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Developmental regulation of axon branching in the vertebrate nervous system

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Summary

During nervous system development, axons generate branches to connect with multiple synaptic targets. As with axon growth and guidance, axon branching is tightly controlled in order to establish functional neural circuits, yet the mechanisms that regulate this important process are less well understood. Here, we review recent advances in the study of several common branching processes in the vertebrate nervous system. By focusing on each step in these processes we illustrate how different types of branching are regulated by extracellular cues and neural activity, and highlight some common principles that underlie the establishment of complex neural circuits in vertebrate development.

Key words: Axon branching, Bifurcation, Collateral branch, Neural activity, Terminal arbor

Introduction

Our ability to perceive, to act and to remember is a reflection of the elaborated synaptic connections of neural circuits. The development of these connections relies on the proper regulation of axon branching, a morphogenetic process that allows a single neuron to communicate with multiple partners via its only axon. Since the pioneering observations by Ramon y Cajal (Ramon y Cajal, 1904), axonal branches have been found throughout the vertebrate nervous system. They help to define the morphology and connectivity of each neuronal cell type and provide alternative strategies for target selection and structural plasticity (O'Leary et al., 1990; Yamahachi et al., 2009). Therefore, understanding the mechanisms that underlie the temporal and spatial control of branching is crucial in the study of neural circuit development.

Axonal branches appear in different shapes, form at different locations, and change with time. Some are long and some are short; some are large, forming exuberant arbors that cover the entire target, whereas some are small, innervating just a small region of the target; some are dense and some are sparse, with only one branch projecting from the main axonal shaft; some are made during early development and form major neuronal pathways that persist throughout life, whereas others form at late stages of development and can be remodeled by neural activity. How are these branches generated? How is such diversity achieved? How is their development tailored to their function? How are they shaped by evolutionary pressures in modern animals? These are fascinating, yet challenging, questions in developmental neurobiology.

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To address these questions, it is practical to consider several common branching processes that are often mentioned in the literature. They can be characterized by the morphology, complexity and function of the branches they generate (Fig. 1). One form of branching is arborization (see Fig. 1A and Glossary, Box 1), which typically occurs at axon terminals in the target region and results in the formation of tree-like arbors with higher-order branches. As discussed in more detail below, examples of arborization are found in retinal ganglion cells and sensory neurons. By contrast, branches with the simplest shape are made by bifurcation (Fig. 1B), a process that often generates two daughter branches that grow away from each other, as seen in the central sensory projections in the spinal cord. Between these two extremes are collateral branches (Fig. 1C), which usually form far from the nerve terminals (see Glossary, Box 1). They extend either orthogonally or obliquely from the axon, and often project to targets that are different from that of the main axon. This form of branching is exemplified by sensory collaterals in the spinal cord and by the descending projections from the cortex (O'Leary and Terashima, 1988).

Studies of these branching processes have recently been carried out in various vertebrates, including fish, amphibians, birds and rodents. With the aid of technological advances in neuronal culture,

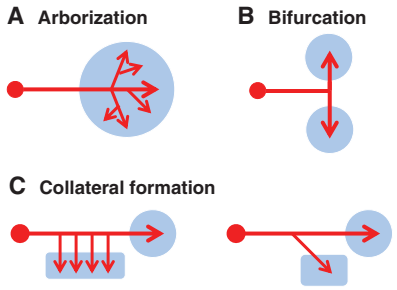

Fig. 1. Common axon branching processes in the vertebrate nervous system. Based on morphology, complexity and function, axon branching is grouped into three classes in this review: arborization, bifurcation and collateral formation. (**A**) Arborization usually occurs at axon terminals in the target region (blue circle), where most elaborated branched structures are generated by repetitive branch formation. (**B**) Axon bifurcation also occurs at axon terminals, but tends to generate two daughter branches that project to targets (blue circles) in opposite directions. (**C**) In collateral formation, daughter branches sprout from the middle of axonal shafts, away from axon terminals, and innervate targets that are usually different from the main axons (blue rectangles versus circles). In some axons, only one collateral branch forms (right), but in others multiple collaterals can form to synapse with similar targets (left). Structurally, collateral formation might be more closely related to axon bifurcation, as only the first-order branches are generated. The main features that distinguish them are the functions of the daughter branches and the angles between the daughter branch and the main axon.

Box 1. Glossary

Afferent. A neuronal process, usually from peripheral sensory neurons, that transmits sensory information from receptors to the CNS.

Collateral branch. A branch that often forms interstitially along the axon, but typically innervates a different target to the parent axon.

Dorsal root entry zone (DREZ). The region where sensory afferents from the DRG enter the spinal cord and form the longitudinal tracts.

Dorsal root ganglia (DRG). A collection of neuronal cell bodies outside of the spinal cord that relay somatic sensory information, such as pain, temperature and touch, from the periphery to the CNS.

Forward and reverse signaling. In forward signaling, Eph proteins act as receptors that are activated by the ephrin ligand to affect the Eph-expressing cell; in reverse signaling, membrane-associated ephrin acts as a receptor and is activated by the Eph ligand to affect the ephrin-expressing cell.

Geniculocortical. This refers to the projections from the lateral geniculate nucleus of the thalamus to the visual cortex.

Growth cone. A dynamic structure at the tip of an axon that is highly motile and leads to the extension of the axon.

Interstitial branch. A branch defined by where it forms along the axon that may become a collateral branch or, as in RGCs, a terminal arbor.

Neurotrophic factor. A molecule that regulates the growth and survival of developing neurons.

Retinal ganglion cells (RGCs). A type of neuron in the inner face of the retina that receives visual information and relays it to various regions in the CNS.

Retinotopic map. The organization of neurons within visual processing centers that maintains the precise spatial relations of cells in the retina.

Self avoidance. A phenomenon in which axonal or dendritic branches from a single neuron avoid contact with each other within the same target field.

Sympathetic ganglion. A ganglion with neurons of the sympathetic nervous system, the function of which is to mediate the 'fight or flight' response. Also known as superior cervical ganglion.

Terminal arborization. A complex branching pattern found at axonal terminals that innervates the target tissue; it can form directly at nerve terminals or, as in RGCs, as interstitial branches followed by axon retraction.

Terminal zone. A specific region of the target tissue in which axons make synaptic connections.

Tiling. A spatial arrangement in which neighboring axonal or dendritic arbors have very little overlap, thereby maximizing the coverage of the target tissue.

Trigeminal ganglion (TG). A ganglion that contains mainly sensory neurons that innervate the head.

Thalamocortical axon. An axon originating from a thalamic neuron that projects to the neocortex.

molecular genetics and cell imaging, some common principles of their developmental regulation have begun to emerge. In this review, we focus on several well-studied branches in the peripheral (PNS) and central (CNS) nervous systems and illustrate how branching morphologies are shaped by extracellular factors (see Table 1) and neural activity. We discuss the unique features of axon branching and explain why it involves more than just forming a branch and why it is more complex than axon growth and guidance. We also highlight the critical steps that are shared among different branching processes during embryonic and postnatal development. We hope that, in combination with concomitant advances in our knowledge of the intracellular regulation of branching (for reviews, see Dent et al., 2003; Kornack and Giger, 2005; Schmidt and Rathjen, 2010), this review will bring us closer to understanding an important developmental process in the assembly of complex neural circuits in vertebrates.

Axon branching: multiple steps to a final form

Axonal branches in mature neural circuits result from the tightly regulated and stepwise cellular processes that occur during development, but their final morphology is not always indicative of how they form. The best illustration of this is the formation of axonal arbors of retinal ganglion cells (RGCs; see Glossary, Box 1) at the terminal zone of the superior colliculus (SC), a brain region in chick and rodents that receives visual inputs from the retina (Fig. 2). In early development, RGC axons initially overshoot their respective terminal zones (see Glossary, Box 1). Later, interstitial branches (see Glossary, Box 1) form preferentially in the region of the appropriate terminal zone and then become terminal arbors after their overextended axons retract (Fig. 2B) (Nakamura and O'Leary, 1989; Simon and O'Leary, 1992). Thus, the RGC terminal arbors are not necessarily initiated at axon terminals but instead arise from previously formed interstitial branches, suggesting that the mechanism that underlies the development of axonal branches can be very different from that implied by their final morphology.

