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WIRING THE BRAIN: THE LOGIC AND MOLECULAR MECHANISMS OF AXON GUIDANCE AND REGENERATION

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Wherein lies that marvelous power which enables the nerve fibers from very distant cells to make contact directly with certain other nerve cells . . . without going astray or taking a roundabout course? . . . I believe that one could . . . think of processes like the phenomenon called . . . chemotaxis.

Santiago Ramon y Cajal, 1893

I. Introduction: The Logic of Axon Guidance

The development of the brain is a wondrous event. In just nine months, the embryonic brain, which starts out the size of a pin-head and contains just a few hundred nerve cells or neurons, undergoes a period of explosive growth that results in the generation of close to a trillion neurons. The right kinds of neurons have to be made at the right time, in the right place, and in the right numbers, and, even more daunting, each of these trillion neurons must connect with an appropriate set of target cells to form the neuronal circuits that underlie the functioning of the brain, for perception, for the control of movement, and for cognition.

We have been interested in elucidating the mechanisms that direct the formation of neuronal circuits. This process starts when each neuron, as it forms, extends a thin cable-like structure, called the axon, that navigates through the embryonic environment to reach its targets to form synaptic connections (diagrammed in Fig. 1A). Remarkably, the growth of axons to their targets is a highly directed process. Individual axons follow very stereotyped trajectories and make very few errors of projec-

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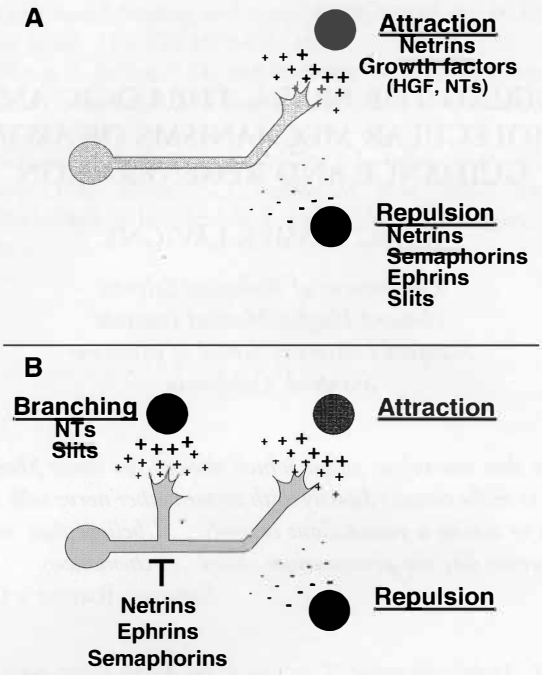


Fig. 1. Axon guidance and branching are regulated by multifunctional wiring cues. (A) Axons are tipped by growth cones, which lead them to their targets under the influence of attractive and repulsive guidance molecules. By 2003, three sets of chemoattractants for developing axons had been identified in vertebrates: the Netrins, which are phylogenetically conserved, and two sets of growth factors (HGF and Neurotrophins), which are not. Four important families of repellents had also been identified: Netrins (which are thus bifunctional), Semaphorins, Ephrins, and Slits. More recently, evidence has been mounting that molecules that are classically thought of as morphogens (Hedgehog, BMP, and Wnt proteins) function in attraction and repulsion (see Fig. 5 and text). (B) Axons connect to multiple targets by sprouting collateral branches, under the influence of positive and negative regulators of branching. Neurotrophins, Slits, Netrins, Ephrins, and Semaphorins also regulate branch formation. See text for details. (See color plates.)

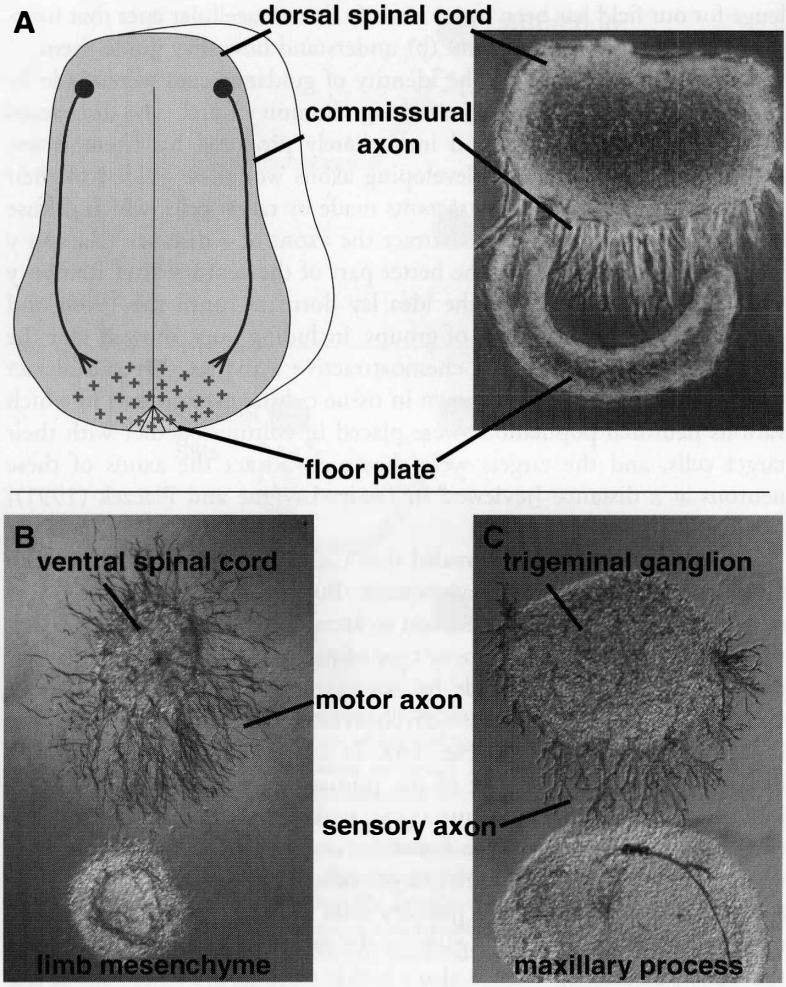
tion. The way this works is that axons are tipped by a motile sensory structure, called the growth cone, that actively probes the environment for guidance information in the form of proteins that function as guidance cues to instruct the axons to migrate in particular directions. Thus, a chal-

lenge for our field has been to (a) identify the extracellular cues that function to guide growth cones and (b) understand how they guide them.

The first speculations on the identity of guidance cues were made by the great Spanish neurobiologist Santiago Ramon y Cajal, who discovered the growth cone in 1893 and immediately proposed his chemotropic theory, according to which developing axons would be guided to their targets by diffusible chemoattractants made by target cells, which diffuse through the environment and attract the axons at a distance (Ramon y Cajal, 1893) (Fig. 1A). For the better part of the century after Ramon y Cajal proposed this theory, the idea lay dormant, until the 1980s and early 1990s, when a number of groups, including ours, showed that the embryo does indeed possess chemoattractive activities that can attract developing axons. This was shown in tissue culture experiments in which various neuronal populations were placed in culture together with their target cells, and the targets were shown to attract the axons of these neurons at a distance [reviewed in Tessier-Lavigne and Placzek (1991)] (see Fig. 2).

These experiments thus revealed that Cajal was right—that axons can be guided by target-derived attractants. But, as it turns out, Cajal was only half right, because, in addition to attractants, it was also found that the embryo contains the opposite type of molecule, chemorepellents that repel axons and that are made by non-target cells and diffuse to create exclusion zones that the axons actively avoid [e.g., Pini (1993); reviewed in Tessier-Lavigne (1994)] (Fig. 1A). In addition, while attention had focused initially on guidance of the primary growth cone of the axon, interest developed in how neurons can make connections with multiple target cells. In the mammalian brain, for instance, neurons make contact on average with over a hundred target cells. The way they do this is by sprouting collaterals from the primary axon shaft to innervate these additional target cells (Fig. 1B). Again, by the early 1990s, it had been found that the branching of axons is also a highly directed process and that it is controlled by branching-promoting as well as by branch-inhibiting activities [reviewed in O'Leary et al. (1990)].

This, then, is where we stood by the early 1990s: We knew that there were attractive, repulsive, and branch-regulating activities, but none of the molecules that mediate those activities were known. The members of my laboratory and I have been interested in identifying the proteins that mediate attraction, repulsion, and branching, and in this chapter I would


A
dorsal spinal cord
commissural axon
floor plate
B
ventral spinal cord
motor axon
limb mesenchyme
C
trigeminal ganglion
sensory axon
maxillary process

like to summarize our work, and that of the field as a whole, in identifying such cues and in deciphering the logic and molecular mechanisms of axon guidance and branching.

This chapter will be divided into two major parts. In the first, I would like to provide a quick overview of what we know about the molecules of axon guidance—the molecules that function as cues and receptors for attraction, repulsion, and branching. This will then provide necessary background information to address in the second part of the chapter how the embryo uses these cues to guide axons and, in particular, how the embryo tackles two important challenges.

The first challenge is to ensure fidelity in axon guidance—that is, to make sure that axons are guided accurately to their targets, making few, if any, errors of projection. The second challenge, related to the first, is to ensure that this fidelity is achieved even for axons that must project over long distances, such as axons that connect the brain and spinal cord. The challenge for these long-projecting neurons is that they can't be guided in one fell swoop, say by a single chemoattractant factor present in their distant target, because the distance over which it would have to act is just too great. Instead, what these axons do is to break up their trajectory into segments. Each of these segments ends in an intermediate target, and the axons extend to their final targets by navigating from one

Fig. 2. Identification of chemoattractant activities for developing vertebrate axons. (A) Netrin-1 is a floor-plate-derived chemoattractant for commissural axons. Left: Diagram of the developing rat spinal cord. Commissural axons project from cell bodies in the dorsal spinal cord to floor plate cells at the ventral midline. Right: When an explant of dorsal spinal cord is cultured in a three-dimensional collagen matrix with a piece of floor plate, a factor secreted by the floor plate stimulates the directed outgrowth of commissural axons. The factor is Netrin-1. [Adapted from Tessier-Lavigne et al. (1988) and Kennedy et al. (1994).] (B) HGF is a limb mesenchyme-derived chemoattractant for motor axons. When a piece of ventral spinal cord containing motoneurons is cultured with a piece of target limb mesenchyme, a factor secreted by the mesenchyme stimulates the profuse and directed outgrowth of motor axons. This factor is HGF. [Adapted from Ebens et al. (1996).] (C) Neurotrophins constitute a maxillary process-derived chemoattractant for trigeminal sensory axons. When a trigeminal sensory explant is cultured with the target maxillary process, a factor secreted by the maxillary process stimulates the profuse and directed outgrowth of trigeminal axons. This factor is combination of the neurotrophins BDNF and NT3. [Adapted from O'Connor and Tessier-Lavigne (1999).] (See color plates.)

intermediate target to the next. Thus, to extend from point A to point Z, the growth cone must navigate points B, C, D, and so forth. Importantly, embryological and genetic evidence has indicated that for each of the segments of its trajectory, the axon must be attracted by the intermediate target: to grow from A to B, it must first be attracted to B. But this immediately raises a paradox: If the intermediate target, point B, is so attractive, why doesn't the axon just stop there, and how can it possibly move on? The answer, we now know, is that the axon can move on because the growth cone possesses a remarkable plasticity, being able to change its responsiveness to cues in the environment: When it reaches the attractive intermediate target, it changes its responsiveness in such a way that this environment, which it previously perceived as attractive, is now perceived as repulsive. This, in turn, results in the growth cone being repelled out of the intermediate target, onto the next leg of its trajectory. In the second part of this chapter, I will describe what we have learned about the mechanisms that underlie this remarkable plasticity—how the growth cone can switch from being attracted to being repelled at an intermediate target.

Finally, at the end of this chapter, I will discuss very briefly how some of the lessons we have learned from studies of embryonic axon guidance are applicable to the problem of axonal regeneration following injury in the adult nervous system, particularly following paralyzing injuries to the spinal cord. When axons connecting the brain and spinal cord are severed by such an injury, they reform growth cones and attempt to regrow to their targets, but they fail to do so, with the consequence that the paralysis that accompanies these injuries is usually permanent. I will address whether what we have discovered about axon growth and guidance in the embryo can help us in attempts to stimulate the regrowth, reguidance, and reconnection of these axons with their targets, to alleviate this paralysis.

Since this chapter is meant principally as a review of our work, most of the references will be to our studies, though key studies by other investigators in the field will also be cited. More comprehensive reviews of work in the field as a whole are provided in Tessier-Lavigne and Goodman (1996), Chisholm and Tessier-Lavigne (1999), and Dickson (2002).

