Articles

Axon Guidance and the Patterning of Neuronal Projections in Vertebrates

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Over the past decade, new insights have been obtained into the cellular strategies and molecular mechanisms that guide axons to their targets in the developing vertebrate nervous system. Axons select pathways by recognizing specific cues in their environment. These cues include cell surface and extracellular matrix molecules that mediate cell and substrate adhesion and axon fasciculation, molecules with contact-dependent inhibitory properties, and diffusible tropic factors. Several guidance cues may operate in a coordinated way to generate the distinct axonal trajectories of individual neurons.

THE FUNCTIONAL PROPERTIES OF THE VERTEBRATE NERVOUS system depend critically on the intricate network of neuronal connections that is generated during development. One of the first steps in this developmental program is the projection of axons to their targets through diverse and changing environments. The accuracy with which axons select pathways and the extent to which the formation of neuronal connections results from the precise guidance of axons to their targets have been subjects of considerable debate. Early proposals that functional circuits are formed despite diffuse and nonselective axon outgrowth (*1*) were largely abandoned in the face of Sperry's evidence (*2*) for specific neuronal recognition. Sperry's studies culminated in the chemoaffinity hypothesis (*2*), which proposed that axon guidance and target recognition are achieved by the operation of highly specific chemical affinities between individual neurons. However, the molecular complexity inherent in Sperry's model, together with accumulating evidence for activity-dependent competitive rearrangements of synaptic connections (*3*), in turn tempered enthusiasm for the chemoaffinity hypothesis (*4, 5*). Despite these reservations, it has become increasingly apparent that there is a high degree of precision and predetermination in the selection of pathways and targets by developing axons. Although some axons are misrouted and fail to reach their appropriate targets, they are frequently eliminated (*6*), with the result that the orderly projection of the remaining axons is reinforced. The patterning of neuronal connections thus emerges, in large part, from precise and coordinated interactions between developing axons and their cellular environment.

In addition, although the superficial plans of vertebrate and invertebrate nervous systems are quite distinct, the basic strategies and molecular mechanisms by which neuronal projections are established may be more strongly conserved than previously supposed (*5*). Support for this view has evolved with improvements in the techniques used to analyze neuronal specificity. In particular, physiological and anatomical tracing methods have revealed that the accuracy with which vertebrate axons select pathways (*7, 8*) can approach that of their invertebrate counterparts (*9*). Conversely, the development of invertebrate neural circuits is not as rigidly programmed as once thought. It is now known, for example, that initial synaptic contacts are refined during the development of neural networks as disparate as those of insect sensory and primate visual systems (*10*). This similarity of cellular strategies is also reflected at a molecular level. Molecular cloning has revealed that many proteins implicated in cell adhesion and axon fasciculation in vertebrates and invertebrates share structural features and are often members of large gene families (*11*). With these advances has come the prospect of defining a set of general principles and molecular mechanisms that underlie the formation of neuronal connections in diverse neural systems.

In this article, we focus on the cellular and molecular mechanisms adopted in vertebrate embryos to guide developing axons to their appropriate targets. The emerging parallels with mechanisms of neuronal recognition in invertebrates can be appreciated by consulting Harrelson and Goodman (*12*).

Strategies in the Guidance of Vertebrate Axons

The guidance of axons to their final destination can be considered as a series of short-range projections to intermediate targets under the influence of local guidance cues. Neurons respond to these cues by means of a motile sensory apparatus at the tip of the advancing axon, termed the growth cone. In both vertebrates and invertebrates, growth cones exhibit striking changes in their morphology in different cellular contexts (*13*), indicating that they respond to, or explore, their local environment.

Many of the cues that guide growth cones have been revealed by cellular analyses that have their experimental origins in the classical observations of Ramón y Cajal, Harrison, and Spiedel (*14*). Not all of these cues operate on the basis of precise molecular interactions with growth cones. Some developing tissues, such as condensing cartilage, may act as physical barriers that prevent the invasion of growth cones, in this way deflecting axons from their trajectory (*15*). Conversely, continuous extracellular spaces in the embryonic neural epithelium may form channels through which growth cones migrate (*16*). At present, however, there is little direct evidence for the existence of such channels in most regions of the nervous system, and growth cones may actively generate spaces in the absence of preformed channels by releasing proteases that modify their imme-

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diate environment (Fig. 1A). In fact, inhibition of the enzymatic function of proteases released from growth cones modifies axon extension in vitro (*17*).

Recent interest in axon guidance has focused primarily on the identification of molecules that regulate growth cone extension and navigation (Fig. 1). General cell adhesion molecules that are expressed on the neural epithelial cells that surround pioneering axons (Fig. 1, B to D) appear to provide permissive substrates that promote axon extension but do not necessarily impart directional information. Other molecules that exhibit restricted or graded patterns of expression may provide directional cues by enhancing or suppressing initial axon extension over certain regions (Fig. 2A). At later stages, when most developing axons are surrounded by other axon tracts, selective fasciculation of axons becomes a significant mechanism of guidance (Fig. 2B). In addition to these contact-mediated cues, growth cones may be oriented by diffusible chemotropic molecules that are secreted by restricted populations of intermediate or final cellular targets (Fig. 2, C and D). Several of these guidance mechanisms may be superimposed in any particular local environment to generate the distinct trajectories of one or more axonal subsets.

Because subsets of growth cones exhibit divergent choices, it is likely that each subset can recognize different cues within the same epithelial or axonal environment. Some of the axonal receptors that mediate responses to these cues have now been identified. Regulation of the spatial and temporal expression of these receptors on individual axons may be necessary to enable neurons to respond appropriately to the changing cellular environments that they encounter en route to their targets (Fig. 3).

