HANDBOOK OF DEVELOPMENTAL COGNITIVE NEUROSCIENCE

Second Edition

Edited by

Charles A. Nelson and Monica Luciana

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1 The Formation of Axons and Dendrites by Developing Neurons

PAUL LETOURNEAU

Introduction

The neuronal circuitry that underlies human behavior and other neural functions develops over a prolonged period lasting from the second fetal month through adolescent years. These circuits arise from the extensive development of elaborate neuronal processes, as neurons express intrinsic morphogenetic behaviors, while interacting with other cells and molecules of the developing nervous system. First, immature neurons migrate from their birthplaces to the sites where they are organized into layers, nuclei, and ganglia of neuronal perikarya. Next, immature neurons sprout axons and dendrites that elongate, sometimes for many centimeters, to make synaptic connections with target neurons or other cells. This chapter describes intrinsic mechanisms of morphogenesis of axons and dendrites and the extrinsic environmental features that regulate where and when axons and dendrites grow to create neural circuits.

The ability to extend neuronal processes, or neurites, is intrinsic to neurons. This is demonstrated when immature neurons, such as from prenatal hippocampus, are placed into tissue culture. Within a few 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 and dendrites and form synapses in vitro. These events in a neutral in vitro environment show that the neuronal phenotype defines the intrinsic behaviors that produce neuronal shape. The most significant cellular components in neuronal morphogenesis are the protein polymers of the neuronal cytoskeleton. In the next section the neuronal cytoskeleton and the intrinsic mechanisms of neurite formation and elongation will be discussed. In the following three sections the regulation of axonal and dendritic growth by extrinsic molecules will be discussed.

The dynamic neuronal cytoskeleton

Neuronal morphogenesis depends on the organization and dynamic properties of two cytoskeletal polymers, microtubules and actin filaments (Dent and Gertler, 2003; Luo, 2002). These cytoskeletal polymers are present in all cell types, although specific mechanisms determine cytoskeletal functions in neurons.

Microtubules Provide Support and a Means of Transport Microtubules are hollow cylinders 25 nm in diameter that extend through the cytoplasm of neuronal perikarya, axons, and dendrites (figures 1.1, 1.2). The wall of a microtubule consists of subunits of highly conserved proteins, alpha tubulin and beta tubulin. Microtubules have no defined length, and single neuronal microtubules can exceed 100 μm (Letourneau, 1982). Microtubules are rigid and resist compression to support the elaborate extensions of axons and dendrites. Microtubules are also the "rails" along which organelles are transported via linkage to the motor proteins, kinesins, and dynein (Hirokawa and Takemura, 2004). These two functions, providing structural support and being rails for intracellular transport, are the functions of neuronal microtubules.

Formation of Microtubules in Cells Tubulin subunits polymerize by endwise addition to form microtubules. Because of 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 (figure 1.1 and plate 1). Microtubules in neurons are formed in the centrosomal region near the nucleus and extend throughout the perikaryon with their minus ends anchored at the centrosome. The plus ends of cytoplasmic microtubules undergo bouts of growing and shrinking called dynamic instability, in which a microtubule end may undergo rapid disassembly, either completely or partially, which is followed by "rescue" and renewed growth (Tanaka, Ho, and Kirschner, 1995).

Regulation of Microtubule Organization by MAPs In neurons, microtubule organization is regulated by a group of proteins called MAPs (microtubule-associated proteins). MAPs bind to microtubules and regulate all aspects of their organization, including assembly and disassembly, stability, and binding to neurofilaments, actin filaments, and other microtubules (Dehmelt and Halpain, 2004; Gordon-Weeks, 2000). Motor proteins, such as kinesin, bind to microtubules and move cargo toward microtubule plus ends, while dynein motors move cargo toward microtubule minus ends. The protein katanin binds microtubules and

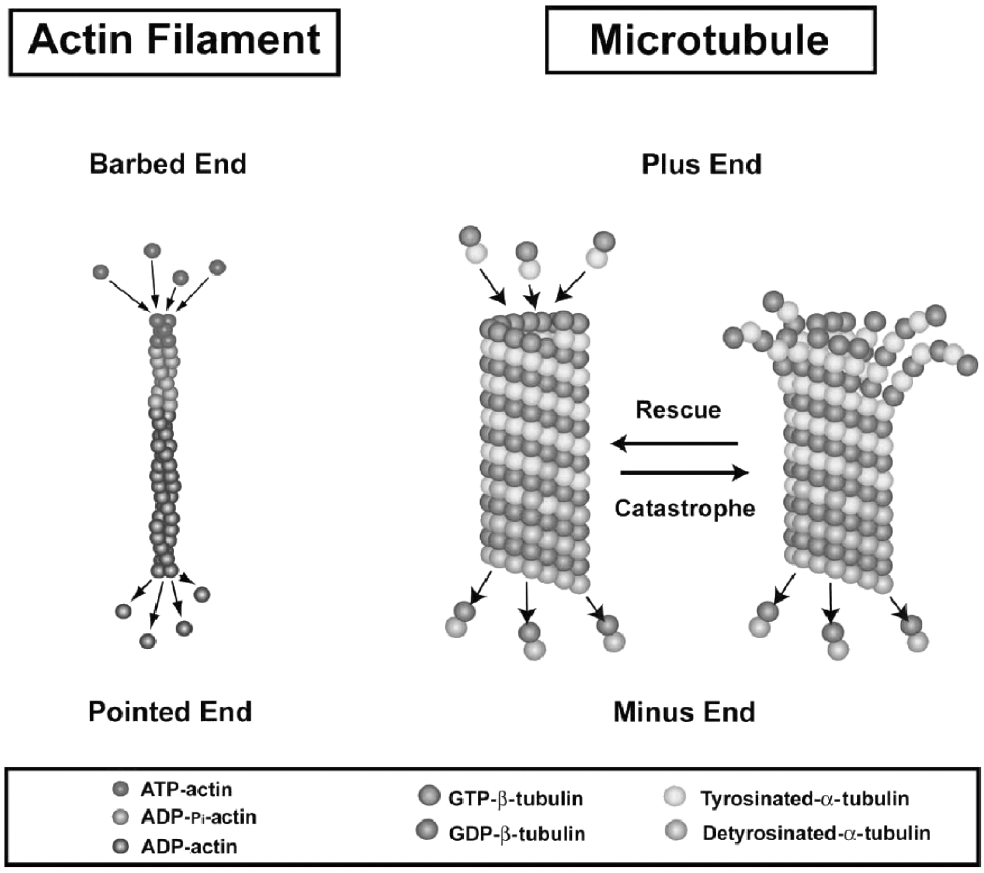

FIGURE 1.1 Actin filaments and microtubules are polarized polymers. Actin filaments are polarized polymers for which the addition of ATP-actin is more likely at the barbed end than the pointed end. After hydrolysis of ATP-actin to ADP-actin, subunits dissociate at the pointed end. Microtubules are also polarized structures with GTP-tubulin dimers adding to the plus or growing end and GDP-tubulin dimers dissociating from the minus end. Microtubules also exhibit posttranslational modifications (detyrosination shown here) that correlate with the age and stability of the polymer. (From Dent and Gertler, 2003.) (See plate 1.)

severs them, promoting reorganization of microtubules and remodeling of neuronal shape (Baas and Buster, 2004). Some maps, such as MAP2, are localized in dendrites, while other MAPs, such as tau and MAP1B, are localized in axons.

Several features distinguish microtubules in axons and dendrites. 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. Microtubules are formed at the centrosome and then transported into axons or dendrites. 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. Many axonal and dendritic microtubules are highly stable as a result of enzymatic modifications of the tubulin protein and from binding of certain MAPs.

