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Gianluca Gallo
Lorene M. Lanier
Editors

Neurobiology of Actin

From Neurulation to Synaptic Function

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Contents

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Chapter 1
Introduction to the Neurobiology of Actin

Gianluca Gallo

Abstract Neurons are arguably the most complex cells in nature and are characterized by a complex, dynamic, and highly polarized morphology. Actin and its regulatory proteins are the most abundant set of proteins within cells and form one of the major cytoskeletal systems, the actin filament cytoskeleton. While much has been learned about the roles of the actin cytoskeleton in non-neuronal cells, our understanding of the full spectrum of the functions of actin in neurons is far from complete. This book is intended to provide the neuroscience community with an introduction to the interface between the actin cytoskeleton and the myriad of issues fundamental to the understanding of nervous system function. The book covers the neurobiology of actin ranging from basic cellular organization and function to the roles of actin in the health and disease states of the nervous system. This chapter provides a primer on actin intended to serve the reader as background and review for the rest of the chapters.

Keywords Actin · Polymerization · Nucleation · Filament · Organization · Primer

1.1 Preamble

Neurons are fascinating and complex cells. In the catalog of metazoan cell types, neurons exhibit the most complicated and varied morphology. The morphology of neurons is certainly related to their function, and the variety of neuronal morphologies likely reflects the varied functions served by different neuron types. The morphology of cells is directly determined by the cytoskeleton. The cytoskeleton is in essence the bones and muscles of a cell. It provides structure to cells and also serves many fundamental physiological functions. While neuroscience researchers have been largely interested in neurons in the context of their information processing

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capabilities, in recent years there has been increasing interest in the cell biology of neurons. For example, biophysicists studying the electrophysiological properties of channels in the neuronal plasma membrane have discovered that many channels are linked to the neuronal actin cytoskeleton and that the interaction of channels with the cytoskeleton can regulate the functions of channels and thus the processing of information in the neuron. Given the increasing interest in the neuronal cytoskeleton by researchers that would classify themselves as neuroscientists, and not cytoskeletal cell biologist, the editors of this book have collected a series of chapters from experts in the fields on the neurobiology of the actin cytoskeleton.

This book is intended to serve as an introductory resource for neuroscientists interested in investigating the actin cytoskeleton in the context of their particular neuroscience research program. We do not intend this book to be an exhaustive resource for the specialist trained in cell biology, but rather an informative introduction to the field of the neuronal actin cytoskeleton. We fully appreciate that the nervous system contains additional important cells types (e.g., astrocytes, oligodendrocytes, and Schwann cells), but in the context of this book the biology of actin in these additional cells types will not be discussed due to space limitations. We hope that this book will prompt, and assist, investigators who consider themselves neuroscientists to further address the functions of the actin cytoskeleton in their work.

1.1.1 Structure of the Book

The book is broadly divided into two parts. The first part (Chapters 1, 2, 3, 4, 5, and 6) reviews the neurobiology of actin at the cellular level. The second part (Chapters 7, 8, 9, 10, 11, and 12) discusses the functions of actin in the context of neurobiological issues ranging from early development to synaptic function and disease states of the nervous system. Readers who do not have a prior understanding of the general concepts regarding the cellular organization and functions of actin in regulating cellular morphology are encouraged to begin the book by reading Chapter 1. This chapter presents the fundamental concepts required to appreciate the details of the molecular machinery that regulates actin in a cellular context presented in Chapters 2, 3, 4, and 5. Readers not familiar with actin are encouraged to read the "primer" that follows this section of the introduction (also see Chapter 11.1.1). The chapters in the second part of the book require a basic understanding of the issues of relevance to neuroscience discussed in each chapter, which should be familiar to a readership trained in the neurosciences. Throughout the book, chapters are cross-referenced in order to assist the reader in finding relevant information that is covered in greater depth in other chapters.

1.1.2 A Primer on Actin and Actin Filaments

In order to provide a background for readers of the book who may not be specialists in the cytoskeleton, we begin the book with a brief, but in the context of this book

we feel sufficient, introduction to actin and actin filaments. For readers interested in learning more in-depth information regarding actin, and the cytoskeleton in general, we provide some additional references at the end of the introduction. The primer focuses on the relevant concepts and not specific molecules.

Actin is a fundamental cytoskeletal protein. Depending on cell type, actin can represent between 5 and 15% of total cellular protein content. The importance of actin in cellular functions is also reflected in the strong conservation of its sequence during phylogeny. Mammals exhibit at least six actin genes, encoding different isoforms. In neurons, two isoforms of actin predominate: β-actin and γ-actin. These isoforms differ from one another in only the first 4–5 amino acids at their N-terminus. Although much remains to be learned, the isoforms of actin may have specific cellular functions.

Actin is a globular protein with a molecular weight of 43 kDa consisting of 375 amino acids. Individual actin molecules are referred to as monomeric actin (also termed G-actin). However, monomeric actin per se does not constitute the cytoskeleton. Monomeric actin is polymerized into actin filaments (usually referred to as F-actin), and it is these polymeric structures that form the actin cytoskeleton proper. Thus, the majority of studies on the actin cytoskeleton focus on actin filaments. However, in order to understand actin filaments it is necessary to appreciate the basics of the biochemistry and structure of monomeric actin.

Monomeric actin is an ATPase, and the hydrolysis of ATP to ADP is the fundamental aspect of F-actin polymerization in cells. Indeed, in neurons the polymerization of F-actin is reported to be a major sink for ATP levels. The loading of monomeric actin with ATP promotes incorporation of the molecule into a polymerizing filament. In a test tube, actin can undergo spontaneous polymerization, given specific ionic conditions (e.g., presence of Mg^{2+}). However, in cells the initial formation and subsequent polymerization of actin filaments is under strict regulation by a variety of additional proteins and signaling pathways.

Actin filaments are approximately 7 nm in diameter and consist of a two-stranded helix of actin monomers. The helix has a right-handed twist with a distance between crossover points of approximately 26–40 nm. F-actin is an example of a polarized filament. The term polarized refers to the fact that one end of the filament exhibits different polymerization dynamics than the other. The two ends of F-actin are referred to as the "barbed" and the "pointed" end, respectively. This nomenclature is derived from the classical method used to visualize the polarity of actin filaments and does not reflect the structure of the filaments themselves. The barbed end is considered to be the fast-growing end of the filament, while the pointed end is thought of as the slow-growing end of the filament. In other words, the addition of monomeric actin to the existing filament, per unit time, is greater at the barbed end than the pointed end. Thus, in terms of filament elongation, the barbed ends are the most active ends of actin filaments.

The generation of an actin filament from a pool of actin monomers involves two steps: (1) the initial nucleation of a "seed" consisting of few actin monomers and (2) the continued polymerization of the nucleated seed into a filament (Fig. 1.1a). In a test tube, actin filaments can spontaneously nucleate and polymerize from a solution of monomeric actin. However, the conditions used for spontaneous F-actin

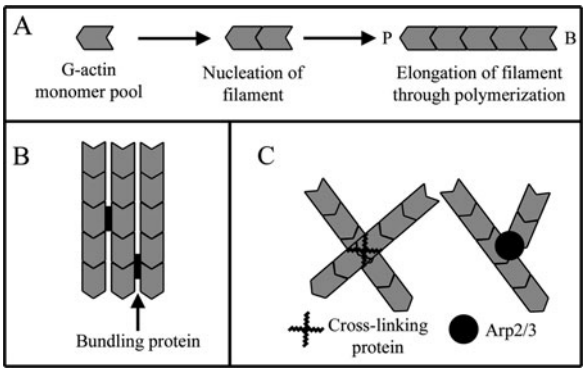

Fig. 1.1 Overview of actin filament nucleation, polymerization, and organizational themes. (**a**) Monomeric globular (G) actin in the cytosol is initially nucleated by forming a "seed" consisting of a few subunits. The "seed" subsequently serves as the basic scaffold for continued polymerization resulting in elongation of the filament. Filaments are polarized and have a pointed end (p) and a barbed end (b). Polymerization of the filament occurs primarily at the barbed end of the filament through continued addition of actin from the G-actin pool. (**b**) Bundles of actin filaments are formed through the binding of actin bundling proteins resulting in the alignment of filaments either with parallel (all barbed ends pointing in the same direction, shown) or anti-parallel (barbed ends pointing in opposite direction) directionality. (**c**) Mesh works of actin filaments can be formed by two separate mechanisms. In the first case, actin cross-linking proteins bind filaments to one another at angles dictated by the molecular structure of the cross-linking protein. In the second case, a multi-molecular complex termed the Arp2/3 complex binds the side of an existing actin filament ("mother" filament) and nucleates a new filament that grows from the side of the mother filament. In this case, the pointed end of the new filament is embedded in the Arp2/3 complex and the new filament elongates with its barbed end growing away from the mother filament

polymerization in a test tube do not reflect those in a cell. The term "critical concentration" refers to the minimum concentration of monomeric actin at which individual actin molecules begin to form multimeric seeds that eventually polymerize into filaments. The barbed and pointed ends of the filaments also have critical concentrations at which the likelihood of monomer addition to the end supersedes that of loss of a monomer from the end. At optimum conditions in a test tube the pointed and barbed ends of filaments are 0.6 and 0.1 μM, respectively. Estimates of the concentration of actin in cells produce values of approximately 150–900 μM. Based on these considerations, one might then conclude that all the actin in a cell would be in filamentous form. However, only approximately 50% of cellular actin is in filamentous form. The reason for this discrepancy is that cells tightly regulate actin monomer availability for polymerization, as well as the nucleation and polymerization of filaments, through a number of additional actin-binding proteins. It is thus paramount to keep in mind that the cell is not a test tube but a highly orchestrated machine with an exquisite system of regulation. Indeed, the cellular regulation of actin nucleation and polymerization is a fundamental topic in the biology of actin.

It is also important to appreciate that cellular actin filaments are often transient structures. Cellular actin filaments go through cycles of polymerization and

depolymerization, termed filament turnover. The average life spans of individual actin filaments in non-muscle cells can range from a few seconds to greater than 30 min, depending on cellular localization of the filaments. The turnover of filaments is of great importance to the physiological functions of actin filaments in cells. Indeed, numerous organisms have developed toxins that affect the dynamics of the cytoskeleton resulting in deleterious effects. For example, some of the commonly used pharmacological tools for altering actin filament dynamics are obtained from bacteria or fungi that use these molecules as toxins. Two examples of these types of toxins are cytochalasins and jasplakinolide, which cause actin filament depolymerization and stabilization, respectively. Thus, when thinking about actin filaments in a cellular context, it is important to consider their turnover rates.

Actin filaments in cells exhibit specific forms of spatial organization, which underlie the functions of the filaments. In a test tube, actin will form filaments, and these filaments can form non-specific filament-to-filament associations through biophysical interactions. However, in cells the organization of filaments is under complex regulation. For example, actin filaments can be organized to form linear bundles (Fig. 1.1b), interconnected networks (Fig. 1.1c), or contractile structures (e.g., cytokinetic ring). The organization of actin filaments in cells is determined by the expression profile of a number of actin filament-binding proteins that determine how filaments are arranged relative to one another.

Actin filaments have many different functions, depending on cellular context. As components of the cytoskeleton, actin filaments serve as scaffolds for the localization of intracellular proteins, and also the localization of membrane proteins. Actin filaments also serve as the substratum for intracellular organelle movement driven by myosin-family molecular motors. The motility of both neuronal and non-neuronal cells is strictly dependent on actin filaments. In particular the dynamic protrusive activity of the cell surface is driven by actin filament polymerization and turnover. These listed functions are simply examples of the range of cellular processes that actin filaments are involved in. As will become clear from reading this book, actin filaments are fundamental structures to a wide range of neurobiological processes.

In general, when thinking about actin and actin filaments in a cellular context it is important to consider the following:

(1) The distribution of filaments and monomers
(2) The turnover rates of filaments
(3) The specific spatial organization of filaments
(4) The distribution of actin filament barbed and pointed ends
(5) The regulation of (1)–(4) by actin regulatory proteins.

1.1.3 Additional Suggested Background Sources

The editors hope that this brief introduction to actin and actin filaments will assist the reader in understanding the chapters of this book. For readers interested

in learning additional information on actin and actin filaments, we suggest the following references:

Books

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Chapter 2
The Neuronal Actin Cytoskeleton and the Protrusion of Lamellipodia and Filopodia

Gianluca Gallo

Abstract The protrusion of filopodia and lamellipodia from the surface of neurons is fundamental to axon extension, guidance, the formation of axon branches, and synaptic structures. Protrusion is driven by the polymerization and controlled organization of actin filament arrays. This chapter provides an overview of the basic mechanisms operative during protrusion. Mechanisms underlying the suppression of protrusive activity are also discussed in relation to the regulation of axonal morphology. The purpose of this chapter is to provide the reader with an understanding of the major concepts underlying the regulation of actin filaments in protrusion without delving deeply into the molecular mechanisms, which are discussed in other chapters of this volume. Focus is placed on the organization of actin filaments in protrusive structures and how the organization relates to the process of protrusion.

Keywords Actin filaments · Arp2/3 · Localized translation · Myosin · Collateral branch · Network convergence · Network contraction · Retrograde flow

2.1 Introduction

The migration of neurons and the extension and guidance of their axons and dendrites are strictly dependent on the actin filament cytoskeleton. The actin cytoskeleton generates protrusive forces that allow the cell to extend its edges forward and thus change shape and extend processes. Filopodia and lamellipodia are two cellular structures that are fundamental to many forms of cell motility. Filopodia are slender finger-like projections (reviewed in Mattila and Lappalainen 2008), while lamellipodia are flat membrane "veils" (Fig. 2.1). Both lamellipodia

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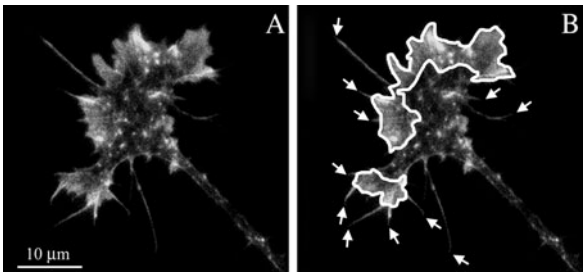

Fig. 2.1 Example of the lamellipodia and filopodia of a cultured embryonic sensory growth cone. In this image, actin filaments are shown as revealed through staining with fluorescently labeled phalloidin (**a**, **b**). **Panel b** denotes filopodial tips (*arrows*) and the lamellipodia (outlined by *white line*). Note that the axonal shaft is largely devoid of actin filaments but characterized by small patches of filaments

and filopodia act as sensors of the extracellular environment and also serve as components of the "engine" that drives cell motility and migration (Kater and Rehder 1995). In recent years, advances have been made in understanding the signaling capabilities of individual filopodia (Gomez et al. 2001). The protrusion of filopodia greatly increases the extracellular area that a cell can sample in order to detect relevant signals. Furthermore, filopodia formed from the surfaces of axons and dendrites are precursors to synapse formation (Sekino et al. 2007). Lamellipodia can also serve as sensors of extracellular signals but are most often thought of as contributing to the motile machinery of the neurons. Both lamellipodia and filopodia exhibit similar types of behaviors, characterized by cycles of extension and retraction. The mechanistic basis for the extension and retraction phases is discussed below.

The paramount role of protrusion in the development of the nervous system is exemplified by the sequence of events underlying the extension of axons (for a review of the cytoskeletal basis of axon extension, see Dent and Gertler 2003; also see Chapter 3). The first step in axon extension, and also the initiation of axons and dendrites from the cell body, is the protrusion of filopodia and lamellipodia (Fig. 2.2). The protrusion step generates new intracellular space for cytoplasm to advance into as the axon extends forward. The movement of cytoplasm, including microtubules and organelles, into newly protruded structures is termed "engorgement" (for details on the interactions of actin filaments and microtubules, see Chapter 5). The final step in axon extension is termed "consolidation" and involves the termination of protrusion from the sides of the advancing growth cone, resulting in the maintenance of a polarized migratory structure at the tip of the axon. Without protrusion, axons and dendrites can still grow to some degree, through microtubule-based mechanisms (engorgement), but the rate of growth is usually decreased and their ability to response to guidance signals required for path-finding is abolished.

This chapter reviews the principles of the cytoskeletal mechanisms underlying the protrusion of filopodia and lamellipodia. The purpose of the chapter is to

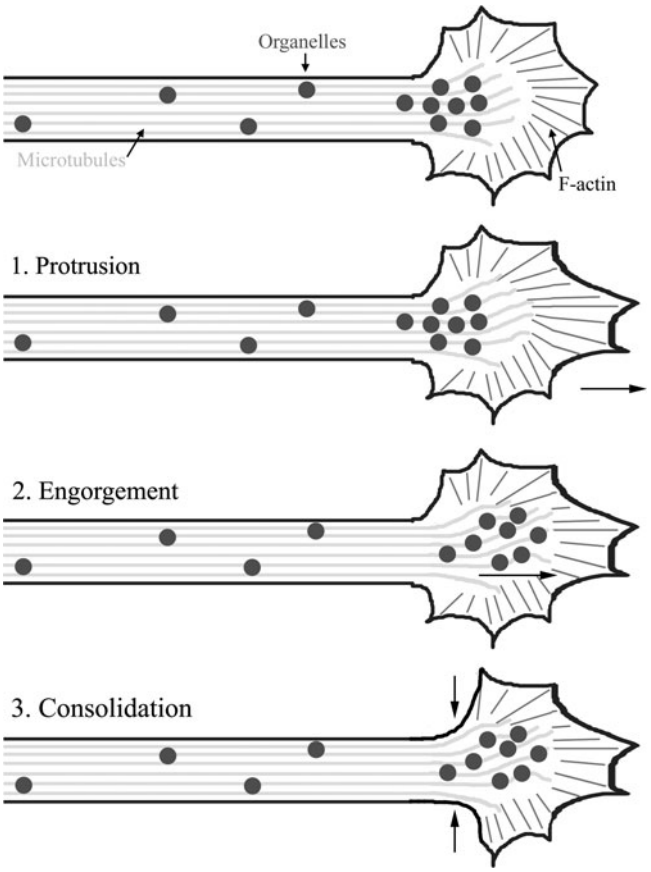

Fig. 2.2 Phases of the mechanism of axon extension. Protrusion of lamellipodia and filopodia to the leading edge of the growth cone is followed by engorgement. Engorgement occurs through the advance of microtubules and organelles into the leading edge of the growth cone. Finally, a new segment of axonal shaft is formed behind the advancing growth cone through the process of consolidation, which suppresses protrusive activity from the axonal shaft and maintains the growth cone polarized to the end of the axon

introduce the reader to general concepts regarding the neuronal actin cytoskeleton and is intended to assist readers in placing the variety of molecular information presented throughout this volume into a cellular context. Thus, the chapter is not intended to be a compendium of literature on the topic but rather focuses on select examples from the literature to emphasize specific points. The author apologizes to colleagues whose work is not referenced in the interest of simplicity and didactics. The formation of axon collateral branches from axonal filopodial protrusions is also discussed as an example of a specific form of protrusion of direct relevance to neurobiology. The chapter ends with a brief discussion on the actin cytoskeleton-based mechanisms used by extracellular signals that guide axons to their targets.

2.2 Actin Filament Organization in Neuronal Filopodia and Lamellipodia

Although both filopodia and lamellipodia are supported by a cytoskeleton of actin filaments, the organization of the filaments in these two structures is fundamentally different. These different organizations of actin filaments provide specific properties to the filopodia and lamellipodia. Within the filopodium, most actin filaments are organized with their barbed (fast growing) ends directed toward the tip of the filopodium (Lewis and Bridgman 1992, Steketee et al. 2001). However, some actin filaments appear to have their pointed end directed toward the tip as well (Lewis and Bridgman 1992). The arrangement of filament's barbed ends provides a system wherein actin filaments in the filopodial bundle will generate a protrusive force in the direction of the tip as the filaments polymerize. The force of filament polymerization is believed to drive the membrane outward as the bundle of filaments elongates (see below). Indeed, if actin filament barbed polymerization is blocked, filopodia immediately stop extending. The presence of actin filaments with their pointed ends directed toward the tip of the filopodium may reflect an organization used by myosin motors to generate contractile forces on anti-parallel actin filaments (see Chapter 4 for a discussion on myosin motor proteins). The filopodium should not be thought of as a simple linear projection hinged at its base. Indeed, at the growth cone, filopodia have been noted to move laterally as a whole (Bray and Chapman 1985) or to exhibit specific substratum attachment along their lengths that allow one part of the filopodium to remain fixed, while the tip can exhibit lateral motions (Sydor et al. 1996).

The bundled actin filaments in filopodia provide a relatively stiff cytoskeletal support for the filopodium. This is important as filopodia act as antennae that reach into the extracellular space. Although on average, both in vivo and in vitro, neuronal filopodia are 5–8 μm long, some filopodia can be as long as 30–40 μm. The distance that filopodia extend from the leading edge of the growth cone establishes the sampling area available to the growth cone for detecting extracellular signals. For example, a 20% increase in filopodial length results in a 45% increase in the area that the growth cone can sample as it extends toward its target (modeling the growth cone and its filopodia as a semicircle with area of $\pi r^2/2$). Thus, even relatively minor changes in filopodial length can result in large changes in the area that the growth cone can sample during axon guidance and regeneration.

Lamellipodia have elongated leading edges and are supported by a complicated geometric arrangement of actin filaments. Similar to filopodia, the nucleation and polymerization of actin filaments is an absolute requirement for the forward extension of lamellipodia. The mechanism of protrusion of non-neuronal lamellipodia has been thoroughly investigated and largely relies on the generation of branched actin filament arrays through the nucleating activity of the Arp2/3 complex (Pollard and Borisy 2003; see Introduction, Fig. 1.1). Although the lamellipodia of non-neuronal cells and neurons were originally thought to be similar, recent evidence suggests otherwise. Initial descriptions of the organization of actin filaments in growth cone lamellipodia suggest that the filaments do not follow the same architectural

principles as those of non-neuronal cells (Lewis and Bridgman 1992, Strasser et al. 2004). At the leading edge of the lamellipodium of growth cones, approximately 50–60% of actin filaments have their barbed ends directed toward the leading edge. Within the body of the lamellipodium the barbed ends of actin filaments also exhibit a similar distribution of barbed ends directed toward the leading edge (Lewis and Bridgman 1992). In the lamellipodia of non-neuronal cells, actin filaments are found in a branched filament type of organization orchestrated by the Arp2/3 filament-nucleating system (Cooper et al. 2001). The Arp2/3 system generates filaments that form at an approximately 70° angle from the side of existing actin filaments. However, in the lamellipodia of hippocampal neuron growth cones, actin filaments were found to be longer than in fibroblast lamellipodia and rarely exhibited a branch filament type of organization (Strasser et al. 2004). Rather, the actin filaments in the lamellipodia of growth cones form a dense network with criss-crossing but not branched filaments. Consistent with these structural observations, in hippocampal neuron growth cones, the Arp2/3 complex was not found at high levels in lamellipodia, as it is in the lamellipodia of non-neuronal cells. Furthermore, inhibition of the Arp2/3 complex does not alter the overall morphology of hippocampal neuron growth cone lamellipodia, although it impairs the dynamics and organization of lamellipodia in non-neuronal cells (Strasser et al. 2004). However, other investigators have reported that the Arp2/3 complex is indeed found in the lamellipodia of growth cones of primary neurons (Mongiu et al. 2007, Korobova and Svitkina 2008) and correlative and experimental data suggest that it may be involved in protrusive activity (Mongiu et al. 2007). Furthermore, Korobova and Svitkina (2008) have provided evidence that siRNA-mediated downregulation of Arp2/3 complex components decreases both lamellipodial protrusion and filopodial formation from growth cones and axons of B35 neuroblastoma cells and also of primary hippocampal neurons. Given the conflicting results in the literature, it will be of great interest to further address the issue of Arp2/3 function in neurons in order to resolve the issue. Additional filament-nucleating systems underlying the protrusion of neuronal filopodia and lamellipodia have not yet been fully elucidated but likely involve members of the formin family (reviewed in Goode and Eck 2007) and an additional nucleating system termed “cordon blu” (Ahuja et al. 2007). It is also possible that the organization and regulation of actin filaments in growth cone lamellipodia and filopodia may represent a variation on a theme of the more generalized non-neuronal cells. Indeed, the expression levels of specific actin regulatory proteins differ significantly between neurons and non-neuronal cells (Strasser et al. 2004), suggesting a differential orchestration of the regulation of the actin cytoskeleton in neurons. The functional reason for this difference across neurons and non-neuronal cells is not clear but likely resides in structure–function relationships unique to the complex morphology of neurons and the issues of axon guidance and synaptic function.

Finally, a recent study using platinum replica electron microscopy in conjunction with immunocytochemical analysis of the localization of Arp2/3 components has revealed unexpected differences in the organization of actin filaments between dendritic filopodia and “conventional” filopodia (Korobova and Svitkina 2010). In dendritic filopodia, actin filaments do not appear to form tight bundles but rather

have a looser organization characterized by a branching pattern that may be reflective of Arp2/3-mediated branching. This organization of the cytoskeleton is also reflected in the shapes of dendritic filopodia which are more polymorphic than conventional filopodia. Thus, a goal for the future will be to further address the differences between growth cone and axonal filopodia relative to dendritic filopodia.

2.3 Polymerization and Turnover of Actin Filaments in Protrusive Structures

The elongation of actin filaments through barbed-end polymerization is required for the forward extension of both filopodia and lamellipodia. Detailed studies in live cells have revealed that the polymerization of actin filament barbed ends is a major determinant of the extension rate of protrusive structures. However, actin filaments are transient structures and undergo turnover. In other words, following nucleation and polymerization, the filaments undergo depolymerization and loss of subunits. The turnover of actin filaments is required for cell motility. Inhibition of actin filament turnover in growth cones results in a block of axon extension followed by retraction of the axon (Gallo et al. 2002). Conversely, increasing actin filament turnover rates by over-expression of ADF/cofilin (see Chapter 11), a major positive regulator of actin filament turnover, increases axon lengths (Meberg and Bamburg 2000). In addition, extracellular signals that regulate axon extension and guidance also regulate actin filament turnover through ADF/cofilin (Gehler et al. 2004, Sarmiere and Bamburg 2004). Collectively, these observations demonstrate that filament turnover is not simply an epiphenomenon of actin cytoskeletal dynamics, but rather a crucial element of the mechanism of cell and axon motility.

The turnover rates of actin filaments differ between filopodia and lamellipodia. The actin filaments in filopodia exhibit slower turnover than do those of lamellipodia (Mallavarapu and Mitchison 1999). The slower turnover of filopodial actin filaments may also be reflected in increased stiffness, as revealed by atomic force microscopy (Grzywa et al. 2006). On the other hand, the slower turnover of filopodial actin filaments may also reflect the need for less dynamic filaments to support an "antenna"-like structure, or perhaps provide a more stable substratum for the directed transport of proteins and small vesicles within the filopodial shaft (Sabo and McAllister 2003).

2.4 Retrograde Flow of Actin Filaments in Protrusive Structures

The polymerization of the barbed ends of actin filaments in protrusive structures is only a component of the mechanism that regulates the extension and retraction of filopodia and lamellipodia. In growth cones, actin filaments undergo retrograde displacement, termed "flow," from the leading edge toward the central domain of the growth cone (Brown and Bridgman 2003a). Retrograde flow has been documented in both the lamellipodia and the filopodia of growth cones (Mallavarapu

and Mitchison 1999). Retrograde flow is driven by forces generated by myosin motors that pull the actin filaments toward the center of the growth cone (Lin et al. 1996; see Chapter 4). The issue of the specific myosin isoform involved in retrograde flow has not been fully resolved, but the weight of the experimental evidence suggests that myosin II is the relevant myosin family member. An involvement of myosin Ic in actin filament retrograde flow in growth cone lamellipodia was suggested by the results of micro-chromophore-assisted laser inactivation experiments (Diefenbach et al. 2002). However, the function of myosin Ic in mediating retrograde flow has been questioned by additional experiments that support a role for myosin II in driving retrograde flow (Brown and Bridgman 2003b, Medeiros et al. 2006). Importantly, myosin Ic does not seem to be targeted to regions of the growth cone consistent with a role in driving retrograde flow (Brown and Bridgman 2003b).

The current model for the role of myosin II in driving actin filament retrograde flow is termed the network contraction model (NCM) (Verkhovsky et al. 1999, Brown and Bridgman 2003a). This model incorporates important aspect of the organization of actin filaments and the distribution of myosin II in lamellipodia. Myosin II is found throughout the growth cone but is most prominent in the central domain of the growth cone, at the base of lamellipodia. The NCM posits that myosin II molecules interacting with a network of interconnected actin filaments will cause the local contraction of the network in regions of highest myosin II motor activity. The contraction of the actin filaments by myosin II in turn will result in displacement of the interconnected network of actin filaments toward the site of contraction. One way to think of the model is to consider a fishing net splayed out on the floor in front of you, representing the interconnected actin filament meshwork. If you then stand at one end of the net and with your hand roll up the net, thus acting as myosin II locally contracting the actin filaments, the whole of the net will move toward you.

A recent study has noted an unexpected component of the rate of actin filament retrograde flow, the force of actin polymerization itself (Medeiros et al. 2006). When barbed-end polymerization was blocked using cytochalasin, a drug that binds to filament's barbed ends and blocks their polymerization, the rate of retrograde flow was diminished. The rate of retrograde flow was maximally inhibited only when both myosin II activity and barbed-end polymerization were both blocked. Additional mechanistic studies will be required to understand how the force of actin filament polymerization contributes to retrograde flow.

2.5 Integration of Actin Polymerization and Retrograde Flow Determines Protrusive Dynamics

The protrusion and retraction of the leading edge of a filopodium or a lamellipodium is determined by both the rate of retrograde flow and the actin filament polymerization. Conceptually, the contributions of retrograde flow and polymerization to protrusion of the leading edge are described by a simple expression (Lin et al. 1996):

$$\text{Rate of protrusion} = \text{rate of polymerization } (R_{\text{poly}}) - \text{rate of retrograde flow } (R_{\text{fl}})$$

Thus, if $R_{poly}>R_{fl}$, then the rate of protrusion will be >0 and the leading edge will advance. If $R_{fl}>R_{poly}$, then the leading edge may retract (negative rate of protrusion). If $R_{fl}=R_{poly}$, the leading edge would be quiescent.

Individual growth cones exhibit regions of the leading edge undergoing protrusion, quiescence, or retraction (Mongiu et al. 2007). This spatial heterogeneity in leading edge behavior is reflected in spatially controlled rates of actin filament polymerization and retrograde flow at the subcellular level. An analysis of these parameters revealed that individual growth cone filopodia exhibit different rates of flow and polymerization (Mallavarapu and Mitchison 1999). Indeed, the subcellular regulation of cytoskeletal dynamics is likely to be of paramount importance to processes such as growth cone guidance wherein one side of the growth cone must behave differently than the other side.

A major mechanism that regulates the rate of retrograde flow is termed the "molecular clutch." The clutch is envisioned as a physical link between a transmembrane protein and its extracellular binding partner to the actin cytoskeleton (Jay 2000; Suter and Forscher 2000). When the clutch is engaged, then the actin filaments become indirectly physically associated with the extracellular environment and the force of this linkage counters the force of retrograde flow, thereby attenuating it. The existence of clutch mechanisms has been clearly demonstrated in elegant experiments using beads coated with antibodies or binding partners to membrane proteins, placed on the surface of growth cones (Suter and Forscher 2001). The beads then indirectly engage the actin filaments undergoing retrograde flow, through molecular links between the membrane proteins they are bound to and the filaments, and are moved in concert with the actin. However, if the bead is prevented from moving after it has engaged the actin filaments, then the retrograde flow of the actin filaments is inhibited. In this way, it is possible to engage the "clutch" on retrograde flow. When the clutch is engaged in the absence of inhibition of actin filament polymerization, the rate of polymerization will prevail and the leading edge will undergo protrusion. The actual molecular basis of the clutch mechanism is not fully understood. However, a general feature of both transmembrane cell–cell adhesion molecules and molecules of the extracellular matrix is that the intracellular domains of these molecules have the ability to directly or indirectly bind to the actin filament cytoskeleton. Recent studies in non-neuronal cells have identified the actin-binding proteins in focal adhesions (e.g., vinculin), one possible form of a clutch mechanism, as exhibiting the highest degree of correlation to retrograde flow, indicating that these proteins may mediate the link between actin filaments and the clutch complex (Hu et al. 2007).

2.6 Mechanisms Underlying the Initiation of Filopodia

Multiple models have been proposed based on work in non-neuronal and neuronal cells, and initially discussed in the context of filopodial formation at the neuronal growth cone. One model is based on branched actin filaments generated through the activity of Arp2/3 being utilized to give rise to a filopodial shaft through the

reorganization of the actin filaments by additional proteins (e.g., fascin; Svitkina et al. 2003). In this model, filaments in a lamellipodial meshwork, formed through an Arp2/3-dependent mechanism, converge in space to form a filopodial shaft. However, for this model to hold true for neurons, an Arp2/3-mediated branched filament meshwork is required, and whether neuronal growth cone protrusive structures contain Arp2/3-mediated actin meshworks is a matter of some controversy (Strasser et al. 2004, Mongiu et al. 2007, Korobova and Svitkina 2008). Furthermore, in some cells the Arp2/3 complex appears to have the opposite role predicted by this model and inhibits filopodial formation (Beli et al. 2008). Regardless, there is evidence that the Arp2/3-dependent network convergence mechanism is operative in neurons (Mongiu et al. 2007, Korobova and Svitkina 2008, Mingorance-Le Meur and O'Connor 2009). Thus, this model although valid for many cells, in many contexts, may not readily explain all instances of filopodial formation and may reflect one of multiple mechanisms for generating filopodia.

An alternative, but not mutually exclusive, model of neuronal filopodial formation involves a cytological structure termed the "focal ring" (Steketee et al. 2001). The formation of a focal ring precedes that of the emergence of a filopodium tip from the leading edge. Focal rings are intriguing donut-shaped cytological structures with a diameter of approximately 120 nm. The focal ring in turn serves as a nucleating/anchoring structure for actin filaments. The barbed ends of actin filaments are found embedded within the focal ring and may provide the population of filaments with pointed end directed toward the tip of filopodia (Steketee et al. 2001). The filaments attached to the focal ring may then serve as a scaffold for recruiting other filaments, perhaps through a network convergence model (see above), into the nascent filopodial filament bundle. These recruited filaments have their barbed end directed toward the tip of the nascent filopodium and thus likely provide the bulk of filament ends that generate a protrusive force through continued polymerization.

The axonal shafts of many cultured neurons contain low amounts of actin filaments. However, as revealed by live imaging of fluorescent actin in living neurons (see Chapter 6), the axons exhibit spontaneously formed transient localized accumulations of actin filaments driven by local polymerization (Loudon et al. 2006, Korobova and Svitkina 2008, Mingorance-Le Meur and O'Connor 2009; Fig. 2.3), which we will refer to as "axonal F-actin patches." Axonal F-actin patches often form and disappear without giving rise to protrusive activity from the axonal shaft. However, when a filopodium or a lamellipodium forms from an axon, it is preceded by the formation of a patch (Fig. 2.3). Thus, these spontaneously formed patches in the axon serve as potential precursors for filopodial formation. In sensory axons, the growth of these axonal actin filament patches is negatively regulated by RhoA and RhoA kinase. The probability that a patch will give rise to a filopodium is also regulated by RhoA, RhoA kinase, and myosin II (Loudon et al. 2006). Similar filament patches have also been reported in the axons of hippocampal neurons, and in these axons protrusive activity from actin filament patches is negatively regulated by calpain-mediated proteolysis of cortactin, an upstream regulator of the Arp2/3 complex (Mingorance-Le Meur and O'Connor 2009). Finally, actin filament precursor patches have also been shown to mediate filopodial formation from dendrites

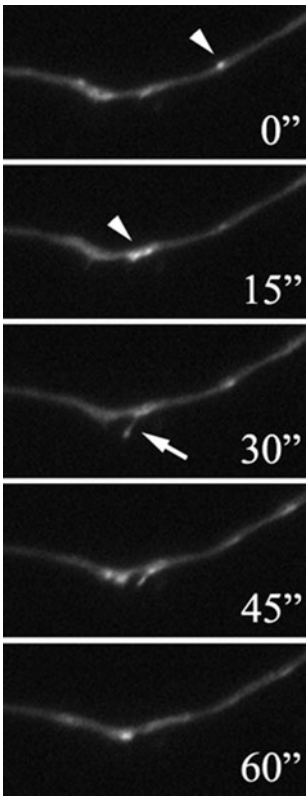

Fig. 2.3 Example of actin dynamics underlying the formation of filopodia from the axon of a cultured sensory neuron transfected with eYFP-β-actin (imaged at 3 s intervals). *Arrowheads* denote a subset of axonal F-actin patches formed during the imaging sequence. Between 15″ and 30″ a filopodial shaft arises from one of the two patches marked at 15″

in vivo (Andersen et al. 2005), indicating that F-actin patches are a shared feature of filopodial formation between axons and dendrites and that these actin structures are of relevance in the in vivo setting. Much remains to be learned about these axonal filament patches, and they may represent mechanism active in the axonal shaft but not the growth cone.

2.7 Relationships Between Filopodial and Lamellipodial Dynamics

Although structurally distinct, both filopodia and lamellipodia are formed by F-actin. At the growth cone, filopodia have been shown to regulate the advance of lamellipodia (Steketee and Tosney 2002). Filopodia have substratum attachment sites along the lengths of their shafts. These attachment sites regulate the ability of lamellipodia to extend between adjacent filopodia. Lamellipodia advance along the filopodial shaft until they reach a filopodial attachment site, at which point lamellipodial advance is hindered. The molecular mechanisms used by filopodial

attachment sites are not currently clear. However, these observations provide a proof of concept demonstration that filopodia can regulate lamellipodial advance. It is reasonable to envision filopodial attachment sites as potential targets of axon guidance factors. These issues remain to be resolved.

2.8 Protrusive Dynamics of the Axonal Shaft: Mechanism of Collateral Branch Formation

The formation of axon collaterals is of great importance to the establishment of neuronal connectivity patterns. Axon collaterals are initiated as filopodial protrusions from the axonal shaft. Subsequent maturation of a stable collateral branch requires the entry of axonal microtubules into the filopodium, thereby providing compressive support for continued growth of the branch, as well as transport of the cargo of axonal transport mechanisms (Dent and Kalil 2001; reviewed in Dent et al. 2003b). Multiple investigators have addressed the issue of cytoskeletal dynamics during collateral branch formation and the results from various studies converge. If axonal filopodia are prevented from forming by depolymerizing actin filaments, then branch formation is blocked. Once an axonal filopodium is formed, it is usually highly dynamic and the majority of axonal filopodia are fully retracted into the axon and do not give rise to collateral branches. However, a small population of filopodia become invaded by axonal microtubules and give rise to branches. The axonal microtubule array undergoes reconfiguration at sites of branch formation evidenced by a splaying apart of microtubules and severing of long microtubules to generate short microtubules. Microtubules can invade an axonal filopodium through either plus end-mediated polymerization or possibly transport by molecular motors. Importantly, microtubules and actin filaments regulate each other's polymerization and stability. Thus, coordination between microtubule dynamics and actin filament dynamics is required for the formation of axonal branches (Dent and Kalil 2001). Ena/VASP proteins are important regulators of filopodial formation in neurons (Lebrand et al. 2004). Depletion of Ena/VASP proteins, by mistargetting the localization in axons, results in growth cones devoid of filopodia and largely blocks the formation of retinal axon branches in vivo (Dwivedy et al. 2007). It will be of interest to determine whether Ena/VASP and Arp2/3 are involved in the formation of axonal F-actin patches, or the ability of a patch to give rise to a filopodium.

2.9 Inhibition of Protrusion by Repellent Guidance Cues

During development, extracellular repellent guidance cues keep axons from entering inappropriate territories. Multiple families of repellents have been identified. Based on the pioneering studies of Fan et al. (1993), it was postulated that repellent guidance cues act by causing F-actin depolymerization in growth cones, resulting in the

loss of protrusive activity, termed growth cone collapse. However, the depolymerization of actin filaments by repellent cues is likely only one aspect of the mechanism of growth cone collapse. Jurney et al. (2002) found that when the GTPase Rac1 is inhibited, retinal growth cones do not undergo collapse in response to ephrin-A2. However, measurements of the actin filament content of growth cones with inhibited Rac1 activity treated with ephrin-A2 revealed that, although the growth cones failed to collapse, the content of actin filaments was decreased to a similar extent as in control growth cones with active Rac1. Thus, a simple decrease in the amount of F-actin in growth cones does not fully explain growth cone collapse. Indeed, phosphorylation and thus inactivation of actin-depolymerizing factor (ADF; see Chapter 11) has been shown to be involved in semaphorin-induced growth cone collapse (Aizawa et al. 2001).

Studies on the cytoskeletal changes induced by repellent guidance cues indicate that changes in the organization of actin filaments may also contribute to growth cone collapse (Brown and Bridgman 2009). Many repellent cues not only cause growth cone collapse but also induce axonal retraction. Repellent cues induce the formation of actin filament cables in axons, and these filaments then serve as a substratum for myosin II-based force generation that drives axonal retraction (Gallo 2006, Brown and Bridgman 2009). However, growth cone collapse is not blocked by inhibiting myosin II, only the ensuing axonal retraction. The formation of actin filament bundles in growth cones, which are distinct from filopodial bundles, has been suggested to underlie the retraction of protrusive structures at the neck of the growth cone during the process of consolidation and lamellipodial retraction (see Fig. 2.2; Loudon et al. 2006, Mongiu et al. 2007, Burnette et al. 2008). Thus, the formation of similar bundles of actin filaments may be a component of growth cone collapse. Consistent with this notion, the introduction of constitutively active RhoA GTPase in axons decreases growth cone size and generates increased numbers of actin filament bundles at the expense of meshworks that normally drive lamellipodial protrusion (Gallo 2006). The RhoA–RhoA kinase signaling axis may be a major regulator of the organization and dynamics of actin filaments in growth cones. High RhoA activity drives the formation of actin filament structures that serve to generate contractile forces, while inhibiting the polymerization of actin filaments that contribute to protrusive activity. In the future it will be important to further elucidate the connections between individual signaling pathways and the various actin filament structures/organizations in growth cones and axons.

2.10 Localized β-Actin mRNA Synthesis as a Regulator of Protrusive Activity

Multiple mRNA species are targeted to axons and dendrites and are locally translated (Bramham and Wells 2007, Lin and Holt 2007). Of direct relevance to this chapter, mRNA for β-actin is targeted to axonal growth cones and synaptic compartments. β-Actin targeting to axons involves a 54-nucleotide, zip code-binding

sequence in the 3′ untranslated region (UTR) of the mRNA. This zip code-binding sequence in turn binds to a protein termed ZBP1, which acts to target the mRNA to cellular domains (Condeelis and Singer 2005). Initial evidence that axonal β-actin mRNA may have functional consequences came from studies determining that neurotrophin-3 induced the localization and localized translation of β-actin in the axons of cultured primary neurons (Zhang et al. 1999). A functional role for localized β-actin translation in the regulation of axonal growth in response to neurotrophin-3 treatment was subsequently demonstrated by inhibiting β-actin localization to axons using antisense oligonucleotide sequences to the zip code-binding sequence (Zhang et al. 2001). Antisense-treated axons exhibited an increased tendency to undergo spontaneous retraction compared to control axons, suggesting that localized β-actin translation is required for normal axonal advance.

Axon guidance by extracellular signals is dependent on the actin cytoskeleton. Recent evidence indicates that localized β-actin translation in growth cones is a component of the mechanism of guidance. Yao et al. (2006) demonstrated that asymmetric presentation of BDNF across a growth cone, which results in growth cone turning toward the highest concentration of BDNF, correlated with redistribution of ZBP1 and β-actin mRNA toward the source of BDNF. In addition, these authors also presented evidence that blocking the interaction between ZBP1 and β-actin mRNA prevented growth cone turning toward BDNF and importantly the establishment of an asymmetry in filopodial protrusion and increases in β-actin protein content across the growth cone during the turning response. Similarly, Leung et al. (2006) also provided strong evidence for localized β-actin translation as being a component of growth cone turning. In addition, Eom et al. (2003) demonstrated that downregulation of ZBP1 and overexpressed β-actin mRNA containing the zip code 3′ untranslated region decreased and increased, respectively, the numbers of dendritic filopodia. Collectively, these studies indicate that the localized translation of β-actin mRNA into protein contributes to the regulation of protrusive activity during axon extension and guidance, as well regulating aspects of synapse formation or morphology. However, care should be taken in not over-emphasizing the exciting possible role of localized translation as a recent study failed to find that net local translation has a significant role in axon extension, guidance, or protrusive activity and the requirement for local translation may depend on the experimental context (Roche et al. 2009).

2.11 Concluding Remarks

Actin filaments are remarkable structures and fundamental to cellular function. While numerous actin regulatory proteins have been discovered, much remains to be learned about their functions in neurons. Moreover, structure and function are intimately related. An important future direction will be to understand how the different types of actin filament organization in neurons are generated. Since cells are extremely complicated systems, it will be necessary to elucidate the redundancies

and cooperation between actin filament regulatory pathways in order to arrive at concrete models for further understating how extracellular signals determine neuronal biology through regulation of the structure–function relationships in the actin filament cytoskeleton.

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Chapter 3
Regulation of Actin Filaments During Neurite Extension and Guidance

Paul Letourneau

Abstract Behavior and other neural functions are based on neural circuits that develop over a prolonged period, which lasts in humans from the second fetal month through postnatal years. These circuits develop as neurons extend complex cytoplasmic processes, long axons and highly branched dendrites, expressing intrinsic morphogenetic behaviors while interacting with other cells and molecules of the developing organism. Actin-based motility dominates this morphogenetic process of developing neural circuits. Actin, always one of the most abundant intracellular proteins in neurons, is expressed at its highest levels during neuronal morphogenesis (Santerre and Rich 1976). An analysis of the actin content of embryonic sympathetic neurons indicated that actin comprises up to 20% of total cell protein (Fine and Bray 1971). Much of this actin is used in the motility that drives the formation of axons and dendrites. This chapter describes the roles of actin in the intrinsic mechanisms of morphogenesis of axons and dendrites and the extrinsic environmental features that regulate where and when axons and dendrites grow.

Keywords Growth cone · Axon guidance · Cytoskeleton · Clutch · Adhesion · Neuronal polarity · Neurite initiation · Actin

3.1 The Dynamic Neuronal Cytoskeleton

The ability to extend neuronal processes, or neurites, is intrinsic to neurons. For example, when immature neurons are placed into tissue culture, within hours, the neurons sprout processes that elongate onto the substrate, each tipped by an adherent motile structure called a growth cone. These neurites mature to become axons

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and dendrites and form synapses in vitro. Thus, in a neutral in vitro environment, neurons express intrinsic morphogenetic behaviors to form axons and dendrites.

Neuronal morphogenesis depends on the organization and dynamic properties of microtubules and actin filaments (Luo 2002, Dent and Gertler 2003). These cytoskeletal polymers are present in all cell types, although specific mechanisms determine their functions in neurons. In the first section the neuronal cytoskeleton and the intrinsic mechanisms of neurite formation and elongation will be discussed. In the subsequent section the regulation of axonal and dendritic growth by extrinsic molecules will be discussed.

3.1.1 Microtubules Provide Support and a Means of Transport

Microtubules are hollow cylinders 25 nm in diameter with walls consisting of subunits of alpha tubulin and ß-tubulin and with no defined length. Single neuronal microtubules can exceed 100 μm (Letourneau 1982). Microtubules are rigid and resist compression to support the elaborately extended axons and dendrites. Microtubules are also the "rails" along which organelles are transported via the motor proteins, kinesins and dynein (Hirokawa and Takemura 2004). Thus, providing structural support and rails for intracellular transport are the functions of neuronal microtubules.

3.1.2 Formation of Microtubules in Cells

Due to inherent asymmetry of the tubulin protein, microtubules are polarized with a distinct molecular face at each end. Tubulin subunits are added more rapidly at one end, called the plus (+) end, while the less likely end for growth is called the minus (–) end. Microtubules in neurons are formed in the centrosomal region and extend throughout the perikaryon with their minus ends facing the centrosome. The plus ends of microtubules undergo bouts of growing and shrinking called "dynamic instability," in which a microtubule end may undergo a period of rapid disassembly, which is followed by "rescue" and renewed growth (Tanaka et al. 1995).

3.1.3 Regulation of Microtubule Organization by MAPs

Microtubule organization is regulated by proteins called MAPs (microtubule-associated proteins), which bind to microtubules and regulate all aspects of their organization, including assembly, disassembly, stability, and binding to neurofilaments, actin filaments, and other microtubules (Gordon-Weeks 2000, Dehmelt and Halpain 2004). Kinesin motor proteins bind to microtubules and move cargo toward microtubule plus ends, while dynein motors move cargo toward minus ends. The protein katanin binds microtubules and severs them, promoting reorganization of

microtubules and remodeling of neuronal shape (Baas and Buster 2004). MAPs, such as MAP2, localized in dendrites, or tau and MAP1B, localized in axons, regulate microtubules to contribute to the specialized properties of these neuronal processes.

Several features distinguish neuronal microtubules. Unlike most cell types, the minus ends of microtubules in axons and dendrites are not anchored to the centrosome, rather microtubules lie entirely within these processes, after being released from the centrosome and transported into axons or dendrites. Many neuronal microtubules are highly stable due to enzymatic modifications of tubulin and from binding of certain MAPs. Nearly all axonal microtubules have their plus ends oriented toward the terminal, while microtubules in dendrites have mixed polarity, some with plus ends and some with minus ends oriented toward dendritic termini.

It is uncertain how microtubules and tubulin subunits are advanced as neurites grow (Baas and Buster 2004). Dynein motor molecules can slide short microtubules along, but long microtubules in axons are stationary, although their plus ends undergo dynamic growth and shrinkage. Possibly, tubulin subunits or short microtubules are transported distally via dynein motors and then disassembled to release tubulin for addition to longer, stable microtubules. This dynamic assembly of tubulin onto existing microtubules at the end of a growing process is a critical event in axonal and dendritic morphogenesis (Tanaka et al. 1995).

3.1.4 Actin Filaments in Neurons

Actin filaments are the other important cytoskeletal components in neuronal morphogenesis (Luo 2002, Dent and Gertler 2003). In mature neurons, actin filaments form a cortical meshwork beneath the plasma membrane that organizes ion channels, vesicles, membrane proteins, and neurotransmitter receptors at nodes of Ranvier and at synapses. However, at the ends of growing axons and dendrites the elaborate and dynamic networks of actin filaments drive the searching behaviors that are necessary for navigation of growth cones to their synaptic targets (Fig. 3.1; Yamada et al. 1971, Letourneau 1979, 1983).

3.1.5 Organization of Actin in Cells

Actin filaments are polymers of the globular protein actin. Actin filaments with a diameter of about 6–7 nm are individually not stiff, but bundles of actin filaments have stiffness. Unlike the limited cortical networks beneath the plasma membranes of mature neurons, actin filament arrays in growth cones are extensive, especially at the motile leading margin, where a dynamic actin filament network fills flattened projections, called lamellipodia, and bundles of actin filaments fill the cores of transient, finger-like projections, called filopodia, which can extend to 100 μm long (Fig. 3.1; Letourneau 1983).

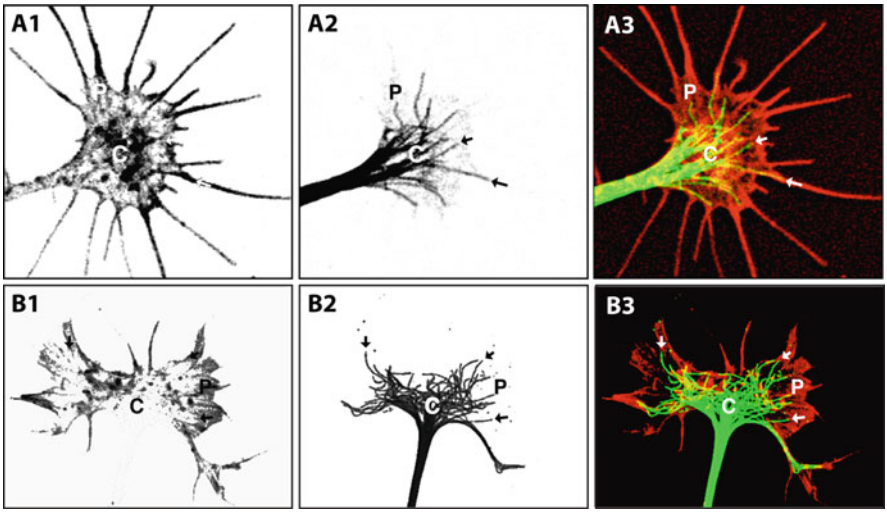

Fig. 3.1 The distribution of microtubules and actin filaments in developing neurons and in axonal growth cones. Actin filaments (A1, B1, *red* in A3 and B3) are arrayed in filament networks and bundles in the peripheral domains (P) of the growth cones and along the shafts of the axons. Microtubules (A2, B2, *green* A3 and B3) are bundled in the axons and branches. In a growth cone the microtubules from the central bundle of the central domain (C) splay apart and individual microtubules extend into the P domain and into filopodia (*arrows*)

Like microtubule polymerization, actin filaments polymerize by endwise addition of subunits. Also, like microtubules, the inherent asymmetry of the actin subunit leads to polarity of actin filaments, in which the "barbed" end is favored for polymerization and the "pointed" end is where actin subunits are lost from filaments. Again, like microtubules, neurons contain many proteins whose function is to regulate the polymerization, stability, and interactions of actin filaments.

3.1.6 Regulation of Actin Filament Organization by ABPs

These actin-binding proteins (ABPs) have numerous functions (Pollard and Borisy 2003). Some ABPs bind actin subunits, regulating their availability for polymerization, other ABPs crosslink actin filaments into meshworks, and bundles. ABPs that bind the barbed and pointed ends of actin filaments regulate the addition and loss of actin subunits. Several ABPs bind actin filaments and sever them, promoting the remodeling of actin filaments. In growth cones, actin filament barbed ends face the leading cell margin, where the addition of actin subunits is promoted by several ABPs. Myosins are motor molecules that move cargos along actin filaments. There are more than 10 myosins, which share a common motor activity but which differ in the direction they move cargos along filaments and in the cargos they move (Brown and Bridgman 2004). Myosins in growth cones interact with actin filaments to move actin filaments, vesicles, or other cargos and to exert tensions on cytoskeletal

components and associated structures (Rochlin et al. 1995). Myosin II in growth cones is particularly important in moving components and reshaping axons and dendrites. In summary, neurons express a unique combination of ABPs, unlike other motile cells like fibroblasts, which are critical to the behaviors of growth cones of developing axons and dendrites.

3.1.7 Regulation of Microtubule and Actin Organization and Dynamics by Cytoplasmic Signaling Pathways

As noted above, the organization of microtubules and actin filaments is regulated by MAPs and ABPs. The dynamic changes in cytoskeletal organization that drive neuronal morphogenesis reflect the dynamic activities of MAPs and ABPs. Certainly, the levels of these proteins are regulated by gene transcription and protein synthesis, but in an immediate fashion, MAPs and ABPs are regulated by intracellular signaling and second messenger pathways.

Cytoskeletal organization is rapidly changed by fluctuating levels of small regulatory molecules, such as Ca^{2+} ions, cAMP, cGMP, and phosphoinositides, which bind MAPs and ABPs and regulate them allosterically (Song and Poo 2001, Dent and Gertler 2003). The addition to and the removal of phosphate groups by protein kinases and phosphates also rapidly regulate MAPs and ABPs. These molecules and pathways are, in turn, regulated by events at the plasma membrane, where adhesive proteins, growth factors, and other ligands bind receptor proteins to trigger events that locally and temporally modulate these regulatory molecules. Thus, cytoplasmic signals that cascade from ligand–receptor interactions at the plasma membrane rapidly and locally regulate cytoskeletal organization during neuronal morphogenesis (Dent and Gertler 2003, Gallo and Letourneau 2004).

The Rho family of small GTPase proteins, in particular, RhoA, Rac1, and Cdc42, are important regulatory proteins that relay signaling from the cell surface to the cytoskeleton (see Chapter 12 for a detailed discussion of Rho signaling; Jaffe and Hall 2005). Rho GTPases bind to and regulate MAPs and ABPs or their upstream regulators, such as protein kinases and phosphatases. A critical feature of GTPases is that their activity is rapidly switched on or off, depending on whether they are bound to the nucleotides GTP (on) or GDP (off). A rich variety of guanine nucleotide exchange factors (GEFs) selectively activate GTPases by exchanging GDP for GTP, GTPase-activating proteins (GAPs) stimulate hydrolysis of GTP to inactive GTPases, and GDP dissociation inhibitors (GDIs) inhibit activation of GTPases by GEFs. These GEFs, GAPs, and GDIs are regulated by cell surface ligand–receptor interactions. Thus, by regulating GTPases, these membrane events regulate cytoskeletal proteins.

Activation of RhoA, Rac1, or Cdc42 has distinct effects on actin filament organization (Jaffe and Hall 2005). Rac1–GTP activates several ABPs to stimulate actin polymerization and formation of lamellipodia, while Cdc42–GTP also stimulates actin polymerization and formation of filopodia. RhoA–GTP activates the kinase ROCK, which phosphorylates several substrates to suppress actin polymerization

and activate the motor protein myosin II, increasing mechanical tensions and rearrangements of actin filaments. If RhoA levels are high, strong contractile forces in the growth cone can cause collapse of microtubule arrays and significant neurite retraction. All three Rho GTPases are present in the growth cone and contribute to growth cone motility.

3.1.8 Microtubule–Actin Interactions Are Important

Two interactions of actin filaments are particularly important in neurite elongation and growth cone migration. As mentioned above, microtubules maintain the shapes of axons and dendrites and resist compressive forces that would collapse or withdraw these processes. Proteins that mediate interactions between microtubule plus ends and actin filaments are significant, because these proteins may be involved in the initiation of neurites from a spherical perikaryon or in directing the advance of a growth cone (Rodriguez et al. 2003). These microtubule–actin interactions connect the microtubule functions of structural support and organelle transport to the dynamic cortical actin filaments and membrane receptors whose signaling regulates the motility of a developing neuron (Fig. 3.1).

3.1.9 Actin Filaments and Adhesive Contacts

In addition to interactions with microtubules, another key interaction of actin filaments involves the adhesive interactions of cells via membrane receptor proteins that form non-covalent bonds between cells or between cells and extracellular matrices (ECMs). The major adhesion receptors of neurons are the cadherins, the adhesion proteins of the immunoglobulin-like superfamily, and the integrin proteins, which mediate cell adhesion to ECM. As cell–cell contacts are initiated, receptors cluster to form discrete adhesive contacts. The cytoplasmic domains of these clustered adhesion receptors create docking sites for signaling enzymes, kinases, GEFs, GAPs, and ABPs that link actin filaments to the adhesive sites, and induce actin polymerization. Thus, adhesive sites are loci from which regulatory signals emanate and where actin filament organization and anchorage is regulated (Zamir and Geiger 2001).

3.2 A Mechanism for Neurite Initiation and Growth

In this section, neuritogenesis, neurite elongation, and growth cone migration will be described, emphasizing the dynamics of actin filaments and microtubules. When developing neurons are placed in culture, the neurons settle on the substrate, and extend and withdraw cylindrical filopodia and flattened lamellipodia, like waves lapping on a beach (Figs. 3.1 and 3.2). This motility is driven by actin filament

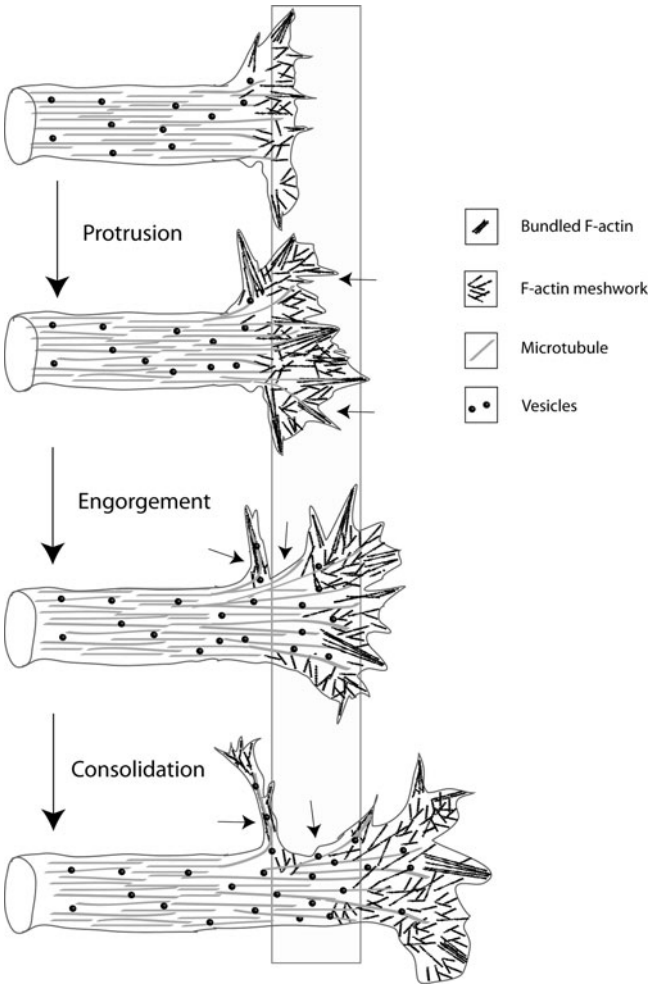

Fig. 3.2 Stages of axon and branch growth. Three stages of axon outgrowth have been termed protrusion, engorgement, and consolidation (Goldberg and Burmeister 1986). Protrusion occurs by the rapid extensions of filopodia and thin lamellar protrusions, often between filopodia. These extensions are primarily composed of bundled and mesh-like F-actin networks. Engorgement occurs when microtubules invade protrusions bringing membranous vesicles and organelles (mitochondria, endoplasmic reticulum). Consolidation occurs when the majority of F-actin depolymerizes in the neck of the growth cone, allowing the membrane to shrink around the bundle of microtubules, forming a cylindrical axon shaft. This process also occurs during the formation of collateral branches off the growth cone or axon shaft. Modified from Dent and Gertler (2003)

polymerization, which pushes the cell margin outward, while myosin II, located behind the cell margin, pulls newly formed filaments backward in a retrograde flow (Fig. 3.3). The rearward transported filaments are severed and depolymerized by ABPs, and if the protrusion and the retrograde flow are equal, these activities

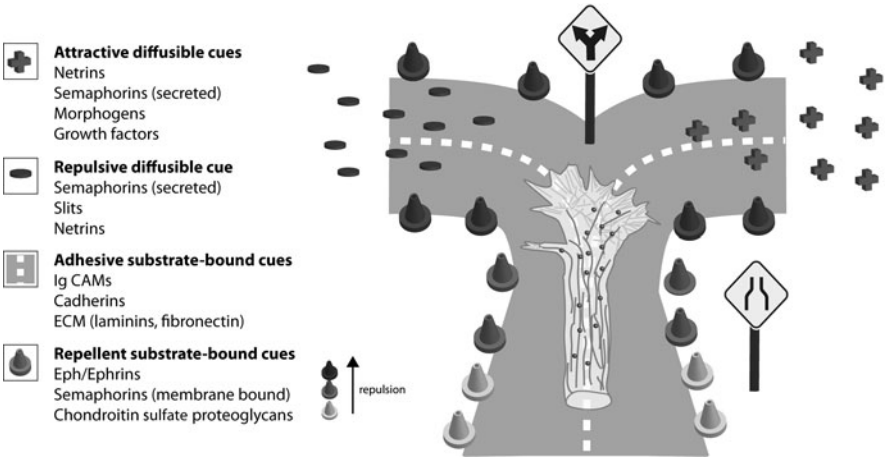

Fig. 3.3 A model of the mechanism of growth cone migration. Actin polymerization pushes the leading margin of the growth cone forward and myosin X transports cargo vesicles to the tips of filopodia. Forces generated by myosin II pull actin filaments backward, where filaments are disassembled. When growth cone receptors make adhesive contacts with a surface, a "clutch" links the adhesive contact to actin filaments of the leading edge, and the retrograde flow of actin filaments stops. This permits the advance of microtubules and organelles and promotes axonal elongation. Intracellular signaling generated by attractive and repulsive axonal guidance cues interacts with the molecular mechanisms of actin polymerization, myosin II force generation, adhesive contacts, and microtubule advance to regulate the paths of growth cone migration

produce no net change. Initially, microtubules remain in a loose network around the nucleus, and any microtubules that enter the protrusions are swept back with the retrograde flow of actin. However, eventually a filopodium or a lamellipodium thickens and moves away from the cell body, tethered by a cylindrical nascent neurite. A critical event in neurite formation is when microtubules and associated organelles enter and remain within a filopodial or lamellipodial protrusion, which then moves forward, ahead of the microtubules and organelles (da Silva and Dotti 2002). Several activities may prompt neurite initiation. An increased expression of MAPs, such as MAP2, tau, and MAP1B, may stabilize microtubules, enhancing their resistance to the myosin-based retrograde forces pulling actin back from the leading margin (Dehmelt and Halpain 2004). At sites where protrusions make firm adhesive contacts with the substrate, actin filaments become anchored to the adhesive apparatus, and retrograde flow stops, creating space into which microtubules can advance (Fig. 3.3). In addition, cytoplasmic signals from the adhesive sites may promote microtubule transport and polymerization. Finally, actin filaments linked to adhesive sites can interact with myosin II motors and pull microtubules and organelles toward the adhesive sites in opposition to the retrograde flow of untethered actin filaments, which carry microtubules backward (Suter and Forscher 2000). The significance of these outwardly directed actomyosin forces in neurite initiation is illustrated by findings that neurites can be initiated and pulled out from a neuron by attaching an adhesive bead to a neuronal surface and then pulling the bead with attached elongating neurite away from the nerve cell body (Fass and Odde 2003).

3.2.1 Organization of Growth Cones and Growth Cone Migration

A typical neurite has a central bundle of microtubules with associated organelles and a motile terminal expansion, the growth cone (Gordon-Weeks 2000; Figs. 3.1 and 3.2). At the growth cone leading margin, called the P domain (peripheral), vigorous actin polymerization pushes the cell margin forward, balanced by the myosin-powered rearward sliding of untethered actin filaments. This retrograde flow is attenuated when actin filaments at the leading edge become linked to adhesive contacts. At the base of a growth cone, microtubule motor proteins move microtubules and organelles from the neurite into the central growth cone, called the C (central) domain. From the C domain, individual microtubules extend into the P domain, sliding forward powered by molecular motors and by adding tubulin subunits to microtubule plus ends. Retrograde flow pulls most of these microtubules back into the C domain (Schaefer et al. 2002). Importantly, some microtubules advance into filopodia or lamellipodia that are stabilized at adhesive sites (Fig. 3.1; Letourneau 1979, Suter and Forscher 2000). If these microtubules persist and are followed by other microtubules and organelles, the C domain advances and the neurite extends. To complete the cycle of growth cone movement, actin filaments and membrane components that are not stabilized by adhesions or associations with microtubules are recycled at the back of the growth cone by the myosin II-powered retrograde flow and by disassembly of actin filaments and endocytosis of plasma membrane.

Thus, neurite elongation proceeds by three activities (Fig. 3.2; Goldberg and Burmeister 1986): (1) the advance, expansion and adhesion of the leading margin of the growth cone, driven by actin polymerization, (2) the advance of microtubules via polymerization, transport, and linkage to actin and adhesive sites (Letourneau 1979), and (3) the advance of organelles via microtubule-based transport. The coordination of actin-driven membrane expansion, the formation of adhesive contacts, and myosin II-powered exertion of tension on these adhesive sites generate a force that pulls the growth cone forward. Thus, neurite elongation involves "push" from the advance of microtubules and "pull" from myosin II-powered tension generated at adhesive sites at the growth cone margin (Letourneau 1981, Letourneau et al. 1987, Lamoureux et al. 1989). Experimental studies show that the "push" of microtubule advance is necessary for neurite elongation, while the "pull" of actin-based motility in growth cones is neither necessary nor sufficient for neurite elongation. However, growth cone "pull" accelerates neurite elongation and, as described later, is necessary for growth cone navigation.