Some of the experimental evidence that forms the basis for this deterministic view of vertebrate axon guidance is discussed in this article. We have categorized different guidance cues, but we do not mean to imply that the mechanisms that operate within vertebrate embryos always conform to these clearly delineated classes. In fact, many of the molecules discussed here are probably used repeatedly within the developing nervous system in a variety of different morphogenetic strategies.

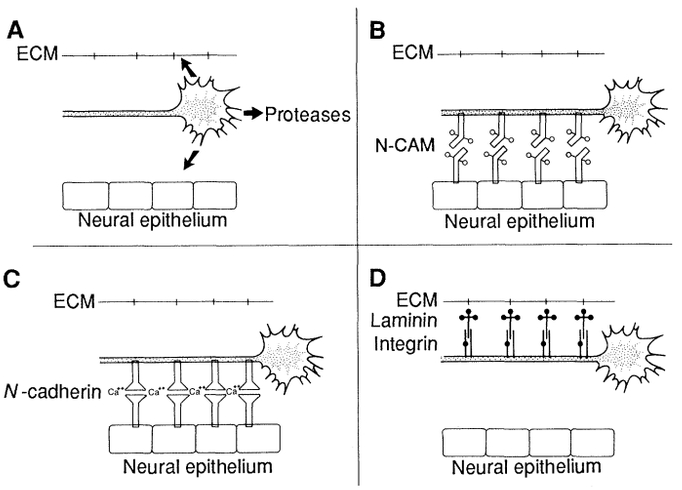

Fig. 1. Axons extend on neuroepithelial and ECM substrates. (**A**) Growth cones may create spaces in their environment by releasing proteases that degrade collagen fibril meshworks and other components of the ECM. (**B** and **C**) Adhesion between axons and neural epithelial cells is mediated by homophilic interactions between surface molecules such as N-CAM and *N*-cadherin. This set of molecules contributes to axon extension but may not provide directional cues. (**D**) ECM glycoproteins such as laminin promote the extension of axons by interacting with receptor molecules termed integrins located on the axonal surface.

Initial Axon Extension on Permissive Substrates

The direction of initial axonal extension of many neurons is highly reproducible (*18*) and has been reported to correlate in vitro with the focus of microtubule polymerization within the cell body (*19*). The organization of the cytoskeleton at the time of neuronal differentiation may thus set the initial orientation of axon extension. At subsequent stages, however, environmental cues rather than the inherent structural features of neurons predominate in the further guidance of axons.

Neurons that differentiate early in development extend axons through mesenchymal or epithelial environments that are devoid of other axons. Three molecules, neural cell adhesion molecule (N-CAM), *N*-cadherin, and laminin, are expressed on or around early neuroepithelial and mesenchymal cells and probably account in large part for the ability of axons to project through these environments (Fig. 1). *N*-Cadherin and N-CAM are integral membrane glycoproteins that are, respectively, the most abundant Ca^{2+}-dependent and Ca^{2+}-independent adhesion molecules present on vertebrate neural cells (*20*). Both molecules promote cell adhesion via a homophilic mechanism; that is, cell binding is mediated by the interaction of the same molecular species on apposing surfaces of interacting cells (Fig. 1). *N*-Cadherin and N-CAM are first expressed on neural ectoderm soon after neural induction (*21*) and may be important in maintaining the cohesiveness of the neuroepithelium before neuronal differentiation (*20, 22*). The axons of differentiated neurons also express high levels of *N*-cadherin and N-CAM, and antibodies to these glycoproteins reduce the outgrowth of central and peripheral axons on cellular substrates in vitro (*20, 22*). These two molecules may therefore permit neurons to adhere to epithelial substrates and to extend axons. The relatively uniform expression of *N*-cadherin and N-CAM in most tissues of the developing nervous system suggests, however, that they do not play primary roles as directional guidance cues.

The extracellular matrix (ECM) glycoprotein laminin is also a major component of substrate pathways over which developing axons project and has been shown to promote axon extension from both central and peripheral neurons in vitro (*23*). Laminin is not as widely expressed as N-CAM or *N*-cadherin and may therefore play a more specific role in promoting directional outgrowth in vivo. For example, the peripheral mesenchyme through which the earliest trigeminal sensory axons project has short, linear arrays of laminin that form a regionally restricted substrate delineating the prospective pathway of these axons to their peripheral targets (*24*). Similarly, the axons of some central neurons extend through the neural epithelium over a transient laminin substrate that precisely marks the initial projection of these axons (*25*).

Laminin promotes axon extension by interacting with axonal glycoproteins that are members of the integrin family of receptors (*26*) (Fig. 1). The integrins are surface proteins consisting typically of noncovalently linked α and β subunits that mediate cell adhesion to other surface and ECM glycoproteins (*27*). Distinct ligand binding specificities result from the particular subunit combinations expressed by individual cells. Antibodies against integrins inhibit the extension of central and peripheral axons on laminin or ECM substrates (*28, 29*). Since laminin is not expressed in all regions of the embryonic nervous system, other cell surface or ECM molecules may also play important roles in axon extension. Several other ECM proteins, in particular collagens, fibronectin, tenascin, and thrombospondin, have been identified in the vicinity of developing central and peripheral axons (*30*). However, these molecules are less

