). Phosphorylation of myosin II by myosin light chain kinase or myosin heavy chain kinase is required for ATP hydrolysis, which drives motor function (Kamm and Stull 2001; Moussavi et al. 1993). Nonmuscle myosin IIb, the main myosin expressed in the developing brain, was identified as important to neuronal migration when mutation in the motor domain of nonmuscle myosin heavy chain IIb was observed to disrupt CGN migration and cerebellar foliation (Ma et al. 2004; Vicente-Manzanares et al. 2009; Rochlin et al. 1995). Actomyosin enrichment of the leading process suggests this compartment may be the main site for actin cytoskeletal dynamics in migrating neurons (Rivas and Hatten 1995; Le Clainche and Carlier 2008).

Migrating CGNs encounter multiple microenvironments and make several types of cell-cell contact as they migrate from the EGL to the IGL (Komuro and Rakic 1998). In CGNs migrating along glial fibers, the dynamic leading process is observed to wrap around Bergmann glia, and junctional adhesion molecule (JAM)-mediated adhesions are shown to form at cell-cell contacts (Famulski et al. 2010). As the neurons encounter different cell types, their modes of migration and

their adhesions change accordingly (Hatten 1990; Fishman and Hatten 1993). Astrotactin provides a receptor system for CGN migration along astroglia, and the integrin β1 receptor promotes migration along laminin fibers (Edmondson et al. 1988; Fishell and Hatten 1991; Fishman and Hatten 1993). Increased astrotactin expression, identified as a general feature of migratory cells, is noted in migratory CGNs in the EGL of the cerebellum (Zheng et al. 1996). *In vitro* assays of CGN migration along glial membrane- and laminin-coated fibers mirrored the saltatory nucleokinesis cycle observed in slice migration assays; however, brief, limited migration was observed on collagen and fibronectin fibers (Fishman and Hatten 1993). Individual cell surface receptors have been identified by *in vitro* migration assays as a requirement for neuronal migration, but it is unclear which combination of receptors is used and how they are anchored to the cytoskeleton in the different migration modes in the developing cerebellum.

Cell biology and genetic studies have created a basic framework to explore how neurons migrate from a GZ to their final laminar positions. However, **several challenges remain**: (1) Current migration models show inconsistencies, how will these differences be resolved? (2) How will the ever increasing array of cytoskeletal regulators be woven into an integrated model of neuronal migration? (3) What mechanisms control migration initiation and migration mode during GZ exit?

As all migrating cells are polarized (i.e., have spatially defined cytoskeletal organizations that are globally coordinated to execute complex motility programs), we will address these three major challenges by examining how polarity signaling globally organizes the neuronal cytoskeleton rather than by the reductionist approach of studying single cytoskeletal components in isolation. The best characterized cell polarity signaling molecules are the evolutionarily conserved partitioning defective (PAR) proteins (Kemphues et al. 1988). The PARD3 and PARD6 adaptor proteins form a complex containing atypical PKC and the CDC42 or Rac1 Rho GTPases (Joberty et al. 2000; Lin et al. 2000). This ternary complex is critical for tight junction formation, mitotic spindle orientation, cell migration and axon specification (Munro 2006; Barnes and Polleux 2009; Nance and Zallen 2011). This chapter will discuss the multiple roles of these proteins in neuronal migration through (1) organelle structure and movement, (2) coordination of cytoskeletal dynamics and associated motors, and (3) interaction with cell-cell focal adhesions. As studies of the PARD3/PARD6/aPKC complex (the PAR complex) progress, the individual roles of the PAR proteins in the centrosome, nucleus, actomyosin cytoskeleton, and focal adhesions are becoming clearer. PAR signaling has been shown to control the two-stroke nucleokinesis cycle of centrosome motion followed by somal translocation (Solecki et al. 2004). During the nucleokinesis cycle, the PAR complex has been shown to regulate myosin II activation and the actin cytoskeleton (Solecki et al. 2009). The role of PAR proteins and their regulation of focal adhesion turnover through the seven in absentia homolog (SIAH) E3 ubiquitin ligase (Famulski et al. 2010) has introduced PAR signaling as being regulated by protein degradation. The question of how PAR controls focal adhesions leads us to investigate how polarity complexes are related to the neuronal cytoskeleton and how these two dynamic structures control cell adhesion and migration.

Taken together, the available evidence indicates that the dynamic PAR complex plays key roles in nucleokinesis and adhesion control. We will now discuss in detail the role of PAR protein in each of these processes in the following sections of the chapter.

2 The PAR Polarity Complex and Microtubule Cytoskeletal Regulation

2.1 Cerebellar Granule Neurons Migrate with Coordinated Organelle Movements

Neurons migrate via a coordinated two-stroke motion of the centrosome and nucleus. Time-lapse imaging of actively migrating CGNs shows

that in the majority of migrating neurons, forward movement of the centrosome is followed by somal translocation (Solecki et al. 2004). As centrosome movement precedes nuclear movement, models have been proposed in which the centrosome acts as a microtubule organizing center, projecting microtubules rearward to the perinuclear tubulin cage and "pulling" the nucleus forward through a dynein-mediated process. A competing model shows microtubules from the nuclear tubulin cage extending past the centrosome and anchoring in the membrane of the leading process (Higginbotham and Gleeson 2007; Tsai and Gleeson 2005). The relationship between centrosome positioning and nuclear translocation may differ among migration modes, as *in vitro* migration assays of CGNs identify a subset of neurons in which the nucleus overtakes the centrosome during active migration (Umeshima et al. 2007). Electron microscopy of CGNs has shown that microtubules extend from the nuclear cage forward to both the centrosome and the leading process membrane, although the anchor point for the microtubule cytoskeleton in the leading process remains unclear. As described in the next section, the PARD6 component of the PAR complex plays an important role in not only regulating the structure the tubulin cage but also the saltatory cadence of centrosome and somal motility.

2.2 PARD6 Signaling Controls Centrosome Positioning and Microtubule Dynamics

Using high temporal–resolution live imaging techniques, Solecki and colleagues (2004) demonstrated that the forward movements of the centrosome and the nucleus are tightly coordinated in migrating neurons. Photobleaching experiments showed the microtubule cytoskeleton to be highly dynamic. Overexpression of PARD6α in granule neurons inhibits neurite extension and disintegration of the perinuclear tubulin cage, showing that PARD6α controls the microtubule dynamics of migrating neurons (Solecki et al. 2004). Disruption of PARD6α signaling also uncoupled the movements of the centrosome and nucleus and prevented migration of granule neurons along Bergmann glial fibers. By using Venus-labeled PARD6α, Solecki et al. (2004) showed PARD6α to colocalize with γ-tubulin and therefore to be a component of the centrosome. PARD6α shows a relationship to centrosome structure, as overexpression of PARD6α reduced levels of centrosomal γ-tubulin (Solecki et al. 2004).

The mechanism by which PARD6 mechanistically controls centrosome positioning and migration has recently been clarified in non-neuronal systems such as epithelial cells. PARD6α siRNA disrupts the microtubule cytoskeleton in epithelial cells (Kodani et al. 2010). PARD6 is also a controlling element of the mitotic spindle, as RNAi of either PARD6α or PARD6γ causes multipolar spindle formation and mitotic failure in epithelial cells (Kodani et al. 2010; Dormoy et al. 2013). In epithelial cell wound healing assays, PARD6γ RNAi-depleted cells were unable to migrate (Dormoy et al. 2013). As overexpression of PARD6α uncouples centrosomal and nuclear movement and disrupts migration, it remains unclear whether the centrosome was acting as an organizer of polarity and migration or a reporter of cellular mechanisms that control migration in these studies.

The PAR polarity complex may play both structural and signaling roles at the centrosome in migrating neurons. In epithelial cells, PARD6α interacts with the centriolar components PCM-1 and dynactin subunit $p150^{Glued}$, as shown through colocalization and immunoprecipitation studies (Kodani et al. 2010). The recruitment of PARD6α to the centrosome requires intact microtubules and dynein, as PARD6α was dispersed from the centrosome when microtubules were destabilized by nocodazole treatment and when dynein function was inhibited by overexpression of dynactin subunit $p150^{Glued}$ (Kodani et al. 2010; Young et al. 2000). Depletion of PARD6α by RNAi reduced microtubule-dependent recruitment of the centrosome proteins pericentrin, PCM-1, centrin, ninein, Cep170, and CPAP, showing that PARD6α promotes centrosome protein recruitment (Kodani et al. 2010). PARD6γ has been found to be a component of the mother centriole

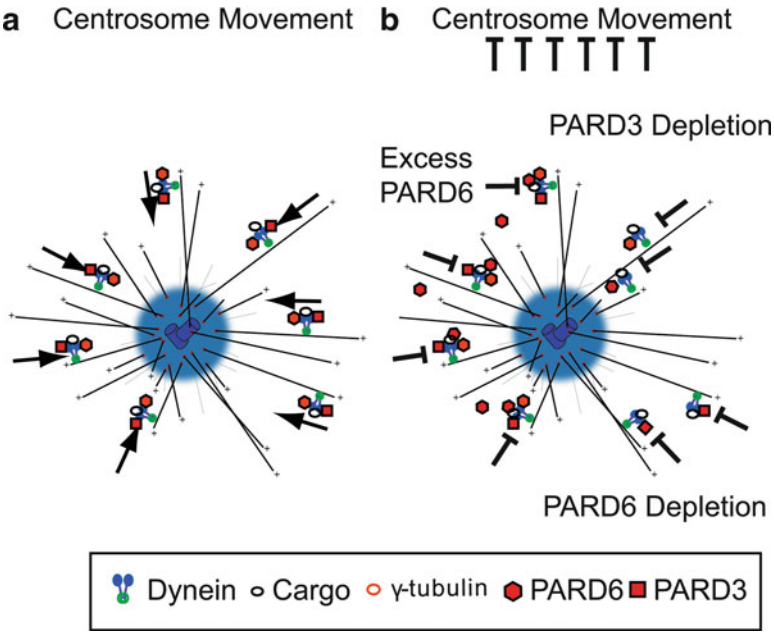

Fig. 7.3 PAR proteins and cytoplasmic Dynein directed minus-end transport. (**a**) Model of Dynein-directed minus-end transport of centrosome components mediated by PAR. This mechanism is responsible for proper centrosome motility. (**b**) Disruption of PAR protein components may result in inhibition of dynein mediated centrosome assembly and centrosome motility by PARD6 overexpression (Solecki et al. 2004), PARD6 RNAi (Kodani et al. 2010), and PARD3 depletion (Schmoranzer et al. 2009)

(Dormoy et al. 2013) and is required for recruitment of centrosome proteins such as PARD6α and $p150^{Glued}$. Interestingly, recruitment of PARD6γ to the mother centriole is microtubule-independent and requires the C-terminus of the PARD6γ protein (Dormoy et al. 2013). These findings show that PARD6 plays both structural and recruitment roles in the centrosome. It is currently unclear where PARD6 lies in the hierarchy of centrosome protein assembly.

2.3 PARD3 Regulates Centrosome Protein Recruitment and Orientation

PARD3 plays roles other than those of PARD6 in regulating centrosomal dynamics. PARD3 associates with dynein, as shown by co-immunoprecipitation of PARD3 with dynein light intermediate chain 2 (Schmoranzer et al. 2009). Dynein is required for assembly of γ-tubulin on centrosomes (Young et al. 2000), supporting the role of the PAR complex in proper centrosome assembly (Fig. 7.3). Removal of PARD3 prevents correct centrosome positioning in relation to the nucleus in epithelial cells (Schmoranzer et al. 2009). Like PARD6, PARD3 plays roles in both directed migration and organelle positioning, and PARD3 RNAi depletion inhibits migration of epithelial cells in wound healing assays (Schmoranzer et al. 2009). Depletion of PARD3 results in increased microtubule dynamics at cell-cell contacts, showing that PARD3 plays a role in microtubule stability in epithelial cells (Schmoranzer et al. 2009). Additional studies have shown PARD3 to stabilize and bundle microtubules both *in vitro* and in hippocampal neurons (Chen et al. 2013). The role of the PAR complex at the centrosome, as observed in several migrating cell types, and its link to dynein, add to our understanding of regulation of the cytoskeleton and neuronal migration by the PAR polarity complex.

The PAR polarity complex and its individual components have been shown to play roles in centrosome structure, protein recruitment, and motility in addition to regulating the dynamics of the microtubule cytoskeleton. After examining the role of the PAR polarity complex in controlling migration through microtubule-based mechanisms, we will explore the role of this complex in regulating the actin cytoskeleton.

3 PAR Complex Regulation of Myosin II Motors

3.1 PARD6α Regulates Myosin II Dynamics in Migrating CGNs

Identification of PAR polarity proteins as regulators of the microtubule cytoskeleton during neuronal migration led to examination of other cytoskeletal elements in migrating neurons. While many migration studies focused on force generation by microtubule-dynein systems, Solecki and colleagues (2009) examined the role of leading-process actomyosin in migrating CGNs. Two opposing models have been proposed for the mechanism by which actomyosin contributes to force generation in neuronal migration: (1) a dynamic forward reach-and-pull model, in which leading-process actomyosin contraction pulls the neuron forward and (2) a rearward contraction model, in which actomyosin contraction at the rear of the cell pushes the migrating neuron forward (Fig. 7.4) (Trivedi and Solecki 2011; Martini and Valdeolmillos 2010; Tsai et al. 2007). Time-lapse microscopy and photobleaching/photoactivation experiments show that leading-process actin is highly dynamic in migrating neurons, and pharmacological stabilization of the actin cytoskeleton or inhibition of the myosin II motor reduces leading-process dynamics, disrupts the two-stroke nucleokinesis cycle, and halts migration of CGNs (Solecki et al. 2009). The centrosome is central to the nucleokinesis cycle, and both actin and myosin light-chain kinase were found to accumulate at the centrosome in the leading edge of migrating neurons. The importance of myosin II to neuronal migration was shown by pharmacological inhibition of the myosin II motor with blebbistatin, which halted centrosome motion and the two-stroke nucleokinesis cycle (Solecki et al. 2009). The PAR complex is a key regulator of actomyosin dynamics in the leading process. In previous studies, overexpression of PARD6α was shown to inhibit neuronal migration (Solecki et al. 2004). The same group (Solecki et al. 2009) later reported that reduced myosin II activation in cells overexpressing PARD6α was one mechanism of migration inhibition. Overexpression of PARD6α or the truncated IQ motif of PARD6α significantly reduced leading-process actin turnover in migrating CGNs (Solecki et al. 2009), showing for the first time that the PAR complex can control myosin II through direct interaction (Fig. 7.5). Co-immunoprecipitation studies revealed that full-length PARD6α binds to myosin light chain and myosin light chain kinase and that overexpression of the PARD6α IQ domain inhibits myosin light chain binding to PARD6α (Solecki et al. 2009). In other studies in *C. elegans* embryos, cortical flow of actin and nonmuscle myosin II transported the PARD3/PARD6/aPKC complex to the anterior of the cell, maintaining polarity (Munro et al. 2004). Myosin IIb-deficient fibroblasts show polarity defects and increased levels of cytosolic PARD3 and PARD6 (Solinet et al. 2011). The mechanism of this relationship remains unclear, although it is possible that myosin II controls proper localization and stabilization of the PAR polarity complex. These results show that the PAR polarity complex regulates actomyosin contractility in the leading process of migrating neurons via PARD6α.

3.2 Actomyosin Dynamics in Migrating Neurons

Further studies examining the dynamics of actin cytoskeletal elements in CGNs buttress the importance of leading-process actin. The forward flow of actin in the leading process plays several roles important to migration. He and coworkers (2010) examined the role of cytoskeletal components

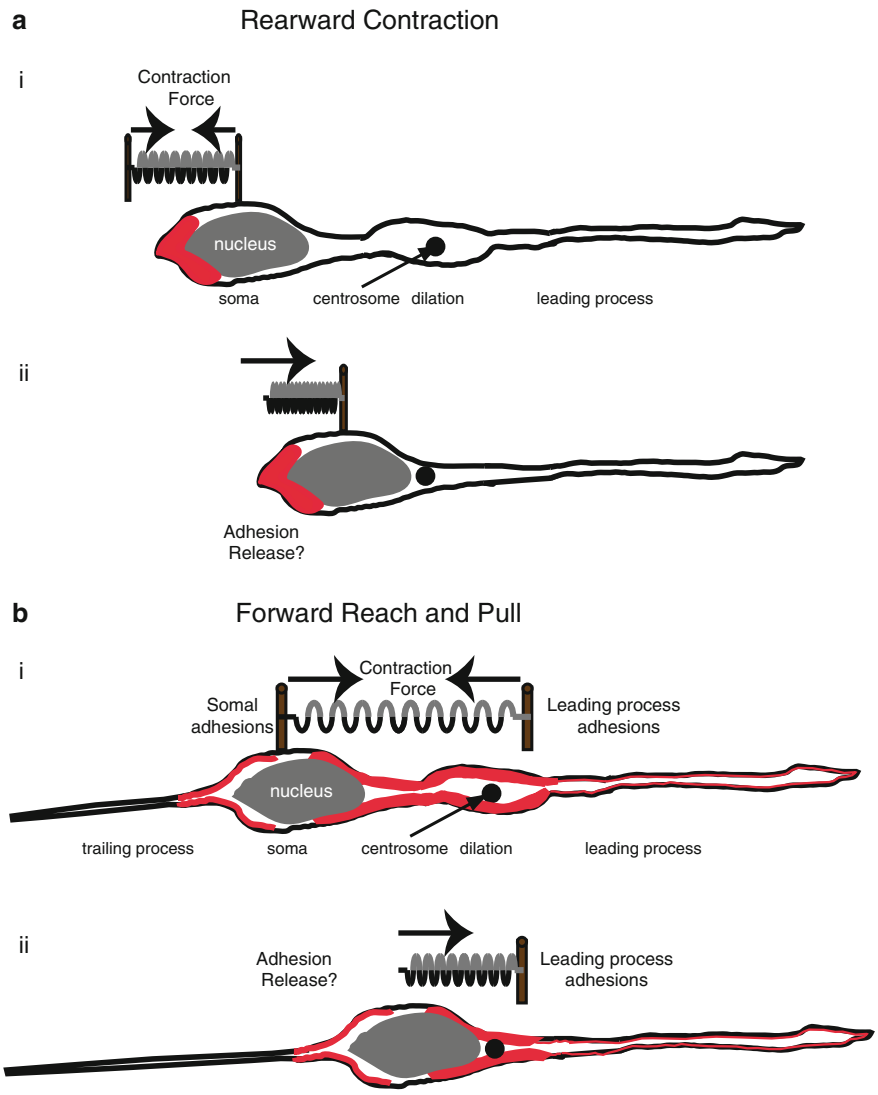

Fig. 7.4 Actomyosin pulling models for Glial-guided neuronal migration. (**a**) Rearward Contraction model. (*i*) Prior to somal movement, actomyosin (*red*) is heavily enriched at the cell rear. (*ii*) During somal movement myosin II squeezing at the rear is thought to "push" the cell body forward. (**b**) Reach and Pull model. (*i*) Prior to somal movement, actomyosin (*red*) is heavily enriched in the leading process from the cytoplasmic dilation to the neuronal soma. Given a muscle-like contraction of the F-actin array by myosin II, a taut spring effectively describes the forces produced when leading process and somal actomyosin anchoring (i.e., adhesions) are balanced before somal movement: one force vector points from the leading process back towards the soma whereas another force vector points from the soma towards the dilation (the future direction of somal movement). (*ii*) Once somal adhesions release, as described in (Gregory et al. 1988), actomyosin tension generated in the leading process primes somal movement towards the cytoplasmic dilation (Reproduced with permission of (Trivedi and Solecki 2011))

and motors *in vitro* in distinct regions of migrating rat CGNs. In their microdissection experiments, severing the distal leading tip of migrating neurons was sufficient to inhibit somal translocation, while a dynamic leading tip contributed to somal translocation by a distance of several cell-body lengths (He et al. 2010). By micropipetting actin-destabilizing drugs (cytochalasin D, latrunculin A)

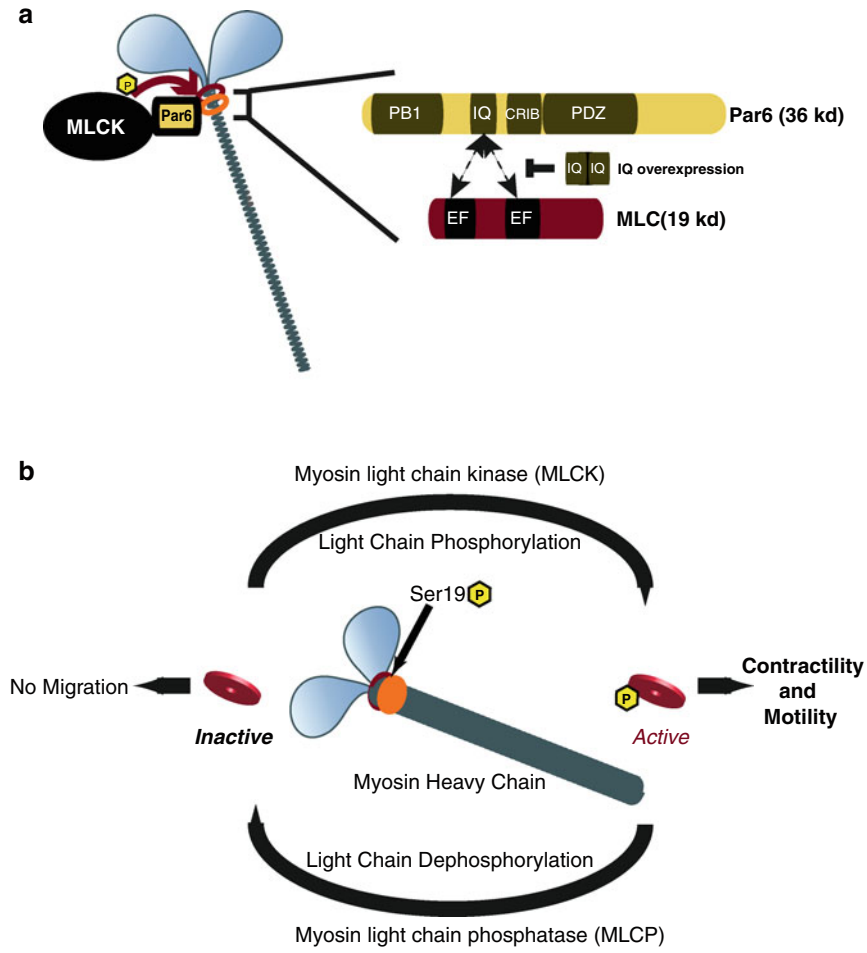

Fig. 7.5 Model of Par6α interaction with the Myosin II motor complex and the Myosin cycle. (**a**) Par6**α** binds to both MLC and MLCK, key signaling nodes regulating actomyosin contractility. *Inset*: The PARD6-MLC interaction may be mediated by the IQ domain of Par6**α** (IQ Motif (aa 104–120) = AFASNSLQRRKKGLLLRPV) and the EF hand domains of MLC. (**b**) Myosin contractility is dependent on Myosin Light Chain (*MLC*) phosphorylation by Myosin Light Chain Kinase (*MLCK*) at Ser19 and is required for neuronal migration. De-phosphorylation of MLC by Myosin Light Chain Phosphatase (*MLCP*) results in MLC inactivity and lack of myosin contractility. MCL and MLCP activity cycles Myosin contractility in migratory cells ((**a**) Reproduced with permission of (Solecki et al. 2009))

or actin-stabilizing drugs (jasplakinolide) into the vicinity of the leading processes of migrating neurons, they also showed that leading-process actin dynamics are required for somal translocation. Pharmacological inhibition of leading-process actin dynamics halted somal translocation; however, when the inhibitor was concentrated in the cell body area, it did not similarly inhibit somal translocation (He et al. 2010). The same group (He et al. 2010) also compared the roles of the microtubule cytoskeleton and of actin in the leading tip and found that the microtubule-destabilizing drug nocodazole did not halt somal translocation, but rather enhanced the rate of nuclear migration. Directed inhibition of myosin II by applying blebbistatin to the leading tip of migrating CGNs halted somal translocation, while blebbistatin treatment at the rear of the cell increased the rate of nuclear migration, demonstrating the importance of the myosin II motor (He et al. 2010). These findings showed that polarized activity of myosin II plays an important role in neuronal migration.