Although microtubules must always be present to support neurites, 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, depending on microtubule length and connections with other structures. Long microtubules in axons are stationary, although their plus ends undergo considerable dynamic instability of 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 is a critical event in the morphogenesis of axons and dendrites (Tanaka and Kirschner, 1995).

ACTIN FILAMENTS IN NEURONS Actin filaments are the other important cytoskeletal components in neuronal morphogenesis (Dent and Gertler, 2003; Luo, 2002). 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, elaborate networks of actin filaments are the organizing component that drives the searching behaviors that are necessary for navigation of

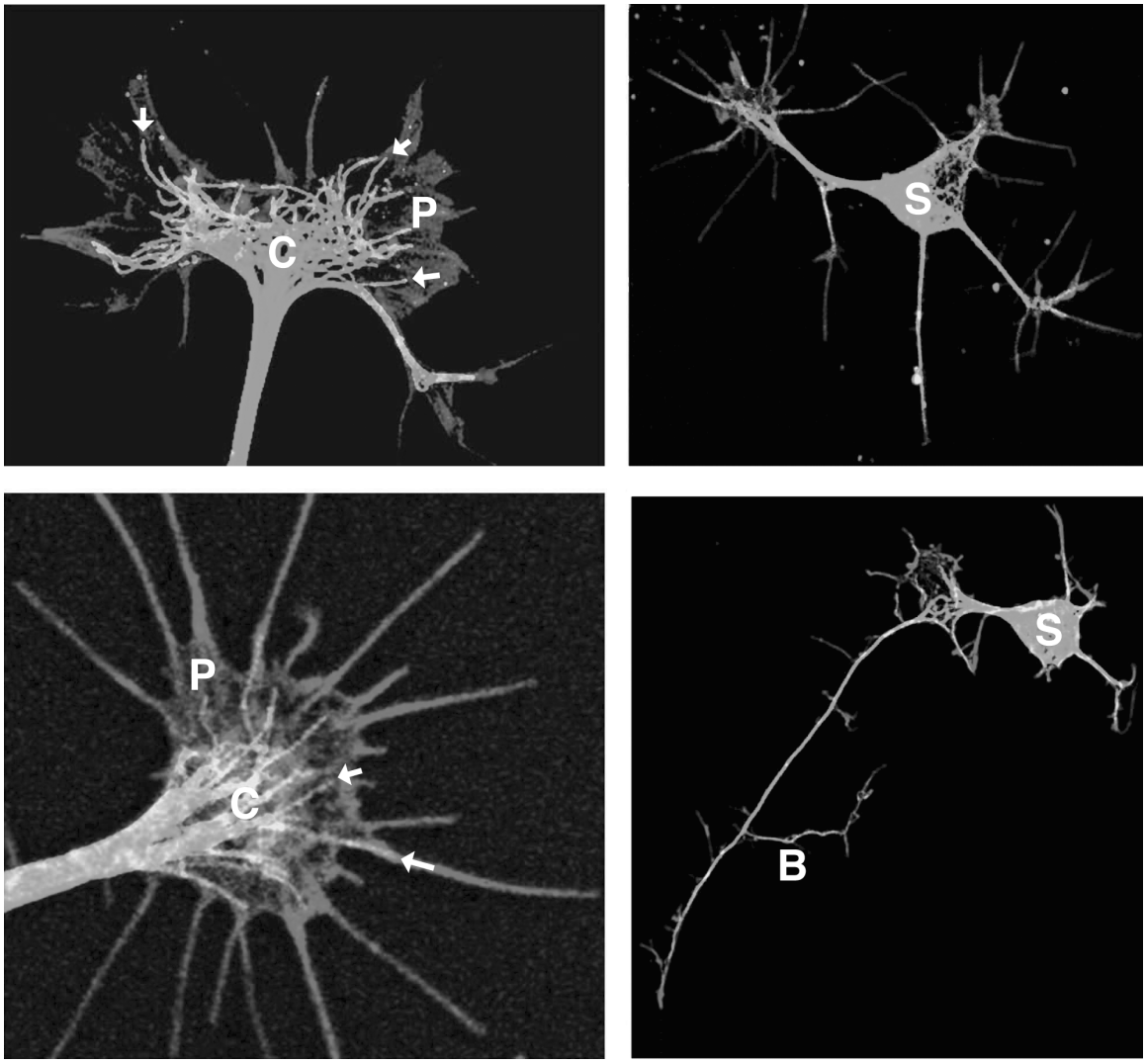

Figure 1.2 The distribution of microtubules and actin filaments in developing neurons and in axonal growth cones. Microtubules (green) are densely packed with the neuronal cell bodies (S) and are bundled in the axons and branches. Actin filaments are arrayed in filament networks and bundles in the peripheral domains (P) of the growth cones and along the shafts of the axons, where small areas of actin filament dynamics may give rise to collateral branches (B). 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). (See plate 2.)

growth cones to their synaptic targets (figure 1.2 and plate 2; Letourneau, 1979, 1983; Yamada, Spooner, and Wessells, 1971).

Organization of Actin in Cells Actin filaments are polymers of the conserved globular protein actin (figure 1.1). Actin filaments with a diameter of about 6–7 nm are individually not stiff, but bundles of actin filaments have stiffness. Unlike the cortical networks in 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, fingerlike projections, called filopodia (figure 1.2; Letourneau, 1983).

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.

Regulation of Actin Filament Organization by ABPs Actin-binding proteins (ABPs) have numerous functions (Dent and Gertler, 2003; Pollard and Borisy, 2003). One class of ABPs binds actin subunits, regulating

their availability for polymerization; other ABPs cross-link 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 to filaments. Several ABPs bind actin filaments and sever them, promoting the remodeling of actin filament arrays. 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 bind and move cargoes along actin filaments. There are more than 10 myosins, which share common features of their motor activity, but which differ in the direction that they move cargoes along filaments and in cargoes that are moved (Brown and Bridgman, 2004). Myosins in growth cones interact with actin filaments and generate forces to move actin filaments, vesicles, or other cargoes and to exert tensions on cytoskeletal components and associated structures (Rochlin et al., 1995). Myosin II in growth cones is particularly important in generating forces to move components and reshape developing axons and dendrites. In summary, ABPs are critical to regulating the behaviors of growth cones of developing axons and dendrites.

Regulation of Microtubule and Actin Organization and Dynamics by Cytoplasmic Signaling Pathways As noted previously, 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 activities of MAPs and ABPs. Certainly, 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 cytoplasmic second-messenger pathways.

Cytoskeletal organization can be rapidly changed by fluctuations in levels of small molecules such as Ca^{++} ions, cAMP, cGMP, and phosphoinositides that bind MAPs and

