Published in final edited form as:

Neuroscientist. 2013 February ; 19(1): 16–24. doi:10.1177/1073858411426201.

Molecular Control of Axon Branching

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Abstract

Axon branching is a complex morphological process, the regulation of which we are just beginning to understand. Many factors known to be important for axon growth and guidance have emerged as key regulators of axon branching. The extrinsic factors implicated in axon branching include traditional axon guidance cues such as the slits, semaphorins, and ephrins; neurotrophins such as BDNF; the secreted glycoprotein Wnt; the extracellular matrix protein anosmin-1; and certain transmembrane cell adhesion molecules—as well as sensory experience and neuronal activity. Although less is known about the intracellular control of axon branching, in recent years significant advances have been made in this area. Kinases and their regulators, Rho GTPases and their regulators, transcription factors, ubiquitin ligases, and several microtubule and actin-binding proteins are now implicated in the control of axon branching. It is likely that many more branching regulators remain to be discovered, as do the links between extrinsic cues and intracellular signaling proteins in the control of axon branching.

Keywords

neuron morphology; axon; branching; arborization

Neurons output information through an astonishing variety of axon morphologies. One of the processes that generates this diversity is axon branching. From reaching multiple targets to defining innervation fields within specific targets, axon branching allows neurons to establish unique patterns of connectivity. Compared with what is known about axon growth, little is known about the molecular regulation of axon branching. Significant advances have been made, however—first in identifying the extrinsic factors that control axon branching, and more recently in identifying the intracellular factors. In both cases, an emerging theme is that many of the cues orchestrating axon growth and guidance also have the potential to promote or inhibit axon branching (Table 1). This overall positive or negative regulation of branching can be the result of changes in distinct cellular events, from branch initiation, elongation, and the development of arbor complexity to termination, retraction, or pruning (Figure 1). In this review, we provide a survey of extrinsic cues and intracellular signaling molecules that control axon branching.

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Declaration of Conflicting Interests The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Extracellular Regulation

Over the last two decades, target-derived factors have been established as potent regulators of axon branching. Slits were one of the earliest target-derived factors found to control branching. Biochemical fractionation of brain and spinal cord extracts promoting the branching of dorsal root ganglion (DRG) neuron axons in culture originally led to the isolation of an N-terminal Slit2 fragment with branch-promoting activity (Wang and others 1999). A subsequent study found that choroid plexus-derived Slit2 similarly induced the branching of trigeminal neuron axons in the brainstem (Ozdinler and Erzurumlu 2002). Recently, studies of knockout mice have elucidated more of the complexities of Slit signaling in regulating branching. For sensory axons in vivo, the branch-promoting activity of Slit is required for peripheral arbor formation, whereas Slit's growth repulsive activity guides the bifurcation of central afferents (Ma and Tessier-Lavigne 2007).

Ephrins represent another example of target-derived factors with a clear influence on axon branching. A key function of ephrin signaling is establishing retinotopic maps, or visual field maps in the retina and brain—and this is accomplished via ephrin's control of axon branching (Feldheim and O'Leary 2010; O'Leary and McLaughlin 2005). In the chick optic tectum, for instance, the glycosylphosphatidylinositol (GPI)-anchored ligand ephrin-A serves as a position-encoding cue, inhibiting the branching of retinal ganglion cell (RGC) axons outside of intended termination zones (Yates and others 2001). Ephrin signaling also regulates the formation of terminal axonal arbors in thalamic neurons making lamina-specific projections to the cortex (Mann and others 2002). In *Drosophila* mushroom body neurons, it is required for the guidance of branches into a specific target—the dorsal lobe of the mushroom body (Boyle and others 2006).

The class III semaphorins are a third major family of axon guidance cues with diverse functions in axon branching. Semaphorin signaling through the Plexin-A3 receptor appears to be critical for the pruning of long hippocampal axon branches in vivo (Bagri and others 2003). In cultures of cortical neurons, semaphorin 3A inhibits axon branching (Dent and others 2004). In addition, through its repulsive activity, semaphorin 3D guides branching of the peripheral, but not central, axon of zebrafish sensory neurons (Liu and Halloran 2005). Together these findings illustrate the intricacies of branch regulation by semaphorins. Similar to the slits and ephrins, semaphorins can regulate both the growth and guidance of branches and can selectively regulate one branch of an axon without affecting another branch.

