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## Beyond the Cytoskeleton: The Emerging Role of Organelles and Membrane Remodeling in the Regulation of Axon Collateral Branches

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### Abstract

The generation of axon collateral branches is a fundamental aspect of the development of the nervous system and the response of axons to injury. Although much has been discovered about the signaling pathways and cytoskeletal dynamics underlying branching, additional aspects of the cell biology of axon branching have received less attention. This review summarizes recent advances in our understanding of key factors involved in axon branching. Here we focus on how cytoskeletal mechanisms, intracellular organelles, such as mitochondria and the endoplasmic reticulum, and membrane remodeling (exocytosis and endocytosis) contribute to branch initiation and formation. Together this growing literature provides valuable insight as well as a platform for continued investigation into how multiple aspects of axonal cell biology are spatially and temporally orchestrated to give rise to axon branches.

### INTRODUCTION

The proper function of the nervous system is dependent upon the establishment of complex patterns of stereotypical synaptic connectivity. As an example, there are an estimated 15–16

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$\times 10^8$  synapses per  $\text{mm}^3$  in the human neocortex (DeFelipe 1999). Although each neuron generates only a single axon, this axon establishes synaptic connections with an expansive array of postsynaptic partners, often in disparate regions of the nervous system. The generation of such complex patterns of synaptic connectivity is in large part due to the developmental branching of axons. Developing axons ramify through two main mechanisms; the bifurcation of the extending growth cone, which initiates two branches of the main axon, and the *de novo* formation of collateral branches from the axon shaft after the growth cone has extended toward its main target region (Gibson & Ma 2010). The vast majority of studies have focused on this latter form of branching, which is the focus of this review. The initiation and stabilization of nascent collateral branches, which begin as plasma membrane protrusions, involves the reorganization of the dynamic cytoskeleton to generate protrusive force against the plasma membrane. This dramatic change in axonal morphology also involves substantial expansion of the axonal plasma membrane, which requires addition and remodeling of membrane material. The cytoskeletal dynamics and membrane turnover underlying branching must be coordinated in space and time, as well as receive support from relevant organelles, such as the endoplasmic reticulum and mitochondria, which control ionic and metabolic aspects of the cytoplasm.

The final pattern of connectivity adopted by a given neuron is ultimately dependent on both the elaboration, maintenance and pruning of axon collateral branches (Kalil & Dent 2014). The formation of axon collateral branches also occurs in response of the nervous system to injury (Hagg 2006; Onifer et al. 2011), in which case the branching can have adaptive or maladaptive consequences (Weaver et al. 2006; Hagg 2006). Here we review the cellular processes that are involved in collateral branch generation, including cytoskeletal dynamics, organelle transport and function, and membrane addition and removal. We also discuss the current understanding of the relationship between the emergence of collateral branches and synapse formation and function.

## The cytoskeletal foundation of axon collateral branches

Localized reorganization of the axonal cytoskeleton underlies the generation of axon collateral branches. The initial step in the formation of a collateral branch is the appearance of an actin filament-based protrusion from the axon shaft (Figure 1A). This protrusive phase of branching takes the form of a filopodium most commonly, or occasionally a lamellipodium (Figure 1A) (Gallo 2011; Kalil & Dent 2014). Filopodia are finger-like protrusions comprising a linear array of bundled actin filaments (Gupton & Gertler 2007), whereas lamellipodia are veil-like protrusions supported by an interconnected array of actin filaments (Mejillano et al. 2004). Relative to the growth cone at the tip of the extending axon, the axon shaft contains fewer actin filaments and exhibits little protrusive activity, due to axonal consolidation (Dent & Gertler 2003; Burnette et al. 2008), which actively suppresses protrusive activity along the axon. Although mechanisms of axonal consolidation maintain the position of the growth cone at the tip of the extending axon, they must be overcome to permit collateral branching of the axon, making collateral axon branch formation distinct from other forms of actin-based protrusions (Loudon et al. 2006; Mingorance-Le Meur & O'Connor 2008), including growth cone splitting. The axonal actin filament cytoskeleton consists of localized patches of actin filaments, sub-membranous rings

of actin filaments circumferential to the main axis of the axon and a smaller population of filaments arranged longitudinally (Spillane et al. 2011; Hu et al. 2012; Xu et al. 2013; Ganguly et al. 2015). Axonal actin patches serve as the precursors to the emergence of localized protrusive activity along the axon shaft (Figure 1A). Whereas the axon generates many actin patches that are transient in nature, only a subset of such patches gives rise to protrusive activity, and the remaining patches dissipate without exhibiting protrusive activity. The formation of axonal actin patches is a major regulatory point in the mechanism used by some branch-inducing signals to increase the level of protrusive activity along the axon (e.g., nerve growth factor/NGF in dorsal root ganglion neurons) (Mingorance-Le Meur & O'Connor 2008; Ketschek & Gallo 2010). Thus, the localized regulation of the cytoskeleton is one of the earliest events in the progression of collateral branch formation.

