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The Perinotochordal Mesenchyme Acts as a Barrier to Axon Advance in the Chick Embryo: Implications for a General Mechanism of Axonal Guidance

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To test the hypothesis that the perinotochordal mesenchyme (the sclerotome ventral to the spinal nerve pathway) is a barrier to axonal advance in the chick embryo, we determined whether axons directly confronted with perinotochordal mesenchyme would turn to avoid it. The initial direction of motor axon outgrowth was altered by rotating the right half of the neural tube after deleting the left half. Perinotochordal mesenchyme was identified histologically or by peanut agglutinin (PNA) binding. We found that axons turned to avoid the perinotochordal mesenchyme and traversed only the dorsal–anterior sclerotome at all stages of outgrowth. When the ventral root was positioned at the midline, axons projected around the perinotochordal mesenchyme and formed spinal nerves on both sides of the embryo. Furthermore, neural crest cells and sensory axons did not penetrate perinotochordal mesenchyme, even in the absence of motor axons. In contrast, perinotochordal mesenchyme did not exhibit inhibitory function and did not differentially bind PNA when the notochord was deleted; axons ramified widely within it. We conclude that the dorsal–anterior sclerotome is permissive and that the perinotochordal mesenchyme is relatively inhibitory for the advance of axons and neural crest cells. Two additional pairs of tissues provide similar permissive/inhibitory contrasts in the embryo, the anterior/posterior sclerotome and the plexus/pelvic girdle mesenchyme. We hypothesize that guidance by all three pairs is mediated by the same set of cellular interactions and has a common molecular basis. We further propose that the transient expression of substances characteristic of these contrasting tissue pairs could serve to guide axons elsewhere, in both the peripheral and the central nervous systems. © 1990 Academic Press, Inc.

INTRODUCTION

Researchers have long sought to define the paths that guide growth cones. The emphasis has been on a search for elements confined to and thus delineating the paths, an emphasis reflected in a commonly accepted synonym for the field of axonal guidance, pathfinding. While this approach has been rewarding, researchers have recently looked up from their close examination of prospective paths to find barriers adjacent to the paths: tissues that help to constrain outgrowth by inhibiting axonal encroachment into surrounding regions (54, 55, 73, 74; see also 45). The new appreciation for the contrast between inhibitory and stimulatory environments within the embryo is likely to give a more realistic view of the complexities that control the patterns of axonal outgrowth.

When speaking of inhibitory interactions in the context of the intact embryo, we operationally define a *barrier as a tissue that growth cones will turn to avoid.* The term barrier is innocent of reference to any particular type of mechanism. It does not necessarily imply that the tissue is completely impenetrable and precludes axon advance. A barrier may be only relatively refractory to axonal invasion in comparison to the tissue that provides the normal pathway and may inhibit axonal outgrowth simply because the choices available to axons are limited within the embryo. A tissue may be suspected to act as a barrier when axons turn to avoid it during normal development or when the tissue lies along the border of a pathway. Position alone is insufficient to demonstrate inhibitory function, since neurites may change course primarily by recognizing an element within their pathway, such as specific landmark cells or axons (see 4, 48, 75). Experiments are required to demonstrate inhibitory function within the embryo. Successful strategies include deletion (removing a tissue that axons turn to avoid during normal development) and transplantation (confronting axons with a tissue placed across their normal pathway).

Despite a new focus on interactions that are relatively or even actively inhibitory, there is little direct experimental evidence that tissues inhibit axonal outgrowth within the developing embryo. One exception is the development of the peripheral projection patterns in the chick embryo, where more than one tissue has been

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shown to be refractory to axonal invasion. The developing pelvic girdle at the base of the limb bud was the first such tissue to be identified. Despite the fact that growth cones might be expected to have equal access to the limb when they gather in a plexus region that extends along the entire limb base, they enter the hindlimb only through hiatuses in the pelvic girdle precursor (73, 74). When novel entryways are experimentally provided by deleting portions of the developing pelvic girdle, axons proceed directly into the limb as though an inhibition has been removed (73; see also 31). The posterior sclerotome is also relatively inhibitory; axons become segmented to form discrete spinal nerves because growth cones traverse anterior but not posterior sclerotome both during normal development and after experimental manipulation (17, 27, 66, 68).

Because anterior sclerotome is commonly considered to be a permissive pathway, it has been surprising to find that a portion of the sclerotome within the anterior of each segment may also inhibit axonal outgrowth. Tosney and Landmesser (74) first suggested that the perinotochordal mesenchyme (the ventral–medial sclerotome that lies adjacent to the ventral border of the developing spinal nerve) may be relatively inhibitory to axonal outgrowth because it stains darkly with Alcian blue, as do the two known barrier tissues. We have also shown that peanut agglutinin lectin (PNA) binds to all three tissues but not to adjacent pathway tissues (41) and we use PNA in this study as a marker for perinotochordal mesenchyme. In addition, motor, sensory, and sympathetic preganglionic axons all grow adjacent to but avoid entering this tissue during normal development (see 68). Direct evidence for inhibitory function has been more difficult to achieve since the perinotochordal mesenchyme cannot be deleted or transplanted directly because it may gain its inhibitory nature secondarily, through an interaction with the notochord (see 10). However, when the notochord has hypertrophied and the perinotochordal mesenchyme is enlarged, axons still extend around (rather than through) the perinotochordal tissue, consistent with an inhibitory function (68).

In the current study we provide direct evidence for inhibitory function in chick embryos by (i) directly confronting outgrowing axons with the perinotochordal mesenchyme, and (ii) preventing the perinotochordal mesenchyme from developing by deleting the notochord. A brief report of some of these results has appeared (41).

METHODS

Embryonic Surgeries

Since the perinotochordal mesenchyme is thought to be induced by the notochord, our basic strategies were to shift the position of the neural tube relative to the notochord or to delete the notochord. Operations were performed at stages before axonal outgrowth (stages 15–18, by Hamburger and Hamilton (22) criteria). White leghorn chick embryos (obtained from David Bilbie, a local farmer) were lightly stained with 0.25% neutral red in distilled water. Since the neural tube is oval rather than round in cross section, it is easier to reposition the presumptive ventral root exit zone after unilateral deletion of the neural tube. The neural tube was opened dorsally with a tungsten needle and the left half of the neural tube was removed from three to eight segments using aspiration through a small micropipet (tip size, 20–40 μm). Extreme rotations of the remaining half of the neural tube were most often obtained when the entire left half had been deleted up to the floorplate. The floorplate may anchor the neural tube in place due to its close interaction with the underlying notochord at these early stages; when this interaction is disrupted, the neural tube commonly shifts its position relative to the notochord without further aid from the surgeon. In additional embryos, the notochord was deleted over two to four segments after opening the neural tube with a needle. The notochord was severed at the anterior and posterior margins of the operation site, freed from adjacent tissues, and removed with a micropipet. Operated embryos were moistened with sterile saline containing 100 units/ml of penicillin–streptomycin (GIBCO) and incubated until stages 19–30. Surgical methods are described in more detail elsewhere (70, 78).

Histology

Three histological preparations were used to examine operated embryos. In the first, operated embryos were fixed overnight in 2% glutaraldehyde in 0.1 *M* cacodylate buffer, dehydrated in graded alcohols, cleared in Hemo-D (Sigma), and embedded in paraplast ($N = 22$ embryos with successful spinal cord rotation in a total of 38 segments and notochord deletion or reduction in 8 segments). Serial, 12-μm sections were stained with cresyl violet. When viewed with fluoroscein epifluorescent optics, the neurons and neurites fluoresce bright yellow and even a few axons can be reliably visualized (see 66, 68).

The second method, described in detail in Tosney and Landmesser (77), gave the best histological preservation. Operated embryos ($N = 14$ with a total of 20 rotated segments) were fixed in 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer for 18–24 h, postfixed in 2% osmium tetroxide in 0.1 *M* cacodylate buffer overnight at 4°C, dehydrated in graded alcohols, and embedded in Epon–Araldite through propylene oxide. The embryos were serially sectioned at 25 μm with a heated steel knife on a standard rotary microtome.

A third, immunocytochemical method was used to identify axons and perinotochordal mesenchyme during

stages before the perinotochordal mesenchyme is histologically distinct. Operated embryos (N = 27 embryos with successful spinal cord rotation in 68 segments and with notochord deletion or significant reduction in eight segments) were fixed in 4% paraformaldehyde with 0.5% cetylperidinium chloride in phosphate-buffered saline (PBS) for 2–4 h at room temperature, given three 10-min washes in PBS, and infiltrated in 5% sucrose for 1–24 h and in 15% sucrose for 18–24 h at 4°C. Embryos were embedded in a solution of 7.5% gelatin and 15% sucrose in PBS after infiltration for 4–6 h at 37°C. After 24 h at 4°C the blocks were rapidly frozen in isopentane cooled on dry ice. Serial 10-μm frozen sections through the operated regions were cut with a Reichert cryostat. Selected sections were reacted with PNA (Vector) and axons were detected using a monoclonal antibody (6-11B1) directed against acetylated α-tubulin (46). PNA binding was detected using goat anti-PNA (Vector). PNA was diluted in Hepes-buffered saline (HBS: 10 mM hepes, 0.15 M NaCl, 0.1 mM $CaCl_2$) containing 1% BSA. Sections were washed three times in PBS to remove gelatin, blocked 30 min in HBS containing 1% BSA and 10% normal rabbit serum, incubated 30 min in PNA (50 μg/ml), washed three times in PBS, incubated 60 min in mixed primary antibodies (anti-PNA, 10 μg/ml; 6-11B-1, 1:10), washed three times in PBS, incubated 60 min in mixed secondary antibodies (rabbit anti-goat FITC, rabbit anti-mouse TRITC, 1:50), washed three times with PBS, and mounted in PBS:glycerol (1:1). The sections were observed and photographed with a Nikon Optiphot microscope using B-2 and G-2A filter sets for FITC and TRITC fluorescence, respectively. All antibodies were diluted in blocking solution containing 0.1% Triton X-100. Secondary antibodies with essentially no cross-reactivity were obtained from Sigma (anti-goat) and Jackson Immunoresearch (anti-mouse). The monoclonal antibody 6-11B-1 was a kind gift of Gianni Piperno.

