# Axonal Guidance: Making Connections

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Peter Kozulin and Linda J. Richards

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**Abstract**

The vertebrate brain contains millions of neuronal and glial cells arranged in a highly organized manner forming functional neural circuits. To form these circuits during brain development, neurons extend an axon from the cell body to make connections with neurons in target brain areas, which can be a considerable distance away from the neuronal cell body. To ensure that axons accurately elongate toward the correct target field over such a distance, specific guidance cues are used to navigate the axons through their environment in a reproducible

P. Kozulin
Queensland Brain Institute, University of Queensland, St Lucia, QLD, Australia

L.J. Richards (✉)
Queensland Brain Institute, University of Queensland, St Lucia, QLD, Australia

School of Biomedical Sciences, The University of Queensland, Brisbane, QLD, Australia
e-mail: richards@uq.edu.au



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pattern of growth. These cues involve guidance molecules that can elicit attractive or repellent guidance on the extending axon and can act over long distances by secretion into extracellular space or over short distances through direct cell contact. In response to the guidance cues, the distal tip of the axon, known as the growth cone, undergoes dynamic structural changes to ensure that it is continually growing in the correct direction. In this chapter, we will discuss the range of highly conserved mechanisms and molecules involved in axon guidance, the biological changes that occur in axons during guidance, and the major assays used to measure the guidance of neuronal axons in vitro.

**Keywords**
Axon guidance • Growth cones • Actin cytoskeleton • Guidepost cells • Pioneering axons

## A Brief History of Axon Guidance

The concept of axon guidance was alluded to in the late nineteenth century by Ramon y Cajal who first observed growth cones in the developing mammalian brain as swellings on the distal tips of growing nerve fibers. Cajal observed a change in growth cone morphology during axon extension and compared this to the motility and dynamic morphology of leukocytes in response to toxic elements. From these observations, Cajal suggested the active involvement of growth cones in steering the direction of axon growth in response to an "intelligent force," which he further hypothesized may be produced by chemical cues in the cellular environment (reviewed in Tamariz and Varela-Echavarría 2015).

It was not until the 1940s that the first breakthroughs in the field of axon guidance occurred, with the pioneering work of Roger Sperry on the organization of regenerating optic nerve and motor neurons. Sperry ruled out the possibility of mechanical or functional factors mediating the correct reconnection of these topographically arranged neurons and proposed that this was instead mediated by "special affinities" within the neurons. This work culminated in Sperry's theory of "chemoaffinity" (reviewed in Sperry 1963), which developed further in the 1950s and 1960s with advances in understanding of the biochemical properties of neuronal development led by Rita Levi-Montalcini. She showed that transplanting mouse sarcoma tissue into chick embryos resulted in an increase in innervation of the embryo's internal organs, with subsequent in vitro experiments leading to the discovery and isolation of the first neurotrophic factor in the vertebrate nervous system, nerve growth factor (NGF) (Cohen et al. 1954). In addition to its enhancement of neuronal survival, NGF was found to be diffusible, driving the hypothesis that it also possessed "chemoattractant" properties for growth cones. These predictions were later supported by the discovery that NGF functions as a chemoattractant for chick dorsal root ganglion axons in vivo (Gundersen and Barrett

1979) and that neurotrophic factors signal through similar molecules that are key to axon guidance signaling, such as Rho GTPases (Yamashita et al. 1999).

More recently, however, the most important piece of evidence to support the theory of chemotaxis was the discovery of Netrins and their homologs in *C. elegans* and mice, which were the first molecules purified based on their chemotactic axon guidance activity (reviewed in Tessier-Lavigne and Goodman 1996). Since this finding, additional families of axon guidance molecules, such as Semaphorins, Slits, and Ephrins, and their receptors and signaling pathways have been identified. With the inclusion of morphogens, proteoglycans and cell adhesion molecules, the latter of which, mediate guidance upon direct cell-to-cell contact, a large number of molecular processes underlying axon guidance have been revealed and will be described in this chapter.

## The Biology of Growth Cones and Modulation of the Axonal Cytoskeleton

Three key components involved in the pathfinding of projecting axons to a target structure are: (1) the axonal growth cone which interacts with (2) guidance molecules secreted by (3) intermediate targets, which direct axons along a reproducible pathway. The neuronal structure that initiates turning of the axon is the growth cone, which contains radiant protrusions known as filopodia that extend into extracellular space. Filopodia are structurally supported by cytoskeletal actin filaments, which are actively rearranged during axon guidance to initiate turning of the growth cone to steer the direction of axon growth. This cytoskeletal rearrangement is initiated in response to guidance cues via an interaction between membrane-bound receptors expressed on the filopodia and external guidance molecules, which can result in an attractive or a repellent response. A schematic of the structure of a growth cone is shown in Fig. 1a, and the mechanisms underlying growth cone responses to guidance cues are described in more detail in Fig. 1b, c.

Growth cones are fan-shaped structures that appear at the terminus of developing axons and are the main structures involved in guiding the axons to their correct target field. They are able to perform this function by detecting guidance cues in the external environment and converting them into signals that initiate changes in the cytoskeleton that determine their direction of growth and motility. In addition, short-range cues can promote growth cone adhesion to nearby cells that provide a substrate for direction-oriented growth.

A key feature of growth cones, which ensures that they remain sensitive to external cues in vivo, is the dynamic nature of their morphology whereby the filopodia are continually advancing and withdrawing to probe their environment. This level of motility is achieved by the continuous restructuring of the primary components of the growth cone cytoskeleton: actin and tubulin (Fig. 1a). Actin microfilaments are primarily found in the peripheral domain of the growth cone