Recent genetic studies (Ma and Tessier-Lavigne, 2007; Schmidt et al., 2009; Schmidt et al., 2007; Zhao and Ma, 2009; Zhao et al., 2009) of the bifurcation of dorsal root ganglion (DRG) sensory axons in the dorsal root entry zone (DREZ) of the spinal cord (see Fig. 3 and Glossary, Box 1) further support this view. These studies suggest that the generation of such a simple structure involves multiple steps that are regulated by different extracellular cues (Fig. 4). The first step is directly linked to the formation of the daughter branch and requires C-type natriuretic peptide (CNP; also known as NPPC), a hormone that binds to the membrane-associated guanylate cyclase natriuretic peptide receptor 2 (NPR2) and stimulates the production of cyclic guanosine monophosphate (cGMP). The genetic ablation of the CNP precursor gene *Nppc*, its receptor *Npr2* or the downstream cGMP target cGMP-dependent protein kinase G1 (*Prkg1*), each leads to a failure in the formation of the second branch (Fig. 4B). This defect suggests that branch formation is regulated by the CNP pathway during bifurcation. This conclusion is supported by studies of dissociated DRG neurons in culture, in which CNP treatment, pharmacological activation of cGMP and overexpression of *Prkg1* all promote branch formation (Zhao and Ma, 2009; Zhao et al., 2009).

The second step involves the guidance of the two daughter branches by the guidance molecules SLIT1 and SLIT2, acting on their receptors ROBO1 and ROBO2 (Ma and Tessier-Lavigne, 2007). In mutant mice lacking both ligands or both receptors, the DRG central afferents (see Glossary, Box 1) still bifurcate but no longer have the normal orientation of the T- or Y-shaped bifurcation fork, as in about half of the neurons one daughter branch follows the normal trajectory whereas the other enters the spinal cord (Fig. 4B). This phenotype suggests that SLITs are normally required to keep the sensory afferents outside of the spinal cord and guide them to the DREZ, presumably through repulsion. This repulsive function for SLITs is supported by the relatively gentle collapsing activity found for SLIT2 on sensory growth cones in culture (Ma and Tessier-Lavigne, 2007).

Table 1. A summary of the molecular cues involved in regulating different types of branching in different neuronal cell types

Molecules	Neurons	Type of branches	Reference(s)
Anosmin	Olfactory neurons	Collaterals	Soussi-Yanicostas et al., 2002
BDNF	RGC	Terminal arbors	Cohen-Cory, 1999; Marler et al., 2008
	SCG	Collaterals	Singh et al., 2008
CNP	DRG	Central bifurcation	Schmidt et al., 2009; Zhao and Ma, 2009
ephrin A/EphA	RGC	Interstitial branches	Feldheim et al., 2000; Rashid et al., 2005; Yates et al., 2001
	Mossy fiber	Terminal arbors	Galimberti et al., 2010
ephrin B/EphB	RGC	Interstitial branches	Hindges et al., 2002; McLaughlin et al., 2003a
FGF2	Pyramidal neurons	Interstitial branches	Szebenyi et al., 2001
netrin 1	Cortical neurons	Interstitial branches	Dent et al., 2004; Tang and Kalil, 2005
	RGC	Terminal arbors	Manitt et al., 2009
NGF	DRG, SCG	Terminal arbors	Glebova and Ginty, 2004; Lentz et al., 1999; Patel et al., 2000
NT3	SCG	Terminal arbors	Lentz et al., 1999
SEMA3A	TG, DRG	Peripheral arbors	Kitsukawa et al., 1997; Taniguchi et al., 1997
	Hippocampal	Collateral	Bagri et al., 2003
SLITs	DRG	Bifurcation	Ma and Tessier-Lavigne, 2007
	TG, DRG	Arbors	Ma and Tessier-Lavigne, 2007; Yeo et al., 2004
WNT3A	DRG	Terminal arbors	Krylova et al., 2002
WNT5A	SCG	Terminal arbors	Bodmer et al., 2009

BDNF, brain-derived neurotrophic factor; CNP, C-type natriuretic peptide; DRG, dorsal root ganglion; FGF2, fibroblast growth factor 2; NGF, nerve growth factor; NT3, neurotrophin 3; RGC, retinal ganglion cell; SCG, superior cervical ganglion; SEMA3A, semaphorin 3A; TG, trigeminal ganglion.

The above phenotypes suggest several interesting features of bifurcation. First, the two daughter branches are not created equally at the beginning, as one daughter branch develops as an extension of the primary afferent, whereas the other branch forms under the regulation of CNP. Second, these two branches have different molecular and biochemical properties as their growth direction is regulated differently. One of them is influenced by SLITs, whereas the other is not. In addition, there seems to be no preference for which branch becomes the ascending or the descending projection, as either one can be affected in the above-mentioned mutants.

Based on these features, we propose a simple repulsion-coupled collateral formation model for sensory axon bifurcation (Fig. 4C). After the sensory afferents reach the DREZ they encounter several environmental cues and respond with the following steps, which might occur very rapidly. First, a stop signal from SLITs, and possibly from other repulsive factors, blocks the growth of the afferents at the DREZ but does not turn them away from it. Instead, the same signal steers the growth cones into the longitudinal track, either anteriorly or posteriorly in a random fashion. Then, CNP stimulates the formation of a new branch at the turning point and promotes the growth of this new branch. Finally, both daughter branches grow in opposite directions along the DREZ, forming the ascending and descending projections. This model suggests that the formation of the second branch might be similar to the formation of collateral branches, rather than involving the growth cone splitting mechanism associated with the final morphology, as previously thought (Acebes and Ferrus, 2000).

Although further studies are needed to validate this model, an intriguing extension of the above discussion is that any branching process, including those commons forms, can be subdivided into several distinct developmental steps. These steps, including branch formation, growth, guidance and pruning, might constitute the core mechanism of axon branching; coordination of their regulation might be sufficient to generate the diverse branching pattern by specifying the location, number, angle/trajectory, size and complexity of branching. For example, collateral formation is dictated by the location of branch formation, followed by guidance of the newly formed branch, whereas for axon bifurcation branch formation is tightly coupled to the guidance of both the nascent branch and the main axon. In addition, arborization could simply be the result of the repetition of these processes in association with branch interactions, such as self avoidance and tiling (see Glossary, Box 1). Therefore, understanding these developmental steps is crucial to elucidating the mechanisms of axon branching. In the following sections we discuss how these steps are regulated by extracellular cues and by neural activity to generate stereotyped branches during development. The cellular mechanisms of their regulation (see Box 2) have been reviewed recently (Dent et al., 2003; Kornack and Giger, 2005; Schmidt and Rathjen, 2010) and will therefore only be discussed in the appropriate context.

Specifying axon branch location

Branches can form at nerve terminals or interstitially along axonal shafts, depending on where branches are initiated. Several mechanisms that specify the location of branching have emerged from recent studies of the extracellular factors involved in branching (Fig. 5).

Target-derived signals acting on axon terminals

Most axonal branches form at nerve terminals in their target regions, as exemplified by both the peripheral and central axons of sensory neurons in the DRG (see Fig. 3). The terminal arbors of these neurons develop after the axons have reached their targets, such as the peripheral skin tissue or the central motoneurons in the spinal cord. Several target-derived factors have been found to provide instructive signals that specify where an axon will arborize.

