Title
The role of the floor plates and netrin-1 in the unique migration of trochlear motor axons

Author
Colamarino, Sophia Alison

Publication Date
1996

Peer reviewed|Thesis/dissertation

THE ROLE OF THE FLOOR PLATE AND NETRIN-1 IN THE UNIQUE MIGRATION OF TROCHLEAR MOTOR AXONS

by

SOPHIA ALISON COLAMARINO

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

NEUROSCIENCE

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

ACKNOWLEDGEMENTS

I must start by giving credit where it is due the most. This thesis would not exist without the tremendously clever ideas of my advisor, Dr. Marc Tessier-Lavigne, whose intellectual capacity I admire enormously. If I can learn to think even half as clearly as he, I shall be a lucky person.

Of course, his input has been augmented by the support and insights of my thesis committee - Dr. John Rubenstein, Dr. David Sretavan, Dr. William Mobley, and Dr. John Ngai - to whom I thank, above and beyond all, for allowing me to graduate.

I would also like to thank Katja Brose and Bob O'Connor for comments on parts of my thesis, and Nick Martin for assistance with the figures. Aaron McGee, a neuroscience student who did a rotation in the lab, worked with me to collect the data shown in Figure 4-1. The work presented in this thesis was funded primarily by a Howard Hughes Predoctoral Fellowship.

I am convinced that no one can experience colleagues as dynamic and intellectually-charged as I have found amongst my lab and the entire 14th floor sub-community (i.e., members of the Bargmann and Martin labs). They have provided the standards for what it means to be a research scientist, and have supplied the pressure to uphold them.

Six years ago, I chose to come to the UCSF Neuroscience Graduate Program because I felt a sense of community that I didn't think I felt at the other programs I visited. At the time, I couldn't have realized how true this was going to be, and how much of a difference it would make in my graduate experience. I particularly need to thank the other neuroscience students, not only for their friendship, but for providing the intellectual backdrop against which all ideas had to hold-up - they have been my best support-system and at times my toughest critics.

The sense of being colleagues is shared also with the faculty, two of whom I must especially thank for coming to my rescue when needed. I thank Drs. Jon Levine and

William Mobley for reminding me to keep seeing the goal in all of this, and more importantly, for teaching me that it is o.k. for that goal to change.

Finally, I share this with my friends and family. From graduate students, to medical students, to technicians and childhood friends, too numerous to name - I could not have lasted without their support, even when it took the form of a not-so subtle prodding to "just finish-up already." This holds 10,000-fold for my parents, Gianni and Gilda Colamarino, who helped me beyond measure. I surely would not be graduating without their boundless love and sacrifice. I wish I knew another method of expressing my gratitude because the words "thank you" do not mean enough.

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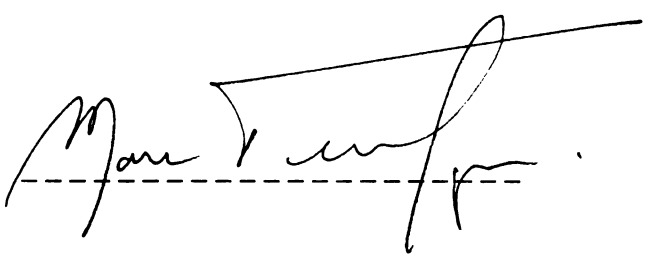

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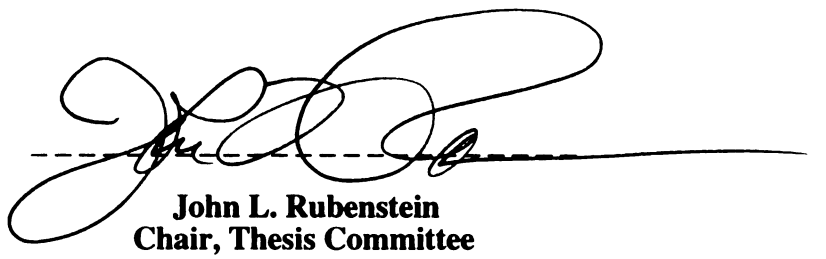

John L. Rubenstein
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ABSTRACT

Proper functioning of the adult nervous system relies on the generation of a precise and intricate network of connectivity between nerve cells. An early step in the establishment of this network occurs during embryonic development when individual axons grow to make contact with their appropriate target cells. The growth cones at the tips of these developing axons migrate in response to molecular guidance cues present in the environment through which they are navigating. Identification of the sources of these guidance cues, and their molecular nature, is central to an understanding of how our nervous system develops.

To investigate this issue we have focused on the role of the floor plate in axon guidance. Recent studies have provided evidence that the floor plate, which comprises the developing ventral midline of the vertebrate neural tube, influences the growth of several different populations of axons. We have used an *in vitro* culture system to demonstrate that the floor plate can act as a chemorepellent for a population of axons, trochlear motor axons, which undergo a unique dorsal migration away from the floor plate *in vivo*. At least part of this repulsion may be mediated by the previously identified

axonal chemoattractant, netrin-1, which we show can also repel trochlear motor axons *in vitro*. These results provide evidence that netrin-1 is capable of acting as a bi-functional axon guidance molecule.

We have analyzed trochlear motor axon projections in mice deficient for netrin-1 production, and found them to be normal. These results indicate that other cues specifying the dorsal migration of these axons must be present *in vivo*. We provide evidence for the existence of at least three different types of netrin-independent axon guidance cues: 1) a second floor plate-derived chemorepellent activity which is distinct from netrin-1, 2) a possible roof plate-derived chemoattractant, and 3) a possible neuroepithelial-derived cue which may designate an area of neuroepithelium as permissive for trochlear motor axon growth. These results suggest that the accurate guidance of trochlear motor axons *in vivo* is a result of a collaboration between many different guidance cues, just one of which may be the molecule netrin-1.

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CHAPTER 1:

Introduction

A Review of Guidance at the Ventral Midline: The Role of the Floor Plate in Axon Guidance

GENERAL INTRODUCTION

Cells at the midline of the embryonic neural tube are strategically positioned to influence the direction of growth of axons near the midline and their decision whether or not to cross to the other side. This introduction will review the guidance role played by the floor plate, the structure that comprises the cells occupying the ventral midline of the developing spinal cord, hindbrain, midbrain, and caudal diencephalon.

In early development, many axonal projections are organized in an orthogonal grid, with axons either growing longitudinally (i.e., rostrally or caudally, parallel to the floor plate) or circumferentially (i.e., in the transverse plane along the circumference of the neural tube, towards or away from the floor plate). Recent studies have provided evidence that the floor plate plays important roles in organizing both longitudinal projections near the midline as well as some circumferential projections. The reason for much of the current interest in the function of the floor plate is that it appears to provide a variety of attractive and inhibitory guidance cues, both diffusible and short-range, that selectively influence the growth of different populations of axons. This property makes the floor plate a potentially rich source for the molecular identification of guidance cues.

The sections that follow will consider a series of choices an axon must make when navigating the midline of the embryo – whether to migrate longitudinally or circumferentially, towards or away from the midline; whether to turn longitudinally before or after crossing the midline; and whether to grow rostrally or caudally along the midline. These issues will be emphasized at the level of the spinal cord, highlighting the role of the floor plate in guiding spinal commissural axons, but what is known about the role of the floor plate in directing axon projections at other axial levels, as well as its potential role in guiding cell migrations, will also be reviewed. The focus will be on the cellular aspects of the guidance events, as in few instances have specific molecules been implicated, though molecular data will also be summarized. This includes the identification of a family of

molecules, the netrins, that are good candidates for directing circumferential migrations in the spinal cord. Remarkably, these molecules are vertebrate homologues of the UNC-6 gene product in the nematode, *C. elegans*, which is required for circumferential migrations of axons and cells in that species (Ishii et al. 1992).

THE FLOOR PLATE

The floor plate in warm-blooded vertebrates

Morphologically the floor plate is made up of columnar ependymal cells, recognizable by their characteristic wedge-shaped appearance, that span the width of the neural tube at its ventral midline (His 1892) (see Figure 1-1). Floor plate cells are among the first to differentiate in the neural tube, apparently under the influence of inductive signals from the notochord (reviewed in Jessell and Dodd 1992). Morphological criteria alone have not permitted a completely unambiguous delimitation of the dimensions of the floor plate either in the transverse plane or along the rostrocaudal axis, but recent molecular studies have suggested more precise criteria for defining these dimensions.

In the transverse plane, many molecular markers appear to be expressed by a group of cells that closely corresponds to what has previously been defined as the floor plate on morphological grounds (Chuang and Lagenaur 1990, Echelard et al. 1993, Keshet et al. 1991, Monaghan et al. 1993, Placzek et al. 1993, Riddle et al. 1993, Roelink et al. 1994, Ruiz i Altaba et al. 1993, Sasaki and Hogan 1993). In the rat at embryonic (E) day 11-12 (i.e., when axons begin to cross the ventral midline), this group is about 15-20 cells wide (Placzek et al. 1993). There may be heterogeneity within this group, however, as some other markers have either a more restricted or a somewhat greater domain of expression than the floor plate proper (McKanna 1992, McKanna and Cohen 1989, Ruiz i Altaba et al. 1993).

The anterior boundary of the floor plate has historically been placed at the hindbrain/midbrain junction (i.e., the fovea isthmii) (Kingsbury 1920, Kingsbury 1930), but there are several reasons for believing that the floor plate actually extends through the midbrain into the caudal diencephalon, ending near the mammillary region. A variety of markers that define the floor plate at spinal cord levels appear to have a rostral limit of expression approximately at the mammillary area (Klar et al. 1992, Matsui et al. 1990, Placzek et al. 1993, Puelles et al. 1987, Ruiz i Altaba et al. 1993, Sasaki and Hogan 1993). Likewise, floor plate chemoattractant activity (see below) is found at all of these axial levels (Placzek et al. 1993), as is netrin-1, a floor plate-derived chemoattractant (Serafini et al. 1994, Kennedy et al. 1994). In addition, prior to the bending of the neural tube to form the cephalic flexure, the mammillary area also marks the rostral extent of the notochord (Puelles et al. 1987), which induces the floor plate.

The floor plate in cold-blooded vertebrates

The floor plate in zebrafish extends from the spinal cord through the hindbrain and midbrain but not apparently into the caudal diencephalon (Hatta et al. 1991). In the transverse plane of the spinal cord, the floor plate comprises a single very large cell at the ventral midline that is distinct morphologically and antigenically from other spinal cord cells (Hatta et al. 1991). It had been proposed that the floor plate also includes the "lateral" cells immediately flanking this "midline" floor plate cell, so that in cross section the floor plate would comprise three cells (Kuwada et al. 1990). However, more recent molecular data (Krauss et al. 1993, A. Klar, K. Hatta and T. Jessell, personal communication) combined with the fact that neuronal cells can apparently be intercalated between the "midline" and these "lateral" cells (Bernhardt et al. 1992b) suggests that the midline cell alone is the closer analogue of the floor plate in warm-blooded vertebrates. On the other hand, the floor plate in the frog had originally been defined as a column of single midline cells based upon morphology (Schroeder, 1970). However, in contrast to Zebrafish, the few molecular

markers for which it has been described now seem to designate a domain approximately 3-5 cells wide as the floor plate in frog (Clarke et al 1991, Ruiz i Altaba et al 1993a, Ruiz i Altaba et al, 1993b). Perhaps, as in Zebrafish, a marker labelling only the midline cell will ultimately be discovered and help to firmly establish whether the floor plate consists of a single cell in most cold-blooded vertebrates or not.

TRAJECTORIES OF AXONS WHOSE PROJECTIONS MAY BE INFLUENCED BY THE FLOOR PLATE

The floor plate is a key landmark for many developing axons as they project to their ultimate targets during embryonic development. Given its midline location and its early differentiation, the floor plate is in a position to act as an intermediate cellular target for extending axons. In this manner it can be thought of as a vertebrate equivalent to the guidepost cells that help direct axon targeting in the invertebrate nervous system (Bovolenta and Dodd 1990, Palka et al. 1992).

Two types of growth patterns - circumferential and longitudinal - are characteristic of early neuronal populations of the vertebrate neural tube. However, any discussion of axonal trajectories in relation to the floor plate is complicated by the fact that slightly different classes of neurons interacting with the floor plate have been identified in different species. For clarity, each species is diagrammed separately, and this discussion will be restricted to neurons that have been shown to be (or are likely to be) influenced by the floor plate, with a particular focus on the earliest developing projections (refer to Figure 1-1 legend for a more detailed description of these neuronal populations).

Circumferential neurons are found in the more dorsal aspects of the spinal cord, and comprise two classes of cells, commissural and association, whose axons initially extend in the same direction ventrally along the lateral edge of the spinal cord, but which diverge upon reaching the ventrolateral marginal zone (see Figure1-1). Axons from

the association neurons turn at right angles to join the ipsilateral longitudinal pathway whereas axons from the commissural neurons cross the basal portion of the floor plate and then turn longitudinally. Upon executing the turn, the commissural axons then fasciculate amongst themselves and with other longitudinally-oriented axons running in the ventral marginal zone.

It appears that two different trajectories may be taken by commissural neurons to reach the floor plate. In chick, the earliest commissural axons follow the lateral edge of the neural tube until they arrive at the ventral midline (Holley 1982, Holley and Silver 1987, Yaginuma et al. 1991, Yaginuma et al. 1990). Similarly, axons of the commissural neurons in Zebrafish (the "CoPA" and "CoSA" neurons) (Kuwada et al. 1990) and those of the commissural neurons in Xenopus (the "dorsolateral commissural interneurons" and "commissural interneurons") (Jacobson and Huang 1985, Roberts and Clarke 1982, Roberts et al. 1988) grow along the circumference of the neural tube to reach the floor plate. However, the later-born commissural neurons in chick and most of those in rodent can follow a slightly different route (Altman and Bayer 1984, Holley 1982, Wentworth 1984, Yaginuma et al. 1991, see Figure 1-1). Although in the dorsal spinal cord they also extend ventrally close to the lateral edge of the neural tube, upon reaching the nascent motor column (which by this stage has become a distinct cellular mass) they break away from the edge of the spinal cord and course ventro-medially to reach the floor plate (see Figures 9E-G in Oppenheim et al. 1988). Many of these later-born axons eventually appear to fasciculate to some extent with other axons, some of which may be the early-born commissural axons that have become passively displaced away from the pial surface in the ventral spinal cord due to the birth and lateral migration of the motoneurons (Holley 1982, Oppenheim et al. 1988) .

Axons that grow to the ventral midline, cross and then turn to project longitudinally are also found at higher axial levels, and have been particularly well-characterized in Zebrafish. Identified vestibulospinal and reticulospinal neurons in the brainstem project to

the spinal cord by sending axons in the bilateral pair of Medial Longitudinal Fascicles (MLF), which run adjacent to the ventral midline (Kimmel et al. 1982, Metcalfe et al. 1986). The majority contribute descending axons to the ipsilateral MLF, but several cell types project in the contralateral MLF. Among those commissural axons are the axons of the Mauthner neurons, which are the first to cross the midline and to pioneer the contralateral MLF in their region of the hindbrain.

Longitudinal neurons differentiating in the ventral neural tube have been described in most species. Their axons extend in the ventral or ventrolateral marginal zone, parallel to the floor plate. The different types of longitudinal neurons include, in chick, the "primitive longitudinal (PL)" neurons (Yaginuma et al. 1990), in Xenopus, the "Kolmer-Agduhr (KA)" neurons (Dale et al. 1987), and in Zebrafish, the "KA" and "VeLD" neurons (Bernhardt et al. 1992b, Kuwada et al. 1990). Longitudinal neurons might also be present in rodents but have not yet been documented.

What do these neurons become in the adult?

Each of these classes of neurons is likely to comprise a heterogenous population of cells. Detailed morphological and immunocytochemical analyses have been used to define many different subtypes of commissural neurons in the cold-blooded vertebrates (Kuwada et al. 1990, Roberts et al. 1988), and of commissural and association neurons in the warm-blooded vertebrates (Oppenheim et al. 1988, Silos-Santiago and Snider 1992, Silos-Santiago and Snider 1994). This heterogeneity within populations no doubt reflects an underlying heterogeneity in function. However, although the axonal trajectories of the early neurons detailed above have been studied in the embryo, their eventual identity in the adult has not been fully established. It has been suggested that some of the commissural axons serve as intersegmental spinal cord interneurons (Oppenheim et al. 1988). In addition, many of these neurons may serve as supraspinal projection neurons. Long-survival [^{3}H] thymidine radiograms in rat identified early commissural neurons as

belonging to the spinothalamic, spinoreticular and spinocerebellar projection neurons (Altman and Bayer 1984).

Cues

Despite superficial differences between species, there are thus two basic patterns of anatomical projections in the early spinal cord - circumferential and longitudinal. These growth patterns exist from the earliest stages of development, and the decision to send axons into either one or the other of these two "modes" of growth appears to be a general feature of axon extension in the developing vertebrate embryo. Cues responsible for generating this stereotyped pattern of axon trajectories not only need to be present very early on, but also must be capable of directing multiple classes of cells. Moreover although many of the late-projecting axons appear to fasciculate with earlier ones, the earliest axonal projections occur in terrain devoid of other axons, so that the cues that direct circumferential and longitudinal projections and the switching between these modes of extension must derive from the early neuroepithelium or adjacent tissues. These cues must instruct the axon (1) to initiate growth in the proper direction (either circumferentially or longitudinally) and (2) once at the midline, first to make the proper choice concerning whether to cross the midline or to remain ipsilateral, and second, when turning longitudinally, to do so in the proper direction and to remain in the appropriate fascicle. The experimental evidence indicating that the floor plate is one source of cues responsible for directing several of these decisions will now be reviewed.

GROWING TO THE MIDLINE ALONG A CIRCUMFERENTIAL TRAJECTORY

Almost all of the different populations of neurons discussed above grow circumferentially at least over a portion of their trajectory, the majority growing all the way to the ventral midline. Only the PL cells in the chick (and possibly the KA cells in Xenopus and zebrafish) are known to initiate longitudinal growth directly (Yaginuma et al. 1990). In this

section the focus is therefore on the cues that guide circumferential growth in the direction of the ventral midline. There is evidence that the mechanisms involved include both short-range local guidance cues derived from non-floor plate cells, as well as long-range chemoattractants emanating from floor plate cells. Short-range cues may serve primarily to direct growth along the edge of the spinal cord, while at least one role for the chemoattractant may be to direct axons that have to grow to the ventral midline through the cellular environment of the motor column.

Attraction of commissural axons to the midline by a floor plate-derived chemoattractant

In warm-blooded vertebrates there is clear evidence that the floor plate has a potent chemotropic effect on commissural axons, a mechanism suggested by Ramon y Cajal (Ramon y Cajal 1909). In 1938 Weber obtained evidence for this type of mechanism through experimental manipulations in chick embryos. Rough transection of the early developing spinal cord was followed by healing of the spinal cord into separate vesicles such that in some cases the dorsal aspect of the spinal cord was separated from the ventral regions by a blood clot. Nonetheless, commissural axons originating from the dorsal cord appeared to home in on the floor plate, growing through the clot as necessary (Weber 1938). Subsequently forgotten, these early studies were revived in recent years when *in vitro* experiments showed that explants of rat floor plate tissue can promote the outgrowth of commissural axons from rat dorsal spinal cord explants into three dimensional collagen matrices (Tessier-Lavigne et al. 1988). Furthermore, *in vitro* experiments with both rodent dorsal spinal cord (Placzek et al. 1990a, Tessier-Lavigne et al. 1988) and chick dorsal spinal cord (S.A.C. and M.T.-L., unpublished observations) have shown that floor plate cells not only possess an outgrowth-promoting activity but can also attract commissural axons, causing them to reorient growth within dorsal explants towards an ectopic floor plate located 100-250μm away. There is a high degree of specificity in the interaction between the floor plate and commissural neurons. The floor plate's ability to induce

turning of commissural axons is not mimicked by explants of any other portion of the neural tube (Placzek et al. 1990a, Tessier-Lavigne et al. 1988) and, within the spinal cord, the chemoattractant selectively affects commissural axons. The axons of association neurons and motoneurons, which do not project to the floor plate *in vivo*, do not reorient in response to the floor plate in culture (Placzek et al. 1990a, Tessier-Lavigne et al. 1988). Moreover, very recent experiments have demonstrated that cerebellofugal axons, which cross the midline at the level of the hindbrain, are also attracted to the floor plate *in vitro*, indicating that chemoattraction of commissural axons to the floor plate may be a fundamental mechanism of axon guidance at all axial levels (Shirasaki et al., 1995).

Studies *in vivo* have confirmed the existence of a floor plate-derived chemotropic activity capable of attracting commissural neurons at a distance. In chick embryos, rotation of a segment of the spinal cord such that the floor plate becomes apposed to the dorsal half of the remainder of the neural tube elicits re-direction of commissural axons towards the ectopic floor plate (Yaginuma and Oppenheim 1991). Similarly, in Danforth short-tail, a mouse mutant lacking the floor plate at the most caudal levels, commissural axons at levels 120μm caudal to the end of the floor plate turn rostrally to grow to the floor plate (Bovolenta and Dodd 1991). More dramatically, a floor plate grafted alongside the spinal cord of a developing chick embryo in ovo causes commissural axons to pierce the external limiting membrane and project abnormally out of the spinal cord towards the graft (Placzek et al. 1990b).

Thus, both *in vitro* and *in vivo* experiments have revealed the ability of the floor plate to attract commissural neurons over hundreds of microns, a mechanism that is likely to contribute to the guidance of commissural axons in the circumferential plane to the ventral midline. The use of a long-range diffusible factor to induce growth of axons to the midline may not be specific to vertebrates. In Drosophila, where midline cells of the CNS have been shown to be important in the formation of the commissures, attraction at a distance for at least some sets of axons has been proposed (Klämbt et al. 1991). In

grasshopper, time lapse recordings have revealed the apparently directed behavior of Q1 axons approaching the midline, leading to the hypothesis that a diffusible cue emanating from the midline region may induce the medial growth of these axons (see Myers and Bastiani 1993b).

Growth parallel to the edge of the spinal cord does not require the floor plate

Although the floor plate clearly has a chemotropic activity, there is also evidence for the existence of a default pathway for axon growth independent of the floor plate along the edge of the spinal cord. *In vitro* experiments have demonstrated that cues specifying a ventral migration must be intrinsic to the dorsal neuroepithelium. Commissural axons in rat dorsal spinal cord explants cultured in the absence of floor plate still grow straight, along their correct dorsoventral trajectory near the edge of the spinal cord, until eventually reaching the cut edge of the tissue (Placzek et al. 1990a, Tessier-Lavigne et al. 1988, see also Nornes et al. 1990). Evidence for the presence of non-floor plate-derived cues has also come from studies *in vivo* in embryos missing a floor plate. At levels that lack a floor plate in the mouse Danforth short-tail mutant, commissural axons succeed in reaching the ventral midline by growing along the circumference of the neural tube (Bovolenta and Dodd 1991). In analogous experiments in chick, where floor plate differentiation is prevented by notochord removal, commissural axons still reach the midline, again by growing along the edge of the spinal cord (Yamada et al. 1991). [However, it is important to note that in both the mouse mutant and the chick notochord-removal studies the commissural axons were navigating in a spinal cord devoid of motoneurons (i.e. a "dorsalized" spinal cord). This occurs because the notochord and/or floor plate are also necessary for motoneuron induction (Bovolenta and Dodd 1991, Yamada et al. 1991). It is not possible to infer from these studies what the behavior of the commissural neurons would have been, had they been challenged to reach the ventral midline in the presence of a differentiated motor column (discussed below)].

Similar observations have been made in the zebrafish mutant *cyc-1*, which lacks the midline floor plate cell at all axial levels (Hatta et al. 1991). Although navigation does go wrong at the midline, almost all circumferential projections to the midline appear the same as in wildtype. In the brainstem, many of the reticulospinal neurons apparently grow to the midline as usual (Hatta et al. 1991). In the spinal cord, the initial short circumferential extension of the VeLD neurons is not disturbed. Similarly, virtually all CoPA and CoSA commissural neurons grow normally around the outer edge of the marginal zone to arrive at the midline (Bernhardt et al. 1992a, Bernhardt et al. 1992b). In fact, their axon trajectories do not display signs consistent with a wandering behavior, as all CoPA and all but 17% of the CoSA axons are directed circumferentially from the point at which the axons exited the cell bodies (Bernhardt et al. 1992b). A similar result was also obtained in UV-irradiated Xenopus embryos lacking both notochord and floor plate (Clarke et al. 1991). Other dorsally-situated cells undergo axonogenesis at the same time as commissural neurons and have axons that grow longitudinally, indicating that the circumferential growth of commissural axons does not reflect the presence of absolute barriers to longitudinal migration (Kuwada et al. 1990). Instead, cues specifying a circumferential migration must be present, even in the absence of the floor plate.

What is the source of the information that directs continued ventral growth along the edge of the spinal cord? It is unlikely that other ventrally-situated cell populations serve to attract the commissural axons since growth along the edge can occur in isolated dorsal spinal cord explants (Placzek et al. 1990a, Tessier-Lavigne et al. 1988) and in embryos lacking motoneurons due to genetic defects or experimental ablation (Bernhardt et al. 1992a, Bovolenta and Dodd 1991, Yamada et al. 1991). Electron microscopy studies of the early neural tube have found no evidence of guidepost cells along the edge of the spinal cord (Holley 1982, Holley and Silver 1987), and have also ruled out the existence of pre-formed channels of extracellular space as a mechanism of circumferential guidance to the midline. Although axons extend close to the pial surface of the spinal cord, their growth

cones rarely, if ever, contact the basal lamina, indicating that it too is not responsible for steering the axons ventrally (Holley and Silver 1987, Yaginuma et al. 1991). Finally, the possibility that other axons are providing the cues is equally unlikely as all studies of axon extension in the circumferential plane at early time points have found these axons to be unfasciculated and independently migrating to the midline (Holley 1982, Holley and Silver 1987, Jacobson and Huang 1985, Kuwada et al. 1990, Yaginuma et al. 1991, Yaginuma et al. 1990).

The local cues directing ventral migration must therefore derive from the neuroepithelial cells themselves. Examination of the circumferentially-growing axons en route to the midline reveals that they make extensive contacts with the neuroepithelial cells as they grow through randomly aligned extracellular spaces (Holley and Silver 1987, Yaginuma et al. 1991). Growth cones of the earliest extending axons exhibit complex morphologies (often used as an indication that they are actively searching their environment for appropriate signals) during the dorsoventral migration (Yaginuma et al. 1991), which supports the notion that circumferentially-directed axons may be reading cues distributed in neuroepithelial cells. By contrast, growth cones of axons extending very late in development are simpler in form (Yaginuma et al. 1991), perhaps indicating that they rely more heavily on fasciculation with other axons as a means of pathfinding along the dorsoventral axis.

Early versus late extension of commissural axons - a hypothesis

From the evidence above it would seem that some circumferential projections do not require long-range cues from the floor plate to grow along the edge to the midline, but that the floor plate is also a source of a diffusible chemotropic factor which can attract some or all axons at a distance. Is there a reason for this apparent redundancy in guidance mechanisms? One hypothesis consistent with the data is that the floor plate-derived chemoattractant is not required for growth along the edge of the spinal cord, but is essential for directing the

commissural axons that grow to the midline through the developing motor column. In this manner it could provide these axons with a mechanism of pathfinding at a time when the environment of the ventral spinal cord has become sufficiently complex that simple growth along the edge is no longer adequate.

There has been no direct test of this hypothesis, which would require examining the ability of later-born commissural axons (that grow when the motor column is already of substantial size) to navigate through motoneurons in the absence of the floor plate. As discussed above, in mouse and chick, the successful arrival of commissural axons at the ventral midline in the absence of the floor plate occurred in a spinal cord lacking motoneurons (Bovolenta and Dodd 1991, Yamada et al. 1991). The axons in this case grew along the edge of the spinal cord, which is analogous to pathfinding at the earliest stages of neural tube development and commissural axon extension. In the cold-blooded vertebrates the only trajectories of commissural neurons described so far are early projections involving growth to the midline along the edge of the spinal cord (Jacobson and Huang 1985, Kuwada et al. 1990, Roberts and Clarke 1982), which, by the model described above, would not be expected to require the presence of the floor plate. This raises the interesting question of whether any later-extending commmissural axons in these species will be found to require the presence of the floor plate to arrive at the ventral midline.