Wang and coworkers (2012) expanded on the role of leading-process actomyosin and its importance in active migration of neurons. They used antibody-coated quantum dots to track the movement of the membrane proteins VAMP2 and endogenous neurotrophin receptor TrkB in actively migrating mouse CGNs and showed that both proteins are non-randomly translocated (in a form of biased drift) in a myosin II–dependent manner toward the leading process. This non-random translocation was not identified in non-migratory cells, leading the authors to hypothesize that the biased forward drift of receptors may be involved in the guidance of migrating neurons. Taken together, these data support the forward flow of the F-actin cytoskeleton in the leading process of migrating neurons observed in our laboratory (Gupton and Waterman-Storer 2006; Vicente-Manzanares et al. 2007; Solecki et al. 2009) and highlight that forward flow is not just important for centrosome positioning but also regulates positioning of receptors within the leading process.

3.3 Potential Role of Actomyosin in Generating Leading Process Traction Forces

The organization of motor proteins and their function in migrating cells is highly regulated during migration. The role of myosin II in both the two-stroke nucleokinesis cycle (Solecki et al. 2009) and biased drift of surface receptors (Wang et al. 2012) provides insight as to how the regulation of actomyosin in the leading process controls migration. It is possible that the actin mechanisms involved in receptor transport in migrating neurons also play a role in the formation and maturation of cell-surface adhesion dynamics. Studies of myosin II in migrating epithelial cells provide an example of the possible roles of these motor proteins in migrating neurons. Active migration of epithelial cells requires the coordination of actin, myosin II, and focal adhesions (Gupton and Waterman-Storer 2006). Gupton and Waterman-Storer examined the migration of Ptk1 cells on various concentrations of extracellular matrix (ECM) and observed the effects of these concentrations on the actin cytoskeleton and cellular adhesion. Migration conditions were optimal at intermediate ECM concentrations, indicating that too much or too little adhesion limits cell migration. Higher ECM concentrations were associated with more pronounced focal adhesion density, yet migration was halted due to lack of focal adhesion turnover. Myosin II activity was highest at intermediate ECM concentrations, showing that migration is optimal when there is high myosin II activity, which is associated with efficient turnover and maturation of focal adhesions; these findings highlight the importance of actomyosin in active cell migration at the level of adhesion. The relation of myosin II activity to focal adhesion stability and maturation of adhesions illustrates how myosin II may control leading process traction. If the leading process of migrating neurons is analogous to the myosin II enriched lamellum of migrating fibroblasts, then myosin II motor activity may fine tune leading process adhesion to neuronal migration substrates. Current studies are further investigating the role of the PAR complex in the balance of actomyosin dynamics in the leading process of migrating neurons.

As actomyosin has a demonstrated role in the leading process of migrating neurons and in nucleokinesis, its interaction with PARD6α provides a mechanism linking polarity complexes with cytoskeletal motor systems in migrating neurons. The role of PARD6α in regulating microtubule dynamics in neurons also provides insight into the interaction of the actin and microtubule cytoskeletal systems in neuronal migration. A molecular clutch model has been proposed to allow transmission of polymerization-driven flow of myosin into traction (Mitchison and Kirschner 1988; Gardel et al. 2010). It is possible that a function of the PAR complex in the leading process of migrating CGNs is as a clutch between the myosin and microtubule networks to generate forward force on the cell and/or individual organelles. As both myosin II and the PAR complex have been shown to play an integral role in neuronal migration, the relationship between polarity, motor proteins, and cellular adhesions must be

considered to further complete our understanding. We will next discuss the relation between PAR proteins and adhesion molecules.

4 The PAR Complex and Adhesion Mechanisms

4.1 CGN Migration Varies in Subsections of the Cerebellum

Neural progenitors proliferate in germinal zones of the brain and must then migrate to their final locations to establish proper neural circuits. A key factor in controlling germinal zone exit is the regulation of cell-cell contacts, especially those that occur as migrating neurons encounter different cell types within distinct regions of the developing cerebellum. During germinal zone exit, CGNs must migrate tangentially among other CGNs in the upper and middle layers of the external granule layer before they transition to the inner layer of the EGL (Komuro et al. 2001). The migration rates differ in these distinct regions of the EGL, suggesting that the motor and adhesion systems of migration may differ as well. Exiting the EGL and moving into the molecular layer, CGNs migrate along glial fibers from the EGL of the cerebellum to their final location in the IGL.

4.2 Antagonistic Interaction of PARD3 and SIAH

The mechanisms controlling the germinal-zone exit of migratory neuronal precursors have revealed novel insights into when and how CGNs initiate the first step in their journey to the IGL. Previously, two competing models were used to explain germinal zone exit: in one model it was thought that a new form of cell-cell adhesion was initiated upon movement of CGNs from the EGL into the molecular layer. The other model proposed that removal of a form of cell-cell adhesion maintained in the EGL allowed maturing CGNs to exit the germinal zone (Métin and Luccardini 2010). Examination of PARD3 function in germinal zone exit suggests that the first model may be active and regulated by polarity signaling cascades.

Famulski et al. identified PARD3 as a novel regulator of cell adhesion through interaction with the E3 ubiquitin ligase SIAH (Famulski et al. 2010). SIAH was identified from a yeast two-hybrid screen of PAR complex interaction partners. Evidence of physical interaction between these proteins led researchers to examine the amino acid sequence of PARD3. Examination of PARD3 motifs identified two SIAH degron recognition sequences as potential regulation sites of germinal zone exit. SIAH and PARD3 interact directly through the SIAH substrate-binding domain targeting the two SIAH-degron recognition sequences of PARD3 in an interaction that requires the catalytic SINA substrate binding domain of SIAH. Ubiquitination of PARD3 by SIAH results in PARD3 degradation by the proteasome, revealing an antagonistic interaction between PARD3 and SIAH (Famulski et al. 2010).

Expression analysis revealed a reciprocal expression profile of SIAH and PARD3. SIAH showed high expression in CGN progenitors, which was extinguished in differentiated CGNs in the developing cerebellum. In contrast, PARD3 was found to be expressed at low levels in the EGL and elevated levels in differentiating CGNs. Systematic necessity/sufficiency testing was then used to test whether these reciprocal expression profiles were functionally relevant to germinal zone exit. Ectopic expression of PARD3 in CGN precursors in the EGL, which normally express low levels of this polarity protein, was sufficient to induce precocious germinal zone exit, while gene silencing of PARD3 blocked migration, showing that its activity was necessary for immature CGNs to exit the EGL and migrate to their final destination (Fig. 7.6) (Famulski et al. 2010). In contrast, ectopic expression of SIAH inhibited germinal zone exit of CGNs and maintained tangential migration paths. Interestingly, co-expression of PARD3 with SIAH was sufficient to restore directed migration and germinal zone exit. Finally, SIAH silencing induced precocious germinal zone exit to a degree similar to that of PARD3 gain of function (Famulski et al. 2010). These results identified PARD3 and SIAH as

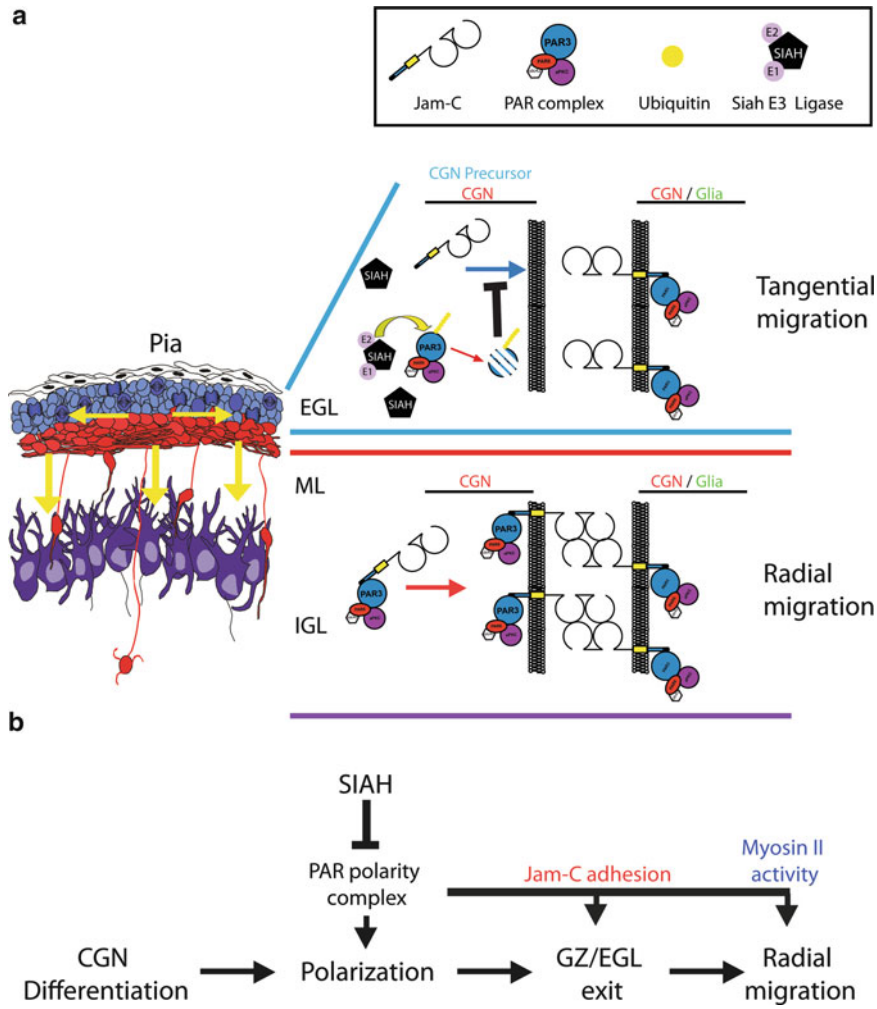

Fig. 7.6 Model of SIAH E3 Ligase regulation of germinal zone exit. (**a**) During cerebellar development CGN precursors migrate tangentially within the EGL. Upon differentiation and polarization, CGNs exit the GZ/EGL and migrate radially to traverse the ML and assume their final position in the IGL. Within the developing postnatal cerebellum SIAH (E3 ubiquitin ligase) is highly expressed in the EGL, where it ubiquitinates PARD3A to target it for proteasome-mediated degradation. PARD3A degradation results in inactivation of the PAR polarity complex, thereby inhibiting recruitment of the JAM-C adhesion molecule to contacts between CGNs or CGN precursors and glial cells. The absence of JAM-C-mediated adhesion prevents GZ exit by restricting the radial migration of CGN precursors. (**b**) The PAR polarity complex is required for differentiated CGNs to polarize, exit the GZ via JAM-C-mediated adhesion, and migrate radially via activation of the myosin II motor. SIAH negatively regulates CGN polarization, GZ exit, and radial migration by inactivating the PAR polarity complex (Reproduced with permission of (Famulski et al. 2010))

novel regulators of the CGN migratory path and germinal zone exit through posttranslational modification of PARD3.

PARD3 had previously been found to localize to cell-cell contacts and therefore to be essential for junction formation in epithelial cells (Hirose et al. 2002). Famulski and coworkers showed that PARD3 regulates germinal zone exit by interacting directly with junctional adhesion molecule C (JAM-C), a cell-cell adhesion molecule whose role in stabilizing cell-cell contacts is required for CGN migration to the IGL (Famulski et al. 2010). Using a JAM-C-pHluorin probe to observe JAM-C junctions in living cells, the

authors characterized JAM-C junctions forming at cell-cell contacts *in vivo* in migrating neurons. SIAH gain of function dissolved JAM-C tight junctions in a manner that was rescued by PARD3, while SIAH silencing greatly enhanced JAM-C adhesion, suggesting that the antagonistic relationship between SIAH and PARD3 controls the surface levels of JAM-C. The critical importance of JAM-C adhesion for germinal zone exit was illustrated by JAM-C gain of function experiments: overexpression of a constitutively active version of JAM-C not only induced precocious germinal zone exit but also fully rescued a SIAH gain-of-function phenotype. Famulski and colleagues identified SIAH as an inhibitor of PARD3-dependent JAM-C adhesion, revealing how reduced polarity signaling within the EGL of the cerebellum prevents the onset of cell-cell contacts that are necessary for germinal zone exit. This was the first demonstration of the direct control of neuron–glial cell adhesion by a polarity complex in the developing nervous system.

4.3 Mechanisms of PAR-Mediated Cell Adhesion

The PAR polarity complex is indirectly related to mechanisms of cell-cell adhesion. The interactions of PAR in epithelial cell junctions and turnover may elucidate the role of PAR proteins in neuronal migration. Myosin II promotes junction formation in epithelial cells by strengthening remodeling adhesions (Vicente-Manzanares et al. 2009; Bertet et al. 2004). As PARD6α has been found to be a regulator of myosin II (Solecki et al. 2009), the PARD3-SIAH complex may be an additional mechanism by which the PAR polarity complex controls polarity and adhesion. Interactions between cell adhesions and cytoskeletal motor systems provide the context in which migrating cells generate force to propel themselves forward. Proteomic analysis identified dynein intermediate chain 2 (DIC2) as a phosphorylation target of aPKC of the PAR complex (Rosse et al. 2012). Regulation of DIC2 by aPKC controls focal adhesion turnover through interaction with focal adhesion complex member paxillin (Rosse et al. 2012). PARD3 was also implicated as a regulator of focal adhesion kinase (FAK) through mass-spectrometry identification of PARD3 binding partners in epithelial cells (Itoh et al. 2010; Xie et al. 2003). Reduction of PARD3 in epithelial cells inhibited adhesion-induced activation of FAK, implicating the PAR polarity complex in the regulation of focal adhesions. Interaction of the PAR complex with both the microtubule and actin cytoskeletons in migrating cells potentially links the two systems, allowing crosstalk between them. As disruption of PAR signaling uncouples the two-stroke nucleokinesis cycle and inhibits recruitment of centrosome proteins, it is possible that interaction between the PAR complex and myosin II (PARD6) and/or dynein (PARD3) is required for proper cytoskeletal rearrangement and is an integral component of neuronal motility.

5 Further Studies of the Compartmental Roles of PAR in Migrating Neurons

PAR proteins and the PARD3/PARD6/aPKC complex have been identified as key regulators of neuronal migration and germinal zone exit. The role of PAR proteins in discrete regions of the cell, interacting with the centrosome or specifically at cell adhesions, may differ from the roles of the PAR complex in migrating cells. Several challenges remain in understanding the mechanisms involved in neuronal migration, and they can be addressed by future studies of the PAR complex. By what mechanism does PARD6 regulate the microtubule and actomyosin cytoskeleton? As the PAR complex has been proposed to regulate actomyosin contraction in the leading edge of migrating neurons, there may be a connection between myosin and dynein motor–generated force in migrating neurons. Does PARD6 or the PAR complex directly link the actin and microtubule cytoskeletons in the leading process of migrating neurons as a molecular clutch? High-resolution co-localization studies of specific components of the PAR complex with cytoskeletal

motor systems in the leading processes of actively migrating neurons may reveal such transient interactions. What additional signals control PARD3's regulation of cell-cell adhesions in the transition from tangential to radial migration? Examining how PARD3 regulates adhesion systems other than JAM-C, and the cytoskeletal systems that use these adhesions to generate propulsive force, will provide a larger context for understanding how the neuronal cytoskeleton interacts with cell-cell adhesions.

Acknowledgements We thank Sharon Naron, Niraj Trivedi, and Shalini Singh for editorial support in preparation of the manuscript. The Solecki Lab is funded by the American Lebanese Syrian Associated Charities (ALSAC), by grant #1-FY12-455 from the March of Dimes, and by grant 1R01NS066936 from the National Institute of Neurological Disorders and Stroke (NINDS). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NINDS or the National Institutes of Health.

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Spinal Motor Neuron Migration and the Significance of Topographic Organization in the Nervous System

8

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Abstract

The nervous system displays a high degree of topographic organisation such that neuronal soma position is closely correlated to axonal trajectory. One example of such order is the myotopic organisation of the motor system where spinal motor neuron position parallels that of target muscles. This chapter will discuss the molecular mechanisms underlying motor neuron soma positioning, which include transcriptional control of Reelin signaling and cadherin expression. As the same transcription factors have been shown to control motor axon innervation of target muscles, a simple mechanism of topographic organisation specification is becoming evident raising the question of how coordinating soma position with axon trajectory might be important for nervous system wiring and its function.

Keywords

Spinal cord • Neuronal migration • Topographic map • Reelin • Cadherin • Transcription

1 Introduction

One of the characteristics of the nervous system thought to underlie its accurate function is its ordered layout. In general, sensory information is received by sensory neurons and relayed to specialised centres, which process it and compute the appropriate output, which activates motor neurons and causes muscle contractions. At each level of this hierarchy, neuronal position is ordered such that adjacent neurons process sensory information gathered from adjacent points on the surface of our bodies, or in the space surrounding our bodies. Hence, the cortical neurons

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L. Nguyen and S. Hippenmeyer (eds.), *Cellular and Molecular Control of Neuronal Migration*, Advances in Experimental Medicine and Biology 800, DOI 10.1007/978-94-007-7687-6_8,

concerned with somatosensory information are ordered into a somatotopic map first described by Penfield (Penfield and Boldrey 1937), similar to the topographic arrangement of neurons reflecting external visual space in the brain's primary visual area. The latter was exploited by Sperry to prove that ordered neuronal connections are important for the accurate functioning of the nervous system (Sperry 1963). Such order is also apparent in the motor system, in particular through the localisation of motor neuron somata in the ventral spinal cord, which are grouped into clusters or pools innervating a particular muscle, and found in register with the position of their target muscles, an idea proposed by early neuroanatomists such as Bikeles (1905), and later elaborated by Romanes (1951).

The developmental origin of highly ordered neural circuits has been under consideration almost since their first descriptions, no doubt inspired by Ramon y Cajal's call to study "the young wood" (Ramón y Cajal 1989). This chapter will attempt to summarise insights into the question of how spinal motor neuron cell bodies are positioned, first by considering cellular level studies, and then by discussing the molecular mechanisms that control spinal motor neuron soma positioning. Finally, the functional implications of ordered spinal motor pools on locomotor function will be explored.

2 Cellular Studies of Migrating Spinal Motor Neurons

One simple answer to the question of how ordered neuronal positioning arises is the idea that neurons can be generated at the exact location where they will reside and function. This appears to be the case in the insect nervous system where for example, neurogenesis involves delamination from ectodermal or neuroblast precursor cells, where the newborn neurons remain relatively static (Dambly-Chaudiere and Gysen 1986). In the vertebrate nervous system, neurons are born from neuronal progenitors lining the nervous system ventricles and migrate radially, perpendicular to and away from the surface of the ventricle and in close contact with radial glia (Rakic 1971), to eventually reach the place where they reside permanently and function. One of the earliest evidences of this process was obtained through radiological labeling of developing cortical neurons (Angevine and Sidman 1961). Additionally, vertebrate neurons also migrate in a tangential direction, approximately parallel to the surface of the ventricular zone (O'Rourke et al. 1992). Over the years, the conclusion that emerged is that this is a highly ordered process, where the eventual position of a neuronal soma within a particular cortical layer is highly correlated with its time of birth, an idea discussed in greater depth in other chapters of this book.

The spinal cord originates as an epithelial sheet, the neural plate, which through a folding process becomes the neural tube: a pseudo-stratified epithelium filled with dividing progenitor cells (Ramón y Cajal 1911; Jacobson 1991). Later, molecular studies revealed that these progenitors are in fact radial glia, which in addition to serving as a source of newborn neurons (Malatesta et al. 2000; Noctor et al. 2001), also act as a migration scaffold (Choi 1981; Gomez et al. 1990). Radiographic studies showed that spinal neurogenesis proceeds in a ventral to dorsal direction, such that motor neurons which are found in ventral regions of the spinal cord are born before the neurons that make up the dorsal horn (Nornes and Das 1974). Early electron microscopy studies confirmed that immature spinal motor neurons have two processes: one reaching the ventricle, and one contacting the pial surface of the spinal cord, the latter becomes the axon and leaves the spinal cord through the ventral root (Chu-Wang et al. 1981; Dorado et al. 1990). Hence, labelling of immature motor neurons by applying a tracer reveals a structure reminiscent of cortical radial glia, featuring an elongated, spindle-shaped soma, and a ventricular zone process (Farel and Bemelmans 1980).

