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Diversity and Pattern in the Developing Spinal Cord

Yasuto Tanabe and Thomas M. Jessell

The generation of distinct neuronal cell types in appropriate numbers and at precise positions underlies the assembly of neural circuits that encode animal behavior. Despite the complexity of the vertebrate central nervous system, advances have been made in defining the principles that control the diversification and patterning of its component cells. A combination of molecular genetic, biochemical, and embryological assays has begun to reveal the identity and mechanism of action of molecules that induce and pattern neural tissue and the role of transcription factors in establishing generic and specific neuronal fates. Some of these advances are discussed here, focusing on the spinal cord as a model system for analyzing the molecular control of central nervous system development in vertebrates.

All neural functions—from simple sensory responses and motor commands to elaborate cognitive behaviors—depend on the assembly of neuronal circuits, a process initiated during embryonic development. An early and fundamental step in this process is the generation of distinct classes of neurons at precise locations within a primitive neural epithelium. Over the past decade, many of the mechanisms that control the identity of specific neural cell types have been defined, in large part through the application of molecular genetics in invertebrate organisms such as *Drosophila* and *Caenorhabditis elegans* but also through cellular and biochemical approaches in vertebrates. Collectively, the study of these diverse systems has provided considerable insight into the relative contributions of environmental signaling and lineage restrictions in neural development and has revealed the identity of many of the extracellular signaling factors and intracellular proteins that direct cell fate.

Some of the most intriguing behaviors depend on the circuits that are formed during the development of the vertebrate brain and spinal cord, yet our understanding of neural development is more fragmentary in the vertebrate central nervous system (CNS) than in other systems (*1*). Here we review recent progress in defining how diverse cell types in the vertebrate CNS are generated, focusing largely on the spinal cord, because it is the simplest and most conserved region of the vertebrate CNS (Fig. 1A). In addition, physiological and anatomical analyses of neuronal circuitry in the spinal cord have provided, from the time of Sherrington, a solid cellular framework for interpreting the neural bases of sensory and motor functions (*2*). Although the functions encoded in spinal cord circuitry are limited by comparison to those of many other brain structures, studies on the development of spinal neurons may reveal general strategies used to establish neuronal diversity and circuitry in more complex regions of the CNS.

We examine the steps involved in the generation of distinct neural cell types through the use of somewhat artificial subdivisions of what is evidently an integrated developmental program.

Induction of the Neural Plate

The development of the spinal cord, as in other regions of the CNS, is initiated by the induction of the neural plate. The classical grafting experiments of Spemann and Mangold in amphibian embryos (*3*) established that the formation of neural tissue depends on signals provided by prospective axial mesodermal cells in the organizer region. Until recently the identity and mechanism of action of these endogenous neural inducing factors have remained obscure. Studies of neural induction in *Xenopus* embryos now suggest that in one major pathway of neural induction, factors antagonize the signals mediated by the transforming growth factor–β (TGFβ)–like protein, bone morphogenic protein4 (BMP4), which represses neural and promotes epidermal cell fate (*4*) (Fig. 2).

BMP signaling and neural induction. The possibility that neural induction might result from the inactivation of a signaling pathway that represses neural fate emerged from the observation that dissociation of blastula-stage ectoderm into single cells, presumably preventing intercellular signaling, was sufficient to elicit the formation of neural tissue (*5*). Members of the TGFβ family were suggested to mediate this repressive signal on the basis of experiments designed initially to test whether the TGFβ-like protein activin was required for the induction of mesoderm (*6*). Injection of transcripts that encoded a dominant negative form of an activin receptor blocked mesodermal differentiation. But ectodermal cells expressing this receptor isoform unexpectedly differentiated into neural tissue, suggesting that the blockade of activin receptor signal transduction is sufficient to trigger neural induction. Two lines of evidence indicate that BMP4 rather than activin itself is likely to be the endogenous TGFβ-like protein that interacts with this receptor and represses neural differentiation. First, BMP4 is widely expressed in the early ectoderm and its expression is extinguished from neural plate cells during neural induction (*7*). Second, BMP4 but not activin can prevent the expression of neural markers and promote epidermal differentiation in dissociated ectodermal cells (*8*). Organizer-derived signals might therefore induce neural tissue by means of endogenous proteins that block signaling mediated by BMP proteins.

Support for this idea has come from the demonstration that three candidate neural inducers expressed by organizer tissue can act in this manner (Fig. 2, B and C). The endogenous activin-binding protein follistatin is expressed by organizer cells, and

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injection of follistatin elicits neural differentiation (9). Follistatin can also bind to BMPs, albeit with lower affinity than to activin (10), and thus its neural inducing activity is likely to result from inhibition of the actions of a BMP rather than of activin. A second protein with neural inducing activity, noggin (11), is also expressed in organizer cells (12) and binds to BMP4 with high affinity (K_d = 19 pM), blocking its biological activity (13). Finally, chordin, a protein identified originally by its expression in induced organizer tissue (14) also has direct neural inducing activity (15) and binds BMP4 (K_d = 320 pM) (16). Strikingly, although follistatin, noggin, and chordin can each antagonize the action of BMPs, they appear to be unrelated structurally.

Additional neural inducers. The neural tissue induced by follistatin, noggin, and chordin is anterior in character, as defined by molecular markers expressed normally in the forebrain (9, 11, 14, 17). A distinct signaling pathway may therefore be required for induction of posterior neural tissue. One class of candidate posterior neural inducers are secreted proteins of the fibroblast growth factor (FGF) family. FGFs can induce neural tissue with characteristics of the spinal cord under conditions in which the repressive action of BMP signaling is reduced or eliminated (17, 18) (Fig. 2A). Moreover, neural tissue characteristic of intermediate levels of the neuraxis, the midbrain, and hindbrain, can be induced by exposure of ectoderm to both noggin and FGF (17). The early regional identity of the neural plate along its anteroposterior axis may therefore be established in part by the coordinated actions of inhibitors of BMP signaling and FGFs.

A second class of molecules that appears to be involved in the generation of posterior neural tissue is the retinoids. Treatment of embryos with retinoic acid leads to an anterior-to-posterior transformation in the regional character of the neural tube and in the identity of specific neuronal cell types (19, 20). Retinoids also decrease the time required for the onset of neuronal differentiation in noggin-induced neural tissue (21). The presence of retinoids in posterior regions of the gastrula embryo (22) may therefore account for the early onset of neuronal differentiation in the spinal cord and hindbrain, and conversely the exclusion of retinoids from more anterior regions (22) may underlie the delay in neurogenesis at forebrain levels.

