Branch management: mechanisms of axon branching in the developing vertebrate CNS

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Abstract | The remarkable ability of a single axon to extend multiple branches and form terminal arbors enables vertebrate neurons to integrate information from divergent regions of the nervous system. Axons select appropriate pathways during development, but it is the branches that extend interstitially from the axon shaft and arborize at specific targets that are responsible for virtually all of the synaptic connectivity in the vertebrate CNS. How do axons form branches at specific target regions? Recent studies have identified molecular cues that activate intracellular signalling pathways in axons and mediate dynamic reorganization of the cytoskeleton to promote the formation of axon branches.

Terminal arbors
Highly branched tree-like structures that are found at the ends of axons that innervate target regions.

Collateral branches
Branches that extend from the sides of an axon, often interstitially, and innervate a target by re-branching to form a terminal arbor.

Cytoskeletal dynamics
Cycles of polymerization and depolymerization that result in growth and shrinkage of microtubules and actin filaments, which enable their reorganization.

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The functions of the vertebrate brain in cognition, learning, memory, sensory perception and motor behaviour require complex neural circuitry. To form this circuitry, individual neurons must connect with multiple synaptic targets, and they do so through extensive branching of their axon and the formation of elaborate terminal arbors[1–6]. Branches establish topographic maps in numerous systems, including the retinotectal[7] and corticospinal[8] systems, in which regions of the retina and sensorimotor cortex are connected to their targets in the optic tectum and spinal cord, respectively. In addition, multiple branches from the same axon can connect widely divergent regions of the nervous system. For example, single descending cortical axons extend branches into the pons and spinal cord[9]; single axons from some regions of the thalamus can ramify widely in the somatosensory, motor and higher-order sensory cortices[10]; and single cortical neurons can send axon collateral branches to homotypic and heterotypic regions of the contralateral cortex[11]. Cajal, after observing the collaterals of callosal axons, commented: "callosal fibres do not simply join structurally and functionally comparable areas in the two hemispheres. They play a broader role, establishing multiple, complex associations that allow activity in one sensory area to influence a number of areas in the contralateral cerebral hemisphere." (REF. 12).

Studies of neural development over the past several decades have focused on mechanisms of axon guidance. Surprisingly, given its importance in establishing neural circuits, axon branching has received less attention. How do axon branches form during development? Branches originate as dynamic protrusions that extend and retract from specific locations on the axon. Some of these protrusions become stabilized into branches that arborize by continued re-branching at target sites, leading to synapse formation. Branching is evoked by local extracellular cues in the target region, which signal through receptors on the axonal membrane to activate intracellular signalling cascades that regulate cytoskeletal dynamics. Axon arbors that form within target regions are highly dynamic but eventually stabilize through competitive mechanisms that can involve neural activity.

In this Review, we examine axon branching in the vertebrate CNS. We present *in vivo* and *in vitro* findings that illustrate modes of axon branching and the role of extracellular cues in the development of branches and the shaping of terminal arbors. Moreover, we discuss the role of cytoskeletal dynamics at axon branch points and how intracellular signalling pathways regulate cytoskeletal reorganization. Last, we consider the role of activity in regulating axon branching and shaping the morphology of terminal arbors and identify areas for future research.

Axon branching and arborization

Growth cones, which are the expanded motile tips of growing axons, respond to extracellular guidance cues to lead axons along appropriate pathways towards their targets[13]. However, axonal growth cones in the vertebrate

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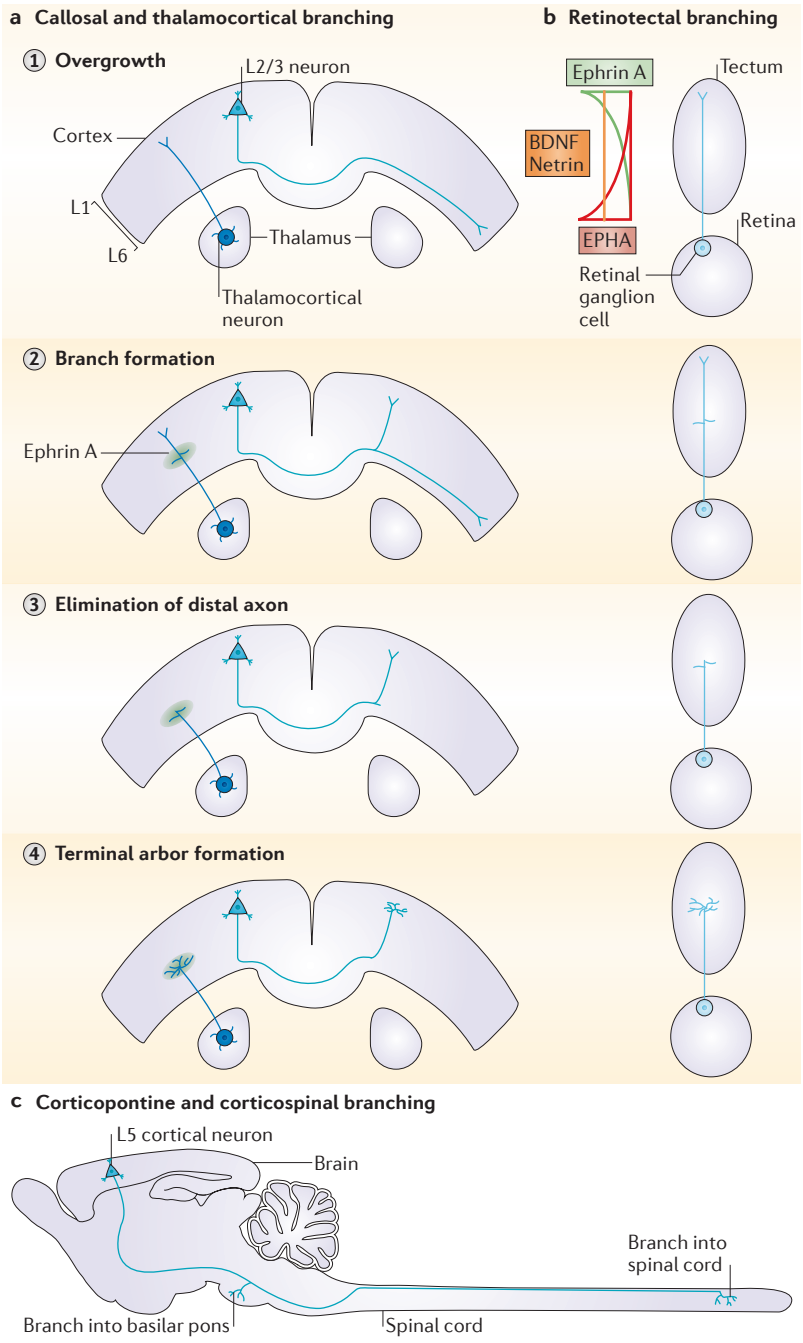

