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Molecular and Cellular Mechanisms of Axon Morphogenesis

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Abstract

Axons allow neurons to transmit electrical information to target cells with synaptic connections. Axons often have stereotypic morphologies accompanied by various branched structures that connect with multiple synaptic targets. Developing these branched morphologies is critical to building complex neural circuits. In this chapter, we discuss our general understanding of axon morphogenesis, with a focus on three steps, growth, guidance, and branching, which are often repeated to generate the diverse morphologies in the nervous system. We summarize some recent findings with the goal of providing a broad, but up-to-date, view of general molecular and cellular mechanisms of this important developmental process.

1.1 Introduction

Neurons are the fundamental functional unit of the nervous system. Each neuron has only one axon to relay the information collected at various synaptic sites on the dendrites and soma. The information is integrated and then propagated along the axons by action potentials generated at the axonal hillock, the specialized region connecting the axon with the soma, and then passed on to target cells via synaptic connections at axonal terminals. Often, axons

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have various types of branches, such as terminal branches that allow synaptic connections with similar targets and collateral branches that connect with different targets. Throughout the nervous system, axons display different morphologies that are defined by the axon length and trajectory as well as branch shape, size, and pattern (Gibson and Ma, 2011; Kalil and Dent, 2014). These diverse morphologies are tailored to the functional needs of neural circuits underlying complex animal behaviors. For example, in the olfactory system, each olfactory sensory neuron has one simple axon that synapses with mitral cell dendrites in a specific glomerulus of the olfactory bulb, while mitral cells send long axons to different regions of the cortex via multiple collateral branches. Another example is found in the reflex arc of the spinal cord. There, sensory neurons in the dorsal root ganglion (DRG) collect somatic sensory information from branched nerve endings of their peripheral projections in the body's periphery. The information is relayed through their central collateral branches that connect directly or indirectly with motor neurons inside the spinal cord. Motor neurons then extend long axons from the spinal cord and reach the muscle targets where they form highly arborized terminal branches.

The diverse axonal morphologies have captivated the attention of neurobiologists studying different regions of the nervous system in the past century. A fundamental question that has been constantly asked is how to generate the stereotypic axonal morphologies for various neural circuits. Past studies have provided a general framework for addressing this seemingly simple question. Studies using live cell imaging in culture or in tissues have demonstrated that axon morphogenesis starts with axonogenesis, the formation of an axon from the soma (Polleux and Snider, 2010), a step that is characterized by neuronal polarization, neurite outgrowth, and axon specification (Figure 1.1A). Several excellent reviews summarized the recent understanding of this critical step (Alfadil and Bradke, 2023; Arikkath, 2020; Funahashi et al., 2020; Schelski and Bradke, 2017; Takano et al., 2019). Following axonogenesis, axons continue to develop through axon growth, guidance, and branching, three steps that are repeated multiple times to generate the eventual axon morphology that is intimately associated with synaptic function (Figures 1.1B and 1.1C) (Gibson and Ma, 2011; Kalil and Dent, 2014). Early cell biological studies have revealed that these steps are highly controlled by the two cytoskeleton components, namely actin and microtubule, which are protein polymers made of subunits that can dynamically assemble and disassemble (Figure 1.1D) (Atkins et al., 2023; Dent et al., 2011; McCormick and Gupton, 2020; Menon and Gupton, 2016). In addition, trafficking of various membrane organelles inside the axon is critical for where to make branches, how to grow or increase branch length, and how to guide them (Bodakuntla et al., 2021; Winkle et al., 2016). Moreover, biochemical and genetic studies in the past three decades

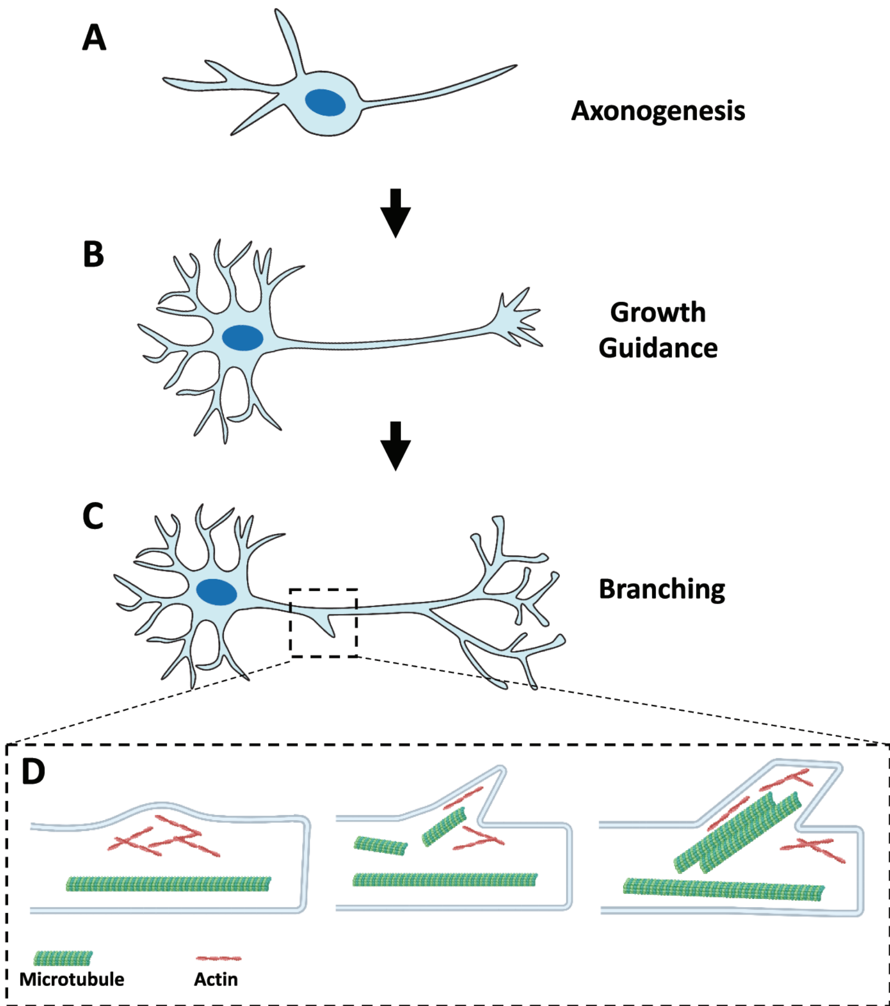

Figure 1.1 Commons steps in axon morphogenesis and the role of cytoskeleton in branch formation. **(A–C)** Schematic drawings depicting different steps leading to the generation of complex axon morphologies. **(D)** Schematic drawings depicting the stages of axon branch formation governed by the cytoskeleton. Actin patches accumulate where a new branch is to be initiated. Actin filaments are then assembled into filopodia-like structures. Microtubules undergo remodeling and invade the newly formed branch.

have identified various molecular cues in the embryonic environment that regulate both cytoskeletal and membrane dynamics via cell surface receptors (Bashaw and Klein, 2010; Chedotal and Richards, 2010; Kolodkin and Tessier-Lavigne, 2011). Many extracellular cues are common in regulating these steps, but the intracellular mechanisms are often unique to each step.

In this chapter, we discuss the general concept related to these steps, with a focus on recent investigations that have spawned new ideas and made disease connections. We hope to provide readers with a broad but more current view of the molecular and cellular mechanisms of axon morphogenesis.

1.2 Regulation of the Cytoskeleton

1.2.1 Actin

The actin cytoskeleton is essential for axon growth, guidance, and branching. It is best studied in the growth cone, the motile tip that explores the environment and leads to the growth and guidance of a growing axon or branch (Lowery and Van Vactor, 2009). Growth cone motility is largely mediated by dynamic assembly and disassembly of actin filaments in two membrane protruding structures: filopodia, a needle-like membrane protrusion that is composed of filamentous actin bundles, and lamellipodia, the veil-like structure composed of filamentous actin networks (Figure 1.2A) (Gomez and Letourneau, 2014). Actin assembly in these structures allows the growth cone to spread out and move forward (Lowery and Van Vactor, 2009). Any asymmetrical protrusion and rearrangement of actin assembly allows the growth cone to turn to a different direction. Actin also plays a role in initiating new branches from the main axonal shaft, where actin polymerization in a structure called “actin patch” precedes branch formation (Gallo, 2011) (Figure 1.1D).