The use of targeted gene disruption in mice can independently test the requirement for neural-inducing molecules identified through gain-of-function assays in *Xenopus*. Mice with mutations in the *BMP4* gene do not exhibit an obvious expansion in neural tissue at the expense of epidermal ectoderm (23), as might be predicted from studies in *Xenopus*. This could be explained by the presence of other BMPs that function in a manner similar to that of BMP4 or by the existence of pathways of neural induction independent of BMP inhibition. In addition, follistatin appears not to have an essential role in neural induction in the mouse. The mouse organizer region, termed the node, possesses neural inducing activity (24) but does not express follistatin (25). Moreover, mice lacking follistatin do not exhibit any obvious defect in neural induction (26). These findings could again be accounted for by the compensatory actions of other organizer-derived molecules that inhibit BMP signaling. Nevertheless, it is also possible that some relevant neural-inducing factors derive from regions other than the node. One hint that there are other sources of neural-inducing factors has come from studies of mice in which the gene encoding hepatocyte nuclear factor (HNF) 3β, a transcription factor expressed in the node, has been inactivated by targeted recombination. HNF3β mutant mice lack overt signs of node differentiation, yet give rise to neural tissue with anteroposterior pattern (27), suggesting that molecules with neural inducing activity reside in tissues other than the node. One potential source of additional neural inducing factors is the endoderm/mesoderm tissue at the anterior end of the gastrula embryo. In *Xenopus*, the *Cerberus* gene is expressed by such endomesodermal cells and encodes a secreted protein that can induce anterior neural tissue (28). It remains uncertain, however, if the neural inducing activity of *Cerberus* is direct or is mediated by other induced cell types.

Thus, many candidate inducers of neural tissue have been identified both in spinal cord and at more anterior levels. To date, however, it has not been possible to separate the induction of neural properties from the acquisition of anteroposterior regional identity. Neural induction and the early regional fate of neural cells appear, therefore, to be linked rather than independent processes.

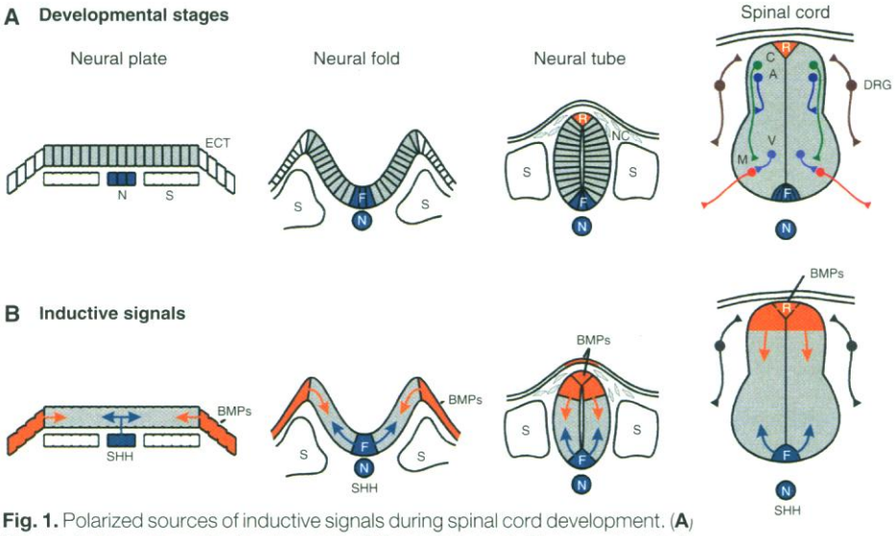

Fig. 1. Polarized sources of inductive signals during spinal cord development. (**A**) Stages in the embryonic development of the spinal cord: The neural plate is generated as a columnar epithelium and is underlain by axial mesoderm cells of the notochord (N), and paraxial mesoderm (later the somites) (S) and is flanked by epidermal ectoderm (ECT). During neurulation, the neural plate buckles at its midline to form the neural folds and a floor plate (F) forms at its midline. The neural tube forms by fusion of the dorsal tips of the neural folds, generating roof plate cells at its dorsal midline and neural crest cells (NC), which emigrate from the dorsal neural tube. Neuroepithelial cells proliferate and differentiate into neurons located at different dorsoventral positions. Subclasses of commissural (C) and association (A) neurons differentiate dorsally, close to the roof plate, whereas motor neurons (M) and ventral interneurons (V) differentiate ventrally near the floor plate. Dorsal root ganglion (DRG) neurons are generated from post-migratory neural crest cells. (**B**) The diagram shows the source of ventralizing [Sonic Hedgehog (Shh), blue] and dorsalizing (BMPs, orange) inductive signals at sequential stages of spinal cord development. Shh is initially expressed in the axial mesoderm, and BMPs originate in the epidermal ectoderm flanking the lateral edges of the neural plate. At neural fold stages, Shh begins to be expressed by floor plate cells at the midline of the neural plate and BMPs by cells in the dorsal tips of the neural folds. After neural tube closure, BMP expression is lost from the epidermal ectoderm except at the dorsal midline but BMPs are now expressed in the roof plate and adjacent dorsal neural tube. At the onset of neuronal differentiation, BMP expression persists in the dorsal neural tube, and Shh expression is maintained in the floor plate.

Patterning the Neural Plate

Two independent signaling systems control the regional fate of induced neural cells (*29*). As discussed above, one system controls pattern along the anteroposterior axis and has a critical role in establishing the subdivisions of the neural tube that prefigure the formation of the forebrain, midbrain, hindbrain, and spinal cord (*30*). The mechanisms that control anteroposterior patterning in the brain are discussed in another article in this issue (*31*). A second signaling system patterns the neural plate along its mediolateral axis, later the dorsoventral axis of the neural tube. In the following sections we discuss the mechanisms by which inductive signals control the diversification of cell types along the dorsoventral axis of the neural tube, focusing on the caudal region of the neural tube that gives rise to the spinal cord. Similar patterning events occur at more rostral regions that give rise to the hindbrain, midbrain, and diencephalic regions of the forebrain (*32*).