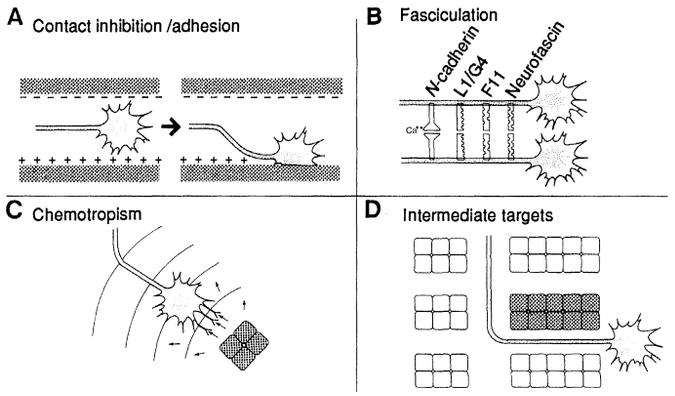

Fig. 2. Growth cones recognize specific guidance cues in their local environment. (**A**) Cell surface or ECM molecules that inhibit growth cones may contribute to their choice of substrate pathways and to selective fasciculation. (**B**) Glycoproteins expressed on developing growth cones and axons may promote fasciculation and growth cone extension on preexisting axon tracts. Homophilic binding is depicted for each of the molecules illustrated, although this has not been established in each case. (**C**) Growth cones may respond to gradients of diffusible molecules that are secreted by discrete cellular targets, thus orienting axonal projections by a chemotropic mechanism. (**D**) Intermediate cellular targets may mediate abrupt alterations in axonal trajectories. Marked changes in fasciculation and axonal projections can also occur in the absence of defined cell groups at "decision regions" (*31*).

effective than laminin in promoting axon growth in vitro, and their pattern of expression does not correlate as well with early axonal trajectories (*30*), suggesting that there are other, as yet undefined, outgrowth-promoting molecules.

Growth Cone Recognition of Specific Cues

Although adhesive interactions between axons and their environment are an important prerequisite for axon extension, the guidance of growth cones is dependent on the recognition of more specific molecular cues that probably do not function solely on the basis of adhesion (Fig. 2). One of the first and most convincing illustrations of specific growth cone recognition in vertebrates occurs during motoneuron pathway selection (*31*).

The axons of motoneurons that exit the spinal cord at particular segmental levels are destined to project to a variety of different muscle targets. Over the initial course of their projection, this mixed population of motor axons projects together in a tight bundle. However, at a discrete site at the base of the developing limb, the plexus region, motor axons defasciculate and reorganize their neighbor relationships such that those destined for the same muscle target emerge from the plexus in a coherent and orderly pattern. Motor axons establish appropriate projections into the limb even after they have been forced, by experimental perturbation, to enter the plexus by novel routes (*32*). The simultaneous segregation of many distinct sets of motor axons indicates that there must be a high degree of specificity in the recognition of cues that exist in the plexus.

In the following sections we point out some of the other experimental systems that have provided support for the idea that the recognition of specific cues is critical to the guidance of vertebrate axons.

Axon guidance by contact inhibition. Molecules that promote axon adhesion and extension play central roles in the initial guidance of vertebrate axons. However, the trajectory of axons can also be influenced by nonpermissive cell surface and ECM substrates and by the contact-mediated inhibition of advancing growth cones (*33*) (Fig. 2A).

Evidence for the existence of cell surface molecules that inhibit axon extension has come from an in vitro analysis of interactions between neurons and oligodendrocytes (*34*). Developing axons will not extend on oligodendrocyte substrates. The inhibitory properties of oligodendrocytes appear to derive from the presence of two cell surface proteins of 35 and 250 kD. Antibodies to these proteins neutralize the inhibitory properties of the oligodendrocyte cell surface. Since mature oligodendrocytes express these proteins, the failure of neurons to regenerate in the adult central nervous system (CNS) could result from the presence of these inhibitory proteins as well as from the absence of permissive molecules such as laminin.

Molecules that inhibit axonal extension are also expressed on axonal surfaces and may contribute to selective fasciculation. The confrontation of growth cones of central neurons with peripheral axons in vitro results in growth cone collapse and axonal retraction (*35*). In contrast, axons do not inhibit the advance of growth cones of the same neuronal class. The collapse of growth cones that encounter foreign axons is dependent on direct contact; moreover, filopodial contacts between growth cones and axons are often maintained even though the axon itself retracts. These observations have provided direct evidence that the growth cones of vertebrate axons can distinguish subsets of axons and suggest that such contact-mediated inhibitory responses contribute to the selection of axonal pathways.

Inhibitory interactions of this type may be important in the initial phases of guidance of motor axons to their peripheral targets. Before motor axons reach the plexus, they project selectively through the anterior half of the adjacent somite (*36*). Experimental manipulation of somites in chick embryos has shown that motor axons never

