Neuron, Vol. 22, 649–652, April, 1999, Copyright ©1999 by Cell Press

The Middle and the End: Slit Brings Guidance and Branching Together in Axon Pathway Selection

Minireview

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A typical developing neuron has a beginning, a middle, and an end: the cell body, the axon, and the growth cone. Those of us who are fascinated by the journey that an axon takes to reach its synaptic partners often focus our attention on the dynamic and talented growth cone, which leads the neuron's exploration of the embryonic landscape. However, it has been clear for a century that connections with targets are often made by collateral branches that extend from the middle of the axon (e.g., Ramon y Cajal, 1911). From an anatomical point of view, guidance of the primary growth cone seems very distinct from the extension of branches. Yet, at the molecular level, it seems entirely plausible that the two processes may share similar mechanisms of control.

Four papers in *Cell* and one in *Neuron* now bring a specific focus to this idea in the context of pathway selection by axons on the way to their target regions. Studies from the groups of Corey Goodman, Marc Tessier-Lavigne, Yi Rao, and Alain Chédotal show that secreted factors known as Slit proteins, named for their *Drosophila* genetic locus, function as axon repellents at the midline, an intermediate target that has been a major model system in axon pathway selection (Brose et al., 1999; Kidd et al., 1999; Li et al., 1999; Nguyen Ba-Charvet et al., 1999). A separate study from the Tessier-Lavigne lab identifies a branch-promoting factor that is likely to be involved in axon pathway selection, where the molecular mechanism of branch control had previously been mysterious. The delightful surprise is that this branch-promoting activity is also one of the vertebrate Slit proteins (Wang et al., 1999). These striking observations unveil Slit proteins as multifunctional regulators of axonal development and raise fascinating questions about the relationship between Slit function at the middle and the end.

The Midline Axon Guidance System

As an axon selects a pathway to reach its synaptic partners, it may encounter intermediate targets that guide its decisions along the way. Among these intermediate targets, the midline that separates the left and right halves of the central nervous system (CNS) has proven to be a powerful model system to dissect axon guidance mechanisms. Specialized cells at the midline have a number of roles in directing growth cone behavior (Figure 1). In the vertebrate spinal cord, in the insect ventral nerve cord, and in *C. elegans*, midline cells produce netrins, long-range chemotropic cues that attract the growth cones of commissural (crossing) axons to grow toward the midline.

The midline also provides repellent information. An early and direct demonstration of this came from studies on the vertebrate forebrain, where Pini (1993) showed by in vitro coculture of neural explants that a diffusible activity from the midline septum can repel axons growing out from the olfactory bulb (Figure 1A). The presence of repellents at the midline was also indicated by genetic analysis in *Drosophila* where, in the *roundabout* (*robo* or *robo1*) mutant, axons fail to respect the midline boundary (Figure 1C). These and other studies (reviewed by Flanagan and Van Vactor, 1998) indicate that cells at the midline choice point act as gatekeepers that have

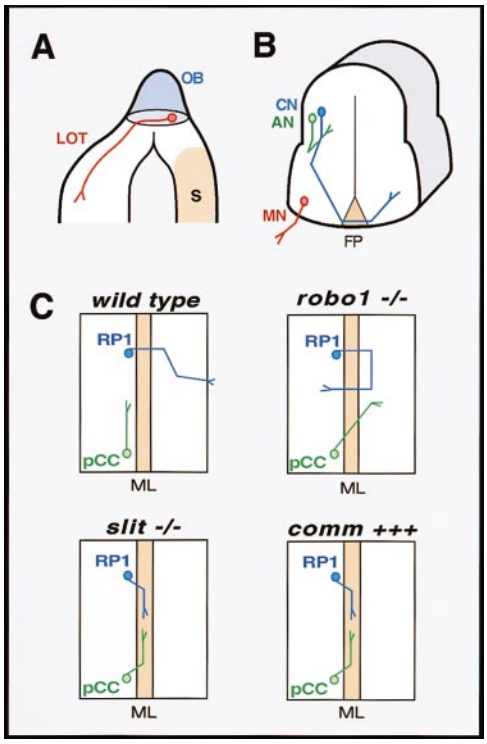

Figure 1. Axon Guidance at the Midline

(A) Cross-sectional diagram of the anterior vertebrate forebrain where axons from olfactory bulb (OB, blue) neurons extend away from the midline septum (S, orange) to form the lateral olfactory tract (LOT, red). Anterior is up.