Along with these three major families of axon guidance cues, neurotrophins—most prominently, brain-derived neurotrophic factor (BDNF)—have emerged as key regulators of axon branching. An early study of RGC axon arbors in the *Xenopus* optic tectum revealed that exogenous BDNF can rapidly increase branching and arbor complexity in vivo (Cohen-Cory and Fraser 1995). This effect has been studied in detail in subsequent years and correlated with increased synapse formation between the RGCs and tectal neurons (Alsina and others 2001; Sanchez and others 2006).

Another extrinsic cue well established as a regulator of axon branching is the secreted glycoprotein Wnt. In sympathetic nerve targets, Wnt5a mediates the neurotrophin-induced growth and branching of incoming axons (Bodmer and others 2009). In the spinal cord, Wnt3 regulates the formation of sensory-motor circuits (Krylova and others 2002). Specifically, Wnt3 produced by spinal motor neurons is thought to control the branching of axons from a subset of DRG sensory neurons; in response to Wnt3, the axons of these proprioceptive neurons stop growing and develop extensive terminal arbors (Krylova and others 2002).

The extracellular matrix protein anosmin-1 also plays a role in axon branching (MacColl and others 2002). Anosmin-1 promotes axon branching of olfactory bulb output neurons by stimulating collateral branch formation (Soussi-Yanicostas and others 2002). Anosmin-1 also promotes axon branching in Purkinje neurons by stimulating both the budding of collateral branches and the extension of terminal arbors (Gianola de Castro and Rossi 2009).

In addition to these secreted and GPI-anchored membrane cues, transmembrane cell-adhesion molecules (Colavita and Tessier-Lavigne 2003; Wang and others 2002; Zhu and Luo 2004) and neuronal activity play key roles in the control of axon branching (Hayano and Yamamoto 2008; Uesaka and others 2006).

Often the extrinsic cues that govern branching regulate each other in complex ways. For instance, slit signaling regulates a semaphorin co-receptor to control sensory axon branching in zebrafish (Miyashita and others 2004), and an interaction between the BDNF receptor TrkB and ephrin-A in the membrane of RGCs regulates axon branching in the chick retina (Marler and others 2008).

Intracellular Regulation

In addition to extrinsic signals that control axon branching, intracellular signaling mechanisms play a critical role in the control of axon branching. Five major classes of intracellular proteins that have been implicated so far are kinases and their regulators, small GTPases of the Rho family and their regulators, transcription factors, ubiquitin ligases, and cytoskeleton-associated proteins (Table 1).

Kinases and their Regulators

Although the nature of evidence implicating specific kinases in axon branching varies, many of the implicated kinases are interconnected in established signaling networks. This observation suggests an important role for kinase signaling in the control of axon branching.

The lipid kinase phosphatidylinositol 3-kinase (PI3K) and the protein kinases AKT and glycogen synthase kinase 3 (GSK3) are components of a well-characterized signaling pathway. When extracellular events lead to the activation of PI3K, AKT is recruited to the plasma membrane and activated. AKT phosphorylates and inhibits GSK3 (Cantley 2002; Doble and Woodgett 2003). All three kinases, PI3K, AKT, and GSK3, are thought to play critical roles in the control of axon branching.

Inhibition of PI3K using pharmacological agents suggested many years ago that PI3K promotes axon branching in DRG neurons—acting as an effector for the branch-promoting neurotrophin nerve growth factor (Gallo and Letourneau 1998). Since then, other studies have also suggested that PI3K mediates axon branching responses (Diaz-Hernandez and others 2008; Drinjakovic and others 2010; Jones and others 2003). Like PI3K, the downstream kinase AKT similarly appears to promote branching in sensory neurons (Grider and others 2009; Markus and others 2002). And, as might be expected from the observation that PI3K/AKT signaling inhibits GSK3, GSK3 inhibits axon branching (Lucas and Salinas 1997; Kim and others 2006; Bilimoria and others 2010).

