Axon guidance at choice points
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The common theme in many recent axonal pathfinding studies, both in vertebrates and invertebrates, is the demonstration of the importance of a balance between positive and negative cues. The integration of multiple and often opposing molecular interactions at each site along the axon’s trajectory, especially at choice points, helps to fine tune the directional response of its growth cone, which continuously samples its environment for guidance cues. The dynamic regulation of the receptors for such cues, in response to extrinsic signals, also enhances the behavioral repertoire of growth cones at different points along their trajectory. Some of the molecules identified as being important for axon guidance at choice points are conserved between invertebrates and vertebrates (e.g. Robo and netrin), whereas other molecules have been identified, so far, only in invertebrates (e.g. Comm) or vertebrates (e.g. axonin-1 and NrCAM).

Introduction
Axon guidance is a complex phenomenon comprising different types of axonal behavior. Growing axons can elongate along pre-existing fiber tracts by selectively fasciculating with axons pioneering these tracts [1,2], or, alternatively, they can respond individually to guidance cues presented by their environment. The leading tip of an axon, the growth cone, can detect gradients of attractants or repellents, and, thus, can navigate toward or away from the source of diffusible guidance cues [3–6]. In addition to these long-range guidance cues, growth cones also use more local, short-range cues to reach their targets [7]. Such cues were traditionally considered to be primarily attractive and to be mediated by cell–cell adhesion [8]; however, repulsive short-range guidance cues have now been shown to be widespread as well [6,9–11].

As mentioned above, growing axons navigate to their target by continuously sampling their environment for guidance cues. A variety of growth cone receptors act as sensors for these cues [12], and growth cones must, therefore, integrate diverse signals derived from multiple molecular interactions at each site along their pathway. Thus, growth cone guidance can be viewed as an infinite series of choice points.

For the purpose of this review, we will focus on only two such choice points: the ventral midline in vertebrates and invertebrates and the optic chiasm. These are points at which the growth cones are forced to make an obvious binary choice, as every axon reaching the midline has to decide whether or not to cross it. Such an apparently simple binary choice appears to involve the integration of both positive and negative signals derived from multiple molecular interactions. For recent reviews on the mechanisms guiding axons to these choice points, see, in this issue, Van Vactor (pp 80–86) and Cook, Tannahill, and Keynes (pp 64–72).

The ventral midline as a choice point for commissural axons
The floor plate in vertebrates
A classic binary choice point for growing axons is the ventral midline of the CNS. In vertebrates, axons either cross the floor plate, the triangular structure formed by specialized cells along the ventral midline, or they project ipsilaterally. In addition to its role as a choice point, the floor plate is a source of netrin-1 [3,4], a long-range chemoattractant for the commissural axons located in the dorsal half of the spinal cord [13]. The function of netrins (and a homologous protein, unc-6) as long-range guidance cues has been shown to be conserved in invertebrates [14,15]. However, in order to cross the midline, commissural axons also require an additional set of short-range guidance cues.

A recent in vivo study in vertebrates has implicated an interaction between two cell adhesion molecules (CAMs) of the immunoglobulin (Ig) superfamily—axonin-1 (homologue of rat TAG-1) on the growth cone and NrCAM on the floor plate—as providing a positive signal for commissural axons to enter the floor plate [16]. In this study, function-blocking antibodies against these CAMs or soluble axonin-1 (to saturate binding sites for membrane-bound axonin-1) were repeatedly injected into the spinal cord of chicken embryos in ovo throughout the period of commissural axon pathfinding. Subsequent analysis of axon trajectories showed that when the function of either axonin-1 or NrCAM was impaired, many axons committed pathfinding errors; specifically, they failed...
In vertebrates, the perturbation of interactions between growth cone axonin-1 and floor plate NrCAM results in pathfinding errors of commissural axons. We analyzed commissural axon trajectories in transverse sections or whole-mount preparations of the embryonic chicken spinal cord by injecting a lipophilic dye into the area of the cell bodies close to the dorsal root entry zone (DREZ) [16]. The whole-mount (open-book) preparations were obtained by cutting the roof plate and flattening the cord, as indicated by the arrows in (a). (a) In control embryos, commissural neurons extend their axons ventromedially toward the floor plate. Their growth cones then enter the floor plate and cross the midline; when they reach the contralateral border of the floor plate, they turn rostrally. This trajectory can easily be followed using the open-book preparation. (b) After perturbing axonin-1 and NrCAM interactions by injecting soluble axonin-1 or function-blocking antibodies, respectively, into the spinal cord in ovo, the commissural axons commit pathfinding errors: up to 50% of the axons turn prematurely along the ipsilateral border of the floor plate and fail to cross the midline.