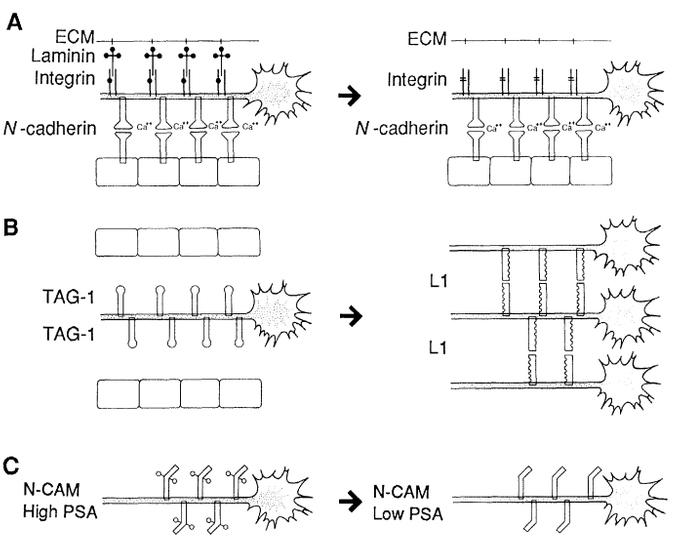

Fig. 3. Transitions in surface glycoprotein expression on developing axons. (**A**) Alterations in integrin function. The axons of many neurons at early stages of neural development use *N*-cadherin and integrins to extend on cell surface and laminin substrates. At later developmental stages integrins are no longer used in the extension of these axons even though they are still expressed on the axonal surface. The involvement of *N*-cadherin in axon extension persists [details in (*28, 29*)]. (**B**) Transitions in the expression of distinct axonal glycoproteins can occur at different stages in the projection of the same set of axons. The TAG-1 glycoprotein is expressed on many axons during initial extension on neural epithelial substrates. TAG-1 expression ceases and the L1 glycoprotein appears as axons begin to fasciculate [details in (*43*)]. (**C**) Regulated changes in the glycosylation of the cell adhesion molecule, N-CAM. The polysialic acid (PSA) content of N-CAM on axons changes with development and in different cellular environments. As axons extend, they express the high PSA form of N-CAM, whereas at late development stages, after axons have reached their target, they express the low PSA form (*85*).

penetrate the posterior somite half. Furthermore, the growth cones of motor axons in vitro grow over sclerotomal cells derived from anterior but not posterior somite halves (*37*); this finding is consistent with the idea that there are inhibitory molecules present in the ECM or on the surface of posterior sclerotomal cells. The segmental patterning of motor nerves and perhaps other peripheral axons may thus derive from contact-mediated inhibitory interactions as well as from adhesion between developing axons.

Axon fasciculation. The first growth cones that extend through epithelial or mesenchymal environments are not guided by other axons. In contrast, neurons that differentiate at later developmental stages send axons through a terrain that contains preexisting fiber tracts on which growth cones may extend. Individual vertebrate axons can make highly selective choices in the fascicles on which they extend (*7, 8*), and several glycoproteins that may be involved in axon fasciculation have been identified.

Many of these axonal glycoproteins were isolated through the generation of antibodies that perturb the adhesion of neural cells in vitro, and as a result they are generally classified as adhesion molecules. Integrins, N-CAM, and *N*-cadherin fall into this class, but they also play more general roles in neural cell adhesion (*20*). Other glycoproteins, such as L1, G4, F11, neurofascin, TAG-1, and contactin (Table 1), tend to be restricted to axonal surfaces (*38–41*) (Fig. 2B). Moreover, glycoproteins such as TAG-1 and TRAP are restricted to functional subsets of developing axons (*42–45*) (Table 1). As with N-CAM and *N*-cadherin, the L1 and G4 glycoproteins appear to mediate interactions between axons by homophilic binding (*38–41*). Although each of these glycoproteins may contribute to the selection of axonal pathways by growth cones, the functions of individual molecules may differ. For example, it has been shown that antibodies to L1, G4, neurofascin, and F11 but not to N-CAM inhibit the extension of growth cones along other axonal surfaces (*39*).

The identification of these axonal glycoproteins has not yet made it possible to elucidate the basic mechanisms underlying selective fasciculation. It remains to be determined whether the selectivity of growth cone–axon interactions is conferred by this class of glycoproteins and whether specificity in pathway selection is generated by differential adhesion. The overlapping expression of these axonal glycoproteins in different combinations (*38–41*) could confer graded differences in adhesive properties to different axonal subsets. Growth cones can discriminate between substrates of markedly different adhesive properties (*46*), but it is not clear how minor differences in the adhesive properties of different axon subsets could result in highly selective growth cone–axon interactions. In fact, after elimination of identified axon fascicles in vertebrate (*7*) and invertebrate (*47*) embryos by laser ablation, growth cones do not extend on the cellular substrates that remain. These results are not consistent with a simple model in which growth cones choose the most adhesive of several different axon fascicles, and they raise the possibility that selective fasciculation may have its basis in more precise recognition events.

Chemotropic guidance of axons. Growth cones may also be guided to their intermediate or final targets by gradients of diffusible factors that are secreted by restricted cell populations within the target (Fig. 2C). The theory that chemotropism contributes to axonal guidance was introduced by Ramón y Cajal after examination of the directed growth of embryonic chick neurons (*48*). The prevalence of chemotactic mechanisms of cell orientation and movement in a wide variety of cells, from bacteria to neutrophils (*49*), has continued to prompt speculation that similar mechanisms might operate within the nervous system. In intact embryos, however, it is difficult to infer from the trajectory of an axon whether it is guided by cues provided by cells of the substrate pathway or by chemotropic factors secreted by distant cellular targets. Evidence for chemotropic guidance in the nervous system has therefore relied on in vitro observations.

Little is known about the molecules that exert chemotropic guidance in the nervous system. Nerve growth factor (NGF) is the only defined molecule for which a chemotropic role has been postulated. In vitro, the growth cones of sensory neurons orient toward a source of NGF (*50*). Moreover, injection of NGF into the brainstem of developing chick embryos evokes a marked ingrowth of sympathetic fibers from the periphery into the CNS (*51*). Thus, NGF may have a tropic function in addition to its well-established neurotrophic role in promoting neuronal survival.