RESULTS

General Morphology of Embryos with Rotated Half-Spinal Cords

We analyzed embryos with rotated half-spinal cords in which all the tissues peripheral to the spinal cord had developed normally. The degree to which the remaining half of the spinal cord was rotated was gauged by the position of the original floorplate, which normally lies at the midline just dorsal to the notochord. Rotations included in the analysis varied in severity from 45° (Fig. 1A) to 90° (Fig. 1B). The position of the ventral root was shifted along the medial–lateral axis as well in some embryos; axons often exited closer to the midline (Fig. 1B) and occasionally exited on the opposite side of the embryo (Figs. 1C and 1D). Operated embryos commonly had spina bifida, since tissues do not reanneal at the midline following surgery. In addition, the altered spatial relationships between the notochord and the neural tube generally led to a variety of abnormalities within the spinal cords, such as an induction of additional floorplates and secondary canals, an alteration in the positions of somata, abnormal trajectories of commissural axons, and the exit of axons from novel positions relative to the lateral motor column. These alterations in the internal morphology of the spinal cord will be the subject of a separate report. Here we analyze the response of axons to the peripheral environment.

Axons from Rotated Half-Spinal Cords Turn to Avoid the Perinotochordal Mesenchyme

We based our approach on the premise that axons confronted with a tissue that is relatively inhibitory to their advance in the embryo should turn to avoid it. To reveal a turning response, we rotated the spinal cord so that axons grew directly toward perinotochordal mesenchyme that had developed in its normal relationship to the notochord. In all cases axons reoriented their trajectories to traverse the outer circumference of the perinotochordal mesenchyme, regardless of the initial direction of their advance upon exiting from the spinal cord. To establish the parameters of the axonal response, we first discuss three classes of turning behavior observed in older embryos in which the perinotochordal mesenchyme was identified by its consistent histological appearance.

Figure 1A illustrates an example of a modest spinal cord rotation in an embryo which is otherwise normal and does not, like the majority, suffer from spina bifida. The ventral root exit zone has been rotated ventrally by about 45°. Despite the shift in their exit point, axons formed a spinal nerve that lies within the dorsal–anterior sclerotome adjacent to the outer circumference of the perinotochordal mesenchyme. Thus axons grew around rather than through the perinotochordal mesenchyme. Regardless of similar shifts in the position of axonal egress from the spinal cord in 83 segments, spinal nerves formed in normal relationship to the perinotochordal mesenchyme in all cases.

A turning response was particularly dramatic when ventral root zones were positioned at the midline as illustrated in Fig. 1B. Axons emerged from the cord with a ventral trajectory, diverged around the perinotochordal mesenchyme, and formed spinal nerves on both sides of the embryo. Split nerves like this commonly formed when the rotation was extreme. We observed 25 segments in which axons from a single lateral motor column diverged at the midline to form two spinal nerves; in seven segments, the axons had emerged from the spinal cord at a common point.

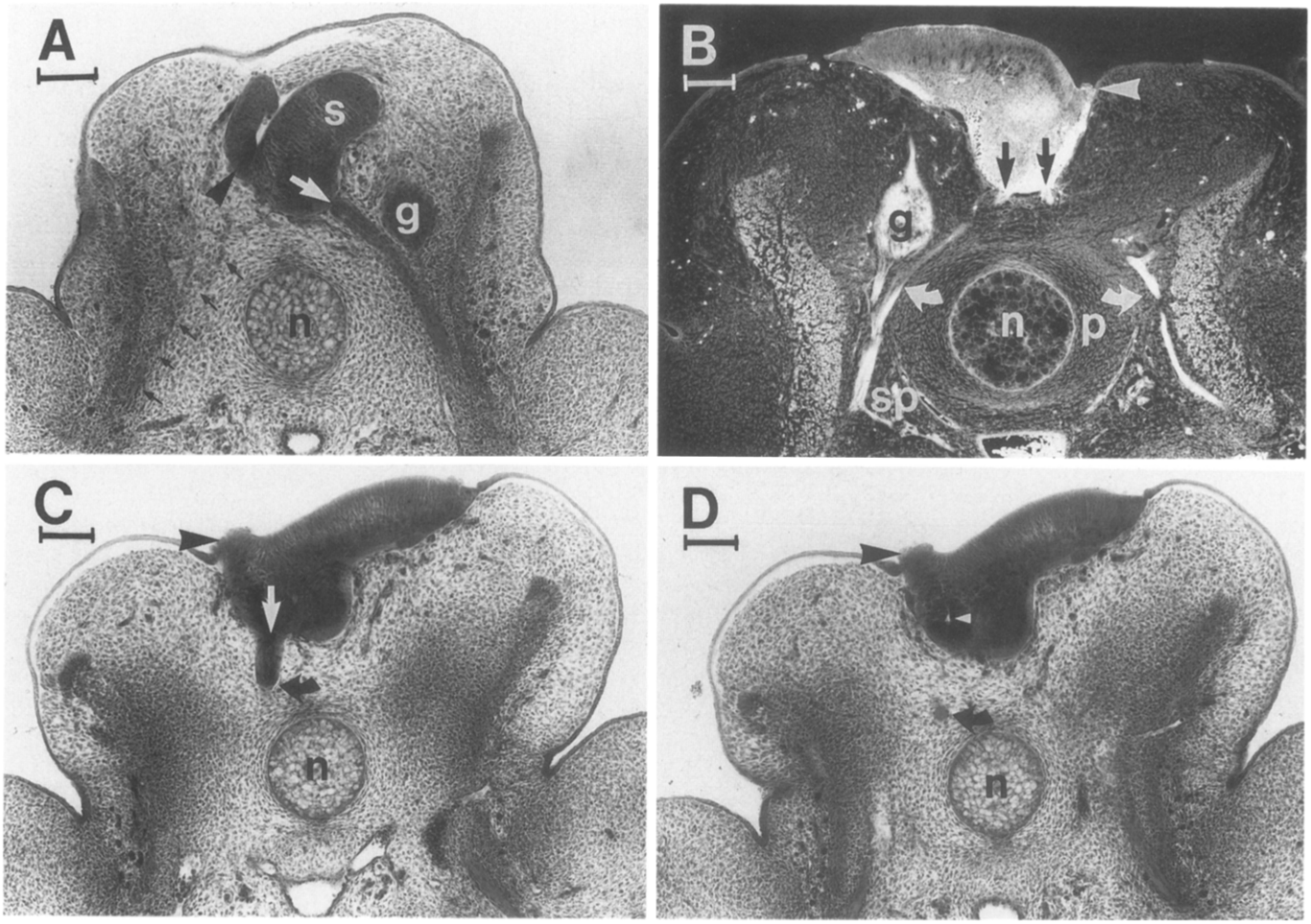


FIG. 1. Axons emerging from rotated spinal cord halves diverge around perinotochordal mesenchyme. The positions of the original floor plate (arrowhead) and the notochord (n) which normally lie adjacent to one another at the midline are marked in all figures to indicate the degree of spinal cord displacement. (A) The left lateral motor column is absent and the remaining spinal cord (s) is slightly rotated in this stage 24 embryo. The border between the dorsal–anterior sclerotome and the perinotochordal mesenchyme is indicated on the left by small arrows. Despite the shifted position of the ventral root (white arrow), axons on the right formed a spinal nerve (curved arrow) within the dorsal–anterior sclerotome that lies along this border. (B) The left half of the spinal cord is rotated 90° but appears histologically normal in this stage 28 embryo. Axons exited ventrally from two ventral root zones (black arrows) and diverged to form two spinal nerves (curved arrows), one on each side of the embryo. Sympathetic preganglionic axons (sp) diverged from the spinal nerves ventral to the perinotochordal mesenchyme. The perinotochordal mesenchyme (p) has begun to form a histologically distinct vertebral body by this stage. In both A and B, the DRG (g) has formed somewhat more ventral–lateral than normal, but is in its normal position relative to the spinal nerve. (C) Axons in the posterior of a segment exited in a ventral direction (white arrow) from the rotated half-spinal cord in this stage 25 embryo and turned toward the anterior just dorsal to the perinotochordal mesenchyme (curved arrow). In D the same nerve (curved arrow) can be seen traveling dorsal to the perinotochordal mesenchyme in a section 40 μm from its exit point. In a more anterior section this nerve diverged laterally around the perinotochordal mesenchyme and joined a spinal nerve that traversed the outer circumference of the perinotochordal mesenchyme in the anterior half of a segment. A secondary canal (small white arrowhead) has formed in this spinal cord. (A, C, D) Bright-field micrographs of plastic sections. (B) fluorescence micrograph of a paraffin section stained with cresyl violet. Calibration bars = 100 μm.