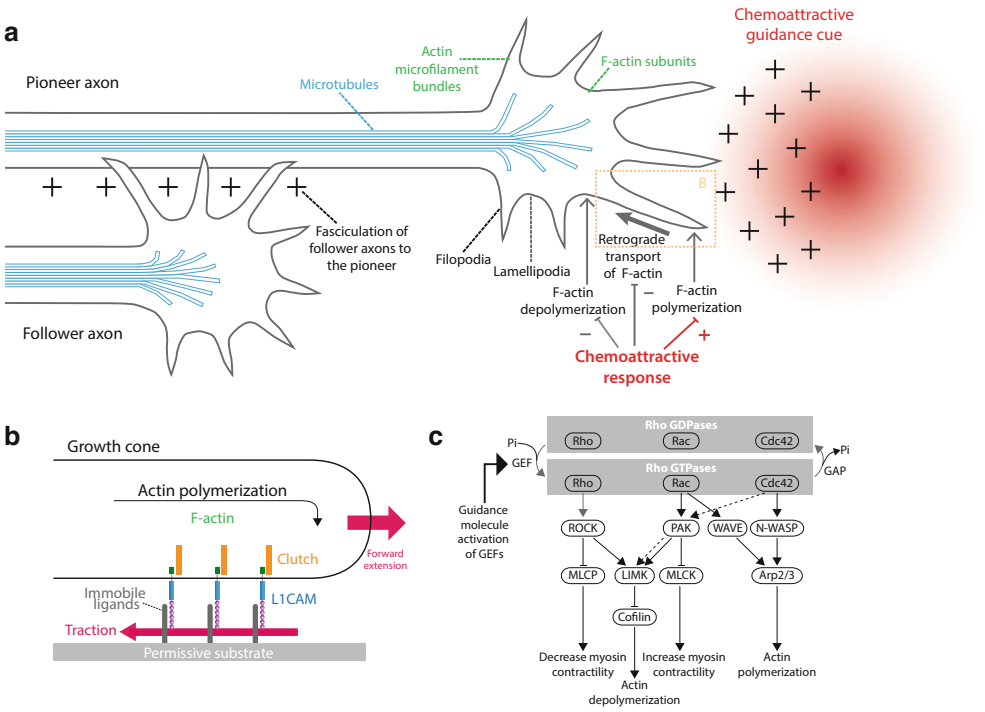


**Fig. 1 Axon guidance by chemoattraction and axon fasciculation** (**a**) Advancement of the pioneer axon towards the external chemoattractive target is initiated by detection of the guidance cue by the growth cone. This detection induces extension of the filopodia proximal to the cue by promoting polymerization of the filopodial actin microfilaments and inhibition of retrograde actin flow. Subsequently, follower axons navigate to the target tissue by fasciculating with the pioneer axons. (**b**) Within a leading filopodia of the pioneer axon, immobile ligands in the extracellular environment interact with the filopodial actin via L1 cell adhesion molecule and a molecular "clutch" such as ankyrin-B. As the actin is retrogradely transported, this molecular interaction creates a traction force that is then transferred to the growth cone to create forward movement. (**c**). Axon guidance receptor/ligand interaction activates guanine nucleotide exchange factors (GEFs) to catalyze the phosphorylation of Rho guanosine diphosphatases (GDPases) such as Rho, Rac, and Cdc42, to guanosine triphosphatases (GTPases). Upon GTPase activation, downstream signaling mediates changes in cytoskeletal organization such as actin polymerization and myosin contractility that are required for filopodial movement. Arp2/3 Actin-related protein 2/3, GAP GTPase activating protein, LIMK LIM kinase, MLCP myosin light chain phosphatase, MLCK myosin light chain kinase, N-WASP Neural Wiskott-Aldrich syndrome protein, PAK p21 protein activated kinase, Pi phosphate, ROCK Rho-associated protein kinase, WAVE Wiskott-Aldrich syndrome protein family verprolin homolog

where they exist as parallel bundles within filopodia or as cross-linked meshworks within peripheral regions between the filopodia, known as lamellipodia. These actin structures are dynamically regulated by a number of simultaneous processes: (1) polymerization of filamentous actin (F-actin) microfilaments at the distal tip of the filopodia, (2) depolymerization of the lamellipodial actin meshwork, and (3) the

retrograde transport of F-actin away from the leading edge to a more central region of the growth cone, known as "treadmilling." Disassembly of more centrally located actin allows F-actin subunits to be recycled to the distal tip of the filopodial microfilaments, whereas the retrograde transport mechanism, driven by myosin-based motor proteins, balances this forward flow of actin. Alterations to the actin transport mechanisms can alter the balance of actin flow and induce changes in the extension or retraction of the filopodia. This is observed, for example, upon growth cone contact with a permissive substrate, which typically induces an extension of filopodia. Substrate contact promotes binding of adhesion proteins with the actin cytoskeleton and a subsequent inhibition of retrograde actin flow that, together with the continued polymerization of actin at the leading edge, produces a net extension of the microfilaments. Concurrently, actin meshworks distal to the site of substrate contact undergo continued depolymerization to provide F-actin subunits to the microfilaments, resulting in retraction of the distal filopodia and extension of the growth cone along the substrate (Fig. 1b).

Tubulin forms the other major cytoskeletal component of growth cones, in the form of long bundles known as microtubules, which extend down the axonal shaft. Microtubules provide structural support for axons and a substrate for the transport of vesicles and organelles toward the growth cone. They also play an important role in axon extension. This occurs through the transport of tubulin to the distal tips of microtubules within the peripheral portions of the growth cone and the subsequent stabilization of these loose and dynamic microtubule ends into tight bundles in its central domain. Microtubules are also directly sensitive to guidance cues and have been implicated in growth cone turning as a result of localized guidance cue responses that produce regional changes in microtubule dynamics and polymerization behind the growth cone.

The growth cone motility that is enabled by the dynamic regulation of cytoskeletal structures is mediated via proteins of the Rho family of small GTPases (guanosine triphosphatases) such as Rho, Rac, and Cdc42 (Fig. 1c). These proteins regulate actin cytoskeletal organization by influencing actin polymerization and depolymerization, as well as retrograde transport within the growth cone by modulating myosin activity. Rho GTPases actively regulate actin and myosin function in their GTP-bound state. Conversely, Rho GTPases become inactive upon hydrolysis to a GDP-bound state via GTPase activating proteins (GAPs) and can be reactivated with guanine nucleotide exchange factors (GEFs). The activity of both GAPs and GEFs can be modulated by the direct coupling or indirect signaling of axon guidance receptors, allowing these receptors to directly influence actin and myosin function. In addition, specific axon guidance receptor/ligand interactions activate GAPs/GEFs and their downstream signaling to induce particular cytoskeletal rearrangements. For example, Netrin-1/DCC (deleted in colorectal carcinoma) activates the GEF Trio to initiate Rac1 and Cdc42 signaling to promote axon growth and a chemoattractive response but does not activate RhoA signaling which inhibits growth and can induce growth cone collapse (Shekarabi and Kennedy 2002) (Fig. 1c).

## Guidance Cues

Pioneering axons are the first to navigate to the target tissue and rely on guidance cues that can be present in the form of diffusible molecules that act over long distances via secretion into the extracellular space or molecules that act over short distances and require cell-to-cell contact. Intermediate guidance targets are also present for some axon tracts, and provide important points of navigation to direct growth cones to their next guidance marker. Furthermore, once the pioneer axons have reached their target, follower axons can use these tracts as an additional intermediate guidance target via fasciculation (bundling; Fig. 1a). In this section, we will discuss the function of pioneer axons and their use of intermediate guidance targets, and the major molecule families of molecules that are known to play a role during axon guidance.

## Intermediate Guidance Targets and Pioneering Axons

Pioneering axons are important to establish the initial trajectory of an axon bundle. Once these initial axons are established they provide the pathway to the target, which subsequent axons can follow by fasciculation. However, to accurately project to the correct target over long distances, intermediate guideposts are used. These typically involve glial cells and corridor neurons. The presence of guidepost cells was first detected in invertebrates during the study of developing limb buds in grasshopper embryos (Bate 1976) and was later demonstrated throughout the central nervous system across a variety of species.

One well-characterized example of an intermediate guidance target is the floor plate of the vertebrate spinal cord, hindbrain, and midbrain. The floor plate is a structure consisting of radial glial cells at the ventral midline that typically secretes a range of chemoattractants to guide the commissural axons toward the midline. In addition, after midline crossing, the growth cones undergo a change in the expression of cell surface guidance proteins that results in repulsion away from the floor plate toward their contralateral target. For example, Slit and Netrin proteins expressed by the floor plate are important regulators of midline crossing in the vertebrate spinal cord or ventral nerve cord in invertebrates. Netrin-1 mediates attraction through DCC expressed on axonal growth cones, promoting growth toward the midline. After midline crossing, interaction between Slit proteins and Roundabout (Robo) receptors expressed by these axons promotes axon growth away from the midline (Keino-Masu et al. 1996; Brose and Tessier-Lavigne 2000).