Much of our understanding of actin dynamics has been derived from neurons cultured on flat substrates (Lowery and Van Vactor, 2009). Two recent studies (Clarke et al., 2020a; Clarke et al., 2020b) used live cell imaging to examine actin assembly in the growth cone of TSM1 pioneer axons from the developing *Drosophila* wing. They revealed that in the *in vivo* three dimensional (3D) environment, the TSM1 axonal growth cone is mainly composed of filopodia-like structures. Here, in the growth cone, actin was highly dynamic with the accumulation of actin filaments at distal portions of the axon, leading to a biased prediction of net outgrowth and protrusion. This actin distribution and distal accumulation were regulated by Abl kinase signaling (Clarke et al., 2020b). Abl is a signaling molecule downstream of some of the most conserved axon guidance molecules including Robo (Bashaw et al., 2000; Yu et al., 2002) and Netrin/Frazzled (Forsthoefel et al., 2005). Blocking Abl signaling altered the growth cone morphology and actin assembly. Using a multitude of parameters, such as number of protrusions, branches, and actin peaks, the study found two distinct correlative clusters that represent the morphological differences with and without Abl signaling

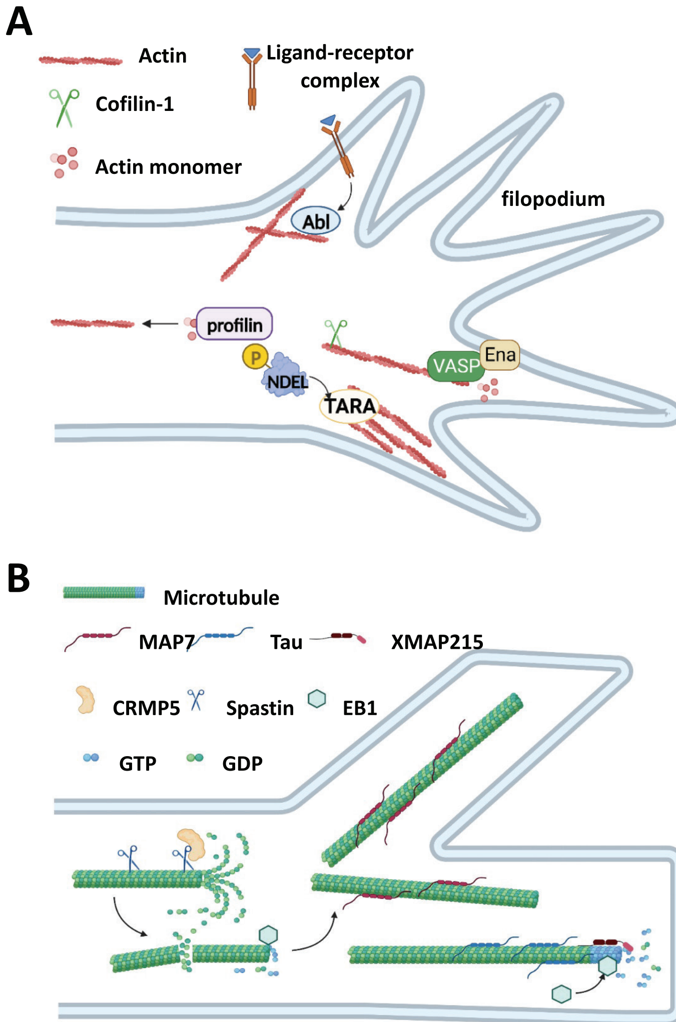

Figure 1.2 Cytoskeletal regulators during axon morphogenesis. **(A)** Actin dynamics contribute to axon morphogenesis through regulation of filopodia and growth cone motility. These processes can be regulated directly by actin binding proteins such as Ena/VASP to promote polymerization or indirectly through various signaling cascades such as NDEL signaling. Abl mediates ligand receptor signaling that regulates actin dynamics ultimately effecting growth cone morphology. **(B)** The dynamics and stability of microtubules contribute to axon branching and morphogenesis. MAP7 binds microtubules to promote axon branching through stabilizing newly formed branches. Other MAPs such as tau stabilizes microtubule polymerizing ends to allow for EB1 and XMAP215 to promote polymerization and axon elongation. CRMP5 and spastin physically interact to control microtubule length and polymerization.

(Clarke et al., 2020a). These studies thus demonstrate the coordinated actin regulation in directing growth cone motility *in vivo* (Figure 1.2A).

Actin assembly is regulated by a number of actin binding proteins (Figure 1.2A). They include: 1) the Arp2/3 complex and formin, which nucleate actin to generate new filaments to form actin networks or bundles respectively; 2) profilin, a protein that binds to actin monomers and aids actin filament polymerization; 3) Ena/VASP, a family of proteins that cooperate with profilin to promote polymerization; and 4) cofilin-1, which destabilizes actin filaments and regulates actin length (Svitkina, 2018). These actin regulators participate in many steps of axon morphogenesis. For example, the Arp2/3 complex is activared by the wave complex, which is recruited by the guidance receptor Robo in midline repulsion (Chaudhari et al., 2021); profilin, Ena/VASP, and formin work in different combinations to control axon regrowth and sprouting in *Drosophila* neurons (Yaniv et al., 2020); and cofilin controls the growth rate of developing motor axons (Frendo et al., 2019).

How these actin regulators are controlled is a key question in uncovering the molecular mechanisms of axon morphogenesis (Figure 1.2A). Recent studies of nuclear distribution element-like 1 (NDEL1) suggest the complexity of such regulation. NDEL1 and its homolog NDEL play many roles in early neural development including neuronal differentiation, migration, and maturation, as well as cytoskeletal reorganization (Sasaki et al., 2005; Hayashi et al., 2010; Ye et al., 2017). NDEL1 expression is first observed in the nervous system during embryonic development and this expression persists throughout adulthood (Pei et al., 2014). Its function is thought to regulate dynein activity and the localization of katanin, both of which are microtubule regulatory proteins (see discussion below) (Shu et al., 2004; Toyo-Oka et al., 2005). However, there is evidence for the interaction of NDEL1 with the actin cytoskeleton (Hong et al., 2016) which is mediated by trio-associated repeat on actin (TARA), an actin bundling protein that directly binds to and stabilizes actin filaments (Hong et al., 2016). A recent study using rat and mouse hippocampal and cortical neurons showed that phosphorylation of NDEL1 at S336 and S332 by DYRK2 and GSK3β kinases is critical for increasing neurite outgrowth. Using biochemical fractionation to distinguish actin filaments vs. monomers, the study demonstrated that phosphorylated NDEL1 was associated with high filamentous actin levels in the growth cone, and when phosphorylation was suppressed, actin filaments were decreased (Woo et al., 2019). As NDEL1 phosphorylation depends on TARA and the recruitment of DYRK2/GSKβ kinases to actin, it is interesting to investigate how NDEL1 regulates actin dynamics via the cordination of actin binding proteins during neurite outgrowth. These studies illustrate the importance of regulating actin assembly by various signaling molecules as

well as the potential connection with the microtubule cytoskeleton. Although it was studied for axon growth, tsuch actin regulation likely plays critical roles in axon guidance and branching during axon morphogenesis.

1.2.2 Microtubule

In addition to actin, microtubules are highly regulated in axon morphogenesis. Made up of α- and β-tubulin heterodimers that assemble into 25-nm hollow cylinders, microtubules provide structural support for mechanical strength and tracks for motor-based transport. Microtubules are not static structures, but rather dynamically polymerize and depolymerize at each end. This inherent property, termed dynamic instability, allows cycles of growth and shrinkage of microtubules, and thereby provides a mechanism to rapidly remodel microtubules during axon growth, guidance, and branching. In the growth cone, most microtubules are concentrated in the center, but some can assemble and extend into the peripheral domain that has active actin remodeling (Figure 1.2B) (Lowery and Van Vactor, 2009). There, dynamic microtubule assembly/disassembly is not only important for the forward advancement of axons but also for controlling their growth direction (Lowery and Van Vactor, 2009). When microtubules polymerize in the center but depolymerize from the edge, the growth cone moves forward; when microtubules polymerize on one side but depolymerize from the other side, the growth cone can reorient and change axon growth direction. When new growth cones are generated from axonal shafts through actin patches (Gallo, 2011), microtubules are critical to generating and stabilizing nascent branches via polymerization or transport as short fragments.

Many molecular players have been identified to control microtubule dynamics and stability at different assembly/disassembly steps (Armijo-Weingart and Gallo, 2017; Kevenaar and Hoogenraad, 2015), and they can regulate axon morphology (Figure 1.2B). Microtubule associated proteins (MAPs), such as tau, MAP1B, and MAP2, bind to the microtubule lattice and stabilize microtubules, and all were shown to function to promote axon morphogenesis (Dehmelt and Halpain, 2005; Kapitein and Hoogenraad, 2015). Severing proteins, including spastin and katanin, differentially participate in axon branch formation by severing microtubules and creating ends to allow for new polymerization to occur (Ghosh et al., 2012; Yu et al., 2008). Also, motor proteins that move cargos along microtubules contribute to axon morphogenesis through anterograde and retrograde transport of proteins, RNAs, vesicles, and organelles (Guillaud et al., 2020). However, how these molecules coordinate to regulate microtubule structure and function during different

stages of axonal morphogenesis remains a major question. Here, we describe several recent studies that illustrate the complexity of such regulation.