Following cell cycle exit, the cell body of a motor neuron begins a migration process, which appears to be similar to the nuclear translocation seen in the cortex. All motor neuron somata initially migrate radially, away from the ventricular zone (Phelps et al. 1991). In general, motor

neurons can be subdivided according to the location of the structures that they innervate, and are generated at rostro-caudal levels of the spinal cord in register with their targets (Altman and Bayer 1984; Oppenheim et al. 1989). This organisation is demonstrated by the fact that body wall and limb muscle innervating somatic motor neurons are generated at non-limb and limb levels of the spinal cord, respectively. Lineage tracing analyses based on infecting single progenitors with a virus encoding an indelible marker suggest that some motor neurons, and spinal neurons in general, migrate in radial, tangential as well as antero-posterior directions (Leber and Sanes 1995; Leber et al. 1990). Electron microscopy studies of such migrating spinal motor neurons suggest that their migration is a process that is very similar to that occurring in the developing cortex (Chu-Wang et al. 1981). As the spinal cord continues to grow, motor neuron positions appear to shift, but it is not clear whether this is an active process or a consequence of increase in soma size.

Somatic motor neurons innervating non-limb and limb muscles end their migration, respectively, in medial or lateral regions of what will become the ventral horn of the spinal cord. The limb innervating motor neurons are eventually confined to the lateral motor column (LMC), whereas body wall and dorsal axial muscle innervating motor neurons are found within the medial motor column (MMC) (Gutman et al. 1993; Landmesser 1978b). These motor columns are themselves subdivided into motor pools which contain motor neurons innervating specific muscles (Romanes 1951, 1964; Hollyday 1980; McHanwell and Biscoe 1981), however the identification of motor pools without molecular markers is restricted to their retrograde labelling from target muscles, which precluded a cellular analysis of their early development. LMC motor neuron somata come to rest near a specialised group of cells called boundary cap cells which lie at the ventral root of the spinal cord and function to allow the exit of motor axons from the central nervous system while at the same time, preventing motor neuron somata from trickling out (Altman and Bayer 1984; Golding and Cohen 1997).

Another motor neuron subtype whose migration has been studied extensively are the visceral pre-ganglionic (PG) motor neurons whose axons leave the CNS through the ventral root, but do not innervate somatic muscles. The primary function of PG motor neurons is to control the action of peripheral sympathetic ganglia (Levi-Montalcini 1950). The initial trajectory of PG somata is similar to that of MMC and LMC neurons: a radial displacement towards the lateral edge of the spinal cord (Phelps et al. 1991), but once near the ventral root, PG motor neurons migrate dorsally in a tangential direction, and eventually populate a medial region of the spinal cord, close to the ventricular zone (Prasad and Hollyday 1991; Markham and Vaughn 1991).

Although spinal motor neuron pools and columns are organised more like nuclei than cortical laminae, spinal motor neuron position can also be correlated with their time of birth (Whitelaw and Hollyday 1983). A significant example of this is the differential migration of lateral LMC and medial LMC motor neurons, which are born from a common progenitor pool but at different times (Hollyday and Hamburger 1977; Whitelaw and Hollyday 1983). Medial LMC neurons innervate muscles of the ventral limb, whereas lateral LMC neurons innervate muscles of the dorsal limb (Landmesser 1978b). Medial LMC neurons are born first and migrate, as all somatic motor neurons do, towards the lateral edge of the spinal cord. Lateral LMC neurons are born at a later time, and end up migrating either around or through the medial LMC neurons, to eventually settle in a position lateral to the medial LMC neurons (Whitelaw and Hollyday 1983; Gould et al. 1999; Hollyday and Hamburger 1977). Interestingly, this inside-out mode of migration also appears to be true for dorsal horn neurons, where later born neurons migrate past those already generated, to occupy more superficial dorsal horn laminae (Nornes and Das 1974; Altman and Bayer 1984). On the other hand, one should not rely excessively on this generalisation since, as suggested by the work of the Sanes lab, many spinal neurons have extensive and circuitous migration patterns (Leber et al. 1990).

3 Early Studies of Motor Axon Guidance in Relation to Soma Migration

As spinal motor neuron cell bodies are migrating within the ventral spinal cord, their axons exit through the ventral root and fasciculate with other motor and sensory axons to form peripheral nerves which follow a pre-determined trajectory to their peripheral targets (Tosney and Landmesser 1985). The invariant nature of peripheral nerve trajectories observed when comparing different individuals of the same species (Landmesser 1978a) strongly argues that motor axon pathways are not random and that axonal growth cones, the sensory structures at the end of axons, are actively sensing some sort of a molecular label. This idea was first proposed by Ramon y Cajal (1911), and was elaborated over many years by cellular studies of Weiss, Sperry and others (Jacobson 1991). Arguably the most studied spinal motor axon trajectory is that linking LMC neuron cell bodies to their limb targets, a process which involves the coalescence of LMC motor axons from a number of spinal segments into a plexus region at the base of a limb, the selection of a dorsal or ventral limb nerve, and then further ramification into ever smaller branches that eventually contain single axons that innervate single muscle spindles (Lance-Jones and Landmesser 1981; Landmesser 1978a; Lu et al. 2009).

The intense interest in the question of how limb axonal trajectory is selected has resulted in many cellular experiments laying the groundwork for molecular studies. One particular experiment relevant to this chapter is the examination of the effect of limb rotation on motor axon trajectory selection (Summerbell and Stirling 1981; Ferguson 1983; Ferns and Hollyday 1993). Originally, these experiments were designed to examine how limb tissues influence the selection of a limb nerve trajectory, and demonstrated that the proximal mesenchyme near the base of the limb contains a source of a short-range acting signal. However, these experiments, as well as more extensive manipulations (Hollyday 1981), also tell us something about the relationship between axon trajectory and the position of motor neuron cell bodies. Namely, motor neuron soma positioning and axon targeting appear to occur independently of one another, such that motor neurons whose axon trajectory is altered settle their cell bodies in their usual location within the ventral spinal cord. Moreover, experiments in which developing neural tubes of a specific rostrocaudal identity are transposed to different antero-posterior locations argue that functional development of spinal motor neurons is independent of the identity of the limb adjacent to it, further suggesting the independence of motor neuron soma positioning and axon trajectory selection (Lance-Jones and Landmesser 1981; Landmesser and O'Donovan 1984).

4 Transcription Factors that Control Spinal Motor Neuron Migration

The above cellular experiments laid down compelling groundwork on which to base a molecular analysis of spinal motor neuron migration. One major advance in this direction was the isolation and characterisation of molecular markers of spinal neurons. Mainly through the work of the Jessell laboratory, it became possible to subdivide spinal motor neurons and their progenitors without the necessity of anatomical handles or generic neurotransmitter markers. Remarkably, transcription factors from the LIM homeodomain family could be used to subdivide LMC and MMC motor neurons: all somatic motor neurons express HB9 (Pfaff et al. 1996; Tanabe et al. 1998), while Lim1 or Lhx1 is expressed in lateral LMC motor neurons, in contrast to the early expression of Isl1 in all motor neurons, and its subsequent restriction to medial LMC neurons (Tsuchida et al. 1994). All LMC neurons express high levels of the forkhead transcription factor Foxp1, while PG motor neurons express it at lower levels (Rousso et al. 2008; Dasen et al. 2008). Some MMC motor neurons can also be identified by their expression of Lim3 or Lhx3 transcription factors (Tsuchida et al. 1994). Additionally, the rostro-caudally restricted

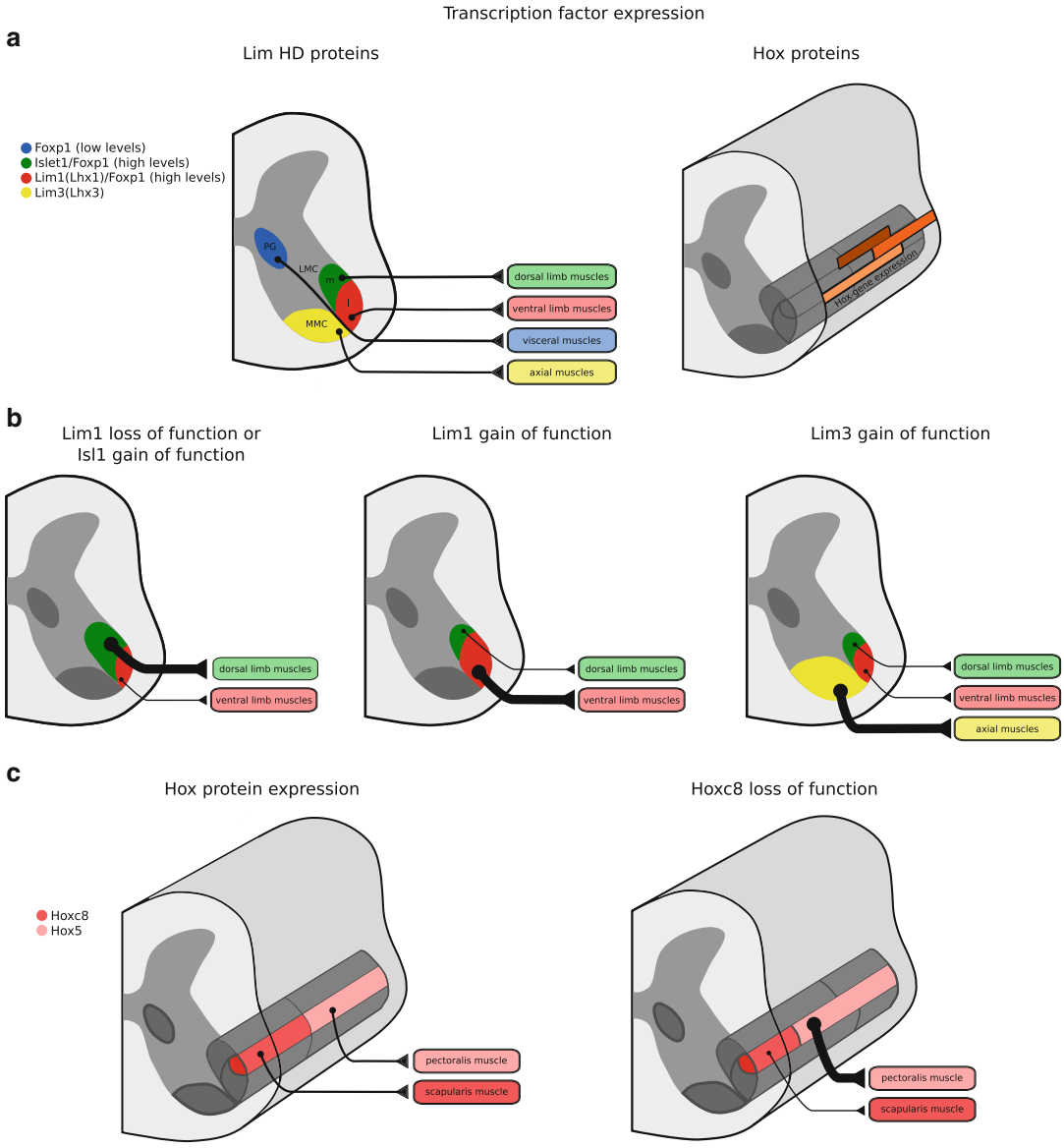

Fig. 8.1 **The function of transcription factors in spinal motor neuron migration**. Summary of expression of LIM homeodomain and Hox transcription factors in spinal motor neuron subpopulations (**a**). Functional evidence of the role of LIM homeodomain (**b**) and Hox transcription factors in spinal motor neuron soma localisation (**c**)

expression of Hox genes involved in providing antero-posterior identity of various tissues and structures also delineates spinal motor neuron columns (Dasen et al. 2003, 2005) (Fig. 8.1).

With the advent of these molecular markers it became possible to ask whether the transcription factors expressed by LMC, MMC and PG motor neurons themselves are contributing to the establishment of myotopic organisation. Thus, when the HB9 transcription factor was knocked out, its importance in the consolidation and maintenance of motor neuron identity became apparent, such that motor neurons lacking HB9 started to express interneuron markers and were found at aberrant positions within the spinal cord (Arber et al. 1999; Thaler et al. 1999). Manipulation of

transcription factors that are confined to a spinal motor neuron column or division leads to a change in motor neuron position specification. Overexpression of the MMC identity marker Lim3 in LMC neurons causes them to adopt a cell body settling pattern like that of MMC neurons, in addition to inducing changes in molecular marker expression and axonal projection (Sharma et al. 2000). Similarly, overexpression of the lateral LMC marker Lim1 in medial LMC neurons causes their cell bodies to settle in lateral regions of the LMC (Kania and Jessell 2003), while loss of Lim1 from lateral LMC neurons causes them to adopt a more medial settling pattern (Palmesino et al. 2010). Ectopic expression of the medial LMC marker Isl1 in lateral LMC neurons causes them adopt a more medial position within the LMC (Kania and Jessell 2003). Together, these experiments argue that the LIM homeodomain transcription factor markers of spinal motor neurons are necessary and sufficient to specify a distinct settling pattern of spinal motor neuron subpopulations.

One of the earliest examples of transcription factors controlling motor neuron migration is that of Hoxb1, which is expressed in a population of motor neurons confined to the hindbrain rhombomere r4, and plays a role in specifying their migration pattern (Studer et al. 1996). In the spinal cord, rostrocaudally-restricted Hox transcription factors are also involved in the specification of motor neuron position. The observation that Hoxc6 is present in LMC motor neurons at brachial level and that Hoxc9 is expressed in PG motor neurons at thoracic levels, suggested that these transcription factors could be important in specifying PG versus LMC motor neuron fate, and thus, dorsomedial versus ventrolateral spinal cord position, respectively. Indeed Hoxc9 overexpression leads to a transformation of LMC neurons into PG neurons, including a shift in their position within the spinal cord. Likewise, Hoxc6 overexpression leads to the conversion of PG neurons into LMC neurons, along with a corresponding cell body position shift (Dasen et al. 2003).

Within a particular domain of the LMC, the rostrocaudal extent of motor pools also coincides with specific domains of Hox protein expression, implying that motor pool identity and therefore motor neuron migration patterns might also be controlled by this class of transcription factors. Hox5 and Hoxc8 expression forms a sharp boundary that coincides with the boundary of scapularis and pectoralis motor pools within the LMC. Overexpressing or inhibiting Hoxc8 shifts Hox5 expression and the boundary of these motor pools such that with the loss of Hoxc8 function, the caudal extent of the scapularis pool shrinks while the pectoralis pool expands more rostrally. Importantly, the mediolateral settling pattern of the ectopic domain of the pectoralis pool as assessed by the expression of its marker Pea3 transcription factor, appears more like the endogenous pectoralis pool and is markedly different from the scapularis pool (Dasen et al. 2005). Thus, not only do Hox genes control the identity of motor neurons and their cell body position pattern at the division level, i.e.: LMC versus MMC versus PG, but also can control it at the level of individual pools. This conclusion raises the question of whether the function of Hox genes at the level of entire motor neuron divisions can be disconnected from their apparently later action at the level of pools, or whether pool identity and soma settling pattern are defined simultaneously at divisional and pool levels. Perhaps the answer to this question might come from a better understanding of the soma position effector molecules whose expression is controlled by Hox genes.

One interesting idea that emerged from studying the development of motor neurons is that the transcription factor Foxp1 is required to mediate the function of essentially all Hox genes. Thus, in *Foxp1* mutants, the above functions of Hox proteins in spinal motor neuron development are abrogated, leading to an interesting consequence on LMC neuron differentiation. In *Foxp1* mutants, general spinal motor neuron identity is specified, along with a general localisation within the ventrolateral region of the spinal cord, and an axon projection through the ventral root. However, in such mutants, none of the above molecular markers of LMC neuron subpopulations are expressed, leading to the conclusion that Foxp1 removal results in the reversion of limb level LMC motor

neurons to a more evolutionary primitive state in which they exist as a homogenous ensemble, undivided into subpopulations such as motor pools. Retrograde labelling of these motor neurons from a particular limb muscle reveals a disruption of their myotopic organisation, such that while motor neurons apparently innervating the same muscle are still located within the approximate position of the LMC, they are no longer located within the same cluster or pool in the ventral spinal cord (Dasen et al. 2008; Rousso et al. 2008). Of course, in the absence of any pool markers, it is difficult to conclude that these motor neurons are indeed part of a molecularly-defined motor pool in *Foxp1* mutants. An alternative possibility is that motor neurons from a particular pool are still clustered, although their axonal projections in the limb are randomised. The resolution between these possibilities will develop as new Foxp1-independent molecular pool markers are uncovered.

The above experiments involve transcription factors that appear to be master regulators of spinal motor neuron identity at the level of divisions or at the level of pools. In addition to controlling cell body position, they sit at the top of a developmental hierarchy, controlling other aspects of motor neuron identity such as axonal projection. Indeed, gain and loss of function experiments of Foxp1, Lim1, Isl1 and HB9 (see above) all demonstrate that concomitant with inducing cell body position change, these manipulations also lead to a change of axon trajectory, but without any evidence of cross-talk between the soma migration and axon trajectory selection. In contrast, ETS transcription factors appear to control cell body position of spinal motor neurons in concert with axon trajectory selection. Initial experiments identifying these proteins as markers of specific motor pools demonstrate that their expression is dependent on signals from the periphery, such that limb bud ablation results in the extinction of expression of these transcription factors (Lin et al. 1998). Further experiments demonstrate that the neurotrophic factor GDNF, expressed in the limb, is actively signalling the maintenance of expression of the Pea3 ETS transcription factor, and that this maintenance is important for specifying the particular migration and dendritic arborisation pattern of the LMC motor neurons that express it (Livet et al. 2002; Haase et al. 2002; Vrieseling and Arber 2006). Thus, the ETS transcription factor Pea3 links axon growth into the limb to soma localisation, and therefore its action is mechanistically different from that of LIM homeodomain transcription factors whose expression does not appear to be induced by extrinsic signals dependent on axonal trajectory (Kania et al. 2000).

The converse of this idea is the dependence of lateral versus medial LMC neuron identity specification on cell body migration. Medial LMC motor neurons are born first, and express the LIM homeodomain transcription factor Isl1. Later-born lateral LMC neurons also initially express Isl1 yet they turn it off relatively quickly, and turn on the expression of Lim1 (Tsuchida et al. 1994). At the cellular level this coincides with the migration of lateral LMC neurons, near or through the cluster of medial LMC neurons, which at this time begin to express the retinoic acid synthesis enzyme retinaldehyde dehydrogenase 2 or RALDH2. Functional experiments argue that this conjunction of lateral and medial LMC neurons is required to turn off Isl1 expression and turn on Lim1 expression, providing an example migratory behaviour in spinal motor neurons that appears to be important for the induction of a particular neuronal identity (Sockanathan and Jessell 1998).

5 Effectors of Spinal Motor Neuron Migration

5.1 Reelin Signaling

The analysis of the role of transcription factors in the specification of spinal motor neurons position certainly yielded some important molecular handles on the problem of motor neuron soma migration. These are now being linked to specific effectors of neuronal migration that mediate signals from the outside environment to the neuronal cytoskeleton. Perhaps the best-known pathway that transduces such a signal is the one

responding to the extracellular matrix protein Reelin (see also Chap. 1). It is a large, secreted protein, originally identified through a mutation in its gene, which leads to a locomotor behavioural phenotype due the aberrant localisation of cerebellar neurons (D'Arcangelo et al. 1995; Hamburgh 1963b). In the cortex, Reelin is deposited in the superficial layer by Cajal-Retzius neurons (Hirotsune et al. 1995; D'Arcangelo et al. 1995) while ApoER2 and Very Low Density Lipoprotein Receptor (VLDLR) are two principal Reelin receptors that transduce the signal to the cytoskeleton through the Dab1 adaptor phosphoprotein (Hiesberger et al. 1999; Trommsdorff et al. 1999). Mice, mutant for genes encoding these components, display severe disruptions in cortical layering, and on the basis of these phenotypes, Reelin has been postulated to act as a neuronal migration stop signal (Falconer 1951; Hamburgh 1963a; Hiesberger et al. 1999; Howell et al. 1997; Sheldon et al. 1997; Tissir and Goffinet 2003; Trommsdorff et al. 1999; Jossin and Cooper 2011). The downstream targets of this pathway include the cytoskeleton-associated proteins Lissencephaly1 (LIS1) and Doublecortin (DCX) (Tissir and Goffinet 2003). Reelin signalling, and its role in cortical projection neuron migration, is described in greater detail in Chap. 1.

Reelin is also expressed in the spinal cord and is required for the normal migration of PG neurons as well as the layering of the dorsal horn laminae (Yip et al. 2000, 2003a, 2004, 2009; Villeda et al. 2006; Phelps et al. 2002). Interestingly, the altered position of PG motor neurons in *Reeler* mutants appears to have no bearing on their connectivity to their ganglionic targets (Yip et al. 2003b) arguing that cell body position and axon projections of motor neurons can be uncoupled. This is also paralleled in more rostral regions of the nervous system where despite being inappropriately positioned, many neurons in the *Reeler* mouse project their axons to appropriate targets (Caviness 1976; Caviness and Frost 1980, 1983). Interestingly, the change in spinal dorsal horn layering organisation seen in *Reeler* mutants does have another important functional consequence: *Reeler* mutants have abnormal nociceptive responses (Wang et al. 2012), although in this case it is somewhat difficult to unequivocally exclude the contribution to this effect of inappropriately-developed rostral regions of the nervous system.