Figure 1 | **Stages of axon branching in developing CNS pathways.** In the mammalian corpus callosum and thalamocortical pathway (part **a**) and in the chick retinotectal pathway (part **b**), axons initially extend past their eventual terminal regions (step 1). After a delay, branches extend interstitially from the axon shaft (step 2), and this is followed by elimination of the distal axon (step 3) and formation of terminal arbors in topographically correct target regions (step 4). Part **c** depicts interstitial branching from an individual layer 5 (L5) sensorimotor cortical axon into the pons and spinal cord. In the retinotectal system (part **b**), opposing gradients of EPHA–ephrin repellents inhibit retinal axon branching anterior and posterior to the correct target, whereas positive cues, such as brain-derived neurotrophic factor (BDNF) or netrin 1, along the tectum promote branching at appropriate topographic positions[7,42]. Callosal axons connect homotypic regions of the cortical hemispheres but can also branch to other cortical areas[11,12] (not shown). Thalamocortical axons form branches that arborize topographically in L4 of the somatosensory cortex[23], but other thalamocortical axons can branch into multiple cortical areas[19] (not shown). Part **c** is adapted, with permission, from REF. 9 © (1988) Elsevier.

CNS do not typically enter their target region. Instead, axons form connections with their target though growth cone-tipped collaterals that branch from the axon shaft and terminal arbors that re-branch from axon collaterals (FIG. 1). In certain circumstances, branches can arise by splitting of the terminal growth cone[4,6], such as at the mouse dorsal root entry zone, where the growth cones of dorsal root ganglion (DRG) axons split to form two daughter branches that ascend or descend and arborize in the spinal cord[14,15].

In the mammalian CNS, axon branches typically extend interstitially, at right angles from the axon shaft behind the terminal growth cone (FIG. 1). This delayed interstitial branching can occur days after axons have bypassed the target[16]. Cortical axons in rodents initially bypass the basilar pons[9], but after a delay, they form filopodia, which are dynamic finger-like actin-rich membranous protrusions that can develop into stable branches that arborize in the pons[17]. Developing corticospinal axons also bypass spinal targets and later form interstitial branches that arborize once they have entered topographically appropriate target sites[18]. Segments of the axons distal to the target are later eliminated[16,19]. Callosal axons, which connect the two cerebral hemispheres, also undergo delayed interstitial branching[20] beneath their cortical targets, where callosal growth cones repeatedly collapse and extend without advancing forward[21]. Growth cone pausing and interstitial branching have also been observed in dissociated cortical neurons[22], where branches develop from dynamic growth cone remnants that remain on the axon shaft in regions in which growth cones paused. Developing thalamocortical axons *in vivo* form layer-specific lower and upper tiers of terminal arbors in the barrel field, which together form a spatial map of the facial vibrissae in the rodent somatosensory cortex[23]. *In vivo* imaging revealed that branches form *de novo* along the shaft of the primary thalamocortical axon[24] and that these give rise to dynamic arbors that extend and retract processes over many days. Thalamocortical axons extend branches at right angles in the target region, which shows that interstitial branching is the dominant form of axonal branching *in vivo*.

In the vertebrate retinotectal pathway, topographic projections maintain spatial information of the visual field from the retina to the tectum. In the avian[25] and rodent[26] retinotectal systems, retinal ganglion cell axons initially overshoot their termination zone in the tectum and later emit interstitial branches at correct tectal positions, which is followed by terminal arborization and regression of the distal axon[7,27] (FIG. 1). However, in frogs and fish, growth cones of retinal axons form arbors as soon as they reach the correct region of the tectum[28] and axons do not emit side branches on their way to their targets. Live-cell imaging of arbor formation at the tips of retinal axons in the frog optic tectum showed that highly dynamic branches repeatedly extend and retract[29]. Thus, in the vertebrate CNS, axon branches, rather than the primary axonal growth cone, extend into targets at appropriate locations and thereby establish topographic specificity. Terminal arbors that develop from these branches then undergo dynamic remodelling (FIG. 1).

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Role of extracellular cues

Axon branching occurs far from the cell body at localized regions of the axon that are close to the axon's targets, suggesting that target-derived cues regulate axon branching. Families of extrinsic axon guidance cues, growth factors and morphogens can regulate axon branching by determining the correct position of branches or by shaping terminal arbors[2,4].

Guidance cues. Netrins, such as netrin 1, are diffusible guidance cues that attract efferent cortical axons *in vivo*[30–33] and that evoke cortical axon branching *in vitro*[34,35]. Focal application of netrin 1 can induce localized filopodial protrusions *de novo* along the axon shaft[34,36] and increase branch length without increasing the extension of the primary axon. Netrin 1 also shapes terminal arbors; in the frog optic tectum, injection of netrin 1 enhances the development of terminal retinal arbors[37] by increasing the dynamic addition and retraction of branches, resulting in an increase in total branch number.

Ephrins are membrane-bound cues that guide axons by repulsion[38,39], and they have a role in specifying the locations of axon branches. *In vitro*, for example, ephrin A5 and its receptor (EPHA5) can promote or repress branching of various cortical axons growing on membranes from specific cortical layers[40]. Ephrin As stimulate the branching of thalamocortical axons that are grown on appropriate layer 4 somatosensory cortical membranes[41], but they repel axons from limbic regions of the thalamus that do not innervate the somatosensory cortex. Ephrin As also evoke topographically specific branching of retinal axons in tectal membrane stripe assays[25]. *In vivo*, the distribution of EPHAs and ephrin As in both the retina and the optic tectum determines the position of retinal axon branches and terminal arbors[7] by forward and reverse signalling[42]. In this system, EPHAs act both as receptors that are activated by ephrin ligands (forward signalling) and as ligands that activate ephrins (reverse signalling). In a model of retinotectal mapping[27], opposing gradients of EPHAs and ephrin As along the anterior–posterior axis of the tectum inhibit axon branching on the retinal axon segments that are anterior and posterior to the correct target region. A molecule with branch-promoting activity, such as brain-derived neurotrophic factor (BDNF), that was expressed uniformly along the tectum would allow branching in a trough of the repellent gradients[43] (FIG. 1). Thus, branching would occur at a unique tectal position for each population of axons because, on the basis of their temporal or nasal origin in the retina, each population would have differential sensitivity to the EPHA–ephrin A repellents. In the dorsal–ventral axis of the tectum, EPHB–ephrin B1 signalling, perhaps in cooperation with additional cues, regulates topographic mapping of retinal axon branches[44]. Thus, repression of axon branching is an important regulatory mechanism, as axons must often traverse non-target regions on the way to their targets, where additional positive cues may evoke branching.