In summary, commissural axons, which normally cross the midline, experience a prevalence of positive cues upon contact with the floor plate, causing them to enter, whereas ipsilaterally projecting fibers, which normally do not cross the midline, fail to enter, presumably because negative cues predominate. The growth cones of ipsilaterally projecting fibers may either lack receptors for some of the positive cues or interpret the same cues differently. How these complex signals are integrated to produce the cytoskeletal alterations underlying growth cone behavior at such choice points is an exciting area for future research.

The ventral midline in invertebrates

A balance between positive and negative cues has also been postulated for growth cone guidance across the midline in Drosophila. In a screen to identify mutations affecting CNS patterning, two mutants—commissureless (comm) and roundabout (robo)—were found to have dramatic alterations in axonal growth across the midline [22]. In the absence of functional Comm, no axons crossed the midline, resulting in the absence of commissures (Figure 3a). In contrast, excessive numbers of axons crossed the midline in robo mutants: these included both axons that normally would not cross the midline as well as commissural axons that normally cross only once but now wandered back and forth across the midline.

The analysis of these mutants was thus consistent with the idea that Comm, expressed by the midline cells, is a ligand for a receptor on commissural axons contributing a positive signal for midline crossing [23°]. In contrast, Robo appears to contribute a negative signal that prevents midline crossing. On ipsilaterally projecting fibers, Robo...
A balance between positive and negative signals determines the behavior of growth cones at choice points. Axon pathfinding across the ventral midline has been studied in both vertebrates [16,17°°] and invertebrates [22,23°°°-26°°°] (see also Figure 3). Although the molecules involved in axon guidance across the midline are not the same, the mechanisms underlying axonal pathfinding are conserved. In both cases, a balance between positive and negative signals derived from multiple molecular interactions between growth cones and the floor-plate/midline determines the behavior of the axons. In vertebrates, the Ig superfamily CAMs axonin-1 and NrCAM provide positive cues for floor-plate crossing. Although evidence for a collapse-inducing activity derived from the floor plate has been found in vitro [17°°°], the molecular nature of this activity is not yet known. (a) Normally, commissural growth cones readily enter floor-plate explants upon contact, as the molecular interactions between the growth cone and the floor-plate surface result in a predominance of positive signals. The interaction of growth cone axonin-1 and floor-plate NrCAM contributes a positive signal, as does the interaction of growth cone axonin-1 with a yet unidentified molecule (see text and [17°°°] for details). (b) The presence of anti-NrCAM antibodies only antagonizes some of the positive signals. As axonin-1 can still interact with an unknown binding partner on the floor plate, the resulting positive signal is sufficient to mask the collapse-inducing activity of the floor plate. Therefore, in the presence of anti-NrCAM, the floor plate is not inhibitory; rather, it is nonpermissive, and commissural growth cones fail to enter the floor plate but do not collapse. (c) A negative signal that induces the collapse of commissural growth cones upon floor-plate contact is derived from the interaction of a receptor on commissural growth cones and a collapse-inducing activity of the floor plate. In the presence of anti-axonin-1, the balance is shifted to a negative signal, because both interactions contributing positive signals are eliminated.

is expressed from the onset of axon growth, whereas commissural axons express high levels of Robo on their growth cones only after crossing the midline [24°°°°] (Figure 4a). This pattern of expression could thus prevent commissural axons from recrossing the midline.