In eight segments with extreme spinal cord rotation, axons exited from the ventral midline in the posterior of a segment. As illustrated in Figs. 1C and 1D, such axons invariably turned upon confronting the more ventral perinotochordal mesenchyme and extended along the anterior–posterior axis ventral to the spinal cord. When these axons reached the anterior portion of a segment, they turned laterally within the dorsal–anterior sclerotome. These patterns of axonal extension support the conclusion that perinotochordal mesenchyme is refractory to axonal advance in both the anterior and the posterior of a segment.

Developmental Time Course of Axonal Outgrowth Relative to Perinotochordal Mesenchyme

We were concerned that the correlation between nerve patterns and perinotochordal mesenchyme that

we observed in the older embryos described above might be an artifact of the spatial pattern of tissue development. For instance, the perinotochordal mesenchyme might physically displace the spinal nerves if it developed its cartilaginous characteristics in a centrifugal direction, from the notochord outward, after the spinal nerves had formed. We therefore examined embryos with rotated half-spinal cords during the stages in which growth cones would have the opportunity to interact with the perinotochordal mesenchyme. The relevant developmental periods are well defined. The first motor axons exit the cord by stage 18 and reach the limb base by stage 21; all motor axons have traversed the sclerotome by stage 25, with the majority growing through this region during stage 22–23 (74 and our unpublished observations). We double-labeled sections with rotated cords to simultaneously visualize axons (with MAB to acetylated α-tubulin) and the perinotochordal mesenchyme (with PNA binding).

At all stages, axons avoided entering the perinotochordal mesenchyme regardless of the degree of rotation of the half-spinal cord. Figures 2A and 2B illustrate a stage 19 embryo in which the spinal cord has been shifted such that axons would be expected to take a ventral–lateral trajectory upon exiting the cord. Instead, axons projected dorsal–laterally and ramified widely within the dorsal–anterior sclerotome. In no case did axons penetrate the tissue that was more intensely labeled with PNA, even at this very early stage of outgrowth. Similarly, as shown in Figs. 2C and 2D, axons remained confined to dorsal anterior sclerotome in a stage 20 embryo, despite a larger shift in their initial position of outgrowth. Figures 2E and 2F illustrate an extreme spinal cord rotation examined at stage 21. Axons from a single lateral motor column have formed spinal nerves on both sides of the embryo that lie exterior to the distinctly labeled perinotochordal region. A more extreme shift examined at stage 25 (Figs. 2G and 2H) also resulted in the formation of two spinal nerves from a single motor column. Axons exited from positions more dorsal and more ventral than normal relative to the lateral motor column and avoided transgressing all regions that bind PNA extensively. In addition, the absence of PNA label along the axonal pathway is independent of the presence of axons (Oakley and Tosney, in preparation). For instance, even though axonal outgrowth was sparse on the left side of the embryo shown in Figs. 2G and 2H, PNA does not bind to the dorsal–anterior sclerotome that comprises the pathway. We conclude that axons turn to avoid regions that exhibit high expression of PNA-binding epitopes at all stages of their outgrowth and ramify freely within the dorsal–anterior sclerotome which expresses PNA binding to a lesser degree.

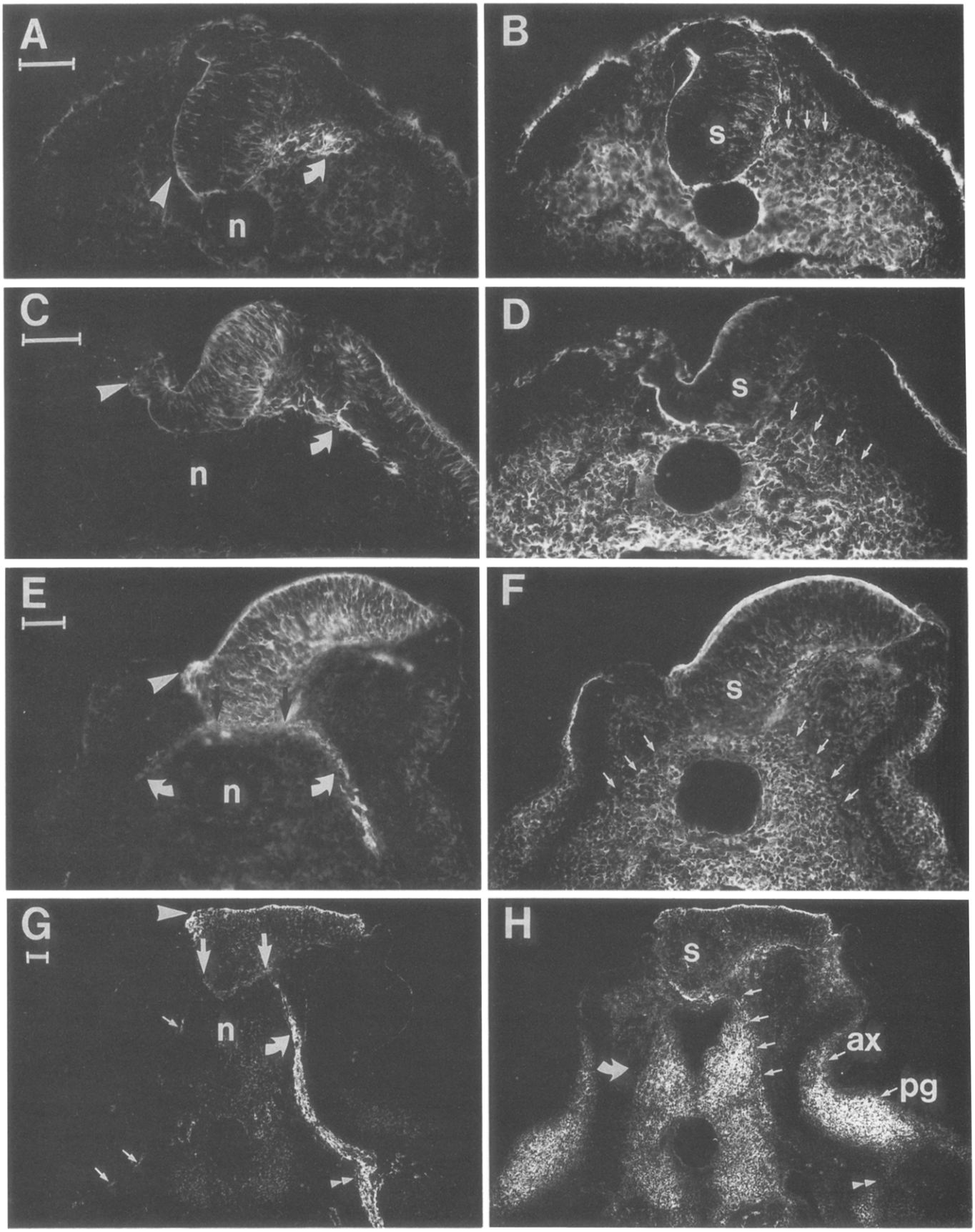
Axonal Outgrowth in the Absence of the Notochord

In segments without a notochord, axons behaved as though there were no barrier to their advance in a ventral direction. As illustrated in Fig. 3A, axons formed several fasciculated bundles that ramified widely throughout both the dorsal–anterior sclerotome and the perinotochordal region. Axons were not confined to a distinct spinal nerve pathway in the absence of the notochord. In addition, PNA binding to perinotochordal mesenchyme was reduced to a level that was more characteristic of dorsal–anterior sclerotome. For instance, the embryo without a notochord shown in Fig. 3B can be compared to an embryo of the same stage shown in Fig. 2H. PNA binds to the distal, axial dermis and the developing pelvic girdle as extensively as it normally does; however, the binding is clearly less extensive than normal in the perinotochordal mesenchyme in the absence of the notochord. There is thus a more uniform pattern of expression of the PNA-binding epitope in the absence of the notochord, and this altered pattern of expression is correlated with the spatial expansion of axonal outgrowth. These results suggest the loss of a dichotomy in the embryonic environment that may normally constrain axonal ramification. These results also suggest that the expression of the PNA-binding epitope in the perinotochordal mesenchyme is dependent on the presence of the notochord and that the PNA-binding epitope may play a role in the inhibitory function.

Neural Crest Migration and Outgrowth of Sensory Axons

During normal development, the motor axons are interposed between the neural crest cells that form the dorsal root ganglion (DRG) and the perinotochordal mesenchyme. We were able to infer the interactions of these neural crest cells with perinotochordal mesenchyme by examining embryos in which all motoneurons but few neural crest cells had been deleted on one side of the embryo. As shown in Fig. 4A, DRG formed much more ventrally than normal when the motor axons were absent, suggesting that the neural crest cells that form this ganglia had the opportunity to interact directly with the perinotochordal mesenchyme. However, the position of the ganglia indicates that the neural crest cells did not traverse this mesenchyme, even when they were given access to it. In all cases the DRG lay adjacent to the perinotochordal mesenchyme; ganglia were never observed within this mesenchyme. These results suggest that the neural crest cells that form the DRG also find the perinotochordal mesenchyme to be an inhibitory environment, in agreement with the findings of Newgreen *et al.* (40).