One such signal is nerve growth factor (NGF), the first neurotrophic factor (see Glossary, Box 1) implicated to be acting as a target-derived signal from the skin to promote terminal branching of sensory axons in the peripheral tissue (Kennedy and Tessier-Lavigne, 1995). To separate its role in cell survival from axon branching, a double deletion of *Ngf* and *Bax* was created to block programmed cell death in mice (Patel et al., 2000). In this mutant, the peripheral projections reach their skin targets but fail to innervate them and to arborize. Although this phenotype might reflect the function of NGF in axon growth, studies of dissociated $Bax^{-/-}$ neurons in culture, in which they survive in the absence of neurotrophic factors but do not develop a branched morphology, indicate that branch elongation and arborization depend on NGF

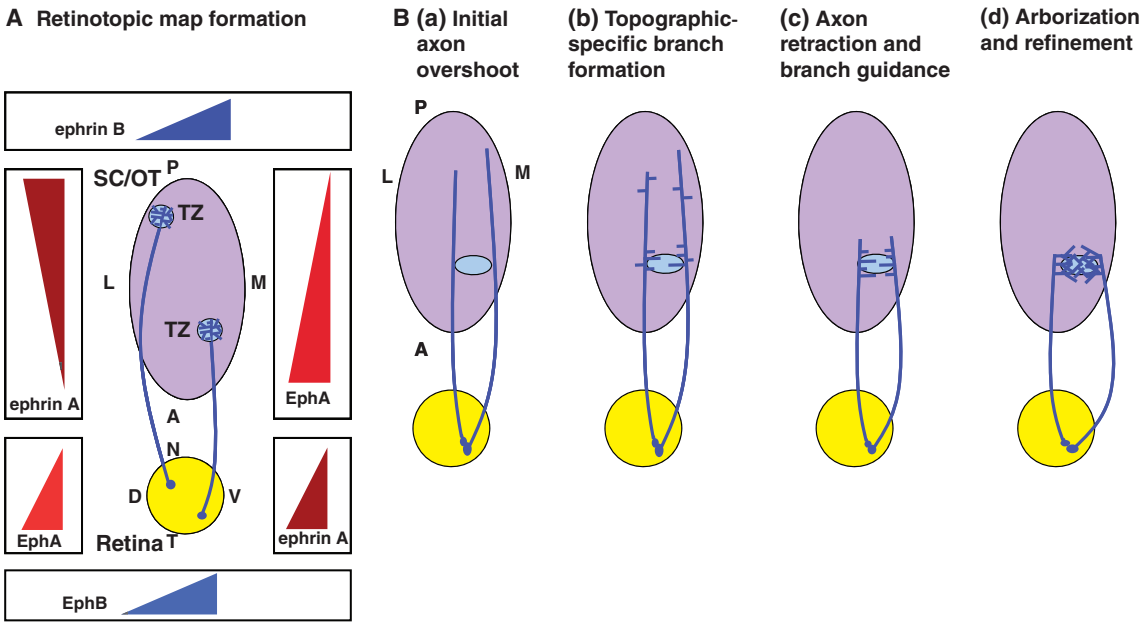

Fig. 2. Development of topographic-specific axonal branches in the visual system. (**A**) Retinotopic map formation is regulated by the interactions of ephrin A/EphA gradients. To preserve the spatial information received in the retina (yellow circle), the retinal ganglion cell (RGC) axons project to the terminal zone (TZ, blue circle) of their cortical target (purple oval) in a topographic fashion to form the retinotopic map. In mammals, the target is the superior colliculus (SC), whereas in *Xenopus*, chick and zebrafish it is the optic tectum (OT). The retinotopic map forms via the repulsive interaction of ephrin A, which is expressed in the target structure, and the EphA receptors, which are expressed on growing RGC axons. As shown in the left gradients, EphAs are expressed in an increasing nasal-temporal (N-T) gradient in the retina, whereas ephrin A ligands are expressed in an increasing anterior-posterior (A-P) gradient in the cortical target. Other signaling gradients, including ephrin A/EphA reverse signaling (right-hand side), as well as ephrin B/EphB (top and bottom), are also shown here and contribute to RGC axon branching. (**B**) RGC terminal arbor development occurs via multiple steps in chick and mouse embryos. (**a**) RGC axons initially overshoot the correct TZ and interstitial branches form along the A-P axis of the axon. (**b**) Branch formation increases at the correct topographic-specific TZ, a process that is regulated by opposing ephrin A/EphA gradients and by global expression of brain-derived neurotrophic factor (BDNF). (**c**) The overextended axons then retract to the correct TZ, and branches are preferentially guided to the TZ, possibly via an EphB gradient on the retinal dorsal-ventral (D-V) axis and an ephrin B gradient on the lateral-medial (L-M) axis of the target. (**d**) An activity-dependent process then eliminates branches outside of the correct TZ while retaining correctly positioned branches. Figures are modified with permission (McLaughlin and O'Leary, 2005).

and other neurotrophic factors such as neurotrophin 3 (NT3; also known as NTF3) (Lentz et al., 1999). This notion is further supported by reduced sensory arborization in the peripheral tissue in mice that lack the low-affinity NGF receptor p75 (also known as NGFR) (Bentley and Lee, 2000).

A role for NGF in axon branching has also been demonstrated in neurons from the sympathetic ganglion (see Glossary, Box 1). Its deletion in a *Bax* null mouse background results in the reduced innervation of a variety of sympathetic targets owing to deficient axonal branching (Glebova and Ginty, 2004). Many of these phenotypes are target dependent, and not all sympathetic targets have the same degree of reduced innervation, indicating the presence of other axon branching factors. A recent study suggests that WNT5A might be a good candidate to mediate NGF-induced branching (Bodmer et al., 2009).

Another Wnt family protein, WNT3, has been implicated in regulating central terminal branching of proprioceptive sensory neurons in the mouse spinal cord (Krylova et al., 2002). These terminals synapse with motoneurons in the ventral spinal cord where *Wnt3* is expressed (Krylova et al., 2002). Growing dissociated embryonic mouse DRG neurons in the presence of WNT3-conditioned medium or next to ventral mouse spinal cord explants increases the number of secondary and higher-order branches, revealing the potential role of WNT3 as a target-derived signal that specifies the location of terminal arborization.

In addition, factors in the target tissue can also regulate the location of bifurcation (Fig. 5A). As discussed above, CNP is expressed in the mouse dorsal spinal cord at the time when sensory axons initially reach the DREZ (Schmidt et al., 2009; Zhao and Ma, 2009), and thus provides a local cue in this intermediate target to specify the location of bifurcation of sensory axons (Fig. 4). It is interesting to note that only two branches form here instead of the exuberant arbors found at the mature DRG axon terminals, indicating that CNP might act differently to other target-derived cues or that other factors are present to restrict the number of branches formed.

Local induction along the axon

Factors from local tissues can provide an instructive cue to promote branch formation along the axon (Fig. 5B). This was first demonstrated by the study of axons that project from layer five of the rat cortex to their subcortical targets through the formation of collateral branches (O'Leary and Terashima, 1988). The target tissues appear to secrete an attractive diffusible factor that promotes branch formation (Heffner et al., 1990; Sato et al., 1994).

