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Neurobiology of Actin

From Neurulation to Synaptic Function

Springer

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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

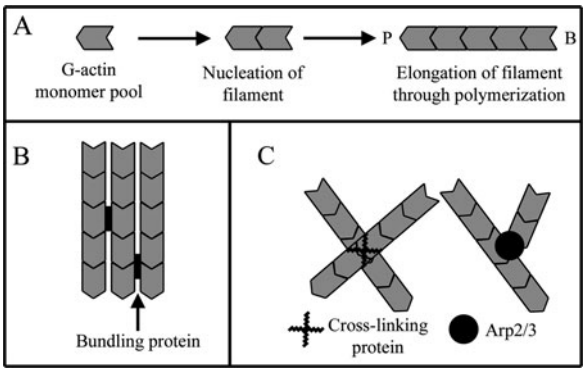

Fig. 1.1 Overview of actin filament nucleation, polymerization, and organizational themes. (**a**) Monomeric globular (G) actin in the cytosol is initially nucleated by forming a "seed" consisting of a few subunits. The "seed" subsequently serves as the basic scaffold for continued polymerization resulting 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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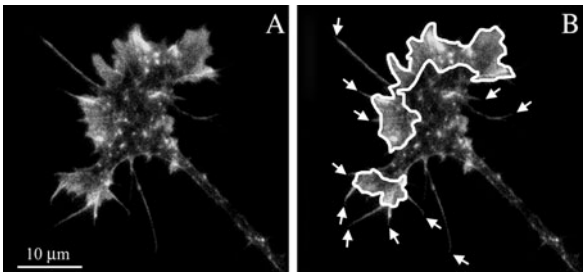

Fig. 2.1 Example of the lamellipodia and filopodia of a cultured embryonic sensory growth cone. In this image, actin filaments are shown as revealed through staining with fluorescently labeled phalloidin (**a**, **b**). **Panel b** denotes filopodial tips (*arrows*) and the lamellipodia (outlined by *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

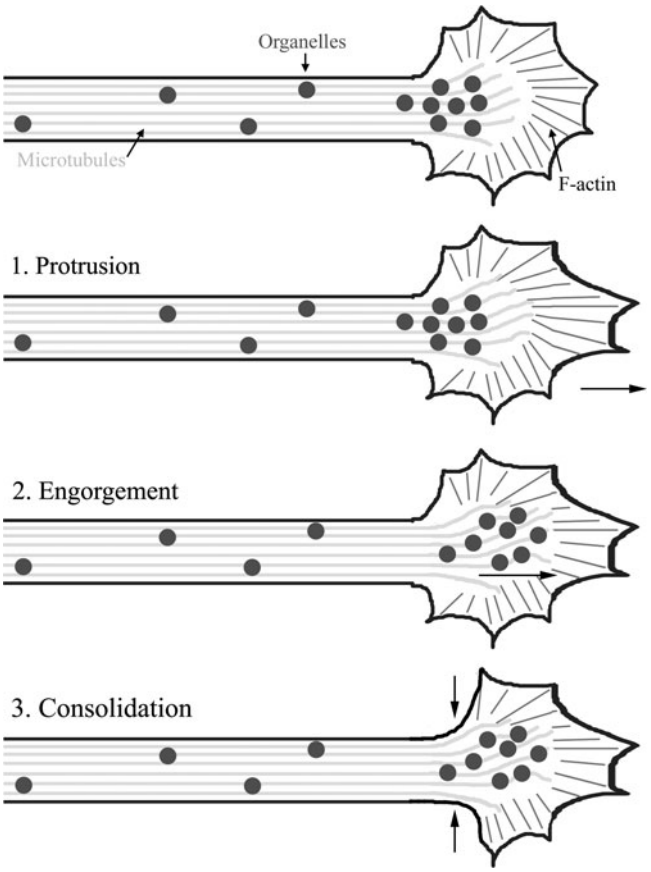

Fig. 2.2 Phases of the mechanism of axon extension. Protrusion of lamellipodia and filopodia to the leading edge of the growth cone is followed by engorgement. Engorgement occurs through the advance of microtubules and organelles into the leading edge of the growth cone. Finally, a new segment of axonal 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

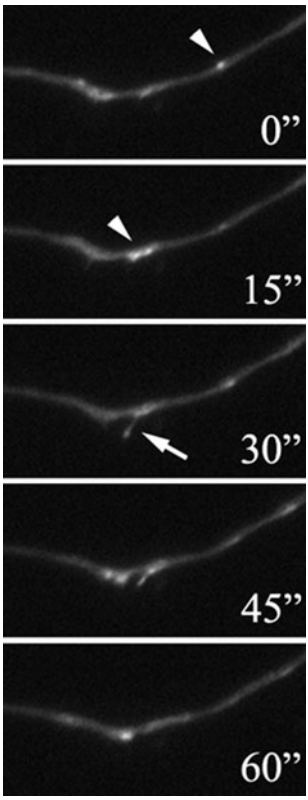

Fig. 2.3 Example of actin dynamics underlying the formation of filopodia from the axon of a cultured sensory neuron transfected with eYFP-β-actin (imaged at 3 s intervals). *Arrowheads* denote a subset of axonal F-actin patches formed during the imaging sequence. Between 15″ and 30″ a filopodial shaft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