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Surrounded by Slit—How Forebrain Commissural Axons Can Be Led Astray

In *Drosophila*, Slit acts as a barrier preventing roundabout expressing axons from entering the midline and sorting contralaterally from ipsilaterally projecting axons. Hutson and Chien, Plump et al., and Bagri et al. (all in this issue of *Neuron*) use Slit knockout mice and zebrafish *astray*/Robo2 mutants to show that in vertebrates, Robo/Slit function to channel axons into specific pathways and determine where decussation points occur. Ipsilaterally and contralaterally projected axons are equally affected.

Roundabout (Robo) was originally identified in *Drosophila* as a gene required to prevent aberrant crossing of the midline. Robo encodes a transmembrane protein with multiple immunoglobulin and fibronectin motifs. Robo has been shown to act as a receptor expressed on axons and migrating cells, and Slit was identified as a repellent ligand for Robo. Robo and Slit proteins are phylogentically highly conserved, with multiple family members having been identified in both vertebrate and invertebrate species. Over the past few years, Robo/Slit proteins have been implicated in a variety of axon guidance events. In particular, there has been a large focus on the role of these proteins in regulating axonal crossing at the midline.

In the longitudinal axis of *Drosophila* and *C. elegans,* commissural axons normally cross the midline only once. However, in Slit (slt1 in *C. elegans* (Hao et al., 2000)) or roundabout (Robo; sax3 in *C. elegans*) mutants of these species, axons either enter the midline and fail to leave, or cross and recross the midline multiple times, suggesting that Slit/Robo interactions normally act to prevent the recrossing of commissural axons in these systems and inhibit ipsilaterally projecting axons from ever crossing the midline. With the identification of Slit/Robo homologs in vertebrate systems and the demonstration that Slit can act as a chemorepellent for vertebrates in axon pathfinding and cell migration (for review, see Brose and Tessier-Lavigne, 2000), it was proposed that Slit/Robo might play similar roles in regulating midline crossing in vertebrates as they do in invertebrate systems.

In vertebrates, the most extensively studied commissural projections are the dorsal sensory neurons of the developing spinal cord that decussate at the ventral floorplate and the retinal ganglion cell (RGC) axons of the visual system that decussate at the optic chiasm. In these systems, interesting questions can be asked not only about how axons cross from one side of the nervous system to the other, but also how ipsilateral and contralateral projections are delineated. In principle, axons make similar choices at the optic chiasm as longitudinal axons make at the invertebrate midline. During development, RGC axons exit the eyes at the optic disc, travelling along the base of the ventral diencephalon toward the midline where they encounter projections from the contralateral eye and form the optic chiasm. At the chiasm, some proportion of these axons cross the midline to project to contralateral targets in the brain, while others grow away from the midline and project to ipsilateral targets. Previous expression analyses and in vitro axon guidance assays in the visual system of mice suggested that Slit and Robo may play a role in regulating whether axons cross or remain ipsilateral at the chiasm. Both Slits and Robos are expressed along the pathway of retinocollicular projections. In the rodent retina, Slit1 and 2 are expressed in the RGC layer of the retina as are Robo1 and to a lesser extent Robo2. Slit2 is also expressed at the optic nerve head on either side of the optic stalk, perfectly situated to surround the optic nerve. In more distal regions of the pathway Slit2 is expressed anterior and dorsal to the optic chiasm in the preoptic area and within the hypothalamus, dorsal thalamus, and epithalamus of the diencephalon (Erskine et al., 2000; Ringstedt et al., 2000). In addition, in vitro Slit causes the collapse and repulsion of retinal ganglion cell (RGC) axons (Niclou et al., 2000; Erskine et al., 2000; Ringstedt et al., 2000).

These in vitro and expression studies suggested the possibility that Slit/Robo may participate in the guidance of RGC axons. The first in vivo confirmation of this came from the identification by Chi-Bin Chien and colleagues of a zebrafish mutant called *astray* (identified as the homolog of Robo2). Due to its transparency and suitability for genetic studies, the zebrafish is an ideal model system for studying vertebrate axon guidance. Chien and colleagues identified *astray* in a large scale screen for defects in retinal axon pathfinding. RGC axons in *astray* mutants display defects in both anterior and posterior RGC axonal pathfinding, recrossing of the midline both at the chiasm and in other regions of the brain, and defasiculation of the optic tract. As in the rodent, *astray*/Robo2 is expressed in RGCs and transplantation studies showed that *astray* is required cell autonomously for the proper pathfinding of RGC axons consistent with the proposed role of *astray*/Robo2 as an axonally expressed guidance receptor (Fricke et al., 2001).