Another example of locally induced branching is the development of sensory collaterals that sprout from the ascending and descending projections (see Fig. 3B). During early spinal cord development these branches do not form immediately after sensory

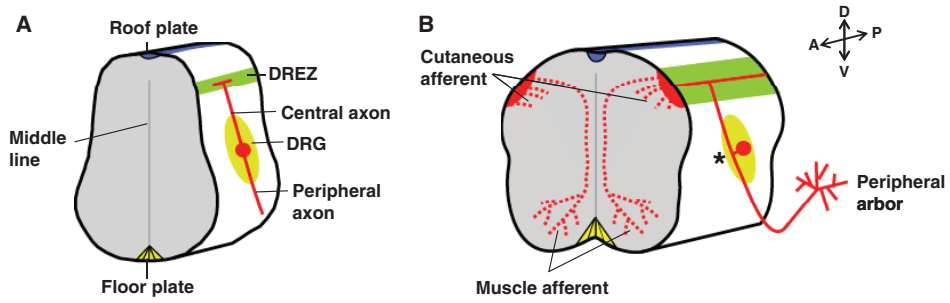

Fig. 3. Development of sensory axon branching in the spinal cord. Schematized cross-sections of a vertebrate developing spinal cord illustrate three branching forms of dorsal root ganglion (DRG) sensory axons (Davis et al., 1989; Mendelson et al., 1992; Mirnics and Koerber, 1995a; Mirnics and Koerber, 1995b; Ozaki and Snider, 1997; Ramon y Cajal, 1904). (**A**) The DRG flanks the spinal cord and contains the cell bodies (red circle) of sensory neurons that initially generate two axons. One axon (the peripheral axon) projects to the skin or muscle, whereas the other axon (the central axon) projects centrally to the spinal cord. (**B**) Later in development, these two axons fuse (asterisk) to form a single axon and establish a pseudo-unipolar morphology (Ramon y Cajal, 1904). The peripheral axons arborize in their targets to form the peripheral arbors, while the central axons bifurcate once they reach the dorsal root entry zone (DREZ; green stripe) of the spinal cord and continue to extend in opposite directions along the anterior-posterior (A-P) axis. There are two functional significances of this bifurcation: it allows sensory information to be transmitted to the high-order relay neurons in the spinal cord or the brain stem via its ascending projections (growing anteriorly); and, both the ascending and descending (growing anteriorly and posteriorly) axons sprout interstitial collateral branches that invade the gray matter of the spinal cord. The collaterals of cutaneous and motor afferents terminate at different lamina on the dorsal-ventral (D-V) axis of the spinal cord and form monosynaptic or polysynaptic reflex circuits. The terminals of these collaterals also arborize, as shown here for the muscle afferents.

afferents bifurcate (Mirnics and Koerber, 1995b; Ozaki and Snider, 1997); instead, there is a 2-day delay before the collaterals sprout. In vitro studies suggest that factors are present in the spinal cord that might be upregulated to directly stimulate the formation of these collaterals (Wang et al., 1999).

Results from in vitro studies indicate that several extracellular molecules can act as local cues to promote branch formation along an axon. One example is NGF, which initiates collateral sprouting from axons of cultured chick DRG neurons when it is presented locally on coated beads (Gallo and Letourneau, 1998). A similar response has been observed to fibroblast growth factor 2 (FGF2) in embryonic hamster pyramidal neuron axons, but in this case the factor acts in close proximity to the growth cone (see Glossary, Box 1) (Szebenyi et al., 2001).

The guidance molecule netrin 1 can also stimulate branch formation of cultured hamster cortical axons when applied locally through a pipette tip (Dent et al., 2004). Filopodial protrusions were initiated at smooth segments of these axons in the vicinity of the pipette tip, followed by filopodial extension towards the tip. The effect of netrin 1 on branching is possibly mediated by calcium signaling, as netrin 1 treatment induces spatially restricted calcium transients in the axon that coincide spatiotemporally with new branch formation (Tang and Kalil, 2005). Moreover, the local application of netrin 1 induces a local Ca^{2+} transient that is accompanied by growth of the stimulated branch (Hutchins and Kalil, 2008).

Finally, the secreted protein anosmin (also known as KAL1) may promote the formation of local collateral branches from projection neurons in the mammalian olfactory bulb. Anosmin is defective in Kallman syndrome, a human disease that is associated with anosmia and linked to the absence of the olfactory tract. In culture, anosmin has been shown to stimulate branch formation in rat olfactory neurons (Soussi-Yanicostas et al., 2002).

Local inhibition coupled with global promotion

Another mechanism that restricts branch formation to certain regions of the axon has been suggested from studies of retinotopic map development (see Glossary, Box 1) in the SC of chick and rodents (see Fig. 2), where interstitial branches form along the axon near the appropriate terminal zone (Luo and Flanagan, 2007; McLaughlin and O'Leary, 2005). A study of chick RGC axons has indicated that the same ephrin/Eph gradients used for map formation might also participate in the generation of topographic-specific arbors (Yates et al., 2001). In culture, the axons of chick RGCs derived from the temporal side of the retina show a distinct preference for forming branches on membrane stripes that are derived from the anterior tectum, their appropriate terminal zone, whereas nasal RGC axons show no preference. The addition of soluble EPHA3 receptors to the culture to sequester ephrin A ligands on the membrane stripes completely abolished this biased branch formation, suggesting that ephrin A-mediated inhibition can restrict branching locally. This notion is consistent with the in vivo observation of ectopic terminal zones in the posterior SC of mice that lack ephrin A2, ephrin A5, or both (Feldheim et al., 2000).

However, this ephrin A/EphA mechanism alone is not sufficient to generate posterior-specific branching by nasal RGC axons. A recent investigation in mice suggests that a complementary gradient of EPHA7 exists in the target structure to regulate this phenomenon (Rashid et al., 2005). *Epha7* is expressed in a decreasing gradient along the collicular anterior-posterior (A-P) axis, whereas ephrin A is more highly expressed on nasal RGC axons than on temporal axons, forming a decreasing gradient along the retinal nasal-temporal axis (Fig. 2A). Loss of *Epha7* in mice leads to the formation of a densely branched ectopic terminal zone in the anterior portion of the SC, in addition to the normal posterior terminal zone (Rashid et al., 2005). Thus, in contrast to the ephrin A forward signaling (see Glossary, Box 1) that is employed in restricting temporal RGC axon branching, ephrin A reverse signaling (see Glossary, Box 1) serves as a negative regulator of nasal RGC axon branching. Thus, a common feature of RGC axon branching is that inhibition provides a key mechanism for generating topographic-specific branches.

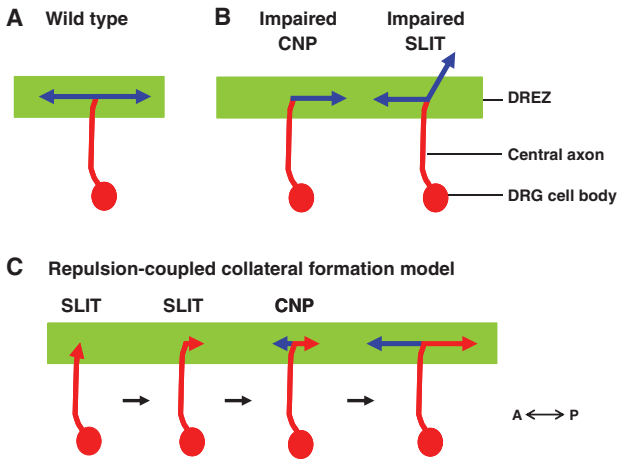

Fig. 4. An illustration of sensory axon bifurcation. A schematic of sensory axon bifurcation in the dorsal root entry zone (DREZ) of the mouse spinal cord. (**A**) In wild-type mouse embryos, the central axon (red) of the dorsal root ganglion (DRG) bifurcates at the DREZ (green stripe) of the spinal cord. The two resulting branches (blue) extend in opposite directions along the anterior-posterior (A-P) axis, perpendicular to the primary axon (red). (**B**) Summary of bifurcation defects in mouse mutants. Mice that lack C-type natriuretic peptide (CNP; also known as NPPC) signaling include: a spontaneous *Nppc* mutant [*lbab* (long bone abnormality)]; a targeted *Nppc* knockout; a spontaneous natriuretic peptide receptor 2 (*Npr2*) mutant; and a targeted deletion of cGMP-dependent protein kinase GI (*Prkg1*) (Schmidt et al., 2009; Schmidt et al., 2007; Zhao and Ma, 2009; Zhao et al., 2009). In these mutants, DRG axons fail to bifurcate in the DREZ, resulting in the loss of the second branch. In mutants with impaired SLIT signaling, such as in *Slit1;Slit2* or *Robo1;Robo2* double mutants, DRG axons still bifurcate, but one daughter branch is misguided and enters the spinal cord (Ma and Tessier-Lavigne, 2007). (**C**) A repulsion-coupled collateral formation model that describes the sequential steps required for sensory axon bifurcation in the spinal cord. After the primary sensory axon (red) first reaches the DREZ (green stripe), it encounters SLIT proteins, which are present next to the DREZ. These cues stop the axon and guide it to make a turn randomly to the A-P track of the DREZ. Immediately after turning, the axon receives an instructive signal from CNP and makes a new branch (blue), which sprouts as a collateral from the turning site and is biochemically different from the other branch (red).