These considerations suggest a model in which growth along the edge occurs in response to floor plate-independent signals but that breaking away from the edge to grow through the motor column requires floor plate-derived signals (Figure 1-2). One limitation of the model is that there is at least one known case, that of a small proportion of the CoSA axons, where initial projections along the edge are disrupted to some degree in the absence of the floor plate. Thus, axons growing along the edge may in some cases be simultaneously responding to the chemoattractant as well.

Molecules involved in directing circumferential projections to the midline

NETRIN-1 IS A FLOOR PLATE-DERIVED CHEMOATTRACTANT. Recent studies have led to the identification of a candidate for the floor plate chemoattractant, netrin-1. The netrins were identified during the purification of an activity in embryonic chick brain extracts that, like the floor plate, can promote outgrowth of commissural axons from explants of rat dorsal spinal cord into collagen gels (Serafini et al. 1994). The outgrowth-promoting activity was due to two proteins, netrin-1 and netrin-2, which each individually possess the activity observed for floor plate *in vitro*. In situ hybridization studies showed that netrin-1 is expressed by floor plate cells, while netrin-2 is expressed at lower levels in roughly the ventral two-thirds of the spinal cord, excluding the floor plate (Kennedy et al. 1994).

Netrin-1 has both the outgrowth-promoting and orienting activities of the floor plate (Serafini et al. 1994, Kennedy et al. 1994, Shirasaki et al 1996) Since it is expressed by floor plate cells, it is likely to contribute to the chemoattractant activity of the floor plate. Biochemically, the netrins partition between the soluble fraction and the membrane fraction of cells. Thus, although netrin-1 can function as a diffusible chemoattractant, its diffusion is likely to be retarded by interactions with the environment. This could conceivably contribute to stabilizing or sharpening the gradient of netrin-1 that is presumed to emanate from the floor plate.

The netrins define a family of vertebrate homologues of the UNC-6 protein of *C. elegans* (Ishii et al. 1992), which is required for circumferential migrations of cells and axons in both dorsal and ventral directions along the body wall. The homology between the netrins and UNC-6 is illustrated in Figure 1-3, which also shows their homology to the amino-terminal end of the B2 chain of laminin. In *unc-6* mutants, both dorsal and ventral circumferential migrations fail at a certain frequency, although axon growth (as opposed to guidance) is not impaired (Hedgecock et al. 1990). *Unc-6* is expressed in a ventral domain of the worm (Wadsworth et al 1996). The UNC-6 protein could be present in a gradient

with its high point at the ventral midline and act as an attractant for cells and axons that migrate ventrally, and perhaps as a repellent for cells and axons that migrate dorsally. This parallel between UNC-6 and netrin-1, not just in sequence but also in potential function, indirectly supports the hypothesis that netrin-1 participates in the guidance of commissural axons to the ventral midline in vertebrates.

THE IDENTITY OF THE CUES THAT DIRECT GROWTH ALONG THE EDGE IS UNKNOWN. The cues that specify growth along the edge of the neural tube may be permissive molecules with a restricted distribution parallel to the edge, or inhibitory molecules distributed in the region from which the axons are excluded (i.e. the ventricular zone). Any mechanism that is postulated, however, must also explain why the axons only grow ventral, not dorsal, and why their growth is confined to the transverse plane.

One candidate for a permissive cue is a complex of laminin and heparan sulfate proteoglycan (Ln-HSPG), detected by the INO antibody (Matthew and Patterson 1983). In the rat, INO labels a corridor along the edge of the spinal cord *in vivo*. Moreover, a substrate containing Ln-HSPG supports commissural axon outgrowth *in vitro*, and INO is capable of inhibiting this outgrowth while antibodies against laminin do not (D. Karagogoes and J. Dodd, personal communication). These observations are consistent with the possibility that a Ln-HSPG complex (or a molecule that cross-reacts immunologically) is a component of a mechanism for guidance along the edge of the spinal cord. More detailed studies on this point are required, however, as laminin itself is not detected in the rat spinal cord (Hunter et al. 1992), nor is INO immunoreactivity detected in the chick spinal cord (Shiga and Oppenheim 1991). A substantial number of other cell-surface and extracellular matrix molecules have been documented in the early spinal cord (see Table 1-1). Although the presence of these molecules has been described, few have been shown to directly affect the growth of dorsal spinal cord neurons. F-spondin, which is expressed by floor plate cells *in vivo*, can mediate attachment of commissural cell bodies

to a substrate *in vitro*, but does not promote commissural axon outgrowth (Klar et al. 1992). While netrin-1 is likely to be a chemoattractant, netrin-2 could conceivably contribute to guidance along the edge. Netrin-2 can promote commissural axon outgrowth into collagen gels (Serafini et al. 1994, Kennedy et al. 1994) and its mRNA is expressed widely by the neuroepithelial cells of the spinal cord (Kennedy et al. 1994). Netrin-2 protein appears to have a more laterally restricted distribution (Kennedy et al, in preparation) and it is at least conceivable that it could contribute to guidance of the axons. Many of the other molecules listed in Table 1-1 may well have effects on commissural neuron outgrowth, but they have yet to be tested.

GUIDANCE AT THE MIDLINE

Guidance events at the midline are more complex than simple growth in the transverse plane and, as a result, are less well understood. The major experimental approach to studying guidance at the midline has been to examine the trajectory of commissural axons in embryos lacking a floor plate. As discussed in the next several sections, in all species it has been found that axon behavior at the midline is abnormal under these conditions. However, the degree to which selective perturbation of the floor plate has been achieved has varied considerably. The most selective perturbation has been obtained in the case of the zebrafish *cyc-1* mutant, in which the major (and perhaps only) defect is the absence of floor plate cells (Hatta et al. 1991). In these embryos, all aspects of guidance at the midline are affected, but the severity is relatively modest. At the other extreme, in the mouse Danforth short-tail mutant and experimentally-manipulated chick embryos, where both the floor plate and notochord are lacking, axon behavior at the ventral midline is seriously disrupted. The generalized perturbation of the entire ventral spinal cord in these embryos may be the cause of the more severe guidance defects. Alternatively, the difference in severity of guidance defects could indicate that the floor plate is simply not as essential for

midline guidance events in cold-blooded vertebrates as it is in warm-blooded vertebrates. In support of this possibility, UV-irradiated Xenopus embryos have a severely compromised ventral midline but may have only relatively minor defects in axon trajectories at the midline (Clarke et al. 1991).

The decision to cross the midline is discussed here separately from the decision to initiate longitudinal growth. This is not to imply that the two decisions are necessarily independent. Crossing and turning may be separable processes which involve distinct cues, but they may also be linked. At the end of these sections two models for guidance at the midline will be discussed in which single cues can direct both the decisions to cross and to initiate longitudinal growth.

The initial decision to cross or not to cross the midline

In contrast to the ability of most early circumferential axons to successfully reach the midline in the absence of the floor plate, the decision whether to cross the ventral midline is in fact perturbed in embryos lacking a floor plate. In the Danforth short-tail mouse mutant and in the chick notochord removal studies, a general confusion in axon behavior is seen at the ventral midline. While some axons do stay in the spinal cord and cross to the contralateral side, many instead project out at the midline, forming an abnormal ventral root (Bovolenta and Dodd 1991, Yamada et al. 1991).

In the zebrafish *cyc-1* mutant, in which the floor plate is more selectively removed, consistent though less severe navigation errors at the midline are observed. These abnormal trajectories can be phenocopied by laser ablation of the midline floor plate cell in normal embryos (Bernhardt et al. 1992a, Hatta 1992). Of the errors, most are mistakes in midline crossing behavior. In the spinal cord, approximately 25% of the commissural axons fail to cross the midline, and instead project longitudinally on the ipsilateral side of the cord (Bernhardt et al. 1992a, Bernhardt et al. 1992b). In the case of the non-crossing axons, 15% of the VeLD axons incorrectly cross the midline in the mutant, suggesting a

role for the floor plate in normally preventing the crossing of these axons (Bernhardt et al. 1992a) This inhibition would likely be through a contact-mediated mechanism, as the VeLD axons appear to contact the floor plate before turning away from the midline during their normal course of extension (Kuwada et al. 1990).

In the brainstem of the *cyc-1* animals a few errors in initial crossing behavior appear to be made by some identified reticulospinal neurons. However, a detailed analysis of the axonal trajectories of the Mauthner cells in the hindbrain revealed that, although there is a gross disorganization of longitudinal pathways (the normally separate and symmetric fascicles of the Medial Longitudinal Fasiculus (MLF) are often fused into one diffuse bundle at the midline), these axons actually rarely made errors in initial crossing decisions (Hatta 1992). The disorganization of the MLF arises principally from the variable behavior of the axons upon the initiation of longitudinal decent after they have crossed the midline (see next section).

It is unclear why the neurons in the brainstem of the *cyc-1* mutant are, for the most part, able to make the correct midline crossing decision in the absence of the floor plate, while those in the spinal cord seem to be more prone to error. Furthermore, although errors are made in the mutant spinal cord, the fact that a still greater percentage of axons makes the correct decision suggests the presence of a second source of signals directing midline crossings. One logical candidate for this source is the underlying notochord. Laser-ablation of the notochord in *cyc-1* mutants does substantially increase the percentage of defects at the midline (Greenspoon et al. 1995). Brainstem pathfinding has yet to be analyzed under these conditions.

Although UV-irradiated Xenopus embryos lacking both the floor plate and the notochord have severe anatomical disruptions they display apparently minor axon guidance defects (Clarke et al. 1991). Despite the fusion of the entire ventral midline area, those commissural neurons that could be examined in detail (the glycine-containing "commissural interneurons") all managed to cross the midline. This crossing did show many abnormal

features, however. Instead of crossing the midline at right angles directly under the pial surface, the axons crossed at variable angles, in some cases running almost parallel with the midline for a distance in the newly-created ventral marginal zone before ultimately crossing. The authors suggest that in Xenopus the floor plate is not needed so much to dictate midline crossing as it is to facilitiate it by directing the angle of crossing. Nonetheless, a detailed analysis of any errors made by other neurons (e.g. the axons of the non-crossing KA neurons) has not yet been performed, and it remains possible that the floor plate or other missing structures plays a more extensive role in directing crossing behavior at the midline of UV-irradiated Xenopus embryos than is apparent from the examination of one population of commissural neurons.

Cellular and molecular biology of midline crossing

The floor plate clearly provides some information necessary to direct the proper routing of axons at the midline. The first growth cones to reach the floor plate may traverse it by inserting themselves between the basal or most ventral surface of the floor plate and the surrounding basal lamina (Kuwada et al. 1990, Yaginuma et al. 1991, Yoshioka and Tanaka 1989). In the grasshopper, contact and fasciculation with the contralateral homologue is involved in successful growth cone crossing of the midline (Myers and Bastiani 1993b), but this does not seem to be the case in vertebrates: although clustering of axons in the floor plate along the rostral-caudal axis has been observed during the earliest periods of floor plate crossing, and growth cones frequently contact each other when in the floor plate, actual fasciculation between them and/or other axons (crossing in either direction) has not been observed (Yaginuma et al. 1990). Furthermore, crossing of commissural axons in zebrafish is achieved in segments in which the contralateral commissural neuron is missing (Kuwada et al. 1990).

Systematic observations of growth cones in fixed tissue have demonstrated significant changes in their shape and complexity as they contact the floor plate (Bovolenta

and Dodd 1990, Yaginuma et al. 1991), suggesting that midline crossing involves more than just passive channeling of axons. These changes could reflect an interaction with signals on the floor plate cells or on the basal lamina underlying the floor plate, which the growth cones contact while crossing (Yaginuma et al. 1991). Processes from the basal surface of the floor plate do tightly enwrap the crossing axons (Glees and Le Vay 1964, Shiga and Oppenheim 1991, Yoshioka and Tanaka 1989). Recently it has been suggested that some signals received by the growth cones are transmitted via a novel mechanism of macromolecular transfer from the floor plate cells. The operation of this mechanism was observed in a transgenic mouse line in which β-galactosidase expression is driven in a subset of floor plate cells; remarkably, the enzyme was also found within adjacent commissural axons (Campbell and Peterson 1993). Previously, dye-coupling between axons and cells had been documented during midline crossing in the grasshopper (Myers and Bastiani 1993a), but the transfer of large cytoplasmic proteins such as β-galactosidase would presumably require a more complex mechanism than simple gap-junctional coupling. A specialized secretory activity for the floor plate had already been suggested (Tanaka et al. 1988, Yoshioka and Tanaka 1989) based on EM observation of numerous vesicles and dense bodies within the cytoplasm of floor plate cells (Glees and Le Vay 1964, Shiga and Oppenheim 1991, Tanaka et al. 1988, Yoshioka and Tanaka 1989).

The first hint at the molecular nature of the signals that direct crossing behavior has come from recent work by Stoeckli et al. (Stoekli et al. 1995). Function-blocking antibodies against Ng-CAM, Nr-CAM, and axonin-1 were injected in ovo throughout the period of commissural axon extension in chick. In each condition, virtually all commissural axons successfully reached the floor plate and executed a longitudinal turn. However, when either axonin-1 or Nr-CAM function was disrupted, this turn occurred on the ipsilateral edge of the floor plate for a significant number of neurons. Injection of a soluble form of axonin-1 as an antagonist produced a greater number of errors (close to 50% of the axons) than was found with either antibody treatment. The authors propose

that this reflects the ability of soluble axonin-1 to act as an antagonist by binding both homophillicly to the axonin-1 on commissural axons and heterophillicly to the Nr-CAM on the floor plate, and would suggest that a direct interaction of axonin-1 with Nr-CAM, documented previously *in vitro* (Suter et al. 1995), may contribute substantially to passage through the ventral midline *in vivo*.

Many other growth-promoting molecules are known to be enriched in the floor plate (Table 1-1) and could possibly aid midline crossing by serving as permissive signals. N-Cadherin and N-CAM are examples of adhesion molecules particularly concentrated on the commissural segment of the crossing axons and which are also expressed by the floor plate cells (Boisseau et al. 1991, Dodd et al. 1988, Krushel et al. 1993, Shiga and Oppenheim 1991). Keratan sulfate proteoglycans in the floor plate have been suggested as possible repellents which prevent passage of non-crossing fibers through the floor plate (Hatta 1992, see Snow et al. 1990a,b for a discussion of inhibition by proteoglycans). Molecules in the basal lamina, such as s-laminin, laminin, heparan sulfate proteoglycan, fibronectin, and collagen type IV, as well as other molecules secreted by the floor plate cells which may become trapped in the basal lamina (e.g., F-spondin and netrin-1), are also all potential signals - either permissive or inhibitory (Hunter et al. 1992, Kennedy et al. 1994, Klar et al. 1992, Shiga and Oppenheim 1991). In the absence of the floor plate, a disruption of the basal lamina or loss of the permissive signals could be responsible for the tendency of some commissural axons to project out of the spinal cord at the midline.

Role of the floor plate in initiating and maintaining longitudinal axonal growth

For many of the early axons, regardless of whether they cross the midline or not, the ventral midline marks the point of termination of a circumferential growth pattern and the site of initiation of a longitudinal one (Bovolenta and Dodd 1990, Kuwada et al. 1990, Roberts and Clarke 1982, Yaginuma et al. 1991, Yaginuma et al. 1990). Where studied in detail, the turn has been found to occur precisely at the edge of the floor plate (Bovolenta

and Dodd 1990). The close correlation of the floor plate with the change in growth pattern suggests a role for the floor plate in influencing longitudinal projections.

In embryos with a severely perturbed ventral midline, axons often fail to turn longitudinally at all. As discussed, many commissural axons in the Danforth short-tail mouse mutant project out of the spinal cord at the midline rather than turning 90^{o} to project longitudinally (Bovolenta and Dodd 1991). In addition, those that stay in the cord may also fail to turn. In the Danforth short-tail mutant and in UV-irradiated Xenopus embryos some axons can apparently be traced which cross the midline and continue to project circumferentially back up the edge of the contralateral spinal cord (Bovolenta and Dodd 1991, Clarke et al. 1991).

In cold-blooded vertebrates, in the absence of the floor plate the vast majority of axons do turn to project longitudinally (even those on the wrong side of the midline). However, errors in the direction of the turns are frequently recorded. In the spinal cord of the zebrafish *cyc-1* mutant, many CoPA and CoSA axons turned caudally instead of rostrally (9% and 24%, respectively) and many VeLD axons turned rostrally instead of caudally (22%) (Bernhardt et al. 1992a, Bernhardt et al. 1992b). Even the KA neurons, which have not yet been documented to contact the floor plate, can project in the wrong direction (17%) (Bernhardt et al. 1992b). A small number of axons display more complicated errors, such as an abnormal bifurcation after crossing the midline and then longitudinal projections in both directions (Bernhardt et al. 1992a). A few Mauthner axons also turned incorrectly in the brainstem (Hatta 1992). In the UV-irradiated Xenopus embryos many more commissural axons are directed caudally than normal (34% vs 13%), and some KA neurons are misdirected as well (Clarke et al. 1991). These data imply that the floor plate may be involved at least to some extent in determining not only when to begin longitudinal growth (before or after crossing), but also the polarity of this growth. The commissural axons of the Danforth short-tail mutants were not analyzed in detail for

turning behavior, and so, if any axons did turn, it is not known whether polarity errors were made in these embryos.

Even after a correct turn has been made, the floor plate also appears to be involved in helping axons seek out and remain in the appropriate longitudinal fascicle. The most common error in axonal pathfinding in the spinal cord of *cyc-1* embryos is the inability of the CoPA axons to directly join the dorsal longitudinal fasciculus (Bernhardt et al. 1992a). The axons instead ascend in the contralateral ventral cord for longer distances, lingering in the ventral longitudinal fasciculus which they normally ignore, before gradually shifting dorsally. Similarly, the principal error of the reticulospinal axons in the brainstem of the *cyc-1* mutants is a disruption of the ability to maintain fasciculation with the correct longitudinal tracts, resulting in disorganized formation of the MLF (Hatta 1992). Axons of the Mauthner cells begin descending as normal but about half of them leave the fascicle at random locations and wander back across the midline. Other reticulospinal axons may join the incorrect longitudinal tract, failing to recognize the MLF as their appropriate target.

Transplantation of wild-type floor plate precursor cells into *cyc-1* embryos only rescues the disorganized phenotype of the MLF at the level of the injected cells (Hatta 1992). Thus, contact with floor plate is not sufficient to completely rectify an axon's projection since axons can aberrantly re-cross the midline after passing through an axial level containing the transplanted floor plate. Because continued presence of the floor plate appears to be required to achieve normal longitudinal projections, the floor plate may serve as a continuous barrier inhibiting midline crossing at all times subsequent to the initial crossing. Further indirect evidence for this can be deduced from chick spinal cord rotation experiments where axons descending in the ventral funiculus also aberrantly crossed the midline upon reaching a discontinuity in the floor plate (see Yaginuma and Oppenheim 1991). These experiments may be interpreted to support a role for the floor plate in providing a short-range inhibitory signal necessary for maintaining laterality of longitudinal projections.

Cellular and molecular biology of longitudinal growth

Evidence from embryos lacking the floor plate thus implies that the floor plate has at least some effect on all decisions regarding longitudinal growth: its initiation, direction and maintenance.

The floor plate may signal crossing growth cones to change their substrate affinities. If this change were one which resulted in an increased propensity of the growth cone to fasciculate with longitudinal axons, then passage through the floor plate would be the key to converting from a circumferential growth mode to a longitudinal one. The discovery that in rat a switch in expression of adhesion molecules, from TAG-1 to L1, occurs as the commissural axons cross the floor plate, has helped to provide a framework for understanding how the commissural axons might successfully disregard all longitudinal pathways prior to crossing the midline (Dodd et al. 1988). Although the appearance of these molecules on the neurons may, in part, be regulated autonomously (Karagogeos et al. 1991), the sharp transition in their expression suggests that the floor plate may be involved in refining the timing or spatial extent of their expression.

Elucidation of signals that instruct the longitudinal axons to turn rostral or caudal, or both, has been more difficult. In rats, growth cones just exiting the floor plate are highly complex, with many filopodia extended both rostrally and caudally in contact with the floor plate, as if they were examining its border for directional cues (Bovolenta and Dodd 1990). Upon executing their rostral turn, the axons extend adjacent to the floor plate edge for the first several hundred microns and then shift laterally in the ventral longitudinal fasciculus. The close association of the longitudinally-extending axons with the floor plate border suggests that it may contain a graded distribution of some signal indicating the rostrocaudal polarity of the neural tube. One attempt to examine this possibility involved *in vivo* rotations of the neural tube. It was found that commissural axons extended normally through a segment of spinal cord which had been inverted along the rostrocaudal axis

(Yaginuma and Oppenheim 1991). These experiments did not, however, distinguish whether directional cues derive from outside the spinal cord or whether the polarity of the neural tube was re-specified after the rotation.

Finally, the signal to switch to a longitudinal growth mode cannot be as simple as a change which results in a generalized fasciculation with any longitudinal axons encountered. This is obvious in the zebrafish spinal cord where, after crossing the midline, the commissural axons choose to ignore longitudinally-directed axons in the ventral longitudinal fasciculus and instead extend dorsally to fasciculate in the dorsal longitudinal fasciculus (Kuwada et al. 1990). A specificity must somehow be imparted to each axon regarding which longitudinal tract to join. It is interesting that in the absence of the floor plate the CoPA commissural axons appeared to have difficulty distinguishing between the ventral longitudinal fasciculus and the dorsal longitudinal fasciculus (Bernhardt et al. 1992a). On the other hand, one cannot exclude that this defect (and all defects in longitudinal growth) arises not directly from the lack of a floor plate but secondarily from the loss or displacement of the longitudinal axons onto which the axons normally fasciculate (Clarke et al. 1991) There are many instances where turns to join longitudinal tracts occur independent of the floor plate (the decision of the association axons to join an ipsilateral longitudinal tract is one such case), which highlights the possible role of early longitudinally-oriented axons, such as those of the PL neurons, in influencing the longitudinal growth of other axon populations.

Models to interpret midline guidance defects

The decisions made at the midline (whether to cross and whether to initiate longitudinal growth) may be directed by distinct cues. However, two simple models can be envisioned in which only one type of signal, either a "crossing" signal or a "turning" signal, is sufficient to direct both decisions.

Axon behavior at the midline could be entirely controlled by signals from the floor plate specifying whether the axons should enter (Figure 1-4A). In this model, the signal would specify the relative attractiveness of the floor plate over the surrounding neuroepithelium and would be positive (more attractive than the neuroepithelium) for the crossing axons, and inhibitory (less attractive than the rest of the neuroepithelium) for the non-crossing axons. Axons barred from entering the floor plate would be forced to turn longitudinally at the ipsilateral floor plate border in order to remain in contact with the more favorable neuroepithelium. Crossing axons enticed to enter the floor plate would be forced to turn longitudinally at the contralateral border of the floor plate to remain in contact with the more favorable floor plate substrate (Bovolenta and Dodd 1990). In this manner, the decision to cross the floor plate would not be separated from the decision to turn, since it is the floor-plate-derived "crossing" signal that would directly result in the choice of when to turn longitudinally. This model requires additional signals to instruct the axons to turn either rostral or caudal, and cannot easily explain why virtually all axons do turn in the *cyc-1* mutant (which presumably lacks these "crossing" signals).

At the other extreme, a model can be proposed in which signals specifying turning behavior (and not crossing behavior) govern the proper routing of axons at the midline (Figure 1-4B). These cues do not necessarily need to be floor plate-derived, but in this model the reception of the signal to turn will directly influence whether the axon crosses the floor plate: if the turn signal is received before crossing the floor plate, an axon will remain ipsilateral; if it is received at the contralateral border of the floor plate, the axon will cross the floor plate before turning. For this model to work, axons that turn only after crossing would somehow have to ignore the turn signal on the ipsilateral side. This would most easily be achieved if the floor plate modulates the ability of the crossing axons to recognize the turn signal (as discussed in the context of the TAG-1/L1 switch). The alternative, that the cues on the left and right sides of the embryo are different, seems quite implausible.

Irrespective of the form of cues the floor plate provides, none of these models require that the floor plate be their only source. In fact, from the data we have reviewed, it would be expected that there is some redundancy, such that cells other than the floor plate (e.g., notochord cells or other axons) are also sources of cues. A strong prediction of both models, however, is that in the absence of all the sources of cues, axons should fail to turn. This is what is observed in the Danforth short-tail mutant and chick embryos lacking both floor plate and notochord. On the otherhand, in the selective absence of the floor plate other sources would be expected to compensate to some extent, resulting in the array of less severe defects found in the zebrafish *cyc-1* embryos.

Regardless of whether all or some of the sources of signals are eliminated, it is more difficult to understand how either of these two models in their simplest form could possibly account for the fact that in the *cyc-1* mutant some commissural axons apparently turn longitudinally *before* crossing. In Model 1, loss of positive signals from the floor plate for crossing axons should not result in an ipsilateral turn. Likewise, in Model 2 the crossing axons should not be able to recognize an ipsilateral turning cue without first contacting the floor plate. One way around this problem would be if the apparently ipsilaterally-projecting commissural axons in the mutant had in fact initially crossed the midline and turned longitudinally before then abberantly wandering back across the midline due to the absence of a barrier to re-crossing. In support of this possibility, aberrant wandering back and forth across the midline in *cyc-1* embryos has been seen for the VeLD axons (J. Kuwada, personal communication). If this interpretation is correct, then cues from remaining sources like the notochord must be sufficient to direct the correct crossing and turning decisions, but not enough to provide the barriers to re-crossing the midline. Although this may seem unlikely, it is indeed what occurs in the brainstem of the zebrafish mutant, where the only serious defects in axon trajectories are errors of re-crossing. Thus, if it were true that errors of re-crossing are also the prime defect in the spinal cord, this

would provide a unifying hypothesis about what occurs at all axial levels when the floor plate is selectively removed.

If, however, in the absence of the floor plate commissural axons do sometimes turn ipsilaterally without ever crossing the midline, then neither Model 1 nor Model 2 can be correct. A more accurate but complicated model would have to be postulated in which crossing axons are not impervious to the turn signal prior to crossing the floor plate, but that the floor plate under normal circumstances provides another signal (perhaps the chemoattractant) that overrides sensitivity to the turning cues (Figure 1-4C). In this new model, passage through the midline would presumably need to modify the axons (perhaps through the TAG-1/L1 switch) such that they could now ignore the "overriding" signal and respond to the contralateral turning cues. This would explain why in *cyc-1* embryos, which lack the floor plate and its "overriding" signal, commissural axons can turn ipsilaterally. To account for all the data from *cyc-1*, however, Model 3 must incorporate several additional assumptions. To explain why only a fraction of the axons turn ipsilaterally in *cyc-1*, it would be necessary to postulate either that there is another source of the "overriding" signal (e.g., the notochord), or that the axons possess only a low-level sensitivity to the turning cue prior to passage through the midline. To explain further why virtually all axons that cross in *cyc-1* do in fact turn, it is also necessary to postulate that the modification of the axons as they pass through the midline (which causes them to ignore the "overriding" signal and respond to turning signals) does not require the presence of floor plate cells. This model could also account for the routing of non-crossing axons, except that contact-dependent signals would be needed to prevent them from entering the floor plate, thus forcing the axons to respond to turning cues on the ipsilateral side. In this way, proper guidance at the midline would result from a combination of "crossing", "turning", and "overriding" signals.