At early stages in the development of the spinal cord, three major classes of cells are generated in the ventral neural tube: floor plate cells at the ventral midline, motor neurons at a ventrolateral position, and ventral interneurons at more dorsal locations (Fig. 1A). Cells in the dorsal neural tube give rise initially to neural crest cells and subsequently to roof plate cells at the dorsal midline and to several classes of dorsal sensory relay interneurons. The inductive signals that control the identity and pattern of these cell types come initially from two distinct groups of non-neural cells. The generation of ventral cell types is controlled by signals from the notochord, an axial mesodermal cell group that underlies the midline of the neural plate. In contrast, dorsal cell types are generated in response to signals derived from the epidermal ectoderm that flanks the lateral margins of the neural plate (Fig. 1).

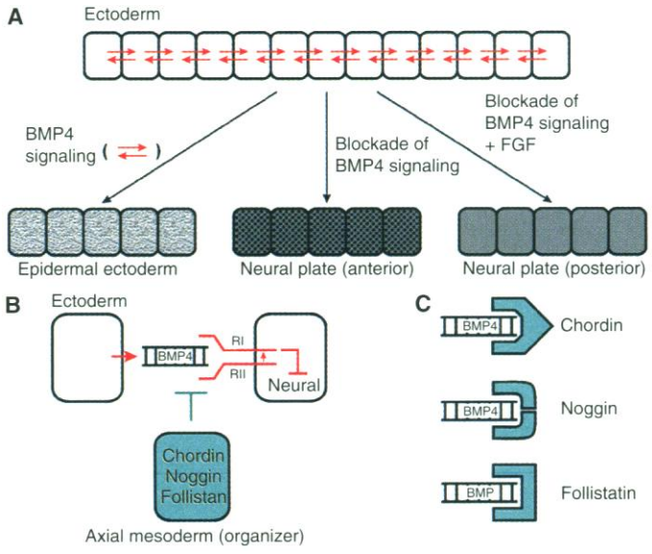

Fig. 2. Mechanisms of neural induction in *Xenopus* embryos. (**A**) Ectodermal cells of the animal pole of gastrula-stage *Xenopus* embryos are subject to tonic BMP4-mediated signaling (red arrows), which promotes their differentiation into epidermal cells. Blockade of BMP4 signaling elicits the formation of anterior neural plate tissue. Exposure of ectoderm to FGFs under conditions in which BMP4 signaling is reduced or eliminated leads to the generation of posterior neural plate tissue. (**B**) A potential mechanism of action of anterior neural inducers derived from prospective axial mesoderm (the organizer region). Chordin, noggin, and follistatin are each secreted by organizer cells and induce neural tissue by blocking BMP4-mediated signaling between ectodermal cells. RI and RII, BMP receptor subunits. (**C**) Both noggin and chordin bind to BMP4. Follistatin can bind to BMP7 and possibly also to other BMPs.

Diversity and Pattern in the Ventral Neural Tube

Tissue grafting assays in chick and *Xenopus* embryos (*33*, *34*), the analysis of mutant mouse and zebrafish embryos (*27*, *35*, *36*), and assays of cell differentiation in neural plate cells grown in vitro (*37–39*) have shown that the notochord is the source of two inductive signals: A local signal that induces floor plate differentiation in midline neural plate cells and a longer-range signal that induces motor neurons. The floor plate, once induced, acquires the ability to generate both of these short- and long-range signals (*35*, *37*). Both short- and long-range inductive activities are mediated by Sonic Hedgehog (Shh), a member of a family of secreted proteins identified initially by their structural similarity to the *Drosophila* segment polarity gene *Hedgehog* (Hh) (*40–43*). *Shh* is synthesized by the notochord and floor plate at the time that these cell groups exhibit their inductive activity (*41–47*) (Fig. 1B). Misexpression of Shh can induce floor plate differentiation in vivo (*41*, *43*), and recombinant Shh can induce floor plate cells and motor neurons in neural plate explants (*45*, *46*). Conversely, antibodies that inhibit Shh signaling in vitro block the ability of the notochord and floor plate to induce ventral cell types (*46*, *47*) and mice in which the *Shh* gene has been inactivated fail to generate ventral structures in the CNS (*48*). Taken together, these studies show that Shh is necessary and sufficient for the induction of ventral cell types.

Patterning actions of Shh. When and how does Shh control the identity and pattern of cell types generated in the ventral neural tube? An early step in this process appears to be the repression of transcription factor expression in cells at medial positions within the neural plate. When the caudal neural plate is formed, cells at all mediolateral positions express the homeodomain-containing transcription factors, Pax3, Pax7, Msx1, and Msx2 (*47*, *49–52*). The expression of these genes is rapidly repressed in medial neural plate cells by a Shh-mediated signal from the notochord (*47*, *49*, *52*) and after neural tube closure their expression is restricted to proliferating cells in the dorsal neural tube (*47*, *49*, *52*) (Fig. 3). The repression of *Pax3* and *Pax7* expression in neural plate cells appears to be a prerequisite for the generation of ventral cell types. Lateral neural plate cells that have never been exposed to Shh maintain Pax7 expression and rapidly lose competence to generate floor plate cells and motor neurons in response to Shh (*47*). Furthermore, misexpression of *Pax3* in ventral regions of the

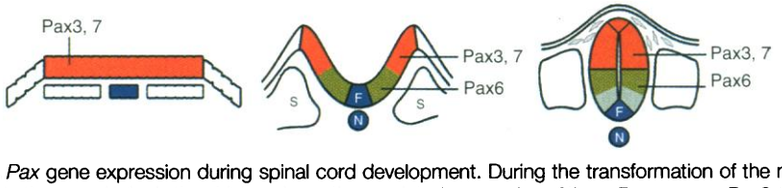

Fig. 3. *Pax* gene expression during spinal cord development. During the transformation of the neural plate into the neural tube in the chick embryo, the regulated expression of three *Pax* genes—*Pax3*, *Pax6*, and *Pax7*—subdivides the neural tube into distinct domains. Caudal neural plate cells at all mediolateral positions initially express *Pax3* and *Pax7*. At neural fold stages, *Pax3* and *Pax7* expression is repressed medially and *Pax 6* expression is detected at all mediolateral positions except at the midlines. After neural tube closure, *Pax3* and *Pax7* expression is restricted to the dorsal half of the neural tube, whereas *Pax6* is expressed by more ventral cells. *Pax6* is also expressed by cells in the dorsal half of the neural tube. N, notochord, F, floor plate.

spinal cord in transgenic mice blocks floor plate differentiation (53). The rapid time course of the repression of *Pax3*, *Pax7*, and *Msx1/2* by Shh (*47*, *49*, *52*) raises the possibility that these genes may be direct targets for the intracellular transduction events triggered in neural plate cells by Shh. The Shh signaling pathway is not completely understood, but appears very similar to Hh signal transduction in *Drosophila* (*54*–*57*). As such, Shh-mediated signaling is likely to culminate in the activation of zinc finger transcription factors of the Gli family (*57*, *58*).