The more ventral position of the DRG in the absence of motor axons also suggests that outgrowing motor axons normally hinder the ventral migration of neural crest cells, as previously proposed by Loring and Erickson (38). A second observation consistent with this proposal is that DRG always developed just dorsal to the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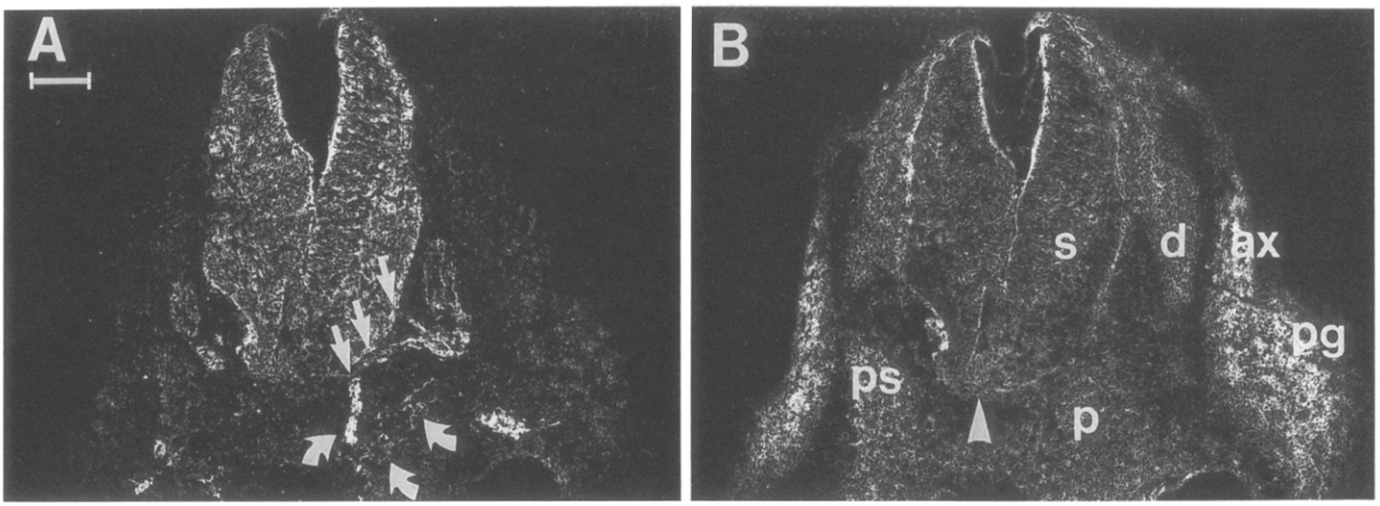

FIG. 3. Patterns of axon outgrowth in relation to PNA binding in the absence of the notochord. The notochord was successfully deleted from two segments of this stage 25 embryo. (A) Axons (curved arrows) have emerged from multiple ventral root zones (straight arrows). Axons formed several fasciculated bundles and ramified widely in the perinotochordal region despite the relatively normal spatial relationship between the spinal cord and other embryonic tissues. (B) PNA binding to the developing pelvic girdle (pg) and axial dermis (ax) is as intense as in other embryos of this stage (compare to Fig. 2H); however, the perinotochordal mesenchyme (p) is less intensely labeled and is not distinct from the dorsal–anterior sclerotome (d). The section is tangential and some differential PNA label is retained in the posterior sclerotome (ps) in the absence of the notochord. arrowhead, floorplate; s, spinal cord. Fluorescent micrographs of frozen sections. Calibration bar = 100 μm.

motor axons in the spinal nerve, even when the position of motor axons was abnormal, and even when the DRG was shifted along the medial–lateral axis (Figs. 1A and 1B).

Analysis of segments without motoneurons on one side also allowed us to assess the response of sensory axons to perinotochordal mesenchyme. These sensory axons extended adjacent to the perinotochordal mesenchyme but never invaded it, despite the more ventral position of the DRG (Fig. 4B). As shown with immunocytochemical labels in Figures 4C and 4D, sensory axons remained within the spinal nerve pathway and did not enter those embryonic tissues displaying extensive PNA binding. Our results are thus in accord with the proposition that the perinotochordal mesenchyme is a barrier to the advance of motor axons, sensory axons, and neural crest cells.

DISCUSSION

Response of Motoneurons to the Perinotochordal Mesenchyme

To test the hypothesis that the perinotochordal mesenchyme acts as a barrier to axon advance, we surgically directed a confrontation between motoneuron growth cones and this tissue *in vivo*. In all cases, axons turned to avoid the perinotochordal mesenchyme. In embryos

FIG. 2. Developmental time course of the relation between axonal outgrowth and the pattern of PNA binding following rotation of half-spinal cords. The same section is labeled to show axons (figures on the left; MAB 6-11B-1 to acetylated α-tubulin and a TRITC-labeled secondary antibody) and the PNA binding pattern (figures on the right; PNA binding indicated with an antibody to PNA and a FITC-labeled secondary antibody). The position of the original floorplate (arrowhead) relative to the notochord (n) indicates the degree of spinal cord displacement in figures on the left; small arrows indicate the approximate border between the dorsal–anterior sclerotome and the perinotochordal mesenchyme in figures on the right. At each stage, axons ramify within the dorsal–anterior sclerotome which exhibits little PNA binding, but not within the more intensely labeled perinotochordal mesenchyme. (A, B) In this stage 19 embryo, the rotation of the spinal cord would predict a somewhat ventral axonal trajectory; however, the first axons (curved arrow) projected dorsal–laterally and ramified widely within the dorsal–anterior sclerotome. (C, D) In this stage 20 embryo, axons (curved arrow) were again confined to the dorsal–anterior sclerotome, despite the spinal cord rotation. (E, F) By stage 21, differential PNA staining of perinotochordal mesenchyme has become quite distinct. Axons have exited ventrally (black arrows in E) and are forming spinal nerves (curved arrows in E) that traverse the outer circumference of the perinotochordal mesenchyme on both sides of the embryo. (G, H) By stage 25, PNA intensely labels perinotochordal mesenchyme, the lateral portions of the axial dermis (ax), and the developing pelvic girdle (pg). Axons exited this rotated half-spinal cord from two positions (straight arrows in G). Despite the reduction in axon outgrowth on the left side of the embryo (small arrows in G), the spinal nerve pathway (curved arrow in H) remains free of PNA binding. On the right side of the embryo, the spinal nerve (curved arrow in G) is in its normal position relative to the perinotochordal mesenchyme. Axons have spread in the plexus region (small double-arrowhead in G) and begun to colonize the limb through a PNA-free hiatus in the developing pelvic girdle (small double arrowhead in H). s, spinal cord. Fluorescent micrographs of frozen sections. Calibration bars = 50 μm.