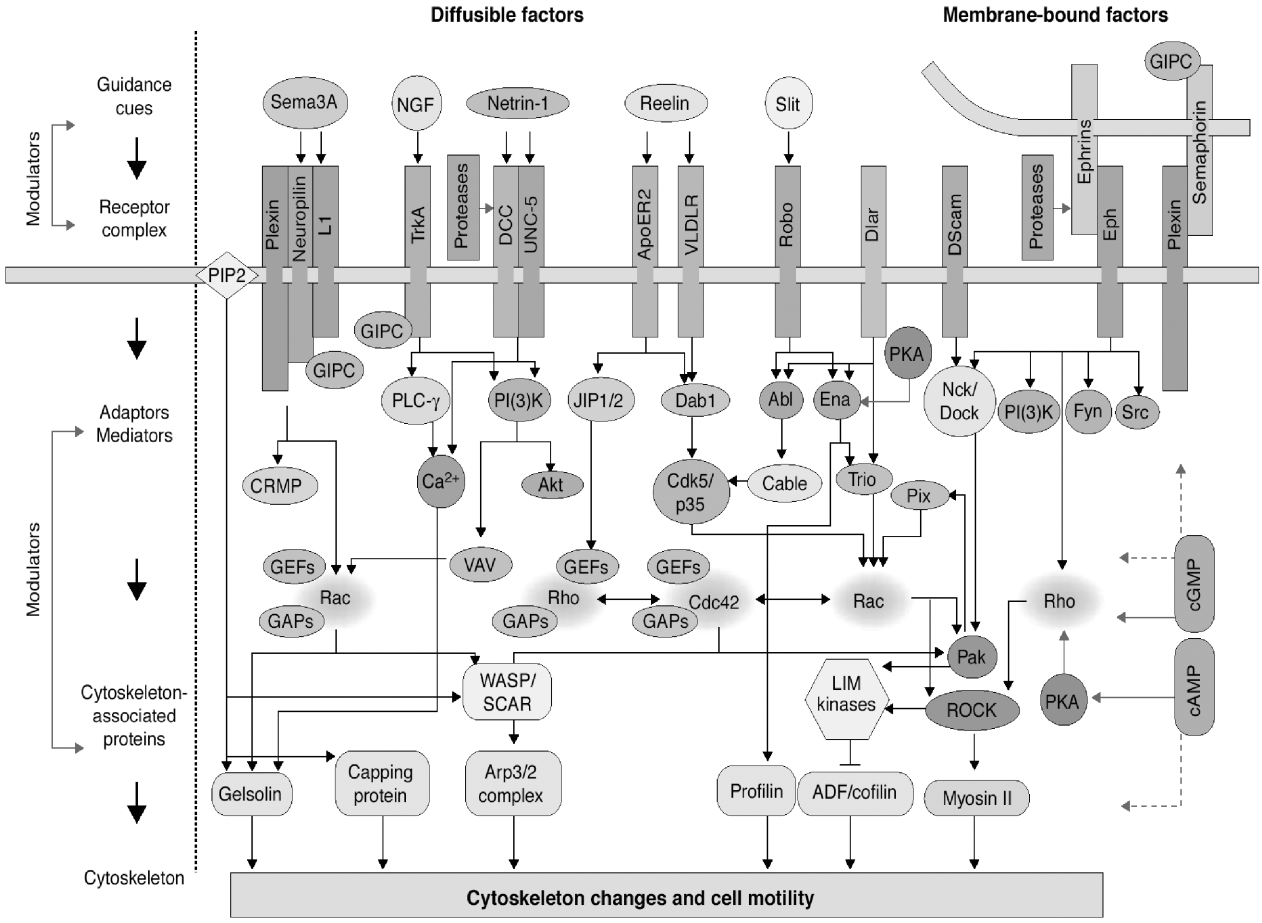

Figure 1.3 The interwoven network of signaling molecules that link guidance receptors with cytoskeletal dynamics underlying growth cone motility. Membrane receptors for extracellular guidance cues may function either alone or in a complex to activate cytoplasmic adaptors and mediators. The Rho family of GTPases may be pivotal links between guidance signals and actin-associated proteins, which are responsible for regulating the assembly and disassembly of actin filaments. Similar types of molecules are represented by symbols of similar color and shape. Lines depict activation pathways that have been demonstrated experimentally in different systems. (From Song and Poo, 2001.)

ABPs and regulate them allosterically (Dent and Gertler, 2003; Song and Poo, 2001; figure 1.3). The addition to and removal of phosphate groups from MAPs and ABPs by protein kinases and phosphates also rapidly regulate their activities. These molecules and pathways are, in turn, regulated by events at the plasma membrane, where adhesive proteins, growth factors, and other ligands bind membrane receptor proteins to trigger events that locally and temporally modulate the levels and activities of these regulatory molecules. Thus cytoplasmic signaling activities 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 guanosine triphosphatase (GTPase) proteins, in particular RhoA, Rac1, and Cdc42, are important regulatory proteins that relay signaling from the cell surface intracellularly to the cytoskeleton (Jaffe and Hall, 2005; figure 1.3). 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 guanosine diphosphate (GDP) (off). A rich variety of guanine nucleotide exchange factor proteins (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 activates the motor protein myosin II, increasing mechanical tensions and rearrangements of actin filaments. If RhoA levels are highly elevated, 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. Microtubule organization and polymerization are also regulated by Rho GTPases, although the mechanisms are less well understood than for actin filaments.

Microtubule-Actin Interactions Are Important

Two particular interactions of actin filaments, which we will describe, are particularly important in neurite elongation and growth cone migration. As mentioned earlier, 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 particularly significant, because these proteins may be important in the initiation of neurites from a spherical perikaryon or in directing the advance of a growth cone (Rodriquez et al., 2003). These microtubule-actin interactions link the microtubule functions of structural support and organelle transport to the dynamic cortical actin filaments and associated membrane receptors that detect extrinsic signals and regulate the cytoskeletal activities that shape the developing neuron (figure 1.4).

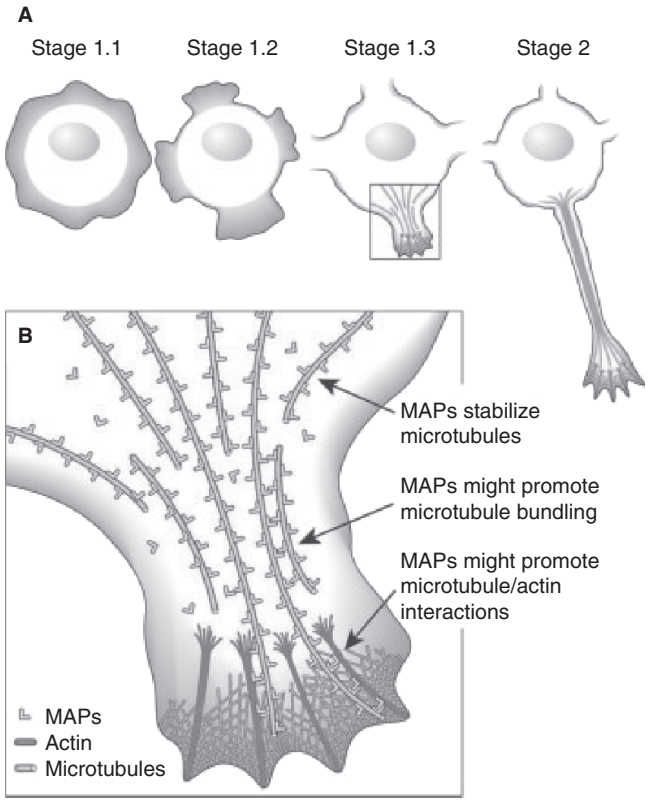

Figure 1.4 A model for cytoskeletal reorganization during neurite initiation. (**A**) Shortly after plating, cultured hippocampal neurons extend a uniform lamellipodium that surrounds the cell soma (stage 1.1). Preceding the initial neurite outgrowth, the lamellipodium becomes segmented at one or more sites (stage 1.2). Then the lamellipodium migrates away from the cell soma to form a growth cone, concurrent with microtubule advance into the initiation site and formation of an ordered microtubule array (stage 1.3). The newly formed protrusion then elongates, and microtubules become tightly packed into parallel arrays inside the nascent neurite (stage 2). Actin-microtubule interactions are present in lamellipodia at all stages. Panel **B** depicts a more detailed view of the proposed cytoskeletal organization in stage 1.3. Interestingly, microtubules preferentially grow along actin bundles in filopodia, suggesting that a physical link between the structures exists. Multifunctional MAPs like MAP1B, MAP2, or plakins are candidates to act as such links. (From Dehmelt and Halpain, 2004.)