The best evidence for chemotropic guidance of axons relates to molecules other than NGF (Fig. 4A). A chemotropic factor appears to guide trigeminal sensory axons to their peripheral target, the maxillary arch (*52*). In three-dimensional collagen gel matrices the maxillary epithelium initiates an oriented outgrowth of developing trigeminal sensory axons by secreting a diffusible molecule that is distinct from NGF and laminin. The initial guidance of trigeminal axons may therefore depend on both a laminin substrate pathway and a chemotropic factor derived from the maxillary epithelial target. NGF is unlikely to play a primary role in guiding trigeminal axons, since it is not expressed by the target epithelium before the arrival of trigeminal axons (*53*). However, it may exert a secondary tropic or trophic role in expanding the peripheral arbor of sensory axons that have been guided to their target epithelium by the maxillary factor. Diffusible molecules may also contribute to the guidance of sensory axons by their aversive actions on growth cones.

Table 1. Molecules with proposed adhesion or recognition functions on developing vertebrate axons.

Glycoproteins	Structure	Ligand	References
	Glycoproteins involved in axon extension on neuroepithelial and ECM substrates		
N-cadherin	Cadherin family	*N*-cadherin	(*20*)
N-CAM	Immunoglobulin gene superfamily	N-CAM	(*20*)
Integrin or integrins	Integrin family	Laminin, ECM glycoproteins	(*27*)
	Glycoproteins involved in axon fasciculation		
L1 (NILE, 69A1)	Immunoglobulin gene superfamily	L1	(*38, 79*)
G4 (Ng-CAM, 8D9)	190- and 135-kD glycoproteins	G4	(*39*)
F11	170- and 135-kD glycoproteins	?	(*39*)
Neurofascin	185- and 160-kD glycoproteins	?	(*40*)
Contactin	Immunoglobulin gene superfamily	?	(*78*)
	Glycoproteins expressed on axonal subsets		
TAG-1	135-kD glycoprotein	?	(*43*)
RB-8	125-kD glycoprotein	?	(*44*)
TRAP	135-kD glycoprotein	?	(*45*)

Sensory axons in vitro appear to avoid epidermal but not mesenchymal cells as a consequence of the secretion of a diffusible factor by the epidermal cells (*54*). This action could contribute to the selective sensory innervation of dermal but not epidermal target fields.

Other chemotropic factors may guide axons within the CNS (Fig. 4B). In the embryonic rat spinal cord, the growth cones of commissural neurons project directly toward the floor plate, a specialized set of neuroepithelial cells at the ventral midline (*43, 55*). Floor plate cells secrete a diffusible factor that evokes the outgrowth of commissural axons from spinal cord explants and orients these axons (*56*). This chemotropic factor is restricted to the floor plate and is distinct from NGF and laminin. Other classes of CNS axons also exhibit directed growth that suggests they may be guided by chemotropism. For example, retinal ganglion axons that grow out after transplantation of amphibian eye primordia to ectopic sites can home to their tectal targets via novel routes despite the displaced origin of the retinal neurons (*57*).

Collectively, these findings suggest that chemotropism plays a more prominent role in the guidance of central and peripheral neurons than previously appreciated. In vertebrate embryos, the distances over which chemotropic factors appear to guide axons is on the order of 100 to 300 μm, well within the theoretical limits of action of diffusible factors in embryonic tissues and consistent with the range over which diffusible morphogens function in nonneural systems (*58*). The sensitivity of growth cones in detecting diffusible gradients has not yet been determined. However, other vertebrate cells, for example neutrophils, can orient in response to soluble gradients of chemotactic factors that generate only 1 percent differences in concentration across the diameter of the cell (*59*). It would not be surprising if growth cones exhibited a similar sensitivity.

Target-associated positional cues. Once vertebrate axons arrive in the vicinity of their targets they often form highly ordered projections within the target field (*60*). The generation of a precise topography in axonal projections has been proposed to result, in part, from the recognition of positional cues that are expressed as molecular gradients within the population of neurons themselves or in their targets (*2, 61*). Analysis of axon guidance in vertebrate systems has provided support for the idea that the topographic projections of developing axons are established by the recognition of graded cues associated with their targets. For example, positional cues along the rostrocaudal axis of vertebrate embryos may contribute to the segmental matching of neurons with their targets. In support of this, electrophysiological studies have revealed a significant preference in the reinnervation, by spinal preganglionic axons, of sympathetic neuron or skeletal muscle targets derived from the same segmental level (*62*).

An even stronger case can be made for the involvement of graded positional cues in the topographic projection of retinal ganglion axons onto the tectum. Retinal axons form an inverted topographic map along both the anterior-posterior (AP) and dorsoventral (DV) axes of the tectum (*60*). The specificity of this map is evident from the time that retinal axons arrive in the tectum (*63*), implying that developing retinal axons recognize positional cues on the tectal surface.

Retinal growth cones can detect the positional origin of tectal membranes or cells in vitro (*64–66*). When temporal retinal axons are confronted with a substrate that is composed of alternating stripes of anterior or posterior tectal membranes, they extend selectively on the anterior membrane stripes (*65*). Moreover, these axons can discriminate the AP origin of tectal membranes in a graded manner that reflects the in vivo topography of the retinal projection. The preferential extension of temporal retinal axons on anterior tectal membranes appears to result from the repellent properties of molecules associated with posterior tectal membranes rather than the predilection of temporal axons for anterior membranes (*66*). Surprisingly, temporal retinal axons extend equally well on uniform substrates composed solely of anterior or posterior tectal membranes, indicating that retinal growth cones exhibit preferences for the positional origin of the tectal substrate only under conditions of choice. The functional properties of the molecules associated with posterior membranes are, therefore, distinct from those of the molecules that produce a context-independent inhibition of axon extension.