Upon exposure to Shh, cells in the medial region of the neural plate are converted to a ventralized state (defined operationally as the repression of *Pax3* and *Pax7* expression) and acquire the capacity to give rise to floor plate cells, motor neurons, or ventral interneurons. The selection of one of these three cell fates appears to be regulated by a second phase of Shh signaling. Cells at the midline of the neural plate appear to be exposed to Shh generated locally by the notochord, which directs floor plate cell fate through the expression of transcription factors of the winged-helix class, notably HNF3β (59). At later stages, Shh signaling in adjacent regions of the ventral neural tube defines whether ventralized progenitors give rise to motor neurons or to ventral interneurons. Shh signaling causes ventralized progenitors to give rise to motor neurons, whereas the blockade of Shh signaling inhibits motor neuron differentiation and leads instead to the generation of ventral interneurons (*47*). Ventralized progenitors require Shh signaling to generate motor neurons until late in their final progenitor cell division. This finding has parallels with studies showing that the laminar identity of cortical neurons is determined late in their final progenitor division cycle (*60*). At the onset of motor neuron differentiation in higher vertebrates, the notochord has been displaced ventrally and is no longer close to the neural tube. Thus, the Shh required to convert ventralized progenitors into motor neurons is likely to derive from the floor plate.

It is unclear whether the generation of motor neurons or ventral interneurons results from a switch in the fate of an individual progenitor cell in response to Shh. The ventral neural tube contains multipotential progenitor cells that give rise both to motor neurons and interneurons (*61*). Moreover, exposure of neural plate cells in vitro to a Shh concentration at the threshold for motor neuron induction leads to the generation of neurons that coexpress markers of motor neuron and interneuron identity (*47*). This observation is most easily explained by the existence of a common progenitor cell for motor neurons and certain ventral interneurons. It remains unclear, however, whether the fates of all ventral interneurons are controlled solely by Shh-mediated signals. A class of ventral interneurons that express the homeodomain protein En-1, but not Isl1, is missing in mice in which motor neuron differentiation has been blocked by elimination of *Isl1* gene function (*62*). This finding suggests that, in vivo, motor neuron–dependent signals may cooperate with Shh signaling to specify certain interneuron fates in the ventral neural tube.

Taken together, these studies suggest that the identity and pattern of cell types generated in the ventral half of the neural tube is controlled in large part by Shh signaling, through actions at multiple concentration thresholds. The early action of Shh to maintain the competence of neural plate cells for later ventral cell type differentiation operates at a concentration threshold two to three times lower than that required later for motor neuron generation (*47*), and floor plate generation requires two to three times more Shh (*45*). Thus, relatively small changes in Shh concentration can elicit the generation of distinct neural cell types. Studies of mesodermal patterning in *Xenopus* have shown similarly that distinct cell types are generated in response to two- to threefold differences in activin concentration (*63*).

If Shh acts at multiple concentration thresholds to control ventral cell fates, what is the range of Shh diffusion and the concentration profile in the ventral neural tube in vivo? The early notochord-mediated repression of Pax7 in the neural plate is observed over a distance of 5 to10 cells (*47*), perhaps the extent of Shh diffusion. Moreover a graded elevation in the expression of *Patched* mRNA is detected in the ventral neural tube at stages at which motor neurons begin to differentiate (*54*). Induction of *Patched* is an indicator of exposure of cells to Hedgehog proteins in *Drosophila* and vertebrates (*54*, *55*), and it is likely therefore that Shh diffuses from floor plate cells and establishes a concentration gradient in the ventral neural tube. These studies on Shh signaling support the idea that the patterning of vertebrate tissues can be controlled by discrete cellular responses to different concentrations of diffusible inductive factors.

The mechanisms that control the extent of Shh diffusion have been clarified in vivo by biochemical studies showing that *Drosophila* and vertebrate Hedgehog proteins are synthesized as larger precursors that are subject to autoproteolytic cleavage to generate biologically active NH_2-terminal (N) fragments (*45*, *64*). During autocatalytic processing, the N fragment is covalently modified by the addition of cholesterol to its COOH-terminus, which results in an increase in hydrophobicity that tethers the protein to the membrane (*65*) and restricts its diffusion. Apparently, as a consequence of this lipophilic modification, the vast majority of Shh-N synthesized by the notochord and floor plate remains closely associated with the surface of midline cells (*45*, *47*, *66*). The diffusible form of Shh-N could derive from the incomplete transfer of cholesterol during autoprocessing, from the regulated cleavage of the cholesterol adduct, or simply from the release of small amounts of cholesterol-modified Shh-N from the plasma membrane.

Future studies on the role of Shh in ventral neural tube patterning will need to investigate how small differences in extracellular Shh concentration generate distinct neural cell types. Defining the intracellular transduction events elicited by Shh in neural cells and the downstream targets of the conserved Shh signaling pathway will be essential steps in resolving this issue.

Diversity and Pattern in the Dorsal Neural Tube

The differentiation of cell types generated in the dorsal neural tube appears to be initiated by a contact-mediated signal from the adjacent epidermal ectoderm (*52*, *67*). Members of the TGFβ family, notably BMPs, are likely mediators of this ectodermal signal. In avian embryos, *BMP4* and *BMP7* are expressed in the epidermal ectoderm at early neural plate stages and can mimic the inductive activity of the ectoderm (*52*) (Fig. 1B). Exposure of neural plate cells to BMPs elevates expression of the same *Pax* and *Msx* genes that are repressed by Shh signaling (*52*). These genes are, however, also expressed by neural plate cells that have not been exposed to ectodermal signals and do not generate neural crest cells or dorsal interneurons. Thus, the expression of *Pax* and *Msx* genes appears insufficient to trigger the differentiation of dorsal cell types. Nevertheless in the mouse the *Pax3* and *Pax7* genes are required for the appropriate differentiation of neural crest cells (*68*), suggesting their involvement in dorsal cell differentiation. One candidate for an intermediate in neural crest cell differentiation is a zinc finger transcription factor, slug (*69*), which is induced in premigratory neural crest cells in response to BMPs (*52*). Antisense oligonucleotide ablation of *slug* expression impairs the migration of neural crest cells from the dorsal neural tube (*69*). After neural tube closure, several *BMPs*—including *BMP4*, *BMP5*, *BMP7*, and *Dsl1*—are expressed in

overlapping domains in and around the dorsal midline (*52*, *70*) (Fig. 1) and induce subsets of sensory relay interneurons that are generated at later stages in the development of the dorsal spinal cord (*70*).