Actin Filaments and Adhesive Contacts In addition to interactions with microtubules, another key function of actin filaments involves the adhesive interactions of cells that are mediated by membrane receptor proteins that form noncovalent bonds between cells or between cells and extracellular matrices (ECM). The major adhesion receptors are the cadherins and the adhesion proteins of the immunoglobulin-like superfamily, which mediate cell-cell adhesions, and the integrin proteins, which mediate cell adhesion to ECM. As cell-cell contacts are initiated by intercellular binding, receptors cluster within the plasma membrane to form discrete adhesive contacts. By way of transmembrane linkage these clustered adhesion receptors create docking sites for signaling enzymes, kinases, GEFs, GAPs, and a number of 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 are regulated (Zamir and Geiger, 2001).

A mechanism for neurite initiation and growth

In this section, neuritogenesis, neurite elongation, and growth cone migration by neurons will be described, emphasizing the dynamic cytoskeleton 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 (see figure 1.4). This motility is driven by actin filament polymerization, which pushes the cell margin outward, while simultaneously myosin II, located behind the cell margin, pulls newly formed filaments backward in a retrograde flow. The rearward transported filaments are severed and depolymerized, and if the protrusion and retrograde flow are equal, these activities 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 lamellipodium thickens and moves away from the cell body, tethered by a cylindrical nascent neurite. The critical step that distinguishes neurite formation from the initial protrusive activity occurs when microtubules and associated organelles enter and remain within a filopodial or lamellipodial protrusion and the protrusive motility moves forward ahead of the microtubules and organelles (Da Silva and Dotti, 2002; figure 1.4). 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. In addition, cytoplasmic signals generated at 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 (Suter and Forscher, 2000). The significance of these outwardly directed forces in neurite initiation is illustrated by findings that neurites can be pulled out from a neuron by attaching an adhesive bead to a neuronal surface and then pulling the bead and attached elongating neurite away from the nerve cell body (Fass and Odde, 2003).

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; figures 1.2, 1.5). At the growth cone's 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. Only when the leading edge forms transient adhesive contacts that link to actin filaments does the retrograde flow attenuate. At the base of a growth cone, microtubule-based motor proteins move microtubules and organelles from the neurite into the central growth cone, comprising the C-domain (central). From the C-domain individual microtubules extend into the P-domain, sliding forward powered by molecular motors and elongating by adding subunits to microtubule plus ends. Retrograde flow pulls most of these microtubules back into the C-domain (Schaefer, Kabir, and Forscher, 2002). Importantly, some microtubules advance into filopodia or lamellipodia, stabilized at adhesive sites (figure 1.2; 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 (Dent and Gertler, 2003; figure 1.5): (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, formation of adhesive contacts, and myosin II–powered exertion of

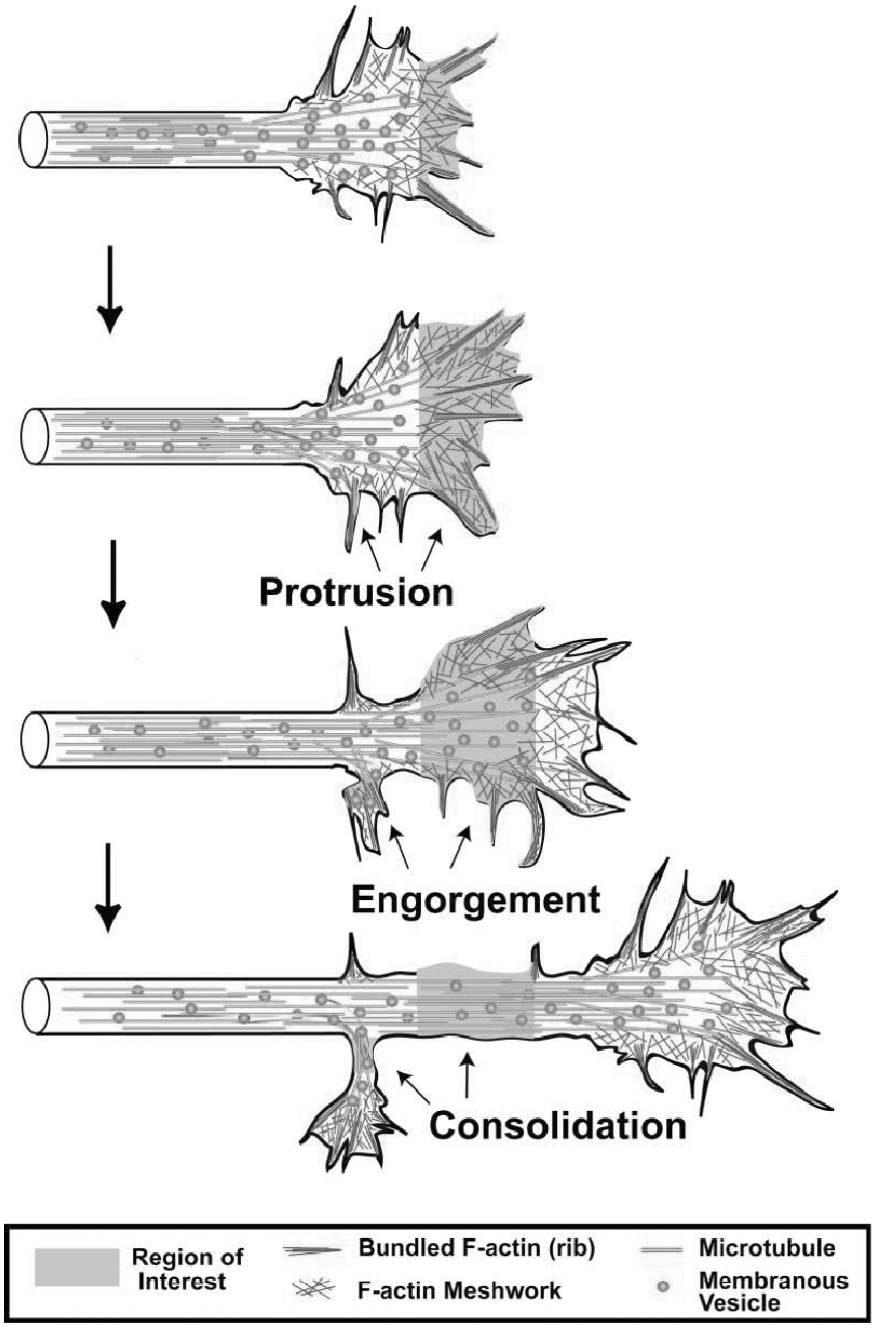

FIGURE 1.5 Stages of axon and branch growth. Three stages of axon outgrowth have been termed protrusion, engorgement, and consolidation (Goldberg and Burmeister, 1986). Protrusion occurs with the rapid extension of filopodia and thin lamellar protrusions, often between filopodia. These extensions are primarily composed of bundled and meshlike 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. (From Dent and Gertler, 2003.)

tension on these adhesive sites generates 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 (Lamoureux, Buxbaum, and Heidemann, 1989; Letourneau, 1981; Letourneau et al., 1987). 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.

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 a growth cone encounters within developing tissues. As described previously, a neurite elongates by the advance of microtubules and organelles from the growth cone C-domain into the P-domain. In a neutral in vitro environment, this elongation may occur first to one side and then to the other, keeping the growth cone on a straight path. In a complex in vivo environment, however, there are local differences in adhesive surfaces, extrinsic factors, or other ligands that interact with growth cone receptors to generate local differences in the activities of Rho GTPases, protein kinases, protein phosphatases, or second messengers, such as Ca^{++} or cyclic nucleotides (Gomez and Zheng, 2006; Guan and Rao, 2003; Song and Poo, 2001). On the one hand, if these local variations in regulatory cues are sufficiently strong or persistent, they produce local differences in actin-based motility and microtubule advance that cause growth cone turning. This outcome might occur because the P-domain expands faster on one side as a result of locally enhanced actin polymerization, reduced retrograde actin flow, or linkage of actin filaments to adhesive sites (figure 1.6, upper panels). Localized signals might directly promote microtubule polymerization or stabilization, so that microtubules preferentially advance to one side of the P-domain (Challacombe et al., 1997; Dickson, 2002; Tanaka and Kirschner, 1995). On the other hand (see figure 1.6, lower panels), 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, microtubule advance on that side will be reduced, and the growth cone will turn toward the other side.