Such a mechanism might also apply to the topographic axonal arborization of dentate gyrus granule cells in mouse hippocampal formation (Galimberti et al., 2010). The axons of dentate gyrus granule cells, termed ‘mossy fibers’, project to the CA3 region of the hippocampus, where they form one or more terminal arborizations at locations in CA3, based on the position of the granule cell soma in the dentate gyrus. The dentate gyrus exhibits an expression gradient of the EPHA4 receptor, which suggests that it could participate in the topographic specification of mossy fiber terminal arborizations. Indeed, disrupting EPHA4 signaling in mouse hippocampal slice cultures increases the number of mossy fiber terminal arborizations, in addition to abolishing the topographic specificity (Galimberti et al., 2010).

However, branching inhibition only restricts where branches form, but does not guarantee eventual branch formation. It is possible that axons are intrinsically capable of making branches, as demonstrated by branch formation of chick RGCs in culture (Yates et al., 2001). In addition, studies of the *Xenopus* optic tectum found that a branch-promoting signal provided by brain-derived neurotrophic factor (BDNF) can complement ephrin A/EphA-mediated branch restriction (Cohen-Cory and Fraser, 1994; Cohen-Cory and Fraser, 1995). BDNF is uniformly expressed in the tectum, and its high-affinity receptor TrkB is uniformly expressed in the retina (Cohen-Cory and Fraser, 1994). Injection of BDNF into the tectum increases the branching and complexity of RGC terminal arbors, whereas injection of specific neutralizing antibodies to BDNF reduces axon arborization and complexity (Cohen-Cory and Fraser, 1995). Furthermore, a recent in vitro study of chick neurons suggests that this branch-promoting activity is mediated by an interaction between TrkB and ephrin A at the cell surface (Marler et al., 2008). TrkB interacts with ephrin A5 via a cysteine-rich domain, and overexpression of this domain in chick RGCs results in a dominant-negative effect on BDNF-induced branch formation. Similarly, addition of soluble EPHA7 to the RGC culture inhibits this activity, as do membrane stripes studded with EPHA7 (Marler et al., 2008). These experiments suggest that a global branch-promoting cue, such as BDNF, coordinates with the ephrin/Eph gradients to control RGC axon branching. Thus, the coupling of global branch promotion with a complex branch-restricting system provides a general molecular mechanism to restrict branching to a specific location in the developing nervous system (Fig. 5C).

Guiding axonal branches

A defining feature of axonal branches is the angle between the daughter branch and the parent axon. This can be determined by the intrinsic properties of each axon (Katz, 1985; Lasek, 1988), but might reflect the interaction of newly formed branches with the environment. Similar to axon guidance, extracellular factors can guide the growth trajectory of daughter branches through both short-range and long-range guidance mechanisms that influence the branching angle and the final branching pattern. As seen during sensory axon bifurcation discussed above, SLITs control the growth direction of the daughter branches (Fig. 4C). In the absence of SLIT signaling, the normal orientation of the bifurcation fork is altered, resulting in one daughter branch entering the spinal cord (Fig. 4B). However, only half of the afferents are affected in these mutants, suggesting the presence of other extracellular signals that guide branch growth during bifurcation (Ma and Tessier-Lavigne, 2007).

The invasion of sensory collaterals into different laminar targets in the spinal cord provides an illustration of how different populations of branches are regulated by the environment. For example, cutaneous afferents from small diameter neurons that convey pain and temperature information are stopped at the dorsal lamina in the rodent spinal cord, whereas muscle afferents from large diameter neurons course through the entire spinal cord and reach the motoneurons in the ventral half of the spinal column (see Fig. 3B) (Mirnics and Koerber, 1995b; Ozaki and Snider, 1997). Interestingly, the muscle afferents follow a hyperbolic trajectory and appear to be attracted directly to the motor column, while avoiding the dorsal gray matter (Snider et al., 1992).

Another example of environmental regulation is provided by RGC axon branches, which, in addition to preferentially forming near the appropriate terminal zone, have a strong preference to extend in either the lateral or medial direction, depending on their relative location (Fig. 2B) (Nakamura and O’Leary, 1989). This preference might be controlled by ephrin B/EphB signaling in mice, as the EPHB receptor is expressed in an increasing dorsal-ventral (D-V) gradient, whereas the ligand ephrin B1 is expressed in an increasing lateral-medial (L-M) gradient in the

Box 2. Cellular regulation of axon branching

In vitro studies have suggested several basic cellular mechanisms for axon branching (Acebes and Ferrus, 2000): growth cone splitting that generates two branches; de novo initiation that generates interstitial branches along the axonal shaft (Heffner et al., 1990; Portera-Cailliau et al., 2005); and delayed sprouting that results from growth cone pausing or collapse (Davenport et al., 1999; Dent et al., 1999; Szebenyi et al., 1998). Although these mechanisms may account for the three forms of branching discussed here (see Fig. 1), recent imaging studies suggest that not all mechanisms are used by developing neurons in vivo (De Paola et al., 2006; Portera-Cailliau et al., 2005).

Branch formation requires both actin and microtubules (Dent et al., 2003). Both microtubule transport and microtubule assembly play important roles in branching (Dent et al., 1999; Gallo and Letourneau, 1999; Yu et al., 1994; Black, 1994; Kornack and Giger, 2005). Microtubule loops are present in regions prior to branch formation (Dent et al., 2004; Dent et al., 1999) and interact with actin at putative branching sites (Dent and Kalil, 2001). Actin assembly is regulated by the Rho family small GTPases (Luo, 2002), which contribute to different aspects of branching regulation (Hall and Lalli, 2010).

Other cytoplasmic factors that regulate axon branching can be divided into several groups: transcription factors (Hippenmeyer et al., 2005; Livet et al., 2002), signaling proteins (Drinjakovic et al., 2010; Guerrier et al., 2009; Kim et al., 2006; Rico et al., 2004; Tang and Kalil, 2005; Wayman et al., 2004), cytoskeleton regulators (Ahuja et al., 2007; Bouquet et al., 2004; Fukata et al., 2002; Homma et al., 2003; Kim et al., 2006; Poulain and Sobel, 2007) and proteins affecting axon stability (Konishi et al., 2004; Stegmuller et al., 2006).

SC (see Fig. 2A) (Hindges et al., 2002). Furthermore, after the normal period of refinement has passed, mutant mice that lack functional EPHB retain ectopic terminal zones at positions lateral to the correct terminal zones as a result of an increase in laterally oriented branches, coupled with a decrease in medially oriented branches (Hindges et al., 2002). A second study using retroviral transfection showed that graded ectopic expression of ephrin B1 results in the formation of laterally oriented branches within the ectopic terminal zone, possibly owing to branch repulsion by the high level of ephrin B1 present on the medial side of the ectopic zone (McLaughlin et al., 2003a).

Thus, the branching angle is determined by the guidance of newly formed branches by mechanisms that are similar to those of axon guidance. Factors present in the local environment or acting from target regions can attract or repel each branch and thereby influence branch morphology and function.

Pruning branches

Axonal branches that form during development are not always retained in the mature neural circuit (Luo and O'Leary, 2005). Many can be eliminated through an active process called pruning. The classical example of pruning is the layer-five projection neurons from the motor and visual cortex (O'Leary and Koester, 1993). During early mammalian cortical development, multiple collaterals form from these projections, but some are eliminated during postnatal development to make the appropriate connection with their targets. RGC development provides another example of pruning (Fig. 2B), as both the overshooting axons and the inappropriately formed interstitial branches are rapidly eliminated over a period of a few days (McLaughlin et al., 2003b).