Glial guidepost populations are essential for midline guidance throughout the brain and spinal cord, as they secrete guidance molecules to direct the midline crossing of commissural axons. In the forebrain of eutherian (placental) mammals, specialized glial populations known as the indusium griseum glia and the glial wedge are present at the dorsomedial and ventrolateral midline, respectively. These populations function as chemorepellent boundaries that direct growing callosal axons via the expression of Draxin, Ephrins, Wnt5a, and Slit2. These glial

populations ensure that callosal axons do not project ventrally into the ipsilateral septum and instead are directed to cross the midline at the cingulate cortex/septum boundary (reviewed in Suárez et al. 2014). In addition, a population of midline glial cells arranged in the shape of a tunnel surrounds and directly influences the projection of the anterior commissure, as well as promoting the packing of these axons. Guidance molecules similar to those that guide the corpus callosum, including chondroitin sulfate proteoglycans (Lent et al. 2005), are secreted by these tunnel glial cells. In many vertebrates, a radial glial cell population at the optic chiasm also mediates the guidance and sorting of ipsi- and contralateral-projecting retinal ganglion cell axons from each eye in order to establish binocular visual circuits. The optic chiasm glia, known as the glial palisade, was one of the first glial populations to mediate axonal guidance using proteoglycans, although these cells also use a large number of other guidance cues, in particular Ephrins, neuronal cell adhesion molecule (NrCAM), and Semaphorins (reviewed in Petros et al. 2008).

Although glial guideposts are highly important mediators of pioneering axons, they are present transiently during axon guidance and disappear later during development. Similarly, transient neural populations can also function as guideposts in the developing brain. Lateral olfactory tract (LOT) cells were one of the first cell populations demonstrated to act as guideposts. LOT cells position themselves horizontally along the pallial-subpallial boundary where they provide short-range guidance for LOT axons as they project caudally from the olfactory bulb toward the anterior olfactory nucleus, the olfactory tubercle, the piriform and entorhinal cortices, and the amygdala (reviewed in Squarzoni et al. 2015). In addition, pioneer cortical subplate neurons function as a guidepost for thalamocortical axons by making contact with and delaying the projection of these axons into the cortical plate (Ghosh and Shatz 1993). Furthermore, transient corridor neurons within the internal capsule, the corpus callosum, and the anterior commissure secrete guidance molecules important for tract formation (Niquille et al. 2009; Benadiba et al. 2012; Garel and López-Bendito 2014; Magnani et al. 2014; Minocha et al. 2015).

Once neuronal or nonneuronal guideposts are in place, pioneering axons can navigate to the correct target tissue and establish a tract where follower axons fasciculate (Fig. 1a). However, with fewer guidance cues to navigate, pioneer axons possess different kinetics to those of the followers. For example, pioneer commissural axons in the zebrafish forebrain have been shown to slow down as they approach the midline, unlike the followers that show no change in their rate of growth (Bak and Fraser 2003). Pioneer axons may also originate from a different area from those of the followers. For example, callosal pioneer axons originate from the cingulate cortex and provide a scaffold across the midline only as far as the contralateral cingulate cortex and not toward the lateral cortical areas (Koester and O'Leary 1993). The stereotypical growth of pioneers would also suggest an exclusive developmental nature of these neurons. However, pioneer axon ablation experiments in zebrafish show that follower axons alter their growth kinetics at the midline to become the new pioneers, suggesting that pioneer axons may exist as a result of exposure to external guidance cues and the lack of a fasciculation target (Bak and Fraser 2003). Similarly, if cingulate axons undergo targeting defects in the forebrain,

cortical axons can assume their role and continue to form the pioneering tract of the corpus callosum (Lim et al. 2015).

## Axon Guidance Molecules

The prominent receptor/ligand families known to be involved in axon guidance signaling include Netrins and UNC/DCC receptors, Ephrins and Eph receptors, Semaphorins and Neuropilin/Plexin receptors, and Slits and Robo receptors. A schematic of the structure of each of these molecules is shown in Fig. 2 and some of their properties and actions in vivo are discussed below.

### Netrins

Netrins were the first family of molecules discovered based on their chemotactic axon guidance activity in developing neural tissue. Four members of the Netrin family have been identified, with at least one Netrin molecule being conserved across invertebrate and vertebrate species, particularly in the amino-terminal VI domain which shows high sequence homology with the amino terminal of the gamma subunit of laminin (Tessier-Lavigne and Goodman 1996). In vertebrates, Netrin-1 and -3 function as chemoattractants in the floor plate where they are required for commissural axon guidance. Additional Netrin family members have been identified; Netrin-2 is present only in chicks and zebrafish, and Netrin-4 is present in some vertebrates but is structurally more divergent than Netrins 1–3 (Moore et al. 2007; Rajasekharan and Kennedy 2009).

Netrin function has frequently been investigated with respect to axon guidance at the spinal cord ventral midline and has been further shown to be important for axon guidance in the midbrain, LOT, and retina, as well as the forebrain, including the corpus callosum, hippocampal commissure, and anterior commissure. Netrin is a multifunctional ligand and can act as a chemorepellent or chemoattractant depending on the type of receptor binding. There are two primary groups of Netrin receptors that are membrane-bound and related to the immunoglobulin superfamily: DCC receptors, which predominantly mediate a chemoattractive response to Netrin, and UNC-5 receptors, which mediate a chemorepellent response. UNC-5 interacts with Netrin via its two extracellular immunoglobulin (Ig) domains that initiate a repulsive response over a short distance, although the response range can increase with a DCC/UNC-5 receptor complex. Conversely, DCC interacts with Netrin via one of its extracellular fibronectin type 3 domains and initiates the signaling cascade for chemoattraction by multimerization of its intracellular P3 domain with cytoplasmic proteins. Evidence that DCC is a primary target for Netrin-1 in vertebrates is reflected in the similarity of the mutant mouse phenotype for these two genes. For example, Netrin1-DCC signaling is essential for the chemoattraction of axons from the cingulate cortex toward the midline to form the pioneer tract of the corpus callosum. Knockout of either of these genes results in failure of the callosal axons to cross the midline and instead misproject ipsilaterally to form Probst bundles, as shown in Fig. 3 for DCC knockout and Fothergill et al. (2014) for both knockouts in mice.