Two other kinases recently implicated in axon branching are the cyclic GMP-dependent protein kinase PrkG1 and UNC-51-like kinase (Ulk). Interestingly, both appear to be connected to the PI3K-AKT-GSK3 signaling axis. PrkG1, which is required for DRG axon bifurcation in vivo, phosphorylates and inhibits GSK3 (Zhao and others 2009). Ulk1 and Ulk2, thought to inhibit DRG axon branching, suppress AKT phosphorylation (Zhou and others 2007).

Yet another kinase with evidence for a role in axon branching is the nonreceptor tyrosine kinase focal adhesion kinase (FAK). Knockout studies elegantly demonstrate that FAK inhibits axon branching in cerebellar and hippocampal neurons (Rico and others 2004).

Since kinases are crucial to the regulation of axon branching, it is not surprising that kinase regulators have also been implicated in the control of this process. MAPK phosphatase 1 (MKP-1) is one clear example. Via its ability to dephosphorylate JNK and diminish its activity, MKP-1 mediates the pro-branching effects of BDNF (Jeanneteau and others 2010).

Another interesting example is the kinase scaffold molecule JNK interacting protein 3 (JIP3), which restricts axon branching in cerebellar granule neurons (Bilimoria and others 2010). Although JIP proteins are best known for their role in scaffolding JNK cascades (Morrison and Davis 2003; Yasuda and others 1999), JNK does not appear to mediate the JIP3-inhibition of branching. Rather, GSK3 appears to operate as a downstream mediator of JIP3 in this pathway (Bilimoria and others 2010).

Transcription Factors

Transcriptional control of axon branching is just beginning to receive notice. The ETS and homeodomain class of transcription factors, both of which belong to the larger family of helix-turn-helix motif DNA-binding proteins, regulate axon branching. In the developing spinal cord and ganglia of the peripheral nervous system, transcriptional networks that establish neuronal identity and differentiation are well-characterized. Pea3 and Er81 are two ETS class transcription factors in these networks that have recently been found to control the branching of motor and sensory neuron axons, respectively (Dasen 2009). Experiments in mice in which the Pea3 gene was disrupted uncovered a role for Pea3 in axon terminal arborization of spinal cord motor neurons (Livet and others 2002). In the absence of Pea3 function, Pea3-dependent motor neuron axons fail to properly arborize within and innervate target muscles (Livet and others 2002). The expression of a cell-surface cadherin and a secreted semaphorin protein is diminished, suggesting a compromised interaction between these motor neurons and the extracellular environment (Livet and others 2002).

Similarly, mice in which the Er81 gene was disrupted revealed that Er81 is required for establishing proper connectivity between proprioceptive sensory neuron axons and motor neurons in the spinal cord. In the absence of Er81 function, the central axonal projections of these sensory neurons fail to terminate in the appropriate zone—compromising the ability of the sensory neurons to innervate their motor neuron targets (Arber and others 2000).

In zebrafish embryos, there is evidence that a Lim homeodomain transcription factor, Islet2, is required for the appropriate development of peripheral branches in primary sensory neurons. Functional repression of Islet2 results in a loss of the peripheral branches, whereas the central (spinal cord or hindbrain) projections of the same sensory neurons remain unaltered (Segawa and others 2001). Analogously to Pea3's control of cadherin and semaphorin expression, Islet2 controls expression of PlexinA4, a co-receptor for semaphorin (Miyashita and others 2004). Intriguingly, the Islet2/PlexinA4 signaling link in sensory neurons appears to be crucial for responses to Slit as an axon branching cue (Miyashita and others 2004; Yeo and others 2004).

In addition to the spinal cord and periphery, there is evidence for regulation of axon branching by helix-turn-helix type transcription factors in the brain. Otx1 is a homeodomain transcription factor expressed in the forebrain and midbrain during early developmental stages (Simeone and others 1992). In mice lacking functional Otx1 protein, specific axon branches of layer 5 visual cortex neurons were compromised (Weimann and others 1999). Although callosal and thalamic projections appeared normal, projections to the inferior

colliculus and spinal cord were aberrant—consisting of exuberant collaterals due to failed pruning (Weimann and others 1999).