The specification of LMC soma position by transcription factors expressed in specific subpopulations of spinal motor neurons raised the question of whether these effects might be mediated by the Reelin pathway. Indeed, Reelin is expressed in the ventral spinal cord, and its receptors as well as the signalling intermediate Dab1 are expressed by LMC neurons (Palmesino et al. 2010). Moreover, *Reelin* and *Dab1* mutants display LMC soma position defects: while LMC cell bodies are found in their appropriate general ventrolateral location in the spinal cord, the lateral LMC and medial LMC neuron positions are inverted along the mediolateral axis. One intriguing explanation for this phenotype is that Dab1 is expressed differentially in these neurons, with lateral LMC neurons having higher levels of the protein, compared to medial LMC. The model that emerged from additional gain and loss of function experiments is that Dab1 expression gates the LMC neurons' sensitivity to Reelin, such that lateral LMC neurons display high Dab1 levels and thus are sensitive to Reelin located close to the ventricular zone. In contrast, medial LMC neurons display lower levels of Dab1, and thus are relatively insensitive to Reelin. This model, which is further supported by Reelin overexpression experiments (E. Palmesino and A. Kania, unpublished), posits that Reelin is not an absolute migration stop signal (D'Arcangelo et al. 1995; Ogawa et al. 1995), but rather a repulsive or migration promoting cue for LMC motor neurons, an idea in line with previous experiments in which ectopic overexpression of Reelin rescues cortical migration defects (Magdaleno et al. 2002).

The differential Dab1 expression in medial and lateral LMC neurons suggested an intriguing possibility that these levels are controlled by the same transcription factors that control LMC soma position. Indeed, Foxp1 and Lim1 gain or loss of function result in, respectively, increase or decrease of Dab1 expression in LMC neurons, and are the first example of the control of Reelin

pathway protein expression by specific transcription factors (Palmesino et al. 2010). Moreover, since the same transcription factors also control LMC axon trajectory in the limb, these observations suggest a molecular hierarchy where specific transcription factors co-ordinately control both cell body position and axon trajectory, and thus establish myotopic organisation. At this point, it is unclear whether this idea can be extended to cortical or cerebellar neurons, two populations in which Reelin signaling and migration as well as molecular differentiation have been studied.

5.2 Cadherin Signaling

This large family of proteins has been associated with many types of adhesive cell-cell interactions (Nollet et al. 2000). Within the nervous system, cadherins are selectively expressed in a combinatorial manner and thus, can be used to subdivide many neuronal populations (Suzuki et al. 1997). This property, combined with their adhesive function, endows them with the ability to control neuronal soma position, as in the case of hindbrain motor neurons (Garel et al. 2000). In the context of spinal motor neurons, type II cadherin expression is used to delineate specific motor pools, suggesting a possible role in motor pool sorting. Indeed, gain and loss of function experiments in this system, where the expression of a single cadherin protein is altered, result in inappropriate clustering of motor neurons such that motor neurons from one transcription factor-defined motor pool are mixed with those from another pool (Price et al. 2002). Additional experiments argue that cadherin-mediated control of LMC neuron migration occurs through catenins, cytoplasmic effectors that link the membrane-bound cadherins to the underlying cytoskeleton (Bello et al. 2012; Price 2012). Extending this idea further, Jessell and his colleagues used mouse genetics to block catenin function and cadherin signalling in spinal motor neurons (Demireva et al. 2011). In such mice, the LMC neuron pool and division positions, as well as those of PG neurons are scrambled, while molecular identity is maintained. Moreover, the limb axonal trajectory of such motor neurons appears normal, providing further evidence that motor neuron soma position and axon trajectory can be uncoupled through the inactivation of specific molecular effectors. Conversely, in such experiments, the link between transcriptional identity and axon trajectory is maintained.

One outstanding question raised by these experiments is how cadherins are acting to selectively position spinal motor neuron cell bodies. In order for the selective adhesion mediated by combinations of cadherins to exert its effect on soma position and thus to allow for the different combinations of cadherins to interact with each other, motor neuron cell bodies need to have an opportunity to interact with one another. For this to happen, cell bodies of motor neurons could be mobilised randomly, like particles exhibiting Brownian motion, or in a more directed fashion, perhaps away from a source of a repellent, such as Reelin. Thus the relative contribution of Reelin signaling and cadherin-mediated adhesion in the specification of motor neuron position becomes an important question. The LMC soma positioning defects in catenin inactivation mutants are apparently more severe than those found in *Reelin* and *Dab1* mutants. It might be that in order for Reelin signaling to occur, close cell-cell contacts need to form, something that could be precluded by inhibition of cadherin signalling. Thus, Reelin signalling in spinal motor neurons, might be dependent on cadherin function, and it will be interesting to evaluate in the future if in motor neurons the intersection of Reelin and cadherin signaling occurs in a mechanistically similar manner like in cortical projection neurons (see also Chap. 1).

5.3 Eph Signaling

The Eph receptor tyrosine kinases and their ephrin ligands are also important protein families that are expressed in many developing tissues, including the nervous system. They have been implicated in cell sorting, where cells that are initially intermingled become sorted into two separate compartments and thus form a boundary

(Klein 2012). One seminal observation has been that of the function of Eph receptors in the compartmentalisation of hindbrain, where molecularly distinct neurons in adjacent rhombomeres form a sharp boundary (Xu et al. 1999). Unlike cadherins, there is ample evidence of the involvement of Eph receptors and their ligands in motor axon trajectory selection (Kao et al. 2012). Thus, ephrin-Eph signaling is well positioned to influence both motor neuron soma position and axon trajectory, coordinating their myotopic organisation and raising the question whether Eph signalling is involved in spinal motor neuron migration. In mice lacking EphA, a receptor important for the correct targeting of lateral LMC axons to dorsal limb muscles, the tibialis motor neuron pool is displaced within the lumbar spinal cord, arguing that this receptor is important for the normal positioning of spinal motor neuron cell bodies (Coonan et al. 2003). Interestingly, in these animals, the tibialis motor neurons still innervate their correct muscle targets, suggesting that Eph receptors can specify LMC soma position independently of axonal trajectory. This is particularly interesting given the expression patterns of ephrins and Eph receptor proteins at the level of spinal motor neuron cell bodies, and is very suggestive of their restriction to specific motor pools (Iwamasa et al. 1999).

5.4 Other Cell Surface Effectors

Although at this point there is no evidence of their function in neuron soma migration, semaphorins and their transmembrane receptors neuropilins have elaborate expression patterns at the level of hindbrain and spinal motor neuron somata (Cohen et al. 2005; Huber et al. 2005). There is accumulating evidence that these families of proteins function in the specification of motor axon trajectory in the limb (Huber et al. 2005; Moret et al. 2007; Huettl et al. 2011; Sanyas et al. 2012). Interestingly, since semaphoring-neuropilin signaling has been previously implicated in cortical neuron migration (Chen et al. 2008), it would not be at all surprising if such signaling also impacts spinal motor neuron soma localisation, although so far no evidence of this function has been reported.

6 Organisation of Spinal Motor Neurons

The above experiments point to a clear conclusion that started to emerge following cellular level studies: spinal motor neuron somata are positioned in a stereotyped manner that is highly correlated with their axonal trajectory, and thus muscle target, as well as their dendritic arborisation. The stereotypy implied that specific molecular pathways position these neurons, as outlined in the preceding section. Moreover, cellular anatomical and structural features that are linked to soma position have a substantial impact on the function of a motor neuron, highlighting the critical nature of soma position specification. The impact of axon trajectory on function is evident: the specificity of synaptic connections is a hallmark of essentially any nervous system, thus, in order to achieve efficient locomotor behavior, motor neurons must be connected to their appropriate muscle targets (Fig. 8.2).

The significance of the location of the cell body of a motor neuron has only recently been addressed in relation to its function. One obvious impact this might have is at the level of sensory-motor connectivity: sensory neurons residing in the dorsal root ganglion innervate specific muscles in the periphery, and transmit information about their proprioceptive properties directly to motor neurons innervating that particular muscle (Eccles et al. 1957). Thus, if motor neuron cell bodies are found in variable positions within the ventral horn of the spinal cord, the specificity of sensory-motor connectivity might be compromised. Indeed, emerging evidence supports this idea: in mice whose spinal motor neuron position is scrambled due to *Foxp1* mutation, proprioceptive sensory axons terminate in stereotyped ventral horn positions, even if their normal motor neuron targets are not found there (Surmeli et al. 2011). This should presumably have a devastating consequence on locomotion, and reveals the

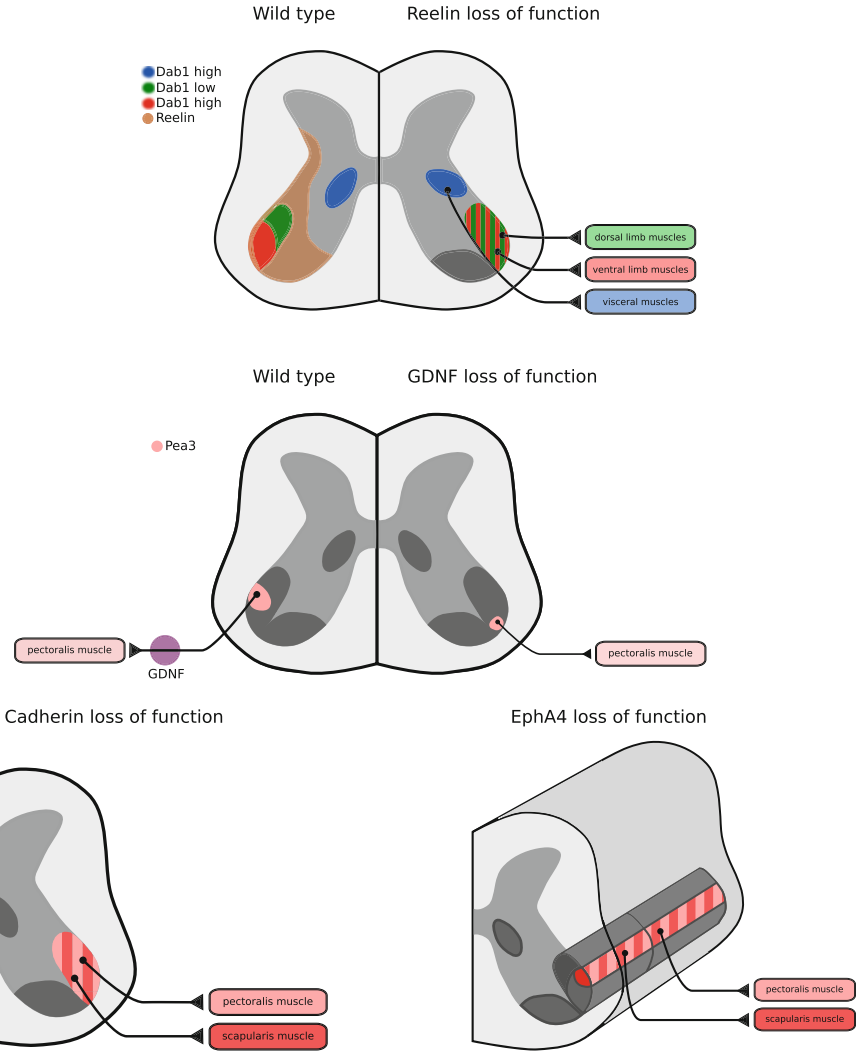

Fig. 8.2 Cell surface effectors of spinal motor neuron migration. Summary of expression of Reelin signaling pathway components, and consequence of Reelin, GDNF, Cadherin and EphA4 loss of function on spinal motor neuron soma localisation

existence of a sensory axon targeting mechanism that operates independently of soma position. These experiments raise a question of the consequence of molecular pathway manipulations, particularly cadherin or Reelin blockade, on sensory-motor connectivity and locomotor function.

Moreover, what about the relationship of dendritic organisation of motor neurons and their spinal cord position? The dendritic arbors of spinal motor neurons are quite complex, enabling them to integrate information arriving not only from direct proprioceptive sensory inputs, but also from spinal interneurons modulating locomotor behaviours or other sensory inputs such as nociception (Brown 1981; Rall et al. 1967). Analyses of mice mutant for the ETS transcription factor Pea3, expressed in selected spinal neuron motor pools (Lin et al. 1998), reveal that in addition to defects in pool position, the dendritic arbor of these motor neurons is also affected (Vrieseling and Arber 2006). The consequence of these changes on locomotor behaviour are

unclear, however, the sensory-motor connectivity in these mutants is compromised. Given that spinal motor neurons whose cell bodies are misplaced due to cadherin signalling defects have aberrant dendritic arbors (Demireva et al. 2011), and that blocking the Reelin signal results in abnormal dendritic morphologies (Senturk et al. 2011; Rice et al. 2001), it is plausible that cell body position could potentially impact how spinal motor neurons receive and integrate the signals normally passing through axon-dendritic synapses. Another recent finding that potentially impacts how we view motor neuron soma position, functional connectivity and dendritic arborisation is the observation that pre-motor interneurons that directly synapse onto spinal motor neurons are organised in a spatially segregated manner that mirrors that of motor neuron divisions (Tripodi et al. 2011).

Another important consequence of soma position on motor neuron development and function could be at the level of their electrical coupling. There is ample evidence that in developing spinal motor neurons, connexin proteins function to maintain electrical synapses that are important for the synchronisation of electrical activity within motor pools (Fulton et al. 1980; Chang et al. 1999). Disruption of gap junctions results in decreased synchronous neuronal activity and precocious neuromuscular synapse elimination, highlighting the importance of such coupling (Personius et al. 2007). Thus, one of the consequences of inappropriate positioning of motor neuron cell bodies would be the disruption of the gap junctions that couple all motor neurons innervating a specific muscle, leading to the aberrant innervation of target muscles. Recording of neuronal activity patterns in spinal cords with disrupted myotopic organisation should reveal whether this prediction holds true.

7 Conclusion

The evidence for the existence of topographic organisation within the nervous system is overwhelming. Pioneering cellular and anatomical studies first brought to the fore this idea with specific examples from the visual system, motor and sensory cortex and the spinal cord. The concept promoted by these observations is that neuronal position is predictive of axonal trajectory, implying that the two are being controlled coordinately. As the molecular logic underlying this coordination is unravelled, we are provided with a toolkit that can be used to finely manipulate specific neuronal populations and can begin to address the functional significance of organisation within the nervous system.

Acknowledgements The author thanks Allison Goodwin-Wilson for comments on the manuscript and figure assembly, and the EJLB Foundation, The SickKids Foundation and the Canadian Institutes of Health Research, whose support enabled his study of the emergence of topographic organization in the nervous system.

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Extracellular Signals Controlling Neuroblast Migration in the Postnatal Brain

9

Giovanna Lalli

Abstract

The most prominent example of long-distance migration in the postnatal brain is the rostral migratory stream (RMS) formed by neuroblasts originating in the subventricular zone (SVZ), one of the main neurogenic niches. Stem cell-derived neuroblasts leave the SVZ and migrate rostrally towards the olfactory bulb (OB), where they ultimately differentiate into inhibitory interneurons. This migration is essential for the proper integration of new neurons into the synaptic network and for the regulation of synaptic plasticity and olfactory memory. SVZ-derived postnatal neuroblasts undergo tangential migration independent of radial glia. They slide along each other in chains, which become progressively encased by an astrocytic tunnel throughout adulthood, while keeping in close contact with surrounding blood vessels. Once in the OB, neuroblasts switch to radial migration before differentiating. While the existence of an RMS is still controversial in the adult human brain, prominent migration of SVZ-derived neuroblasts towards the OB is observed in human infants, where it may play an important role in plasticity in a crucial period for the formation of synaptic networks. Moreover, SVZ neuroblasts are able to deviate from their migratory path to reach areas of injury and neurodegeneration. Identifying the extracellular factors and the intracellular mechanisms regulating neuroblast migration can therefore not only clarify a fundamental aspect of postnatal neurogenesis, but can also become relevant for therapeutic strategies exploiting the recruitment of endogenous stem cell-derived neural progenitors. This chapter presents an overview of the wide range of extracellular factors guiding neuroblast migration that have emerged over the last two decades.

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L. Nguyen and S. Hippenmeyer (eds.), *Cellular and Molecular Control of Neuronal Migration*,
Advances in Experimental Medicine and Biology 800, DOI 10.1007/978-94-007-7687-6_9,

Keywords
Neuroblast migration • Rostral migratory stream • Postnatal neurogenesis • Subventricular zone • Olfactory bulb

1 Introduction

The subventricular zone (SVZ) lining the lateral ventricles and the subgranular zone of the dentate gyrus of the hippocampus are the main neurogenic niches in the postnatal brain (Zhao et al. 2008). Here, stem cell-derived neural progenitors acquire the ability to migrate to their final destinations (either the granular zone of the hippocampus or the olfactory bulb – OB), where they will ultimately integrate into a pre-existing neuronal network. Increasing experimental evidence supports the crucial role of postnatal neurogenesis in maintaining functional plasticity associated with learning and memory in the hippocampus and with olfactory memory and processing in the OB (Lepousez et al. 2013; Lazarini and Lledo 2011; Zhao et al. 2008). Importantly, proper migration of neuronal progenitors is essential to determine their final fate and maturation as functional neurons (Belvindrah et al. 2011). This is especially evident along the so-called rostral migratory stream (RMS), made of chains of neuroblasts migrating from the SVZ towards the OB (Fig. 9.1a). The RMS displays the most substantial, long-distance neuronal migration in the mammalian postnatal brain. Slowly dividing neural stem cells in the SVZ ("B" cells) give rise to highly proliferative transient amplifying progenitors ("C" cells), which in turn generate migratory neural progenitors (or neuroblasts, or "A" cells) (Doetsch et al. 1997) (Fig. 9.1b). These highly dynamic progenitors move rostrally towards the OB by sliding along each other in chains running tangentially (i.e. parallel) to the brain surface (Fig. 9.1c). Once in the core of the OB the chains disperse, allowing neuroblasts to migrate radially to their final destination, the glomerular and periglomerular cell layers of the OB. Within their target area, neuroblasts will finally differentiate into functional mature GABAergic interneurons by extending a short basal dendrite and a long, branched apical dendrite and by making dendrodendritic synapses with mitral and tufted cells integrating olfactory sensory inputs (Fig. 9.1d) (Lledo et al. 2006). These new neurons will participate in complex processes such as olfactory memory formation, odorant discrimination, and social interactions (Lazarini and Lledo 2011).

Many studies have conclusively proven the existence of neurogenesis in the human brain (Goritz and Frisen 2012; Spalding et al. 2013). Whereas the RMS is highly prominent in rodents, its presence in the adult human brain is still a subject of controversy (Curtis et al. 2007b; Sanai et al. 2007). A recent report monitoring levels of nuclear bomb test-derived ^{14}C in genomic DNA has shown very limited olfactory bulb neurogenesis in the adult human brain (Bergmann et al. 2012). However, a prominent RMS is visible in the human infant brain up to 18–20 months after birth (Sanai et al. 2011). The extensive migration of neural progenitors observed in this early postnatal stage could be linked to the need to maintain a high level of functional synaptic plasticity at this crucial stage of human development. Indeed, it has been suggested that this migration may also be the target of a number of neurological conditions developing in early infancy (Sanai et al. 2011).

Interestingly, several rodent and human studies have shown that SVZ-derived neuroblasts have the ability to be re-routed to sites affected by injury, trauma, stroke or neurodegeneration (Curtis et al. 2007a; Sundholm-Peters et al. 2005; Arvidsson et al. 2002; Ohab et al. 2006). Studying neuroblast motility may therefore be therapeutically relevant not only for devising endogenous stem cell-based repair strategies but also to promote recruitment of transplanted progenitors in neuroregenerative approaches. Finally, many factors and intracellular regulators of neuroblast

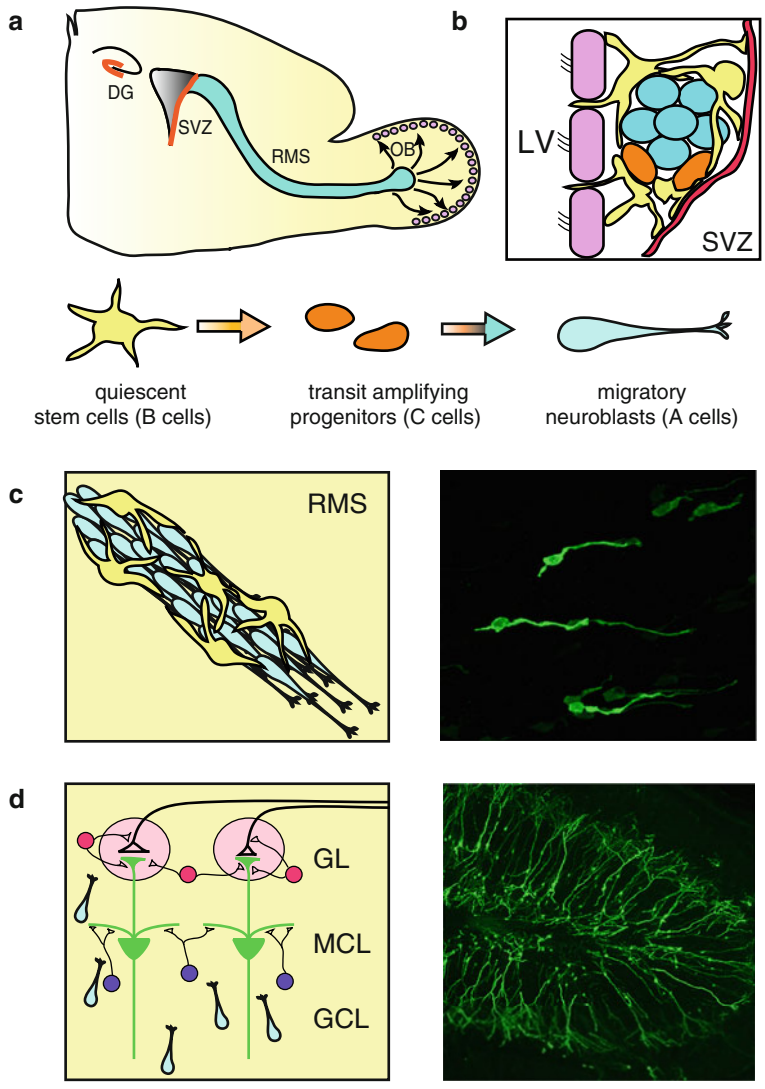

Fig. 9.1 Neurogenesis in the SVZ. (**a**) Schematic brain sagittal section showing the two main neurogenic niches in the postnatal brain, the dentate gyrus of the hippocampus and the SVZ (*orange*). SVZ-derived migrating progenitors (*light blue*) move tangentially along the RMS towards the OB, where they migrate radially and differentiate into inhibitory interneurons. (**b**) Anatomy of the SVZ. Ependymal ciliated cells (*purple*) line the wall of the lateral ventricle, and are in contact with quiescent stem cells ("B" cells, *yellow*), which in turn give rise to transit amplifying cells ("C" cells, *orange*). The latter produce migratory neuroblasts ("A" cells, *light blue*). The SVZ niche is rich in blood vessels (*red*). (**c**) (*Left panel*) Migratory neuroblasts slide along each other in chains surrounded by an astrocytic network. (*Right panel*) Typical RMS migratory neuroblasts extend a long protrusion oriented towards the OB, as shown by GFP-labeling via *in vivo* postnatal electroporation. The OB is located to the right of the image. (**d**) (*Left panel*) Following radial migration in the OB, neuroblasts (*light blue*) differentiate into either granular cells (*purple*) making dendrodendritic synapses with mitral cells in the GCL (*green*) or into periglomerular cells (*dark pink*), making synapses with mitral cells and olfactory neurons in the GL. (*Right panel*) Fluorescent SVZ-derived inhibitory interneurons are visible in the OB 14 days after *in vivo* electroporation of a GFP-encoding plasmid in the SVZ. *DG* dentate gyrus, *OB* olfactory bulb, *RMS* rostral migratory stream, *SVZ* subventricular zone, *GCL* granule cell layer, *MCL* mitral cell layer, *GL* glomerular layer

migration may also influence pathological cell migration, as in the case of highly metastatic brain tumour cells. This chapter provides an overview of the main extracellular factors regulating RMS neuroblast migration in the postnatal brain.