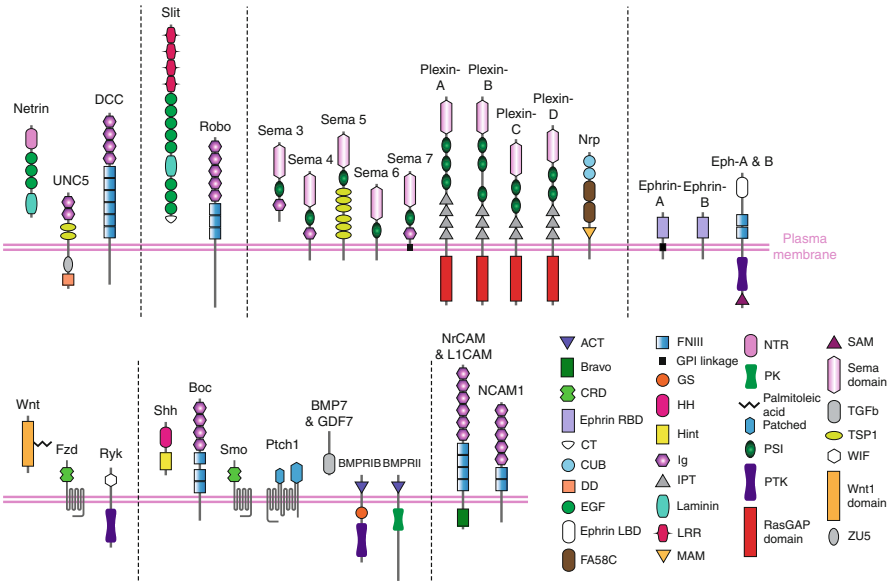


**Fig. 2 Vertebrate axon guidance molecule families and their conserved domains** The major vertebrate axon guidance molecules are arranged in their families, with the ligands presented as the leftmost molecules and the receptors as the rightmost molecules within each family. Dashed lines separate the molecule families, and phylogenetically conserved domains are identified in the figure key. ACT Activin types I and II receptor domain, BMP7 Bone morphogenetic protein 7, BMPRIB Bone morphogenetic protein receptor type IB, BMPRII Bone morphogenetic protein receptor type II, Boc Biregional cell adhesion molecule-related/downregulated by oncogenes (Cdon) binding protein, Bravo Bravo-like domain (contains conserved motif FIGEY), CRD Cysteine-rich domain, CTCK C-terminal cysteine knot domain, CUB Complement component C1r/C1s, uEGF and BMP1 domain, DCC Deleted in colorectal carcinoma, DD Death domain, EGF Epidermal growth factor-like domain, Ephrin LBD Ephrin receptor ligand binding domain, Ephrin RBD Ephrin receptor binding domain, FA58C Coagulation factor V/VIII C-terminal (discoidin)-like domain, FNIII Fibronectin III domain, Fzd Frizzled, GDF7 Growth differentiation factor 7, GPI glycosylphosphatidylinositol, GS Transforming growth factor beta GS domain (GS refers to the peptide sequence within this motif: TTSGSGSG), HH Hedgehog amino-terminal signaling domain, Hint Hedgehog/Intein domain, Ig Immunoglobulin-like domain, IPT Ig-like, Plexins, transcription factors, LRR Leucine-rich repeat, MAM Meprin, A-5 protein, and receptor protein-tyrosine phosphatase mu domain, NrCAM Neuronal cell adhesion molecule, Nrp Neuropilin, NTR Netrin (Tissue inhibitor of metalloproteinase-like) domain, PK Protein kinase domain, PSI Plexin-Semaphorin-Integrin domain, Ptch1 Patched-1, PTK Protein tyrosine kinase domain, Robo Roundabout receptor, SAM Sterile alpha motif, Sema Semaphorin, Shh Sonic hedgehog, Smo Smoothened receptor, TGFb Transforming growth factor beta-like domain, TSP1 Thrombospondin-1 domain, WIF Wnt inhibitory factor-like domain, ZU5 Zona occludens-1 and Unc5-like Netrin receptor domain

## Ephrins

Eph tyrosine kinase receptors consist of two classes (A and B) according to the type of Ephrin ligand they bind, with each class of receptor able to mediate chemoattractive or chemorepellent responses. In addition, Ephs and Ephrins are

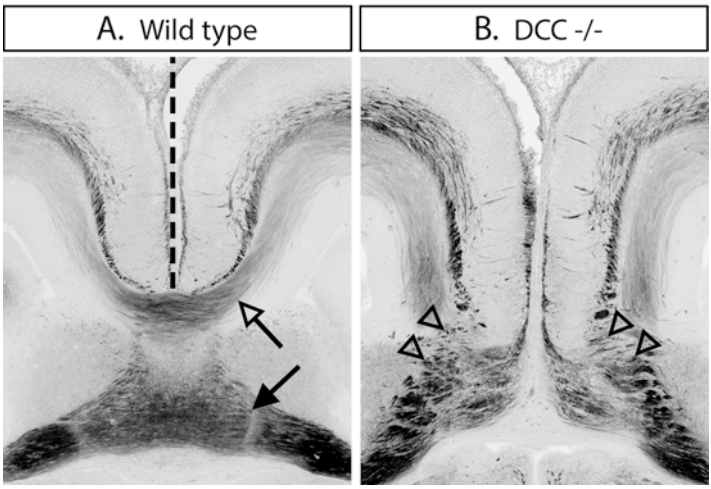


**Fig. 3 DCC-mediated axon guidance at the forebrain midline influences formation of the corpus callosum** DCC is one of a number of molecules that mediate guidance of corpus callosum axons across the midline in the forebrain of eutherian mammals. (**a**) A magnified view of the midline of an embryonic day 17 wild type mouse forebrain. The horizontal section is stained for GAP-43, a marker of growing axons (black). In the wild type forebrain, commissural axons of the corpus callosum (*open arrow*) and hippocampal commissure (*black arrow*) cross the midline and connect the contralateral sides of the brain. (**b**) The forebrain midline of an E17 DCC knockout mouse. The callosal and hippocampal commissural axons fail to cross the midline and instead project ipsilaterally to form GAP-43-positive Probst bundles (*arrowheads*)

membrane-bound and, upon interaction, result in both downstream signaling in the receptor-bearing cell and upstream signaling in the ligand-bearing cell. For a review of this bidirectional signaling of Ephs and Ephrins see Cowan and Henkemeyer (2002).

Ephs and Ephrins are best known for their roles in axon guidance, particularly in the topographic mapping of the retinotectal sensory system. Graded Eph-A expression has been described in many regions of the developing vertebrate central nervous system, including the retina, superior colliculus, dorsal lateral geniculate nucleus (dLGN), and cortex. Repellent Ephrin-mediated signaling along opposing gradients of ligands and receptors is the principal mechanism underpinning topographic mapping of the retina onto visual targets in mammals. The onset of neural activity in Ephrin-expressing retinal ganglion cells during development is also important in forming eye-specific layers in the dLGN (Pfeiffenberger et al. 2005). Furthermore, gradients of Eph expression are dynamic during development. For example, Eph-B receptors are uniformly expressed along the dorsoventral axis of the mouse retina during the early phase of retinal development, when ganglion cell axons are finding their way to the optic disk, but are found in a distinct low-to-high dorsoventral gradient later in development, when axons are mapping to their visual targets.

### Semaphorins

The Semaphorins are a large family of secreted and transmembrane guidance proteins, which were first discovered in grasshopper (Fasciclin IV) and later in vertebrates (Sema3A). Semaphorins contain a phylogenetically conserved 500 amino acid domain, and in vertebrates consist of more than 20 members, divided into five classes (no. 3 to 7) based on structure and sequence homology (Jongbloets and Pasterkamp 2014). Classes 4 to 7 are membrane bound (either transmembrane or glycosylphosphatidylinositol (GPI) linked) and class 3 is secreted. The Semaphorins mediate many important chemorepellent interactions during brain development; however, further analyses have shown that they can also mediate chemoattractive and other neurotrophic functions such as axon branching, fasciculation and targeting, neuronal migration, and synaptogenesis. The primary receptors of Semaphorins are the Plexins, of which there are nine members in vertebrates, divided into four families (A, B, C and D). Plexins typically mediate chemorepellent responses by initiating actin filament disassembly, potentially leading to the collapse of the growth cone. In addition, Neuropilins are cell surface glycoproteins that function as coreceptors for Semaphorins by forming complexes with Plexins, although they can also form homo- or heterodimers that interact directly with Semaphorins, such as the interaction between Sema3D and Neuropilin-1 (Nrp1) that mediates chemorepellent guidance of the medial longitudinal fasciculus.