As the majority of axonal filopodia do not give rise to a branch and are instead retracted, the subset that proceed to branch formation are somehow stabilized. The invasion of axonal filopodia by the polymerizing tips of axonal microtubules, or their active transport into filopodia, is a requisite step in this stabilization and thus the formation of collateral branches (Gallo & Letourneau 1999; Dent & Kalil 2001)(Figure 1A). However, entry of a microtubule into a filopodium is not sufficient for the maturation of the filopodium into a branch. This is likely due to the dynamic nature of microtubules, where their plus ends switch between phases of polymerization and depolymerization, a process termed dynamic instability (Mitchison & Kirschner 1984; Brouhard 2015). Microtubule depolymerization can result in the removal of the microtubule from the filopodium, and destabilization of the nascent axonal protrusion. Thus, the stabilization of microtubules within axonal filopodia is also considered a necessary step in the subsequent maturation of the filopodium into a branch (Gallo & Letourneau 1999; Dent & Kalil 2001; Dent et al. 2004; Gallo 2011). Also notable, the axonal microtubule array, which is normally characterized by linearly aligned microtubules, undergoes splaying at sites of axonal protrusive activity and branching (Gibney & Zheng 2002; Ketschek et al. 2015). This localized reorganization of the axonal microtubule array likely promotes interactions between actin filaments and microtubules during the early steps of branching, however this aspect of branching requires further investigation to elucidate its mechanisms.

The defining event in the maturation of a filopodium into a collateral branch is the reorganization of its actin filament array from a bundle of aligned filaments into a polarized structure. This structure is supported by microtubules and exhibits actin filaments localizing predominantly at the tip of the nascent branch in a growth cone-like structure (Figure 1A). The maturation of a branch can thus be considered a form of cell polarization at the level of a single filopodium. However, the mechanism that drives this polarization remains poorly understood. The maturation of an axonal filopodium into a branch shares similarities with the initial establishment of the main axon. In both cases, a filopodium is converted into a microtubule containing structure with a polarized actin filament cytoskeleton (Smith 1994; Dehmelt et al. 2003; Dent et al. 2007).

## Beyond the cytoskeleton

Collateral branching increases the total volume and surface area of the axon, thus the delivery of cellular components, such as membrane material and organelles are critical to axon branch generation and maturation (Pfenninger & Johnson 1983; Pfenninger 2009). Collateral branches eventually mature into a functional compartment of the main axon, capable of supporting synapse formation and activity. As such, axonal transport cargoes must be delivered into the branch from the main axon, and organelles must populate the nascent branch in order to maintain normal physiology. Sites of axon branching exhibit accumulation of vesicles and also organelles (e.g., mitochondria) (Yu et al. 1994; Greif et al. 2013; Courchet et al. 2013; Tao et al. 2013; Spillane et al. 2013), indicating that membranous organelles and membrane recycling systems likely have roles in axonal branching. For the balance of this review, the role of organelles, pre-synaptic exocytic machinery, and membrane recycling machinery will be considered vis-à-vis the delineated cytoskeletal-based steps of the mechanism of axon branching described above (a summary of proteins discussed in the review is presented in Figure 1B).

## Mitochondria

The lengthening and branching of axons necessitates high ATP production to power local protein synthesis and changes in neuronal morphology. Given that distal axon branches are positioned far from the soma, local energy production is a chief function of mitochondria found within the axon. Mitochondria undergo active bi-directional transport in axons through a kinesin-mediated mechanism (Saxton & Hollenbeck 2012; Sheng 2014). However, between 60–70% of axonal mitochondria remain stalled at specific locations along the axon for prolonged time periods (Miller & Sheetz 2006; Roossien et al. 2014). Mitochondrial density increases in growing axon branches as the influx of the organelles into the branch outweighs efflux from branches (Ruthel & Hollenbeck 2003). This mitochondrial influx is due to organelle specific motility and not passive axoplasmic flow, suggesting that mitochondria may play a key role in axon branch maturation. In embryonic mouse cortical neurons, deletion of the kinase *Lkb1* (Par4), its downstream effector kinase *Nuak1*, or the microtubule and mitochondria linker protein syntaphilin, decreases the percentage of stalled mitochondria present along the axon and the number of mature axon branches (Courchet et al. 2013). Overexpression of any of these three proteins increases the number of stalled mitochondria and associated axon branches. These data suggest that LKB1, NUA1 and syntaphilin control the stalling of mitochondria within the axon, which allows for local energy production and consequently the extension of axon branches.

In embryonic chick sensory neurons, axonal sites containing both a previously stable filopodium and a stalled mitochondrion mature into stable axon branches in response to bath application of NGF (Spillane et al. 2013), suggesting mitochondrial derived energy is necessary for this transition (Figure 1A). Inhibiting mitochondrial respiration, or locally ablating subsets of mitochondria using chromophore assisted light inactivation (CALI) on neurons expressing a mitochondrially-targeted killer-red (KR) construct blocks maturation of filopodia into branches. Similarly, reversal of nascent branches to their previously

filopodial-like morphology occurs in response to targeted ablation of stalled mitochondria at their branch point bases.

The induction of axon branching by NGF requires the intra-axonal translation of mRNAs coding for actin regulatory proteins (e.g., cortactin and Arp2; (Spillane et al. 2012). Localized mRNA translation can be visualized in axons using a myrGFP mRNA that includes the 3'UTR of Arp2 or cortactin mRNAs. Mitochondria colocalize with areas of active mRNA translation, suggesting that mitochondria may fuel local translation of cytoskeletal components involved in axon branching (Spillane et al. 2012; Spillane et al. 2013). Accordingly, the formation of translational 'hotspots' associated with mitochondria decreases upon CALI ablation of mitochondria or pharmacological inhibition of mitochondrial respiration. Taken together these results suggest that stalled, actively respiring mitochondria are required for the formation of 'hot spots' of localized axonal translation, which are in turn required for localized branch formation (Spillane et al. 2012).