Common themes of dorsoventral patterning. There are both common features and significant differences in the principles by which cell fate and pattern appear to be regulated in the dorsal and ventral halves of the neural tube. The strategy of inductive transfer of secreted signaling factors expressed initially by non-neural tissues (the notochord and epidermal ectoderm) to cells at the midline of the neural tube (the floor plate and roof plate) (Fig. 1B) is used to propagate patterning signals within both the ventral and dorsal neural tube. Ventral patterning is, however, at least in higher vertebrates, regulated by the activities of a single Hedgehog protein, Shh, whereas several BMPs are expressed in the epidermal ectoderm and dorsal neural tube. How are distinct dorsal cell types generated in response to structurally related inductive signals? By analogy with the mechanisms of Shh-mediated signaling in the ventral neural tube, different dorsal cell types may be triggered at different BMP concentration thresholds. Alternatively, members of the TGFβ family may possess qualitatively distinct inductive activities by virtue of their interaction with different BMP receptors (*71*). Temporal changes in the response of neural plate cells to the same BMP signal could also contribute to the generation of distinct dorsal cell types.

BMPs influence the patterning of many tissues in vertebrates and *Drosophila*. Both diffusible BMP gradients (*72*) and a BMP-initiated cascade of signaling molecules (*73*) have been suggested to underlie long-range patterning. Differentiation of ventral cell types appears to be controlled by Shh through both its local and direct long-range actions. However, the initial source of BMPs, the epidermal ectoderm, requires contact with target cells to induce dorsal cell types (*52*, *70*). BMPs may therefore control cell pattern in the dorsal neural tube only by a local action, achieving long-range patterning through the propagated, cell-to-cell induction of *BMP* gene expression in responsive neural cells.

Whether the early dorsoventral patterning of the neural tube can be explained solely by Hedgehog and BMP activities is unclear. Several *Wnt* genes are expressed in restricted domains along the dorsoventral axis of the neural tube (*74*). There is currently no evidence that Wnt proteins contribute to dorsoventral patterning in the spinal cord (*75*), but they do contribute more rostrally and in non-neural tissues (*76*) and may therefore have as yet unappreciated roles in cell patterning in the spinal cord. In addition, chordin, noggin, and follistatin are each expressed by the notochord or floor plate (*9*, *12*, *14*, *77*), raising the possibility that ventral sources of proteins that antagonize BMP signaling might also contribute to the patterning of the ventral neural tube.

Diversification of Motor Neuron Subtypes

Hedgehog and BMP proteins have an early role in specifying the identity of cell types along the dorsoventral axis of the spinal cord, but as development proceeds more specialized neuronal subtypes are generated. Additional signaling steps that appear to be independent of the early dorsoventral patterning signals are involved in the generation of neuronal diversity at later stages in spinal cord development. Evidence for this has emerged most clearly from the analysis of the cellular interactions that control the differentiation of motor neuron subclasses.

Motor neurons in the developing spinal cord can be subdivided on the basis of the position at which their cell bodies are located and also by their axonal projection patterns. In higher vertebrates, subclasses of motor neurons are organized into longitudinally oriented columns that occupy distinct and, in some cases, discontinuous domains along the rostrocaudal axis of the spinal cord. Motor neurons within a single column send their axons to a common peripheral target (*78*). Motor neurons in the medial subdivision of the median motor column (MMC) project their axons to axial muscles that lie close to the vertebral column; motor neurons in the lateral subdivision of the MMC project their axons to body wall muscles; and motor neurons in the lateral motor column (LMC) innervate muscles in the limb (Fig. 4). Motor neurons in the LMC are further organized into pools, each of which innervates a specific muscle in the limb (*79*). In lower vertebrates such as the zebrafish, three major subclasses of primary motor neuron can be identified by the distinct rostrocaudal positions at which they are generated within a single segment and by their selective projections to different axial muscle domains in the periphery (*80*). The columnar subclasses of motor neurons in the chick and the primary motor neuron subclasses in zebrafish can be distinguished by the combinatorial expression of transcription factors of the LIM homeodomain class (*81*) (Fig. 4). LIM homeodomain proteins control cell fate decisions in both *C. elegans* and *Drosophila* (82, 83), and in vertebrates they may control the expression of molecules that are involved in the guidance of motor axons along different pathways to their muscle targets in the periphery.