3.2.2 Growth Cone Turning

Growth cone navigation to synaptic targets occurs by the selective turning, advance, or retreat of a growth cone in response to guidance cues that growth cones encounter within developing tissues. As described above, a neurite elongates by the advance of microtubules and organelles from the growth cone C domain into the P domain of the growth cone. In a neutral in vitro environment, this occurs randomly to one side

and then to the other, as the mass of the trailing neurite keeps the growth cone on a relatively straight path, once termed "a leukocyte on a leash" (Pfenninger 1986). However, in complex in vivo environments there are local differences in adhesive surfaces, extrinsic factors, and other ligands that interact with growth cone receptors to generate local differences in the activities of the factors that regulate actin filament dynamics, Rho GTPases, protein kinases, protein phosphatases, or second messengers, such as Ca^{2+} or cyclic nucleotides (Song and Poo 2001, Guan and Rao 2003, Gomez and Zheng 2006). If these local variations in regulatory cues are sufficiently strong or persistent, they produce local differences in actin-based motility that causes growth cone turning. This might occur because the P domain expands faster on one side due to locally enhanced actin polymerization, reduced retrograde actin flow, or by linkage of actin filaments to adhesive sites. Localized signals might directly promote microtubule polymerization or stabilization so that microtubules preferentially advance to one side of the P domain (Tanaka and Kirschner 1995, Challacombe et al. 1997, Dickson 2002). On the other hand, if local differences in signals triggered by extrinsic cues reduce actin-mediated protrusion on one side of a growth cone or if myosin II-powered retrograde flow of actin filaments increases on one side of a growth cone, microtubules advance on that side will be reduced, and the growth cone will turn toward the other side.

3.2.3 Mechanisms of Branching

Branches of neurites, axons, or dendrites are formed in two ways: by a growth cone splitting or by a new branch sprouting from the neurite shaft behind a growth cone. In either case, the acquisition of stable microtubules is key to forming a branch (Figs. 3.1 and 3.2). A portion of the P domain and associated C domain of a growth cone may separate from the whole and establish an independent growth cone and a new branch of the parent neurite. This may happen when a growth cone "pulls" in two directions. Branch formation along the shaft of a neurite is initiated by an increase in localized actin-based protrusion of filopodia or lamellipodia (Fig. 3.2; Gallo and Letourneau 1998). This mechanism is particularly prevalent in the branching morphogenesis of dendrites. This localized actin-based motility may occur until microtubules enter an actin-filled nascent branch by transport or by polymerization of microtubules from the main neurite (Gallo and Letourneau 1999). Microtubule ends in the main neurite may become linked to the actin filaments of the protrusion and be pulled into the branch. The microtubule severing protein katanin may promote branch formation by locally severing microtubules in the neurite shaft to create nearby microtubule ends that can be moved into a nascent branch (Baas and Buster 2004). Once stable microtubules are established, the advance of microtubules and organelles into the branch sustains its growth, as actin-based motility leads the branch forward. This sprouting of branches along axonal shafts may involve similar activities in linking microtubule advance to dynamic actin arrays as during neurite initiation from a neuronal soma.

3.2.4 The Differentiation of Axons and Dendrites: Polarization of Neuronal Form

A hippocampal neuron in vitro initially sprouts several neurites that extend slowly. After 18–24 h one neurite increases actin motility, expands its growth cone, and elongates significantly faster than the others. This neurite has become the axon, and it accumulates proteins typical of axons, such as MAPs tau and MAP1B, and GAP43, a protein involved in actin motility (Mandell and Banker 1996). Several molecules and pathways may be critical to axonal specification, including PI3 kinase, the Par complex, and small Rho GTPases (Arimura and Kaibuchi 2005, Wiggin et al. 2005). These molecules concentrate at the tips of newly specified axons and are implicated in regulating key activities, such as actin filament organization and polymerization, microtubule polymerization or stability, and transport and addition of plasma membrane components. It is unclear whether axonal specification always begins with the same upstream event, such as concentration of PI3 kinase activity in a neurite tip, or whether concentration of any of the above-mentioned molecules or signals is sufficient to confer axonal character. In vitro manipulations, such as focally pulling on a neurite or presenting adhesive proteins to one neurite, will induce that neurite to become the axon. Thus, extrinsic signals can influence the intrinsic mechanism of axonal specification, perhaps by locally activating PI3 kinase, key ABPs, Rho GTPases, or other components of the mechanism. After one neurite becomes the axon, the other neurites become dendrites. Less is known about the mechanisms of dendrite specification. Acquisition of microtubules with mixed polarities may be important, as well as localization of cytoskeletal, membrane, and signaling components that regulate dendritic characteristics.

3.3 Regulation of Neuronal Morphogenesis In Vivo

The previous section focused on the intrinsic mechanisms of neurite initiation and elongation, growth cone migration and turning, neurite branching, and the specification of axons. This section will discuss the roles of extrinsic molecules and signaling events in regulating neuronal morphogenesis in vivo. The neutral environment of a tissue culture dish facilitates investigating these intrinsic mechanisms. However, the in vivo environment is never neutral, and spatial and temporal patterns of distribution of molecular cues in the developing brain shape these intrinsic mechanisms to generate neural circuits (Tessier-Lavigne and Goodman 1996).

3.3.1 Neuronal Migration

Immature brain neurons arise from proliferating neural precursors in the ventricular zone. From their birth, immature neurons are polarized by the asymmetry of the local cues, including the adhesive protein laminin in the underlying extracellular

matrix (ECM) of the ventricular layer, and growth factors, morphogens, and guidance molecules, such as sonic hedgehog and netrin, produced by the surrounding neuroepithelial cells. These newly born neurons migrate from the ventricular zone and encounter additional cues as they migrate.

3.3.2 Neuronal Polarization and the Initial Growth of Axons and Dendrites

Neurons sprout axons soon after ceasing migration. In a homogenous neutral tissue culture environment, it is a random decision as to which neurite sprouted from a neuron becomes the axon, but cortical neurons in vivo always sprout their axon in the same direction that the axon will grow. In the model organism, *Caenorhabditis elegans*, a diffusible molecule, netrin, produced by ventrally located cells causes localized activity of PI3 kinase in young neurons, which then develop ventrally directed actin-based protrusions and sprout their axon toward the netrin source (Adler et al. 2006). PI3 kinase is also involved in axonal specification of mammalian neurons, and, thus, localized PI3 kinase in response to a local cue may both specify axonal identity and regulate actin motility to control the direction of axonal initiation.

Other factors are implicated in regulating the initial direction of cortical axonal growth. Immature cortical pyramidal neurons first extend an axon toward the ventricle, followed by an apical dendrite, which grows toward the pial surface. Unexpectedly, these opposite directions of axonal vs. dendritic growth are regulated by the same extracellular molecule semaphorin 3A (Sema3A), produced by cells at the outer pial surface and released to create an extracellular gradient (Whitford et al. 2002). Axons are repelled by Sema3A, while apical dendrites of the same neurons are attracted by Sema3A. The different directions taken by these processes are not due to local difference in expression of membrane receptors for Sema3A but rather due to a local difference in the signaling proteins that modulate levels of the cyclic nucleotide cGMP. The combination of Sema3A signaling and high cGMP levels in the apical dendrite promotes actin polymerization and dendritic growth, while Sema3A signaling in the axon combined with low cGMP activates the GTPase RhoA, which depresses actin dynamics and activates myosin II contractility, so the axonal growth cone migrates away from the Sema3A source. Thus, the opposite responses of axons and dendrites to Sema3A are due to an asymmetric distribution of cytoplasmic signaling in dendrites vs. axons, leading to locally different regulation of actin.

3.3.3 Axonal Guidance

Once sprouted from neuronal perikarya, axons follow stereotypical routes to their targets. This pathfinding is called growth cone navigation, that is, a growth cone

detects and responds to physical and chemical features in its environment. The dynamic actin polymerization and protrusion of filopodia and lamellipodia from the growth cone of a 1-μm-diameter axon allows exploration of an expanded search area 25 μm or more across. When filopodial and lamellipodial protrusions are experimentally suppressed by limiting actin polymerization, axons grow, but they do not navigate accurately, because without filopodial and lamellipodial protrusions a growth cone's search area is too small to localize guidance cues.

If the path of a growth cone to its target is long, the path is divided into several segments, each ending at an intermediate target to which the growth cone navigates (Fig. 3.4). Often, these intermediate targets represent a choice point at which a growth cone turns or changes direction as it enters the next segment of its journey. Pathways for growth cone navigation contain molecules that promote actin polymerization, adhesion, and growth cone migration. These molecules generally activate Rac1 and Cdc42 GTPases and elevate PKA activity and cytoplasmic [Ca^{2+}]. Although these "positive" molecules may be expressed outside the pathway, molecules that repress adhesion or actin dynamics are expressed outside to these pathways, acting like "guard rails" to keep growth cones on the proper path. Several proteins have been identified as negative guidance cues, including slit proteins, Sema3A, and several ephrin-As. Although each negative cue is detected by a different receptor with different signaling mechanisms, common features of these

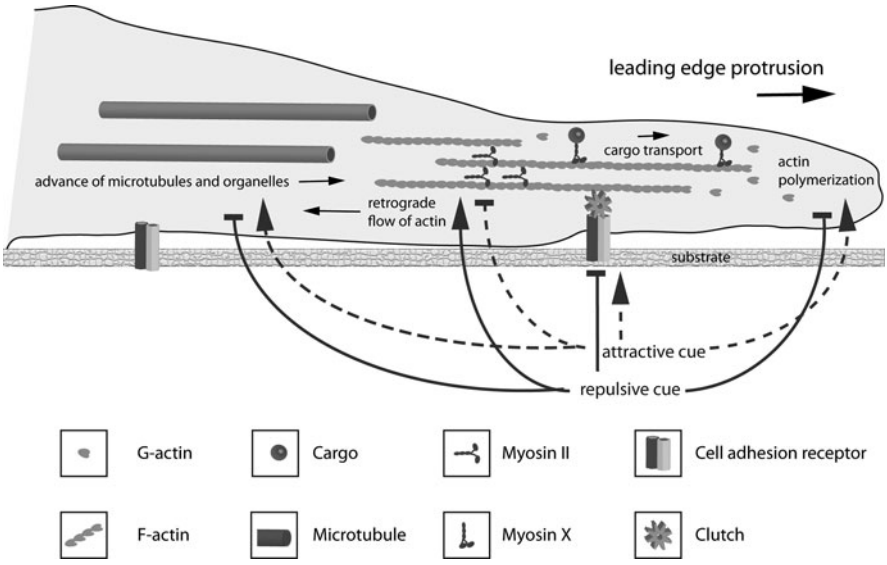

Fig. 3.4 Summary of the action of guidance cues that are involved in growth cone navigation. Short range cues on surfaces that growth cones contact act to promote or inhibit growth cone adhesion and migration. Long range cues are diffusible molecules released from intermediate or synaptic targets that attract or repel migrating growth cones. Growth cones integrate information coming simultaneously from multiple cues during navigation

mechanisms include suppression of actin polymerization, activation of RhoA to stimulate myosin II-mediated contraction, and often disruption of adhesive interactions, leading to growth cone collapse and sometimes retraction of entire axonal branches or segments (Guan and Rao 2003). Some molecules mark a path as positive or negative without providing directional information, while other molecules are soluble, released by navigation targets, and are distributed in gradients that provide directional information to growth cones (Fig. 3.4). At any instance a growth cone is detecting several guidance molecules, so growth cone migration depends on integrating the intracellular signals simultaneously triggered from multiple receptors (Fig. 3.4). For example, guidance cues like Sema3A and ephrin-A2 induce growth cone collapse by activation of RhoA. However, the effect of these "repulsive" cues is prevented or blunted by simultaneous exposure of growth cones to neurotrophins, such as NGF or BDNF, whose signaling reduces RhoA activity within growth cones.

This discussion has emphasized the importance of regulating actin dynamics and growth cone motility through allosteric control of protein interactions and activities in growth cones. However, recent studies show that protein synthesis in growth cones contributes to guiding growth cone migration. Messenger RNA for β-actin and proteins that regulate actin dynamics, for example, RhoA and cofilin, are detected in axonal growth cones, and translation of these mRNAs is regulated by guidance cues, such as netrin and semaphorin 3A. Certainly, the vast bulk of axonal protein mass is synthesized in neuronal somata and transported distally, and axonal growth continues for hours when protein synthesis is selectively inhibited in distal axons (Blackmore and Letourneau 2007). The significance of protein synthesis in growth cones is not to sustain axonal growth but to provide a mechanism for local responses to extrinsic cues over a period of time, or when growing axons enter new environments (Brittis et al. 2002) or undergo transitions to new activities, such as becoming a presynaptic terminal.

3.3.4 Navigation of Corticofugal Axons

The growth of axons from the cerebral cortex provides an example of guidance by multiple cues, as cortical axons trace long pathways from their origins to their targets. As stated above, cortical neurons sprout their axons away from the pial surface in response to the repellent cue Sema3A. The earliest corticofugal axons reach their first target, the intermediate zone, attracted by Sema3C, expressed in the subventricular zone (Fig. 3.5). The intermediate zone is rich in ECM and contains laminin, an adhesive protein that binds growth cone integrin receptors to form adhesive contacts that promote actin polymerization and give growth cones traction to migrate. The intermediate zone is a choice point for corticofugal axons, as they encounter the repellant Sema3A, expressed by the underlying ventricular zone (Bagnard et al. 1998). Corticothalamic and corticospinal axons turn laterally to exit the dorsal telencephalon through the internal capsule, while corticocortical axons turn medially. The molecules or cells that mediate this first decision are unknown. The internal capsule contains the attractant netrin-1 (Braisted et al. 2000)

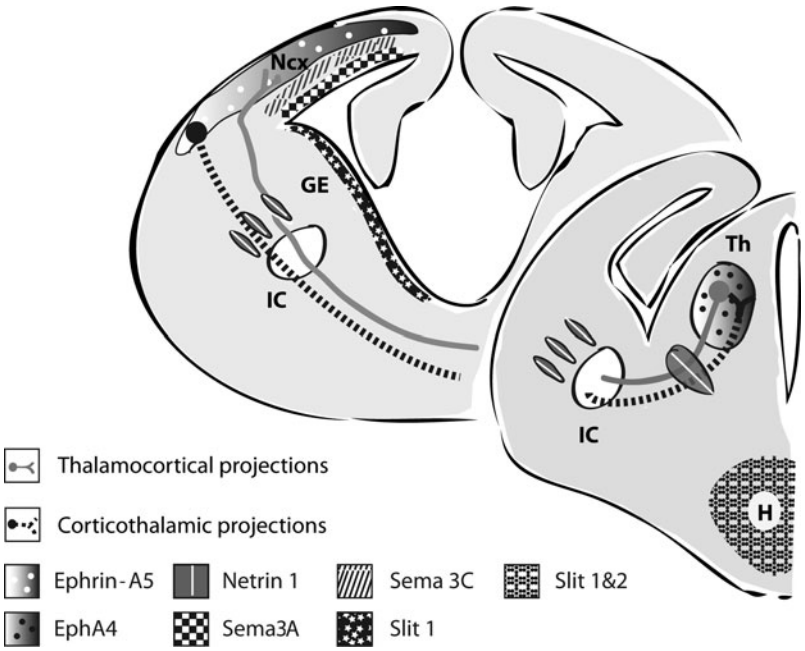

Fig. 3.5 The trajectory of growing thalamocortical and corticothalamic fibers involves multiple steps and both attractive and repulsive guidance cues. The expression of guidance molecules is related to each of these steps: Slit is a repellent that steers thalamic axons emerging from the diencephalon and in the ventral telencephalon. Ephrin-A5 is involved in sorting thalamocortical axons in the ventral telencephalon. Netrin-1 is an attractive factor for both populations of fibers in the internal capsule. Semaphorins 3A and 3C steer cortical fibers to penetrate the intermediate zone and then turn. EphA4 in the thalamus and ephrin-A5 in the cortex are involved in the establishment of topographic connections; Th, thalamus; H, hypothalamus; IC, internal capsule; GE, ganglionic eminence; Ncx, neocortex. Modified from Uziel et al. (2006)

which along with laminin promotes growth through the internal capsule. As these axons traverse the internal capsule, they are prevented from moving medially by the repellent cue slit-1 produced in the ganglionic eminence (Bagri et al. 2002). At the telencephalic–diencephalic boundary, corticofugal axons reach another choice point and split into two groups. Corticothalamic axons turn toward the thalamus, while corticospinal axons continue caudally, avoiding slit proteins expressed in ventromedial diencephalon.

Growth cones of corticothalamic axons may turn in response to attractants released from their thalamic target or they may recognize early thalamocortical axons and grow along them to reach the thalamus. Axons express several adhesion molecules, including L1 and N-cadherin, that bind homophilicly to the same molecules on growth cones to form adhesive contacts that promote growth cone migration. Growth cone migration along previously extended axons is a major means of axonal growth, and it is common that the first axons that establish a path become “pioneer fibers” that are followed by subsequent growth cones.

Corticospinal axons continue rostrally to the decussation area in the hindbrain (Ramakers 2005). Although corticospinal axons were previously repelled from the midline by slit proteins and other negative cues, these repellents are not expressed in the decussation area, and corticospinal axons now respond to midline attractants such as netrin, completing the decussation. Expression of midline repellents, slit and ephrin-3B, caudal to the decussation, prevents corticospinal axons from recrossing as they grow down the spinal cord. Once in the spinal cord growth cones delay entering the gray matter, perhaps because of the presence of repulsive molecules. Innervation of target areas of gray matter by corticospinal axons occurs in an interesting manner. Corticospinal axons initially extend beyond their target areas. Eventually, target cells in the gray matter release attractants and express adhesive ligands that activate Rho GTPases and ABPs to induce localized actin-based protrusive activity from the axonal shafts, followed by collateral branches that grow into the targets. Axonal segments that extend beyond the innervated target are then eliminated via retraction involving increased RhoA activity and myosin II contraction with cortical actin filaments of the axonal shaft. This exuberant growth of axons followed by retraction of mistargeted axonal segments is a common feature in the development of many cortical circuits (Innocenti and Price 2005).

The corticocortical fibers that form the corpus callosum make several guidance decisions after they turn medially in the intermediate zone (Richards 2002). The molecules that guide these decisions are unknown, although the callosal path may be "pioneered" by earlier axons, creating a path to the midline. The growth cones of corticocortical axons are attracted by netrin-1, produced by midline cells, and they are simultaneously channeled across the midline by repulsion from slit proteins expressed by cells above and below the developing corpus callosum. After navigating into the contralateral hemisphere, the axons reach the cortical subplate where they extend before sprouting collateral branches into their appropriate final target regions of the cortex. Neurotrophins, such as NT3, may be local cues that induce localized actin protrusion along axonal shafts preceding these collateral sprouts.

3.3.5 Patterning Axonal Distribution Within Targets

Once a group of axons reach their synaptic target, they become organized into arrays that represent physiologically relevant topography or sensory parameters. The distribution of retinal ganglion cell axons in their midbrain target (optic tectum or superior colliculus) is a model system in understanding this process (Mclaughlin and O'Leary 2005). Gradients in the distribution of ephrin ligands and their Eph receptors on cells across the optic tectum (or colliculus) and the incoming retinal growth cones are key features that organize the topography of retinal inputs to the tectum. Ephrin-A2 and A5 are expressed in an increasing gradient from the anterior to posterior tectum. EphA receptors bind ephrin-A ligands and trigger decreased Rac1 and Cdc42 activities and increased RhoA activity, stimulating growth cone repulsion by limiting actin dynamics and activating myosin II. Growth cones of temporal retinal axons express high levels of EphA receptors, so they stop soon after entering the anterior tectum and begin innervation of tactal neurons, while

nasal retinal growth cones, expressing lower levels of EphA receptors, extend to the posterior tectum, because they are less repelled by the ephrin-A gradient. Retinal mapping along the medial–lateral tectal axis involves gradients in the distribution of ephrin-B's and their EphB receptors among retinal axons and tectal cells. Unlike ephrin-A ligands, both ephrin-B ligands and EphB receptors can activate cytoplasmic signaling domains to regulate axonal targeting along the medial–lateral tectal axis.

The initial distribution of axons, as regulated by these gradients, is not final, and subsequent remodeling of axons due to further interactions and physiological activities refines the neural circuits. The patterning of inputs to a target depends on activities distributed along the afferent axons, in addition to the growth cones. Local signaling by guidance cues or other physiological events along axonal shafts can rapidly regulate activities of RhoA or Rac1 and Cdc42 to regulate actin dynamics and myosin II activity to induce retraction or addition of collateral or terminal branches along developing axonal shafts (Gallo and Letourneau 1998, Mclaughlin and O'Leary 2005).

The accessibility and the simple anatomy of the retinotectal projection have allowed much progress in understanding the patterning of developing neural circuits. The discovery of gradients in the distributions of ephrin-A and EphA receptors in the neocortex and thalamus, respectively, indicates that gradients of interacting ephrins and their receptors have similar roles in regulating axonal guidance and patterning of thalamocortical connections to their targets in the primary sensory regions of the cerebral cortex (Gao et al. 1998, Mackarehtschian et al. 1999, Vanderhaeghen et al. 2000, Uziel et al. 2006). Similar mechanisms may operate in patterning the development of other CNS circuits (Flanagan 2006).

3.4 Development of Dendrites

Like the formation of axons, dendrite formation is intrinsic to the neuronal phenotype. In fact, different neuronal types in a neutral tissue culture environment will form dendritic arbors that are reminiscent of their characteristic in vivo morphologies. The same basic mechanisms of actin filament and microtubule dynamics operate to drive the formation of dendrites, although dendrites are more numerous, shorter, and more elaborately branched than axons, due to expression of dendritic-specific cytoskeletal, membrane, and signaling proteins.

Generally, a neuron initiates dendrites after it is actively engaged in axonal elongation. This lag may be due to both environmental factors and intrinsic factors, such as changes in the expression of specific cytoskeletal proteins. The sites of dendrite initiation from a neuron may be determined by previous cell interactions, e.g., the apical dendrites of cerebral cortical neurons are formed from the leading process with which immature neurons had migrated from the ventricular lining of the cortex. As described previously, the apical dendrites of cortical neurons are oriented by an attractive response to Sema3A, produced at the pial surface. Other extrinsic proteins produced by neighboring cells or afferent axons promote the formation

of dendrites, including osteogenic protein-1 (BMP7) and neurotrophins BDNF and NT-3 (Whitford et al. 2002).

Thus, intrinsic regulation of cytoskeletal and membrane components combined with availability of extrinsic factors, such as osteogenic protein-1 and neurotrophins, orchestrates the initiation and elongation of dendritic arbors. However, the formation of dendrites is a prolonged activity and the final shaping of dendritic arbors depends on afferent inputs and interactions with axon terminals (Van Aelst and Cline 2004). Visualization of the morphogenesis of individual dendrites in developing brains of living frogs and zebrafish has revealed rapidly changing addition and loss of small branches and arbors as dendrites interact with afferent axons. Filopodia transiently extend from dendritic shafts and termini, and if contacts are made with axonal growth cones, the dendritic filopodium may be stabilized and nascent synapses may form. However, many of these contacts and synapses are brief, and the terminal axonal and dendritic branches may be retracted. Synaptic activity is a factor in dendritic morphogenesis, and activation of NMDA receptors at nascent synapses regulates Ca^{2+} signaling and Rho GTPases in a manner similar to the actions of guidance cues to modulate actin filament dynamics that underlies the extension and retraction of dendritic filopodia (Van Aelst and Cline 2004). The roles of synapses in regulating dendritic growth may also change as the synapses mature. Postsynaptic activation at early synapses may stimulate formation of more dendritic filopodia and elaboration of dendritic branches, while signaling at more mature synapses may generate stop-growing signals to stabilize dendritic arbors. New excitatory synapses contain only NDMA receptors, and AMPA receptors are added later. Addition of AMPA receptors to synapses may be required for retention of synapses and stabilization of dendritic arborizations. The final shaping of axonal terminals is also dependent on interactions with dendrites and postsynaptic contacts. Retrograde synaptic interactions may signal growth cones to reduce their dynamic actin activity, stop, and transform to a presynaptic ending. Motor axons growing on muscle fibers of mice that lack the acetylcholine (Ach) receptor-aggregating protein, agrin, or the agrin receptor component, MUSK, extend abnormally long distances across muscle surfaces, implicating MUSK and agrin in an axonal "stop signal." The neuromuscular junction contains a laminin isoform, S-laminin, that inhibits axonal growth. Nitric oxide, which is released by dendrites in response to synaptic activity, may be a retrograde signal that stops axonal growth in synaptic regions. This transition from a growth cone to an axonal terminal involves a significant reduction in actin-related motility. Little is known about this transition, concerning changes in actin filament turnover, filament stability, and the activities of ABPs, Rho GTPases, and the other signals that regulate actin filament dynamics and organization.

3.5 Axonal Regeneration

When axons are injured by trauma or disease, neural circuits can be disrupted, resulting in dysfunction, unless neuronal connections are regenerated or readjusted to recover normal function. When long projection axons that travel the spinal cord

are damaged, the challenge of regeneration is daunting. Unlike the developing nervous system, the environment of the adult CNS does not promote axonal growth. Axonal regeneration in the damaged spinal cord is inhibited by proteins released from degenerating myelin and by formation of glial scars that contain repulsive molecules, such as semaphorin 3A, ephrin-A2, and chondroitin sulfate proteoglycans. These proteins of the scars and the myelin inhibit growth cone adhesion and activate RhoA signaling to limit actin polymerization, trigger myosin II contractility, and inhibit growth cone advance (Sandvig et al. 2004). Approaches to improve the environment for axonal regeneration include adding antibodies and enzymes to block the inhibitors, adding agents that inhibit RhoA GTPase, and adding growth factors or reagents that promote growth cone motility, perhaps through stimulating actin dynamics. These interventions can improve axonal regeneration by a few percent of injured projection axons. However, these adult neurons are not in a growth mode, and the ends of their axons do not express robust dynamic actin-based motility to drive growth cone migration and axonal growth. In fact, spinal cord projection neurons lose an intrinsic capacity for growth cone migration after axons reach their targets during development, and this capacity is not re-expressed after injury (Blackmore and Letourneau 2006). On the other hand, peripheral neurons, which can regenerate axons, do increase the expression of genes for β-actin, tubulin, and GAP43, to promote axonal regeneration (Bisby and Tetzlaff 1992). Some mRNAs for cytoskeletal proteins, including β-actin, cofilin, and tropomyosin, have been detected within regenerating axons, suggesting that axonal protein synthesis may contribute to increasing actin dynamics and growth cone motility of regenerating axons (Twiss and van Minnen 2006). Other studies have shown that axon regeneration is promoted by increased activity of PKA and ERK kinase, which promote growth cone activity of embryonic neurons (Chierzi et al. 2005). Thus, success in promoting regeneration of CNS axons requires strategies to deter the inhibitory molecules of the CNS environment, increase growth-promoting molecules of the environment, and change gene expression in the injured neurons to re-express the cytoskeletal components and signaling activities that characterize developing neurons, as their axons navigate to targets (Filbin 2006, Pearse and Bunge 2006).

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Chapter 4
Functions of Myosin Motor Proteins in the Nervous System

Daniel M. Suter

Abstract The myosin superfamily consists of 24 classes of actin-based molecular motors that carry out a diverse array of cellular functions ranging from cell motility and morphology to cytokinesis, signal transduction, membrane trafficking, RNA and protein localization. The development and functioning of the nervous system strongly depends on the proper establishment of complex networks of neurons with highly specialized morphologies and molecular composition. Thus, it is not at all surprising that multiple classes of myosin motors are expressed in the nervous system, including classes I, II, III, V, VI, VII, IX, X, XV, and XVI. This review discusses the current knowledge on myosin functions in both neurons and specific sensory neurons, the hair cells of the inner ear, and the photoreceptors in the eye. The role of myosin II in growth cone motility and neurite outgrowth is the best characterized myosin function in neurons. However, there is increasing evidence that different myosin motors are also involved in protein and organelle trafficking underlying synaptic function. Multiple myosin motors have been localized to hair cells and photoreceptors and associated with genetic diseases; however, a future challenge will be a better characterization of the cellular functions of these various motor proteins.

Keywords Motility · Transport · Contractility · Sensory · Stereocilia

4.1 Introduction

Myosins are molecular motors that use the energy derived from ATP hydrolysis to move along actin filaments. Myosins carry out a large variety of cellular functions including cell movements, maintenance of cell shape, cytokinesis, organelle transport, signal transduction, phagocytosis, membrane trafficking, RNA and

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protein localization, and transcription (Brown and Bridgman 2004, Gillespie and Cyr 2004, Krendel and Mooseker 2005, Sweeney and Houdusse 2007). Over the past 20 years the number of newly identified myosin molecules and classes has increased significantly. The myosin superfamily includes at least 24 classes in species ranging from *Saccharomyces cerevisiae* to *Drosophila* and humans (Berg et al. 2001, Foth et al. 2006). Not all classes are present in each species. The human genome encodes approximately 40 myosin genes grouped into 12 classes (Berg et al. 2001). Since the nomenclature for myosin homologs from different species has been confusing in the past, I am following the system suggested for class I myosins; thus, Myo1a will be used as abbreviation for the myosin Ia protein (Gillespie et al. 2001).

Myosins contain at least one heavy chain with an N-terminal conserved catalytic domain of around 80 kDa, followed by a neck region with one or more putative light chain-binding motifs, sometimes known as the IQ motif (Fig. 4.1).

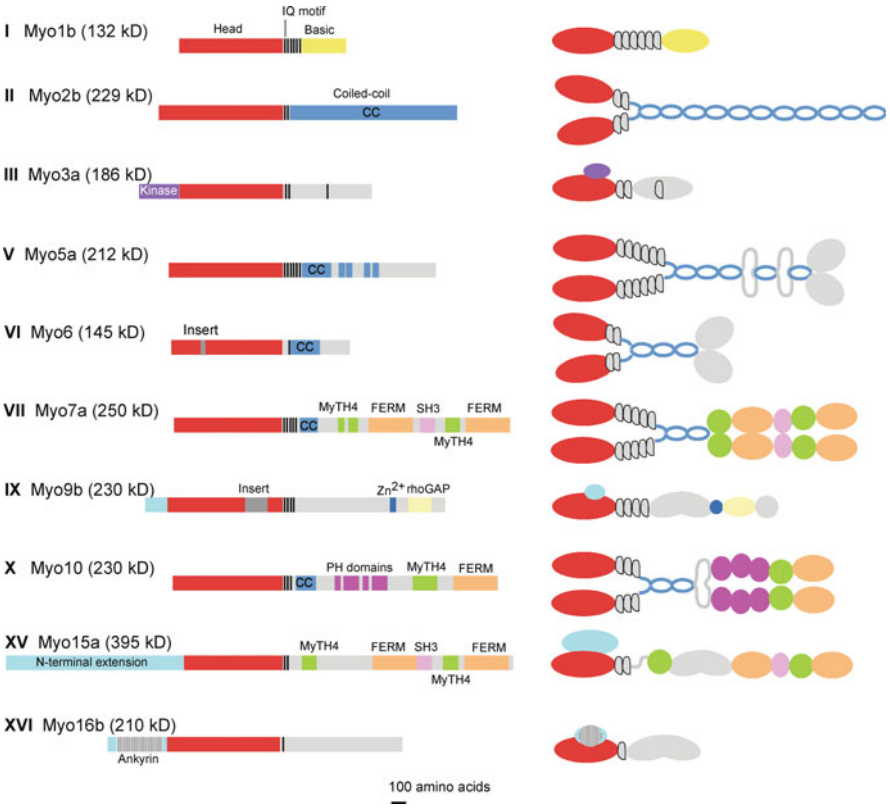

Fig. 4.1 Structures of myosins in the nervous system. Domain organization of the myosin heavy chains represented in the nervous system. Domains are *color coded* and discussed in the text. The *blue* coiled-coil tail domains allow dimerization. The *right panel* shows schematics of the corresponding myosin structures as either single- or double-headed motors. Myo6 and Myo7a can exist in both monomer and dimer form. Light chains (*grey ellipsoids*) are indicated based on the number of IQ motifs. Figure was modified from Krendel and Mooseker (2005)

The most conserved residues of the IQ motif conform to the consensus sequence IQxxxRGxxxRK (Cheney and Mooseker 1992). The following C-terminal tail contains specific domains for cargo binding or heavy chain dimerization that results in a double-headed motor. Class II myosins have been termed as "conventional myosins" since they were discovered first and their role in muscle contraction has been extensively characterized. Beginning with *Acanthamoeba* myosin I (Myo1) (Pollard and Korn 1973), all newly characterized myosins with a structure different from Myo2 have been classified as "unconventional myosins" (Cheney and Mooseker 1992, Mooseker and Cheney 1995). The classes of the myosin superfamily are mainly defined by differences in the head structure. More recently a novel myosin classification scheme has been proposed that is based on the combinations of various tail domains (Fig. 4.1; Richards and Cavalier-Smith 2005). For a more detailed discussion of the diversity of structural motifs and related functions, I refer to several recent articles and reviews (Berg et al. 2001, Krendel and Mooseker 2005, Richards and Cavalier-Smith 2005, Foth et al. 2006).

Structural diversity among myosin heavy chains evolved not only in the tail region but also in the N-terminal head region, resulting in different motor properties, such as number of heads, processivity, duty ratio, and directionality (Krendel and Mooseker 2005). The duty ratio indicates the proportion of the ATPase cycle that the motor spends strongly bound to the actin filament, while the processivity is a measure for how long the motor can walk on the track without detaching (Fig. 4.2). Motors involved in organelle transport such as Myo5 have a higher processivity compared to motors mediating actomyosin contractility such as Myo2 (Mehta et al. 1999). The majority of myosin motors walk toward the barbed end (Fig. 4.2). Myo6 is the only myosin that has been widely accepted as pointed end-directed motor (Wells et al. 1999).

This book chapter provides an overview on the numerous functions of the 10 classes of myosin motor proteins identified in the nervous system to date, classes I, II, III, V, VI, VII, IX, X, XV, and XVI. For some of these myosins (e.g., classes IX and XVI), the functions have not been well characterized in the nervous system. In the first part, I will discuss the functions of myosins in neurons, focusing on classes

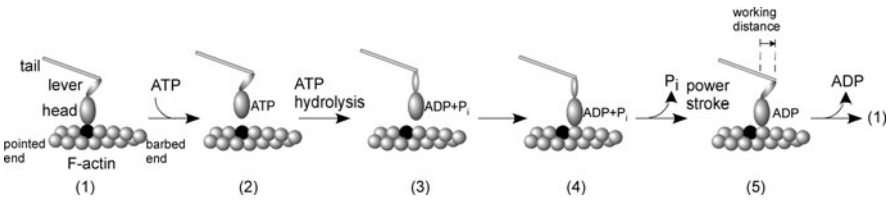

Fig. 4.2 ATPase cycle and actin binding of myosin motors. (*1*) In the absence of ATP, myosin binds tightly to F-actin. (*2*) ATP binding to the head weakens the affinity for actin and causes the motor to detach. (*3*) ATP hydrolysis results in the recovery stroke that moves the head forward. (*4*) The ADP/P_i state rebinds to the actin filament. (*5*) Following the release of phosphate, the power stroke moves the lever forward. Then ADP is released and the cycle begins again at a new position on the actin filament

I, II, V, and VI. In the second part, I will summarize the different myosin motor functions in the best-studied sensory cells, the hair cells of the inner ear, and the photoreceptors in the eye.

4.2 Myosins in Neurons

Neurons possess a high degree of polarity, and this enables them to effectively transmit signals in a directed fashion. The initial establishment and the maintenance of the axonal and somatodendritic compartments in neurons depend on both the F-actin and microtubule cytoskeleton (Chapter 5). As discussed in Chapter 2, 3, 5, and 10, specific neuronal compartments such as peripheral domains of growth cones, presynaptic terminals, and dendritic spines are particularly rich in F-actin. While the polarity of the actin filaments in some of these compartments is not completely understood, different myosins have been implicated in growth cone motility, axonal outgrowth, organelle transport, synaptic function, and plasticity.

4.2.1 Class I Myosins

Myo1 is a single-headed, nonfilament-forming motor with a neck domain and a tail. The tail can be short and basic (tail homology 1, TH1 domain) or longer and include an SH3 domain (Fig. 4.1; Mooseker and Cheney 1995, Berg et al. 2001). In humans and mice, eight different class I myosin heavy chain genes have been identified, *Myo1a–Myo1h*, two of which (*Myo1e* and *Myo1f*) are long tailed and include the SH3 domain (Berg et al. 2001). Four of the eight *Myo1* genes are expressed in the nervous system, *Myo1b* (rat myr 1), *Myo1c* (rat myr 2), *Myo1d* (rat myr 4), and *Myo1e* (rat myr 3). Myo1b is widely expressed in the rodent brain and spinal cord (Ruppert et al. 1993, Sherr et al. 1993). Its peak levels of expression occur in the late embryonic and early postnatal stages, declining thereafter. In growth cones of cultured rat superior cervical ganglion (SCG) neurons, Myo1b has been localized close to the upper and lower plasma membrane and on both F-actin bundles and meshwork (Lewis and Bridgman 1996). Thus, Myo1b could have a role in growth cone structure, adhesion, and motility (Fig. 4.3). Although Myo1b is associated with tubulovesicular structures in rat SCG neurons (Lewis and Bridgman 1996) and moves bi-directionally in neurites (Bridgman 1999), there are no functional data to indicate that Myo1b is required for organelle transport in axons.

Myo1c exhibits a wider tissue expression pattern than does Myo1b, but at lower levels in the brain (Wagner et al. 1992, Sherr et al. 1993, Ruppert et al. 1995). Very little Myo1c staining was reported for growth cones of rat SCG neurons (Brown and Bridgman 2003b). However, micro chromophore-assisted laser inactivation (CALI) of Myo1c in chick dorsal root ganglion (DRG) growth cones implicated Myo1c in driving F-actin flow and growth cone motility (Diefenbach et al. 2002). On the other hand, CALI of Myo2b had little effect on retrograde flow (Diefenbach et al. 2002),

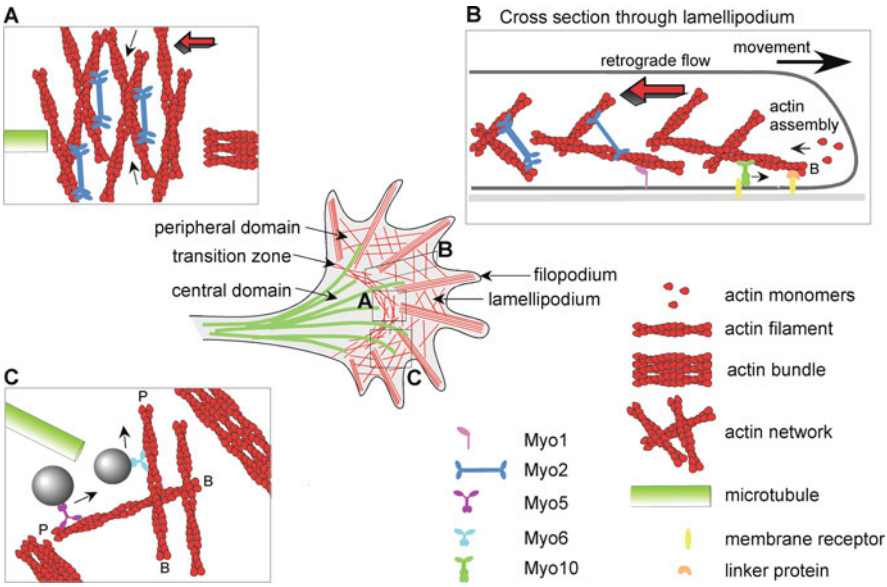

Fig. 4.3 Myosin functions in growth cones. Schematic in the center shows regional organization of the growth cone and the distribution of the actin (*red*) and microtubule (*green*) cytoskeleton. Insets reveal well-described and potential functions of myosin motors. (**A**) In the transition zone, Myo2 forms bipolar filaments mediating network contractions that drive retrograde F-actin flow. (**B**) Cross section through lamellipodium depicting actin networks undergoing retrograde flow. Membrane receptor (*yellow*) and linker protein can couple to actin network. Membrane-bound Myo1 could drive retrograde flow. Receptor-bound Myo10 could move receptor anterogradely or drive retrograde flow. (**C**) Vesicle transport on actin filaments by either Myo5 [barbed (B) end directed] or Myo6 [pointed (P) end directed]

which is at variance with increased retrograde actin flow rates in growth cones from Myo2b knockout mice (Brown and Bridgman 2003b). To better dissect the role of Myo1c and other myosins in growth cone motility, it will be essential to visualize where each motor protein resides relative to the substrate during outgrowth.

While Myo1b and Myo1c are present in axons and growth cones, Myo1d has been localized to neuronal cell bodies and dendrites (Bahler et al. 1994). It is expressed in most parts of the nervous system, and expression levels reach maximal values during adulthood (Bahler et al. 1994). Myo1e has been detected in brain tissue (Stoffler et al. 1995), but not at the subcellular level. Myo1e interacts with both synaptojanin-1 and dynamin in biochemical assays, and expression of the Myo1e tail inhibits receptor-mediated endocytosis in HeLa cells (Krendel et al. 2007). Thus, Myo1e could be involved in actin-dependent membrane turnover and endocytosis at the synapse, particularly since myosin I proteins regulate endocytosis in yeast (Jonsdottir and Li 2004, Sun et al. 2006). In summary, expression patterns and subcellular locations of class I myosins indicate that they may have a role in growth cone motility, organelle transport, and membrane trafficking; however, more

isoform-specific knockdown approaches and high-resolution imaging studies are needed to confirm these potential functions.

4.2.2 Class II Myosins

Myo2 is the conventional double-headed motor protein, comprised of heavy chains that contain two IQ motifs followed by a long coiled-coil tail domain, which allows the formation of bipolar filaments (Fig. 4.1). Nonmuscle Myo2 is present in all eukaryotic cells and involved in essential cellular functions that require sliding of adjacent actin filaments against each other, such as cytokinesis, contraction of the actin ring at adherens junctions, and cell migration. Nonmuscle Myo2 is without doubt the best-studied neuronal myosin motor with well-established functions in neuronal migration, axonal growth, and guidance (Brown and Bridgman 2003a, 2004). Three different nonmuscle Myo2 heavy chains have been identified in vertebrates: Myo2a, Myo2b, and Myo2c (Kawamoto and Adelstein 1991, Golomb et al. 2004). Both Myo2a and Myo2b have overlapping but distinct expression patterns in the nervous system, Myo2b being more abundant than Myo2a (Kawamoto and Adelstein 1991, Itoh and Adelstein 1995). Myo2a and Myo2b are present in cell bodies, axons, and growth cones of cultured rat DRG neurons (Miller et al. 1992). In rat SCG growth cones, Myo2a and Myo2b staining is concentrated in the central and transition domains (Rochlin et al. 1995). The low Myo2c expression levels during development suggest that Myo2c most likely is not involved in neuronal migration or axonal outgrowth (Golomb et al. 2004).

4.2.2.1 Myosin II in Neuronal Migration and Cell Adhesion

Myo2b knockout mice have major heart and brain defects and die between embryonic day 14.5 (E14.5) and birth (Tullio et al. 2001). Two recent studies revealed that Myo2 activity is critical early in CNS development for interkinetic nuclear migration of neuronal progenitor cells in both cortex and retina (Schenk et al. 2009, Norden et al. 2009). Aberrant neuronal cell migration in Myo2b knockout mice caused accumulation of facial neurons into the fourth ventricle, resulting in hydrocephalus (Tullio et al. 2001, Ma et al. 2006). Myo2b may have a scaffolding function independent of its functional motor domain, affecting cell adhesion of neuroepithelial cells facing the spinal canal (Ma et al. 2007). Reduced cell adhesion in the Myo2b mutant causes neuroepithelial cells to invade the spinal canal and obstruct cerebrospinal fluid flow. Less is known about in vivo nervous system function of Myo2a, since knockout mice die much earlier (E6.5), probably due to a decreased cell–cell adhesion (Conti et al. 2004). More recently, experiments with the chemical inhibitor blebbistatin have revealed that Myo2 plays a critical role in forward pulling of both the centrosome and the soma during glial-guided neuronal cell migration in vitro and in vivo and that this response is regulated by the cell polarity protein Par6α (Schaar and McConnell 2005, Solecki et al. 2009).

4.2.2.2 Myosin II in Axonal Growth

Both F-actin assembly/disassembly and actomyosin contractility control growth cone motility and axonal outgrowth (Chapters 2 and 3). F-actin assembly in growth cones occurs at the filopodial tips and the leading edge by addition of G-actin to filaments in both polarized bundles and less polarized F-actin networks, respectively (Fig. 4.3). After assembly, F-actin structures undergo retrograde translocation by a process referred to as retrograde F-actin flow, followed by severing and depolymerization in the transition zone (Forscher and Smith 1988, Lin and Forscher 1995, Mallavarapu and Mitchison 1999, Schaefer et al. 2002). What is the driving force of retrograde flow? Initial studies in *Aplysia* growth cones using general myosin inhibition approaches revealed a strong reduction of retrograde flow (Lin et al. 1996). Using a more specific Myo2 inhibitor, blebbistatin, the Forscher lab recently demonstrated that actin flow is reduced by 50% (Medeiros et al. 2006). When actin assembly was blocked in combination with Myo2 inhibition, flow was further reduced, indicating that, in addition to Myo2 activity, actin assembly pushing against the plasma membrane contributes to retrograde flow. All these findings, along with the presence of bipolar Myo2a and Myo2b mini-filaments in the transition zone (Bridgman 2002), suggest that a mechanism similar to the dynamic network contraction model established in fish keratocytes (Svitkina et al. 1997) may drive retrograde flow in growth cones (Fig. 4.3).

What is the role of retrograde flow in cell movement? When retrogradely moving actomyosin networks are linked to extracellular substrates via cell adhesion receptors, flow can be harnessed for forward movement according to the substrate–cytoskeletal coupling model (Fig. 4.3; Mitchison and Kirschner 1988, Suter and Forscher 2000). According to this model, actin flow is specifically reduced in the axis of growth. Simultaneously, there is an increase in both protrusive growth at the leading edge and tension between the growth cone peripheral and central domain, which ultimately pulls the central domain forward. This was experimentally observed in growth cones interacting with specific adhesion protein substrates presented on microbeads (Suter et al. 1998). Consistent with this model, acute Myo2 inhibition results in reduced actin flow, reduced growth cone motility, and increased leading edge advance (Lin et al. 1996, Bridgman et al. 2001, Medeiros et al. 2006), while chronic Myo2 inhibition results in reduced axonal growth and guidance (Wylie et al. 1998, Wylie and Chantler 2001, Turney and Bridgman 2005).

What is known about Myo2a- and Myo2b-specific functions in growth cone motility and axonal growth? Application of Myo2a-specific antisense oligonucleotides to Neuro-2A neuroblastoma cells resulted in reduced neuronal adhesion and reduced lysophosphatidate (LPA)- or thrombin-induced neurite retraction, while neurite length was unaffected (Wylie and Chantler 2001, 2003). Repulsive cues such as LPA, semaphorin 3A, and ephrin A2 activate the Rho–Rho kinase pathway, which results in increased Myo2 light chain phosphorylation and Myo2-dependent axonal retractions (Amano et al. 1998, Gallo et al. 2002, Wylie and Chantler 2003, Gallo 2006), which are likely involved in refining neuronal connections. A recent study provided evidence that semaphorin 3A causes Myo2a redistribution from the

growth cone into the neurite, thereby facilitating collapse, while Myo2b mediates semaphorin 3A-induced neurite retraction (Brown et al. 2009). On the other hand, Myo2b has also been implicated in neurite outgrowth and turning (Wylie et al. 1998, Tullio et al. 2001, Wylie and Chantler 2001, Turney and Bridgman 2005), traction force production, and growth cone motility (Bridgman et al. 2001), as well as retrograde flow (Brown and Bridgman 2003b). The mechanism of Myo2-regulated axonal growth is dependent on the substrate: on laminin, Myo2 mediates adhesion, while on poly-lysine, Myo2 prevents microtubule advance into the growth cone periphery (Ketschek et al. 2007). Furthermore, Myo2 negatively regulates the development of neuronal polarity (Kollins et al. 2009). In summary, a large body of evidence indicates that Myo2 regulates various aspects of neuronal growth cone motility and axonal growth.

4.2.2.3 Myosin II in Synapse Function

Myo2b has been detected in different neurons both pre- and postsynaptically (Miller et al. 1992, Mochida et al. 1994, Takagishi et al. 2005, Ryu et al. 2006). In cultured rat SCG neurons, neurotransmitter release was reduced when a recombinant Myo2b heavy chain tail fragment was injected, but not when a corresponding Myo2a fragment was used (Takagishi et al. 2005), confirming earlier studies that implicated Myo2 in transmitter release (Mochida et al. 1994). In the CNS, dynamic dendritic spine morphology, motility, and synaptic function of hippocampal neurons depend on Myo2b activity (Ryu et al. 2006); however, the details of how Myo2b is involved in dendritic spine morphology as well as in synaptic vesicle release are not known. A recent ultra-structural localization study by the Svitkina lab identified Myo2 in the neck of dendritic spines of hippocampal neurons, suggesting a role of actomyosin contractility in spine development (Korobova and Svitkina 2010).

4.2.3 Class V Myosins

The polarized morphology of neurons and the extremely long axons require an efficient organelle and protein transport system for both neuronal development and function. While axonal long-range transport is mainly mediated by the microtubule-based motors kinesins and dyneins, short-range transport at specific locations such as growth cones, presynaptic terminals, and dendritic spines is achieved by actin-based myosin motors. Myo5 is a barbed end-directed, two-headed motor whose heavy chains contain six IQ motifs followed by a coiled-coil domain and a globular tail (Fig. 4.1; Cheney et al. 1993). Myo5 has several properties that make it an effective organelle motor (Fig. 4.1): (1) the globular tail binds cargo via adaptor proteins; (2) the coiled-coil domain allows the formation of a double-headed motor; (3) the high affinity for F-actin results in attachment of at least one head at all times; (4) the long neck allows Myo5 to take large (36 nm) steps, making it a highly processive motor (reviewed in Sellers and Veigel 2006). A variety of biochemical, structural, genetic, and cellular studies largely performed in neurons, melanocytes, and yeast

have made Myo5 the best characterized organelle motor within the myosin superfamily (Reck-Peterson et al. 2000, Langford 2002, Bridgman 2004). Many studies addressing the organelle motor function of Myo5a have used the *dilute-lethal* mice, which carry a null mutation in the *Myo5a* gene, exhibit a lightened coat color, seizures and other neurological defects, and die around 3 weeks of age (Mercer et al. 1991).

Three different members of class V myosins have been identified in vertebrates: Myo5a, Myo5b, and Myo5c, all exhibiting wide but distinct expression patterns (Rodriguez and Cheney 2002). Myo5a is strongly expressed in the brain and in other parts of the nervous system (Espindola et al. 1992, Espreafico et al. 1992, Rodriguez and Cheney 2002). Both Myo5b and Myo5c are less abundant than Myo5a in the brain but exhibit higher expression in kidney, liver, colon, and placenta (Myo5b), and in colon, pancreas, salivary gland, and stomach (Myo5c) (Zhao et al. 1996, Rodriguez and Cheney 2002, Lise et al. 2006).

4.2.3.1 Myosin V in Axonal Transport, Growth Cones, and Presynaptic Terminals

Several studies provided evidence for actin-based organelle transport in axons and growth cones (Kuznetsov et al. 1992, Evans and Bridgman 1995, Morris and Hollenbeck 1995), and implicated Myo5a as the relevant motor (Prekeris and Terrian 1997, Evans et al. 1998, Tabb et al. 1998, Bridgman 1999). Myo5a was detected predominantly in the central domain and transition zone of growth cones (Evans et al. 1997, Suter et al. 2000). Evans et al. (1997) showed that neurite outgrowth, growth cone morphology, and cytoskeletal organization of *dilute-lethal* SCG growth cones are normal. A different growth cone phenotype was reported in another study, which showed that local Myo5a inactivation by micro-CALI affects filopodia extension dynamics (Wang et al. 1996). The filopodia effects in the CALI study could be due to reduced membrane traffic to the filopodia. On the other hand, Myo5a might have a role in axonal extension, since climbing fibers of *dilute* Purkinje cells exhibit reduced innervation (Takagishi et al. 2007).

With which organelles is Myo5a associated in axons and growth cones? In vitro motility, immunolocalization, and biochemical studies revealed Myo5a association and Myo5a-mediated transport of large endoplasmic reticulum (ER) vesicles (Tabb et al. 1998), synaptic vesicle precursors (Evans et al. 1998, Miller and Sheetz 2000), and potential synaptic vesicles (Prekeris and Terrian 1997). In addition, mitochondria are good candidates for cargo organelles in axons (Hollenbeck and Saxton 2005). Finally, Myo5a associates with neurofilaments, implicating a role of this interaction for the proper distribution of neurofilaments in axons (Rao et al. 2002). This hypothesis was confirmed when green fluorescent protein (GFP)-tagged neurofilaments were observed in axons of dilute-lethal mice (Alami et al. 2009). Fluorescent live cell imaging in both normal and *dilute-lethal* axons further revealed that Myo5a-associated vesicles move along microtubules over long distances and use actin/Myo5a-based transport for local movements, such as in presynaptic terminals (Bridgman 1999). Interestingly, the presynaptic terminals in *dilute-lethal*

mice have increased cross-sectional area and greater numbers of synaptic vesicles (Bridgman 1999). These findings suggest that Myo5a may act as modulator of vesicle transport slowing down microtubule/kinesin-based anterograde transport into the presynaptic terminal. In conclusion, studies in both neurons and melanocytes have shown that multiple microtubule- and actin-based motors reside on organelles that may become engaged/activated at specific subcellular sites during the journey of an organelle (Bridgman 1999, Gross et al. 2002, Levi et al. 2006). This model is particularly intriguing in light of the fact that the cargo-binding domain of Myo5a directly binds to kinesin, thus forming a hetero-motor complex for fast switching of the cytoskeletal track system (Huang et al. 1999).

Based on the studies discussed above, one might expect a potential role for Myo5a in synaptic vesicle release. Both pre- and postsynaptic function of *dilute* hippocampal CA3-CA1 synapses appeared normal when compared to littermate controls (Schnell and Nicoll 2001). In agreement with this study, no effects of Myo5a on synaptic transmission were reported for SCG neurons from Myo5a mutant rats (Takagishi et al. 2005). On the other hand, Myo5a binds to the t-SNARE syntaxin-1A and mediates Ca^{2+}-induced exocytosis of vesicles in chromaffin cells (Watanabe et al. 2005). Thus, the presynaptic function of Myo5a is still controversial and may depend on the specific neuronal connection. In addition, other myosins present in presynaptic terminals of Myo5a mutant animals may compensate for the lack of functional Myo5a.

4.2.3.2 Myosin V in Dendrites and Postsynaptic Function

The roles of Myo5a and Myo5b are better defined on the postsynaptic side and in dendrites where both myosins have been localized (Espindola et al. 1992, Naisbitt et al. 2000, Walikonis et al. 2000, Lise et al. 2006). Indirect binding of Myo5a to postsynaptic density-95 (PSD-95) suggests a potential role for Myo5a in the transport of the PSD-95 complex (Naisbitt et al. 2000). Myo5a activity is required for smooth ER localization in dendritic spines, local calcium release, and postsynaptic functions such as long-term synaptic depression (Dekker-Ohno et al. 1996, Takagishi et al. 1996, Miyata et al. 2000). Furthermore, Myo5a facilitates the transport of the mRNA-binding protein TLS and its target RNA Nd1-L in a Ca^{2+}-dependent manner into dendritic spines of mouse hippocampal neurons, implicating a role for Myo5a in synaptic plasticity (Yoshimura et al. 2006). Myo5a and Myo5b have been implicated in dendritic trafficking and recycling of the AMPA-type glutamate receptor subunit GluR1, the dendritic K^+ channel Kv4.2, as well as of the muscarinic acetylcholine receptor subunit M4, although the functional involvement of these two myosin V isoforms in synaptic plasticity has remained somewhat controversial (Volpicelli et al. 2002, Lise et al. 2006, Correia et al. 2008, Lewis et al. 2009). Myo5b appears to be an important Ca sensor in synaptic plasticity. Ca influx after NMDA receptor activation causes rapid recruitment of Myo5b to recycling endosomes and thereby exocytosis of AMPA receptors (Wang et al. 2008). How is the specificity between motor proteins and cargo receptors achieved? Increasing evidence from both neuronal and melanocyte systems indicates that the

small GTPases of the Rab family and linker proteins may provide such specificity (Seabra and Coudrier 2004). In summary, class V myosin motors modulate long-range organelle transport and control short-range transport to specific neuronal compartments, thereby supporting the development and function of neuronal connections.

4.2.4 Class VI Myosins

Myo6 contains a head, a short neck region with one IQ motif, and a C-terminal tail domain that is capable of dimerization (Fig. 4.1; Kellerman and Miller 1992, Hasson and Mooseker 1994, Buss et al. 2004). Despite the presence of only one IQ motif, each heavy chain binds two calmodulins (Bahloul et al. 2004). Myo6 has some special structural features that result in mechanochemical properties that are unique from other myosins and enable Myo6 to perform special functions. The most intriguing feature of Myo6 is the fact that it moves "backward" toward the pointed end of actin filaments (Wells et al. 1999). Another special feature of Myo6 is that it appears to exist both as a monomer and a dimer, perhaps with cargo binding inducing dimerization (Park et al. 2006, Sweeney and Houdusse 2007). This mechanism would allow the formation of the processive, dimerized motor at the right subcellular location, e.g., the plasma membrane, enabling Myo6 to carry out its well-described function in endocytosis (Buss et al. 2004, Sweeney and Houdusse 2007). Myo6 was originally identified in *Drosophila melanogaster* (Kellerman and Miller 1992), where it has been implicated in several functions, including transport of cytoplasmic particles in the embryo (Mermall et al. 1994). In mammalian cells, Myo6 has been localized to membrane ruffles, endocytotic vesicles, the cytosol, and the Golgi complex, where it has both transport and anchoring roles in membrane traffic, cell adhesion, and migration (Buss et al. 2004, Sweeney and Houdusse 2007).

4.2.4.1 Myosin VI in Nervous System Development

What is known about Myo6 functions in the nervous system? It is expressed in all major parts of the mouse brain at similar levels from postnatal day 1 (P1) into adulthood (Osterweil et al. 2005). In chick brains, Myo6 was detected as early as E6, while in DRGs expression starts from E10 on (Suter et al. 2000). Within cultured neurons, Myo6 is found in all subcellular compartments (Suter et al. 2000, Osterweil et al. 2005). In chick DRG growth cones, Myo6 shows the highest concentration in the central domain similarly to Myo5a; however, Myo6a is less cytoskeleton-associated than Myo5a (Suter et al. 2000). Could Myo6 be involved in growth cone motility and axonal outgrowth? Considering that Myo6 is a pointed end-directed motor that can bind to plasma membrane (Spudich et al. 2007), one could imagine a mechanism by which membrane-bound Myo6 moves actin filaments forward in the direction of growth; however, there is as yet no experimental evidence for this model. Myo6 is also involved in early neuronal development in *Drosophila.* Myo6

is required for basal localization of Miranda, an adaptor protein which in turn localizes cell fate determinants Prospero and Staufen to the basal side of the neuroblast (Petritsch et al. 2003).

4.2.4.2 Myosin VI in Synaptic Function

Because of its transport and anchoring properties, Myo6 could mediate complex membrane-trafficking events at the synapse. A number of binding partners have been identified for Myo6, including Dab2, GIPC, and SAP97 (Buss et al. 2004). GIPC is an adapter protein between the BDNF receptor TrkB and Myo6 in hippocampal neurons (Yano et al. 2006). This interaction is important for specific presynaptic functions, including BDNF–TrkB-mediated long-term potentiation and enhancement of glutamate release (Yano et al. 2006). A recent study implicated Myo6 in postsynaptic structure and AMPA-type glutamate receptor endocytosis (Osterweil et al. 2005). Homozygous *Snell's waltzer* mice that lack Myo6 exhibit fewer synapses and shorter dendritic spines in the hippocampal CA1 region when compared with heterozygous mice. Furthermore, hippocampal neurons from *Snell's waltzer* mice show reduced AMPA-type receptor GluR1 subunit internalization because of disrupted clathrin-mediated endocytosis (Osterweil et al. 2005). The interaction between Myo6 and the GluR1–AMPA receptor is mediated by the adapter protein SAP97 and important for AMPA receptor trafficking (Wu et al. 2002, Nash et al. 2010). In summary, Myo6 forms specific complexes with membrane proteins at the pre- and postsynaptic sites and thereby regulates both synaptic vesicle release and neurotransmitter receptor trafficking. Considering the well-established role of Myo6 in membrane trafficking in other cellular systems, more discoveries on Myo6's actions in the nervous system are expected in the future.

4.2.5 Classes IX, X, and XVI Myosins

I discuss myosins of these three classes briefly, since they have been detected in neuronal tissues, but are not well characterized with respect to functions in the nervous system.

4.2.5.1 Class IX Myosins

The hallmark structural feature of class IX myosins is the GTPase-activating protein (GAP) domain in the tail, which activates Rho but not Rac or Cdc42 (Fig. 4.1; Muller et al. 1997, Post et al. 1998). Additional structural features are an N-terminal extension of the head domain, a highly basic insertion at the presumed actin-binding site, a neck region with 4–6 IQ motifs, and a zinc-binding motif in front of the GAP domain. The best-characterized class IX myosins include Myo9b (rat myr 5) and Myo9a (rat myr 7) (Reinhard et al. 1995, Wirth et al. 1996, Chieregatti et al. 1998). Biophysical and biochemical studies suggest that Myo9b is a single-headed, processive motor that binds actin with high affinity (Post et al. 1998, 2002, Kambara

and Ikebe 2006). In the developing and adult brain, Myo9a is expressed at higher levels than Myo9b (Chieregatti et al. 1998). While no data on potential class IX myosin functions in the nervous system are currently available, the developmental expression pattern and the Rho GAP activity indicate that at least Myo9a may be involved in actin-dependent developmental processes, e.g., neuronal migration, axonal growth, or synaptogenesis.

4.2.5.2 Class X Myosins

Only one member of class X myosins (Myo10) has been identified in vertebrates so far (Berg et al. 2001). Following the head domain, the neck with three IQ motifs, and a short coiled-coil region, the Myo10 tail contains three pleckstrin homology (PH) domains implicated in phosphoinositide signaling, a myosin tail homology 4 (MyTH4) domain, and a band 4.1/ezrin/radixin/moesin (FERM) domain for binding to transmembrane proteins (Fig. 4.1; Berg et al. 2000). Expression of both full-length and headless isoforms of Myo10 in the brain is developmentally regulated (Sousa et al. 2006). Myo10 is enriched at the leading edge of lamellipodia, tips of filopodia, and membrane ruffles in several cell lines, including the neuronal CAD cells (Berg et al. 2000, Sousa et al. 2006). Furthermore, Myo10 undergoes intrafilopodial motility and functions in filopodia formation (Berg and Cheney 2002, Bohil et al. 2006). Interestingly, it can act as a linker between actin filaments and β-integrin receptors (Zhang et al. 2004). This linker function may likely play role in axonal outgrowth; thus, the actin/Myo10/integrin complex may be involved in the redistribution of adhesion receptors or in substrate–cytoskeletal coupling (Fig. 4.3). In support of a role for Myo10 in axonal guidance, it has been shown that the FERM domain of Myo10 interacts with the DCC, a receptor for the guidance molecule netrin-1 (Zhu et al. 2007). Importantly, both expression of a headless Myo10 and suppression of Myo10 expression by siRNA reduced DCC localization at the tips of neurites and affected netrin-induced neurite outgrowth and axonal guidance. Because of its linker function, Myo10 is likely to mediate additional interactions between receptors and the cytoskeleton that are important for axonal growth and guidance.

4.2.5.3 Class XVI Myosins

Myo16 was identified as rat myr 8 during a search for myosins involved in cerebellar granule neuron migration (Patel et al. 2001). The motor domain is preceded by eight ankyrin repeats and followed by a single IQ domain and either a short or a longer C-terminal tail in the Myo16a or Myo16b isoform, respectively (Fig. 4.1; Patel et al. 2001). Myo16b is expressed in various brain regions, particularly between P0 and P10, and has been detected in cerebellar granule neurons both in vitro and in vivo (Patel et al. 2001). Heterologous expression of Myo16b in COS-7 cells revealed that the C-terminal tail can target this motor to the nucleus during the interphase and that it may have a role in cell cycle control (Cameron et al. 2007); however, it remains to be determined if Myo16b indeed plays a role in neuronal migration.

4.3 Myosins in Sensory Cells

Sensory cells have developed a high degree of functional specialization to convert various stimuli into electrical signals that are transmitted to the nervous system. In the case of hair cells of the inner ear and photoreceptors of the retina, special cytoskeletal and membranous compartments support these signal transduction functions. The following section summarizes the key functions of several myosins that have been extensively characterized in these two sensory cells. I refer to a number of reviews for a more detailed discussion of the experimental evidence for myosin functions in hair cells and photoreceptors (Brown and Bridgman 2004, Gillespie and Cyr 2004, El-Amraoui and Petit 2005, Lin et al. 2005).

Inner ear hair cells are probably the best-characterized cell type with respect to the number of different myosins. At least six myosins are essential for hearing and balance based on mutants characterized in both mice and humans, including Myo1a, Myo2a, Myo3a, Myo6, Myo7a, and Myo15a (Friedman et al. 1999, Gillespie and Cyr 2004, El-Amraoui and Petit 2005). To transduce mechanical forces into electrical signals, hair cells develop highly organized F-actin-rich protrusions called stereocilia that are interconnected with cadherin molecules. Classes I, II, III, VI, VII, and XV myosins have been localized to specific subcellular regions in the hair cell where they have distinct roles for the development, maintenance, and sensory function of this cell (Fig. 4.4). In the photoreceptor cell, classes II, III, V, VI, and VII myosins have been detected, of which classes III and VII have been characterized the best with respect to a role in vision.

4.3.1 Class I Myosins

Three of the eight class I myosins in higher vertebrates, Myo1b, Myo1c, and Myo1e, are strongly expressed in cochlea and vestibular organs of rodents at birth (Dumont et al. 2002). While mutations in the *Myo1a* gene have been linked to deafness in humans but not in mice, there is no information available about the localization of Myo1a in the inner ear (Donaudy et al. 2003, Tyska et al. 2005). Myo1b is mainly expressed in the supporting cells that surround the hair cells and in an apical ring of the hair cell (Dumont et al. 2002). Myo1c is enriched at tips of stereocilia and in the pericuticular necklace of vestibular hair cells, and uniformly distributed along the stereocilia of auditory hair cells (Fig. 4.4; Gillespie et al. 1993, Hasson et al. 1997, Dumont et al. 2002, Schneider et al. 2006). Myo1c is an important component of the hair cell's adaptation complex that allows closing of the transduction channel in both the slow and fast adaptation mechanisms by releasing tension (Holt et al. 2002, Gillespie and Cyr 2004, Stauffer et al. 2005). Myo1c is reported to interact via its neck domain with PIP_2 and cadherin 23, the presumptive tip link, at the tips of stereocilia (Siemens et al. 2004, Hokanson and Ostap 2006, Phillips et al. 2006). This interaction enables Myo1c to reposition the tip complex during adaptation. Myo1e was detected in the cuticular plate of cochlear and vestibular hair cells (Dumont et al. 2002); however, there are no experimental data on the role of Myo1b or Myo1e in hair cells.