Semaphorins are repellent axon guidance cues that function in the assembly of neuronal circuits[45]. For example, semaphorin 3A (SEMA3A) repels cortical axons[46] and, *in vitro,* inhibits cortical axon branching[34,47], decreasing cortical branch length without affecting the length of the primary axon[34]. SEMA3D has no effect on the central branches of Rohon–Beard axons *in vivo* but does repel and induce branching of the peripheral branches of the same axons[48]. SEMA3A, through its neuropilin–plexin receptors, regulates the pruning of hippocampal axons *in vivo* and *in vitro* by promoting the selective retraction of transient branches to septal targets[49]. However, SEMA3A can also positively influence axon branching. Recently, SEMA3A was shown to promote branching of cerebellar basket cell axons onto Purkinje cells in the cerebellar cortex[50]. *In vivo*, mice deficient in SEMA3A show layer-specific reductions in terminal branching of basket cell axons, and, *in vitro*, SEMA3A increases the complexity of basket cell axon arbors. Localized signalling mechanisms in basket cell axons[50] ensure that SEMA3A promotes specific axon branching without affecting overall axon organization. This might be a general regulatory mechanism by which molecular cues can influence local branching without affecting axon outgrowth or guidance.

Like semaphorins, SLITs, which act on ROBO receptors, are repulsive cues, and they function, for example, in the guidance of commissural axon pathways in the mammalian forebrain[51]. However, SLITs can also promote the collateral axon branching of mammalian sensory DRG axons *in vitro*[52] and the branching of zebrafish trigeminal axons *in vivo*[53]. By contrast, Slit1a inhibits arborization of retinal ganglion cell axons in the zebrafish optic tectum, thereby preventing the premature maturation of terminal arbors[54]. In this study, *in vivo* imaging showed that genetic removal of Slit–Robo signalling increases the size and complexity of axon arbors by decreasing branch dynamics and increasing stable branch tips. SLITs, like semaphorins, exhibit context-dependent promotion or repression of axon branching, depending on the particular population of neurons. Moreover, the balance between factors that promote and inhibit arborization may determine final arbor size.

Growth factors. Growth factors, such as fibroblast growth factor (FGF), and neurotrophins, such as nerve growth factor (NGF) and BDNF, can also evoke axon branching and arborization. For example, *in vitro*, NGF promotes DRG axon branching[55,56], BDNF evokes protrusions called filopodia and lamellipodia along frog spinal axons[57], and FGF2 and BDNF stimulate branching in cortical axons[58,59]. In hippocampal slice cultures, BDNF transfection specifically increases axon branching in dentate granule cells[60]. Moreover, BDNF has been shown to have a prominent role in axon arbor formation in retinal axons in the frog optic tectum[61,62]. For example, *in vivo* imaging[63] showed that acute BDNF application induces rapid extension of new branches on retinal axon arbors. Thus, target-derived cues shape terminal arbors by regulating the dynamic extension and retraction of their branches. Interestingly, comparisons between the effects of netrin 1 and BDNF on retinal arbors[37] suggest that although both factors

Filopodia
Finger-like membrane protrusions that contain bundled actin filaments. Filopodia extend transiently from the growth cone, the axon shaft and axon branches.

Neurotrophic factor
A type of molecule, such as brain-derived neurotrophic factor or nerve growth factor, that regulates neuronal growth and survival.

Lamellipodia
Thin sheet-like veils of cytoplasm at the growth cone periphery that are comprised of actin filament networks.

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increase arbor complexity, BDNF promotes the addition and stability of axon branches, whereas netrin 1 induces new branch growth but not branch stabilization. Different cues can, therefore, produce a similar outcome on final arbor morphology by different dynamic strategies.

Morphogens. WNTs comprise a diverse family of secreted morphogens that shape embryonic development. Several WNTs also function as axon guidance cues[64,65] or regulate axon branching. For example, WNT7A[66], which is secreted by cerebellar granule cells, induces remodelling of pontine mossy fibre axons by inhibiting their elongation, enlarging their growth cones, inducing spreading at regions along the axon shaft and consequently increasing axon branching. WNT3A, which is secreted by motor neurons, regulates terminal differentiation of a subset of presynaptic arbors of spinal sensory neurons by inducing axon branching[67,68], whereas WNT5A, a repulsive axon guidance cue for cortical axons *in vivo*[69–71], increases the elongation of cortical axons and branches[72] but not the numbers of axon branches[73]. However, WNT5A increases axon branching of developing sympathetic neurons acutely and, over the long term, also enhances axon extension[74].

These findings suggest that extracellular cues can promote or repress axon branching without affecting the growth and guidance of the parent axon. Differences between local signalling and cytoskeletal mechanisms at the growth cone and along the axon shaft could account for different effects of the same molecule on axon guidance, elongation and branching.

Reorganization of the cytoskeleton

The initiation, growth and guidance of axon branches in response to extracellular cues require the regulation of actin and microtubule dynamics (FIG. 2), which share similarities[5,75,76] with cytoskeletal dynamics during growth cone navigation[75,77–79]. Filopodial and lamellipodial protrusions along the axon serve as precursors to branches. Filopodia contain long unbranched, bundled actin filaments, whereas lamellipodial veils contain a meshwork of branched actin filaments. An initial step in branch formation is the focal accumulation of actin filaments at axon membrane protrusions[34,80]. Filopodia can emerge from transient accumulations of actin filaments (which are termed actin patches) on sensory axons[81–83], although only a fraction of actin patches give rise to axonal filopodia[3].

Actin dynamics. Actin filaments are polymers with barbed ends that face the plasma membrane and pointed ends that face away from the membrane; actin monomers are added to the barbed end, and actin filaments disassemble at the pointed end. Membrane protrusion and motility are driven by dynamic cycles of actin polymerization and depolymerization[77,78]. In growth cones and filopodia, actin filaments undergo continual cycles of retrograde flow and polymerization[84]. The balance between these two processes determines whether membrane protrusions extend or withdraw.

Actin arrays and dynamics are highly regulated by actin-associated proteins, which have important roles in axon guidance and branching[77] (FIG. 2). For example, ENA/VASP (enabled/vasodilator-stimulated phosphoprotein) proteins bind to the barbed ends of actin filaments and enhance actin filament elongation by antagonizing the capping proteins that inhibit F-actin elongation[85]. When ENA/VASP proteins are sequestered to the mitochondria in hippocampal neurons, the axons of these neurons have fewer filopodia that contain fewer actin filament bundles[35]. Interestingly, depletion of ENA/VASP proteins in frog retinal ganglion neurons reduces filopodial formation, retinotectal axon branching and arborization in the tectum *in vivo*[86]. The formins, which nucleate actin filaments at barbed ends (that is, they promote the formation of actin filaments *de novo* rather than the addition of actin monomers to existing filaments), are essential in neurite initiation and the formation of filopodia. In cortical neurons that lack ENA/VASP proteins, filopodia can be restored by ectopic expression of the formin MDIA2 (also known as DIAPH3)[87]. A recently identified actin nucleator, cordon-bleu[88], promotes actin polymerization of unbranched filaments and, when overexpressed in hippocampal neurons *in vitro*, increases axon branching without affecting axon length. Actin-related protein 2/3 (ARP2/3) nucleates new branched filaments from the sides of existing actin filaments[77] and is required for NGF-induced actin patch formation, filopodia formation and branching of sensory axons[82,83]. One study has shown that ARP2/3 depletion in hippocampal neurons[89] reduces filopodial and lamellipodial dynamics, although another study[90], using a dominant negative approach to inactivate ARP2/3, saw no decrease in axonal filopodia.