II. Identifying Ligands and Receptors for Axon Guidance

A. The Netrins

Let us turn, then, to the identification of the cues that guide axons. The approach we took initially in my laboratory was to try to identify chemoattractants for developing axons by characterizing chemoattractive activities described in tissue culture. The first activity that we focused on was an activity that Marysia Placzek and I had described when we were postdoctoral fellows with Tom Jessell and Jane Dodd, and which operates in the embryonic spinal cord (Tessier-Lavigne et al., 1988; Placzek et al., 1990). This activity is made by a specialized group of cells at the ventral midline of the spinal cord called floor plate cells, and it functions to attract the axons of a specialized set of neurons, so-called commissural neurons, whose cell bodies are located in the top, or dorsal, half of the spinal cord and which extend along a dorso-ventral trajectory to the floor plate cells at the ventral midline (Fig. 2A). We discovered the existence of a chemoattractive activity of floor plate cells for these axons in tissue culture experiments in which we cut out the dorsal half of small pieces of spinal cord from embryonic rats and placed them in culture alone, with control tissues, or with target floor plate. As illustrated in Fig. 2A, the target floor plate, but not other tissues, stimulates the profuse and directed outgrowth of commissural axons from these explants, showing that the floor plate makes a chemoattractive activity.

When I set up my laboratory at UCSF in 1991, I decided to try to identify this activity because, at the time, no chemoattractant for developing axons was known. We weren't able to purify the factor directly from floor plate cells, because it is too small a tissue source, but fortunately we found that a similar activity is present in extracts of embryonic brain, which provides a more abundant tissue source. Indeed, we were able to purify this activity through six steps of purification to homogeneity starting from 25,000 embryonic chick brains. This purification showed that the activity in brain extracts is due to two related proteins of molecular weight 78 and 75 kDa, which we named Netrin-1 and Netrin-2, from the Sanskrit word "netr," meaning "one who guides" (Serafini et al., 1994). Pleasingly, although these proteins were purified from brain, we found that one of them, Netrin-1, is made by floor plate cells, leading us thus to propose that Netrin-1 functions to attract commissural axons to the ventral midline in the developing spinal cord in vivo (Kennedy et al.,

1994). We have, indeed, verified this theory through genetic analysis (Serafini et al., 1996), as summarized below. Before discussing this, however, I must first describe how this discovery revealed a remarkable conservation of guidance mechanisms across evolution. Indeed, in parallel and independent experiments, Ed Hedgecock and colleagues were studying an analogous guidance event in a more primitive organism, the nematode worm *C. elegans*. They focused on a set of sensory neurons with cell bodies in the dorsal half of the worm that extend axons to the ventral midline of the worm, and they screened for mutants in which these axons are misrouted. In this way, they discovered a mutant, the *unc-6* mutant, first isolated by Sydney Brenner (Brenner, 1974), in which many of these axons failed to project appropriately to the ventral midline (Hedgecock et al., 1990). When they cloned the *unc-6* gene, they found that it encodes a secreted protein (Ishii et al., 1992), and they later showed that this protein, UNC-6, is made by cells in the ventral midline region (Wadsworth et al., 1996), leading them to propose that, as with Netrin-1 in the spinal cord, UNC-6 attracts axons to the ventral midline in *C. elegans*.

Importantly, UNC-6 and Netrin-1 are species homologs, demonstrating a remarkable conservation of guidance mechanisms. Of note, it isn't simply that members of the Netrin family function as guidance cues in both species. Instead, what is most striking is that Netrins are used for the very same purpose in both, namely to attract axons to the ventral midline. We can therefore think of the worm as a miniature spinal cord; we can also think of the spinal cord as the worm within us.

I believe it is fair to say that at the time when we made these observations, they were quite unexpected. Indeed, it was widely believed at the time that the mechanisms involved in wiring the mammalian brain, which is so much more sophisticated and complex than that of invertebrates, would be distinct from those operating in those lower organisms (Easter et al., 1985). These results proved that assumption wrong by showing that at least some of these mechanisms are highly conversed across species.

B. Other Attractants: Growth Factors

We were naturally interested next in trying to identify additional chemoattractants for developing axons, and, ironically, although the first one we identified was highly conserved, the next two we identified were not. We first sought to identify a chemoattractant made by the limb mes-

COLOR PLATES

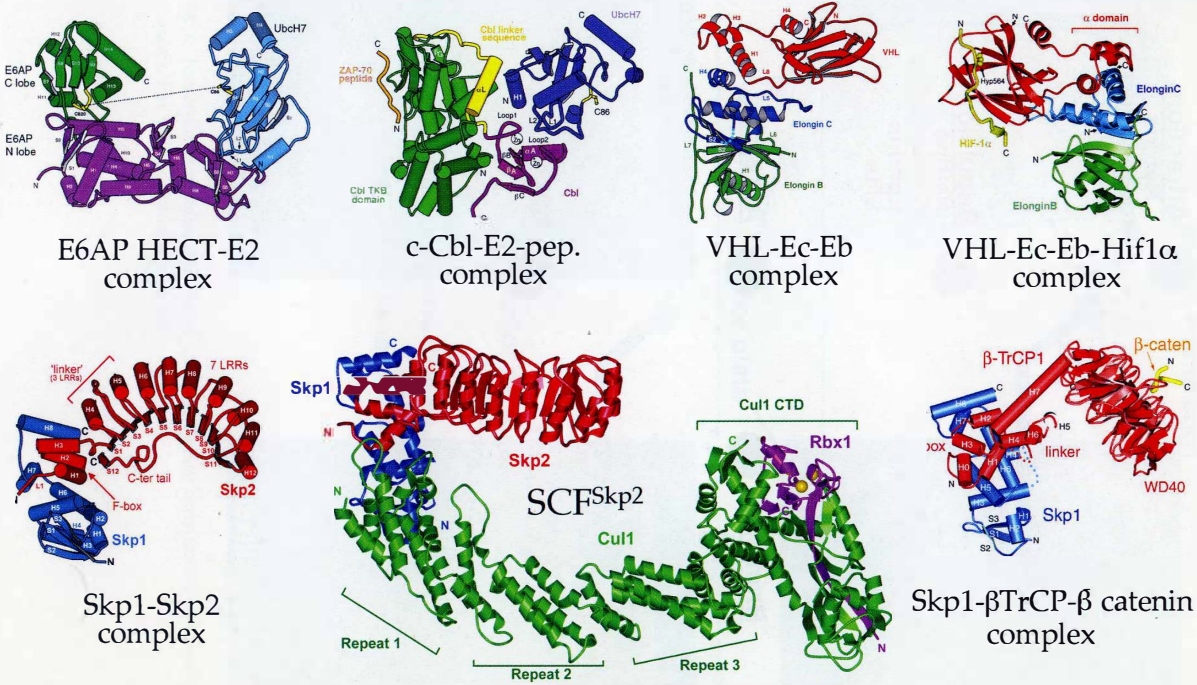


Fig. 2, Pavletich. Crystal structures of ubiquitin ligases or of their subunits discussed in this chapter. For full caption, see page 52.

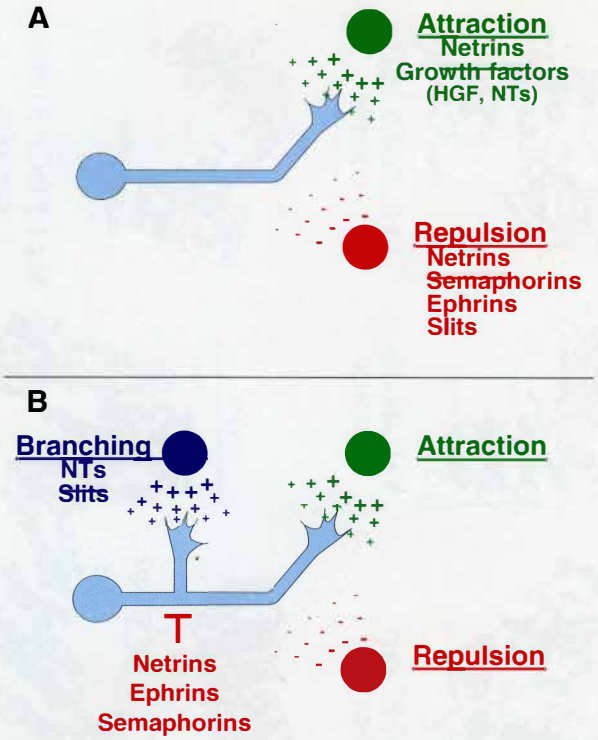


Fig. 1, Tessier-Lavigne. Axon guidance and branching are regulated by multifunctional wiring cues. For full caption, see page 106.

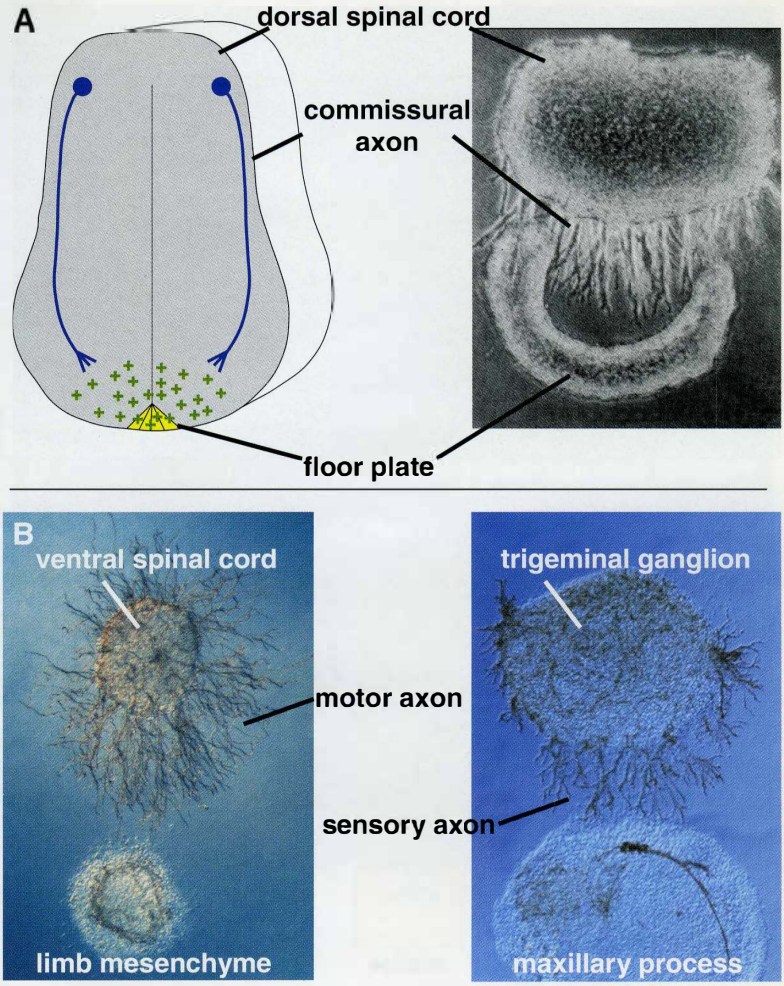


Fig. 2, Tessier-Lavigne. Identification of chemoattractant activities for developing vertebrate axons. For full caption, see page 109.

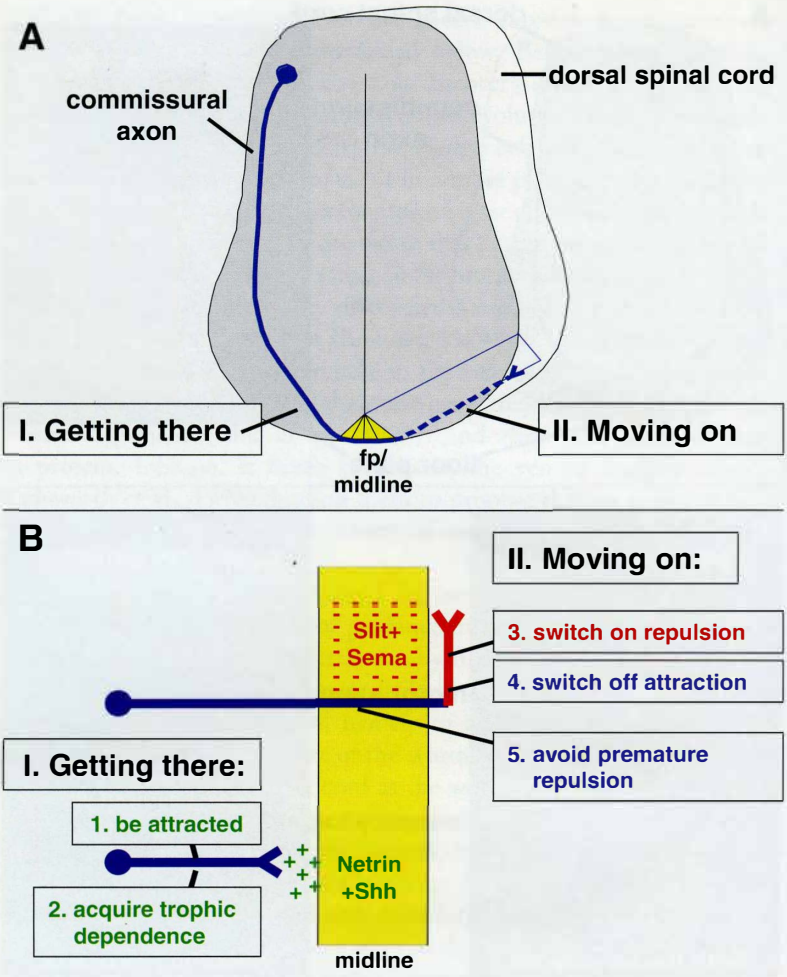


Fig. 4, Tessier-Lavigne. Gerting there and moving on: Five key regulatory mechanisms. For full caption, see page 119.