Mitochondrial accumulation also contributes to activity-dependent changes in axonal morphology, indicating that synaptic function can create feedback to modulate the morphology and connectivity of the axon. In rat postnatal granule cells, KCl-induced depolarization increases the length and number of axon branches (Tao et al. 2013). Both the initiation and maintenance of longer and more numerous branches are associated with accumulations of mitochondria. The induction of axon branch formation by depolarization and correlated mitochondrial presence requires AMPK activation. These data indicate that mitochondria involved in axon branching are not only regulated by extracellular cues like NGF, but also by membrane depolarization, which may play an integral role in defining which potential synaptic partners are innervated by the axon during developmental activity-dependent circuit formation.

## Endoplasmic Reticulum

The endoplasmic reticulum (ER) is a network of membranous cisternae critical in the synthesis and transport of proteins and membrane lipids, many of which are destined for the axonal compartment and axonal membrane. In addition to supplying cellular materials, the ER maintains  $\text{Ca}^{2+}$  homeostasis, and interacts with and regulates mitochondria. The ER pervades the axon as parallel 20–40 nm wide interconnected tubules (Broadwell 1984). Whereas the ER has been implicated in the establishment of axonal polarity, axon guidance and dendritic arborization, little is known regarding its role in axon branching (Renois  & Blackstone 2010). However, the ER accumulates at the base of axon branches, where it colocalizes with mitochondria. As axon branches elongate, the ER reaches further into their bases, suggesting the ER may play a role in branch stabilization (Spillane et al. 2013). Current data suggest that the close interaction between the ER and the microtubule cytoskeleton is requisite in both axon branching and elongation. For instance, in primary cortical mouse neurons, silencing Protein 600, a microtubule associated protein that binds to the ER, destabilizes neuronal processes and decreases neurite extension (Shim et al. 2008). These data suggest that the scaffolding of ER to microtubules within neuronal processes is required for process maintenance, presumably including axon branches. Whereas axons have been presumed devoid of rough ER, recent work suggests that developing axons contain

components functionally equivalent to rough ER for local protein synthesis (Merianda et al. 2009). Further, in cultured rat dorsal root ganglion neurons and *Xenopus* retinal ganglion cells, intra-axonal immunoreactivity and colocalization was observed for ER-specific proteins including SRP54, Ribophorin II and TRAP $\alpha$  (Merianda et al. 2009). As the literature points to a potential role for both smooth and a specialized version of rough ER in the axon (Tang & Kalil 2005), but does not examine axon branching in depth, this is a research area open to new development that warrants a functional analysis of the role of the ER in branching.

## Characterization of synaptic proteins

A substantial amount of cellular material must reach the plasma membrane during axon branching. The fusion or exocytosis of secretory vesicles not only secretes soluble cargo, but also delivers membrane material and membrane proteins to the expanding plasma membrane during axonal elongation and branching (Pfenninger 2009). Mature synaptic vesicles are rich in proteins, particularly those associated with membrane trafficking, which are present in multiple copies (Takamori et al. 2006). Interestingly, presynaptic exocytic machinery is present well before the onset of synaptogenesis and some components contribute to the growth cone-mediated extension and branching of the primary axon (Greif et al. 2013; Winkle et al. 2014). Developing axons are capable of neurotransmitter release before target contact, indicating that the fundamental molecular mechanism of synaptic vesicle clustering and Ca<sup>2+</sup> dependent exocytosis is already in place in growing axons (Kraszewski et al. 1995; Verderio et al. 1999; Ahmari et al. 2000). Activity-associated fusion events contribute to the modeling and remodeling of developing axonal arbors, suggesting that neurotransmitter release via early-expressed synaptic machinery contributes to morphology of axon terminals. Whereas neurotransmitter release at the synapse is a highly regulated form of exocytosis, it represents an elaboration of the more general process of vesicle trafficking and fusion with the plasma membrane known as the SNARE hypothesis (Söllner et al. 1993; Rothman 1994; Südhof & Rothman 2009).

The discovery of homologous proteins to SNARE components in yeast and non-neuronal tissue (Bennett & Scheller 1993; Aalto et al. 1993; Brennwald et al. 1994) and the high degree of conservation of other vesicle proteins across species suggests that shared mechanisms control synaptic and other forms of secretory vesicle fusion (Söllner et al. 1993; Bennett & Scheller 1994; Rothman 1994), including in developing neurons. Among the large group of proteins implicated in vesicular trafficking and fusion, the SNARE complex components synaptobrevins (also known as vesicle associated membrane proteins, or VAMPs), presynaptic membrane syntaxins and SNAP-25, and vesicle-associated synaptotagmins (syts) are among the most well-studied. VAMPs form a stable, SDS-resistant SNARE complex with syntaxin and SNAP-25 that promotes the close apposition of the vesicular and destination membrane. This complex has been proposed to provide the energy necessary for fusing two membranes (Hanson et al. 1997; Wiederhold & Fasshauer 2009).