Although multiple Plexins and Neuropilins are present in vertebrates and can form different combinations of complexes, current evidence suggests that Semaphorin signaling on specific cells is not indiscriminate and that interactions with specific receptors or complexes are required to elicit particular responses in neurons. One of the most extensively studied Semaphorins to mediate chemorepellent responses and fasciculation in vertebrates is Sema3A. Of the Neuropilin types present in vertebrates, Sema3A binds only with Nrp1 but can interact (in a complex with Nrp1) with several Plexins including Plexin-A1, A3, and A4. Null mutations of Sema3A in mice result in defasciculation and guidance defects of the cranial nerves and major axon tracts in the spinal cord. Sema3A has also been shown to locally regulate cytoskeletal proteins within the growth cones of Xenopus retinal axons. Upon treatment with Sema3A, there is a local increase in translation of RhoA in growth cones to stimulate cytoskeletal rearrangement for filopodial retraction and an increase in the translation of *cofilin*, an actin depolymerization protein that can trigger growth cone collapse (Piper et al. 2006).

Semaphorins also have been described to mediate chemoattractive responses in the development of the anterior commissure. Sema3B provides chemoattractive guidance for axons linking the olfactory bulbs and anterior piriform cortex, which form the anterior tract of the commissure. In addition, it has been reported in zebrafish that Sema3D is present at the midline and provides a chemoattractive cue for anterior commissural axons expressing Nrp1-Nrp2 heterodimers (Mann et al. 2007). The modality of Sema3D guidance in the anterior commissure can, however, be altered by interaction only with Nrp1, further highlighting the distinct functional relationship Semaphorins share with their receptors. Semaphorin signaling can also be modified by interaction with additional molecules such as integrin

receptors, glycosaminoglycans such as chondroitin or heparin sulfate, and members of the Ig superfamily of CAMs such as L1CAM.

### Slits

Slits are secreted glycoproteins that were discovered in a screen for genes that control midline axon guidance in *Drosophila*. In flies mutant for Slit, axons collapse onto the midline and fail to leave, making the fly appear to have a single "slit" of axons. The primary family of Slit receptors comprises the Robo proteins, members of the Ig superfamily of cell adhesion molecules, which mediate a repellent interaction with Slits and promote branching of axons and dendrites in sensory neurons. Slit-Robo binding occurs via the second N-terminal leucine-rich repeat (D2) domain in Slits and the first and second extracellular Ig domains in Robos. There are three Slit and four Robo types in vertebrates, whereas there is less variation in *Drosophila* with three Robos and only one Slit, although the binding domains between all invertebrate and vertebrate species tested are highly conserved. Cleavage of the 140 kDa N-terminal fragment of Slit proteins is still sufficient to bind Robos and stimulate axon elongation and branch formation (Ypsilanti et al. 2010).

In the precerebellar neuron populations of the vertebrate hindbrain, Slit-Robo signaling provides an important repellent guidance cue required for post-midline crossing axons. This signaling, however, is also used for directing the migration of these neurons prior to axon extension. These neuron populations migrate from the dorsal edge of the fourth ventricle (known as the rhombic lip) in a rostroventral direction toward several cranial motor nuclei and then reorient toward the ventral midline. During normal development, the neuronal cell bodies of the lateral reticular nucleus and the external cuneate nucleus cross the midline and project mossy fiber axons to cerebellar granule cells. In contrast, the neuronal cell bodies of the pontine nucleus and inferior olive nucleus (ION) cease migration before the midline and project their mossy fiber axons or climbing fiber axons for the pontine nucleus and ION, respectively, to contralateral cerebellar granule cells or Purkinje cells. Disruption of the repellent Slit-Robo interaction in the vertebrate hindbrain results in the premature migration of pontine neurons to the ventral midline and the extended migration of ION cell bodies across the midline.

Slit-Robo signaling is modulated in *Drosophila* by a regulatory protein called commissureless (Comm). Comm is expressed by midline glia in *Drosophila* where it functions as an important inhibitor of repellent Robo signaling in pre-crossing axons at the ventral nerve cord but is downregulated post-crossing to promote axon projection away from the midline and toward the contralateral target. Although no orthologs of Comm exist in vertebrates, regulatory proteins such as the Robo3 receptor have arisen to promote midline crossing of commissural axons via interaction with other guidance molecules. In mammals, Robo3 has evolved a divergent function from the other Robos, whereby disruption of Robo3 results in a severe phenotype that reduces the ventral migration of precerebellar neurons and prevents their contralateral projection. Unlike the repulsive interaction between Robo1/2 and Slits in other animals, Robo3 has lost its affinity for Slits in mammals because of amino acid substitutions in the first extracellular Ig domain. Instead, Robo3 interacts

with DCC, with their intracellular domains forming a receptor complex that potentiates chemoattractive binding with Netrin-1 at the floor plate to promote neuronal cell migration toward the midline (Zelina et al. 2014). Furthermore, in the spinal cord Robo3 is expressed in pre-crossing commissural neurons and promotes axon guidance toward the midline by exhibiting a repellent interaction with the diffusible glycoprotein NELL2 in the ventral horns, thereby preventing axons from entering the motor columns (Jaworski et al. 2015).

Slits have also evolved multiple interactions with additional molecules. For example, heterophilic interactions with molecules such as heparin sulfate proteoglycans (HSPGs) promote Slit-Robo-mediated repulsion. It has been shown that HSPGs can act as coreceptors for Slits by binding the fourth N-terminal leucine-rich repeat (D4) domain to induce Slit homodimerization, which potentiates Robo signaling. Overall, although there have been changes across species, Slit-Robo signaling has remained a potent mediator of axonal midline crossing throughout evolution.

### Morphogens

Morphogens are secreted proteins that play critical roles in cell fate specification and tissue patterning early in development. Morphogens such as Wnts, Sonic hedgehog (Shh), and the bone morphogenetic proteins (BMPs) provide early cues for axon guidance, often via noncanonical signaling pathways, and can function in parallel with other guidance molecules.

The function of Wnts as guidance molecules is highly conserved across vertebrates and invertebrates, particularly with regards to guidance along the anterior-posterior axis. The Wnts are a large and complex family of proteins, with at least 12 subfamilies present in vertebrates. Early studies in chick showed that Wnt4 and Wnt7 are expressed in a decreasing anterior-posterior gradient in the ventral midline of the spinal cord during gestation and have a chemoattractive interaction with Frizzled-3-positive commissural axons to control their rostral turning after midline crossing. During the postnatal stage, Wnt1 and Wnt5a are expressed in a similar expression gradient at the dorsal midline of the chick spinal cord to control the posterior extension of corticospinal tract axons via a chemorepellent interaction with Ryk receptor. In hamsters, Wnt5a-Ryk-mediated repulsion was also found to control guidance of the major commissure of the forebrain, the corpus callosum, using a noncanonical downstream signaling pathway involving an influx of extracellular calcium through transient receptor potential (TRP) channels. Similarly, work in rat spinal cord showed that Wnt4-mediated guidance of post-crossing commissural axons uses a noncanonical pathway involving atypical protein kinase C (aPKC) and signaling by phosphatidylinositol-3-kinases (PI3Ks) and heterotrimeric G-proteins. Wnt5 is also expressed along the anterior-posterior axis of each segment of the ventral nerve cord in *Drosophila* to control repellent axon guidance at the midline; however, it is expressed in the opposite gradient to that found in chick (increasing anterior-posterior gradient) and signals via the *Drosophila* ortholog of Ryk, known as Derailed (Onishi et al. 2014).