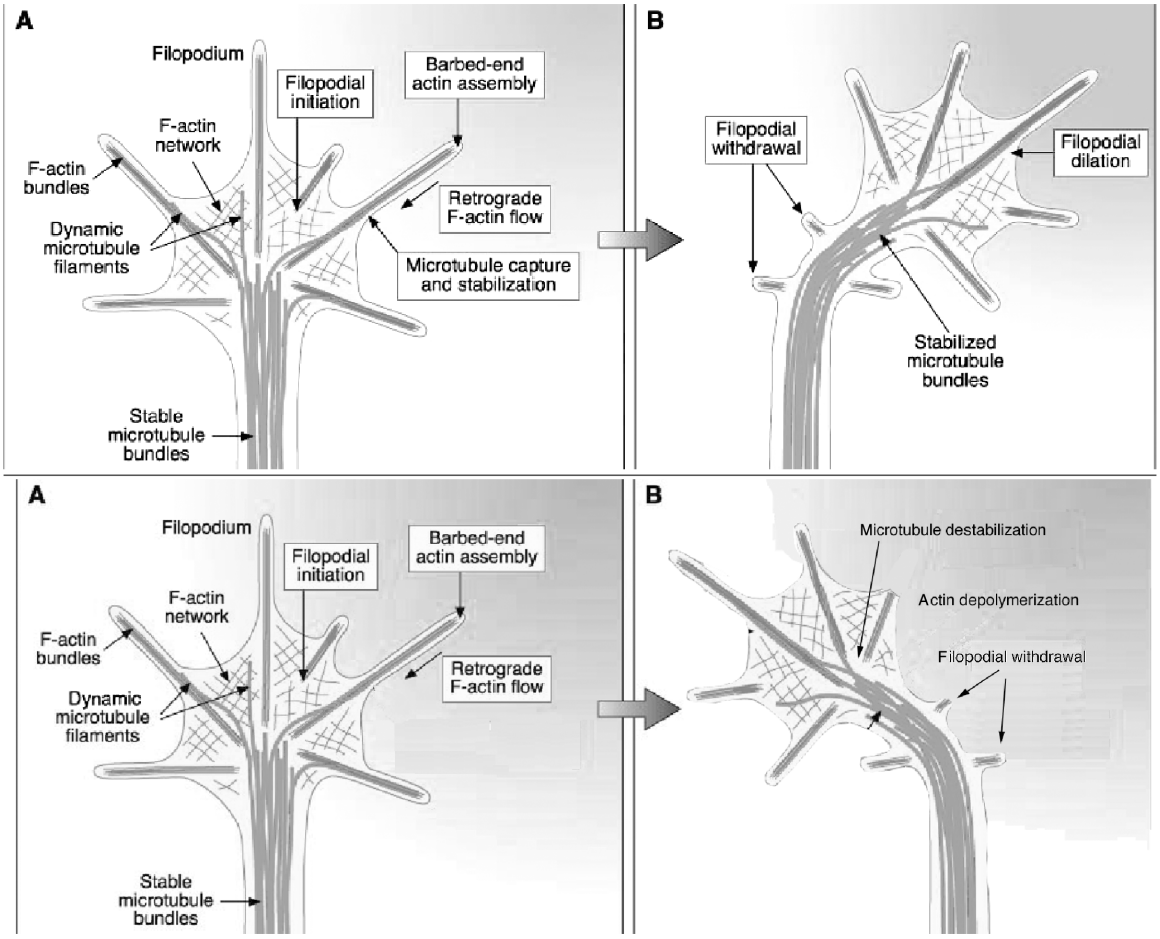

Figure 1.6 Summary of reorganization of actin filaments and microtubules in the peripheral domain of growth cones that is involved in turning toward an attractant and turning away from a repellent guidance molecule. An attractant promotes actin polymerization, adhesion, and microtubule advance, while a repellent inhibits actin polymerization and advance of microtubules. (Adapted from Dickson, 2002.)

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 (figures 1.2, 1.5). In a growth cone, part of the P-domain and associated C-domain may separate from the whole and establish an independent growth cone and a new branch of the parent neurite. This result may occur when a growth cone "pulls" in two directions (figure 1.2). Branch formation along a neurite is initiated by localized protrusion of filopodia or lamellipodia (figure 1.2; 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 actin filaments of the protrusion and be pulled into the branch. The microtubule-severing protein katanin may promote branch formation by severing microtubules in the neurite shaft to create 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.

The Differentiation of Axons and Dendrites; Polarization of Neuronal Form A hippocampal neuron in vitro initially sprouts several similar neurites that extend slowly. After 18–24 hours one neurite expands its growth cone and elongates significantly faster than the others. This neurite becomes the axon, and it accumulates proteins typical of axons, such as the 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, 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 previously mentioned molecules or signals is sufficient to specify axonal character. In vitro manipulations, such as focally pulling on a neurite or presenting adhesive proteins to one neurite will induce a neurite to become the axon. Thus extrinsic signals can influence the intrinsic mechanism of axonal specification, perhaps by locally activating PI3 kinase 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.

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 the developing human brain. The neutral environment of a tissue culture dish facilitates understanding these intrinsic mechanisms. However, the in vivo environment is never neutral, and spatial and temporal patterns of distribution of axonal guidance cues in the environment of the developing brain shape these intrinsic morphogenetic mechanisms to generate neural circuits (Tessier-Lavigne and Goodman, 1996).

Neuronal Migration Immature neurons arise from proliferation of neural precursors in the ventricular zone of the developing brain. From their birth immature neurons become polarized by asymmetry in local cues, including the adhesive protein laminin in the underlying extracellular matrix (ECM) of the ventricular layer, as well as growth factors, morphogens, and guidance molecules, such as sonic hedgehog and netrin, produced by the surrounding neuroepithelial cells. These newly born neurons migrate out of the ventricular zone of the telencephalon to establish the cortical plate in a wave of migration between 6 and 18 gestational weeks (Ramakers, 2005). Migrating neurons retain their initial polarization and encounter additional cues as they migrate upward. Neural migration stops at the outer marginal zone, where reelin, produced by Cajal-Retzius cells of the marginal zone, triggers neurons to cease expressing integrin adhesion receptors. Younger neurons migrate past older neurons to reach the marginal zone, so the upper layer II contains the youngest neurons, while the oldest neurons inhabit the lowest layer VI.

Neuronal Polarization and the Initial Growth of Axons and Dendrites Neurons sprout axons soon after ceasing migration, as early as the seventh week in the cortex. In a 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 sprout

their axon toward the netrin source (Adler et al., 2006). PI3 kinase is involved in axonal specification of mammalian neurons, and thus localization of 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 versus dendritic growth are regulated by the same extracellular molecule, semaphorin 3A (Sema3A), produced by cells near the pial surface and released to create an extracellular gradient (Whitford et al., 2002). Axons are repelled by Sema3A, while the subsequently formed apical dendrites of these neurons are attracted by Sema3A. The difference in directions of these processes lies not in a local difference in expression of membrane receptors for Sema3A, but rather in a local difference in distribution of 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 activated 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 components in dendrites versus axons.