Components of the SNARE complex interact with presynaptic membrane components of the syt, Munc/Sec and exocyst families (Li & Chin 2003; Südhof & Rothman 2009; Deák et al. 2009) to mediate regulated and localized vesicle fusion and neurotransmitter release. Syt1 is

proposed to serve as a central regulator of  $\text{Ca}^{2+}$ -triggered SNARE-mediated vesicle fusion at the presynaptic terminal (Rizo & Rosenmund 2008; Rizo & Südhof 2012). Syt1 also interacts with phospholipids in the plasma membrane via its C2 domains (Perin et al. 1990; Perin et al. 1991; Brose et al. 1992; Bai et al. 2003), suggesting that it may promote interactions between vesicles and the plasma membrane. Newly-developing axons contain clusters of vesicles (Matteoli et al. 1992; Kraszewski et al. 1995; Foletti et al. 2000), and syt1 is expressed during axon outgrowth of cortical neurons *in vivo* (Chun & Shatz 1988). In addition, Rab GTPases, synaptophysin, synapsins and several other presynaptic terminal proteins are implicated in regulated exocytosis. Synapsin I and synaptophysin, are also expressed prior to neurite outgrowth (Fletcher et al. 1991) and are observed in the branches of developing axon arbors in the retinotectal system of *Xenopus* embryos (Pinches & Cline 1998). Together these findings suggest that an extensive number of exocytic proteins may play roles in axon branching and development (Chen & Scheller 2001; Südhof 2004; Rizo & Rosenmund 2008; Südhof & Rothman 2009; Rizo & Südhof 2012)

## Synaptic proteins and the development of axons

Recent evidence suggests that membrane vesicles may be targeted to selected sites by interactions with the cytoskeleton, and that both exocytic and endocytic events contribute to the dynamic developmental remodeling of processes (Zakharenko & Popov 1998; Sabo & McAllister 2003; Alberts et al. 2006; Meyer & Smith 2006; Tojima et al. 2007; Erez et al. 2007; Gupton & Gertler 2010; Tojima et al. 2014). Growing axons navigate through a complex extracellular environment, changing direction in response to cues (Gallo & Letourneau 2000; Dent et al. 2003; Horton & Ehlers 2003; Lowery & Van Vactor 2009; Gallo 2013). Evidence suggests that response to positive/attractant cues might involve addition of membrane by controlled exocytosis via SNAREs (Cotrufo et al. 2011; Winkle et al. 2014; Tojima et al. 2014; Ros et al. 2015), contributing to the steering of growth cones towards positive signals, while repulsive signals might be avoided in part by regulated endocytosis of membrane material (Tojima & Kamiguchi 2015; Pfenninger 2009). Selective asymmetric insertion of membrane into growth cones in response to guidance cues suggests a role for controlled exocytosis in axonal outgrowth and guidance (Sakisaka et al. 2004; Tojima et al. 2007; Tojima et al. 2014)

Synaptic proteins are implicated in the regulation of axon elongation. For example the family of synapsins is linked to regulation of axon outgrowth (Ferreira et al. 2000; Kao et al. 2002; Fornasiero et al. 2009). The TC10- exo70 exocyst complex is reported to be an essential component for axon outgrowth (Vega & Hsu 2001; Dupraz et al. 2009). The results of manipulation of SNAREs and related proteins on axon outgrowth are variable (Tojima & Kamiguchi 2015). Whereas VAMP2-mediated exocytosis is observed in growth cones (Sabo & McAllister 2003; Tojima et al. 2007), cleavage of VAMP by tetanus toxins does not alter axon outgrowth (Osen-Sand et al. 1996), although it does block intrinsic neuritogenesis (Gupton & Gertler 2010). In contrast, overexpression of VAMP2 in the neuronal-like PC12 cell line promotes neurite elongation (Kimura et al. 2003). The varied roles suggest specific vSNAREs may function at distinct developmental stages or in response to various extracellular signals, and may deliver distinct cargo to the expanding plasma membrane. Cleavage of SNAP-25 by Botulinum toxins or reductions in expression by antisense

oligonucleotides inhibits axon growth (Osen-Sand et al. 1993; Osen-Sand et al. 1996). However, thalamo-cortical axon outgrowth from *Snap25*<sup>-/-</sup> knockout mice observed in E16-18 organotypic cultures is indistinguishable from that in *Snap25*<sup>+/+</sup> cultures (Blakey et al. 2012). Knockout of *Munc18* and *Munc13* reduces axon outgrowth in organotypic cultures and in dissociated hippocampal neurons (Broeke et al. 2012). Blocking the C2A domain of sytI/II with antibodies reduces neurite outgrowth in PC12 cells, whereas overexpression promotes neurite outgrowth (Kabayama et al. 1999). Possible explanations for observed inconsistencies include functional redundancy among members of protein families or high levels of expression of synaptic proteins localized within growth cones. Thus, while the synaptic machinery is strongly implicated in multiple aspects of axon development and guidance, the roles of specific components remain to be elucidated.