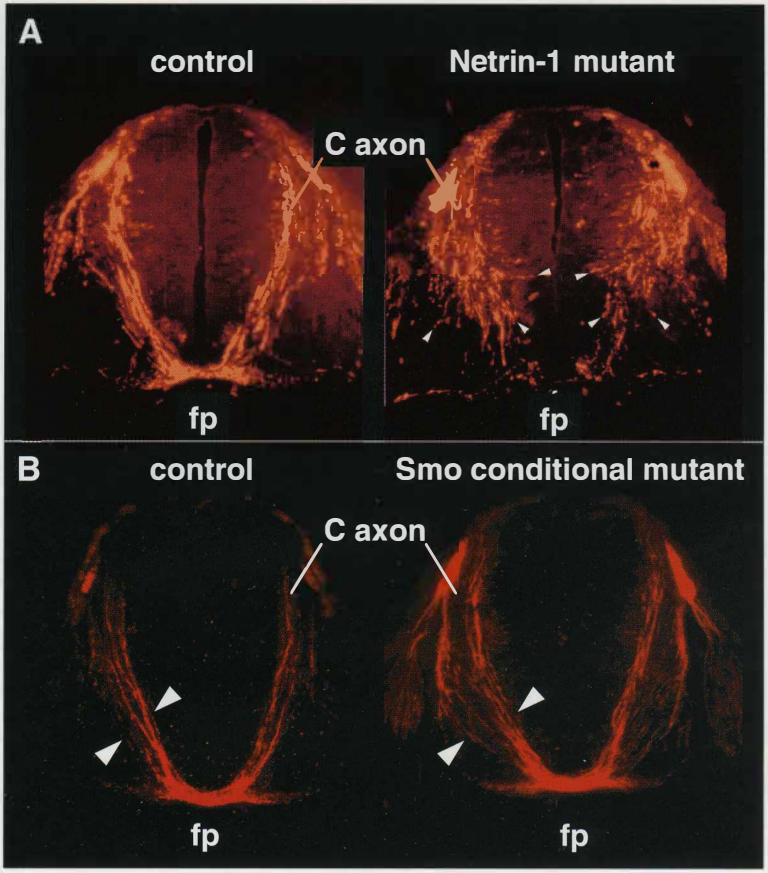


Fig. 5, Tessier-Lavigne. A,B

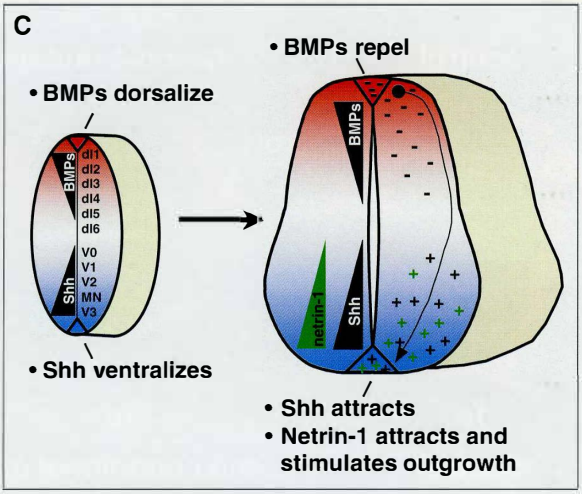


Fig. 5, Tessier-Lavigne. The morphogen Shh collaborates with Netrin-1 in midline attraction. (A) Netrin-1 is required for commissural axon guidance to the midline. Left: Control embryo illustrating the trajectory of commissural (C) axons to the floor plate (fp) in a mouse embryo, visualized with an antibody to TAG-1. Right: In a Netrin-1 knockout embryo, commissural axon guidance is normal in the dorsal spinal cord, but when the axons reach the developing motor column, they get profoundly confused, with many projecting medially or laterally (arrowheads). Note that a few do make it to the ventral/midline, indicating the existence of collaborating cues. [Adapted from Serafini et al. (1996).] (B) Shh signaling is also required for commissural axon guidance to the ventral midline. Right: A mutant mouse in which the Shh signaling component Smoothened is selectively disrupted in the dorsal spinal cord shows defects in commissural axon guidance. The axons again project normally in the dorsal spinal cord, but when they get to the motor column many continue to grow straight. Some do still project normally, and even those that make errors appear to correct them—guidance that is attributable to Netrin-1. The role of Shh signaling appears to be to direct the sharp turn so that the axons can make a bee-line to the floor plate. [Adapted from Charron et al. (2003).] (C) Axon guidance by morphogens. BMPs and Shh initially pattern the spinal cord, with BMPs dorsalizing and Shh ventralizing. They then appear to be reused at later stages for axon guidance, with BMPs repelling commissural axons away from the dorsal midline and Shh attracting them to the ventral midline, collaborating with Netrin-1. [Adapted from Charron et al. (2003).]

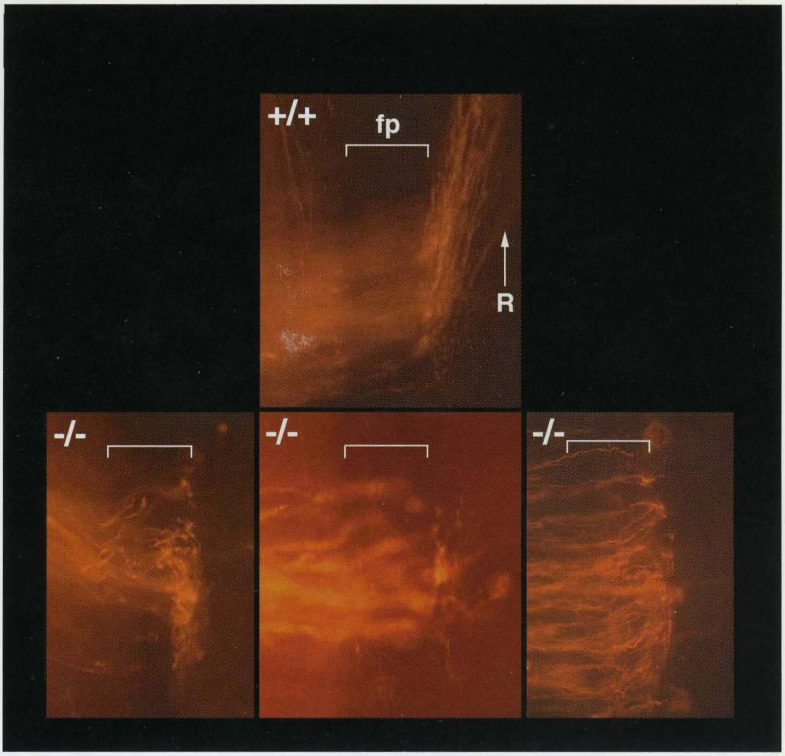


Fig. 7, Tessier-Lavigne. Leaving the midline requires switching on repulsion. Shown are the trajectories of commissural axons at the floor plate (fp) of embryonic mice, visualized with DiI injections. In control mice (top panel), the axons cross the floor plate and then leave it, turning rostrally (R). In mice lacking the repellent receptor Neuropilin-2 (bottom three panels), the axons stall out at high frequency in the floor plate. [Adapted from Zou et al. (2000).]

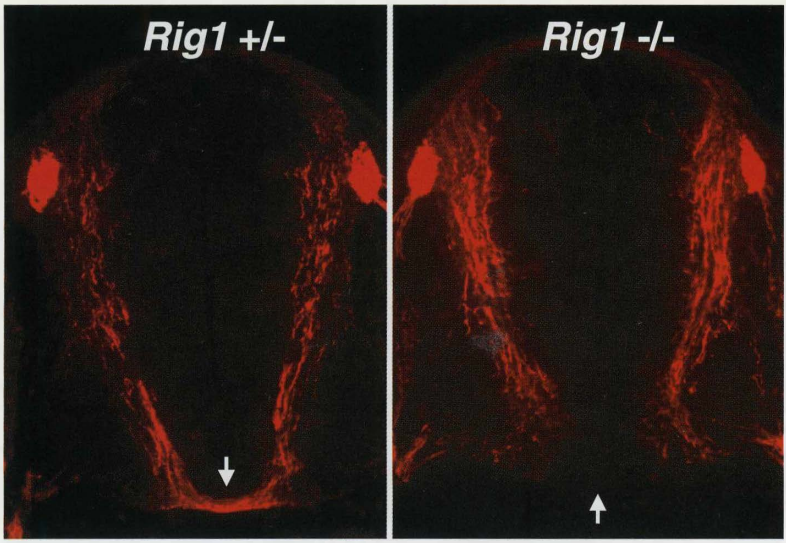


Fig. 9, Tessier-Lavigne. Rig-1 is required to prevent premature Slit responsiveness. For full caption, see page 133.

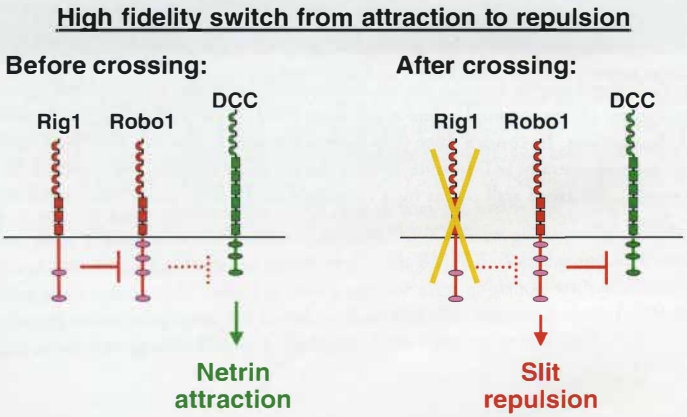


Fig. 10, Tessier-Lavigne. A high-fidelity switch from attraction to repulsion at the vertebrate midline. For full caption, see page 134.

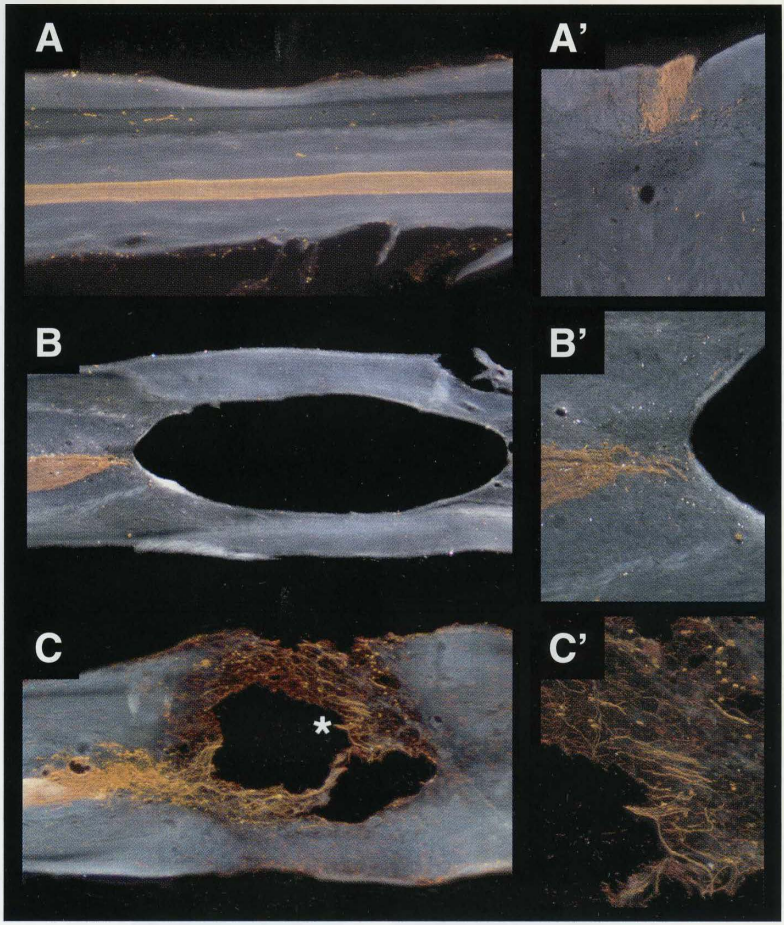


Fig. 11, Tessier-Lavigne. Stimulating regeneration by kicking adult sensory neurons into a growth state. (A–C) Dorsal columns visualized in an intact rat spinal cord (A), the spinal cord of a rat six weeks after a dorsal hemisection (B), and the spinal cord of a rat in which the cell bodies of sensory neurons were exposed to a single pulse of dibutyrl cAMP several days before a dorsal hemisection (again, axons were visualized 6 weeks later) (C). No regeneration is observed in the control lesioned animal (B), whereas extensive regeneration is observed through the lesion site after the cAMP treatment. Panels B′ and C′ show higher magnification views of panels B and C. Panel A′ shows labeling in the dorsal column nuclei in the control animal (A). [Adapted from Neumann et al. (2002).]