There is also evidence for an adhesive gradient along the DV axis of the retinotectal system (*67*). In addition, several molecules with graded distribution along the DV axis of the retina and tectum have been defined by monoclonal antibodies (*68*). A 47-kD glycoprotein,

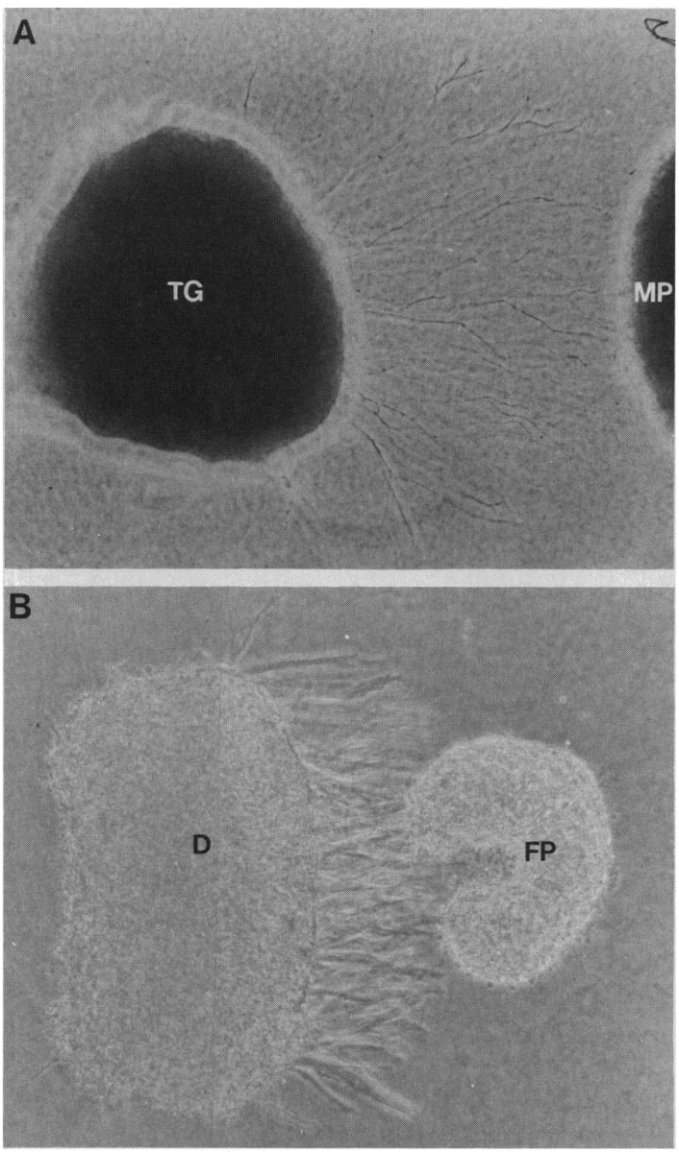

Fig. 4. Chemotropic factors guide extending axons in the developing mammalian nervous system. Photomicrographs of explants of neuronal and target tissue cocultured in vitro in three-dimensional collagen gels. (**A**) Peripheral neurons: neurites of sensory neurons emerge from the embryonic trigeminal ganglion (TG) and grow toward their peripheral target tissue, the epithelial cells of the maxillary process (MP). [Courtesy of A. G. S. Lumsden; (*52*)] (**B**) CNS neurons: commissural neurons in the embryonic rat dorsal spinal cord (D) extend bundles of axons toward the floor plate (FP), an intermediate target in their pathway [details in (*56*)]. Scale bar: A, 200 μm; B, 130 μm.

the TOP antigen, is distributed in a marked D to V gradient in the retina and in an inverted, V to D, gradient on the tectal surface (*69*). Although the functional roles of this and other antigens have not been defined, studies of retinotectal specificity provide support for the concept that positional information, in the form of molecular gradients, is involved in the patterning of neuronal projections within target fields.

Signaling mechanisms in growth cones. Some progress has been made in defining the mechanisms by which growth cones transduce signals provided by substrate and target-associated molecules. Soluble and surface molecules that influence axonal growth may bind to receptors on growth cones that trigger the activation of second messenger systems. One class of chemical mediators that could function in development, neurotransmitters, have been shown to regulate the morphology of growth cones and the rate of axon elongation in vitro by modulating intracellular Ca^{2+} levels in growth cones (*70*). It seems likely that contact-dependent interactions of growth cones can also be mediated by changes in intracellular Ca^{2+} (*71*). The mobilization of intracellular Ca^{2+} or other second messengers may regulate growth cone morphology via the activation of protein kinases. For example, NGF activates several different protein kinases that may mediate its trophic and tropic actions (*72*). The regulation of growth cone morphology by protein kinase activation could involve changes in the function of adhesion molecules. Phosphorylation of the cytoplasmic domain of integrins by tyrosine kinases such as $pp60^{src}$ disrupts interactions between ECM glycoproteins and the cytoskeleton and alters the shape and adhesive properties of fibroblasts (*73*). At present, insufficient information is available on most of the glycoproteins found on developing axons to determine how they are involved in or regulated by intracellular signaling. The different cytoskeletal associations of the variable cytoplasmic domains of N-CAM (*74*), however, suggest that each form of this adhesion molecule could have distinct intracellular functions. These examples illustrate the importance of resolving the mechanisms by which extracellular signals and intracellular messengers confer changes in growth cone structure and motility. Advances in this area are covered in two recent reviews (*75*).