## Synaptic proteins and axon branching

As outlined above, synaptic proteins are heavily implicated in axon extension, guidance and neurotransmitter release, but their roles in axon branching are less well documented. *In vivo* live imaging of axon branch formation in retinal ganglion cells reveals a correlation between sites of branch formation and the accumulation of the synaptic vesicle marker synaptobrevin II (Cohen-Cory & Lom 2004). Axon branching in cortical and hippocampal neurons is stimulated by the secreted axon guidance cue netrin-1 (Dent et al. 2004; Winkle et al. 2014). Total internal reflection fluorescence microscopy (TIRFM) of murine cortical neurons expressing VAMP2-pHluorin or VAMP7-pHluorin revealed that netrin-1 treatment stimulates the fusion of vesicles positive for either v-SNARE particularly in neurites (Winkle et al. 2014). TeNT-mediated cleavage of VAMP2 or longin-mediated inhibition of VAMP7 individually fails to decrease netrin-dependent axon branching independently, however simultaneous inhibition of both vSNAREs decreases axon branching, indicating the function of either vSNARE is sufficient for plasma membrane expansion and axon branching. Acute Botulinum Neurotoxin A-mediated cleavage of SNAP25, which forms a SNARE complex with either VAMP2 or VAMP7 decreases netrin-dependent axon branching, in a similar fashion to the inhibition of both v-SNAREs, suggesting that exocytic vesicle fusion is required for plasma membrane expansion during axon branching (Winkle et al. 2014). These data suggest that VAMP2 and VAMP7 may function during netrin-stimulated axon branching (Winkle et al. 2014).

SNAP47 is a SNARE protein also present in the nervous system, which forms SNARE complexes with syntaxin-1A and VAMP2 in cultured neurons, and facilitates exocytic fusion events (Holt et al. 2006). VAMP2, SNAP25 and SNAP47 mediate the local vesicular release of the neurotrophic factor BDNF. Moreover, ablation of the function of SNAP47, BDNF or the BDNF receptor TrkB disrupts callosal axon branching both *in vitro* and *in vivo*, suggesting that SNAP47 may also function in both exocytosis and axon branching (Shimojo et al. 2015). SNAP47 interacts with VAMP7 and Trans-Golgi associated VAMP4, suggesting that SNAP47 pairs with diverse SNARE proteins to potentially fulfill multiple distinct trafficking requirements in developing neurons (Kuster et al. 2015). As such, silencing SNAP47 dramatically changes the cellular localization of VAMP4, whereas SNAP47 overexpression inhibits VAMP7-dependent exocytic activity. As the literature regarding



SNAP47 is relatively sparse, further investigations of its roles in the regulation of secretory transport in the developing neuron are warranted.

The synaptotagmin syt1 is implicated in axon branching as well. Syt1 mRNA is enriched in immature axons and growth cones compared to more mature axons (Gumy et al. 2011). Syt1 immunoreactivity is present within filopodia in embryonic chicken forebrain neurons *in vitro* (Greif et al. 2013). Overexpression of syt1 increases filopodial initiation and stability, leading to increased numbers of axonal filopodia and subsequent primary axon branches. Conversely, knockdown of endogenous syt1 by RNAi reduces the number of axonal filopodia and branches (Greif et al. 2013). Axonal branching is also reduced in thalamo-cortical co-cultures by a dominant-negative construct of syt1 with a mutated C2B Ca<sup>2+</sup>-binding domain, which reduces vesicle cycling (Granseth et al. 2013). Transfection with the mutant construct also blocks increases in axonal branching in response to BDNF.

The numerous other synaptotagmin proteins likely play critical roles in axon outgrowth as well. For example, both process outgrowth and branching *in vitro* are defective in sympathetic neurons from syt7-defective mice (Arantes & Andrews 2006). Ca<sup>2+</sup>-dependent translocation of syt1 to the plasma membrane of dendrites of developing rat hypothalamic neurons also suggests that syt1 specifically and synaptotagmins in general also may contribute to dendritic morphology as well (Schwab et al. 2001). Syt1 also contributes to the morphology of neuronal-like processes in non-neuronal cells. Ectopic expression of syt1 in non-neuronal cells produces extensive filopodia-like structures (Feany & Buckley 1993; Johnsson & Karlsson 2012). The polybasic sequence on syt1 directly stimulates actin polymerization via interactions with anionic phospholipids in non-neuronal cells (Johnsson & Karlsson 2012), suggesting a potential interaction between synaptic vesicles and the cytoskeletal reorganization associated with filopodial development that represents the initial phase of axon branching (Figure 1A).

Axon branching requires membrane addition for increased surface area. Whether vesicle fusion occurs locally or globally during axon branching and how this changes over development remains to be seen. However, if the plasma membrane is as fluid as suggested in the literature this membrane may not necessarily need to be inserted locally. Since local increases in Ca<sup>2+</sup> are associated with filopodia and branch development, local control of SNARE-mediated membrane fusion may provide an effective mechanism to couple cytoskeletal reorganization with membrane addition. Furthermore, the proteins involved in the docking and fusion of vesicles with the presynaptic membrane are general components of synapses, regardless of transmitter type (Chen & Scheller 2001; Südhof 2004; Südhof & Rothman 2009). Since early neural activity can modulate the organization, architecture and strength of synapses and this is associated with the regulation of axonal branching morphogenesis in multiple systems (O'Leary & McLaughlin 2005; Uesaka et al. 2006; Hayano & Yamamoto 2008; Yamamoto & López-Bendito 2012), future work is necessary to further define the spatial and temporal regulation of neural activity as it relates to arborization.