How might Robo, which is conserved in vertebrates and invertebrates, mediate a negative signal for midline crossing? As Robo is a member of a new subgroup of the Ig CAMs that contain five Ig and three fibronectin type III (FnIII) domains [24°°°°], a direct interaction with Ig CAMs such as axonin-1 and NrCAM is possible, at least in vertebrates. Robo might compete with axonin-1 for floor-plate NrCAM. Thus, the strong positive signal for midline crossing, provided by the axonin-1/NrCAM interaction, could be decreased or even made negative by expression of Robo. The same effect could be achieved by a cis-interaction of Robo and axonin-1 in the growth cone membrane, resulting in a decrease in the trans-binding affinity of growth cone axonin-1 for NrCAM on the floor plate. Alternatively, Robo could function simply as a receptor for a midline repulsive molecule (Figure 4b). Interestingly, a Robo homologue (Sax-3) has been found in the nematode Caenorhabditis elegans, whose ventral nerve cord normally lacks commissures [25°°°°] (Figure 3b). Thus, the function of Robo in preventing midline crossing could be phylogenetically very old. The development of commissures in arthropod and chordate lineages could have evolved by different molecular mechanisms that produce the same effect, such as the generation of a positive signal to override Robo’s action.

In Drosophila, Comm is required to suppress the inhibitory Robo signal. Thus, in the absence of Comm, no axons are able to cross the midline. However, when Robo is lacking, Comm is not needed for midline crossing, as
In invertebrates, as in vertebrates (Figure 2), the balance between positive and negative signals derived from the multiple molecular interactions between growth cones and the midline determines the behavior of the growing axons. (a) In Drosophila, the guidance of commissural axons (C) across the midline depends on a balance between positive and negative signals. (i) Normally, Comm, which is expressed by the midline cells, contributes a positive signal for midline crossing, whereas Robo acts as a receptor for the inhibitory activity derived from the midline. Ipsilaterally projecting fibers (I) express Robo from the onset of axon elongation, and are thus prevented from crossing the midline. Commissural axons express Robo only after contact with the midline on the distal part of the axon and the growth cone (see also Figure 4). This upregulation of Robo prevents the growth cone from recrossing the midline. (ii) In comm mutants, no commissures are formed, because in the absence of Comm, the inhibitory activity of the floor plate cannot be overcome. (iii) In contrast, in robo mutants, the sensory apparatus for the detection of the inhibitory activity of the floor plate is missing, resulting in additional axons crossing the midline, as well as commissural axons crossing the midline more than once (not shown). Interestingly, Robo is conserved from invertebrates to vertebrates [24]. (b) An ortholog of Robo, Sax-3, has recently been described in C. elegans [25]. In the nematode, axons normally do not cross the midline, consistent with the identification of this Robo-like protein (i.e. a receptor for the inhibitory activity), but not of a Comm-like protein (i.e. a positive cue for midline crossing). Therefore, it appears that the inhibitory influence of the midline is phylogenetically very old, and that invertebrates and vertebrates have solved the problem of overcoming this repellent force using different mechanisms.

shown by double mutants [22,23,26]. The mechanism underlying the function of Comm, which is expressed by the midline cells and apparently transferred to the commissural axons as they cross, is not fully understood [23]. Comm could antagonize the effect of Robo by providing an independent positive signal, as postulated for axonin-1/NrCAM. However, genetic studies have revealed that Comm actually regulates the expression of Robo on commissural axons [26]. Flies overexpressing Comm show reduced levels of Robo on their axons, and, consistent with the lack of repulsive activity, too many of their axons cross the ventral midline. Flies lacking Comm appear to express increased levels of Robo on their axons, preventing them from crossing the midline. The mechanism by which comm regulates robo expression is not yet known, nor is it known whether vertebrates use a similar mechanism, as no homologue of comm has yet been identified.