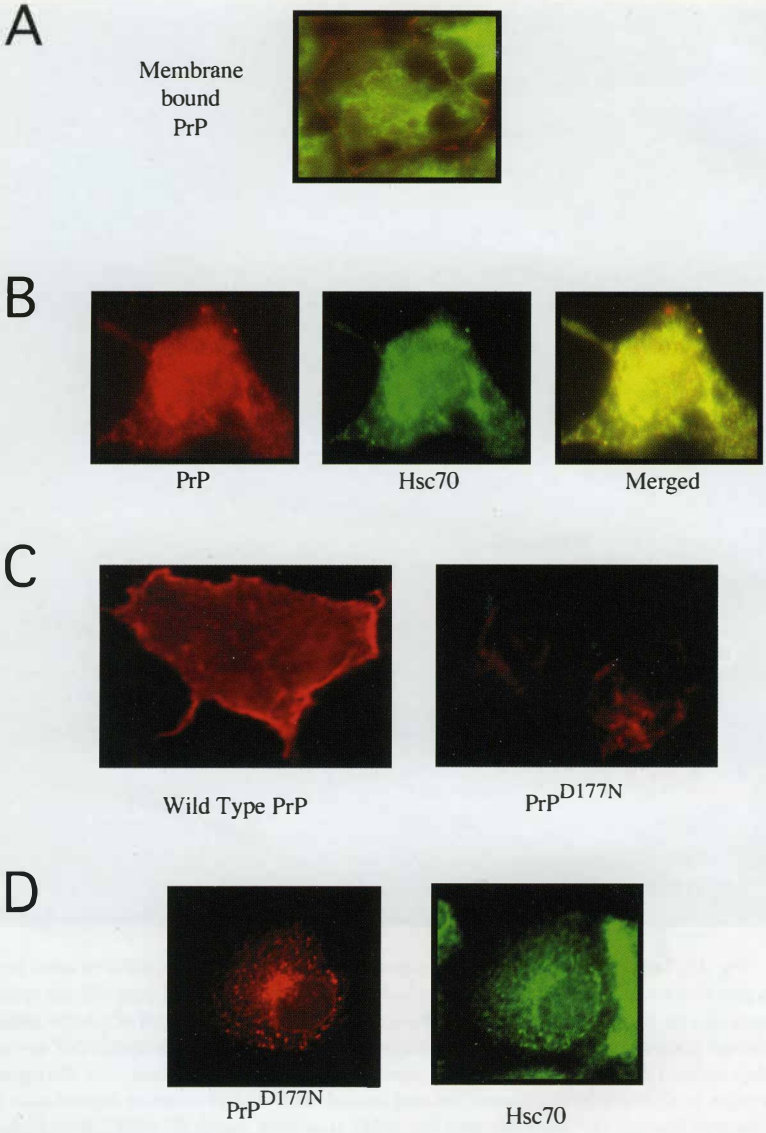


Fig. 4, Lindquist. Localization of PrP within the cell under different conditions. For full caption, see page 191.

enchyme that attracts the axons of motoneurons [e.g., Pollack and Liebig (1977); see also references in Ebens et al. (1996)]. We used the same approach to identify it, namely biochemical purification based on a bioassay, but it wasn't necessary to purify to homogeneity in this case because the factor turned out to be none other than hepatocyte growth factor or scatter factor (HGF/SF) [Ebens et al. (1996)], a factor that had been previously identified but not previously implicated in axon guidance and which functions to attract the axons by activating the Met receptor tyrosine kinase (Fig. 2B). The next chemoattractive activity we tackled is one first described in the early 1980s by Lumsden and Davies, which is made by the maxillary process of the upper jaw and which functions to attract the axons of trigeminal sensory neurons (Lumsden and Davies, 1983). Lumsden and Davies christened this factor max factor, because it is made by the maxillary process. We sought to identify max factor through purification based on a bioassay, but again this wasn't necessary as we realized in the course of the purification that max factor is none other than a combination of two neurotrophins, BDNF and NT3 (O'Connor and Tessier-Lavigne, 1999) (Fig. 2C). This study showed that neurotrophins can function in the guidance of developing axons, in addition to their other well-characterized functions in nervous system development, and put to rest the hotly debated question of whether neurotrophins could function in developmental axon guidance. Indeed, as early as the late 1970s, evidence had been provided that the neurotrophin nerve growth factor (NGF) could attract axons *in vitro* and *in vivo* (Menesini-Chen et al., 1978; Gundersen and Barrett, 1979). However, the relevance of those observations was questioned by the finding that developing axons are not NGF responsive until after they reach their targets, and NGF is in any case not made until the axons reach their targets (Lumsden, 1988). Even today there is no evidence implicating NGF itself in guiding developing axons, though it does contribute to sprouting of axons at their targets (Kennedy and Tessier-Lavigne, 1995). However, our finding that BDNF and NT3 can contribute to developmental axon guidance has emphasized that neurotrophins can indeed contribute to early wiring events.

This, then, is where we stood until very recently. We had three chemoattractants: the Netrins, which are phylogenetically conserved, and two sets of growth factors (HGF and neurotrophins), which are not (Fig. 1A). Although this represents significant progress, it should also be emphasized that this is still a relatively small number of chemoattractants

given the immense complexity of the mammalian brain. Do other chemoattractants remain to be identified? The answer is yes, as discussed in detail below.

C. Repellents: Netrins, Semaphorins, Ephrins, Slits, and Their Receptors

The 1990s also saw the identification of several families of repellents. In our own studies, we found, to our surprise, that although we had purified the Netrins as attractants for commissural axons, they also turn out to be repellents for other classes of axons (Colamarino and Tessier-Lavigne, 1995) (Fig. 1A). Thus the Netrins are bifunctional: They attract some axons and repel others; these different effects, we now know, are due to activation of different receptors for attraction and repulsion (Fig. 3) (Hamelin et al., 1993; Chan et al., 1996; Keino-Masu et al., 1996; Leonardo et al., 1997; Hong et al., 1999). In this chapter, however, I will focus primarily on the attractant effects of the Netrins.

In the 1990s, three other very important families of chemorepellents were identified: the Semaphorins, the Ephrins, and the Slits (Fig. 1A). The Ephrins were identified as important repellents by Friedrich Bonhoeffer, John Flanagan, and their colleagues [reviewed in Drescher et al. (1997); Flanagan and Vanderhaeghen (1998)], but will not be discussed further. I will, however, refer to the two other families of repellents, the Semaphorins and the Slits, which I will therefore introduce briefly (see Fig. 3 for diagrams).

The Semaphorins are a large family of factors, with over 20 members in vertebrates. The first were identified in grasshoppers by Corey Goodman and in vertebrates by Jonathan Raper [reviewed in Kolodkin (1998)]. We were interested in trying to identify the receptors through which they produce their effects. The best characterized Semaphorin is Sema3A (also known as collapsin), a diffusible chemorepellent with profound effects on a variety of classes of axons. To identify its receptor, we used an expression cloning approach, isolating a binding protein for Sema3A that turned out to be an orphan receptor called neuropilin-1. Through loss-of-function studies, we went on to show that neuropilin-1 not only binds Sema3A but is also required for mediating its repellent actions (He and Tessier-Lavigne, 1997). A related Semaphorin, Sema3F, which is also a potent repellent for other classes of axons does not, however, bind neuropilin-1 with high affinity, but we found a second neuropilin, neuropilin-2, that does bind Sema3F with high affinity, and we

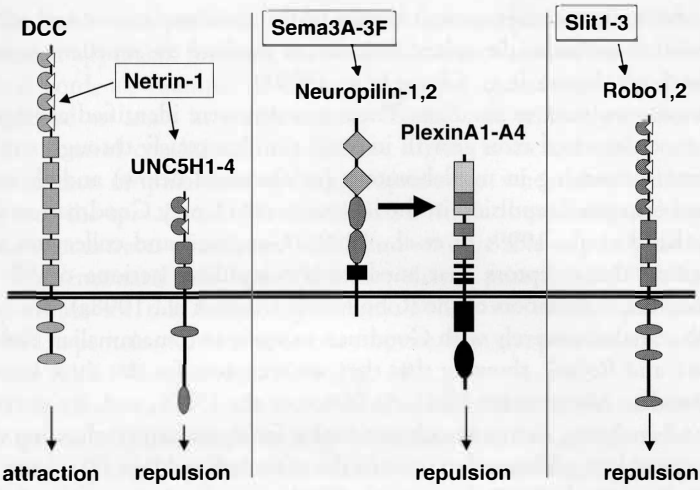


Fig. 3. Receptors for Netrins, Semaphorins, and Slits. Left: DCC family members (of which there are two in vertebrates) mediate attractive actions of Netrins, whereas members of the UNC5 family (of which there are four in vertebrates: UNC5H1–4) mediate repulsive responses, either alone or in combination with DCC family members. Middle: Class 3 Semaphorins (Sema3A-Sema3F) elicit repulsive responses by binding Neuropilin-1 or -2, which then complex with members of the Plexin family which function as signal transducers. There are nine Plexins in vertebrates; the Neuropilins are thought to interact with A class Plexins (PlexinA1-A4). Right: Slit proteins (of which there are three in vertebrates, Slit1–3) that function as repulsive receptors elicit repulsive responses by activating receptors of the Robo family, of which there are two that function as repulsive receptors in vertebrates. A third one, Rig-1/Robo3, actual blocks slit responsiveness (see Fig. 10).

went on to show also that it is required for mediating Sema3F's effects (Chen et al., 1997, 2000) (Fig. 3). Similar results were obtained independently by Alex Kolodkin, David Ginty, and their colleagues (Kolodkin et al., 1997).

The neuropilins have very short cytoplasmic domains and therefore did not appear to be signaling molecules, so the hunt was on to identify their signaling co-receptors. Together with a consortium of other investigators, we, as well as Steven Strittmatter and colleagues, identified Plexin proteins as candidate signaling co-receptors for the neuropilins (Fig. 3) (Tamagnone et al., 1999; Takahashi et al., 1999). There are nine Plexins in vertebrates, and we have knocked out several of them. Through such

loss-of-function studies, as well as gain-of-function studies, we and others now feel that Plexins do indeed function to mediate the repellent actions of the Semaphorins [e.g., Cheng et al. (2001)].

Let us now turn to the Slits. These proteins were identified as important modulators of axon growth in 1999 simultaneously through studies on axon branching in my laboratory (as discussed below) and through studies on axonal repulsion in the laboratories of Corey Goodman and Yi Rao (Kidd et al., 1999; Li et al., 1999). Goodman and colleagues also identified the receptors that mediate the repulsive actions of Slit in Drosophila as members of the Robo family (Kidd et al., 1998a). We have collaborated extensively with Goodman to study two mammalian Robos, Robo1 and Robo2, showing that they are receptors for the three known mammalian Slit proteins (Slit1–3) (Brose et al., 1999), and, by generating and analyzing mouse knock-out strains for these factors, showing that they direct key guidance decisions in the mammalian brain (Plump et al., 2002; Bagri et al., 2002; Long et al., 2004) (Fig. 3). An example of this, taken from our collaborative work with Carole Mason, is provided by the visual system, where we focused on the growth of retinal axons from the eye to the optic chiasm, down the optic nerve. We found that Slit1 and Slit2 are expressed in regions that flank the optic nerve (Erskine et al., 2000), and they actually function to confine these axons to this path through repulsion. This was shown by knocking them out and by showing that when we take away both Slit1 and Slit2, the axons now invade the region where the Slits are normally expressed, consistent with this repulsive action of Slits on those axons (Plump et al., 2002).

Studies like these have thus demonstrated important roles for Netrins, Semaphorins, Ephrins, and Slits in repelling axons.

D. Axon Branching Regulators, and the Multifunctionality of Guidance Cues

Let us now turn to axonal branching. It has been known for some time that neurotrophins play a role in stimulating the branching of axons in their target fields, so-called terminal arborization [reviewed in Kennedy and Tessier-Lavigne (1995)], but it is not known whether neurotrophins also stimulate branching of axons before their targets, so-called interstitial branching, involved in projections to multiple targets (O'Leary et al., 1990). In fact, molecules that regulate interstitial branching were not known in the 1990s. We were thus intrigued to know what other kinds of molecules might function in branching.

To tackle this problem, we decided again to use a functional biochemical approach, focusing on the branching of sensory axons into the spinal cord, an example of interstitial axonal branching. We therefore developed an assay in which sensory axons were relatively unbranched under control conditions, but became highly branched when cultured with extracts of spinal cord of the appropriate age, when axons are branching into the spinal cord. We found that a similar branching activity was present in extracts of neonatal brain, and we were able to purify the activity from neonatal calf brain, showing that a protein of 140 kDa was responsible for this branching activity. When we microsequenced the 140-kDa protein, we found, to our surprise, that the protein was a Slit—bovine Slit2 (Wang et al., 1999) (Fig. 1B).