## Endocytosis and endocytic trafficking in branching

After synaptic vesicle fusion, vesicle membrane is recycled via dynamin-mediated endocytosis and subsequently vesicles are refilled with neurotransmitter (Südhof 2004). Similarly, axon outgrowth involves a balance between addition and removal of membrane; evidence suggests that endocytosis of membrane components also play a role in axonal morphogenesis (Lasiecka & Winckler 2011). Although the regulation of endocytosis and trafficking of endosomes is crucial for the development and maintenance of axons (Yap & Winckler 2015), in comparison to exocytosis, there is relatively little published on the function of endocytosis in axon branching. Endocytosis and protrusion can be considered opposing cellular behaviors. The first results in the removal of plasma membrane and the formation of an endocytic vesicle within the cytoplasm, whereas the second results in the formation of a filopodium or lamellipodium supported by membrane expansion, which could mature into an axon collateral as outlined (Figure 1A). In the growth cone, endocytosis can modulate growth cones turning away from a repellent signal. In elegant studies, Kamiguchi and colleagues have demonstrated that asymmetric endocytosis on one side of the growth cone is sufficient to induce turning away from repulsive signals (Tojima & Kamiguchi 2015; Tojima et al. 2010). They propose that axon guidance may be a balance between exocytosis and endocytosis, acting in an antagonistic fashion. If these findings are extrapolated to axon branching and collateralization then one might expect that endocytosis plays a role in the inhibition of branching through the endocytic retrieval of cell adhesion molecules and growth factor receptors.

Consistent with these findings, inhibition of endocytosis is thought to increase axon branching. For example, inhibition of fibroblast growth factor receptor (FGFR1) endocytosis results in increased axon branching of adult DRG neurons (Hausott et al. 2011). Another study in thalamo-cortical neurons, demonstrated that BDNF stimulation increases axon branching and the inhibition of clathrin-mediated endocytosis, via overexpression of AP180C, has a biphasic effect, decreasing branching at low BDNF concentrations and increasing branching at high BDNF concentrations (Granseth et al. 2013). Since high concentrations of AP180C result in sequestration of clathrin, it follows that inhibiting clathrin-mediated endocytosis in thalamo-cortical axons results in increased branching. Presumably, inhibition of endocytosis allows growth factor receptors to build up on the axonal plasma membrane and increases the likelihood of activation by growth factors such as FGF-2 and BDNF. Consistent with these data, knockdown of Utk1 and/or Utk2, two UNC-51-like kinases impaired endocytosis of NGF, resulting in increased axon branching of embryonic sensory neurons (Zhou et al. 2007). Thus, inhibiting endocytosis appears to leave more receptors on the membrane surface and results in increased receptor signaling, leading to increased branching.

Conversely, disrupting endosomal pH can decrease axon branching. Mutation of NHE6, the endosomal Na<sup>+</sup>/H<sup>+</sup> exchanger that is mutated in Christianson syndrome, decreases axon branching in cultured hippocampal neurons (Ouyang et al. 2013). NHE6 is present on early, late and recycling endosomes and opposes endosomal luminal acidification by the V-ATPase. Knockout of the Na<sup>+</sup>/H<sup>+</sup> exchanger abrogates axon branching and is rescued by re-expression of a functional exchanger or excess BDNF. The authors propose that the over-

acidification and increased pH-sensitive proteolysis in endosomes and/or the expansion of lysosomes that occurs upon knockout of NHE6, results in decreased signaling and premature degradation of TrkB receptors. Thus, the loss of functional TrkB receptors in endosomes leads to a decrease in axon branching, presumably because subsequent exocytosis of these over-acidified vesicles results in fewer functional TrkB receptors on the membrane surface.

Two recent studies by Halloran and colleagues demonstrated that endocytic trafficking is also important for peripheral sensory neuron branching *in vivo* (Ponomareva et al. 2014; Ponomareva et al. 2016). Studying the Rohan-Beard neurons in living zebrafish, this group showed that constitutively active Rab5, which favors enlargement of early endosomes and disrupts the conversion to late endosomes, has no effect on axon outgrowth but decreases axon branching (Ponomareva et al. 2014). By photoactivating GFP-Rab5 and imaging its transport, they detected Rab5-labeled vesicles accumulating at axon branching points. Disruption of Rab5 transport by knockdown of the kinesin adapter protein calyntenin-1 similarly results in decreased axon branching, suggesting that transport of Rab5-containing vesicles is important for peripheral axon branching. Moreover, constitutively active mutations in Rab7 and point mutations that give rise to Charcot-Marie Tooth syndrome, also decrease axon branching in these peripheral sensory axons (Ponomareva et al. 2016). Together, these studies indicate a properly functioning endocytic trafficking system is crucial to branch formation during development. Presumably, this occurs by targeting specific endocytic populations with their associated receptor cargo to distinct regions along the axonal arbor and subsequent insertion of these receptors results in a localized signaling cascade, cytoskeletal polymerization and formation of a new collateral branch.