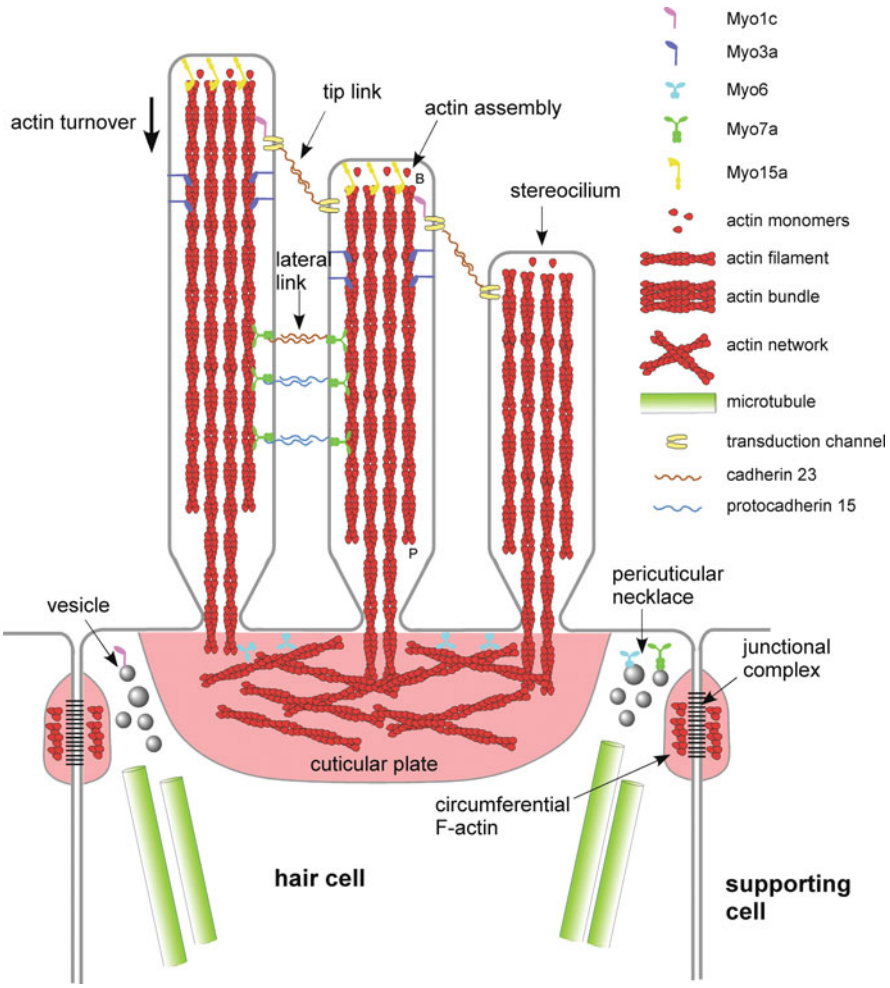

Fig. 4.4 Myosin functions in hair cells. Schematic cross section through the apical side of an inner ear hair cell depicting the location of different myosins with important functions in hair cell development and function. Myo15a forms a cap on top of the F-actin bundles where it controls the length of stereocilia. Actin filaments are oriented with barbed (B) end toward the tip of stereocilia and the pointed (P) end toward the base. Myo1c is found at the tip of stereocilia where it adjusts the position of the transduction channel during adaptation. Myo3a is located in a ring around the tip of stereocilia and controls their shape. Myo6 may connect the apical plasma membrane to the F-actin network in the cuticular plate and connect the stereocilia to the plate. Myo7a is located along the side of the stereocilia and stabilizes them via lateral links. Myo1c, Myo6, and Myo7 are also found in the pericuticular necklace, a vesicle-rich area around the cuticular plate where they could mediate organelle traffic

4.3.2 Class II Myosins

A mutation in Myo2a results in the human nonsyndromic deafness DFNA17 (Mhatre et al. 2006). Myo2a has been localized along the stereocilia of mouse cochlear hair cells and may stabilize the stereocilia (Mhatre et al. 2006). A similar function for class II myosins has been found in the retina. In *Drosophila* photoreceptors, nonmuscle myosin II *zipper* forms two stripes of actomyosin extending along the sides of each of the R1–6 rhabdomeres, suggesting that this cytoskeletal system may be important for the alignment of the rhabdomeres with the optical axis (Baumann 2004). This hypothesis was supported by the distorted rhabdomeres in *zipper* flies carrying a mutation in the motor domain (Baumann 2004).

4.3.3 Class III Myosins

Class III myosins, Myo3a and Myo3b, are single-headed motors that have an N-terminal kinase domain, a neck with variable IQ motifs, and a short tail (Fig. 4.1; Dose and Burnside 2000, Berg et al. 2001). The kinase domain has serine–threonine kinase activity (Ng et al. 1996) and is essential in *Drosophila* phototransduction (Porter and Montell 1993). Class III myosins were originally identified in *Drosophila* as two proteins encoded by the *ninaC* gene (Montell and Rubin 1988). NinaC mutant flies exhibit reduced amounts of rhodopsin in the photoreceptors and abnormal electroretinograms. The exact role of Myo3 in the photoreceptor rhabdomeres is still unclear; it may have structural role (Hicks et al. 1996), a signaling (Porter and Montell 1993, Wes et al. 1999), or transport function for components of the phototransduction machinery (Cronin et al. 2004, Lee and Montell 2004, Liu et al. 2008). An essential role for Myo3a in inner ear function is indicated by the fact that point mutations in the motor domain lead to the human nonsyndromic hearing loss DFNB30 (Walsh et al. 2002). Myo3a has recently been localized in a thimble-like pattern around the tips of the hair cell stereocilia (Fig. 4.4; Schneider et al. 2006). Deletion of the kinase domain caused lengthening of stereocilia and bulging of the tips, suggesting that Myo3a might regulate actin bundle maintenance, transport of the transduction complex to the stereocilia tip, or be part of the tip complex itself (Schneider et al. 2006). A recent study provided new insights into the role of Myo3a in hair cells showing that this motor regulates the length of stereocilia by transporting the actin-bundling protein espin 1 to the plus end of actin filaments (Salles et al. 2009).

4.3.4 Class V Myosins

Elegant genetic studies in *Drosophila* by the Ready lab have recently revealed that the organelle motor Myo5 with the help of the small GTPase Rab11 and the linker protein dRip11 transports rhodopsin-containing secretory vesicles to the photoreceptor rhabdomeres, which are critical for photoreceptor morphogenesis

(Li et al. 2007). Furthermore, the same laboratory also reported that calcium-activated Myo5 transports pigment granules to the rhabdomeres in response to bright light in order to close the fly pupil (Satoh et al. 2008). Thus, Myo5 is important for both photoreceptor development and physiology.

4.3.5 Class VI Myosins

Myo6 has been localized to the actin-rich cuticular plate of hair cells, indicating that it could be important for the anchoring of the stereocilia or membrane–actin interactions (Fig. 4.4; Hasson et al. 1997). A role for Myo6 in hair cell function was originally established when a recessive mutation in Myo6 was identified as the cause of deafness observed in the *Snell's Waltzer* mouse in which hair cells degenerate (Avraham et al. 1995). Myo6 mutations have also been linked to deafness in humans (Melchionda et al. 2001, Ahmed et al. 2003). During hair cell development in both *Snell's Waltzer* mice and zebrafish Myo6b mutants, the apical surface protrudes and stereocilia fuse together, suggesting that Myo6 is critical for anchoring the apical plasma membrane to the underlying actin-rich cuticular plate (Self et al. 1999, Kappler et al. 2004, Seiler et al. 2004). In photoreceptor cells, Myo6 has been localized to the inner segments, and *Snell's Waltzer* photoreceptors show a decreased responsiveness (Kitamoto et al. 2005). In summary, evidence from several systems implicates Myo6 in the development and function of inner ear hair and photoreceptor cells.

4.3.6 Class VII Myosins

Class VII myosins have a head, five IQ motifs, a short coiled-coil domain, and two sets of MyTH4 and FERM domains in the C-terminal tail that are separated by an SH3 domain (Fig. 4.1; Berg et al. 2001). Like Myo6, the short coiled-coil domain could dimerize upon cargo or actin binding and thereby generate a processive motor (Yang et al. 2006). Class VII myosins are expressed in a wide range of organisms from dictyostelium to humans and are involved in adhesion, phagocytosis, and organelle transport (Berg et al. 2001, Krendel and Mooseker 2005). Myo7a is expressed in retina, cochlea, kidney, and testis (Hasson et al. 1995). Mutations in Myo7a result in the most frequent and severe form of deafness and blindness in humans, called Usher 1 syndrome (Weil et al. 1995, El-Amraoui and Petit 2005), nonsyndromic deafness in humans (Liu et al. 1997, Weil et al. 1997), as well as deafness in mice (*shaker-1*) (Gibson et al. 1995). Stereocilia of *shaker-1* mice hair cells do not develop normally, implicating Myo7a in the assembly of the actin bundles (Self et al. 1998). Myo7a is localized to the pericuticular necklace and along the stereocilia in close association with the lateral connections mediated by cadherin 23 and protocadherin 15; thus, Myo7a may be an intracellular anchor for these linkages in order to maintain the integrity of the hair bundle (Fig. 4.4; Hasson et al. 1997, El-Amraoui and Petit 2005). Recent mosaic complementation experiments provided

evidence that Myo7a regulates the length of stereocilia by affecting actin dynamics (Prosser et al. 2008). Finally, hair cell currents are affected in certain *shaker-1* mutants, suggesting that Myo7a could also participate in hair cell adaptation (Kros et al. 2002).

In photoreceptors, Myo7a has been localized to the cilium that connects the inner and outer segments, where it transports opsin into the outer segment (Liu et al. 1999, Wolfrum and Schmitt 2000). Myo7a is highly expressed in the pigment epithelial cells, which constantly absorb the shed-off membrane discs of the outer photoreceptor segments. In *shaker-1* mice, these cells exhibit both delayed phagocytosis of outer segment membranes (Gibbs et al. 2003). Thus, Myo7a has important functions in the maintenance of the outer segments, which are severely affected in Usher 1 syndrome patients (El-Amraoui and Petit 2005). The lack of retinitis pigmentosa in the *shaker-1* mouse could be explained by the shorter life span of mice or by the differences in the photoreceptor anatomy and physiology between mice and humans.

4.3.7 Class XV Myosins

Vertebrate Myo15a is a 395-kDa motor with a large N-terminal extension of unknown function and a C-terminal tail similar to Myo7a, which allows interactions with membrane and scaffolding proteins (Liang et al. 1999). This motor is expressed in the cochlea and vestibular systems in the developing mouse inner ear, and mutations result in severe hearing loss both in mice (*shaker 2*) and humans (DFNB3) (Probst et al. 1998, Wang et al. 1998). In wild-type hair cells, Myo15a localizes as a cap on the top of stereocilia, and the amount of Myo15a correlates with the length of the bundles (Fig. 4.4; Belyantseva et al. 2003, Rzadzinska et al. 2004, Delprat et al. 2005). *Shaker 2* stereocilia are considerably shorter than wild type and do not exhibit the staircase pattern (Belyantseva et al. 2003, Rzadzinska et al. 2004). Thus, Myo15a controls the length of stereocilia, for example, by regulating the rate of F-actin assembly at the top of the bundles that slowly turn over by retrograde flow (Rzadzinska et al. 2004, Belyantseva et al. 2005, Delprat et al. 2005). On the other hand, the formation and function of the hair cell transduction complex does not require Myo15a (Stepanyan et al. 2006). Future biophysical and biochemical studies will provide important insights regarding if and how this large motor protein regulates actin dynamics and/or membrane/actin interactions.

4.4 Summary and Perspectives

Neurons and sensory cells of the nervous system have harnessed the wide spectrum of functionality of all the major myosin classes for morphogenesis, cell movement, cell maintenance, and signaling. Probably the best-characterized motor with respect to function is Myo2 in actomyosin contraction and neurite outgrowth. However, it

Table 4.1 Summary of proposed myosin functions in neurons and sensory cells

	Myo1 b c e	Myo2 a b	Myo3 a	Myo5 a b	Myo6	Myo7 a	Myo10	Myo15 a
Neuroblast diff.					x			
Neuronal migration		x						
Growth cone motility, axonal growth	x x	x x		x			x	
Axonal transport	x			x	x			
Presynaptic function	x	x		x	x			
Postsynaptic function		x		x x	x			
Hair cell development		x	x		x	x		x
Hair cell function	x		x		x	x		
Photoreceptor development		x	x	x	x	x		
Photoreceptor function			x	x	x	x		

has become evident that myosins have many additional roles in the nervous system, most of them involving one type of membrane–cytoskeletal interactions, such as endocytosis, organelle transport, membrane protrusion and stability, cell adhesion, and signaling (Table 4.1). The functional diversity of myosins within a single cell is particularly intriguing in the inner ear hair cell, where at least six different myosins have specific roles in cellular morphogenesis and mechanotransduction. While most of the earlier reports implicated myosins in various steps of nervous system development, recent studies have also provided accumulating evidence for both pre- and postsynaptic functions, as well as for morphogenesis and signal transduction in sensory cells.

Despite the recent progress there are still open questions and challenges. What are the functions of classes IX and XVI myosins in the nervous system? Do class I myosins play a role in organelle transport and growth cone motility? Because of the presence of multiple myosin proteins within one cell, it will be essential to use more specific chemical inhibitors, as well as temporally and spatially restricted knockdown approaches to further dissect functions. In addition, while several motors have been well characterized biochemically and genetically, future cellular studies involving live cell imaging techniques will advance our understanding of the various myosin functions. It will be important to simultaneously visualize membrane, F-actin, and myosin movements in processes where myosins have been implicated as coupling agents. Furthermore, the polarity of several subcellular actin structures in the nervous system needs to be determined. Finally, identification of additional cargo and linker proteins will benefit our understanding of the multiple roles of myosins in the nervous system.

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Chapter 5
Microtubule–Actin Interactions During Neuronal Development

Kenneth A. Myers and Peter W. Baas

Abstract Neuronal development involves many morphological changes and events that are intimately dependent upon microtubules. These include neuronal migration, axonal and dendritic differentiation, growth, and branching, the navigation of the axon to its target, and the retraction of overgrown axons. Within the various compartments of developing neurons, microtubules take on a variety of different lengths and configurations, and undergo behaviors such as dynamic assembly and disassembly, stabilization, and transport. A growing body of evidence suggests that the microtubule behaviors that underlie neuronal morphogenesis may be regulated in part by interactions of the microtubules with the actin cytoskeleton. The purpose of this chapter is to provide an overview of some of these microtubule–actin interactions and to discuss how these interactions may be important during the development of the neuron. The chapter includes discussions on signaling pathways, molecular motor proteins, classical and non-classical microtubule-associated proteins, and +TIPS.

Keywords +TIPs · Microtubule-associated proteins · Motor proteins · Axon retraction · Filopodia

5.1 Introduction

Typical vertebrate neurons consist of a small rounded cell body, several dendrites, and a single axon. Dendrites are typically tapered and highly branched so that they can serve as a receptive field for incoming information. Axons can also become highly branched but are specialized to transmit information outward. They are

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effectively unlimited in their growth potential and typically much longer than dendrites. Axons are heralded at their tip by a structure called the growth cone, which transforms into an axon terminal after reaching an appropriate target tissue. Many neurons, prior to the development of bona fide axons and dendrites, undergo a great deal of migration through the embryo. Migratory neurons extend a leading process that tows the cell body along and also display a trailing process behind the moving cell body. The leading processes of migratory neurons and the growth cones of axons are both highly sensitive to environmental cues that direct them to their final destinations. At all stages of neuronal development, the morphological features of the neuron have the potential for plasticity. Perhaps the best example of such plasticity is the fact that developing neurons extend many more axonal branches than needed during development, after which a great many of them are pruned away by a process called axonal retraction.

The morphological features of neurons are intimately related to the properties of architectural elements collectively termed the cytoskeleton. Interestingly, while the morphology of the terminally post-mitotic neuron is distinct from that of proliferating cell types, the same cytoskeletal elements, which include intermediate filaments, actin filaments, and microtubules, are utilized in both cases. Microtubules have been of particular interest over the years because they are such prominent structures within developing axons (Mukhopadhyay et al. 2004). Microtubules are hollow structures and the largest in diameter of the three major cytoskeletal filaments. They are thought to provide a great deal of structural rigidity needed to support the outgrowth of axons and dendrites and to prevent them from retracting. In addition to their structural role, microtubules facilitate intracellular transport and trafficking of cargo by serving as a substrate for motile proteins known as molecular motors. Microtubules are intrinsically polar filaments with a “plus” end that is favored for assembly and disassembly over a “minus” end. Microtubule-based motor proteins, namely the kinesins and cytoplasmic dynein, are able to interpret the polarity of the microtubule and move specifically toward one end or the other. These motors also interact with various cargos including membranous organelles that are transported in concert with the motor protein (Welte 2004).

Distinct patterns of microtubule organization enable motor proteins to traffic cargo to specific cellular destinations, and alterations of microtubule configurations in various regions of the cell provide a foundation for neuronal asymmetries. In taking advantage of these properties, neurons establish unique patterns of microtubule organization in different cellular compartments that account for both the regional morphology of the cell and the cytoplasmic composition attributable to cargo delivery. In the axon, microtubules are uniformly oriented with their plus ends distal to the cell body, whereas in the dendrite, the microtubules are typically of mixed orientations (Heidemann et al. 1981, Baas et al. 1989). These differences in microtubule configuration contribute significantly to organelle and cargo composition, as well as to the length and shape of these processes. Other regions of the neuron including growth cones and branch points display characteristic patterns of microtubule organization that are critical for defining the morphologies of these regions (Dent and Gertler 2003). In addition, migratory neurons display characteristic patterns

of microtubule organization that are crucial for the morphological changes and transport events that underlie their directed cellular motility during embryogenesis (Higginbotham and Gleeson 2007).

One of the most important questions in neuronal cell biology is how the microtubule arrays of neurons are organized and regulated. How are the distinct polarity patterns of axonal and dendritic microtubules established? How are microtubules in growth cones organized and re-organized to enable the axon to navigate toward appropriate targets? How are the microtubules in migrating neurons orchestrated so that these neurons can extend a leading process, retract a trailing process, and move the nucleus coordinately forward as the neuron translocates through its environment? How are microtubules in the parent axon and dendrite reconfigured during the formation of branches? All of these questions have been under intensive study for the past several years in many different laboratories. One of the key hypotheses that has emerged from studies on neurons and other cell types is that microtubules are highly influenced by the organization of another cytoskeletal element, namely actin filaments. Early studies led to the conclusion that microtubule behaviors often follow as a consequence of actin behaviors. More contemporary studies suggest that the two filament systems actually influence one another through a variety of molecular interactions.

5.2 Microtubule–Actin Interactions in Developing Neurons

Like microtubules and their tubulin subunits, actin is present in cells in both filamentous and subunit form. The filaments are referred to as F-actin, microfilaments, or simply as actin filaments. The subunits are referred to as G-actin or simply as actin subunits. As with microtubules, the assembly and the disassembly of actin filaments are regulated in part by a process called dynamic instability, which involves GTP in the case of microtubules but ATP in the case of actin. Compared to microtubules, however, actin filaments take on a far vaster array of configurations which range from simple straight bundles to very complex reticular networks. The configuration of the actin filaments is determined by the specific actin-associated proteins with which they affiliate. In the axonal shaft, some of the actin filaments exist as individual unbundled filaments that are often aligned with microtubules (Bearer and Reese 1999). However, a substantial portion of the actin in the axonal shaft exists in the form of a cortical meshwork that lies just beneath the cell membrane. In the presence of repellent extracellular signals, the axonal actin meshwork is re-organized into filamentous bundles of actin. Myosin-II motors are triggered to generate contractile forces against the actin bundles, and this is the major force underlying axonal retraction (Gallo et al. 2002, Gallo 2004, 2006). In the distal region of the axon, the cortical meshwork fans out to form the peripheral domain of the growth cone (Kabir et al. 2001, Schaefer et al. 2002, Zhou et al. 2002). Each filopodium extending from the peripheral domain contains a straight bundle of actin filaments. During axogenesis, the lamellae that coalesce into early neurites contain a meshwork of actin

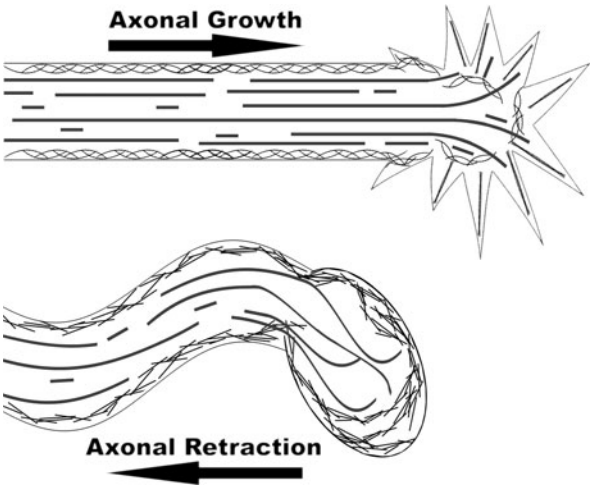

Fig. 5.1 Microtubule and actin organization within the growing and retracting axon. (*Top panel*) In the elongating axon, long compression-bearing microtubules function to offset the contractility of the cortical actin meshwork, while the transport of shorter microtubules provides a source of tubulin subunits as well as nucleating sites for further microtubule polymerization as the axon grows. (*Bottom panel*) During axonal retraction, there is a dramatic change in the organization of both the cortical actin and the microtubule array. These changes may relate to enhanced actin contractility and/or a reduction in microtubule–actin interconnectivity

similar to the peripheral domain of the growth cone, and the same is true of the leading edge of migratory neurons. Dendritic spines are also known to be rich in filamentous actin (Schubert and Dotti 2007).

There are several ways in which the functions of microtubules and actin filaments are thought to complement one another (see Fig. 5.1). For example, within the axonal shaft, long compression-bearing microtubules are critical for offsetting the contractility of the cortical actin meshwork, thus enabling the axon to overcome its tendency to retract. In addition, short microtubules are highly mobile within the axon, and actin filaments are thought to provide one of the substrates against which microtubule-based motors generate forces to enable the transport of these microtubules (Hasaka et al. 2004, He et al. 2005, Myers et al. 2006a, b). In migratory neurons, microtubules are thought to transmit actomyosin-generated forces from the leading edge back to the cell body and nucleus to enable them to move coordinately as the neuron migrates (Brown and Bridgman 2004).

The process of growth cone turning is perhaps the best known example of microtubule/actin coordination in the neuron (see Fig. 5.2). As paraxially aligned microtubules from the axonal shaft enter the growth cone, they are mainly confined to its central domain. The mass of microtubules is prevented from engorging the peripheral regions of the growth cone by a powerful backward flow of actin filaments, fueled by myosin-II (Lin and Forscher 1993). However, individual microtubules can, under certain conditions, splay apart from the microtubule bundle,

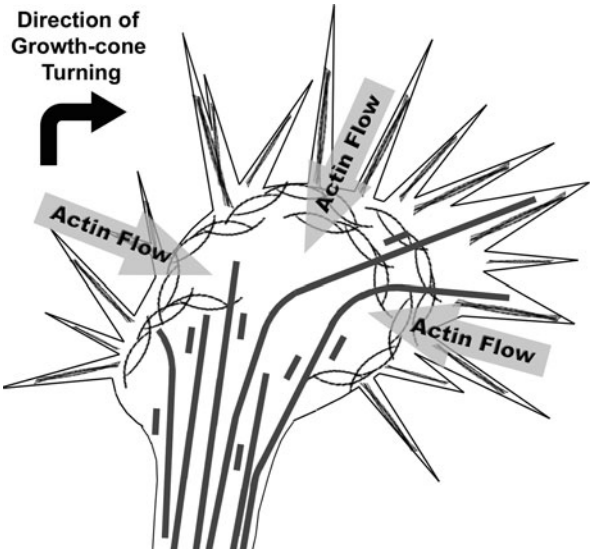

Fig. 5.2 Microtubule and actin filaments within the axonal growth cone. Within the growth cone, the molecular motor myosin-II generates contractile forces against peripherally localized F-actin and in doing so, establishes a retrograde flow of F-actin. The retrograde flow of actin restricts the advance of the microtubules, constraining a majority of the microtubule array to the central domain of the growth cone. During growth cone navigation, individual microtubules overcome this retrograde flow of actin and selectively invade individual filopodia. Having entered the filopodia, microtubules can then polymerize along filopodial actin bundles. The successful and directed invasion of filopodia by microtubules facilitates growth cone turning in the direction of those particular filopodia which were invaded by microtubules

overcome the retrograde forces, and extend from the central domain into the peripheral domain of the growth cone, to invade filopodia. The invasion of microtubules into filopodia occurs to a greater degree on the side of the growth cone facing the direction of turning. The selective invasion of microtubules into certain filopodia enables these filopodia to then be engorged by cytoplasm, for the body of the growth cone to be shifted in the direction of the turn, and then for the mass of microtubules to advance in that direction as the axonal shaft elongates (Tanaka and Kirschner 1995, Challacombe et al. 1996, Gordon-Weeks 2004, Zhou and Cohan 2004, Myers et al. 2006b). In order for this to happen, the invading microtubules must be able to overcome the retrograde flow of the actin meshwork and also align appropriately with the straight bundles of actin in the filopodia.

Perhaps the simplest (and oldest) view of microtubule–actin interactions is that the configuration of the actin filaments determines the means by which the microtubules can interact with them (Forscher and Smith 1988, Bearer and Reese 1999, Bradke and Dotti 1999). For example, if the actin filament is present in the form of a straight bundle, then a microtubule can align with it, potentially assemble coordinately with it, or move relative to it using a molecular motor protein. However, if the

actin is present as a particularly dense meshwork, then it may physically constrain the distribution of the microtubules. In this view, the regulation of actin filament organization by actin-regulatory proteins indirectly impacts the organization and distribution of the microtubules. Studies over the past several years suggest that there is actually far greater complexity with regard to how microtubules and actin filaments interact. For example, the retrograde flow of actin filaments, fueled by myosin motors, imposes a force on the microtubules that they need to overcome in order to invade the peripheral domain of the growth cone (Fig. 5.2). In addition, there is a growing list of proteins that are known to interact with both types of filaments and hence could provide for structural and/or functional linkages between them. Finally, studies on signaling cascades indicate that microtubules are not always passive followers of actin filaments but that both filament systems actively "inform" one another.

5.3 Signaling Cascades Integrate Dynamic Properties of Microtubules and Actin

The most obvious thing that microtubules and actin filaments can do is to undergo dynamic bouts of assembly and disassembly. Early studies on cultured neurons suggested that the dynamic properties of microtubules and actin filaments may be coordinately regulated (Yamada et al. 1971). More recent live-cell imaging experiments on growth cones have revealed that dynamically assembling tips of microtubules can induce changes in actin filament assembly (Rochlin et al. 1999) and that microtubules and actin filaments can assemble coordinately alongside one another (Dent et al. 1999). These phenomena appear to relate to signaling cascades involving small G proteins. Studies from a variety of cell types suggest that RhoA instigates the formation of reticular networks of actin filaments capable of contraction while also stabilizing microtubules. In addition to influencing actin organization, Rac activation is also able to cause dramatic reductions in the frequencies of microtubule catastrophe, resulting in enhanced numbers of microtubules that actively invade and interact with actin-rich filopodia and lamellipodia (Wittmann et al. 2003). The pathways involved in these responses involve a variety of other proteins including the Arp2/3, ADF/cofilin, and kinases (Rodriguez et al. 2003). In neurons, it has been shown that Rac and Cdc42 are required for neurite formation, as their inhibition prevents neurons from extending axons (Sarner et al. 2000, Aoki et al. 2004). RhoA appears to function antagonistically to Rac and Cdc42, by preventing axonal elongation, and constitutively active RhoA has been shown to induce neurite retraction (Tigyi et al. 1996).

In response to extracellular growth signals, both Rac and Cdc42 work at the leading edge of cells to initiate actin polymerization in lamellipodia and filopodia, whereas RhoA induces the maturation of focal adhesion behind leading edges and the dissociation of focal adhesion at the rear (Kaverina et al. 2002, Fukata et al. 2003, Raftopoulou and Hall 2004). These roles are important for the formation and

advance of both elongating axons and their growth cones, as well as during the active mobility of migrating neurons. Control of the Rho G proteins is dependent upon the hydrolysis of GTP, which is in turn regulated by the activity of GTPase-activating proteins (GAPs), which facilitate the exchange of G-protein bound GTP for GDP, and guanine nucleotide exchange factors (GEFs), which perform the reverse function, providing usable energy to the G protein. Because they can be both spatially and temporally regulated, the neuron utilizes these activator proteins in order to fine-tune pathways involving Rac, Rho, and Cdc42 (Takai et al. 2001). Recent investigations have identified at least one GEF whose function is to regulate Rac activity toward enhanced microtubule stability through the inactivation of the Op18/stathmin protein, which is known to destabilize microtubules. The activated Rac then leads to the promotion of axonal formation in a manner that can be locally regulated during neuronal development (Watabe-Uchida et al. 2006). Thus, the same upstream regulators of the Rho family of small GTPases, which for many years have been known to regulate actin organization, now appear to be important for the control of microtubule stability. Thus, these cascades may afford the developing neuron a powerful mechanism for the coordinated control of microtubules and actin filaments by extracellular signaling cues.

5.4 Integration of Microtubules and Actin via Motor-Driven Forces

A major focus of our own laboratory has been to study the molecular motors that impinge upon neuronal microtubules in order to transport and configure them during events such as axonal growth, retraction, branching, and navigation. Recent studies have revealed that the only microtubules that are in active transit within the axon are quite short, just a few micrometers in length (Wang and Brown 2002). In developing axons, roughly two-thirds of these short microtubule movements occur in the anterograde direction while roughly one-third occur in the retrograde direction (Wang and Brown 2002, Hasaka et al. 2004). Our hypothesis is that the molecular motors that transport the short microtubules are not selective for the short microtubules but rather impinge upon microtubules of all lengths. When they act on long immobile microtubules, these motor-driven forces serve many roles, including functional integration of the microtubules with the substrate against which the short microtubules would be transported. Our studies suggest that short microtubules can be transported against longer microtubules, but also against actin filaments (Hasaka et al. 2004, Myers et al. 2006b). Thus, we contend that motor-driven forces are among the factors responsible for interactions between microtubules and actin.

Cytoplasmic dynein, a multi-functional motor protein responsible for trafficking vesicular cargo and organelles toward microtubule minus ends (Vallee et al. 2004), is probably the best candidate for generating forces between microtubules and actin in the axon. In mitotic cells, cytoplasmic dynein appears to generate forces between astral microtubules and the actin-rich cortex in order to assist in the separation of the

half-spindles (O'Connell and Wang 2000, McCartney et al. 2001, Yamamoto et al. 2001). Such forces may also serve to align the spindle to determine the axis of cell division (Huisman and Segal 2005, Pecreaux et al. 2006). On this basis, it makes sense that cytoplasmic dynein may transport microtubules down the axon with their plus ends leading by generating forces against actin filaments that are configured in a form that is less resistant to movement than the short microtubules. Such a scenario was originally proposed on the basis of studies showing that most of the dynein that is transported anterogradely down the axon is transported at the same rate as actin rather than tubulin (Dillman et al. 1996a). This led to the idea that dynein somehow interfaces (via its cargo domain) with the actin cytoskeleton. Presumably this interaction is indirect and involves the dynactin complex (Dillman et al. 1996b) and perhaps other actin-binding adaptor proteins. The motor domain of the dynein molecule would then be available to intermittently interact with microtubules. It should be noted that, while a host of functional studies support such a scenario, there is still little information on the precise nature of the molecular interactions that would be required for this mechanism to ensue.

Recent studies from our laboratory sought to directly test the role of cytoplasmic dynein on the transport of short microtubules in the axon. For these studies, we depleted the heavy chain (motor domain) of cytoplasmic dynein using RNA interference. In cultured neurons depleted of dynein heavy chain, the frequency of short microtubule transport in the axon was reduced by nearly 50%, and this effect was specific for anterograde microtubule movements. Interestingly, pharmacologic depletion of filamentous actin produced a nearly identical effect on the movements of short microtubules, with a reduction in anterograde transport frequency of about half, and again no effect on the frequency of retrograde transport. These findings demonstrate that the dynein/actin model can account for a substantial fraction of the transport of microtubules in the anterograde direction.

We suspect that the dynein-driven forces would not be selective for microtubules of a particular length. It seems more reasonable that these forces would apply to all microtubules, regardless of length, but that these forces would only result in rapid transport of microtubules if they are quite short. A principal task of the long microtubules is to act as compression-bearing elements that prevent the axon from retracting in response to the actomyosin-based contractility of the cortical actin. Interestingly, when cytoplasmic dynein is depleted from the axon (or when its functions are indirectly inhibited), the axon becomes more susceptible to retraction (Ahmad et al. 2000). The microtubules do not display wholesale disassembly during retraction but rather are severely contorted as if, in the absence of dynein, they are no longer able to bear the compressive forces imposed upon them by the actomyosin. Thus, we have posited that the dynein-driven forces between long microtubules and the actin cytoskeleton serve to attenuate, offset, or balance the myosin-II-driven forces on the cortical actin (Fig. 5.3).

Dynein-driven forces generated between microtubules and actin also appear to play a major role in growth cone turning. In neurons depleted of dynein heavy chain, growth cone microtubules are less able to gain entry into the peripheral domain and filopodia of the growth cone, and this severely inhibits the ability for the growth

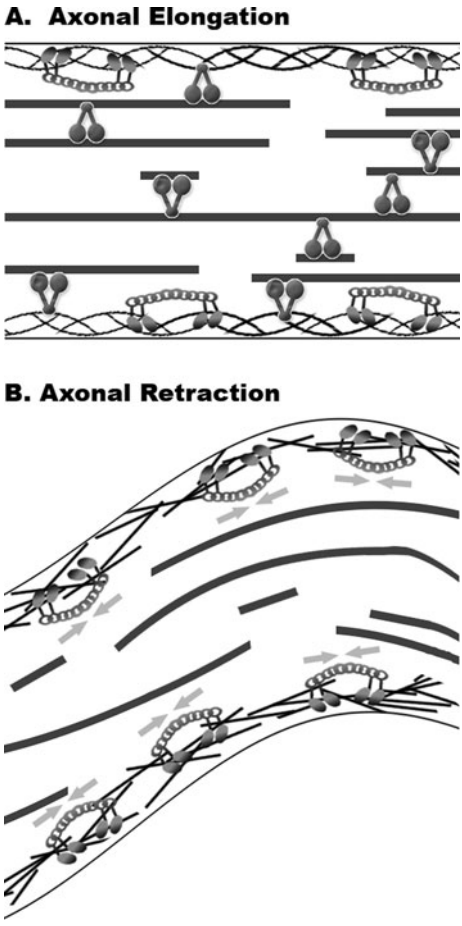

Fig. 5.3 Cytoplasmic dynein and myosin-II are critical determinants of axonal growth and retraction. (*Top panel*) Forces generated by cytoplasmic dynein serve to transport short microtubules down the axon. The same dynein-driven forces that transport the short microtubules act on the long immobile microtubules to offset the contractility of the cortical actin that would otherwise cause the axon to retract. (*Bottom panel*) In the absence of dynein-driven forces, the unopposed actomyosin forces result in the retreat of the microtubule array and the retraction of the axon

cone to turn. However, when myosin-II is inhibited as well, the microtubules in the dynein-depleted neurons freely enter all the peripheral domain of the growth cone and all of the filopodia (Myers et al. 2006b). On this basis, we concluded that the myosin-II-driven retrograde flow of actin filaments is sufficiently powerful to restrict the entry of microtubules from the central domain into the peripheral domain of the growth cone but that dynein-driven forces are sufficient to enable microtubules to overcome these myosin-II-driven forces. This would presumably be a highly selective process in which the balance between the two motor-driven forces is altered in response to environmental cues that dictate the direction of growth cone turning (Fig. 5.4).

We should note that there is an abundance of evidence suggesting that the dynamic properties of the microtubules are critical for growth cone turning (Buck and Zheng 2002, Suter et al. 2004, Zhou and Cohan 2004), which begs the question of how the antagonistic force relationship described here is coordinated with the

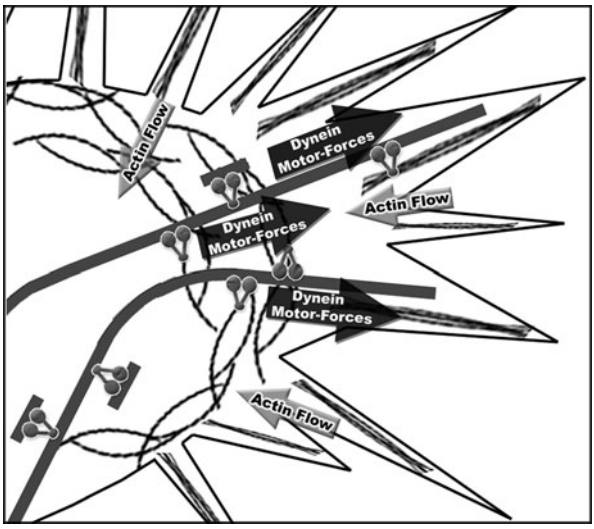

Fig. 5.4 Cytoplasmic dynein and myosin-II are critical determinants of growth cone turning. Retrograde flow of actin in the peripheral region of the growth cone restricts the microtubule array to the central domain (see Fig. 5.2). Forces generated by cytoplasmic dynein enable individual microtubules to overcome the retrograde flow and invade the peripheral domain of the growth cone so that they can enter filopodia. The competition of forces is presumably regulated by factors relevant to growth cone turning so that microtubules can invade filopodia on the side of the growth cone in the direction of turning

dynamic properties of the polymers. This will be a topic of study in the future, but for now, we suspect that the transition zone between the central and peripheral domains of the growth cone may be the chief "battleground" for the motor-driven forces. The battle is fought at the level of individual microtubules; when the dynein-driven forces on individual microtubule are able to overcome the myosin-II-driven forces, the microtubule is then able to penetrate into the peripheral domain where it can subsequently assemble into a filopodium. In this scenario, still to be tested, there are distinct roles for the motor-driven forces and the assembly dynamics of the microtubules in different regions of the growth cone.

5.5 Proteins that Interact with Both Microtubules and Actin

5.5.1 +TIPs

Over the past several years, one of the most fascinating developments in microtubule biology has been the discovery of a class of proteins that associate with the plus ends of microtubules during bouts of assembly. These proteins, termed +TIPs,

avidly interact with the plus end of the microtubule as it assembles but then gradually lose association as the microtubule either continues to assemble or undergoes a bout of a disassembly. For this reason, GFP fusions for the +TIPs, when expressed in cells, appear on the plus ends of microtubules in the shape of a comet, with the tail of the comet directed toward the minus end of the microtubule. The localization of the +TIPs is of particular interest to microtubule–actin interactions because the plus ends of microtubules very often extend into the actin-rich cortical regions of cells, including axonal growth cones. The current list of microtubule +TIPs consists of a variety of proteins that perform very different functions and interact with microtubules in various ways. Studies to date have revealed the existence of at least three families of +TIP proteins, including the CLIP-170 family (Perez et al. 1999), the end-binding (EB) family (Mimori-Kiyosue et al. 2000, Tirnauer and Bierer 2000), and the dynein–dynactin motor complex (Schuyler and Pellman 2001, Vaughan 2005).

Experiments on non-neuronal cells designed to investigate CLIP-170 have identified that this protein is integral to the formation of asymmetric microtubule–actin linkages as a pre-requisite for polarized migration (Watanabe et al. 2005). This is accomplished through the formation of a multi-protein complex consisting of CLIP-170, the adenomatous polyposis coli (APC) protein, and IQGAP1, one downstream target of Rac1 activation. Following activation of Rac1 or Cdc42 GTPases, the binding of IQGAP1 to CLIP-170 is greatly enhanced, resulting in the capture of CLIP-170 in the actin-rich regions of filopodia. This capture is most likely facilitated by the IQGAP1 protein and associated APC, which commonly colocalizes to actin-rich leading edges in mitotic cell types. Thus, it appears that there is a flow of molecular signaling from the Rho family of GTPases that result in the integration of dynamic actin and microtubules producing the polarized cytoskeletal organization that underlies cellular migration.

From a developmental perspective, it appears that the formation of the CLIP-170/IQGAP1/APC multi-protein complex is essential but not sufficient in and of itself for sustained microtubule–actin interactions during axonal growth and growth cone navigation. While +TIPs strongly influence microtubule dynamics, these proteins also provide a physical mediator for another family of linker proteins, the cytoplasmic linker protein (CLIP)-associated proteins (CLASPs) (Akhmanova et al. 2001). Interactions of CLASPs, themselves technically classified as +TIPs, with microtubules are spatially regulated, with a clear preference for microtubule binding within polarized regions of the cell (Wittmann and Waterman-Storer 2005). Thus far, CLASPs have been shown to bind to the CLIP-170 and EB1, but unlike these plus-end-tracking proteins, the CLASPS have a higher propensity to bind along the length of the microtubule. Similar to CLIP-170 and EB1, CLASPs visualized by GFP labeling show a preference for microtubules undergoing active growth, but other investigators have shown that CLASPs can bind microtubules regardless of their polymerization state (Lee et al. 2004). These authors also identified a propensity for CLASP localization to a subset of microtubules within the leading edge of the growth cone and extending into actin-rich filopodia, regions of the neuron where Rac1-mediated actin remodeling, coupled with microtubule–actin dynamics,

is thought to drive axonal navigation and growth (Kirschner and Mitchison 1986, Schaefer et al. 2002, Lansbergen et al. 2006).

The EB family of proteins consists of three members in vertebrates: EB1, EB2 (RP1), and EB3. EB1 and EB3 are able to directly interact with CLIP-170 and a closely related form of the cytoplasmic linker proteins known as CLIP-115. When cellular levels of EB proteins are reduced, there is greatly reduced CLIP binding to microtubules as well as enhanced rates of CLIP dissociation from microtubule plus ends (Komarova et al. 2005).

The EB proteins are believed to be the central family of +TIPs because they directly interact with all of the other known +TIP families, including dynactin large subunit p150glued, APC, CLASPs, RhoGEF2, and even the kinesin-13 family of depolymerizing kinesins. Moreover, the removal of any one of these interacting proteins does not affect the binding characteristics of the EB proteins, suggesting that EB associations with microtubules are independent of other +TIP-associated proteins (Komarova et al. 2005). Current evidence related to EB function suggests that the EB proteins work to promote either the removal or the inhibition of other microtubule-interacting proteins that function to block +TIP binding or that the EB proteins stabilize microtubule plus ends in a conformation that is better suited for binding to the CLIP +TIPs. Neither of these EB functions is mutually exclusive, and it is certainly possible that both scenarios are effective simultaneously. What is certain is that the association of EB proteins with the microtubule plus end results in changes in CLIP dynamics by reducing the dissociation rates of CLIP +TIPs (Komarova et al. 2005). Increased association of CLIPs with microtubule plus ends would then promote microtubule tip interactions with the CLIP-associated proteins IQGAP1, APC, and the actin-associated CLASPs.

A more recent addition to the category of microtubule +TIPs is the molecular motor cytoplasmic dynein. The current mechanistic understanding of dynein's association with the microtubule plus end involves associations with subunits of the dynactin complex. The dynactin complex is unique in that it contains two regions (Arp1 and Arp 11) that maintain remarkable structural similarity to actin and may represent the physical link between the dynein motor domain and the actin cytoskeleton (Eckley and Schroer 2003). The largest member of the dynactin complex is the p150glued subunit, which is particularly important for dynein–microtubule plus-end activity. P150glued is a large protein (~150 kDa) and is able to bind the microtubule +TIPs EB1 and CLIP-170, and there is evidence that p150glued is also able to bind to multiple molecular motors in addition to the dynein motor component (Zhang et al. 2003). Current data suggest two possible models of dynein interactions with microtubule plus ends. The first model proposes that the phosphorylation of the dynactin complex (by protein kinase A) causes its dissociation such that the localization of dynactin (and dynein) is dependent upon the temporal phosphorylation state of the complex. The second model suggests that dynactin associates with the microtubule plus ends through CLIP-170. This model is supported by data showing that overexpression of CLIP-170 enhances dynactin accumulation at the microtubule plus end (Valetti et al. 1999), and small-interfering RNA-based depletion of CLIP-170 has the reverse effect (Lansbergen et al. 2004).

Given these findings it appears likely that the CLIP-170 protein is responsible for recruiting dynein to microtubule plus ends, which may be dependent on the phosphorylation state of the dynactin complex.

The fact that all of the microtubule +TIP proteins are specifically focused at the dynamic plus ends of microtubules raises questions as to how their functions are integrated and what cellular outcome is the result of this integration. As with the CLIPs and CLASPs, it appears that the association of cytoplasmic dynein with the microtubule plus end provides for the specific localization of this motor protein to cortical regions of the cell where individual actin–microtubule interactions will take place. This method of dynein targeting may function to provide the highest probability for cortical microtubule capture while enabling cortical motor force capabilities. During early neuronal development, for example, when the cooperative organization of actin and microtubules is essential, microtubule +TIP associations begin with extracellular cues that either stimulate or inhibit the activity of the Rho family of small G proteins. As dynamic microtubules invade the cortical regions of the axon and enter the peripheral regions of the growth cone, G-protein activity regulates the dynamic associations of the EB family of proteins with the microtubule plus ends. The EBs then facilitate plus-end associations with CLIP-170, APC, and IQGAP1, which together establish actin–microtubule capture through interactions with actin-associated CLASPs. At the same time, polymerizing microtubule plus ends, bound to CLIP-170, form temporal binding interactions with p150glued and the dynactin complex (Fig. 5.5). The dynactin complex then recruits cytoplasmic dynein, which, following cortical microtubule capture, generates motor-driven forces between the cortical actin meshwork and captured microtubules (see Fig. 5.5 and Section 5.4).

5.5.2 Classical Microtubule-Associated Proteins

Any protein that binds to microtubules can justifiably be termed a microtubule-associated protein (MAP). However, the term MAP is most closely associated with a family of structural proteins, discovered decades ago, now often referred to as “fibrous” or “classical” MAPs. This group of MAPs is able to bind along the length of the microtubule lattice, and at least in vitro, the binding of these proteins promotes the assembly and stabilization of the polymer. In addition to a microtubule-binding domain, these MAPs generally also consist of a projection domain that emanates away from the microtubule. The projection domain contributes to the spacing between neighboring microtubules and affords opportunities for interaction with other proteins relevant to cytoskeletal organization (Chen et al. 1992, Chen and Chalovich 1992, Teng et al. 2001). In neurons, the classical MAPs include tau, MAP2, MAP1b, and MAP1a. The MAP originally called STOP is now known as MAP6 (Bosc et al. 2003), and another MAP termed MAP8 has recently been discovered (Ding et al. 2006).

MAP1A and MAP1B are often considered together because of their similar mobility on electrophoretic gels and because they utilize the same light chains

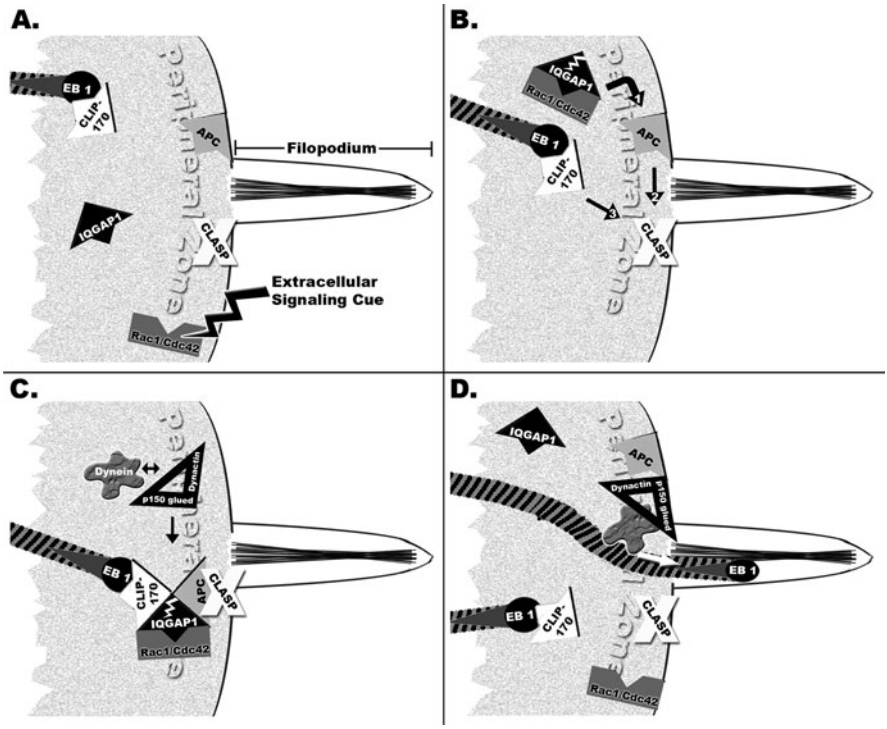

Fig. 5.5 Microtubule +TIPs coordinate microtubule–actin interactions within the growth cone. (**a**) A pioneering microtubule polymerizing into the peripheral zone of the growth cone forms associations with the EB1 and Clip-170 +TIPs. These +TIP associations are typically transient interactions until Rho-family GTPases (Rac1/Cdc42) are activated by extracellular signaling cues. (**b**) Rac1/Cdc42 activity stimulates IQGAP1 activation and a chain of events ensues. These events involve membrane-associated APC and CLASPs, as well as the IQGAP1 protein, which may link membrane-localized APC–CLASPs with EB1-CLIP-170 located at the microtubule plus end. (**c**) The assembly of the +TIP complex enables the temporary association of the polymerizing microtubule and the actin meshwork near the peripheral growth cone plasma membrane. Integration of the +TIP complex with motor-driven forces may be achieved through p150glued (a member of the dynactin complex), which is able to bind and interact with the EB1–CLIP-170 complex, and in doing so may recruit cytoplasmic dynein to microtubule plus ends. (**d**) The dynein–dynactin complex is then able to generate forces that enable the microtubule to enter the filopodium, where the microtubule can assemble along the filopodial actin bundle. The +TIP complex is recycled as a new "pioneering" microtubule advances into the peripheral zone

as accessories. While MAP1A is predominantly expressed in the adult nervous system, MAP1B expression levels are highest during the early developmental processes associated with neuronal axogenesis. There appears to be a switch from MAP1B to MAP1A expression as the neurons age (Noiges et al. 2002). Interestingly, both MAP1 proteins contain N-terminal microtubule-binding regions as well as C-terminal F-actin-binding regions, and this organization is thought to

elicit cross-linking properties to the MAP1 proteins based on their elongated, rod-like orientation when bound to the microtubule lattice (Shiomura and Hirokawa 1987, Sato-Yoshitake et al. 1989). More recent investigations have shown that MAP1B may be directly involved in microtubule–actin cross-linking and that this effect is likely important in axonal elongation (Cueille et al. 2007). In another study, MAP1B was shown to directly interact with the Lis1 protein, in a manner dependent upon the phosphorylation state of MAP1B (Jimenez-Mateos et al. 2005). Lis1 (lissencephaly-related protein 1) was originally identified as a dynein-associated protein in cases of brain lissencephaly, a disease caused by abnormal neuronal migration (Jimenez-Mateos et al. 2005). The interaction between Lis1 and MAP1B was observed to disrupt the ability for Lis1–dynein binding, which has been a proposed mechanism for dynein–actin communication during neuronal migration (Kholmanskikh et al. 2006), as well as during axonal elongation and growth cone navigation (Grabham et al. 2007). In addition, the sequestering effect of MAP1B on Lis1 could also work to indirectly influence microtubule–actin interconnectivity by disrupting Lis1-mediated Cdc42 activation. This MAP1B influence has recently been shown to cause reductions in the perimembrane localization of IQGAP1 and CLIP-170, ultimately producing reduced tethering of microtubule plus ends to the cortical actin cytoskeleton (Kholmanskikh et al. 2006). These effects of MAP1B are likely important during the migratory phases of neuronal development, where migratory neurons utilize the coordinated movements of both the leading process and the nucleus. Accumulating migration data in several migratory neuronal cell types provide supportive evidence that neuronal movement utilizes MAP1B-mediated linkage of microtubules to actin filaments (Gonzalez-Billault et al. 2001, Del Rio et al. 2004, Gonzalez-Billault et al. 2005). These studies suggest that the ability of the MAP1B protein to bind to both microtubules and actin may provide an organizational advantage in which both cytoskeletal systems become less dynamic (i.e., more stabile), while at the same time, the linked microtubules and actin filaments are coordinately positioned alongside one another through the structural rigidity afforded by the actin–microtubule–MAP1B complex (Noiges et al. 2002, Cueille et al. 2007).

This type of activity could produce a variety of effects during neuronal development. In the migrating neuron, for example, once the MAP1B-mediated linkage of microtubules to actin is achieved, the neuron may be afforded the opportunity to enlist molecular motor proteins, whose forces against microtubules linked to actin filaments may enable the coordinated movement of the nuclear compartment with the neuronal cytoskeleton. In the case of growth cone navigation, MAP1B-driven positional organization of microtubules and actin could work to enable cortical capture of microtubule plus ends through +TIP proteins or to facilitate the proper positioning of growth cone microtubules so as to enable the motor-driven advance of individual microtubules into peripheral growth cone filopodia. Once the microtubules gain entry into actin-rich filopodia, MAP1B (perhaps in combination with other MAPs) could presumably exert these same organizational effects such that microtubules might continue to polymerize in close proximity to filopodial actin bundles.

The MAP2/tau family of proteins consists of various isoforms of MAP2 and tau, as well as the non-neuronal family member MAP4. These MAPs consist of a similar microtubule-binding domain with multiple repeats. Three major isoforms of MAP2 are expressed during different time periods of neuronal development, beginning with MAP2c expression in juvenile neurons and throughout early development (Leclerc et al. 1996). MAP2b is expressed throughout the life of the neuron, and MAP2a expression rises as MAP2c expression levels fall, making MAP2a the predominant MAP2 family member in adult neurons (Chung et al. 1996). During development, MAP2 proteins become concentrated on the microtubules in neuronal dendrites and cell bodies, while tau is more concentrated on the microtubules in growing axons. In theory, MAP2 is particularly well suited for interactions between microtubules and actin filaments because the microtubule-binding domain of MAP2 incorporates an actin-binding domain (Rogers et al. 2004). Indeed, MAP2c has been shown to bind and bundle filamentous actin, and expression of MAP2c alone in neuronal precursor cells is sufficient to promote neurite formation (Dehmelt et al. 2003). Axonal development correlates with a transition from a MAP2–actin filament interaction to a MAP2–microtubule association and binding of tau to the distal axon (Kwei et al. 1998).

Interestingly, despite the lack of a known actin-binding domain, functional studies suggest that tau also interacts with actin filaments as well as microtubules. A potential tau–actin interaction is suggested by studies showing that tau protein colocalizes and is co-immunoprecipitated with filamentous actin (Cross et al. 1993, Henriquez et al. 1995). Other experimental investigations of tau localization revealed that the tau distribution in the distal region of young axons could be disturbed both by the pharmacologic disruption of the neuronal microtubule array and by the pharmacologic depletion of filamentous actin (Kempf et al. 1996). The structural organization that actin provides to the cortical regions of the growth cone also appears to be influenced by the tau protein. In tau-suppressed neurons, both growth cone area and filopodia number are substantially reduced, while the length of the remaining filopodia is greatly enhanced. Moreover, this phenomenon does not require the removal of growth cone microtubules but rather is accompanied by considerable changes in the staining pattern for filamentous actin (DiTella et al. 1994). Such developmental roles for tau may relate, in part, to interactions between tau and the Src kinases, which are known to play roles in actin remodeling (Sharma et al. 2007). In the adult, a potential membrane-bound functional complex has been proposed to include both tau and actin on the basis of their interactions with α-synuclein (Esposito et al. 2007).

More recent investigations have suggested that the influences of actin–tau interactions, in particular the tau-induced formation of filamentous actin rods, may be critical to the development and phenotypic morphologies of tau-based neuropathies including Alzheimer's disease and Pick's disease. Important findings regarding the tau–actin relationship come from studies in *Drosophila*, where flies co-expressing tau and actin displayed substantial increases in the amount of F-actin and in the formation of actin-rich rods in the fly brain, but no change in the levels of soluble G-actin (Fulga et al. 2007). Taken together, the current data support a model in

which the tau protein functions in developing neurons to regulate features of the axonal microtubule array, while at the same time participating in the normal organization of the neuronal actin array. The identification of actin as a tau-interacting protein and as an important downstream effector of tau-mediated degeneration provides support to the idea that the actin cytoskeleton is an important mediator of toxicity in tauopathies. A correlation between F-actin accumulation and the formation of actin-rich rods in the fly brain suggests that tau-dependent F-actin accumulation may directly lead to rod formation and potentially to analogous structures typically observed in human tauopathies.

5.5.3 Non-classical Microtubule-Associated Proteins

The non-classical MAP family is considerably larger than is the classical MAP family and by definition contains all of the newly discovered MAP proteins that have distinct structural and/or functional variation from the classical MAPs. One group of non-classical MAPs are the plakins, a family of rod-like proteins that contain the largest number and the most likely candidates for mediating actin–microtubule interactions (Roper et al. 2002). The plakin family members include desmoplakin, plectin, microtubule–actin cross-linking factor 1 (MACF1), bullous pemphigoid antigen 1 (BPAG1), envoplakin, periplakin, and epiplakin. For our purposes we will restrict this discussion to the MACF1 and BPAG1 proteins (commonly referred to as spectraplakins), because these proteins are evolutionarily conserved and because they are non-classical MAPs that have been shown to bind to both the actin and microtubule cytoskeletons.

Spectraplakins are relatively large proteins (200–600 kDa) and because they maintain a rod-like structure, many are predicted to bind microtubules and filamentous actin at sites that are spatially well separated. The MACF1 and BPAG1 proteins are structurally similar, containing a C-terminal microtubule-binding domain and an N-terminal F-actin-binding domain. They are also able to associate independently with either microtubules or actin filaments in a manner that can influence cell motility and morphogenesis (Andra et al. 1998). Because these two proteins maintain high structural and seemingly high functional similarities, it has been suggested that there is a partial redundancy of function between them.

The mammalian spectraplakin protein MACF1 (also known as ACF7, or actin cross-linking family 7) is expressed ubiquitously in mouse embryos, with the highest levels in the nervous system and skeletal muscle. Interestingly, the MACF1 null mutation is lethal in mice, and endodermal cells taken from these mice display abnormal microtubule dynamics. In particular, the microtubules are no longer able to tether to cortical actin, nor are they able to polymerize along actin filaments as is observed in the presence of MACF1 (Kodama et al. 2003). Advances in the understanding of MACF1 function have been most forthcoming in experimental investigations of the MACF1 *Drosophila* homologue termed *Shot* (also known as *shortstop/kakapo*). *Shot* mutants display markedly abnormal neuronal morphologies

during development including defects in axonal growth, growth cone navigation, dendritic sprouting, and neuromuscular arborization (Roper and Brown 2003). In vitro studies have revealed that the entire *shot* protein is required for early axonal extension and that the removal of either the microtubule or actin-binding regions from *shot* is sufficient to inhibit axon initiation. The *shot* protein is also expressed at equally high levels within the neuronal growth cone, supporting a role for shot-based microtubule–actin coordination during growth cone navigation and target finding (Lee and Kolodziej 2002).

Data from higher eukaryotic cell systems support the *Drosophila* findings but add complexity to the story. Interestingly, *shot* represents the only spectraplakin gene in *Drosophila*, and disruption of the *shot* gene produces neuronal effects similar to but much more dramatic than those seen when a mutation is introduced into either MACF1 or BPAG1. Thus, both structural analysis and genetic manipulation studies support at least a partial functional redundancy between the MACF1 and BPAG1 proteins in vertebrate cells, although experiments designed to directly test this redundancy have yet to be performed.

In vertebrate HeLa cells, the ACF7 (synonymous with MACF1) protein has been shown to be involved in mediating proper CLASP localization to the cell cortex. Using small interfering RNA-based knockdown of ACF7, it was found that partial reductions in ACF7 protein levels resulted in reduced levels of cortical CLASP signal and an accompanied loss of microtubule +TIP capture at the cortical microtubule–actin juncture (Drabek et al. 2006). Moreover, in mice the MACF1 protein has also been found to interact with a complex consisting of β-catenin and GSK3beta, responsible for indirectly affecting microtubule stability, and APC, which is able to achieve physical interactions with microtubule plus ends through a complex with the EB1 protein, in order to enhance the stability of the dynamic microtubule array.

A closely related family member of MACF1 the BPAG1 spectraplakin shares homology with the murine dystonin protein, which was first identified as a neuronal form of bullous pemphigoid antigen 1 (Dalpe et al. 1998). In neurons, the dystonin protein is able to bind and link microtubules, neurofilaments, and actin filaments. Inhibition of this activity does not prevent cultured neurons from initiating axons, but those axons that do elongate display disorganized arrays of all three cytoskeletal elements (Dalpe et al. 1998). Some aspects of this phenotype are visible in mice possessing mutations in the *BPAG1* gene locus, the cause of the cytoskeletal disorder *dystonia musculorum* (*dt*). Homozygous dt/dt mice suffer from progressive sensory neuron degeneration and loss of motor activity shortly after birth (Dowling et al. 1997, Bernier et al. 1998, Dalpe et al. 1999). These BPAG1 mutant mice also display a severe skin-blistering phenotype, attributable to the separation of epidermal and muscle layers resulting from the loss of cytoskeletal interconnectivity.

More recent investigations have identified a potential role for BPAG1 activity in and around the cell nucleus. In mouse myoblast cell lines it has been shown that N-terminal modifications to the BPAG1 protein may be one mechanism for altering cell localizations of this cytoskeletal cross-linker protein. Previous knowledge of BPAG1 activity at cortical sites of microtubule–actin boundaries, coupled with

localization studies of perinuclear BPAG1, supports a role for this protein–nucleus association in the structuring of the nuclear envelope and nuclear tethering (Young et al. 2006). The nuclear tethering functions could prove critical during neuronal migration, where the advance of the nucleus in the direction of migration is known to be a pre-requisite for migratory advance of the cell body and associated cytoplasmic organelles and membrane structures. One physiological advantage of establishing a BPAG1-mediated linkage of perinuclear actin with cytoplasmic microtubules may be to tie nuclear movements and cell migration to molecular motor forces, including forces generated by the cytoplasmic dynein motor, that play an important role in pulling the nucleus in the direction of cell migration (Tsai and Gleeson 2005). Such migratory coupling of nucleus and cytoplasm is an evolutionarily conserved mechanism utilized by mitotic cell types and migratory neurons, both of which ubiquitously express at least one variant of the BPAG1 protein.

5.6 Concluding Remarks

Microtubule–actin interactions in both neurons and non-neuronal cells comprise one of the most rapidly expanding areas of modern cell body. Here, our goals were to provide an overview of some of these interactions and to discuss how they may be important during the development of the neuron. We apologize to authors whose work there was insufficient space to discuss.

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Chapter 6
Working with Actin: Methodological Approaches for the Study of Actin in Neurons

Erik W. Dent

Abstract Throughout the life of an organism, the cytoskeleton plays fundamental roles in many physiological processes. An important component of the cytoskeleton is actin, in both filamentous (F-actin) and globular form (G-actin). In the nervous system, actin functions in many processes from neuronal determination to synapse formation. Actin is dynamic, continuously transiting between polymer and monomer with the aid of myriad actin-associated proteins. Filamentous actin can produce several secondary structures within the cell, including filopodia, lamellipodia, veils, and dendritic spines. Actin can also be manipulated with a number of peptide toxins that stabilize or destabilize the actin cytoskeleton in specific ways. This chapter will discuss several techniques for studying actin in the nervous system.

Keywords Actin · Cytoskeleton · Growth cone · Live cell imaging · Fixation · TIRF · Transfection

6.1 Introduction

The cytoskeleton plays an important role in the developing and mature nervous system. Three of the primary components of the cytoskeleton in neurons are actin filaments (F-actin), microtubules, and neurofilaments. These cytoskeletal elements are crucial for many processes, including cell division, polarization, process outgrowth, guidance, synaptogenesis, and modification of synapses throughout the life of the organism. Actin, in both filamentous and monomeric form, plays a central role in many of these processes. An important aspect of actin biology is that, like the neuron, it too forms a polar structure. Generally, actin monomers add to one end of the filament and are removed from the other end (Pollard and Borisy 2003). When actin monomers self-associate, they form a polar polymer that, when joined

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to other actin polymers, is capable of making several secondary structures that are key for neuronal differentiation. Actin filaments are thought to be nucleated near the membrane and new monomers are added at the distal ends of existing filaments at the membrane (Pollard and Borisy 2003). This produces a protrusive force that gives rise to actin-rich structures such as lamellipodia, veils, filopodia, and dendritic spines. Many of these structures are concentrated in the neuronal growth cone, at the tip of extending processes, but can also occur at other places on axons and dendrites.

A brief summary of actin-based structures provides an appreciation for their complexity and the important roles they play in neurons. Lamellipodia, the flat sheet-like protrusions, consist of a meshwork of actin filaments. In non-neuronal cells this meshwork is formed through the nucleation of actin filaments off existing filaments by activation of the Arp2/3 complex, to produce a Y-shaped structure with a stereotyped branching angle of 70° (Pollard and Borisy 2003). Interestingly, in neurons, although lamellipodia can contain these Y-shaped actin structures (Mongiu et al. 2007), they are dominated by a cross-hatched network of longer actin filaments (Strasser et al. 2004). When a meshwork of actin filaments polymerize between two filopodia, they are referred to as veils. Filopodia, the finger-like projections that emanate from the growth cone and along axon and dendrite shafts, are thought to arise primarily from several cross-hatched lamellipodial actin filaments that coalesce to form a lambda (λ) shape and elongate in tandem (Svitkina et al. 2003). Filopodia are composed of about a dozen or so actin filaments that polymerize parallel to one another and are subsequently linked together to form narrow (0.1–0.4 μm in diameter) tube-like extensions that can extend several tens of micrometers (Lewis and Bridgman 1992). The filopodium is an important sensory-motor apparatus for neurons. They are sensory structures because they contain receptors for guidance cues and are usually the first structures of a neuron that encounter extracellular cues. Filopodia also have a high surface area-to-volume ratio; so they are very efficient at conducting information from the periphery to the more central regions of the neuron. They also serve a motor function because they transduce force through the formation of adhesions with the substrate.

Unlike lamellipodia, veils and filopodia, actin arcs, and intrapodia are much more recently described actin structures that occur in the growth cone. Actin arcs are also formed from cross-hatched actin filaments but in contrast to filopodia, which are formed at the membrane, arcs are formed more proximally in the lamella through compaction of actin filaments into anti-parallel arrays (Schaefer et al. 2002). Neuronal actin arcs are structurally similar to stress fibers that are formed in many other cell types in culture. It is not yet clear if arcs are formed in growth cones growing in vivo, but they are prominent in paused, enlarged growth cones in vitro. Intrapodia, like filopodia, are formed at the membrane but intrapodia "surf" along the dorsal surface of growth cones (Katoh et al. 1999, Rochlin et al. 1999, Dent and Kalil 2001). They translocate at a higher velocity than filopodia extend and are short lived (Rochlin et al. 1999). Their structure appears to be a cross between a filopodium and a lamellipodium, resembling a "comet tail" of actin filaments that form from the distal end of invasive bacterial or viral pathogens. Like actin arcs, it is not known if intrapodia form in vivo or what function they may serve.