Spastin is an AAA-ATPase severing protein that regulates microtubule dynamics by producing small microtubule segments and thus promotes neuronal branching possibly via increased fragment transport and new microtubule assembly (Kuo et al., 2019; Yu et al., 2008). However, the underlying mechanism for this role has remained elusive. One study suggests spastin accumulates at the ends of microtubules, severs the shrinking ends, and then increases the total mass and number of microtubules through an ATP-independent regrowth mechanism (Kuo et al., 2019). How does severing allow for the regrowth of microtubules? A recent study has identified a connection between spastin and CRMP5, a member of the collapsin-response mediator protein (CRMP) family proteins that are known to promote microtubule assembly and neuronal development (reviewed in (Hotta et al., 2005; Nakamura et al., 2020; Yamashita et al., 2011)). The group found a direct physical interaction, through immunoprecipitation, between the N-terminal of spastin and the C-terminal of CRMP5. This interaction is important for increasing microtubules in HeLa cells in which co-transfection of CRMP5 and spastin reduced the loss of total tubulin levels caused by spastin alone. Based on rescue and overexpression analysis in hippocampal neurons, the study showed that this interaction is important for axon growth and branching (Ji et al., 2018). This new study supports the notion that spastin-induced severing could then be closely followed by another microtubule polymerization stimulating factor to promote overall microtubule assembly needed for axon growth and branching.

The notion that different microtubule regulators work in a coordinated manner to mediate axon morphogenesis is supported by another recent study of the interplay between three microtubule regulators, XMAP215, tau, and EB1 (Hahn et al., 2021). XMAP215 is a microtubule polymerase that promotes microtubule growth and elongation *in vitro* by catalyzing the addition of tubulin dimers at the plus end (Brouhard et al., 2008). XMAP215 was shown to play a critical role in growth cone morphology and axon guidance by regulating microtubule dynamics downstream of guidance cues (Slater et al., 2019). Tau is most widely known for stabilizing microtubules in neurons. However, recent evidence suggests that tau is not required for microtubule stabilization but enables microtubules to have long lived labile domains for growth and polymerization (Kadavath et al., 2015; Qiang et al., 2018). Additional work in hamster neurons has shown that tau is enriched in growth cones and associated with dynamic and stable microtubules as well as actin filaments to promote neurite outgrowth and growth cone turning (Biswas and

Kalil, 2018). EB1 (also known as end binding protein 1) is a microtubule plus end tracking protein associated with microtubule extension, and it localizes at the distal axons and growth cones to track the growing microtubule plus end (Akhmanova and Hoogenraad, 2005). The current study screened individual microtubule regulator in cultured *Drosophila* neurons (Hahn et al., 2021) and found that genetic ablation of any one of these genes resulted in reduced polymerization, curling of the microtubules into the formation of bundles, and defects in axon growth. Trans-heterozygous and epistatic analysis further showed a role of their genetic interaction in promoting microtubule bundle formation and polymerization based on shared mutant phenotypes, i.e., reduced axon growth, EB1 comet sizes, numbers, and velocities (Hahn et al., 2021). Using several truncation mutants in rescue experiments, the study suggested a model in which tau outcompetes EB1 for space on GDP tubulin, therefore allowing EB1 to bind and stabilize the GTP tubulin cap that is needed for XMAP215 mediated polymerization. The study also linked these three proteins to spectraplakin-dependent guidance of microtubule bundle formation needed for axon growth and maintenance.

While the above study illustrates the importance of local regulation of microtubule assembly via multiple regulators during axon growth, local regulation is also important for microtubule stability and transport function during axonal branch development. This is illustrated by recent studies of MAP7, a MAP that upregulates its expression during the development of collateral branches of sensory neurons from the DRG (Tymanskyj and Ma, 2019; Tymanskyj et al., 2017; Tymanskyj et al., 2018). Knockout of MAP7 in cultured DRG neurons reduces branch formation, whereas overexpression of MAP7 increases branches (Tymanskyj and Ma, 2019; Tymanskyj et al., 2017). Interestingly, endogenous MAP7 localizes at mature branch junctions, but in nascent branches, MAP7 follows the newly arrived microtubules (Tymanskyj et al., 2017; Tymanskyj et al., 2018). This delay is attributed to its ability to bind to acetylated microtubules and avoid tyrosinated microtubules at the dynamic plus ends. This unique binding feature is mediated by two microtubule binding domains, thus allowing MAP7 to stabilize microtubules and prevent nascent branch retraction. Furthermore, MAP7 is shown to interact with the motor protein kinesin-1 via its carboxyl domain and affect kinesin recruitment to microtubules (Monroy et al., 2018; Tymanskyj et al., 2018). This unique ability allows MAP7 to change intracellular transport via a potential track switching mechanism to promote branch growth (Tymanskyj et al., 2018). In fact, analyses of MAP7 domains that interact with microtubules or kinesins in DRG neurons are consistent with its role in regulating branch formation and growth, two steps that are essential to axon

morphogenesis. MAP7 has two additional gene homologs expressed in mammalian neurons. MAP7D1 localizes in the proximal region of cortical neurons and enhances outgrowth of callosal neurons through phosphorylation by DCLK1 *in vivo* (Koizumi et al., 2017). MAP7D2 was recently shown to localize at the proximal region of cortical neurons and recruit kinesin-1 to enhance transport and cortical branching (Pan et al., 2019). Since other MAPs, such as tau, not only localize to certain parts of microtubules but also interacting with kinesin motors (Dixit et al., 2008), these studies raise the importance of localized control of transport by various MAPs in supporting axon morphogenesis.

1.3 Regulation of Intracellular Membrane Organelles

1.3.1 Energy metabolism and mitochondria

The creation of various axon morphologies is an energetically demanding process. Led by motile growth cones, axons can travel great distances and navigate through many different tissues in order to find their correct targets. Depending on local cues, branches can be formed and retracted along this developmental journey. As mentioned earlier in this chapter, axon growth, guidance, and branching invovle cytoskeletal rearrangement and motor-based organelle transport, both require energy. Where is the energy produced to support these processes and how does the neuron use energy to create the diverse array of neuronal subtypes and specific compartments such as axons and dendrites?

ATP is the main energy source for many cellular processes such as signaling cascades, actin and microtubule re-reorganization, and motor-based long distance transport (Figure 1.3) (reviewed in (Gallo, 2020)). Cellular ATP is primarily generated through oxidative phosphorylation in mitochondria, which relies on the movement of electrons through the electron transport chain to produce large amounts of ATP with the consumption of oxygen. Mitochondria are transported through the cell by kinesin-based transport to sites of high energy demand. This role of mitochondria at developing branch junctions has been shown by live imaging of cultured DRG neurons where mitochondria stall prior to sites of branch formation (Spillane et al., 2013). Disruption of mitochondria transport and localization is implicated in numerous development and degenerative diseases such as Parkinson's disease (Liu et al., 2012). Therefore, transport and localization of mitochondria to areas of high energy demand is particularly important for neuronal morphogenesis.