(B) Cross-sectional diagram of the embryonic vertebrate spinal cord illustrating commissural axon pathways (CN, blue) that cross the midline floorplate (FP, orange), association pathways (AN, green) that remain ipsilateral, and motor pathways (MN, red) that extend away from the midline. Once CN axons cross the floorplate, they turn anteriorly and extend between the floorplate and the motor neurons. Dorsal is up.

(C) Axonal phenotypes of *Drosophila* mutants. In wild-type embryos, the ipsilateral neuron pCC (green) extends an axon anteriorly without crossing the midline (ML, orange), whereas the contralateral neuron RP1 (blue) crosses the ML once. In *robo*−/− embryos, pCC now crosses the midline, while RP3 can cross multiple times. In *slit*−/− mutants, all axons collapse into one central pathway. Although moderate overexpression of *comm* leads to a phenotype similar to *robo*, strong *comm* overexpression (+++) leads to a phenotype similar to *slit*. Anterior is up.

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two important functions: (1) to prevent axons from inappropriately crossing (or recrossing) the midline, and (2) to induce a switch in growth cone responsiveness to guidance cues beyond the gateway, thus allowing axons to begin the next step in their journey.

The first molecular clues about the gatekeeper mechanism came from *robo* gene cloning and sequencing, which showed that the encoded protein is a receptor-like protein of the immunoglobulin superfamily with highly conserved counterparts from *C. elegans* to mammals (Kidd et al., 1998a; Zallen et al., 1998). In the *Drosophila* embryo, Robo protein is abundant on noncrossing axons, whereas crossing axons express low levels before they reach the midline and high levels after they cross (Kidd et al., 1998a). As one would expect of a receptor, Robo functions cell autonomously in neurons that should avoid the midline (Kidd et al., 1998a; Zallen et al., 1998). Thus, the function, structure, and expression of Robo family members led to the hypothesis that these molecules are receptors for a ligand that acts as a repellent midline gatekeeper. However, proof of this model required identification of the ligand.

Slit and the Genetic Logic of the Midline Choice Point

Slit, first characterized in *Drosophila*, is a large secreted protein containing four leucine-rich and seven epidermal growth factor repeats. It is expressed at the CNS midline, is associated with the extracellular matrix, and accumulates on the axons of neurons that do not express the gene (Rothberg et al., 1990).

Identification of Slit as a ligand for Robo represents a triumph of reason, largely because the *slit* phenotype in *Drosophila* is far more severe than that of *robo* alone (Figure 1C). In *slit* mutants, all CNS growth cones extend toward the midline where they form one giant axon fascicle, as if the midline retains its chemoattractive function but has lost both the gatekeeper repellent and the ability to orient axons to the next set of cues. In contrast, in *robo* mutants only axons most proximal to the midline cross inappropriately, and although these axons can cross the midline multiple times, they retain the ability to escape the grasp of the midline cells, forming two axon bundles on either side of the midline.

The logic that links Robo and Slit involves another midline player, *Drosophila commissureless* (*comm*), which acts to allow contralateral axons to cross the midline by downregulating the expression of Robo (Kidd et al., 1998b, and references therein). Kidd and colleagues now show that while moderate overexpression of Comm protein results in a *robo*-like phenotype, high levels of Comm misexpression yield a phenotype very similar to *slit* (Kidd et al., 1999). This observation, coupled with the existence of a second Robo family receptor in *Drosophila*, Robo2 (Kidd et al., 1998a), suggests that Comm regulates both receptors and also that Slit might function as a ligand for both receptors. This model predicts that double mutants lacking both Robo1 and Robo2 should appear identical to *slit* mutants; however, mutations in *robo2* have yet to be described.