Dendritic spines are another structure resulting from a concentration of actin filaments. Dendritic, in this context, can refer to both the structure from which spines protrude (the dendrite) and the distribution of actin filaments within the spine (a dendritic array of filaments). Spines are thought to form from filopodial precursors that condense into a mushroom-shaped structure (Yuste and Bonhoeffer 2004). The actin cytoskeleton within a spine is dynamic (Fischer et al. 1998) but also contains a small population of relatively stable actin filaments (Star et al. 2002). Again, in the case of the spine, structure gives rise to function. In diseases of the nervous system that result in several types of mental retardation, it is thought that the dendritic spines often lack a mature mushroom-shaped structure (reviewed in Calabrese et al. 2006). This mature spine structure appears to be important for cell-to-cell communication because immature, filopodial-like spines appear to be less efficient at conducting information from one neuron to another.

To continue to elucidate the function of actin in the nervous system, it is important that we have means to reliably study its structure and interaction with the many actin-binding proteins that are essential for its function. This chapter will provide several methodologies for studying actin in the nervous system. Although I will focus on the study of actin in dissociated neurons in cell culture, many of the methods discussed here will serve to inform researchers that study neurons in tissue slices or in the animal as well.

6.2 Fixation and Immunocytochemistry of Actin in Neurons

An important aspect of studying any protein is to develop a faithful and repeatable method for fixation and labeling of the protein. We will restrict the procedures covered here to cultured embryonic neurons grown on glass coverslips. Neurons from the central nervous system (CNS) and the peripheral nervous system (PNS) are very sensitive to fixation conditions. Several methods of fixation that may be used in more adherent cells, such as fibroblasts, are not appropriate for the fixation of neurons. An example of such a fixative is methanol. Usually, 100% methanol is used that has been dehydrated by the addition of molecular sieves (Aldrich) and kept at –20°C until just prior to fixation. Methanol is added to the cultures after washing them in PBS and removing as much of the PBS as possible. We have found that neurons fixed by this method retain their gross morphology; cell bodies, minor processes, and axons are generally intact. However, much of the fine morphological detail such as growth cone morphology and lamellipodia and filopodia along the neurite shafts are usually destroyed. We therefore favor an aldehyde cross-linking fixative (see below). However, antibodies, especially polyclonal antibodies made to peptide antigens, may not react well with aldehyde-cross-linked proteins. In such circumstances we have successfully fixed neurons for a very short period of time in a paraformaldehyde fixative (10–30 s) followed by careful removal and addition of cold, dehydrated methanol. The paraformaldehyde fixative applied for a short period of time stabilizes labile structures followed by precipitation of proteins by methanol. This two-step

fixation maintains the morphology of the neuron while preserving the antigenicity of epitopes that may be disrupted during paraformaldehyde fixation alone.

We have tested many types of fixatives on both CNS and PNS neurons and have found one that we feel best preserves both the morphology of the neurons and the structure of actin filaments (recipe below). This is a paraformaldehyde fixative in a Krebs-based solution, containing sucrose. We determined this formulation by making up many types of fixatives and slowly perfusing them into chambers containing dissociated neurons, while imaging the growth cone at high resolution (100×/1.4NA DIC objective). The purpose of these experiments was to find the fixative that faithfully maintained the morphology of the growth cone between the last living image and the fixed image. This procedure also allowed us to observe how fast each fixative "fixed" the growth cones in place. An example of a living and fixed growth cone with this fixative is shown in Fig. 6.1 and the recipe for this fixative in Fig. 6.2.

As mentioned above, actin is present in both filamentous (F-actin) and globular (G-actin) forms. Therefore, during fixation both forms of actin are fixed in place. However, during fixation a small percentage of F-actin and G-actin are lost. We have found that after microinjecting cortical neurons with fluorescent phalloidin, a toxin with nanomolar affinity for F-actin, we lose on average about 5–10% of the fluorescence. This may be due to some loss of filaments, dissociation of fluorescent phalloidin from the filaments, and some photobleaching of the fluorophore.

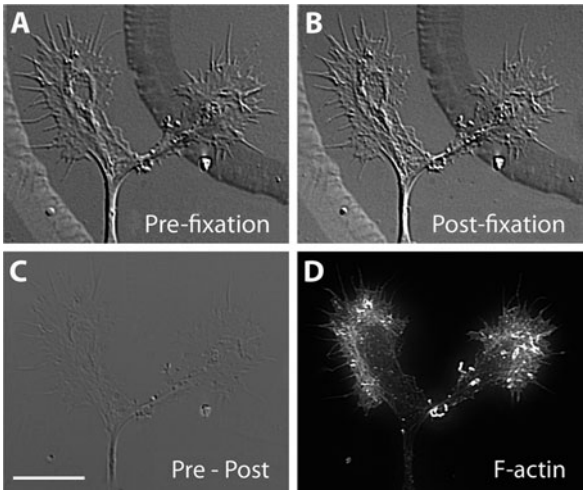

Fig. 6.1 Morphology of neurons fixed in 4% PKS is faithfully maintained. (**a**) Living growth cones from a cultured cortical neuron imaged with DIC microscopy. This is the last frame imaged before addition of 4% PKS fixative (see text for formulation). These neurons were cultured on etched coverslips for identification after fixation (etchings in background of image). (**b**) The same two growth cones shown in (**a**) after fixation with 4% PKS. (**c**) An image resulting from subtraction of (**a**) from (**b**). Differences are manifest as *bright* or *dark regions*. Note that there is little difference in the pre-fixation and post-fixation images. (**d**) The same two fixed growth cones labeled with fluorescent phalloidin to stain filamentous actin

4% Paraformaldehyde in Krebs+Sucrose Fixative (4% PKS)

4% (w/v) Paraformaldehyde (EM Sciences)
145mM NaCl
5mM KCl
1.2mM $CaCl_2$-$2H_2O$
1.3mM $MgCl_2$-H_2O
1.2mM NaH_2PO_4-H_2O
10mM Glucose
20mM HEPES
0.4M Sucrose

(All other reagents are purchased from Fisher or Sigma)

- Heat ~80% final volume of ddH_2O in beaker in fume hood to ~60°C.
- Add about 10 drops of 10N NaOH while heating.
- Add paraformaldehyde while heating.
- Make sure most of paraformaldehyde goes into solution and cool down to near room temperature.
- Add all remaining ingredients and dissolve (there will be a white flocculent precipitate – that will go into solution when pH is adjusted)
- pH to 7.4 with pH paper
- Top up to final volume with ddH_2O
- Sterile Filter to remove debris
- Aliquot and store at –80°C
- Thaw in 37°C water bath and fix at 37°C
- Note: if a white precipitate appears upon thawing and doesn't go into solution upon vortexing discard all frozen fix and make new.

We generally store this fixative for several months at –80°C. We may occasionally add 0.25% glutaraldehyde (EM Sciences) to this fixative as well, which tends to stabilize the cytoskeleton slightly better than the paraformaldehyde-only fixative. However, glutaraldehyde, even at this low concentration, can increase autofluorescence.

Fig. 6.2 Preparation of fixative optimized for preservation of the neuronal cytoskeleton

For labeling F-actin, Alexa-labeled phalloidin (Molecular Probes/Invitrogen) is our probe of choice. Phalloidin is a bicyclic heptapeptide toxin produced by the death cap mushroom. Because it is specific for F-actin, there will be very little background labeling in fixed neurons. Actin antibodies, such as a β-actin antibody (Sigma), can be used but will generally label both filamentous and globular actin and result in a much more punctate labeling of actin filaments compared to phalloidin. This may be due to the decreased accessibility of the large antibody compared to the small phallotoxin. If one is interested in labeling G-actin specifically, a fluorescently labeled DNase I (Sigma) can be used. In previous work, we have ratioed the amount of staining with phalloidin and DNase I in fixed neurons to determine a measure of F-actin:G-actin in the growth cone (Dent and Kalil 2001). Taking the ratio of the two fluorescent labels also corrects for artifacts due to changes in thickness in different regions of the cell. It should also be noted that methanol fixation destroys the epitope

for phalloidin, so it should not be used as a fixative if one desires to label F-actin with phalloidin. Therefore, when double labeling with an antibody that requires methanol fixation, one must use an anti-actin antibody in place of phalloidin.

6.3 Microinjection of Labeled Actin or Phalloidin to Monitor F-Actin in Living Neurons

At present, we have found it easier to transfect neurons with actin coupled to versions of green fluorescence protein (GFP) or mCherry (Shaner et al. 2004) than to microinject neurons with fluorescent phalloidin. We will discuss how to transfect cortical neurons below but have also included this section on microinjection as another methodology for visualizing actin filaments in neurons. One advantage of microinjection of labeled phalloidin is that you can chose the cells you want to inject based on their morphology and robustness. A further advantage is that microinjection of labeled phalloidin is better than transfection of labeled actin for imaging stable actin structures, such as actin arcs (Zhang et al. 2003).

We have successfully used Alexa-labeled phalloidin (Molecular Probes/Invitrogen) for labeling F-actin in living cortical neurons (Dent and Kalil 2001). We have also described the methodology for preparing the phalloidin for injection and the process of injection in detail in another chapter (Dent and Kalil 2003) so we will describe only the process briefly here. Alexa-labeled phalloidin is solubilized in anhydrous methanol to a concentration of 6.6 μM and stored at –20°C. An aliquot (200 μl) is removed to a microfuge tube and dried under a stream of nitrogen. The phalloidin is then solubilized in 0.5 μl of DMSO. Injection buffer (100 mM PIPES, 0.5 mM $MgCl_2$, pH 6.9) is added to the phalloidin/DMSO to a final concentration of 132 μM. This solution is centrifuged at 15,000 rpm for 5 min to pellet any particulates. The solution is removed and loaded into microinjection pipets. These pipets must be pulled to submicrometer tip diameters on a quality pipet puller such as a Sutter P97.

CNS or PNS neurons are injected at high magnification (100×/1.4NA objective) with the aid of a precision microinjector (Eppendorf). For purposes of locating neurons after microinjection, we plate them on etched grid coverslips (Bellco). Recording the coordinates of the injected neurons allows us to return to them after letting them recover for about 30–60 min before imaging. It is curious that phalloidin does not seem to affect actin dynamics because it does stabilize actin filaments in cell-free systems. However, it has been used with success in several types of neurons (Schaefer et al. 2002, Dent and Kalil 2003). Alternatively, we have tried injecting fluorescently labeled non-muscle actin (Cytoskeleton, Inc.) but have found that it tends to clog the small diameter microinjection pipets.

As mentioned above, an advantage of microinjection of phalloidin is that one is able to choose a robust neuron at a certain stage of development and image the neuron within less than 1 h after injection. Also, since fluorescent phalloidin binds only

F-actin at high affinity, it gives a good signal-to-noise ratio and little background fluorescence. It also appears to label stable actin structures better than labeled actin. Disadvantages of this technique are that it takes time to become proficient at injection of neurons and the microinjector and pipet puller are quite expensive. However, if one is studying peripheral neurons, such as dorsal root ganglion neurons (DRGs), they are easier to inject because they have larger cell bodies (20–30 μM in diameter, compared to 10–15 μM for cortical/hippocampal neurons) than CNS neurons at a similar stage of development. Another caveat is that it can be more difficult to study very dynamic actin-driven processes because phalloidin does not appear to label the very distal ends of elongating filaments as readily as fluorescently labeled actin (Zhang et al. 2003).

6.4 Transfection of Neurons with EGFP or mCherry–β-Actin

Because of the disadvantages associated with microinjection, transfection is our preferred method for fluorescently labeling actin in neurons. Until recently, however, it was not possible to reliably transfect/infect cortical neurons with high efficiency. Lentivirus and adenovirus have been used to effectively deliver proteins into CNS neurons (Lebrand et al. 2004, Strasser et al. 2004, Janas et al. 2006). These viruses generally take a day or two to express sufficient levels of protein for fluorescence detection, so they may not be appropriate to use for visualizing actin dynamics during early neuronal differentiation. However, a way to circumvent this lag in expression is to infect and grow neurons in a non-adherent dish for several days, during which time adenoviral/lentiviral delivered proteins are expressed, followed by dissociation and plating on an adherent substrate (L.M. Lanier, personal communication). Also, viral infection can produce stable, long-term expression of the fluorescent protein of interest and thus could be quite useful for labeling the actin cytoskeleton for the study of dendritogenesis and synaptogenesis, which occur later than axon outgrowth. A disadvantage with any viral infection is that the virus itself is composed of many foreign proteins that may have undesired effects on the infected neuron. I will consider only methods of transfection in this article.

One of the primary difficulties in studying early events in neuronal development, such as neurite formation, is that transfection techniques usually take a day to produce sufficient levels of protein for visualization. Furthermore, we have found that mouse cortical neurons are especially difficult to transfect, in comparison with cortical neurons from other rodents. It is not clear why this is the case, but personal experience has shown that cultured embryonic mouse cortical neurons are refractory to transfection via calcium phosphate and lipofection at early times in culture. Nevertheless, calcium phosphate and lipofection are often used to label small percentages of neurons in older cultures (>1 week in culture) and are often preferred methods for studying synapse development and function. However, we have found that it is possible to use electroporation to transfect embryonic cortical neurons with relatively high transfection efficiencies (20–50%). With the aid of the Nucleofector

electroporator (Amaxa) we have successfully transfected many different cytoskeletal and cytoskeletal-associated proteins into embryonic cortical and hippocampal neurons (Dent et al. 2007, Hu et al. 2008). A disadvantage of this system is that it requires the initial purchase of a Nucleofector device and the continued purchase of the manufacturer's kits to transfect specific primary cells, which can be quite expensive. However, for hard to transfect cells, like murine cortical neurons, it has really been a breakthrough technology.

Another advantage of the Nucleofector system is that neurons are transfected in solution, just after dissection, which results in the expression of measurable levels of fluorescent protein in just a few hours. Other transfection or infection techniques (calcium phosphate/lipofection or adenoviral/lentiviral) usually require adherent cells and take about a day to produce sufficient quantities of fluorescently labeled protein to image. Therefore, we have found nucleofection to be our method of choice for producing fluorescently labeled proteins in neurons with high efficiency and with a short time to expression. We have also kept neurons in solution in uncoated plastic dishes (Falcon) for several days after transfection, which allows the neurons to express the fluorescently labeled protein of interest. We then plate these neurons on poly-D-lysine-coated glass and can observe very early events during initial attachment and spreading. We have also used nucleofection of dissociated neurons to transfect cortical neurons for the study of dendritic spine formation in cultures several weeks old. Most cytoskeletal proteins, including β-actin, are expressed at relatively high levels for as long as we have cultured these cells (1 month). Thus, this technology can be useful to study much later events in neuronal differentiation, such as synapse formation and function.

I will briefly describe what is involved in the transfection of CNS neurons with the Amaxa Nucleofector to give readers a sense for whether it would be appropriate for their purposes. Briefly, primary neurons are isolated from embryonic mouse fetuses. We use E14.5 fetuses for isolation of cortical neurons or E15.5 fetuses for hippocampal neurons. Generally, we have found that isolating neurons as early as possible is best because of increased death after isolation and electroporation of older neurons. This is not to say that older neurons cannot be successfully electroporated, just that there tends to be significantly more death in these cultures.

After euthanization of a pregnant female mouse with an appropriate technique, the uterine horns are removed to a sterile dish containing ice-cold dissection medium (DM). DM is HBSS without calcium and magnesium (Invitrogen), with 10 mM HEPES added. Fetuses are removed from the uterus and placed in a fresh dish containing ice-cold DM. Fetuses are decapitated with microforceps and cortical hemispheres are isolated and stripped of meninges in cold DM. The cortex and/or the hippocampus are carefully dissected away from other areas of the brain and placed in a microfuge tube containing 1.0 ml of DM. Trypsin (110 μl) is added from a 10× stock solution (Invitrogen) and the microfuge tube containing the cortex or the hippocampus is placed at 37°C for 20 min. We prefer to use the incubator rather than a water bath because of the increased likelihood of contamination when microfuge tubes are placed in 37°C water.

After 20 min at 37°C, the DM/trypsin solution is removed and warmed plating medium (PM) is added to the tissue. PM is Neurobasal solution with B27 supplements (Invitrogen), glutamine (Invitrogen), and 5% fetal bovine serum (Hyclone). We also add extra glucose (0.3%) and NaCl to increase the osmolarity of the Neurobasal solution (~225 mOsm) to ~310 mOsm (Dent and Kalil 2003). Initially, we did this because we found it difficult to microinject cortical neurons in the low-osmolarity Neurobasal medium but have found that the neurons appear healthier at osmolarity similar to other basal solutions (i.e., DMEM). Containers of both PM and SFM (see below) are placed, with lids ajar, in the incubator for several hours prior to the dissection to come to proper temperature and pH. The cortical or the hippocampal tissue is rinsed two more times in PM and the tissue is dissociated by gentle trituration (10–15× with a P1000 pipet). Any remaining tissue chunks are allowed to settle and the dissociated cells are removed to 4 ml of PM and spun at low speed (350 rpm/16×*g*) for 7 min. If the neurons are not transfected, they are resuspended by trituration in PM. However, if they are to be transfected, they are resuspended in Amaxa electroporation solution. If we use the dissected neurons for Western blotting, we do not spin the cells down and plate them at high concentrations ($1–2\times10^5$ cell/cm^2).

We have found that we can transfect up to five different plasmids into tissue from one cortex (two hemispheres) or a single plasmid into four hippocampi (from two brains). These amounts of tissue are equivalent to about 5×10^5 cells per transfection. However, if tissue is not limiting, we prefer to transfect about $5–8\times10^5$ cells at a time. This is substantially fewer neurons than the manufacturer recommends (4×10^6 neurons) and may result in more death, but also result in higher transfection efficiency. Generally, we assume we will isolate about 1.5×10^6 neurons from a pair of cortical hemispheres or 3×10^5 neurons from a pair of hippocampi. We add 100 μl of transfection solution per transfection condition. For example, if we wanted to electroporate five different plasmids, we would isolate two pairs of cortices (3×10^6 neurons) and add 500 μl of transfection solution, gently dissociate the neurons again, and separate them to five microfuge tubes containing the appropriate amount of DNA (see below). Working quickly, we electroporate the neurons, add 500 μl of PM to the cuvette after electroporation, remove the neurons to another microfuge tube, and bring the total volume to 1.0 ml with PM. Neurons are counted on a hemocytometer and plated at a density of $5–10\times10^3$ cells/cm^2 for short-term studies (1–3 days in culture) or $2–4\times10^4$ cells/cm^2 for long-term studies. For preparation of coverslips, see Dent and Kalil (2003).

For DNA purification we usually use Qiagen Endo-Free DNA purification kits but have also used standard Qiagen kits as well. It is likely that DNA purified from other manufacturer's kits (Invitrogen, Clontech, etc.) would work as well. We have empirically found that expression of genes following electroporation is dependent primarily on the promoter and the size of the gene being expressed. We generally find a CMV promoter or a β-actin promoter with a CME enhancer work the best. Also, we discovered that we need to use substantially more DNA than recommended by the manufacturer to get optimal transfection efficiencies. Assuming that the fusion protein is driven by a CMV or β-actin/CMV$_{enh.}$ and GFP or mCherry is

approximately a 30-kDa protein, we use the following formula for determining the amount of DNA to use for each transfection:

$$\text{DNA}(\mu\text{g}) = (30\ \text{kDa} + \text{kilodalton of protein fused to GFP})/10$$

For example, for β-actin, which is 42 kDa, a GFP–β-actin fusion protein would be about 72 kDa; so we would use 7–8 μg DNA per transfection. We usually prefer to use DNA at 1 μg/μl or more in TE buffer so that we do not dilute the transfection solution. We have transfected proteins up to 250 kDa (using ~30 μg of DNA) but have found that with large proteins even using a lot of DNA does not necessarily result in more transfected cells. If multiple plasmids are used to transfect primary neurons, we use the above calculation for each construct. At maximum we have used 40 μg of DNA per transfection.

6.5 Imaging Actin Dynamics in Neurons

After nucleofection we have been able to visualize GFP-labeled β-actin within 4 h of plating but usually do not image the neurons until 16–48 h post plating, depending on the stage of development in which we are interested. One should always be concerned with how the expression of an exogenous protein affects the physiology and morphology of the cell being studied. Even if one is interested in visualizing a GFP-tagged version of a protein not present in the cell of interest, it is always possible to produce phenotypes that result from overexpression of the gene of interest. One of the primary artifacts that one observes in cells overexpressing a GFP-tagged protein is aggregation. The amount of aggregation varies with the type of protein and the levels of expression and may or may not be exacerbated by the fusion of GFP to the protein. We have found that expression of GFP–, mRFP–, or mCherry–β-actin at high levels can lead to some aggregation, especially in the cell body. Furthermore, there is research indicating that GFP-labeled actin, at concentrations above 5% of endogenous actin, has the potential for disrupting actin-associated processes, such as the binding of myosin to actin filaments (Choidas et al. 1998). Injecting fluorescently labeled phalloidin can circumvent these problems because of the small size of the label (<1 kDa compared to ~30 kDa for GFP), but high concentrations of phalloidin can produce artifacts as well by overstabilizing actin filaments. It is therefore important to titrate the amount of labeling of actin, regardless of the label, to a minimal level sufficient for imaging.

The minimal level of labeled actin that one can image will depend on the method of visualization. We have imaged labeled actin with wide field, spinning disk confocal, and total internal reflection fluorescence microscopy (TIRFM). One can also use scanning confocal microscopy and two-photon confocal microscopy. These latter two techniques are especially good for use with thicker samples. However, with any type of light microscopic imaging of a fluorescently labeled protein within a living cell, there is always a trade-off in resolution vs. health of the cell. Each of the

fluorescent light microscopy techniques mentioned above has their place in imaging of the actin cytoskeleton. All of these techniques use epifluorescence illumination: the exposure of cells to light of a given wavelength and collection of longer wavelength light from the sample through the objective. In epifluorescence microscopy, the objective acts as both the objective and the condenser. The benefits and limitations of several of these methods are covered below. Scanning and two-photon microscopy will not be covered because these systems are usually purchased as a unit from the manufacturers (Olympus, Nikon, Zeiss, Leica, etc.) and we have little experience with these methods of visualization.

Wide-field fluorescence microscopy is probably the least costly and easiest technique for imaging actin dynamics in living neurons. It requires a high-power lamp (usually mercury, xenon, or metal halide), an epifluorescent light path, filters for selection of wavelength, and high numerical aperture objectives for exposing and collecting light from the sample. It also requires a sensitive, high-resolution CCD camera for detection of the fluorescence signal. The standard light source for fluorescent illumination is usually a mercury lamp (100 W HBO) in a housing designed by the manufacturer of the microscope. A major drawback of mercury arc lamps is that they are not capable of evenly illuminating the field of view, even when aligned properly. They are also usually mounted directly on the microscope and can therefore transfer excessive heat to the microscope and the sample. Also, the lamp life of mercury arc lamps is limited to about 300 h. To overcome these limitations a number of manufacturers have designed metal halide lamps coupled to liquid light guides. These are very bright light sources that produce even illumination because the liquid light guide randomizes the light from a metal halide light source. Two versions that we have used with success are the X-Cite 120 fluorescent illumination system, comprised of a 120-W metal halide lamp with liquid light guide (EXFO Life Sciences) and the Lumen 200 fluorescent illumination system that has a 200-W metal halide lamp with liquid light guide (Prior Scientific). Both of these units provide intense, evenly illuminated light for exciting fluorophores in living and fixed neurons.

Regardless of the type of illumination used, the epifluorescent light path is designed by the microscope manufacturer. Therefore, the next piece of equipment that can be chosen for a given system are the fluorescent filters for excitation of the sample and collection of the emitted light. Filters can be purchased from a number of manufacturers including Chroma, Omega, and Semrock. Filter sets should be chosen carefully. If one is exciting one fluorophore, then a filter set containing an excitation filter/dichroic mirror/emission filter set should be chosen for that fluorophore. However, if one wishes to image the actin cytoskeleton at one wavelength and other labeled proteins at a different wavelength, it is necessary to purchase a multi-band-pass dichroic/polychroic mirror and filter wheels for both excitation and emission filters. Each fluorescent wavelength can then be collected by leaving the dichroic filter in place and rotating the excitation and emission filter wheels accordingly (Salmon et al. 2003). We have used filter wheels from a number of manufacturers including Sutter, Ludl, and Prior. It is important to have both an excitation and an emission filter wheel because GFP and mCherry excitation and emission spectra overlap to some extent and therefore need to be selected with

individual filters so that "bleed-through" of one channel to another does not occur. Using dual-filter wheels with a single dichroic mirror also allows one to false-color images and directly overlay them to determine colocalization between proteins in a single neuron.

The selection of the objective is also an important factor in imaging cells containing fluorescently labeled proteins. Generally, 60×/1.4 NA or 100×/1.4 NA objectives are used because they provide the maximum numerical aperture for both efficient excitation of cells and collection of the fluorescent light from the cells. We prefer to use apochromatic objectives because they allow one to collect images in two wavelengths from a sample without changing focus. It should be noted, however, that even apochromatic objectives focus light of different wavelengths at slightly different places in the *z*-axis. Therefore, if one is imaging fluorophores whose emission wavelengths are widely separated on the electromagnetic spectrum, such as Alexa 350 and Alexa 647, the focus will have to be adjusted between exposures. If one were to use non-apochromatic objectives, it would be impossible to get even GFP and mCherry in focus without adjusting the position of the objective.

Another, more recently developed technique for imaging fluorescence in living cells is spinning disk confocal microscopy. This technique consists of illumination of cells through a spinning disk that contains thousands of small pinholes (Inoue and Inoue 2002). When this disk is rotated rapidly, the entire field of view is exposed to light but results in a confocal image because of the small aperture of holes in the disk. Generally, laser light is required to adequately expose the sample because the spinning disk attenuates a significant amount of light reaching the sample and the detector. Interestingly, spinning disk confocal microscopy tends to do less damage to cells than does wide-field microscopy. Reasons for this are not entirely clear but probably are the result of exposure of only part of the sample in the z-axis due to the apertures in the spinning disk but also may be due to the averaging of the thousand points of light that penetrate the disk (Inoue and Inoue 2002). By averaging the light over the entire sample, less light may be needed to acquire an image sufficiently above the background noise. A disadvantage of this technique is the cost of the spinning disk head (Yokagawa) and the laser system for illumination. We have used a two-watt, multi-line krypton/argon laser (Coherent) and selected wavelengths with dual excitation/emission filters. With this system we found that we could image living neurons for about 25–50% longer than when using standard wide-field illumination. It is therefore a preferable system for live cell imaging of neurons because they tend to be very sensitive to photodamage.

Total internal reflection fluorescent microscopy (TIRFM) has also been developed recently to image fluorescently tagged proteins within living cells. Commercially available TIRFM systems are now available from the major microscope manufacturers. We have found that TIRFM, like spinning disc confocal microscopy, produces less photodamage to neurons, allowing one to image cellular events with increased signal-to-noise and for longer periods of time, compared to wide-field microscopy. Briefly, TIRFM excites fluorophores only in the several hundred nanometers closest to the bottom membrane of the cell as a result of an evanescent wave of energy created by deflecting high-power focused laser light

at an angle greater than the critical angle of the objective. Importantly, TIRFM does not excite any of the fluorophores in the upper regions of the cell; so one can acquire very high-quality images even in very thick regions of cells. However, one is limited to imaging the few hundred nanometers near the bottom surface of the cell. Nevertheless, TIRFM microscopy is particularly useful for imaging adhesion complexes or regions of the cell where exocytosis or endocytosis occurs, precisely because it only images the bottom region of the cell. Interestingly, one can also image a great deal of cytoskeleton (both actin and microtubules) in the lower regions of a cell or a growth cone (Fig. 6.3). This makes TIRFM a good microscopic technique for imaging the actin cytoskeleton.

Actin is a very dynamic polymer and we have discovered that using TIRFM microscopy we can image actin dynamics for relatively long periods of time (20–60 min) while acquiring images at frequent intervals (3–10 s). This is about three times as many images as we can acquire using wide-field microscopy. However, we have found that growth cones of both CNS and PNS neurons are rarely flush against the surface of the coverslip across the entire growth cone. Therefore, using TIRFM

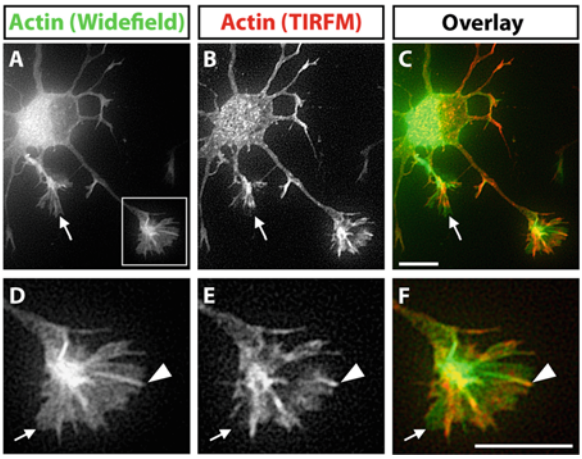

Fig. 6.3 Comparison between fluorescent wide-field illumination and total internal reflection fluorescent microscopy (TIRFM). (**a**) A single cortical neuron transfected with mCherry–β-actin and imaged in wide-field illumination. The *boxed region* from this image and **(b)** and (**c**) are digitally magnified in (**d**–**f**). The *arrow* points to the distal extent of a growth cone. (**b**) The same neuron as in (**a**) imaged in TIRFM. Note that the distal veil of the growth cone (*arrow*) cannot be seen in this imaging mode indicating it is off the surface of the coverslip. Also note the lack of blooming around the cell body and the higher signal to noise of the actin present there. (**c**) An overlay image of (**a**) and (**b**). The wide-field image is in *green* and the TIRFM image is in *red*. There are several areas in the growth cone that contain little signal in the TIRFM image (*green regions*), while other regions are more prominent in the TIRFM image (*red regions*). (**d**–**f**) Digitally zoomed images of the growth cone shown in (**a**–**c**). The *arrow* points to a region of the distal growth cone that is present in the wide-field image but absent from the TIRFM image indicating it is off the substrate. The *arrowhead* points to a region of the growth cone with a higher signal in the TIRFM image, indicating that actin is concentrated near the cell membrane in that region

to image growth cones oftentimes results in dark regions where there is obviously actin present but where the growth cone is well above the glass coverslip (Fig. 6.2). This oftentimes occurs in the distal lamellipodia and filopodia of the growth cone. At present we use a microscope system that is capable of acquiring both TIRFM and wide-field images in quick succession. When TIRFM and wide-field images are false colored and merged, one can appreciate regions of the growth cone or the neuron that are near the substrate and those regions that are not (Fig. 6.3).

6.6 Pharmacological Manipulation of Actin

Although one can gain a great deal of information from high-resolution, time-lapse imaging of the actin cytoskeleton in living neurons, it is often useful to be able to perturb actin in neurons to determine if actin dynamics is necessary for the process being studied. There are a plethora of actin-binding proteins that regulate actin polymerization, stability, and depolymerization, and disruption of these proteins will affect actin in specific ways. There are also several small molecule toxins that can specifically influence actin in reproducible ways (Spector et al. 1999).

To depolymerize actin the two primary drugs used are cytochalasin and latrunculin (Sigma/Molecular Probes–Invitrogen/Calbiochem). These drugs come in several varieties. Cytochalasins are cell-permeant fungal toxins. We generally use cytochalasin D because it is more potent than other cytochalasins (B and E). Latrunculin, a marine toxin from the Red Sea sponge, comes in two varieties, A and B. We use latrunculin A because it is usually sold at higher purity than latrunculin B. Cytochalasin D when used at low concentration (<100 nM) specifically caps actin filaments at their growing (barbed) end. However, when cytochalasin is used at higher concentrations (1–10 μM), it is also capable of severing actin filaments in addition to capping their ends. Capping and severing of actin filaments are two ways to reduce the pool of F-actin in cells. Capping does not allow actin filaments to elongate, and if the actin polymers are dynamic, they will depolymerize over time from their pointed ends. Severing by cytochalasin, in addition to capping, speeds up this process. Latrunculin A is a compound that binds to actin monomers (G-actin) and sequesters them, effectively removing them from the actin cycle. Thus, latrunculin A enhances depolymerization of filamentous actin. Generally, latrunculin is used at low micromolar concentrations to depolymerize much of the actin in developing neurons (Gallo et al. 2002). Cytochalasin and latrunculin have also been used to disrupt the actin architecture within dendritic spines in older (2–4 week) cultures (Fischer et al. 1998, Star et al. 2002).

There are also toxins that exist that are capable of stabilizing filamentous actin within cells. As mentioned earlier, phalloidin can stabilize actin filaments in cell-free systems, but when injected into neurons, at least at the concentrations we and others have used, it appears to act as a filamentous actin label rather than an actin-stabilizing drug. However, when bath applied to cultures of neurons, phalloidin can affect the actin cytoskeleton, even though it does not effectively cross the plasma membrane (unpublished results). Another actin-stabilizing compound is

jasplakinolide. Jasplakinolide is a potent, cell-permeable macrocyclic peptide from another type of sea sponge that stabilizes actin filaments at nanomolar concentrations by binding to the sides of actin filaments. Jasplakinolide is also capable, at micromolar concentrations, of inducing actin nucleation (Bubb et al. 2000). This drug can be used in both developing and older neurons to show that ongoing actin polymerization and depolymerization are essential for axon extension and synaptic function (Gallo et al. 2002, Star et al. 2002). One caveat to keep in mind when using jasplakinolide is that it binds competitively to the same site on the actin filament as phalloidin (Bubb et al. 1994). Therefore, fixation of jasplakinolide-treated neurons and subsequent labeling with fluorescent phalloidin would show very little to no staining. To get around this issue, some investigators have washed out the jasplakinolide just before fixation (Cramer 1999) or simply used an actin antibody in place of phalloidin to label actin filaments in the treated cells. Others have actually used the mutually exclusive binding of phalloidin and jasplakinolide to F-actin to their advantage to determine the amount of jasplakinolide bound to F-actin by incubating fixed neurons with labeled phalloidin and comparing them with neurons pretreated with varying concentrations of jasplakinolide (Gallo et al. 2002).

There are also several other toxins and drugs that are known to bind and affect actin dynamics. However, I have limited my discussion to these few compounds because they have been the most studied and their functions are well known. It is likely that many more natural toxins and synthesized compounds that affect actin chemistry are likely to be discovered in the ensuing years, providing researchers with specific tools to study actin-dependent processes in neurons.

6.7 Future Directions for Studying Actin in Neurons

Actin has been studied extensively in neurons and non-neuronal cells over the past decades, but there is still much to be learned. Many of the signaling cascades that begin with receptor activation lead eventually to the cytoskeleton. Actin is probably the primary cytoskeletal component that is affected downstream of receptor-activated signaling cascades. Furthermore, there is much we do not know about the actin cytoskeleton in neurons. Recent work indicates that local translation of β-actin is important for axon guidance but not necessary for outgrowth per se (Leung et al. 2006, Yao et al. 2006). These studies also raise a number of interesting questions. Is newly translated actin somehow special? Is it better at polymerizing or is more stable/dynamic? It is also unclear how actin may be posttranslationally modified in neurons? Furthermore, there is little known about why there are two forms of actin (β and γ) present in approximately equal levels in neurons. Do they serve distinct purposes? There have also been relatively few studies on actin dynamics in neurons; however, with ever improving light microscopy techniques such as TIRFM and two-photon confocal microscopy we are likely to be able to discover much more about this interesting protein/polymer and how it plays a central role in the nervous system function.

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Chapter 7
Functions of the Actin Cytoskeleton in the Early Embryology of the Nervous System

Jeffrey D. Hildebrand

Abstract This chapter outlines the role that the actin cytoskeleton plays during the various stages of neural tube morphogenesis. While a great deal has been learned about this topic, there is still much to be uncovered. The large number of mutants that have been established to date is an excellent starting point, but a large-scale genetic analysis of these genes has yet to be performed. Based on the fact that either many of the proteins discussed above interact directly or the pathways in which they function interact, it will be important to use genetics to uncover the relationship between these proteins and pathways in vivo. Understanding how these pathways interact genetically may provide critical data that could explain the complicated etiology of human neural tube defects. To date no loci have been definitively identified as being causative for human neural tube defects. However, most studies indicate that there is a genetic basis for human birth defects of this nature and that they are multigenic in origin. This would suggest that heterozygosity at several different loci might be sufficient to cause neural tube defects. Currently, the only preventative agent for NTD is dietary supplementation with folic acid. However, the mechanism by which folic acid reduces the risk of birth defects is unclear. Therefore, it will be important to determine if folic acid can ameliorate NTDs caused by errors in actin dynamics, PCP, or ciliogenesis or if lesions in these pathways make animals more susceptible to folic acid deficiency. It appears that understanding how cytoskeletal organization is regulated in vivo during neurulation will be critical for future efforts to prevent NTD as a source of embryonic lethality and human suffering.

Keywords Actin · Cytoskeleton · Neurulation · Neural plate · Planar cell polarity · Neural tube defect

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7.1 The Basics and Clinical Significance of Neural Tube Morphogenesis

In vertebrates, the central nervous is derived from an embryonic structure called the neural tube. Several studies have estimated that 1 out of every 1,000 human births exhibits complications arising from errors in the formation of the neural tube, making it the second most common birth defect in human pregnancy (Campbell et al. 1986, Copp et al. 1990). Neural tube defects (NTDs) cause conditions such as spina bifida, acrania/anencephaly, and craniorachischisis. The majority of these defects result in lethality, with the exception of spina bifida which is compatible with post-natal viability. This number does not take into account other human syndromes, such as holoprosencephaly, that result from mutations in genes that encode critical regulators of neural tube patterning (Roessler and Muenke 2001). The neural tube is formed following induction of neural identity in the ectoderm by the activity of numerous secreted BMP inhibitors expressed primarily by the underlying mesoderm (De Robertis and Kuroda 2004). This chapter will not cover neural induction but will concentrate on the cellular basis for the morphological changes that occur following specification of the neural ectoderm. Once induced, the neural ectoderm undergoes a series of well-characterized morphogenetic steps to form the neural plate and subsequently convert the neural plate into the neural tube. The conversion of a flat sheet of cells into a closed neural tube that extends along the entire length of the embryonic anterior–posterior axis requires a significant degree of coordination between cell behaviors such as adhesion, migration, proliferation, differentiation, and morphology (Colas and Schoenwolf 2001). This chapter will focus primarily on the role of the actin cytoskeleton in mouse neural tube morphogenesis. The emphasis is being placed on mouse as a model system because the majority of the genetic analysis that has been performed has been done in mice and because this system probably best recapitulates that of human neural tube formation. However, conclusions drawn from experiments from other model systems, including *Drosophila*, *Xenopus*, chick, and zebrafish, will also be included if those systems suggest different possible interpretations or provide additional insights.

In both mice and humans, the neural tube closes in a defined and reproducible pattern along the rostro-caudal axis of the embryo beginning by embryonic (E) day 8.5 in mice or day 21–22 in humans. The first site of closure (point 1) is located approximately at the midbrain–hindbrain boundary (arrowhead 1 in Fig. 7.1a). Initial closure at this position facilitates closure in a "zipper-like" fashion in both the anterior and posterior directions (arrowhead in Fig. 7.1a). The second fusion point in mice is at the midbrain–forebrain boundary, while the third closure point is in the rostral-most position of the neural ectoderm, also called the anterior neuropore. The final closure point is at the posterior neuropore, located in the future lumbar–sacral region of the spine. Closure at these individual sites can occur independent of each other. This premise is based on the observation that a defect in one of these closure events does not necessarily affect closure at any other region of the embryo. This is the basis for the differing classes of birth defects that can arise from aberrant neural tube formation (Fig. 7.1b–e). Defects in closure points 2 are the

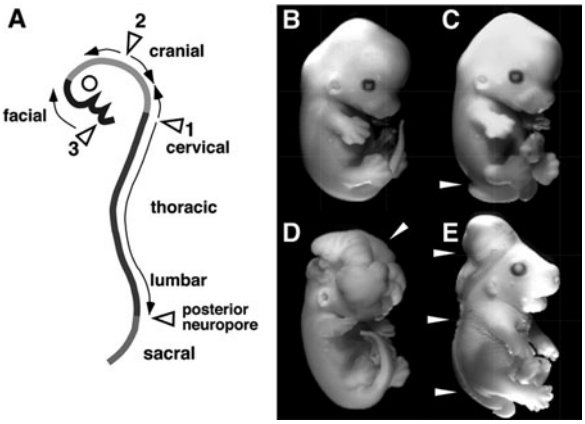

Fig. 7.1 Zones of neural tube closure and neural tube defects in mice. (**a**) Linear representation of the rostro-caudal axis of the mouse neural tube. Each *shaded area* represents an independent closure zone. The *numbered arrowheads* indicate the sites of initial fusion between the *lateral edges* and *arrows* indicate the direction of closure following fusions. (**b**)–(**e**) Mouse embryos displaying defects in neural tube closure, including spina bifida (**c**), exencephaly (**d**), and craniorachischisis. A normal embryo is shown in (**b**). *Arrowheads* indicate an open neural tube

most common and result in exencephaly/anencephaly, a condition where portions, or the entirety, of the cranial neural tube fail to close. In mice the brain continues to develop, but it is not enclosed in the bones of the skull (Fig. 7.1d). Failure in closure point 1 results in craniorachischisis, the most severe (and most rare) class of neural tube defect. This is typified by an open neural tube that extends from the midbrain to the caudal end of the neural tube (Fig. 7.1e). The final form of neural tube defect that is seen in humans and mice is spina bifida, a defect typified by an open neural tube in the lumbar–sacral region (Fig. 7.1c). This is caused by failed closure of the posterior neural pore. The role of the actin cytoskeleton in these closure events will be discussed in more detail below.

7.2 The "Classic" Steps of Neural Tube Closure

The ability to successfully execute the developmental program that converts the neural plate into the neural tube depends on highly choreographed interactions between numerous cellular processes, including proliferation, cell death, differentiation, cell movement, and cell morphology (Schoenwolf and Smith 1990, Smith and Schoenwolf 1997, Colas and Schoenwolf 2001). The fact that such a large number of events must be regulated, either coordinately or independently, likely accounts for the observation that, in mice, there are approximately 200 genes that have been implicated in neural tube morphogenesis based on the phenotypes associated with mutations in specific genes (Harris and Juriloff 2007). As might be expected, the molecular function of proteins encoded by these genes covers the entire gamut of

cellular and biological processes. This chapter will focus on those mutations that target either actin-binding proteins, regulators of actin dynamics, or components of cellular pathways that direct actin-based cellular processes.

Conversion of the neural plate into the neural tube has classically been divided into four basic steps: formation of the neural plate (step 1), shaping of the neural plate (step 2), neural plate bending (step 3), and neural tube closure (step 4) (Colas and Schoenwolf 2001). The basic morphogenic steps are depicted schematically in Fig. 7.2. The formation of the neural plate occurs during or shortly after specification of the neural ectoderm. The neural ectoderm becomes phenotypically distinct from the surrounding ectoderm as these cells adopt a more columnar appearance and become distinctly taller along their apical–basal axis relative to the surrounding ectodermal cells that will form the dermis. To date, there is no evidence that the actin cytoskeleton plays a direct role in the early formation or the specification of the neural plate.

The second step, shaping of the neural plate, is a critical stage in forming the neural tube. When first specified, the neural plate is wide along the medio-lateral (left–right) axis and short along the rostro-caudal (or anterior–posterior) axis. During this step, the neural plate narrows along the medio-lateral axis and lengthens along the rostro-caudal axis (Fig. 7.2). This change in shape is often referred to as convergent extension (CE) because cells move, or converge, toward the midline and, as a consequence, the tissue elongates, or extends, caudally (Davidson and Keller 1999, Keller et al. 2000, Keller 2002). CE is regulated by the planar

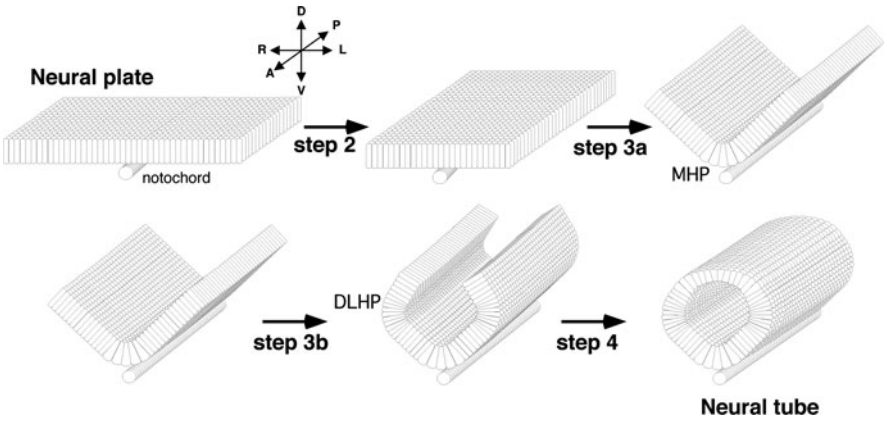

Fig. 7.2 Schematic representation of neural tube morphogenesis. Once specified (step 1, not shown), the neural plate is shaped by CE movements, becoming narrower in the medio-lateral axis and longer in the rostro-caudal axis (step 2). Concomitant with shaping, furrowing at the midline to form the medial hinge point (MHP) and elevation of the *lateral edges* creates the neural groove (step 3a). A second furrowing event in each neural fold creates DLHP that allows for rotation of the lateral edges; so they become juxtaposed at the dorsal midline (step 3b). Once positioned at the midline, the neural folds fuse at defined regions and then close along the remainder of the neural epithelium to form the closed tube

cell polarity (PCP) pathway (also called the non-canonical Wnt signaling pathway) in vertebrates (Montcouquiol et al. 2006, Seifert and Mlodzik 2007). This pathway will be discussed in more detail below. In vertebrates, molecular lesions that effect the expression or the activity of components of the PCP pathway all result in craniorachischisis (Fig. 7.1e) (Copp et al. 2003). The physiological basis for this phenotype lies in the inability of the neural plate to adopt the correct shape. The plate remains wider in the medio-lateral dimension and shorter in the rostro-caudal dimension. Consequently, the neural folds can elevate properly but are not close enough to fuse at the dorsal midline.

The role of the actin cytoskeleton is probably best understood during epithelial bending and furrowing in the neural ectoderm. It has long been predicted that the role of the actin cytoskeleton is primarily two-fold during this third step. In order to convert the neural plate into the neural tube, two sequential furrowing and bending processes must occur. The first of these furrowing events occurs at the midline of the neural plate in those cells positioned directly above the notochord. Signals from the notochord (likely the secreted factor sonic hedgehog) cause the adjacent neural cells to furrow by inducing them to adopt a wedge-shaped morphology (Ybot-Gonzalez et al. 2002). To accomplish this, cells constrict their apical surface while widening at the basal surface. This structure forms the medial hinge point (MHP). It should also be noted that the cells within the MHP constitute a specialized set of cells termed the floor plate, which play an essential role in patterning the ventral region of the neural tube later in development. Simultaneously, the lateral edges of the neural plate begin to elevate, causing the plate to bend at the MHP. The elevation of the neural folds in the cranial region is likely to be assisted in part by the rapid expansion of the cranial mesenchymal cells by both proliferation and increase in extracellular space (Morriss and Solursh 1978). The combined action of the formation of the MLP and the elevation of the neural folds converts the neural plate into a “V”-shaped structure often referred to as the neural groove (Fig. 7.2, step 3a).

The second furrowing and folding event serves to position the lateral edges of the neural folds at the dorsal midline. Paired hinge points (one in each neural fold) form approximately midway between the lateral edges of the folds and the MHP. Similar to the MHP, these dorso-lateral hinge points (DLHP) form via cell wedging. The lateral edges of the neural folds then swing around the DLHP until they are juxtaposed at the dorsal midline (Fig. 7.2, step 3b). The force provided to push the lateral edge of the neural folds around the DLHP appears to come from both internal and external sources. Internally, the contraction of the actin cytoskeleton appears to provide force to pull the edges together, although this point is controversial (Schoenwolf and Smith 1990, Ybot-Gonzalez and Copp 1999). Externally, the adjacent epidermal ectoderm provides a force that pushes the folds together (Alvarez and Schoenwolf 1992). As will be discussed in more detail below, this step appears to be more critical for cranial neural tube closure than for spinal neural tube closure and is exquisitely sensitive to perturbation in the actin cytoskeleton.

The final step is fusion of the neural folds. It has been assumed that initial contact between the paired lateral edges causes an initial cell–cell adhesion event that then strengthens and matures. Unfortunately, identifying the cell surface and intracellular

molecules that mediate these adhesion events has proved difficult. This may stem from the idea that defects in fusion might result in phenotypes that mimic failure in either elevation or folding if incorrectly fused neural folds rapidly splay open.

Based on the above changes in cellular behavior and morphology, it seems obvious that the cytoskeleton would play an important role in neurulation. However, what is the evidence for that supposition? There are primarily three lines of experimental evidence that support the notion that the actin cytoskeleton plays a direct role in the regulation and control of neural tube closure in vertebrates. First is the spatial distribution of actin and its putative regulators in the neural epithelium at the time of neural tube closure. F-actin and several regulators of actin dynamics and cell adhesion are localized in a dense, sub-apical belt of actin that encircles each cell of the neural epithelia (Fig. 7.3). It has been predicted that this actin belt is actually a contractile actomyosin network. Second, and perhaps the best evidence, is the

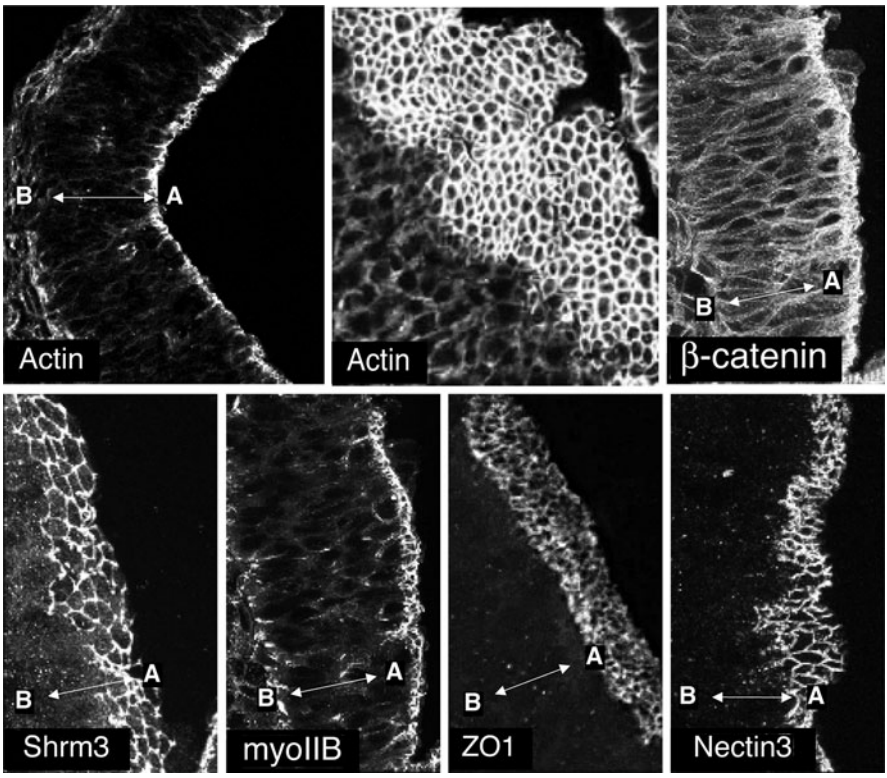

Fig. 7.3 Subcellular localization of proposed regulators of epithelial morphogenesis. Frozen sections of neural epithelium from embryonic day 10.5 mouse embryos were stained to detect the indicated proteins. Note that all of these proteins are either restricted to (Shrm3, nectin3, ZO-1) or enriched in (F-actin, b-catenin, and non-muscle myosin IIB) the apical domain of the cells. These proteins form a circumferential ring in an apical lateral position that is likely the AJC. The ring of actin and myosin is thought to generate mechanical forces that cause apical constriction or tension across the apical surface of the epithelium to facilitate cell wedging or tissue rigidity, respectively. The apical (A)–basal (B) axis is indicated in each *panel* by a *double-headed arrow*

fact that there are myriad mutations, either alone or in combination, in well-known cytoskeletal regulators that control neural tube closure. These genes include Epb4.1l5 (Lee et al. 2007b), *Abl1/Abl2* compound mutants (Koleske et al. 1998), *Nap1* (Rakeman and Anderson 2006), *Dlc1* (Durkin et al. 2005), *Grlf1* (p190RhoGAP) (Brouns et al. 2000), *Mena/Vasp* compound mutants (Menzies et al. 2004), *Mena/profilin* compound mutants (Lanier et al. 1999), *Shroom3* (Hildebrand and Soriano 1999), Marcks (Stumpo et al. 1995), *Marcksl1* (Chen et al. 1996, Wu et al. 1996), *Vinculin* (Xu et al. 1998), *Palladin* (Luo et al. 2005), and *n-cofilin* (Gurniak et al. 2005). Third, several in vitro experiments using pharmacological inhibitors of cytoskeletal elements further support the hypothesis that the actin cytoskeleton is essential for proper neural tube morphogenesis. Specifically, the actin inhibitor cytochalasin D has been shown to block aspects of neural tube closure (Lee and Nagele 1985, Morriss-Kay and Tuckett 1985, Ybot-Gonzalez and Copp 1999, van Straaten et al. 2002). These data will be discussed in great detail below.

7.3 The Function of the Cytoskeleton in Neurulation

To date, very little is known about the actual function of F-actin regulatory proteins during the process of neural tube closure and morphogenesis. However, based on their defined functions in biochemical or cell-based assay systems, the characterization of the mutant phenotypes, and experiments in other developmental systems, it is possible to speculate on the roles that specific proteins play during neural tube morphogenesis. These proteins fall into a wide range of functional categories, including actin polymerization, actin severing, cell–cell and cell–ECM adhesion, actin bundling, and the regulation of myosin II activity or distribution. How each of these activities might participate in neural tube closure will be discussed in the context of the four steps of neural tube morphogenesis.

The best place to begin the discussion of how the actin cytoskeleton functions during this developmental process is with a description of the cellular and cytoskeletal organization exhibited by neural ectodermal cells. The cells that comprise the neural ectoderm are polarized, with a basal surface facing the basal lamina and the apical surface facing the future lumen of the neural tube (Colas and Schoenwolf 2001). The cells adopt a pseudo-stratified appearance because the nuclei are at various positions along the apical–basal axis. The cells are adherent to both the basal lamina and their neighboring cells. The cell–cell interactions are mediated by a number of cell surface receptors that are linked indirectly to the actin cytoskeleton, including cadherins and nectins. During the time of neurulation, F-actin is highly enriched in a dense circumferential band that is located at the junction between the lateral side of the cell and the apical surface of the cell (Figs. 7.3 and 7.4). Along with actin, this dense ring also contains the actin-associated or regulatory proteins Abl1 and Abl2 (Koleske et al. 1998), afadin (Manabe et al. 2002), Par-3 (Takekuni et al. 2003), Mena (Menzies et al. 2004), Epb4.1l5 (Rakeman and Anderson 2006), ZO1, Shroom3 (Hildebrand 2005), non-muscle myosin IIB (Hildebrand 2005), and

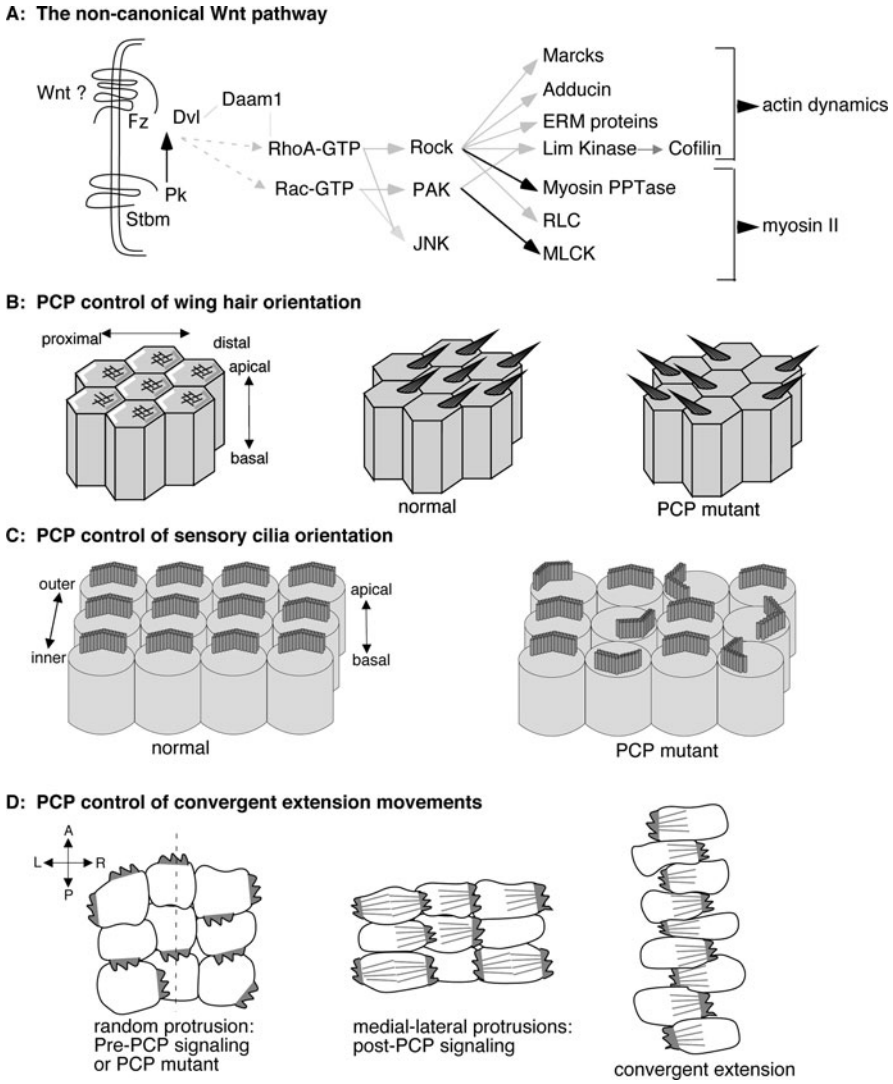

Fig. 7.4 The planar cell polarity pathway in flies and vertebrates. (**a**) Schematic of the non-canonical Wnt pathway depicting some of the "core" PCP proteins and the downstream effectors of Dvl. *Grey arrows* denote activating interactions or modifications and *black arrows* indicate inhibitory modifications or interactions. (**b**) PCP signaling in the *Drosophila* wing establishes the position and orientation of the actin-based wing hairs. The wing hair starts as an accumulation of F-actin (*grey meshwork*) that then organizes into the actin bundles (*grey lines*) that form the hair. The *white line* at the proximal side of the cell indicates the Stbm–Pk complex and the *grey line* at the distal side of the cell indicates the Fz–Dsh–Dgo complex. In PCP mutants, the hairs form normally but they are either in the wrong position or oriented incorrectly. (**c**) The orientation of sensory cilia in the outer hairs cells of the inner ear of mammals. Cells exhibit a planar polarity from inner to outer, with the stereocilia adopting a final position at the outer face of the cell cortex. In PCP mutants, the stereocilia form normally but are in randomized positions. Actin-rich

beta-catenin (Fig. 7.3). This region of the cell likely corresponds to the apical junctional complex (AJC), a specialized site of cell–cell adhesion that plays a critical role in establishing cell adhesion, defining apical–basal polarity, the formation of tight junctions, and the regulation of protein sorting (Roh and Margolis 2003).

7.3.1 Cytoskeletal Regulators that Act Early in Shaping the Neural Plate

One of the ways that neural plate shaping is achieved is by the movement of cells from lateral to medial positions. This process likely requires cells to dynamically modulate their cytoskeletal architecture and adhesive properties. Therefore, it could be predicted that mutations that prevent cell movement during shaping of the neural plate would have dramatic defects in neural tube closure. Currently only *Epb4.1l5* (*erythrocyte protein band 4.1 like 5*) and *Nap1* mutant embryos appear to exhibit this "catastrophic" defect in neural tube morphogenesis and both of these genes encode proteins that regulate actin dynamics (Rakeman and Anderson 2006, Lee et al. 2007b). Epb4.1l5 colocalizes with actin in the apical region of the neural ectoderm, while Nap1 co-distributes with actin in both the apical and basal domains of these cells. Epb4.1l5 contains a FERM domain (4.1, ezrin, radixin, moesin), a motif found in a number of known cytoskeletal regulatory proteins that is predicted to target these proteins to the plasma membrane via the direct or the indirect association with transmembrane proteins (Bretscher et al. 2002). Many proteins of this family also contain an actin-binding sequence at their C terminus. In vitro-based cell culture assays have shown that Epb4.1l5 localizes with the plasma membrane and induces the robust formation of a cortical actin network. Consistent with these data, neural epithelial cells lacking Epb4.1l5 exhibit a disorganized cytoskeletal architecture with mis-localized F-actin, myosin IIB, and activated forms of ERM-related proteins (Lee et al. 2007b). One caveat to these observations is that these mutants display a variety of additional phenotypes, thus the neural tube defects may be more severe due to the simultaneous failure of other developmental processes. Interestingly, Epb4.1l5 is orthologous to the zebrafish gene *mosaic eyes (moe)*, which is required for proper polarization of the retinal epithelia and formation of the ventricles of the brain (Jensen and Westerfield 2004). In addition, Moe exhibits a very similar subcellular distribution as Epb4.1l5. Moe has been shown to associate

Fig. 7.4 (continued) stereocilia are depicted as projections from the surface and microtubule-based kinocilium are not shown. (**d**) Oriented membrane protrusions and directional movements during CE in response to PCP signaling in vertebrates. Prior to initiation of CE or if PCP signaling is blocked, cells exhibit randomized actin-rich lamellipodia (*grey*). Following PCP signals, the lamellipodia become oriented along the medial–lateral axis. In *Xenopus*, mesoderm cells usually exhibit bipolar protrusions, while ectoderm cells have monopolar, medially directed protrusions. Membrane protrusions attach to matrix or neighboring cells and provide traction, via the action of the cytoskeleton and myosin II (*grey lines*) to move toward the midline

with the Crumbs and Pals proteins, both of which are key determinants of apical–basal polarity, suggesting that this protein is required for establishing apical–basal polarity in epithelial cells (Hsu et al. 2006). Consistent with this result is the observation that several epithelial populations in Moe-deficient embryos display defects in apical–basal polarity. This phenotype in not shared in Ebp4.1l5 null mouse embryos, as the apical–basal polarity is seemingly normal. Based on the data from mice and zebrafish, it appears that this protein plays an important role in cytoskeletal organization or polarity early in the process of neural plate morphogenesis in mice.

The second actin regulator that is required for early neural morphogenesis is the Nap1 protein, a component of the Wave complex (Steffen et al. 2004). Wave family proteins are scaffolds that convert upstream signals into dynamic actin remodeling via the activation of the Arp2/3 complex (Takenawa and Suetsugu 2007). Arp2/3-induced actin polymerization functions in a wide range of cellular processes that occur at the plasma membrane, including membrane ruffling, formation of lamellipodia, cell movement, and vesicle trafficking. The precise role of Nap1 in the Wave complex is unclear, but two activities of Nap1 appear to be critical (Steffen et al. 2004). First, Nap1 plays an essential role in the stability of the complex, as removal of Nap1 by either mutation or RNAi-mediated knockdown results in loss of the Wave complex. Secondly, Nap1 indirectly links the Wave complex to active Rac1. Rac1 has been shown to activate the Wave-related protein Wasp, but the role of Rac1 binding in the activation of Wave is unclear. However, the activation of Rac1 causes the relocalization of the Wave complex to the plasma membrane and the Wave complex is required for the actin remodeling that occurs following Rac1 activation. Therefore, the defects seen in Nap1 mutants could result from the loss of the Wave complex. Based on the function of Wave in the activation of F-actin polymerization, it appears that the neural tube defects seen in the Nap1 mutants could stem from aberrant movement of ectodermal cells during shaping of the neural plate. In addition, Wave2 is required for the formation and maintenance of cadherin-based adhesions in cell culture (Yamazaki et al. 2007). This suggests that the Nap1 phenotype may also reflect a lack of adhesion between neural ectodermal cells.

Taken together, these results suggest that proteins that function in many processes, such as Nap1 in the Wave complex, or in determining the basic cellular architecture or polarity of a cell, such as Epb4.1l5, will act early in neurulation. In addition, it is very likely that loss of these proteins due to mutation will have dire consequences on embryogenesis.

7.3.2 The Role of the Planar Cell Polarity in Neural Morphogenesis

A wealth of experimental and genetic evidence has shown that the PCP pathway is essential for proper neural tube closure and morphogenesis in vertebrates (Montcouquiol et al. 2006). The concept of PCP was first put forth as a way to

explain phenotypic anomalies in tissues, such as the wing in *Drosophila*, that normally display a clear polarity in the orientation of structures across the surface of a tissue. In its most simplistic terms, PCP serves as a cellular compass that provides spatial cues to cells so that they know where they are and how they should behave relative to neighboring cells, their location within a tissue, and their position along the embryonic axes (Seifert and Mlodzik 2007). This concept is demonstrated very nicely in the *Drosophila* wing, in which every cell produces a single wing hair that emanates from the distal side of the cell and points toward the distal tip of the wing. Wing hairs are actin-based structures that form in the apical domain of the cell and project from the apical surface of the polarized ectodermal epithelium that forms the wing. In flies with PCP mutations, these wing hairs often project from different regions of the cells and point in random directions (Fig. 7.4). The key step in positioning this hair at the distal edge of the cell is the generation of cytoskeletal asymmetry within each cell such that the distal and proximal sides of the cell are different from each other. The PCP pathway functions to establish this asymmetry.

In vertebrates, this same concept holds true, but the pathway also regulates cells that are moving during CE as well as those developing in a more "static" environment. In mammals, the hair cells within the organ of Corti produce and extend an array of actin-rich stereocilia from their apical surface. After formation and maturation, this array adopts a final position at the outer cortex (these cells are polarized along an inner to outer axis as opposed to proximal–distal axis as in the *Drosophila* wing). Mice that lack PCP signaling exhibit defects in the orientation of these projections such that they form at random positions along the inner–outer axis (Montcouquiol et al. 2006). It is important to note that the stereocilia form normally, they are just in the wrong position. While it may seem that the involvement of PCP in cells undergoing dramatic morphogenesis is not that similar to the asymmetric formation of actin-rich projections from the cell surface, there is a common theme: cellular asymmetry. In CE, cells move in a directed way from lateral to medial. Analysis of cells undergoing CE indicates that these cells are asymmetric such that they elongate along their medial–lateral axis and extend actin-rich lamellipodia along the medial–lateral axis. Importantly, blocking the PCP pathway blocks cell elongation and the formation of spatially restricted lamellipodia (Fig. 7.4).

Primarily through the power of genetic screens and genetic analysis, the core components of planar cell polarity have been identified and a mechanism by which they function established. These core factors include the transmembrane proteins Frizzled (FZ), Strabismus (Stbm, also called Van Gogh), and Flamingo (Fmi, also called Starry Night or Celsr in vertebrates) and the cytoplasmic proteins Dishevelled (Dsh, Dvl in vertebrates), Diego (Dgo), and Prickle (Pk) (Seifert and Mlodzik 2007). In brief, the PCP pathway is initiated by activation of the Fz receptor, presumably by a secreted Wnt ligand. Fz activation results in the activation and translocation of Dsh to the plasma membrane. It is at this point that the pathway diverges from the canonical pathway, and instead of activating beta-catenin, the pathway activates the Jun kinase (JNK) cascade and the small GTPases Rac and Rho. Upon activation of the pathway, Fz, Dsh, and Dgo become asymmetrically localized to the distal, apical region of the cell. The Fz–Dsh–Dgo complex appears to act positively to promote

signaling. In contrast, a Stbm–Pk complex becomes localized to the proximal, apical region of the cell and works to antagonize the Fz–Dsh–Dgo complex. The Fmi protein is localized to both the proximal and distal sides of the cell and may act to stabilize both complexes. It is this initial asymmetry in the signaling components that establishes the planar polarity of the cell. This mechanism of establishing early asymmetry appears to be conserved in vertebrates, as Vangl2 (also called looptail, the mouse ortholog of Strabismus/Van Gogh), Dvl, Frizzled 3, Frizzle 6, and Prickle all display asymmetric distribution in sensory cells of the ear (Wang et al. 2006a, b, Deans et al. 2007, Qian et al. 2007).