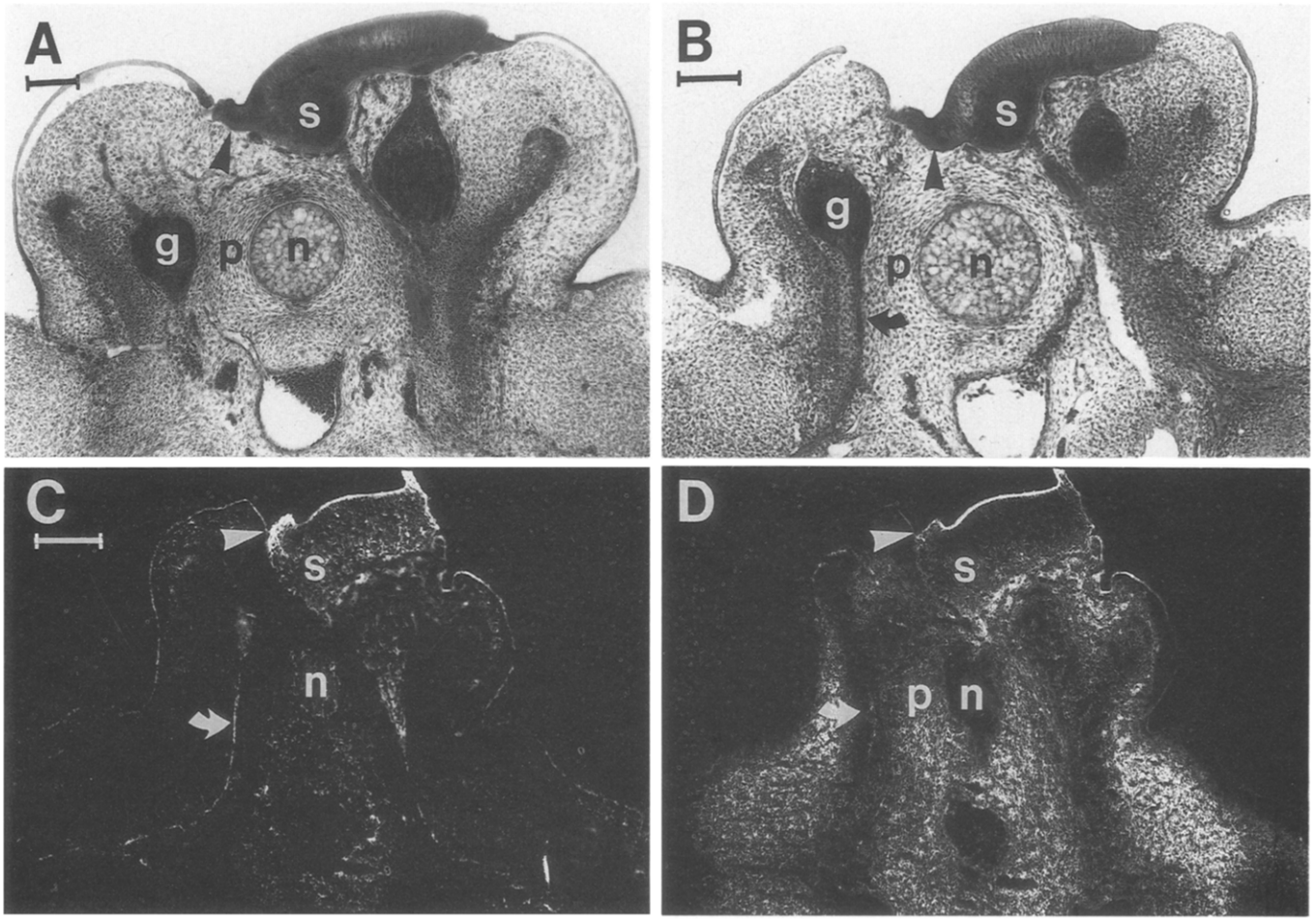

FIG. 4. Relation of DRG formation and sensory axon outgrowth to perinotochordal mesenchyme in the absence of motor axon outgrowth. (A) In the absence of motor axon outgrowth on the left side of this stage 24 embryo, the DRG (g) has formed farther ventral than normal, just lateral to the perinotochordal mesenchyme (p). (B) Sensory axons (curved arrow) from a DRG (g) in another stage 24 embryo remain in the spinal nerve pathway and do not transgress the perinotochordal tissue (p), despite the more ventral position of the ganglion. A section labeled for axons (C) and for PNA binding (D) shows a stage 25 embryo in which motor axons are absent on the left side of the embryo. Sensory axons (curved arrow in C) form a spinal nerve in a region with low PNA label (curved arrow in D) and do not extend into the intensely labeled perinotochordal mesenchyme (p). arrowheads, original floorplate; n, notochord; s, spinal cord. (A, B) Bright-field micrographs of plastic sections. (C, D) Fluorescent micrographs of frozen sections. Calibration bars = 100 μm.

with moderate spinal cord rotations, the avoidance behavior was characterized by a deviation from the expected trajectory. In embryos with severe spinal cord rotations, single or multiple ventrally directed motor roots from a single lateral motor column projected to either side of the perinotochordal mesenchyme. In no case did motor axons penetrate the perinotochordal mesenchyme. Moreover, this avoidance behavior was detected at very early stages of axon outgrowth, which indicates that the patterns described at later stages of development are not the result of differential growth and axon displacement. These results strongly suggest that the perinotochordal mesenchyme is a relatively inhibitory environment, particularly in contrast to the dorsal anterior sclerotome which supports extensive axonal ramification.

We also found that the inhibitory nature of the perinotochordal mesenchyme may be dependent on the presence of the notochord. In the absence of the notochord, some axons projected ventrally through the perinotochordal region. This apparent lack of barrier function was correlated with an altered PNA-binding pattern such that the perinotochordal mesenchyme no longer differentially bound the lectin, thus leading to a uniform and diffuse pattern of PNA binding within the entire anterior half of the sclerotome while PNA binding remained normal in other tissues. These results suggest that an important contrast in the environment is lost in the absence of the notochord and are consistent with the possibility that the PNA-binding epitope plays a role in the inhibitory function. However, the notochord has

been shown to have an inductive effect on the differentiation of the sclerotome and to increase the synthesis and deposition of proteoglycans and glycosaminoglycans (GAGs) typical of chondrogenic differentiation (10, 82). Any or all of these components may be involved in barrier function. It is also noteworthy that several GAGs are poor substrata for axonal outgrowth (9) and that DRG axons will turn to avoid a keratan sulfate/chondroitin sulfate proteoglycan in order to remain on a laminin substratum *in vitro* (55).

Response of Neural Crest Cells and Their Derivatives to Perinotochordal Mesenchyme

Newgreen and his colleagues (40; see also 38) were the first to show that neural crest advances into dorsal–anterior sclerotome but avoids the region around the notochord. In addition, *in vitro* studies have shown that the extracellular matrix elements that are produced by the notochord are poor substrata for neural crest cells (19, 40, 81). Our results are also in accord with the proposition that neural crest cells, like motor axons, find the perinotochordal mesenchyme to be a relatively inhibitory environment.

An inhibition of advance by perinotochordal mesenchyme is not, however, the only environmental interaction that prevents neural crest cells from advancing completely through the sclerotome rather than stopping to form DRG. As suggested by Loring and Erickson (38), motor axons may normally occlude the ventral pathway, prevent these neural crest cells from interacting directly with the perinotochordal tissue, and help to assure that ganglia condense in the proper position. Our demonstration that DRG form farther ventrally in the absence of the motor axons is in full accord with this suggestion. These results further suggest that the dependence of DRG development on the spinal cord reported by Kalcheim and Le Dourain (25) may be due to the outgrowth of axons from the spinal cord as well as to a chemotrophic interaction. Erickson and her colleagues (personal communication) are currently testing this hypothesis more completely.

The perinotochordal mesenchyme may also be a barrier to the advance of sensory axons, but the evidence is less direct. In the absence of motoneurons, sensory axons extended adjacent to the perinotochordal mesenchyme but never invaded it, even though the DRG was in a more ventral position and was in direct contact with the perinotochordal mesenchyme. These results suggest that the response of sensory axons to their environment is independent and is not a function of their propensity to fasciculate with motor axons (see 76).

COMMENTARY

Differences within the sclerotome populations have heretofore been almost exclusively interpreted in terms of segmentation. Our demonstration that a portion of the sclerotome acts as a barrier to axonal advance even in the anterior of a segment leads us to reinterpret such studies and to put forth a more encompassing hypothesis. We find it compelling that three pairs of tissues provide contrasting inhibitory/permissive environments, the perinotochordal mesenchyme/dorsal–anterior sclerotome, the plexus/pelvic girdle mesenchyme, and the anterior/posterior sclerotome (see Fig. 5). We think it unlikely that each pair of tissues guides populations by a different mechanism. We think that the mechanisms are the same and, moreover, that the mechanisms are not confined to these examples alone. We hypothesize that *the general guidance of a variety of cells and axons is mediated by the same set of cellular interactions and has a common molecular basis.* In this commentary, we marshall evidence in support of this unifying hypothesis.

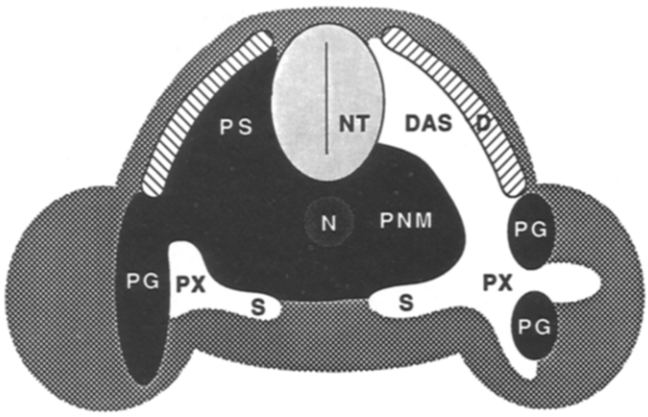

FIG. 5. Schematic cross section showing the relationships among permissive and inhibitory tissues in the hindlimb region of the chick embryo. The distribution of permissive (white) and relatively inhibitory (black) tissues and the sequence of events described are summarized from references in the text and from our unpublished studies. The posterior sclerotome in each segment (PS; left side of diagram) exhibits inhibitory activity first as neural crest cells emerging from the dorsal neural tube avoid it and enter the more permissive environment of the dorsal–anterior sclerotome (DAS; right side of diagram). Some of these neural crest cells migrate quickly to the sympathetic region (S) shortly before motor axons grow out and before the perinotochordal mesenchyme expresses differential PNA binding. The motor axons then traverse the dorsal–anterior sclerotome at the outer margin of the perinotochordal mesenchyme and form the spinal nerves of each segment; however, these lose their segmental organization as they spread within the unsegmented plexus region (PX) that extends along the anterior–posterior axis at the limb base. Axons enter the limb in accord with the distribution of the pelvic girdle (PG) mesenchyme. The right side of the diagram shows the crural plexus region in which axons extend through a hiatus in the pelvic girdle to form the dorsal nerve trunk and extend ventral to the girdle to form the ventral nerve trunk. The left side shows a region of the limb in which the pelvic girdle bars access to the limb along the entire dorsal–ventral axis. N, notochord; NT, neural tube/spinal cord; D, dermamyotome/myotome.