This simultaneous discovery of Slits as branching factors in our laboratory, and of Slits as repellents by Goodman and Rao, in one fell swoop established that Slit proteins are multifunctional, capable of guiding some axons and regulating branching of others (compare Fig. 1B to Fig. 1A). These studies therefore extended the concept of bifunctionality that we first developed with the Netrins, and, interestingly, it has now been extended quite widely. Indeed, most or all of the molecules implicated in attracting or repelling axons have also been found to regulate axonal branching as well [see, e.g., Bagri et al. (2003) for effects of Semaphorins on branching] (Fig. 1B).

In fact, based on these results, it appears that we shouldn't think of these molecules as attractants or repellents or branching regulators per se, but rather as wiring cues that can be interpreted in different ways by different axons, or even by the same axons at different stages of their development. These studies have also underscored the importance of the "big four" of axon guidance—Netrins, Semaphorins, Ephrins and Slits, as well as some growth factors—in wiring the brain. The question remains, however, whether these represent most or even all of the molecules involved in wiring the brain, or whether others remain to be identified, a point I will come back to again below.

III. Navigating Intermediate Targets

After this brief introduction to the molecules of axon guidance, I would like to focus for the remainder of this chapter on how the nervous system uses these cues and these receptors to wire the brain, ensuring that growth cones can project accurately to their targets. In particular, I would like to

address how axons can navigate from one intermediate target to the next in order to extend over long distances. For this, I will focus on one particular biological system that I have already introduced to you: the developing spinal cord. We will focus on the so-called commissural neurons introduced above, which have cell bodies in the dorsal spinal cord and send their axons initially to floor plate cells at the ventral midline of the spinal cord. What was not mentioned above is that the floor plate is not the final destination of these axons, but rather an intermediate target. The axons will actually cross the midline at the floor plate, and then, immediately upon crossing, make a sharp right angle turn and project alongside the floor plate to different axial levels in the embryo, eventually leaving the midline to seek out their final destinations (Fig. 4A). The next sections will discuss these three legs of their trajectory: getting to the midline, crossing, and leaving.

A helpful way of looking at this guidance involves opening the spinal cord at the dorsal midline and flattening it out like a book, with the floor plate, the midline, running down the center (a so-called "open-book preparation"). Figure 4B illustrates that commissural neurons face two challenges. First, they must send their axons to the midline. Then, upon reaching it, they must cross, and then they must leave the midline on to the next leg of their trajectory.

A. Five Important Regulatory Mechanisms to Get There and Move On

We have been studying five important regulatory mechanisms that are involved in getting there and moving on. These will be introduced briefly here, before being discussed in greater detail. They are also depicted in Fig. 4B.

First, "getting there" involves being attracted by Netrins—as mentioned above, it is in this context that we identified Netrin-1. What we subsequently discovered is that Netrin-1 isn't the whole story. We have evidence for a second chemoattractant made by midline cells that collaborates with Netrin-1 to attract the axons to the midline, which is an old friend in a new guise—as discussed below.

Second, once the axons reach the midline, we found, remarkably, that the neurons become dependent for their continued survival on a trophic factor made by midline cells. We proposed that this dependence might actually help eliminate neurons that misproject and fail to reach the

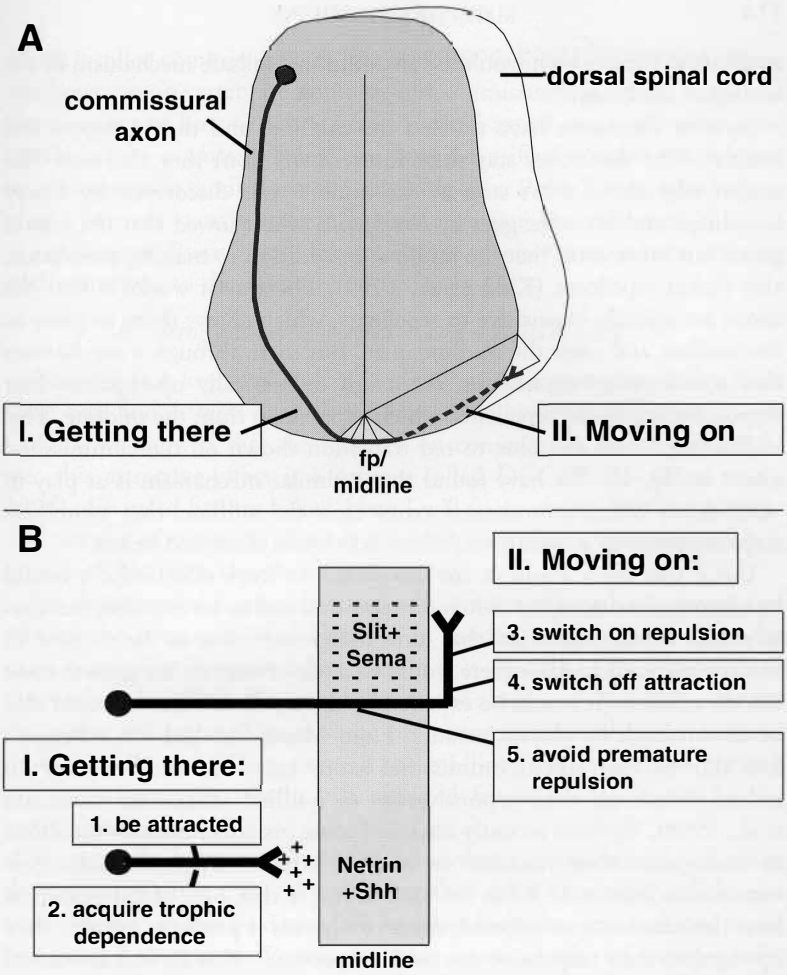


Fig. 4. Getting there and moving on: Five key regulatory mechanisms. (A) The floor plate is not the final destination of commissural axons, just an intermediate target. Commissural axons project to the floor plate, then enter the floor plate to cross the midline. Upon crossing, they leave the midline, make a sharp right angle turn, and project to other axial levels in the embryo. (B) The same trajectory viewed in an "open-book"preparation, in which the spinal cord is opened at the dorsal midline and flattened, with the midline running down the middle. Five regulatory mechanisms are involved in getting to the midline and then moving on: (1) The axons must first be attracted by Netrin-1 and Shh; (2) upon reaching the midline, they become dependent for their continued survival on a floor-plate-derived trophic factor; (3) upon crossing, they switch on responses to the repellents Slit1–3 andSema3B made by floor plate cells; (4) they also switch off responsiveness to the attractants that got them to the midline; (5) they also avoid switching on responsiveness to midline repellents before crossing the midline. See text for a detailed discussion. (See color plates.)

midline on time, which would be an additional failsafe mechanism in the wiring of the brain.

So now the axons have reached the midline, and they're happy and healthy. Why don't they stay there forever, why don't they stall out? The reason why axons don't stop at the midline was discovered by Corey Goodman and his colleagues in *Drosophila*, who showed that the reason axons can move on is that the midline, in addition to making attractants, also makes repellents (Kidd et al., 1999). The way it works is that the axons are initially insensitive to repellents, which allows them to grow to the midline and cross the midline once. But then, through a mechanism that is still rather mysterious, the axons dramatically up-regulate their responsiveness to the repellents, which expel them from the midline. This is illustrated with the blue to red transition shown on the commissural axons in Fig. 4B. We have found that a similar mechanism is at play in vertebrates: When commissural axons cross the midline, they switch on responsiveness to a midline repellent activity, as discussed below.

But if you think about it, for this switch to work effectively, it would be important not only to switch on repulsion and to be expelled, but also to switch off the attraction that lures the growth cone to the midline in the first place. Otherwise there would be a tug-of-war: As the growth cone left the midline, it would be expelled by the repellents, but it would also be drawn back by the attractants. Fujio Murakami and his colleagues first showed that when commissural axons cross the midline, they do indeed switch off their reponsiveness to midline attractants (Shirasaki et al., 1998). We have recently obtained some insights into how the axons down-regulate their response to midline attractants, particularly their response to Netrin-1. What we have found is that part of the reason at least the neurons lose responsiveness to Netrins is precisely because they up-regulate their responsiveness to the repellents—that is, that activation of one of the repellent receptors actually results in switching off of the Netrin response. Again, this will be discussed below.

Finally, one can easily appreciate that it is essential to choreograph this switch precisely, such that repulsion is switched on only after the axons cross the midline, not before—otherwise the axons wouldn't be able to cross the midline in the first place. It should come as no surprise, then, that elaborate regulatory mechanisms exist to ensure that the axons don't become repelled prematurely. In *Drosophila*, Goodman and colleagues have identified a protein that plays an important role in avoiding premature repulsion,

which is called commissureless (Tear et al., 1996; Kidd et al., 1998b). There are, however, no vertebrate homolog of Commissureless, which has raised the question of how vertebrate commissural axons can avoid premature repulsion. As discussed below, we have identified a novel mechanism involving a Robo family member called Rig-1 (or Robo 3) which we believe plays a key role in preventing premature repulsion in vertebrates.

Let us now discuss these five regulatory mechanisms in turn, starting with attraction to the midline.

B. Being Attracted: Netrin-1 and a Collaborator, Shh

Netrin-1 is a key mediator of midline attraction: it is made by floor plate cells and attracts commissural axons. In collaboration with Joseph Culotti, we showed that the receptor on commissural axons that mediates this attractive effect is a protein called DCC (Keino-Masu et al., 1996), discussed further below. Here I will focus on the fact that Netrin-1 is not the whole story: there is a second chemoattractant that collaborates with Netrin-1 in attracting the axons.

We discovered this second attractant in studies in which we were determining the precise role of Netrin-1 in guiding commissural axons by examining what happens when Netrin-1 is deleted in knock-out mice (Serafini et al., 1996). We found that in the absence of Netrin-1 many of the axons get confused, with some projecting more medially and others more laterally and failing to reach the midline (Fig. 5A), thus verifying our hypothesis that Netrin-1 is required for normal guidance to the ventral midline.

Importantly, however, we also observed that a few axons do make it to the ventral midline (Fig. 5A), showing that other cues must collaborate with Netrin-1 in this guidance. We wondered whether one such cue could be a second attractant. We reasoned that we could take advantage of the Netrin knock-out mouse to ask whether floor plate cells from this animal still possess chemoattractant activity, which we could test by dissecting them from the knock-out animals and testing whether they still possess chemoattractant activity in our invitro assay.

We therefore first turned to the outgrowth assay described above, in which a piece of dorsal spinal cord is cultured in a collagen matrix together with floor plate, which stimulates the directed outgrowth of commissural axons into the collagen. We found that floor plate tissue from Netrin-1 knock-out animals completely lost this outgrowth-promoting activity,

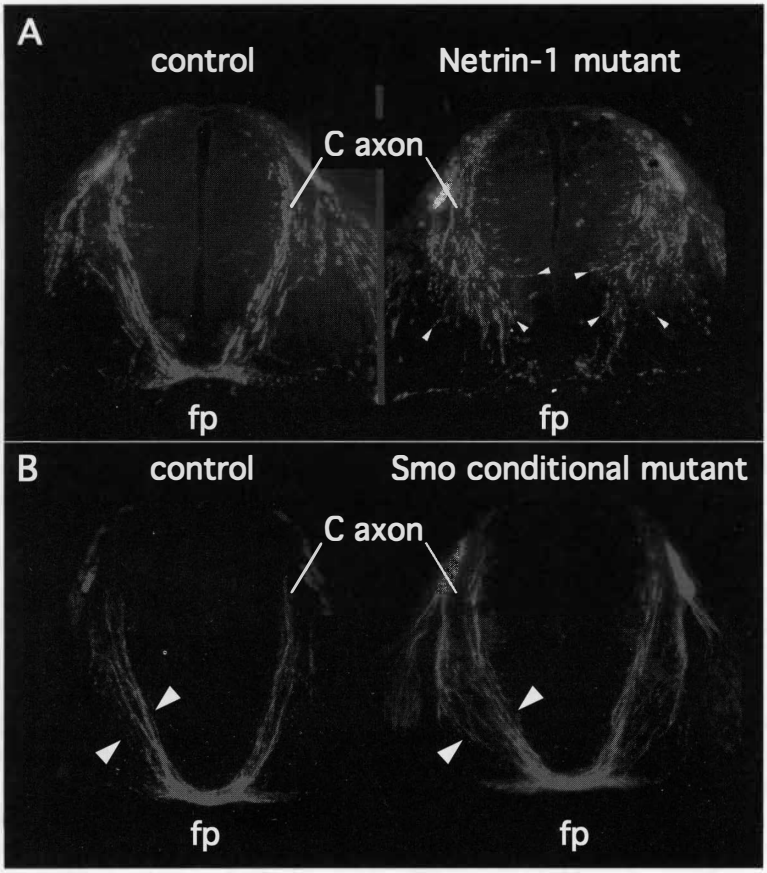


Fig. 5. A,B

showing that Netrin-1 is absolutely required for the outgrowth effect and presumably mediates the outgrowth-promoting activity completely. Does this experiment also show that there is no other attractive activity? On closer inspection, we realized that this particular experiment can't test whether there is another factor that can attract the axons if this factor is unable itself to stimulate axon outgrowth—without outgrowth, we couldn't test for turning. To test whether there might be such a factor,