In order to investigate the mechanisms that cause the phenotypic defects observed in the *astray* mutant, Hutson and Chien (2002) used both fixed tissue and time lapse imaging of live RGC axons as they grew toward and across the midline. Interestingly, Hutson and Chien find that not only does *astray*/Robo2 regulate pathfinding by "guiding" axons in the proper direction (as would be consistent with previous repulsive effects in vitro), but *astray*/Robo2 also appears to act to correct pathfinding errors. By imaging wild-type and mutant labeled growth cones, Hutson and Chien show that even in wild-type embryos, a proportion of axons misroute at the midline, but that these navigation errors are corrected by a process involving growth cone collapse and retraction. However, in *astray*/Robo2 mutants, axons make more

errors than in wild-type and most of these misroutings are not corrected (Hutson and Chien, 2002). These experiments support the model that Slit/Robo (*astray*) interactions are required for axons to remain within the main axon bundle of the optic nerve. It is also of note that although there is no ipsilateral visual projection in zebrafish, these defects occur even in axons which never cross the midline. This was best illustrated by crossing *astray* mutants to *bel* mutants (in *bel* mutants, RGC axons project only to the ipsilateral tectum), where Hutson and Chien (2002) found that RGC axons still defasciculated from the main axon bundle even though they had not crossed the midline.

To determine if Slit proteins act in a similar way in mammals, Tessier-Lavigne and colleagues have generated Slit1, Slit2, and Slit1/2 knockout mice (Plump et al., 2002) and examined the development of the optic chiasm in such mutants. Plump et al. find that in mice defective in either Slit1 or Slit2, RGC axons show few or no pathfinding defects. In contrast, in Slit1/2 double knockout mice, RGC axons display a variety of guidance errors, including the formation of an ectopic chiasm in more anterior regions where Slit would normally be expressed, as well as other misprojections at the chiasm, phenotypes which are remarkably similar to those observed in the *astray*/Robo zebrafish mutant. In both species, ipsilateral and contralateral visual projections were affected.

The defects in the Slit1/2 double knockout mice and the *astray* mutants suggest that Robo/Slit at the chiasm may be acting in a manner differently from the way these proteins function at the fly midline. In vertebrates, Robo/Slit does not appear to function to sort ipsilateral from contralateral projections. Rather, Slit1/2 appear instead to be required to maintain axons within the optic nerve in a tightly fasciculated bundle. In addition, Slit/Robo also appear to play a role in regulating RGC pathfinding prior to reaching the midline. This is in contrast to *Drosophila*, where contralaterally projecting axons are only responsive to Slit after crossing the midline, and is also different from the results that have been obtained in vitro with dorsal commissural axons of the spinal cord which are also responsive to Slit only after crossing the floorplate (Zou et al., 2000). In both *astray* mutants and Slit1/2 double knockout mice, guidance defects were also found prior to crossing the midline (Fricke et al., 2001; Hutson and Chien, 2002; Plump et al., 2002).

These results confirm that Slit/Robo interactions are required for axonal pathfinding on the ipsilateral side of the brain, before crossing the midline (as well as the contralateral side). In parallel experiments, Tessier-Lavigne and colleagues also examined the phenotype of Slit1/2 double knockouts in other regions of the forebrain and show that the same "channeling" mechanism is operating within other developing axonal bundles of the forebrain. Bagri et al. (2002) show in Slit1/2 double knockouts that cortical and thalamic axons deviate within the internal capsule and form an ectopic commissure at the level of the anterior commissure. This data, coupled with Slit expression in the cortex and thalamus/hypothalamus indicate that Slit acts to channel these axons into the correct path. In the developing corpus callosum, Slit expression in the glial wedge and indusium griseum glia has been suggested to provide a repulsive/suppressive signal that was proposed to provide a barrier that maintains axons within the tract of the corpus callosum (Shu and Richards, 2001). Consistent with this, in Slit2 knockout mice, callosal axons fail to cross the midline and instead grow down through the glial wedge and into the septum (Bagri et al., 2002). Together with the analysis at the chiasm, these results suggest that Slit/Robo proteins control multiple aspects of axon guidance in the brain, including the maintenance of correct dorsoventral positioning of axon tracts, the channeling of axons into specific regions and tracts, and in preventing and correcting normally occurring axonal misroutings.

In each of these vertebrate systems, Slit appears to maintain these axons within the correct tract by a mechanism similar to that described in the peripheral nervous system and called surround repulsion (Keynes et al., 1997). In the periphery, DRG axons are repelled by secreted molecules from tissues located either directly medial or lateral to the growing axons. Such surround repulsion maintains the sensory axons in their dorsoventral trajectory. In Slit1/2 mutants, surround repulsion acts in the medial growth of axons within the optic tract and corpus callosum and Slit channels axons within the internal capsule that project in both the rostrocaudal and ventrodorsal axes by repelling them from the midline and ventral regions. Therefore surround repulsion may be a more generalized guidance mechanism not restricted to particular neuronal types or to axons growing within a specific axis of the embryo.