General Guidance by Contrasting Permissive versus Barrier Tissues

Axonal guidance cues can be classified as general or specific (see 33 for review). *General cues* comprise a

"public highway system" that channels a variety of populations down common paths. In contrast, *specific cues,* like signposts along highways, are essential for particular populations to diverge into appropriate branches of a path (at intersections) and at targets (exit ramps). General cues are by definition nonspecific since they guide a variety of axons. Nevertheless, they contribute significantly to the specificity of innervation. They assure that populations are channeled into regions where they can respond to population-specific cues and, conversely, restrict the response to inappropriate cues by spatially restricting axonal outgrowth.

It is the contrast between perinotochordal mesenchyme and dorsal–anterior sclerotome that provides general guidance to establish the dorsal–ventral position of the spinal nerves. Likewise, the contrast between anterior and posterior sclerotome establishes the anterior–posterior position of spinal nerves, and the contrast between pelvic girdle and plexus mesenchyme establishes the nerve trunk position (see Fig. 5). All three contrasting pairs of tissues are vital for the general rather than for the specific pattern of axon outgrowth; limb muscles can be innervated specifically even when all sclerotome populations or the pelvic girdle mesenchyme are deleted (68, 73; see also 31). We propose that all three pairs of tissues present a *dichotomy* that gives axons a choice between two environments, one of which supports outgrowth and one of which does not and that it is the contrast between these environments that establishes the gross anatomical pattern of outgrowth.

A variety of axonal populations respond similarly and independently to each of these contrasting tissue pairs, consistent with a common mechanism. Sensory axons travel the same pathways in the absence of motor axons; motor axons respond to sclerotome populations in the absence of sensory axons or of neural crest cells (34, 49, 68, 72, and the current report). In no case do sensory, motor, or sympathetic preganglionic axons, regardless of their precise target specificity, find a relatively inhibitory tissue to be permissive for their outgrowth in the embryo (see 68).

Neural crest cells respond to the same strictures as well (see Fig. 5). For instance, neural crest cells advance into anterior but not posterior sclerotome (8, 38, 40, 49), into dorsal–anterior sclerotome but not perinotochordal mesenchyme (38, 40, and the current report) and avoid the pelvic girdle mesenchyme (Erickson, personal communication). Moreover, neural crest cells respond independently to sclerotome populations (68, and the current report). The similar response of a variety of axons and of neural crest cells to these contrasting tissue pairs is suggestive of a common mechanism of guidance.

Common Environments Due to Common Developmental Fates

A basic similarity in these contrasting tissues is suggested by embryonic surgeries. Deletion of one set of tissue pairs may alter but not prevent patterned outgrowth because axons then have access to a more distal set of tissues. For instance, when all sclerotome populations are removed, axons and neural crest cells still advance; they enter the unsegmented plexus mesenchyme where they form correspondingly unsegmented patterns (68). We propose that the contrasting tissue pairs can substitute for one another because they present essentially the same physical and chemical environment and differ in respect to general guidance only by their position and orientation in the embryo.

These tissues do have characteristic and contrasting developmental fates and we propose that the dichotomy between the permissive and inhibitory environments is due to the different sets of molecules that are expressed as a consequence of these different fates. The barrier tissues exclusively form a single derivative—cartilage which later forms bone. The pelvic girdle is an obvious example; the perinotochordal mesenchyme forms the vertebral body (see 1, 3, 68, 80). The relative contributions of the more dorsal portions of both posterior and anterior sclerotome to the vertebral arches have not been examined systematically, although current studies suggest that the dorsal anterior sclerotome contributes relatively little to this derivative (1, 3, 14).

The permissive tissues also exhibit considerable similarity. Although their developmental fates have not been studied in detail, all three may give rise to loose connective tissue. For instance, the dorsal–anterior sclerotome has been suggested to form the loose connective tissue of the interarch space surrounding the DRG and the ventral roots (14). The plexus mesenchyme is derived from lateral plate which also contributes to the connective tissue of the limb (see 32). An additional similarity is the widespread death and phagocytosis of cells within all three permissive tissues during the period of axonal outgrowth (78). The phagocytes in these tissues could contribute directly to their permissiveness by removing substances or cells or by secreting growth factors or other substances.

Common Molecular Mechanisms

Permissive tissues and barrier tissues also express distinct molecular profiles (Table 1) that are likely to be related to their different developmental fates and to their differential function in guidance. It should be noted that in many cases the references listed in Table 1 do not directly address the issues raised here and the information in brackets is based on our examination of published figures.

A variety of early chondrogenic markers are expressed by barrier tissues during or just after axon outgrowth. Intense Alcian blue staining of GAGs typifies precartilaginous tissues and is characteristic of all three barrier

TABLE 1

Molecular Profiles of Pathway and Barrier Tissues

Molecular marker	Probe (reference)	Distal pathway	Sclerotome		PNM	Girdle	Early cartilage
			Ant	Post			
Gal β 1-3 GalNAC	PNA (41, 57)	−	−	+	+	+	+
CTB proteoglycan	pAB (61, 24)	?	−	+	[+]	+	+
Hylauronectin	pAB (16)	?	*	*	+	?	+
Hylauonic acid	Hylauronectin (20) Link protein (50)	[−]	*	*	+	[+]	[+]
Type II collagen	mAB 2B1 (29) cDNA (59, 60) pAB (43)	[−]	[−]	[+]	+	[+]	+
KS/CS proteoglycan	pAB (43)	?	?	?	+	?	+
Cytotactin/tenascin[a]	pAB (13, 39, 58, 61) mAB M1 (11)	[−]	−/+	+/−	[+]	[+]	+
Fibronectin	pAB (49, 51, 64, 65)	?	+	+	+	[+]	+
Laminin	pAB (49, 51)	?	+	+	[+]	?	?
N-CAM	mAB (79)	+	+	+	−	−	−
Butyrylcholinesterase	Histochemical (35)	?	+	−	[−]	?	?
70-kDa membrane protein	mAB M7412 (62)	+	+	−	−	−	−

Note. This table summarizes molecular differences between tissues known to comprise peripheral axon paths or barriers. Note that barrier tissues (posterior sclerotome, perinotochordal mesenchyme, and girdle precursor) all share similarities with early cartilage, whereas pathway tissues (anterior sclerotome and more distal pathways do not (lines 1–7). Note also that two epitopes (entries 11 and 12) mark pathway but not barrier tissues. Three adhesive molecules (entries 8–10) do not obviously correlate with either paths or barriers. Abbreviations used: Ant, anterior; CTB, cytotactin binding; KS/CS, keratan sulfate/chondroitin sulfate; mAb, monoclonal antibody; N-CAM, neural cell adhesion molecule; pAB, polyclonal antibodies; PNM, perinotochordal mesenchyme; Post, posterior. Symbols used: [ ] indicates our interpretation of published figures; ? indicates that information is not available; * indicates that this molecule is present in early sclerotome but that a differential distribution in anterior or posterior has not been assessed.

[a] Stern and colleagues (58) have recently reported that cytotactin/tenascin is initially expressed in posterior sclerotome and only becomes polarized to the anterior sclerotome following neural crest migration.

tissues but not of permissive tissues (74). As suggested under Discussion, proteoglycans and GAGs are likely to contribute directly to the inhibitory nature of barrier tissues. In addition, all three barrier tissues preferentially bind PNA, whereas the adjacent permissive tissues do not bind this lectin (Oakley and Tosney (41) and in preparation). The differential binding of PNA to posterior but not anterior sclerotome was first reported by Stern *et al.* (57) and this reagent has recently been recognized as an early but not exclusive marker of chondrogenic differentiation (2, 83). The early expression of PNA-binding epitopes in barrier tissues at developmental stages relevant to axonal guidance (41) and the coordinated loss of PNA binding and barrier function that we document in the present report raise the possibility that these epitopes are directly involved in constraining axonal pathways. The recent isolation of several PNA-binding molecules (7, 15, 24) provides an excellent opportunity to assess the role of such molecules in guidance. Since chondroitin sulfate proteoglycans (CSPGs) are generally characteristic of early chondrogenic differentiation (43), it is particularly interesting that one PNA-binding molecule is a CSPG that is preferentially distributed in posterior sclerotome (61) and that also appears to be differentially expressed in the perinotochordal mesenchyme of the anterior sclerotome (see Table 1).

Although the permissive tissues are less well characterized at the molecular level, two antigens have been identified as being differentially distributed in the anterior sclerotome (Table 1). The epitope identified by Tanaka and his colleagues (62) is especially interesting since it is present in all of the permissive tissues, including the distal pathways, prior to axonal outgrowth.

Common Cellular Interactions

Documenting an inhibitory role for a tissue within the embryo does not define the type of cellular interaction responsible. It is important to directly establish what cellular interactions mediate guidance to eventually understand how environmental cues act on the growth cone to control its trajectory and to hone the search for the most appropriate molecular candidates. Only by systematically examining the interactions of a variety of invasive populations with the contrasting tissue pairs can we determine whether similar cellular mechanisms do in fact mediate general guidance.