How might the subtype identities of motor neurons be established? Experimental

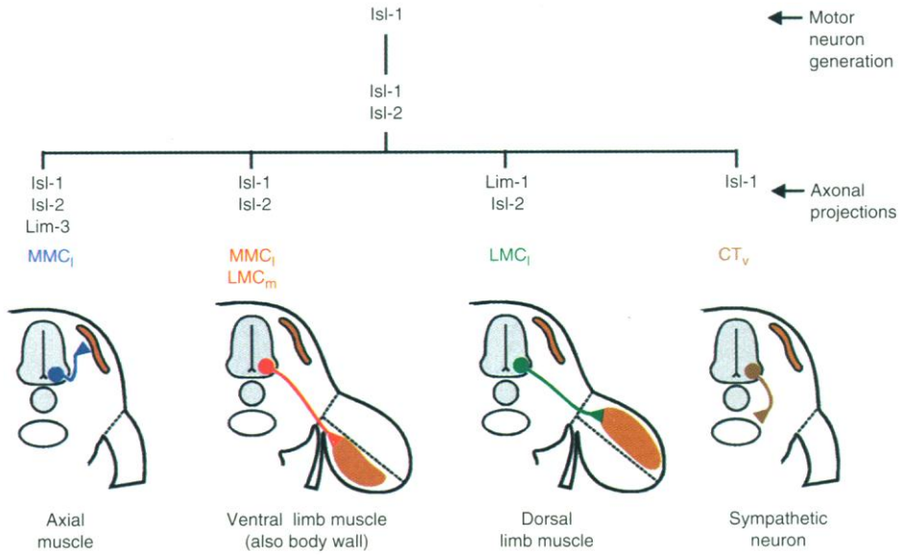

Fig. 4. LIM homeodomain protein expression in motor columns in the chick spinal cord. The temporal sequence of expression of LIM homeodomain proteins by newly differentiating motor neurons. All classes of motor neuron initially express Isl1 and Isl2, soon after their birth. Differential expression of LIM homeodomain proteins occurs at around the time of axon extension. The lower diagram shows transverse sections through stage 22 to 25 chick embryos at different segmental levels, indicating the projection of motor neurons located in different motor columns to their peripheral targets. The medial division of the median motor column is shown in blue (MMC$_m$); the lateral division of the median motor column is shown in red (MMC$_l$); the medial division of the lateral motor column (LMC) in red (LMC$_m$); the lateral division of the LMC in green (LMC$_l$) ; and the column of Terni in brown (CT).

manipulations in chick and zebrafish embryos have suggested that motor neuron diversification depends on local signals that act on neural tube cells over restricted domains along the rostrocaudal axis of the spinal cord. Transplantation of segments of the chick neural tube to different rostrocaudal positions results in a transformation in the columnar identity of motor neurons and in the expression of combinations of LIM homeodomain proteins appropriate for their new position (*84*, *85*). Similarly, inversion of the neural tube at lumbar levels leads to a respecification of motor pool identity, as defined by changes in the pattern of motor axon projections in the periphery (*86*). These neural tube translocations and inversions also change the expression of *Hox* genes (*85*, *87*), raising the possibility that the expression of *Hox* genes is involved in defining motor neuron subtype identity in the spinal cord as well as in the hindbrain (*31*). In zebrafish, transplantation of individual primary motor neurons to a different intrasegmental position also results in a change in motor neuron identity as defined both by altered LIM homeodomain protein expression and by the respecification of axonal trajectory (*81*, *88*).

Taken together, these observations have suggested the existence of rostrocaudally restricted signals that control the subtype identity of motor neurons. The signals appear to act initially on progenitor cells (*79*, *85*), although motor neuron subtype identity may be modifiable after cells have left the cell cycle (*80*, *81*). The cellular origin and identity of these local signals is not known, although a possible source is the paraxial mesoderm that flanks the neural tube. Thus, inductive signals from the axial mesoderm may help to establish the generic identity of motor neurons and signals from the paraxial mesoderm may define their subtype.

Neurogenesis

Studies on the contribution of inductive signaling to the specification of cell fate in the spinal cord have not addressed the mechanisms that operate more generally to control the differentiation of progenitor cells into postmitotic neurons. The molecular genetic dissection of neurogenesis in *Drosophila* has yielded clues to the mechanisms that operate in vertebrates.

In *Drosophila*, the selection of a single neuron from a large population of equivalent ectodermal cells requires a series of cell interactions that progressively restrict cell fate (*89*). The initial step in this process is the generation of a proneural region, a small cluster of cells that acquires the potential to give rise to neural precursors. This process involves the induction of expression of a group of transcription factors of the basic helix-loop-helix (bHLH) class, termed proneural genes, the most notable of which are members of the *achaete-scute* complex (*89*). Within each proneural region, however, not all cells generate neurons and this selection process involves a lateral inhibitory signal mediated by neurogenic genes (*90*), key amongst which are the cell surface proteins Delta and Notch (*91*). Expression of *Delta* is controlled by proneural genes (*89*, *91*), and the Delta protein encodes a transmembrane ligand that activates Notch, initiating intracellular signals that lead to the repression of proneural gene expression and, as a consequence, to the down-regulation of Delta (*89*, *91*). Thus, ectodermal cells are subject to a local feedback cycle that amplifies an initially minor difference in the level of Notch signaling. Cells in which Notch signaling is relatively weak give rise to neurons, whereas cells in which Notch signaling is relatively strong acquire alternate fates. Notch signaling, however, is a general mechanism for imposing differences in equivalent cell groups rather than a process dedicated to the selection of neuronal identity (*92*).

The mechanisms that control neurogenesis in vertebrates appear to have been coopted in a remarkably conserved manner from those that operate in *Drosophila* (*93*) (Fig. 5). bHLH proteins expressed in vertebrate neural tissue have been identified, and many of these share structural features with *Drosophila* proneural proteins (*93–95*). Similarly, vertebrate Notch proteins and ligands of the Delta and Serrate class have been identified (*96–101*). Evidence that the vertebrate Notch and Delta proteins regulate a core program of neurogenesis in a manner similar to that of their *Drosophila* counterparts has emerged in large part from the analysis of the primary (early born) neurons at caudal levels of the neural plate in *Xenopus* embryos. Overexpression of Delta or of an activated form of Notch inhibits the generation of primary neurons. Conversely, expression of a dominant negative form of Delta results in the generation of additional primary neurons (*99*) (Fig. 5).

In *Xenopus*, primary neurons are not generated uniformly in the neural plate but are confined to three longitudinally arrayed stripes; the medial stripe corresponds to motor neurons, the intermediate to interneurons, and the lateral to sensory neurons (*102*). Delta expression in the neural plate is centered on these three stripes prior to