To test this model, Kidd et al. (1999) used the genetic strategy of transheterozygote analysis. They found that 2-fold reduction in either protein alone (*slit*$-/+$ or *robo*$-/+$) has little effect on midline axon guidance, but a 2-fold reduction in both simultaneously causes dramatic midline crossing defects, implying that they act in the same molecular pathway. As a confirmation of the hypothesis that Slit repels axons, Slit was ectopically expressed in developing muscles. As expected, motor growth cones fail to extend across muscles that express Slit (Kidd et al., 1999).

Slit Binds to Roundabout and Controls Growth Cone Guidance

While *Drosophila* genetics served to identify Slit as a likely ligand for Robo receptors, confirmation of the model requires a direct biochemical demonstration of binding. This was answered for both *Drosophila* and vertebrate proteins by cell surface binding assays, which showed that Slit proteins do bind to Robo proteins (Brose et al., 1999; Li et al., 1999).

Supporting a functional relationship in vivo, Robos and Slits display complementary patterns of embryonic expression. This is true in *Drosophila* (Rothberg et al., 1990; Kidd et al., 1998a) and also in vertebrates, where three Slit proteins have been identified (Brose et al., 1999; Li et al., 1999; Nguyen Ba-Charvet et al., 1999; and references therein). In particular, it is notable that all the known Slits are expressed at the CNS midline.

Repellent activity was directly shown by assays on mammalian neural explants in vitro. These showed that Slit2 can repel motor axons, though the biological significance of this finding is not entirely clear since motor neurons themselves express high levels of *slit2* (Brose et al., 1999). They also showed that Slit2 strongly repels olfactory bulb axons (Li et al., 1999; Nguyen Ba-Charvet et al., 1999). This latter finding is particularly satisfying since Slit2 is expressed prominently in the septum (Li et al., 1999; Nguyen Ba-Charvet et al., 1999) and is a strong molecular candidate for the activity that had been identified several years before by Pini (1993) as the first diffusible chemorepellent.

These studies of Slit and Robo, as well as other recent observations, make our understanding of the midline choice point much clearer. We have Netrin to bring crossing axons to the midline and Slit, the gatekeeper, to drive noncrossing axons away. Once crossing axons have passed the midline barrier, Slit can now act to keep them on the opposite side through midline repulsion. In addition, as Li and colleagues point out, the expression of Slit2 by vertebrate motor neurons may prevent commissural axons from extending too far beyond the midline, thus restricting their subsequent longitudinal pathway to its correct location between the floorplate and the motor columns (Figure 1B; Li et al., 1999).

Other questions remain. After being attracted to the midline, how do crossing axons escape from it, and what is the molecular switch that makes crossing axons interested in a different set of guidance cues on the opposite side of the gateway? In vertebrates, contact with midline cells makes commissural growth cones nonresponsive to the chemoattractant netrin, a switch in behavior that presumably helps them escape from the midline (reviewed by Flanagan and Van Vactor, 1998). Since no axons escape the midline in *Drosophila slit* mutants, it is possible that Slit itself has something to do with this switch. An alternative model is simply that the repellent action of Slit may normally make the midline an unappealing place to linger, providing a counterbalance to the attraction of netrin. It is also not yet

known whether Slits have any effect on vertebrate commissural axons (Brose et al., 1999)—for example, one might suspect a repulsion, but one that would depend on a prior switch in responsiveness induced by floorplate crossing.