The transmembrane signaling properties of axonal glycoproteins could underlie the ability of growth cones to interact with subsets of axon fascicles and cell surfaces. In particular, graded differences in the density of these glycoproteins on axonal surfaces might impose absolute selectivity in growth cone interactions if the intracellular signals triggered by surface binding events have thresholds for their activation.

Adaptation of Axons to Different Environments

Many of the guidance cues outlined here are encountered sequentially. An individual growth cone may first migrate on epithelial surfaces, later extend on axonal substrates, and finally interact with graded target cues. To achieve this, growth cones and axons may have to adapt to their changing cellular environment. Recent findings have indicated three mechanisms by which such adaptation might occur (Fig. 3).

Alterations in integrin function. In vitro studies have demonstrated that soon after neuronal differentiation, the axons of many central and peripheral neurons, for example, retinal and ciliary ganglion neurons, use a combination of integrins and *N*-cadherin to mediate their extension on cell surface and ECM substrates (*28, 29*) (Fig. 3A). Later in development, the ability of axons of the same neurons to extend on laminin substrates decreases dramatically, and their extension is no longer inhibited by antibodies to integrins. In contrast, *N*-cadherin continues to be required for axon extension (*29*). The loss of laminin responsivity is not, however, accompanied by the loss of integrins from the axonal surface. Integrins may therefore change their ligand-binding properties in response to changing environmental cues, perhaps by switching α subunits (Fig. 3A). The binding specificities of the integrins that remain on axons that are no longer responsive to laminin have not yet been determined. Some integrins can interact with adhesion molecules that belong to the immunoglobulin gene superfamily (*76*). Integrins that are expressed on axons at later times could therefore interact with other ECM proteins or with axonal glycoproteins such as N-CAM, contactin, and L1 that possess immunoglobulin-like domains (*77–79*) (Table 1).

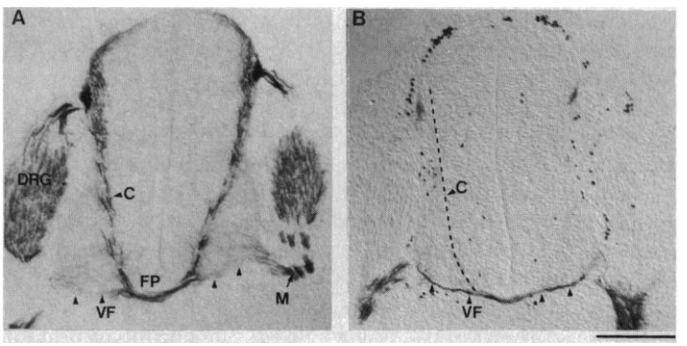

Fig. 5. Transitions in axonal surface glycoprotein expression in the mammalian CNS. (**A**) The TAG-1 glycoprotein, visualized by immunoperoxidase staining, is expressed on commissural axons (C) as they project to the floor plate (FP). As commissural axons join the ventral funiculus (VF), indicated by arrowheads, expression of TAG-1 ceases. Motor axons (M) and sensory neurons of the dorsal root ganglion (DRG) also express TAG-1 at this stage. (**B**) L1 is not detected on the proximal segment of commissural axons during their extension toward the floor plate (the position of the unlabeled proximal segment of commissural axons on one side of the spinal cord is indicated with a dashed line). L1 is expressed on the contralateral segment of commissural axons that have joined the ventral funiculus. This transition in axonal glycoprotein expression occurs at the floor plate. Scale bar: A, 140 μm; B, 130 μm.

Transitions in axonal glycoprotein expression at intermediate targets. A potential mechanism by which axons adapt to changing cellular environments involves spatially regulated transitions in glycoprotein expression on different segments of the same axon. For example, the expression of the TAG-1 glycoprotein coincides with the nonfasciculated extension of commissural axons through the neuroepithelium (*43*) (Fig. 5A). After commissural axons pass through one of their intermediate targets, the floor plate, they undergo an abrupt change in trajectory and begin to fasciculate (Fig. 6). At this point, TAG-1 expression ceases and the L1 glycoprotein is induced (Figs. 3B and 5B). Once L1 appears, it is restricted to the distal segment of the axon that has passed through the floor plate. This transition in TAG-1 and L1 expression on commissural axons coincides with and perhaps contributes to the change in axonal trajectory and the onset of fasciculation. These changes may be triggered by interactions with the floor plate. Consistent with this idea, experimental manipulation or genetic mutations in vertebrates that result in the absence of the floor plate are associated with marked perturbations in axonal projection patterns at the ventral midline (*80, 81*). In effect then, floor plate cells function as vertebrate counterparts to the guidepost or landmark cells (*82*) that influence the trajectory of pioneering invertebrate axons.

Changes in N-CAM sialylation. Alterations in the molecular form of adhesion molecules in different environments may also regulate axon extension (Fig. 3C). The highly sialylated form of N-CAM is expressed on most neurons over the period that they are actively

extending. The arrival of axons at their final targets is correlated in many cases with a switch to the low sialic acid form of N-CAM (*83*). Since a decrease in the extent of sialylation of N-CAM increases the rate of N-CAM–mediated adhesion in vitro (*84*), expression of the highly sialylated form of N-CAM may reduce the potential for axonal interactions with neural epithelial cells or other axons and thus facilitate axon elongation (*85*). Analysis of the projection of retinal ganglion axons has also provided evidence that the degree of sialylation of N-CAM may differ on separate segments of individual axons (*86*). It is unclear how N-CAM sialylation is modulated in vivo. The expression of neuraminidases on cells in restricted regions of a prospective axonal pathway would provide one mechanism for achieving such local regulation.