Regulation of guidance signals and receptors

An additional level of complexity at choice points results from the fact that receptors for guidance signals can also be regulated in a highly dynamic manner, in some cases in response to signals that the growth cones encounter at choice points—Robo is a good example (Figure 4). Another example is the switch in expression from TAG-1 to L1 as rat commissural axons cross the floor plate [27]. In vertebrates, Robo is upregulated following midline
The behavior of commissural axons changes as a result of their contact with the midline. Because the midline is the source of a chemoattractant for commissural axons, it is not clear why they would extend beyond the midline to project longitudinally rather than stall or linger in the midline region. (a) In invertebrates, Robo is upregulated after the growth cone contacts the midline [24**,26**]. As Robo is a receptor for the inhibitory cue of the midline the upregulation can prevent axons from recrossing the midline. Furthermore, Comm, which is expressed by the midline cells and provides a positive cue for midline crossing, is removed by transfer to the commissural axons as they cross the midline [23**]. Overexpression studies have shown that Comm can regulate the expression of Robo [26**]. Surprisingly, however, it was found that Comm overexpression causes downregulation of Robo rather than upregulation. Therefore, it is not clear how the transfer of Comm to the commissural axon is connected to the expression of Robo in this situation. (b) In vertebrates, Robo is also found upregulated on the distal part of the axon after contact with the floor plate (FP), and, thus, could prevent the commissural axons from recrossing the midline. It could help to make growth cones leave the floor plate area by tipping the balance to a more negative signal. Furthermore, the loss of responsiveness to netrin as a result of contact with the floor plate has been suggested as a mechanism for preventing axons from recrossing the midline [28**]. The molecular basis of this observation, which was made in a series of co-culture experiments [28**], is not yet known. Therefore, it is not known whether the loss of netrin responsiveness and the upregulation of Robo are linked or whether they just cooperate to get axons out of the floor plate and prevent them from recrossing.

Finally, these recent studies also emphasize that selective targeting and/or stabilization of guidance receptors on defined portions of the axon will also play important roles in guidance at choice points. For example, Robo is only concentrated on the surface of the portion of the axon that has crossed the midline. In addition, genetically induced upregulation of Robo mRNA does not cause increased cell-surface expression on commissural axons before crossing the midline. Thus, an important area for future research will be to learn how such local cell-surface expression is achieved and about the extrinsic signals that trigger it.

**Axon guidance at the optic chiasm**

At the optic chiasm of many vertebrates, one population of retinal axons crosses the midline to project contralaterally, while a population of uncrossed axons is deflected at the midline, projecting ipsilaterally. Neuronal cells within the chiasm are postulated to provide guidance cues for the earliest axons [30,31]. When these neurons are destroyed by complement-mediated cytolysis, the growth of all axons entering the chiasm is halted [32]. Later axons, however, appear to derive guidance cues from a midline palisade of radial glia. The cells in this region appear to provide a generalized negative signal, as the growth cones of both crossed and uncrossed axons pause when entering this region in vivo [33] and both types of axons have their growth retarded by the chemosuppressant effect of chiasm cultures in collagen gels [34**,35]. Following the pause in vivo, crossed axons extend across the chiasm,
presumably by responding to a positive signal sufficient to overcome any negative signals.

While this behavior has been replicated in culture (i.e. uncrossed axons were found to be repelled by clusters of chiasmatic neurons and glia, whereas crossed axons grew across these clusters [36]), the molecular nature of the signals is unknown. It would appear that crossed and uncrossed axons must themselves exhibit molecular differences that allow them to respond differently to signals emanating from the optic chiasm [37]. The findings of a recent study of the albino mouse, which has fewer than normal numbers of ipsilaterally projecting retinal fibers, support the idea that the albino mutation affects the crossed/uncrossed identity of retinal axons [38*].