As indicted above, PCP has been implicated in CE movements during neural tube closure. This is based on the phenotypes caused by mutations in several of the core PCP components, including *Vangl2* (looptail, lp) (Kibar et al. 2001), *Celsr* (Curtin et al. 2003), *Fz3/Fz6* (double mutants) (Wang et al. 2006b), and *Dvl1/Dvl2* (double mutants) (Wang et al. 2006a). Embryos of these genotypes all fail to close the hindbrain and spinal region of the neural tube, resulting in craniorachischisis. Genetic analysis has also indicated that there might be either additional core components or cross-talk between other pathways in vertebrates, as embryos homozygous for a mutation in Scribbled1 (Scb1, circletail) cause craniorachischisis and show defects in the polarity of sensory cilia in the ear. In *Drosophila*, Scribble is required for establishing apical–basal polarity. Genetic analysis suggests that these genes are functioning in the same pathway as $Lp^{+/-}$; $Celsr^{+/-}$, $Lp^{+/-}$; $Crc^{+/-}$ and $Lp^{+/-}$; $Dvl2^{-/-}$ embryos all exhibit the same neural tube phenotype and errors in the orientation of stereocilia in the ear (Montcouquiol et al. 2003, Murdoch et al. 2003, Wang et al. 2006).

If the outcome of the PCP signal is to establish the asymmetric distribution of the Fz–Dsh–Dgo complex and cytoskeletal asymmetry appears to mediate both orientation of cellular projects (hairs or stereocilia) and directional cell migration, what is the link between the Fz–Dsh–Dgo complex and actin? The key to answering this question will be to identify all of the effectors of this complex as well as those of the Stbm–Pk complex. One of the critical downstream factors in the non-canonical Wnt pathway is activation of the GTPases RhoA and Rac1. Both of these proteins are well-defined regulators of cytoskeletal dynamics that are activated downstream of Dvl during PCP signaling (Habas et al. 2003). Activated RhoA causes the robust formation of actin fibers in cultured cells, while active Rac1 regulates cortical actin during events such as membrane ruffling and lamellipodia extension. It should be noted that unlike several other cytoskeletal proteins that modulate the cytoskeleton during neurulation, Rho and Rac are not direct regulators of the cytoskeleton but instead activate a cadre of effector proteins that subsequently control cytoskeletal dynamics and cellular behavior.

Two important questions that have yet to be solved are how RhoA and Rac1 become activated by the Fz–Dvl–Dgo complex and what are their relevant effectors during neurulation. One critical link between RhoA and Dvl was uncovered with the identification of Daam1. Daam1 encodes a Formin Homology protein that binds directly and simultaneously to GTP–RhoA and Dvl, thus linking the two proteins (Habas et al. 2001). In *Xenopus* it was shown that activation of Fz via Wnt results

in the activation RhoA and CE. Blocking the expression of Daam1 inhibits both of these events (Habas et al. 2001, 2003). It is not known how activation of non-canonical Wnt signaling results in the observed increases in the amount of active Rac1 and RhoA. While the number of identified effectors of both RhoA and Rac is extensive, there are a number of particularly attractive targets as they relate to regulation of the actin cytoskeleton during neural tube morphogenesis (Bustelo et al. 2007). This includes the Rho-associated serine/threonine kinase Rock. In both vertebrates and *Drosophila*, Rock appears to be a key downstream effector of RhoA. The binding of active RhoA to Rock results in the activation of Rock and the subsequent phosphorylation of its substrates, many of which go on to regulate cytoskeletal dynamics (Lim kinase), cytoskeletal architecture (Marcks, ERM proteins), and the contractility of non-muscle myosin II (myosin phosphatase and myosin regulatory light chain) (Riento and Ridley 2003).

Two of the Rock substrates are antagonistic regulators of non-muscle myosin II. A central step in regulating the contractile activity of myosin II is phosphorylation of the associated regulatory light chains (RLCs) (Bresnick 1999). Phosphorylation of the RLC promotes myosin II binding to F-actin and subsequent contractile activity. Several kinases have been shown to directly phosphorylate the RLC, including myosin light chain kinase (MLCK) and Rock. The RLC is de-phosphorylated and inactivated by the myosin phosphatase. Rock is capable of directly inhibiting this phosphatase by phosphorylating the myosin-binding subunit. Therefore, Rock regulates the activity of myosin II by two different mechanisms. It has been proposed that the activity of myosin II promotes cell movements by stimulating the formation of stress fibers and generating force and traction to move cells as they adhere to either the ECM or to each other. This idea is also consistent with CE movements during gastrulation in *Drosophila*, where myosin II activity is required for proper reorganization of cell–cell adhesion in the ectoderm (Bertet et al. 2004).

While the majority of data implicating the Rho–Rock pathway in CE and neural tube closure have come from *Drosophila* and *Xenopus*, the role of this pathway in mouse neurulation was recently established using a combination of genetics and a pharmacological inhibitor for Rock (Ybot-Gonzalez et al. 2007). Treatment of wild-type mouse embryos with low doses of the Rock inhibitor Y-27632 had little to no effect on neural tube morphogenesis. In contrast, treatment of $Lp^{+/-}$, $Celsr^{+/-}$, $Crc^{+/-}$ embryos with the drug blocked fusion at closure point 1. In contrast, treatment of embryos of the same genotype with a drug that blocks JNK activity did not result in neural tube defects. These results contrast those in *Xenopus*, where JNK activity was shown to be required for CE. This suggests that in mice the PCP pathway goes through the small GTPase RhoA and its downstream target Rock to regulate neurulation. Importantly, all of these components are expressed in the neural ectoderm at the time of neural tube closure in mice.

Another actin regulatory protein that seems to act downstream of Dvl is the Marcks protein. Marcks is a myristoylated protein (and is thus targeted to the plasma membrane) that has the capacity to cross-link actin filaments. Blocking the expression of Marcks in *Xenopus* embryos using morpholinos results in dramatic CE defects (Iioka et al. 2004). Several lines of evidence suggest that Marcks acts at

the level of cortical actin and membrane dynamics during PCP-regulated CE. First, cells that lack Marcks function do not orient along the medial–lateral axis and do not move toward the midline. Second, knockdown of Marcks causes a loss of the actin-rich membrane protrusions normally displayed by cells undergoing CE. Third, Marcks is required for the dramatic membrane protrusive activity induced by activation of Frizzled. It should be noted that Marcks is phosphorylated and activated by Rock and is also required for cranial neural tube closure in mice (see below).

7.3.3 The Role of Actin Regulatory Proteins in Bending and Folding of the Neural Tube

While shaping of the neural plate is occurring, it begins to undergo the furrowing and bending steps required to generate the tube that is seen at the end of neurulation. This step of neural tube closure probably has the best-characterized requirement for an intact actin cytoskeleton. This is based on the observations that both pharmacological agents that disrupt the actin cytoskeleton and mutations in genes encoding cytoskeletal proteins block neural tube closure at this step. Two morphological events are occurring at this time that account for this dependence on the cytoskeleton. The first is the formation of the MHP and DLHP via cell wedging. The second is the bending of the neural plate, which positions the lateral edges at the dorsal midline prior to fusion. Both of these processes are likely regulated by the contractile actin–myosin network located at the apical surface of the neural ectoderm that was described above (Fig. 7.5). Cell wedging is thought to occur by two mechanisms. First, contraction of the apical actin ring would cause cells to decrease their apical surface while leaving the basal surface unchanged, thus converting columnar-shaped cells in wedge-shaped cells with a narrower apical surface. Additionally, it appears that the nuclei of cells within the hinge points tend to be located in the basal portion of the cell, thus increasing the basal area (Smith and Schoenwolf 1987, 1988).

The second predicted role for the cytoskeleton also invokes force generated by contraction of the actomyosin network, but instead of necessarily causing a constriction event, the force functions as a “purse string” to pull the lateral edges upward and toward the midline. The outcome of this purse-string model may not be seen as a change in tissue or cellular morphology but as a change in tension across the population of epithelial cells and increase in tissue rigidity (Fig. 7.5). By maintaining a constant tension across the surface of the neural ectoderm via myosin contraction, the neural folds can maintain the integrity needed to withstand the actual morphogenetic process. It is simple to predict that there are essential requirements for maintaining a specific organization to the cytoskeleton in the neural ectoderm and that this is regulated at the level of F-actin distribution, turnover, higher order architecture (cross-linked or bundled), and contractile activity (via myosin motors).

What is the experimental evidence that actin participates in these processes? The first line of evidence was outlined above in discussing the subcellular distribution of actin, cytoskeletal regulators, and adhesion proteins during neurulation.

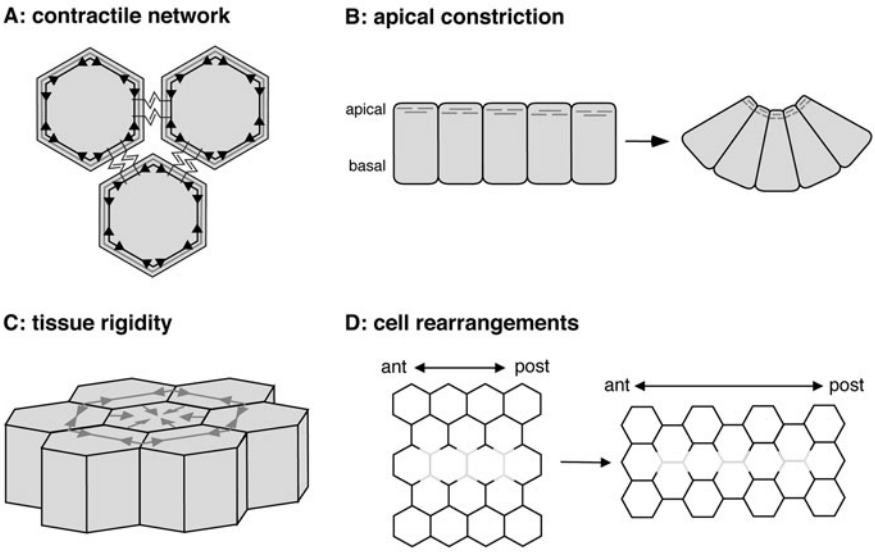

Fig. 7.5 Model for possible functions of apical actin in neurulation. (**a**) Schematic of an apical view of cells showing a circumferential ring of contractile actomyosin (*black* and *grey*) that is anchored to transmembrane receptors in the apical junctional complex. Contraction of the actomyosin network generates force around the circumference of the cells. (**b**) Myosin II-dependent constriction of the apical surface of the cells can convert a relatively flat plate of cells into a curved tissue. This action is thought to function at least in part during hinge point formation or in folding of the neural plate. (**c**) Force generated by the contraction of the actomyosin ring, denoted by *grey arrows*, provides tension across the entire field of epithelial cells and provides the tissue rigidity required to undergo morphogenesis. (**d**) The action of myosin II is required for cell to change adhesion dynamics and cellular interactions, facilitating cell movement and migration during CE. This has been shown to occur during germ band extension in *Drosophila*. Model based on that proposed by Bertet et al. (2004). Similarly, the action of actomyosin may be needed basally for cells to migrate via integrin-mediated adhesion

The second and perhaps most convincing evidence comes from genetic analysis in mice harboring mutations in genes that encode cytoskeletal regulators. As mentioned above, mutations in *Abl1/Abl2* (double mutants)*, Dlc1, Grlf1 (p190RhoGAP)*, *Mena/Vasp* (compound mutants), *Mena/profilin* (compound mutants), *Shroom3*, *Marcks*, *Marcksl1* (*Marcks-like 1*), *Vinculin*, *Palladin*, and *n-cofilin* all cause neural tube defects. Interestingly, the majority of these mutations affect only the rostral neural tube and cause exencephaly. The exceptions to this are mutations in *Shroom3* and *Marcksl1*, which cause spina bifida (at a lower frequency) in addition to exencephaly. How is it possible that deficits in these proteins with differing functions give very similar phenotypes? Based on both cellular and biochemical assays, it appears that all of these proteins, with the exception of Shroom3 and Vinculin, function either directly or indirectly to regulate the dynamics of actin.

The Ena/Vasp family of proteins has been shown to promote the growth of actin filaments by preventing the addition of capping protein to the barbed end (Bear

et al. 2002). Profilin promotes actin polymerization by facilitating the addition of actin monomers to the fast-growing barbed ends of actin filaments. The genetic interaction between Mena and profilin likely reflects the fact that these proteins form a complex and are predicted to act together to promote actin polymerization (Krause et al. 2003). The ability of these proteins to promote the formation of actin filaments may be critical to maintaining the apical populations of actin. In addition, Mena has been shown to be an important regulator of actin polymerization during the formation of new cadherin-based sites of cell–cell adhesion (Vasioukhin et al. 2000). Thus, this complex may participate in the formation and breakdown of cell–cell adhesion structures as the epithelial cells change position and neighbors within the neural plate during morphogenesis. Along these same lines, the protein Palladin has been shown to co-distribute with F-actin and induce the formation of large actin bundles when expressed in cells (Rachlin and Otey 2006). Therefore, it can be predicted that Palladin might regulate the organization of actin in the actin belt. However, Palladin binds a number of other cellular proteins, including Mena (Boukhelifa et al. 2004). Therefore, Palladin's main function may be to help localize the Mena/profilin complex. The reason that both Mena and Vasp must be mutated in order to see neural tube defects probably stems from the observations that they exhibit similar expression patterns and function (Menzies et al. 2004).

As discussed above, RhoA is a central player in regulating actin dynamics. It is interesting to point out that mutations in two proteins that act to turn off RhoA, the Rho GTPase-activating proteins Dlc1 and p190RhoGAP, cause defects in cranial neural tube closure. This suggests that too much Rho activity may be as detrimental as too little Rho activity. In some cells, activation of RhoA can lead to precocious F-actin formation. Consistent with this observation, there is a dramatic increase in the amount of F-actin present in the neural ectoderm of p190RhoGAP null embryos (Brouns et al. 2000). The hyperactivation of RhoA in these cells could also act at the level myosin II. An interesting attribute of p190RhoGAP is that it appears to integrate multiple cellular pathways. It becomes activated in response to signaling via receptor and non-receptor tyrosine kinases, it associates with p120RasGAP and Abl2 (Bradley et al. 2006), and it regulates cell–cell adhesion via interaction with p120 catenin (Wildenberg et al. 2006).

Unlike several of the above factors that appear to directly control actin dynamics, the Abl non-receptor tyrosine kinases might work directly by interacting with F-actin and indirectly by modulating the activity of other proteins whose function is to control actin dynamics. Both Abl1 and Abl2 possess actin-binding domains that regulate actin organization (Wang et al. 2001). Alternatively, these domains could target the proteins to the correct subcellular compartment. These kinases phosphorylate proteins that control actin dynamics and adhesion, including p190RhoGAP (Bradley et al. 2006), Wave2 (Leng et al. 2005), and cortactin (Boyle et al. 2007). It should also be pointed out that Enabled (the *Drosophila* ortholog of Mena) was identified as a substrate and modifier of *Drosophila* Abl in neuronal development (Gertler et al. 1995). Subsequently, Abl and Ena have been shown to function together in several

actin-dependent cellular events, including epithelial morphogenesis in *Drosophila* (Gates et al. 2007). The last interesting aspect of Abl is its interaction with Abi1, a component of the Wave complex described above. Abi1 recruits Abl to the complex where it phosphorylates and activates Wave (Leng et al. 2005).

Marcks, Marcksl1, and *n*-cofilin also act to directly regulate actin dynamics and architecture and are required for cranial neural tube closure in mice. Furthermore, each of these has been linked to at least one of the other proteins identified above. Specifically, both Marcks and cofilin are downstream of Rock, with Rock directly phosphorylating Marcks and indirectly regulating the activity of cofilin via Lim kinase.

The Shroom3 protein appears to function differently than any of the above proteins in the context of neural tube closure. Recent works have shown that Shroom3 is both necessary and sufficient for cells to undergo apical constriction (Haigo et al. 2003, Hildebrand 2005). When expressed in either the epithelial ectoderm of *Xenopus* embryos or in polarized epithelial cells in culture, Shroom3 causes cells to constrict their apical surface and adopt a wedge-shaped morphology. This happens in the absence of new gene expression, suggesting that Shroom3 can reprogram the cytoskeletal machinery that is present in these polarized cells. In addition, blocking Shroom3 function in *Xenopus* or mouse embryos results in failed neural tube closure. Shroom3 is localized to the apical actin belt in the neural epithelium and to the AJC in cultured epithelial cells. This localization is mediated by the direct binding of Shroom3 to F-actin. The ability of Shroom3 to bind actin appears to be critical only because the protein must be localized to this actin population in order to trigger apical constriction. A second domain of Shroom3 actually causes the constriction event and it does so by recruiting myosin II to the AJC in cells (Hildebrand 2005). Additionally, a significant amount of myosin IIB is lost from the apical region of neural ectoderm in Shroom3-deficient embryos. Recent work in *Xenopus* suggests that Shroom3 plays a role in regulating microtubules in the neural ectoderm (Lee et al. 2007a). Specifically, Shroom3 causes the apical distribution of gamma-catenin and the organization of microtubules along the apical–basal axis. This promotes the lengthening of cells along the apical–basal axis that is associated with neural plate bending in *Xenopus*. The role of this change in cell morphology in mouse neural plate bending is not clear.

Of the proteins listed above, only Palladin, Dlc1, and p190RhoGAP have not been shown to directly bind actin (although Palladin and p190RhoGAP have been shown to localize with F-actin in cells). Unfortunately, it is not clear at present whether or not the actin-binding activity of any of these proteins is directly responsible for their function in neurulation. Binding of actin may simply recruit these proteins to the apical region of the cell where other activities are required for neural tube closure. In the case of Vinculin, it may function to anchor F-actin to the adherens junction as it can bind F-actin and alpha-catenin, a component of the adherens junction, simultaneously.

7.3.4 Regional Differences in the Requirement for F-Actin

A feature of the above examples is that they all cause defects in the cranial region of the neural tube (closure 2) and do not affect spinal neural tube closure. This suggests that either F-actin is not an essential participant in spinal neural tube closure or there is a different set of actin regulators that function in this region of the neural tube. Based on studies using cytoskeletal poisons, the former appears to be the case. Treatment of mouse, chicken, or rat embryos with the drug cytochalasin D, which binds to the barbed ends of actin filaments and prevents the addition of actin monomers, blocks cranial but not spinal neural tube closure (Morriss-Kay and Tuckett 1985, Ybot-Gonzalez and Copp 1999, van Straaten et al. 2002). It should be noted that even in the presence of cytochalasin D, the DLHP still seem to form but the remainder of the neural epithelium losses its morphology. This suggests that F-actin may be more important for maintaining tissue rigidity than for causing cell wedging. Alternatively, the contraction of the apical actin ring may be refractory to the effects of cytochalasin D if this population of F-actin is very stable. These results indicate that in non-bending regions of the neural tube (spinal) the cytoskeleton may play a stabilizing role, while in regions that undergo extensive bending (cranial) the actin cytoskeleton plays an active role in altering tissue morphology. This is supported by the observation that in many of the mutant embryos described above, even though the spinal neural tube has closed per se, the neural epithelium is often misshapen, suggesting that the tissue integrity is not maintained in the absence of an intact cytoskeleton.

7.4 Cilia Formation and Neural Tube Morphogenesis

An exciting new area of study in regard to neural tube patterning and morphogenesis is that of cilia formation and the requirement of cilia for proper signaling by sonic hedgehog and PCP. In vertebrates, it now appears as if all hedgehog signaling through Gli-type transcription factors requires intact cilia on the cell surface. Several recent reports stemming from a forward genetic screen to identify genes required for neural tube morphogenesis isolated numerous mutants whose embryonic phenotypes mirror those seen when components of the hedgehog pathway are missing (Huangfu et al. 2003, Huangfu and Anderson 2005, Zohn et al. 2005). These defects include exencephaly, degrees of holoprosencephaly (errors in midline patterning), polydactyly (excess digits), and aberrant dorsal–ventral patterning of the neural tube. The subsequent cloning of these mutations has identified a number of genes that encode vertebrate orthologs of intraflagellar transport (IFT) proteins that are required for proper ciliogenesis in other organisms. Genetic analysis of these IFT mutants with known hedgehog signaling components has shown that cilia are clearly required for proper regulation of the hedgehog pathway. Based on what has been learned about cilia assembly from other organisms, it appears that the majority of these proteins function in the actual assembly or maintenance of the cilia.

So what then is the relationship between the cilia, a microtubule-based structure, and the actin cytoskeleton? Early steps in the assembly of cilia require the formation of centrioles from gamma-catenin at the apical surface. These centrioles dock at the apical plasma membrane and become the basal bodies of the cilia. Following docking, the microtubule-based axoneme is assembled. The critical regulator or facilitator of centriole docking is the assembly of an actin network just below the apical plasma membrane. Current data suggest there are at least two pathways that might be working together to control the formation of this actin network. The first pathway involves the transcription factors Foxj1 and RhoA. The second involves the PCP proteins Inturned and Fuzzy. Foxj1 is an epithelia-specific transcription factor that is required for ciliogenesis (Chen et al. 1998, Brody et al. 2000). Using an in vitro model system, primary ciliated airway epithelial cells, it was demonstrated that Foxj1 expression leads to the activation of RhoA and RhoB and that both Foxj1 and RhoA are required for the formation of the apical actin network. Importantly, it was shown that blocking the activity of RhoA after the network was established did not inhibit ciliogenesis, showing that activity of RhoA is needed for the genesis of the actin network (Pan et al. 2007). To date, it is unclear how Foxj1 activates RhoA, but it could do so by modulating the expression of genes that encode regulators of Rho activity.

A particularly interesting finding is that two proteins Inturned and Fuzzy, originally identified as PCP determinants in *Drosophila*, may link PCP and cilia formation (Park et al. 2006). Inhibiting the expression of either Inturned or Fuzzy in *Xenopus* embryos causes defects in both midline patterning and CE, consistent with errors in hedgehog signaling and PCP, respectively. The midline defects stem from perturbations in the formation of cilia in the neural tube. The inability of these cells to make cilia results from a block in the formation of the apical actin network. Interestingly, these cells still accumulate apical microtubules; however they are not organized correctly. The link between PCP signaling and ciliogenesis may come at the level of Dvl, as both Inturned and Dvl are recruited to the apical actin network. How or if these proteins interact at any level is yet to be determined. It is easy to speculate that Dvl may regulate the action of Inturned and Fuzzy or that Inturned and Fuzzy may form a scaffold to cluster the PCP signaling network. In light of the fact that RhoA is necessary for ciliogenesis and is also a downstream effector of the PCP pathway, this is another place that these two processes could intersect.

7.5 The Cytoskeleton and Fusion of the Lateral Edges

As indicated above, virtually nothing is known about how the lateral edges contact and fuse to close the neural tube. Imaging of the neural ectoderm has shown that cells at the lateral edge exhibit robust membrane ruffling and the extension of cellular processes, likely driven by actin remodeling. It is probable that these cellular processes contact cells on the opposing neural fold and form an initial contact site that then matures into a stable cell–cell adhesion event.

Two recently generated targeted mutations in the genes encoding the cell surface ligand Ephrin-A5 and its receptor EphA7 may shed some light on this process. Both of these mutations cause exencephaly and appear to act at the level of fusion (Holmberg et al. 2000). In EphA7 mutants, the neural folds undergo all aspect of neural tube morphogenesis normally but do not fuse. Eph receptor and their Ephrin ligands regulate cell–cell interactions and they work through the cytoskeleton to control these events. It should be noted that Mena/Vasp proteins have been shown to act downstream of Eph receptors and mediate aspects of cytoskeletal remodeling that result following activation of the receptors (Evans et al. 2007).

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Chapter 8
Actin Cytoskeletal Regulation in Neuronal Migration

M. Elizabeth Ross and Stanislav Kholmanskikh

Abstract Polymerization of actin has many functions in motile cells, ranging from assisting with subcellular localization of proteins important to polarization, generation of motive forces, cell adhesion, alteration of membrane shape, and even intracellular protein trafficking. While the broad strokes of these events are similar among cell types, recent studies indicate significant differences for neuronal motility compared with epithelial and fibroblast cells. This chapter discusses current insights into the role and regulation of F-actin dynamics in the steps required for neuronal migration.

Keywords F-actin · Neuronal migration · GTPases · Arp2/3 complex · Formins · Cofilin · Cell adhesion · Endocytosis · Actin dynamics · Brain malformations

8.1 Introduction

Regulation of the many-faceted functions of actin has been a focus of intense investigation for over a decade, yet there remain unresolved questions, particularly with regard to the role of actin in neuronal migration. On its own, actin has a strong tendency to polymerize but within the cell, less than 50% of it is in the filamentous (F-actin) form (Hall 1994). This is due to the multiple protein interactions that sequester actin monomers (G-actin) and that cap actin filaments. Such protein interactions must be exquisitely regulated to affect the organization and remodeling of F-actin in response to signals and (1) provide the cell with contractile forces through the combination of actin with myosin II, (2) control cell adhesion needed for cell–cell and cell–matrix interactions, and (3) transduce signals at the plasma membrane

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and reshape the cell surface. At the plasma membrane, F-actin comprises a cortical meshwork to which microtubule ends are captured in a dynamic interaction that bridges these major cytoskeletal systems (see Chapter 5), coordinating the actions of actin and microtubules in motile cells.

The central importance of filamentous actin in neuronal migration was recognized in early experiments by Rivas and Hatten (1995), who used time-lapse imaging of cerebellar granule neurons in culture to show that inhibition of actin depolymerization with cytochalasin B caused a loss of lamellipodial motility in the leading process and halted neuronal advancement. Moreover, organelles in the juxtanuclear area were no longer polarized in the direction of migration (Rivas and Hatten 1995). The importance of actin to neuronal migration has also been evident in several human brain malformation syndromes and their mouse models, in which gene mutations have been associated with actin deregulation (Tables 8.1 and 8.2). In bilateral periventricular nodular heterotopia (BPNH), neurons fail to migrate out of the ventricular zone. One of the genes that can cause BPNH is filamin A (FLNA), which is an actin-cross-linking phosphoprotein (Fox et al. 1998, Parrini et al. 2006). In classical lissencephaly ("smooth brain") associated with loss of function mutations in Lis1, the F-actin cytoskeletal compartment is diminished in leading processes and neurites of migrating and newly post-migratory neurons (Kholmanskikh et al. 2003, 2006). Moreover, restoration of F-actin in Lis1$^{+/-}$ neurons restores their migration capacity in vitro. Associated with human lissencephaly with cerebellar hypoplasia (LCH), the reelin (RLN) gene encodes an extracellular ligand that not only binds lipoprotein receptors VLDLR and ApoER2 but also interacts with α3β1-integrin (Dulabon et al. 2000, Schmid et al. 2005). Integrins are well-known components of focal adhesions connecting the extracellular matrix with F-actin within the cell (Hall 1994). Thus, we know that F-actin must be important for neuronal migration, but the devil is in the details. In this chapter, we discuss current insights into the role and regulation of F-actin in the steps required for neuronal migration.

8.1.1 Features of Neuronal Locomotion

Neurons move in several modalities, including a saltatory pattern when migrating on a glial process or a neurite, starting and pausing as the neuron "inches" along its guide (Edmondson and Hatten 1987, Hatten 1990). In this mode, neurons extend a highly motile leading process in the direction of movement that wraps around the process guide (Rakic 1971, Hatten 1990). The leading process elaborates filopodia but, unlike the growth cone, few or no lamellipodia and the nucleus and soma translocate relatively independently of the extension or the retraction of this leading process (Hatten 1990, Solecki et al. 2006). Neurons can also move in a roving pattern on a flat surface like a coated glass coverslip or the extracellular matrix during tangential neuronal migration in brain (Marin and Rubenstein 2001). This pattern is more similar to fibroblast movement in that the leading process includes both

Table 8.1 Human mutations in actin regulatory genes associated with clinical syndromes of brain malformation and mental retardation

Gene	Human disorder	Function in actin regulation	Clinical phenotype	References (clinical/basic)
Reelin pathway				
REELIN (RLN)	Lissencephaly with cerebellar hypoplasia b LCHb	Reelin impacts actin cytoskeleton through a direct mDab–N-WASP interaction and Rho GAP and –GEF phosphorylation	Frontal > occipital pachygyria (AP gradient) with mild cortical thickening (~5 mm) Severe cerebellar hypoplasia	Hong et al. (2000) Ross et al. (2001)
VLDLR	LCHb	Reelin receptor	Mild cerebral cortical thickening Gyral simplification Cerebellar hypoplasia	Boycott et al. (2005)
CDK5	Focal cortical dysplasia	Phosphorylates mDab and FAK independent of reelin, inhibits Pak1 and Rac1 activity	Foci of abnormally placed neurons (heterotopia) in cortex	Sisodiya et al. (2002)
Rho GTPases and associated proteins				
LIS1	Classical lissencephaly Miller–Deiker syndrome LCHa	Activation of Rac1 and Cdc42, inhibition of RhoA, localization of Iqgap1 Multi-function protein including interactions with cytoplasmic dynein and microtubules	"Smooth brain" with lissencephaly or pachygyria Occipital > frontal (PA gradient) Marked thickening of cortical grey matter Head circumference at –2 SD Paralysis, mental retardation, epilepsy	Lo Nigro et al. (1997) Leventer et al. (2001) Kholmanskikh et al. (2006)
ARGEF2	Microcephaly with periventricular heterotopia	Regulates vesicle trafficking	Primary microcephaly (≤ –3SD at birth) Neuronal heterotopia	Sheen et al. (2004)
FMRP (FMR1)	Fragile X mental retardation	Genetically linked to Rac1 pathway and Rac1-dependent regulation of cofilin	Mental retardation syndrome CGG expansion in 5′ UTR of FMR1 Periventricular heterotopia	Moro et al. (2006) Schenck et al. (2003) Castets et al. (2005)

Table 8.1 (continued)

Gene	Human disorder	Function in actin regulation	Clinical phenotype	References (clinical/basic)
OPHN 1	X-linked mental retardation syndrome	A Rho–GAP protein oligophrenin 1 modulates length of dendritic spines in hippocampus via modulation of RhoA activity	Mental retardation Epilepsy (partial complex seizures) Cerebellar hypoplasia	Billuart et al. (1998) Philip et al. (2003) des Portes et al. (2004) Govek et al. (2004)
Actin-associated proteins				
Filamin (FLN-A)	Bilateral periventricular nodular heterotopia (BPNH)	F-actin-binding protein	Impaired migration of neural cells leaves clusters of neurons at lateral ventricular surface in a "string of pearls" pattern	Fox et al. (1998) Parrini et al. (2006)
DCX	Females: double cortex/SBH (subcortical band heterotopia) Males: classical lissencephaly or LCHa	Like other microtubule-associated proteins (MAPs), when DCX is phosphorylated, it loses affinity for microtubules and instead binds actin	DC/SBH in females with consequences from normal appearing brain with mild MR, to frontal subcortical band to complete AP band and normal IQ, mild, moderate, or severe MR Males are more affected and severe inactivating mutations cause classical lissencephaly (while severe mutations in females cause complete band due to random X-inactivation)	Matsumoto et al. (2001) Ross et al. (2001) Guerrini et al. (2003) Tanaka et al. (2004b) Tsukada et al. (2005) Saplr et al. (2008)

Table 8.2 Genes implicated in neuronal migration disorders and actin regulation in mouse models

Gene	Model	Function in actin regulation	Neuronal migration phenotype	References
Reelin pathway				
Reelin	*Reeler* autosomal mutation, transgene insertion	mDab and Src family kinases acting downstream of reelin impact actin cytoskeleton through a direct mDab–N-WASP interaction and via phosphorylation of Rho GAP and –GEF molecules	Neurons migrating from the ventricular zone of the developing cortex fail to split the pre-plate and newer born neurons fail to migrate past older born neurons resulting in the reversal of cortical lamination. Purkinje cells in the cerebellum fail to migrate depriving granule neuron precursors of trophic support resulting in cerebellar hypoplasia and ataxia, hence *reeler* phenotype	D'Arcangelo et al. (1995)
mDab	*Scrambler* and *Yotari* autosomal mutations			Sheldon et al. (1997)
ApoER2 VLDLR	Double knockout	Reelin receptor		Trommsdorff et al. (1999)
Src Fyn	Double knockout	Principal mediators of reelin signaling		Kuo et al. (2005)
Cdk5	Knockout	Phosphorylates mDab and FAK independent of reelin, inhibits Pak1 and Rac1 activity	Reeler-like, except the earliest born neurons do manage to split the pre-plate into sub-plate and marginal zone	Gilmore et al. (1998)
p35 p39	Double knockout	Obligatory activators of Cdk5		Kwon and Tsai (1998) Ko et al. (2001)
α3β1 integrin	α3 knockout	Reelin receptor	Abnormal migration and cortical layering due to impaired neuron–glia interactions and mDab levels	Anton et al. (1999) Dulabon et al. (2000)
Rho GTPases and associated proteins				
Cdc42	Brain-specific knockout	Activation of N-WASP and inhibition of cofilin	Severe brain malformation partly attributable to migration defects	Garvalov et al. (2007)

Table 8.2 (continued)

Gene	Model	Function in actin regulation	Neuronal migration phenotype	References
Pafah1b1	Knockout	Activation of Rac1 and Cdc42, inhibition of RhoA, localization of Iqgap1	Impaired cortical migration in vivo and cerebellar granule neurons in vitro, reduction in F-actin at the leading edge, failure to polarize	Hirotsune et al. (1998) Kholmanskikh et al. (2003)
	RNAi			Tsai and Gleeson (2005)
Iqgap1	Knockout	Activation of Rac1 and Cdc42, activation of N-WASP and actin polymerization	Impaired migration of undifferentiated neural progenitor cells in response to VEGF in vitro	Balenci et al. (2007)
	RNAi		Impaired migration of cerebellar granule neurons in vitro	Kholmanskikh et al. (2006)
Actin-associated proteins				
n-cofilin	Knockout	F-actin-severing protein. Induces F-actin depolymerization or polymerization, via new polymerization sites and replenishing pool of monomers	Impaired migration of neural crest cells that fail to polarize and do not contain F-actin bundles	Gurniak et al. (2005)
n-cofilin	Brain-specific knockout		Impaired radial migration, resulting in the lack of intermediate cortical layers	Bellenchi et al. (2007)

filopodia and lamellipodia. As neurons make their tangential migrations, for example, from the ventral forebrain to the cortex, they may extend first one process and then branch out a second process, with the nucleus then moving to and pausing at the bifurcation point. One process then retracts and the nucleus translocates toward and into the base of the remaining process (Marin et al. 2006).

In both of these modes of locomotion, there is a nuclear cage or a network of microtubules surrounding the nucleus that is tethered to the microtubule-organizing center or centrosome (Rivas and Hatten 1995, Xie et al. 2003, Tanaka et al. 2004a, Xie and Tsai 2004). Just prior to nuclear translocation, the leading process develops a swelling, which the centrosome invades, and the nucleus advances into in order to maintain its position relative to the centrosome (Tanaka et al. 2004a, Schaar and McConnell 2005, Tsai and Gleeson 2005). The migration of neurons can be halted by interfering with either microtubule or actin polymerization (Rivas and Hatten 1995). Indeed, a posterior "blebbing" of the soma that can be inhibited by the myosin II antagonist blebbistatin has been associated with nuclear translocation in migrating neurons, suggesting a requirement for interaction of actin and myosin II (Schaar and McConnell 2005). Thus, in an emerging view of neuronal migration, the translocation of the nucleus requires both traction created by the nuclear microtubule cage tethered to the centrosome and a "squeezing"/contractile force created by actin–myosin II contraction that propels the nucleus forward.

Neuronal migration may be somewhat arbitrarily divided into a series of subcellular events. First, the cell must polarize to distinguish the front from the back of the cell so that the Golgi apparatus and the centrosome are at one pole with the nuclear cage forming a concentration of microtubules oriented from the centrosome to surround the nucleus. Second, a leading process is extended at the front in the direction of migration. Third, the centrosome moves into a swelling in the proximal portion of the leading process. Fourth, the nucleus and large organelles including the Golgi apparatus move toward the centrosome. As these steps occur, cell adhesion is regulated to become strong at the front and weak at the back of the cell to create a net force forward when the actin–myosin elements within the cell contract. In addition, extracellular signals including soluble factors and matrix proteins provide directional cues, motogenic and inhibitory signals. Actin dynamics has potential roles in each of these steps of neuronal migration.

8.1.2 Regulation of Actin Dynamics

The major second messenger system regulating actin polymerization comprises the Rho-family GTPases RhoA, Cdc42, and Rac1, which use their effector proteins to directly impact the cycling of actin. Binding proteins that coordinate actin filament severing and capping of the filament are principally responsible for actin polymerization. The major factors in this process are actin-related proteins Arp2, Arp3, formins including mDia (mammalian Diaphanous), and Spire. Arp2/3 are part of a heptameric complex that initiates peripheral actin nucleation associated

with the sides of existing actin filaments to form branches that are rapidly capped at their barbed ends (Cooper and Pollard 1985, Millard et al. 2004). Cdc42 catalytically promotes actin polymerization through de novo nucleation, branching from the sides of actin filaments, that requires the Arp2/3 complex (Mullins and Pollard 1999). Activation of the Arp2/3 complex occurs largely through the proteins of the WASP (Wiskott–Aldrich syndrome protein) family, including the Scar (a.k.a. WAVE) proteins (Miki et al. 1998, Mullins et al. 1998, Machesky et al. 1999). In its GTP-bound active form, Cdc42 binds to the CRIB domain of WASP (or neuronal, N-WASP), leading to the release of autoinhibition of WASPs (Kim et al. 2000). Activation of the Arp2/3 complex by Cdc42 in particular promotes protrusion of F-actin to form finger-like filopodia (Nobes and Hall 1995, Bishop and Hall 2000), while Arp2/3 activation through Rac1 promotes lamellipodia formation, a sheet-like projection from the leading edge of a motile cell (Ridley and Hall 1992). In contrast, RhoA promotes the formation of focal adhesions and, in non-neuronal cells, stress fibers through its actions on profilin and formins (Ridley and Hall 1992, Watanabe et al. 1997). Profilin forms a 1:1 complex with G-actin, promoting the ADP/ATP exchange and subsequent assembly of actin into filaments (Theriot and Mitchison 1993). The RhoA effector protein, mDia, can bind to both profilin and GTP–RhoA at different sites, providing a means of localizing actin polymerization (Watanabe et al. 1997). The formins are a large family of multidomain proteins containing formin homology domains (FH1 and FH2), involved in actin-based processes ranging from formation of actin stress fibers, to contractile rings during cytokinesis, to filopodial formation. Unlike the Arp2/3 complex, the formins nucleate actin from the barbed ends of filaments and remain bound to the barbed ends to antagonize capping protein and gelsolin and so promote extended growth of the actin filament (Harris and Higgs 2004, Schirenbeck et al. 2005, Faix and Grosse 2006). In addition to direct interaction with RhoA, mDia1 can interact with Cdc42 toward the formation of filopodia (Peng et al. 2003). Interestingly, the mDia1/Cdc42 interaction is not essential for filopodial formation, since another Rho GTPase Rif can induce filopodia in combination with mDia2 (Pellegrin and Mellor 2005).

In addition to nucleation and extension, actin dynamics is regulated through capping of growing ends accomplished by capping protein and gelsolin. This capping is antagonized by mammalian-enabled (mEna) and vasodilator-stimulated phosphoprotein (VASP), which associate with the barbed ends of actin filaments to promote polymerization (Bear et al. 2002, Mejillano et al. 2004). In contrast, others have found no anti-capping activity for Ena/VASP proteins, reporting instead that VASP is required for bundling and stabilization of actin to promote filopodial formation (Schirenbeck et al. 2006). In neurons, mEna binds to profilin concentrated at growth cone lamellipodial and filopodial tips. Actin severing at the rear of the actin meshwork is accomplished by cofilin and actin-depolymerizing factor (ADF) proteins. Actin polymerization is also promoted by cofilin-mediated actin severing, since severed F-actin fragments are the preferred substrates for Arp2/3 (Ghosh et al. 2004, Huang et al. 2006). There is evidence that precise spatial regulation of the depolymerizing/severing activity of cofilin is crucial for directed cell movement (Ghosh et al. 2004, Gurniak et al. 2005, Bellenchi et al. 2007, Garvalov et al. 2007).

8.1.3 Peculiarities of Actin Regulation in Neuronal Migration

8.1.3.1 Polarization and Centrosome Positioning

As noted above, neuronal polarization in preparation for migration is reflected in the positioning of the centrosome and inhibition of actin depolymerization with cytochalasin B obliterates the asymmetric localization of the centrosome and Golgi complex (Rivas and Hatten 1995). This positioning in fibroblasts is dependent upon Cdc42 and Par6 along with atypical PKCζ, through control of APC (adenomatous polyposis coli) and Dlg1 (discs large 1) at the cortical actin meshwork to capture microtubules (Etienne-Manneville et al. 2005). Cell polarization also requires the action of non-canonical Wnt signaling through Wnt5a, Dvl, and axin (Schlessinger et al. 2007). In neurons, it is established that Par6 is required for centrosome positioning, which in turn is needed along with Par3 for axon initiation and migration (Solecki et al. 2004, Solecki et al. 2006). Although cooperation between microtubules and cortical actin is likely required for centrosome positioning and leading process extension, the precise molecular interactions leading to this initial polarization in neuronal motility are still largely uncharacterized.

8.1.3.2 Leading Edge Extension

In all motile cells examined, forward movement requires extension of the leading edge (Lauffenburger and Horwitz 1996, Mitchison and Cramer 1996). The general mechanism of leading edge extension is largely conserved among different cell types and consists of plasma membrane protrusion in the direction of movement driven by actin polymerization underneath the membrane. The motive force of this protrusion is actin polymerization in which incorporation of new actin subunits occurs at the barbed end of the growing filament just underneath the membrane. The disassembly of the filament at the pointed end provides actin subunits for recycling into further polymerization of the barbed end (Pollard 2003, Pollard and Borisy 2003). Cells contain a pool of unpolymerized actin monomers bound to profilin and sequestering proteins such as thymosin-β4 (Yarmola and Bubb 2004). New filaments arise when signaling pathways activate nucleation-promoting factors such as members of the WASP/Scar family of proteins (Takenawa and Suetsugu 2007). Active nucleation-promoting factors then stimulate Arp2/3 complex proteins to initiate a new filament as a branch on the side of an existing filament. Using actin–profilin derived from the subunit pool, new branches grow rapidly and push the membrane forward. The rate of filament growth is determined by capping proteins like CapZ and gelsolin that bind to the actin barbed ends and halt incorporation of new subunits into a growing actin polymer, and by proteins of the Ena/VASP family that antagonize capping (Cooper and Schafer 2000, Krause et al. 2003). Actin subunits in this branched network hydrolyze their bound ATP quickly but dissociate the γ-phosphate slowly. Dissociation of γ-phosphate initiates disassembly reactions by inducing debranching and binding of ADF/cofilin, which, in turn,

promotes severing and dissociation of ADP subunits from filament ends. Profilin, the nucleotide exchange factor for actin, catalyzes exchange of ADP for ATP and returns subunits to the ATP–actin–profilin pool, ready for another cycle of assembly. Furthermore, gelsolin and ADF/cofilins may also help initiate new protrusions and actin remodeling by severing filaments to expose barbed ends for elongation (Ono 2007).

A detailed analysis of the leading edge ultrastructure and the biochemistry of actin dynamics performed in large flat epithelial cells and fibroblasts is lacking for migrating neurons. However, functional studies looking at the role of actin-binding proteins indicate that the same basic mechanism of leading edge extension also operates in migrating neurons. WASP/Scar proteins are expressed in the nervous system throughout development and knockout of the neuron-specific WAVE1 in mice causes notable neuroanatomical malformations without overt histopathology of peripheral organs (Fukuoka et al. 1997, Benachenhou et al. 2002, Dahl et al. 2003). The first step in the stimulation of actin polymerization and leading edge extension by WASP/Scar proteins is their activation through signaling pathways triggered by extracellular factors at the plasma membrane. IQGAP1 and mDab play an important role in neuronal migration as shown by a reduction in migration velocity in IQGAP1 knockdown neurons (Kholmanskikh et al. 2006), delayed migration of neural progenitors in IQGAP1-null mice (Balenci et al. 2007), and severe malformation of the cerebral cortex caused by failed neuronal migration in mDab1 (mouse Disabled) mutant *scrambler* mice (Sheldon et al. 1997). IQGAP1 and mDab mediate Rho GTPase and reelin signaling, respectively, that may impact cellular motility on many levels (Bar et al. 2000, Brown and Sacks 2006). Intriguingly both IQGAP1 and mDab directly bind to and activate N-WASP to promote actin polymerization (Suetsugu et al. 2004, Bensenor et al. 2007, Le Clainche et al. 2007). Genetic and cell biological evidence indicates a critical role for Ena/VASP/Evl family of proteins in neuronal migration in organisms ranging from *Caenorhabditis elegans* to *Drosophila* to mammals. For example, simultaneous disruption of WSP-1 and Unc-34, worm homologues of WASP and Ena, respectively, resulted in defects in neuronal migration in *C. elegans* (Withee et al. 2004). In *Drosophila,* Ena genetically interacts with the neuronal migration gene *Dab* (Gertler et al. 1990). Finally, abnormal neuronal migration resulted when Ena/VASP were inactivated through their mislocalization to mitochondria (Goh et al. 2002).

Actin remodeling at the leading edge induced by gelsolin and ADF/cofilin is necessary for cell motility (Southwick 2000). Interestingly, both proteins are targets of the transcription factor serum response factor (SRF), whose deficiency impedes tangential chain migration along the rostral migratory stream from the subventricular zone into the olfactory bulb (Alberti et al. 2005). Specifically, SRF deficiency results in the reduced expression of gelsolin and dramatically elevated inhibitory phosphorylation of cofilin. Inactivation of the mouse *n*-cofilin locus results in impaired migration of neural crest cells that fail to polarize and lack F-actin bundles (Gurniak et al. 2005). Neuron-specific knockout of *n*-cofilin, but not ADF, severely impairs radial migration of neurons in the developing cortex (Bellenchi et al. 2007). Furthermore, cofilin plays an important role in neuronal motility as a target in at

least two kinase pathways with established roles in neuronal migration, PAK1 and Cdk5 (Kawauchi et al. 2006, Jacobs et al. 2007). Therefore, actin remodeling is critically important for neuronal locomotion.

In summary, inactivation of key components in the mechanism of actin polymerization and recycling of actin subunits underlying leading edge extension leads to impairment of neuronal migration. Direct evidence that inactivation of these proteins in migrating neurons induces defects in actin polymerization and leading edge extension is largely lacking. However, conservation of mechanisms of cytoskeletal regulation and dynamics among different cell types allows one to hypothesize that disruption in the elements of actin dynamics inhibits neuronal migration through the impairment of leading edge extension.

At least some aspects of the regulation and organization of actin cytoskeleton in migrating neurons appear to be neuron specific. These features include the absence of detectable stress fibers, the presence of point contacts rather than large focal adhesions (Kuczmarski and Rosenbaum 1979, Arregui et al. 1994), and the presence of neuron-specific signaling pathways that impact the actin cytoskeleton, for example, mediated by Cdk5 (Xie et al. 2006) or triggered by glutamate or GABA receptors (Manent and Represa 2007). The precise role of leading edge extension for neuronal migration is also in question. In non-neuronal cells, protrusion of the leading edge provides an advanced, adhesive "anchor" against which motive force is generated. However, recent data suggest that the strongest points of neuronal adhesion during migration may not be at the tip of the leading process, but rather at a cytoplasmic expansion in the proximal third of the leading process, into which the centrosome invades (discussed in the next section) (Solecki et al. 2004, Schaar and McConnell 2005, Solecki et al. 2006, Tsai et al. 2007). Regardless of its mechanics, neurons do not move beyond displacing the nucleus a few micrometers without a leading process (Schaar and McConnell 2005). It seems inescapable that this leading edge is required at least for neuronal guidance. Though the centrosome may have a special importance in the migrating neuron, actin polymerization in the leading process is likely involved in producing locomotive force for these distinctive cells.

These unique features are indeed expected considering the unique morphology of the migrating neuron and its unique developmental program with distinct actin-dependent processes being regulated by only partly overlapping cascades as neuroblasts are switched in rapid succession from cell proliferation to migration to neurite outgrowth and synaptogenesis. The challenge of future studies will be to definitively confirm the role of the actin polymerization and subunit recycling in leading edge extension in migrating neurons and to identify additional neuron-specific mechanisms that allow migrating neurons to adopt their unique morphology, adapt to multiple substrata on which leading edge extends, and allow for responses to a multitude of extracellular signals.

8.1.3.3 Translocation and Extrusion of the Nucleus

In order for a cell to advance, its subcellular organelles must be displaced in the direction of movement. This is a particularly daunting task for neurons whose

nuclei comprise the majority of the volume of the cell soma. It is not surprising then that neurons have devised particular methods for translocating their nuclei, capitalizing on evolutionarily ancient and more recent proteins. Current models of neuronal nuclear translocation require the cooperation of traction and propulsive forces. Traction is provided by the microtubule nuclear cage that surrounds the nucleus and its tethering to the microtubule-organizing center or centrosome. This must be coordinated with contractile forces governed by actin–myosin II interactions at the rear of the nucleus that propel it forward. Though still incompletely understood, a number of proteins involved in nuclear translocation are known.

The cage of microtubules surrounding the neuronal nucleus was recognized over a decade ago (Rivas and Hatten 1995). The nuclear cage is organized with its microtubule minus ends tethered to the centrosome and the plus ends wrapped about the nucleus. Neuronal migration proteins Lis1 and doublecortin (Dcx) interact with cytoplasmic dynein, the nuclear cage, and centrosome to maintain a relatively constant distance between the nucleus and the centrosome during migration (Shu et al. 2004, Tanaka et al. 2004a). In the migration model, the centrosome advances into a dilatation in the proximal shaft of the leading process, while the nucleus is pulled toward the centrosome, largely through the actions of the minus end-directed protein motor dynein and assisted by myosin II, which presumably provides contractile force (Tsai et al. 2007). The involvement of myosin II in the process strongly suggests that F-actin must play a role as well, though exactly how remains to be elucidated. One possibility is that the dynein motor complex forms contacts that bridge microtubules and the actin meshwork, using myosin II to form the traction needed to advance the centrosome and to facilitate nuclear translocation.

Along with the pull on the neuronal nucleus, contractile forces appear to propel and extrude it toward the centrosome. The nucleus is seen to elongate and deform, seeming to be squeezed into the space formed by the proximal portion of the leading process. In addition, pharmacological manipulation with the myosin inhibitor blebbistatin indicates that forward movement of the nucleus and dissolution of microtubules at the back of the soma activate membrane myosin II-dependent blebbing and loss of cell adhesions to the rear of the nucleus (Schaar and McConnell 2005). In their model, the facilitation of microtubule depolymerization or myosin II activity (by inference, actin–myosin II contraction breaking focal adhesions at the rear of the cell) creates a kind of slingshot effect, adding a propulsive element to nuclear displacement.

8.1.3.4 Generation of Motile Force—Microtubule Capture to Cortical Actin and Contractile Elements

The fundamentals of the mechanical forces generated in motile cells, including neurons, are relatively well understood (Lauffenburger and Horwitz 1996, Mitchison and Cramer 1996). However, the molecular details, especially in neurons, are only recently coming to light. It seems clear that motive force can be derived from the antagonism of contractile proteins acting against more rigid structures like

focal adhesions that link extracellular matrix, plasma membrane, and intracellular F-actin, as discussed in the next section. This antagonism can build potential energy for release upon disrupting the adhesive contact. Another opportunity for generating motive force is the interaction between the cortical actin meshwork and microtubules.

Capture of microtubules by the cortical actin meshwork requires the recruitment of several proteins to bridge those that bind filamentous actin to those that bind microtubules, and in particular microtubule tips (Rodriguez et al. 2003). In non-neuronal cells, it has been established that microtubule tips are captured to cortical actin through the cadherin-modulated binding of Cdc42 effector protein IQGAP1, which binds both F-actin and the microtubule +TIPs protein CLIP170 and also requires participation of the adenomatous polyposis coli (APC) protein (Watanabe et al. 2004, Noritake et al. 2005). In migrating neurons, the Lis1 protein which promotes F-actin polymerization and Cdc42/Rac1 activity participates in complexes with IQGAP1, CLIP170, and GTP-Cdc42 (Kholmanskikh et al. 2006), suggesting that similar complexes are required for microtubule capture in motile neurons. Moreover, this role of Lis1 is regulated by calcium-dependent cell signaling via the NMDA receptor (Kholmanskikh et al. 2006), providing a regulatory mechanism for Lis1 participation in these complexes. Interestingly, in neurites of post-migratory neurons, the more important scaffold protein may be IQGAP3 (Wang et al. 2007).

Evidence suggests that these complexes linking microtubule ends to cortical F-actin also include non-muscle myosin II to provide contractile force along the stabilized microtubules. In addition, protein motor complexes including cytoplasmic dynein and kinesin motors may use this microtubule–actin bridging complex to transport their cargoes retrogradely or anterogradely, depending on the motor (Rodriguez et al. 2003). In neurons, proteins associated with the dynein light chain, for example, Tctex, may provide an activity-dependent means of coordinating microtubule binding of dynein with F-actin interaction (Sachdev et al. 2007). Microtubules must extend and resist the retrograde flow of F-actin in neuronal growth cones and probably during the advancement of the leading process of a migrating neuron. This appears to require the cooperation of Lis1 and dynein, as targeted reduction of these proteins with blocking antibodies or RNAi limits the extension of microtubules into the growth cone (Grabham et al. 2007).

8.1.3.5 Adhesion and Guidance

In order for neurons to polarize and move in a directional fashion, they must be able to form adhesive contacts with their migration surface and to detect differences from front to back in the information gradient that guides their path. In motile non-neuronal cells, adhesive contacts with the migration surface are typically organized into focal adhesion complexes. Focal adhesion complexes form when pro-migratory extracellular proteins induce clustering of the integrin receptors (Gupton and Waterman-Storer 2006, Gupton et al. 2007). Clustering of the integrin receptors recruits signaling machinery that promotes migration and provides a physical link between the extracellular matrix and the cytoskeleton. Actin filaments are a major

cytoskeletal component connected directly and through adaptor proteins to the cytoplasmic tails of integrin receptors. However, recently it was shown that microtubules are targeted to focal adhesion complexes as well. The actin cytoskeleton is a major target of signaling triggered by integrin clustering. Focal adhesion complexes recruit and activate a number of tyrosine kinases, including Src and FAK (focal adhesion kinase), that through phosphorylation of GAP and GEF molecules control activation of Rho GTPases, in turn regulating actin polymerization. Existence of well-defined large focal adhesion complexes similar to those observed in fibroblasts has not been demonstrated in migrating neurons. Nonetheless, individual components of focal adhesions are essential for neuronal migration. Inactivation of alpha3 integrin in the developing cortex causes retarded radial and tangential neuronal migration associated with aberrant actin cytoskeletal dynamics at the leading edges (Schmid et al. 2004). Moreover, the extracellular domain of alpha3beta1 integrin binds to reelin, while its cytoplasmic tail binds to Dab in a reelin-dependent manner (Schmid et al. 2005). Inactivation of major tyrosine kinases associated with focal adhesions causes distinct neuronal migration phenotypes. Targeted deletion of integrin-linked kinase (Ilk) from embryonic mouse dorsal forebrain neuroepithelium results in severe cortical lamination defects resembling cobblestone (type II) lissencephaly (Niewmierzycka et al. 2005). Focal adhesion kinase (FAK) is a target of Cdk5 and FAK phosphorylation by Cdk5 at S732 is important for microtubule organization, nuclear movement, and neuronal migration (Xie et al. 2003). The Src family kinases Src and Fyn mediate Dab1 phosphorylation downstream of VLDLR and ApoE2R and phosphorylated Dab1 in turn further activates Src family kinases (Bock and Herz 2003). The combined absence of Src and Fyn almost abolishes tyrosine phosphorylation of Dab1 and causes reeler-like phenotype in the fetal cortex and cerebellum (Kuo et al. 2005). A number of RhoGAP and GEF molecules are targeted by Src family kinases in focal adhesion complexes; however, their specific involvement in neuronal migration has not been tested.

Many other cell adhesion molecules are expressed by migrating neurons and connections with the actin cytoskeleton have been shown in some cases. Inactivation of N-CAM gene expression causes impaired migration in the rostral migratory stream (Tomasiewicz et al. 1993). Loss of polysialic acid that plays an essential role in brain development by modifying the neural cell adhesion molecule (NCAM) affects both tangential and radial migration of neural precursors during cortical development, resulting in aberrant positioning of neuronal and glial cells (Angata et al. 2007). Clustering of NCAM activates FAK and Fyn tyrosine kinases that are important for actin remodeling as discussed above and promotes neuronal growth cone migration (Beggs et al. 1997, Bodrikov et al. 2005).

The cell adhesion molecule L1 was originally identified as an antigen for an antibody disrupting neuronal migration in developing cerebellum (Lindner et al. 1983). L1 gene mutations in humans result in a severe neurological phenotype that is attributed in part to impaired neuronal migration (Wong et al. 1995). Participation of L1-CAM in neuronal migration indeed involves effects on the actin cytoskeleton through potentiation of integrin-dependent signaling that involves beta1 integrin endocytosis (Thelen et al. 2002, Panicker et al. 2006). Moreover, clustering of L1

adhesion molecules directly activates GEF Vav2 leading to Rac1 activation (Schmid et al. 2004).

8.1.3.6 Endocytosis, Actin Regulation, and Neuronal Migration

One way that adhesion may be regulated in a gradient from the front to the back of the cell is through endocytosis of receptors from the plasma membrane, a process that requires tight regulation of actin dynamics to govern endocytosis of clathrin, and probably caveolin-coated vesicles (Yarar et al. 2005, Kaksonen et al. 2006). Although most information derives from studies of *Saccharomyces cerevisiae* and epithelial cells, the same principles are likely to apply to neurons. For example, in neuronal synapses, sites of clathrin-mediated endocytosis co-localize with F-actin and disruption of presynaptic actin impairs this endocytic process (Shupliakov et al. 2002). Moreover, the advance of growth cones requires regulated adhesion that is stronger at the front, and an important mechanism for this is clathrin-dependent endocytosis and movement of L1-CAM to the leading edge of the advancing growth cone (Kamiguchi and Lemmon 2000, Dequidt et al. 2007). Not only is this rapid recycling of L1-CAM important for growth cone motility, but it is also likely to be important for neuronal migration as well. Indeed, interference with endocytic machinery in epithelial cells impairs their ability to polarize and move in a wounding model of cell migration (Proux-Gillardeaux et al. 2007).

Evidence to date in mammalian cells favors a model of endocytosis in which F-actin dynamics are required for invagination and constriction of the clathrin-coated pit. Actin dynamics, centered around nucleation and Arp2/3 regulation, may also regulate scission and translocation of the endocytic vesicle (Yarar et al. 2005). In this model, WASP works with myosins to activate the Arp2/3 complex around the clathrin-coated pit and growth of actin filaments near the plasma membrane leads to invagination of the coated membrane. Interaction of actin–myosin with dynamins around the neck of the vesicle pinches the membrane in-pocketing to release the vesicle for internalization. Once free off the membrane, the endosome moves within the cell, driven by the Arp2/3-regulated polymerization of actin, forming a comet-tail like structure in a process sometimes referred to as "rocketing" (Merrifield et al. 1999, Orth et al. 2002, Kaksonen et al. 2006). Recognition of endocytotic/exocytotic processes in neuronal migration has only recently become evident and will be an area of increasing interest in this field.

8.2 Concluding Remarks

This chapter outlines some of the features and functions of actin filaments in motile neurons. While much is now understood, many questions, new and remaining, will be investigated as methodological approaches such as TIRF and speckle microscopy become more widely used. Unresolved issues include the mechanisms by which the microtubule and actin–myosin components of the cytoskeleton are coordinated

and where within the neuron are the major motive forces generated? What protein complexes are formed at these critical subcellular locations and how is their assembly ordered? What molecular engines—such as small GTPases, protein motors, ATP hydrolysis—drive the dynamics at these spots? How does endocytosis contribute to neuronal migration and how does actin dynamics regulate this process? Do actin polymerization or protein motors or both power endocytic scission? How is actin nucleation regulated at clathrin- or caveolin-coated vesicle sites? While these aspects are no doubt universally important for motile cells, the details will also likely be different for neurons than for other cell types.

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Chapter 9
Actin and Neuronal Polarity

Annette Gärtner and Carlos G. Dotti

Abstract Mechanisms determining neuronal polarity have been most extensively studied using dissociated hippocampal neurons which develop in a very stereotypic manner in the absence of differential extrinsic cues. Polarity is established in two major steps: first, an initial deformation of the spherical cell gives rise to the first neurite, and later, after multiple neurites grew from the sphere, one neurite will be selected for fast axonal outgrowth. Both steps are under the tight control of cytoskeletal rearrangements; sub-membranous local actin remodelling will support the disruption of the spherical architecture to thus allow the formation of the first neurite and from this, later on, rapid growth. Axonal growth from a multipolar neuron at later stages has been shown to be supported by local actin destabilization and that could be achieved either by intrinsic or extrinsic cues, demonstrating that polarity establishment is a complex endogenously controlled remodelling process, under active control by extrinsic factors.

Keywords Hippocampal neurons · Permissive · Instructive · Growth cone · Minor neurite · Dendrite · Axon

9.1 Introduction

Neuronal polarity refers to the striking differences in the molecular and supramolecular organization of the principal neurites, i.e., axons and dendrites, which reflect their different functions: axons transmit signals, while dendrites receive those signals. The most characteristic feature of axons is the presence of voltage-gated sodium channels and synaptic vesicles, while dendrites are equipped with all the molecules and cellular structures for the reception and computation of incoming information, for instance, neurotransmitter receptors (for a precise list of axonal

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and dendritic polarized molecules, the reader should consult the reviews of Craig and Banker 1994, Horton and Ehlers 2003). Neurons, however, establish a clearly polarized phenotype much earlier in development, before they have acquired the capacity to transmit and receive electrical signals. This is essential for the correct wiring of the brain and therefore effort has been taken in order to understand by which mechanisms this polarity is achieved, regulated and maintained. Among the multiple supra-molecular events involved in organizing the development of polarized cells in general, the precise spatial and temporal regulation of the structure and dynamics of the actin cytoskeleton is essential. This chapter will provide a broad overview on the mechanisms which regulate actin dynamics during the development of a polarized neuron and those via which actin could influence the establishment of neuronal polarity.

9.2 Model System to Study Neuronal Polarity

9.2.1 Neuronal Development In Vivo

The establishment of polarity is most extensively studied in hippocampal or cortical excitatory, pyramidal neurons. In vivo cortical excitatory neurons are generated in the ventricular or the subventricular zone of the dorsal telencephalon and migrate radially towards the pial surface, where they occupy the most superficial layer of the developing cortical plate and populate the cortex in an inside first, outside last sequence, generating the six-layered cortex (for more in-depth review, see Gotz and Huttner 2005, Noctor et al. 2004). Young neurons display a polar phenotype already during their migration with a leading and trailing edge (Hatten 1999, Kriegstein and Noctor 2004, Nadarajah et al. 2003, Parnavelas et al. 2002, Rivas and Hatten 1995). Final axon–dendritic polarity becomes manifest only when neurons reach their final destination in the upper cortical layers, where they extend a ventricle-directed axon and a number of dendrites, with the principal apical dendrite directed towards the pial surface. However, in approximately half of the neurons, an axon-like neurite elongated already during the migration, in the intermediate zone (Noctor et al. 2004, Shoukimas and Hinds 1978), showing that the mechanisms participating in directional migration and axonal dendritic specification are strongly intertwined. This mechanistic connection makes the selective study of the specific process of axon/dendrite fate determination difficult, since disruptions in the signalling to the actin cytoskeleton will always have an impact on migration before polarity can establish (Rivas and Hatten 1995). Moreover, the complex nature of extracellular cues in vivo makes it difficult to distinguish between cell autonomous and non-autonomous effects. Therefore, most studies on mechanisms leading to the establishment of neuronal polarity relied on in vitro models in which neurons polarize in the absence of migration and without any differential distributed extracellular cues. Typically, studies were undertaken in embryonic (E16–E19)-dissociated hippocampal neurons in low-density culture which develop their polarity in a very

similar way as described in vivo (Dotti et al. 1988). Therefore we will briefly introduce this model and focus this article on the knowledge drawn from those studies. For more information on the regulation of actin during neuronal migration, the reader should refer to Chapter 8.

9.2.2 *Dissociated Hippocampal Neurons as Model System to Study Polarity*

Hippocampal neurons provide an excellent model to study neuronal polarity since they develop in such a stereotyped manner that their development was classified in distinct stages (Dotti et al. 1988), which are illustrated in Fig. 9.1. In the first stage, immediately after plating, neurons attach and sometimes develop some lamellipodia. In stage 2 which develops within the first 4–24 h, neurons sprout between three to five morphologically identical neurites. Those are called minor neurites and are highly dynamic, extending and retracting without any major net growth. Interestingly this stage of extension and retraction lasts up to 24 h in individually

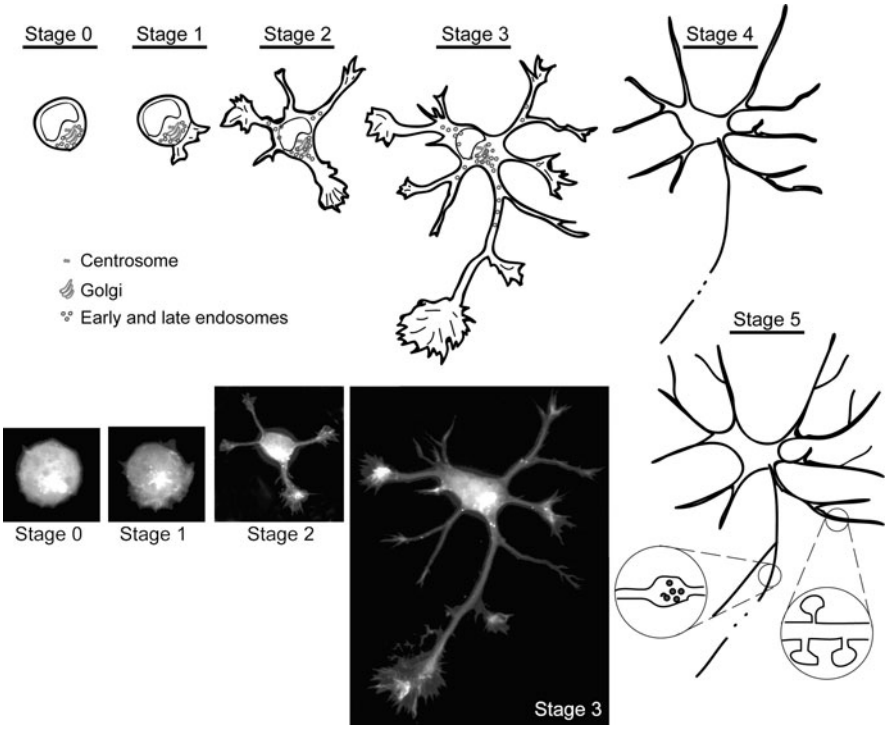

Fig. 9.1 The favoured model system for the study of neuronal polarity are hippocampal neurons in low-density culture which develop their axonal dendritic polarity in the absence of any polarized extrinsic cues in a very reproducible pattern (see Section 9.2.2)

observed neurons and parallels the multipolar stage neuron display in the intermediate zone in vivo (Tabata and Nakajima 2003). The most striking morphological event is the fast (five times faster) outgrowth of one of the minor neurites to become the axon (stage 3), while the other neurites are inhibited and do not show major net growth. In stage 4, dendritic outgrowth occurs from the remaining minor neurites and stage 5 describes the maturation of neurons which makes them capable of establishing synaptic contacts.