Together these data underscore the importance of transcriptional control in various aspects of axon branching. One emerging mechanism by which transcription factors control branching is regulating the expression of axon guidance receptors or other molecules involved in the extrinsic control of axon development.

Rho GTPases and their regulators

Highly conserved members of the Rho family of small GTPases include RhoA, Rac, and Cdc42 (Hall and Lalli 2010; Heasman and Ridley 2008). These are a group of molecules intimately linked to dynamics of the actin cytoskeleton (Hall and Lalli 2010; Heasman and Ridley 2008). A number of studies suggest that Rho GTPases and their regulators control various aspects of axon development, including axon branching (Hall and Lalli 2010). For instance, a study of Rac proteins in *Drosophila* mushroom body neurons uncovered defects in axon development contingent upon the degree of Rac loss. Axon branching defects occurred with partial loss of Rac activity, whereas guidance and growth deficits occurred with more severe loss of Rac activity (Ng and others 2002). A study of branching in the rat cortex suggests that RhoA mediates activity-dependent axon branching (Ohnami and others 2008). In addition, regulators of the Rho family, such as p190RhoGEF, are thought to mediate the inhibition of axon branching by FAK (Rico and others 2004). Fittingly, p190RhoGAP, with its function opposing p190RhoGEF, positively regulates axon branching—promoting branch stabilization by repressing a retraction pathway (Billuart and others 2001).

Ubiquitin Ligases

Similar to kinases and transcription factors, ubiquitin ligases have influence over a large pool of intracellular targets. Recent evidence implicates specific ubiquitin ligases in the control of axon branching. *Drosophila* studies reveal that the ubiquitin ligase *highwire* restrains axon arborization (Wan and others 2000). The zebrafish ortholog, Esrom, also regulates axon arborization. However, instead of controlling the number of branches, Esrom controls the position of branches (D'Souza and others 2005). The *Caenorhabditis elegans* ortholog, RPM-1, is required for different aspects of axon morphogenesis in different types of neurons, including branch stabilization and inhibition (Schaefer and others 2000). The mammalian ortholog, Phr1, regulates axon outgrowth through modulation of microtubule dynamics (Lewcock and others 2007). Specifically, it controls growth cone morphology, restrains presynaptic sprouting at the neuromuscular junction, and is required for the formation of major CNS axon projections and tracts (Bloom and others 2007; Lewcock and others 2007). Together, these studies suggest an evolutionarily conserved role for the ubiquitin ligase Phr1 and its orthologs in the regulation of axon morphogenesis, including the control of axon branching. Recently, another ubiquitin ligase, Nedd4, has been found to promote axon branching in *Xenopus* (Drinjakovic and others 2010), suggesting that ubiquitin signaling plays a critical role in the control of axon branching. However, the mechanisms by which ubiquitin ligases control axon branching remain to be elucidated.

Cytoskeleton-Associated Proteins

The regulation of actin and microtubule dynamics is undoubtedly crucial for effecting changes in axon branching. Much remains to be determined in terms of which cytoskeleton-associated proteins govern the formation, growth, stabilization, or retraction of axonal branches and the mechanisms by which these molecules enact changes. Some examples

identified to date include members of the kinesin superfamily, other microtubule-binding proteins, and actin-binding proteins.

KIF2a is a kinesin superfamily protein recently found to regulate axon branching. As part of the Kin1/Kinesin-13 family, KIF2a differs from the conventional kinesin motor proteins that transport cargo along microtubules. Instead, this kinesin destabilizes microtubules, promoting their depolymerization from both ends in an ATP-dependent fashion (Ovechkina and Wordeman 2003). Experiments in KIF2A knockout mice reveal that KIF2A suppresses collateral branch extension. In the absence of KIF2A, branches are overextended—suggesting that microtubule depolymerization is necessary for branch elongation (Homma and others 2003).

Microtubule-binding proteins outside the kinesin superfamily that are known to regulate neurite branching include the microtubule-severing protein spastin (Yu and others 2008), the stathmin family member SCG10-like (Poulain and Sobel 2007), doublecortin (DCX) (Kappeler and others 2006; Friocourt and others 2007), collapsin response mediator protein 2 (CRMP2) (Yuasa-Kawada and others 2003), and microtubule-associated protein 1b (MAP1b) (Bouquet and others 2004).