These considerations show that if some commissural axons do indeed turn ipsilaterally in *cyc-1* embryos, then their normal routing probably requires the operation of

multiple signals. The other interpretation we have suggested - that apparent ipsilateral turns actually result from an initial crossing followed by a re-crossing - is more easily explained as being due to the simple absence of a barrier to re-crossing in *cyc-1*. Time-lapse analysis of vitally-labelled commissural axons could determine whether their aberrant ipsilateral projections do indeed result exclusively from initial crossing followed by re-crossing of the axons, or whether some turn ipsilaterally without crossing.

CELL MIGRATIONS THAT MAY BE AFFECTED BY THE FLOOR PLATE

Pathfinding is usually discussed in the context of axonal elongation and growth cone guidance, but it also occurs at the level of cell body migrations. There are suggestions that the floor plate may contribute to directing cell body as well as axonal migrations across the midline.

The fact that many targets receive bilateral innervation has long been appreciated. That the contralateral component can arise not by a crossing of the axon to the contralateral side, but instead by a midline crossing of the cell body, would not, however, have been obvious a priori. Previously this had been inferred to occur for a subpopulation of oculomotor neurons (see for example Naujoks-Manteuffel et al. 1991, Puelles and Privat 1977). A more direct observation of cell body crossing comes from recent studies in chick on the sensory receptor fields of the inner ear, which receive bilateral innervation from the hindbrain. The efferent axons to the receptor fields arise from early-born neurons located in rhombomere 4, adjacent to the motor nuclei of the VII and VIII nerves, and they exit the brainstem through the same combined nerve root. A recent study found that the commissure of the contralateral component is generated by cell body migration across the floor plate, which is initiated at some point after the axons have already reached the nerve exit point (Simon and Lumsden 1993). Retrograde labelling with DiI application to the nerve root allowed the progression of the cell bodies to be followed from their original

location in the ventral motor column near the ipsilateral floor plate border, through their presence in the floor plate, to their ultimate settling near the contralateral border of the floor plate.

Why only this select population initiates this migration and what cues instruct them to back across the floor plate and stop in the contralateral ventral cord are unresolved. The role the floor plate plays, if any, in providing these cues has yet to be examined, but given its central position in the path of this migration it is hard to imagine that the floor plate plays only a passive role in the event. Interestingly, the axons of these neurons are found to be fasciculated from the nerve exit point to the ipsilateral ventral motor column but unfasciculated along the length through the floor plate to the cell body on the contralateral side. It would thus appear that when the cells are propelling themselves backward and adding new axon cylinder at the proximal end, they are probably not using other axons of the same class as a cue to their migration.

A more complex example of cell body migration is represented by the fascinating case of rat precerebellar neurons. Generated in the primary precerebellar neuroepithelium (the "rhombic lip") located in the dorsal wall of the IVth ventricle, they actually migrate away from their cerebellar target and circumnavigate the entire ventrolateral aspect of the medulla before ultimately settling as the neurons of the Inferior Olivary Nucleus (ION), the Lateral Reticular Nucleus (LRN) and the External Cuneate Nucleus (ECN), among others (Bourrat and Sotelo 1990a, Bourrat and Sotelo 1990b). What is extraordinary about this extensive migration is that the cells which become olivary neurons migrate and coalesce to become the ION near the ipsilateral ventral midline, but those destined to become neurons of the LRN and ECN migrate across the midline and continue dorsally to populate the territories of the future LRN and ECN on the side contralateral to which they were born. By the time the migrations are completed, the LRN neurons will have migrated three quarters of the way around the circumference of the medulla to form the contralateral LRN (in the lateroventral medulla) Those of the ECN will have migrated around the entire

margin of the brainstem to form the contralateral ECN which actually lies dorsally alongside the rhombic lip, the site at which these cells are generated.

The purpose of this impressive migration is not at all clear, but it provides an accessible system to study some of the cellular and molecular mechanisms of pathfinding by cell bodies. In particular, given that the neurons of the ION, LRN, and ECN are generated at the same time and dorsal location, what are the cues which direct all the cells ventrally? What cues are used to halt the ION neurons before they cross the midline, in the ipsilateral ION territory, yet ensure that the LRN and ECN neurons will bypass their domains ipsilaterally and only settle in them after they cross the midline? The authors have proposed the floor plate's involvement in all of the above processes.

SUMMARY

The data reviewed above have implicated the floor plate in directing axonal growth towards the midline, in directing the behavior of axons at the midline, and finally in directing the longitudinal growth of axons alongside the midline.

In the case of growth to the midline, there is clear evidence for the existence of two types of cues that collaborate to direct growth - short-range cues that can direct axons along the edge of the spinal cord and a long-range chemoattractant secreted by the floor plate cells whose main function may be to direct commissural axons that must migrate through the complex environment of the developing motor column. Much less is known about the exact role of the floor plate in directing axon growth at the midline, though it is clearly required for accurate guidance. In the absence of the floor plate a range of errors has been found, the most prominent of which are aberrant midline crossings and errors in longitudinal growth near the ventral midline. The severity of these errors does vary with species, which could be due either to the variable importance of the floor plate in the different species or to the fact that so far quite different manipulations of the ventral midline

region have been performed in different species. The most specific perturbation of the ventral midline occurs in the zebrafish *cyc-1* mutant, where the selective loss of the floor plate leads to stereotyped misrouting events. Perhaps surprisingly, in this mutant most all axons grow to the midline and turn longitudinally, although often on the wrong side and sometimes in the wrong direction. The observed defects in the hindbrain can be interpreted to result primarily from the loss of a barrier to re-crossing provided by the floor plate, so that axons do not become confined to the appropriate side of the embryo. We have speculated that perhaps this can account for most of the errors observed in the spinal cord too. However, other cues from the floor plate directing navigation at the midline may certainly be present, but the exact form of these cues (e.g. "crossing", "turning", or "overriding" signals, as we have discussed) remains elusive. Furthermore, studies such as those on the precerebellar neurons, serve to remind us that the floor plate is undoubtedly participating in many more guidance events than have been directly tested.

Determining the precise contribution of the floor plate-derived cues will require identifying them and perturbing them *in vivo*. With the exception of very recent work on the chick proteins Nr-CAM and axonin-1, there has been little functional data on the molecular nature of any of the cues that direct the guidance events taking place at the midline. There is also no data on the identity of the cues that direct growth along the edge of the spinal cord toward the ventral midline; their identification in the spinal cord would be of quite general significance since the growth of axons parallel to (but not in contact with) the pial surface is a widespread feature of early axon growth at all axial levels of the neural tube. Finally, a strong candidate for the long-range chemoattractant is netrin-1, a homologue of the UNC-6 protein of *C. elegans* and a distant relative of laminin, which is expressed by floor plate cells and which can both promote and orient commissural axon outgrowth. Netrin-1 may also influence growth of the many other populations of neurons that exhibit stereotyped behaviors near the ventral midline.

Studies on the role of the floor plate in axon guidance have highlighted the difficulty of analyzing such intricate guidance events and elucidating their molecular basis. Sharp turns made by axons at particular landmarks like the floor plate are made in many other regions that are less amenable to experimental analysis. It is for this reason that lessons learned from the further study of axon guidance at the floor plate are likely to offer insights into similar types of decisions made elsewhere in the nervous system. This thesis will describe the identification of a novel floor plate-mediated guidance event, and will begin to establish its molecular nature both *in vitro* and *in vivo*.

ACKNOWLEDGMENTS

We thank Tom Jessell and Jane Dodd for many helpful discussions and comments on this manuscript, and Erin Peckol, Timothy Kennedy, Kimberly Tanner, Jen Zallen, and Lindsay Hinck for their critical reading of the manuscript. Many thanks to Caroline Murphy for assistance in preparing the manuscript. Supported by grants from the Lucille P. Markey Charitable Trust, the Searle Scholars' Program/Chicago Community Trust, the McKnight Endowment Fund for Neuroscience and the Esther A. and Joseph Klingenstein Fund. S.A.C. is a Howard Hughes Medical Institute Predoctoral Fellow; M.T.-L. was a Lucille P. Markey Scholar in Biomedial Sciences and is currently an Assistant Investigator of the Howard Hughes Medical Institute.

Figure 1-1. Schematic Diagram Summarizing Early Axonal Populations in the Developing Spinal Cord Whose Growth May be Affected by the Floor Plate.

Note the two patterns of axonal projections--circumferential and longitudinal--characteristic of the earliest neurons. The shaded area represents the floor plate. Dorsal is up, caudal is to the left. Dashed lines indicate rostrally-directed projections. The developmental stage listed for each species is that at which the first axons begin to extend. **A.** *Chick* (st 15) (Holley 1982, Holley and Silver 1987, Oppenheim et al. 1988, Yaginuma et al. 1991, Yaginuma et al. 1990). Axons of the circumferential neurons grow ventrally along the lateral margin of the spinal cord (but not in direct contact with the external limiting membrane) in the transverse plane. Upon reaching the ventrolateral cord, axons of the ipsilaterally-projecting association neurons (●) turn at right angles and project longitudinally. Axons of the contralaterally-projecting commissural neurons (O) grow to the floor plate and cross the ventral midline before turning to project longitudinally. The earliest-born commissural neurons have axons which reach the floor plate by growing along the edge of the spinal cord (like the Zebrafish commissural neurons in **C**). The later-born commissural axons (illustrated here) break away from the edge in the ventral spinal cord and grow ventromedially to the floor plate. Primitive Longitudinal (PL) cells (□) have longitudinally-directed axons which extend either rostrally or caudally in the ventrolateral spinal cord. It is not known whether they contact the floor plate cells or instead grow parallel to them at some distance. **B.** *Rodent* (rat E11; mouse E9) (Altman and Bayer 1984, Holley 1982, Wentworth 1984). Commissural neurons (O) in rodent are similar to the later-born commissural neurons described in (**A**) for the chick, except that those in rodent have so far only been documented to project rostrally, while those in chick have been found to turn both rostrally and caudally. Association neurons (●) are also similar except that the earliest ones in rodent project longitudinally in the lateral, rather than ventrolateral, marginal zone. **C.** *Zebrafish* (16hrs) (Bernhardt et al. 1992, Kuwada et al. 1990). Commissural ("CoPA" and "CoSA") neurons (O) project circumferentially along

the edge of the spinal cord to the ventral midline. After crossing the floor plate they turn rostrally and ascend obliquely (dorso-rostrally) for approximately one segment to join the dorsal longitudinal pathway. Association-like neurons (not shown), whose axons turn ipsilaterally, have been described ("CiA" and "CiD" neurons) but are not well-characterized. Axons of the VeLD neurons (Δ) initially extend circumferentially to contact the floor plate but do not cross the midline and instead turn to project caudally. Kolmer-Agduhr (KA) neurons (■) have longitudinally-directed axons which extend rostrally in the ventral spinal cord. Whether they too contact the floor plate before turning is unknown.

D. *Xenopus* (st 25) (Dale et al. 1987, Jacobson and Huang 1985, Roberts and Clarke 1982, Roberts et al. 1988). Commissural neurons ("Dorsolateral commissural interneurons" and "Commissural interneurons") (O) project circumferentially along the edge of the spinal cord to the ventral midline. After crossing the floor plate they project longitudinally either rostrally, caudally or with branches in both directions. See Zebrafish (**C**) for description of KA neurons (■).

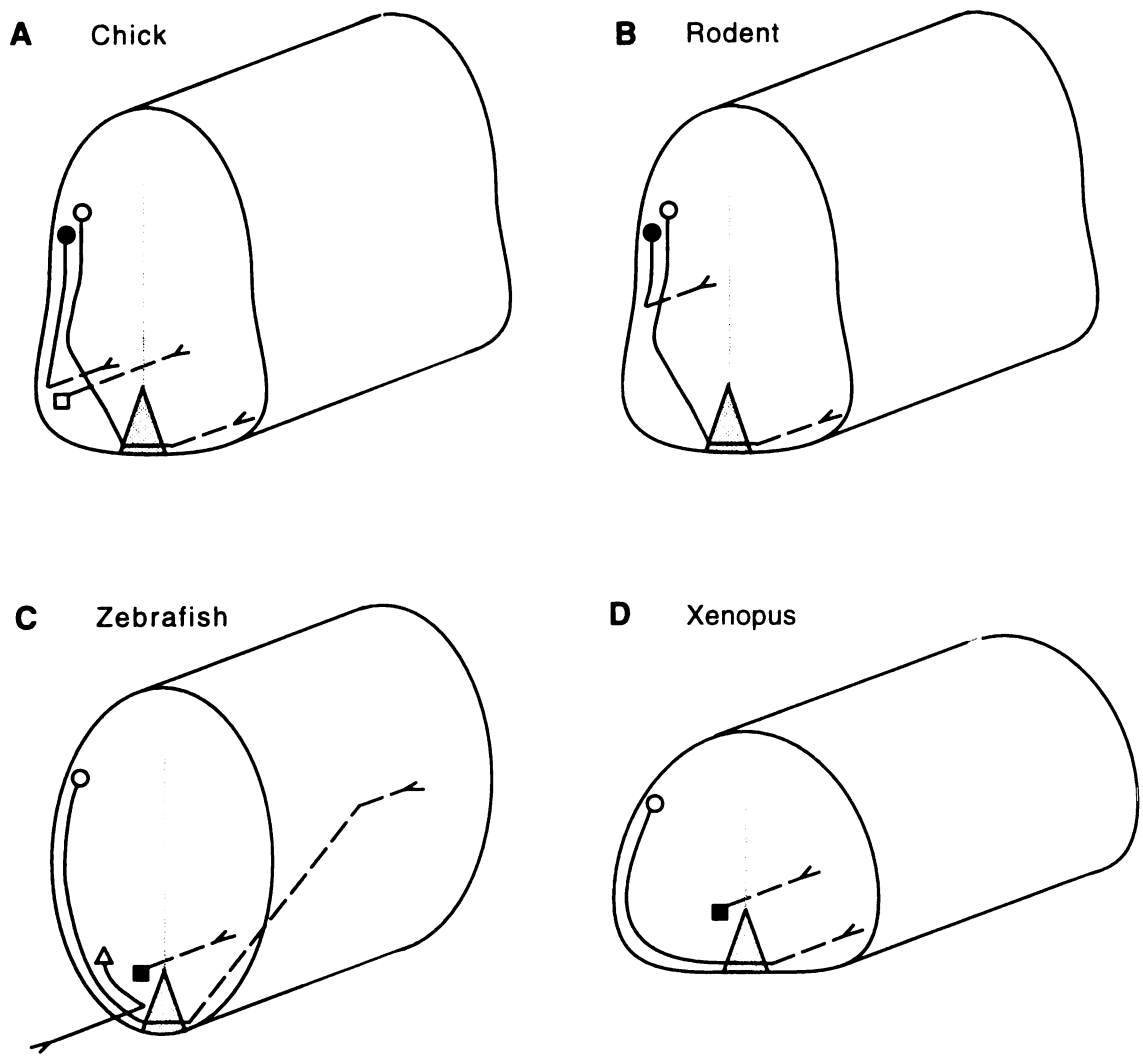


Figure 1-1

Figure 1-2. Different Types of Cues May Collaborate to Guide Commissural Axons to the Ventral Midline.

A. Commissural axons which grow along the edge (such as the earliest born in chick and those in Zebrafish and Xenopus, as well as all Circumferential axons in the dorsal spinal cord) may be primarily directed by an immobilized corridor of molecules distributed along the lateral edge of the spinal cord. The ventricular zone may also contain inhibitory cues restricting axon growth to the edge (not shown). **B.** Commissural axons which break away from the edge in the ventral spinal cord (such as those in rat and the later-born ones in chick) may be directed by a gradient of a diffusible chemoattractant released by the floor plate. The operation of these cues is not mutually exclusive.

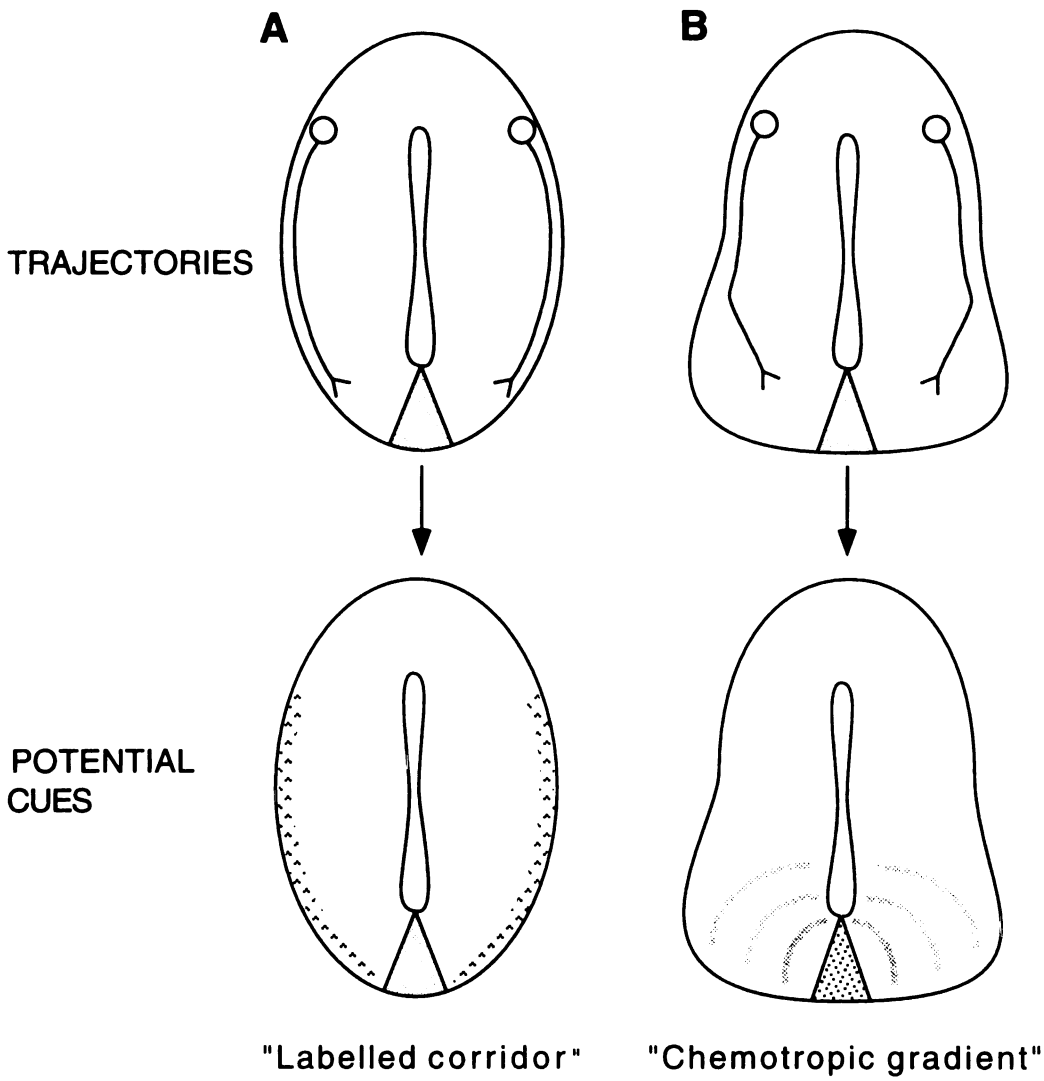

A
B
TRAJECTORIES
POTENTIAL
CUES
"Labelled corridor"
"Chemotropic gradient"

Figure 1-2

Figure 1-3. Structures of the Netrins (from chicken) Compared to UNC-6 and the B2 Chain of Mouse Laminin.

All proteins are homologous in domains VI and the three EGF-like repeats of domain V. The C-terminal domain of the netrins and UNC-6 are homologous, but diverge completely from the laminin sequence. Percent identity between sequences is indicated. See (Serafini et al. 1994) for details.

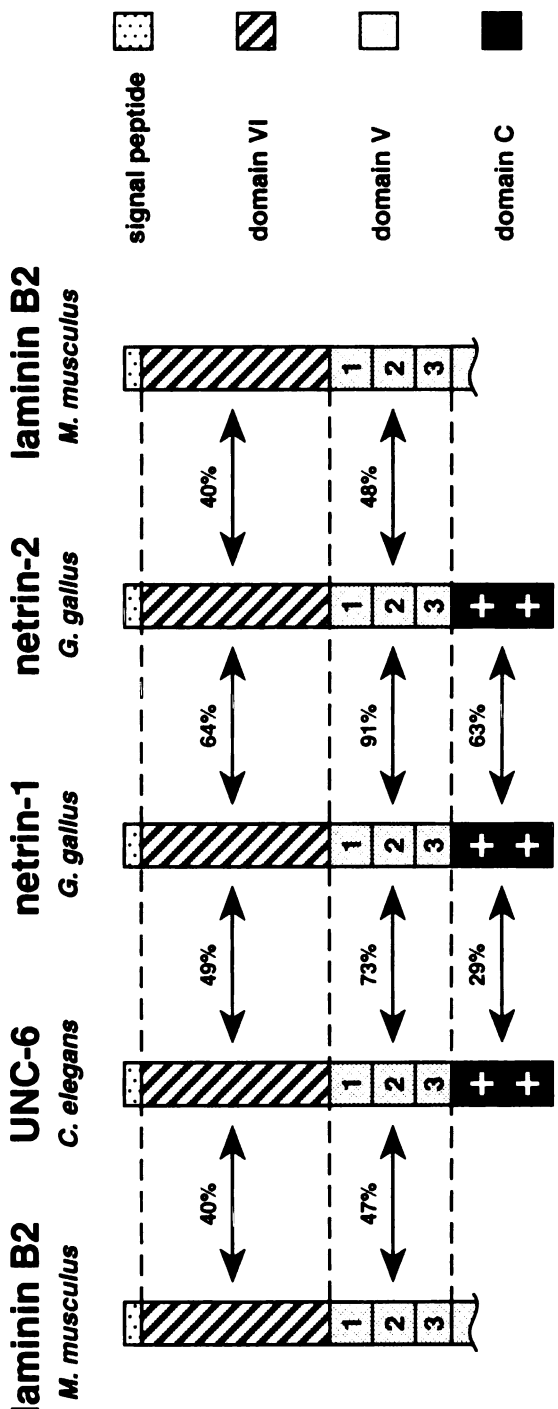

signal peptide
domain VI
domain V
domain C
laminin B2
M. musculus
netrin-2
G. gallus
netrin-1
G. gallus
UNC-6
C. elegans
laminin B2
M. musculus
1
2
3
+ +
40%
48%
64%
91%
63%
49%
73%
29%
40%
47%

Figure 1-3

Figure 1-4. Possible Models for Axon Guidance at the Ventral Midline.
(See text for details) In (A) and (B), crossing and turning events result from the operation of a single type of cue. In each diagram, an axon projecting circumferentially approaches the floor plate and either turns longitudinally on the ipsilateral side (left) or crosses the floor plate before turning (right) **A.** *Model 1*: The floor plate (thick vertical lines) serves as a more (+) or less (-) favorable substrate than the surrounding neuroepithelium (+) for contralaterally- and ipsilaterally- projecting axons, respectively. Longitudinal turns occur because the axons grow to remain in contact with their most favored substrate. **B.** *Model 2*: Signals that direct turning (arrows) on or near the floor plate borders indicate when the axons should turn longitudinally. Contralaterally-projecting axons ignore turning signals on the ipsilateral side (which are therefore not represented), until passage through the floor plate somehow up-regulates their ability to respond to the signals. **C.** *Model 3*: This model is designed to account for defects observed in the *cyc-1* mutant. "Crossing", "turning" and "overriding" signals together guide axon routing at the midline. Axons are sensitive to the turning signals (arrows) but initially ignore them due to an overriding signal (dots) released by the floor plate that masks the turning signals and drives the axons to the midline. Contact with the midline then modifies the axons such that they are no longer sensitive to this overriding signal and can respond to the turning signals. The floor plate must also provide contact-dependent crossing signals which repel the ipsilaterally-projecting axons from entering.

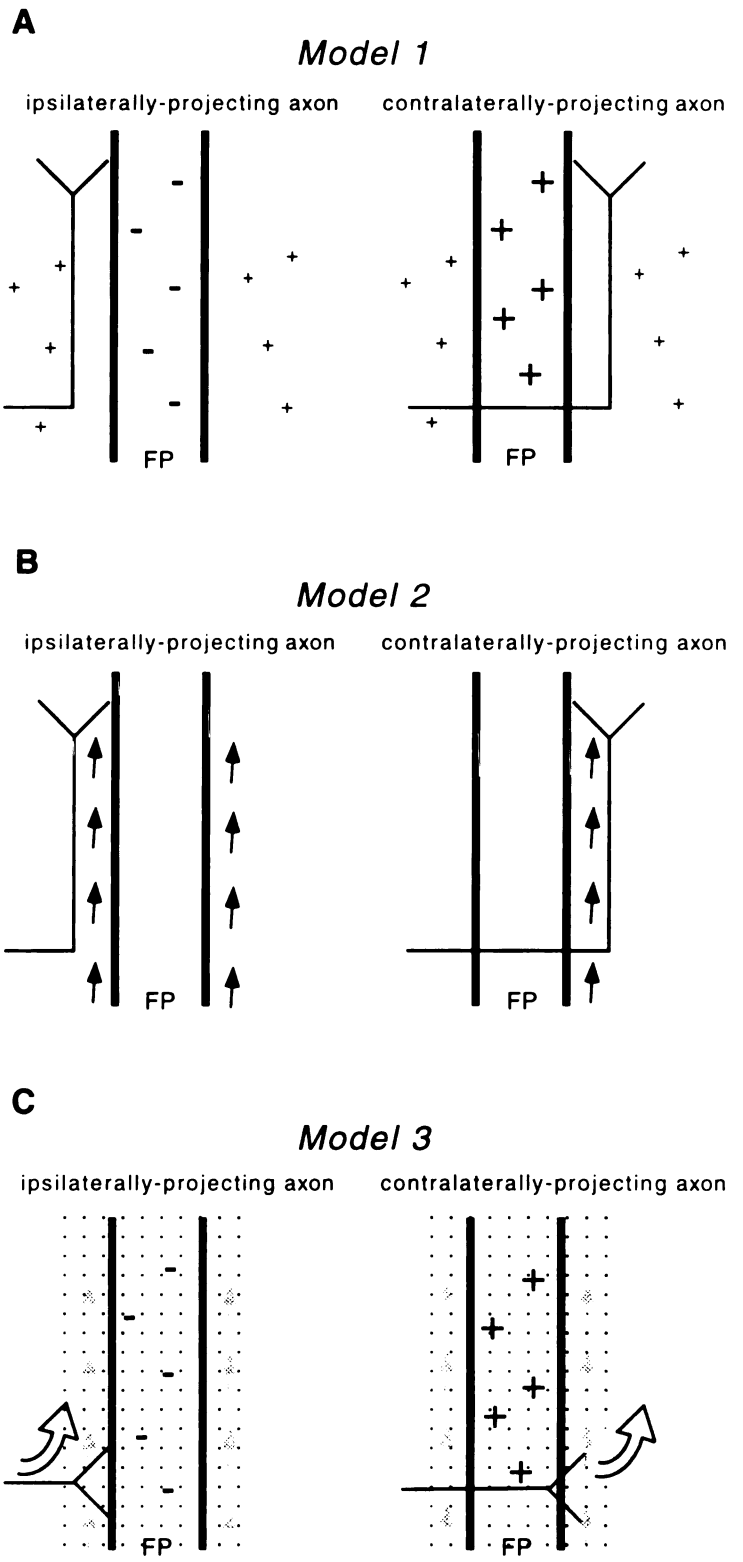

A
Model 1
ipsilaterally-projecting axon
contralaterally-projecting axon
FP
FP
B
Model 2
ipsilaterally-projecting axon
contralaterally-projecting axon
FP
FP
C
Model 3
ipsilaterally-projecting axon
contralaterally-projecting axon
FP
FP

Figure 1-4

Table 1-1 Expression of Selected Cell-Surface and Extracellular Matrix Molecules in the Early Developing Spinal Cord

Molecule	neuroepithelial cells[a]	FP	commissural cells	References
TAG-1	–	–	++[b,c]	(Dodd et al. 1988, Shiga & Oppenheim 1991)
L1	–	–	++[d]	(Dodd et al. 1988)
NgCAM/G4	+[e]	+[e]	++	(Shiga & Oppenheim 1991, Shiga et al. 1993)
Nr-CAM	–	++	++[f]	(Krushel et al. 1993)
Neurofascin	+	–	++[g]	(Shiga & Oppenheim 1991)
F11	–	–	++[d]	(Shiga & Oppenheim 1991)
SC-1	–	++	–	(Tanaka & Obata 1984, Yamada et al. 1991)
F84.1	–	++	–	(Prince et al. 1992)
p84	–	++	–	(Chuang & Lagenaur 1990)
IGFBP-2	+[h]	++	–	(Wood et al. 1992)
C-kit	–	–	++	(Keshet et al. 1991)
Steel factor	–	++	–	(Keshet et al. 1991, Matsui et al. 1990)
BMP-1	–	++	–	(Sasaki & Hogan 1994)
BMP-6/DVR-6	–	–	++	(Wall et al. 1993)
Sonic hedgehog/vhh1	–	++	–	(Echelard et al. 1993, Krauss et al. 1993, Riddle et al. 1993, Roelink et al. 1994)
T-Cadherin	++	++	++[g]	(Kanekar & Ranscht 1992) ; B. Ranscht, personal communication
N-Cadherin	++	+[i]	++[g]	(Hatta et al. 1987, Shiga & Oppenheim 1991)
netrin-1	–	++	–	(Kennedy et al. 1994, Serafini et al. 1994)
netrin-2	+	–	–	(Kennedy et al. 1994, Serafini et al. 1994)
N-CAM (PSA)	–	++	++	(Boisseau et al. 1991, Dodd et al. 1988)
F-spondin	+[j]	++	–	(Klar et al. 1992)
Thrombospondin	++	N.D.	N.D.	(O'Shea & Dixit 1988)
S-Laminin	–	++[k]	–	(Hunter et al. 1992)
Fibronectin	–	++[k,l]	–	(ffrench-Constant 1989, Shiga & Oppenheim 1991)

Collagen IV	–	++[k,l]	–	(Shiga & Oppenheim 1991)
β-1 integrin	+[m]	++[l]	++[f]	(Shiga & Oppenheim 1991)
keratan sulfate proteoglycan	N.D.	++	N.D.	(Hatta 1992)
tPA	–	++	–	(Sumi et al. 1992)

[a] Excludes differentiated neurons

[b] + indicates relative amount of expression for each molecule

[c] Concentrated on pre-commissural and commissural segments (i.e. circumferential portion) of axons

[d] Concentrated on post-commissural segment (i.e. longitudinal portion) of axons

[e] Only when contacted by NgCAM-positive axon or growth cone

[f] Concentrated on commissural segment of axons (i.e. under the floor plate)

[g] Concentrated on commissural and post-commissural segments of axons

[h] At very early stages of development, persists slightly longer in caudal neural tube

[i] Weakly present on basal surface of floor plate cells

[j] In ventral ventricular zone

[k] On basal lamina underlying floor plate as well as on floor plate cells

[l] Concentrated on apical surfaces of floor plate cells

[m] Concentrated on ventricular and pial endfeet

N.D. not determined

CHAPTER 2:

The Axonal Chemoattractant Netrin-1 is Also a Chemorepellent for Trochlear Motor Axons

Abstract

Extending axons are guided in part by diffusible chemoattractants that lure them to their targets and by diffusible chemorepellents that keep them away from non-target regions. Floor plate cells at the ventral midline of the neural tube express a diffusible chemoattractant, netrin-1, which attracts a group of ventrally-directed axons. Here we report that floor plate cells also have a long-range repulsive effect on a set of axons, trochlear motor axons, that grow away from the floor plate *in vivo*. COS cells secreting recombinant netrin-1 mimic this effect, suggesting that netrin-1 is a bifunctional guidance cue which simultaneously attracts some axons to the floor plate while steering others away. This bifunctionality of netrin-1 in vertebrates mirrors the dual actions of UNC-6, a C. elegans homolog of netrin-1, which is involved in guiding both dorsal and ventral migrations in the nematode.