2 The Migration of SVZ-Derived Neuroblasts

RMS neuroblasts display a very characteristic mode of migration, which does not rely on other cell types (like, for example, glial-guided or axon-guided migration in the developing brain) (Lois et al. 1996). Indeed, neuroblasts slide along each other in chains, which can be visualized by immunostaining for neuroblast markers like the polysialilated form of neural adhesion molecule (PSA-NCAM) (Fig. 9.7a). The integrity of the chains is also favoured by the presence of an astroglial "tunnel" encasing the RMS (Fig. 9.1c), which acts not only as a physical barrier but also as a signaling system contributing to efficient migration (see also below) (Bozoyan et al. 2012). In the intact brain, a complex balance of chemoattractant, chemorepellent and motogenic factors ensures the directed migration of RMS neuroblasts towards the OB, even though the presence of the OB is not absolutely necessary for the directed migration of neuroblasts (Kirschenbaum et al. 1999). The typical chain migration can be recapitulated *in vitro* by embedding RMS explants in a three-dimensional Matrigel matrix (Wichterle et al. 1997) (Fig. 9.7g), showing that neuroblasts have an intrinsic capacity to migrate even when they are isolated from their native environment.

Time-lapse imaging studies have highlighted distinct phases in neuroblast migration (Nam et al. 2007; Schaar and McConnell 2005) (see also Chaps. 1, 4, 6 and 7) (Fig. 9.2): (1) extension of a leading process in the direction of migration, followed by its stabilization via contacts with other cells and/or the extracellular matrix; (2) formation of a dilation in front of the nucleus, where membranous organelles and the centrosome are located, and where endocytic trafficking

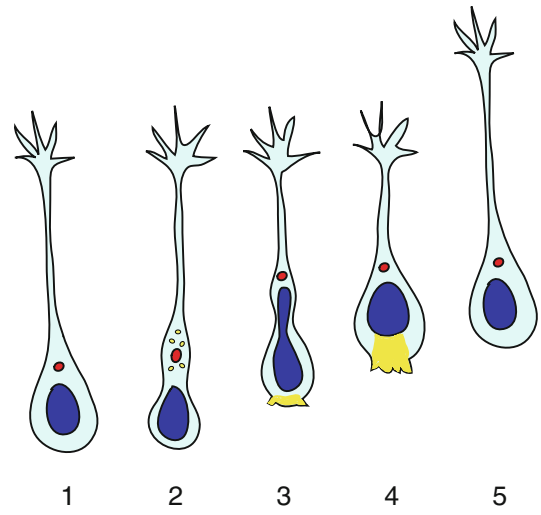

Fig. 9.2 Stereotypical phases of RMS neuroblast migration. *1* Neuroblasts extend a leading process tipped with a dynamic growth cone forming transient adhesions. *2* The process stabilizes by strengthening adhesions and a dilation forms in front of the nucleus containing trafficking vesicles (*yellow dots*) and the centrosome (*red dot*) connected to microtubules encasing the nucleus (not shown). *3–4* Endocytic events in the dilation weaken adhesion, while myosin II-mediated contraction (*yellow shading*) at the cell rear promotes nucleokinesis. *5* The cell starts the cycle again by extending a new leading process

weakens adhesion; (3) advancement of the nucleus in the dilation, helped by myosin II-mediated contraction at the cell rear (nucleokinesis step). Continuous repetition of this cycle results in the forward movement of neuroblasts.

Localization of the centrosome in front of the nucleus and the establishment of a protrusion in the direction of the OB are key events requiring the involvement of polarity regulators, such as the Partitioning defective (Par) proteins (Goldstein and Macara 2007). Among these, Par1/MARK2 is required for polarizing the neuroblast leading process, thus contributing to the directed migration towards the OB (Fig. 9.3). Depletion of MARK2 via shRNA disrupts migration directionality and results in poor integration of interneurons in the OB (Mejia-Gervacio et al. 2012). MARK2 may function by phosphorylating several microtubule-associated proteins such as tau, or MAP2/4 and doublecortin (DCX), a crucial molecule required for both stabilization of the leading process and

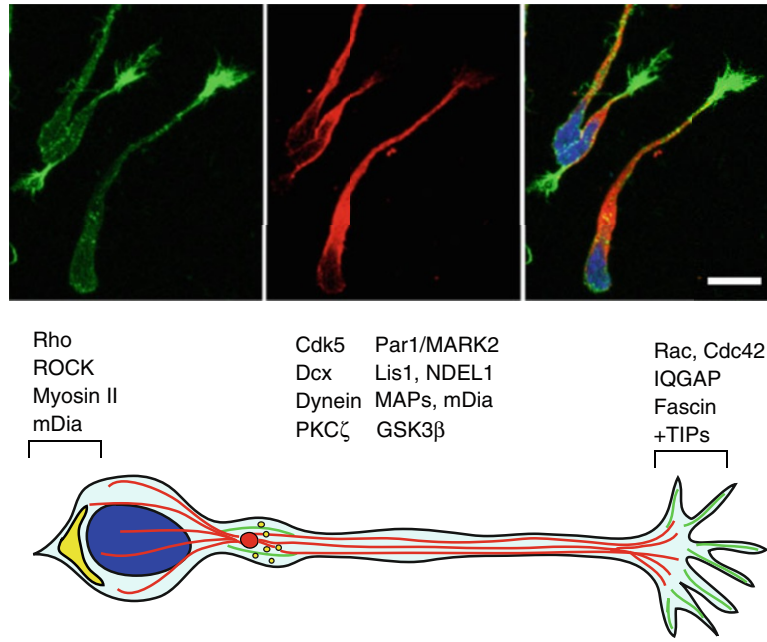

Fig. 9.3 Intracellular mechanisms regulating RMS neuroblast migration. (*Top*) Confocal images of postnatal rat migratory neuroblasts. Microtubules (visualized by βIII tubulin immunostaining, *red*) fill in the neuroblast leading processes, which are tipped with a growth cone-like structure enriched in actin filaments (visualized by fluorescent phalloidin, *green*). Cell nuclei are stained with Hoechst (*blue*). Scale bar, 10 μm. (*Bottom*) Schematic diagram showing important intracellular regulators of neuroblast migration. The microtubule cytoskeleton (*red lines*) is necessary for maintenance and growth of the leading process, whereas an intact actin cytoskeleton (*green*) is necessary for repolarization and centrosome reorientation, which is dependent on GSK3β and PKCζ activity. Par1/MARK2 phosphorylates MAPs and regulates the activity of other microtubule regulators, such as DCX and Cdk5. DCX may also promote coupling between nucleus and centrosome, together with the Lis1/Ndel1/dynein complex. Cdk5 contributes to the dynamic organization of the microtubule cytoskeleton by phosphorylating a number of substrates including Ndel1, DCX, and FAK. Centrosomally anchored microtubules may become captured in the leading process through interactions between the Rac1/Cdc42 effector IQGAP and plus-end MT tip proteins. Actomyosin-based contractility mediated by mDia and ROCK at the cell rear (*yellow*) promotes nucleokinesis. The actin-bundling protein fascin regulates actin bundling in peripheral filopodia, and may participate in controlling adhesion during migration. Localized clathrin-mediated endocytosis of adhesion components (*yellow dots*) occurs in the dilation forming ahead of the nucleus after extension of the leading process, weakening adhesion before nucleokinesis

nuclear translocation. Indeed, DCX suppresses secondary branching and promotes coupling between nucleus and centrosome during migration (Koizumi et al. 2006). The latter role may involve the coordination of DCX function with the dynein protein complex. Given that the centrosome acts as an anchor to the minus end of microtubules encasing the nucleus, dynein could promote translocation of the nucleus via its minus end-directed motor activity. Indeed, disruption of dynein or dynein-associated components such as Lis1 or Ndel1 impairs nucleus-centrosome coupling and ultimately affects neuronal migration (Shu et al. 2004; Tanaka et al. 2004) (see also Chaps. 1 and 7).

Par1/MARK2 may also regulate Cyclin-dependent kinase 5 (Cdk5), another crucial molecule regulating the tangential migration of neuroblasts along the RMS. Cdk5 deletion impairs chain formation, speed, directionality and leading process extension of SVZ-derived neuroblasts in a cell-autonomous manner (Hirota et al. 2007). This important kinase may contribute to the dynamic organization of the microtubule cytoskeleton by phosphorylating a number of substrates involved in neuronal migration,

such as Ndel1, Pak1, CRMP-2, DCX, and FAK (Hirota et al. 2007).

Also involved in the directed migration of neuroblasts is protein kinase C ζ (PKCζ), a member of the Par3-Par6-PKCζ complex, a master regulator of polarization in a variety of cellular contexts (McCaffrey and Macara 2009). Upon binding of active Cdc42 to Par6, activation of the polarity complex results in PKCζ-mediated phosphorylation and inhibition of glycogen synthase kinase 3β (GSK3β), ultimately affecting proper centrosome localization and microtubule stability. Indeed, both GSK3β and PKCζ inhibition block Slit-induced reorientation of the centrosome and process stabilization (Higginbotham et al. 2006), suggesting a conserved role for these molecules in the regulation of neuroblast polarization. Importantly, disruption of the actin but not microtubule cytoskeleton impaired centrosome movement within the cell body. This suggests that while an intact microtubule cytoskeleton is necessary for maintenance and growth of leading process, an intact actin cytoskeleton is necessary for repolarization and centrosome reorientation.

The centrosomally anchored microtubules may become captured at the edge of the leading process through interactions between the Rac1/Cdc42 effector IQGAP and plus-end MT tip proteins. Consistent with this model, IQGAP is required for neuroblast motility (Balenci et al. 2007). The essential role of tightly regulated actin dynamics for migration along the RMS is also emerging from studies in knockout mice lacking actin regulators, such as the Rho effectors mDia1 and mDia3 and the actin-bundling protein fascin. Both animal models have an abnormal RMS with a caudal accumulation of migratory neuroblasts and a smaller OB (Shinohara et al. 2012; Sonego et al. 2013). mDia is required for anterograde F-actin movement towards the leading process during nucleus-centrosome separation and for the formation of an "F-actin cup" at the cell rear during nuclear translocation, probably by generating actin filaments in actomyosin bundles for nuclear translocation. At the cell rear, activation of Rho-associated protein kinase ROCK downstream of Rho would lead to phosphorylation and activation of myosin light chain kinase (MLCK), thus promoting actomyosin-based contractility and nuclear movement over large distances (Shinohara et al. 2012), as shown in cortical interneurons (Godin et al. 2012). Instead, F-actin condensation ahead of the nucleus may ensure centrosomal movement before nuclear translocation. The same area displays an accumulation of fascin, an actin bundling protein also localized on peripheral filopodia of the leading process tip. Consistent with a role in regulating the directed motility of RMS neuroblasts, fascin depletion disrupts neuroblast morphology, causing ectopic branching and impairing migration both *in vitro* and *ex vivo* (Sonego et al. 2013). The ability of fascin to cycle on-off actin filaments via PKC-dependent phosphorylation on Ser39 is crucial for neuroblast migration, and may provide a molecular link between actin and adhesion dynamics (Anilkumar et al. 2003).

Together with cytoskeletal regulation, membrane trafficking is likely to play an important role in the directed migration of neuroblasts. Clathrin-mediated endocytic events frequently occur in the dilation forming ahead of the nucleus after extension of the leading process (Shieh et al. 2011). Localized endocytosis of adhesion components such as β1 integrins (see also below) or N-cadherin (Kawauchi et al. 2010) could weaken adhesions to facilitate forward translocation during neuronal migration. Blocking endocytosis by pharmacological inhibition or expression of either dominant negative dynamin or clathrin impairs migration by affecting the ability of the cell body to advance. From these experiments a model emerges where anchoring adhesions in the leading growth cone provide traction forces, a gradient of adhesive strength enables the cell rear to detach from the substrate during somal translocation, while endocytosis in the dilation weakens adhesive contacts and prepares the cell for the nuclear translocation into the dilation (Shieh et al. 2011). Recent studies on embryonic neuronal migration have started to highlight the importance of Rab GTPases in trafficking of cell adhesion molecules (Kawauchi et al. 2010), which could play similar roles also

in postnatal neuroblast migration. Exploring the function of these and other membrane traffic regulators will help us understand how localized signalling is achieved during the polarized migration of postnatal neuroblasts and how this can be coordinated with cytoskeletal and centrosome dynamics.

3 Factors Controlling RMS Neuroblast Migration

The directed migration of RMS neuroblasts relies on the complex combination of a variety of extracellular factors with chemorepellent, chemoattractant, and motogenic activities. In the last two decades in vitro migration assays using RMS explants and a variety of transgenic mouse models have been instrumental to identify several regulators of neuroblast migration, including cell adhesion/ECM and axon guidance molecules, growth factors, and neurotransmitters (Figs. 9.4, 9.5, 9.6 and 9.8). The following section describes the roles of key extracellular signals controlling the different phases of neuroblast migration, including detachment from the SVZ, tangential migration along the RMS, and the final switch to radial migration in the OB.

3.1 Adhesion Molecules/ECM Molecules

PSA-NCAM: A function for PSA-NCAM in the formation of neuroblast chains was initially suggested based on the high expression of this molecule by migrating neuroblasts (Rousselot et al. 1995). Indeed, migration along the RMS is hampered in mice lacking NCAM and PSA (Tomasiewicz et al. 1993; Cremer et al. 1994). Although chains are still visible in *NCAM*$^{-/-}$ mice, they appear disorganized and more sparse compared to wild-type animals (Chazal et al. 2000). However, migration may rely on PSA-NCAM more in the early postnatal RMS compared to later stages. PSA-NCAM may actually serve more as an important mediator in favouring the interaction of neural progenitors with their

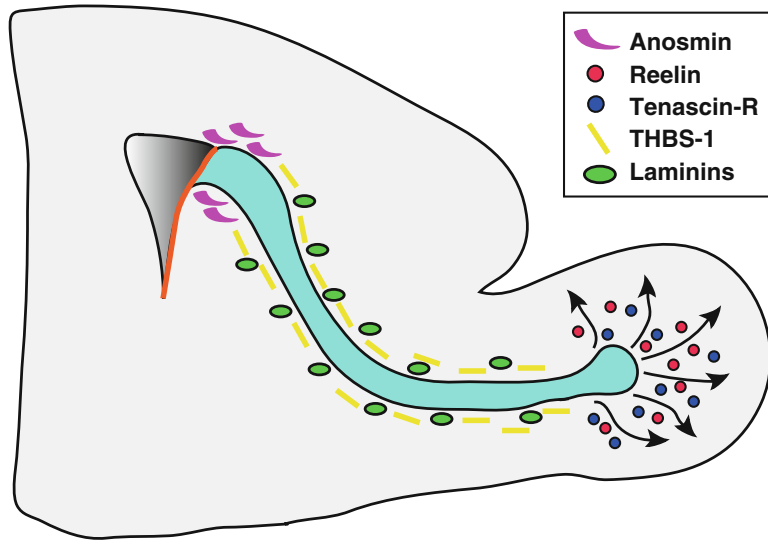

Fig. 9.4 Cell adhesion and ECM molecules involved in neuroblast migration. PSA-NCAM and various integrin subunits modulate neuroblast motility along the RMS. Integrins can interact with a number of laminins present along the RMS. The ECM glycoprotein anosmin secreted by neuroblasts may cooperate with FGFR1 signalling to promote detachment of neuroblasts from the SVZ. Secreted thrombospondin-1 (THBS-1) and Reelin act on the same receptors (ApoER2 and VLDLR) to modulate tangential and radial migration, respectively. Expression of the ECM component Tenascin-R is activity-dependent and controls chain dispersion in the OB

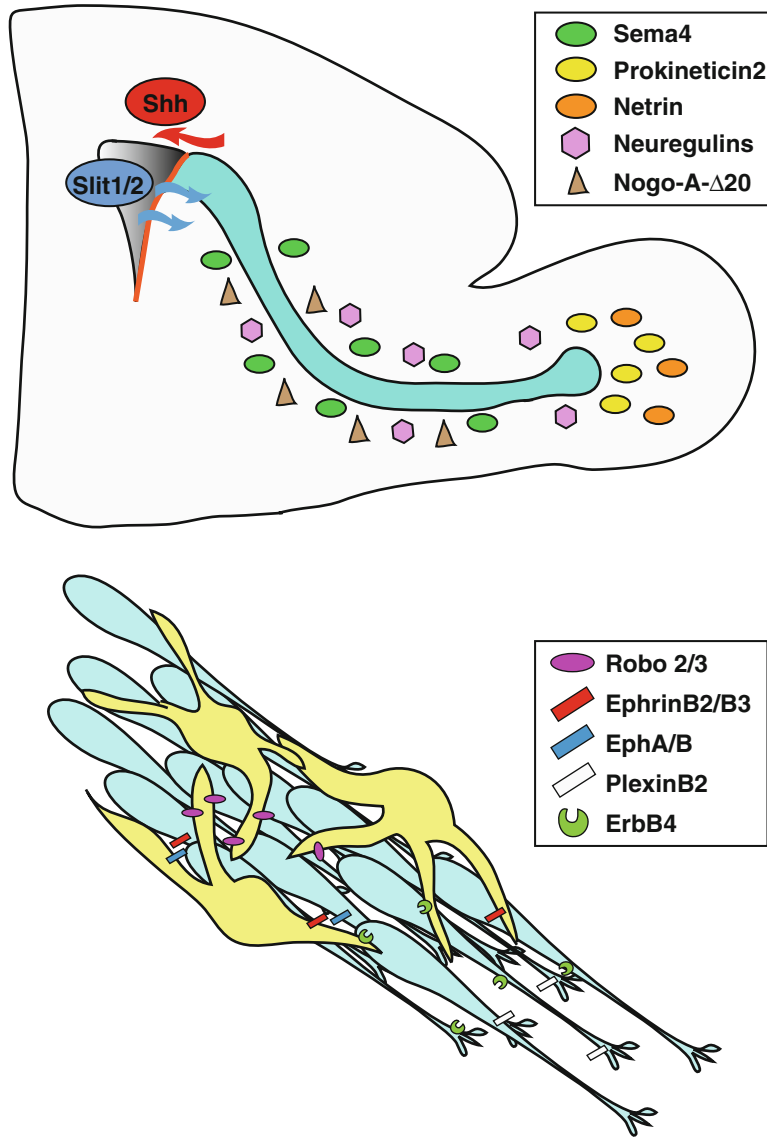

Fig. 9.5 Axon guidance molecules regulating neuroblast migration. (*Top*) Slits1/2 derived from the choroid plexus function as chemorepellents for RMS neuroblasts, while Sema4 acts on PlexinB2 receptors to restrict tangentially migrating neuroblasts to the RMS, forcing cells to move along the caudo-rostral axis. The Nogo-A-Δ20 domain may contribute to ensure proper adhesion/detachment cycles in neuroblasts along the RMS. Prokineticin 2 expressed in the OB acts as a chemoattractant for neuroblasts along the RMS and as a dissociation signal at the OB entry. Netrin secreted by mitral cells in the OB may promote the directed migration of neuroblasts expressing the DCC receptor. (*Bottom*) Axon guidance molecules also function in neuroblast-astrocyte cross-talk along the RMS. Neuroblasts secrete Slit-1 to remodel the surrounding glial tubes via Robo2/3 receptors present on astrocytes. Eph receptors in the RMS may act upon binding of EphrinB2/B3 ligands present on astrocytes to regulate chain maintenance. NRG/ErbB4 signalling regulates neuroblast-astrocyte interaction, chain organization and both tangential and radial migration

environment, rather than between migrating precursors themselves. Indeed, enzymatic removal of PSA by intraventricular injection of neuraminidase causes a major disorganization of the RMS, disruption of astrocyte/neuroblast alignment and most of all enhances dispersion of cells into neighbouring areas, like the striatum or the cortex (Battista and Rutishauser 2010). This is likely due

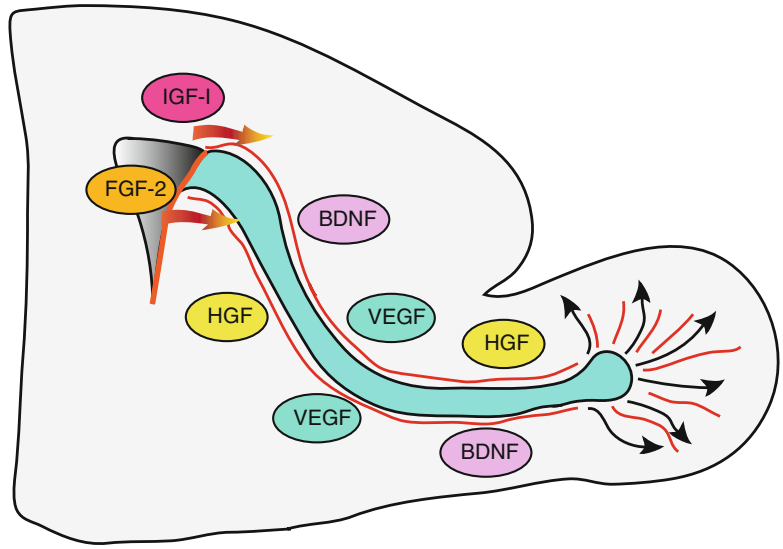

Fig. 9.6 Growth factors involved in neuroblast migration. IGF-I and FGF-2 promote the exit of neuroblasts from the SVZ while VEGF may favour tangential migration via VEGFR2. VEGF secreted by astrocytes may also indirectly regulate the motility of neuroblasts by remodeling blood vessels functioning as scaffold for migration. BDNF secreted by blood vessels promotes neuroblast motility via p75NTR, while HGF acts as a motogenic factor by binding the Met receptor present on neuroblasts. See text for details

not only to abnormalities in the astrocytic "tunnels" encasing the neuroblast chains, but also to the unveiling of some new, PSA-independent migratory properties coupled with NCAM-dependent triggering of neuroblast differentiation.