Branch pruning may involve cellular factors that are important for branch degeneration (Luo and O'Leary, 2005), and its regulation may employ the same molecules that are used for axon guidance (Vanderhaeghen and Cheng, 2010). For example, an early study of the SEMA3A receptor, plexin A3 (PLXNA3), suggests that pruning of specific hippocampal mossy fibers and of pyramidal branches requires this pathway (Bagri et al., 2003), as both pruning events are defective in the *Plxna3* mouse knockout. Moreover, a recent study has suggested that mossy fiber pruning can be mediated by ephrin B/EphB reverse signaling (Xu and Henkemeyer, 2009). In addition to such molecular regulation, an activity-dependent mechanism has been proposed for branch pruning in RGCs as well (see below).

Regulating arbor size

Terminal arbors are responsible for the innervation of the target field with a high degree of fidelity. Their morphology influences the function and plasticity (see below) of neural circuits and is often defined by branch number, length and order, as well as by arbor size. These parameters are commonly used to distinguish different neuronal cell types and to describe changes in arbor morphology during development. They reflect the interplay of branch formation, growth and guidance, as well as interactions between branches.

Recent studies of the peripheral projections of sensory neurons from the DRG and of the trigeminal ganglion (TG; see Glossary, Box 1) have illustrated how arbor size is regulated by competing factors in the environment. One factor is the SLIT family of secreted proteins, which can positively regulate the peripheral branching of TG axons in mice (Ma and Tessier-Lavigne, 2007). In wild-type embryos, the TG axons form a major arbor above the eye, but in *Slit2;Slit3* double mutants the arbor is greatly reduced in size owing to a reduction in both branching and growth, whereas in *Slit1;Slit2;Slit3* triple mutants the entire arbor is missing. The same defect was also found in embryos that lack the SLIT receptors ROBO1 and ROBO2. These results are consistent with a study in zebrafish embryos, in which *slit2* overexpression dramatically increased the branching of peripheral sensory axons (Yeo et al., 2004).

This positive role of SLITs is counterbalanced by the semaphorin (SEMA) family of guidance molecules, particularly SEMA3A. These molecules are known for their activity in inhibiting axon growth and repelling growth cones (Dickson, 2002). Genetic studies have revealed enhanced peripheral axon growth and branching in both DRG and TG neurons from mouse embryos that lack SEMA3A (Kitsukawa et al., 1997; Taniguchi et al., 1997). Similar defects are found in mice in which the genes encoding the SEMA3A co-receptor neuropilin 1 or plexin A3/A4 have been deleted (Gu et al., 2003; Yaron et al., 2005). Such an activity is consistent with the inhibitory activity of SEMA3A on axon branching of dissociated cortical neurons in culture (Dent et al., 2004). Therefore, the negative regulation by SEMA3A cooperates with the positive regulation by SLITs to determine the arbor size of peripheral sensory axons.

The repulsive action of SEMAs and of other inhibitory cues might also play a role in self avoidance and tiling, two processes that reflect the interactions between branches within the terminal arbors. Live in vivo imaging of sensory axons in zebrafish has shown that removing one axon arbor allows the neighboring neurons to expand their arbor (Sagasti et al., 2005), indicating the existence of a repulsive mechanism to prevent the formation of overlapping receptive fields. Although the molecular mechanisms that underlie these processes have been extensively studied in the

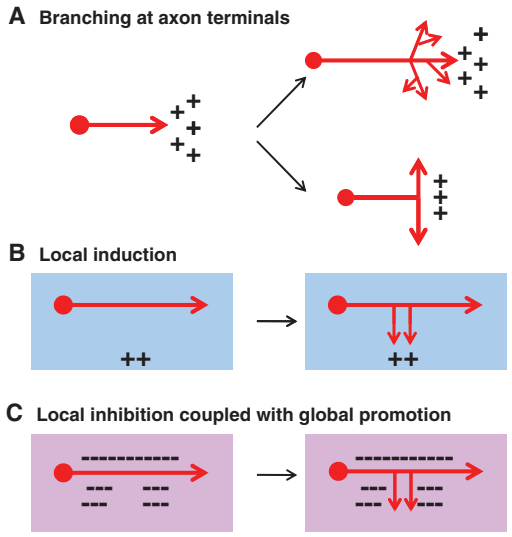

Fig. 5. Mechanisms that specify the location of axon branching. (**A**) Branches often occur at the end of axonal terminals. The presence of branch-promoting factors (represented by ++), such as nerve growth factor (NGF), wingless-related MMTV integration site (Wnt) factors and C-type natriuretic peptide (CNP), in the target region, can promote the formation of both terminal arbors (top) and bifurcation (bottom). However, the mechanisms that underlie these different types of branching remain to be determined. (**B**) Collateral branches can be promoted by a positive cue interstitially along the axon. When navigating to find their targets during early development, many axons either cannot form branches or are inhibited by the local environment (blue box). A positive branch-promoting cue (++) present in the target area can stimulate the formation of an interstitial branch at a specific location along the axon and promote its growth to become a collateral branch. (**C**) A mechanism involving local inhibition (represented by dashed line) coupled with global activation can also specify the location of interstitial branch formation along the axon. In this mechanism, branch formation can occur anywhere along the axon, either by an intrinsic mechanism or by a positive branch-promoting factor that is present along the route of axonal growth (purple box). Inhibitory factors, such as ephrin A/EphA, present near the axons specify branching location by restricting branch formation to certain regions of the axon.

dendritic development of non-vertebrates, it would not be surprising if some of the factors identified there play similar roles in axonal tiling and avoidance in vertebrates (Grueber and Sagasti, 2010). For example, the cell adhesion molecule DSCAM that regulates these processes in axons and dendrites of *Drosophila* neurons (Grueber and Sagasti, 2010) has recently been implicated in dendritic avoidance in the rodent retina (Fuerst et al., 2009; Fuerst et al., 2008) and might well regulate axonal arbor morphology in vertebrates.

Regulating axon branching by neural activity

Developing neurons experience patterns of activity – both spontaneous and experience dependent – that contribute to the initial development and subsequent refinement of the functional circuit (Spitzer, 2006). Following the classic work of Wiesel and Huber (Wiesel and Hubel, 1963), many early studies convincingly demonstrated that activity is required for the segregation of visual inputs to produce so-called ‘eye stripes’, or regions of the target structure that are innervated by axons carrying information from one eye and are adjacent to regions innervated by axons carrying information from the other eye (Antonini and Stryker, 1993; Reh and Constantine-Paton, 1985; Shatz and Stryker, 1988; Sretavan et al., 1988). Importantly, the loss of segregation in the absence of neural activity is the direct result of increased arborization by the afferent terminals, demonstrating that activity can potently influence branched structures. More recent work has revealed that activity-dependent axon branching is mediated by a variety of mechanisms, including the relative rates of branch addition and retraction, competitive interactions between neighboring branch arbors, and synapse-dependent branch stabilization (Fig. 6). These different mechanisms are likely to cooperate to produce the final arbor morphology and provide multiple means by which axon branching can be influenced by neural activity.

Regulation of branching dynamics

Axonal arbors often form as a result of a dynamic process that consists of the ongoing addition and retraction of nascent branches. These so-called ‘branching dynamics’ can be influenced by neural activity to produce a bias toward branch addition or retraction, thus contributing to the final arbor size and shape (Fig. 6A).