Introduction

An early step in the establishment of neuronal connections during embryonic development is the growth of each axon from its site of origin on the neuronal soma to its synaptic partners. The growth cone at the tip of the developing axon migrates in response to molecular guidance cues in the embryonic environment, which can be attractive (encouraging migration in a particular direction) or repulsive (discouraging migration in other directions). Some guidance cues are expressed by cells along the axons' paths and operate over a short-range. In addition, there is evidence for the existence of longer-range guidance cues, i.e. diffusible chemoattractants that emanate from intermediate or final targets of the axons, and diffusible chemorepellents that are secreted by cells in regions that the axons avoid (reviewed in Goodman and Shatz, 1993; Tessier-Lavigne, 1994).

The identity of long-range axon guidance cues is largely unknown. Recently, a family of chemoattractants for developing axons, the netrins, has been identified (Serafini et al., 1994; Kennedy et al., 1994). Netrin-1 and netrin-2 function as chemoattractants for developing spinal commissural axons *in vitro*, and netrin-1 is expressed by an intermediate target of these axons, the floor plate, during the period that commissural axons grow along a ventral circumferential trajectory to the floor plate (Kennedy et al., 1994). Thus, netrin-1 secreted by floor plate cells likely plays a role in directing the circumferential migrations of commissural axons, attracting them to the ventral midline of the spinal cord. In the case of diffusible chemorepellents, the Semaphorin family of axon guidance cues (Kolodkin et al., 1992; Luo et al., 1993; Kolodkin et al., 1993) includes members that have recently been implicated as chemorepellents (Messersmith et al., 1995) or diffusible inhibitors (Matthes et al., 1995).

The netrins are homologs of the UNC-6 protein of C. elegans, which is required for circumferential migrations of cells and axons in the nematode, and which has been proposed to function as a guidance cue that may directly control these migrations (Hedgecock et al., 1990; Ishii et al., 1992). Importantly, loss of UNC-6 function disrupts

both ventrally-directed and dorsally-directed circumferential migrations; moreover, UNC-6 appears to be concentrated in the ventral portion of the nematode (Wadsworth et al., 1996). One model consistent with these observations is that UNC-6 is present in a decreasing ventral-to-dorsal gradient in the nematode, and that ventrally-directed axons are attracted by increasing concentrations of UNC-6 whereas dorsally-directed axons are repelled.

The conservation between netrin and UNC-6 sequences, and the apparent conservation in the function of these proteins in directing ventral migrations, raises the question of whether the netrins also contribute to directing dorsal migrations away from the floor plate by repelling dorsally-directed axons. To test this possibility, we have focused on the development of the trochlear nerve (cranial nerve IV). Trochlear motor axons originate from cell bodies located near the floor plate and extend along a distinctive dorsally-directed circumferential trajectory away from the floor plate, to the dorsal midline of the neural tube and then into the periphery. The circumferential trajectory to the dorsal midline is conserved within all vertebrate species in which it has been examined, and is unique among motoneurons, as well as among all other neurons in the midbrain, hindbrain and spinal cord (Sinclair, 1958; Fritzsch and Sonntag, 1988; Matesz, 1990; Fritzsch and Northcutt, 1993; Szekely and Matesz, 1993; Chedotal et al., 1995). Although the trochlear motoneuron population has been studied extensively as a model system for cell death and axonal regeneration (Cowan and Wenger, 1967; Sohal and Holt, 1977; Sohal et al., 1985; Sonntag and Fritzsch, 1987; Fritzsch and Sonntag, 1990; Murphy et al., 1990; Sohal et al., 1991a, 1991b; Derouiche et al., 1994) the mechanisms that direct its unique dorsal projection are unknown. Here, we have explored possible interactions between floor plate cells and trochlear motor axons. We show that the floor plate and heterologous cells secreting netrin-1 can repel trochlear motor axons at a distance *in vitro*. These results suggest that netrin-1 secreted by floor plate cells may function as a chemorepellent to guide trochlear motor axons away from the ventral midline *in vivo*.

Results

The axons of trochlear motoneurons follow a stereotyped trajectory to the dorsal midline *in vivo* and *in vitro*

The cell bodies of neurons in the trochlear (IVth) nerve nucleus differentiate ventrally near the floor plate at the level of the junction between the hindbrain and midbrain around embryonic day 11 (E11) in the rat (Altman and Bayer, 1981). Their axons extend circumferentially away from the floor plate along a dorsal trajectory, cross the midline in the roof of the hindbrain (the anterior medullary velum), and then project to their final target, the superior oblique muscle of the contralateral eye (Figure 2-1A), reaching it by E13 (B. Fritzch, personal communication). The dorsal trajectory and emergence of these axons from the CNS at the dorsal midline can be visualized in transverse sections through the hindbrain-midbrain junction (HMJ) using the marker F84.1 (Figure 2-1B), which labels both motor axons and floor plate cells (Prince et al., 1992), or by backlabelling the axons with the fluorescent dye DiI injected into the mesenchyme between the eye and the anterior medullary velum (Figure 2-1C). The dorsal projections of trochlear motoneurons contrast with the projections of motoneurons of the oculomotor (cranial nerve III) nucleus which are born less than 150 μm rostral to trochlear motoneurons and also project to eye muscles, but whose axons (which also express F84.1) exit the neural tube near their cell bodies (Puelles and Privat, 1977; Altman and Bayer, 1981; Prince et al., 1992; Szekely and Matesz, 1993; Chédotal et al., 1995; Fritzsch et al., 1995; discussed in legends to Figures 2-1 and 2-2).

To study the factors that influence trochlear motor axon growth, we first examined whether the characteristic dorsal migration of these axons can be replicated in explant culture. Explants of the entire hindbrain-midbrain junction (HMJ explants) were isolated from rat embryos at E11 (i.e, around the time when trochlear motoneurons begin to differentiate), freed of surrounding mesenchyme, and cultured in three-dimensional

collagen matrices for 48 hr. At that time, a bundle of axons could be observed that had emerged from the dorsal surface of explants during the culture period (data not shown). DiI applied to the bundles retrogradely labelled axons with cell bodies located in the ventral (basal) aspect of the explants in a position characteristic of trochlear motoneurons (Altman and Bayer, 1981), and which had extended along a dorsal trajectory similar to that followed by trochlear motor axons *in vivo* (compare Figures 2-1D and 2-1C). The axons in the bundles also expressed F84.1, consistent with their being motor axons (data not shown; see Figure 2-2C below). Thus, the developing neuroepithelium appears to contain all the cues necessary to direct the differentiation of trochlear motoneurons and the dorsal migration of their axons *in vitro*.

The ventral neuroepithelium contains cues that can instruct trochlear motor axons to grow dorsally

This dorsal trajectory could result from the attraction of these axons by dorsal structures, an intrinsic bias in the direction of neurite outgrowth from these neurons, or the repulsion of these axons by ventral structures, among others. To test whether dorsal structures are required for the dorsal migration *in vitro*, we removed the dorsal two thirds of E11 HMJ explants and cultured the remaining portions (referred to below as "ventral HMJ explants") in isolation (diagrammed in Figure 2-2A). A cluster of axons emerged from the cut dorsal-most edge of the ventral HMJ explants after ~ 20 - 24 hr in culture (data not shown) and continued to grow within the collagen matrix along a trajectory that paralleled the original dorso-ventral axis of the explant. This resulted in the presence after 40 hr of a characteristic loose bundle of axons, ~ 50 - 90 μm wide, oriented roughly perpendicular to the dorsal edge of the explants (41/42 explants, Figure 2-2B). In what follows, we refer to this bundle (exiting the dorsal edge and more than 30 μm wide) as a "dorsal bundle". The axons in the dorsal bundle were likely trochlear motor axons, as they expressed F84.1 (Figure 2-2C), and derived from cell bodies in the characteristic location of trochlear

motoneurons (as assessed by F84.1 staining (Figures 2-2C and 2-2D) and retrograde labelling with DiI (data not shown)). Other isolated axons and thin axon bundles were sometimes observed projecting into the collagen from apparently random sites in the explants (Figure 2-2B). These axons could be labelled with anti-neurofilament antibodies but did not usually express F84.1 and did not follow any obvious trajectory within the collagen matrix (data not shown); we presume that they derived from other neurons such as medial longitudinal fasciculus neurons in the explants (Altman and Bayer, 1981). In some cases, one or two thin F84.1+ fascicles that appeared to originate from the trochlear nucleus were observed separate from the large F84.1+ bundle (see Figure 2-2C).

These results indicate that trochlear motor axons do not require signals from the dorsal two-thirds of the neuroepithelium to direct their initial dorsal migration. In addition, although most of the trochlear motor axons observed within cultured ventral HMJ explants exited the dorsal pole of the cell bodies and appeared to have migrated along a straight dorsal trajectory, cases were also observed of F84.1+ axons whose initial segments were found on the ventral side of the cell bodies and which were directed ventrally towards the floor plate for a short distance. However, all such axons then appeared to make a sharp U-turn to project dorsally (Figure 2-2D). These observations show that the dorsal migration of trochlear motor axons *in vitro* does not result simply from polarized outgrowth from the cell bodies of origin and indicates that the ventral neuroepithelium contains cues that can redirect the axons to grow dorsally.

The floor plate suppresses the formation of dorsal bundles of trochlear motor axons

We next examined whether the floor plate provides trochlear motor axons with a cue(s) that directs them along a dorsal trajectory. It was not possible to test this by removing the floor plate from the explants and scoring the axons' trajectories, because this manipulation resulted in apparent failure of trochlear motoneurons to differentiate (data not shown; we

presume that these motoneurons, like spinal cord motoneurons (Yamada et al., 1991), require inductive signals from ventral midline structures for their initial development).

We therefore examined whether the floor plate can influence the growth of trochlear motor axons by culturing two ventral HMJ explants in tandem such that the floor plate of one explant (top) was opposite the dorsal cut edge of another explant (bottom), with the explants separated by 100 - 450 μm (see Figure 2-3A). Axon outgrowth from the top explant in such cultures was comparable to that in control cultures (i.e. explants grown alone), with the characteristic dorsal bundle of trochlear motor axons projecting from all explants (35/35 explants with bundles, Figure 2-3B). In contrast, dorsal bundles were not observed projecting from the bottom explant (0/35 with bundles, Figure 2-3B). Monitoring of explants during the culture period indicated that absence of a dorsal bundle was due to a failure of the bundle to form rather than to its extension and subsequent retraction (data not shown). The presence of a dorsal bundle was not affected when the top explant was replaced by an explant of dorsal spinal cord (12/12 with bundles, Figure 2-3C) or ventral spinal cord (without floor plate) (11/11 with bundles), indicating that its absence in tandem cocultures of ventral HMJ explants did not result from a non-specific effect such as a distortion of the collagen matrix by the top explant. The floor plate region microdissected from ventral HMJ explants prevented the appearance of this bundle (0/9 with bundles), as did explants of floor plate from spinal cord levels (1/16 with bundles, Figure 2-3D). These results indicate that the floor plate secretes a diffusible factor(s) that suppresses the formation of a dorsal bundle of trochlear motor axons, and that expression of this factor is not restricted to the axial level of the trochlear nucleus.

COS cells secreting netrin-1 suppress the formation of dorsal bundles of trochlear motor axons

The long-range inhibitory effect of the floor plate on dorsally-directed trochlear motor axons contrasts with its long-range attractive action on ventrally-directed spinal

commissural axons, which is thought to be mediated by netrin-1, an UNC-6 homolog. In C. elegans, UNC-6 contributes to guiding both dorsally-directed and ventrally-directed axons, raising the question of whether netrin-1 – which is expressed in the floor plate at all axial levels (Kennedy et al., 1994) – contributes to the inhibitory action of the floor plate. To examine this possibility, COS cells were transfected with a netrin-1 expression construct, and aggregates of the transfected COS cells were placed opposite the dorsal cut edge of a trochlear explant and co-cultured for 40 hrs. Netrin-1-secreting COS cells suppressed the appearance of the dorsal bundle of trochlear motor axons, whereas control COS cells did not (Figure 2-4). The netrin-1-secreting cells were slightly less effective than the floor plate, since dorsal bundles were observed in about 30% of cases (Figure 2-4C). However, the mean length of the bundles in these cases was significantly shorter than with control COS cells ($p = 0.002$, t test; Figure 2-4D; see also Figures 2-5K and 2-5L), showing that even in these cases netrin-1 affected trochlear motor axon growth.

The floor plate and netrin-1 repel trochlear motor axons

To determine what happened to trochlear motor axons that failed to form dorsal bundles in the presence of floor plate or netrin-1-secreting cells, we visualized such axons using F84.1. Trochlear motor axons were unimpeded by control tissues (e.g. dorsal spinal cord: Figure 2-5A) or control COS cells (Figure 2-5C) and, as seen in transverse views, appeared to grow over them (Figures 2-5B and 2-5D). In contrast, the extension of trochlear motor axons was perturbed in a variety of different ways in explants cultured opposite three different sources of netrin-1: ventral HMJ explants, spinal cord floor plate explants, or COS cells secreting netrin-1. The axons displayed a wide range of behaviors even within a single explant, though all behaviors were observed in all three types of coculture, and all of these behaviors resulted in the failure of a dorsal bundle to form (each type of behavior will be illustrated here for only one of the coculture conditions). In some cocultures, numerous F84.1+ axons grew dorsally to the edge and exited the explant, and

then turned to project ventrally within the collagen, often hugging the explant (illustrated for a coculture with COS cells secreting netrin-1: Figures 2-5E and 2-5F) and sometimes lifting off as they approached the endogenous floor plate in the bottom explant (illustrated for a coculture with a ventral HMJ explant: Figures 2-5G and 2-5H). In other explants, thin fascicles of F84.1+ axons continued to grow dorsally after exiting the explant, but were mostly deflected away from the top explant as they progressed dorsally (illustrated for a coculture with a spinal cord floor plate explant: Figures 2-5I and 2-5J). In cases where a short dorsal bundle was observed in explants cultured opposite netrin-1-secreting cells (see Figure 2-4D), F84.1 staining suggested that the axons had stalled (Figures 2-5K and 2-5L). Finally, in some cocultures the axons from the bottom explant appeared to have grown to the edge of the explant and stopped there, as no axons were seen growing into the collagen or over the edge (data not shown). Thus, the apparent absence (as viewed in dark field optics (Figures 2-3 and 2-4)) of a dorsal bundle projecting from explants cultured opposite floor plate cells or COS cells secreting netrin-1, seems to reflect both an inhibition of axon outgrowth into the collagen gel and a redirection away from the top explant of those axons that do exit.

Discussion

Developing axons are guided by diffusible chemoattractants and chemorepellents, but the identity of these long-range guidance cues is largely unknown. Netrin-1 is a long-range chemoattractant expressed by floor plate cells which is thought to guide commissural axons along a ventrally-directed circumferential trajectory. The finding that netrin-1 is a vertebrate homolog of UNC-6, which is involved in guiding both dorsally-directed and ventrally-directed circumferential migrations, led us to examine the involvement of netrin-1 in directing dorsal migrations away from the floor plate. Our results indicate that the floor plate and netrin-1 repel trochlear motor axons at a distance. Thus, netrin-1 appears to be a bifunctional long-range guidance cue, attracting some ventrally-directed axons, while steering some dorsally-directed axons away. These results indicate a striking conservation in the function of UNC-6/netrin family members despite the 600 million years of evolution that separate present day nematodes from chordates.

Guidance of trochlear motor axons by the floor plate and netrin-1

We have shown that the floor plate suppresses the formation of a bundle of trochlear axons that normally projects from the dorsal aspect of ventral HMJ explants, and that this involves a redirection of the axons away from the source, i.e. a repulsion. Netrin-1 may likely mediate this effect either partly or entirely, since *netrin-1* is expressed by floor plate cells in the midbrain and hindbrain (Kennedy et al., 1994), and since COS cells secreting netrin-1 mimic the effect of floor plate cells. In our experiments, COS cells were slightly less effective than floor plate cells (Figure 2-4C). It is possible that floor plate cells secrete additional factors that contribute to its repulsive effect (e.g. Netrin Synergizing Activity (Serafini et al., 1994)). Alternatively, the transfected COS cells used here may simply secrete less netrin-1 than do floor plate cells.

Our experiments have not resolved how netrin-1 exerts its long-range repellent effects. Repulsion could result from a concentration-dependent inhibition of axon

extension; when encountering a threshold concentration of netrin-1, the axons may reorient growth at random and eventually extend away from the source. Alternatively, the axons may be capable of detecting a gradient of netrin-1 and of turning to grow down gradient. One consideration that favors a gradient detection mechanism is that in our experiments the developing axons were actually confronted with two sources of netrin-1: an endogenous floor plate within the explant, located 50-150 μm from the axons as they emerged from the cell bodies, and an exogenous floor plate or aggregate of transfected COS cells that could exert a repulsive effect on the axons even when positioned over 400 μm away from the dorsal edge of the explant. Thus, trochlear motor axons have no difficulty extending in the vicinity of a source of netrin-1 provided they are growing away from it, but they are inhibited from extending towards a source, even a relatively distant one. The simplest interpretation of this result is that the axons can grow down a gradient of netrin-1 but are inhibited from growing up gradient.

A gradient-detection mechanism has been shown to underly the responses of temporal retinal axons to a membrane-associated repellent in posterior chick tectum (Baier and Bonhoeffer, 1992). When growing up gradients of this repellent, these axons showed a wide range of behaviors: under conditions where they were permitted to turn around, many different angles of deflection were observed and only a few axons reversed their trajectory entirely, but when constrained to grow up gradient in a narrow corridor, the axons stalled (Baier and Bonhoeffer, 1992). This range of behaviors is reminiscent of that observed for trochlear motor axons growing towards a source of netrin-1 (Figure 2-5); the fact that a wide variety of behaviors was observed in our experiments may presumably be ascribed to the confusion wrought by the simultaneous operation of the repulsive force of the exogenous source of netrin-1 and of other forces within the neuroepithelium that tend to make the axons grow dorsally (see below).

The precise role of netrin-1 in the guidance of trochlear motor axons *in vivo* may depend on the mechanism of its repulsion. If netrin-1 functions through a concentration-

dependent inhibitory mechanism, then *in vivo* it may simply create an inhibitory barrier that prevents trochlear motor axons from ever approaching the ventral midline; its main role might then be to redirect the few axons that initially project in a ventral direction. However, if netrin-1 functions through a gradient detection mechanism, it could participate more directly in guiding all trochlear motor axons *in vivo* by being present in a decreasing ventral-to-dorsal gradient that instructs the axons to grow dorsally. Even in this case, however, it is unlikely that netrin-1 is the sole cue guiding the axons. For example, the action of netrin-1 cannot easily explain why the axons are confined to a narrow corridor and do not fan out in a rostral or caudal direction as they grow dorsally. The axons are presumably channeled by permissive cues that mark out the corridor and/or by inhibitory cues that surround it. In addition, while our experiments show that attractive cues from more dorsal structures are not required for the initial dorsal guidance of trochlear motor axons, it is possible that such cues guide the axons as they start to approach the dorsal midline (see Chapter 4).

Guidance of other motor axons by the floor plate

The axons in dorsal bundles that were repelled in our experiments were identified as trochlear motor axons by the location of their cell bodies and by their expression of F84.1. Our experiments do not, however, address whether axons other than trochlear motor axons – such as the F84.1+ oculomotor axons in some of our explants – are also repelled. The floor plate has, in fact, recently been reported to repel other motor axons in the hindbrain and spinal cord (S. Guthrie and A. Pini, personal communication), and as yet unidentified axons from mesencephalic alar plate and basal plate (A. Tamada, R. Shirasaki and F. Murakami, personal communication). Thus, it is conceivable that the floor plate has a generalized repulsive effect on all axons that normally grow away from the floor plate, in particular motor axons. Whether these repulsions are mediated by similar molecular mechanisms is unknown. In addition, this generalized repulsion on its own cannot explain

the observation that, although no motor axons project towards the floor plate, different classes of motor axons do have distinct trajectories: dorsally- or ventrolaterally-directed (see references cited in the Introduction, and Puelles and Privat, 1977; Altman and Bayer, 1984; Lumsden and Keynes, 1989). The axons of trochlear motor (nerve IV) and branchiomotor (nerves V, VII and IX) neurons all project along a dorsal trajectory (though branchiomotor axons exit before growing all the way to the dorsal midline). In contrast, the other cranial motor axons (nerves III, VI and XII) and spinal motor axons extend ventrolaterally to exit the neural tube close to the cell bodies of origin. Floor plate repulsion cannot on its own easily account for ventrolateral projections. One possibility is that dorsal portions of the neural tube secrete another repellent that selectively prevents the ventrolaterally-directed axons from projecting dorsally, without effect on trochlear motor or branchiomotor axons.

Molecular basis of the distinct axonal responses to netrin-1

Our experiments do not make it possible to distinguish whether the attractive and repulsive effects of netrin-1 are mediated by a single type of receptor coupled to distinct transduction mechanisms, or by distinct types of receptors. In C. elegans, studies of the *unc-5* gene have suggested that distinct receptors direct dorsal and ventral migrations involving UNC-6. Mutations in *unc-5*, which encodes a putative transmembrane protein (Leung-Hagesteijn et al., 1992), disrupt circumferential migrations in the dorsal direction without effect on ventral migrations (Hedgecock et al., 1990). Ectopic expression of *unc-5* in neurons that normally project ventrally or longitudinally redirects their axons dorsally in an *unc-6*-dependent manner (Hamelin et al., 1993). Together, these results strongly suggest that UNC-5 is the receptor (or a component of the receptor) that mediates dorsal migrations, and that a distinct receptor mediates ventral migrations. By analogy to the nematode, distinct receptors may also mediate the attractive and repulsive actions of netrin-1.

Chemorepulsion and the bifunctionality of axon guidance cues

Whereas axons were postulated to be guided by chemoattractants over a century ago (Ramon y Cajal, 1892), it was only recently appreciated that axons can also be guided by diffusible chemorepellents (Pini, 1993; Fitzgerald et al., 1993). Netrins are the only chemoattractants for developing axons so far identified; our results have now also identified netrin-1 as a diffusible chemorepellent. In addition to netrin-1, collapsin/Sema III, a soluble protein that can cause collapse of sensory growth cones (Luo et al., 1993), has recently been shown to function as a diffusible chemorepellent (Messersmith et al., 1995), and Sema II has been been found to function as a diffusible inhibitor of terminal arborization (Matthes et al., 1995).

The demonstration of dual effects of netrin-1 extends to diffusible factors the previous observations made on dual effects of non-diffusible cell surface and extracellular matrix molecules. The ECM molecule tenascin promotes the outgrowth of spinal motor axons (Wehrle and Chiquet, 1990), but it provides an unfavorable substrate for a variety of CNS axons (Faissner and Kruse, 1990). Myelin-associated glycoprotein (MAG), a transmembrane member of the immunoglobulin gene superfamily, has been shown to promote the extension of some axons but to inhibit the growth of others (Mukhopadhyay et al., 1994; McKerracher et al., 1994). In Drosophila, the cell-surface protein connectin, which is expressed on a subset of muscle cells, appears to have dual actions, repelling motor axons that do not normally innervate them (Nose et al., 1994), and promoting innervation by the appropriate motor neurons (A. Nose, personal communication). These studies suggest that axon guidance cues may quite generally be both attractive and repulsive, and that some guidance cues are best thought of simply as signposts, bearing directional information that can steer axons in different directions depending on the interpretive machinery in the growth cone.

Materials and Methods

Explant Cultures

E11 rat embryos (E0 = day of vaginal plug) were dissected in L15 medium (Gibco) with 5% heat-inactivated horse serum after incubation in a 1:1 mixture of STV (saline, 0.25% trypsin, 0.02% versene) and 10X Pancreatin (Gibco) on ice for 20 min. Explants were embedded in collagen gels as described (Tessier-Lavigne et al., 1988) and cultured in a 75:25 mixture of OptiMEM and F12 medium (Gibco) supplemented with Glutamax (Gibco), 1% penicillin/streptomycin, 40mM glucose, and 5% fetal calf serum. In cocultures, explants were separated by 100 - 450 μm. We did not systematically test repulsive activity for larger separations.