Dynamic axonal filopodia not only protrude but also withdraw. Gelsolin severs actin filaments and caps free barbed ends, and its depletion in hippocampal neurons increases filopodia through reduced retraction[91]. The actin-severing protein family actin-depolymerizing factor (ADF)/cofilin increases actin filament depolymerization, but it also promotes filament assembly by increasing the available pool of actin monomers and free barbed ends. Thus, ADF/cofilin activity contributes to the regulation of BDNF-induced filopodia on retinal growth cones[92]. Myosin II, a motor protein involved in actomyosin contraction of antiparallel filaments, drives retrograde actin flow and aids in turnover of F-actin networks[84]. Although the exact role of many actin-associated proteins remains to be determined in vertebrate axon development, dynamic remodelling of the actin cytoskeleton during cycles of polymerization and depolymerization is an essential event in the initiation of branches by filopodia and lamellipodia as they probe the extracellular environment.

Microtubule dynamics. Actin filament polymerization is essential for the pushing force that drives membrane protrusion, but further development and stabilization of axon branches require the entry of dynamic microtubules into filopodia[5,93]. Microtubules are hollow

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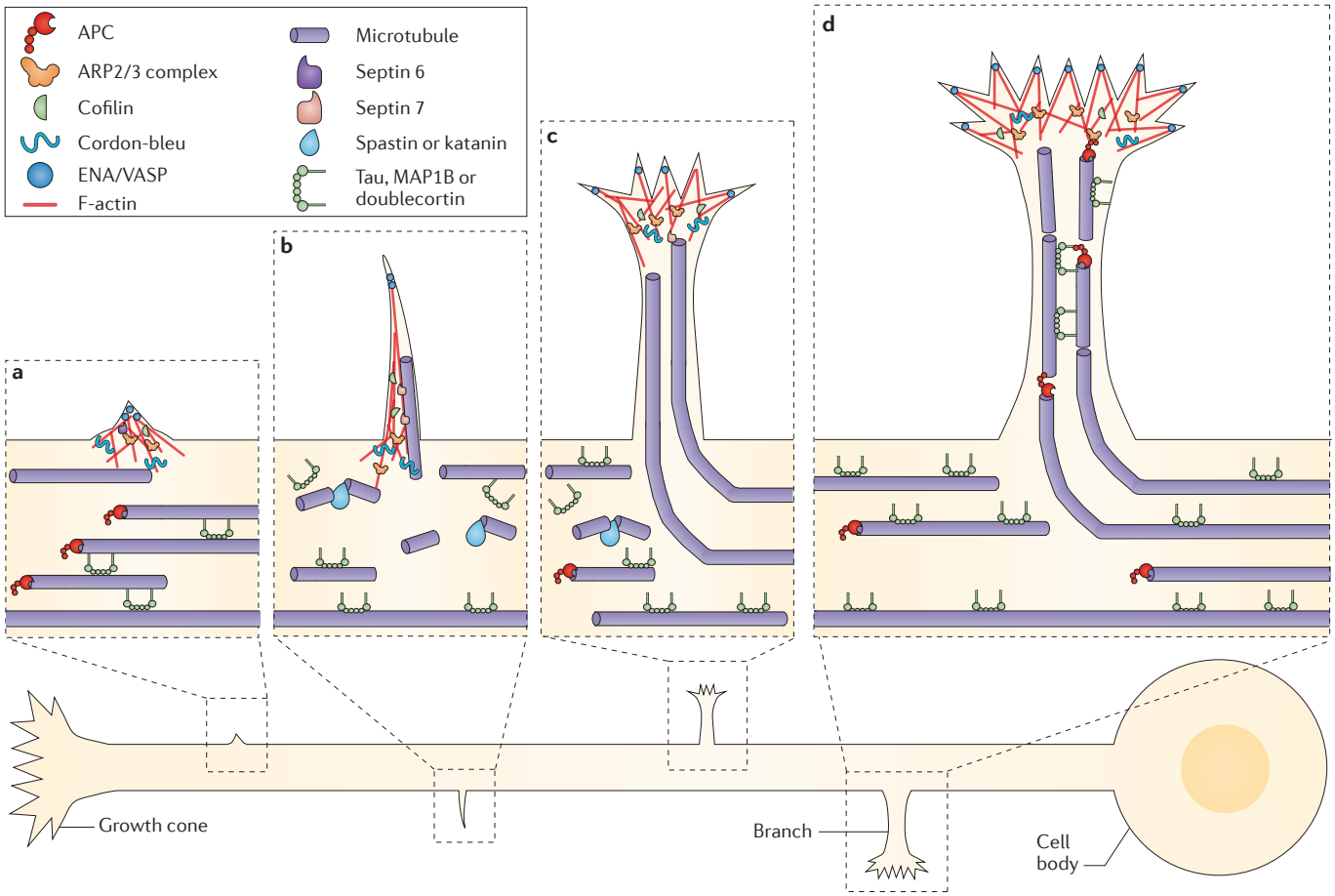

Figure 2 | **Cytoskeletal reorganization at different stages of axon branching.** **a** | Membrane protrusion requires the accumulation of actin filaments (F-actin), which can form patches[81–83]. Membrane protrusion also requires actin nucleation (which is mediated by actin-related protein 2/3 (ARP2/3)[82,83] and cordon-bleu[88]), actin branching (which is mediated by ARP2/3) and actin elongation (which is mediated by ENA/VASP (enabled/ vasodilator-stimulated phosphoprotein) proteins)[35]. Cofilin is important for the turnover of actin filaments[92], and septin 6 is localized to the actin patch[110]. Microtubules are stabilized by microtubule-associated proteins (MAPs) (tau and doublecortin)[78,99,103] in the axon but are also capable of extending at their plus ends (a process that requires adenomatous polyposis coli protein (APC) and end-binding proteins)[100,101]. **b** | Filopodia, which contain bundled actin filaments, emerge from the axon shaft accompanied by localized splaying and fragmentation of microtubules[55,80,93,96,97]; these invade the filopodium and extend along actin bundles. Microtubules are severed by spastin and katanin[98], and septin 7 promotes the entry of microtubules into the filopodium[110]. **c**,**d** | Stable microtubules enter the nascent branch, which continues to mature and extend; this growth is led by a motile growth cone. MAPs protect some of the microtubules in the axon shaft from severing. Within the axon branch, microtubules become bundled and stabilized by MAPs.

tubular structures that are composed of linear arrays of α- and β-tubulin[78,94]. In growth cones and developing branches, tubulin dimers are added and subtracted at the microtubule plus ends, which face outward towards the membrane, whereas microtubules are generally stabilized at their minus ends, which are orientated away from the membrane. Microtubules undergo dynamic instability[95] that results in continuous cycles of growth and shrinkage at their plus ends. These cycles of polymerization and depolymerization enable microtubules to reorganize and explore the growth cone periphery and extend into developing axon branches. Microtubules in axons are organized in stable parallel bundles, so important early steps in axon branch formation are the splaying of bundled microtubules, their local fragmentation at axon branch points, followed by entry of short microtubule fragments into filopodia. This dramatic microtubule reorganization, which has been observed in chick sensory axons as well as in rodent hippocampal and cortical neurons[55,80,93,96,97], leads to the stabilization of elongating axon branches that contain microtubules. As shown by time-lapse imaging, only those filopodia that contain microtubules develop into branches[93], although even long branches containing microtubules can eventually retract.