Previous studies have shown that Lkb1, a tumor-suppressor protein, regulates mitochondrial motility and axon morphogenesis of the central

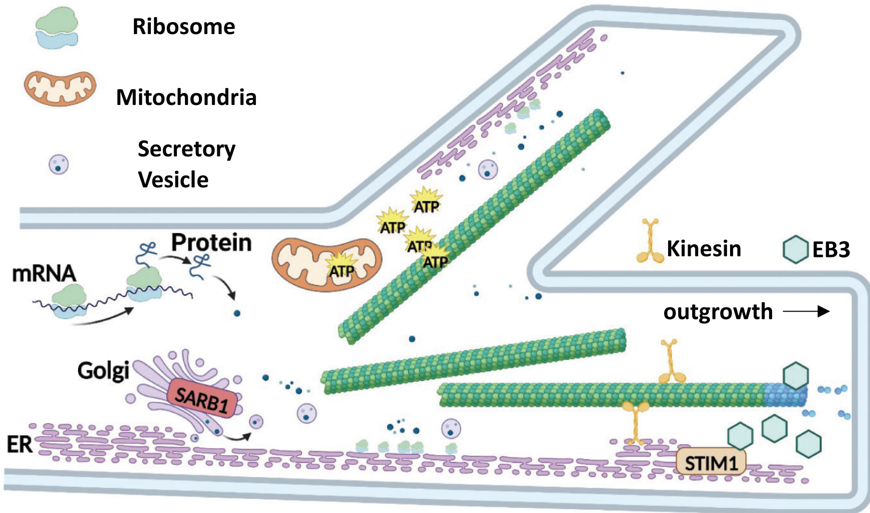

Figure 1.3 Intracellular organelles enriched within a branch junction contribute to axon morphogenesis. Schematic drawings depicting the energy and local translation machineries that are enriched within a branch junction. Endoplasmic reticulum (ER), Golgi apparatus, mRNA transcripts, ribosomes, and mitochondria are present to support protein synthesis and secretory vesicle formation during branch formation and extension. Interplay between microtubules, kinesins, and ER could shape neuronal morphology as ER interacting proteins, such as STIM1, promote the recruitment of EB3 to plus ends of microtubules allowing for outgrowth.

nervous system (CNS) neurons *in vivo* (Courchet et al., 2013; Spillane et al., 2013). A recent study of the transcriptome of Lkb1 knockout neurons has identified Efhd1, a calcium-binding protein in the inner mitochondrial membrane to be the downstream effector of Lkb1. Utilizing both *in vitro* DRG explants and *in vivo* limb analysis in knockout mice, the authors showed that loss of Efdh1 led to decreased ATP production, shortened mitochondria, and aberrant axon morphology such as reduced axon elongation and reduction in axon branching (Ulisse et al., 2020). Interestingly, however, these defects are not related to mitochondrial motility, but rather caused by increased stress responses mediated by two kinases (AMPK and Ulk) (Ulisse et al., 2020). Since mitochondria are known to be involved in many metabolic functions and critical for energy production, this new study demonstrates additional roles that mitochondria play in axon morphogenesis (Figure 1.3).

ATP can be also produced by glycolysis, which enzymatically adds phosphate to ADP with the conversion of glucose into pyruvate. Glycolysis is oxygen independent and is faster but less efficient than oxidative phosphorylation. Recent studies demonstrate the requirement of the glycolytic pathway for high energetic needs of morphogenesis in a faster and local manner.

Axons and growth cones of sensory DRG axons in culture were shown to have glycolysis enzymes. With the necessary materials present, the authors showed that growth cone dynamics and axon extension depend on glycolysis (Ketschek et al., 2021). Considering its additional role in synaptic development (Jang et al., 2016), the non-mitochondrial sources of ATP could provide another way to regulate axon morphogenesis, specifically in the creation of new branches.

1.3.2 Local translation, endoplasmic reticulum, and the Golgi apparatus

In addition to energy demands, the highly asymmetric structures of neurons contain different compositions of proteins and organelles in their axons. How is a neuron able to produce the needed proteins to maintain its function across vast distances? The emerging idea of local protein synthesis (LPS) suggests that specialized neuronal branches and distal regions of axons locally produce necessary proteins instead of relying on transport-based protein delivery. The presence of the protein synthesis machinery at distal regions and distinct branch sites provides evidence of local translation away from the soma (reviewed in (Holt et al., 2019)). Supported by proteomic analysis, fluorescent-based live cell imaging, and *in vivo* knockdown of ribosomal proteins (Holt et al., 2019; Wong et al., 2017), specific regulation of local protein synthesis is recognized as an important characteristic of highly polarized and morphologically complex neurons (Figure 1.3).

New techniques such as *in situ* cryo-electron tomography (CryoET) have allowed for a finer look at the axon branch to understand the complexity of the morphological distinction of subcellular components. CryoET examines protein 3D structures at high resolution in their intact, cryo preserved, native environment by repeated exposure of electrons to the sample at rotating angles. Recently, CryoET was utilized for the identification of organelles and molecular machinery present in two morphologically distinct stages of axonal branches (Nedozralova et al., 2022). It revealed that immature branches contained mainly actin patches, while mature branches contained microtubules and more structured actin. Small mitochondria were often localized at branch sites. Complex association of microtubules, mitochondria, and endoplasmic reticulum (ER) was found at mature branches, suggesting their important roles. Interestingly, clusters of ribosomes were also found in dense patches at branches, supporting the idea of locally synthesized proteins and providing evidence that branches are hubs of axon development and outgrowth (Nedozralova et al., 2022). Identification of these ribosomal clusters further supports the concept that instead of being transported to long

distances, proteins are able to be made locally. The co-presence of these LPS machineries in the axon branch junction reinforces the necessity of cytoskeleton and membrane remodeling needed for branch morphogenesis.

The interplay between microtubules and ER in determining neuronal polarity and axon specification is further supported by additional studies. The ER has two main subtypes: tubules and cisternae. In unpolarized cells, the ER is organized in perinuclear cisternae that are connected with tubular structures in the cell periphery. These two ER subtypes were found to localize in developing neurons based on marker staining, with ER tubules in the axons and ER cisterna in the soma and dendrites. This differential distribution depends on the interaction of ER with microtubules via the kinesin motor KIF5A (Figure 1.3). Conversely, ER tubules also regulate microtubule organizations by facilitating the stabilization of microtubules and correct localization of proteins required for the development of the axon initial segment. Importantly, recruitment of ER tubules into one nascent neurite is required for axon specification and it involves an ER shaping protein P180, which regulates microtubule stabilization (Farías et al., 2019). The result suggests a greater role of the concerted effort of ER and microtubules in axon morphogenesis. Indeed, remodeling of the growth cone also requires a concerted effort of reorganization of microtubules and ER. The ER-calcium sensor stromal interacting molecule 1 (STIM1) was shown to regulate the recruitment of polymerizing microtubules through EB3 movement into filopodia of sensory DRG neurons *in vitro* and *in vivo* (Pavez et al., 2019). Neurons with STIM1 knockdown were unable to recruit microtubules to the steering edge of the growth cone. This study provides a potential mechanism not only linking ER and microtubules but also connecting calcium signaling to growth cone dynamics, which ultimately can regulate branching and outgrowth, leading to the formation of the diverse morphologies of neurons (Pavez et al., 2019).

Trafficking from the ER to the Golgi apparatus is part of normal cellular function, pivotal in secreting proteins throughout the neurons to specified destinations. Recent studies investigating disruption of this process suggest a role of the Golgi apparatus in early stages of neuronal development such as axon polarization and axonogenesis (Figure 1.3). This is demonstrated by the study of SAR1B, a member of the Arf GTPase family involved in the fusion and budding of vesicles in the secretory pathway from the ER to the Golgi (Li et al., 2020). SAR1B was shown to express in the developing cortex. SAR1B knockdown in the developing mouse cortex led to defects in radial migration and axon morphogenesis of cortical neurons, as neurons were unable to orient in the proper layers of the cortex and had altered processes with more unpolarized morphologies, and disrupted Golgi localization

(Li et al., 2020). Another example is from the study of Efa6, an Arf activator (Qu et al., 2019). Interestingly, Efa6 is a membrane-associated protein that inhibits microtubule outgrowth by binding to tubulin directly through its microtubule elimination domain. This ability allows Efa6 to exclude microtubules from filoppdial protrusions and inhibit microtubule polymerization, thus controlling the location of branching (Qu et al., 2019).

Since the Golgi apparatus plays a role in cellular trafficking of proteins, axonal specification and outgrowth have been linked to Golgi dynamics (Rosso et al., 2004). One example of this regulation is demonstrated by SCY1-like pseudokinase 1 (SCYL1), a catalytically inactive protein kinase that interacts with the COP protein and controls Golgi morphology. SCYL1 is expressed in the brain and its variants cause brain developmental delay (Schmidt et al., 2007b). A recent study further demonstrated that SCYL1 knockdown altered Golgi morphology and inhibited axon outgrowth in Rat1-cells. Interestingly, it was identified that arginine methylation of SCYL1 is pivotal in normal Golgi morphology and subsequent outgrowth of hippocampal neurons (Amano et al., 2020). Biochemical analysis showed that SCYL1 was methylated in brain tissues, and pharmacological inhibition of protein arginine methyltransferase 1 (PRMT1) led to the similar Golgi and axon outgrowth defects. Finally, wild-type SCYL1, but not the arginine methylation deficient SCYL1 mutant, rescued the SCYL1 knockdown defects. Although the mechanism mediating this methylation regulation remains to be resolved, the organization of membrane structures within the developing axon will continue to show their critical albeit complex roles in axon morphogenesis.