Integrins: Several studies have confirmed the presence of different integrins along the RMS, even though a thorough characterization of the range of integrins present at different ages is still lacking. For example, in neonatal mice α1 integrin subunit is expressed along the entire stream, but then is downregulated from early postnatal stages onwards (Murase and Horwitz 2002). In contrast, the β8 integrin subunit is expressed throughout development and adulthood and its genetic deletion causes severe defects in chain migration, with the appearance of disorganized neuroblast clusters and increased numbers of GFAP-positive astrocytes throughout the brain (Mobley and McCarty 2011). αv, β3 and β6 subunits are observed along the RMS from early postnatal/young adulthood stages onwards (Murase and Horwitz 2002). The presence of different integrin subunits in the RMS may reflect the diverse range of extracellular matrix (ECM) components surrounding the neuroblast chains. Indeed, integrins act as receptors for several laminins, which also display a specific distribution in the different cell types composing the SVZ niche (Kazanis et al. 2010). In the RMS, prominent expression of several α, β and γ laminin subunits can be detected, including α1, α2, and α4 (Belvindrah et al. 2007). While the requirement of many laminin subunits for neuroblast migration is difficult to test due to the early lethality of knockout mouse models (such as α1, β1 and γ1-deficient mice), α2/α4 double knockout animals could be examined thanks to their ability to survive to adulthood. These mice display a less compact RMS, consistent with the idea that laminin/integrin signaling ensures the formation of organized neuroblast chains. Since the α2 and α4 subunits are components of several laminin isoforms (laminin-2, -4, -12 for α2 and laminin-8 and -9 for α4, respectively) and given the lack of RMS defects in the single α2 and α4 knockouts, it is likely that several laminin isoforms perform redundant roles in the maintenance of neuroblast chains (Belvindrah et al. 2007).

Genetic deletion or blocking of β1 integrin disrupts RMS architecture, causing defects in the glial tubes surrounding neuroblast chains. As a consequence, ectopic migration is observed into the tissue surrounding the RMS and also the medial side of the lateral ventricle, an area usually almost completely lacking migratory neuroblasts (Kazanis et al. 2010). However, quite surprisingly cells lacking β1 are still able to extend dynamic protrusions and migrate towards the OB. The disruption in chain migration caused by blocking or genetically ablating β1 suggests that this subunit is specifically required to ensure proper cell-cell interactions during migration (Emsley and Hagg 2003; Belvindrah et al. 2007). In absence of β1 integrin, cells no longer form compact chains also *in vitro*, and this influences their mode of migration, which becomes more random and less directional. The crucial role of the β1 subunit suggests that several laminin-binding integrins expressed on neuroblasts (such as α1β1, α2β1, α3β1, α6β1, and α7β1) may cooperate with each other, with additional ECM and other secreted signaling molecules/surface receptors to ensure migration of neuroblasts in compact chains (Belvindrah et al. 2007). Interestingly, integrins can also control the activity of N-cadherin, a cell adhesion molecule highly expressed along the RMS and downregulated in the OB, where cells radially disperse (Yagita et al. 2009). In this regard, it is also worth pointing out that gangliosides such as ganglioside 9-O-acetyl GD3 (9acGD3) are markedly expressed in the RMS from embryonic until adult stages. This acetylated ganglioside displays a punctate distribution along the surface of cultured neuroblasts, which partially colocalizes with β1 integrin (Miyakoshi et al. 2012). Even though blocking 9acGD3 *in vitro* does not disrupt neuroblast chains, it decreases the speed of migration and leads to chain retraction, suggesting that 9acGD3 could regulate neuroblast motility by modulating integrin-dependent adhesion. Interestingly, the deacetylated form of GD3 can interact with Tenascin-R (Probstmeier et al. 1999), another ECM molecule regulating the radial migration of neuroblasts in the OB (see below), which highlights the potential ability of this glycolipid to mediate distinct modes of migration when interacting with different adhesion molecules present in the extracellular environment.

Galectin-3: Galectin-3 is a member of a family of 15 proteins identified by their similar carbohydrate recognition domains, with a conserved sequence of approximately 130 aminoacids able to bind galactoside residues. Among galectins, Galectin-3 can regulate cell-ECM interactions through laminin and integrins, but is also able to shuttle between the cytoplasm and the nucleus, where it regulates pre-mRNA splicing (Elola et al. 2007). Interestingly, Galectin-3 is expressed selectively in the RMS but not in the OB, suggesting a specific role in tangential migration. More detailed analysis showed that it can be found in GFAP$^+$ astrocytes and ependymal cells of the SVZ and in the astrocytes lining the RMS, but is excluded from PSA-NCAM$^+$ migratory neuroblasts. *Gal3* ko mice display distorted and thicker process of GFAP$^+$ cells, but chain integrity is not altered, even though the number of individually migrating cells appears increased. However, BrdU labeling experiments clearly showed a significantly impaired neuroblast migration, resulting in a marked decrease of neuron number in the OB (Comte et al. 2011). Galectin-3 may regulate neuroblast migration in several ways, given its ability to bind β-galactoside residues on Epidermal Growth Factor Receptor (EGFR), laminin, integrins, NCAM and tenascin, all involved in controlling neuroblast motility. First, the lack of Galectin-3 decreases and disrupts cilia, crucial structures present in the ependymal cells lining the wall of the ventricle. The correct orientation and beating of ependymal cilia ensures proper flow of cerebrospinal fluid (CSF), establishing gradients of chemorepellents in the SVZ regulating the directed migration of neuroblasts (Sawamoto et al. 2006), which may therefore be affected by the deletion of Galectin-3. Moreover, the disrupted astrocytic tunnels observed in *Gal3* ko mice are likely to have a profound effect on neuroblast migration, given that these structures play a fundamental role not only in physically constraining the migratory neuroblasts, but also in controlling the speed of

migration through important cell-cell interactions (see also below). Finally, Galectin-3 may also decrease the activation state of epidermal growth factor receptor (EGFR), which inhibits neuronal migration (Kim et al. 2009). It will be important to investigate the potentially multiple signaling pathways operating downstream of Galectin-3 in the control of neuroblast motility.

Thrombospondin-1 (THBS-1): Thrombospondins are secreted proteins involved in cell-cell and cell-matrix interactions in a variety of cellular contexts. Thrombospondin-1 (THBS-1) can interact with integrins, proteoglycans, and growth factors like PDGF and TGFβ (Lawler 2000), and is found in both the SVZ and the RMS. THBS-1 contributes to the stabilization of RMS chains by binding to apolipoprotein E receptor 2 (ApoER2) and very low-density lipoprotein receptor (VLDLR) present on migrating neuroblasts. Addition of THBS-1 to RMS explants increases chain length, while *THBS-1* ko mice exhibit higher number of individual migrating cells *in vitro*, a widened, less compact RMS, and a delayed migration to the OB *in vivo* (Blake et al. 2008). However, the RMS phenotype in *THBS-1* ko is milder than that observed in mice lacking both ApoER2 and VLDLR, where chain formation is completely absent (Andrade et al. 2007), suggesting that other extracellular factors may act on ApoER2 and VLDLR in the RMS. Remarkably, while ApoER2 and VLDLR maintain chains upon binding THBS-1 along the RMS, in the OB they promote chain dispersion and radial neuroblast migration downstream of Reelin (Herz and Chen 2006; Hack et al. 2002). Intriguingly, the interaction of ApoER2 and VLDLR with both THBS-1 and Reelin lead to a common initial event, the intracellular phosphorylation of the adaptor protein Disabled (Dab1). How the ApoER2 and VLDLR downstream signaling pathways diverge in mediating such different effects (tangential versus radial migration) is still unclear.

Reelin: Reelin is a large secreted glycoprotein essential for the development of laminated structures in the brain (Tissir and Goffinet 2003), and is best known for its role in promoting radial neuronal migration in the neocortex and in the hippocampus. In the postnatal brain, Reelin causes the detachment of RMS neuroblast chains, allowing proper radial migration within the OB (Hack et al. 2002). Consistent with this function, Reelin mutant animals (*Reeler* mice) display abnormal neuroblast accumulation at the end of the RMS, pointing to a severe radial migration defect resulting in the loss of the stereotypical layer organization of the OB and in an overall significant reduction of newly generated neurons in this structure (Hack et al. 2002; Kim et al. 2002). Reelin is highly expressed by mitral cells, the principal neuronal targets for the interneuron precursors that arrive via the RMS in the OB. Moreover, a descending Reelin protein gradient can be detected from the mitral cell layer (MCL) through the entire granule cell layer (GCL) as far as the RMS, which instead is negative for reelin. A recent study has proposed that reelin, which has a higher affinity for ApoER2 and VLDLR than THBS-1, might displace THBS-1 and other putative ligands from ApoER2 and VLDLR once neuroblasts start to enter the OB, resulting in the dissolution of chains and helping achieve correct positioning of cells in the OB layers (Blake et al. 2008). *In vitro* studies using neuroblast cultures suggest that reelin may induce neuroblast detachment via a Dab1/PI3K pathway leading to MAPK activation and ERK phosphorylation (Simo et al. 2007), but the functional relevance of these events still needs to be explored *in vivo*.

Tenascin-R: Among the members of the tenascin gene family is Tenascin-R, an ECM component containing a cysteine-rich amino terminal region, epidermal growth factor (EGF)-like domains, fibronectin type III homologous repeats and a domain homologous to fibrinogen (Jones and Jones 2000). This molecule promotes the detachment of neuroblasts from chains at the end of the RMS and their radial migration in the OB. Consistent with this function, Tenascin-R is detectable exclusively in the deep layers of the OB and around the rostral section of the RMS. *TNR* ko mice have normal RMS architecture and tangential migration, but display neuroblast accumulation at

the rostral RMS when cells enter the OB, indicating a failure in chain dispersion. Interestingly, expression of Tenascin-R is activity-dependent, as it is significantly reduced by odor deprivation (Saghatelyan et al. 2004). Tenascin-R therefore may act as an important signal linking network activity with recruitment of newborn neurons in the OB. The Tenascin-R receptors involved in this function remain to be identified. They could include contactin (or F3) (Pesheva et al. 1993), acetylated gangliosides like GD3 (Probstmeier et al. 1999), and receptor protein-tyrosine phosphatases belonging to the family of chondroitin sulfate proteoglycans (CSPGs) (Xiao et al. 1997). Another possible mode of function may involve the ability of Tenascin-R to capture and present extracellular factors promoting radial migration. In this regard, the possibility of cross-talk between Tenascin-R and the reelin signaling pathway, also involved in radial migration in the OB, remains to be investigated. Interestingly, grafting experiments of Tenascin-R-expressing cells showed that this molecule not only promotes the detachment of neuroblasts from chains, but is also sufficient to re-route tangentially migrating neuroblasts towards non-neurogenic areas (Saghatelyan et al. 2004), which could become an important aspect in therapies exploiting endogenous stem cell repair.

3.2 Axon Guidance Molecules

Slit/Robo: Slits are diffusible proteins involved in multiple aspects of neurodevelopment, such as axon guidance and neuronal migration (Ypsilanti et al. 2010). Two Slit members, Slit1 and Slit2 are expressed in the adult brain septum and choroid plexus and are sufficient to repel SVZ-derived neuroblasts *in vitro* (Hu 1999; Wu et al. 1999). Further studies using *Slit1* and *Slit2* ko mice identified the nature of the septum-derived chemorepulsive activity as a mixture of Slit1 and Slit2, while Slit2 is the choroid plexus-derived chemorepulsive factor (Nguyen-Ba-Charvet et al. 2004), demonstrating that both Slits play an important role in orienting the migration of SVZ-derived neuroblasts. However, Slits do not seem to act purely as chemorepellents. Indeed, Slit1 is also present in type C and A cells in the SVZ/RMS, while is absent from GFAP$^+$ astrocytes. In *Slit1* ko mice, neuroblasts leave the RMS prematurely to migrate dorsally and caudally to the SVZ, throughout the corpus callosum (Nguyen-Ba-Charvet et al. 2004). In the same study, genetic deletion of *Slit1* markedly increased the migration of neuroblasts out of SVZ explants, leading the authors to propose a cell-autonomous role for Slit in neuroblast migration. A second report more directly analyzed the dynamics of *Slit1* ko neuroblasts labeled with DiI in brain slices and found that they were 40 % slower than wild-type, consistent with a marked reduction in the proportion of cells able to reach the OB (Kaneko et al. 2010). *Slit1* ko neurons transplanted in a wild-type RMS showed significantly slower speed compared to Slit1-expressing wild-type cells, supporting a cell-autonomous role for Slit1 in neuroblast migration. In addition, Slit1-expressing wild-type cells displayed a slower and more irregular migration when transplanted in a Slit1-deficient RMS compared to a wild-type RMS, revealing also a non-autonomous function for Slit-1 in neuroblast migration. Interestingly, the Slit receptors Robo2 and Robo3 are present on both migrating neuroblasts and on the surrounding astrocytes in the RMS. Elegant experiments have shown that Slit1 secreted by the migrating neuroblasts "repels" and changes the morphology of the surrounding astrocytes via Robo signaling, thus contributing to the formation and maintenance of the astrocytic tunnels that ensure proper long-range migration (Kaneko et al. 2010).

Slit can also bind the secreted factor Netrin-1, which is present on migrating neuroblasts together with its receptor DCC (Murase and Horwitz 2002) (see below). In other contexts, Slit can modulate DCC-mediated responses by silencing Netrin-1 signalling, but it is still unclear whether such mode of action is preserved along the RMS. It is intriguing that while all PSA-NCAM$^+$ cells in the RMS express Slit1, radially migrating cells in the OB appear to downregulate Slit1 levels, suggesting that the switch from

tangential to radial migration may involve modulation of Slit signaling. The absence of Slit favours independent migration of neuroblasts, and a possible cross talk with other signaling molecules promoting the dispersion of chains in the OB such as reelin or Tenascin-R would be worth investigating.

Netrin-1/Dcc: Both Netrin and the netrin receptors neogenin and DCC are strongly expressed by migrating neuroblasts in the RMS during E15 until P5, a time of massive migration from the SVZ to the OB (Murase and Horwitz 2002). Moreover, mitral cells express high levels of netrin-1, at least in late embryonic and early postnatal stages, but downregulate netrin-1 after P5. This suggests that a chemogradient of netrin-1 secreted by the mitral cells may help guiding neuroblast migration at least in the early postnatal brain. Time-lapse imaging experiments in brain slices performed in the presence of a function-blocking antibody against the netrin receptor DCC show a loss of neuroblast directionality and a significant decrease in their speed, pointing to a role for the Netrin-DCC signaling system in regulating the directed migration of neuroblasts along the RMS. A more recent report has highlighted the presence of high levels of netrin-4 in some astrocytes located in the OB and along the border of the anterior RMS in adult mice (Staquicini et al. 2009). These netrin-4-producing astrocytes may have a role in restricting the migratory pathway, promoting the entry of neuroblasts into the OB. However, whether this is indeed the case or whether there may be an additional role for netrin-4 in controlling the proliferation of migratory neuroblasts remains to be investigated.

ErbB4/Neuregulin NRG1-NRG3: Neuregulins (NRGs) are multiple EGF-like domain-containing ligands, which act on receptor tyrosine kinases ErbB2, ErbB3 and ErbB4. NRGs are well-characterized regulators of synaptic plasticity (Buonanno 2010), oligodendrocyte/Schwann cell lineages (Garratt et al. 2000), and muscle spindle formation (Hippenmeyer et al. 2002). Moreover, they can also control glial-guided neuronal migration in the developing cortex and cerebellum (Anton et al. 1997; Rio et al. 1997). NRG1-3 are all expressed in the postnatal CNS and they activate ErbB4, which is selectively expressed at high levels in the SVZ and RMS, mainly in type A migratory neuroblasts, a subset of GFAP$^+$ astrocytes and type C proliferating progenitors (Anton et al. 2004). Conditional deletion of ErbB4 in nestin-expressing stem cells or in GFAP-expressing stem cells and astrocytes leads to similar phenotypes displaying fragmented neuroblast chains and an RMS with jagged boundaries. The close interaction between neuroblasts and ensheathing astrocytes along the RMS was disrupted when ErbB4 was deleted in GFAP-expressing cells, suggesting that this receptor is required in both neuroblasts and astrocytes to ensure proper formation and organization of the RMS *in vivo*. Live cell-imaging also revealed impaired orientation and decreased speed of neuroblast migration along the RMS in *Erbb4*$^{lox/-}$;*hGFAP-Cre* mice. The impaired migration in *Erbb4*$^{lox/-}$;*hGFAP-Cre* mice leads to disrupted organization of the OB, which displays a reduction in interneuron number, abnormal interneuron morphology and distribution and a tendency of cells to accumulate at the end of the RMS in the internal granular layer (Anton et al. 2004). Together, these observations indicate that lack of ErbB4 affects both tangential and radial migration. Immunolocalization analysis of the different NRGs suggests that NRG1 type III isoform (containing cysteine-rich/SMDF domains) is the predominant ErbB4 ligand acting in the RMS, where it is detectable especially in early postnatal stages. Indeed, NRG1 exhibits almost complete overlap with PSA-CAM$^+$ neuroblasts in the RMS (Ghashghaei et al. 2006). Consistent with this, *in vitro* stripe assays indicate that NRG1 type III on the cell surface may provide a permissive guidance substratum with a motogenic effect for ErbB4$^+$ migrating neuroblasts (Anton et al. 2004). Other NRGs such as NRG2 and NRG3 are mostly expressed in the OB, where they could influence the radial migration and final differentiation of neuroblasts. How ErbB4 signalling system can influence migration is still unclear, but possible mechanisms include its ability to cross-talk with integrin-linked pathways and to

regulate transcription following proteolysis and entry into the nucleus of its cytoplasmic tail.

Ephrin-B2/EphB2: The Eph family of receptor tyrosine kinases and their transmembrane-associated ephrins are divided into two subclasses, A and B, based on their binding specificities. During development Eph/ephrin signalling contributes to the regulation of a multiple events such as axon guidance and neural crest cell migration (Klein 2012). Eph receptors signal through their tyrosine kinase domain upon ligand binding (Hall and Lalli 2010). However, Ephrin-B ligands also transduce intracellular signals through their cytoplasmic tyrosine residues, thus allowing bidirectional signaling (Holland et al. 1996). EphB/EphA4 and ephrin-B bi-directional signaling favours cell-cell repulsion, for example to restrict cell intermingling during boundary formation. However, it may also mediate attraction in other contexts such as endothelial assembly in blood vessels (Yancopoulos et al. 1998). Ephrin B2 and B3 ligands are present on astrocytes in both the SVZ/RMS and OB. Infusion of truncated EphB2 and ephrin-B2 proteins into the lateral ventricle to perturb normal EphB/ephrin-B signaling increases cell proliferation in the SVZ and disrupts neuroblast chain migration (Conover et al. 2000). Since Ephrin ligands are present on astrocytes both in the SVZ and along the RMS, they may help to restrict the migratory path that also contains the ephrin receptors EphA4, B1 and B2. It is still unclear which of these receptors are actually expressed by the migratory neuroblasts in the RMS, and whether disruption of chain migration may be a secondary effect following the abnormal proliferation caused by disruption of ephrin signaling in the SVZ. However, the presence of both ephrin ligands and Eph receptors along the RMS migratory route suggests that this system plays an active role in regulating cell-cell signaling both during proliferation in the SVZ and in migration towards the OB. The molecular details of this event and the functional role of a potential Ephrin-B/EphB bidirectional signaling in this process remains to be clarified.

Semaphorin/Plexin-B2: Semaphorins are axon guidance molecules controlling neuronal migration in the developing nervous system (Renaud et al. 2008; Kerjan et al. 2005; Tran et al. 2007). They act by binding to Plexin receptors, which are divided into four subgroups (A-D) based on structural features. Type B Plexins are expressed in the postnatal CNS but their function is still largely unclear. A recent report analyzing *Plxnb2* ko mice highlighted an important role for this semaphorin receptor in postnatal neurogenesis. In the SVZ, Plexin-B2 is highly expressed in GFAP+ astrocytes and Mash1^{+} transit amplifying progenitors. In the RMS, Plexin-B2 is present in migrating neuroblasts but absent in the surrounding astrocytic tunnels. Interestingly, in the OB Plexin-B2 expression is not detectable in the radially migrating neuroblasts cells, while it is found at lower levels in all periglomerular cells, mitral cells and olfactory axons (Saha et al. 2012). The presence of the class IV Semaphorins (Sema4A, 4C, 4D, 4G) acting as ligands for Plexin-B2 along the RMS, granular cell and mitral cell layers suggests that a classical Semaphorin-Plexin signaling may provide a way to fine-tune neuroblast migration in "decision points" such as the RMS exit and the different layers of the OB. Indeed, besides disruption in proliferation, *Plxnb2* ko mice display ectopic neuroblast migration in non-neurogenic areas like the corpus callosum and septum. Moreover, *Plxnb2* ko neuroblasts move faster, are less directed and leave the RMS more rapidly once in the OB. Sema-Plexin-B2 interactions may therefore help neuroblasts to inhibit radial migration in the CNS as well as polarize the tangential migration along the RMS according to the so-called "surround repulsion" model (Keynes et al. 1997), forcing cells to move along the caudorostral axis. Plexin-B2 can also bind to receptor tyrosine kinases such as MET and RET and modulate their activation by their respective ligands, Hepatocyte Growth Factor (HGF) and Glial cell Derived Neurotrophic Factor (GDNF), two growth factors also involved in neuroblast migration (see below). However, Plexin-B2 does not seem to be necessary for HGF or GDNF-induced neuroblast migration, at least *in vitro* (Saha et al. 2012).