How are microtubule dynamics and organization regulated during axon branch formation? Microtubule-associated proteins (MAPs) — such as stabilizing, severing, destabilizing and motor proteins and plus-end-tracking proteins — have roles in growth cone

Plus-end-tracking proteins
Plus-end-tracking proteins, such as end-binding protein 1 (EB1) and EB3, associate with the growing plus ends of dynamic microtubules.

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steering[77,78,94], and some of these MAPs have also been investigated for a role in axon branching. For example, overexpression of spastin or katanin (both of which are microtubule-severing proteins) in cultured hippocampal neurons increases axon branching[98], whereas depletion of either protein reduces axon branching. Interestingly, tau, a MAP that stabilizes microtubules by preventing depolymerization, protects microtubules against the severing effects of katanin, so that when tau is depleted axon branching is increased[99]. This suggests that detachment of tau from microtubules might be a mechanism for regulating branch formation. One possibility is that as short microtubule fragments enter nascent branches, structural MAPs such as MAP1B, which also interacts with actin filaments[78], could stabilize them.

Further growth of microtubules at their dynamic plus ends is regulated by a family of plus-end-tracking proteins, such as end-binding protein 1 (EB1), EB3 and adenomatous polyposis coli protein (APC), which contribute to microtubule elongation and axon outgrowth[100]. WNT3A-induced remodelling of DRG axons[68] — comprising a decrease in the growth rate of the axon and increases in branching and growth cone size — results from changes in the directionality of microtubules that lead to their looping; such looping has also been observed in large paused cortical growth cones[80,93]. As WNT3A decreases the levels of APC, APC is thought to have a role in determining the direction of microtubule growth, although exactly how this contributes to the regulation of axon branching is unclear. *In vivo*, APC-deficient mice show severe misrouting of cortical axons, and *in vitro* cortical axons lacking APC show excessive axon branching, which is caused by microtubule instability and disruption of microtubule organization at branch points[101]. The growth cones of APC-deficient cortical neurons frequently split to produce multiple branches[102], and this results from microtubule splaying and disorganization. Deletion of the gene encoding doublecortin (DCX), another MAP, not only causes migratory defects in adult mouse forebrain neurons but also evokes excessive branching of the primary neurite[103], which has been attributed to a failure in the maintenance of microtubule crosslinking. The microtubule-destabilizing kinesin, KIF2A, also has a role in axon branching[104]. Hippocampal and cortical neurons from mice lacking KIF2A have abnormally long axon collateral branches that form more secondary and tertiary branches *in vivo* and *in vitro*. Concomitantly, in neurons lacking KIF2A, microtubule-depolymerizing activity is diminished, which suggests that KIF2A normally suppresses collateral growth. These results emphasize the importance of regulated cytoskeletal dynamics in the repression of axon branching during the development of neural circuitry.

Actin–microtubule interactions. Neither actin filaments nor microtubules act alone during the formation of axon branches. Dynamic microtubules and actin filaments are thought to interact bidirectionally in growth cone motility[78,105], as dynamic microtubules extend along and co-polymerize with actin filaments in the growth cone periphery[80,106,107]. Actin–microtubule interactions are also essential for branch formation, as microtubules extend into filopodia along actin filament bundles (FIG. 2). Thus, drugs that attenuate the dynamics of either actin filaments or microtubules inhibit branch formation but not extension of cortical axons *in vitro*[80]. Mechanisms that link actin filaments and dynamic microtubules may have implications for axon branch formation. For example, in cultured cortical neurons, the interaction between the F-actin-associated protein drebrin and EB3 occurs at the base of filopodia, which is important for the exploration of filopodia by microtubules during neuritogenesis[108]. Genetic ablation of ADF/cofilin in cortical and hippocampal neurons disorganizes the actin network and impairs microtubule bundling and protrusion into filopodia, which leads to inhibition of neurite initiation[109]. As ADF/cofilin regulates actin retrograde flow by actin disassembly and turnover, the ability of microtubules to coalesce and form neurites was thought to depend on actin retrograde flow driven by actin-severing proteins. In this study, the authors concluded that microtubules follow the lead of dynamic actin filaments; coordination of actin and microtubule dynamics is also required for axon branching in chick DRG neurons and mammalian hippocampal neurons. For example, branch formation in sensory axons involves several septin proteins that interact with both cytoskeletal components[110]. Septin 6 (SEPT6) localizes to actin patches and recruits cortactin, an ARP2/3 regulator, to trigger the formation of filopodia, whereas SEPT7 promotes the entry of axonal microtubules into filopodia, which enables branch formation through as-yet-unknown mechanisms. It seems likely that future studies will reveal additional molecules that coordinate the reorganization of the actin and microtubule cytoskeleton to promote the development of axon branches.

Signalling pathways

During axon pathfinding, the growth cone must simultaneously interpret and integrate numerous extracellular cues through activation of multiple signal transduction pathways[111,112]. Some of these pathways also regulate axon branching. RHO GTPases[78,113] and the protein kinase glycogen synthase kinase 3β (GSK3β)[114–117] have emerged as signalling nodes that regulate actin and microtubule dynamics through multiple extracellular cues and cytoskeletal effectors. Although no single pathway from receptor to the cytoskeleton has been completely defined, we present several examples of signalling pathways that regulate axon branching (FIG. 3).

NGF promotes the formation of filopodia and branches in chick sensory axons[55] through an increase in the rate of patch formation[81]. This effect is driven by phosphoinositide 3-kinase (PI3K)[82,83] and requires the actin-nucleating complex ARP2/3. In a model of the NGF signalling pathway[82], the NGF receptor TRKA (also known as NTRK1) activates PI3K, which in turn activates RAC1 to drive activity of WAVE1 (also

RHO GTPases
A family of molecules that regulate cytoskeletal dynamics downstream of guidance cue receptors.