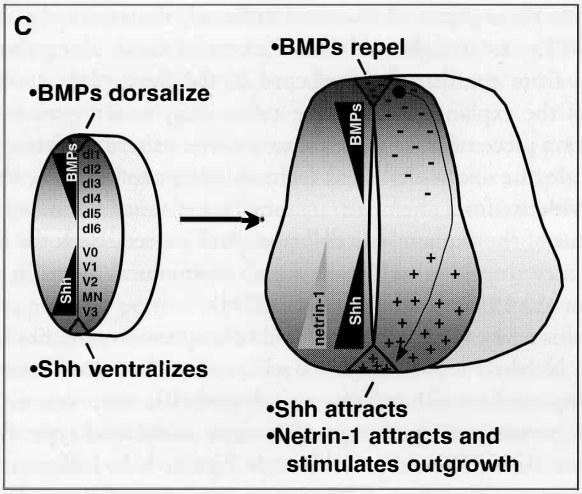


Fig. 5. The morphogen Shh collaborates with Netrin-1 in midline attraction. (A) Netrin-1 is required for commissural axon guidance to the midline. Left: Control embryo illustrating the trajectory of commissural (C) axons to the floor plate (fp) in a mouse embryo, visualized with an antibody to TAG-1. Right: In a Netrin-1 knock-out embryo, commissural axon guidance is normal in the dorsal spinal cord, but when the axons reach the developing motor column, they get profoundly confused, with many projecting medially or laterally (arrowheads). Note that a few do make it to the ventral midline, indicating the existence of collaborating cues. [Adapted from Serafini et al. (1996).] (B) Shh signaling is also required for commissural axon guidance to the ventral midline. Right: A mutant mouse in which the Shh signaling component Smoothened is selectively disrupted in the dorsal spinal cord shows defects in commissural axon guidance. The axons again project normally in the dorsal spinal cord, but when they get to the motor column many continue to grow straight. Some do still project normally, and even those that make errors appear to correct them—guidance that is attributable to Netrin-1. The role of Shh signaling appears to be to direct the sharp turn so that the axons can make a bee-line to the floor plate. [Adapted from Charron et al. (2003).] (C) Axon guidance by morphogens. BMPs and Shh initially pattern the spinal cord, with BMPs dorsalizing and Shh ventralizing. They then appear to be reused at later stages for axon guidance, with BMPs repelling commissural axons away from the dorsal midline and Shh attracting them to the ventral midline, collaborating with Netrin-1. [Adapted from Charron et al. (2003).] (See color plates.)

we thus needed a different way of assessing attractive activity that doesn't require stimulation of outgrowth. The assay we turned to is what we had previously termed the "turning assay" (Placzek et al., 1990). This involves taking pieces of spinal cord and laying them on their side. What we found

is that when these pieces of tissue are cultured, the axons of commissural neurons will grow straight within the pieces of tissue along their normal trajectory, from the dorsal spinal cord to the floor plate at the ventral midline of the explant. Because the axons all grow straight, we can ask: If we place a piece of floor plate tissue or cells secreting Netrin-1 over to the side, are the axons deflected from this trajectory? If so, that would provide evidence for a chemoattractant effect without requiring the axons to grow out of the explant into collagen. And indeed, both the floor plate and cells secreting Netrin-1 can attract commissural axons in this assay (Placzek et al., 1990; Kennedy et al., 1994).

Using this assay, we could thus tackle the question whether floor plate cells from Netrin-1 knock-out mice still possess chemoattractant activity. Remarkably, and to our surprise, we found that they do—in fact, the degree of turning is similar to that seen with wild-type floor plate (Serafini et al., 1996). Thus, although Netrin-1 is sufficient to cause turning, it is not necessary: There must be a second floor-plate-derived chemoattractant. Note, however, that this second attractant is different from Netrin-1: Although it can attract in the turning assay, unlike Netrin-1 it cannot stimulate outgrowth in the collagen gel outgrowth assay.

We naturally next wanted to identify this second attractant, a challenge we tackled initially by testing candidates, focusing on proteins made by floor plate cells. In this way we rapidly came to focus on none other than the morphogen Sonic Hedgehog (Shh). Shh is made by floor plate cells and plays a key role patterning the ventral spinal cord, as shown by Tom Jessell and his colleagues. Shh protein is inferred to be present in the spinal cord in a gradient and has been shown to pattern the ventral spinal cord, inducing in a dose-dependent fashion various classes of interneurons and motoneurons (Jessell, 2000). Because Shh expression persists in the floor plate at later stages, when commissural axons are growing to it, we were interested in whether Shh might function in attraction as well. Quite remarkably, we found that cells secreting Shh can fully mimic the effect of floor plate cells in stimulating the turning of commissural axons (Charron et al., 2003). Thus, Shh is in the right place, at the right time, and has the right activity for being the Netrin-independent chemoattractant activity of floor plate cells. We went on to show that the chemoattractant activity of floor plate cells in the turning assay could be essentially completely abrogated if we simultaneously blocked both Netrin-1 signaling (using floor plate cells from Netrin-1 knock-out mice) and Shh sig-

naling (using cyclopamine, a selective alkaloid antagonist of the Shh signaling component Smoothened (Smo)) (Charron et al., 2003). These experiments thus provided evidence that Shh mediates the non-Netrin turning activity of floor plate cells.

We next wanted to define the contribution of Shh to the normal guidance of commissural axons *in vivo*. We couldn't test this simply by examining commissural axon trajectories in Shh knock-out mice, since the ventral spinal cord does not form normally in those animals, so any defects would be difficult to interpret. It was therefore necessary to use a more selective approach, perturbing Shh signaling in the dorsal-most spinal cord (including in commissural neurons) but leaving it intact in the ventral spinal cord, so that any defects that were seen could be attributed to loss of an attractive effect on the axons. To achieve this, we performed a region-specific knock-out of the Shh signaling component Smo in the dorsal-most spinal cord using the cre-loxP system, in collaboration with Andy McMahon. For this, we crossed a floxed allele of Smoothened to a cre driver line in which cre is expressed in the dorsal most spinal cord (including in commissural neurons) by being expressed under the control of the Wnt-1 promoter. What we observed was a clear, reproducible, and highly penetrant defect in commissural axon guidance. Specifically, in the mutants, the axons projected normally in the dorsal spinal cord, but upon reaching the level of the motor column many failed to turn and make a bee-line towards the floor plate, instead continuing to extend straight along their dorso-ventral trajectory (Fig. 5B) Thus, Shh is required to make the bee-line toward the floor plate. As can be seen in Fig. 5B, however, many, indeed perhaps most, of the axons that make a mistake and continue to grow ventrally eventually correct their errors and turn toward the floor plate—and many do not make errors at all. But this is expected, since Netrin-1 is still present in these mutants. Thus, we interpret the trajectories of commissural axons observed in the mutants to reflect the guidance that Netrin-1 can achieve on its own—which is quite good, though not perfect. To get all axons to make a bee-line toward the floor plate, Netrin-1 needs to be assisted by Shh, its collaborator in chemoattraction.

C. Morphogens as Guidance Cues

From these results, I would therefore like to draw three major conclusions.

First, they support a model in which Shh, after performing its first job of patterning the ventral spinal cord, is subsequently reused to attract commissural axons, in collaboration with Netrin-1. What is particularly pleasing about this finding is that it dovetails nicely with work from Jane Dodd's lab that has focused on members of the Bone Morphogenetic Protein (BMP) family. These proteins are expressed in the dorsal midline and function to pattern the dorsal spinal cord at early stages, inducing multiple classes of dorsal neurons (Jessell, 2000). Dodd and colleagues have obtained evidence that the BMPs later get reused to repel commissural axons away from the ventral midline (Augsburger et al., 1999). Together, these results suggest a model in which Shh and BMPs initially pattern the spinal cord through their antagonistic actions, with Shh ventralizing and BMPs dorsalizing, then later get reused in axon guidance, with Shh attracting and BMPs repelling (Fig. 5C). Although Shh and BMPs have opposite effects on guidance, these effects synergize, because Shh is placed in front of the growth cone providing a pull, whereas BMPs are placed behind the growth cone, providing a push. Thus, it appears that the embryo, sensibly, recycles the morphogen gradients that are used at early stages to pattern the spinal cord, reusing them at later stages for axon guidance.

The second conclusion is that Netrins came first: They represent a more ancient guidance mechanism on which the morphogens were layered later in evolution. The reason for thinking this derives from the fact, mentioned above, that Netrins are conserved throughout evolution, from vertebrates to worms to flies. In each of these organisms, complete loss of Netrin function results only in a partial defect in guidance to the midline, showing that in each of these organisms other cues must collaborate with the Netrins. I have argued that in the spinal cord, Shh is the other attractant collaborating with Netrin-1. In nematodes, we can be certain that this is not the case, for the simple reason that nematodes don't have Hedgehog genes—those genes arose after the divergence of nematodes from both insects and vertebrates six hundred million years ago. This supports the idea that the Netrin mechanism is an ancient one involved in guiding axons to the midline, and that morphogens like Shh were added later. In fact, in nematodes, we have also found that the collaborating cue with the Netrin UNC-6 is none other than the repellent Slit, which is expressed dorsally and provides a push from behind, assisting the Netrin pull (Hao et al., 2001).

The third conclusion I would like to draw is that these results provide a partial answer to a question posed earlier: What other molecules function as axon guidance cues? These studies on cues that collaborate with Netrin-1 suggest that we must also take seriously the possibility that molecules that we think of more classically as morphogens, like Hedgehogs, WNTs, and BMPs, might later be reused in axon guidance. Indeed, there is also mounting evidence from multiple labs, including ours, that these three sets of molecules can be used in all of these guises (Colavita et al., 1998; Trousse et al., 2001; Yoshikawa et al., 2003; Lyuksyutova et al., 2003). It will be very interesting to examine to what extend this is the exception and to what extent it is the rule—whether morphogens are widely used in guidance and are as important or even possibly more important than the classical guidance molecules: Netrins, Semaphorins, Slits, and Ephrins.

Be that as it may, in the more specialized context of the developing spinal cord, the evidence implies that attraction to the midline involves not just Netrin-1 but also the morphogen Shh.

D. En Passant Neurotrophic Action of an Intermediate Target

As mentioned earlier, we found that once the axons have reached the midline, they become dependent for their continued survival on a trophic or survival signal provided byfloor plate cells. We discovered this trophic effect in studies in which we cultured the explants of dorsal spinal cord for more extended periods (Wang and Tessier-Lavigne, 1999). Whereas commissural neurons are healthy at 24 hr in culture, after 48 hr they are all dead, as assessed by the extensive blebbing of their axons, as well as by the death and disintegration of their axons (Fig. 6). This sudden death occurs unless they are provided with a trophic activity secreted by floor plate cells, in the form of floor plate-conditioned medium (Fig. 6). We still don't know what the trophic factor is.

However, this dependence seen *in vitro* has led us to propose that commissural neurons *in vivo* are dependent for their continued survival on trophic support from their intermediate target, the floor plate (Wang and Tessier-Lavigne, 1999). This finding extends the classical neurotrophic hypothesis, according to which neurons become dependent for their continued survival on trophic support from their final targets. We have also argued that a dependence on trophic support from an intermediate target would provide a potential mechanism for eliminating axons that mispro-

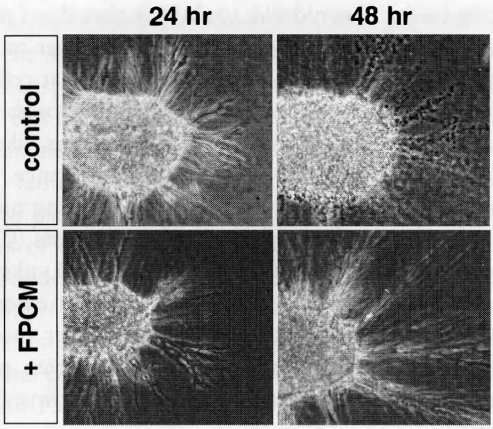


Fig. 6. Commissural axons require a survival factor from floor plate. Top panels: When an E13 rat dorsal spinal cord is cultured for 24 hr in a collagen matrix in the presence of netrin-1, commissural axons grow out and are healthy. At 48 hr, however, the neurons have all died, as evidenced by the blebbing of their axons. Bottom panels: Addition of floor-plate-conditioned medium prevents the death of commissural neurons, revealing the existence of an en passant trophic factor for commissural axons from the floor plate. [Adapted from Wang and Tessier-Lavigne (1999).]

ject and fail to reach the intermediate target on time, thus providing an additional fail-safe mechanism for the proper wiring of the brain.