9.2.3 Intrinsic and Extrinsic Regulation of Neuronal Polarity

The most important implication from that model, and the subsequent work based on it, was that polarity first was determined at the transition stage 2–3. In a homogenous environment, free of asymmetric cues, this selection was thought to be stochastically determined (Bradke and Dotti 2000), while under conditions of differential applied external cues the axon maybe selected by the local activation of signalling by extracellular receptors (Esch et al. 1999, Polleux et al. 1998). However, recently it was shown that the selection of the axon in a homogenous environment is not a stochastic event but that the neurite that appears first from the cell body of the new neuron later becomes the axon (de Anda et al. 2005). This finding implies that axonal fate, at least partly, is endogenously determined. On the other hand, the maintenance of growth, consolidating such fate, requires the activation of signalling cascades from external cues. Actin remodelling plays the most essential role in both intrinsic definition and extrinsic consolidation of axonal fate.

9.3 Which Are the Critical Steps in Polarity Establishment Which Could Be Regulated by Actin?

The previous description of the developmental events shows that there are two main critical steps in the establishment of neuronal polarity: (1) the selection of the first sprout from the spherical cell and (2) the transition from a minor neurite to an axon. In the following section we will illustrate these steps in the light of a possible regulation by the actin cytoskeleton.

9.3.1 First Bud Formation and Control by the Actin Network

Initially, in grasshopper neurons (Lefcort and Bentley 1989) and later in mammalian neurons (de Anda et al. 2005, Rivas and Hatten 1995) it was shown that the asymmetric positioning of the centrosome marks the site from which the first neurite extends, which will become the axon. The site of the centrosome could simply be defined by the cleavage plane of the last neurogenic division as shown by de Anda

et al. (2005). In the vicinity of the centrosome, from which microtubules sprout, membrane organelles and the Golgi are clustered, which deliver the membrane and proteins necessary for the increase of the membrane surface at this site. It is known that endocytosis and exocytosis can be regulated by the actin cytoskeleton (Morales et al. 2000, Smythe and Ayscough 2006) and thus it is possible that intrinsically or extrinsically activated actin regulatory molecules participate in axon axis definition at this level. This however, has not been investigated. On the other hand, it is clear that sub-membranous actin remodelling plays a role in polarity. This becomes quite evident by comparing the homogeneous organization of F-actin bundles at the spherical stage with the rather disorganized arrangement in one pole as soon as the sphere brakes in order to generate the first sprout (Fig. 9.2). This is reminiscent of what has been observed during bud formation in *Saccharomyces cerevisiae*. In *S. cerevisiae* the bud site can be selected in the absence of extracellular cues by recruiting a cascade of small GTPases to this site, which coordinates the actin and microtubule cytoskeleton to permit a localized docking/fusion of vesicles at that site of membrane growth (Nelson 2003). An indication that the requirement of small GTPases at this step is conserved in mammalian cells also came from a recent study which demonstrated that the general selection of bud sites in hippocampal neurons is achieved by the local inactivation of the small GTPase RhoA and Rho kinase which leads to an increase in the dephosphorylation of the actin-stabilizing protein

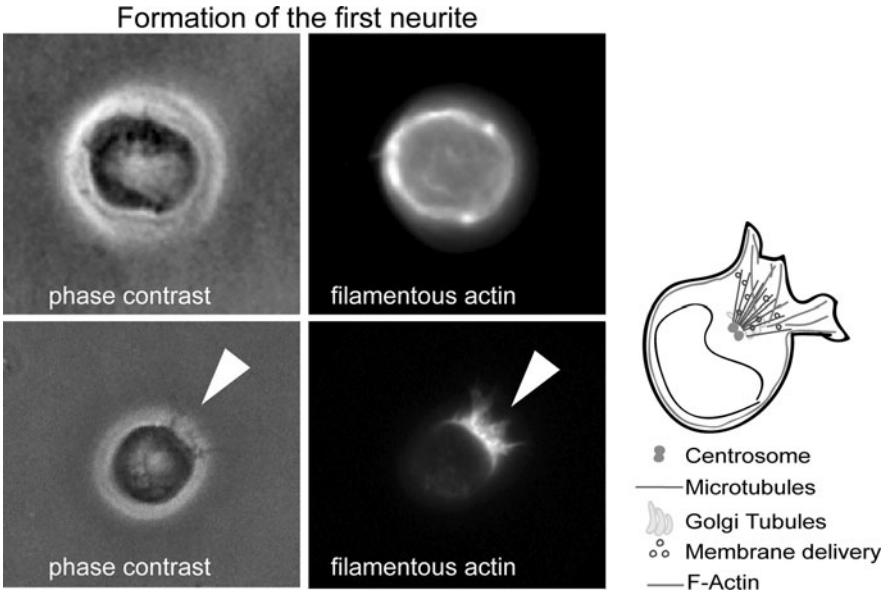

Fig. 9.2 The formation of the first neurite is accompanied by a breakage of the cortical actin (*lower panels*) which in a round neuron is localized quite homogenously at the cell cortex (*upper panels*). The *right hand* shows the arrangement of intracellular components during that process (see Section 9.3.1)

profilin IIa, resulting in the destabilization of the actin cytoskeleton (Da Silva et al. 2003). However, these last results were obtained in cells that had sprouted all the neurites and thus it remains to be investigated whether the RhoA-inactivating signal is localized to the area of the membrane where cells sprout the first neurite, which, as mentioned above, contains the information to later become the axon. Due to the sparse data on this topic, we will in the following section rather focus on the second step in polarity establishment, the stage 2–3 transition.

9.3.2 Stage 2–3 Transition and Actin Control

The most obvious polarity change in the developing neuron is the fast extension of only one of the minor neurites (transition from stage 2 to stage 3). In addition to changes in actin dynamics at the growth cone of the selected neurite, the actual extension involves a preferential and directed polymerization of microtubules (Dent and Gertler 2003), the polarization of mitochondria for energy provision, the directed transport of membrane compartments for polarized protein and lipid transport as well as the polarization of components of the machinery involved in their fusion, retrieval and degradation (Bradke and Dotti 1997). This means that any molecule that, directly or indirectly, participates in the structural or functional regulation of any of all the above pathways will, to a different extent, participate in neuronal polarity. We will focus here on actin regulation.

The differential regulation of actin dynamics can support fast outgrowth of one neurite by different ways: (1) retraction/extension frequency and duration of the growth cone, (2) rate of exocytosis and endocytosis and (3) cross talk with microtubules (see Chapter 5). One must keep in mind, however, that such events must happen before the fast outgrowth commences, in order to selectively prepare only one growth cone for fast growth. These are the true polarizing mechanisms, different from the similar events taking place during elongation of the already specified axon. That polarized actin dynamics controls the establishment of polarity was demonstrated in a series of experiments in stage 2 hippocampal neurons (Bradke and Dotti 1999). In this work the stability of actin in different growth cones, exposed to short treatments with the actin-depolymerizing drug cytochalasin D, was measured. This experiment revealed that actin is less stable in the growth cone of the future axon before axonal outgrowth becomes visible. Moreover, this growth cone was shown to be larger and more motile just before its fast outgrowth, again reflecting a difference in actin dynamics. Most strikingly, selective depolymerization of F-actin by local transient perfusion with cytochalasin D of one growth cone triggered axonal outgrowth from that particular growth cone. Bath application of cytochalasin D initiated fast axonal outgrowth from all minor neurites, demonstrating that destabilization of actin in growth cones is sufficient for fast growth. The destabilization of actin could possibly promote fast neuronal outgrowth by allowing the protrusion of microtubules in the peripheral zones of the growth cone (Forscher and Smith 1988) or alternatively by facilitating vesicle and membrane turnover (see above) and most likely through both. Those results indicate that any natural stimulus

capable of reducing actin stability in a spatially and temporally restricted manner in a developing neuron will act as a polarizing stimulus.

Different from the Bradke and Dotti (1999) results, a global application of another actin-depolymerizing drug cytochalasin E only supported the further growth of the axon but not that of the other neurites (Ruthel and Hollenbeck 2000). The different outcome is still not fully explained but could be due to slightly different actions or stability of these two toxins or by the differences in culture conditions. In any case, because the cytochalasin E treatment eliminated the growth cones yet axonal growth continued, the authors of this work concluded that the growth cone does not determine axonal fate, indirectly supporting the view that fate specification is controlled by an intrinsic, centrifugal mechanism, as shown by De Anda et al. (2005). Of particular interest in this regard is that the application of cytochalasin E to round, stage 0 neurons resulted in the outgrowth of only one neurite with axonal characteristics (Ruthel and Hollenbeck 2000).

Moreover, it is not clear how the appearance of a larger growth cone in the future axon (Bradke and Dotti 1997) correlates with the destabilization of the actin cytoskeleton, as this is associated rather with growth cone shrinking or collapse (Gallo and Letourneau 2004). On the other hand, one has to take into account that growth cone collapse involves more than actin depolymerization alone and so one can assume that, in a physiological context, actin-regulating mechanisms would destabilize actin in a more subtle way than does the addition of cytochalasins, therefore resulting in neurite extension without growth cone collapse. Thus the accompanying increase in membrane addition observed by Bradke and Dotti (1997) in the growth cone of the neurite that later becomes the axon can be a consequence of such subtle actin remodelling. In agreement with that possibility it was shown that the destabilization of actin can increase membrane exocytosis at presynaptic terminals (Morales et al. 2000) and exocytosis also from non-neuronal cells (Muallem et al. 1995). Since local cytochalasin D application, as described above, can specify the axon, the destabilization of the actin cytoskeleton seems to be sufficient for axonal fate regulation at this stage. In such scenario, increased membrane transport would be a consequence of actin destabilization and contribute to the further growth of the selected neurite.

In summary, these data demonstrate that actin is a key regulator of neuronal polarity and thus a number of molecules controlling actin dynamics will have an influence on polarity establishment.

9.3.3 Instructive or Permissive Role of Changes in Actin Dynamics?

The above data showing that local destabilization of actin in a selected growth cone of stage 2 neurons can select specifically that neurite as the axon suggests an instructive role of actin in controlling polarity. Yet, the fact that polarity is predefined at earlier stages, by the polarized distribution of the centrosome, endosomes and the Golgi (de Anda et al. 2005), would indicate that actin regulation at stage 2 played

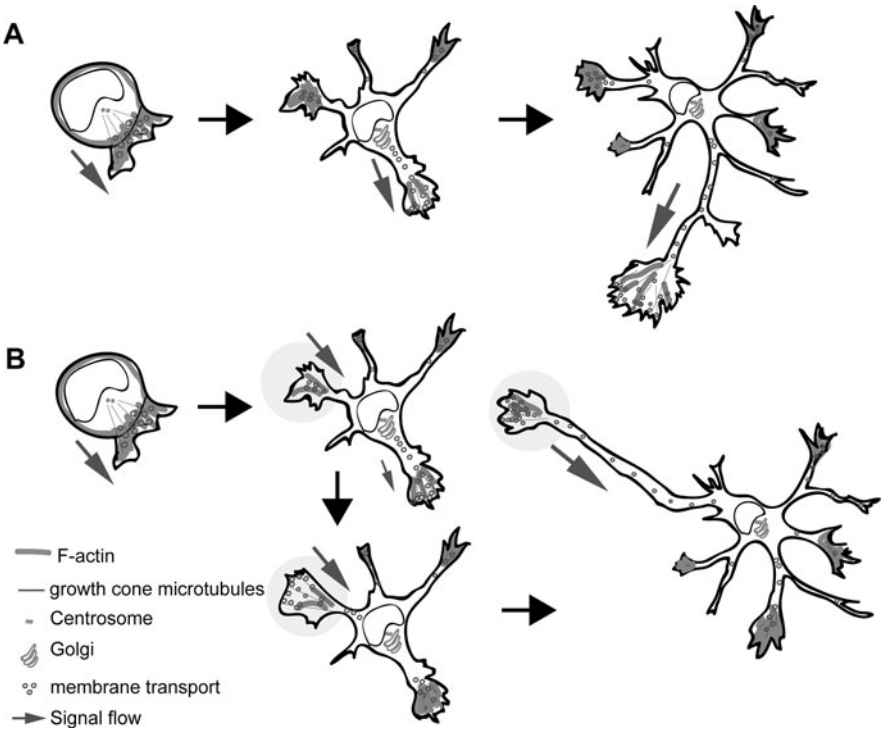

Fig. 9.3 Actin destabilization in one growth cone precedes its axonal outgrowth. (**a**) This selective actin regulation can be triggered by intrinsic cues acting on the formerly selected axon. (**b**) Locally applied strong extracellular cues (*light grey circle*) inducing signals which result in the destabilization of actin can override the intrinsic programme (see Section 9.3.3)

a permissive role. In this scenario, how would alterations in actin stability confer growth permission to the first neurite so that only this grows efficiently at later stages? One possibility, depicted in Fig. 9.3, is that the first neurite contains, because of temporal hierarchy, higher amounts of the molecules that support neurite growth via the modification of actin dynamics. This excess can determine restricted growth once this area of the cell gets in contact with appropriate extracellular cues, even if these are equally presented to all neurites.

9.4 Molecular Regulation of the Actin Cytoskeleton During the Establishment of Neuronal Polarity

In view of the above discussed mechanisms, a fundamental question to be resolved is from where the signals involved in actin destabilization originate. In general terms, actin destabilization at a single place of the cell can occur by an extrinsically regulated, outside-in process or by an intrinsically determined inside-out event (Fig. 9.4).

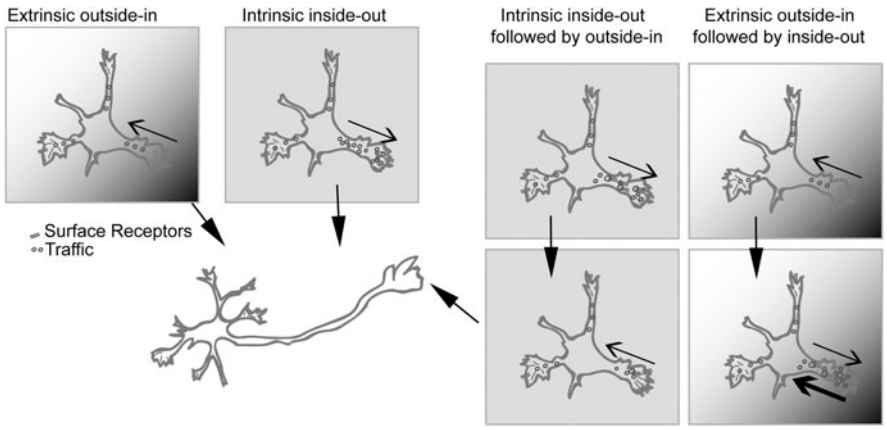

Fig. 9.4 Different possible signalling pathways regulating neuronal polarity (see Section 9.4)

A regulation of polarity exclusively by extrinsically triggered outside-in signalling pathways would refer to the asymmetric distribution of extracellular cues which signal to a uniformly organized cell. In contrast, intrinsic regulation of polarity would require the asymmetric arrangement of molecules in the neuron, with polarized growth occurring in a homogenous environment. Inside-out signalling pathways confer asymmetry without the need of external signals by, for instance, regulating directionality of transport events or by directional control of the cytoskeleton. However, the intrinsically determined polarized insertion of membrane receptors or downstream signalling components makes the cells asymmetrically responsive to uniformly distributed extracellular cues.

Although in the past, a number of molecules and pathways were described which can influence the development of a polarized neuron (Arimura and Kaibuchi 2007, Wiggin et al. 2005), most of these can be only attributed to a role in axonal elongation and are therefore not necessarily involved in polarity specification. Therefore, in the following section we will describe only those which could work through the regulation of the actin cytoskeleton before axonal growth when the cell is still at stage 2 of differentiation.

9.4.1 Extrinsic Cues Regulating the Actin Cytoskeleton

The peripheral actin cytoskeleton is the primary target for the regulation of growth cone advance by extrinsic cues, which allows the advance of microtubules in this area. However, in order to control a polarized outgrowth from only one growth cone, polarity-inducing molecules must be present or signal asymmetrically at the different growth cones.

In an elegant experiment demonstrating that extracellular matrix molecules regulate neuronal polarity, neurons were placed on alternating stripes of poly-lysine and

the axon growth-promoting substrate laminin (Esch et al. 1999). Neurons developing at the interface of these two substrates extend their axon always on laminin showing a very strong impact of integrin signalling on axonal specification. That the axon was really determined by the substratum became clear by the observation that the axon always grew from the neurite closest to the laminin substrate and not from a random neurite. Laminins could in theory also be relevant for neuronal polarity establishment in vivo since they and their receptors, the integrins, are expressed in the early stages of nervous system development (Venstrom and Reichardt 1993). There are a number of molecules interacting with integrin receptors which regulate the actin cytoskeleton (Wiesner et al. 2006, 2005). Recently it was also described that laminin triggers the bundling of microtubules in an actin-independent manner and this in turn mediates the accumulation of F-actin in front of the growth cone (Grabham et al. 2003). However, which particular pathway is involved downstream of laminin–integrin signalling leading to polarized growth has not yet been clarified but it must act in a different manner than just inducing actin destabilization as cytochalasins do (see above), since neurons in which all growth cones are exposed to high concentrations of laminin do not form multiple axons but only one (Lochter and Schachner 1993).

Apart from extracellular matrix molecules, soluble molecules play a role in patterning final polarity. A prominent one, shown to play a role in vivo, is semaphorin 3A. This was demonstrated by observing the development of dissociated, labelled neurons, which are seeded on top of cortical slices, thus allowing neurons to polarize in their natural environment. Under such conditions, the final place of axonal and dendritic outgrowth is selected with respect to the semaphorin 3A gradient in the cortical plate (Polleux et al. 2000). Interestingly, semaphorin 3A repels axonal growth and attracts later dendritic growth, which was explained by the asymmetric distribution of the downstream molecule soluble guanylate cyclase, demonstrating the mutual dependence of both extra- and intracellular polarity cues. Since semaphorin 3A is expressed in the developing cortex, initially in the ventricular zone (Bagnard et al. 1998) and at late embryonic development in the cortical plate (Skaliora et al. 1998), it may be a very relevant molecule for patterning axonal dendritic projections during development. Semaphorin 3A signals via the tubulin-binding protein Crmp2 (Uchida et al. 2005), a molecule involved in axon specification (Inagaki et al. 2001). Crmp2 has primarily a very pronounced impact on the actin cytoskeleton, by depolymerizing the axonal cytoskeleton and leading to growth cone collapse. The effects are mediated by the binding of semaphorin 3A to its receptors neuropilin and plexin, which signal via Rac1 and Crmp proteins (Nakamura et al. 2000). It was also shown that semaphorin 3A-induced growth cone collapse is mediated via Lim kinase and cofilin (Aizawa et al. 2001; see Chapter 11 of this book).

A number of further extracellular molecules exist, which will differently affect neurons during their developmental progression in vivo migrating through the diverse zones of the cortex, and it awaits future research in order to understand their action onto polarity and the cytoskeleton.

9.4.2 Intracellular Pathways Which Change Actin Dynamics in Neurons

Despite the strength of the above data, polarized growth occurs in the homogenous environment of a tissue culture dish, as it is the case in hippocampal neurons developing at low density in vitro (Dotti et al. 1988), clearly demonstrating that polarization does not require a graded extracellular environment. One way by which this could occur is through the intrinsic asymmetric organization of the cell, by delivering, for instance, actin-regulating molecules to a specific site of the cell. This would establish an intracellular gradient that then can give a polarized architectural response in a homogeneous environment. This would, in turn, result in the selective addition of membrane, the selective protrusion of microtubules and, as consequence of these, in further enhancement of the signalling to the actin cytoskeleton coming from the homogeneous environment. A large number of actin regulatory molecules could regulate polarization following asymmetric delivery or retention. This can be membrane receptors that signal to actin upon binding its ligands or soluble molecules which are either direct modulators of actin (actin binding proteins) or that act indirectly such as, for instance, small GTPases of the Rho family.

9.4.2.1 Small Rho GTPases and Polarity Control in Neurons

The most prominent group of molecules regulating actin dynamics are the Rho GTPases (Hall 1998, Jaffe and Hall 2005), with its main representatives Cdc42, Rac1 and RhoA. Rho GTPases act like molecular switches being in an active GTP-bound state or an inactive GDP-bound state. This process is tightly controlled by a large number of guanine nucleotide exchange factors (GEFs) that catalyse exchange of GDP for GTP (Schmidt and Hall 2002), GTPase-activating proteins (GAPs) that stimulate the intrinsic GTPase activity to inactivate (Bernards and Settleman 2004) and guanine nucleotide dissociation inhibitors (GDIs), which can block spontaneous activation (Olofsson 1999). The large number of neuron-specific isoforms of these regulating molecules suggests an important role for Rho GTPases in nervous system development and function.

Cdc42 has a quite central function in polarity control in other systems such as in yeast bud formation and in mammalian cells. It regulates, via interaction with the polarity complex Par6/Par3/aPKC, the formation of tight junctions in cultured epithelial cells and the polarization during migration in fibroblasts and astrocytes (Nelson 2003). Rac1 activity is controlling the apical basolateral polarity of epithelial cells and all three GTPases are downstream of dishevelled, a molecule involved in the regulation of planar tissue polarity on the level of the actin cytoskeleton and gene transcription (Jaffe and Hall 2005).

There are a number of indications that all three GTPases are as well involved in the regulation of neuronal polarity in mammalian neurons. The overexpression of dominant-negative or constitutive active constructs of Rac1 or Cdc42 leads to the

inhibition of axon formation (Nishimura et al. 2005). The downregulation of Cdc42 by small interfering RNAs prevents axon formation, while the overexpression of a fast cycling (between the active and inactive states) variant of Cdc42 leads to the outgrowth of super-numerous axon-like neurites (Schwamborn and Puschel 2004). In this study the authors proposed that local activation of Cdc42 in the future axon is triggered by the local activation of another small GTPase Rap1B. However, in those studies only the peak accumulation of the total protein was shown to occur in selected growth cones, not that of the active, GTP-bound Cdc42. Since GTPase activity does not depend on their abundance but on the presence of regulating factors and upstream signals, the localization of specifically active forms of Rho GTPases during the development of neurons will be a future challenge. However, there are some hypotheses how Cdc42 or Rac1 activity could be localized to one neurite only. Rac can be locally activated via the local action of the Rac GEF Stef1, which is activated by the polarity complex Par3/Par6 (Nishimura et al. 2005), while Cdc42 can be locally activated by Rap1B (Schwamborn and Puschel 2004). The localized inactivation of Rap1B was thought to occur via the site-specific ubiquitination and degradation (Schwamborn et al. 2007). Apart from Stef, other GEFs in particular for Rac were also described to be involved in polarity regulation. Tiam-1, for instance, which is expressed at high levels in the developing brain, is involved in the regulation of actin organization in the axonal growth cone (Kunda et al. 2001). Overexpression of Tiam-1 promotes the formation of supernumerous neurites, while the downregulation prevents axon formation. Another recently described Rac-GEF, DOCK7, regulates axon formation in a similar manner but is thought to exert its effect via the phosphorylation of the microtubule-destabilizing protein stathmin in the axon (Watabe-Uchida et al. 2006). Thus the involvement of Rac1 in polarity control is via the parallel regulation of the actin and microtubule cytoskeleton.

RhoA, in agreement with its generally inhibitory role in neurite outgrowth (Luo 2000), has an antagonistic effect with respect to Rac and Cdc42 in regulating neuronal polarity: RhoA activation leads to the block in axon formation, while the inhibition of the downstream effector Rho kinase triggers multiple axonal outgrowth in cerebellar granule neurons (Bito et al. 2000). However, what restricts the activity of all those molecules under natural conditions to selected neurites only is still a matter of future research.

9.4.2.2 How Can Rho GTPases Change Actin Dynamics?

In spite of the numerous studies on the involvement of Rho GTPases in neuronal polarity development, only few studies show the detailed action downstream of Rho GTPases on the actin cytoskeleton. Since Rho GTPases can modulate actin but also tubulin in many diverse ways (Jaffe and Hall 2005), studies on that could be interesting in the future, especially in the light of the complex interactions and interdependence of the actin and microtubule cytoskeleton (Waterman-Storer and Salmon 1999).

In general, Rho GTPases coordinate actin polymerization and actin filament bundling, processes which are controlled by a number of actin filament-capping and

-severing proteins and actin polymerization factors. Rac1 and Cdc42 initiate peripheral actin polymerization and actin branching by the WAVE- or WASP-induced activation of the Arp2/3 complex, while RhoA activates formins of the mDia family, leading to the linear elongation of actin filaments (Jaffe and Hall 2005). ADF/cofilin which severs and disassembles actin filaments can be regulated by phosphorylation of all three Rho GTPases via Lim kinase, which in turn is activated either by Cdc42/Rac1-dependent PAK kinases or by Rho kinase (Jaffe and Hall 2005). Until now there are no studies demonstrating that any of these components is directly involved in controlling neuronal polarity. However, recently it was shown that axonal outgrowth in *Drosophila* neurons is controlled by cofilin, which is regulated by a number of pathways involving Rho GTPases (Ng and Luo 2004). Cofilin in this pathway is thought to be regulated by phosphorylation by the Lim kinase and dephosphorylation by slingshot phosphatase. Lim kinase in turn, as described above, is regulated by the coordinated action of all three Rho GTPases. Moreover, and in support of the concept of actin regulation of inside-out signalling pathways, it was shown in hippocampal neurons that Lim kinase in addition to its role in the growth cone regulates Golgi dynamics and that its presence in the Golgi is critical for axonal outgrowth (Rosso et al. 2004). It remains to be established how relevant this pathway could be in the earlier process of polarity determination before axonal outgrowth starts.

A further way to control the spatial arrangement of the actin cytoskeleton is via RhoA and Rho kinase and via the phosphorylation of the myosin light-chain phosphatase. This leads to the phosphorylation of the myosin light chain, which then confers increased actin cross-linking activity to myosin II (Jaffe and Hall 2005). The components of this pathway are therefore interesting candidates for further investigations.

9.5 Outlook

The establishment of polarity is achieved by the polarized regulation of cytoskeletal dynamics and structure and of membrane traffic. All three mechanisms are closely linked and the main goal will be to understand how these actions are polarized in order to trigger axonal outgrowth in a temporally and spatially correct manner. Even though it is known that actin plays a role in coordinating this process, we still lack a detailed description of the mechanisms and signals by which actin controls polarity and how the fine structure and dynamics of actin is changed during that process. Little is known about the very first step of polarity establishment when the first neurite sprouts from a spherical cell. The second step the fast elongation of the axon is preceded by the destabilization of actin in the selected growth cone, which seems to be sufficient for axon fate selection. Real-time in vivo studies of labelled cytoskeletal components and fluorescently tagged actin-regulating factors will help in the near future to better understand the temporal and spatial changes in actin and tubulin dynamics.

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Chapter 10
Actin at the Synapse: Contribution to Pre- and Postsynaptic Functions

Dezhi Liao

Abstract Synapses are specialized intercellular junctions through which one neuron sends signals to another neuron or a non-neuronal excitable cell. In the central nervous system (CNS), electrical signals can be propagated across neurons via either electrical or chemical synapses. An electrical synapse is a direct electronic coupling between two adjacent cells via a specialized apparatus called gap junction (Bennett and Zukin 2004. The term "synapses" often simply refers to chemical synapses because they are the most common synapses in animals and the term "synapse" was historically introduced to describe chemical synapses (Cowan and Kandel 2001, Boeckers 2006). A typical chemical synapse contains the presynaptic terminus, synaptic cleft, and postsynaptic membrane. Electrical signals are transmitted from a presynaptic neuron to a postsynaptic neuron when neurotransmitters released from the presynaptic terminus cause postsynaptic depolarization in excitatory synapses or hyperpolarization in inhibitory synapses. Actin and its binding partners can modulate presynaptic functions by altering the release of neurotransmitters (Doussau and Augustine 2000, Schweizer and Ryan 2006) and can also modulate the strength of postsynaptic responses by altering the morphology and function of dendritic spines (Carlisle and Kennedy 2005, Kopec and Malinow 2006, Tada and Sheng 2006).

Keywords Actin · Cytoskeleton · Postsynaptic density · PSD · Synapse · Dendritic spine

10.1 Presynaptic Roles of Actin

Quick-freeze, deep-etch microscopic studies reveal that presynaptic termini of neuromuscular junctions and synapses in CNS are enriched in both microtubules and actin filaments (Hirokawa et al. 1989, Gotow et al. 1991, Landis et al. 1998).

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Microtubules are mainly present in the center of a presynaptic terminus, whereas actin filaments are peripherally present and close to the active zones (Walker et al. 1985, Gotow et al. 1991), although some exceptions have been reported (Gray 1983). The presence of presynaptic actin filaments is further confirmed by fluorescent phalloidin staining of isolated synaptosomes (Drenckhahn and Kaiser 1983, Bernstein and Bamburg 1989), presynaptic termini of retinal bipolar cells (Job and Lagnado 1998), and cultured chick sympathetic neurons (Bernstein et al. 1998). In a recent live imaging study, GFP-tagged actin was about twofold more concentrated in presynaptic termini relative to adjacent axon cylinder (Schweizer et al. 1995, Schweizer and Ryan 2006). The cycling of presynaptic vesicle is classified into several classical steps: the storage (clustering), docking, priming, fusion, and endocytosis (Fig. 10.1; Hilfiker et al. 1999, Sudhof 2004, Becherer and Rettig 2006). Although multiple roles of actin have been proposed in almost every step, the best characterized role of actin is that it serves as a molecular scaffold in the storage step of vesicle cycling (Fig. 10.1; Schweizer and Ryan 2006). In addition, actin is also important for clathrin-dependent "pit-coating" endocytosis (Smythe and Ayscough 2006). However, the role of actin in vesicle cycling in presynaptic termini is still unclear as vesicles can be recycled through the "kiss-and-run" pathway (Fig. 10.1; Sudhof 2004).

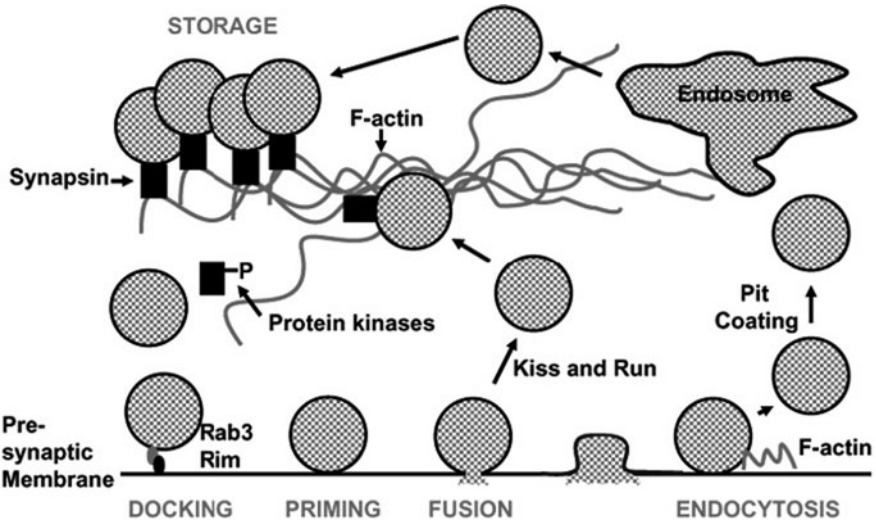

Fig. 10.1 Actin regulates the release of presynaptic neurotransmitters. The best characterized presynaptic role of actin is the storage of synaptic vesicles (*top left*). In addition, actin might also participate in clathrin-dependent, "pit-coating" endocytosis (*bottom right*)

10.1.1 Storage

The strongest evidence that actin cytoskeleton can sequester synaptic vesicles as reserve pools comes from extensive studies of synapsin I, II, and III. Synapsin is

postulated to be the protein that anchors synaptic vesicles to the actin cytoskeleton because synapsin binds to both synaptic vesicles and actin filaments (Hilfiker et al. 1999). All three isoforms of synapsins have three highly conserved domains (A–C) in the N-terminal half and two less conserved domain (D–E) in the C-terminal half (Hilfiker et al. 1999, 2005) Domain A can be phosphorylated by both cAMP-dependent protein kinase (PKA) and calcium calmodulin-dependent protein kinase I (CAM kinase I) and domain D can be phosphorylated by CAM kinase II. Dephosphorylated synapsin I strongly binds to synaptic vesicles and nucleates actin polymerization, whereas phosphorylated synapsins have lower binding affinities (Hilfiker et al. 1999, 2005). The widely accepted model is that actin filaments sequester synaptic vesicles as reserve pools with synapsins as anchors, which can be unanchored after phosphorylation triggered by an activity-induced increase in intracellular calcium (marked as "–P" in Fig. 10.1; Schweizer and Ryan 2006).

This model is directly supported by electron microscopic studies showing that immuno-labeled synapsin I indeed links synaptic vesicles to actin filaments in the presynaptic termini (Hirokawa et al. 1989, Landis et al. 1988). Functional disruption of synapsin by microinjection of anti-synapsin antibodies to presynaptic termini disperses the clustering of synaptic vesicles in the cytoplasm but not in areas immediately adjacent to the active zone (Pieribone et al. 1995). Antibody injection has little effect on synaptic transmission at low stimulus frequencies but profoundly suppresses presynaptic release of vesicles at high stimulus frequencies (Hilfiker et al. 1998). High-frequency, stimulation-induced synaptic depression is also enhanced in transgenic mice lacking genes for synapsin I and/or II (Li et al. 1995, Rosahl et al. 1995). Furthermore, an increase in presynaptic intracellular calcium by strong electrical stimulation enhances the phosphorylation of synapsin by CAM kinase II and results in conformational changes in synapsins (Benfenati et al. 1990). Injection of dephosphorylated synapsin I to presynaptic terminus decreased the amplitude and rise time of the postsynaptic potentials, whereas injection of calcium/calmodulin-dependent protein kinase II had opposite effects, increasing the rise time and amplitude of the potentials (Llinas et al. 1985). Domain C and E peptides, which inhibit the interaction between synapsin and F-actin by competitive binding, decrease the size of a reserve pool of vesicles and cause parallel synaptic depression (Hilfiker et al. 2005). With the accumulation of tremendous amount of data, there is little doubt that actin cytoskeleton plays an essential role in maintaining the reserved pools of presynaptic vesicles (Doussau and Augustine 2000, Schweizer and Ryan 2006).

10.1.2 Vesicle Docking and Mobilization

Presynaptic termini contain many vesicles, some of which are docked to the presynaptic plasma membrane in "active zones," the releasing sites of neurotransmitters (Pfenninger et al. 1972, Harlow et al. 2001). A synaptic vesicle docks to the "active zones" by binding Rab3 in the vesicle to Rim in the presynaptic membrane (Fig. 10.1; Lonart 2002). Actin is probably not required for vesicle docking itself

because the disruption of actin filaments by cytochalasin does not affect the release of docked vesicles, even though cytochalasin treatment decreases the reserve pool of vesicle (Wang et al. 1996).

Actin filaments are, however, postulated to indirectly regulate vesicle docking by altering the mobilization of synaptic vesicles from storage sites to docking sites (Schweizer and Ryan 2006). The roles of actin in the mobilization of vesicles are still controversial due to many conflicting experimental results, some of which support a facilitatory role (Kuromi and Kidokoro 1998, Cole et al. 2000) and some indicate a negative regulatory role (Wang et al. 1996, Bernstein et al. 1998, Morales et al. 2000). Only recently, has it been possible to measure the details of synaptic vesicle dynamics within a single bouton using fluorescence fluctuation spectroscopy (Jordan et al. 2005, Shtrahman et al. 2005, Yeung et al. 2007). These spectroscopic studies support a "stick and diffuse" mechanism for synaptic vesicles to travel across the presynaptic terminus. Vesicles transiently bind to an immobile actin filament, unbind and diffuse, and then bind again to repeat the whole circle (Shtrahman et al. 2005, Yeung et al. 2007).

Inhibitors of myosin light-chain kinase (MLCK) reduced vesicle mobility, although disruption of actin filaments and tubulins by cytochalasin D, latrunculin B, and colchicine had little effect on this mobility, indicating that actin filaments might only serve as a molecular scaffold (Sankaranarayanan et al. 2003, Jordan et al. 2005). The cellular mechanism underlying the activity-induced mobilization of vesicles close to plasma membrane is still largely unknown, although abundant evidence supports that myosin and MLCK are involved in this process (Prekeris and Terrian 1997, Ryan 1999, Hasson and Mooseker 1997, Jordan et al. 2005). Myosin-V transports vesicles on actin filaments in many types of cells including neurons (Langford 2002), and thus actin has been proposed to serve as a "track" for the movements from reserved vesicle pool to the active zones in the presynaptic terminus (Doussau and Augustine 2000). However, a recent live imaging study of GFP-tagged actin does not support this "track" role of actin at presynaptic termini (Sankaranarayanan et al. 2003).

10.1.3 Priming and Fusion

Injection of anti-synapsin antibody or domain E peptides significantly slows the kinetics of neurotransmitter release, indicating that synapsins might play roles downstream of vesicle docking by affecting the priming and fusion of vesicles (Hilfiker et al. 1998, Humeau et al. 2001). As domain E is important for the interaction between synapsin and F-actin (Hilfiker et al. 2005), we would expect that actin might play important roles in vesicle priming and fusion. The priming process is defined as the stage when vSNARE and tSNARE form a "zipper" to pull the vesicle and plasma membrane in close apposition to allow vesicle fusion (Hasson and Mooseker 1997). It is still unknown what roles actin might play in vesicle priming.

Two types of fusions have been proposed. In the classical model, vesicles fully collapse to the plasma membrane; in the "kiss-and-run" model, only a fusion pore opens to allow neurotransmitter discharge (Fernandez-Peruchena et al. 2005, Harata et al. 2006). Synaptic vesicles at small synapses in the CNS might be retrieved through the "kiss-and-run" recycling pathway, in which the fusion pore does not dilate and instead rapidly reseals with the secretory vesicle almost fully intact; actin probably plays little role in this process (Fig. 10.1; Murthy and Stevens 1998). A recent study using the calyx of Held in the medial nucleus of the trapezoid body from rats reveals that both full collapse fusion and "kiss-and-run" exocytosis occur in CNS synapses (He et al. 2006). A third form of exocytosis, dubbed "kiss-and-coat", has been reported in *Xenopus* eggs, although it is unknown whether this form of exocytosis occurs in the CNS (Sokac and Bement 2006). In the third form of exocytosis, the prolonged dilation of fusion pore is maintained through assembly of actin filament (F-actin) coats around the exocytosing secretory vesicles (Sokac and Bement 2006). As it is still controversial which form of exocytosis plays the major role in synapses in the brain (Klyachko and Jackson 2002, He et al. 2006), the role of F-actin in the vesicle cycling is difficult to know.

10.1.4 Endocytosis

It has been well known for more than three decades that synaptic vesicles undergo endocytosis to be reused after exocytosis (Ceccarelli et al. 1973, Heuser and Reese 1973). Two hypothetical models of synaptic vesicle cycling have been proposed: the classical clathrin-mediated, pit-coating endocytosis pathway and the "kiss-and-run" recycling pathway (Schweizer et al. 1995, Sudhof 2004). The essential role of actin in the pit-coating endocytosis is clearly illustrated in budding yeast (Smythe and Ayscough 2006). In mammalian cells, actin instead plays some regulatory roles in the clathrin-mediated endocytosis. Dynamin, a crucial player in endocytosis, can interact with many actin-binding proteins including profilin, Abp1, cortactin, and syndapin (Orth and McNiven 2003). Dynamin can participate in many specific actin-mediated processes of endocytosis including tabulation and vesiculation via its interaction with actin. Recent evanescent field (EF) microscopic studies directly demonstrated that internalization of a clathrin-coated pit is accompanied by recruitment of both actin and dynamin (Merrifield et al. 2002). The expression of dominant-negative dynamin is shown to block the internalization of opioid receptors in many cell types including HEK293 cells and neurons (Chu et al. 1997, Puthenveedu and von Zastrow 2006, Liao et al. 2007). Based on data in other systems, the actin cytoskeleton is believed to play many important roles in endocytosis by providing scaffolds or movement tracks in synapses (Doussau and Augustine 2000). Probably due to technical difficulties, the role of actin filaments in endocytosis has not yet been specifically demonstrated at the tiny apparatus of the presynaptic terminus.

10.2 Postsynaptic Roles of Actin in Inhibitory Synapses

The principal neurotransmitter that mediates fast inhibitory synaptic transmission in the brain is γ-aminobutyric acid (GABA), whereas the principal inhibitory neurotransmitter in the spinal cord is glycine (Rang et al. 1995). The fast component of GABA responses is mediated through the ionotropic $GABA_A$ channel receptors that gate inward Cl^- currents (Michels and Moss 2007). The $GABA_A$ and glycine receptors are often clustered in the postsynaptic membrane of "non-spiny" synapses with the presynaptic terminus directly apposed to the shaft of dendrites (Kirsch et al. 1993, Harris and Kater 1994, Craig et al. 1996). The importance of actin cytoskeleton in the anchoring and clustering of both GABA and glycine receptors largely comes from the extensive studies of gephyrin (Kneussel and Loebrich 2007). Gephyrin, which interacts with tubulin, was originally found to be essential in anchoring glycine receptors to the subsynaptic cytoskeleton (Fig. 10.2a; Prior et al. 1992, Kirsch et al. 1993). Later on, gephyrin was also found to be important for the clustering and transport of GABA receptors and is believed to be the backbone of the inhibitory postsynaptic scaffold (Kneussel and Betz 2000, Michels and Moss 2007).

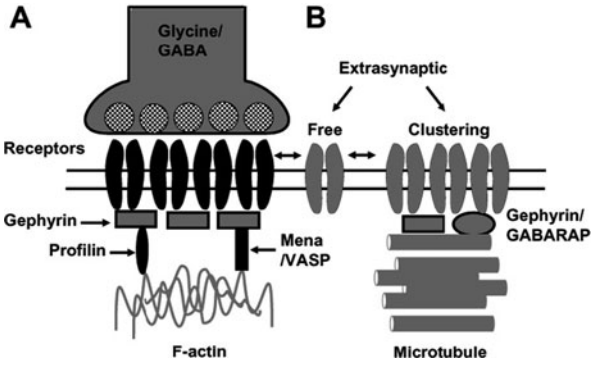

Fig. 10.2 Actin anchors glycine and GABA receptors to the postsynaptic membrane of inhibitory synapses. (**a**) Gephyrin serves as a scaffold that links inhibitory neurotransmitter receptors to actin cytoskeleton at synaptic sites. (**b**) Microtubule might be important for the clustering of glycine or GABA receptors at "extrasynaptic" sites

One commonly proposed model is as follows: gephyrin provides a postsynaptic scaffold by binding to the β subunits of glycine or the γ_2 subunits of $GABA_A$ receptors, causing the clustering of these receptors at a synapse; at the same time, gephyrin binds to profilin and Mena/VASP, both of which can bind to actin cytoskeleton (Fig. 10.2a). Although there is little doubt that gephyrin can directly bind to glycine receptors (Prior et al. 1992, Kirsch et al. 1993), it is still controversial whether gephyrin can directly bind to GABA receptors (Kneussel and Loebrich 2007, Michels and Moss 2007). Functional studies demonstrated that both the γ_2 subunits of $GABA_A$ receptors and gephyrin are required for receptor clustering and targeting (Essrich et al. 1998). However, the direct interaction between these two

proteins has yet been demonstrated in published literatures, and thus some groups still believe that there might be an unidentified link protein between gephyrin and the γ_2 subunits of $GABA_A$ receptors (Kneussel and Loebrich 2007).

Live imaging studies of Venus yellow fluorescent protein (YFP)-tagged gephyrin directly demonstrate that gephyrin molecules can move laterally away from the gephyrin cluster at a synapse to extrasynaptic membrane (Fig. 10.2b; Hanus et al. 2006). Treatment of latrunculin diminished the clustering of gephyrin, providing direct evidence that the stability of inhibitory postsynaptic scaffold is F-actin dependent. A recent live imaging study further reveals that disruption of F-actin by latrunculin increases the diffusion of glycine receptors at synapses, whereas disruption of microtubule by nocodazole has little effect on the synaptic clustering of glycine receptors, indicating that F-actin is the more important component of cytoskeleton in stabilizing inhibitory neurotransmitter receptors on postsynaptic membrane (Charrier et al. 2006). In contrast, nocodazole increases the diffusion of F-actin from "extrasynaptic" clustering sites (Charrier et al. 2006), supporting the previously proposed hypothetical model that microtubule and GABA receptor-associated protein (GABARAP) might play some roles in anchoring $GABA_A$ receptors to extrasynaptic clustering sites (Fig. 10.2b; Coyle and Nikolov 2003). Although the synaptic clustering of neurotransmitter receptors is known to be correlated with the strength of synaptic responses, the physiological significance of "extrasynaptic" clustering of glycine and GABA receptors remains to be determined (Nelson and Turrigiano 1998, O'Brien et al. 1998, Wiens et al. 2005).

10.3 Postsynaptic Roles of Actin in Excitatory Synapses

The principal and ubiquitous neurotransmitter that mediates fast excitatory synaptic transmission both in the brain and the spinal cord is glutamate, which depolarizes the postsynaptic membrane (Rang et al. 1995). As previously discussed, most inhibitory synapses are non-spiny synapses with the presynaptic terminus directly apposed to the shaft of dendrites (Harris and Kater 1994, Craig et al. 1996). In contrast, over 90% of excitatory synaptic responses transmit through the release of glutamate and these transmissions occur in dendritic spines, tiny postsynaptic membranous protrusions that extend from the dendritic shaft (Rang et al. 1995, Kennedy 2000, Nimchinsky et al. 2002). Although dendritic spines can have various sizes and shapes, almost all presynaptic termini that make synapses on dendritic spines release glutamate and a typical spine contains α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)-type glutamate receptors and/or *N*-methyl-D-aspartate (NMDA)-type glutamate receptors (Liao et al. 1999, Kennedy 2000). Furthermore, virtually all of them contain a postsynaptic density (PSD), a 50-nm-thick structure apposed to the cytoplasmic side of postsynaptic membrane (Harris and Kater 1994, Kennedy 2000). In the 1970s, two groups developed methods for the isolation of subcellular fractions of PSD (Cotman et al. 1974, Cohen et al. 1977). The subsequent biochemical and cell biological analyses reveal the

presence of many PDZ domain-containing scaffolding proteins including PSD95, GRIP, SAP97, Shank, and synGAP in the PSD (Kennedy 2000, Sheng and Sala 2001). A variety of interactions between PSD scaffolding proteins and the actin cytoskeleton have been supported by numerous experiments, indicating that actin cytoskeleton is important in the maintenance and remodeling of the PSD composition (Wyszynski et al. 1997, Bockers et al. 2001, Shirao and Sekino 2001, Hering and Sheng 2003, Kuriu et al. 2006).

It is well known that actin is clustered and highly concentrated in dendritic spines (Fischer et al. 2000, Matus 2000, Okamoto and Hayashi 2006). Three decades ago, electron microscopic studies have revealed that the cytoskeleton of dendritic spines is mainly composed of a loose network of actin filaments (Gray 1983, Landis and Reese 1983, Cohen et al. 1985, Fifkova 1985). The filamentous network of dendritic spines contains almost no microtubules, whereas the cytoskeleton in the middle of dendrites is enriched with microtubules (Westrum et al. 1980, Chicurel and Harris 1992). A recent live imaging study further confirms that GFP-tagged actin is concentrated in dendritic spines and microtubule is likely to be concentrated in the middle of dendrites (Kaech et al. 2001). The high enrichment of actin in dendritic spines and the extensive interaction between actin and PSD scaffolding proteins enable actin to play diverse roles in dendritic spines, including spine motility, receptor anchoring, spine morphogenesis, and synaptic plasticity. These diverse roles of actin will be discussed in more detail in later sections of this chapter.

10.4 Roles of Actin in the Motility of Dendritic Spines

Two-photon live imaging studies reveal that dendritic spines in the cerebellum, the cerebral cortex, and the hippocampus are highly dynamic and exhibit rapid morphological changes over several seconds in brain slices (Dunaevsky et al. 1999). This rapid morphological change in dendritic spines is believed to arise from the remodeling of the actin cytoskeleton and actin-based protrusive activity from the spine head (Fischer et al. 2000, Matus 2000, Zito et al. 2004, Pilpel and Segal 2005). Interestingly, the rapid form of spine motility is more pronounced during early development and progressively decreases as neurons become more mature, suggesting that spine motility might play some important roles during the critical period of neuronal development (Dunaevsky et al. 1999; Korkotian and Segal 2001). Other slower forms of morphological changes in dendritic spines have also been reported: changes in the length of spines over minutes (Dailey and Smith 1996, Dunaevsky et al. 1999, Lendvai et al. 2000, Portera-Cailliau et al. 2003) and emergence and retraction of spines over hours, days, or months (Engert and Bonhoeffer 1999, Maletic-Savatic et al. 1999, Grutzendler et al. 2002, Trachtenberg et al. 2002, Zuo et al. 2005, Holtmaat et al. 2006). As these slow forms of morphological changes were observed in a variety of preparations and occurred under very different experimental conditions, it is difficult to know whether the cellular mechanisms underlying these diverse forms of spine mobility are the same as the rapid spine motility.

It has been hypothesized that synaptic activities may stabilize dendritic spines by inhibiting actin-based rapid spine mobility during synaptic plasticity and neuronal development (Lippman and Dunaevsky 2005). In support of this hypothesis, activation of either AMPA or NMDA receptors strongly inhibited rapid actin-based spine mobility (Fischer et al. 2000). Furthermore, blockade of presynaptic activities by tetrodotoxin (TTX) increased spine motility, which was inversely correlated with developmental age and contact with active presynaptic termini (Korkotian and Segal 2001). Consistent with this hypothesis, chronic inhibition of presynaptic activities with TTX increased the number of filopodia and dendritic spines (Petrak et al. 2005, Richards et al. 2005). However, this hypothesis seems to be in conflict with the consistent observation that activity-dependent synaptic plasticity such as long-term potentiation strengthens synaptic responses by increasing the number of dendritic spines (Maletic-Savatic et al. 1999, Matsuzaki et al. 2004; also see Section 10.7 for more details). A plausible explanation for this conflict is that basal synaptic activities are important for homeostatic regulation of the morphology and function of dendritic spines, and a suppression of synaptic activity would induce compensatory growth of new dendritic spines (Turrigiano and Nelson 2004).

10.5 Roles of Actin in the Anchoring of AMPA and NMDA Receptors

At least three types of glutamate receptors including the AMPA, NMDA, and kainate receptors have been cloned (Hollmann and Heinemann 1994). In the CNS, the majority of excitatory synaptic transmission is mediated via the AMPA- and NMDA-type glutamate receptors (Malinow et al. 2000). NMDA receptors are known to be essential for synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD) of glutamatergic synapses (Bliss and Collingridge 1993, Malenka 1994). Both LTP and LTD can modulate synaptic strength by regulating AMPA receptor trafficking (Shi et al. 1999, Brown et al. 2005). However, AMPA receptors ultimately determine synaptic strength of glutamatergic synapses under normal conditions because NMDA receptors are not activated at a normal resting potential (Mayer et al. 1984, Isaac et al. 1995, Liao et al. 1995, Durand et al. 1996).

One common distinct feature of AMPA and NMDA receptors is that both of them are clustered in dendritic spines (Fig. 10.3a; Liao et al. 1999). In order to maintain the clustering of these receptors on the postsynaptic membrane of excitatory synapses, a wide variety of scaffolding proteins are needed to anchor these glutamate receptors to the cytoskeleton (Allison et al. 1998, Hering and Sheng 2001, Kim and Sheng 2004, Boeckers 2006). Two major tools that are used to initially identify these PSD scaffolding proteins are the yeast two-hybrid screening and the biochemical purification of PSD fractions (Kennedy 2000, Kim and Sheng 2004). Mainly through these two approaches and other follow-up functional analyses over the past 30 years, it is now believed that the PSD contains several

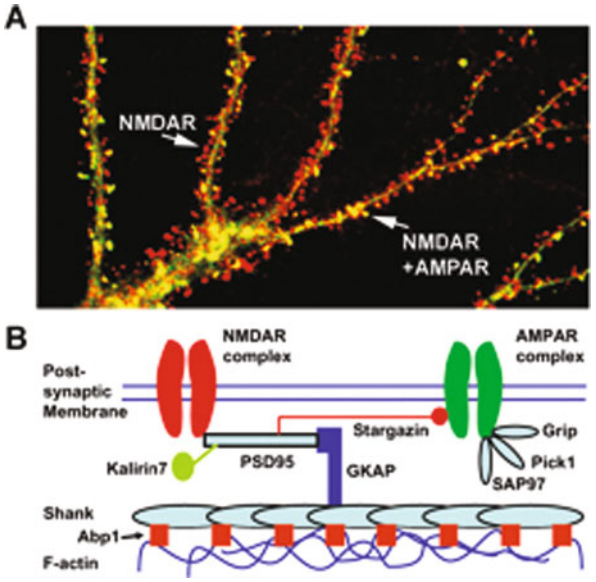

Fig. 10.3 Actin anchors glutamate receptors to dendritic spines. (**a**) An overlay image of immunostaining of AMPA (*green*) and NMDA (*red*) receptors, both of which are clustered in dendritic spines of cultured dissociated hippocampal neurons. (**b**) PSD95 is the "organizer" of the NMDA receptor complex, whereas Shank is the "master organizer" of the deep layer of postsynaptic scaffold. AMPA receptor complex is probably linked to actin cytoskeleton via several PDZ domain-containing proteins including Grip, Pick1, SAP97, and stargazin

hundreds of different proteins and at least 30 proteins have already been fully or partially sequenced (Boeckers 2006). How are these proteins linked to cytoskeleton? Among these numerous proteins, PSD95 and three members of ProSAP/Shank family, Shank 1–3, stand out as the major proteins that construct the mainframe of the PSD scaffold. PSD95 is the organizer of NMDA receptor complex, which is located in the superficial layer of PSD scaffold (Fig. 10.3b, upper left), whereas Shank 1–3 are the "master organizers" of PSD, which are located in deep layers of the PSD and are linked to actin cytoskeleton via Abp1 (Fig. 10.3b, lower; Naisbitt et al. 1999, Sheng and Kim 2000, Kim and Sheng 2004, Qualmann et al. 2004). GKAP is the intermediate link between the PSD95-enriched superficial layer and the Shank-enriched deep scaffolding layer (Kim et al. 1997). In contrast, multiple scaffolding proteins from distinctly different families including Grip, stargazin, Pick1, and SAP97 contribute to the clustering and targeting of AMPA receptors (Fig. 10.3b, upper right).

10.5.1 NMDA Receptor Complex

PSD95 is widely believed to be the main organizer of the NMDA receptor complex because it is an abundant component of PSD and can directly bind to NMDA

receptors (Kim and Sheng 2004). PSD95 proteins, which are encoded by four genes, are probably the best characterized scaffolding proteins in dendritic spines. These proteins contain three PDZ domains, a SRC homology 3 (SH3) domain, and a guanylate kinase-like (GK) domain (Funke et al. 2005). PDZ domains are frequently found in scaffolding proteins and are important for protein–protein interactions. The C terminus of NR2 subunits of heteromeric NMDA receptors binds to the first/second PDZ domain of PSD95 (Cho et al. 1992, Kistner et al. 1993, Kornau et al. 1995, Niethammer et al. 1996) and, at the same time, PSD95 forms multimers by self-association through PDZ domains (Hsueh and Sheng 1999, Christopherson et al. 2003). Proteomic analysis reveals that PSD95, GKAP, and the NR1, NR2A, and NR2B subunits of NMDA receptors are indeed located in the same sub-compartment of PSD, supporting the scaffolding role of PSD95 in NMDA receptor complex (Husi et al. 2000). Postembedding immunoelectron microscopy confirms that PSD95 is indeed the best candidate for the organizer of the first layer of scaffold immediately attached to NMDA receptors (Fig. 10.3b; Valtschanoff and Weinberg 2001).

10.5.2 AMPA Receptor Complex

Proteomic analysis of PSD proteins reveals that AMPA receptors are located in a separated sub-compartment that is distinctly different from NMDA receptor complex (Husi et al. 2000). This might provide a biochemical explanation why the level of AMPA receptors varies greatly among individual dendritic spines (Carroll et al. 1999, Liao et al. 1999, Luscher et al. 2000). There are even synaptic structures without "stable" AMPA receptors localized at the postsynaptic membrane, supporting the existence of "silent synapses" (Isaac et al. 1995, Liao et al. 1995, Groc et al. 2006). Two-photon imaging studies reveal that AMPA receptors are very mobile and can move into dendritic spines within minutes (Shi et al. 1999).

The high mobility of AMPA receptors suggests that these receptors might be anchored to the postsynaptic membrane via mechanisms distinctly different from NMDA receptors (Fig. 10.3b, right; Malinow et al. 2000). The C-terminal tails of the GluR2 and GluR3 subunits of AMPA bind to the fifth PDZ domain of the scaffolding protein GRIP, which was shown to cluster AMPA receptors (Dong et al. 1997). PICK1 (protein interacting with C kinase 1), another PDZ domain-containing protein, can also bind to the C terminus of GluR2 and GluR3 subunits (Xia et al. 1999). The lipid-binding BAR domain of PICK1 is probably important for anchoring AMPA receptors to the lipids in the postsynaptic membrane (Jin et al. 2006). Interestingly, the C-terminal end of the GluR1 subunit binds to another PDZ domain-containing protein SAP97 (Leonard et al. 1998). Although the majority of AMPA receptor-binding proteins including SAP97, GRIP, and PICK1 do not directly bind to NMDA receptor complex, as an exception, stargazin (or TARPs: Tomita et al. 2004, Fukata et al. 2005) has been shown to interact with both AMPA receptors and the PDZ domains of SAP90/PSD-95 (Chen et al. 2000). As AMPA

receptors are very mobile, not surprisingly, all of the above AMPA receptor-binding proteins have been shown to be important for AMPA receptor trafficking and targeting during synaptic plasticity (Malinow et al. 2000, Derkach et al. 2007). Although it is unknown how actin cytoskeleton modulates AMPA receptor trafficking and targeting, the actin cytoskeleton is frequently implicated to interact directly and/or indirectly with many AMPA receptor-interacting proteins including GRIP, AMPAR-binding protein (ABP), protein 4.1 N, and SAP97 (Derkach et al. 2007), suggesting that actin might regulate AMPA receptor trafficking via these interacting proteins.

10.5.3 Master Scaffolding

As discussed above, PSD95 is the organizer of the superficial layer of PSD structure that anchors glutamate receptors. What are the molecules that link this PSD95-enriched layer of scaffold to actin cytoskeleton? Three members of the ProSAP/Shank family, shanker1, Shank 2 and Shank 3, are called the "master organizers" of the scaffold of PSD, serving as the link between the PSD95 and actin filaments (Sheng and Kim 2000, Boeckers 2006). A Shank protein often has an Ank domain, a SH3 domain, a PDZ domain, and a proline-rich region (Lim et al. 1999). GKAP serves as a link protein between the superficial PSD95 scaffold and the deep Shank scaffold by simultaneously binding to the PDZ domains of Shank and PSD95. The proline-rich region or the ankyrin repeats of Shank are affixed to the actin filaments by binding to several classical actin-binding and modulating proteins including α-fodrin, cortactin, and Abp1 (Fig. 10.3b).

10.6 Roles of Actin in Morphological Changes in Dendritic Spines

Changes in the morphology of dendritic protrusions and spines might affect the strength of synaptic responses by altering the number of active synapses, the electronic filtering at a spine, and the amount of postsynaptic AMPA receptors per spine (Malinow et al. 2000, Kopec and Malinow 2006). The actin cytoskeleton is believed to be the basic structural foundation of dendritic protrusions and spines (Shirao and Sekino 2001, Nimchinsky et al. 2002). Dendritic spines contain many actin-binding and actin-regulatory molecules (for example, α-actinin, drebrin, spinophilin/neurabin II, adducing, SPAR, and cortactin), which regulate spine density and spine geometry (Luo 2000). Therefore, not surprisingly, three Rho GTPases (RhoA, Cdc42, and Rac1) are the major regulators of morphogenesis of dendritic spines as they are well-known modulators of the actin cytoskeleton in fibroblasts, budding yeast, and neurons (Adams et al. 1990, Ridley et al. 1992, Nakayama et al. 2000, Carlisle and Kennedy 2005).

10.6.1 Rac1/Pak1 Pathway

The best characterized signaling pathway for dendritic morphogenesis is the EphB/Kalirin-7/Rac1/Pak1/LIMK pathway (Fig. 10.4, the second left pathway marked by black symbols). It is well documented that axons express the B subtype of ephrins, which are cell-surface, membrane-bound ligands to EphB receptors (Henkemeyer et al. 1996, Klein 2004, Carlisle and Kennedy 2005). EphB receptors are postsynaptic tyrosine kinase-linked receptors (Torres et al. 1998). It has now been well established that the ephrin-B/EphB receptor signaling pathway is essential for the formation of normal dendritic spines (Fig. 10.4a from Liao et al., unpublished; also see Henkemeyer et al. 2003, Penzes et al. 2003). Henkemeyer et al. (2003) demonstrated that in EphB1, EphB2, and EphB3 triple knockout mice, dendritic spines are absent in cultured hippocampal neurons and are grossly

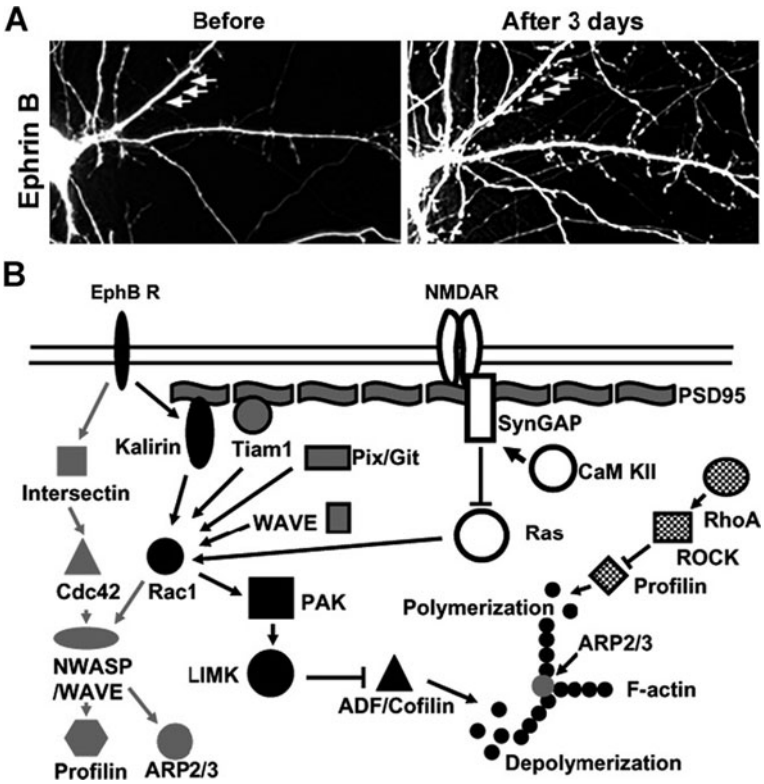

Fig. 10.4 Four signaling pathways that mediate the morphogenesis of dendritic spines. (**a**) Time-lapse images of a cultured GFP-expressing hippocampal neuron (7 days in vitro) before and 3 days after treatment with ephrin-B. *Arrows* denote emergence of new dendritic spines. (**b**) *Gray symbols*, the Cdc42/N-WASP/WAVE pathway; *black symbols*, the Rac1/PAK pathway; *open symbols*, the SynGAP/Ras pathway; *dotted symbols*, the RhoA/ROCK pathway

abnormal in vivo. Penzes et al. (2003) showed that treatment of neurons with ephrin-B1 causes the clustering and activation of EphB receptors and Kalirin-7, and increases the density of dendritic spines. These Kalirin-7-induced changes in dendritic spines are mediated through the Rac1/PAK1 pathway (Penzes et al. 2003). Kalirin-7, a Rac1-specific GEF, is likely to be anchored to PSD95 scaffold and is essential for the normal formation of dendritic spines (Penzes et al. 2001). The activation of Rac1 increased the density and size of dendritic spines and could also enhance excitatory synaptic transmission by increasing the clustering of AMPA receptors (Nakayama et al. 2000, Wiens et al. 2005). Finally, LIMK, a kinase downstream of PAK1, inhibits the depolymerization by phosphorylating and inactivating ADF/cofilin (Arber et al. 1998, Meng et al. 2002).

10.6.2 Cdc42/N-WASP/WAVE Pathway

This disease-related signaling pathway has recently attracted great attention from a wide range of research fields (Fig. 10.4b, the left pathway marked by gray symbols). Wiskott–Aldrich syndrome is an X-linked recessive primary immunodeficiency disease. The Wiskott–Aldrich syndrome protein (WASP) was initially identified as the causative gene of this disease (Derry et al. 1994). Thereafter, five members of WASP family and WASP-family verprolin homologous protein [WAVE; also called suppressor of cAMP receptor (SCAR) mutation] family are identified in mammals: WASP, neural (N-) WASP, WAVE1, WAVE2, and WAVE3. WASP is restricted to hematopoietic cells; WAVE2 is ubiquitous; N-WASP, WAVE1, and WAVE3 are enriched in the brain (Derry et al. 1994, Miki et al. 1996, Suetsugu et al. 1999, Takenawa and Miki 2001). Although N-WASP and WAVE proteins can interact with many other proteins, the two major actin-modulating effectors are profilin and actin-related protein Arp2/3 (Mullins 2000, Takenawa and Suetsugu 2007). Loss of WAVE1 function decreases the number of mature dendritic spines and this deficit can be reversed by the expression of dephosphorylated WAVE1 (Kim et al. 2006, Soderling et al. 2007). This WAVE1-induced change in dendritic spines is regulated by cyclin-dependent kinase 5 (Cdk5) and Arp2/3 complex (Kim et al. 2006).

WASP and N-WASP are believed to be the effectors of Cdc42 because actin polymerization induced by these proteins is inhibited by dominant-negative mutants of Cdc42, but not of Rac1 or RhoA (Miki et al. 1996, Symons et al. 1996). In contrast, WAVE proteins (also called SCAR) are believed to be the effectors of Rac1 because the expression of truncated WAVE inhibits Rac-mediated actin polymerization but has no effect on Cdc42-mediated polymerization (Miki et al. 1998). Intersectin is a scaffolding protein which might serve as the signaling link between EphB receptors and Cdc42, and thus EphB receptors might act via Cdc42/N-WASP pathway (Carlisle and Kennedy 2005). However, some experiments reveal that Cdc42 seems to have little effect on the density and size of dendritic spines (Tashiro et al. 2000). Therefore, Cdc42/N-WASP pathway might not be required for spine morphogenesis and EphB receptors are more likely to induce spine morphogenesis

through Rac1/PAK1/LimK pathway (Fig. 10.4b, black symbols) or the alternative Rac1/WAVE pathway (Fig. 10.4b, gray symbols).