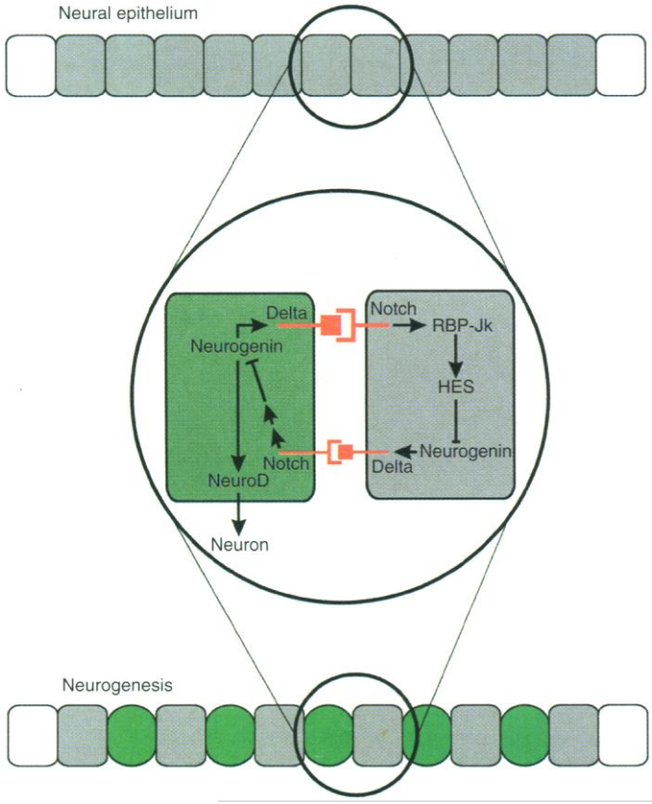

Fig. 5. Model for the role of Neurogenin, NeuroD, and Notch signaling in the determination of neuronal fate. The conversion of a neural epithelium consisting exclusively of proliferating progenitor cells (gray) to one in which certain cells have adopted a neuronal identity (green) is shown. The acquisition of neuronal identity requires the action of bHLH proteins and Notch signaling. The model indicates that Neurogenin expression in the left hand cell induces expression of Delta, which in turn activates Notch signaling in the right-hand cell, leading to the repression of Neurogenin expression and consequently to a decrease in Delta expression. By analogy with similar signaling events in *Drosophila*, the inhibition of Neurogenin expression may be mediated by RBP-Jk, a vertebrate homolog of Suppressor of Hairless [Su(H)] and through HES proteins, vertebrate bHLH proteins of the Hairy/enhancer of split [E(spl)] class (*113*, *114*). Neurogenin expression above a certain threshold leads to the induction of NeuroD, which promotes neuronal differentiation. Modified with permission from (*93*).

the onset of neuronal differentiation (99). After experimental manipulations that suppress Notch signaling, the generation of additional neurons is restricted to these three stripes, resulting in an increase in local neuronal packing density rather than an expansion in the proportion of the neural plate occupied by neurons or the generation of ectopic neurons in non-neural ectoderm (99). These findings indicate the existence of a program of neurogenesis that functions at earlier stages to define regions of the neural plate within which cells are competent to generate neurons. Within these domains the selection of neuronal fate appears to depend on the state of Notch signaling.

What genes activate the core program of neurogenesis in vertebrates? One vertebrate bHLH protein, Neurogenin, is expressed prior to Delta in regions of the *Xenopus* neural plate destined to generate primary neurons (95). Overexpression of Neurogenin leads to an expansion of the domain of expression of *Delta* and to an increase in the number of neurons. Importantly, neurogenesis is no longer restricted to the original three stripes, and ectopic neurons are also detected in non-neural ectoderm (95). Neurogenin overexpression also results in the induction of a second and later appearing bHLH protein, NeuroD (94), which can also induce ectopic neuronal differentiation in non-neural ectoderm. These and other results (95) suggest that Neurogenin may be an important early activator of neurogenesis and more generally that neurogenesis in vertebrates, as in *Drosophila*, involves the sequential activation of distinct bHLH factors that either determine neuronal fate or promote later aspects of neuronal differentiation (93) (Fig. 5).

Studies in *Drosophila* have also shown that in certain neural cells, the Notch-mediated control of neurogenesis is itself subject to regulation by proteins that are asymmetrically inherited during the division of the progenitor cell. Notable amongst these is the *Drosophila* protein Numb, which confers neuronal identity to cells that inherit the protein by inhibiting the intracellular transduction of Notch-mediated signals (*103*). Numb-related proteins have now been isolated in vertebrates (*104*), and in the ventricular zone of the mammalian cerebral cortex both Numb and Notch proteins are localized asymmetrically during certain progenitor cell divisions (*104, 105*). Analysis of the function of Numb and other localized determinants should help to clarify the extent to which proteins segregated during cell division control neuronal identity in the vertebrate CNS.

The emerging evidence for a core program in neurogenesis leaves unresolved the issue of how glial cell fates are defined. In the developing spinal cord, floor plate and roof plate cells can be considered specialized classes of early differentiating glial cells. Some of the environmental signals that control the differentiation of more conventional classes of glial cells—astrocytes, and oligodendrocytes—have also been identified (*106, 107*) but the cell intrinsic factors that specify glial cell type in the CNS remain to be defined. In *Drosophila*, nuclear proteins required for glial cell differentiation have been identified (*108*), and the isolation of their vertebrate counterparts could reveal whether elements of the biochemical machinery that controls gliogenesis have also been conserved during evolution.

Integration of Neurogenic and Patterning Mechanisms

How might the core program of neurogenesis controlled by bHLH proteins and Notch signaling be integrated with Hedgehog- and TGFβ-dependent signaling systems to generate distinct classes of neurons in the spinal cord? One possibility is that the neurogenic program controls solely the decision of progenitor cells to become neurons or remain undifferentiated, with the establishment of neuronal subtype identity depending on the transcription factors controlled by Hedgehog and TGFβ signaling. The spatially restricted expression of *Pax3*, *Pax6*, and *Pax7* along the dorsoventral axis could then primarily determine neuronal identity in the spinal cord (Fig. 6).

Arguing against this view is the finding in *Drosophila* that structural differences in distinct bHLH proteins do contribute to the specification of the subtype identity of neurons (*109*). Moreover, in vertebrates three different bHLH proteins—neurogenin, Mash-1, and Math1/Atonal—are expressed in complementary, non-overlapping domains of the ventricular zone along the dorsoventral axis of the spinal cord (*93, 110*) (Fig. 6). Similarly, two different Notch ligands, Delta and Serrate/Jagged, are also expressed in complementary subdomains of the ventricular zone (*97, 98*) (Fig. 6). Distinct bHLH proteins and Notch ligands could therefore participate, together with *Pax* genes, in the control of neuronal subtype identity in the developing spinal cord.