Wnts have also been implicated as important guidance cues in the formation of neurosensory topographic maps. In particular, Wnt3 is expressed in a dorsal-ventral

gradient in the chick optic tectum and provides repellent and attractive guidance cues for ganglion cell axons projecting from the dorsal-ventral axis of the retina. An EphrinB1 expression gradient is also present in the tectum and provides an additional and balancing guidance force with Wnt3 for the retinal ganglion cell axons to achieve the accurate termination pattern required to form a retinotectal map (Schmitt et al. 2006).

Shh is another morphogen that provides directional cues to multiple neuronal populations using noncanonical signaling pathways during vertebrate development. Shh provides a chemoattractive cue to pre-crossing commissural axons in the spinal cord by binding with the receptors Boc and Ptch1 and activating the Smoothened (Smo) protein and Src family kinases locally within the growth cone to mediate the attractive response. Shh also influences the guidance of retinal ganglion cells at the optic chiasm by providing a repellent guidance cue that directs contralaterally projecting retinal ganglion cells, which do not express Boc, through the chiasm. Retinal ganglion cells that do express Boc, however, do not undergo repellent guidance through the chiasm which, in combination with other guidance cues, such as Nrp1 and EphB1, results in their ipsilateral projection (Fabre et al. 2010).

Finally, BMPs are members of the transforming growth factor beta (TGF-ß) family of molecules that provide an important chemorepellent guidance cue for commissural axons along the dorsal-ventral axis of the spinal cord. BMP7 and BMP family member growth differentiation factor 7 (GDF7) are expressed in the roof plate and form a heterodimer that functions as a chemorepellent cue that guides spinal cord commissural axons away from their site of origin adjacent to the dorsal midline and directs them toward the ventral midline. BMP-mediated guidance is initiated by BMP receptors type II and subsequently type IB, which are both expressed on the dorsal commissural neurons, and downstream signaling occurs via a noncanonical pathway involving PI3K. There is also evidence to suggest BMPs can modulate axon growth, and possibly axon guidance, by activating LIM kinase and *cofilin* signaling to control the level of actin polymerization and growth cone motility (Yam and Charron 2013).

### Cell Adhesion Molecules

Cell adhesion molecules (CAMs) play an important role in mediating the interaction between developing neurons and their environment, including adjacent cells and extracellular molecules. It is known, for example, that the adhesion properties of CAMs are required for axon fasciculation during pathfinding along pioneer axons and that CAM signaling can stimulate axon growth and elongation. CAMs are also an important component of axon guidance systems in the developing central nervous system and can function in association with or without axon guidance molecules to mediate this process. Of the different classes of known CAMs, the Ig superfamily is the most extensively studied with respect to axon guidance and includes subfamilies of molecules such as the L1 subfamily. Ig CAMs contain an extracellular region of Ig domains and fibronectin type III repeats and are either transmembrane or GPI-anchored. It has been proposed that CAMs enable axon elongation and movement by transmitting the retrograde movement of depolymerized F-actin from the

peripheral to the central domain of the growth cone. In particular, membrane-localized CAMs are linked to actin subunits via a molecular "clutch," and upon retrograde transport of the actin by myosin, CAMs interact with immobile ligands in the extracellular environment to transmit the transport force into forward traction for the growth cone. Ankyrin-B is one example of a clutch used for L1CAM-actin coupling during neurite growth (Kamiguchi 2007; Hashimoto et al. 2009) (Fig. 1b).

The association of CAMs with Semaphorins has been observed in several systems of vertebrate axon guidance. For example, NrCAM, a member of the L1 subfamily of Ig CAMs, forms a receptor complex with Nrp2 to mediate the chemoattractive and chemorepellent responses of Sema3B in commissural axons. In the anterior commissure of the forebrain, the modality of Sema3B-mediated guidance varies between the anterior and posterior tracts and is dependent on the localization of the downstream signaling molecules focal adhesion kinase (Fak) and the non-receptor tyrosine kinase Fyn, intracellularly or at the plasma membrane. In the spinal cord, however, NrCAM has a more dynamic role acting as a molecular switch to modulate the guidance of commissural midline crossing at the ventral floor plate. Plexin-A1 and Nrp2 are expressed in spinal cord commissural axons and are important receptors for Sema3B, thereby controlling axon guidance at the floor plate. Prior to midline crossing, however, Plexin-A1 is inhibited by calpain activity, and the axons are free to enter the floor plate via chemoattractants such as Netrins and Shh. Upon midline crossing, NrCAM inhibits calpain processing of Plexin-A1 and allows for an upregulation of this receptor and its repellent interaction with Sema3B to project axons away from the midline (Nawabi et al. 2010). Furthermore, NrCAM is known to interact with other Ig CAMs such as axonin-1/TAG-1 to mediate the guidance of chick spinal cord commissural axons without axon guidance molecules, indicating that CAM-CAM interactions are sufficient to control axon guidance in some neural systems (Stoeckli et al. 1997).

The function of L1CAM has also been extensively studied in the development of the central and peripheral nervous systems. L1CAM interacts with Nrp1 in *cis* to form a receptor complex that mediates Sema3A repellent signaling. In mice lacking L1CAM, there is a guidance error of corticospinal axons at the pyramidal decussation because axons do not respond to Sema3A at the ventral floor plate, resulting in a large portion of the axonal tract projecting ipsilaterally. If the L1CAM mutation involves the sixth Ig domain, which mediates L1CAM-L1CAM homophilic interactions, ventriculomegaly can also occur possibly because of hypoplasia of the axon tracts. In addition, L1CAM is required for the guidance and fasciculation of thalamocortical axons and for the topographic mapping of retinocollicular projections in mice. The L1CAM ortholog Neuroglian has also recently been identified as an important coordinator of synapse and mushroom body formation, as well as axon growth and fasciculation in *Drosophila*.

### Proteoglycans

Proteoglycans are involved with many important functions in the developing nervous system, such as cell differentiation, migration, and synaptogenesis, and are known to also influence axon guidance. The basic structure of proteoglycans

consists of a core protein with one or more glycosaminoglycans covalently attached, such as heparin sulfate (HS) or chondroitin sulfate (CS), after which the proteoglycans are named. HSPGs have been shown to interact with many receptors/ligands of the families of axon guidance molecules mentioned so far, and inhibition of HSPGs or the enzymes that synthesize them can result in phenotypes similar to mutations of the guidance molecules. For example, HSPGs are required for the regulation of Slit/Robo function during guidance of retinal ganglion cells. Genetic screens in developing zebrafish showed that mutations in *dackel* and *boxer* cause defects in sorting of axons in the optic tract, and subsequent work showed these genes code for the exotosin glycosyltransferases, Ext2 and Extl3, required for the synthesis of HS. In addition, these mutations only affect retinal ganglion cell axon sorting and not their topographic mapping in the tectum in vivo, similar to the phenotype of Robo2 mutants, suggesting HSPGs have a specific guidance function in the optic tract that is achieved via interaction with Robo2 signaling (Lee et al. 2004).