## F-BAR proteins in plasma membrane remodeling and branching

One large family of proteins implicated in endocytosis, cell migration and morphogenesis is the FER/CIP4 Homology Bin-Amphiphysin-RVS (F-BAR) superfamily of proteins. F-BAR proteins are present in many tissues and are involved in sensing and inducing plasma membrane curvature through their N-terminal F-BAR domain (Chitu & Stanley 2007; Aspenström 2008; Suetsugu et al. 2014). F-BAR proteins also contain other domains involved in binding active Rho GTPases (HR1 domains) or domains that act as GTPase-activating domains (GAP domains). Many F-BAR proteins also contain a C-terminal SH3 domain(s) that binds dynamin, the major vesicle scission protein, as well as a host of actin-associated proteins, such as N-WASP, WAVE and the formin DAAM1 (Suetsugu et al. 2014). F-BAR proteins exist as homo and heterodimers in cells (Coutinho-Budd et al. 2012) and act to elongate endocytic tubules by associating as end-to-end polymers that form helical coats around vesicles (Shimada et al. 2007; Frost et al. 2008). Their ability to bridge the plasma membrane to actin polymerization positions them as potentially important actors in filopodia, lamellipodia and subsequent axon branch formation (Figure 1A).

Several studies implicate F-BAR proteins as negative regulators of axon branch formation, consistent with their roles in endocytosis and tubulation. For example, knockdown of TOCA-1, a CIP4 family F-BAR protein, increases axon branching slightly in cultured hippocampal neurons (Kakimoto et al. 2006). Knockdown of syndapin I, another F-BAR family member, results in a pronounced increase in axon branching, whereas overexpression

of syndapin I resulted in fewer axon branches in hippocampal neurons (Dharmalingam et al. 2009). Together, these data suggest that decreasing endocytosis increases branching, while driving endocytosis decreases branching. Although these studies did not delineate the pathways between endocytosis and branching the results are consistent with studies mentioned in the previous section.

Conversely, the inverse F-BAR (iF-BAR) protein srGAP2 promotes axon arborization (Guerrier et al. 2009). Instead of resulting in elongation of endocytic vesicles, overexpression of the iF-BAR protein srGAP2 resulted in filopodial-like structures that require actin polymerization to form, but surprisingly do not require F-actin for their maintenance (Guerrier et al. 2009; Carlson et al. 2011). Thus, srGAP2, as well as family members srGAP1 and 3 (Coutinho-Budd et al. 2012), are thought to form helical polymeric rings along the entire inner surface of the extended filopodial-like structure. Since filopodia are important for branch formation (Figure 1A), it follows that knockdown of srGAP2 decreases axon branching in cortical neurons (Guerrier et al. 2009). Although studies of F-BAR proteins in branching are limited, the few mentioned here suggest these proteins may be acting to bridge plasma membrane deformation and cytoskeletal rearrangements. Since both endocytosis and protrusion play key roles in the regulation of branching, further studies are likely to implicate other F-BAR proteins in the process of axon branching.

## Concluding Remarks

In conclusion, axon branching is a complex cell biological problem involving the coordination of the cytoskeleton, the secretory pathway, and the organelles that provide the material and energy to elaborate the axon. We now have a working “parts lists” of the many cellular components that fuel this important stage of neuronal morphogenesis, although surely more are to be uncovered. Future work to determine how these different systems are coordinated and regulated is a critical area of research. Although we have touched upon many proteins that regulate both axonal outgrowth and axonal branching, further delineation of the similarities and differences between these dynamic stages of axonal morphogenesis is an important area of investigation. As noted in a prior review (Table 1 in (Gallo 2011)), experimental manipulation of specific proteins can have disparate effects on axon branching and axon outgrowth. In the case of *syt1*, reviewed herein, along the axon *syt1* was found to promote the formation of filopodia and thus branching, but had no effect on axon length. This might reflect the role of *syt1* in mediating filopodia formation which is crucial to branching but dispensable, if not antagonistic, for axon extension (Argiro et al. 1984; Kleitman & Johnson 1989). Furthermore, axons can extend in the absence of actin filaments but collateral branching from the same axons is strictly dependent on actin filaments and protrusive activity (Dent & Kalil 2001). These considerations indicate that it will be important to dissect the roles of specific proteins in the morphogenetic events leading to axon branching or outgrowth, beyond the characterization of a function for the protein of interest in axon branching or outgrowth.

Axonal branching likely involves a number of scaffolding components at the plasma membrane that concentrate these different cellular machines in spatially clustered units, such as F-BAR proteins. Furthermore, there are likely a plethora of enzymes that catalyze post-

translational modifications or otherwise alter the function, localization or stability of the cellular machines that promote stages of axon branching both spatially and temporally. As in non-neuronal cells, members of both the Rho and Rab families of small GTPases are integral to this function. There are also likely a number of neuronal-specific proteins necessary for this unique cellular shape change. For example, the brain enriched E3 ubiquitin ligase TRIM9 inhibits SNARE complex formation and exocytosis through binding to SNAP25 (Li et al. 2001; Winkle et al. 2014). Additionally TRIM9 ubiquitinates and inhibits the actin polymerase VASP, which reduces filopodial stability (Menon et al. 2015). Interestingly, TRIM9 also directly interacts with the netrin receptor DCC, and netrin stimulation inhibits these negative regulatory functions of TRIM9 in order to promote axon branching axon branching (Winkle et al. 2014). In addition to netrin, a number of other axon guidance cues can promote or block axon branching (Kennedy & Tessier-Lavigne 1995; Dent et al. 2004). Elucidation of the signaling pathways activated downstream of these distinct cues and how they converge upon the cellular machines necessary for branching is a key component to how these shape changes are spatially and temporally controlled *in vivo*.