Having revealed a role for Slit in regulating crossing at the midline in the forebrain and chiasm, it will, of course, be interesting to see whether the Slit1/2 double mutants (or ultimately knockouts of the receptors) reveal defects in spinal cord projections. Zou et al. (2000) showed, using in vitro explant assays, that dorsal commissural axons, having crossed the floorplate, are repelled by Slit2. In addition, spinal motor neurons have been shown to be repelled by Slit-expressing cells, and this effect is blocked by the addition of Robo ectodomain proteins. However, recently, Pini and colleagues (Patel et al., 2001) found that the repulsion of either spinal motor axons by the floor plate, which strongly expresses Slit, or olfactory axons by the septum, cannot be blocked by the addition of Robo ectodomain proteins. This suggests that either Slit is not the floorplate or septum-derived repellant for these axons or, at the very least, that another factor from the floorplate or septum either compensates for Slit or masks Slit's activity in the collagen gel assay. One caveat to keep in mind when comparing the effects of Slit in various in vitro assays, is that what is not apparent is the amount of Slit protein expressed by various tissues, such as the floorplate, compared with the transfected cell lines. A more precise method of presenting a quantified amount of factor may reveal more about how axons respond to differing amounts, or gradients, of Slit. If Slit expressed by the floorplate (for motor axons) or septum (for olfactory axons) does not cause complete growth cone collapse, then it may also act as a channeling mechanism in these systems, but in conjunction with an additional molecule. The surround repulsion assay may be used to test multiple molecules and the analysis of these

systems in the Slit1/2 double knockout and other mutant mice may reveal the answer.

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AEXpulsing a Retrograde Signal

A variety of secreted components have been identified as retrograde signals mediating diverse aspects of synaptic development, maintenance, and plasticity; however, little is known about the mechanisms mediating the release of secreted retrograde signals. Doi and Iwasaki (this issue of *Neuron*) implicate AEX-1, a protein distantly related to the UNC-13/Munc13 family, as an attractive candidate regulator of the retrograde release machinery in muscle.

Though the highly specialized ultrastructure of the presynapse focuses one's attention to the anterograde signaling properties of synapses, retrograde signaling also plays critical roles in the development, maintenance, and plasticity of synapses both in vertebrates and invertebrates (Sanes and Lichtman, 1999; Schaefer and Nonet, 2001; Tao and Poo, 2001). Physiological, molecular, biochemical, and genetic dissection of the vesicular release pathways in the last decade have provided a wealth of information that outline the molecular details of anterograde signaling. By comparison, defining retrograde signaling pathways has been more challenging. Many physiologically defined signals, such as those mediating synaptic elimination at the vertebrate neuromuscular junction (Sanes and Lichtman, 1999), remain molecularly undefined. Furthermore, the molecular mechanisms controlling the regulated release of many retrograde signals are largely unknown. In this issue, Doi and Iwasaki have defined a retrograde signaling pathway operating in *C. elegans* at the neuromuscular junction (and other cell types). Furthermore, though the evidence remains largely indirect, they identified a distantly related homolog of the UNC-13/Munc-13 family, AEX-1, as a potential regulator of the retrograde signal secretion machinery.

The key player in this new chapter in synapse "regulation" is *aex-1*, a *C. elegans* gene identified initially in a genetic screen for mutations that lead to defects in the defecation motor program (Thomas, 1990). Defecation in the worm involves a series of coordinated muscle contractions. These events occur repetitively with a 45 s cycle time and are associated with waves of calcium in the intestine. Signaling from intestine to muscle is likely regulated by these calcium oscillations (Dal Santo et al., 1999). In this issue of *Neuron*, Doi and Iwasaki (2002) now show that *aex-1* mutants also have defects in neuromuscular function. Secondly, they find that *aex-1* is required in muscle, rather than neurons, to regulate synaptic functions. Thirdly, they position *aex-1* in a retrograde signaling pathway that modulates the activity of the presynaptic regulator UNC-13.

Until quite recently, direct physiological analysis of synaptic transmission in *C. elegans* had not been feasible, and it remains technically very difficult. Because of this roadblock, several sensitive pharmacological assays for synaptic function have been developed. The most common of these is characterizing sensitivity to the cholinesterase inhibitor aldicarb (Miller et al., 1996). Virtually every *C. elegans* mutant that disrupts a component associated with the synaptic release apparatus shows altered sensitivity to this drug, making aldicarb responsiveness a potent indirect assay for synaptic dysfunction. Likewise, *aex-1* mutants exhibit reduced sensitivity to the cholinesterase inhibitor aldicarb, suggesting that cholinergic synaptic transmission is reduced in the absence of AEX-1.

Mechanistically, the alteration in presynaptic function in *aex-1* appears to be, at least in part, attributable to disrupting the activity of the synaptic vesicle priming factor UNC-13. UNC-13 and its vertebrate homologs of the Munc13 family are highly conserved synaptic proteins that regulate the priming step of the synaptic vesicle cycle (Brose et al., 2000). Several differentially localized forms are present at the synapse, a long form that is highly concentrated at synapses and a short form (UNC-13S) whose localization is more dynamic (Kohn et al., 2000; Nurrish et al., 1999). GFP-tagged UNC-13S is recruited to synaptic sites in response to activation of G protein pathways coupled to DAG production (Nur-