HSPGs also mediate Slit/Robo function during guidance of forebrain commissures in mice, under the influence of HS-modifying enzymes called heparan sulfotransferases, Hs6st1 and Hs2st. In Hs6st1 knockout embryos, the retinal ganglion cells show a reduced sensitivity to Slit2-mediated repulsion at the optic chiasm, resulting in aberrant projections toward the contralateral retina. Similarly, Hs2st knockout embryos show aberrant and ectopic axonal projections along the midline. In mice, mutations for Hs6st1 and Hs2st phenocopy mutations for Slit2 and Slit1, respectively, suggesting an interaction between the heparan sulfotransferases and Slit signaling. Furthermore, Hs6st1 and Hs2st knockout also produce severe phenotypes in the corpus callosum, completely preventing midline crossing of callosal axons and forming ipsilateral Probst bundles (Conway et al. 2011). The sulfation of HS induced by Hs6st1 and Hs2st is one of a large range of modifications that can be made on glycosaminoglycans after they are synthesized. In addition to other enzymatic modifications such as deacetylation and epimerization, the number of combinations of possible HS modifications is vast and modulates a diverse range of molecular interactions, of which many are not yet known. This diversity of interactions is also thought to contribute to establishing the complex guidance systems present in the nervous system from a relatively small number of axon guidance molecules.

HSPGs are also important for modulating the function of morphogens during axon guidance. In *Drosophila*, mutations of HS-synthesizing enzymes, such as the exotosin glycosyltransferases Ext1 (Ttv) and Ext2 (Sotv) required for HS chain polymerization, show similar phenotypes to mutations for Wingless (Wnt), hedgehog (Hh), and decapentaplegic (BMP) that involve malformations of nerve fiber tracts. Subsequent experiments show that Ttv and Sotv interact with the signaling of these morphogens by promoting their expression and graded extracellular distribution required for axon guidance function (Bornemann et al. 2004). In addition, HSPGs can facilitate morphogen signaling by promoting the concentration of ligands around their receptors in order to enhance their binding. This ligand presentation function is also dependent upon lipid raft domains, which are thought to

recruit and aggregate receptors after ligand binding, thereby promoting and localizing the downstream signaling (Guirland et al. 2004).

## In Vitro Assays of Axon Guidance

In vitro assays have elucidated the principle mechanisms of axon guidance that we understand today. For example, the generation of concentration gradients in vitro has been used to demonstrate directed axon growth up or down a molecular gradient. This has enabled the identification of new guidance molecules and the demonstration of axon guidance properties of known growth promoting and patterning molecules. These techniques range from simple assays involving cocultures of developing and target tissues to more elaborate assays involving multiple chambers, producing carefully controlled gradients, and live imaging of single axon growth. Once guidance molecules are discovered in neural systems, in vitro assays are instrumental in determining their mechanism of action on axon growth, guidance and growth cone function. The in vitro assays described below are the most commonly used in this field to investigate the function of both secreted and membrane-bound guidance cues.

### Coculture Assay

Coculture assays are simple assays where explants of the developing neural tissue and the target tissue are cultured together in close proximity within an extracellular growth matrix, usually consisting of collagen (Lumsden and Davies 1983) (Fig. 4a). This setup allows for direct observation of the growth of a large number of axons simultaneously to determine if the target tissue promotes directed axon growth. Coculture assays also provide an indication of the guidance properties of the cues released by the target tissue (attractive or repellent) by measuring axonal length and direction of growth. However, coculturing is most commonly used as a qualitative assay of guidance given it is a delicate setup that can be difficult to reproduce results based on the specific positioning of the explants with respect to each other. In addition, the concentration of guidance molecules released by the target tissue into the collagen matrix is not known, although the technique can be modified to replace the target tissue with filter paper or gel soaked in purified protein or stripes of protein microprinted on the gel which dissolve into the matrix at known concentrations (Dupin et al. 2013). Alternatively, the assays described below can be used to provide a more quantitative measure of the association between guidance molecule concentration and its effect on axons.

### Micropipette Assay

The micropipette assay, also known as the growth cone turning assay, uses a micropipette to release guidance molecules proximal to an axonal growth cone to

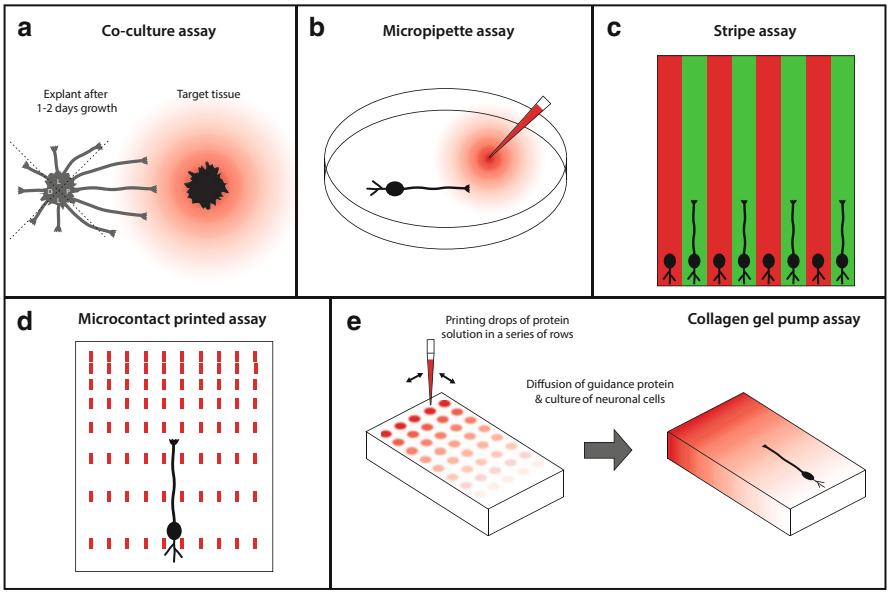


**Fig. 4 In vitro assays of axon guidance** (**a**) Coculture assay showing axon growth from an explant after 1–2 days of culture. In the case of chemoattraction, the longest axons extend from the side of the explant proximal (P) to the target tissue, and project toward this tissue which secretes a chemoattractant into the medium. Shorter axons project from the lateral (*L*) and distal (*D*) sides of the explant. (**b**) Micropipette assay showing a chemoattractant protein solution being injected onto a permissive culture surface to influence guidance of the developing axon. (**c**) Stripe assay showing axon growth after 1–2 days of culture on a substrate with two different guidance proteins shown in alternate colored stripes. The axons show preferential growth on the green substrate, indicating that it is chemoattractive or that the green substrate is permissive and the red substrate is chemorepellent. (**d**) Microcontact printed assay showing an array of lines of a chemorepellent protein printed in a pattern of increasing density. The growing axon extends along the substrate until it encounters a line with a high enough repellent concentration that it prevents further growth. (**e**) Collagen gel pump assay. The left image shows the minipump printing drops of chemorepellent protein solution onto the collagen gel in a series of lines of increasing concentration. The right image shows the diffusion of the protein solution across the gel to create a smooth gradient and the subsequent neuronal cell culture that shows a reduction of axon extension once the chemorepellent concentration gradient is sufficiently high

determine the response of a single axon (Fig. 4b). Primary cultures of neurons are grown on a poly-D-lysine and laminin coated coverslip substrate until the neurons extend axons. The micropipette tip is usually placed at an angle of 45° from the axon's longitudinal axis about 100 μm from the growth cone, and the guidance cue is released in a pulsatile manner into the culture medium to form a gradient across the growth cone. Accurate formation of concentration gradients depends on several factors including the molecular weight and charge of the guidance molecule and the pulse duration and frequency, which can result in variability in the guidance cue gradient and area of diffusion. However, once this technique has been optimized it allows for highly detailed analysis of a single axon and has

been used extensively to investigate the role of molecules, such as calcium and cAMP, in growth cone signaling networks during axon guidance (Forbes et al. 2012).