1.4 Cell Surface Signaling

Axon morphogenesis involves the integration of information in the surrounding environment with temporal–spatial specificity. Neighboring cells and extracellular matrix often provide molecular cues. Neurons can detect these cues through ligand-receptor mediated signaling cascades. During development, these signaling cascades control axon growth, guidance, and branching, ultimately giving rise to the diverse shapes tailored for specialized neuronal functions (reviewed in (Bashaw and Klein, 2010; Gibson and Ma, 2011; Kalil and Dent, 2014)).

The classical signaling cascade involves transmembrane receptors bound by specific extracellular ligands activating downstream proteins to alter growth cone responses, typically through cytoskeletal rearrangement (Figure 1.4) (Bashaw and Klein, 2010). Many of these receptors are type I transmembrane proteins that, once bound to ligands, dimerize and engage

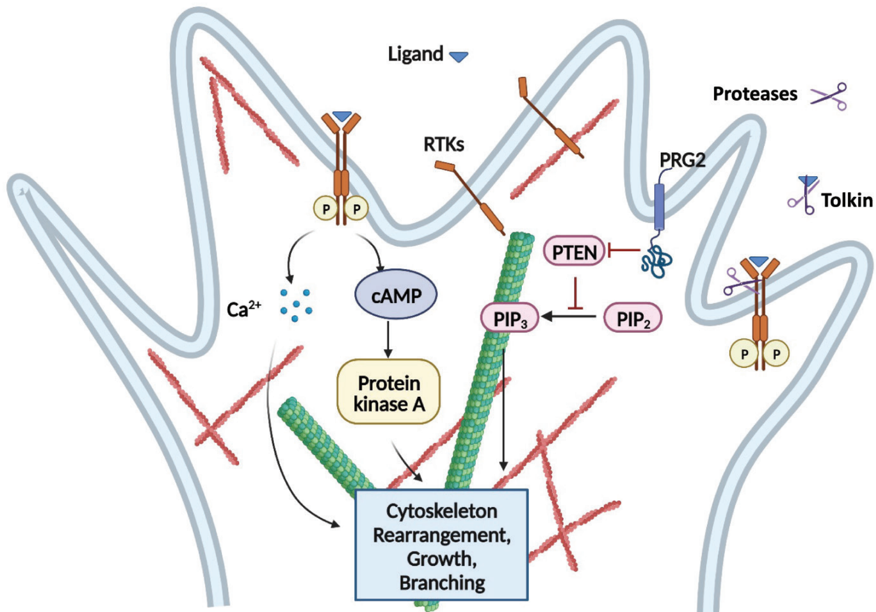

Figure 1.4 Signaling from cell surface receptors in axon morphogenesis. Schematic drawing of extracellular ligands binding to receptor tyrosine kinases (RTKs), which leads to receptor autophosphorylation and downstream signaling via second messengers, such as calcium (Ca^{2+}), cAMP and lipids (PIP_2 and $PIPI_3$). This signaling leads to rearrangement of cytoskeletal components and ultimately axon growth, guidance, and branching. Interacting proteins, such as PRG2/PTEN, balance positive and negative regulators to reorganize the cytoskeleton. Cell surface receptors can be regulated by other factors, including proteases, such as Tolkin, that can cleave both ligands and receptors.

downstream signaling molecules either through phosphorylation or direct binding. Four main families of ligands and receptors were initially identified for guiding axons: netrins and their DCC, Unc5, and neogenin receptors (Kennedy, 2000); Slits and their Robo receptors (Brose and Tessier-Lavigne, 2000); semaphorins and their plexins and neuropilins receptors ((Pasterkamp and Kolodkin, 2003); and ephrins and their Eph receptors (Kullander and Klein, 2002). Since the initial identification of these families, additional factors like the morphogens (BMP, sonic hedgehog, and Wnt) as well as growth factors (such as neurotrophins and neuregulin) have been shown to mediate axon guidance and morphology (Augsburger et al., 1999; Charron et al., 2003; Lyuksyutova et al., 2003; Markus et al., 2002). Although additional factors may be found, there is a finite number of genes encoding these factors in the genome. What are the mechanisms that increase the versatility of

molecular pathways to generate complex axon morphologies in the developing nervous system?

One mechanism is to regulate receptor function. This can be illustrated by Slit/Robo signaling involved in midline crossing during commissural axon development in the spinal cord. Commissural axons project across the floorplate at the midline making connections on the contralateral side. Prior to crossing, secreted Slit ligands at the ventral midline are unable to influence the axons. This is partly due to the inhibition of the surface expression and the activity of the Slit receptors, Robo1/2, by other proteins such as Robo3. Post crossing, Robo3 expression is downregulated and Slits are able to interact with Robo1/2 receptors and repel the growth cone from the floorplate to prevent recrossing (Chedotal, 2019).

Additionally, proteolytic regulation of ligands and receptors could provide a mechanism for specificity (Figure 1.4). This can be again illustrated by Slit/Robo signaling in axon guidance. A previous biochemical purification study has suggested that Slit is cleaved into N and C fragments (Wang et al., 1999). While the N fragment functions similar to the full length (FL) Slit to mediate repulsive guidance of commissural axons in the midline, the C fragment is shown to bind to PlexinA1 to regulate postcrossing commissural axons (Delloye-Bourgeois et al., 2015). In *Drosophila*, commissural axons expressing a cleavage-blocking Slit mutant caused a longitudinal guidance defect but not midline repulsion. Further biochemical and genetic analyses of the two Slit receptors, Robo and Dscam, suggest a model in which FL-Slit binds Robo complex to cause repulsion while the cleaved N fragment binds Dscam and Robo complex to promote axonal outgrowth after midline crossing (Alavi et al., 2016). Recently, the protease responsible for the cleavage was identified to be Tolkin (Tok) based on both *in vitro* and *in vivo* analyses (Kellermeyer et al., 2020). Utilizing *Drosophila* S2 cells, the study identified that only co-transfected cells with Slit-FL and Tok had processed Slit. *In vivo*, genetic manipulation of Tok through knock-out and rescue demonstrated that Slit-FL processing into Slit-N and Slit-C is critical for spinal cord midline guidance. While Tok may be a Slit-specific protease, understanding how extracellular ligands undergo cleavage to alter the guidance response can shed light on how morphology can be shaped as circuits develop.

Another proteolytically regulated family of cell surface proteins is neuregulins (Nrgs), which are implicated in having a neural developmental role since mutations of Nrgs pose as a high-risk factor and are associated with greater incidences of psychiatric disorders (reviewed in (Mei and Nave, 2014)). Nrgs are transmembrane proteins that exist in two topologies and undergo proteolytic cleavage by extracellular proteases including BACE1.

In type I topology, proteolysis causes membrane tethered Nrgs to release a diffusible epidermal growth factor (EGF)-like containing polypeptide that interacts with ErbB receptors to elicit a conical signaling cascade. In type II topology, BACE1 cleavage generates a transmembrane form that contains the EGFL domain to interact with ErbB to elicit transmembrane-dependent signaling. A recent study showed that in cortical GABA positive interneuron cultures, the soluble form of three Nrg homologs (Nrg1–3) was able to increase neurite outgrowth of interneurons by increasing branch number, complexity, and length (Rahman-Enyart et al., 2020). Another study aimed at understanding the sites of transmembrane forms of Nrgs using an optogenetic cleavage reporter, LA^{143}-NRG3 (Ahmad et al., 2022). It was found that mature membrane Nrg3 emerges on the somatodendritic plasma membrane where it is re-endocytosed and transported into axons via transcytosis. Mature Nrg3 also accumulates on the presynaptic membrane through a "transsynaptic retention model". Thus, the mechanism mediating the processing of cell surface signaling molecules could diversify the regulation of their normal function in axon outgrowth and the ability to make proper connections.

While the regulation of ligands and their cell surface receptors is important for the diversity of responses from axons, dissecting the downstream signal transduction pathways provides a more complete understanding of how the axon can navigate its environment, giving rise to the necessary morphologies. Second messengers, including cAMP, cGMP, $Ca2^{+}$, and lipid-based messengers such as phosphoinositides, are important components of the pathways to propagate signals more broadly across the navigating axon (Figure 1.4). For example, most receptor tyrosine kinases, when they dimerize after ligand binding, undergo a conformational change allowing them to autophosphorylate to provide binding sites as well as to activate downstream targets through phosphorylation. The targets include membrane voltage gated $Ca2^{+}$ channels, Ryanodine receptors that release calcium from intracellular stores, cyclic nucleotides, phosphoinositides such as phosphatidylinositol 3,4,5-trisphosphate (PIP3) at the plasma membrane, and other second messengers (Newton et al., 2016). These second messengers allow for the membrane localized signaling to propagate away from the cell surface to more precise mediators like cytoskeletal regulators needed for creating the diverse neuronal morphologies (Figure 1.4).