A recent study of axon regeneration in explants of adult DRG neurons noted an increase in axon collateral and higher order terminal axon branching in MAP1b knockout mice (Bouquet and others 2004). MAP1b is a MAP enriched in growing axons, a protein that binds the microtubule lattice—the helical arrangement of tubulin heterodimers that makes up the surface of a microtubule—and stabilizes microtubules (Halpain and Dehmelt 2006). Therefore, like the KIF2A results, these findings provide *in vivo* evidence for the concept that regulating axon branching requires control of microtubule stability (Bouquet and others 2004).

Actin-binding proteins known to regulate axon branching include Ena/VASP and UNC-115. Ena/VASP proteins control the branching of individual actin filaments (Bear and Gertler 2009), and in *Xenopus* they are necessary for the terminal arborization of RGC axons in the optic tectum (Dwivedy and others 2007). UNC-115 controls lamellipodia and filopodia formation (Yang and Lundquist 2005) and appears to specify branch routing and termination (Lundquist and others 1998).

Overall, the examples presented here illustrate the key role played by cytoskeleton-associated proteins in axon branching. In addition to revealing new molecular regulators of the process, many of these studies have contributed to our mechanistic understanding of the changes that occur in microtubule and actin dynamics when axons branch.

Summary and Conclusions

One of the main themes emerging in our molecular understanding of axon morphogenesis is the observation that many of the factors important for axon growth and guidance also play important roles in axon branching. Extrinsic factors implicated in axon branching include the traditional axon guidance cues slit, semaphorin, and ephrin; the neurotrophin BDNF; the secreted glycoprotein Wnt; the extracellular matrix protein anosmin-1; and certain transmembrane cell adhesion molecules, in addition to sensory experience and neuronal activity (Table 1). Intracellular proteins of various classes are also crucial regulators of axon branching; these consist of kinases and their regulators, transcription factors, Rho GTPases and their regulators, ubiquitin ligases, and various cytoskeleton-associated proteins, including the kinesin superfamily, other microtubule-binding proteins, and actin-binding proteins (Table 1).

It is likely that many more regulators of axon branching remain to be identified, as do the links among different known regulators, both extrinsic and intrinsic. Many questions remain regarding the cytoskeletal basis of branching processes such as branch formation, growth, retraction, and stabilization—as well as how these processes vary in different neurons and different branch types. Additionally, much work also remains to be done in terms of linking the functional insights gained from molecular studies of axon branching (i.e., whether a protein promotes or inhibits branching) with our existing knowledge of the cytoskeletal mechanisms of branching. At present there are not enough data to categorize all of the branching molecules listed in Table 1 into the specific modes of branching regulation depicted in Figure 1.

Ultimately, expanding our knowledge of axon branching enhances our understanding of circuit development in the nervous system. For instance, uncovering the mechanisms by which the ephrins control axon branching has advanced our understanding of topographic map formation—particularly the formation of visual field maps in the brain (Feldheim and O'Leary 2010). Uncovering the roles of specific transcription factors, such as *Pea3* and *Er81*, in axon branching has strengthened our understanding of sensory-motor circuit assembly (Dasen 2009). Finally, uncovering the contribution of semaphorins to stereotyped axon branch pruning has improved our understanding of the way hippocampal circuits are refined (Bagri and others 2003).

Beyond what it reveals about normal nervous system development, elucidation of the mechanisms of axon branching is likely to have important consequences for the treatment of neurodevelopmental disorders and nervous system injuries. Disrupted patterns of synaptic connectivity are thought to underlie numerous developmental disorders, and axon branching clearly plays a key role in establishing patterns of synaptic connectivity. Apart from developmental disorders, in nervous system injuries—such as spinal cord injury—where the inability to regenerate lost or damaged axonal fibers remains a major obstacle to treatment, promoting axon collateral sprouting in surviving neurons is potentially a strategy for restoring function (Hagg 2006).

Acknowledgments

The authors thank members of the Bonni laboratory for helpful discussions.

Funding The author(s) disclosed receipt of the following financial support for the research and/or authorship of this article: This work was supported by National Institutes of Health Grants NS041021 and NS047188 (A.B.).