Recently a number of non-canonical membrane delivery schemes have been identified which involve the direct coupling of the ER to the neuronal plasma membrane (Giordano et al. 2013; Petkovic et al. 2014). Although the coupling of the ER to the membrane has not been implicated in axon branching as of yet, the ER accumulates at sites of branching (Spillane et al. 2013) and since ER-based mechanisms may contribute to the delivery of plasma membrane material to the axolemma, they are well poised to also modulate axon branching. Continued investigation into the mechanism of axon branching is likely to yield novel insights into the coordination and regulation of the cytoskeleton, membranous compartments and membrane turnover.

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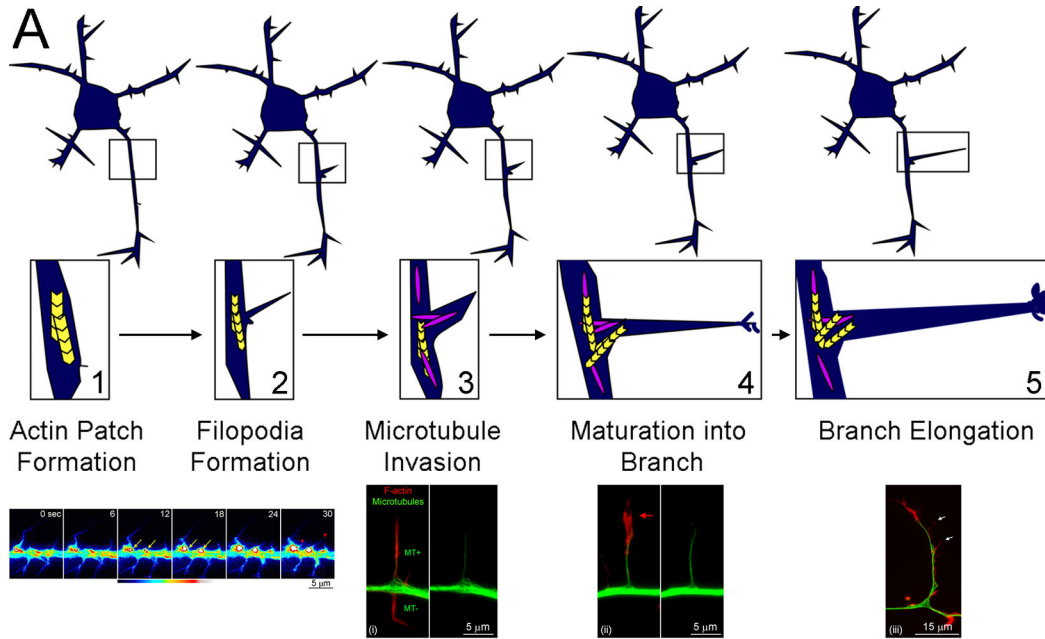


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**B**

Synaptic Proteins	Endocytic Proteins	Mitochondria Related Proteins	ER proteins
VAMP2 (+)	AP180C (+/-)	LKB1 (Par4) (+)	Protein600 (+)
VAMP7 (+)	UIK 1 (-)	NUAK1 (+)	SRP54 (unk)
Syntaxin1A (+)	UIK2 (-)	Syntaphilin (+)	Ribophorin II (unk)
SNAP47 (+)	NHE6 (+)	AMPK (+)	TRAP $\alpha$ (unk)
SNAP25 (+/no change)	TrkB/BDNF (+)		
TC10-exo70 (+)	Rab-5 (-)		
Synaptotagmin-1(+)	Rab-7 (-)		
Ankyrin-B (+) (Transport of vesicles)	F-BAR Family (-)		
SrGAP1,2,3 (+)			

**Figure 1.** Sequence of cytoskeletal events underlying the collateral branching of axons. (A) Schematic of the sequence of events leading to the formation of a collateral branch (top, A1–A5). Examples of cytoskeletal dynamics and organization during the branching of embryonic sensory axons are also provided (bottom). Axonal filopodia are the first morphogenetic step in the formation of a branch (A2). Filopodia emerge from pre-existing patches of axonal actin filaments (A1). The time-lapse sequence (A1 bottom) shows the formation of axonal actin patches and the emergence of filopodia from the patches (eYFP- $\beta$ -actin imaging; time

is shown in seconds in each panel). Actin patches form and elaborate (i.e., grow brighter and larger) as denoted by the yellow arrows at 12–18 sec. The red arrowheads and 30 sec denote filopodia that have emerged from the two patches labeled in prior panels. The targeting of one or more microtubules (MT+) into a filopodium is a required event for the maturation of the filopodium into a branch (A3). Filopodia that are targeted by microtubules can then mature into a branch (A4). The maturation of the filopodium is characterized by the development of a polarized distribution of actin filaments (red arrow, A4 bottom), which form a small growth cone-like structure at the tip of the nascent branch. As the branch elongates actin filaments remain mostly polarized at its tip, although the branch can also give rise to filopodia along its shaft (white arrows, A5 bottom), and in turn secondary branches. **(B)** Table featuring the proteins discussed in the text and denoting whether they promote (+) or impede (–) branching. (unk) denotes proteins with insufficient data regarding their role in axon branching.