Another way to generate diverse axonal morphologies in developing axons is the regulation of competing signaling pathways leading to both positive and negative outcomes. The lipid signaling involving PIP3 provides a good example to illustrate this mechanism. PIP3 is generated by phosphoinositide 3-kinase at the plasma membrane and promotes axon elongation

and axon branching by reorganizing the actin cytoskeleton. Interestingly, this positive signaling can be attenuated by PTEN, a phosphatase that dephosphorylates PIP3 and reverses axon growth and branching. As PTEN is expressed at high levels in neurons, how does PIP3 overcome this antagonizing molecule? A recent study (Brosig et al., 2019) found a PTEN interacting protein that can resolve this conflict. It involves plasticity-related gene 2 (PRG2), a transmembrane protein that inhibits the phosphatase activity of PTEN and thus supports localized PIP3 production needed for actin remodeling. Overexpressing PRG2 in embryonic stem cell-derived neurons induced filopodia and branches along axons, whereas PRG2 knockout in hippocampal neurons reduced filopodia and branch formation. Importantly, the branching defect in the PRG2 knockout neurons can be rescued by the knockdown of PTEN or expressing FL-PRG2 but not the deletion mutant that does not bind PTEN. Although it remains to be determined how PRG2 is controlled, the inhibitory release model demonstrates the balancing act to control positive and negative signaling pathways in developing axons. Here, a mechanism to attenuate the PTEN block allows PIP3 signaling to facilitate the cytoskeletal rearrangement that is required for axon growth, guidance, and branch formation.

1.5 Posttranslational Modifications

Axon morphogenesis is a complex and dynamic process that requires tight regulation of a variety of protein functions. Posttranslational modifications (PTMs) increase the diversity of a protein through the covalent modification of their structures after protein synthesis. Here, we focus on PTMs that add functional groups, such as phosphates, small molecules, amino acids, peptides, or lipids. These types of PTMs affect protein structure, function, localization, stability, and proteolytic cleavage (Ramazi and Zahiri, 2021). Each of these PTM processes can modify the neuronal cytoskeleton such as microtubules, as well as signaling molecules that play a role in different steps of axon morphogenesis. The following section will discuss several recent studies and provide examples of four PTMs that play important roles in axon morphogenesis.

1.5.1 Phosphorylation

Phosphorylation is the addition of a phosphate group onto a protein by a protein kinase; this results in the protein being more negatively charged. Phosphorylation is a common method of protein regulation in the nervous

system and can occur on a variety of proteins, structures, and other molecules. This PTM functions to reversibly mediate a wide variety of protein functions during axon development and morphogenesis such as: microtubule stability, energy and mitochondrial metabolism, and neuronal polarization (Barbier et al., 2019; Igarashi et al., 2020; Ulisse et al., 2020). Each of these events is required for axon development and morphogenesis as previously mentioned in this chapter. For example, as discussed above, phosphorylation of AMPK and Ulk in the mitochondrial inner membrane decreases energy production and metabolism in *Efhd1* knockout neurons and results in decreased branching, demonstrating that phosphorylation plays a role in axon morphogenesis (Ulisse et al., 2020) (Figure 1.5A).

1.5.2 Acetylation of tubulin

Acetylation is the transfer of an acetyl group onto the lysine residue of a protein by the lysine acetyltransferase, resulting in the change of protein functionality and localization. Acetylation is widely known to occur in many cell types and cellular processes, but how it contributes to axon morphogenesis depends on the modified site in the target protein. Here, we use microtubule acetylation as an example to illustrate this point. Microtubules can undergo a variety of modifications such as acetylation, tyrosination, phosphorylation, and ubiquitination (Wloga et al., 2017). Acetylation of tubulin is known to be associated with microtubule behaviors in cells. Among multiple acetylation sites, lysine 40 (K40) on α-tubulin has been well characterized. This site is inside the lumen of microtubules, and its acetylation is correlated with stable and long-lived microtubules. Interestingly, recent studies have identified >12 other sites, and one of them, lysine 394 (K394), is highly conserved and located at the α–β tubulin interface (Figure 1.5B). Using the *Drosophila melanogaster* neuromuscular junction (NMJ) as a model system, a recent study (Saunders et al., 2022) examined the role of K394 in neuronal morphogenesis. They found that genetically blocking acetylation in a K394 mutant reduced microtubule stability and increased the number of synaptic boutons associated with the growth of axonal terminals. This mutant has decreased MAP1B-induced microtubule loops in axon terminals as well as an increased sensitivity to nocodazole, suggesting a role of microtubule stability supported by K394 acetylation. Consistent with the idea, increasing microtubule stability by various methods, including taxol treatment and overexpression of MAP1B (Futsch) or a tubulin chaperon, rescued the mutant phenotype similar to the wild-type level. They were also able to show that HDAC6, a deacetylase, is likely the key enzyme that controls the level of

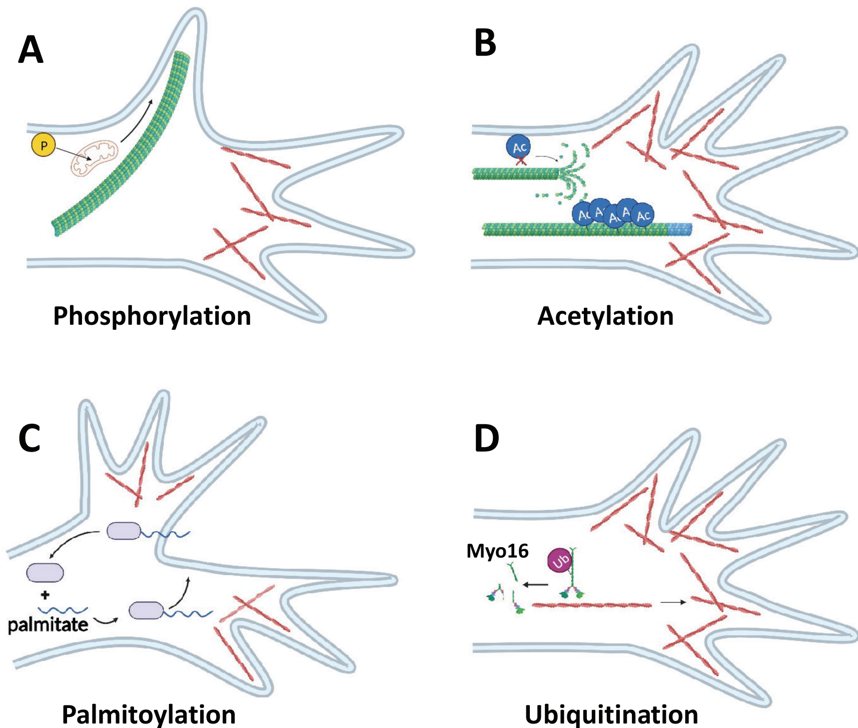

Figure 1.5 Posttranslational modifications in axon morphogenesis. (A) Phosphorylation of the mitochondrial inner membrane regulates energy production and plays a role in axon branching. **(B)** Acetylation (Ac) of tubulin is an important PTM regulating microtubule dynamics. Tubulin acetylation stabilizes microtubules allowing for axons and branches to be maintained. **(C)** Palmitoylation signals for proteins to be associated with the membrane. Palmitoylation functions in growth cone splitting allowing for the formation of two daughter branches. **(D)** Some proteins can be tagged for ubiquitination and subsequent degradation during axon development. A family of TRIM E3 ligases interact with unconventional Myo16 to promote axon growth.

K394 acetylation and consequently microtubule stability in the boutons. The study demonstrates the importance of microtubule acetylation in neuronal morphogenesis. Additional work determining other acetylation targets such as molecular motors, synaptic proteins, and receptors, as well as specific sites in these proteins could help provide a more detailed insight into how acetylation plays a role in axon morphogenesis.