The cellular interactions that mediate the response of axons to pairs of contrasting tissues have been addressed

only in regard to the anterior and posterior sclerotome and these studies form a baseline for tests of the hypothesis. It is particularly interesting that cellular interactions that *actively* inhibit growth cone motility have in general been ruled out. Posterior sclerotome is not a totally nonpermissive substratum, since growth cones will extend on it, though reluctantly (42, 57). Physical barriers or channels are not present (Tosney (68) and unpublished observations). Toxins and diffusible substances that inhibit or repel outgrowth are ruled out by analysis of motor axon outgrowth in a relatively intact culture preparation (Oakley and Tosney (42) and in preparation). Contact paralysis, an active inhibition that is associated with physical collapse of the growth cone (see 26), has been ruled out as well by time-lapse microscopy of dissociated cells (67 and Oakley and Tosney, unpublished observations). The barrier function that the posterior sclerotome plays within the embryo is evidently not due to its direct and active inhibition of growth cone motility. If common interactions do mediate the general guidance, then such actively inhibitory interactions should be ruled out in other cases as well. We therefore and with due caution refer to tissues that act as barriers within the context of the embryo as being *relatively* rather than actively inhibitory.

Three types of interactions provide promising candidates for general guidance by the contrasting tissue pairs. First, there is direct evidence for substratum preference; motor growth cones prefer anterior over posterior sclerotome as a substratum both in dissociated culture and when contacting a border between populations in culture (42, 67). Neural crest cells could be guided by a similar preference, since cells as well as growth cones exhibit substratum preference in culture (23) and since both DRG axons and neural crest cells find several types of GAGs and proteoglycans to be relatively poor substrata (9, 19, 40, 55, 81). Second, the anterior sclerotome may supply a diffusible substance that orients and stimulates axonal outgrowth (Oakley and Tosney (42) and in preparation). Third, a tissue may be more permissive because it is more susceptible to degradation by the advancing population. Growth cones secrete proteases such as plasminogen activator (30, 47) and protease activity is essential for neural crest cells to advance through gels in culture (18). The hypothesis predicts that the same *sets* of cellular interactions that mediate the segmentation of motor axons will mediate the response to all three contrasting tissue pairs. There is no *a priori* reason to suppose that there is only one essential interaction, and all three of the above interactions may contribute.

A common set of cellular interactions does not directly imply common molecular mechanisms. A common cellular interaction could be mediated by the same or by different subsets of molecules within each functional class of tissues. Even when the cellular interactions and candidate molecules are identified, the precise molecular mechanism must still be more directly elucidated by using further experimental approaches. For instance, if substratum preference is identified as an important and common mechanism, then it is likely that the cartilaginous components of barrier tissues contribute to the poverty of these environments as substrata for advance. However, such molecules could inhibit axonal advance by a variety of molecular mechanisms. For example, they could decrease the average adhesivity of the substratum, they could limit access to more preferred substrata such as laminin and fibronectin (which may be present in both types of tissues; see Table 1), or they could be less susceptible to degradation. In addition, different neuronal populations could be differentially susceptible to the various molecular components that are common to the barrier tissues. The identification of the cellular interactions that mediate general guidance is thus seen as a first step in the test of our hypothesis; knowledge of the pertinent interactions at the cellular level will in turn direct a more focused search for the relevant molecules and their mechanism of action and will establish to what extent the cellular and molecular mechanisms are common to general guidance.

The Utility and Implications of the Hypothesis

Hierarchies of guidance cues with a range of permissiveness have been suggested to contribute to axonal guidance in nearly every system studied (for examples, see 5, 6, 33, 44, 74, 75). What is new here is the proposal that a single set of definable cues channels a wide variety of populations. If this is so, the problem of general guidance would be considerably simplified and fewer population-specific cues would need to be posited. To discuss additional implications with brevity, we primarily focus on the premise that one or more molecules characteristic of early cartilage differentiation are responsible for the relatively inhibitory nature of a tissue. Programing growth cones and neural crest cells to avoid regions where such molecules are expressed would have profound consequences.

First, spatial patterns of axonal and neural crest outgrowth would be *adapted to the architecture of the organism.* A seemingly trivial example emphasizes this point: without axonal segmentation, spinal nerves would ramify within the vertebrae (rather than through interarch spaces) and be damaged during normal movements of the organism.

Second, *evolutionary* changes in the shape or position of bones would not require reprogramming of neuronal or neural crest properties. The hypothesis thus predicts that ontological correlations between axonal outgrowth and the development of cartilaginous structures would be widely conserved.

Third, the inhibitory substances may *control the temporal accessibility of permissive environments.* If these substances were expressed in permissive tissues at later times, they could decrease the permissiveness of the environment and consequently cause the cessation of axonal and cell advance and promote the condensation of neural crest cells to form ganglia. This proposal is consistent with reports that cytotactin/tenascin, which is known to inhibit neural crest migration (21), is expressed only in the posterior half of the sclerotome during the early period when neural crest cells and axons begin to advance, but is later expressed in the dorsal–anterior sclerotome during the period when neural crest cells are beginning to condense to form DRG (58, 61) (see Table 1). This suggests that the same inhibitory molecules may be responsible both for the segmentation of axonal and neural crest populations and for the condensation of ganglia.

A fourth and very intriguing implication is that *the same mechanism could operate throughout the embryo.* If a wide variety of axons avoid tissues expressing early cartilage markers, then the *transient* expression of such markers in tissues like the central nervous system (CNS) could establish many of the general projection patterns. Two observations in accord with this possibility are that keratan sulfate is transiently expressed in the roof plate of the spinal cord during the period when it is thought to act as a barrier to the outgrowth of commissural and sensory axons (55) and that PNA epitopes are transiently expressed in the mouse somatosensory barrel cortex and in a variety of other CNS sites where they have been hypothesized to form boundaries (12, 56). In addition, a temporal change in the environment that correlates with a transition in the expression of extracellular matrix elements has been shown to be essential for the advance of neural crest and for the outgrowth of cutaneous axons (37, 71).

Fifth, the hypothesis easily integrates with an additional layer of complexity to *predict the nature of additional, more specific cues.* While axons prefer anterior over posterior sclerotome as a substratum, the choice seems to be between bad and worse, since axons extend with much greater facility on a wide range of other cell types and substrata than they do on anterior sclerotome (42). If the basic choice between these permissive/inhibitory environments is near the bottom of any hierarchy of preferences, then axonal and cell populations that have entered any of the permissive tissues would be within a permissive but still rather hostile environment. Consequently, they would readily respond to additional, more positive guidance cues. We expect, therefore, that only specific cues that actually stimulate advance would cause populations to choose between two adjacent permissive environments. Conversely, actively inhibitory cues would be required for axons to leave a more preferred substratum and advance into an environment that was permissive, but only poorly so.

A final implication concerns the adult nervous system. The same inhibitory molecules might also play a role in *stabilizing interactions by preventing inappropriate sprouting,* a possibility consistent with the binding of PNA to the neuromuscular junction (28). Moreover, if such molecules were expressed anomalously as the result of disease or injury, they would *contribute to the generation of pathological barriers* that prevent regeneration and recovery. This possibility is consistent with reports of GAGs such as keratan sulfate and other inhibitory molecules in the adult or injured CNS (see 36, 52, 53).

This commentary integrates divergent observations on axonal guidance, cell migration, morphogenesis, and molecular analysis to establish a unifying and novel hypothesis. This hypothesis identifies a promising line of research that should help to reveal the cellular and molecular mechanisms that establish general patterns within the central and peripheral nervous systems.

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REFERENCES

1. AOYAMA, H., AND K. ASAMOTO. 1988. Determination of somite cells: Independence of cell differentiation and morphogenesis. *Development* **104:** 15–28.
2. AULTHOUSE, A. L., AND M. SOLURSH. 1987. The detection of a precartilage, blastema-specific marker. *Dev. Biol.* **120:** 377–384.
3. BAGNALL, K. M., S. J. HIGGINS, AND E. J. SANDERS. 1988. The contribution made by a single somite to the vertebral column: Experimental evidence in support of resegmentation using the chick–quail chimaera model. *Development* **103:** 69–85.
4. BASTIANI, M. J., J. A. RAPER, AND C. S. GOODMAN. 1984. Pathfinding by neuronal growth cones in grasshopper embryos. III. Selective affinity of the G growth cone for the P cells within the A/P fascicle. *J. Neurosci.* **4:** 2311–1328.
5. BENTLEY, D., AND M. CAUDY. 1984. Navigational substrates for peripheral pioneer growth cones: Limb–axis polarity cues, limb–segment boundaries and guidepost neurons. *Cold Spring Harbor Symp. Quant. Biol.* **48:** 573–585.
6. BERLOT, J., AND C. S. GOODMAN. 1984. Guidance of peripheral pioneer neurons in the grasshopper: Adhesive hierarchy of epithelial and neural substrates. *Science* **233:** 493–496.
7. BONHOEFFER, F. 1989. Remarks made during a symposium talk entitled "Inhibitory influences on growth cones and cells." *Soc. Neurosci. Abstr.* **15:** 1106.
8. BRONNER-FRASER, M. 1986. Analysis of the early stages of trunk neural crest migration in avian embryos using monoclonal antibody HNK-1. *Dev. Biol.* **115:** 44–55.