Axon Branching

Work by O'Leary and his collaborators, beginning in the mid 1980s, played an important role in advancing the understanding of the development of axon collateral branches. Particularly informative results came from their studies of cortical layer 5 neurons, which send axons all the way down to the spinal cord and on the way establish a connection with the basilar pons in the hindbrain. They were able to show that this connection to the basilar pons is formed by neither guidance nor bifurcation of the axon tip, but rather by "delayed interstitial branching"—sending out side shoots from the shaft of the primary axon, long after its tip has passed (reviewed by O'Leary et al., 1991).

Just as with Pini's studies of olfactory axon guidance, a key step for O'Leary's group involved exploiting the collagen gel coculture assay, growing cortical explants, and placing pieces of basilar pons nearby, either before or after the cortical axons had grown out (O'Leary et al., 1991). These experiments demonstrated for the first time the existence of a diffusible target-derived branch-promoting activity. However, the molecule(s) responsible for this activity remain unknown.

Early studies on the neurotrophins indicated that these molecules can act as both chemoattractants and branch-promoting factors (Levi-Montalcini, 1987, and references therein). The significance of these findings in a physiological context has been uncertain until recent years, however, when neurotrophins have been found to promote axon branching within targets; for example, brain-derived neurotrophic factor (BDNF) in the optic tectum promotes the branching of retinal axons (Cohen-Cory and Fraser, 1995). While these studies indicate roles for neurotrophins in branching within targets, they have not so far indicated functions in controlling branching before target entry, during pathway selection, where the identity of branching factors has remained a mystery.

Slit Molecules and the Control of Axon Branching

Tessier-Lavigne and his colleagues set out to address the branch factor question using as a model system dorsal root ganglion (DRG) neurons, which provide the sensory connections from the periphery to the spinal cord (Figure 2). Rather than plunging directly into the spinal cord during development, when the DRG axons first reach the cord, they bifurcate and grow along its surface in the anterior and posterior directions. After a delay, entry into the cord is then accomplished by collaterals that sprout from these axons (Ozaki and Snider, 1997; Wang et al., 1999). The process thus appears to fit the description of delayed interstitial branching.

Wang et al. (1999) addressed this modern problem with a classical approach—coupling a bioassay with a biochemical purification scheme. Since no convenient bioassay was available, they developed their own. By dispersing DRG neurons in a collagen gel at low density, they were able to show that formation of prominent collateral branches is promoted by a homogenate of embryonic spinal cord. The next phase was a series of biochemical fractionation steps, using calf brain as an abundant source rather than embryonic spinal cord. This relies on a leap of faith, of course, that the same molecules will be present. The result of the biochemistry was the purification of a protein with branch-promoting activity, which was microsequenced to find its identity.

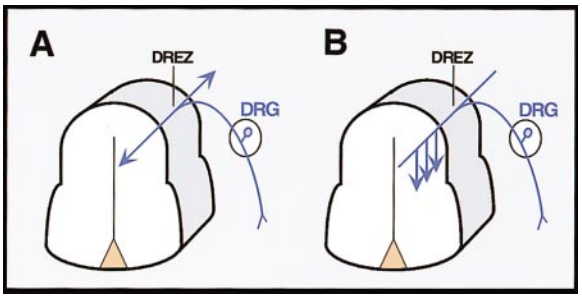

Figure 2. Branching of DRG Axons as They Enter the Spinal Cord

(A) Sensory axons from neurons in the dorsal root ganglia (DRG, purple) first contact the spinal cord at the dorsal root entry zone (DREZ) and then extend anteriorly and posteriorly along the surface of the spinal cord (embryonic day E14 in rat).

(B) As development proceeds, ventrally directed collateral branches sprout from the DRG axons toward targets within the spinal cord (E17 in rat).

In one of the remarkable coincidences of molecular biology, the branch-promoting activity turned out to be none other than Slit2—which was being studied as a chemorepellent ligand in a totally independent project in the same lab! At a stroke, these studies therefore established Slit2 as a bifunctional molecule, which can cause chemorepulsion of some axons, while having branch-promoting activity on others. In fact, Wang et al. (1999) also showed that Slit2 promotes DRG axon elongation, so one might even call it multifunctional.