Axonal Guidance Once sprouted from neuronal perikarya, axons follow stereotypical routes to their targets. This pathfinding occurs by growth cone navigation; that is, a growth cone detects and responds to physical and chemical features in its environment (figure 1.7). The 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 protrusion is suppressed, 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. 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 adhesion and growth cone migration. Molecules that repress adhesion or actin dynamics are expressed adjacent to these pathways, acting like "guard rails" to keep growth cones migrating on the proper path. Several proteins have been identified as negative guidance cues, including slit proteins, Sema3A, and several

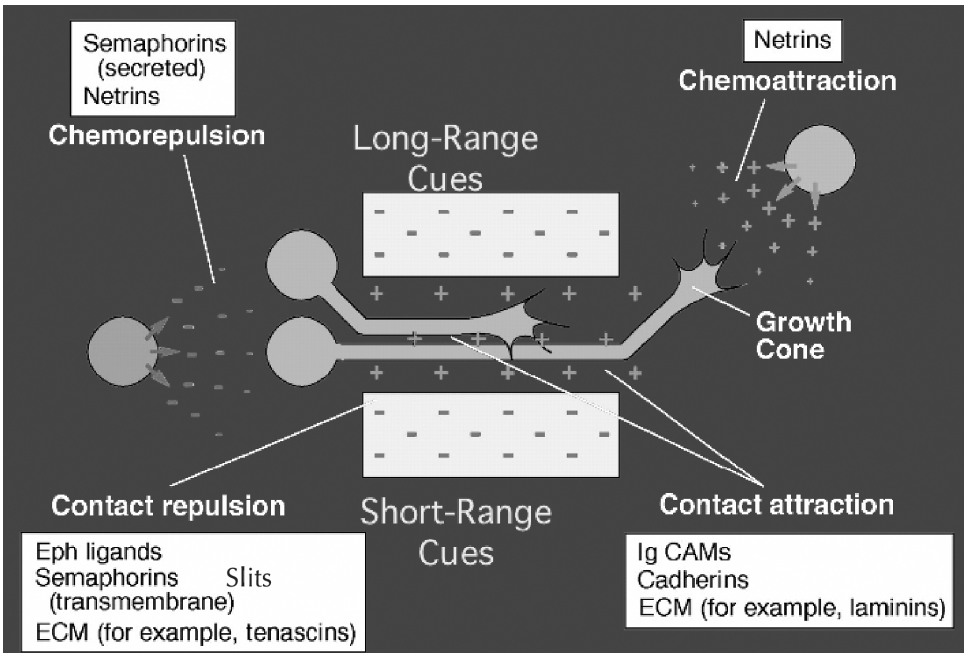

Figure 1.7 Summary of the action of guidance cues that are involved in growth cone navigation. Short-range cues on surfaces that growth cones come into contact with act to promote or inhibit growth cone adhesion and migration. Long-range cues are diffusible molecules released from intermediate or synaptic targets that attract or repel migrating growth cones. Growth cones integrate information coming simultaneously from multiple cues during navigation. (From Tessier-LaVigne and Goodman, 1996.)

ephrinA's. Each negative cue is detected by a different specific receptor with specific signaling mechanisms, although common features of these mechanisms include disruption of growth cone adhesions, suppression of actin polymerization, and activation of RhoA to stimulate myosin II–mediated contraction, leading to growth cone collapse and sometimes retraction of entire axonal branches or segments (Guan and Rao, 2003). Some molecules simply mark a path as positive or negative without providing directional information, while other molecules are soluble, are released by navigation targets, and are distributed in gradients that provide directional information to growth cones. At any instant a growth cone is detecting several guidance molecules, so growth cone migration depends on integrating the intracellular signals simultaneously triggered from multiple receptors. The following section describes specific features of growth cone guidance in the developing CNS. Most of the molecular information about growth cone guidance comes from studies of model vertebrate systems, but the timing of the events in human brain development is included (Ramakers, 2005).

Growth cone navigation along major pathways during cerebral cortical development

Navigation of Corticofugal Axons As stated earlier, cortical neurons sprout their axons away from the pial surface in response to a gradient of the repellent cue Sema3A. At eight weeks the earliest corticofugal axons reach their first target, the intermediate zone, attracted by Sema3C, expressed in the subventricular zone (figure 1.8 and plate 3; figure 1.9 and plate 4). The intermediate zone is rich in extracellular matrix 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 the first choice point for corticofugal axons, as they encounter the repellent Sema3A, expressed by the underlying ventricular zone. Growth cones of corticothalamic and corticospinal axons turn laterally to exit the dorsal telencephalon through the internal capsule, while growth cones of corticocortical axons turn medially. The molecules or cells that mediate this first decision are unknown. The internal capsule contains the attractant netrin-1, which along with laminin promotes growth through the internal capsule. As these axons traverse the internal capsule, they are prevented from moving medially by expression of the repellent cues, slit-1 and slit-2, in the ganglionic eminence (Bagri et al., 2002). At the telecephalic-diencephalic boundary these 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.

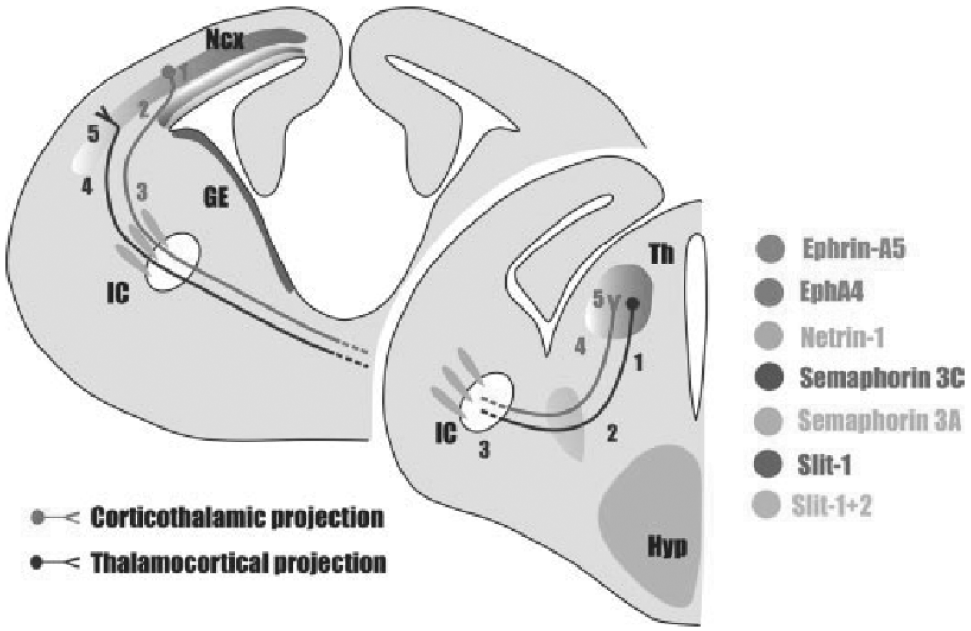

Figure 1.8 The trajectory of growing thalamocortical and corticothalamic fibers involves multiple steps and both attractive and repulsive guidance cues. The expression of guidance molecules is related to each of these steps: Slit is a repellent that steers thalamic axons emerging from the diencephalon 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; Hyp, hypothalamus; IC, internal capsule; GE, ganglionic eminence; Ncx, neocortex. (From Uziel et al., 2006.) (See plate 3.)