COS Cell Transfections

COS cells were transfected with a netrin-1 expression construct, and aggregates of transfected or mock-transfected cells were prepared by the hanging-drop method as described (Kennedy et al., 1994). In all experiments, the secretion of netrin-1 by cell aggregates was monitored by testing the ability of the cells to evoke robust commissural axon outgrowth from E11 dorsal spinal cord explants (Kennedy et al., 1994).

Immunostaining and DiI labelling

After fixation with 4% paraformaldehyde in PBS, immunostaining was carried out with F84.1 supernatant (Prince et al., 1992; 1:20) or an anti-neurofilament antibody (NF-M; Lee et al., 1987; 1:5000), and an HRP-conjugated secondary antibody (Boehringer-Mannheim; 1:500) in PHT (PBS, 1% heat-inactivated normal goat serum, 1% Triton X-100). Staining of explants in collagen gels was as described previously (Kennedy et al., 1994). For sections, the HMJ of an E13 embryo was fixed, dissected and stained in whole-mount prior to cutting 50μm vibratome sections. Axons were labelled retrogradely with DiI in

fixed tissue (Godement et al., 1987; Honig and Hume, 1989) by placing small crystals either into the mesenchyme posterior to the eye (in intact E13 embryos), or in contact with the dorsal bundle of axons projecting from complete HMJ or ventral HMJ explants cultured for 48 or 40 hr, respectively, and allowing the dye to diffuse through the axons for two to seven days at 37°C.

Quantification of Inhibitory Effects

To quantify the presence and length of dorsal bundles of trochlear motor axons (Figures 2-3, 2-4C and 2-4D), a dorsal bundle was defined as a loose cluster of axons, over 30 μm wide, projecting from the dorsal aspect of the ventral HMJ explant. All quantification was performed prior to immunolabelling of the explants.

Acknowledgements

We thank C.M. Fan, M. Galko, L. Hinck, G. Martin, C. Mirzayan and T. Serafini for critical reading of this manuscript, N. Martin, G. Martin, C. Murphy and L. Bauer for assistance with figures, C. Mirzayan for helping with COS cell transfections, W. Stallcup and V. Lee for the generous gifts of F84.1 supernatant and anti-NF-M, and B. Fritzsch for many enjoyable and helpful discussions on the trochlear nucleus. Many thanks to A. Pini and S. Guthrie, to A. Tamada, R. Shirasaki and F. Murakami, and to D. Matthes, H. Sink, A. Kolodkin and C. Goodman for sharing their results prior to publication. This work was supported by the Lucille P. Markey Charitable Trust. S.C. is a Howard Hughes Medical Institute Predoctoral Fellow. M. T.-L. is an Assistant Investigator of the Howard Hughes Medical Institute.

Figure 2-1. Trajectory of Trochlear Motor Axons *in vivo* and *in vitro*.

(A) Diagram illustrating this trajectory in the E11-E12 rat. The cell bodies of trochlear motoneurons (red circle) are located in the ventral neural tube at the junction between hindbrain (Hb) and midbrain (Mb) (approximate level of arrowheads). Their axons project dorsally within the neural tube to the dorsal midline (dashed red line), then exit the neural tube and project in the periphery (full red line) to their target, the superior oblique muscle of the eye (blue), by E13. Arrowheads also indicate the plane of section in (B).

(B) Cross section of the hindbrain-midbrain junction (HMJ) at E13 showing the trajectory of trochlear motor axons (visualized by F84.1 immunolabelling) from their cell bodies in the trochlear nucleus (IV) to the exit point at the roof plate (rp) (or anterior medullary velum). F84.1 also labels the basal aspect of floor plate cells (fp). Additional abbreviation: D, dorsal; V, ventral.

(C and D) Trajectories of trochlear motor axons, visualized by retrograde labelling with DiI, in the E13 rat brain (C) and in explants of the entire E11 HMJ after 48 hr in culture in collagen gels (D) (sagittal views; the explants were ~ 400 - 700 μm long and sometimes included neurons of the neighboring oculomotor nucleus). The trajectory taken by axons during the 48 hr culture period (D) is similar to that of trochlear axons *in vivo* (C) (only one side of the neural tube is shown in (C), whereas both sides are included in (D), explaining the more dense appearance of the nucleus). In some cultured E11 HMJ explants, a few of the labelled axons originated in the neighboring IIIrd nerve nucleus and had grown through the explant or around its edge (data not shown; most IIIrd nerve axons that exited the explant did so near their nucleus). Note that, although trochlear motoneurons differentiate on schedule and extend axons along a normal trajectory *in vitro*, the neural tube tissue does not increase in size to the same extent in culture as *in vivo* (compare scale bars in (C) and (D)).

Scale bars: (B), 90 μm; (C), 115 μm; (D), 40 μm.

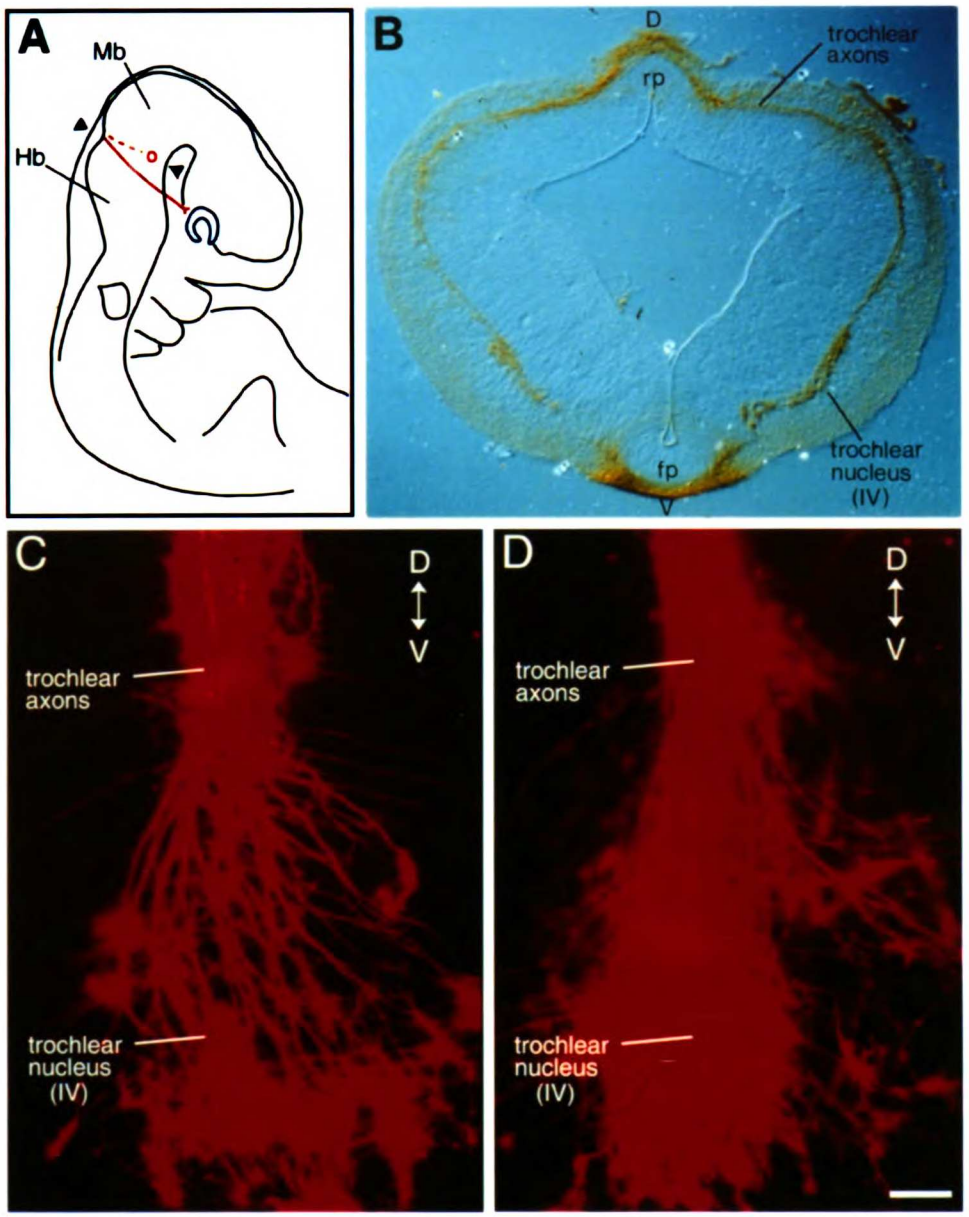

A
Mb
Hb
B
D
rp
trochlear
axons
fp
V
trochlear
nucleus
(IV)
C
D
V
trochlear
axons
trochlear
nucleus
(IV)
D
D
V
trochlear
axons
trochlear
nucleus
(IV)

Figure 2-1

Figure 2-2. Trochlear Motor Axons are Guided Along a Dorsal Trajectory in Ventral Hindbrain-Midbrain Junction (HMJ) Explants in Culture.

(A) Diagram illustrating the dissection of ventral HMJ explants which contain a floor plate (fp) and differentiating motoneurons in the trochlear nucleus (IV) (dotted line indicates what will be the dorsal-most edge of the explant). In this and all subsequent panels, explants are oriented with dorsal to the top and ventral to the bottom.

(B) A ventral HMJ explant (side view) cultured for 40 hr and visualized with dark-field optics to show the projection of a characteristic bundle of axons from the cut dorsal edge of the explant ("dorsal bundle", arrow). Arrowhead indicates some of the other axons or thin axon bundles that project from the explant.

(C) A ventral HMJ explant cultured for 40 hr as in (B) and stained for expression of F84.1, showing that axons in the dorsal bundle (arrow) express F84.1. A thin F84.1+ bundle (arrowhead) also originates in the trochlear nucleus (IV) but travels separately. Note expression of F84.1 by cells in the trochlear nucleus, the caudal portion of the oculomotor nucleus (III) which was included in the explant, and floor plate cells (fp). Although not visible in this micrograph, F84.1+ axons from the oculomotor nucleus exited the explant near the nucleus.

(D) High power view of F84.1+ neurons in the trochlear nucleus in a ventral HMJ explant cultured for 40 hr, showing that the axons of some of these cells initially grow ventrally but subsequently turn (arrows) to project dorsally.

Scale bars: (B), 120 μm; (C), 90 μm; (D), 25 μm.

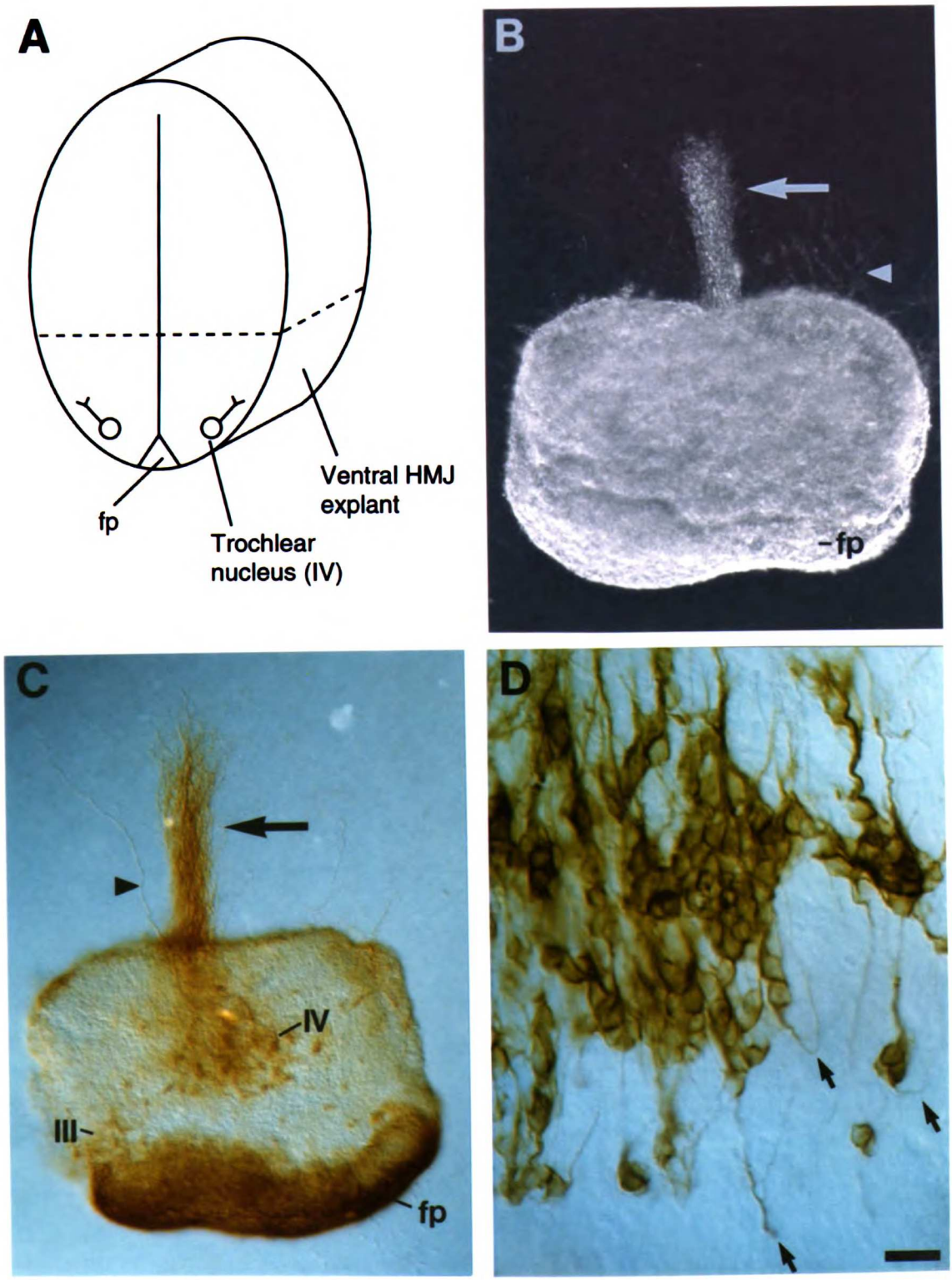

A
Ventral HMJ
explant
fp
Trochlear
nucleus (IV)
B
fp
C
IV
III
fp
D

Figure 2-2

Figure 2-3. The Floor Plate Suppresses the Formation of a Dorsal Bundle of Trochlear Axons in Ventral HMJ Explants at a Distance *in vitro*.

(A) Diagram illustrating the coculture paradigm: a ventral HMJ explant (bottom) is cultured for 40 hr in a collagen gel with its cut dorsal edge opposite a test explant (top; in this case, another ventral HMJ explant). Schematic drawing of neurons projecting dorsally indicates the approximate location of the trochlear nucleus (IV) in each explant. fp, floor plate.

(B-D) Explants cultured opposite the floor plate edge of a ventral HMJ explant (B) or opposite a spinal cord floor plate explant (D) failed to develop a dorsal bundle of trochlear axons, whereas explants cultured opposite a dorsal spinal cord explant (C) developed a dorsal bundle (arrow). Note in (B) that the top explant serves as an internal control and has a dorsal bundle (arrow); the mean length of the bundles from top explants was not significantly different from those projecting from explants grown alone (data not shown). d, dorsal spinal cord; s-fp, spinal cord floor plate.

Scale bars: (B - D), 190 μm.

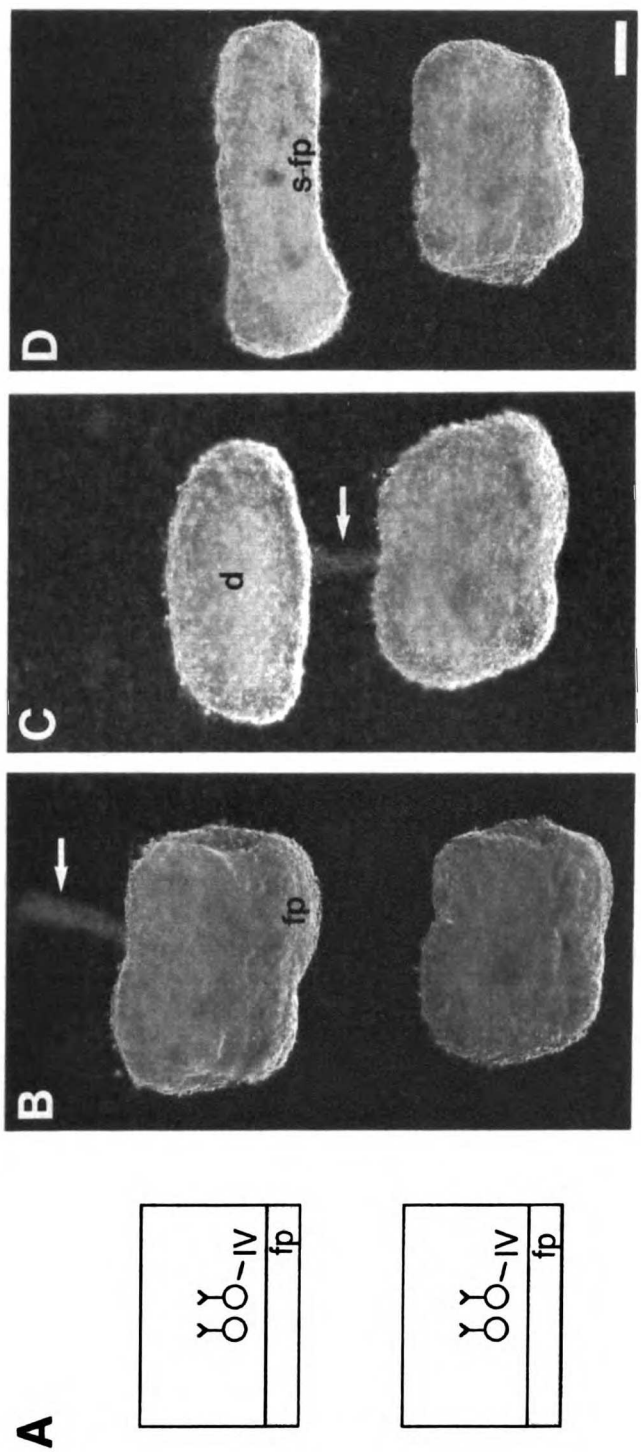


Figure 2-3

Figure 2-4. COS Cells Secreting Netrin-1 Suppress the Formation of a Dorsal Bundle of Trochlear Axons in Ventral HMJ Explants.

(A, B) COS cells secreting netrin-1 (B), but not control COS cells (A), suppress the formation of a dorsal bundle of trochlear motor axons (arrow in (A)) from ventral HMJ explants cultured for 40 hr in collagen gels (ventral HMJ explants are oriented as in Figure 2-3A). c, control COS cells; n, netrin-1-secreting COS cells.

(C) Fraction of ventral HMJ explants with dorsal bundles when grown alone ("alone"), in the presence of control COS cells ("control COS"), or of netrin-1-secreting cells ("netrin-1 COS"). Values shown are means ± S.E.M. for three experiments (at least five explants per condition in each experiment).

(D) Mean length (± S.E.) of the dorsal bundles observed projecting from ventral HMJ explants cultured alone (n = 19), with control COS cells (n = 19), or with netrin-1 expressing COS cells (n = 6) (values were from explants with bundles in the three experiments shown in (C) and were pooled). Note that the bundles that projected from explants cultured with control COS cells often grew into the cells. This explains why the lengths of these bundles were, on average, shorter than those in explants cultured alone (the length of the bundle was measured to the point of contact with the cells). If we consider only those cocultures where the control COS cells were more than 300 μm from the explants (i.e. a distance equal to the average length of bundles in explants cultured alone), we find that the lengths of bundles with control COS cells were not significantly different from the lengths of bundles in explants cultured without COS cells ($p > 0.1$, t test). Abbreviations as in (C).

Scale bars: (A, B), 190 μm.

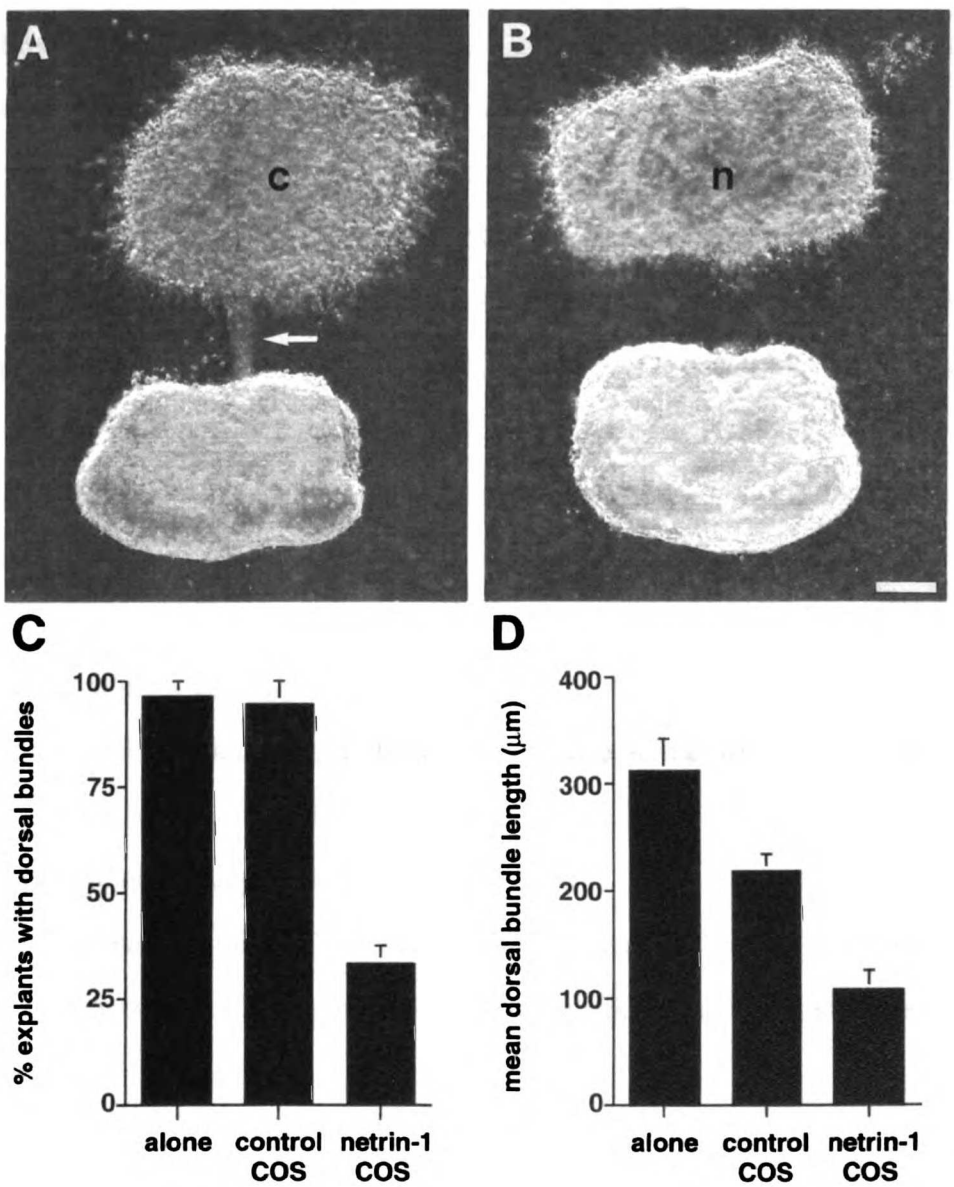

A
c
B
n
C
% explants with dorsal bundles
100
75
50
25
0
alone
control COS
netrin-1 COS
D
mean dorsal bundle length (μm)
400
300
200
100
0
alone
control COS
netrin-1 COS

Figure 2-4

Figure 2-5. Inhibition and Repulsion of Trochlear Motor Axons by the Floor Plate and Netrin-1-secreting COS Cells.

Side views (A, C, E, G, I, K) and transverse views (B, D, F, H, J, L) of ventral HMJ explants (bottom explant in each panel, oriented as in Figure 2-3A) stained for expression of F84.1 after 40 hr of culture in collagen gels opposite the following tissues (top explant in each panel): a dorsal spinal cord explant (A, B); control COS cells (C, D), another ventral HMJ explant (G, H); a spinal cord floor plate explant (I, J); or COS cells secreting netrin-1 ((E, F) and (K, L)). Each of the bottom panels shows a transverse view of the culture that is viewed from the side in the corresponding top panel. In explants cultured opposite a source of netrin-1 (E - L), the absence of a dorsal bundle is associated with a variety of different behaviors of F84.1+ axons. In (E, F) and (G, H), axons grow ventrally in the collagen, hugging the explants (arrowhead in (F)); in (H) the axons lift off the explant (arrowhead). In (J), some axons continue to grow dorsally but are deflected away from the floor plate (arrowheads point to two such fascicles). In (K, L), the axons continue to grow dorsally but are foreshortened. Note that in some cases ((C, D) and (K, L)) the bundles projecting from the trochlear nuclei in the two leaves of the explant did not merge into a single bundle; this was observed in a small proportion of cocultures irrespective of the target tissue.

Abbreviations: c, control COS cells; d, dorsal spinal cord explant; fp, floor plate in ventral HMJ explants; n, netrin-1-secreting COS cells; s-fp, spinal cord floor plate.

Scale bars: 160 μm.