## Stripe Assay

Stripe assays were initially developed to study topographic mapping of retinotectal axons in the chick. Cell membranes of different regions in the embryonic tectum were used to establish alternating stripe-patterned substrates upon which retinal tissue was cultured. By providing retinal axons with a binary choice of substrates, Walter et al. (1987) observed the growth preference of the developing axons and began to decipher the guidance properties of the different tectal regions with respect to retinal axon mapping. Subsequent work showed that Ephrin-As are prominent guidance molecules in this system.

The stripes of this assay are created either from purified proteins or cell membrane fractions isolated from tissue or cells. The proteins or membrane fractions are deposited onto a surface of glass, plastic, or nucleopore membrane using a silicone matrix with parallel microchannels imprinted in the surface and, once the matrix is removed, leaves a pattern of stripes adhered to the surface. In addition, a second membrane fraction can be added to the gaps between the first stripes by applying it to the whole surface, resulting in the second protein/membrane fraction only adhering to the parts of the surface not blocked by the first, provided the two proteins show low affinity toward each other (Fig. 4c). Laminin is also applied to these striped "carpets" to facilitate permissive axonal growth and interaction with the plated guidance molecules. Once neurons are cultured in the stripe assay, they encounter the two different substrates and show preferential growth on the substrate that is permissive and chemoattractive and avoid stripes that are chemorepellent and/or nonpermissive. These results provide direct measurement of the axonal response to the substrates that can be easily quantified and reproduced.

## Microcontact Printed Assay

Microcontact printing was initially developed for printing alkanethiolates onto gold surfaces to generate electrically conductive microcircuits. This method was later adapted to printing proteins on substrates for studying cell biology in vitro, including neurodevelopmental systems, to investigate neuronal outgrowth and synaptogenesis as well as axon guidance. Given the inherent difficulty in establishing and maintaining diffusible gradients, microcontact printing provides a fixed, substrate-bound discontinuous protein gradient that can be used to study long-term cell growth. It can also generate reproducible gradients where the spatial dimensions such as the density, gradient, concentration, and shape of the printed pattern can be controlled. It is this precise control of parameters that allowed microcontact printing

to be successfully used in the study of Ephrin-A gradients during guidance of retinal axons in chick to differentiate the response profiles between temporal and nasal retinal regions (Philipsborn et al. 2006).

This assay involves the microprinting of a solution of guidance protein onto a permissive substrate, which is achieved via a micrometer-sized stamp made of PDMS (polydimethylsiloxane). The stamp is inked in the protein solution and used to imprint a pattern of any desired spatial density or distribution, with the stamp itself commonly having the shape of a dot or line. A common pattern produced by this technique is a discontinuous gradient where the pattern of stamps decreases in density along the length of the substrate (Fig. 4d). Further design variations include printing different shapes and solutions onto the same substrate, for example, to test the effect of different guidance molecules on the same cultured axons, to print non-guidance molecules, such as laminin, to test permissive growth, and to use smaller stamps to make submicron patterns to investigate other events such as synaptogenesis. Finally, microprinted substrates can be relatively fast to make, particularly if multiple replica silicone stamps are created from the master mold, allowing multiple prints to be made on the substrate simultaneously.

## Collagen Gel Pump Assay

The pump assay is used to create a constant and continuous gradient of guidance protein within a collagen gel, upon which neurons are cultured. The gradient is created with a diffusive printing process using a piezoelectrically controlled minipump to "print" drops of the protein solution, commonly in a series of lines of increasing concentration, onto the surface of the collagen gel. The solution then diffuses across the gel to create a smooth gradient that remains stable for at least one day, making it compatible with long-term culture experiments (Fig. 4e). For example, this assay was first used to investigate the gradient sensing properties of postnatal rat dorsal root ganglion axons to NGF over a 36–40 h culture period. The final shape and steepness of the gradient is dependent on the diffusive properties of the solution on the collagen gel, but it can also be modified by altering other parameters of the printing process, such as the density and concentration of the droplets. For example, the printing can be modified to produce nonlinear concentration gradients or gradients of different shapes. Furthermore, pump assay gradients require only a small amount of protein to produce and can be generated quickly and precisely, allowing for a high level of reproducibility. The highly efficient control of the protein gradient generation achieved in collagen gel pump assays allowed this to be used in highly sensitive experiments measuring the threshold of responsiveness of axonal growth cones. These experiments have shown that growth cones are able to detect as little as one guidance molecule across their structure and that this sensitivity is present across only a small range of guidance molecule concentrations (Rosoff et al. 2004).

## Outlook

Axon guidance research has traditionally focused on unraveling the mechanisms controlling the guidance of individual neurons or explants of neurons, by one molecule and/or its receptor. One established mechanism that allows growth cones to navigate their environment, and which can be studied in vitro, is the presence of molecular gradients. Growth cones are able to accurately and reliably detect the direction of a gradient and then convert this into guided movement, although it is still unclear exactly how this is achieved. Guidance proteins are present in very low amounts in vivo and have inherent fluctuations in the concentration of their molecular gradients, yet growth cones can reliably respond to these stimuli. The in vitro assays described above have been important in determining the nature of the interactions mediated by guidance molecules. Attractive or repellent interactions are produced by specific ligand-receptor pairs, which initiate changes in the polymerization and distribution of cytoskeletal components within the growth cone that extend or retract filopodia, respectively. The modality of guidance can also be altered by the formation of receptor protein complexes that influence downstream signaling and the specificity of ligand interactions. Understanding the intracellular signaling in axonal guidance is an active area of research and one that may lead to new therapeutics for promoting axonal growth.

The current major challenge in axon guidance research is to understand how axon guidance systems operate in vivo. Many guidance molecules are expressed simultaneously and affect the same neuronal population during development, raising the question of how multiple guidance cues are integrated by the neuron to provide a coherent and accurate guidance signal. Furthermore, guidance gene expression is highly dynamic, and the expression profile is regulated over time to ensure changes in ligands/receptors occur at critical developmental stages to allow appropriate responses to local environmental cues. To study these complex guidance systems that function in parallel in vivo, it is possible to examine them across species given that guidance genes are highly conserved between vertebrate, as well as invertebrate, species. Functional analyses have shown that many of the same molecules act to guide similar groups of neurons between species and suggest that during evolution guidance signaling pathways and their molecular interactions have also, to some extent, been conserved. Furthermore, technological advances will allow for the creation of improved methods for studying more complex axon guidance systems in vitro, such as new techniques for creating accurate and continuous concentration gradients that can be maintained over even longer periods of time. Combined with improvements in computational and analytical methods, these new techniques will provide greater insight into the cellular mechanisms involved in gradient-mediated axon guidance.

Finally, understanding axon guidance not only provides an insight into the evolution of nervous system development, but it has also been the foundation for understanding axonal regeneration after injury or disease. Injured neurons are known to be responsive to axon guidance cues in a similar way to developing axons, and it is known that upregulation of repellent guidance molecules can occur after neuronal injury to mitigate their regeneration. These observations demonstrate that multiple

axon guidance systems are not only present in the mature nervous system but that they play an important role in its function. An improved understanding of the spatiotemporal and molecular signaling aspects of guidance systems active during development can help to inform genetic interventions for injured mature neurons without causing adverse effects.

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