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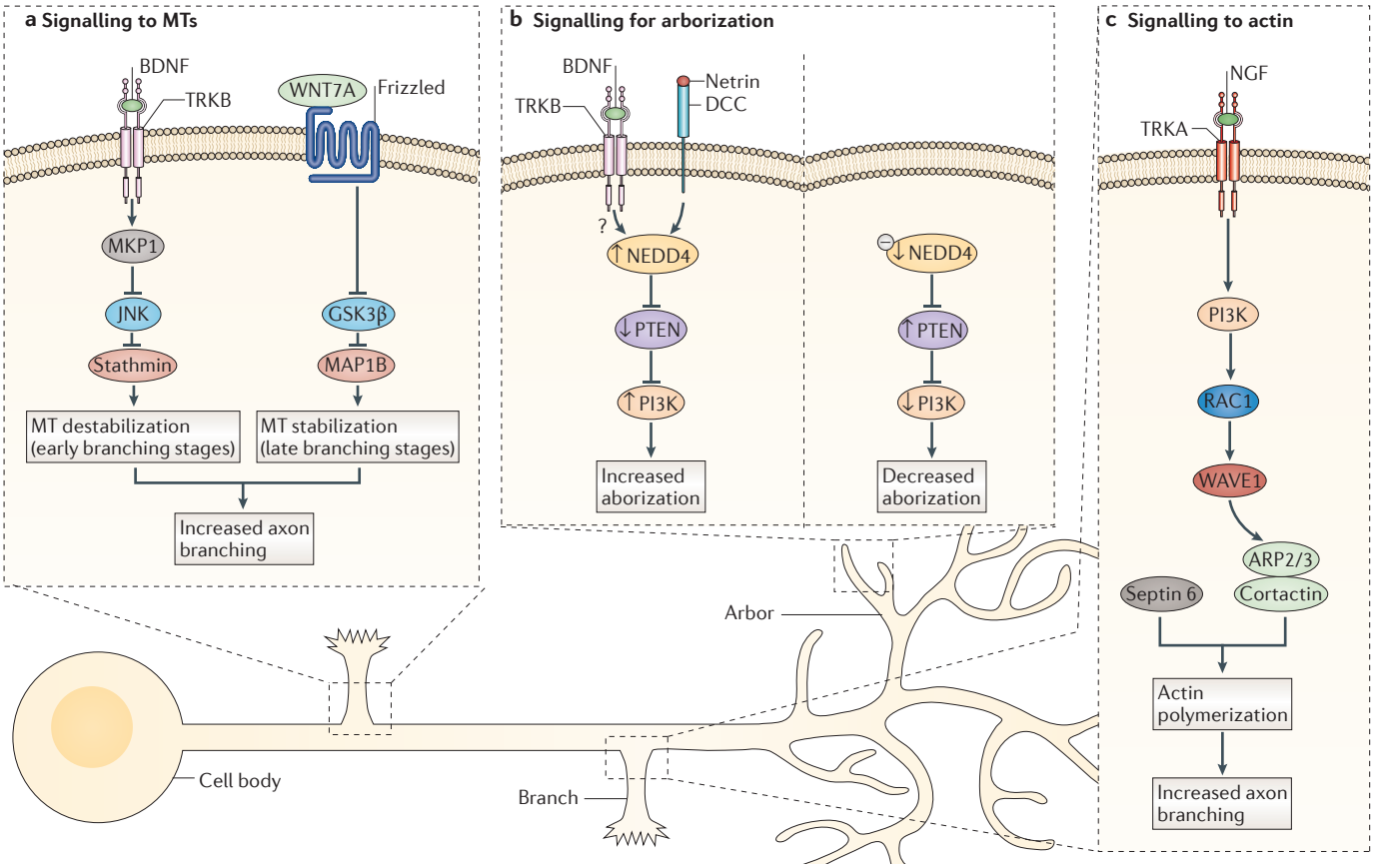

Figure 3 | **Signalling pathways that promote axon branching.** **a** | Different pathways that lead to microtubule (MT) destabilization (left) at early branching stages or to MT stabilization at later branching stage (right) can each promote axon branching by opposing effects on MT stability. Differential effects on MT stability are shown in two examples: in the first example, branching is induced by brain-derived neurotrophic factor (BDNF) and its TRKB receptor (left) in cortical neurons[58] ; and in the second example, branching is induced by the morphogen WNT7A (right) in pontine mossy fibres[66,120]. **b** | BDNF and netrins increase terminal arborization of frog retinotectal axons[37,63]. In this signalling pathway[123], ubiquitin protein ligase E3 NEDD4 ubiquitylates phosphatase and tensin homologue (PTEN), and this PTEN degradation leads to increased terminal axon branching by promotion of phosphoinositide 3-kinase (PI3K), which is known to regulate cytoskeletal dynamics (direct activation of NEDD4 by TRKB has yet to be shown and this is denoted by the question mark). By contrast, inhibition of NEDD4 leads to an increase in PTEN levels and hence inhibition of axon branching. **c** | Nerve growth factor (NGF)-induced TRKA signalling promotes axon branching in chick sensory dorsal root ganglion axons[82]. It does this by activating PI3K and, in turn, RAC1, which activates actin-associated proteins to increase actin polymerization and the formation of actin patches. Cortactin, which is recruited by septin 6, promotes the emergence of filopodia from actin patches. ARP2/3, actin-related protein 2/3; GSK3β, glycogen synthase kinase 3β; JNK, JUN amino-terminal kinase; MAP1B, MT-associated protein 1B; MKP1, mitogen-activated protein kinase phosphatase 1.

known as WASF1), which activates ARP2/3. The actin-associated protein cortactin, which stabilizes branched actin filaments that are nucleated by ARP2/3 (REF. 118), does not regulate actin patch formation but promotes the emergence of filopodia from actin patches. Cortactin is also involved in axon branching[119] by promoting actin polymerization and membrane protrusion. In hippocampal neurons, calpain, through proteolysis of cortactin, represses actin polymerization and maintains the neurite shaft in a consolidated state, which can be de-repressed by factors such as netrin 1 and BDNF to permit branching. These examples illustrate how actin-nucleating proteins and associated stabilizers regulate specific steps in branch formation as well as the suppression of axon branching.

BDNF induces axon branching of mouse cortical neurons through its receptor, TRKB (also known as NTRK2). Recently, negative regulation of mitogen-activated protein kinase (MAPK) by the MAPK phosphatase 1 (MKP1; also known as DUSP1) was shown to have a role in BDNF-induced cortical axon branching[58]. MAPK is also involved in cortical axon branching downstream of netrin 1-induced calcium signalling[36]. *In vivo* overexpression or downregulation of MKP1 in layer 2/3 cortical neurons increases or decreases their terminal axon branching, respectively, in the contralateral cortex. MKP1 is transiently induced at sites of BDNF activity and inactivates the MAPK JUN amino-terminal kinase (JNK) by dephosphorylation, which in turn reduces the phosphorylation of the JNK substrate stathmin (STMN1), thereby

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activating STMN1. This study also showed that MKP1 overexpression increased tyrosinated tubulin (which is associated with dynamic microtubules) and decreased detyrosinated and acetylated tubulin (which is associated with stable microtubules). As STMNs destabilize microtubules[94], in this model[58], the authors propose that activating STMNs through BDNF signalling destabilizes microtubules and, consequently, increases cortical axon branching (FIG. 3).