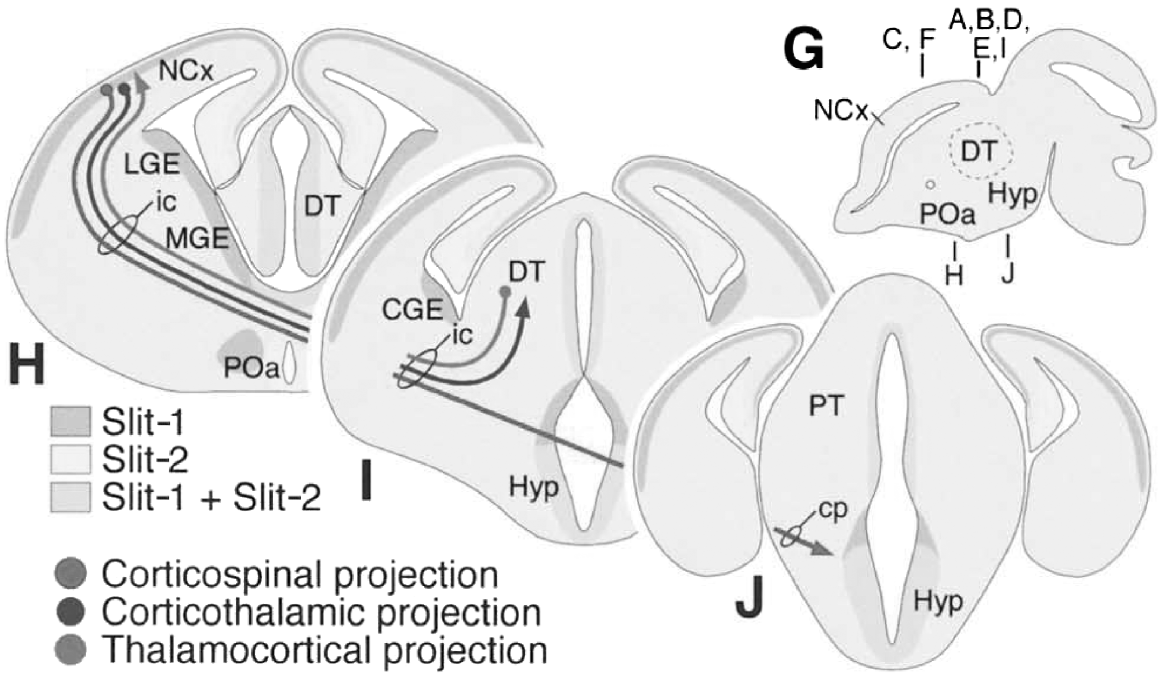

FIGURE 1.9 Schematic diagrams of coronal sections through the developing forebrain showing the trajectory of corticospinal (red), corticothalamic (blue), and thalamocortical (purple) axons in relation to regions that express slit-1 (blue) and slit-2 (yellow) at selected levels. Regions depicted in green express both slit-1 and slit-2. CGE, caudal ganglionic eminence; H, hippocampus; ic, internal capsule; NCx, neocortex. (From Bagri et al., 2002.) (See plate 4.)

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 homophilically 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 in many tracts, 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 toward the hindbrain until they reach the decussation area at 10 gestational weeks in humans (Ramakers, 2005; Ten Donkelaar et al., 2004). 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 attractants such as netrin, to cross the midline, completing the decussation by week 17. Renewed expression of midline repellents, slit and ephrin3B, caudal to the decussation, prevents corticospinal axons from recrossing as they grow down the spinal cord. The lumbrosacral area is reached by 29 weeks, but growth cones do not enter spinal cord gray matter for several weeks. 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 release attractants and express adhesive ligands that specifically activate local regions along the afferent axons. Activation of Rho GTPases and ABPs induces localized actin-based protrusive activity from the axonal shafts, followed by collateral branches that grow into the targets. The axonal segments that extend beyond the innervated target are then eliminated via retraction involving myosin II. 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 their first decision to turn medially in the intermediate zone (Richards, 2002). The molecules that guide these decisions are unknown, although the callosal path may be "pioneered" by axons from the cingulated cortex, creating an axonal path that is followed by neocortical axons to the midline. The growth cones of corticocortical axons are attracted by netrin-1, produced by midline cells, and channeled to cross the midline by repulsion from slit proteins expressed by cells above and below the developing corpus callosum. In humans the corpus callosum begins forming by 11–12 weeks and is well developed by 18–20 weeks. After navigating dorsally and into the contralateral hemisphere, the axons reach the cortical subplate where they extend and branch, remaining for several

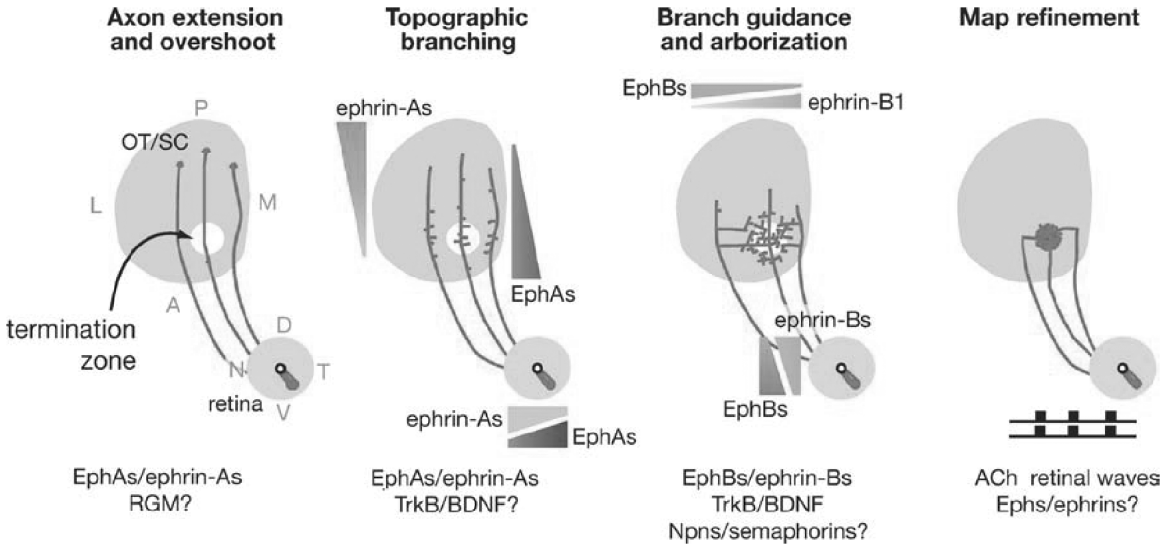

FIGURE 1.10 Mechanisms and molecules controlling retinotopic mapping in chicks and rodents. The names and/or distributions of molecules known, or potentially able, to control the dominant mechanisms at each stage are listed. The gradients represent the consensus distribution for a combination of related molecules (i.e., ephrin-A's), which are not listed individually owing to distinctions in the individual members expressed and the precise distributions between species. Molecules other than those listed are likely to participate. (From McLaughlin and O'Leary, 2005.) (See plate 5.)

weeks before sprouting collateral branches at 28 weeks into their appropriate final target regions of the cortex.

NAVIGATION OF THALAMOCORTICAL GROWTH CONES Thalamocortical afferent axons begin their navigation by growing ventrally until they are stopped by repulsion from slit proteins expressed by the underlying hypothalamus (Lopez-Bendito and Molnar, 2003; Uziel et al., 2006; figure 1.8). Then the growth cones turn laterally, being attracted by netrin-1 expressed by cells in the internal capsule. The growth cones turn dorsally and migrate toward the cortex within the internal capsule, keeping lateral in response to slit proteins expressed by the ganglionic eminence (Bagri et al., 2002). Within the internal capsule, thalamocortical axons meet corticofugal fibers, which they follow toward their cortical targets. Thalamocortical axons penetrate the cortical subplate between 9 and 18 weeks in developing humans. By 24 weeks they fill the upper subplate and extend branches exploring for their correct cortical targets. Thalamocortical axons finally enter the cortex between 26 and 28 weeks, prior to the entry of callosal axons.