10.6.3 RhoA/ROCK/Profilin Pathway

The expression of constitutive active RhoA decreases the density and size of dendritic spines, effects that are opposite to the activation of Rac1 (Fig. 10.4b, far right, *dotted symbols*; Nakayama et al. 2000, Tashiro et al. 2000). The upstream regulators are postulated to be EphA/Cdk5/ephexin1 (Fu et al. 2007), whereas one of the downstream effectors that mediate RhoA-induced loss of dendritic spines is the neuronal-specific profilin IIa (Schubert et al. 2006).

10.6.4 SynGAP/Ras Pathway

In addition to Rho GTPase, other small GTPases such as Ras might also regulate the morphogenesis of dendritic spines (Fig. 10.4b, second right, open symbols; Gartner et al. 2005). SynGAP is clustered in dendritic spines and is a major inhibitor of Ras in excitatory synapses (Chen et al. 1998, Kim et al. 1998). The size of dendritic spines is increased and the development of spines is accelerated in SynGAP knockout mice, supporting the role of synGAP/Ras pathway in modulating spinogenesis (Vazquez et al. 2004).

10.7 Roles of Actin in Synaptic Plasticity of Dendritic Spines

Synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD), is widely believed to be the cellular mechanism underlying learning and memory (Martin et al. 2000). Although it is controversial whether LTP can alter the release of presynaptic neurotransmitters, there is now almost no doubt that both LTP and LTD can change the amount of AMPA receptors in dendritic spines. The size of the spine head is positively correlated with the number of AMPA receptors in the postsynaptic membrane (Nusser et al. 1998, Matsuzaki et al. 2001). Therefore, in order to maintain this correlation, synaptic plasticity should also be expected to alter the morphology of dendritic spines. Indeed, many experiments demonstrate that spine morphology is profoundly influenced by synaptic plasticity (Engert and Bonhoeffer 1999, Maletic-Savatic et al. 1999, Toni et al. 1999, Matsuzaki et al. 2004).

As previously discussed in Section 10.3, the cytoskeleton of dendritic spines is mainly composed of actin filaments. Several groups have investigated the role of actin cytoskeleton in functional and morphological changes in dendritic spines during synaptic plasticity (Kopec and Malinow 2006). Inhibition of actin filament

assembly impairs the maintenance of LTP (Kim and Lisman 1999, Krucker et al. 2000). LTP causes a rapid shift of actin equilibrium toward F-actin in the dendritic spines and increases in the amount of rhodamine–phalloidin-labeled polymerized actin (Lin et al. 2005, Okamoto and Hayashi 2006).

Synaptic plasticity regulates the activity of Rho GTPases, the major regulators of actin-dependent morphogenesis (Li et al. 2002). The Rac1–GEF Tiam1 has been reported to couple the NMDA receptor to the activity-dependent development of dendritic arbors and spines (Tolias et al. 2005, 2007). A recent study demonstrates that LTP increases the number of spines that contain phosphorylated PAK1 and its downstream target cofilin, indicating that LTP-induced morphological changes in dendritic spines are mediated via the Rac1/Pak signaling pathway (Fig. 10.4, black symbols). The second major actin-regulatory signaling pathway with a clearly identified role is the RhoA/ROCK/profilin pathway (Fig. 10.4, dotted symbols; Schubert et al. 2006, Schubert and Dotti 2007). A recent study revealed that WAVE1 and WRP signaling complex might also regulate synaptic plasticity (Fig. 10.4, gray symbols; Soderling et al. 2007). In addition, postsynaptic actin-bound neurabin-I–PP1 complex and integrin-driven actin polymerization might consolidate actin cytoskeleton at the later stage of LTP (Hu et al. 2006, Kramar et al. 2006). Cortactin, which is an F-actin-binding protein and activator of the Arp2/3 actin nucleation machinery, participates in the regulation of activity-dependent morphological plasticity of dendritic spines (Hering and Sheng 2003).

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Chapter 11
Actin and Diseases of the Nervous System

Barbara W. Bernstein, Michael T. Maloney, and James R. Bamburg

Abstract Abnormal regulation of the actin cytoskeleton results in several pathological conditions affecting primarily the nervous system. Those of genetic origin arise during development, but others manifest later in life. Actin regulation is also affected profoundly by environmental factors that can have sustained consequences for the nervous system. Those consequences follow from the fact that the actin cytoskeleton is essential for a multitude of cell biological functions ranging from neuronal migration in cortical development and dendritic spine formation to NMDA receptor activity in learning and alcoholism. Improper regulation of actin, causing aggregation, can contribute to the neurodegeneration of amyloidopathies, such as Down's syndrome and Alzheimer's disease. Much progress has been made in understanding the molecular basis of these diseases.

Keywords Actin-depolymerizing factor · Cofilin · Rod · Alzheimer · Neurodegeneration · Down's syndrome

11.1 Introduction

Most of the work described in this chapter is based on the assumption that the neuron is the functional unit of the nervous system. This is despite the fact that discoveries over the last few decades have made us increasingly aware of the importance of ensembles of neurons as the structural and functional unit (Bullock et al. 2005) for both cognition (Lin et al. 2006) and behavior (Insel 2007). Among these developments are the following: (1) the widespread nature of gap junctions throughout the CNS even among neurons with chemical synapses, (2) the plasticity of these electronic synapses, and (3) qualitative enhancement, both temporally and spatially,

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of simultaneous recording and activity imaging of ever larger numbers of neurons. The importance of neuronal ensembles certainly does not facilitate a detailed molecular understanding of neuronal disease, but ultimately the understanding we reach should include neuronal ensembles as the brain's functional unit.

As we proceed section by section, enumerating the specific mutations or environmental stresses that affect actin function and are known to be associated with neurological or psychiatric illnesses, we hope that we will provide sufficient understanding of the role of actin in neurophysiology and development that the reader will have no problem understanding how even mild perturbation of normal actin regulation could lead to a diseased state. We will not describe every neuronal disease but rather try to provide an appreciation for the extensive range of known actin-based neuronal pathologies, limiting our discussion to those situations in which actin is thought to be, or is likely to be, one of the initial systems affected by a particular genetic defect or environmental stress. Many of these problems involve virtually every system in the body; the prime example of this is cancer. We will concentrate only on those illnesses that affect primarily the nervous system and will attempt to explain mechanistically the involvement of actin.

A short review of actin and the actin-binding proteins featured in this chapter will be provided. For more detailed background information about actin and the myriad actin-binding proteins in healthy neurons, the reader is referred to other chapters in this volume (see Chapters 3, 4, and 5 and suggested reading material in Chapter 1). The importance of actin in neuronal physiology is emphasized by the demonstration that actin dynamics modulates the activities of ion channels, ion transporters, and receptors (Maguire et al. 1998). Interfering with filament formation extends the duration of action potentials and neuronal calcium transients evoked by depolarization (Houssen et al. 2006). In addition there are multitudes of enzymes for which actin filaments provide a regulatory viscoelastic scaffold whose area is about 60× that of the plasma membrane of the average neuron (Janmey 1998).

We will end this chapter with a brief summary of some less well-recognized biochemical properties of the actin cytoskeleton that have not yet been directly tied to disease. Intuitively their potential for involvement in disease should be clear. The obvious complexity of interdependent functions within individual cells and between cells of neuronal ensembles should explain the failure thus far to understand fully any disease on a detailed molecular basis.

11.1.1 Overview of Actin Dynamics and Superstructures

The actin monomer (G-actin) spontaneously undergoes self-assembly into a double-stranded helical filament (F-actin) when exposed to physiological ionic conditions. The rate-limiting step for this reaction is nucleation, requiring three subunits to assemble into the proper structure. In the cell, spontaneous nucleation is suppressed by G-actin-sequestering proteins (e.g., thymosin β4, profilin, calbindin) and promoted by others: Arp2/3 complex, Spir proteins, formins, or, under some conditions, cofilin. The barbed end has a lower equilibrium concentration than the pointed

end. Thus at steady state (or when actin monomer is between these two equilibrium concentrations), actin subunits will add onto the barbed end and depolymerize from the pointed end without changing filament length, thus producing treadmilling of the filament. Polymerization at the barbed end can do work, driving protrusive membrane expansion (lamellipodia and filopodia) and, in some cases, vesicle motility. Actin contains a bound adenine nucleotide (ATP on most G-actin) which is rapidly hydrolyzed upon assembly; then the inorganic phosphate is slowly released with the release modulated by actin-binding proteins. Nucleotide hydrolysis provides the energy required for the filament to maintain different equilibrium concentrations at each end, named the pointed and barbed ends for the arrowhead decoration of actin with proteolytic fragments of the myosin II motor. Filament turnover in the cell, driven mainly by cofilin, profilin, and Cap1, is about 100–150 times faster than for pure actin in vitro (Fig. 11.1). Cofilin can sever filaments, creating more ends to enhance subunit dissociation, and at high concentrations can nucleate filament growth, saturate filaments, and stabilize them (Andrianantoandro and Pollard 2006). Cyclase-associated protein 1, Cap1, can bind the ADP–actin–cofilin complex, releasing the cofilin and enhancing actin nucleotide exchange. Profilin also enhances nucleotide exchange and binds to the ATP–actin pool, enhancing

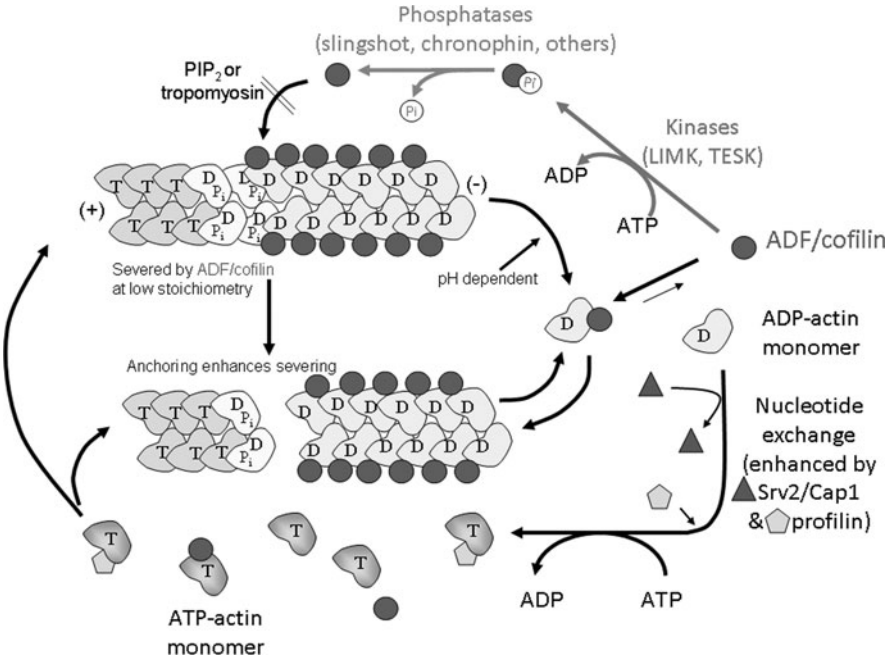

Fig. 11.1 The enhancement of actin filament turnover by ADF/cofilin proteins. Included are several modes of ADF/cofilin regulation including roles for other actin-binding proteins in nucleotide exchange

the growth of filament barbed ends, especially those nucleated and elongated by formins.

Cofilin is regulated in mammalian cells in multiple ways; a major one is inactivation by phosphorylation on ser 3. There are two LIM kinases and two TES kinases that phosphorylate cofilin at this site. The LIM kinases are themselves activated by phosphorylation via the p21-activated kinase, Pak, downstream of the Rho family GTPases, Rac and Cdc42, or by the Rho-activated kinase, ROCK. Cofilin is reactivated by phosphatases in the slingshot family (Niwa et al. 2002) or by chronophin (Huang et al. 2006). Complicating our understanding of cofilin's role is the fact that its phosphorylation has two distinct effects on its activity: (1) blocking its interaction with actin, thus slowing actin turnover, and (2) rendering cofilin an activator of phospholipase D1 activity (Han et al. 2007). The products of this enzymatic activity (e.g., phosphatidic acid) are broadly active in cytoskeletal regulation.

Actin filaments in cells form a variety of superstructures that utilize other actin-binding proteins to create gels or bundles of different types (Pak et al. 2008). The following are cross-linking proteins discussed herein: filamin, a protein that stabilizes the interactions between two orthogonal filaments; α-actinin, a protein that cross-links anti-parallel filaments into a linear array such as in stress fibers; fascin, a protein that cross-links parallel filaments, such as those in filopodia. Often specific isoforms of tropomyosin, a coiled-coil helical protein that binds F-actin, may restrict the selection of other proteins with which F-actin interacts. Cofilin and tropomyosins usually compete for filament binding but prefer forms of F-actin with different subunit rotation (twist). Both bind cooperatively along an actin filament and can drive the other off the filament (Kuhn and Bamburg 2008).

11.1.2 Developmental Disorders

11.1.2.1 Neuronal Migration and Cortical Development

The complexity of organization of the mammalian forebrain derives from the intricately orchestrated neuronal migration that forms it during development. This organization is critical for cognition. Deviations in it lead to severe learning handicaps and epilepsy (Fig. 11.2; Marin and Rubenstein 2003). Over 26 such syndromes have been linked to the actin cytoskeleton through mutations in filamin A (aka actin-binding protein 280; ABP280). Structural abnormalities include schizencephaly, porencephaly, lissencephaly ("smooth brain"), agyria, macrogyria, and pachygyria. Periventricular heterotopia (PH) refers to a disorder in which an otherwise normal-looking cortex shows gray matter nodules along the lateral ventricular walls (Sheen et al. 2004). Severe truncation in the X-linked filamin A gene can make the male occurrence lethal, while mild missense mutations have been seen in males with normal intellect, strongly suggesting a correlation between mutation type and the severity of neurological problems ranging from poor motor function, seizures, developmental delays to mental retardation, hearing loss, and even epilepsy and schizophrenia (Masruha et al. 2006).

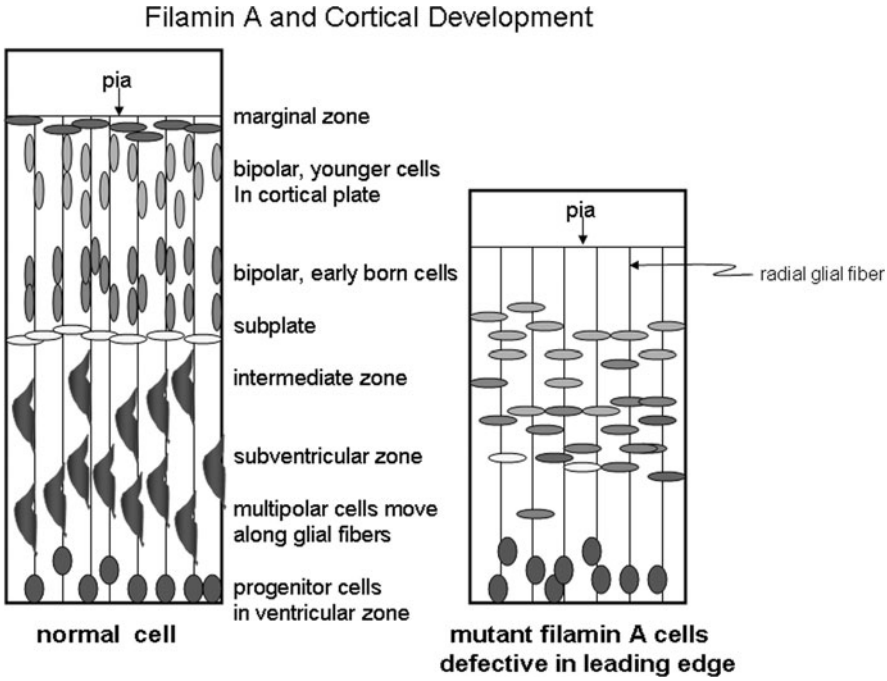

Fig. 11.2 Mutations in filamin A (aka actin-binding protein 280; ABP280) cause defects in neuronal migration that can be devastating for the development of the forebrain. Filamin is an actin filament cross-linking protein that also links transmembrane proteins to the underlying cytoskeleton. The effects of its mutation range from mental retardation to schizophrenia (adapted from Lambert and Goffinet 2001)

Many environmental factors, such as radiation, cocaine, and alcohol, can interfere with neuronal migration. We can only speculate about the mechanistic overlap these factors may share with mutated filamin A. Filamin is widely expressed in all brain cortical layers, cross-links actin filaments into orthogonal networks in cortical cytoplasm, and helps anchor membrane proteins such as integrin and transmembrane receptor complexes to the underlying cytoskeleton. Filamin is an additionally potent regulator of actin dynamics because it has a bidirectional role with Pak1, an effector of Rho family small GTPases (Fig. 11.3). Filamin not only is phosphorylated by Pak1 physiologically but also stimulates autophosphorylation and the kinase activity of Pak1 (Vadlamudi et al. 2002). Pak1 phosphorylates and activates LIM kinase 1, which inactivates cofilin, a major regulator of filament turnover. Thus abnormal filamin function could readily interfere with neuronal migration since cofilin is necessary for radial migration (Bellenchi et al. 2007) and growth cone guidance after the cell finds its final position (Fig. 11.4; Wen et al. 2007).

Neuronal polarity, the establishment of one neurite as the axon, is also essential for correct wiring of the cortex, hippocampus, and cerebellum. Pak1 is distributed throughout all neurites of the differentiating neuron, but active Pak1 is restricted

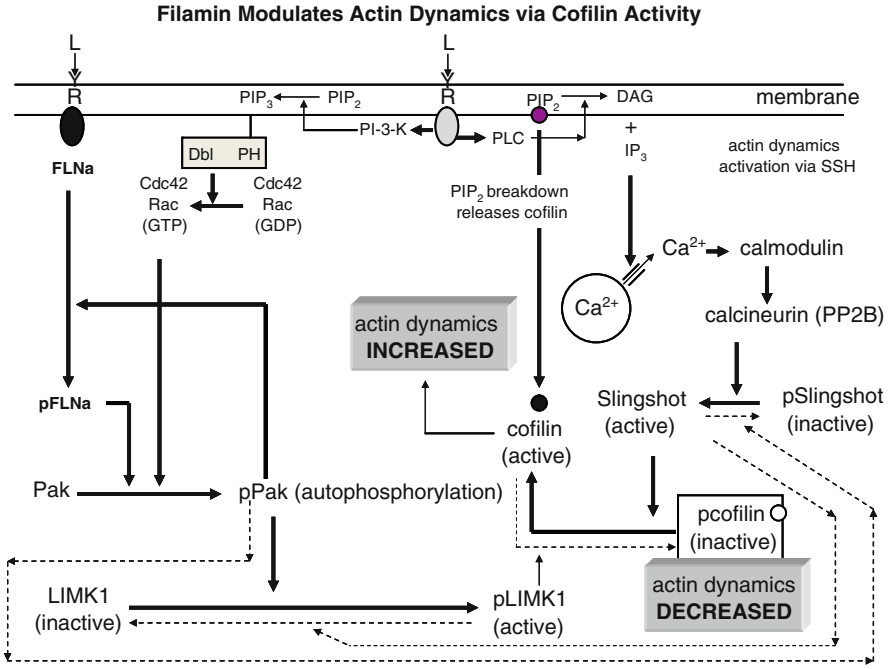

Fig. 11.3 Filamin A (FLNa) could seriously impact actin dynamics in the leading edge of migrating neurons by stimulating Pak autophosphorylation and hence Pak activity. Pak has multiple effects on ADF/cofilin phosphorylation: it phosphorylates slingshot (an inactivation) and phosphorylates LIMK (an activation). The phosphorylation of both slingshot and LIMK reduces ADF/cofilin activity. Adding to the complexity of these interactions is the fact that pPak feeds back on the phosphorylation of FLNa. *Bold arrows* symbolize activation; *dashed arrows* symbolize inactivation

to the presumptive axon (Jacobs et al. 2007) where it likely is needed for cofilin regulation via Cdc42 (Garvalov et al. 2007).

11.1.2.2 Developmental Disorders of the Neural Crest

Neuronal migration dependent on Pak1 is also known to be clinically disrupted at the level of gene expression if Pax3 is mutated. Pax3, a highly conserved developmental transcription factor essential for normal embryonic development in a wide range of organisms (Dahl et al. 1997), contains two DNA-binding motifs: a conserved 128-amino-acid paired box and a homeobox domain. Each motif binds different DNA sequences. There are nine *Pax* genes in humans which, if mutated, are associated with diverse human diseases. These include Waardenburg syndrome, an autosomal dominant disease of tissues derived from migratory neural crest precursors. It occurs in humans with *Pax3* mutations. In mice, homozygous mutations to *Pax3* cause numerous developmental anomalies including spina bifida, loss of skeletal muscles, and skeletal abnormalities. The somite defects of Pax3-deficient

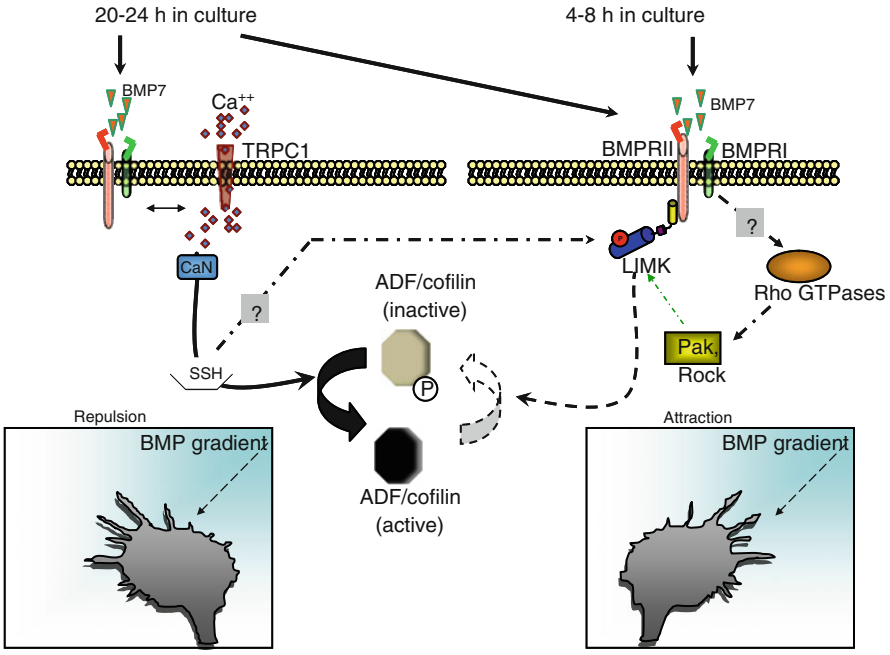

Fig. 11.4 In 4–8 h *Xenopus laevis* cultures, growth cones are attracted by a BMP7 gradient via LIMK which inactivates ADF/cofilin. However, the appearance of TRPC1 after overnight culture tilts the LIMK/slingshot balance toward slingshot (SSH), thus converting the BMP7 gradient to repulsion of the growth cone. The repulsion requires Ca^{2+} activation of slingshot via calcineurin (CaN). *Bold arrows* symbolize activation; *dashed arrows* symbolize inactivation (adapted from Wen et al. 2007)

mice are consistent with Pax3 being required for the mesenchymal condensation and/or *m*esenchymal to epithelial *t*ransition (MET; Schubert et al. 2001, Wiggan et al. 2006).

In an in vitro model of MET, Pax3 was shown to regulate Pak1 and Pak2 (Wiggan et al. 2006). Pak2 plays a particularly important role in the actin cytoskeletal organization required for MET, probably via cofilin, which is inactivated by Pak2 phosphorylation of LIMK1. Pak2 null mice have been generated (Chernoff and Rogers 2004) but have not yet been extensively characterized. However, Pak2 null mice and cofilin null mice (E10.5) are embryonic lethal at about the same day. Together these results suggest a linkage between Pax3 defects in neural crest cell migration, neural tube closure defects, and Pak2 regulation of cofilin, although other targets of Pak2 are undoubtedly involved in cytoskeletal organization. However, it is possible that Pak1 is acting through LIMK1 and stathmin phosphorylation to stabilize microtubules (Gorovoy et al. 2005, Yang et al. 2004).

11.1.2.3 Hereditary Spastic Paraplegias (HSPs) and BMP

This diverse group of diseases is characterized by degeneration of corticospinal tract axons and lower extremity spasticity and was recently linked to actin regulation through a bone morphogenetic protein (BMP7) via a protein that internalizes the BMP receptor. Over 20 causative loci have been mapped including the ichthyin family protein NIPA1 (aka SPG6), which is widely expressed but especially heavily in brain tissue. Wang et al. (2007) studied NIPA1's *Drosophila* homolog, spichthyin (Spict), to elucidate how NIPA1's mutated normal function might produce corticospinal degeneration. Spict blocks the signaling of the transforming growth factor family member BMP, which stabilizes presynaptically the neuromuscular junction and many other aspects of neuronal morphogenesis. Spict binds to and promotes the internalization of the BMP receptor, thus linking genes responsible for hereditary spastic paraplegias to effectors of BMP which were recently reported to include regulators of neuronal growth cone guidance. Wen et al. (2007) found that a BMP7 gradient can guide growth cones by balancing the phosphorylation of ADF/cofilin through LIMK and slingshot phosphatase activation (Fig. 11.4). Reversible phosphorylation is critical for ADF/cofilin's regulation of actin dynamics underlying neurite behavior (Chen et al. 2000, Meberg et al. 1998, Meyer and Feldman 2002, Gungabissoon and Bamburg 2003) and growth cone turning (Wen et al. 2007). Earlier Lee-Hoeflich et al. (2004) determined that the binding of LIMK1 to BMP receptor II was necessary for BMP induction of dendritic arbor in cortical neurons. The physical interaction of LIMK1 with BMPRII synergizes with the Rho GTPase, Cdc42, to activate LIMK1 catalytic activity (Lee-Hoeflich et al. 2004). Hence the association of mutations in BMP regulators, such as NIPA1, strongly implicates actin regulation in paraplegias.

The fact that BMP regulates cofilin activity suggests that actin dysregulation may be involved in other diseases associated with BMP function besides hereditary spastic paraplegias. These possibilities are listed below, and BMP's role in neuronal plasticity is cited in the Section 11.1.3.3.

1. Inhibition of BMP by overexpression of noggin, a physiological inhibitor, prevents clustering of neural crest cells and formation of enteric ganglia. These are the hallmark of two human intestinal obstruction disorders Hirschsprung's disease and intestinal neuronal dysplasia (Goldstein et al. 2005).
2. BMP6 and 7 control the induction and differentiation of dorsal roof plate interneurons (Lee and Jessell 1999, Liu and Niswander 2005).
3. BMP7 signals rapid morphometric changes in the guidance of developing spinal cord neuronal growth cones (Augsburger et al. 1999, Yoshikawa and Thomas 2004, Bovolenta 2005).

11.1.3 Post-embryonic Disorders

These lifetime problems often arise after embryological development and can be induced by genetic and non-genetic factors. This is particularly true for mood or

behavioral disorders that often manifest in adulthood. Some of these have been traced to specific genetic defects, but others have not and are known only to be gross failures of brain regions (neurological defects) or circuitry disturbances in particular regions (psychiatric disorders; Insel 2007). In addition many types of mental retardation have idiopathic causes, e.g., lead poisoning, traumatic head injury, malnutrition (Ramakers 2002), or prenatal exposure to drugs or alcohol. Some of these disorders, such as fragile X syndrome, are known to arise from abnormal mRNA transport and local translation. These will be considered separately.

11.1.3.1 Dendritic Spine Dysgenesis and Synaptic Defects

Most of the diseases classified under this heading are defects within the hippocampus that affect the formation of the important postsynaptic structure the dendritic spine. Studies on biopsied human cortical tissue have linked morphological anomalies in dendrites and dendritic spines to various forms of mental retardation (Huttenlocher 1970, 1975, Marin-Padilla 1972, Purpura 1974) and anomalies in dendrite complexity to cognitive function (reviewed in Benavides-Piccione et al. 2004, Lewis 2004). We include these conditions in this chapter because of the clear dependence of pre- and postsynaptic function and morphology on a highly dynamic actin pool (Matus 1999) and an assortment of actin-binding proteins (e.g., Arp2/3, cortactin, ADF/cofilin, profilin, gelsolin, drebrin, neurabin, α-actinin) (for a review, see Ethell and Pasquale 2005).

11.1.3.2 Dendritic Spine Development and Function

Dendrite arbor complexity and spine morphology contribute to the theoretical complexity of postsynaptic signal integration and processing (London and Hausser 2005). Dendritic spines, the major site of excitatory glutamatergic synaptic transmission, are highly specialized, micrometer-long protrusions, found in selected neuronal populations of vertebrates that include hippocampal pyramidal neurons and in some invertebrate neurons (reviewed in Nimchinsky et al. 2004). The classical view of the mature dendritic spine is an enlarged, mushroom-shaped head that is connected to the dendrite shaft by a thin neck (Nimchinsky et al. 2004, Matsuzaki et al. 2004), but there is growing awareness of spine shape heterogeneity in adult brain (Jontes and Smith 2000): mushroom, thin, and stubby (Fig. 11.5; Peters and Kaiserman-Abramof 1970, Fiala et al. 1998, Vaughn 1989). The spine shape heterogeneity may represent not only distinct functional classes but also developmental stages (Dailey and Smith 1996, Fiala et al. 1998, Harris 1999, Ziv and Smith 1996). The dominant dendritic protrusion type shifts during development, but all are present in the adult brain (Holtmaat et al. 2005).

It has long been thought that functions of spines, including conductance, are shape and hence actin cytoskeleton dependent (Somogyi et al. 1983). For example, sequestration of calcium (Allbritton et al. 1992, Majewska et al. 2000) and compartmentalization of structural protein ensembles and organelles require F-actin (Axelrod et al. 1976, Kennedy et al. 2005, Koch and Zador 1993, Nimchinsky et al. 2002, Svoboda et al. 1996). F-actin is highly enriched in the spine (Okamoto et al.

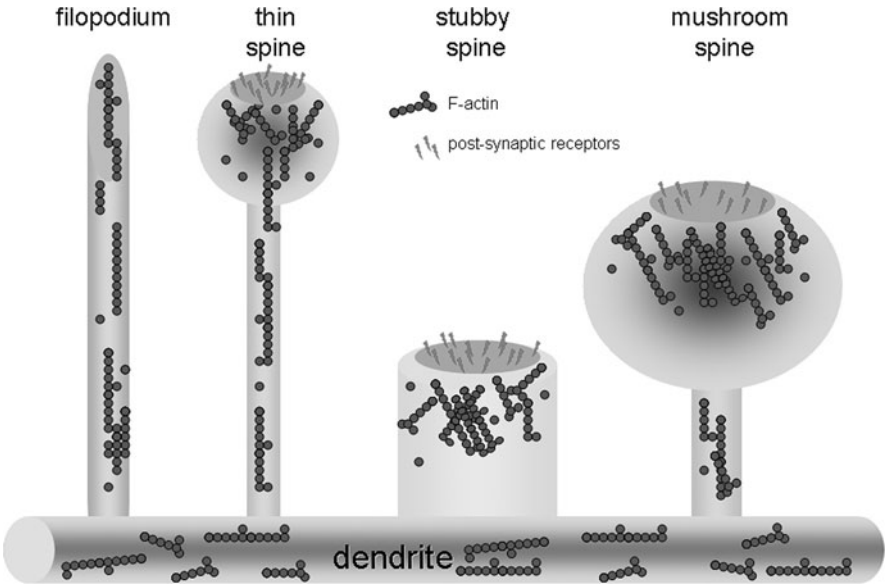

Fig. 11.5 This cartoon depicts the heterogeneity of dendritic spine morphology in both mature and developing brain. Alterations in spine morphology have been correlated with significant changes in human behavior and are largely a function of the regulation of actin, the major cytoskeletal protein found within these structures (adapted from Sekino et al. 2007)

2004) and largely determines the spine's growth and morphology since it is the major cytoskeletal element in the spine (reviewed in Matus 1999, 2000, Zito et al. 2004).

The delivery of newly synthesized proteins to specific synapses may also be actin dependent since the translocation of GFP-CaMKII and even 3-kDa dextran diffusion is inhibited by pharmacological stabilization of F-actin (Ouyang et al. 2005). They also found that neuronal activity depolymerizes actin and dephosphorylates cofilin. Additionally, it was recently reported that CaMKII, long known to be critical for synaptic plasticity and more abundant in spines than expected solely for enzymatic activity, is in fact capable of bundling F-actin through a stoichiometric interaction (Okamoto et al. 2007).

11.1.3.3 Long-Term Depression (LTD) and Long-Term Potentiation (LTP)

Two groups recently succeeded in testing the critical hypothesis that LTP stores information. Pastalkova et al. (2006) did this by demonstrating that reversing LTP in vivo causes the loss of 1-day-old spatial information; LTP was reversed by microinjecting a protein kinase inhibitor. Whitlock et al. (2006) actually showed that inducing inhibitory avoidance learning in rats produced the same glutamate receptor potentiation in hippocampal CA1 neurons as high-frequency stimulation.

F-actin generation is required for the induction of LTP, although the underlying mechanisms regulating these changes remain mysterious. Potentiation that produces actin-rich spines with large synapses requires LIMK1 phosphorylation of ADF/cofilin (Lisman 2003). High-frequency stimulation that induces LTP is correlated with an increase in dendritic spine density and/or enlargement, and LTD, induced by low-frequency stimulation, is correlated with marked spine shrinkage (Zhou et al. 2004). Subsequent high-frequency stimulation can reverse the shrinkage which is mediated by cofilin, but not via PP1. PP1 is essential for LTD. This suggests that LTD and spine shrinkage involve different downstream pathways. A rapid, sustained increase in presynaptic vesicle-associated proteins is also seen with LTP (Antonova et al. 2001).

Addition of phalloidin (an F-actin stabilizer) or a cofilin inhibitory peptide blocked LTD of NMDA receptor EPSCs but not AMPA receptor EPSCs. These findings suggest that the same pattern of afferent activity elicits depression of AMPAR and NMDAR responses through distinct triggering and expression mechanisms (Morishita et al. 2005). Together the above results suggest a role for cofilin in synaptic plasticity of hippocampal circuits which are critical for generating memory and learning.

Further evidence for a likely actin/cofilin role in LTP comes from studies with BMP and BDNF. Above we discussed BMP7's specific part in growth cone guidance via its spatial/temporal regulation of cofilin phosphorylation. BMP7, actin, and cofilin also have roles in synaptic plasticity and cognition as removal of chordin, an inhibitor of BMP7 and other BMPs (Wilkinson et al. 2003), increases transmitter release, paired-pulse facilitation, and LTP in cultured hippocampal neurons (Sun et al. 2007b). BDNF is a positive modulator of LTP induced uniquely by theta burst stimulation (TBS), enhancing the number of spines staining densely for F-actin and the phosphorylation level of Pak1 and cofilin in these spines (Rex et al. 2007). The effect of BDNF on cofilin activity was previously reported in growth cone filopodial expansion (Gehler et al. 2004). Furthermore, spines with phospho-cofilin staining are associated with larger synapses following unsupervised learning (Fedulov et al. 2007).

Memory impairment caused by hippocampal seizures in patients suffering from epilepsy may be due in part to decreases in F-actin and increase in cofilin activation (Ouyang et al. 2007) since the seizure-inducing agent 4-aminopyridine has these effects. Moreover, seizures disrupt the finely regulated biphasic sequence of actin depolymerization and repolymerization induced by LTP-inducing stimulation (Ouyang et al. 2005). The sequence is responsible for contradictory reports on the effects of LTP-inducing stimulation on F-actin levels (Fukazawa et al. 2003, Okamoto et al. 2004, Lin et al. 2006, Kim and Lisman 1999, Ouyang et al. 2005, Shen and Meyer 1999).

11.1.3.4 Corticobasal Degeneration

Actin is likely involved in corticobasal degeneration (CBD), an adult onset progressive neurodegenerative disorder, that includes the frontoparietal cortex and several

subcortical nuclei, causing symmetrical rigidity, bradykinesia, myoclonus, and dystonia (Kurz 2005). Proteome profiles, based on two-dimensional gel electrophoresis, showed that cofilin-1 (non-muscle) was one of the only two proteins whose expression was upregulated in CBD brain. Six proteins declined in expression (Chen et al. 2005). Although it is not possible to draw definitive conclusions from this single sample, developmental changes in cofilin expression or activity could underlie synaptic dysfunction. Modified cofilin activity could directly alter synapse function through any combination of actin's neuronal functions discussed in the introduction of this volume and expanded upon in this chapter: spine morphology dynamics, ion channel activity, and vesicle cycling which increases during LTP (Malgaroli et al. 1995) and depends on actin dynamics (Bernstein et al. 1998).

11.1.3.5 Williams Syndrome

Williams syndrome (WS) is a rare neurodevelopmental disorder occurring in approximately 1 in 7,500 births (Meyer-Lindenberg et al. 2005). Affected individuals display craniofacial dysmorphology, dental abnormalities, cardiovascular disorders, hypertension, neonatal hypercalcemia, delayed language and motor development, abnormal sensitivity to certain sounds (Keating 1997, Bellugi et al. 1999), "elfin-like" facial features with a broad forehead, oval ears, and a wide mouth (Preus 1984), and striking impairments in areas such as general intelligence (IQ 40–79), visuospatial constructive cognition, and attention (Hoogenraad et al. 2004). The effects of WS are not all negative; these patients are more skilled than normal at face processing, social engagement, and expressive language (Bellugi et al. 1999).

The genetic cause of WS is deletion of approximately 1.6 Mb of DNA, the WS critical region, that accounts for about 28 genes at Chr band 7q11.23. To better understand the relationship between genotype and phenotype in WS patients, the DNA sequences of the 83.6-kb deleted region of two families with a WS phenotype were analyzed. These families showed supravalvular aortic stenosis, WS facial features, and impairment in visual–spatial constructive cognition (Frangiskakis et al. 1996). The analyses revealed the deletion of only one gene normally expressed in adult brain: *LIMK1*. In addition, *LIMK1* heterozygosity cosegregated with impaired visual–spatial constructive cognition (Frangiskakis et al. 1996). These data imply that abnormal cofilin regulation of actin may be responsible for the deficits in visual–spatial constructive cognition of WS patients.

Mice with the following *LIMK1* mutations have been developed to elucidate its role in neuronal development: homozygous *LIMK1* null, *LIMK2* null, and double null (Meng et al. 2002). Despite a decrease in levels of phospho-cofilin in the *LIMK1*-/- mice, they were grossly normal. Hippocampal neurons from *LIMK1* null mice have no growth cones, increased actin aggregates along dendrites in mature neurons, and spines with thicker necks and smaller heads. They show normal LTD, but increased LTP. Spatial learning performance in the water maze was normal except for relearning with changes in the visual cues (Meng et al. 2002).

The *LIMK1/2* double-knockout mice are severely affected in both cofilin phosphorylation and excitatory synaptic function in the hippocampal CA1 region (Meng et al. 2004). Their surprising normalcy and residual phospho-cofilin level are still unexplained.

The above data indicate that LIMK plays important roles in brain development, spine morphology, and synapse stability. However, it is not certain that any or all of these effects are mediated by cofilin or even that kinase activity is required, but the regulation of cofilin by LIMK is important for spine remodeling. Mutations in two tumor suppressor genes of the tuberous sclerosis complex (*TSC1* and *TSC2*) often lead to mental retardation, epilepsy, and autism, and loss of the Tsc1 and Tsc2 proteins increases LIMK phosphorylation of cofilin and enlargement of spines (Tavazoie et al. 2005). These changes were blocked by non-phosphorylatable cofilin but not wild type.

Another gene of the WS critical region *CYLN2* encodes 115-kDa CLIP-115 which binds microtubule-growing ends, promoting persistent growth and non-persistent shortening (Schuyler and Pellman 2001, Komarova et al. 2002). Mice with haploinsufficiency in CLIP-115 have mild growth deficiency, brain abnormalities, hippocampal dysfunction, and particular deficits in motor coordination (Hoogenraad et al. 2002). Thus WS hippocampal abnormalities arise from both actin filament- and microtubule-dependent processes during development.

11.1.3.6 Mental Illness

Mood disorders alone affect ~20% of the population of industrialized countries (Goodwin and Jamison 1990). An accepted, but yet to be published, model of functional gene pathways involved in neuropsychiatric diseases suggests that disruption of synaptic transmission caused by cytoskeletal dysfunction is responsible for mood disorders and schizophrenias. The model cited above is based on identified genes involved in such disorders or genes residing in chromosomal segments associated with the disorders, or on the probable targets of pharmacological treatments that seem to help patients. In support of this idea, a pharmacological model for the psychiatric disorder of schizophrenia identified the RNA-binding protein, TLS, from a screening (Takumi 2007). TLS moves to dendritic spines with mGlu5 activation and probably regulates their morphology, at least in part, by facilitating the transport of an mRNA that encodes an F-actin-stabilizing protein, Nd1-L (Fujii and Takumi 2005).

Further supporting the importance of balanced actin assembly in mood, Cap1, an accelerator of filament turnover, was identified as a cogent quantitative trait gene for depression in mice. In the frontal cortex of a depressive mouse strain, Cap1 protein level was downregulated relative to a strain resistant to depression (Nakatani et al. 2007). Cap1 forms a trimeric complex with actin and cofilin. Cap1 forms a trimeric complex with actin and cofilin. It enhances both cofilin dissociation and exchange of actin ADP for ATP, priming actin for reassembly (Moriyama and Yahara 2002). Pak1 is also downregulated and cofilin 1 and profilin 1 are upregulated in these

depressive mice. Neuregulins have been pinpointed as potential susceptibility genes for schizophrenia (Corfas et al. 2004), which appears to involve glutamatergic transmission through NMDA receptors. Systemic application of NMDA receptor antagonists mimics schizophrenia symptoms. Of particular interest to us, Gu et al. (2005b) reported that neuregulin inhibits NMDA receptor current by internalizing these receptors via an actin-dependent means (Gu et al. 2005a). Moreover, the cytoplasmic tail of neuregulin, a transmembrane protein, interacts with an inhibitor of actin turnover, LIMK1 (Wang et al. 1998).

11.1.3.7 Addiction: Ethanol and Cocaine

Alcohol addiction afflicts ~300 million people. Individuals who fail to respond acutely to consumption are considered at risk to this genetically determined disease (McQuaid et al. 2000). The NMDA receptor is one of the most sensitive targets of ethanol, and adaptive changes to ethanol are thought to occur as responses in this receptor. Eps8, an actin barbed-end capping protein, is part of the evolutionarily conserved, nearly 100-protein complex that modulates ethanol consumption and NMDA receptor activity (Offenhauser et al. 2006). *Eps8* null mice consume excessive quantities of ethanol presumably because they are resistant to the intoxicating acute effects. Furthermore their cerebellar granule neurons, when cultured, do not show the postsynaptic actin depolymerization and increase in active cofilin of wild-type neurons. Eps8 has a dual structural/signaling role in actin dynamics: it is a barbed-end capping protein (Disanza et al. 2004) and part of a Rac–GEF complex that could modulate cofilin phosphorylation through slingshot (Innocenti et al. 2003, Kligys et al. 2007). Hence proper actin regulation seems needed for cellular and behavioral responses to ethanol consumption.

In an in vivo study of cocaine addiction, withdrawal from repeated administration of the drug produced a 3-week sustained increase in F-actin in the nucleus accumbens of rats (Toda et al. 2006), a region involved in regulating behavioral responses to rewards. Cocaine administration and withdrawal also induced changes in actin cycling and in the level and phosphorylation state of several actin-binding proteins (profilin, cofilin, fascin, arp3, mena, *p*-VASP, cortactin, *p*-adducin).

11.1.3.8 Parkinson's Disease

Early onset Parkinson's disease is associated with mutations in a gene encoding parkin, an ubiquitin (E3) ligase which binds LIMK1 and enhances its ubiquitination and proteasomal degradation (Lim et al. 2007). In a neuroblastoma cell line, but not a kidney cell line, overexpression of parkin reduced the amount of LIMK1-induced phosphorylation of cofilin. Levels of LIMK1 in cultured cells may be self-limiting through normally functioning parkin since LIMK1 decreases parkin self-ubiquitination, extending the link between actin regulation and Parkinson's disease.

11.1.4 Diseases of mRNA Delivery and Translation

11.1.4.1 Overview of mRNA Delivery and Regulation

Because of their shape, organization, and compartmentalization, neurons are one of the most challenging cell types for delivery to and maintenance of compartments which are thousands of cell body diameters from the nucleus. Localized synthesis of many cytoskeletal proteins is important in migrating and polarized cells, such as fibroblasts and epithelial cells (Shestakova et al. 2001), but neurons have a far more daunting task. Defects in the delivery of protein and mRNA down axons and dendrites or local control of translation develops into neurodegenerative diseases (Bassell and Kelic 2004).

The idea that proteins are locally synthesized in axons has only recently been accepted despite evidence for it presented more than 25 years ago (Koenig and Adams 1982). Locally synthesized proteins include β-actin, ADF (Lee and Hollenbeck 2003), cofilin, and other cytoskeletal proteins (Bassell and Kelic 2004, Willis et al. 2005). Neurotrophin treatment of axons results in a very rapid increase in mRNA transport into the axon (Willis et al. 2005) and local synthesis of actin and cofilin. Indeed synthesis of proteins within growth cones is important for axonal guidance (Campbell and Holt 2001) and regeneration (Verma et al. 2005). "Zip code-binding" proteins usually recognize mRNAs through sequences in their 3′-untranslated regions (3′-UTR). Defects in binding can cause compartmental mistargeting of mRNAs in cells and neurodegenerative diseases (Huttelmaier et al. 2005).

11.1.4.2 Spinal Muscular Atrophy

Spinal muscular atrophy, an often severe autosomal recessive form of a motor neuron disease, is caused by defects in SMN, part of a multiprotein complex responsible for splicing, targeting, and transport of RNAs (particularly β-actin mRNA) to the axon through interactions with the 3′-UTR zip code-binding protein (Rossoll et al. 2003, Zhang et al. 2004). Motor neurons, isolated from a mouse expressing defective SMN, exhibit normal survival but have reduced axon growth, which correlates with reduced levels of β-actin mRNA and β-actin protein in distal axons and growth cones.

11.1.4.3 Fragile X Syndrome (FXS) and Autism

The most common cause of inherited mental retardation and autism is loss of the fragile X mental retardation protein (FMRP) which is encoded by the *FMR1* gene (Verkerk et al. 1991, O'Donnell and Warren 2002). Patients may also display anxiety, attention deficit, hyperactivity, stereotypy, and seizures. FMRP and its mRNA are found in dendritic spines where the protein selectively regulates mRNA translation of proteins thought to be necessary for long-term synaptic plasticity and spine morphology in the cortex and hippocampus (Darnell et al. 2001, Laggerbauer et al.

2001). FXS patients and *FMR1* knockout mice have an unusual abundance of dendritic spines, particularly long immature ones (McKinney et al. 2005, Greenough et al. 2001).

Because FMRP negatively regulates pp2Acβ translation (Castets et al. 2005) and this phosphatase directly or indirectly dephosphorylates cofilin, levels of phosphorylated, but not total cofilin, are lower in the absence of wild-type FMRP. Thus, a change in cofilin activity is possibly a contributing factor to the increase in filopodial and balloon-shaped spines observed in brains of patients with fragile X syndrome (McKinney et al. 2005). It is interesting to note that murine fibroblasts, lacking FMRP or carrying point mutations in the RNA-binding domain, fail to relocalize four FMRP-binding partners to the actin ring area, as do normal cells.

The *Drosophila* homolog of FMRP binds the mRNA for the GTPase Rac which is upstream of Pak and actin regulation. In a transgenic mouse, expressing a dominant negative form of Pak, both the behavioral and spine morphogenetic effects of knocking out *FMR1* are reversed, further supporting the neurological importance of spine morphogenesis (Hayashi et al. 2007).

While the exact molecular mechanism of the above phenomenon remains to be elucidated, additional evidence for the role of Pak and actin regulatory proteins in cognitive disease comes from a study on Alzheimer's disease (Zhao et al. 2006). In Alzheimer's patients, total Pak and its activity are reduced, and cofilin is increased in the cytoskeletal fraction, whereas drebrin, an F-actin-stabilizing protein with an ADF homology domain, is decreased. Cofilin removes drebrin from actin, and oligomers of Aβ directly influence the Pak-related pathway. Pharmacological inhibition of Pak produced cofilin abnormalities, drebrin loss, and memory impairment in mice.

11.1.5 Actin Inclusions and Disease

The following neuronal problems can be caused by either genetic factors or, what appear to be, purely environmental stresses. Whether or not environmental induction is exacerbated by genetic predisposition remains for future study.

11.1.5.1 ADF/Cofilin–Actin Rods and Other Actin Inclusions

Cofilin–actin-enriched inclusions are a common pathological feature in a broad spectrum of neurodegenerative diseases. These inclusions appear as either irregular aggregates and sheets, paracrystalline lattices (as in Hirano bodies), or rod-shaped bundles of filaments (so-called "rods") that can appear in either the nucleus or the cytoplasm (Nishida et al. 1987, Sanger et al. 1980). It is unclear whether they share a common origin or arise through independent mechanisms all ultimately generating cofilin–actin aggregation.

Actin rods and stress fibers have distinctly different protein compositions. Fluorescent antibody staining shows that many proteins associated with stress fibers

are not found associated with nuclear or cytoplasmic actin rods: myosin, TM, α-actinin, filamin, vinculin, vimentin, and tubulin. Rod-like structures were noted in disease tissues more than 100 years ago (Mann 1894). They form in the cytoplasm or the nucleus of many types of cultured cells in response to 10% DMSO (Fukui 1978, Fukui and Katsumaru 1979), heat shock (Iida et al. 1986, Nishida et al. 1987, Ohta et al. 1989, Iida et al. 1992), osmotic stress (Iida et al. 1986, Nishida et al. 1987), overexpression of cofilin (Minamide et al. 2000), and ATP rundown (Bershadsky et al. 1980, Minamide et al. 2000, Ashworth et al. 2003, 2004). In fibroblasts or epithelial cells, rods are generally reversible and do not appear to cause permanent damage to the cell. However, in neurons, rods form primarily in the axons and dendrites where they compromise synaptic function (Jang et al. 2005).

ADF and cofilin are also major components of Hirano bodies (Maciver and Harrington 1995), first described in patients in Guam with amyotrophic lateral sclerosis and parkinsonism–dementia (Hirano et al. 1968, Hirano 1994). These unique intracellular inclusions consist of a paracrystalline-ordered array of parallel regularly spaced 6–10 nm filaments in orthogonal layers, encircled by a less structured actin-dense region (Schochet and McCormick 1972, Tomonaga 1974).

Because Hirano bodies are found in aged human brain from individuals with normal cognitive abilities, their significance is somewhat mysterious when frequently present in brains of individuals with the following diseases: Alzheimer's (Mitake et al. 1997, Gibson and Tomlinson 1977, Schmidt et al. 1989), parkinsonism–dementia (Hirano et al. 1968), Pick's disease (Schochet et al. 1968), amyotrophic lateral sclerosis (Hirano et al. 1968), ataxic Creutzfeldt–Jakob disease (Cartier et al. 1985) scrapie (Field and Narang 1972), Kuru (Field et al. 1969), papovavirus (Hadfield et al. 1974), cancer (Fu et al. 1975, Gessaga and Anzil 1975), diabetes (Sima and Hinton 1983), and chronic alcoholism (Laas and Hagel 1994). Hirano bodies have been found in multiple areas of the brain but most frequently in the Sommer's sector of Ammon's horn (Hirano 1994), a region in which Alzheimer's neurofibrillary tangles and Pick bodies are also enriched (Hirano 1994). Because Sommer's sector is involved in the development of new memories, Hirano bodies may contribute to cognitive impairment.

In addition to neurons, Hirano bodies appear in astrocytomas, aged extraocular muscle fibers, inflammatory cells of a leptomeningeal vessel (Ho and Allevato 1986), skeletal muscle fibers (Fernandez et al. 1999), and testis (Setoguti et al. 1974). They contain microtubule-associated proteins, e.g. tau, but primarily they contain actin and actin-related proteins: α-actinin, vinculin, tropomyosin, and ADF/cofilin. However, it is unclear in many cases whether the antibodies are staining the Hirano body core or the material surrounding the core, so the antigens may not be part of the organized cross-linked filament array. Although the mechanism of Hirano body formation from endogenous proteins is unknown, expression in mammalian cells of a C-terminal fragment (CT) of the 30-kDa *Dictyostelium discoideum* actin cross-linking protein induces structures morphologically identical to Hirano bodies (Maselli et al. 2002).

11.1.5.2 ADF/Cofilin–Actin Rods and Alzheimer's Disease (AD)

The amyloid beta (Aβ) peptide induction of cofilin–actin rods suggested a possible feed-forward mechanism for improper regulation of actin assembly leading to AD (Fig. 11.6; Maloney et al. 2005, Maloney and Bamburg 2007). We will describe the feed-forward mechanism after first presenting some essential background information. Proteolytic cleavage of the full-length amyloid precursor protein (APP) by β- and γ-secretases gives rise to amyloid beta (Aβ) peptides which are major components of the extracellular senile plaques characteristic of AD (Price et al. 1995b, Sisodia and Price 1995, Hardy and Selkoe 2002, Mattson 2004, Tanzi and Bertram 2005). Mutations linked to early onset familial AD lead to increased production of amyloidogenic $A\beta_{1-42}$ species (Chartier-Harlin et al. 1991a, b, Goate et al. 1991, Murrell et al. 1991, Price et al. 1995a) and increased accumulation of amyloid plaques.

Cofilin immunostaining showed linear arrays of rods in human AD brains, not seen in control human brain (Minamide et al. 2000). A similar cofilin-staining pattern was observed in brains of rapidly perfusion-fixed transgenic mice (Tg2576; also called the *Alzheimer mouse*) expressing the Swedish mutation of human APP, but not in control mouse brains fixed identically (Maloney et al. 2005). These findings

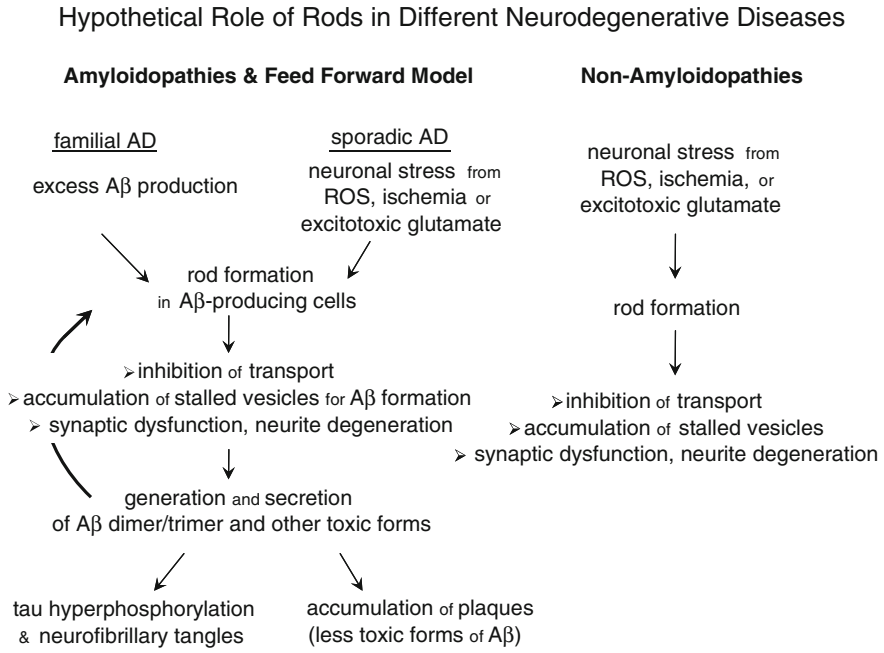

Fig. 11.6 This schematic illustrates the hypothetical feed-forward model of neurodegeneration that may occur when actin-cofilin rods are generated in neuronal amyloidogenic conditions. In the absence of amyloid accumulation, rod formation may also contribute to synaptic dysfunction

and others strongly suggest that these cofilin-staining aggregates are features of the diseased brain, are not postmortem artifacts, and probably appear before plaques appear.

Rods composed of actin and cofilin are induced in both axons and dendrites of cultured hippocampal neurons by ATP depletion, reactive oxygen species, excess glutamate (Minamide et al. 2000), and $A\beta_{1–42}$ (Maloney et al. 2005), all common mediators of neuronal stress. Although rods may be transient and disappear when the stress is removed, they often return within 24 h in a subset of neurites but are not lethal to the cell (Minamide et al. 2000). However, rods that enlarge to occlude the neurite disrupt distal microtubules and eliminate growth cones by causing neurite degeneration distal to the rod site.

Rods develop in 15–19% of $A\beta_{1–42}$-treated cultured hippocampal neurons with about 50% of the maximum response occurring within 6 h. Significant rod formation occurs with as little as 10 nM $A\beta_{1–42}$, the minimum synthetic Aβ reported to induce a physiologically relevant response. The feed-forward mechanism, linking actin deregulation and AD, is based on the following: (1) soluble forms of synthetic $A\beta_{1–42}$ oligomers induce the formation of rods in E18 rat hippocampal neurons in a time- and concentration-dependent manner in up to 19% of exposed neurons (Maloney et al. 2005) and (2) vesicles containing APP, β-secretase, and presenilin-1, a component of the γ-secretase complex, accumulate at rods, particularly over the distal portion. The β-C-terminal fragment of APP, the immediate precursor to $A\beta_{1–42}$, or the Aβ peptide itself, also localizes at rods, suggesting that blockage in transport of these vesicles could enhance Aβ production. Transport inhibition is one of the earliest defects in the transgenic mouse model for AD (Stokin et al. 2005). Production of Aβ occurs preferentially following endocytosis of APP (Ehehalt et al. 2003). These results suggest that rods, formed in response to either $A\beta_{1–42}$ or other stress, block APP transport and provide a site for producing more $A\beta_{1–42}$ peptides or assembling them into more toxic oligomers, such as the soluble dimers/trimers (Cleary et al. 2005), which can then be released from the cell. The additional peptide may induce rods in surrounding neurons, expanding the degenerative zone and eventually causing plaque formation. Taken together, these data strongly support a role for cofilin–actin rods in the blockage of transport associated with the synaptic loss, plaque formation, and a feed-forward mechanism of degeneration.

$A\beta_{1–42}$ dimers and trimers, but not monomers, even at physiological levels reduce spines from pyramidal neurons in brain slices (Townsend et al. 2006). The proposed Aβ pathway is through NMDA receptor-increased calcium influx and calcium activation of calcineurin which in turn activates slingshot through dephosphorylation (Wang et al. 2005). Slingshot activates cofilin through dephosphorylation, elevates cofilin/actin complex, and thus induces rod formation.

If rod formation is detrimental to neurons, why do rods form? Actin dynamics rely heavily on ATP hydrolysis and nucleotide exchange to drive the filament turnover process. ATP rundown leads to an accumulation of ADP–actin and active unphosphorylated cofilin. Rods may serve as a protective mechanism for cells mildly stressed by sequestering virtually all cofilin and a large fraction of the

more abundant actin into these non-dynamic aggregates. The remaining actin turns over much more slowly (Bernstein and Bamburg 2003, Bernstein et al. 2006) as seen in latrunculin depolymerization assays and fluorescence recovery after photobleaching experiments. Because actin dynamics can account for up to 50% of total ATP consumption in growing neurons and in resting platelets (Daniel et al. 1986, Bernstein and Bamburg 2003), slowing actin turnover significantly conserves ATP and transiently protects cultured neurons (Bernstein et al. 2006).

In addition to slowing filament turnover, sequestration of cofilin by actin rods might protect the cell from apoptosis through a totally different mechanism. Cofilin in highly stressed cells can translocate to mitochondrial outer membrane, release cytochrome c, and thus initiate apoptosis (Chua et al. 2003). Cofilin sequestration minimizes this translocation (Bernstein et al. 2006). The signaling pathways that target cofilin to mitochondria in neurons are unknown.

11.1.5.3 LIMK1 and Alzheimer's Disease (AD)

The reported effects of fibrillar and oligomeric Aβ on cultured hippocampal neurons are quite different (Heredia et al. 2006). *Fibrillar* $A\beta_{1–40}$ increases phosphorylated (active) LIMK1, phosphorylated (inactive) cofilin, and phalloidin-stainable F-actin, whereas *oligomeric* $A\beta_{1–42}$ dephosphorylates and activates cofilin, inducing rods. These differences could be due to the different concentrations used, a subset of neurons reacting to each differently, different mechanisms activated, peptide compositions, or particle size. Fibrillar Aβ is thought to act through paxillin and focal adhesions, structures that link the extracellular matrix to the actin cytoskeleton, whereas small Aβ oligomers may interact with the external portion of the amyloid precursor protein (Lorenzo et al. 2000), influencing its cleavage and the release of the transcriptionally active APP-intracellular domain (Lu et al. 2003, Galvan et al. 2002). A synthetic S3 peptide that competes with LIMK1 phosphorylation of cofilin prevents the fibrillar Aβ elevation of phospho-cofilin and the subsequent neurodegeneration (Heredia et al. 2006; reviewed in Maloney and Bamburg 2007). Fibrillar $A\beta_{1–40}$ induces a calcium increase and signals through Rac1/Cdc42 Rho GTPases with the involvement of a guanine nucleotide exchange factor, Tiam1 (Mendoza-Naranjo et al. 2007). In mice overexpressing the Swedish mutation of human APP, senile plaques of $A\beta_{1–42}$ show the deposits of cofilin but no phospho-cofilin, suggesting that in this model system, cofilin activation has occurred (Mendoza-Naranjo et al. 2007). Fibrillar $A\beta_{1–40}$ and $A\beta_{1–42}$ may have different cellular targets.

An upstream regulator of LIMK1 activity is Pak5 which through MARK also controls the phosphorylation of tau, a microtubule-associated protein (Timm et al. 2006). Hyperphosphorylation of tau leads to neurofibrillary tangles of tau, the intracellular hallmark of AD. Curiously the reduction of tau prevents behavioral deficits of mice with mutant human *App* gene (Roberson et al. 2007). Perhaps Pak5-regulated phosphorylation of cofilin increases in the absence of tau. With less activation of cofilin by Aβ, there is less chance of long-lasting rod formation, synaptic loss, and subsequent plaque generation.

11.1.5.4 Stroke

Actin dysregulation is likely to play a major role in stroke since cofilin–actin rods form rapidly in cultured hippocampal neurons subjected to chemical ischemia (<20 min after start of ATP synthesis blocking; Minamide et al. 2000). To study this phenomenon in a readily manipulated system in which neurons maintain close to their normal interconnections, rod formation was followed in organotypic hippocampal slices infected with adenovirus for expression of a cofilin–GFP (Davis et al. 2009). Four to five minutes after making the slice anoxic by sandwiching it between coverslips, the normal diffuse distribution of cofilin–GFP was transformed throughout the slice into fluorescently dense rods. The number of rods peaked in 10 min. Thus hippocampal rod formation has kinetics similar to the onset of irreversible ischemic brain injury.

Alternatively, since rods are reversible, we hypothesize that microischemic incidents are likely associated with transient rod formation which may improve the chances of mitochondrial membrane potential not falling below an irreversible threshold of apoptosis (Bernstein et al. 2006, Ankarcrona et al. 1995).

11.1.5.5 Dystonia with Dementia

A distorted or twisted posture or repetitive movements characterize dystonia, a disease of prolonged contraction of agonist and antagonist muscle groups (Yanagisawa and Goto 1971). Classical dystonia is an affliction of irregular interneuronal signaling rather than neurodegeneration (Berardelli et al. 1998). It involves primarily the basal ganglia circuit (Bhatia and Marsden 1994). However, there is a diverse set of neurodegenerative disorders that are classified as subtypes of dystonia, such as hereditodegenerative dystonia. Here patients display fundamental brain degeneration (Fahn et al. 1998).

An interesting study analyzed the brains of twins that suffered from dystonia with dementia (Gearing et al. 2002). The genetic cause of this dystonia remains unknown. The *TOR1 A* gene, which is responsible for the most severe forms of early onset dystonia, was not mutated. The twins displayed a normal karyotype, and mitochondrial DNA sequencing uncovered no irregularities. However, the twins had multiple developmental maladies suggestive of an actin-related problem in embryogenesis: cleft lip and palate, skeletal abnormalities, cataracts, blindness, and deafness. At age 12 they suffered from rapidly progressive and dopa-unresponsive generalized dystonia, characterized by motor disorder, progressing to leg dystonia by age 14, and followed by loss of fine and subsequent gross motor skills by age 15. At age 17, progressive intellectual decline and dementia were observed as the twins began to lose their ability to communicate. They died at ages 21 and 22 with no major macroscopic defects. Microscopic brain analysis revealed neither neuronal loss nor neurofibrillary tangles. It did reveal eosinophilic, ovoid, or rod-like cytoplasmic inclusions in the neocortex and thalamus. These inclusions were labeled with antibodies to cofilin but not actin. Additionally, eosinophilic spherical structures in the striatum, globus pallidus, and substantia nigra were immunostained for actin and

cofilin, but failed to stain for tau, neurofilament protein, glial fibrillary acidic protein, α-synuclein, Aβ, and APP. Within the globus pallidus and substantia nigra, some of the aggregates were rod-like. In the neocortex, oblong filament aggregations appeared sometimes associated with axonal swelling; electron microscopy revealed myelin sheath disruption. Normal individuals, as well as people with neurological diseases, alcoholics, myotonic dystrophy, patients, and aging mice, have shown similar eosinophilic, rod-like structures (Culebras et al. 1973, Fraser 1969, Kawano and Horoupian 1981, Ono et al. 1987, Pena and Katoh 1989). These structures did not appear in the nucleus as did similar rod-like structures in one case of Meige disease (Kulisevsky et al. 1988). Although the case of the twins is phenotypically distinct from all other reported cases of hereditodegenerative dystonia, this study does provide evidence for the role of cofilin and actin containing rod-like aggregates in a human neuronal dysfunction that is distinct from AD. In addition, these data support the idea that cofilin–actin aggregation contributes to neurodegenerative disease in vivo.

11.1.5.6 Other Actin-Related Changes Associated with AD

AD can be genetically caused as in familial occurrence or environmentally induced as we suggest in the case of stress induction through rod generation. Additionally, there is another mechanism for the association of AD and actin which was recently reported (Yokota et al. 2007). Suzuki et al. (2000) found only one gene that was markedly depressed in an expression profile analysis of thousands of sporadic AD-affected brains. It encodes the orthologue of rat Nap1 (Nck-associated protein 1) which maps to human chromosome 2q32 and is expressed primarily in neurons. Interestingly, Nap1 is thought to form a pentameric complex with four other proteins (WAVE, PIR121, Abi1/2, and HSPC300) that modulate actin nucleation (Suzuki et al. 2000, Baumgartner et al. 1995). Since NAP1 is selectively expressed in the developing cortical plate when neurons stop migrating and begin differentiating, it is not surprising that its loss of function mutation disrupts differentiation and its premature expression causes still migrating neurons to begin post-migratory differentiation. Whether Nap1's reduced expression level occurs at an early or late stage of AD remains to be determined.

11.1.6 Concluding Remarks

11.1.6.1 Critical, Often Overlooked Actin Cytoskeletal Properties

We have described known mutations in actin regulatory proteins or known environmental sources of direct deleterious effects on actin itself that can lead to disease. There are several well-documented processes dependent on actin which one can imagine, if disrupted by mutation or a stress, would be so profound as to be pathological. We end with an overview of these processes to sharpen the reader's

awareness of the importance of actin in healthy neuronal function and to peak his/her imagination.