1.5.3 Palmitoylation

Palmitoylation is the addition of a lipid group, such as palmitic acid, to a protein that results in membrane association and trafficking of the protein

through intracellular membrane compartments. A recent study (Dumoulin et al., 2018) suggests that palmitoylation contributes to neuronal morphogenesis through growth cone shaping and subsequent axon bifurcation (Figure 1.5C). The study used embryonic DRG neurons that exhibited a stereotyped axon bifurcation in the dorsal spinal cord (Gibson and Ma, 2011). This process is controlled by cGMP-dependent signaling, which involves a secreted peptide hormone CNP, its receptor Npr2, a membrane guanylyl cyclase that produces cGMP, and the cGMP-dependent protein kinase 1 (cGKI) (Schmidt et al., 2009; Schmidt et al., 2007a; Zhao and Ma, 2009; Zhao et al., 2009). In culture, CNP induced DRG growth cone enlargement, a potential step leading to bifurcation, through activation of cGKI (Dumoulin et al., 2018). Interestingly, cGKI is localized to vesicular structures that are associated with palmitoylated proteins, and cGKI activation leads to increased levels of protein palmitoylation. Using pharmacological reagents, the study showed that blocking S-palmitoylation, a reversible modification via thioester linkages, attenuated growth cone enlargement stimulated by CNP, whereas blocking de-palmitoylation increased growth cone size and axon elongation. These results suggest that S-palmitoylation could function downstream of cGKI to mediate CNP-mediated growth cone remodeling and subsequent axon bifurcation.

While this study is the first to explore the role of S-palmitoylation in growth cone remodeling, it is important to note that palmitoylation can also occur via amide-linkages (N-palmitoylation) to generate irreversible attachment of palmitate to the N-terminus of a protein. N-palmitoylation has previously been shown to regulate sonic hedgehog (Shh), a morphogen involved in cell proliferation and differentiation (Ji et al., 2016; Pepinsky et al., 1998). Therefore, many proteins involved in axonal development, such as guidance cues and receptors, could be regulated this way, and identifying the palmitoylation target proteins will provide useful clues to this type of regulation during axon morphogenesis.

1.5.4 Ubiquitination

Ubiquitination is an enzymatic reaction that adds a small protein ubiquitin to a cellular protein that is typically destined for degradation. The target protein is recognized by a substrate-specific E3 ligase, which in conjunction with the E2 ubiquitin-conjugating enzyme catalyzes the addition of single or multiple ubiquitins (Stack et al., 2000). Ubiquitination is known to play a role throughout the life of a neuron, from neurite outgrowth to presynaptic formation as well as degeneration (Pinto et al., 2021). Interestingly, ubiquitination can also enhance neurite outgrowth by tagging and degrading proteins that

inhibit axon growth (Konishi et al., 2004; Yang et al., 2010). A recent study showed that a family of E3 ubiquitin ligases contributes to axon guidance and morphology through destabilizing actin in response to extracellular cues (Figure 1.5D) (Pinto et al., 2021).

The TRIpartitite Motif (TRIM) proteins are a family of ubiquitin E3 ligases found to have critical roles in morphogenesis of cortical and hippocampal neurons. Two members, TRIM9 and TRIM67, interact with DCC, the netrin-1 receptor, to promote axon guidance and exhibit contrasting aberrant phenotypes when deleted (Boyer et al., 2018; Winkle et al., 2014). Deletion of TRIM9 in the cortex results in the thickening of the corpus callosum, which could be due to aberrant branching (Boyer et al., 2018). However, cortical deletion of TRIM67 results in the opposite phenotype, leading to thinning of the corpus callosum. Additional studies have demonstrated that TRIM9 and TRIM67 regulate cytoskeletal dynamics by modulating the ubiquitination of VASP, an actin polymerase (Menon et al., 2015). Interestingly, TRIM9 and TRIM67 have opposite roles in branching. *Trim9*$^{-/-}$ neurons have increased basal level branching and no change in Netrin-1-induced branching, whereas *Trim67*$^{-/-}$ neurons have no change in basal level branching but affect Netrin-1 branching, suggesting additional substrates for these ligases that could mediate such differences. Using a BioID-based proteomics approach, a recent study (Menon et al., 2021) identified a host of new candidates that TRIM9 and TRIM67 interact with based on their proximities. One candidate is an unconventional myosin, Myo16, which is localized along the axon as well as the tip of growth cone filopodia. Myo16 was found to interact with TRIM9 and TRIM67 at specific sites, and knockdown analysis suggests that Myo16 regulates netrin-dependent axon branching. Interestingly, Myo16 knockdown blocked netrin-induced branching seen in *Trim67*$^{-/-}$ neurons as well as basal branching seen in *Trim9*$^{-/-}$ neurons. These data suggest a possible regulatory mechanism in which TRIM9 and TRIM67 recognize Myo16 for ubiquitination for subsequent basal-level and netrin-dependent branching (Menon et al., 2021). Because the study has also revealed critical interactions with several other cytoskeletal components such as MAPs and tubulin isoforms, it is not surprising that additional regulation of cytoskeletal components by ubiquitination is present to fine-tune the cytoskeletal organization during neuronal morphogenesis (Menon et al., 2021).

1.6 Transcriptional Control

The complexity and diversity of neuronal subtypes is a hallmark of the mammalian nervous system. Historically, the classification of neurons has been

determined by morphology or functional roles. The morphologically specific features are associated with neuronal differentiation that is often accompanied by transcriptional regulation. Recent technological advances including single-cell isolation and next-generation sequencing allow for transcriptomic analysis of individual cells. Identification of the precise transcriptional signature of each neuron helps to understand the diversity of neurons and their morphologies (reviewed in (Armand et al., 2021)). An emerging idea is that defined neuronal cell types are determined not only by their distinct morphological features but also by their transcriptional profiles (Winnubst et al., 2020; Yuste et al., 2020). Additionally, studying distinct compartments (axon vs. soma) can link transcriptional profiles of axons or synapses to neurological diseases (reviewed in (Di Paolo et al., 2021)).

Alternative splicing is another mechanism that can expand the diversity of proteins produced for building axon morphologies. Pre-mRNA constructs can be spliced into a multitude of mRNAs that encode protein isoforms that shape neuronal subtypes. The *Drosophila* down syndrome cell adhesion molecule (Dscam) uses splicing to generate >19,000 isoforms with variable extracellular domains. These isoforms provide a unique self-surface recognition or tagging mechanism that is critical to self-avoidance, a patterning mechanism to generate non-overlapping branches (Grueber and Sagasti, 2010). A recent study examined not just the isoform expression, but their localization through lipid trafficking between dendritic and axonal compartments. When DSCAM trafficking and localization were disrupted through perturbed sphingolipid biosynthesis, *Drosophila* mushroom body axons lost the ability to segregate axon branches at distal axonal regions (Goyal et al., 2019). Alternative splicing and distinct localization of those isoforms thus can give neurons the diverse protein-building blocks to form the complex array of patterning of neuronal circuits.

Recent advances in transcriptomics allow for identification of alternative splicing in neuronal subtypes for a specific gene. Using cultured cortical neurons pre- and post-axon specification, a recent study found a strong association of axon specification and neuron-specific alternative splicing (Zhang et al., 2019). PTBP2, an RNA binding protein that is enriched in neurons at the time of axonogenesis, is required for proper axon formation both *in vitro* and *in vivo*. The importance of splicing is further demonstrated by one of the PTBP2 targets, Shtn1, which is involved in neuronal polarization. Normally, Shtn1 switches from a long isoform (Shtn1L) to a short one during axonogenesis. This switching depends on PTBP2 and is critical to axon formation because each isoform has different activities in actin polymerization (Zhang et al., 2019).

The complexity of vertebrae neuronal cells has also been expanded by the study of non-coding genomic regions (Closser et al., 2022). Enhancers, the gene regulatory sequences that alter gene transcription, were shown to have varying promoter interaction profiles between neuronal and non-neuronal cell types. Additionally, the use of mouse embryonic stem cells and *in vivo* mouse embryonic spinal cord allowed a closer examination of motor neurons at different developmental stages. During these stages with distinct connectivity and morphological shapes, motor neurons have unique gene regions that are accessible for transcriptional factors. In addition, the authors showed the evolutionary differences in the complexity of regulatory elements, providing a possible explanation to how the nervous system increases its complexity with a relatively small difference in the total number of genes (Closser et al., 2022). The diverse array of neuronal cell types requires some intrinsic mechanisms to dictate them and, as the understanding of genetic regulation expands, these mechanisms can add additional control of distinct axonal morphologies and hence diverse cell types.

1.7 Axon Morphogenesis in Neurodegeneration and Nerve Regeneration

Neurodegeneration can lead to drastic changes in neuronal morphology, due to activation of abnormal cell death pathways and cell death (Jellinger, 2001). There are a wide variety of neurodegenerative diseases, including, but not limited to, amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), and Parkinson's disease (PD). While it is known that the key contributors to each of these disease result in neuronal death, less is known about their role, if any, in neural development and maintenance. Not surprisingly, though, the recent identification of an evolutionary conserved Wnk kinase demonstrates the cross-function of a developmental gene in neurodegeneration (Izadifar et al., 2021). Identified through reverse genetic screening in *Drosophila* and corroborated in mouse cortical neurons *in vivo*, Wnk was found to be required for branch extensions and stabilization, as well as maintenance of adult neurons with a role in programed degeneration. Interestingly, downstream factors of Wnk include Nmnat, Axed, and Sarm1, proteins known to have opposite functions in axonal maintenance and destruction, suggesting that axonal development and maintenance share intrinsic mechanisms. Thus, in this section, we discuss some recent investigations of key neurodegeneration players in axonal growth and morphology. Understanding their roles in axon morphogenesis may provide insights and potential therapeutic targets for neurodegenerative diseases. In addition, we will expand the discussion to nerve regeneration, the opposite of

degeneration, when axon morphogenesis is crucial to functional recovery after nerve injury.