3.3 Other Factors

Sonic Hedgehog (Shh): The morphogen Shh has recently been implicated in the regulation of neuroblast migration. *In vivo* perturbation of Shh signaling significantly alters the amount of $BrdU^+$ nuclei found in the SVZ and OB, without altering the total amount of $BrdU^+$ cells. Specifically, overexpression of Shh led to an increase in $BrdU^+$ cells in the SVZ at the expense of the OB, while overexpression of the Shh inhibitor Hip led to opposite effects, suggesting an involvement of Shh signaling in neuroblast proliferation and migration (Angot et al. 2008). Indeed, RMS migratory neuroblasts express both the 12-pass transmembrane protein Patched (Ptc) and the G protein-coupled receptor Smoothened (Smo), which are the main mediators of Shh signaling. *In vitro* migration assays using RMS explants point to a chemoattractive role for Shh, confirmed by the fact that *in vivo* grafting of Shh-expressing cells attracts migrating neuroblasts away from the RMS (Angot et al. 2008). Since Shh is highly expressed in the SVZ and in the CSF, this morphogen may act as a molecular cue to control the exit of neuroblasts out of the SVZ, retaining them within the niche. It remains to be seen how Shh exerts this effect, whether classically through attenuation of Gli transcriptional repressors or through modulation of integrin/N-cadherin adhesion, as shown in neural crest cells and in the neuroepithelium (Jarov et al. 2003). Thus, Shh signaling is likely to contribute to the complex balance between attractive, repulsive and motogenic factors regulating the detachment of neuroblasts from the SVZ and the initiation of their migration in the RMS.

Nogo: A recent study has highlighted the contribution of Nogo signaling to the regulation of neuroblast migration. Nogo-A and its receptor NgR1 are well-known regulators of neurite growth and synaptic plasticity in the CNS (Pernet and Schwab 2012), but they have also been implicated in controlling neural stem cell proliferation (Li et al. 2011). Interestingly, while Nogo-A/NgR1 signalling reduces neural stem cell proliferation *in vivo*, the Nogo-A-Δ20 domain supports tangential migration in the RMS independent of NgR1, which is absent from neuroblasts. This effect appears to rely on the ability to affect cytoskeletal dynamics by stimulating a Rho-ROCK signaling cascade, which is likely to ultimately contribute to the balance of adhesion/detachment cycles during migration (Rolando et al. 2012). Consistent with this, *in vivo* infusion of a Nogo-A-Δ20 domain blocking antibody causes a marked accumulation of DCX+ neuroblasts in the SVZ, supporting a role for Nogo-A in promoting neuroblast mobilization via its Δ20 domain, facilitating their sliding onto each other. Interestingly, this Nogo-A effect is lost at the rostral end of the RMS, when cells switch from tangential migration to radial migration along vascular scaffolds, suggesting that Nogo-A-Δ20 may act by modulating homotypic interactions between neuroblasts along the RMS. The receptor(s) mediating this Nogo-A-Δ20 dependent effect are still unknown.

Prokineticin 2: Prokineticin (1 and 2) are secreted bioactive molecules acting via two closely related G-protein-coupled receptors (Prokr1 and Prokr2), and are involved in controlling gut motility, reproductive function and circadian output from the suprachiasmatic nuclei (Prosser et al. 2007). Both prokineticin receptor transcripts are highly expressed in the SVZ and RMS, and prokineticin 2 plays an important chemoattractant role for RMS neuroblasts (Ng et al. 2005). Prokr2 is highly expressed by RMS neuroblasts, but is also present on transit amplifying cells, suggesting it could play an additional role in regulating the transition from the transit amplifying "C" cell state to the migratory neuroblast "A" cell state (Puverel et al. 2009). $Prokr2^{Brdm1}$ mice (with a null prokineticin receptor 2) show severe defects in neuroblast migration, with substantial accumulation of cells in the RMS and a drastic decrease in the size of the OB, which also displays disrupted organization and failure in differentiation (Prosser et al. 2007). Both *Prok2* and *Prokr2* genes play an essential role also during the terminal steps of the migration of neuroblast chains in the RMS, since both $Prokr2^{-/-}$ and $Prok2^{-/-}$ mice display defective dissociation of chains at the exit of the RMS. These data,

together with the specific expression of *Prok2* in the OB (Ng et al. 2005) strongly support a role for prokineticin2 as a chemoattractant for SVZ-derived neuroblasts along the RMS and as a dissociation signal at the entry of the OB. Importantly, *Prokr1* ko mice retain a normal OB, supporting a specific role for *Prokr2* in neuroblast migration. Interestingly, the effects on the OB observed in *Prokr2* null mice are more severe compared to those caused by the lack of its ligand Prokineticin2, suggesting that the loss of *Prok2* is at least partially compensated *in vivo* by other mechanisms.

3.4 Neurotransmitters

SVZ and RMS neuroblasts are characterized by a typical "ionic electrophysiological signature" distinct from that observed in mature neurons. Indeed, they have high-input resistances, with an estimated mean resting potential of −59 mV. They also express Ca^{2+}- and voltage-dependent K^+ channels and delayed rectifying K^+ channels, but they lack inward K^+ currents and transient A-type outward K^+ currents. About 80 % of RMS neuroblasts express Na^+ channels at a lower density than K^+ channels (Wang et al. 2003). Expression of Na^+ channels by a higher percentage of neuroblasts in the RMS compared to the SVZ suggests that the presence of Na^+ channels is an early event characterizing the differentiation of neural progenitors to a migratory phenotype. However, although Na^+ channels participate to the generation of action potentials in mature neurons, their function in migrating neuroblasts is still unclear, since these cells are unable to generate action potentials.

Because of the high input resistance observed in neuroblasts, induction of small current flows due to local changes in extracellular signals can significantly modulate cell membrane potentials, which could contribute to influence cell behavior. These current flows may be associated with K^+ channel opening/closing or the activation of $GABA_A$ receptors, which are found in neuroblasts (Wang et al. 2003; Bolteus and Bordey 2004; Stewart et al. 2002).

GABA is the main inhibitory neurotransmitter in the brain acting through the activation of ionotropic ligand-gated $GABA_A$ or $GABA_C$ receptors and G-protein-coupled $GABA_B$ receptors. Application of GABA in acute brain slices from young and adult mice significantly reduces the migration speed of RMS neuroblasts, an effect mediated by $GABA_A$ receptors (Bolteus and Bordey 2004). In contrast, pharmacological blockade of $GABA_A$ receptor activity increased migration rate, suggesting the presence of an endogenous GABAergic tone controlling neuroblast migration. Following high K^+ application, neuroblasts release GABA, which can act in an autocrine-paracrine manner on their $GABA_A$ receptors and decrease migration speed by interfering with intracellular Ca^{2+} signaling independent of cell depolarization, most likely by affecting the release of Ca^{2+} from intracellular Ca^{2+} stores. Since GABA release is promoted by cell depolarization and GABA is able to depolarize neuroblasts, this would create a positive feedback loop causing further increase in extracellular GABA. Interestingly however, astrocytes surrounding the migratory neuroblasts are able to modulate GABA levels in the environment by controlling GABA uptake via the high-affinity GABA transporter subtype GAT4, which can be detected on the astrocytic processes ensheathing GABA-containing neuroblasts (Bolteus and Bordey 2004) (Fig. 9.8). Therefore, astrocytes help to maintain appropriate GABA levels in the microenvironment to ensure proper neuroblast motility.

Other neurotransmitter receptors progressively appear on neuroblasts during migration along the RMS, including the metabotropic glutamate receptor mGluR5, the Ca^{2+} permeable Glu_{K5} kainate receptor and NMDA receptors (Platel et al. 2008b, 2010). Also in this case astrocytes surrounding the RMS seem to play a major role by releasing glutamate in a Ca^{2+}–dependent manner to activate NMDA receptors on neuroblasts, thus providing an important *in vivo* survival cue for neuroblasts before their differentiation in the OB. However, NMDA activity does not seem to regulate neuroblast migration. Similarly, there is some evidence that mGluR5 promotes neuroblast survival, but has no effect on motility

(Platel et al. 2008a). In contrast, pharmacological inhibition of Glu_{K5} kainate receptor significantly promoted neuroblast migration in acute slices, suggesting that Glu_{K5} receptors are tonically activated in migrating neuroblasts to decrease their speed, somehow cooperating with the action of GABA (Platel et al. 2008b). Additional experiments examining neural progenitor proliferation support the idea that glutamate and GABA provide a homeostatic system regulating neuroblast production and migration along the RMS. How the functions of these two neurotransmitters are coordinated in migrating neuroblasts is still not understood, and is made more complex by the heterogeneous expression of $GABA_A$, Glu_{K5} and mGluR5 in these cells (Platel et al. 2008a; Young et al. 2011) and by the ability of growth factors and other extracellular signals to regulate expression of multiple neurotransmitter transporters and receptors.

3.5 Growth Factors

Insulin-like Growth Factor-I (IGF-I): Several growth factors are present in the SVZ/RMS/OB system and their actions interplay to achieve optimal neural progenitor proliferation, neuroblast migration and differentiation. *Igf-I*$^{-/-}$ mice display impaired radial migration in the OB, with lower amounts of neuroblasts found in the glomerular layer and a parallel increase in neuroblast density at the exit of the RMS (Hurtado-Chong et al. 2009). In addition, a significant accumulation of DCX^+ neuroblasts was observed in the SVZ, suggesting a defect in the ability of neuroblasts to leave the SVZ to initiate migration. Interestingly, while wild-type and *Igf-I*$^{-/-}$ neuroblasts migrate similarly *in vitro*, addition of IGF-I significantly stimulated migration of cells out of wild-type RMS explants, an effect that was blocked by PI3 kinase and Src kinase inhibitors. Based on these observations and on the fact that IGF-I is released by the choroid plexus of the lateral ventricle, IGF-I is likely to modulate neuroblast motility by influencing adhesion during migration. Besides promoting neuroblast exit from the SVZ, a second putative role for IGF in the OB would be to maintain adequate levels of phosphorylated Dab1, a scaffold protein operating downstream of Reelin to guarantee proper radial migration and positioning of differentiating interneurons.

Vascular Endothelial Growth Factor (VEGF): VEGF belongs to a family of glycoproteins playing a fundamental role in the development of blood vessels, angiogenesis and hematopoiesis. The VEGF family includes six different homologous factors (VEGF-A-E). VEGF-A binds to the receptor tyrosine kinases VEGF receptor-1 (VEGFR-1, or fms-related tyrosine kinase-1, Flt-1) and VEGF receptor-2 (VEGFR-2 or fetal liver kinase 1). A first study showed that SVZ-derived neural progenitors express both VEGFR-1 and -2 and that VEGF-A stimulated the migration of progenitors from SVZ explants by acting via VEGFR-2 (Zhang et al. 2003). A second report analyzing the phenotype of VEGFR-1 signaling deficient mice (*Flt-1TK*$^{-/-}$) revealed lower amounts of $DCX^+/BrdU^+$ cells in the RMS and a significant increase in the amount of $BrdU^+$ cells found in the outer layers of the OB, consistent with a greater supply of newly formed cells to the OB compared to wild-type mice. Deleting *Flt-1* specifically enhances the proliferation of neural progenitor cells in the SVZ, affects the cell type composition of the OB by promoting differentiation into dopaminergic olfactory neurons, but also promotes faster movement of neuroblasts, as indicated by *in vitro* migration assays and by a bigger OB size (Wittko et al. 2009). Interestingly, these effects are phenocopied by intracellular infusion of VEGF-A in wild-type mice, supporting the idea that deletion of VEGFR-1 signalling leads to increased amounts of VEGF-A protein in the SVZ/RMS, which in turn cause higher levels of phosphorylated VEGFR-2 in migratory neuroblasts. Intriguingly, VEGFR-2 phosphorylation is abolished when neuroblasts detach from the RMS and enter the OB, suggesting an important role for VEGF-A/VEGFR-2 in tangential migration. VEGF-A is expressed by glial cells in the SVZ and RMS, and in the same study VEGFR-1 also appears to be confined to GFAP+ astrocytes. While VEGF-A may have a paracrine role in regulating migration by acting on VEGFR-2

present in migratory neuroblasts, the potential roles of VEGF signaling on remodeling the astrocytic tunnels guiding neuroblasts towards the OB and the signaling pathways modulating VEGFR-2 activation at the RMS exit need further elucidation. In this regard, a recent study on the function of astrocyte-produced VEGF proposes that this factor may have an indirect role on neuroblast motility by modulating the vasculature used as a scaffold by migrating neuroblasts (Bozoyan et al. 2012). Therefore, a direct effect of VEGF on neuroblast migration along the RMS remains to be conclusively demonstrated.

Brain Derived Neurotrophic Factor (BDNF): BDNF is highly expressed along the SVZ/RMS/OB, even though both BDNF mRNA and protein seem to be present at higher level in the OB. TrkB is found in the RMS, particularly on astrocytes, while migratory neuroblasts express the low affinity BDNF receptor p75NTR at adult stages (Snapyan et al. 2009). Interestingly, infection with adenoviral BDNF or BDNF intraventricular infusion cause a substantial increase in the number of newly generated migratory neuroblasts in the RMS and OB (Zigova et al. 1998; Grade et al. 2013) while removal of BDNF with infusion of TrkB-Fc drastically decreases the number of $BrdU^+$ cells found in the OB (Snapyan et al. 2009). These observations support a major role for BDNF in neuroblast migration, which is further corroborated by the finding that BDNF haploinsufficient mice have a decreased number of $BrdU^+$ cells in the OB 28 days after BrdU injection (Bath et al. 2008). Blood vessels outlining the RMS have recently been identified as a source of BDNF (Snapyan et al. 2009) (see also below), which could then modulate neuroblast migration in a paracrine manner. Interestingly, neuroblasts appear to regulate local levels of BDNF via a complex interplay with the astroglial network and the vasculature scaffold (see below). Similar to other growth factors like Glial-Derived Neurotrophic Factor (GDNF), BDNF appears to have a motogenic effect on neuroblasts *in vitro*, since the number of migrating cells but not the actual migration distance is increased following exposure of RMS explants to BDNF (Chiaramello et al. 2007).

Hepatocyte Growth Factor (HGF): HGF is a pleiotropic factor regulating migration, morphogenesis and proliferation in a variety of developmental events (Birchmeier and Gherardi 1998). It binds to its tyrosine kinase receptor Met and is expressed in the brain both during development and in adulthood. HGF appears to be produced both in the SVZ and RMS, while high levels of the Met receptor are localized on $PSA\text{-}NCAM^+$ neuroblasts, but not on $GFAP^+$ astrocytes (Garzotto et al. 2008). Supplying HGF to RMS explants significantly promotes motility on larger number of cells, without affecting total migration distance. HGF therefore has a motogenic effect on neuroblasts, and classical Boyden chamber assays indicate that it may also have a chemoattractant effect, even though the actual distribution of this factor in the SVZ/RMS has not been characterized *in vivo*. Upon HGF binding, Met can activate a MAPK cascade directly through the Grb2 binding site (Ponzetto et al. 1996), ultimately leading to ERK phosphorylation. Importantly, mice carrying a point mutation in Met causing selective uncoupling from the Grb2 adaptor ($Met^{Grb2/Grb2}$) are impaired in their ability to trigger downstream Ras/MAPK signaling. Consistent with this, RMS explants derived from $Met^{Grb2/Grb2}$ mice have lower levels of phosphorylated ERK after exposure to HGF. This signaling cascade can promote neuroblast motility, since $Met^{Grb2/Grb2}$ RMS explants show reduced neuroblast migration. Even though other HGF-dependent intracellular pathways involving other adaptors could also control neuroblast motility, these experiments strongly suggest that a full HGF/Met signaling is required for proper migration along the RMS.

Glial cell Derived Neurotrophic Factor (GDNF): Members of the GDNF family bind to specific glycosyl phosphatidylinositol (GPI)-anchored co-receptors (GFRα1-α4) but signal together with the RET tyrosine kinase or NCAM transmembrane receptors. GDNF appears to be expressed all along the RMS and in the OB, similar to the GFRα1 receptor. Results from *in vitro* migration assays suggest that GDNF has a chemoattractant role on neuroblasts derived from both caudal and rostral RMS. This effect may

involve Cdk5, since incubation with GDNF enhances Cdk5 activity monitored by *in vitro* kinase assays using extracts from dissociated RMS cells. In addition, the chemoattractant effect of GDNF is abolished in presence of the Cdk5 inhibitor roscovitine. Interestingly, RMS explants from NCAM$^{-/-}$ mice fail to respond to the chemoattractant effect of GDNF *in vitro*, suggesting that NCAM could act as an alternative receptor for GDNF in the stream (Paratcha et al. 2006). Evidence of this role for GDNF *in vivo*, however, is still lacking.

Meteorin and Cometin: Recent reports have highlighted a function for two newly discovered secreted molecules defining a new family of neurotrophic factors, meteorin and cometin, as regulators of neuroblast migration. Meteorin is highly expressed by neural stem cells, astrocytes and discrete neuronal populations in the postnatal brain. This factor can regulate axonal extension, glial cell differentiation and angiogenesis, and has a chemokinetic effect on SVZ-derived neuroblasts, increasing the length of chains migrating out of SVZ explants (Wang et al. 2012). Moreover, a possible role for meteorin in regulating neuroblast migration following stroke has been proposed (see below). The receptor for meteorin and the downstream signaling pathways still need to be identified, even though there is some evidence that meteorin can act via the Jak-STAT3 cascade (Lee et al. 2010). Similarly, cometin also induces neurite outgrowth and appears to act via Jak-STAT3 and MEK-ERK signaling (Jorgensen et al. 2012). Incubation with cometin significantly stimulates chain migration out of SVZ explants. More detailed characterization of both cometin and meteorin activity is needed to confirm their role on neuroblast migration *in vivo*.

Epidermal Growth Factor (EGF): EGF controls a variety of processes during development, including proliferation and cell migration, by binding to its receptor (EGFR, also known as ErbB1) (Wells 1999). In the context of neurogenesis, EGF is required to drive proliferation in the SVZ via EGFR, which is expressed on stem cells and particularly on transit-amplifying progenitors (Doetsch et al. 1997, 2002). However, low levels of EGFR can also be detected on a subset of migratory PSA-NCAM^{+} and DCX^{+} neuroblasts, suggesting a role for EGF in controlling neuroblast motility. Indeed these EGFR^{low} cells exhibit slower and less directed movement than EGFR-negative neuroblasts, and perfusion of brain slices with TGF-α, an EGFR selective agonist, decreases the percentage of motile cells in the RMS (Kim et al. 2009). It is possible that the EGFR^{low} cells are recently born neuroblasts that have not yet acquired a fully mature migratory phenotype, and that EGF inhibits the motility of these cells. In contrast, infusion of EGF or TGF-α in pathological contexts stimulates the emigration of neuroblasts out of the RMS towards the adjacent septum and striatum (Craig et al. 1996; Doetsch et al. 2002). The precise molecular mechanisms underlying such different EGFR-dependent effects on neuroblast motility still need to be clarified.

Fibroblast Growth Factor (FGF): FGFs are members of a large family of structurally related polypeptides regulating growth and differentiation in a wide variety of structures of endodermal, mesodermal and ectodermal origin (Itoh and Ornitz 2004). In particular FGF-2 has a fundamental role in the niche, where it regulates proliferation, self-renewal and differentiation of neural precursors (Mason 2007). In early postnatal stages FGF-2 is present in the SVZ/RMS in a caudo-rostral gradient, while later it is predominantly found in the SVZ, suggesting a possible role in regulating neuroblast motility. Indeed, a recent study showed that FGF-2 acts as a motogenic factor for SVZ-derived embryonic and early postnatal neuroblasts without exerting a chemotropic effect (Garcia-Gonzalez et al. 2010). Based on pharmacological experiments, FGFR1 appears to be the main FGF receptor responsible for the FGF-2-dependent motogenic effect. The FGF-2 action may be modulated by anosmin, a cleavable extracellular matrix glycoprotein involved in axon guidance and neuronal migration during development (Soussi-Yanicostas et al. 2002; Cariboni et al. 2004), which can bind to FGFR1 or other ECM components to regulate adhesion and migration (Gonzalez-Martinez et al. 2004; Dode et al. 2003; Bribian et al. 2006). Like FGF-2, anosmin is localized in a rostro-caudal gradient along the

SVZ/RMS particularly in early postnatal stages. *In vitro* migration assays with SVZ explants have highlighted a potential chemotropic role for anosmin in neuroblast migration, which seems particularly dependent on FGFR1 activity at early postnatal stages and other FGFRs like FGFR2 and 3 during development (Garcia-Gonzalez et al. 2010). The emerging picture from these observations identifies FGF-2 and anosmin as potential motogenic cues regulating the early migration of SVZ-derived neural progenitors (from E14 to P15), particularly in the initial RMS. The presence of anosmin only in PSA-NCAM$^+$ neuroblasts suggests that these cells produce and secrete this glycoprotein, which could then act in a paracrine fashion on other neuroblasts. However, the molecular details of anosmin function and its potential additional interactions with other ECM components along the RMS remain to be elucidated.