In other models of axon branching, extracellular cues have been shown to regulate axon branching by influencing microtubule dynamics through GSK3β signalling. For example, WNT7A[66] induces filopodia and spread regions along the axon shaft of pontine mossy fibres, where stable microtubules become unbundled[120], leading to increased axon branching. WNT7A inhibits activity of GSK3β, which normally phosphorylates MAPs such as tau, APC and MAP1B[65]. Activation of the WNT pathway therefore decreases MAP1B phosphorylation. As phosphorylated MAP1B maintains microtubules in a dynamic state[121], decreased MAP1B phosphorylation would increase microtubule stability, leading to terminal remodelling of mossy fibres and axon branching. This seems to contradict results showing that axon branching is dependent on pathways that destabilize microtubules[58]. However, axon branching initially involves destabilizing microtubules at branch points (FIG. 2), but further growth of branches requires the entry of stable microtubules into the nascent branch. GSK3β has also been shown to function in a novel signalling pathway that inhibits axon branching of granule cell neurons in the rodent cerebellar cortex[122]. In this study, knockdown of the JNK-interacting protein kinase 3 (JIP3) stimulates granule cell interstitial axon branching by decreasing the levels of GSK3β. Suppression of axon branching by JIP3–GSK3β signalling involves phosphorylation of a novel GSK3β substrate, DCX. However, the downstream cytoskeletal mechanisms by which the JIP3–GSK3β–DCX signalling pathway actively suppresses axon branching are unknown. Manipulation of GSK3α and GSK3β levels and their association with different MAP substrates in DRG and hippocampal neuronal cultures[117] have led to various outcomes on axon outgrowth and axon branching, highlighting the complexity of GSK3 function in the regulation of microtubule organization and dynamics.

Netrin 1 and BDNF regulate terminal branching of retinal axons in the frog optic tectum[37,63]. A recent *in vivo* study[123] identified a signalling pathway involving the ubiquitin protein ligase E3 NEDD4 and phosphatase and tensin homologue (PTEN) that negatively regulates the PI3K signalling pathway (FIG. 3). Inhibition of NEDD4 increases the levels of PTEN, resulting in simple, sparsely branched retinal axon arbors in the tectum but has no effect on axon navigation. Thus, PTEN overexpression inhibits axon branching, whereas PTEN knockdown restores axon branching. In this study, the authors proposed that, in response to external signals, NEDD4 ubiquitylates PTEN, thereby targeting it for degradation, which promotes the PI3K pathway and the downstream cytoskeletal reorganization that leads to axon branching. Importantly, this study shows how signalling pathways can specifically regulate terminal branching without affecting long-range axon pathfinding.

Activity-dependent mechanisms

Neuronal activity can also regulate axon branching (FIG. 4). Spontaneous electrical activity during early development[124] generates transient fluctuations in the levels of intracellular calcium, an essential second messenger. Calcium transients, which are activated by cues such as netrin 1 and BDNF in neuronal growth cones, regulate axon outgrowth and guidance[125] by signalling to downstream effectors such as calcium/calmodulin-dependent protein kinase II (CaMKII) and the calcium/calmodulin-dependent phosphatase calcineurin that ultimately regulate the growth cone cytoskeleton. Calcium signalling also has an important role in axon branching. For example, local application of netrin 1 to cortical axons *in vitro*[36] increases the frequency of calcium transients in small regions of the axon and simultaneously evokes rapid localized filopodial protrusion and axon branching through CaMKII and MAPK activation. Thus, local calcium transients can promote branching within discrete regions of the axon.

Downstream of calcium activity and activation of CaMKII, which acts as an intracellular calcium sensor, the RHO-family of small GTPases regulates actin filament assembly and organization in growth cones[78,125]. The RHO GTPase RHOA has also been shown to have an important role in cortical axon branching[126]. In this study, in which branching of horizontal axons arising from upper-layer cortical neurons was observed in cortical slices, the introduction of constitutively active RHOA increased axon branching through the increased dynamic addition and loss of branches, whereas RHOA inhibition decreased branching. Blockade of neural activity decreased the levels of active RHOA, suggesting that RHOA is a positive regulator of activity-dependent axon branching in cortical neurons. This is only one possible regulator of activity-dependent axon branching, and further research will be required to understand the molecular mechanisms that mediate activity-dependent processes.

Branches can extend while their axons stall or retract[19]. *In vivo*, axons and branches are likely to encounter different types or concentrations of factors that evoke different levels of calcium activity, which could independently regulate the outgrowth of different processes from the same axon. For example, in NGF-mediated axon outgrowth of sympathetic neurons, axon branches that are depolarized to induce calcium entry are favoured for growth at the expense of unstimulated branches of the same axon[127]. In cortical neurons, imposition of calcium activity in an axon versus its branches causes a process with higher-frequency calcium transients to grow faster at the expense of another process with lower levels of calcium activity, which stalls or retracts[128]. How growth of one axonal process is coordinated with retraction of another is unknown.

Calcium transients
Transient increases in the level of intracellular calcium that can occur repetitively at different frequencies.

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Activity-dependent mechanisms are also important in shaping terminal axon arbors[129,130]. For example, patterned neural activity in the frog retinotectal pathway shapes the morphology of retinal axon arbors by governing axon branch dynamics[131]. Callosal axons in mice expressing an inward-rectifying potassium channel to suppress neural activity extend through the contralateral cortex with little branching and end in immature growth cone-like structures[132]. Similarly, suppression of spontaneous firing of thalamic or cortical neurons in organotypic co-cultures reduces thalamocortical axon branching[133]. Conversely, neural activity enhances the branch dynamics of thalamocortical axons that are biased towards branch addition and elongation in cortical target layers[134].