E. Moving On: Becoming Repelled

So now the axons have reached the midline, and they are happy and healthy, exposed to both attractants and trophic factors. Why don't they just stay there, stall out; why do they move on? Again, as described above, they move on because they change their responsiveness upon crossing the midline, up-regulating responsiveness to midline repellents. In *Drosophila*, there is a single midline repellent provided by the Slit protein. In mammals, we have evidence that the repellent activity is due to a combination of three Slits, Slit1–3, and a Semaphorin protein, Sema3B, which repel the axons by activating Robo receptors (in the case of Slit proteins) or complexes of neuropilins and plexins (in the case of Sema3B). This model is supported by *in vitro* studies showing that commissural axons

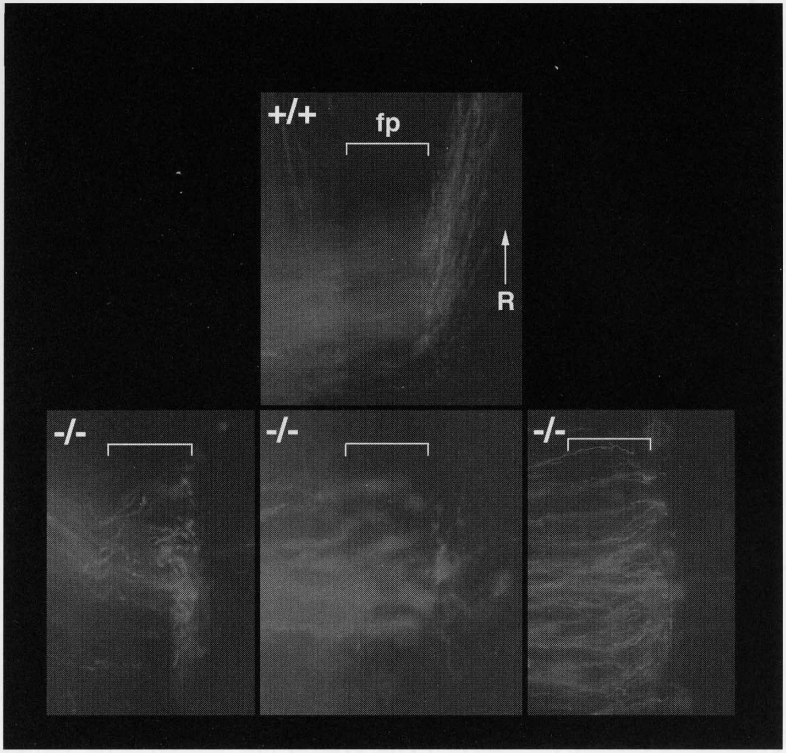


Fig. 7. Leaving the midline requires switching on repulsion. Shown are the trajectories of commissural axons at the floor plate (fp) of embryonic mice, visualized with DiI injections. In control mice (top panel), the axons cross the floor plate and then leave it, turning rostrally (R). In mice lacking the repellent receptor Neuropilin-2 (bottom three panels), the axons stall out at high frequency in the floor plate. [Adapted from Zou et al. (2000).] (See color plates.)

switch on responsiveness to these factors after crossing the midline (Zou et al., 2000). It is also supported by loss of function studies. We predict that if the repulsion of postcrossing axons is lessened, then the axons should be expelled less efficiently and stall out in the floor plate. Indeed, that is what we observed both in Neuropilin-2 knock-out mice (Zou et al., 2000) (Fig. 7) and in Slit triple mutant mice (Long et al., 2004). These data are thus consistent with the idea that the reason the axons move on

is because they switch on reponsiveness to repellents, which expel them out of the midline.

F. Moving On: Losing Attraction

As mentioned, however, for this expulsion to be effective, it is not only important to switch on repulsion after crossing the midline, it is also important to switch off attraction; otherwise the growth cones would get confused. We have obtained some insight into how attraction might be switched off at least in the case of Netrins. Specifically, we found that activation of the Slit receptor Robo can switch off Netrin-mediated attraction by binding and inactivating the Netrin receptor DCC. The way we discovered this dominant effect of Slit on Netrin was in experiments in which we studied the responses of individual *Xenopus* spinal growth cones to gradients of Netrin-1. The growth cones normally turn toward the source of Netrin over a period of tens of minutes, but if the experiment is performed in the presence of Slit in the bath, however, the attraction is completely abolished (silenced) (Stein and Tessier-Lavigne, 2001) (Fig. 8). This is a specific effect: If the axons are exposed to a different attractant, the neurotrophin BDNF, the attraction is not at all affected by Slit. Through extensive biochemical and physiological experiments, we found that this specific silencing effect of Slit is mediated by a direct receptor–receptor interaction: When Slit binds the receptor Robo, some change occurs in Robo (presumably conformational) that results in the Robo cytoplasmic domain latching on to the cytoplasmic domain of the Netrin receptor DCC, thereby silencing attraction (Stein and Tessier-Lavigne, 2001) (Fig. 8).

Thus, the mechanism of silencing involves a direct receptor–receptor interaction. The logic of this interaction is that it provides a guidance cue hierarchy, in which the repellent Slit dominates over the attractant Netrin. I believe that it makes eminent sense biologically to have such a hierarchy. Indeed, the aim is to activate repulsion and to inactivate attraction, and to do this in a coordinated manner to avoid growth cone confusion. What better way to ensure that the two events are coordinated than to have one, the switching on of repulsion, cause the other, the switching off of attraction?

We conclude, therefore, that moving on involves both switching on repulsion and switching off attraction. In this way the interaction of growth cones and midline cells is reminiscent of some human relations

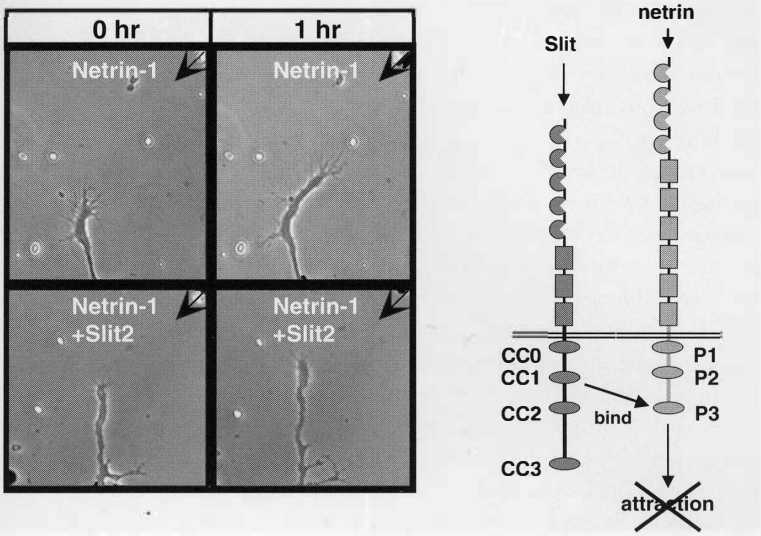


Fig. 8. Switching off attraction: Silencing of Netrin attraction by Slit. The left panels show individual *Xenopus* spinal growth cones exposed to a gradient of Netrin-1 protein emanating from a glass micropipette. Normally, the axons will turn toward the source over a period of an hour (top panels). However, in the presence of Slit2 protein (bottom panels), the attractive effect of Netrin-1 is blocked (silenced). As shown in the right-hand side of the figure, the evidence indicates that silencing of Netrin attraction involves Slit binding its receptor Robo, whose cytoplasmic domain then latches on to the cytoplasmic domain of the Netrin receptor DCC, thereby silencing DCC-mediated attraction. [Adapted from Stein and Tessier-Lavigne (2001).] (See color plates.)

in which, sadly, an initial attraction or infatuation is later destabilized by a growing repulsion, which not only pushes the two parties apart, but also, if it gets strong enough, can actually counteract the attraction that got them together in the first place, until all memory or trace of the attraction is erased, causing the parties to move on forever.

G. Preventing Premature Repulsion

But let us return to growth cones. For this guidance to occur accurately, it is evident that the switch from attraction to repulsion much be carefully choreographed. In particular, it is essential to ensure that the switch-

ing on of repulsion occurs only after crossing the midline, not before; otherwise the growth cones would fail to enter the midline. As I mentioned above, we have recently discovered a protein, Rig-1, that plays an essential role in mammals in preventing premature repulsion. Specifically, we have proposed that Rig-1 functions to keep commissural axons in a Slit nonresponsive state prior to midline crossing. The evidence that supports this notion is as follows (Sabatier et al., 2004).

First, Rig-1, which is a transmembrane protein, is present on the surface of commissural axons before and during midline crossing, but gets down-regulated rapidly after crossing, coincident with the upregulation of Slit responsiveness. This can be seen by immunohistochemistry using an anti-Rig-1 antibody, and it is consistent with a role for Rig-1 in commissural axon guidance prior to midline crossing.

Second, and most importantly, if we take away Rig-1 in a Rig-1 knock-out mouse, commissural axons extend normally to the floor plate, but then they all fail to cross the midline (Sabatier et al., 2004) (Fig. 9). This is, to my knowledge, the most highly penetrant axon guidance phenotype observed to date in a mammal. It is also consistent with the possibility that Rig-1 prevents the axons from becoming Slit-responsive prior to midline crossing.

A direct demonstration that Rig-1 functions to prevent premature Slit responsiveness comes from *in vitro* experiments using dorsal spinal cord explant cultures. When explants are taken from wild-type animals, Netrin-1 stimulates the outgrowth of these axons, an effect that is not impeded by the presence of Slit protein because the axons have not crossed the midline and hence are not yet Slit responsive. In contrast, when explants of dorsal spinal cord from Rig-1 knock-out mice are cultured, Netrin-1 still stimulates outgrowth of commissural axons, but addition of Slit protein can completely block this outgrowth, indicating that the axons are prematurely Slit responsive prior to midline crossing (Sabatier et al., 2004).

Thus, taken together, these results support the model that Rig-1 functions to prevent the axons from being responsive to Slit before crossing the midline, and that, upon crossing, Rig-1 gets down-regulated, allowing the axons to become Slit responsive.

What is Rig-1, and how did we come across it? Rig-1 is actually a divergent member of the Robo family, also known as Robo3, first identified by Lee and colleagues as an Rb-inducible gene (Yuan et al., 1999), hence

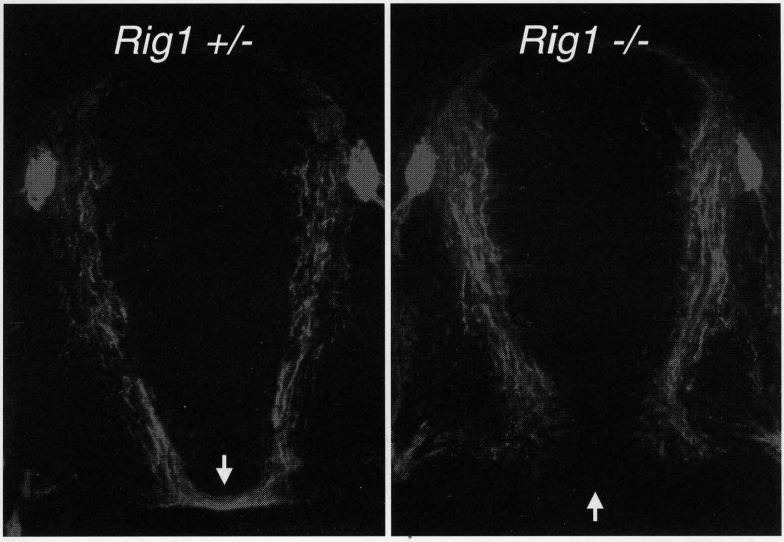


Fig. 9. Rig-1 is required to prevent premature Slit responsiveness. In a Rig-1 knockout mouse (right panel), commissural axons (labeled with an anti-TAG-1 antibody) all fail to cross the midline, in contrast to their normal behavior seen in wild-type mice or Rig-1 heterozygous mice (left panel). Floor plate is indicated by arrow. This failure to cross results from premature sensitivity of commissural axons to repellent Slit proteins at the midline. [Adapted from Sabatier et al. (2004).] (See color plates.)

its name "Rig." It is homologous to classical Robos on the outside but more divergent in its cytoplasmic domain. We were initially studying it simply because we thought it was just another Robo family member, but we rapidly found that it does not behave like a classical Robo protein: We expect a classical Robo to be absent from commissural axons before crossing, then switched on after crossing. However, we see exactly the opposite with Rig-1: It is on before, and off after. Similarly, in classical Robo knock-outs we expect the axons to stall out in the midline because they find it more attractive, but in the Rig-1 knockout they avoid the midline entirely. Put another way: Classical Robos are positive regulators of Slit function—they transduce a repulsive Slit signal—but Rig-1 is a negative regulator of Slit function—it blocks responses to Slits. Thus, rather than being a classical Robo, Rig-1 is an anti-Robo protein.