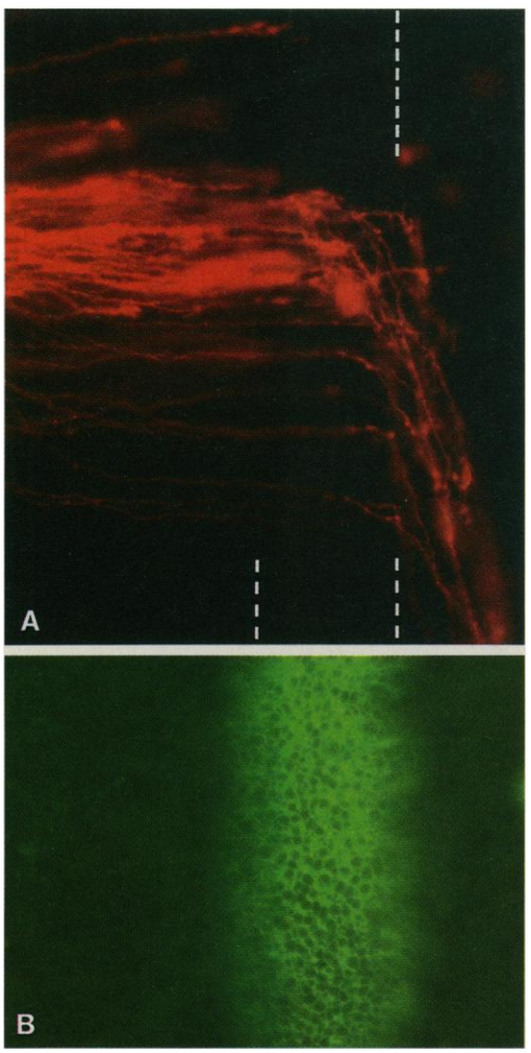

Fig. 6. Axon guidance at intermediate cellular targets. Commissural axons alter their trajectory at the floor plate. (**A**) A group of commissural axons labeled with the fluorescent carbocyanine dye Di-l (Molecular Probes) project toward and across the floor plate (viewed from above). The position of the lateral borders of the floor plate is marked with dashed lines. (This structure extends along the length of the spinal cord.) At the contralateral edge of the floor plate, commissural axons make an abrupt right-angled turn and project in the longitudinal plane along the lateral surface of the floor plate. [Courtesy of P. Bovolenta] (**B**) Floor plate cells express surface properties that distinguish them from adjacent neural epithelial cells. The fluorescence micrograph illustrates the expression of a floor plate–specific cell surface antigen (K1). The specialized surface properties of the floor plate cells may contribute to the changes in axonal trajectory that occur at the floor plate. Scale bar: A and B, 75 μm.

Future Directions in Axon Guidance

We have attempted to summarize recent studies supporting the idea that there is considerable precision and selectivity in the guidance of developing vertebrate axons. The first clear evidence for this view emerged from the analysis of motor axon pathfinding in the chick embryo by Landmesser and colleagues (*31*). The demonstration that motoneuron growth cones recognize selective guidance cues effectively dispensed with earlier ideas of axon outgrowth as a random process (*1*) and at the same time marked a resurgence of interest in Sperry's concept of chemoaffinity (*2*). The in vitro studies of retinotectal specificity by Bonhoeffer and colleagues (*65, 66*) have provided more direct cellular evidence for selectivity in growth cone recognition. The development of intricate in vitro assays has also been crucial in the isolation and characterization of molecules that mediate neural adhesion and recognition. Many of these proteins belong to multigene families, other members of which serve similar recognition and adhesive functions in nonneural cells. Chemotropism and contact-mediated inhibition have also been established as viable mechanisms of guidance, implying that signal transduction across the growth cone membrane is likely to be as important as adhesion in orienting axon trajectories.

Despite these advances, there are still deficiencies in the understanding of the mechanisms by which growth cones interact with their environment. Current approaches to defining adhesion and recognition molecules are heavily reliant on in vitro assays, and in few cases is there clear evidence that these molecules operate in the same way in vivo. Moreover, the identification and classification of adhesion molecules have been based largely on antibody perturbation experiments, which are indirect and may be misleading. The molecular cloning of genes that encode neural adhesion molecules has, however, begun to permit genetic dissection of the function of these molecules in vitro (*87*) and offers the prospect of assessing their role in the developing embryo. Other unresolved issues include (i) the identification of cell surface and diffusible recognition molecules that at present are only inferred on the basis of cellular assays, (ii) the more detailed molecular dissection of interactions between adhesion molecules and their receptors, and (iii) the delineation of signal transduction mechanisms in growth cones.

The existence of these multiple regulatory cues does not prevent apparent errors in navigation from occurring. Superimposed on the order imparted by molecular cues are mechanisms for eliminating axons that persist in projecting along aberrant pathways (*6*). However, these regressive events may operate only after axons have failed to respond to ancillary guidance cues that can reestablish projections to appropriate targets.

Finally, although research during the past decade has provided important insights into the strategies used in axonal pathfinding, it has not been possible to provide a complete description of the guidance mechanisms that operate for a single vertebrate neuron from the time of its differentiation to the establishment of its synaptic connections. Only when this information is obtained will the ingenuity and versatility of the morphogenetic plan used by the developing nervous system become fully apparent.

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