**Conclusions**

Recent pathfinding studies have demonstrated that a balance between positive and negative guidance cues determines the response of growth cones at choice points. Both long-range and short-range guidance have been shown to depend on attractive and repulsive cues. The balance between opposing influences rather than single signals allows for the fine tuning of pathfinding using a finite number of molecules. Thus, even small shifts in the balance between positive and negative cues can result in dramatic changes in axonal behavior.

The behavior of growth cones at the midline, a major choice point in both vertebrates and invertebrates, emphasizes the considerable conservation that has occurred during evolution. In some cases, the molecular signals are conserved (i.e. netrin as a chemoattractant, Robo as a receptor for a midline repulsive signal). Even when the molecular basis (i.e. the positive signal that overcomes the inhibitory midline cue) differs, the strategy of using a positive signal to effectively overcome an inhibitory one has been conserved. Similarly, the dynamic regulation for such guidance cues in response to the extrinsic signals that the growth cone receives, especially at choice points, appears to be common, and contributes to both the complexity and fidelity of choices that a growth cone is capable of making.

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**References and recommended reading**

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

necessary for guidance toward or away from the midline. Neuron 1993, 10:409-426.


Comm protein is transferred by an unknown mechanism from midline cells to commissural axons as they cross the ventral midline. Comm appears to be part of the mechanism that helps commissural axons overcome the negative cues emanating from the floor plate, as commissures fail to form in Comm mutants.


The cloning of the robo gene in Drosophila and its subsequent comparison with human and rat genes revealed that Robo is a member of a new Ig superfamily subgroup with five Ig and three fibronectin type III (FnIII) domains. Robo is expressed on ipsilaterally projecting axons from the onset of growth, whereas on commissural axons, it is upregulated only after they have crossed the midline and turned longitudinally.


A genetic screen for C. elegans mutants with aberrant axonal pathfinding has resulted in the identification of the sax-3 gene. sax-3 is homologous to Drosophila, human, and rat robo (see [24**]). Consistent with the function postulated for robo, sax-3 mutants have ectopic axons crossing the midline. Interestingly, no comm homologue has been identified so far, indicating that the almost complete absence of commissures in wild-type nematodes may be explained by a lack of positive cues for midline crossing.


Robo acts as a receptor for a midline-derived inhibitory activity; thus, it prevents ipsilaterally projecting fibers, which express Robo from the onset of elongation, from crossing the midline. On commissural axons, Robo is upregulated on the distal part of the axon after the axon crossing the midline, and therefore prevents axons from recrossing the midline. Loss-of-function and gain-of-function experiments have revealed that the expression of Robo is coupled to the expression of Comm. Overexpression of Comm downregulates Robo, resulting in additional axons crossing the midline.


Commissural axons are attracted toward the midline by netrin; however, as shown in a series of co-culture experiments, they lose their responsiveness to netrin by crossing the floor plate. Commissural axons that had been in contact with floor-plate cells no longer respond to netrin coming from a second floor-plate explant or to a cluster of netrin-expressing 293 cells.


The midline expresses a diffusible factor with a chemosuppressant effect on retinal, but not other CNS axons tested in a collagen gel assay. As crossed and uncrossed axons are equally affected by this factor, it is unlikely to have a direct effect on sorting out the two classes of retinal axons. However, it is conceivable that the slowing down of axons at the chiasm allows the growth cones to respond to more specific guidance cues associated with the midline.


The finding that albino mice have a smaller number of ipsilaterally projecting retinal fibers than normal mice is correlated to a change in the retinal axons rather than to the optic chiasm in these animals. The results are consistent with the hypothesis that crossed and uncrossed axons diverge at the chiasm because they experience a different balance between positive and negative cues. In albino mice, more retinal axons are specified as contralaterally projecting. Either they lack the receptors to respond to negative cues or they overexpress the molecules that provide positive cues for crossing the midline.