1.7.1 Amyotrophic lateral sclerosis

TDP-43 is a transcriptional repressor well-known for its involvement in ALS and the related disorder, frontotempoal dementia (FTD). Mutations in TDP-43 produce cytosolic aggregates in post-mortem tissue, a hallmark of ALS and FTD (Suk and Rousseaux, 2020). The aberrant expression and mislocalization of TDP-43 are associated with neurite abnormalities and subsequent degeneration of motor neurons (Brettschneider et al., 2014; Vickers et al., 2009; Zhou et al., 1998). Although TDP-43 is predominantly localized to the nucleus, it is involved in transporting messenger ribonucleoprotein particles along axons. A recent study (Atkinson et al., 2021) links TDP-43 expression to neuronal morphology and cytoskeleton regulation during development. It shows that changes in TDP-43 expression levels might affect the neuronal cytoskeleton by upregulating actin cytoskeletal proteins. Using primary cortical mouse neurons that expressed either one or two copies of human wild-type TDP-43, the group used a proteomic approach to show that two copies of TDP-43 significantly upregulated actin-binding proteins, which are required for axon guidance, receptor trafficking, and synaptic plasticity. Interestingly the group found that expressing one copy of human TDP-43 increased the number of branches in DIV3 cortical neurons compared to wild-type neurons; however, no change in the overall length or density of neurites was observed. Additional data established that neurons expressing two copies of TDP-43 did not show any morphological changes compared to the wild-type neurons potentially due to an increase in cytoplasmic TDP-43. This suggests that changes in TDP-43 expression can result in changes in neuronal branch complexity but do not result in changes in overall neuron growth. Together with the proteomics data, TDP-43 expression could contribute to changes in branch complexity through the interaction with the cytoskeleton (Atkinson et al., 2021). Other molecules such as FUS and SOD1 have been previously described to have similar branching phenotypes (Garone et al., 2021; Osking et al., 2019). As many actin proteins are involved in growth cone dynamics and outgrowth, further studies are required to parse out the relationship and the mechanisms governing the regulation of the actin cytoskeleton by these ALS-linked genes.

1.7.2 Alzheimer's disease

Amyloid precursor protein (APP) is most widely known for its association with AD. APP typically undergoes cleavage by β and γ secretase. Aberrant

cleavages caused by genetic mutations result in the accumulation of APP and subsequent AD symptoms. Under normal conditions, APP is a transmembrane protein that contributes to a wide variety of cellular events including axonal outgrowth, synaptic development, and plasticity as well as receptor-like signaling (reviewed by (Muller et al., 2017)). APP is highly expressed during early development and is also found in stem cells in the adult brain, suggesting that it might be critical for neurogenesis (Wang et al., 2014). While the pathological role of APP is widely studied, its role in axon growth was only investigated recently. Using cultured hippocampal neurons, a recent study (Southam et al., 2019) provides the evidence for the role of APP in axonal morphogenesis, as APP knockout neurons displayed a decrease in the distal axonal and dendritic outgrowth compared to wild-type neurons. However, immunohistochemistry and Western blots showed no alteration in synaptic markers. Interestingly, APP mutant neurons adhered more strongly to poly-L-lysine substrates in culture, raising the question about the role of APP as a cell adhesion molecule. This potential role could be important for axon growth and development since axons need to be attached to a substrate prior to growth (Southam et al., 2019).

While this study lays a solid framework for the role of APP in axon and dendrite morphogenesis, further studies are required to parse out the relationship between synaptic loss and axonal or dendritic loss. Furthermore, as studies have begun to characterize a myriad of biological and physiological roles of APP, loss of APP expression in hippocampal neurons could result in impaired intracellular signaling that could impact axon growth and branching.

1.7.3 Parkinson's disease

Alpha-synuclein (α-synuclein) is well-known for its role in PD. The pathogenic form of α-synuclein accumulates to form aggregates called Lewy bodies that are typically the precursor to neuronal degeneration. Many studies have shown under normal physiological conditions that α-synuclein localizes in the presynaptic terminals and alterations in localization result in changes of neurotransmission (reviewed in (Stefanis, 2012)). Additional studies have shown that α-synuclein associates with cellular membranes, but its functions in axonal morphogenesis are not well understood. A recent study (Schechter et al., 2020) showed that α-synuclein plays a significant role in axon growth and elongation. In cultured α-synuclein null mouse cortical neurons, overexpressing either wild-type (WT) α-synuclein or the A53T α-synuclein mutation that is associated with familial early-onset PD, led to longer axons and more collateral branches compared to control neurons. Interestingly,

A53T α-synuclein branch length was also significantly longer than WT collaterals. The authors further established a relationship between α-synuclein and $PI4,5P_2$, a phosphoinositide found at neuronal membranes and known to function in axon growth. The study revealed that α-synuclein expression increased together with $PI4,5P_2$ levels, which corresponded with increased axon growth and collateral branching. They also observed increased density of striatal white matter tracts *in vivo* consistent with the phenotypes seen *in vitro* (Schechter et al., 2020). These results support a role for α-synuclein in axon growth and branching. Further studies are necessary to better understand the role of membrane-associated α-synuclein in axon growth and guidance and parse out the functional difference between the axonal and synaptic pools of α-synuclein.

1.7.4 Nerve regeneration

Understanding the cellular and molecular mechanisms of axon regeneration is important for treating devastating human conditions following traumatic injury. Most research attempts to parse out the mechanisms governing regrowth of injured nerves in the central and peripheral nervous system. Extracellular factors such as proteins associated with myelin have been found to inhibit axonal regeneration, whereas intrinsic mechanisms can be altered to overcome such inhibition and increase the growth capacity (Mukhopadhyay et al., 1994; Qiu et al., 2002). The details of those mechanisms specifically related to the central nervous system can be found in a comprehensive review by Frank Bradke (2022). Interestingly, however, another way to promote nerve regeneration is through axon branching from injured or spared nerves. This is often referred to as "nerve sprouting" in the regeneration field and has been shown to be a potentially effective way to achieve functional recovery (Tuszynski and Steward, 2012). Relatively little is known how sprouting can be achieved and whether it can be promoted by targeting developmental mechanisms. Regardless, recent analysis using single-cell RNA sequencing (scRNA-seq) in various injury models (Matson et al., 2022, Li et al, 2022, Jacobi et al, 2022) could provide new insights into this important problem.

1.8 Concluding Remarks

Recent studies have provided many new insights into axon morphogenesis. Building on the key framework, we have begun to appreciate the complexity of molecular and cellular regulation of axon growth, guidance, and branching. Aided by new technologies, such as super resolution imaging and

single-cell sequencing, future studies will continue to explore the mechanisms that endow the morphological diversity that are essential for building complex neural circuits. Several key questions need to be addressed. How does transcriptional regulation specify axon morphology? What controls the expression of a specific set of genes that are critical to defining axon morphology? How do neurons respond to environmental cues? And how are the expression and localization of these cues controlled? A recent study of the transcriptional regulation of dendrite development provides a good example of how to address these questions (Xie et al., 2022).

The molecular and cellular mechanisms of axon morphogenesis would help understand human brain disorders. For example, many axon guidance molecules are associated with autism and schizophrenia, whereas animal models with mutations in these molecules have revealed neural deficits found in human disorders (Van Battum et al., 2015). As described above, many genes related to neurodegeneration have begun to be found to play roles in axon growth, guidance, and branching. Further investigation of their roles in axonal development may provide a better understanding of their contribution to various neurodegenerations in adult brain. Finally, the mechanism identified during development would help find ways to promote axon regeneration after injury and regain the normal morphology for functional recovery (Hoersting and Schmucker, 2021). The recent study of the Wnk kinases and its evolutionary conserved role in axon branch development, maintenance, and degeneration (Izadifar et al., 2021) provides an excellent example. Therefore, continuing investigation of axon morphogenesis using a combinatorial approach will not only provide a better answer of a basic science question but also shed lights on many clinically relevant problems.

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