Particularly in view of this remarkable coincidence, one might wonder if branch promotion is a rather nonspecific property that might be shared by numerous factors. However, before committing themselves to the purification, Wang and colleagues had tested an impressive array of 39 other candidate factors, none of which had branch-promoting activity. It therefore seems, at least in this assay, that branch promotion is a very specific action of Slit2.

Are Slit proteins the key regulators of DRG axon branching in vivo? Expression studies by Wang et al. (1999) support this idea, placing Slit2 expression at an appropriate time and place in the dorsal spinal cord to promote collateral entry. Slit1 is also expressed in the dorsal spinal cord and might additionally contribute to branch control. A more formal demonstration of the role of Slit proteins in vivo will require further studies, presumably including loss-of-function experiments. However, the in vitro activities and expression patterns already indicate that Slit proteins are very likely to play at least some role in the control of DRG axon branching.

The relationship between the structure of Slit2 and its various actions has already been partly worked out, though other aspects remain as interesting questions. Slit2 is proteolytically cleaved to produce two large fragments, Slit2-N and Slit2-C (Brose et al., 1999; Wang et al., 1999). These two differ in their tendency to remain

cell associated in culture, with Slit2-N being more tightly bound, suggesting the possibility of two active factors with different ranges of action. It is already known that branch-promoting activity resides in Slit2-N, though it is not yet clear whether the repellent functions are also associated with Slit2-N or instead with Slit2-C. In another unusual twist to the story, Slit2 was found to bind at high affinity to laminin-1 and netrin-1, two other molecules that can affect axon outgrowth or guidance (Brose et al., 1999). So far, there is no evidence that the binding of these molecules affects their activities, but it is at least an intriguing possibility that in vivo they may form a localized complex with multiple functions in the control of axon connectivity.

What Next? The Beginning?

The multitalented nature of Slit raises interesting questions about the mechanisms downstream. Do Robo1 and Robo2 have similar or different functions? Are all the actions of Slits mediated by Robo family receptors? More generally, bifunctionality is now a common theme among axon guidance cues, though we still understand little about the mechanisms that translate signals at the cell surface into differential responses within the cell. Studies in vitro show that intracellular cyclic nucleotide levels can interconvert responses between attraction and repulsion, implying that these opposite types of guidance share a common signaling machinery (Song et al., 1997). The identification of neurotrophins and Slit proteins as molecules that can control guidance, and can also promote prominent collateral branches, suggests a common machinery for guidance and branching too. Intriguingly, careful in vitro observations have indicated that sprouts, which might be precursors to collateral branches, can form in response to collapse of the primary growth cone (Davenport et al., 1999), or at axonal positions that have been previously marked by growth cone arrest (Szebenyi et al., 1998). These studies suggest potential mechanisms for a direct link between the middle and the end, though it is not yet known whether this may be relevant to sprouting in vivo (e.g., Ozaki and Snider, 1997). It will also be interesting to know whether the machinery that regulates the middle and the end also controls the beginning—the initial polarity of axon outgrowth.

These new studies in axon pathway selection may also provide a glimpse of another kind of beginning. Up to now, research on axon guidance molecules has been in the realm of basic science, but one can increasingly begin to see the potential for clinical applications. Wang and colleagues point out that target-derived branch promoting factors might prove useful in the regeneration of connections after spinal injury (Wang et al., 1999). Another example is tactile allodynia, where nerve injury or inflammation can induce collateral sprouting of mechanoreceptor axons within the spinal cord, switching the perception of innocuous stimuli to that of painful ones (Woolf, 1997). Here, there might be a clinical use for branch inhibition. Eventually, Slit proteins or other factors that can modulate axon branching or guidance might find applications in many conditions where the outcome could be improved by the regulation of neural plasticity.

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