3.6 Endocannabinoid Signaling

The endocannabinoid (eCB) system plays a fundamental role in the developing nervous system by regulating axon guidance, neuronal migration, and synaptic plasticity (Oudin et al. 2011b). To date, the lipid arachidonylethanolamide (also known as anandamide) and 2-arachidonoylglycerol (2-AG) are the best characterized candidate eCBs acting on the G protein-coupled seven-transmembrane-spanning cannabinoid receptors CB1 and CB2, widely distributed in the brain. Synthesis of 2-AG is achieved by two regulated enzymes, the diacylglycerol lipases (DAGLα and β), which use diacylglycerol (DAG) as a substrate (Reisenberg et al. 2012). DAGLs and CB receptor expression patterns are highly correlated in the brain during development and in adulthood, and the major cannabinoid responses require at least one of the DAGL enzymes, as shown by studies of *DAGL*α and β$^{-/-}$ mice (Gao et al. 2010). These observations strongly support the idea that DAGL and CB receptors cooperate to drive physiological eCB responses. Recent studies have highlighted the requirement for eCB signaling in postnatal neurogenesis (Goncalves et al. 2008; Aguado et al. 2006; Palazuelos et al. 2006; Molina-Holgado et al. 2007; Jin et al. 2004), further supported by the significant decrease in neural stem cell proliferation in both dentate gyrus and SVZ in *DAGL* ko mice (Gao et al. 2010). In addition, administration of a CB2 agonist stimulates SVZ neural progenitor proliferation *in vivo*, counteracting the decline in neurogenesis in older ages. As a result, CB receptor activation causes a substantial increase in the amount of new neurons found in the OB, while inhibition of CB receptors or DAGL has the opposite effect (Goncalves et al. 2008). Interestingly, DAGL and CB receptors are detected not only on actively proliferating progenitors, but also in their PSA-NCAM$^+$ migrating progeny, while they are not detected in the surrounding GFAP$^+$ astrocytes along the RMS (Fig. 9.7a–f). Stimulation of both CB1 and CB2 receptors has a motogenic and chemokinetic effect on RMS neuroblasts, which are able to migrate longer distances (Fig. 9.7g–h). Time-lapse imaging experiments show that CB agonists promote neuroblast motility by increasing process length and the frequency of efficient nucleokinesis steps (Oudin et al. 2011a). Importantly, specific pharmacological block of CB receptors is sufficient to significantly inhibit neuroblast migration out of RMS explants, supporting the existence of an endogenous endocannabinoid tone in RMS cultures. CB antagonist-treated neuroblasts lose their typical

Fig. 9.7 (continued) and JTE-907, respectively both at 1 μM). (**h**) A GFP-encoding plasmid was electroporated in P3 mouse pups to label migrating neuroblasts. Fourteen days later animals received a single i.p. administration of CB1 or CB2 antagonists (AM251 and JTE-907, respectively both at 5 mg/kg). Representative pictures of GFP-labelled migrating neuroblasts from electroporated animals. Neuroblasts from CB antagonist-treated animals (*centre and right panels*) display disrupted morphology, with shorter processes and increased branching compared to unipolar control cells (*left panel*). Scale bar: (a–b), 30 μm; (c–e), 10 μm; (f), 4 μm; (g), 200 μm; (h), 20 μm (Figure adapted from (Oudin et al. 2011a))

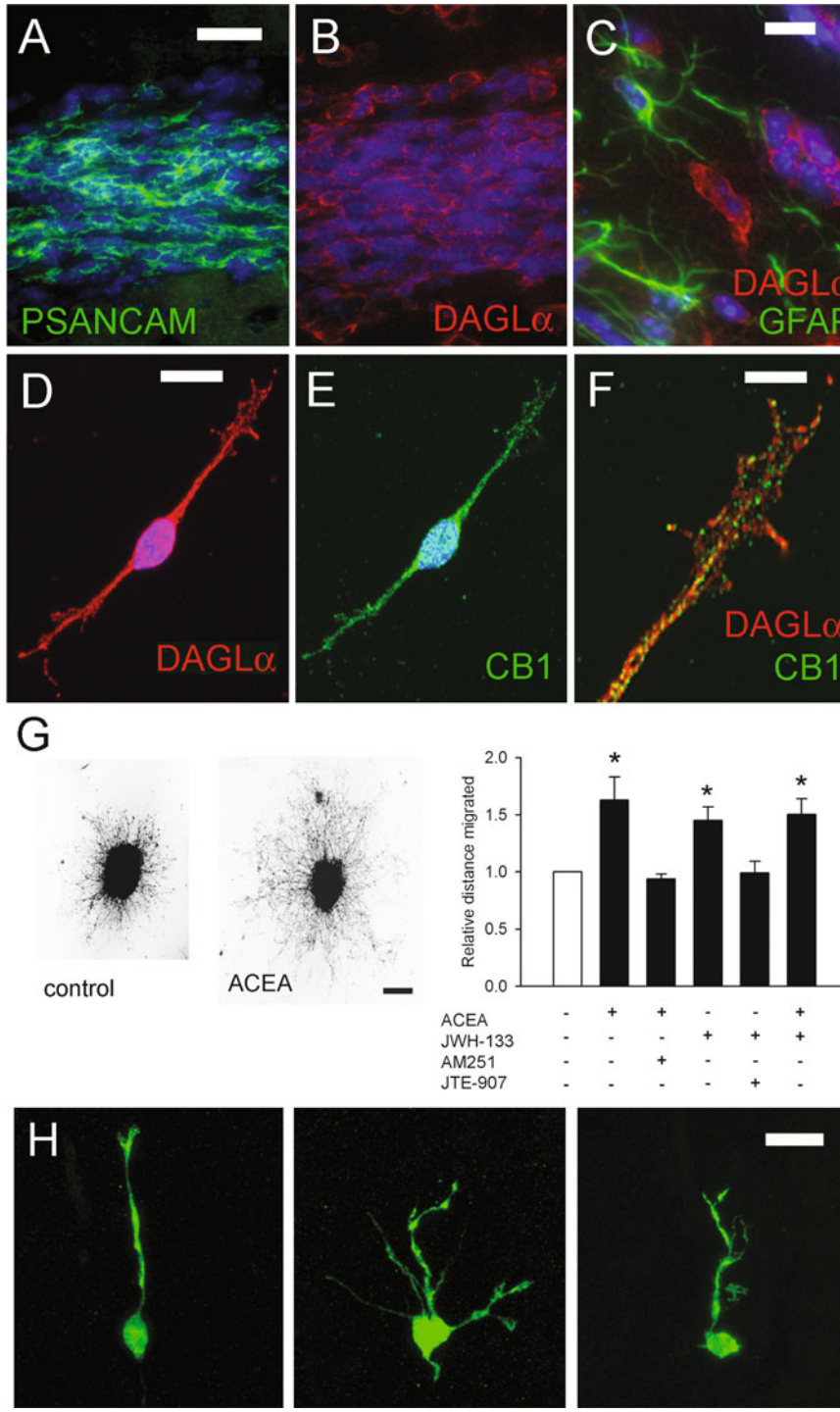

Fig. 9.7 Cannabinoid signaling regulates neuroblast migration. (**a**–**c**) The 2-AG synthesizing enzyme DAGLα is present in PSA-NCAM+ neuroblast chains but not in the surrounding GFAP+ astrocytes. (**d**–**f**) Both DAGLα and the CB1 receptor display a punctate distribution in migrating neuroblasts. (**g**) Activation of CB receptors by the CB1 agonist ACEA or CB2 agonist JWH-133 (both at 0.5 μM) significantly enhances the migration of mouse neuroblasts out of P7 RMS explants embedded in Matrigel. This effect is blocked by preincubation with CB1 and CB2 antagonists, (AM251

unipolar morphology and become highly branched, suggesting that eCB signaling is required to maintain their polarized morphology. These effects were confirmed *in vivo* by intraperitoneal administration of a single dose of different CB1 or CB2 antagonists, which markedly affected the morphology of RMS neuroblasts labeled by *in vivo* postnatal electroporation of GFP in the lateral ventricle (Fig. 9.7h). Endogenous CB signaling can therefore have several roles in postnatal neurogenesis, by regulating neural progenitor proliferation and neuroblast migration. Besides CB receptors and DAGL, neuroblasts also express monoacylglycerol lipase (MAGL), the enzyme responsible for 2-AG degradation, suggesting they are able to synthesize, respond and degrade 2-AG (Oudin et al. 2011a). This, together with the notion that 2-AG has a relatively short half-life (Rouzer et al. 2002), supports the idea of an autocrine cannabinoid signaling regulating neuroblast motility. It will be important to identify not only the factors driving DAGL activation and triggering CB signaling, but also the intracellular downstream players mediating the effects of eCB on neuroblast motility. Interestingly, CB1 agonists can promote axonal growth (Williams et al. 2003; Keimpema et al. 2010) and DAGL-dependent eCB signaling is required for neurite growth promoted by FGF-2 and a number of CAMs (Bisogno et al. 2003; Williams et al. 2003). PLCγ activation downstream of receptor tyrosine kinases like FGFR can lead to production of DAG, which in turn functions as a DAGL substrate for the synthesis of 2-AG. Similarly, CB receptors on neuroblasts may have the potential to cross-talk with a number of factors and cell adhesion molecules found along the RMS. In developing neurons, the CB-dependent axon growth response relies on N- and L- calcium channels (Williams et al. 2003). Whether this is true also for neuroblasts remains to be clarified.

Endogenous CB signaling ultimately influences cytoskeletal dynamics during neuroblast migration. One piece of evidence comes from recent Fluorescence Lifetime Imaging Microscopy (FLIM) imaging studies showing that altering CB signaling modulates the localization of fascin, an actin-bundling protein cycling on and off actin filaments depending on PKC-mediated phosphorylation (Sonego et al. 2013). By regulating the interaction between PKC and fascin, cannabinoid signaling may participate to the tight regulation of fascin localization, which can influence both actin cytoskeleton dynamics and adhesion in migrating neuroblasts. It will be important to investigate how CB signaling is regulated at a cellular level, and whether polarized recycling CB receptors or spatio-temporal regulation of DAGL activity play a role in controlling neuroblast motility.

4 The Influence of the SVZ-RMS Architecture on Neuroblast Migration

The complex architecture of the SVZ/RMS plays a major role in regulating neuroblast migration. The SVZ ependymal layer in contact with the lateral ventricle is polarized with oriented bundles of motile cilia protruding into the ventricle lumen (Mirzadeh et al. 2008) (Fig. 9.1b). The coordinated beating of these cilia ensures proper flow of CSF, which is secreted by the choroid plexus located in the caudal region of the lateral ventricle. The organized ciliar distribution helps to create gradients of factors guiding neuroblast migration along the SVZ network, such as member of the Slit family. Indeed, disrupting cilia structure as in the *Tg737*^{orpk} mutant mice impairs ependymal flow and disorients SVZ neuroblast migration (Sawamoto et al. 2006), proving that polarized ciliated cells play an important role in conveying directional information.

Chain formation of neuroblasts occurs late in postnatal development (Pencea and Luskin 2003; Peretto et al. 2005). Neuroblast chains become progressively ensheathed by a network of astrocytic processes forming “glial tubes”, resembling tunnel structures. Moreover, blood vessels become aligned in the direction of the RMS, supporting the migration of neuroblasts (Bozoyan et al. 2012). Recent studies have shed some light on how astrocytes and blood vessels modulate neuroblast motility, especially after the initial postnatal stages (Fig. 9.8).

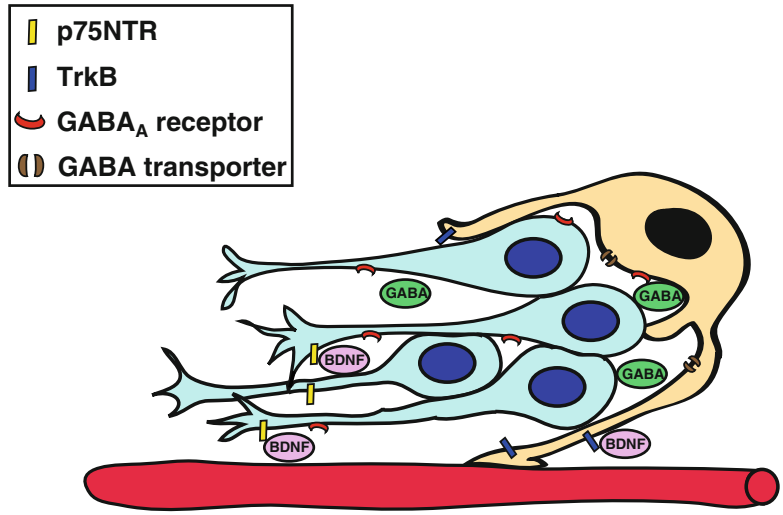

Fig. 9.8 Role of the RMS architecture in neuroblast migration. Neuroblasts (*light blue*) are in close proximity with blood vessels (*red*), which serve as a scaffold for their migration. Neighbouring astrocytes (*orange*) are positioned between neuroblasts and the endothelial cells of blood vessels, which synthesize and release BDNF to promote neuroblast migration via p75NTR. Activation of astrocytes by GABA released by neuroblasts promotes the insertion of TrkB receptors on the astrocyte membranes, which can trap BDNF, causing neuroblasts to pause. GABA can also be cleared by astrocytes via the high-affinity GABA transporter subtype GAT4, promoting neuroblast migration. Astrocytes can therefore influence neuroblast motility by modulating the amount of local BDNF and creating a microgradient of GABA in the RMS chains

4.1 Neuroblast-Astrocyte Interaction

The major components of the parenchyma around the RMS are GFAP$^+$ astrocytes, which in adult stages separate the neuroblast chains from their surrounding environment (Jankovski and Sotelo 1996; Chazal et al. 2000; Lois et al. 1996). Several studies have described a close interaction between astrocytes and migrating neuroblasts along the RMS. Lack of NCAM perturbs neuroblast-astrocyte interactions, which ultimately contributes to inhibit neuroblast migration (Chazal et al. 2000). Similarly, lack of β1 integrin subunit disrupts glial tubes surrounding the RMS, causing neuroblast chain disorganization (Belvindrah et al. 2007). Recent evidence indicates that neuroblasts can dynamically remodel the astroglial tunnel network by releasing Slit, which acts on Robo2/3 receptors on astrocytes and repels their processes, "clearing" their migratory path (Kaneko et al. 2010).

Even though astrocytes are not absolutely required for chain migration (Wichterle et al. 1997), disrupting the astrocytic tunnels causes neuroblasts to prematurely leave the RMS and ectopically migrate towards surrounding regions. Astrocytes may therefore create a physical boundary by defining a 'corridor', where secreted signalling molecules would influence chain formation and migration speed. Indeed, several reports have started to clarify some of the molecular events involved in this process, such as for example GABA clearance by astrocytes (Bolteus and Bordey 2004), or glutamate release by astrocytes influencing the activation state of GABA$_A$, Glu$_{k5}$ and NMDA receptors on migrating neuroblasts, promoting their survival and modulating their motility (Platel et al. 2007, 2008b, 2010). Astrocytes could help to create a microgradient of GABA and glutamate in the neuroblast chains, thus the relative distance between neuroblast and astrocyte may influence neuroblast speed and motile behaviour in the RMS. Changes in the functional state of astrocytes (linked for example to injury,

disease or influence by growth factors) are likely to affect their ability to regulate neurotransmitter levels, ultimately influencing neuroblast motility.

4.2 Vasculature

Emerging evidence points to a fundamental role for the vasculature surrounding the RMS in regulating neuroblast migration. Blood vessels, which are usually randomly distributed throughout the brain, progressively align parallel to the RMS from birth onwards (Snapyan et al. 2009). Importantly, the RMS astroglial network plays an essential role in this process by secreting the angiogenic factor VEGF at early postnatal stages. Selective *in vivo* downregulation of VEGF expression in astrocytes markedly altered the morphology of blood vessels at the outer border of the RMS, and as a consequence disrupted neuroblast migration, increasing stationary periods and decreasing displacement without affecting speed (Bozoyan et al. 2012). Interestingly, neuroblasts migrating along the vasculature scaffold migrate more efficiently, consistent with the fact that migration is faster in adult compared to early postnatal stages, when the vascular network is still developing along the RMS. Blood vessels not only provide a physical scaffold for migration, but are also likely to provide factors promoting migration. Recent evidence has shown a close interplay between blood vessels, astrocytes and neuroblasts in maintaining proper neural progenitor migration along the RMS. Efficiently migrating neuroblasts in the adult RMS and OB are positioned very close to blood vessels, and surrounding astrocytic processes are always present between endothelial cells and neuroblasts (Bovetti et al. 2007; Snapyan et al. 2009). While astrocytes modulate blood vessel growth and rearrangement via VEGF (Bozoyan et al. 2012), endothelial cells in the blood vessels in turn secrete BDNF that promotes migration via p75NTR on neuroblasts (Snapyan et al. 2009). However, GABA released by neuroblasts activates Ca^{2+}-dependent insertion of high-affinity TrkB receptors on the astrocyte plasma membrane. This results in "trapping" of extracellular BDNF, promoting the entrance of neuroblasts into a stationary period. This dynamic modulation of the local levels of available BDNF would contribute to the control of the long-distance saltatory migration of neuroblasts from the SVZ to the RMS.

5 Neuroblast Migration in Injury/Disease

Recent evidence has shown that the SVZ can "reactivate" and respond to insults like ischemia, epilepsy, and neurodegenerative conditions by modulating neurogenesis (Curtis et al. 2007a). SVZ-derived neuroblasts have the ability to migrate towards sites of injury, stroke and neurodegeneration, as shown in some Huntington's and Parkinson's disease models (Arvidsson et al. 2002; Sundholm-Peters et al. 2005; Ohab et al. 2006). Endogenous neural progenitors therefore appear capable to leave their normal migratory path by responding to signals triggered by damage and inflammation. These include chemokines like monocyte chemoattractant protein-1 (MCP-1), stromal-derived factor-1 (SDF-1), meteorin. MCP-1 and SDF-1 act via their receptors, CCR2 and CXCR4 to attract neuroblasts towards the ischemic striatum (Belmadani et al. 2006; Imitola et al. 2004; Robin et al. 2006; Yan et al. 2007). It is still unclear whether SDF-1 regulates RMS migration in the normal brain, where it is thought to regulate homing of SVZ progenitors to endothelial cells and support lineage progression (Kokovay et al. 2010). Instead, inflammation at the site of infarct triggers SDF-1 production by reactive astrocytes, microglia and endothelial cells which persists even weeks after stroke. SDF-1 promotes chain migration out of RMS explants and acts as a chemoattractant both *in vitro* and *in vivo*, since a neutralizing anti-CXCR4 antibody significantly reduces stroke-induced neuroblast migration (Robin et al. 2006). Similarly, the chemokine MCP-1 is upregulated after injury in astrocytes and microglia, and ko mice lacking either MCP-1 or CCR2 display a

decrease in neuroblast migration towards the ischemic striatum in the middle cerebral artery occlusion model of stroke (Yan et al. 2007). Moreover, infusion of MCP-1 into the normal striatum re-directs neuroblasts from the RMS, showing that neuroblasts can be chemoattracted by this chemokine both in the normal and in the infarcted brain.

Injury may also regulate neuroblast migration indirectly by perturbing the delicate balance of neurotransmitters in the microenvironment, which ensures proper movement of neuroblasts in the healthy brain (Young et al. 2011). Moreover, after an ischemic episode ectopic migration may be facilitated by the breakdown of the glial tubes surrounding the RMS and consists of neuroblasts moving either in chains or individually. In the diseased brain neuroblasts may employ several migration modes using myelinated fibre tracts, astrocytes and blood vessels to target injured sites (Cayre et al. 2009). Indeed, peri-infarct sites display high levels of angiogenesis, and blood vessels provide factors promoting neuroblast migration like BDNF and matrix metalloproteinases (MMP) 2 and 9, which facilitate neuroblast displacement and recruitment (Lee et al. 2006; Grade et al. 2013). Moreover, vasculature-derived SDF-1 and angiopoietin (Ang1) contribute to recruit neuroblasts to infarct areas, and gain-of-function and loss-of-function approaches for these factors and their receptors have further supported their requirement for vasophilic migration of neuroblasts towards stroke-affected areas (Ohab et al. 2006).

Other studies have shown that infusion of TGF-α in a 6-hydroxydopamine lesion model of Parkinson's disease can partially restore SVZ proliferation and migration towards the injury site. Whether SVZ-derived neural progenitors can target infarct areas in the human brain is still debated (Ekonomou et al. 2011; Macas et al. 2006). However, the therapeutic exploitation of endogenous neural progenitors for brain repair relies not only on the efficient recruitment, but also on the differentiation of neuroblasts to the appropriate neuronal cell type, which still remains a substantial challenge for current and future research.

6 Conclusive Remarks

Studies over the last two decades have clearly identified the SVZ/RMS/OB system as a powerful model to examine different stages of neurogenesis and neuronal migration in the postnatal brain. Much progress has been made in understanding the complex range of extracellular factors regulating stem cell-derived neuroblast migration. More recent reports have started to address how the disruption of migration affects neuroblast fate, functional maturation and integration into pre-existing synaptic circuits. Several aspects deserve future investigation, for example what is the nature of the coupling between network activity in the OB, proliferation in the SVZ and migration along the RMS. Furthermore, it will be important to explore how different extracellular signals co-operate in modulating neuroblast migration and identify the intracellular molecular pathways involved. Also, do any of these signals regulating migration play a role in the subsequent morphological and functional maturation of newborn neurons? Conditional approaches allowing loss/gain of function or genetic deletion at specific time points would help to answer these points.

Basic cell biology questions relevant to the characteristic collective migration of neuroblasts are still outstanding, including how cytoskeletal dynamics, adhesion and membrane trafficking are coordinated in these cells. Investigating these aspects is also likely to provide clues on the metastatic properties of invasive brain tumour cells, which like neuroblasts have the ability to migrate long distances in the brain.

Given the complexity of the RMS architecture, exploring the close interaction among migratory neuroblasts, surrounding astrocytes and blood vessels will be essential to better understand how neuroblast motility is regulated both in health and disease. This type of studies will likely benefit from recent technological advances, including for example two-photon time-lapse imaging of whole mount preparations to study migration at the population level, *in vivo* postnatal electroporation and

calcium imaging to visualize activity along the RMS (James et al. 2011; Lacar et al. 2012; Sonego et al. in press-b).

In conclusion, elucidating how neuroblast migration is orchestrated will not only help to clarify a fundamental aspect of postnatal neurogenesis, but will also contribute to identify molecular mechanisms underlying the recruitment of endogenous neural progenitors to injury sites and the metastatic potential of stem cell-derived brain tumours.

Acknowledgments Work in the Lalli lab is funded by the Wellcome Trust. I thank Patrick Doherty and Giovanni Lesa for valuable comments on this chapter, Madeleine Oudin and members of the Lalli and Doherty labs for providing neuroblast images.

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L. Nguyen and S. Hippenmeyer (eds.), *Cellular and Molecular Control of Neuronal Migration*, Advances in Experimental Medicine and Biology 800, DOI 10.1007/978-94-007-7687-6,

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