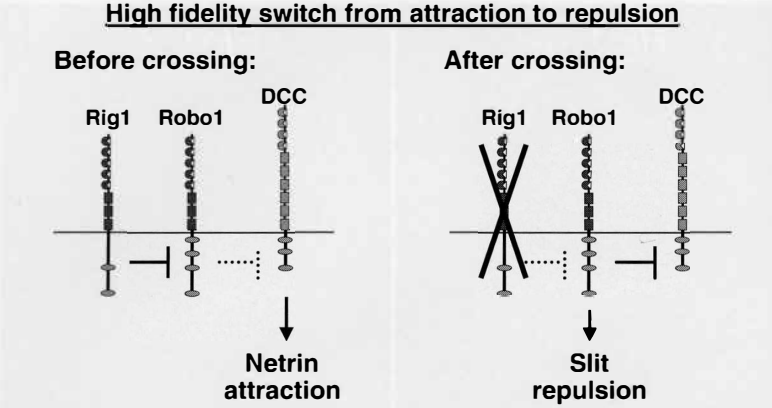


Fig. 10. A high-fidelity switch from attraction to repulsion at the vertebrate midline. This switch involves at least three proteins, DCC, Robo1, and Rig1. Left panels, before crossing: Netrin attraction, mediated by DCC, would be silenced by Robo1 if it weren't for the fact that the axons express Rig1, which inhibits Robo1, disinhibiting DCC. Right panel, after crossing: Netrin attraction would continue inappropriately after crossing if it weren't for the fact that Rig1 is down-regulated (indicated by the X), disinhibiting Robo1, with two consequences: DCC is silenced, and Slit repulsion can proceed. [Adapted from Sabatier et al. (2004).] (See color plates.)

How does Rig-1 produce its anti-Robo effect? We don't know at present, but one possibility is that, given its structure, Rig-1 functions as an endogenous dominant negative, blocking the function of the classical Robos. Future studies will determine whether it produces its effects through this or through another mechanism.

H. Summary: A High-Fidelity Switch from Attraction to Repulsion

The preceding sections have thus shown that there is a high-fidelity switch that occurs in the growth cone at the midline, from being attracted to being repelled, which involves at least three proteins: Rig-1, Robo-1, and DCC. The mechanism of the switch is illustrated in Fig. 10. Before midline crossing, Netrin attraction, mediated by DCC, would be blocked by Robo-1 if it weren't for the fact that Rig-1 is expressed on the axons, blocking Robo-1 function, thereby disinhibiting DCC, which allows Netrin attraction to proceed. After midline crossing, Netrin attraction

would continue inappropriately if it weren't for the fact that Rig-1 gets down-regulated, disinhibiting Robo-1, with two consequences: first, this results in the silencing of Netrin attraction by direct binding of Robo-1 to DCC; second, it results in Robo-1 transmitting a repulsive Slit signal, allowing the axon to be repelled out of the midline and to move on to the next leg of its trajectory.

There are of course many unanswered questions that remain. How is Rig-1 protein switched off? How does Rig-1 block Robo-1 function? How do other attractants like Shh and repellents like Sema3B fit in? Is Shh also silenced by Slit, and how is Sema3B activated? And what is the identity of the en passant trophic factor, and what is its normal physiological role? Clearly, much more work remains in order to understand fully the mechanisms of midline guidance.

IV. First Conclusion: The Logic and Molecular Mechanisms of Axon Guidance

Despite the many holes in our knowledge, studies like those summarized above already support some tentative conclusions regarding the logic and molecular mechanisms of axon guidance.

First, axons are guided by the combined actions of attractants and repellents acting in concert. It is their combined actions that ensure the high degree of fidelity in axon guidance—the fact that axons make few, if any, errors of projection.

Second, axons can be guided by both short-range and long-range guidance cues—in this chapter I have focused primarily on the latter.

Third, we should think of these cues not as attractants, repellents, or branching factors per se, but rather as wiring cues, that can be interpreted in different ways by different axons, or by the same axon at different times.

Fourth, the plasticity of guidance responses at intermediate targets, involving a switch from attraction to repulsion, is key to the ability of axons to move on from one intermediate target to the next, thus enabling them to extend accurately over long distances.

Finally, we must take seriously the possibility that, in addition to the "big four" of axon guidance—Netrins, Semaphorins, Ephrins, and Slits, as well as growth factors—we must also consider morphogens—Hedgehogs, Wnts and BMPS—as good candidates for wiring the brain as well.

V. Second Conclusion: Axon Development and Axon Regeneration

At the beginning of this chapter, I promised I would end by discussing how these insights obtained in the embryo might assist in attempts to stimulate regeneration following injury, so I would now like to turn to the issue of regeneration. Before I do this, however, I must first discuss one last aspect of development, because it is immediately relevant to regeneration.

Until now, I have focused exclusively on the effects of cues like the Netrins on growth cones and axons in stimulating axon growth and in guiding axons. However, evidence has been mounting in recent years that how axons respond to these cues is conditioned by dedicated transcriptional programs active in the nucleus of the cells that specify the expression of receptors and signal transduction pathways by the neurons. Very recently, we and our collaborators made the unexpected discovery that some of these transcriptional pathways are activated by cues like the Netrins and neurotrophins themselves. Specifically, what we showed is that Netrins and neurotrophins activate a signaling cassette involving calcineurin–NFAT signaling in the nucleus of neurons, and that activation of this signaling pathway is absolutely essential to the ability of neurons to respond to these factors with extensive axon growth in a sustained fashion: If we block this cassette (genetically or pharmacologically), the axons can only respond for a short period of time, presumably because this cassette activates expression of genes required for sustained outgrowth (Graef et al., 2003). This provides a potential transcriptional gate at the level of which axon elongation can be regulated. The main message of this study is that the growth of axons involves both (a) the action of extracellular cues on growth cones, which was the major focus of this chapter, and (b) the action of dedicated transcriptional programs in the nucleus of the neurons that determines how the neurons respond to these cues.

This message is directly relevant to regeneration. Whereas during development, axons grow to their targets under the influence of attractants and repellents, as described above, in the adult central nervous system (i.e., the brain and spinal cord), when axons are severed, they will reform growth cones and try to regrow, but fail to do so. For example, following a spinal cord injury, axons connecting the brain and spinal cord are

severed and fail to regrow, so the paralysis that accompanies spinal cord injury is usually permanent.

Injured axons in the adult central nervous system (CNS) fail to regrow for two reasons [reviewed in Filbin (2003)]. First, the environment is hostile to regrowth: There are factors that actively inhibit regrowth. A major focus in the field is thus to try to identify the major inhibitors, in order to neutralize their effects. Candidates for inhibitors of adult axon regeneration include molecules like Nogo, chondroitin sulfate proteoglycans, and the classical axon guidance molecules like the Semaphorins, Slits, Ephrins, and Netrins, which we and others are studying. But in addition to inhibitors in the environment, evidence has been mounting that another reason adult CNS neurons fail to regenerate is because they have a decreased intrinsic ability to regrow. In the case of retinal ganglion cells, for example, elegant studies have indicated that around the time of birth these neurons lose the ability to send axons out in an efficient way, even when placed in a highly permissive environment (Goldberg et al., 2002). Thus, to obtain efficient regeneration, it may also be necessary to kick the neurons back into a more embryonic-like growth state.

The idea that it might be possible to kick adult neurons back into a growth state derives support from work on adult sensory neurons, to which we and our collaborators have contributed. Sensory neurons in the dorsal root ganglia have both a peripheral axon, which regenerates after injury, and a central branch, which normally does not. Interestingly, if the central branch is lesioned a few days after the peripheral branch is lesioned (a so-called preconditioning lesion), the central branch will now regenerate. This finding, and others, have been interpreted to show that the preconditioning peripheral lesion kicks the neuron into a growth state through some sort of transcriptional switch in the cell body, and that once it is in that state, both the peripheral and central branches benefit from the new growth state, being capable of regrowth; in contrast, lesioning the central branch does not trigger the switch [reviewed in Filbin (2003)]. We and our collaborators, and, independently, Marie Filbin's group, have shown that regeneration of the central branch can also occur if the central lesion is performed after the cell bodies in the dorsal root ganglia have been exposed to a membrane-permeable analog of cAMP, consistent with a model in which cAMP activates a program of gene expression that puts the neuron into a growth state (Neumann et al., 2002; Qiu et al., 2002) (Fig. 11).

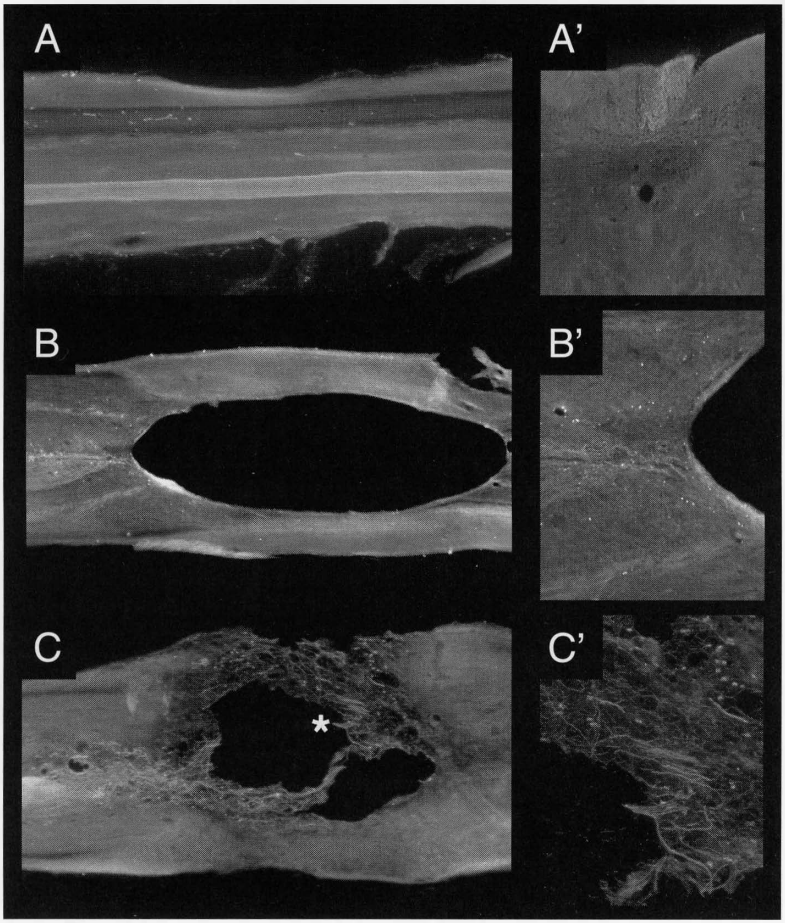


Fig. 11. Stimulating regeneration by kicking adult sensory neurons into a growth state. (A–C) Dorsal columns visualized in an intact rat spinal cord (A), the spinal cord of a rat six weeks after a dorsal hemisection (B), and the spinal cord of a rat in which the cell bodies of sensory neurons were exposed to a single pulse of dibutyrl cAMP several days before a dorsal hemisection (again, axons were visualized 6 weeks later) (C). No regeneration is observed in the control lesioned animal (B), whereas extensive regeneration is observed through the lesion site after the cAMP treatment. Panels B′ and C′ show higher magnification views of panels B and C. Panel A′ shows labeling in the dorsal column nuclei in the control animal (A). [Adapted from Neumann et al. (2002).] (See color plates.)

Experiments like this provide hope that some manipulations of the intrinsic growth state of neurons, together with manipulations to make the environment less inhibitory, will some day provide sufficient regenerative capacity to spinal neurons to allow significant recovery from paralysis in spinal cord injury patients.

VI. Ramon y Cajal, Scientist and Prophet

These considerations bring me back to Ramon y Cajal, because he was not only the discoverer of the growth cone and the first person to propose the chemotropic theory of axon guidance, he was also a pioneer in the analysis of the degeneration and regeneration of the nervous system. The limitations on the regenerative capacity of the nervous system, which he documented so extensively, led him to lament in his classic treatise on the "Degeneration and Regeneration of the Nervous System" (Ramon y Cajal, 1913) that:

. . . the functional specialization of the brain imposed on the neurons two great lacunae: proliferative inability and irreversibility of intraprotoplasmic differentiation. It is for this reason that, once the development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In adult centers the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated.

This might appear to be a very pessimistic conclusion. However, Ramon y Cajal was at heart and optimist, and he continued as follows in the very next paragraph:

It is for the science of the future to change, if possible, this harsh decree. Inspired with high ideals, it must work to impede or moderate the gradual decay of the neurons, to overcome the almost invincible rigidity of their connections, and to re-establish normal nerve paths, when disease has severed centers that were intimately associated.

The studies summarized in the closing paragraphs of this chapter, will, I hope, provide some justification for believing that Ramon y Cajal's optimism was indeed warranted, and that therapies for stimulating repair and regeneration of injured axons in the central nervous system will see the light of day before long.

Acknowledgments

In closing, I would like to thank the many collaborators who have contributed to this work, including first and foremost the many students, postdoctoral fellows, and research staff in my laboratory, whose work is mentioned in the articles cited in this chapter. We have also benefited from a wonderful set of collaborators, particularly in the laboratories of Corey Goodman, Cori Bargmann, Joseph Culotti, Mu-ming Poo, Fujio Murakami, Christine Holt, and Allan Basbaum, whom I would like to thank for their friendship and collaboration.

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