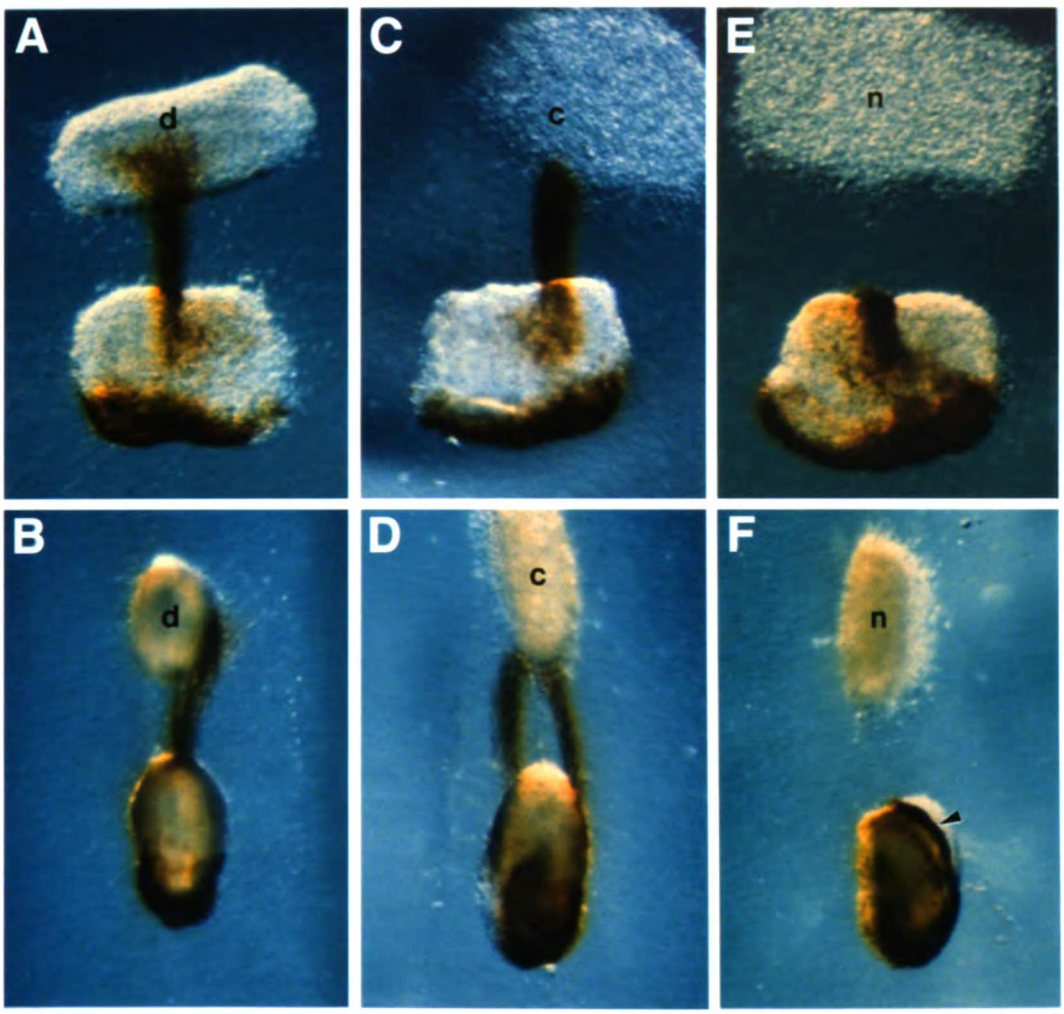


Figure 2-5

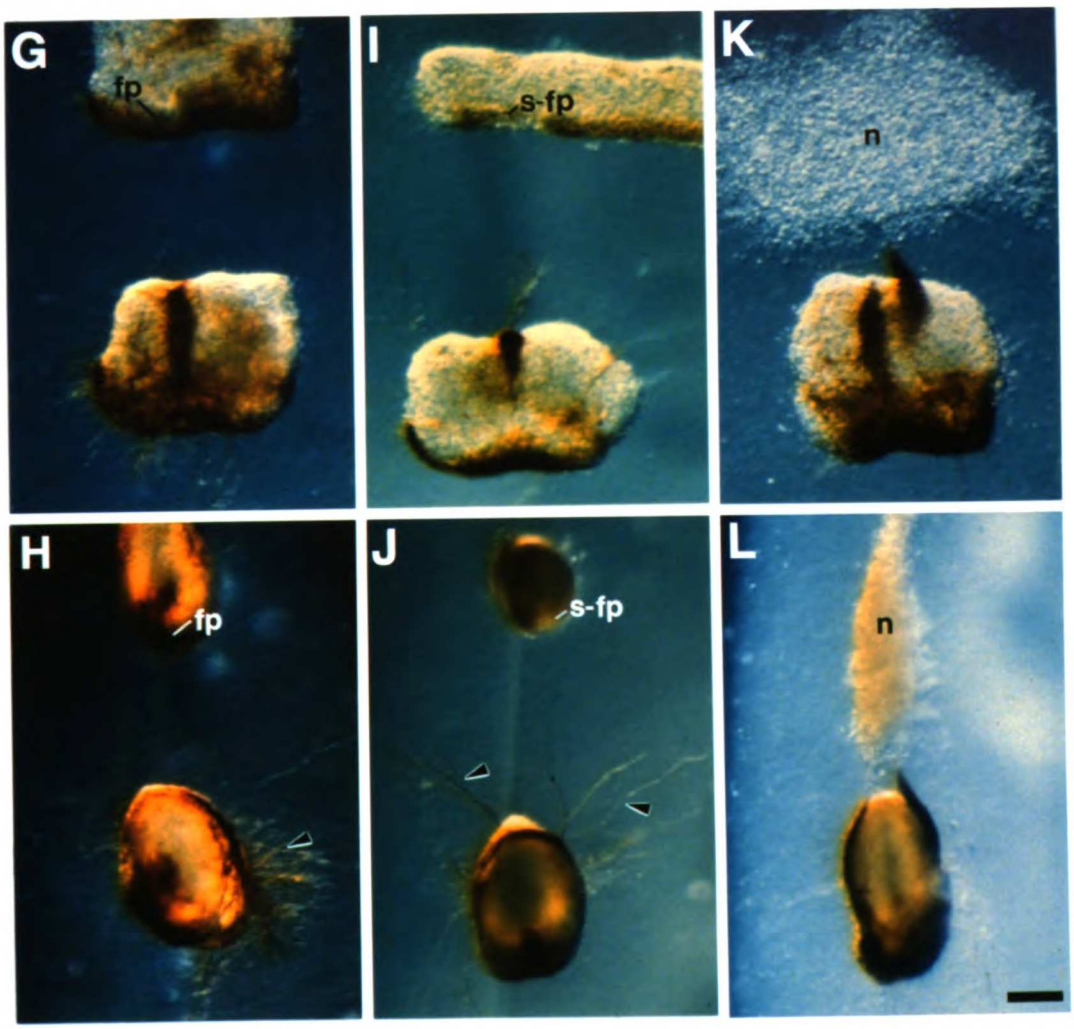


Figure 2-5

CHAPTER 3:

Netrin-1 is Not Required In Vivo for the Dorsal Projection of Trochlear Motor Axons

Abstract

In vertebrates, commissural axons pioneer a circumferential pathway to the floor plate at the ventral midline of the developing spinal cord, and trochlear motor axons extend dorsally away from the floor plate in the hindbrain. Netrin-1, a diffusible protein made by floor plate cells, can attract the growth of commissural axons *in vitro*. Mice deficient in netrin-1 function exhibit severe defects in commissural axon projections that are consistent with netrin-1 guiding these axons to the midline *in vivo*. Netrin-1 can also repel the growth of trochlear motor axons *in vitro*, but its role in directing the migration of these axons *in vivo* is unknown. An analysis of the trochlear nuclei from the netrin-1 mutant mice revealed that major defects are not observed in trochlear motor axon projections, predicting the existence of additional cues that guide these axons. Evidence is provided that one of these cues is a chemorepellent produced by floor plate cells which is distinct from netrin-1.

Introduction

Embryological experiments in both vertebrates and invertebrates have provided evidence that developing axons are guided to their targets in the nervous system by the combined actions of attractive and repulsive guidance cues, but the identity of these cues and their precise contributions to axon guidance are only now being elucidated (reviewed in Goodman, 1996). One family of putative guidance cues for developing axons are the netrins, a family of large (~ 70-80 kD) soluble proteins which show homology in their amino termini to portions of the extracellular matrix molecule laminin, and which have been implicated in axon guidance through complementary lines of evidence in worms, flies and vertebrates (reviewed in Culotti and Kolodkin, 1996).

In the nematode *C. elegans*, the netrin family member UNC-6 was implicated in axon guidance through loss-of-function studies that demonstrated that UNC-6 is required for accurate circumferential migrations of axons in both a dorsal and a ventral direction (Hedgecock et al., 1990; Ishii et al., 1992). The finding that the UNC-6 gene product is concentrated in the ventral portion of the nematode during the period of axon guidance has suggested a model in which UNC-6 functions to attract ventrally-directed axons and to repel dorsally-directed axons (Wadsworth et al., 1996). Although a direct guidance role for UNC-6 is supported by the loss-of-function phenotype of *unc-6* mutants, it has not yet been excluded that UNC-6 participates less directly in guidance by playing a permissive rather than an instructive role (discussed in Ishii et al., 1992). In addition, the fact that a substantial fraction of circumferential migrations are normal even in homozygous null mutants for *unc-6* suggests the existence of other factors that work with UNC-6 to direct these migrations (Hedgecock et al., 1990).

Netrin proteins have been implicated in directing circumferential axonal migrations in vertebrates as well. Commissural axons pioneer a circumferential trajectory to the floor plate at the ventral midline of the neural tube during embryogenesis (reviewed in Colamarino and Tessier-Lavigne, 1995b). Floor plate cells secrete a diffusible factor

that can promote the outgrowth of commissural axons from explants of dorsal spinal cord into collagen matrices *in vitro* and reorient these axons within the neuroepithelium (Tessier-Lavigne et al., 1988; Placzek et al., 1990). Two netrin proteins were purified from embryonic chick brain on the basis of their ability to mimic the outgrowth-promoting effect of floor plate cells. Recombinant versions of these proteins are capable of reorienting commissural axon growth *in vitro* (Serafini et al., 1994; Kennedy et al., 1994), and one of them, *netrin-1* is expressed by floor plate cells (Kennedy et al., 1994). These results have suggested a model in which a gradient of netrin protein contributes to directing the growth of commissural axons towards the ventral midline (Kennedy et al., 1994). Further evidence has suggested that this mechanism operates for commissural axons in the hindbrain as well (Shirasaki et al., 1995).

In addition to playing an attractive role, there is also evidence that netrin-1 may have a repulsive role. The floor plate and netrin-1 were found to be capable of repelling *in vitro* a group of axons, trochlear motor axons, that grow away from the floor plate *in vivo* (Colamarino and Tessier-Lavigne, 1995a), suggesting that a gradient of netrin protein may contribute to directing the growth of some axons away from the ventral midline as well. These studies did not, however, determine whether and how netrin proteins contribute to guiding these different classes of axons during vertebrate development *in vivo*. Does the ability of netrin-1 to attract commissural axons *in vitro* actually reflect a role for netrin-1 in attracting these axons to the ventral midline *in vivo*? Is its ability to repel trochlear motor axons important for setting the trajectory of those axons?

To begin to address these questions, we have taken advantage of the results of a novel gene-trapping study (Skarnes et al., 1995) which has provided the means to isolate a loss-of-function allele of the murine *netrin-1* gene (Serafini et al., 1996). To generate the mouse, a β-galactosidase encoding gene trap vector was used in embryonic stem cells to selectively recover mutations in genes encoding proteins with signal sequences

(Skarnes et al., 1995). It is worth noting that the mutant allele generated is likely to be a severe hypomorph rather than a complete null because very low levels of wildtype transcript (presumably produced by splicing over the inserted sequences) are detected in mice homozygous for the mutation.

Homozygous mice are born but die within a week after birth (Serafini et al., 1996). Immunohistochemical examination of the developing spinal cords of mutant embryos during the period of commissural axon outgrowth (E10.5-E11.5) demonstrated that, although the overall morphology of the spinal cord was relatively normal, the trajectories of commissural axons displayed profound disturbances. Instead of growing in a directed manner toward the floor plate as in wildtype embryos, the commissural axons projected in many abberant directions, with only a few that successfully invaded the ventral spinal cord, and even fewer that reached the floor plate (Serafini et al., 1996). The misrouting of spinal commissural axons observed in homozygous mutant embryos thus strongly supports the guidance role postulated for netrin-1. In addition to the defects in axonal projections in the spinal cord, serial sections of brains from late gestation mice (E18) revealed that the mutant animals also display multiple selective defects in brain development, including a complete absence of the corpus callosum, the hippocampal commissure, and the anterior commissure (Serafini et al., 1996). The defects did not reflect a generalized defect in brain commissure formation, since other commissures were unaffected. Although, it is not known at present whether the defects reflect a direct role for netrin-1 in guiding any of the affected commissural axons, the distribution of netrin-1 mRNA along the paths of the affected axons and at the points where they cross the midline is at least suggestive of a role for netrin-1 in guiding these axons.

Netrin-1 also effects the growth of trochlear motor axons *in vitro*, and is expressed in the appropriate place and time (Kennedy et al., 1994; and data within) to be involved in their development *in vivo*. We therefore examined whether the severe reduction of netrin-1 protein in these mutant mice results in any disturbances in the trajectories of

trochlear motor axons. We found that the axons still grew dorsally as in wild type, implying that other cues exist to steer trochlear motor axons away from the ventral midline *in vivo*. With the use of *in vitro* cultures and netrin-1 function-blocking antibodies, we show that one other cue may be a second floor plate-derived chemorepellent which is distinct from netrin-1.

Results

Mouse *netrin-1* is expressed at the level of the developing trochlear nucleus

Chick netrin-1 is known to be expressed along the entire length of the floor plate, which extends from the most caudal spinal cord to the ventral diencephalon (Kennedy et al., 1994; reviewed in Colamarino and Tessier-Lavigne, 1995b). To verify that in mouse netrin-1 is indeed also expressed in the rostral hindbrain at the level of the trochlear nucleus, we took advantage of the fact that the translation product of the mutant *netrin-1* gene includes a cytoplasmic β-galactosidase reporter construct. Antibodies against β-galactosidase were used to perform wholemount immunostaining of the hindbrain-midbrain junction of heterozygous and mutant embryos throughout the ages E9-E10.5, the period during which mouse trochlear motoneurons first differentiate and extend axons along a dorsal trajectory away from the floor plate. The resultant staining was punctate in nature, consistent with the expected localization of the fusion protein to a cytoplasmic compartment (Skarnes et al., 1995). A wide band of *netrin-1* expressing cells was found along the ventral midline of the entire hindbrain-midbrain junction, confirming that in mouse *netrin-1* is expressed in the floor plate at the level of the developing trochlear nucleus (Figure 3-1). Like many other floor plate markers (Sasaki and Hogan, 1993; Echelard et al., 1993; Krauss et al., 1993), however, the domain of strong *netrin-1* expression at this axial level appears to expand dorsolaterally into the ventral cord, beyond the confines of the floor plate proper. Much fainter labelling was detected in cells in even more dorsal regions, such that *netrin-1* expression was seen throughout almost the entire ventral half of the neural tube (Figure 3-1A). Notably, at what appears to correspond precisely to the level of the rhombic isthmus, which is where the trochlear motor axons exit the neural tube, there is a sharp gap in the labelling of ventral neural tube cells (Figures 3-1B and 3-1C), creating a small corridor of non-netrin-1 expressing neuroepithelium bounded medially by *netrin-1* expression in the floor plate, and caudally

and rostrally by *netrin-1* expression in the basal plate of the hindbrain and midbrain, respectively.

Trochlear motor axon trajectories are largely normal in mice deficient for netrin-1

Netrin-1 functions as a chemorepellent for trochlear motor axons *in vitro* (Colamarino and Tessier-Lavigne, 1995a). To determine if this *in vitro* effect is also relevant *in vivo*, we examined the trajectory of these axons in the *netrin-1* mutant embyros during their early development. Axons were visualized in embryo wholemounts by labelling with antibodies to neurofilament (NF-M, Lee et al., 1987), which label both trochlear motor axons as well as other axons that project longitudinally in the hindbrain (Figure 3-2). It was not possible to use the more selective marker of trochlear motor axons, F84.1 (Prince et al., 1992), that was used in our previous study on rat tissue, as it does not label axons in the mouse.

Trochlear motor axon trajectories were apparently largely unaffected by loss of *netrin-1* function, throughout the period E9.5 - E11.5 (Figure 3-2 and data not shown). However, a minor, but consistent defect, was the presence of a larger number of cell bodies of trochlear motoneurons in the floor plate region of the mutant embryos compared to wildtype or heterozygous littermates (arrows in Figure 3-2). In addition, axons extending from these cells appeared often to have wandered ventrally before reorienting their growth to project dorsally. While these results suggest that netrin-1 might play a role in the placement of the cell bodies of these neurons or in the initial extension of their axons, netrin-1 does not appear to be required for the dorsally-directed guidance of these axons.

Mutant floor plates retain the ability to repel trochlear motor axons *in vitro*

The observation that trochlear axon trajectories are largely normal in the homozygous mutants suggested that other guidance cues might function with netrin-1 to guide these

axons. One possibility we considered is that the floor plate might express additional diffusible guidance molecules for these axons. Although previous studies have shown that both floor plate cells and netrin-1 possess chemorepellent activity *in vitro*, these studies did not determine whether netrin-1 accounts for all of the repellent activity produced by floor plate cells.

To address this issue, we examined whether floor plate cells from mutant embryos possessed the *in vitro* activity of wild-type floor plate cells, that is, the ability to repel the growth of trochlear motor axons extending into a collagen matrix from explants of rat ventral hindbrain-midbrain junction (HMJ) (Colamarino and Tessier-Lavigne, 1995a). As compared to floor plates from wildtype or heterozygous embryos, mutant spinal cord floor plate explants were as effective in repelling the growth of trochlear motor axons at a distance (Figure 3-3). These results suggest that floor plate cells also secrete a repellent activity for trochlear motor axons which is distinct from netrin-1. This result was not unexpected given the largely normal trajectory of these axons in the mutant.

Wild-type floor plate can repel trochlear motor axons in the presence of netrin-1 function-blocking antibodies

While these experiments suggest the existence of other chemorepellents in addition to netrin-1, it remains a formal possibility that the ability of homozygous mutant floor plates to repel trochlear axons *in vitro* is due to the small amount of netrin-1 being produced by mutant floor plate cells (Serafini et al., 1996). We therefore sought an independent method of determining whether the floor plate produces a chemorepellent for these axons which is distinct from netrin-1.

To address this issue, we decided to use anti-netrin-1 function-blocking antibodies. Polyclonal antibodies against a truncated form of netrin-1 (Kennedy et al., manuscript in preparation) were screened for their ability to block a floor plate activity that is known to be completely netrin-1 dependent, namely the ability to block floor plate-

induced outgrowth from explants of E13 dorsal spinal cord. Indeed, although floor plate from the netrin-1 deficient mice retains its ability to repel trochlear motor axons, it suffers a near complete loss of the ability to provoke outgrowth from E13 rat dorsal spinal cord explants (Serafini et al., 1996), suggesting that perhaps all of the outgrowth-promoting capabilities of the floor plate can be accounted for by netrin-1. We therefore decided to use this assay as the basis for determining which of the polyclonal netrin-1 antibodies are function-blocking.

Explants of E13 rat dorsal spinal cord were co-cultured with floor plate explants in the presence of either anti-netrin antibodies, purified control IgG, or no antibody. At the concentration of 100μg/ml antibody, antibody 65II#2 completely blocked spinal cord floor plate-induced commissural axon outgrowth when added directly to the medium (Figure 3-4A). The results were dose-dependent, with a partial block observed at 50μg/ml (data not shown). Control IgG produced no effect (Figures 3-4B and 3-4C). These same results were obtained when E11 rat dorsal spinal cord was cultured with E11 spinal cord floor plate (data not shown). Finally, to assure that this antibody was sufficient to block the netrin-1 activity produced by the floor plate at the level of the trochlear nucleus, we confirmed that 100μg/ml can also block the outgrowth of E11 dorsal spinal cord explants when co-cultured with floor plate taken from the hindbrain-midbrain junction (Figures 3-4D - 3-4F). Most importantly, the antibody also blocked the repulsion of trochlear axons by netrin-1 secreting 293 cells (Shirasaki et al., submitted), proving that this antibody is capable of blocking both the sites required for outgrowth promotion and those required for chemorepulsion (Figures 3-4G - 3-4I).

To determine whether netrin-1 alone accounts for all of the chemorepellent activity for trochlear motor axons produced by the floor plate, we tested whether antibody 65II#2 could block the ability of wild-type floor plate to repel trochlear motor axons *in vitro*. Rat ventral HMJ explants were cultured in tandem, (described in Colamarino and Tessier-Lavigne, 1995; see Chapter 1), such that the floor plate edge of one ventral HMJ

explant (top) was placed opposite the dorsal-most cut edge of a second (test) ventral HMJ explant. In the absence of antibody, or in the presence of 100μg/ml control antibody, this resulted in a repulsion of trochlear axons from the test explant away from the floor plate of the top explant (Colamarino and Tessier-Lavigne, 1995a). Addition of 100μg/ml of the netrin-1 function-blocking antibody 65II#2 to this tandem assay did not affect the ability of the floor plate to repel the trochlear motor axons (Figure 3-5). Thus, titrating out the netrin-1 activity produced by the floor plate does not rid the floor plate of its repulsive capabilities, suggesting that at least one other chemorepellent for trochlear motor axons is being made by the floor plate cells. These results corroborate those derived from the *in vitro* study of floor plate taken from the *netrin-1* mutant mice.

Discussion

There is considerable evidence that axons can be guided *in vitro* by diffusible chemoattractants and chemorepellents, and in recent years progress has been made in identifying candidates for the molecules that mediate these effects. However, the *in vivo* functions of diffusible guidance cues, including netrin-1, have yet to be defined in vertebrates. The generation of an insertional mutation in the mouse *netrin-1* gene has made it possible to assess the function of netrin-1. Our findings demonstrate that although netrin-1 appears to guide many axons during development of the vertebrate central nervous system (Serafini et al., 1995), other cues must exist which collaborate with netrin-1 to effect accurate guidance, including other diffusible cues.

Expression of mouse *netrin-1* at the hindbrain-midbrain junction

Netrin-1 transcripts are detected in the ventral midline cells of the hindbrain-midbrain junction before, during, and after the development of the trochlear motor neuron projections (i.e. E9 - E10.5). This expression domain clearly includes the floor plate. However, transcripts are found in an area slightly larger than the prospective floor plate, such that the domain of high-expressing cells expands to cover the adjacent lateral regions of the neural tube. Intriguingly, this expansion is absent at the axial level of the rhombic isthmus, which is precisely where the trochlear motor axons funnel together before exiting the neural tube at its dorsal edge (Sinclair, 1958; Fritzsch and Northcutt, 1993; Chedotal et al., 1995). The cell bodies of trochlear motoneurons are spread out in the rostral hindbrain over a few hundred microns, which necessitates a mechanism to assure their axons will gather together to exit the neural tube as a single bundle. In vitro, trochlear motor axons can bundle together in explants of isolated ventral HMJ tissue, demonstrating that cues from their exit point are not required for their convergence. There is some evidence that "channeling" cues are present in the neuroepithelium through which the axons are growing (see Chapter 4).

It is therefore tempting to speculate that the absence of *netrin-1* expression at the rhombic isthmus is the neuroepithelial-derived cue that serves to channel the axons together and perhaps to guide them toward their appropriate dorsal exit point. However, there are several reasons to argue against this model. Firstly, although the axons always gather together at the axial level of the isthmus, their actual point of convergence does not correspond to the beginning of the corridor - the axons actually funnel together more laterally (see Figure 3-1). Secondly, and more importantly, if netrin-1 repulsion serves to "hem" the axons together, then convergence of the axons should be disrupted in the *netrin-1* mutant, which does not appear to be the case. Unfortunately, use of the β-galactosidase transgene as our marker of *netrin-1* expression does not allow us to make a direct comparison between the point of axon convergence and the exact location of the corridor in wild type (which by definition would not express the transgene), so that we were unable to contrast this to what is seen in the mutant. However, comparisons of the size, shape and axon trajectories from trochlear nuclei of all 3 genotypes reveal no obvious differences (see Figure 3-2). Therefore, the fact that the rhombic isthmus is both the site of this corridor devoid of *netrin-1* expression and the point at which trochlear motor axons converge, likely reflects a response to an underlying patterning event at this junctional region, rather than a direct causal relationship.

Netrin-1 is not essential to trochlear motor axon guidance

Netrin-1 can repel trochlear motor axons *in vitro* (Colamarino and Tessier-Lavigne, 1995a) and is expressed in the floor plate at the hindbrain-mindbrain junction *in vivo*. However, unlike the dramatic effect which has been seen on spinal commissural axons (Serafini et al., 1996), loss of *netrin-1* function does not significantly alter the projections of trochlear motor axons. A disorganization of the placement of the cell bodies of trochlear motor neurons and possibly of the initial direction of migration of their axons is observed in homozygous mutant embryos, but the axons seem otherwise to grow along a

largely normal trajectory. Thus, other cues must be present that can guide the axons in the absence of netrin-1. One candidate is a second chemorepellent for trochlear motor axons, whose existence is indicated by *in vitro* experiments demonstrating that floor plate depleted of netrin-1, either genetically or with the use of function-blocking antibodies, can still repel rat trochlear motor axons *in vitro*. Neither loss of function experiment is conclusive: in the first case, we know that the mutant floor plate produces low levels of netrin-1, and in the second case, we cannot be absolutely certain that we have added enough antibody to completely block all of the repellent activity of netrin-1 when it is being produced by the ventral hindbrain-midbrain tissue. Taken together, however, these experiments strongly argue for the presence of a second chemorepellent activity produced by the floor plate. Our finding of a floor plate-derived chemorepellent distinct from netrin-1 is consistent with the recent demonstrations that floor plate cells, but not netrin-1, can repel migrating olfactory interneuron precursors (Hu and Rutishauser, 1996) and posterior commissure axons (Shirasaki et al., submitted). Whether the same factor can produce all three of these repulsive effects remains to be determined. Furthermore, it will be interesting to see whether these chemorepellents act redundantly, or serve different purposes *in vivo*.

In C. elegans, loss of *unc-6* function causes misrouting of both ventrally- and dorsally-directed axons, showing that the (presumed) repulsive effect of UNC-6 is essential for dorsally-directed axons (Hedgecock et al., 1990; Wadsworth et al., 1996). So far all ventrally-directed axons in vertebrates that are attracted to the floor plate have been found to be netrin-1 sensitive (Serafini et al., 1994; Kennedy et al., 1994; Shirasaki et al., 1995). Our results indicate that there must also be netrin-independent floor plate factors involved in establishing dorsally-directed circumferential migrations in vertebrates. Further characterization of *netrin-1* mutants will be required to determine whether the ability of netrin-1 to guide through repulsion is essential *in vivo* for the guidance of any vertebrate axonal class.

Materials and Methods

Explant Culture and *In Vitro* Assays

In vitro assays of floor plate-derived commissural axon outgrowth and trochlear motoneuron repulsion activities were performed as previously described (Tessier-Lavigne et al., 1988; Serafini, et al. 1994; Kennedy et al., 1994; Colamarino and Tessier-Lavigne, 1995a), except that in the assays demonstrating netrin-1 repulsion of trochlear motor axons, stably-transfected human 293 cells secreting netrin-1 (generated by C. Mirzayan; Shirasaki et al., submitted) were used in place of transiently-transfected COS cells.

Immunohistochemistry

Wholemount immunohistochemistry was performed on E11 rat ventral hindbrain-midbrain junction (HMJ) explants using antibody F84.1 as described previously (Colamarino and Tessier-Lavigne, 1995a), and on E9.5-11.5 mouse HMJ regions using an antibody to NF-M (kind gift of Dr. Virginia Lee), essentially as described except that it was performed in PBSMT (PBS with 2% nonfat milk and 0.1% Triton X-100) using 2 d incubations in antibody and 1-2 d washes at 4 °C.

Polyclonal Antibodies

Rabbit polyclonal antibodies were raised against purified expressed domain VI and V protein of chick netrin-1 and affinity purified as described (Kennedy et al., in preparation). Control antibodies were affinity purified from preimmune rabbit serum using protein A sepharose as described (Kennedy et al., in preparation).

Figure 3-1. Expression of Mouse *Netrin-1* at the Hindbrain-Midbrain Junction During the Development of Trochlear Motor Axon Projections.

(A-C) The hindbrain-midbrain junction of a homozygous mutant mouse was dissected at E10.5 and subjected to double wholemount immunohistochemistry to show *netrin-1* expression (as revealed by the localization of the fusion protein with a polyclonal antiserum against β-galactosidase (green)) in relation to trochlear motor axons (red). The tissue was cut open at the dorsal midline, and is shown with the ventricular surface facing down. Rostral is up, and dorsal is lateral.

(A) *Netrin-1* is strongly expressed by a wide band of cells at the ventral midline, corresponding to the floor plate (fp) and the cells immediately adjacent. Lower-levels of expression are also seen throughout the ventral half of the neural tube, but drop-off in the dorsal neural tube (not shown). There is a distinct corridor of cells at the rhombic isthmus which lacks *netrin-1* expression.

(B, C) Close ups of the region boxed in (A), showing that the corridor of cells lacking *netrin-1* expression (B) is exactly where trochlear motor axons gather and grow dorsally through the neuroepithelium (C). Notice in (B) the bleed-through of axonal staining (arrowhead).

scale bars: 100 μm.