Thalamocortical (TC) axons (see Glossary, Box 1) typically arborize early in mammalian development in cortical layer four to establish contacts with their target cells. An early study in cats showed that blocking neuronal activity with tetrodotoxin (TTX) significantly reduces the area occupied by TC axonal arbors, in addition to reducing the total branch number (Herrmann and Shatz, 1995). Recent studies have investigated this further using rat organotypic slice cultures in which high levels of spontaneous activity are present (Uesaka et al., 2007). TC axons in a thalamic explant grown next to the ventral surface of a cortical explant innervate and preferentially arborize in their normal layer-four target. Time-lapse analysis of fluorescent protein-labeled axons in culture indicates that arborization is a result of the bias in branching dynamics toward branch addition in the target layer. Furthermore, blocking all neuronal activity with pharmacological inhibitors causes arbor growth to decrease significantly, owing to a reduction in branch addition but not in branch extension.

An earlier study of rat cortical neurons that arborize in layer 2/3 of the cortex is also consistent with this conclusion (Uesaka et al., 2005). Axons traced by fluorescent proteins in cortical slices formed branches that are similar to those found during normal development in vivo, again as a result of branching dynamics being biased toward branch addition. However, the number of branches was reduced by pharmacological treatments that block neuronal activity, probably as a result of reduced branch addition (Uesaka et al., 2005). Thus, the biased regulation of branching dynamics can lead to a net increase or decrease in arbor size and complexity by shifting the dynamics toward branch addition or branch retraction, respectively. These slice culture results must be interpreted with care, however, as they are derived from ex vivo preparations in which many long-range inputs have been lost, and as such might not recapitulate development in vivo.

Competitive interactions between axonal branches

The branches of neighboring axons often innervate a shared or adjacent target field. Initially, there is an overabundance of branches, some of which are then retracted to produce mutually exclusive innervation fields. In addition to self avoidance and tiling, there is increasing evidence that this developmental pruning occurs via a competition-based mechanism in which ‘winning’ axons are retained and ‘losing’ axons are retracted (Fig. 6B).

Studies of zebrafish retinas in which subsets of RGCs are deficient in either electrical or neurosecretory activity have provided evidence that activity contributes to the interactions between neighboring RGC terminal arbors as they compete for limited target space (Gosse et al., 2008; Hua et al., 2005). Specifically, individual RGCs with suppressed activity via the expression of an exogenous potassium channel or of a dominant-negative SNARE protein show decreased axon terminal growth and reduced formation of new terminal arbors, but this inhibition can be relieved if the activity of neighboring RGCs is also suppressed (Hua et al., 2005). A more recent zebrafish study complements this finding using an elegant transplant approach to produce retinas that contain only a single RGC (Gosse et al., 2008). In the absence of any competition from neighboring RGCs, and thus no inhibition of branching, these single RGCs develop terminal arbors that occupy a greater area of the target and are more complex than RGCs in wild-type animals (Gosse et al., 2008). Thus, activity-dependent competitive interactions between neighboring RGCs may restrict arbor size and complexity.

This model is consistent with an earlier observation made in mouse RGCs that lack the β2 subunit of the nicotinic acetylcholine receptor (CHRNB1), which is required for the generation of spontaneous activity in developing retinal neurons prior to eye opening. These mutant RGCs fail to refine their terminal arbors, indicating that the absence of spontaneous activity eliminates any potential activity-dependent competition between neighboring RGCs, allowing their terminal arbors to grow unfettered by neighboring inhibition (Grubb et al., 2003; McLaughlin et al., 2003b).

However, a very recent study in zebrafish (Fredj et al., 2010) using tetanus toxin to suppress synaptic activity suggests that this activity-dependent branch regulation might be more complex. Here, the loss of activity in a single RGC led to enlarged terminal arbors that invaded the territory of neighboring RGCs, whereas suppressing the activity of neighboring RGCs at the same time resulted in normal terminal arbors. This effect is opposite to that found by Hua et al. (Hua et al., 2005), which might be due to the different approaches used to suppress synaptic activity. Nonetheless, activity-dependent competition is a critical determinant of RGC terminal arbor morphology.

A potential molecular mechanism that mediates activity-dependent competition has been suggested from studies of rodent sympathetic neurons (Singh and Miller, 2005; Singh et al., 2008). In culture, electrically stimulated sympathetic axon collaterals exhibit a distinct growth advantage compared with unstimulated collaterals. This effect appears to be mediated by BDNF, which is produced by electrically active axons in response to depolarization, as well as by its receptor p75, which is upregulated in unstimulated axons. Moreover, both BDNF and p75 are required for sympathetic axon competition in vivo. In wild-type mice, single sympathetic neurons of the superior cervical ganglion (SCG), the source of sympathetic innervation of the head, initially innervate two regions of the eye but are then pruned so that each neuron innervates only a single region. In $p75^{-/-}$ mice this pruning does not occur, resulting in single neurons that innervate two compartments and single compartments innervated by multiple neurons (Singh et al., 2008). Similarly, mice bearing an activity-insensitive mutant *Bdnf* gene show the same deficit. Interestingly, the axon pruning appears to be the result of axon degeneration, as opposed to simple retraction, and this process requires BDNF and p75. Thus, these findings suggest a model (Fig. 6B) in which the most active axonal branches – the 'winners' – secrete factors such as BDNF, which then act on the less active neighboring branches – the 'losers' – to directly induce axonal degeneration and regulate branch stability (Singh et al., 2008).

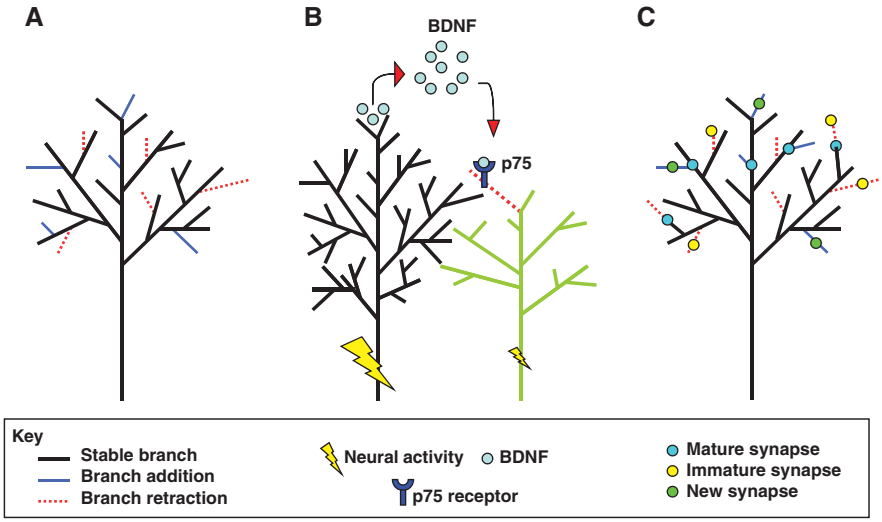

Fig. 6. Mechanisms underlying activity-dependent regulation of axon branching. (**A**) Axon branching often occurs as a dynamic process that involves branch addition and branch retraction. Branching dynamics can be modulated by neural activity to produce a bias toward branch addition or retraction, leading to a net change in the arbor morphology. (**B**) Neighboring axonal branches compete for innervation territory in the target structure. Typically, the axon experiencing the greatest amount of activity 'wins' the competition by inhibiting the arborization of neighboring axons. In sympathetic neurons, this phenomenon might be mediated by a brain-derived neurotrophic factor (BDNF)-nerve growth factor receptor (p75) interaction, in which the 'winning' branch secretes BDNF that then binds to the p75 receptor on the 'losing' axon, resulting in the loss of that branch due to axon degeneration (illustrated here as branch retraction for simplicity). (**C**) Axon branching is tightly coupled to synapse development. The maturity of new synapses is a significant criterion for determining which branches will be retained or retracted, in addition to where new branches will form. Mature synapses can halt branch retraction, whereas immature synapses are associated with retracting branches. New synapses preferentially form on new branches, and new branches preferentially form near mature synapses.