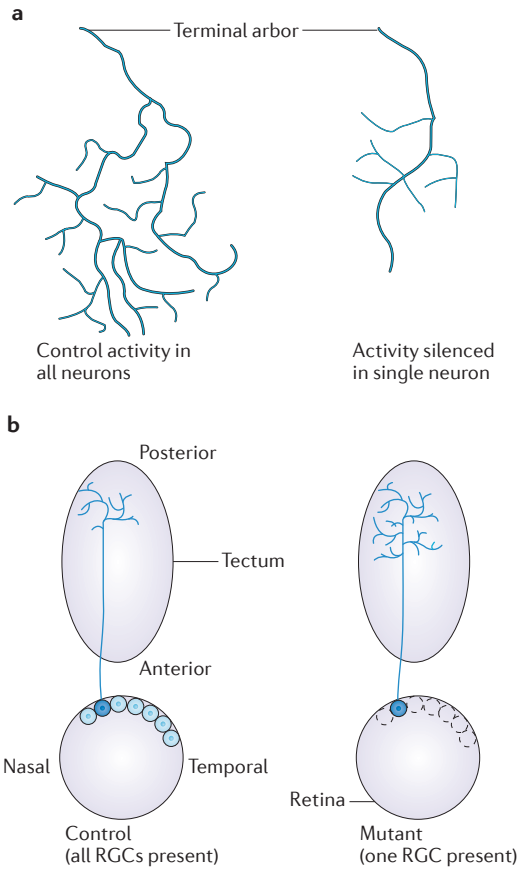

Figure 4 | **Competition shapes the morphology of terminal arbors.** **a** | The terminal arbor of a retinal axon in the zebrafish optic tectum with normal electrical activity has a complex, highly branched morphology (left), and it can outcompete a neighbouring retinotectal arbor in which electrical activity has been silenced (right), resulting in a smaller, less elaborate morphology in the silenced axon[134]. **b** | A retinal axon arbor in a control zebrafish tectum (left) has a smaller less, complex morphology when all other retinal ganglion cells (RCGs) are present. In the mutant zebrafish with only a single RCG axon (right), arbors terminate in appropriate tectal regions but are larger and more complex in the absence of competitive interactions with neighbouring axons. Figure is adapted, with permission, from REF. 137 © (2008) Macmillan Publishers Ltd. All rights reserved.

The effects of neural activity can also involve competition among neighbouring axon arbors. For example, in the zebrafish retinotectal system[135], silencing electrical activity in one axon relative to neighbouring axons reduces the size of the terminal arbor of the 'silenced' axon (FIG. 4). Arbor size and complexity of the neuron with reduced activity is restored when activity of neighbouring axons is silenced, demonstrating a competitive, activity-dependent mechanism. However, another study in the zebrafish retinotectal system[136] showed that singly silenced axons have larger than normal arbors, although silencing neighbouring axons also restores the normal arbor size of the activity-suppressed axon. In this study, suppression of presynaptic activity in retinal ganglion cells and live-cell observations of axon arbor formation over days showed that presynaptic activity is not required for the development of arbors with an appropriate number of stable branches. However, presynaptic activity is required for arbor maturation, as shown by increased production of dynamic filopodia. These results suggest that activity normally arrests the formation of filopodia during arbor maturation. Moreover, singly silenced axons fail to arrest growth of branches, leading to expansion of the arbor territory on the tectum. Restoration of normal arbor size by silencing of neighbouring retinal axons demonstrates that, although the cellular mechanisms underlying the competition are unknown, the ability of axons to arrest their growth is an activity-dependent, competitive process. To further demonstrate effects on axon arbors in the absence of competition, zebrafish with a single retinal ganglion cell were created[137]. Arbors of this single axon terminate in appropriate tectal regions but are larger and more complex than normal. Although an understanding of the exact mechanisms requires further study, these examples show the importance of competitive interactions in shaping axon terminal arbors.

Conclusions and future directions

Over the past decade, we have gained considerable insight into mechanisms that regulate axon branching. The discovery of new branching mechanisms, such as the kinase signalling pathway that controls immobilization of mitochondria at nascent presynaptic sites to regulate cortical axon branching[138], will continue to expand and modify our understanding of axon branching. However, many issues remain unresolved. First, we still lack a complete understanding of why individual axons branch only at specific target locations. The presence of gradients of molecular cues and the suppression of inappropriate branching have been postulated as mechanisms that underlie the specificity of branching in the retinotectal system[7], but whether these apply more generally to vertebrate systems will require further study. Second, although the cytoskeleton is the final target for the convergence of signalling pathways, we still lack a clear understanding of how the consolidated axonal cytoskeleton is transformed into dynamic microtubules and actin filaments that drive membrane protrusion at axon branch points. The improvements in genetically encoded labelling techniques and high-resolution time-lapse microscopy should now make it possible to observe longer-term cytoskeletal reorganization and dynamics that

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Box 1 | **New technologies for studying axon branching**

Much of the organization of axon branch points is obscured because of the small diameter and cylindrical structure of the axon. Many of the live-cell imaging studies of cytoskeletal dynamics at branch points and the growth cone described in this Review have used transfection of neurons with plasmids encoding fluorescent fusion proteins, such as enhanced green fluorescent protein and mCherry, fused to cytoskeletal components such as actin and tubulin. To visualize fluorescently labelled actin filaments and microtubules and their associated proteins, many investigators are currently using high-resolution microscopy such as total internal reflection fluorescence (TIRF) microscopy and spinning disk confocal microscopy. However, new advances in imaging — specifically, structural illumination microscopy (SIM)[139], stimulated emission depletion (STED) microscopy[140], photoactivated localization microscopy (PALM)[141] and stochastic optical reconstruction microscopy (STORM)[142] — will allow future studies of cytoskeletal dynamics and signalling at branch points at resolutions on the order of tens of nanometres. Importantly, all of these imaging techniques can be used on living samples. The study of axon branching would also greatly benefit from the availability a greater diversity of 'caged' proteins and compounds, so that local uncaging could be performed at specific sites along an otherwise consolidated axon in a similar way to the uncaging of neurotransmitters at individual dendritic spines. Optogenetic control over genetic or functionally defined populations of neurons or individual neurons will undoubtedly allow much more sophisticated experiments that investigate the role of activity in axon branching at high temporal and spatial precision[141]. Such techniques would also allow researchers to test the role of activity in individual branches or arbors of the same neuron. To understand axon branching, it is also important to reconstruct, with high precision, the full extent of an axon arbor. In the past, this has required laborious thin-section and/or electron-micrograph reconstruction. The recent advent of tissue-clearing techniques, such as ScaleA2 (REF. 143), CLARITY[144], SeeDB[145] and Clear$^{T/T2}$ (REF. 146), which allow fluorescently labelled axons and branches to be reconstructed to their full extent, will help in understanding how axon branching is affected in normal, transgenic and disease models.

occur at specific stages of axon branching (BOX 1). Third, to understand mechanisms of axon branching *in vivo*, it will be important to investigate signalling events and cytoskeletal changes during branching in preparations of the CNS, such as slices or explants that recapitulate the complex *in vivo* environment. These approaches can be combined with genetic manipulations of signalling components and cytoskeletal effectors. It is clear from this Review that our understanding of how signalling pathways transduce the effects of extracellular cues on the axonal cytoskeleton to promote axon branching is at an early stage. At present, it is unclear how the same guidance cue can have different effects on branching in different neuronal populations or differentially affect the growth of an axon and branches of the same neuron. In future studies, it will be important to connect intracellular signalling to its effects on cytoskeletal dynamics at branch points in living neurons of different types. Moreover, mechanisms for the suppression of axon branching to create topographic specificity are poorly understood. Live-cell imaging has provided an exciting window into the dynamic remodelling of axon terminal arbors *in vivo*, and this will be important in revealing how electrical activity through calcium signalling regulates arbor formation and competitive interactions among neighbouring axons in a target region. Addressing such questions with new techniques over the next decade will inform our understanding of the remarkable ability of a single axon to branch into specific regions of the developing nervous system.

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Competing interests statement
The authors declare no competing interests.

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