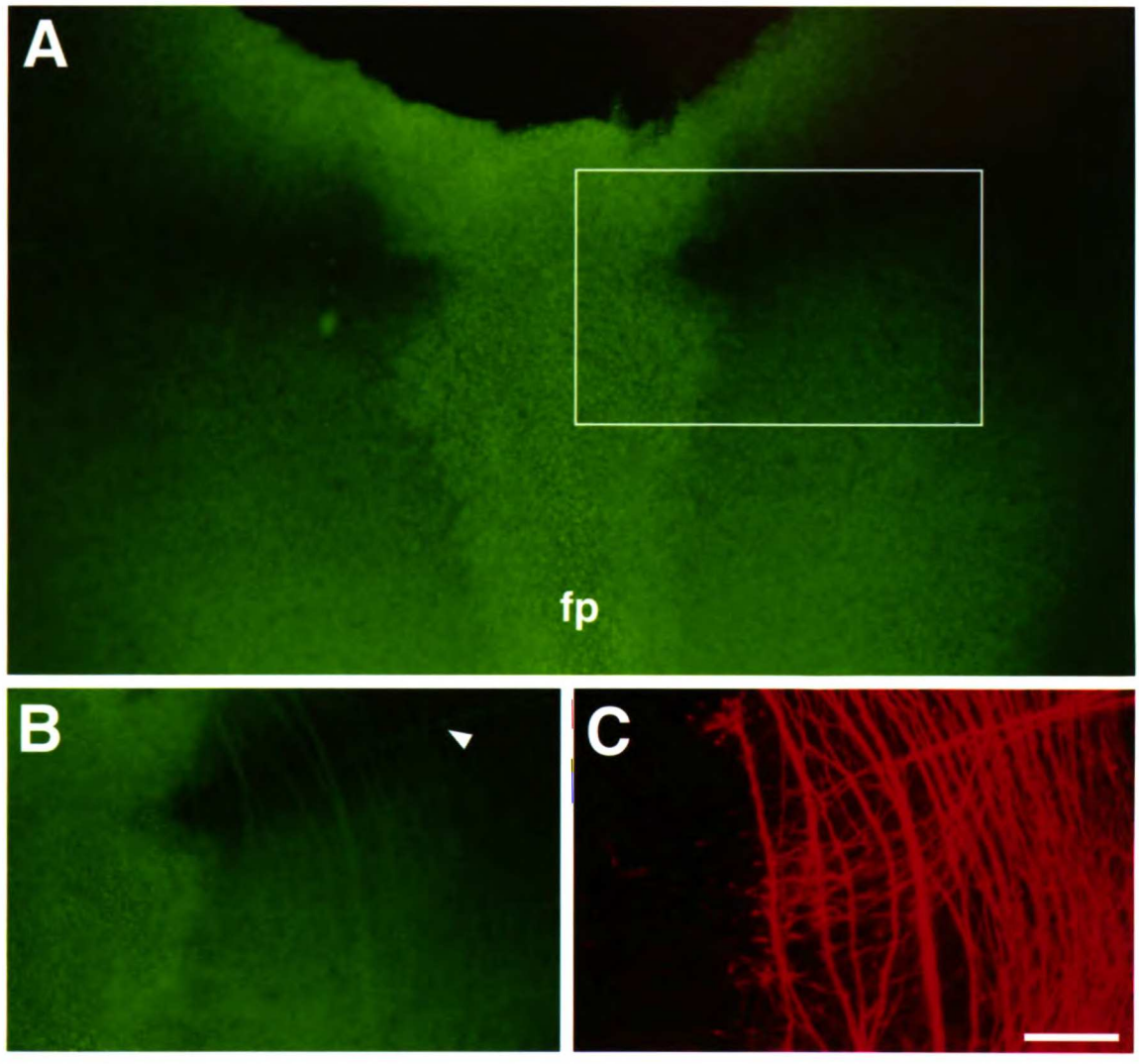


Figure 3-1

Figure 3-2. Trochlear Motoneurons Project Normally in *Netrin-1* Homozygous Mutant Mice.

Sagittal (side) views of the region of the trochlear nucleus, in (A) wildtype, (B) heterozygote, and (C) homozygous mutant E10.5 mice, visualized after wholemount immunohistochemistry with an antibody recognizing neurofilament-M (NF-M). Dorsal is up, rostral to the right. Trochlear axons run toward the top of the figure and coalesce (asterisk) before exiting the neural tube to form the trochlear nerve. Their trajectory is partially obscured by bundles of axons coursing longitudinally (i.e., horizontally in the figure). Several trochlear axons among those present are indicated (white arrowheads). When compared to wildtype and heterozygote littermates, mutants show no obvious defects in trochlear axon trajectories (n=12, 27 and 14 trochlear nuclei examined at E10.5, respectively). However, more trochlear neurons are present within the floor plate region in the mutants (compare green arrows in (A) and (C)). In addition, longitudinal axon tracts are disorganized.

Scale bars: 60 μm.

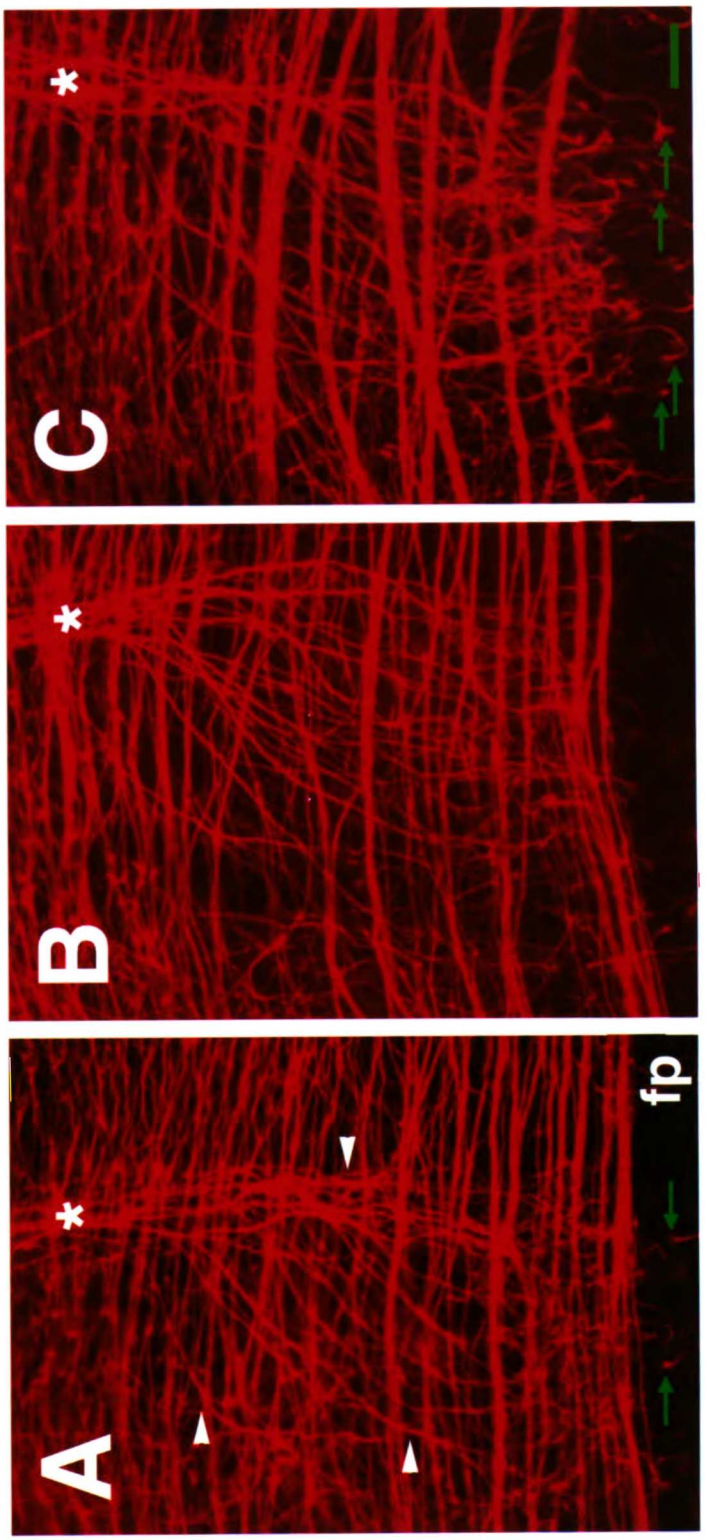


Figure 3-2

Figure 3-3. Floor plate Tissue from Homozygous Mutants Can Repel the Growth of Trochlear Axons *in vitro*.

Trochlear motoneuron axon repulsion assays (A-C) performed without any floor plate present (A), with floor plate from a wildtype E11.5 embryo present (B), and with floor plate from a homozygous mutant E11.5 embryo present (C). Explants of ventral hindbrain-midbrain junction (HMJ) from E11 rat embryos were cultured for 40 hr in collagen gels, and stained with antibody F84.1 to visualize trochlear motor axons (Colamarino and Tessier-Lavigne, 1995a). The trochlear axons (blue arrowhead) extend from these explants in the absence of floor plate (A), but are repelled by floor plate from both wildtype (B) and homozygous mutant (C) embryos. Wildtype, n = 10; mutant, n = 7; heterozygote (data not shown), n = 22.

Scale bars: 180 μm.

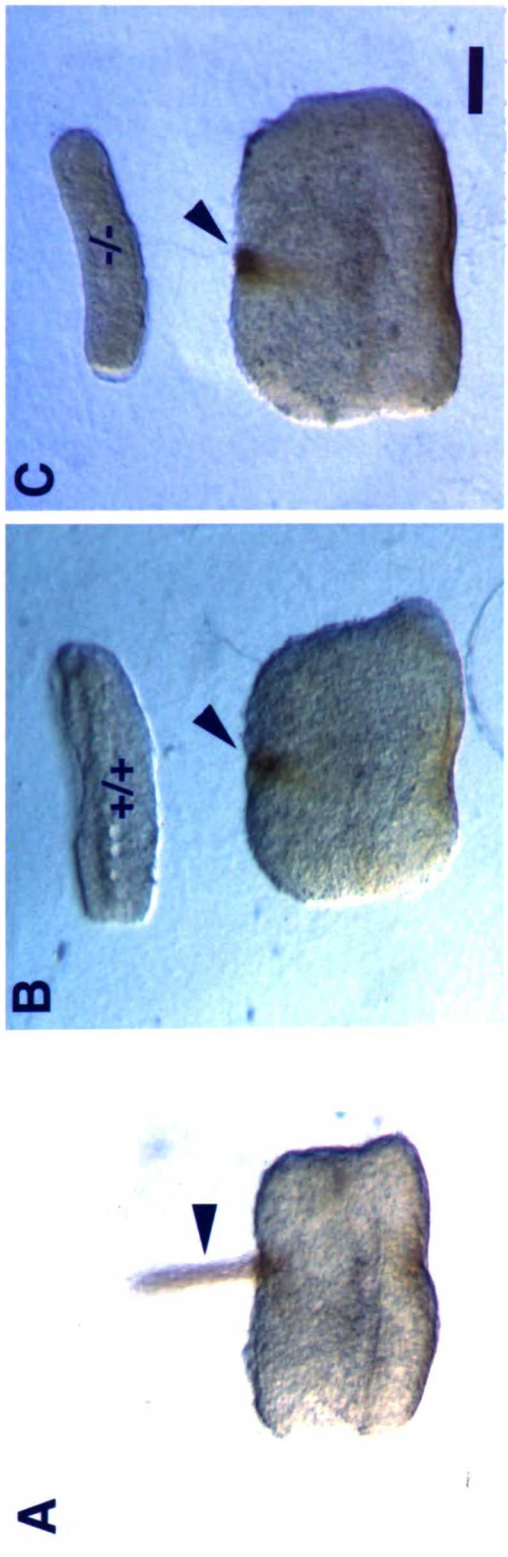


Figure 3-3

Figure 3-4. Polyclonal Antibodies Can Block Netrin-1 Function in *in vitro* Assays.

Antibodies to netrin-1 can block both its outgrowth-promoting and its chemorepellent activities. (A, D, G) 100μg/ml anti-netrin antibody 65II#2; (B, E, H) 100μg/ml purified control IgG; (C, F, I) no antibody.

(A-C) Anti-netrin antibody blocks outgrowth from E13 dorsal spinal cord explants elicited by E13 spinal cord floor plate explants (n = 6).

(D-F) Anti-netrin antibody blocks outgrowth from E11 dorsal spinal cord explants elicited by E11 floor plate from the hindbrain-midbrain junction (n = 6).

(G-I) Anti-netrin antibody blocks the repulsion of trochlear motor axons caused by the presence of netrin-1 secreting 293 cells (n = 5).

Scale bars: 400 μm.

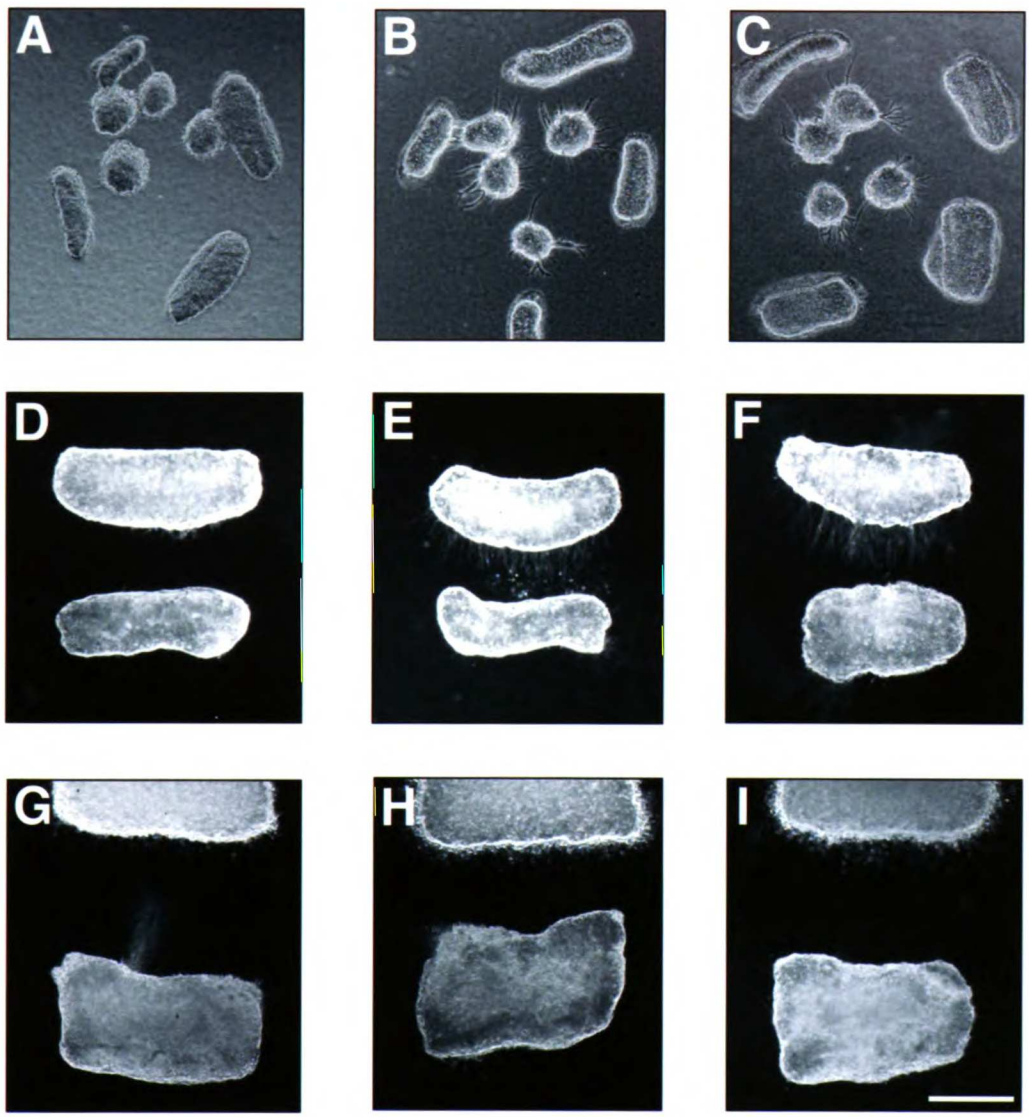


Figure 3-4

Figure 3-5. Netrin-1 Function-Blocking Antibodies Do Not Inhibit Floor Plate Repulsion of Trochlear Motor Axons *in vitro*.

Anti-netrin antibody 65II#2 is not sufficient to block the repulsion of trochlear motor axons caused by floor plate from the hindbrain-midbrain junction, suggesting that other chemorepellents are produced by the floor plate. (A) 100μg/ml anti-netrin antibody (n = 11) (B) 100μg/ml purified control IgG (n = 11) (C) no antibody (n = 7). Scale bars: 170 μm.

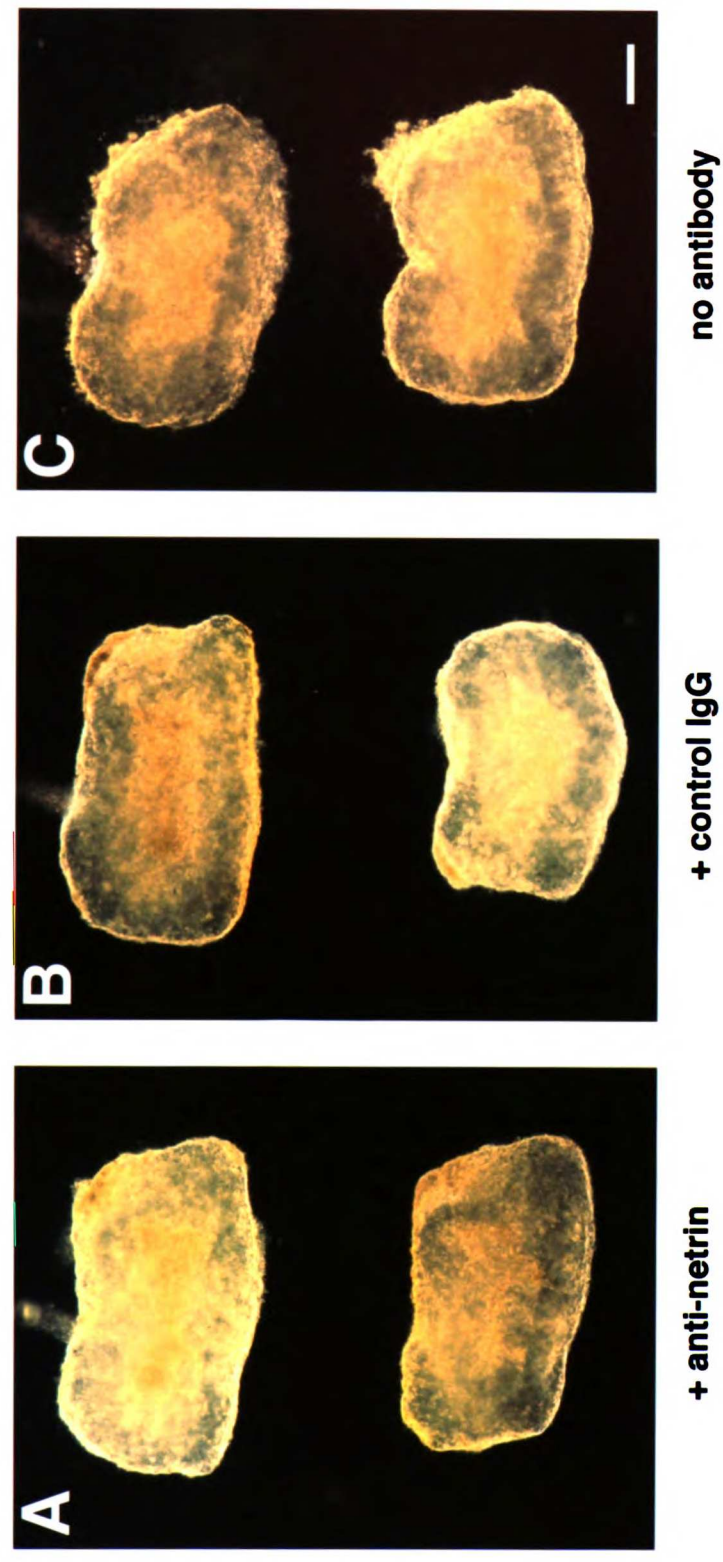


Figure 3-5

CHAPTER 4:

Future Directions

Other Cues Which May Direct The Unique Migration of Trochlear Motor Axons

Abstract

In vivo, the presence of netrin-1 does not appear to be crucial for the dorsal guidance of trochlear motor axons. This is most likely due to the presence of other cues which also specify the dorsal migration of these axons. Here we report that the neuroepithelium and roof plate may be sources of other cues which guide trochlear motor axons to the dorsal midline *in vivo*. Preliminary *in vitro* experiments suggest that the neuroepithelium at the rhombic isthmus contains cues which help channel the axons together, and that the roof plate may attract trochlear motor axons over a distance. The relationship of these cues to the generation of different motoneuron trajectories is also discussed.

Introduction

The work presented in Chapter 2 demonstrated that netrin-1 can act as a chemorepellent for trochlear motor axons *in vitro*, and the work in Chapter 3 suggested that other cues exist besides netrin-1 to guide these axons dorsally *in vivo*, one of which may be a second, as yet unidentified, floor plate-derived chemorepellent. Floor plate chemorepulsion in general may serve to steer the axons during the initial part of their trajectory away from the ventral midline, but this mechanism alone does not explain why motor axons of the trochlear nucleus are the only ones which grow all the way to the dorsal midline of the neural tube. Preliminary data described in this chapter suggest the existence of at least two other cues which may act in concert with the floor plate chemorepellents to guide trochlear motor axons along their unique trajectory.

As discussed in Chapter 2, motor axons may take one of 3 different routes in leaving the neural tube to project to their target muscles. Somatic motoneurons, which include all spinal motoneurons and those of the oculomotor, abducens, and hypoglossal nuclei in the brainstem (which comprise cranial nerves III, VI, and XII), project their axons ventrolaterally to exit the neural tube close to their cell bodies of origin (Altman and Bayer, 1984; Lumsden and Keynes, 1989). Axons of the branchial motoneurons in the trigeminal, facial, and ambiguus nuclei (which contribute to cranial nerves V, VII, and IX), project dorsolaterally within the neural tube until they reach their exit points which lie approximately midway along the dorsovental axis (Lumsden and Keynes, 1989; Guthrie and Lumsden, 1992). Finally, the trochlear motoneurons (cranial nerve IV), which are somatic, are exceptional in that their axons continue dorsally to circumnavigate the neural tube and exit at the dorsal midline, the side opposite to their cell bodies (Sinclair, 1958; Fritzsch and Sonntag, 1988; Matesz, 1990; Fritzsch and Northcutt, 1993; Szekely and Matesz, 1993; Chedotal et al., 1995). Much recent progress has been made in the study of the mechanisms which establish these different trajectories.

All of the trajectories described above are similar in their initial choice of direction i.e., away from the ventral midline. In fact, no motor axons are known to cross the midline - the crossed projection of the few motoneurons which have contralateral targets (other than the trochlear motoneurons) is achieved via a migration of the cell body across the ventral midline, rather than the axon (Puelles and Privat, 1977; Naujoks-Manteuffel et al., 1991). Thus, the first choice that all motor axons need to make is to avoid the ventral midline. One simple mechanism to achieve this would be through a general repulsion of all motor axons by ventral midline structures such as the floor plate and/or the ventral ventricular zone. Evidence for this has now been documented for motor axons that follow each of the three different trajectories described above (Colamarino and Tessier-Lavigne, 1995a; Guthrie and Pini, 1995). Therefore, it is possible that at least the initial directionality of all motor axons may be set by a ventral midline-derived repellent.

Subsequent to orientation away from the ventral midline, the motor axons are required to make a second choice between one of two different paths - growth straight out of the neural tube (somatic motoneurons), or growth dorsally within the neural tube along its lateral edge (branchial motoneurons and trochlear motoneurons). The differential behavior probably reflects a differential reaction to a cue(s) present in their environment, and indeed, the two types of axons can be distinguished *in vitro* by a differential sensitivity to netrin-1. Only those axons which grow dorsally within the neural tube are repelled by the molecule netrin-1 (Colamarino and Tessier-Lavigne, 1995a; S. Guthrie, personal communication), even though, as mentioned, all of them can be repelled by the ventral midline (Colamarino and Tessier-Lavigne, 1995a; Guthrie and Pini, 1995). Although we know that netrin-1 itself cannot be the only cue responsible for directing the choice of a dorsal migration (or if so, we would expect to see trochlear motor axons exiting ventrally with the other somatic motoneurons in the netrin-1 deficient mice), this does provide evidence that motor axons which grow dorsally for any amount of time, regardless of their origin or exit points, behave similarly at a molecular level.

The third choice involved in establishing the different trajectories requires that branchial motor axons halt their dorsal growth to leave the neural tube at their exit points, while trochlear motor axons must forge onward into the dorsal half of the neural tube. Many mechanisms can be postulated to account for the divergence in pathways. There is some evidence, though not conclusive, that the exit points of branchial motor axons release a chemoattractant for the axons (Chang et al., 1992; Guthrie and Lumsden, 1992), to which the trochlear motor axons would presumably be unresponsive or are not exposed. It is also conceivable that a repellent or inhibitor exists which selectively prohibits the growth of branchial motor axons into the dorsal neural tube. The continued dorsal growth of trochlear axons may also result from cues, either short- or long-range, which are actively enticing growth of these axons all the way to the dorsal midline. The following unpublished experiments will provide some support for this possibility by providing evidence of 1) short-range cues distributed within the neuroepithelium which may channel and constrain trochlear motor axons to growth in a dorsal direction, and 2) a chemoattractant activity for trochlear motor axons which is produced by the roof plate of the hindbrain-midbrain junction.

Results

The neuroepithelium provides channeling cues for trochlear motor axons

Simple observations of the growth pattern of trochlear motor axons *in vitro* are consistent with the presence of guidance cues within the neuroepithelium through which they are growing. *In vivo,* trochlear motor axons grow as a single bundle toward the dorsal edge of the rhombic isthmus (Chedotal et al., 1995). The convergence of the axons into a large group, which occurs at a point approximately a third of the way along their dorsal migration, can also be seen in explants of the entire hindbrain-midbrain junction (HMJ) cultured *in vitro* for 48 hours (Colamarino and Tessier-Lavigne, 1995a; Figure 4-1A). Removal of the dorsal 2/3 of the HMJ, which deprives the axons of cues from their endogenous exit point, does not affect the convergence of the axons and they emerge from the ventral HMJ explants as a particularly striking, single bundle of axons (Chapter 2, Figure 2-2B). Wholemount immunolabelling of the axons reveals that they funnel together within the neuroepithelium prior to projecting into the collagen matrix (Chapter 2, Figure 2-2C). This suggests that the axons have access to a cue present within the neuroepithelium that clusters them into a single bundle along the rostrocaudal axis.

To address whether these *in vitro* observations truly reflect the presence of neuroepithelial-derived channeling cues, we performed a series of neuroepithelium rotation experiments. However, in order to better view the behavior of axons growing within the neuroepithelium, we first modified our dissection protocol. Explants of the ventral HMJ were isolated as usual (Colamarino and Tessier-Lavigne 1995a, see Chapter 1), but then further sub-dissected by splitting them open along the ventral midline (i.e., cutting along the middle of the floor plate). This generated explants containing only one trochlear nucleus, which made it easier to visualize individual axons. We found that trochlear motoneurons also differentiated in these half-ventral HMJ ("half vHMJ") explants and extended axon bundles into the collagen matrix in the same way as with full ventral HMJ

explants (although the bundles were thinner because they consisted of axons from only one nucleus - data not shown).

We cultured these half vHMJ explants with explants of neuroepithelium under a variety of circumstances. As a control, explants of neuroepithelium taken from the intermediate neural tube at the HMJ ("iHMJ" explants) were placed in contact with the dorsal-most edge of half vHMJ explants, taking care to maintain their original polarity and alignment, and were co-cultured in collagen gels for 40 hours (diagrammed in Figure 4-1B). Immunostaining of the trochlear axons revealed that they grew through the iHMJ explants as an intact bundle, without showing signs of deviating from their normal dorsally-directed trajectory (Figure 4-1E). During the time in culture the two explants appeared to have sealed together and, although the border between the two explants was often still visible, the axons apparently grew from the half vHMJ explants into the iHMJ explants in seamless fashion, without appearing to recognize the border as such.