The classes of genes that act downstream of the neurogenic and patterning programs active in progenitor cells to specify neuronal subtype identity remain unknown. Members of many classes of transcription factors are expressed in subsets of neurons in the embryonic spinal cord (*111*). In particular, members of each of the five subclasses of LIM homeodomain proteins (*82*) are expressed in the developing spinal cord and define functional subsets of interneurons as well as motor neurons (*70, 81*) (Fig. 6). The possibility that LIM homeodomain proteins regulate neuronal subtype identity in the vertebrate CNS has received preliminary support from the analysis of Isl1, a LIM homeodomain protein expressed at an early stage in the differentiation of all spinal motor neurons. Mice in which *Isl1* function has been eliminated by gene targeting fail to generate motor neurons (*62*). However, the relation between LIM homeodomain proteins and the expression of genes that define neuronal connectivity and function—those involved in axonal pathfinding, synapse formation and neurotransmission—remains unknown. In peripheral sympathetic neurons, separate classes of transcription factors have been suggested to control neuronal identity and transmitter phenotype (*93, 112*), and it is likely that the diverse phenotypic properties of individual neurons in the CNS will also depend on the combined actions of multiple transcription factors.

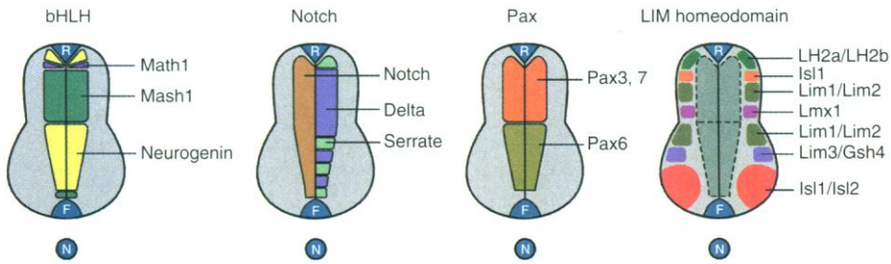

Fig. 6. Dorsoventral subdivisions of the ventricular zone of the developing spinal cord and early neuronal patterning. At the onset of neuronal differentiation, the ventricular zone of the embryonic spinal cord is subdivided into dorsoventral domains that express different combinations of bHLH proteins, Notch ligands, and Pax proteins. The right-hand diagram shows that subsets of neurons derived from different domains of the ventricular zone can be distinguished by the expression of LIM homeodomain proteins. Motor neurons (red) express Isl1/Isl2, certain dorsal commissural neurons (green) express LH2a/LH2b; and dorsal ipsilateral interneurons (orange) express Isl1. The axonal projection patterns of the remaining interneuron classes have not been established. Motor neurons can be further subdivided into columnar subsets on the basis of a more complex LIM homeodomain protein code (see Fig. 4).

Prospects

Progress in clarifying the mechanisms that control cell diversity and pattern in the spinal cord has accelerated appreciably over the past few years but there are many unresolved issues. It is still unclear how neural cells sense small differences in the concentration of inductive factors and respond with the generation of distinct cell types. Similarly, the relation between the combinatorial expression of transcription factors and the generation of neuronal subtypes is not yet apparent. There has also not been a satisfying integration of the patterning mechanisms controlled by molecules such as Sonic Hedgehog and BMPs with the core program of neurogenesis. Nevertheless, the molecular genetic methods now being developed in the mouse and zebrafish and the ongoing cellular analyses of avian embryos offer considerable promise for extending the insights we now have into the mechanisms of neural cell specification in the spinal cord.

The extent to which the principles that emerge from studies of spinal cord development will prove generally relevant to other regions of the CNS also remains uncertain. From what is already known, it seems likely that similar principles operate in the hindbrain, midbrain, and even in the diencephalic region of the forebrain. Within the telencephalon, however, regional differentiation and neurogenesis occur at a significantly later stage, when the dimensions of the telencephalic neuroepithelium are much greater than that of the caudal neural tube. At present, the nature and mechanism of action of signals that control regional pattern and the generation of distinct neuronal subtypes within the embryonic cerebral cortex is not known. In the future, a comparison of the strategies and mechanisms used to generate diversity and pattern in the spinal cord and cerebral cortex might, therefore, provide a more complete molecular solution to the problem of early neural differentiation. Whether such solutions will contribute to a deeper understanding of the organization and function of neural circuits in the CNS may take more time to evaluate.

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The Molecular Biology of Axon Guidance

Marc Tessier-Lavigne and Corey S. Goodman

Neuronal growth cones navigate over long distances along specific pathways to find their correct targets. The mechanisms and molecules that direct this pathfinding are the topics of this review. Growth cones appear to be guided by at least four different mechanisms: contact attraction, chemoattraction, contact repulsion, and chemorepulsion. Evidence is accumulating that these mechanisms act simultaneously and in a coordinated manner to direct pathfinding and that they are mediated by mechanistically and evolutionarily conserved ligand-receptor systems.

The remarkable feats of information-processing performed by the brain are determined to a large extent by the intricate network of connections between nerve cells (or neurons). The magnitude of the task involved in wiring the nervous system is staggering. In adult humans, each of over a trillion neurons makes connections with, on average, over a thousand target cells, in an intricate circuit whose precise pattern is essential for the proper functioning of the nervous system. How can this pattern be generated during embryogenesis with the necessary precision and reliability?

Neuronal connections form during embryonic development when each differentiating neuron sends out an axon, tipped at its leading edge by the growth cone, which migrates through the embryonic environment to its synaptic targets, laying down the extending axon in its wake (Fig. 1). Observations of developing axonal projections in vivo have revealed that axons extend to the vicinity of their appropriate target regions in a highly stereotyped and directed manner, making very few errors of navigation. They do so apparently by detecting molecular guidance cues presented by cells in the environment (*1*). Studies in the past two decades have provided a detailed understanding of the cellular interactions between growth cones and their surroundings that direct pathfinding, which we summarize in the first section of this review. Our understanding of the molecular biology of axon guidance is, however, much more fragmentary. Molecules implicated as guidance cues or as receptors for these cues are introduced in the second section. Many of these molecules have only recently been identified, and it seems likely that additional guidance cues and receptors remain to be discovered. Moreover, in most cases the precise guidance functions of candidate ligand-receptor systems in vivo are poorly understood. In the third section we discuss specific guidance decisions in which the roles played by some of these molecules are beginning to be defined. As will become apparent, despite the many gaps in our knowledge the picture that is starting to emerge is that pathfinding is directed by the coordinate action of multiple guidance forces that are mediated by mechanistically and evolutionarily conserved ligand-receptor systems. A considerable body of evidence supports these conclusions (*2*).

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Cellular Interactions That Guide Axons

The appearance that axons give of unerring navigation to their targets is all the more