The preceding paragraphs have described how axons navigate to their targets by detecting and responding to guidance molecules that regulate growth cone motility. It may seem that the relatively limited numbers of guidance molecules, laminins, ephrins, semaphorins, netrins, slits, and immunoglobulin-like adhesion molecules are too few to account for the complexity of neural circuitry (Yu and Bargmann, 2001). However, this diversity of axonal pathways arises from cell-type–specific differences in expression of receptors for guidance cues, in downstream cytoplasmic signaling activated by guidance cues, and temporal and spatial differences in the expression of guidance cues and their receptors by developing tissues and neuronal populations. An interesting recent finding is that growth cone responses to guidance cues may depend on bursts of local protein synthesis of receptors or signaling components within a growth cone. For example, Sema3A rapidly stimulates synthesis of the GTPase RhoA from mRNA within growth cones (Wu et al., 2005). RhoA activity is necessary for Sema3A induction of growth cone collapse. Some growth cones cross the ventral spinal cord and only then synthesize and express EphA receptors that mediate a repulsive response to midline ephrins, preventing recrossing the midline (Brittis, Lu, and Flanagan, 2002). Much remains to be learned about how growth cones detect guidance cues and integrate complex signals to navigate to their intermediate and final targets.

PATTERNING AXONAL DISTRIBUTION WITHIN TARGETS Once a group of axons reach their synaptic target, they become organized into patterns that represent physiologically relevant topography or sensory parameters. The distribution of retinal ganglion cells' 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 ephrins and their Eph receptors on cells across the optic tectum (or colliculus) and the incoming retinal axons and growth cones are key features that determine the topography of retinal inputs to the tectum (figure 1.10 and plate 5). 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. Growth cones of temporal retinal axons express high levels of EphA receptors, so they stop and innervate the anterior tectum, 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. The distances that retinal growth cones migrate along the anterior-posterior tectal gradient of increasing ephrin-A expression are determined by the relative levels of EphA expression by growth cones. Retinal mapping along the medial-lateral tectal axis involves gradients in the distribution of ephrin-Bs and their EphB receptors among retinal axons and tectal cells. The identification of signaling activity from the cytoplasmic domain of ephrin-B ligands indicates that both ephrins and EphB receptors can activate cytoplasmic signaling to regulate axonal targeting along the medial-lateral tectal axis.

This mechanism for topographic mapping of connections by gradients of cell surface ligands and receptors was proposed by Roger Sperry (1963) as the chemoaffinity hypothesis. The discovery of gradients in expression of ephrins-A2 and -A5 confirmed Sperry's hypothesis. It has become clear that the initial distributions of axons, as regulated by these gradients, is not final, and that subsequent remodeling of axons due to further cellular interactions and physiological activities is necessary to create more precise 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).

The accessibility and 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 (Uziel et al., 2006; figure 1.8). Similar mechanisms may operate in patterning the development of circuits in other regions (Flanagan, 2006).

In addition to the development of the correct distribution of axons within a target, axons must recognize the target neurons with which they make synapses. Several cell surface and extracellular molecules are expressed in a lamina-specific manner in the developing cortex, including cadherins, Eph receptors, ephrin ligands, proteoglycans, and neurotrophins (Lopez-Bendito and Molnar, 2003). These molecular differences may provide cues for thalamocortical and corticocortical axons to terminate in the correct layer.

Development of dendrites

The dendritic arborization of a neuron contains the synaptic inputs to the neuron and is where synaptic inputs are integrated before the initiation of action potentials. Thus dendritic arbors are critical to the processing of neural information for behavior and other neural activities. 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. As described earlier, 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 several days, and may be due to both environmental factors and intrinsic factors, such as changes in expression of specific cytoskeletal proteins. The sites of dendrite initiation from a neuron may be determined by previous cell interactions; for example, 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 branched dendritic arbors. However, as described in the following paragraphs, the formation of dendrites is a prolonged activity, and the final shaping of dendritic arbors depends heavily 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 zebra fish 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 may regulate Rho GTPases to modulate actin filament dynamics that underlie the extension and retraction of dendritic filopodia (Van Aelst and Cline, 2004). The roles of these 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 NDMA receptors only, 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 activity, stop, and transform to a presynaptic ending. Motor axons growing on muscle fibers of mice that lack the 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.

Dendritogenesis in the Prenatal and Postnatal Human Brain Neurons begin to form dendrites soon after they initiate axon formation, although dendrites are initially short and slow growing. Apical dendrites are present on cortical pyramidal neurons by 12–13 weeks' gestation. However, once innervating axons arrive in the cortical plate at 26–28 weeks, dendrite formation accelerates as a result of synaptic contacts, electrical stimulation by axons, and the release of neurotrophins and other factors from axons. In humans, most dendritic growth occurs postnatally in conjunction with synaptogenesis and the increased physiological experience and activity of postnatal life. Dendrite formation in the developing human brain has been examined most thoroughly in the visual cortex and prefrontal cortex (Ramakers, 2005). In the visual cortex most dendritic branches develop prenatally, and postnatal growth involves dendritic lengthening by terminal growth of branches as synapses are added. Total dendrite length of pyramidal neurons in the visual cortex increases rapidly in the first few postnatal months, increasing two- or threefold and reaching the adult levels by 1–2 years. In the prefrontal cortex, synaptogenesis and dendritic growth proceed more slowly than in the visual cortex. During the first postnatal year the length of dendrites increases 5- to 10-fold by branching and elongating, while after the first year most growth occurs by elongation of branches. By two years of age the total dendritic length per pyramidal neuron is only half the adult level. Yet, at age two the average dendrite length per neuron in the prefrontal cortex is longer than dendritic length in the visual cortex, consistent with the greater dendritic and synaptic complexity in the more integrative cortical regions, compared to unimodal primary cortical regions.

These measures of dendrite elaboration in the developing human brain are mostly based on anatomical studies involving Golgi staining of fixed neurons. These data are static and fail to account for the dynamic activities of dendritic elongation, branching, and retraction that are revealed from real-time visualization of dendrite growth and synaptogenesis in living embryos, as mentioned earlier. Much remains to be learned about how axonal and dendritic shapes are sculpted over a period of years, as the result of interactions between genetically defined mechanisms of neuronal growth and a dynamic flux of intercellular molecular signaling, synaptogenesis, and the unpredictable physiological activity of postnatal experience.

Summary

Neural circuits arise by a morphogenetic process in which axons and dendrites are formed according to intrinsic neuronal mechanisms that respond to extrinsic regulatory interactions with molecules, cells, and features of the developing organism. The driving force for axonal and dendritic growth is the advance of microtubules and associated organelles, while the actin-based motility of growth cones at the ends of elongating processes allows exploration of local tissue environments for molecular guidance cues. Binding of guidance cues to their receptors on growth cones triggers cytoplasmic signaling that regulates actin filament organization, mechanical forces, and microtubule advance to locally direct growth cone migration, turning, and branching. Axonal growth cones reach their synaptic targets by navigating to a series of intermediate targets, guided by positive and negative responses to surface-bound and soluble molecular cues. Axonal projections within a target are initially patterned according to gradients in the expression of molecules, such as ephrins and Eph rceptors, on axons and target cells. Synaptogenesis, other cellular interactions, and physiological activities adjust and refine axonal growth and branching within a target to achieve more accurate axonal topography. Formation of dendrites begins before afferent axons arrive and involves interactions of intrinsic and extrinsic mechanisms that regulate the orientation and rates of dendritic growth. Dendritic growth accelerates when axons arrive and initiate synaptogenesis. The final shaping of arborizations of dendrites and axons depends on mutual interactions, and

physiological activity has a major role in this final phase of the formation of neural circuits. In the developing human brain, axonal navigation to targets begins in the first trimester and continues throughout the second and into the third trimester. Dendrite growth begins in the second trimester, accelerates in the third trimester, and continues most vigorously through the first 2–3 years and then for years afterward, as dendrites and axonal terminal arbors are sculpted and refined by experience.

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