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Figure 1.

Modes of axon branching regulation. Changes in axon branching patterns are achieved through regulation of a variety of morphological processes—including branch initiation, branch elongation, the development of arbor complexity, branch retraction/pruning, and terminal arborization.

Table 1

Molecular Regulators of Axon Branching

Molecule	Net Effect on Branching	Branching Mechanism	Type of Neurons*	References
Extrinsic cues				
Slits	+	Promote branching but also guide branches via repulsive activity	DRG and trigeminal sensory neurons	Wang and others 1999; Ozdinler and Erzurumlu 2002; Ma and Tessier-Lavigne 2007
Class III semaphorins	–	Inhibit branching, promote retraction of long branches, and guide branches via repulsive activity	Hippocampal and cortical neurons, zebrafish sensory neurons	Bagri and others 2003; Dent and others 2004; Liu and Halloran 2005
Ephrins	–/+	Control organization of axon terminal arbors within targets; also guide branches	Thalamocortical projection neurons, chick retinotectal neurons, <i>Drosophila</i> mushroom body neurons	Yates and others 2001; Mann and others 2002; Boyle and others 2006
Wnts	+		DRG sensory neurons, sympathetic neurons, cerebellar granule neurons	Lucas and Salinas 1997; Krylova and others 2002; Bodmer and others 2009
BDNF	+	Promotes terminal arborization that is correlated with synaptogenesis	<i>Xenopus</i> retinotectal neurons	Cohen-Cory and Fraser 1995; Alsina and others 2001; Sanchez and others 2006
Anosmin-1	+	Stimulates collateral formation and extension of terminal arbors	Olfactory bulb and Purkinje neurons	Soussi-Yanicostas and others 2002; Gianola and others 2009
Kinases/kinase regulators				
PI3K	+		DRG sensory neurons	Gallo and Letourneau 1998
AKT	+		DRG sensory neurons	Markus and others 2002
GSK3	–		DRG sensory neurons, hippocampal neurons, cerebellar granule neurons	Kim and others 2006, Lucas and Salinas 1997, Bilimoria and others 2010
PrkG1	+		DRG sensory neurons	Zhao and others 2009
Ulk5	–		DRG sensory neurons	Zhou and others 2007
FAK	–		Hippocampal and Purkinje neurons	Rico and others 2004
MKP-1	+		Cortical neurons	Jeanneteau and others 2010
JIP3	–		Cerebellar granule neurons	Bilimoria and others 2010
Transcription factors				
Pea3	+		Spinal motor neurons	Livet and others 2002
Islet2	+		Zebrafish sensory neurons	Segawa and others 2001
Otx1	–	Eliminates exuberant collaterals	Cortical projection neurons	Weimann and others 1999
Rho GTPase/RhoGTP regulators				
Rac	+		<i>Drosophila</i> mushroom body neurons	Ng and others 2002
RhoA	+		Cortical neurons	Ohnami and others 2008
p190 RhoGEF	–		Hippocampal neurons	Rico and others 2004
p190 RhoGAP	+	Represses branch retraction	<i>Drosophila</i> mushroom body neurons	Billuart and others 2001

Molecule	Net Effect on Branching	Branching Mechanism	Type of Neurons*	References
Ubiquitin ligases				
Highwire/Esrom/RPM-1	-/+	Restraints growth of terminal arbor, controls branch position, promotes branch stabilization, inhibits branching	Drosophila NMJ, zebrafish retinal neurons, <i>C. elegans</i> sensory and motor neurons	Wan and others 2000; D'Souza and others 2005; Schaefer and others 2000
Nedd4	+	Promotes terminal branching	Xenopus retinal ganglion neurons	Drinjakovic and others 2010
Cytoskeleton-associated proteins				
KIF2A	-	Suppresses extension of collateral branches	Hippocampal and cortical neurons	Homma and others 2003
MAP1b	-	Inhibits collateral and higher order terminal branching	Adult DRG neurons	Bouquet and others 2004
Xena/XVASP	+		Xenopus retinal ganglion neurons	Dwivedy and others 2007

* Where species is not indicated, neurons are from rodents.