1. *Nuclear functions*. The cytoplasmic functions of actin have been a subject of investigation far longer than its nuclear ones, but it is now clear that these too are fundamentally important. Nuclear actin is 3–6% of the total interphase nucleus protein and subserves vital functions such as chromatin remodeling, gene transcription by all RNA polymerases, and transport and maintenance of nuclear structure (Zhu et al. 2004, Percipalle and Visa 2006). The importance of the fine-tuning of G-/F-actin equilibria has been suggested by several recent findings: (1) actin-binding proteins that stabilize and nucleate actin facilitate elongation mediated by RNA polymerase II and (2) a myocardin family transcriptional co-activator (MAL) for serum response factor requires binding to G-actin to exit the nucleus and release from G-actin binding to accelerate entry into the nucleus (Vartiainen et al. 2007, Posern and Treisman 2006). Actin is both an upstream regulator of transcription and a downstream effector because many of the targeted genes are actin regulatory proteins.
2. *Membrane cholesterol.* An increase in cell adhesion and a decrease in membrane diffusion constant follow cholesterol depletion but only if the cytoskeleton remains intact (Sun et al. 2007a). Because cholesterol is known to affect β-secretase activity (Frears et al. 1999) and high serum cholesterol indicates increased midlife AD risk (Solomon et al. 2007), this cholesterol finding may have specific involvement in AD. It also has broad implications for a wide range of brain function.
3. *Potassium channels.* The actin cytoskeleton corrals Kv.2.1 potassium channels (Tamkun et al. 2007) whose activity is regulated by cortactin, an actin-binding protein (Williams et al. 2007). These channels are of heightened interest because of their newly discovered property of facilitating transmitter release through a direct interaction with a t-SNARE [soluble NSF (*N*-ethylmaleimide-sensitive factor) attachment protein receptor], a component of vesicle release machinery (Singer-Lahat et al. 2007). These channels were previously thought to affect neurotransmitter release only through their ion-conducting influence on membrane potential.
4. *Ca^{2+} homeostasis.* The actin cytoskeleton mediates the control of Ca^{2+} release from intracellular stores such as those controlled by inositol 1,4,5-triphosphate (Shin et al. 2000).
5. *Membrane fluidity.* Polymerization of actin, linked to the membrane by PIP_2-NWASP, can separate two coexisting liquid phases in membranes initially homogeneous, demonstrating an *active* role for actin in membrane organization and suggesting another widespread influence of actin assembly regulation (Liu and Fletcher 2006).

The above five examples should indicate the enormous possibility for disease that exists if actin, itself, or any one of the myriad proteins that regulate actin assembly or binding is mutated. The known examples of the dependence of specific diseases on

proper regulation of actin dynamics strongly suggest that any abnormal functioning of an actin regulatory protein has the likely potential of inducing a disease state.

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Chapter 12
Signal Transduction Pathways: From Receptor to the Actin Cytoskeleton

Catherine Irene Dubreuil and David L. Van Vactor

Abstract In order to make functional connections in the developing nervous system, neurons must be actively guided through the extracellular environment to reach their appropriate targets. The growth cone, a motile structure located at the tip of axons, responds to extracellular guidance cues that can act as either attractants or repellents thereby steering the axon. As in motile non-neuronal cells, motility is achieved through actin and microtubule cytoskeletal rearrangements. The driving forces in growth cone advance, turning, and retraction all require changes in actin dynamics (Tessier-Lavigne and Goodman 1996, Dent and Gertler 2003, Dontchev and Letourneau 2003, Huber et al. 2003). Rho GTPases are proteins that have been shown to be principal actin regulators in both neuronal and non-neuronal cells (Mackay and Hall 1998, Burridge and Wennerberg 2004, Govek et al. 2005, Hall 2005, Jaffe and Hall 2005). These GTPases can regulate actin through effectors that can directly bind to or activate more downstream actin-binding proteins (Van Aelst and D'Souza-Schorey 1997, Takai et al. 2001). Activation of Rho GTPases can thereby coordinate actin dynamics by enhancing polymerization or by inhibiting monomer binding to filaments as well as by regulating actin disassembly and acto-myosin contractility. These GTPases have been shown to be essential in guidance decisions and, through their effects on actin and microtubules, orchestrate directional motility in growth cones.

Keywords Rho GTPase · Ephrin · Netrin · Slit · Myelin · Semaphorin actin cytoskeleton · Growth cone

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12.1 Rho GTPase Effectors: Signaling Pathways Induced by Activated GTPases to Regulate Actin

Rho GTPases are ubiquitously expressed proteins of the Ras superfamily that cycle between an active GTP-bound state and an inactive GDP-bound state and are best characterized for their roles in regulating actin. They have been shown to be involved in most intracellular processes that require changes in actin dynamics such as cell migration, cytokinesis, exocytosis, and endocytosis but have also been shown to regulate microtubule dynamics and even activation of other mechanisms such as transcription (Aznar and Lacal 2001, Li et al. 2002b, Burridge and Wennerberg 2004, Raftopoulou and Hall 2004, Govek et al. 2005, Hall 2005, Jaffe and Hall 2005, Watanabe et al. 2005). The three most studied Rho GTPases are RhoA, Rac, and Cdc42. In non-neuronal cells, active RhoA had been shown to lead to stress fiber and focal adhesion formation and acto-myosin contraction, whereas activated Rac and Cdc42 induce lamellipodial and filopodial formation, respectively. Rho GTPases are themselves regulated by three distinct protein families: GEFs (guanine nucleotide exchange factors), GAPs (GTPase-activating protein) and GDIs (guanine nucleotide dissociation inhibitors). In order to activate Rho GTPases, GEFs catalyze GDP release and induce GTP binding, thereby switching on the GTPase so that it can subsequently interact with its effectors (Rossman et al. 2005). Rho GTPases are inactivated upon GTP hydrolysis. Although GTPases have the weak intrinsic capacity to hydrolyze GTP to GDP, GAPs promote this reaction by increasing the hydrolysis frequency by a factor of 10^5 (Bernards 2003, Moon and Zheng 2003). As with GAPs, Rho GDIs define a family of regulators that also ensures Rho inactivation (DerMardirossian and Bokoch 2005, Dovas and Couchman 2005, Dransart et al. 2005). GDIs interact mainly with the inactive GDP-bound GTPases and inhibit GDP dissociation. GDIs also inhibit Rho activation by GEFs, inactivation by GAPs, and block GDP to GTP exchange by inhibiting GTP hydrolysis. GDI binding to Rho induces a Rho–GDI cytosolic complex by extracting it from the membrane, thereby sequestering the GTPase. Upon receptor stimulation this complex is translocated to the cell membrane, where the GTPases can then be activated. Therefore GDIs not only regulate Rho GTPase activity but also regulate their subcellular localization (Sasaki and Takai 1998, Olofsson 1999).

12.1.1 RhoA

RhoA signaling can lead to actin polymerization, depolymerization, and acto-myosin contraction. These different effects are achieved by activation of multiple effectors (Fig. 12.1a), the best characterized being Rho-associated kinase ROCK (or p160ROCK) and a member of the formin homology family mDia (for reviews on Rho GTPase signaling pathways, see Van Aelst and D'Souza-Schorey 1997, Takai et al. 2001, Burridge and Wennerberg 2004, Govek et al. 2005, Hall 2005, Bustelo et al. 2007). ROCK is a serine–threonine kinase that interacts with GTP-bound

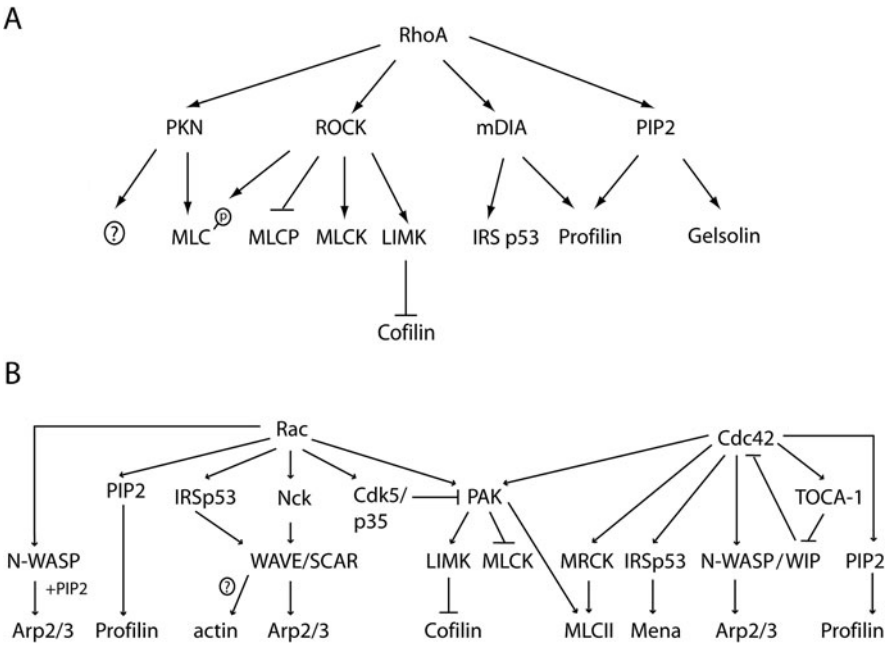

Fig. 12.1 Schematic of the best characterized Rho GTPases signaling pathways leading to actin reorganization. (**a**) RhoA signaling pathways. RhoA can induce actin remodeling by activating actin-binding proteins and by influencing acto-myosin contractility through different subsets of effector proteins. (**b**) Rac and Cdc42 pathways. Rac and Cdc42 activate common and distinct pathways leading to actin remodeling. Both Rac and Cdc42 can activate effector PAK, Rac, and Cdc42 can also induce actin remodeling through well-established pathways via effectors WAVE and WASP, respectively

active RhoA and induces acto-myosin contraction through phosphorylation of MLC (myosin light chain) and MLCP (myosin light chain phosphatase), thereby ensuring MLCP inactivation. MLC phosphorylation induces myosin II ATPase activity and promotes its association to actin filaments. Two other less studied RhoA effectors PKN and citron kinase can also stimulate MLC phosphorylation, myosin activation, and contraction. ROCK has also been shown to induce actin filament stabilization (important for myosin contraction) by its effects on the actin-severing protein ADF/cofilin (Fig. 12.1a). Cofilin-dependent actin severing leads to an increase in uncapped ends that may serve as actin polymerization sites; however, cofilin can also induce dissociation of actin monomers, thereby inducing depolymerization (dos Remedios et al. 2003). ROCK's effects on cofilin are mediated by LIMK. RhoA activation of ROCK leads to ROCK-dependent LIMK phosphorylation; LIMK can then phosphorylate and inactivate cofilin, thereby stabilizing the actin filaments.

The formin family member p140mDia stimulates actin polymerization. Once activated by GTP-bound RhoA, mDia can bind profilin and IRSp53 (Fujiwara et al. 2000), leading to actin nucleation and assembly. RhoA can also affect profilin through the phosphoinositol family proteins by stimulating PIP2 synthesis

(or PI4,5p2: phosphatidylinositol 4,5-bisphosphate) that in turn can bind directly to actin interactors such as profilin and the actin-severing and actin-capping protein gelsolin (Fig. 12.1a). PIP2 binding to profilin induces the dissociation of the profilin–actin monomer complex when in proximity to actin filaments, thereby facilitating incorporation of the actin monomers to the filament. PIP2 binding to gelsolin induces its dissociation from actin, thereby leading to filament extension (Lassing and Lindberg 1985, Skare and Karlsson 2002, dos Remedios et al. 2003, Hilpela et al. 2004; Fig. 12.1a).

The specific neuronal pathways activated by RhoA in neurons remain poorly elucidated, although key effectors such as ROCK have been shown to play important roles in actin remodeling in the neuronal growth cone. In neurons, activation of RhoA is mainly thought to lead to collapse through myosin contraction, but may also do so by affecting cofilin activation (through ROCK–LIMK) (Govek et al. 2005) or by effecting actin-capping proteins. However, RhoA was also shown to induce the formation and stabilization of actin structures at the base of the growth cone (Zhang et al. 2003), indicating a role for RhoA-dependent actin polymerization in neurons. The specific neuronal RhoA pathways leading to actin reorganization in response to specific stimuli will be discussed in detail in the sections on how guidance cues induce actin organization.

12.1.2 Rac

Activation of Rac induces actin polymerization through its two main effectors PAK and WAVE (Fig. 12.1b). PAK can influence both actin polymerization and acto-myosin contraction through distinct pathways. PAK activation can lead to actin reorganization through LIMK phosphorylation and inhibition of cofilin, thereby enhancing actin filament elongation. PAK can influence contraction in two ways: (1) by phosphorylating and inactivating MLCK to restrict myosin contraction and (2) by phosphorylating MLCII inducing acto-myosin contractility (Bokoch 2003, Rudrabhatla et al. 2003, Zhao and Manser 2005). Rac activation can also lead to actin filament polymerization by the activation of Arp2/3 by WAVE–SCAR proteins (Machesky et al. 1999, Suetsugu et al. 1999, Smith and Li 2004). Arp2/3 leads to actin branching by associating to pre-existing filaments. Although WAVE does not directly interact with Rac, it has been found to form a complex that includes HSPC300 and the Rac-binding proteins Nck, Nap 125, and PIR121 (Kobayashi et al. 1998, Eden et al. 2002, Govek et al. 2005). IRSp53 binding to Rac can also achieve WAVE-dependent actin polymerization (Miki et al. 2000, Miki and Takenawa 2002, Smith and Li 2004; Fig. 12.1b; Rac pathways). A recent study identified a new Rac pathway-inducing actin polymerization. It was found that Rac could activate N-WASP, an effector that was until now thought to be regulated by Cdc42, and that in the presence of PIP2, Rac enhanced the Arp2/3 actin polymerization engaged by this pathway (Tomasevic et al. 2007). However, in neurons, Rac-mediated actin polymerization can occur independently of PAK (Ng and Luo

2004) and inhibition of Arp2/3 enhances rather than suppresses axon elongation (Strasser et al. 2004). These findings suggest that Rac-induced actin polymerization may occur through a PIP2–profilin pathway or by a WAVE pathway that is Arp2/3 independent (Sasaki et al. 2000). In contrast to Rac-induced actin polymerization, Rac-dependent inhibitory effects (retraction and collapse) have been shown to be PAK dependent. Moreover, Rac signaling has been shown to be regulated by Cdk-5/p35. Cyclin-dependent kinase 5 (Cdk-5) is a cyclin-dependent, proline-directed serine–threonine kinase that was previously shown to co-immunoprecipitate with microtubules (reviewed in Shelton and Johnson 2004, Xie et al. 2006). Cdk-5 activators p35 and p39 are restricted to the CNS, and therefore Cdk-5 activation and kinase activity are predominant in neurons. Cdk-5 activity has been shown to be important in CNS development, neuronal differentiation, axon guidance, and neurodegeneration, to name a few, but is not directly involved in cell cycle progression. Cdk-5 was shown to interact with activated Rac and inactivate PAK (Fig. 12.1b; Rac pathways), thus silencing Rac–PAK signaling. This Rac autoregulatory pathway has been shown to be important in modulating Rac signaling by neuronal guidance cues. Furthermore, this regulation lends insight as to how Rac can be temporally and spatially regulated by guidance cues during different stages of collapse. This may also serve as a mechanism to ensure PAK-independent actin polymerization by Rac-activating guidance cues.

12.1.3 Cdc42

Actin polymerization by activated Cdc42 is induced through three major subsets of effector proteins: PAK, WASP, and IRSp53 (Govek et al. 2005). Activation of PAK by Cdc42 activates the same pathways as Rac, leading to cofilin phosphorylation by LIMK (Zhao and Manser 2005), which have been shown to induce both actin polymerization and growth cone collapse. Cdc42 also binds to and activates the N-WASP protein, leading to actin polymerization by the Arp2/3 complex (Millard et al. 2004, Tomasevic et al. 2007). The Cdc42/WASP pathway has been shown to be autoregulatory. N-WASP (neuronal WASP) was shown to interact with the protein WIP, which can suppress Cdc42-induced WASP activity. This mechanism is tightly regulated as Cdc42 also binds Toca-1 (transducer of Cdc42-dependant actin assembly), which will inactivate WIP, thereby promoting WASP activity (Ho et al. 2004; Fig. 12.1b; Cdc42 pathways). Cdc42 can also induce polymerization through the IRSp53 interaction with Mena (mammalian Ena) (Krugmann et al. 2001; Fig. 12.1b; Cdc42 pathways). The Ena/VASP proteins, which antagonize the capping proteins at the barbed ends of actin filaments and ensure actin polymerization into long unbranched filaments (Bear et al. 2002), have been shown to regulate growth cone filopodial dynamics (Lebrand et al. 2004).

RhoA, Rac, and Cdc42 not only use multiple effectors but also share common effectors to accomplish similar outputs (myosin contraction and actin polymerization/depolymerization). This may be due to subtleties in the regulation of actin

dynamics by these effectors. The activation of a same subset of effectors (e.g., PAK, LIMK, PIP2) may be explained by the spatial–temporal regulation of Rho GTPases; activation of the same effectors can ensure proper actin regulation by one GTPase when others are not present or are inactivated.

12.2 From Receptor to Actin: Growth Cone Guidance Cues Activate Rho GTPases to Induce Cytoskeletal Remodeling

Growth cone steering and directed motility are achieved by constant actin remodeling as well as myosin-dependant contraction in response to attractive or repellent cues (Tessier-Lavigne and Goodman 1996, Dickson 2001, Guan and Rao 2003, Huber et al. 2003). Attractive guidance cues lead to increased actin polymerization in the growth cone at the site adjacent to the attractive source, resulting in forward movement toward the attractant. Repellent guidance cues have the opposite effects in growth cones, resulting in decreased polymerization and myosin contraction at the site proximal to the repellent source. Repellent cues inhibit lamellipodial and filopodial formation on the side of contact, resulting in movement away from the negative source (Mueller 1999, Song and Poo 2001, Yu and Bargmann 2001, Gallo and Letourneau 2002, Wen and Zheng 2006, Rossi et al. 2007). Repellent guidance cues induce both collapse and axonal retraction. Growth cone collapse is characterized by the inhibition of actin polymerization and consequent inhibition of lamellipodial and filopodial formation. Axonal retraction requires F-actin bundling as well as myosin to induce contractile forces that drive retraction to pull back the axon and the growth cone (Gallo and Letourneau 2002, 2004, Luo and O'Leary 2005, Gallo 2006). Attractive and repulsive guidance cues signal through different classes of receptors that activate downstream pathways, converging on Rho GTPases to regulate actin (Dickson 2001, Huber et al. 2003, Govek et al. 2005, Chilton 2006).

This section will focus on the classic guidance cues and receptors and the best understood signaling pathways they induce. Guidance receptor signaling is context dependent and these receptors play other roles in such processes as neuronal migration, axonal outgrowth, and regeneration and apoptosis. These pathways and guidance decisions can also be modulated by calcium, cAMP, and in some cases by other guidance receptors. For further detail on these and other mechanisms, we refer the readers to excellent previously published reviews (Tessier-Lavigne and Goodman 1996, Guan and Rao 2003, Huber et al. 2003, Govek et al. 2005, Chilton 2006).

12.2.1 Semaphorin Signaling Through Plexin Receptors

Semaphorins are membrane-bound or secreted repulsive guidance cues that have been shown to mediate their effects through Rho GTPases and signal through plexin transmembrane receptors (Guan and Rao 2003, Huber et al. 2003). To

date, 30 semaphorins have been identified, all containing a conserved N-terminal domain. Sema3A does not bind plexin-A directly but induces the formation of a plexin-A1 receptor complex with the neuropilin-1 (Npn-1) and the L1 adhesion molecule receptors to induce chemorepulsion. Sema3A (first identified as collapsin-1) was the first to be associated with actin dynamics (Fan and Raper 1995) and it was later shown that Sema3A induced collapse through the activation of the Rho GTPase Rac1 but not RhoA (Govek et al. 2005). Sema3A-stimulated neurons show increased phospho-cofilin levels and the presence of dominant-negative LIMK inhibits this increase as well as growth cone collapse (Aizawa et al. 2001), indicating that Sema3A growth cone collapse may be achieved through Rac-dependent LIMK activation (by PAK) and subsequent phosphorylation and cofilin inactivation (Fig. 12.2a).

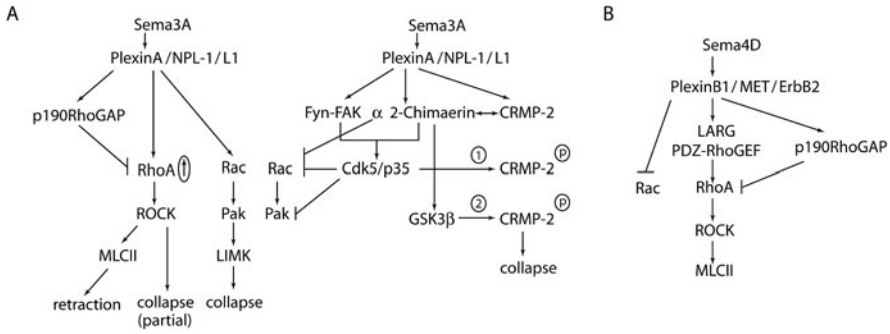

Fig. 12.2 Semaphorin signaling. (**a**) Signaling pathways induced by Sema3A via receptor complex plexin A/neuropilin/L1. Sema3A can also induce collapse via activation of the Rac and PAK to induce actin-dependent growth cone collapse. Sema3A signaling can also lead to Rac inactivation and CRMP-2 activation to induce collapse by influencing the microtubule cytoskeleton. (**b**) Sema4D-induced signaling pathways via the receptor complex plexin-B/MET/ErbB2. Sema4D induces collapse through activation of RhoA ROCK and inactivation of Rac

Other studies have linked Sema3A-dependent collapse to Fyn and Cdk5 activation. Fyn (the Src family non-receptor tyrosine kinase) can associate with and phosphorylate the cytoplasmic plexin-A2 domain. Cdk5 was also shown to associate with the plexin–neuropilin complex in the presence of activated Fyn to induce collapse (Sasaki et al. 2002). As Cdk5 can interact with Rac and inhibit PAK activity, Sema3A may also negatively regulate Rac signaling through a Fyn/Cdk5 pathway (Fig. 12.2a, left). Interestingly, transient Rac inactivation was shown to precede Sema3A collapse (Jurney et al. 2002); Sema3A may achieve this through Cdk5 activation. Together these data show two Sema3A-dependent collapse pathways that converge on Rac, leading to insight on how this guidance cue may temporally regulate Rac activation during growth cone collapse.

Sema3A has also been shown to induce collapse in Rac-independent pathways through the phosphorylation of CRMP-2 (Goshima et al. 1995, Arimura et al. 2000,

Brown et al. 2004). CRMP-2 can bind to actin and microtubules, but phospho-CRMP-2 has been shown to preferentially bind actin. This leads to its dissociation from tubulin and produces a subsequent decrease in microtubule integrity. These results suggest that Sema3A chemorepulsion can also be achieved by influencing microtubule dynamics. Sema3A regulation of CRMP-2 is dependent on both α2-chimaerin, a Rac GAP, and Cdk-5. CRMP-2 was shown to bind α2-chimaerin and both these proteins can associate with the activated plexin-A receptor (Brown et al. 2004). This association was shown to be required for collapse, as mutation in the GAP domain of α2-chimaerin inhibits growth cone collapse in DRG neurons. Moreover, α2-chimaerin can associate with both Cdk5/p35 and Gsk3β; these two proteins then sequentially phosphorylate CRMP-2, inducing collapse (Brown et al. 2004; Fig. 12.2a, right). The role of α2-chimaerin and Cdk5 in Sema3A-dependent repulsion may be twofold; first, they may both serve to inactivate Rac in the early stages of collapse and second to induce CRMP-2-dependent microtubule reorganization required for collapse. Because both α2-chimaerin, a Rac GAP, and Cdk5 can silence Rac activation and signaling, these data indicate that Sema3A effects on microtubules are Rac independent. It is of interest to note that the CRMP-2 phosphorylation induced by Sema3A is also ROCK (RhoA pathway) independent (Arimura et al. 2000). In this context, CRMP-2 phosphorylation may or may not be dependent on Cdc42 or other Rho GTPases. Together, these data show that Sema3A can induce growth cone collapse by synergistically affecting both the actin (via Rac–PAK–LIMK–cofilin) and the microtubule (via CRMP-2) cytoskeleton.

The above-described data support a role for Rac in Sema3A signaling; however, recent evidence also shows a role for RhoA in Sema3A growth cone collapse and retraction. Strong evidence for this came from a study showing that Sema3A induces RhoA mRNA upregulation (not ROCK or Rac) in axons and growth cones. Local translation of these transcripts was shown to be required for Sema3A growth cone collapse (Wu et al. 2005). In a separate study, RhoA activation was shown to be involved in Sema3A-induced neurite retraction (Gallo 2006). RhoA retraction, but not collapse, was dependent on MLCII phosphorylation by ROCK (Fig. 12.2a, left). Interestingly inhibition of either RhoA or ROCK also partially prevents Sema3A growth cone collapse in a myosin-independent manner (Gallo 2006). The RhoA pathway involved in collapse may be due to ROCK activation of effectors such as LIMK, but not MLCII, as is the case for Sema3A-induced neurite retraction.

Sema3A-dependent growth cone collapse was shown to be followed by axonal retraction (Gallo 2006). These results may reconcile the divergent data on whether Sema3A activates RhoA or Rac to induce collapse. Initial collapse is Rac independent and may require RhoA and ROCK (MLCII independent). Later stages of collapse may thereby be mainly dependant on Rac activation (PAK–LIMK) and the subsequent retraction dependent on RhoA, ROCK, and MLCII. Together, these data indicate that guidance cues can control growth cone navigation by coordinated temporal activation of multiple Rho GTPases that act synergistically and sequentially.

Sema4D also induces growth cone collapse through the plexin-B receptor. This is achieved through the activation of RhoA. Plexin-B1 has been shown to be

associated with the tyrosine kinase receptor MET (scatter factor 1/hepatocyte growth factor receptor) to mediate Sema4D repulsion (Giordano et al. 2002) as well as with the transmembrane tyrosine kinase ErbB2 (Swiercz et al. 2004). Sema4D induces the phosphorylation and activation of ErbB2 and dominant-negative ErbB2 blocks Sema4D-dependent RhoA activation and hippocampal growth cone collapse. Plexin-B can interact with both RhoA and Rac; however, Rac binding to the receptor inhibits it from interacting with its effectors, thereby sequestering it and preventing Rac-dependent signaling (Driessens et al. 2001, Vikis et al. 2002). Although plexin-B can bind directly to RhoA in *Drosophila* (Hu et al. 2001), this does not seem to be the case in vertebrates, where the receptor interacts with Rho GEFs, PDZ–Rho GEF, and LARG to activate the GTPase (Aurandt et al. 2002, Hirotani et al. 2002, Perrot et al. 2002, Swiercz et al. 2002). In hippocampal neurons, dominant-negative PDZ–Rho GEF prevents Sema4D growth cone collapse (Swiercz et al. 2002). Moreover, plexin-B clustering in fibroblast was shown to increase actin–myosin assembly (Driessens et al. 2001), indicating that plexin inhibition may also be dependent on Rho activation of MLC (Fig. 12.2b).

In cells expressing both PDZ–Rho GEF and plexin-B, constitutively active Rac can induce the phosphorylation of plexin-B in a Sema4D-dependent manner (Swiercz et al. 2004). These data indicate that although Sema4D does not seem to mediate repulsion through Rac, Rac can modulate the receptor (Swiercz et al. 2004). Moreover, a study in differentiated PC-12 cells also identified a Rho GAP important in Sema4D growth inhibition: p190RhoGAP associates with plexin-A and -B and p190RhoGAP knockdown significantly decreases neurite length (Barberis et al. 2005). As p190 Rho GAP can also interact with plexin-A, it may mediate RhoA inactivation in response to Sema3A during Rac-dependent collapse.

12.2.2 Slit Signaling Through Robo Receptors

Slits are repulsive guidance cues that bind the Robo (Roundabout) transmembrane receptor. Slits are best characterized for their role as a repellent cue at the CNS midline. The Robo receptor intracellular domains have conserved CC (0–3) motifs that are important in mediating Slit repulsive effects (Guan and Rao 2003, Huber et al. 2003). Slits have been shown to mediate their effects through Robo and the actin-interacting protein Ena (Bashaw et al. 2000). Ena can bind the CC1–CC2 domain of Robo, thus inducing growth cone collapse. The precise mechanism by which Ena contributed to repulsion is currently unknown. As Rho GTPases have been linked to Ena and Slit signaling, they may play a part in their activation but this remains to be examined. The involvement of Ena in Slit repulsion is paradoxical as it typically functions to drive actin polymerization and filament extension and it remains to be understood how it contributes to collapse.

Slit-mediated repulsion has also been linked to the modulation of Cdc42, Rac, and RhoA. Slit signaling negatively regulates Cdc42 to induce repulsion. Slit was shown to inhibit Cdc42 and cells expressing constitutively activated Cdc42 are

insensitive to Slit repulsion. The Slit-dependent Cdc42 inactivation is due to the Slit–Robo GAP (srGAP). Robo was found to associate with srGAP at the intracellular CC3 motif upon Slit stimulation. Moreover, dominant-negative srGAP prevents Slit repulsion and inhibits Cdc24 inactivation (Wong et al. 2001). Together these results indicate that Slit through the Robo receptor can induce repulsion through inactivation of Cdc42 by srGAP (Fig. 12.3).

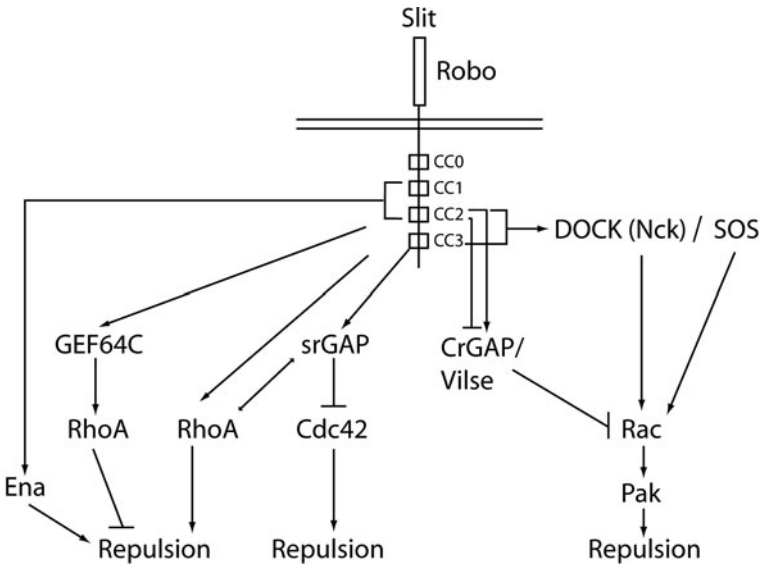

Fig. 12.3 Signaling pathways induced by Slit signaling through the Robo receptor. Slit induces actin-dependent growth cone collapse by inactivation of Cdc42 and activation of Rac and RhoA. Both Rac and RhoA can be regulated by GEFs activated by this signaling pathway

More recent evidence indicates that Slit can modulate the activation of both Rac and RhoA to mediate repulsion. The role of activated Rac in Slit repulsion is becoming clearer with the emergence of several Rac regulators and effectors in this pathway. In *Drosophila*, Slit was shown to recruit adaptor protein DOCK (vertebrate homologue Nck) as well PAK to Robo, and activate Rac leading to collapse (Fan et al. 2003). Furthermore, in Robo mutants (mutations in the CC2 and CC3 domains) that are not able to bind DOCK, no PAK recruitment to the receptor or increases in Rac activity were observed. Together, these data suggest a pathway by which Slit through Robo (CC2/CC3 domains) recruits DOCK and PAK to activate Rac and induces repulsion (Fig. 12.3, right).

Recent studies have focused on the roles of specific Rac regulators in the Slit-induced chemorepulsive pathways. The Rac GEF Son of sevenless (Sos) and the Rac GAP CrossGAP/Vilse (crGAP/Vilse) have been identified in Slit-induced repulsion (Lundstrom et al. 2004, Hu et al. 2005, Yang and Bashaw 2006). Sos was shown to be enriched in axons and interact with the Robo receptor. In vivo, Sos

can act as a Rac-specific GEF but may also exert slight effect on RhoA as both dominant-negative Rac and RhoA show increased guidance defects in Sos null species arguing for a role for activated RhoA in Slit-mediated repulsion. Sos was shown to be recruited to the plasma membrane and form a protein complex with Robo and DOCK, and Sos GEF activity toward Rac was shown to be required for Slit repulsion through Robo (Yang and Bashaw 2006). Together, this suggests a pathway where Slit positively regulates Rac activation to induce collapse by inducing the formation of a protein complex made up of Robo, DOCK/Nck, Sos, Rac, and PAK (Fig. 12.3, right). The proteins activated downstream of PAK in this pathway are yet unknown, but are likely to include LIMK–cofilin.

Two groups simultaneously identified CrossGAP/Vilse (crGAP/Vilse) as a Rac GAP involved in Slit chemorepulsion. Both groups found that crGAP/Vilse was a Rac GAP that could interact with Robo (at the CC2 motif). However, Lundstrom and colleagues (2004) found that Vilse mutants showed the same phenotypes as Slit/Robo mutants, suggesting that crGAP/Vilse activity induced repulsion through potential inactivation of Rac. In contrast, Hu and colleagues (2005) found that overexpression of crGAP/Vilse inhibits repulsion in the same way as inhibition of either Robo or Rac, suggesting that crGAP/Vilse is antagonistic to Slit/Robo repulsion. The authors argue that Robo downregulates crGAP/Vilse activity to induce Rac activation. The Bashaw group later showed that the Sos Rac GEF has opposing effects to those of crGAP/Vilse in Slit repulsion, further supporting a role for Robo inhibition of crGAP/Vilse (Yang and Bashaw 2006). Nevertheless, these data are not mutually exclusive. Rac inactivation to induce Slit repulsion may be cell type specific. This idea is supported by data showing that inhibition or activation of Rac can suppress Slit–Robo repulsion in different cell types. This GAP may also serve to inactivate Rac when RhoA activation mediates Robo repulsion (see below). Lastly, this GAP may serve to temporally regulate Rac during the different phases of collapse and retraction.

The role for RhoA in Slit-mediated repulsion is the least characterized in this pathway. Nevertheless, there is some evidence suggesting that activated RhoA can induce Slit-dependent repulsion. In a study showing the role of activated Rac in Slit repulsion, both dominant-negative Rac and RhoA (to a lesser extent than Rac) were shown to enhance (i.e., make the mutant phenotype stronger) Robo null or Slit–Robo heterozygote phenotypes, indicating that RhoA may also have a role in Slit repulsion through Robo (Fritz and VanBerkum 2002, Fan et al. 2003). Additionally, srGAP can interact with RhoA (but to a lesser extent than Cdc42) and, unlike for Cdc42, Slit stimulation leads to a decreased interaction between srGAP and RhoA (Wong et al. 2001). However, as srGAP does exert GAP activity toward RhoA, it may also negatively regulate Rho in cell type-dependant contexts or during the different phases of collapse and retraction (Hu et al. 2005). Another study indicates that GEF64C, a RhoA-specific GEF, can overcome Robo repulsion if overexpressed (Bashaw et al. 2001), indicating that RhoA may also negatively regulate Slit–Robo signaling.

Slit repulsion through Robo receptor is complex and tightly regulated. This guidance cue coordinates the activity of RhoA, Rac as well as Cdc42 to mediate

repulsion. It remains to be determined if these events are sequential or simultaneous, if Rho GTPase activation is temporally regulated over different phases of collapse and retraction, and if RhoA and Rac have the same or distinct functions in Slit repulsion.

12.2.3 Netrin Signaling Through UNC and DCC Receptors

Netrins are secreted guidance cues that are important attractants for spinal commissural neurons to the floor plate. Depending on the receptors activated, netrins can induce both attractive and repulsive effects (Fig. 12.4). Netrins can bind both DCC and UNC receptors. Signaling through the DCC–UNC5 receptor complex leads to repulsion, whereas activation of DCC alone leads to attractive responses (Huber et al. 2003, Barallobre et al. 2005, Garbe and Bashaw 2007, Round and Stein 2007). The ligand-dependent association of UNC-5 and DCC's cytoplasmic domains has been shown to mediate long-range repulsion. Short-range repulsion can be achieved independently of DCC as shown by experiments where expression of UNC-5 cytoplasmic domain is sufficient to induce repulsive effects.

In DCC-expressing cells, netrin-1 has been shown to activate both Rac and Cdc42 to induce netrin attraction (Li et al. 2002a, Shekarabi and Kennedy 2002). In the same conditions, netrin attraction was RhoA independent (Li et al. 2002a). DCC

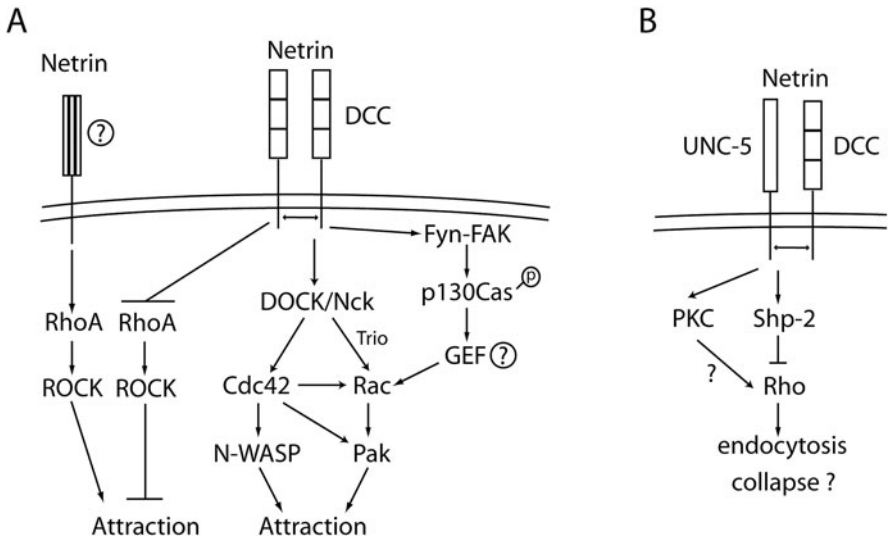

Fig. 12.4 Signaling pathways activated by netrins. (**a**) Netrin induces attractive responses via the activation of DCC receptors. Netrin-dependent attraction through DCC is achieved by the activation of Cdc42 and Rac and their effectors N-WASP and PAK. Netrin can also induce attraction via the activation of RhoA through an unknown receptor. (**b**) Netrin-mediated repulsive effects via the UNC-5/DCC receptors. These events are dependent on Shp-2 and PKC

was shown to associate with the adaptor protein Nck in commissural neurons leading to Rac activation (Li et al. 2002a). In mammalian (rat) commissural neurons, netrin stimulation induces Rac, Cdc42, Pak, and N-WASP to complex with DCC and Nck leading to the activation of these four proteins. Activation of Rac and Cdc42 in these cells was found to regulate growth cone expansion (defined as increased growth cone surface and filopodia) through PAK and N-WASP in response to netrin (Shekarabi et al. 2005). In this experimental setting, Cdc42 was shown to bind both PAK and N-WASP and to activate Rac, suggesting that Cdc42 is upstream of Rac in this netrin-dependent pathway (Fig. 12.4a).

DCC has been previously shown to interact with the Src family tyrosine kinase Fyn and focal adhesion kinase FAK to induce netrin-dependent attraction (Li et al. 2004, Liu et al. 2004, Ren et al. 2004). Liu and colleagues have recently linked this pathway to Rac and Cdc42 (Liu et al. 2007). They show that DCC can interact with and phosphorylate FAK, Fyn, and $p130^{CAS}$ (a known FAK interactor) upon netrin stimulation. $P130^{CAS}$ knockdown abolished netrin attraction in commissural neurons, both in vitro and in vivo, indicating a role for $p130^{CAS}$ in netrin-attractive signaling. Moreover, $p130^{CAS}$ was shown to be downstream of FAK and Fyn since FAK was shown to be required for $p130^{CAS}$ phosphorylation. Activation of $p130^{CAS}$ was also shown to be upstream of Rac and Cdc42 activation, as inhibition of the protein blocks GTPase activation (Fig. 12.4a). However, $p130^{CAS}$ does not directly activate the GTPases, suggesting that it signals to activate GEFs or inactivate GAPs in this pathway. It is probable that the DCC activation of FAK–Fyn and $p130^{CAS}$ serves to activate specific GEFs leading to Rac activation (possibly Trio) in a pre-existing protein complex made up of Nck, Cdc42, Rac, and effectors PAK and N-WASP.

There is some evidence showing a possible role for RhoA in both netrin-induced attraction and repulsion. Recent findings indicate that netrin can also mediate attraction through a DCC-independent pathway that is dependent on RhoA and ROCK activation but not on Rac or Cdc42 (Causeret et al. 2004; Fig. 12.4a). The neogenin receptor has been shown to mediate netrin attraction (Cole et al. 2006, Wilson and Key 2006, 2007) and recent evidence indicates that neogenin can activate RhoA- and ROCK-dependent repulsion by the ligand RGM (Conrad et al. 2007). It is possible that netrin-dependent RhoA–ROCK-mediated attraction may be achieved through the neogenin receptor. There is currently no further evidence supporting a positive role for RhoA in netrin attraction. Circumstantial evidence for a role of RhoA in netrin-induced repulsion does exist. Netrin induces repulsion through the UNC-5/DCC receptor complex (Fig. 12.4b). The protein tyrosine phosphatase SHP-2 and kinase PKCα have been shown to associate with and phosphorylate UNC-5 (Tong et al. 2001, Williams et al. 2003). UNC-5 phosphorylation by PKCα induces receptor endocytosis. Clearance of UNC-5 from the membrane was shown to be sufficient to switch from a repulsive to an attractive response, indicating that receptor endocytosis is a limiting factor in netrin repulsion. Both SHP-2 and PKC have been shown to negatively and positively regulate RhoA activation, respectively (Schoenwaelder et al. 2000, Sivasankaran et al. 2004). It is possible that regulation of RhoA by PKC and SHP-2 may mediate netrin repulsion by regulation of UNC-5 receptor

endocytosis (Fig. 12.4b). It remains to be determined if Rac and Cdc42 play a role in netrin repulsion.

12.2.4 Ephrin Signaling Through Eph Receptors

Ephrins are repulsive guidance cues that are either transmembrane or GPI-linked molecules that signal through Eph tyrosine kinase receptors (Fig. 12.5). The Eph family can be separated into two distinct categories: EphA receptors that bind GPI-linked ephrin-A and EphB receptors that bind transmembrane ephrin-B (Wilkinson 2001, Kullander and Klein 2002, Guan and Rao 2003, Huber et al. 2003, Huot 2004, Chilton 2006). Ephrin-mediated repulsion is mainly achieved through RhoA activation and Rac inhibition (Wahl et al. 2000). RhoA activation by Eph is achieved by the GEF ephexin (Eph-interacting exchange factor) (Shamah et al. 2001). Ephrin stimulates ephexin to directly bind to the receptor. Moreover, ephrin induces ephexin phosphorylation that in turn increases RhoA activity, leading to growth cone collapse (Sahin et al. 2005).

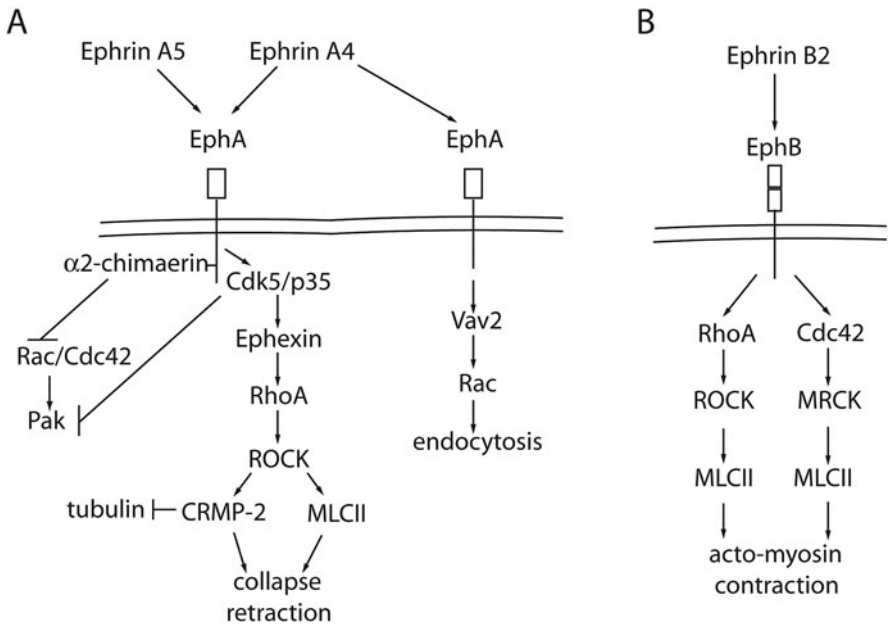

Fig. 12.5 Signaling pathways activated by ephrins. (**a**) Ephrins induce collapse and retraction through the Eph receptors via the activation of RhoA by GEF Ephexin and subsequent ROCK activation. This leads to growth cone collapse by affecting both microtubules and actin. Ephrin-dependent activation of Rac also induces receptor endocytosis. (**b**) Ephrin-B induces repulsion through the activation of MLC via both RhoA and Cdc42 activation

Recent findings have linked ephrin-mediated RhoA activation to previous data showing a role for Cdk5/p35 in ephrin-induced collapse (Cheng et al. 2003, Fu et al. 2007). Inhibition of Cdk5 was found to inhibit ephrin-A4-dependent RhoA activation and retraction. EphA4 activation recruits Cdk-5 to the receptor leading to its tyrosine phosphorylation, which enhances ephexin1 activity and RhoA activation (Fu et al. 2007). Few studies have examined ephrin-dependent repulsive signaling pathways downstream of RhoA. However, ephrin-mediated RhoA activation was shown to induce retraction through ROCK and MLCII (Fig. 12.5a; Harbott and Nobes 2005, Parri et al. 2007). In these cells, retraction was also dependent on Abl (Harbott and Nobes 2005); it remains to be determined if Abl and RhoA induce retraction in the same or distinct pathways.

The ephrin-signaling pathways described above pertain to actin dynamics, but recent finding suggest that ephrins may also induce collapse by affecting microtubule integrity. Ephrin collapse was linked to the actin- and tubulin-binding protein CRMP-2 via a RhoA–ROCK pathway. Ephrin 5 was shown to induce collapse through CRMP-2 phosphorylation by ROCK (Arimura et al. 2005). CRMP-2 can associate with both actin and microtubules; however, phospho-CRMP-2 was shown to only localize to actin (and no longer binds tubulin), whereas CRMP-2 co-localized with actin, microtubules, and clathrin-coated pits (Arimura et al. 2005). These data indicate that ephrins can induce RhoA-dependent growth cone collapse by regulating both actin (via MLCII) and microtubules (via CRMP-2) (Fig. 12.5a).

The role of Rac in the ephrin-repulsive pathways remains unclear. Three separate studies have recently placed the Rac GAP α2-chimaerin that was previously described in the semaphorin 3A pathway, in the ephrin-signaling pathway. They show that EphA4 receptor directly binds α2-chimaerin, and cells in which this GAP is absent or inactive show decreased levels of growth cone collapse upon ephrin-B3 and ephrin-A1 stimulation (Beg et al. 2007, Iwasato et al. 2007, Wegmeyer et al. 2007). Together these data indicate that the repellent guidance receptor EphA4 induces collapse in part through α2-chimaerin-dependent inactivation of Rac. These studies and those pertaining to ephexin-dependent growth cone collapse would indicate that ephrin-repellent effects are Rac independent. However, there are some studies linking activated Rac to ephrin-induced repulsion. One such study found that ephrin-A2 could transiently inactivate Rac1 followed by a period of Rac activation that correlated with collapse (Jurney et al. 2002). Furthermore, Rac inactivation blocks F-actin depolymerization and reorganization during collapse. The upstream and downstream Rac pathways activated here remain uncharacterized. This regulation may serve to ensure actin depolymerization (through Rac) during potential phases of collapse that are RhoA independent. A second link to Rac activation in ephrin collapse shows a role for activated Rac in receptor endocytosis (Jurney et al. 2002, Cowan et al. 2005). Blocking Eph receptor endocytosis induces defects in growth cone collapse, indicating that this step is necessary to mediate ephrin repulsion. Vav2, a Rho GEF, was shown to regulate EphA4 receptor endocytosis in a Rac-dependent manner (Cowan et al. 2005), strengthening a role for activated Rac in ephrin collapse (Fig. 12.5a, left).

Little is known about signaling pathways induced by transmembrane ephrin-B as opposed to those induced by ephrin-A (Fig. 12.5b). Although few studies in neurons have been performed to date, EphB has been shown to induce repulsion and cell retraction in endothelial cells in a RhoA- and Cdc42-dependent manner (Groeger and Nobes 2007). Inhibition of either GTPase only partially inhibits retraction; however, combined inhibition leads to total inhibition of retraction. In these cells, retraction was apparently due to myosin activity regulated by the combined effects of ROCK and the Cdc42 effector MRCK (myotonic dystrophy kinase-related Cdc42-binding kinase, a protein that has been shown to induce MLCII phosphorylation; Dong et al. 2002) (Fig. 12.5b) (Dong et al. 2002, Groeger and Nobes 2007). Recently, ephrin-B-dependent receptor endocytosis and repulsion was shown to involve Mena, an Ena/VASP family member, in a pathway that may be parallel to that of RhoA (Evans et al. 2007). Rac and Cdc42 may be involved in this pathway, as they have previously been linked to Ena/VASP proteins (Krugmann et al. 2001), where they may possibly act by destabilizing lamellipodia and ruffles at the leading edge of the growth cone (Evans et al. 2007).

12.2.5 Myelin-Derived Growth Inhibitory Proteins Signaling Through the NgR-p75NTR-TROY/TAj Receptor Complex

Myelin-derived growth inhibitory proteins have been best described in post-CNS injury settings pertaining to axonal regeneration studies (Bandtlow 2003, David and Lacroix 2003, He and Koprivica 2004, Schwab 2004). In vitro, these proteins inhibit axonal extension, cause neurite retraction, and lead to growth cone collapse. As they are expressed after injury (Tang et al. 2001), they are ideal target candidates to examine the regenerative block present after CNS lesions. However, not only are these proteins present in the PNS, where regeneration occurs after injury, but also are they present during neural embryogenesis, suggesting that they may also function as negative guidance cues. To date the three best characterized myelin-derived growth inhibitory proteins are MAG (myelin-associated glycoprotein), a transmembrane protein of the immunoglobulin superfamily; Nogo, a member of the reticulon family; and OMgp (oligodendrocyte myelin glycoprotein; Fig. 12.6) (He and Koprivica 2004), a GPI-linked protein that has also been shown to play a role in Ranvier node formation (Huang et al. 2005, Nie et al. 2006).

Myelin inhibitors have all been shown to bind a common receptor NgR (Nogo receptor) (Fournier et al. 2001, Domeniconi et al. 2002, Liu et al. 2002, Wang et al. 2002a). NgR is a GPI-linked protein and must therefore associate with other transmembrane receptors in order to activate inhibitory intracellular signaling cascades. The p75 neurotrophin receptor ($p75^{NTR}$) was shown to interact with and act as a co-receptor to NgR (Wang et al. 2002a, Wong et al. 2002). Subsequently, the nervous system-specific transmembrane receptor LINGO (LRR and Ig domain containing Nogo receptor-interacting protein) was also shown to interact with both NgR and $p75^{NTR}$ (Mi et al. 2004) to mediate the effects of myelin inhibitors. More recent

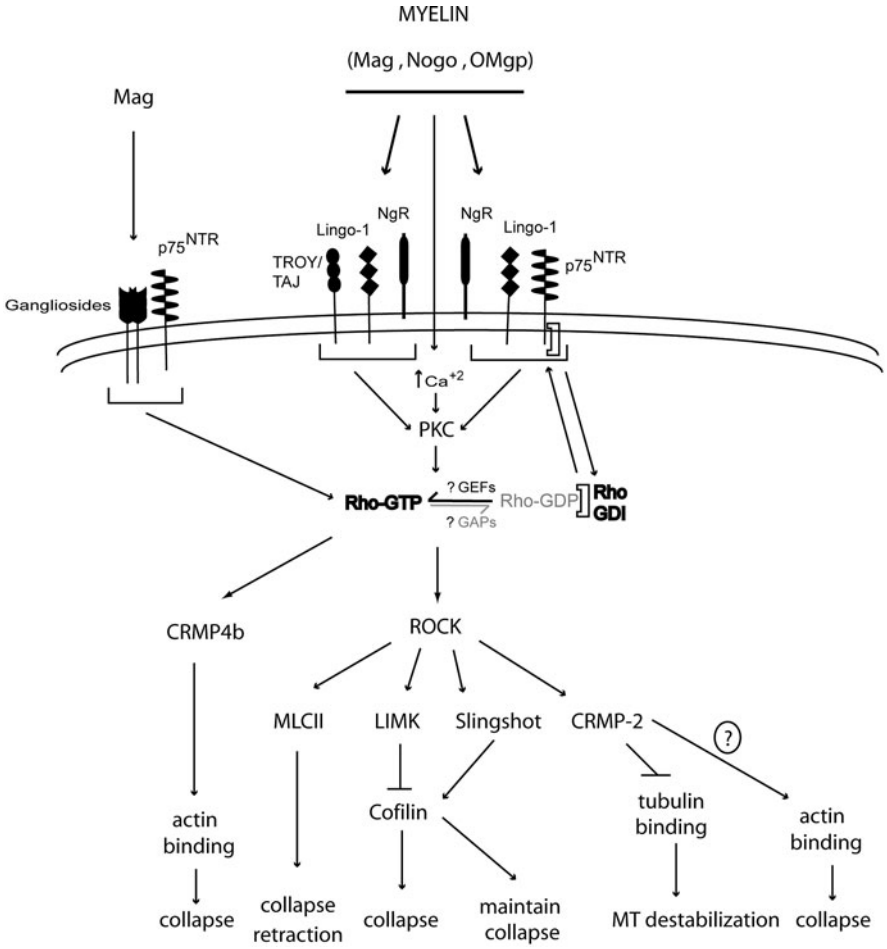

Fig. 12.6 Inhibitory signaling achieved by myelin-derived growth inhibitory proteins. Myelin inhibitors signal via receptor complexes NgR/Lingo-1/p75^{NTR} or NgR/Lingo-1/Troy-TAJ to induce collapse and retraction. Myelin inhibitors induce repulsion through the activation of RhoA

studies have also identified the TROY/TAJ receptor, a member of the TNF receptor superfamily, as being able to mediate myelin inhibitor effects in neurons devoid of p75^{NTR} (Park et al. 2005, Shao et al. 2005).

Interestingly, MAG can also induce growth inhibition via a second receptor complex containing p75^{NTR}. Before the identification of NgR, ganglioside receptors (GD1a and GT1b) were identified as receptors for MAG (Vinson et al. 2001, Vyas et al. 2002). It was later shown that in the absence of p75^{NTR}, neurons were not inhibited by MAG and that the GT1b receptor can associate with p75^{NTR} (Yamashita et al. 2002). This association was shown to be necessary to induce MAG's inhibitory effects.

Myelin-derived growth inhibitory proteins induce growth inhibition and growth cone collapse through the activation of RhoA and inactivation of Rac. Specific inactivation of the GTPase and its effector ROCK leads to neurite outgrowth in the presence of these inhibitors (Winton et al. 2002, Fournier et al. 2003). To this effect, it has been shown that both NgR receptor complexes (with either p75NTR or TROY) and the GD1b/p75NTR complex activate RhoA to signal inhibition (Wang et al. 2002b, Yamashita et al. 2002, Park et al. 2005, Shao et al. 2005). Although the specific GEFs and GAPs in these pathways still remain unknown, evidence for the modulation of RhoA activation states by Rho GDI was recently elucidated. Although $p75^{NTR}$ does not directly bind RhoA, the intracellular domain binds Rho GDI in the presence of myelin inhibitors, thereby inducing the dissociation of the RhoA–GDI complex and permitting RhoA activation (Yamashita and Tohyama 2003). PKC has also been shown to modulate the inhibitory effects of myelin by modulating RhoA activation (Sivasankaran et al. 2004). PKC inhibitors prevent RhoA activation in the presence of myelin, indicating that PKC is required for RhoA activation in this pathway. It is of interest to note that the PKC effects are calcium dependant; this suggests that myelin-dependant calcium influx can modulate growth inhibition through PKC-dependant RhoA activation.

Recent work from the Fournier group has elucidated the molecular pathways downstream of RhoA that induce myelin-dependant growth cone collapse (Alabed et al. 2006, Hsieh et al. 2006, Alabed et al. 2007). Inhibition of the RhoA effector ROCK significantly rescues myelin inhibition. Moreover, ROCK inhibition prevented Nogo-dependent increases in MLCII phosphorylation (Alabed et al. 2006), indicating that myelin can induce inhibition via a RhoA–ROCK–MLCII pathway (Fig. 12.6). In a subsequent study this group also showed that myelin inhibitors stimulate LIMK and Slingshot phosphatase activity to regulate cofilin in a ROCK-dependant manner (Hsieh et al. 2006). The authors showed that the myelin inhibitor Nogo induced early cofilin phosphorylation. Dominant-negative LIMK prevented myelin-dependant growth cone collapse as well as myelin-dependant cofilin phosphorylation. Interestingly, Nogo was also shown to sequentially activate both LIMK and Slingshot phosphatase in a time frame that correlates with Nogo-dependent cofilin phosphorylation. Moreover, inhibition of ROCK was shown to block the activation of both LIMK and Slingshot by Nogo, as well reversely influence cofilin phosphorylation states. The temporal changes in cofilin activation by myelin may correlate with its actin-severing activity; early inactivation (by LIMK) may serve to diminish actin turnover as less monomers are available to be incorporated into growing filaments and later activation (by Slingshot) may activate cofilin's actin-severing activity inducing actin filament dissolution, thereby sustaining growth cone collapse.

In a separate study, CRPM4b was found to interact with RhoA and lead to inhibition in a Nogo-dependent manner (Alabed et al. 2007; Fig. 12.6). CRMP4, an actin-binding protein that co-localizes with actin rib-like structures and promotes F-actin bundling, was previously shown to influence neurite outgrowth. Interfering RNA directed toward CRMP4 abolished myelin inhibition and induced neurite outgrowth. CRPM4b binding to RhoA was shown to be direct and phospho

dependent. Moreover, the CRMP4b–RhoA interaction does not alter RhoA binding to ROCK, and CRMP4 is not a ROCK substrate. These data indicate that RhoA could induce myelin-dependant growth cone collapse by simultaneously activating ROCK and CRMP4 pathways. The above-described pathways pertain to myelin inhibition through actin-based mechanisms. Recent data show that myelin inhibitors also induce collapse by affecting microtubules. To this effect, MAG and Nogo through RhoA activation were shown to induce ROCK regulation of CRMP-2 (Mimura et al. 2006; Fig. 12.6). Together these data show a clear role for activated RhoA in mediating myelin inhibition and present molecular signaling pathways by which this GTPase can produce inhibitory effects by acting on both actin and microtubules.

12.3 Spatial Regulation: Orchestration of Rho GTPases in the Growth Cone

This chapter has focused on the regulation of actin in the neuronal growth cone by guidance cues. There is still much work to be done in elucidating the specific pathways downstream of Rho GTPases in guidance signaling. Although it is clear that Rho GTPases regulate actin and microtubule dynamics by these cues, the majority of Rho GTPase regulators, GEFs GAPs, and GDIs involved in these pathways remain unknown. Identification of these proteins will help to understand not only the specific effects of the guidance cues but also how the activation of common effector (such as LIMK) can have differential effects on actin.

One of the most interesting questions that remains to be examined in these systems is how does the activation of the same subset of proteins (e.g. cofilin) lead to opposing effects in actin regulation This can be thought of as a question of specificity: How can inactivation of cofilin by Rac and Cdc42 induce both growth cone attraction and repulsion by guidance cues? Another question of interest is: How does one GTPase regulate both attraction and repulsion? As Rho GTPases can play on both cofilin kinases and phosphatases, it is possible that these different effects can be achieved in part by differential cofilin regulation or by GTPase spatial restriction in the growth cone.

Other key findings are now beginning to emerge pertaining to the spatial–temporal regulation of the Rho GTPases in the neuronal growth cone. It was long thought that the growth cone activation of RhoA induced collapse by regulating myosin and myosin-dependent actin retrograde flow. However, recent evidence indicates that this may not be the case. To this effect, the Forscher group showed that myosin II-dependent retrograde actin flow was not dependent on RhoA, Rac, or Cdc42. Instead, RhoA regulates growth cone collapse by stabilizing actin arcs in the transition domain of the growth cone (Zhang et al. 2003).

The idea that RhoA activation is important in transition and central domains but not at the leading edge/peripheral domain is supported by studies on RhoA ubiquitination (by SMURF1) and degradation at the leading edge of migrating fibroblast

(Wang et al. 2003). SMURF1, a protein that binds ubiquitin, is only located at the front of migrating cells. This indicates that RhoA is degraded at the leading edge, producing intracellular asymmetric distribution of RhoA thereby spatially restricting it to the rear of migrating cells. RhoA was also shown to be ubiquitinated by SMURF1 in neuronal cells, promoting axonal extension in vivo (Bryan et al. 2005). It has been proposed that the function of RhoA degradation at the leading edge may be to ensure the activation of Rac and Cdc42 by GEFs that can activate all three GTPases (Jaffe and Hall 2003). It may also serve as a measure to inhibit RhoA-dependant actin depolymerization that may impede advancement of migrating cells (Jaffe and Hall 2003) and neurons. This may not hold true in neurons however, as Rac and Cdc42 have been shown to be involved in both attractive and repulsive guidance (Fig. 12.7).

In response to guidance cues such as myelin, semaphorins, and ephrins, RhoA activation has been shown to induce neurite retraction as well as complete or partial growth cone collapse (Govek et al. 2005; see above section on guidance). If RhoA degradation induces a decrease in RhoA protein levels in neuronal growth cones, would the leftover pool of RhoA be sufficient to induce collapse? If RhoA-dependent neurite retraction could be solely due to RhoA spatial restriction to the base of the growth cone, RhoA-induced growth cone collapse may not require spatial restriction. Interestingly, a study by Wu and colleagues shows that Sema3A stimulation increases RhoA mRNA levels (but not ROCK or Rac) in axons and growth cones and that local RhoA mRNA translation is sufficient to induce growth cone collapse (Wu et al. 2005). This supports the notion that as RhoA is ubiquitinated and degraded (Wang et al. 2003, Bryan et al. 2005), there would be a need to replenish RhoA levels in neurons in order to induce collapse. RhoA upregulation was shown to be required for Sema3A repulsion (Wu et al. 2005) but it remains to be seen if other guidance cues can regulate RhoA or Rac and Cdc42 local translation to induce collapse.

Together these results indicate a strict regulation of RhoA at the mRNA and protein level in the growth cone. Therefore the role of local RhoA translation could be to spatially restrict RhoA activation and signaling to sites of RhoA translation. Together these results are the first demonstration of spatial regulation of Rho GTPases expression and are a step in explaining how pathways that have similar effects on actin are spatially segregated. These data also indicate that actin regulation at the leading edge is mostly RhoA independent, thereby indicating that Rac and Cdc42 may be solely responsible for actin remodeling at the leading edge of growth cones. Rac and Cdc42 activation would then induce both actin polymerization and depolymerization in response to attractive and repulsive cues at the growth cone periphery. Spatial limitation of Rho GTPases may not only help to understand why multiple effectors are needed to accomplish very similar outputs but also further explain how and why these GTPases can regulate both attraction and repulsion.

Other data indicate that the spatial regulation of RhoA as well as Rac and Cdc42 can be dependent upon stimulation and activation states. In imaging studies, high levels of RhoA, Rac, and Cdc42 were found in the growth cone periphery; however, RhoA activation induced RhoA activity in the neurite shaft as well as

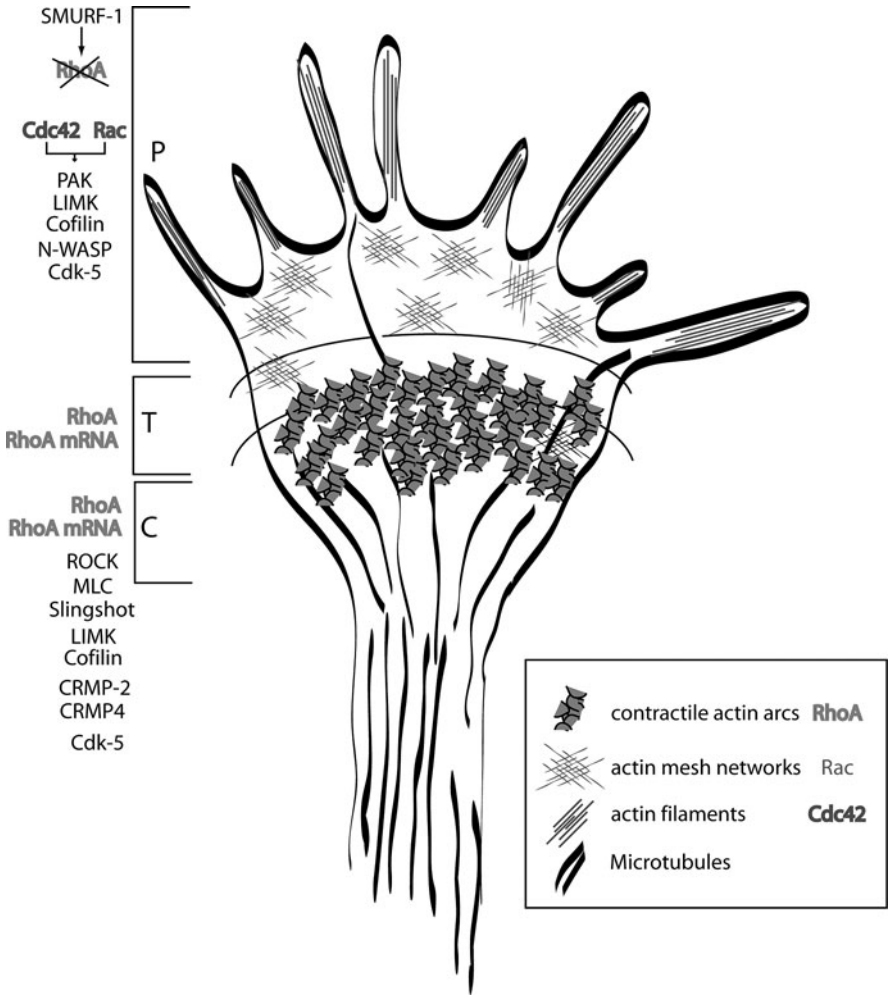

Fig. 12.7 Spatial regulation of Rho GTPases in the growth cone. Schematic representation of a growth cone illustrating the peripheral and central domains as well as the transition zone and the sub-localization of RhoA, Rac, and Cdc42 in these domains. Ubiquitination of RhoA restricts it to C domain, whereas Rac and Cdc42 are more present in the P domain

retraction (Nakamura et al. 2005). In a separate study, randomly migrating cells were shown to have increased levels of RhoA at the periphery, whereas upon PDGF-stimulated directed motility, RhoA activity was decreased and Rac activation was increased at the periphery (Pertz et al. 2006). Together these data indicate that the spatio-temporal Rho GTPase localization may also be dependent on specific stimulation and also supports the notion that RhoA activation may be spatio-temporally restricted to the base and Rac activation at the leading edge of the growth cone during specific signaling events.

The coordination of the different Rho GTPases in the growth cone to achieve either repulsion or attraction has recently shed light on the specific pathways activated by guidance cues. In order to fully comprehend the distinct roles of each Rho GTPase in the signaling pathways of each cue, it will be important to determine how they are spatially and temporally regulated during the different stages of growth cone collapse and neurite retraction and outgrowth.

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Index

Note: The letters 'f' and 't' following the locators refer to figures and tables respectively.

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