To test our hypothesis that there are cues within the HMJ neuroepithelium which make only a select corridor of neuroepithelium permissible for growth, we tested how the axons would behave if the iHMJ explants were displaced either rostrally or caudally relative to the half vHMJ explants (diagrammed in Figure 4-1C). Although in a few cases the bundles grew as in the controls (data not shown), the majority showed abnormal behavior upon reaching the border of the displaced iHMJ explants. In most instances, the axons avoided entering the displaced HMJ neuroepithelium. Sometimes they stalled (Figure 4-1F), but usually they grew along the border between the two explants. In two of these cases, the axons then turned at a right angle at a specific location to grow through the neuroepithelium (Figure 4-1G). In other co-cultures, upon contacting the displaced iHMJ explant, the axons formed a tight fascicle and appeared to grow over the top of the explant (Figure 4-1H). These results suggest that the axons cannot grow equally well through neuroepithelium at every position along the rostrocaudal axis.

A similar set of results was obtained when the axons were challenged to grow through neuroepithelium that had been rotated 90° (diagrammed in Figure 4-1D). Again, the axons tended to avoid entering the iHMJ explant, growing along its border before forming a bundle which either projected into the collagen (Figure 4-1I) or tracked along the edge of the rotated iHMJ explant. We did see two instances where after growing along the edge of the neuroepithelium for a short distance, the bundle turned 90° to grow through the neuroepithelium along what would have been the original dorsoventral axis (Figure 4-1J). In one co-culture the trochlear axons entered the rotated iHMJ explant and grew through it as individual axons (Figure 4-1K), behavior which is highly unusual for trochlear motor axons since they usually grow as a single group. This may indicate that growth as a bundle is not an absolute requirement for trochlear motor axon extension.

Finally, to ask what would happen to the axons if they were confronted with growing through foreign neuroepithelium, we substituted the iHMJ explant with an explant of the dorsal spinal cord ("DSC explant" - the roof plate was also included in these explants but was always positioned so that it faced away from the half vHMJ explant). In every case, the trochlear axon bundles immediately broke up into many fascicles upon contact with the DSC explant. The many axon fascicles then grew randomly throughout the neuroepithelium, encircling the entire explant and only rarely leaving it to enter the surrounding collagen (Figure 4-1L). This further suggests that the normal bundling of trochlear motor axons must be due to some constraint placed upon their growth by cues found in the neuroepithelium at the level of the rhombic isthmus.

The roof plate attracts trochlear motor axons

Trochlear motor axons grow to the roof plate, which occupies the dorsal midline of the neural tube. Although there is evidence that the floor plate repels trochlear motor axons away from the ventral midline, the roof plate also may encourage their migration to the dorsal midline by secreting a chemoattractant for trochlear motor axons. To investigate this

possibility, we asked whether explants of roof plate are capable of re-directing the growth of trochlear motor axons *in vitro*.

Bundles of trochlear motor axons generally emerge from ventral HMJ explants growing along a straight trajectory, and in a direction perpendicular to the floor plate edge of the explant (Colamarino and Tessier-Lavigne, 1995a). Although there is an occasional deviation in their angle of outgrowth, an analysis of this angle of deflection (see Figure 4-2A, inset) showed that on average, there does not appear to be a bias in the direction of deflection (based on an arbitrary assignment of left and right - the original rostrocaudal polarity of the explants becomes randomized upon dissection and thus we can not exclude a bias based upon polarity of the tissue). The majority of explants show no deflection at all (Figure 4-2A, mean angle < 1°). Although deflections are sometimes seen, turning of the axon bundles within the collagen is never seen in explants cultured alone.

With the assurance that the bundles of trochlear axons maintain a strong tendency to grow straight when cultured on their own, we examined the angle of bundle deflection when ventral HMJ explants were co-cultured with either roof plate or control tissue. Roof plate (RP) explants were obtained by removing the top 1/3 of the HMJ, which had been dissected at E11 as previously described (Colamarino and Tessier-Lavigne, 1995a), and then embedded in collagen alongside ventral HMJ explants at an approximate angle of 90° (Figure 4-2A, inset). The cut-edge of the RP explant was always placed facing away from the ventral HMJ explant to ensure the proximity of the roof plate, and to eliminate the chances that axonal growth from the RP explant might interfere with the growth of trochlear axons. After 48 hours in culture, the angle of deflection of the trochlear axon bundles was measured as above. In the presence of RP explants, there was both a large increase in the angle of deflection and in the percentage of bundles with a deflection (Figure 4-2A). Ten out of twelve of the ventral HMJ explants co-cultured with RP explants had bundles pointing toward the roof plate (Figure 4-2B; mean angle = 23°), with the bundles from the remaining two explants growing straight. Moreover, approximately 60% of the

bundles that grew in the presence of the roof plate actually turned within the collagen to re-orient towards the roof plate (Figure 4-2C).

To control for the possibility that the deflections and turning of trochlear axon bundles reflected a mechanical distortion of the collagen gel by the presence of the second (roof plate) explant, we replaced the roof plate explants with explants of intermediate neural tube (iHMJ) from the HMJ (i.e., the middle third of the HMJ). In this case, there was an increase in the percentage of explants with deflected bundles although the average deflection was much smaller than in the presence of RP explants, and actually in the direction away from the iHMJ explants (Figure 4-2A and 4-2D; mean angle = -7^{o}). In addition, in two out of the nine cultures, the bundles may have turned slightly within the collagen, but again we observed that this was in the direction away from the iHMJ explants.

Discussion

The neuroepithelium provides cues which channel trochlear motor axons

Many guidance molecules are known to act as short-range cues, either permissive or inhibitory, for extending axons (reviewed in Goodman, 1996). Precedence for the existence of short-range cues which guide circumferential migrations within the neural tube has come from experiments that showed that spinal commissural axons grow along their correct pathway - in both the dorsoventral and mediolateral axes - even in isolated explants of dorsal neuroepithelium (reviewed in Colamarino and Tessier-Lavigne, 1995b). It is therefore not unreasonable to assume that trochlear motor axons, which undergo a migration similiar to that of commissural axons, albeit in the opposite direction, are benefitting from the presence of similar short-range guidance cues.

A single pioneer trochlear motor axon has been described in chick, and it was suggested that fasciculation with the pioneer was the mechanism of creating the bundle of axons (Chedotal et al., 1995). We have never seen evidence of a single, early-extending axon in either rat or mouse (S.A.C. and M.T.-L., unpublished observations). Although we cannot rule out axon-axon interactions, these observations favor the hypothesis that the axons grow together due to some constraint within the neuroepithelium. In support of this, we have found that trochlear axons do not grow through neuroepithelium whose orientation or source has been altered.

From this preliminary data it is not possible to positively ascertain the nature and distribution of these neuroepithelial-derived cues. Although we did not quantitate the axon behavior, in the majority of experimental conditions the axons avoided growing into the neuroepithelium. This indicates that neuroepithelium from the hindbrain-midbrain junction (HMJ) is not an entirely permissive substrate. We did see occasions where the axons turned 90° to grow through neuroepithelium which had been rotated 90°, suggesting that a defined corridor within the neuroepithelium may exist. This is further supported by the

discovery that the axons will grow randomly in a piece of neuroepithelium taken from a different axial level. Although this could be a function of a permissive cue which is present in the neuroepithelium of the dorsal spinal cord but not in that of the rhombic isthmus, it does at the very least show that trochlear motor axons are not always contrained to grow as a group. Hence, the most likely interpretation of these pilot experiments is that cues, perhaps inhibitory, are located within the HMJ neuroepithelium and serve to make only the rhombic isthmus permissive for trochlear axon growth. One function for these cues may be to prevent the axons from deviating from a dorsal trajectory.

The roof plate may attract trochlear motor axons *in vitro*

The roof plate lies at the dorsal midline of the neural tube and is composed of cells that have many structural similarities to those of the floor plate (Snow et al., 1990b). However, a definitive role for its participation during development has not been established. It may have inductive capacities and help confer dorsal cell identities (Basler et al., 1993). It may also play a role in axonal decussation at the dorsal midline. During the period of sensory axon ingrowth to the spinal cord, the roof plate expresses high levels of keratan sulfate and chondroitin sulfate, both of which are non-permissive for sensory neurite growth *in vitro* (Snow et al., 1990a). This has led to the hypothesis that the roof plate acts as a barrier which prevents axons from crossing the dorsal midline through a contact-mediated inhibition of outgrowth (Snow et al., 1990a,b).

Here we provide evidence that the roof plate may also exert a long-range influence on the patterning of axonal projections within the neural tube. We have shown preliminary data that trochlear motor axons appear to be attracted to the roof plate *in vitro*. At this point, however, the effect does not seem to be as robust as other *in vitro* assays demonstrating the existence of a chemotropic activity (e.g., the floor plate attraction and repulsion of commissural and trochlear motor axons, respectively (Tessier-Lavigne et al., 1988; Placzek et al., 1990; Colamarino and Tessier-Lavigne, 1995a)). The fact that not all

trochlear axon bundles re-oriented extensively towards the roof plate may be attributable to several difficult-to-control-for factors in our assay system, such as the distance of the trochlear nucleus from the roof plate explant, and the exact angle between the two explants. Furthermore, it is possible that the chemoattractant activity resides only within a specific portion of the roof plate, which may explain the variability in results. This could be confirmed through microdissections of the HMJ roof plate. We have also not conclusively determined the tissue specificity of the effect. It appears that the control tissue (intermediate neural tube (iHMJ)) also has an effect on the growth of trochlear axon bundles. In the case of the data presented here, the iHMJ had a tendency to repel the axons, although in other experiments we have sometimes seen that the iHMJ can attract the axons (although neither effect is as strong as that of the roof plate). In hindsight, the effects of the iHMJ are not surprising given that *netrin-1* is expressed not only by the midline floor plate cells, but by cells throughout the ventral half of the neural tube (see Chapter 3). A roof plate chemoattractant may similarly have a more widespread expression, in which case our iHMJ explants most likely contained some combination of netrin- and attractant- expressing cells. Therefore, the use of the iHMJ as the control tissue may not have been the most optimal choice.

Although more controls clearly need to be done, the rather dramatic turning of trochlear axon bundles towards the roof plate is nonetheless suggestive that the effect is not just an artifact. Recent evidence has also been obtained for roof plate repulsion of spinal commissural axons *in vitro* (J. Dodd, personal communication), which further supports the idea that the roof plate plays a more active role in axon guidance than has previously been thought (Snow et al., 1990a,b).

Molecular basis of the roof plate- and neuroepithelial- devived cues

Although it is admittedly premature to attribute these guidance activities to any particular molecules, the rhombic isthmus region is known to possess special signaling capabilities

(Marin and Puelles, 1994). It is also the site of unique patterns of gene expression. For example, the isthmus is one of only two regions of the nervous system where a Wnt-1/FGF-8 border exists (Crossley and Martin, 1995), and as described in Chapter 3, it is also in precisely this region that *netrin-1* expression appears to be down-regulated. Detailed expression studies will likely find other guidance molecules that have distinctive expression patterns at the rhombic isthmus and may help illuminate the molecular basis of trochlear guidance to the dorsal midline.

Finally, it is not clear whether the unique trajectory of trochlear motor axons in relation to the other motor axons is a function of the selective expression of guidance molecules at a particular axial level, or of the selective expression by trochlear motor neurons of receptors for more ubiquitously distributed guidance cues. Although, as mentioned above, there is evidence for the regionalized expression of guidance molecules, there is also a growing body of evidence that populations of motoneurons may be uniquely specified. In the spinal cord, expression of different members of the LIM homeobox transcription factor family by distinct subsets of motor neurons has been correlated to their particular target of innervation (Tsuchida et al., 1994). The diversity in transcription factor expression makes it likely that motoneurons which share a common trajectory ultimately express a different array of receptors, which in turn may result in specific recognition events. This could provide an explanation, for example, of why only those motor axons which grow dorsally are repelled by netrin-1 (Colamarino and Tessier-Lavigne, 1995a; S. Guthrie, personal communication). Thus, analysis of transcription factors expressed selectively in various cranial motor nuclei may provide new insight into how the trochlear motor axons accomplish their unique migration.

Summary and General Conclusions

We have used a novel *in vitro* system of culturing explants of the hindbrain-midbrain junction to examine several cues involved in the guidance of trochlear motor axons. These

axons grow away from the ventral midline *in vivo*, and we have shown that *in vitro* they are repelled by the floor plate, the structure that comprises the ventral midline. It had previously been shown that the floor plate influences axon growth at the ventral midline through permissive and inhibitory contact-mediated mechanisms, as well as through a mechanism of long-range chemoattraction (reviewed in Colamarino and Tessier-Lavigne, 1995b). This work now adds chemorepulsion to the repertoire of mechanisms that the floor plate can use to influence the growth of axons in its vicinity. Interestingly, just as the discovery of guidance by inhibitory molecules lagged behind that of guidance by permissive molecules, chemorepulsion as a guidance mechanism has been overshadowed by chemoattraction. However, since the description of the chemorepulsion of olfactory projection axons (Pini, 1993), examples for the operation of chemorepulsion in the development of the nervous system have now been documented for motor axons (Colamarino and Tessier-Lavigne, 1995a; Guthrie and Pini, 1995), primary sensory axons (Fitgerald et al., 1993; Messersmith et al., 1995), axons of central nervous system interneurons (Tamada et al., 1995; Shirasaki et al., submitted), and even neuronal cell bodies (Hu and Rutishauser, 1996).

We have identified one floor plate-derived chemorepellent for trochlear motor axons as the axonal chemoattractant netrin-1. This demonstrates that netrin-1 possesses the capacity to act as a bi-functional guidance cue, at least *in vitro*, and illustrates the rather amazing conservation in presumed function of the UNC-6 / netrin family of guidance molecules. These results should now allow for future study of the molecular nature of chemorepulsion, which is currently poorly understood. For instance, it is not known whether chemorepulsion is brought about through a gradient detection mechanism. To address this, trochlear motor axons can be tested for their ability to recognize and orient in gradients of netrin-1 protein *in vitro*. Moreover, given that netrin-1 can act both as an attractant and a repellent, a molecular dissection of the protein can be performed to

determine exactly which sites mediate the two activities, and how this relates to the binding of different netrin-1 receptors.

In vivo, the presence of netrin-1 does not appear to be crucial for the dorsal guidance of trochlear motor axons. This is most likely due to the presence of other cues which also specify the dorsal migration of these axons. We have identified several of these potential other cues, including a likely second floor plate-derived chemorepellent, a possible roof plate-derived chemoattractant, and a possible neuroepithelial-derived cue which may channel the axons together to assist their dorsal growth. The molecules responsible for these guidance events are not known. It will be interesting to learn not only their identity, but also where the specificity for their effects lies, and how the multiple cues act together to effect accurate guidance of trochlear motor axons in relation to other motor axons. For example, is the presence of a roof plate chemoattractant for motor axons restricted to the rhombic isthmus? If not, do trochlear motor axons alone possess the attractant receptor, or are all motor axons capable of responding to the roof plate? If other motor axons besides trochlear axons are found to be attracted to the roof plate, then this should initiate a search for other cues, perhaps neuroepithelial-derived cues, which must serve to keep all but the trochlear motor axons from being in a position to sense the attractant *in vivo*. A combined molecular and cellular approach may provide answers to these issues.

In conclusion, the unique migration of trochlear motor axons may result from a combination of three different types of cues: a "push" cue from the ventral midline; a "pull" cue from the dorsal midline; and a "channeling" cue which hems the axons together. Together with the knowledge that dorsal spinal cord commissural axons can be "pulled" to the ventral midline, and possibly "pushed" from the dorsal midline (J. Dodd, personal communication), this work begins to point toward a more complete symmetry of the axon guidance mechanisms involved in circumferential migrations in the vertebrate neural tube.

Materials and Methods

Explant Culture and *In Vitro* Assays

Explants of the neural tube at the hindbrain-midbrain junction (HMJ) were isolated as described (Colamarino and Tessier-Lavigne, 1995a). The HMJ explants were dissected into approximate thirds along the dorsoventral axis (see Figure 4-1A): the ventral-most third was designated as the ventral HMJ explants (as previously described in Colamarino and Tessier-Lavigne, 1995a); the dorsal-most third was used as the roof plate (RP) explants; and the remaining intermediate third was used as the intermediate neuroepithelium (iHMJ) explants. For the neuroepithelium rotation experiments, the ventral HMJ explants were further dissected into the two halves of the neural tube by splitting them open along the ventral midline. These half ventral HMJ explants were then either cultured with iHMJ in various configurations, or with explants of E11 rat dorsal spinal cord (Tessier-Lavigne et al., 1988). All explants were embedded in collagen gels and cultured as previously described (Colamarino and Tessier-Lavigne, 1995a).

Immunohistochemistry

Wholemount immunostaining of explants with F84.1 (gift of W. Stallcup) was carried out as described in Colamarino and Tessier-Lavigne, 1995a.

Quantitation of the Angles of Deflection of Trochlear Motor Axon Bundles

For the experiments documenting roof plate chemoattraction, after 48 hours in culture pictures were taken of the ventral HMJ explants cultured either alone, with iHMJ explants, or with RP explants. The "angle of deflection" of the bundle of trochlear motor axons was then measured by drawing a series of three lines: 1) a line parallel to the floor plate edge of the ventral HMJ explant, 2) a line perpendicular to the floor plate line and which ran through the center point of the trochlear bundle at its point of origin from the ventral HMJ

explant and, 3) a line connecting the center of the trochlear bundle at its origin with the center at its point of termination. The angle between these last two lines was designated as "the angle of deflection" to refer to the deviation from growth perpendicular to the floor plate. This angle was assigned a positive or negative value depending on whether the bundle pointed towards or away, respectively, from the RP/iHMJ explants. In the case of ventral HMJ explants cultured alone, bundles pointing to the left were arbitrarily assigned negative values. In all conditions, ventral HMJ explants without bundles were excluded from the quantification (although in the case of explants cultured with RP, after immunostaining for axons we often found that they did indeed have bundles, but that the bundles had entered the RP explant by growing along the dorsal-most edge of the ventral HMJ tissue).

Figure 4-1. The Neuroepithelium at the Hindbrain-Midbrain Junction (HMJ) Contains Short-Range Guidance Cues for Trochlear Motor Axons.

(A-L) Neuroepithelium rotation experiments indicate that cues may exist to constrain trochlear motor axon growth. In all panels dorsal is up. (A, E-L) Trochlear axons and floor plate are stained with antibody F84.1. (A) Explant of entire HMJ cultured for 48 hours. Trochlear axons extend dorsally and coalesce to form a single bundle of axons which grows directly toward the dorsal edge of the neural tube at the rhombic isthmus. Dotted lines represent (for all the subsequent panels) where the HMJ explants were cut to generate half vHMJ explants (bottom line, plus one other cut (not shown) along ventral midline) and iHMJ explants (middle line to bottom line). (B) Schematic diagram of "control" condition where the half vHMJ and iHMJ explants are aligned. (C) Schematic diagram of experimental ("displaced") condition in which the iHMJ explant is displaced in either the rostral or caudal direction. (D) Schematic diagram of experimental ("rotated") condition in which the iHMJ is rotated 90^{o} so that its original rostrocaudal axis is now aligned with the dorsoventral axis of the half vHMJ explant. (E) Control - axons entered and grew through the iHMJ piece as normal. (F) "Displaced" - when the axons reached the border of the displaced iHMJ they avoided entering it, and in this case stalled. (G) "Displaced" - axons first grew along the border between the two explants before turning and growing through the displaced iHMJ. (H) "Displaced" - axons appear to have avoided growing through the displaced iHMJ explant and formed a tight fascicle to grow over its surface. (I) "Rotated" - axons avoided entering the rotated iHMJ and grew instead along the border between the two explants. (J) "Rotated" - axons originally avoided growing into the rotated iHMJ explant and instead tracked along its edge before turning 90^{o} to grow through it. (K) "Rotated" - axons did not coalesce at all and instead grew randomly through the rotated iHMJ as individual axons. (L) When the iHMJ explant was replaced with an explant of dorsal spinal cord neuroepithelium, the axons formed many different fascicles and grew readily throughout the entire explant. scale bars: 160μm.

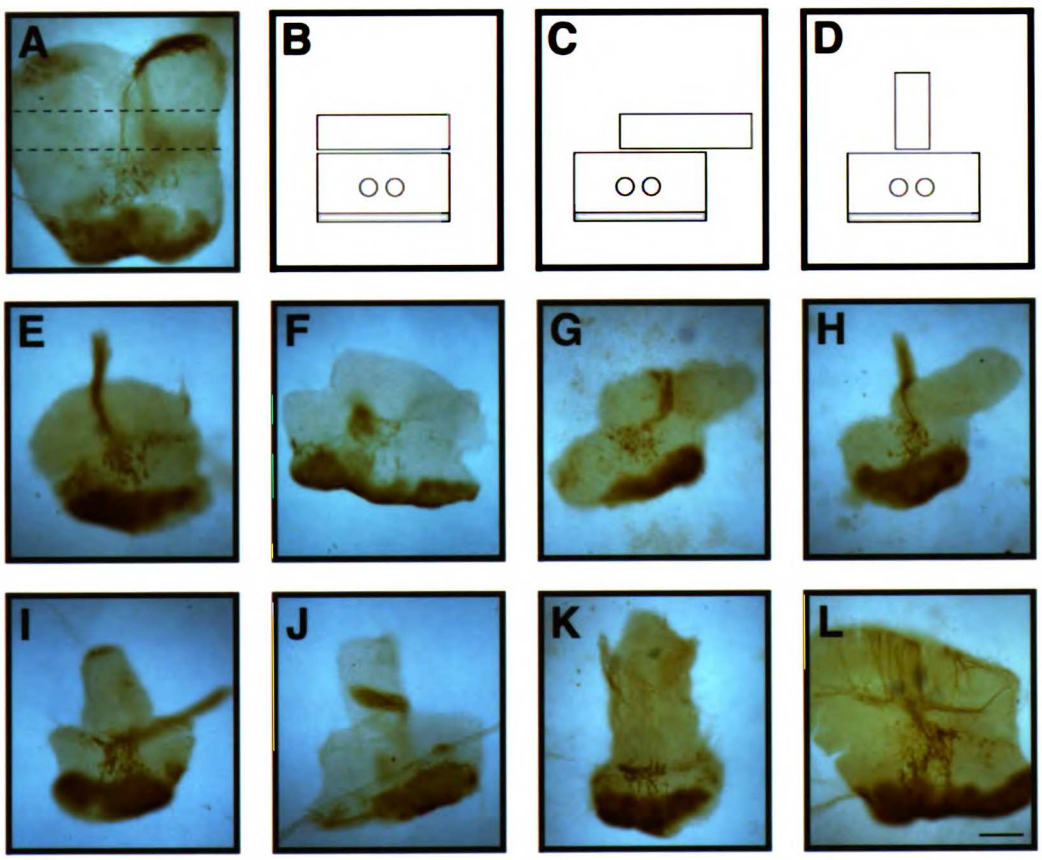


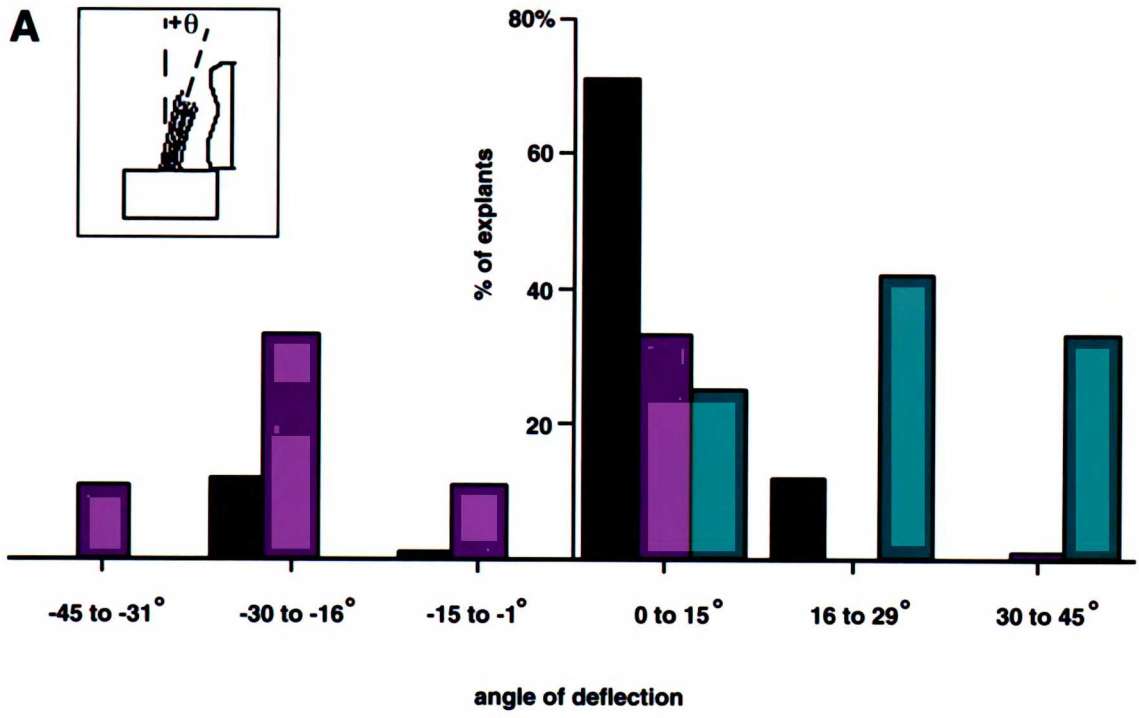
Figure 4-1

Figure 4-2. The Roof Plate Attracts Trochlear Motor Axons *in vitro*.

(A) Trochlear motor axons generally emerge from ventral HMJ explants as a bundle of axons growing perpendicular to the floor plate edge of the tissue. Deviations from this trajectory were measured for explants grown either alone, or in the presence of second explants which were placed at a 90^{o} angle to the ventral HMJ explants (see inset). Angles of deflection (θ) were determined as diagrammed in the inset (see also Materials and Methods), and the results were binned in increments of 15^{o} and plotted as a percentage of the total number of explants for each condition. Negative values were used to designate deflections pointing away from the added explants, or in the case of ventral HMJ explants cultured alone, to designate bundles pointing to the left. Trochlear motor axon bundles from explants grown alone tend not to deviate much, if at all, from the perpendicular (black bars), whereas those grown for 48 hours in the presence of roof plate (RP) explants (green bars) deflect toward the RP. Those grown in the presence of control intermediate neural tube (iHMJ) explants (purple bars) showed a small tendency to point away from the iHMJ explants (see Discussion). Mean angle of deflection: alone: <1^{o} (n=18); +RP: 23^{o} (n=12); +iHMJ: -7^{o} (n=9).

(B-D) Examples of trochlear axon outgrowth in the presence of RP (B, C), or control iHMJ explants (D). Notice in (C) the bundle is turning within the collagen to point toward the RP explant.

scale bars: 350 μm.


A
+θ
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% of explants
-45 to -31°
-30 to -16°
-15 to -1°
0 to 15°
16 to 29°
30 to 45°
angle of deflection

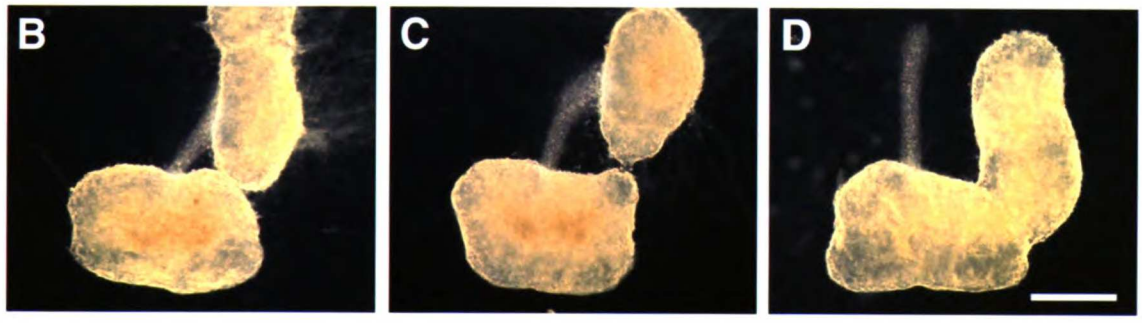
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Figure 4-2

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