Terminal arbor remodeling and synapse regulation

Early work in the cat convincingly demonstrated that neural activity is required for the remodeling of terminal arbors (Antonini and Stryker, 1993). Occluding one of the eyes to block visually evoked neural activity resulted in a dramatic reduction in the complexity of geniculocortical terminal arbors (see Glossary, Box 1) over a period of only a few days. Interestingly, the time frame of this reduction correlates well with the period over which the physiological response to the deprived eye is lost in the cortical neurons, suggesting that synapses on the retracted branches are lost and that the remaining synapse-bearing branches can rapidly remodel. Thus, the regulation of synapses provides another mechanism by which neural activity may influence arbor morphology.

Such a mechanism has been examined in a number of studies of the retinotectal terminal arbors in *Xenopus* and zebrafish. The relationship between synaptogenesis and branch remodeling was first demonstrated by a study in *Xenopus* tadpoles, in which time-lapse imaging of fluorescent protein-labeled synaptobrevin II was used to visualize synapses in RGC axons (Alsina et al., 2001). This revealed that during the period of normal remodeling, most synapses are relatively stable; however, new synapses are both added and eliminated as the terminal arbor is reshaped. Interestingly, new branches usually originate at synaptic sites, whereas retracted branches tend to have few synapses present. This dynamic correlation may involve BDNF, which has been shown to modulate activity-dependent branching (Cohen-Cory, 1999). Microinjection of BDNF into the tectum increased both arborization and synapse number, but had stronger effect on synapses, suggesting that synapse formation might directly contribute to the regulation of axon branching (Alsina et al., 2001). In addition, neutralizing endogenous BDNF with function-blocking antibodies eliminates both synapses and branches, indicating the involvement of BDNF in the stability of both structures (Hu et al., 2005).

Like BDNF, another extracellular factor, netrin 1, has also been implicated in regulating both synaptogenesis and branching in *Xenopus* (Manitt et al., 2009). Microinjection of netrin 1 protein into the tectum increased both branch dynamics and synapse formation, whereas blocking the netrin 1 receptor DCC with antibodies specifically prevented the addition of new branches and synapses, without affecting existing branches and synapses. However, the manner in which these two molecular cues – and any as yet unidentified cues – coordinate their actions to regulate RGC terminal arborization and synaptogenesis remains to be determined.

Two recent studies have further explored the intimate relationship between branch regulation and synaptogenesis by live imaging of zebrafish and *Xenopus* retinotectal projections (Meyer and Smith, 2006; Ruthazer et al., 2006). Using fluorescently labeled synaptophysin, a synaptic vesicle protein in the presynaptic compartment, both studies followed the maturation of developing synapses based on label intensity and simultaneously examined the behavior of developing terminal branches. New branches were found to preferentially form in the region of highest fluorescence intensity, presumably reflecting the presence of mature synapses, whereas new synapses were preferentially added to new branches. In addition, stabilized branches consistently had bright puncta along their length, indicative of the presence of mature synapses, whereas mature synapses halted retracting branches (Meyer and Smith, 2006; Ruthazer et al., 2006).

Furthermore, because RGC terminal arbors are continually remodeled on a small scale in response to visual experience, the *Xenopus* study also tested the structural response to evoked activity using patterned visual stimulation (Ruthazer et al., 2006). This paradigm increased the stability of the branches that bear intense puncta and induced the retraction of branches with faint or no puncta, strongly suggesting that experience-dependent activity is capable of regulating the retention of branches with mature synapses while inducing the retraction of branches with unstable synapses (Ruthazer et al., 2006). Interestingly, however, very recent work in zebrafish suggests that the presence of a synapse, but not its activity, is important for branch stabilization, as individual neurons that have been silenced by tetanus toxin are still capable of generating a normal number of stable branches (Fredj et al., 2010). Thus, the effect of visual stimulation is likely to be due to an effect of sensory-induced activity on the maturation of synapses, rather than on their function.

Taken together, these studies demonstrate that the maturity of developing synapses can regulate axon branching by promoting the growth of nascent branches and determining the selective stability of extended branches, and that visual activity can induce terminal arbor remodeling in a synapse-dependent fashion, providing a mechanism by which ongoing sensory experience can regulate axon branching (Fig. 6C).

Box 3. Outstanding questions

- Do the general principles discussed here apply to other types of axonal branches?
- What is the physiological significance of those molecular cues that can stimulate branching locally in culture? Can other molecules serve the same function?
- How does branching occur? Most studies discussed here are based on static images in mouse mutants, but in vivo time-lapse imaging (De Paola et al., 2006; Liu and Halloran, 2005; Portera-Cailliau et al., 2005; Sagasti et al., 2005) can reveal the dynamics of each branching process.
- What are the molecular mediators of activity-dependent branching regulation, besides BDNF and Netrin?
- How do synapses regulate branch development independently of neural activity? As the maturational state of the synapse determines branch stability (Meyer and Smith, 2006; Ruthazer et al., 2006), investigations into cytoskeletal regulation between synapses and branching might provide insight into this question.
- How does branching contribute to circuit function? The perturbation of factors that are implicated in branching regulation should allow us to test this critical question in circuit development.
- What is the contribution of branching regulation to overall brain plasticity? The adult brain can alter its structure and function in finely tuned ways in response to ongoing experience (Holtmaat and Svoboda, 2009). To what extent do the structural rearrangements of axonal branches contribute to this plasticity?
- How does branching regulation adapt to evolutionary pressure? Is it constrained by the genetic program or does it have the capacity to remodel depending on the environment?

Conclusions

The formation of proper axonal branches is a critical step in the establishment of functional neural circuits. The studies that we have discussed here illustrate important concepts in the developmental regulation of axon branching. Although some ideas need to be tested further (see Box 3), the complex regulation of axon branching can be broken down into several developmental steps, which include branch formation, growth, guidance and pruning, as well as branch interactions, such as competition, self

avoidance and tiling. The spatiotemporal coordination of these key steps by both extracellular factors and neural activity at different locations on the axon could potentially generate the many types of branches found in the vertebrate nervous system.

Since these principles are derived from several well-studied neurons with stereotypic branched morphology, future studies will need to validate them in other types of neurons and branches. In addition, many extracellular cues have roles in the multiple processes discussed above. Distinguishing these activities requires a fuller understanding of the intracellular signaling mechanisms of axon branching, such as those involving GSK3 and PTEN, as well as their connections to the cytoskeleton (see Box 2) (Dent et al., 2003; Drinjakovic et al., 2010; Zhao et al., 2009). Furthermore, the intrinsic properties of neurons can influence axonal behaviors and modulate their responses to extracellular cues. Detailed cell biological studies of branch formation will provide a complete picture of how branches develop, how genetic programs dictate branching regulation, and how activity modulates branches. Finally, although different in structure and function, axons and dendrites both develop branches and might share molecular and cellular mechanisms of branching that are evolutionarily conserved (Grueber and Sagasti, 2010; Jan and Jan, 2010). Recent studies of axonal and dendritic development in flies and worms (Alexander et al., 2010; Hao et al., 2010; Jan and Jan, 2010; Oren-Suissa et al., 2010) will thus provide useful insights into the many outstanding questions regarding the regulation of axon branching in vertebrates (see Box 3).

Axon branching is crucial to vertebrate circuit development and contributes to guidance, targeting and plasticity (O'Leary et al., 1990; Yamahachi et al., 2009). The detailed molecular mechanisms identified here and in the future will provide useful tools to test circuit function. More importantly, many neurological and psychiatric disorders have been associated with defects in synaptic connections during development, which may result from the perturbation of branching regulation due to both genetic and environmental factors (Geschwind and Levitt, 2007). Indeed, several molecules described above have already been linked to these diseases, including ROBO in autism and anosmin in Kallmann syndrome (Anitha et al., 2008; Franco et al., 1991; Legouis et al., 1991). Understanding their regulation will provide new insights into the development and etiology of these disorders. Finally, branch sprouting has been suggested to be a viable means for regenerating nerve connections following injury (Cafferty et al., 2008), and understanding how they are formed during development will help to identify new ways to stimulate functional recovery in injured adults.

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Competing interests statement

The authors declare no competing financial interests.

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