9. CARBONETTO, S., M. M. GRUVER, AND D. C. TURNER. 1983. Nerve fiber growth in cultures on fibronectin, collagen and glycosaminoglycan substrates. *J. Neurosci.* **3:** 2324–2335.
10. CHENEY, C. M., AND J. W. LASH. 1981. Diversification within embryonic chick somites: Differential response to notochord. *Dev. Biol.* **81:** 288–298.
11. CHIQUET, M., AND D. M. FAMBROUGH. 1984. Chick myotendinous antigen. I. A monoclonal antibody as a marker for tendon and muscle morphogenesis. *J. Cell Biol.* **98:** 1926–1936.
12. COOPER, N. G. F., AND D. A. STEINDLER. 1986. Lectins demarcate the barrel subfield in the somatosensory cortex of the early postnatal mouse. *J. Comp. Neurol.* **249:** 157–169.
13. CROSSIN, K. L., S. HOFFMAN, M. GRUMET, J.-P. THIERY, AND G. M. EDELMAN. 1986. Site-restricted expression of cytotactin during development of the chicken embryo. *J. Cell Biol.* **102:** 1917–1930.
14. DALGLEISH, A. E. 1985. A study of the development of thoracic vertebrae in the mouse assisted by autoradiography. *Acta Anat.* **122:** 91–98.
15. DAVIES, J. A., S. A. WAJED, G. M. W. COOK, C. D. STERN, AND R. J. KEYNES. 1989. Isolation of a peanut agglutinin-binding glycoprotein fraction which inhibits axon outgrowth in the chick embryo. *Soc. Neurosci. Abstr.* **15:** 331.
16. DELPECH, A., AND B. DELPECH. 1984. Expression of hyaluronic acid-binding glycoprotein, hyaluronectin, in the developing rat embryo. *Dev. Biol.* **101:** 391–400.
17. DETWILER, S. R. 1934. An experimental study of spinal nerve segmentation in *Amblystoma* with reference to the plurisegmental contribution to the brachial plexus. *J. Exp. Zool.* **67:** 395–441.
18. ERICKSON, C. A., AND R. R. ISSEROFF. 1989. Plasminogen activator activity is associated with neural crest cell motility in tissue culture. *J. Exp. Zool.* **251:** 123–133.
19. ERICKSON, C. A., AND E. A. TURLEY. 1983. Substrata formed by combinations of extracellular matrix components alter neural crest cell motility in vitro. *J. Cell Sci.* **61:** 299–323.
20. GIRARD, N., A. DELPECH, AND B. DELPECH. 1986. Characterization of hyaluronic acid on tissue sections with hyaluronectin. *J. Histochem. Cytochem.* **34:** 539–541.
21. HALFTER, W., R. CHIQUET-EHRISMANN, AND R. P. TUCKER. 1989. The effect of tenascin and embryonic basal lamina on the behavior and morphology of neural crest cells in vitro. *Dev. Biol.* **132:** 14–25.
22. HAMBURGER, V., AND H. L. HAMILTON. 1951. A series of normal stages in the development of the chick embryo. *J. Morphol.* **88:** 49–92.
23. HARRIS, A. 1973. Behavior of cultured cells on substrata of variable adhesiveness. *Exp. Cell Res.* **77:** 285–297.
24. HOFFMAN, S., K. L. CROSSIN, AND G. M. EDELMAN. 1988. Molecular forms, binding functions, and developmental expression patterns of cytotactin and cytotactin-binding proteoglycan, an interactive pair of extracellular matrix molecules. *J. Cell Biol.* **106:** 519–532.
25. KALCHEIM, C., AND N. M. LE DOURAIN. 1986. Requirement of a neural tube signal for the differentiation of neural crest cells into dorsal root ganglia. *Dev. Biol.* **116:** 451–466.
26. KAPFHAMMER, J. P., AND J. A. RAPER. 1987. Collapse of growth cone structure on contact with specific neurites in culture. *J. Neurosci.* **7:** 201–213.
27. KEYNES, R. J., AND C. D. STERN. 1984. Segmentation in the vertebrate nervous system. *Nature (London)* **310:** 786–789.
28. KO. C-P. 1987. A lectin, peanut agglutinin, as a probe for the extracellular matrix in living neuromuscular junctions. *J. Neurocytol.* **16:** 567–576.
29. KOSHER, R. A., AND M. SOLURSH. 1989. Widespread distribution of type II collagen during embryonic chick development. *Dev. Biol.* **131:** 558–566.
30. KRYOSTOSEK, A., AND N. W. SEEDS. 1981. Plasminogen activator release at the neuronal growth cone. *Science* **213:** 1532–1534.
31. LANCE-JONES, C. J. 1986. Motoneuron projection patterns in chick embryonic limbs with a double complement of dorsal thigh musculature. *Dev. Biol.* **116:** 387–406.
32. LANCE-JONES, C. J. 1988. Motoneuron axon guidance: Development of specific projections to two muscles in the embryonic chick limb. *Brain. Behav. Evol.* **31:** 209–217.
33. LANDMESSER, L. T. 1985. The development of specific motor pathways in the chick embryo. *Trends Neurosci.* **7:** 336–339.
34. LANDMESSER, L., AND M. G. HONIG. 1986. Altered sensory projections in the chick hind limb following the early removal of motoneurons. *Dev. Biol.* **118:** 511–531.
35. LAYER, P. G., R. ALBER, AND F. G. RATHJEN. 1988. Sequential activation of butyrulcholinesterase in rostral half somites and acetylcholinesterase in motoneurones and myotomes preceding growth of motor axons. *Development* **102:** 387–396.
36. LIU, H. M., K. ANDERSON, AND B. CATERSON. 1987. Demonstration of a keratan sulfate proteoglycan and a mannose-rich glycoconjugate in corpora amylacea of the brain by immunocytochemical and lectin-binding methods. *J. Neuroimmunol.* **14:** 49–60.
37. LOFBERG, J., A. NYNAS-MCCOY, C. OLSSON, L. JONSSON, AND R. PERRIS. 1985. Stimulation of initial neural crest cell migration by tissue grafts and extracellular matrix transplanted on microcarriers. *Dev. Biol.* **107:** 442–459.
38. LORING, J. F., AND ERICKSON, C. A. 1987. Neural crest cell migratory pathways in the chick embryo. *Dev. Biol.* **121:** 230–236.
39. MACKIE, E. J., R. P. TUCKER, W. HALFTER, R. CHIQUET-EHRISMANN, AND H. H. EPPERLEIN. 1988. The distribution of tenascin coincides with pathways of neural crest cell migration. *Development* **102:** 237–250.
40. NEWGREEN, D. F., M. SCHEEL, AND V. KASTNER. 1986. Morphogenesis of sclerotome and neural crest in avian embryos. In vivo and in vitro studies on the role of notochordal extracellular material. *Cell Tissue Res.* **244:** 299–313.
41. OAKLEY, R. A., AND K. W. TOSNEY. 1988. Peanut agglutinin (PNA) binds to tissues that act as barriers to axon advance in the chick embryo. *Soc. Neurosci. Abstr.* **14:** 870.
42. OAKLEY, R. A., AND K. W. TOSNEY. 1989. Analysis of the cellular interactions that mediate the development of motor axon segmentation. *Soc. Neurosci. Abstr.* **15:** 875.
43. OETTINGER, H. F., G. THAL, J. SASSE, H. HOLTZER, AND M. PACIFICI. 1985. Immunological analysis of chick notochord and cartilage matrix development with antisera to cartilage matrix macromolecules. *Dev. Biol.* **109:** 63–71.
44. PALKA, J. 1987. Axon guidance in the insect periphery. *Development* **99:** 307–309.
45. PATTERSON, P. H. 1988. On the importance of being inhibited, or saying no to growth cones. *Neuron* **1:** 263–267.
46. PIPERNO, G., M. LEDIZET, AND X. CHANG. 1987. Microtubules containing acetylated α-tubulin in mammalian cells in culture. *J. Cell Biol.* **104:** 289–302.
47. PITTMAN, R. N. 1985. Release of plasminogen activator and a calcium-dependent metalloprotease from cultured sympathetic and sensory neurons. *Dev. Biol.* **110:** 91–101.
48. RAPER, J. A., M. BASTIANI, AND C. S. GOODMAN. 1983. Pathfinding by neuronal growth cones in grasshopper embryos. II. Selective fasciculation onto specific axonal pathways. *J. Neurosci.* **3:** 31–41.

49. RICKMANN, M., J. W. FAUCET, AND R. J. KEYNES. 1985. The migration of neural crest cells and the growth of motor axons through the rostral half of the chick somite. *J. Embryol. Exp. Morphol.* **90:** 437–455.
50. RIPELLINO, J. A., M. M. KLINGER, R. U. MARGOLIS, AND R. K. MARGOLIS. 1985. The hyaluronic acid binding region as a specific probe for the localization of hyaluronic acid in tissue sections. Application to chick embryo and rat brain. *J. Histochem. Cytochem.* **33:** 1060–1066.
51. ROGERS, S. L., K. J. EDSON, P. C. LETOURNEAU, AND S. C. MCLOON. 1986. Distribution of laminin in the developing peripheral nervous system of the chick. *Dev. Biol.* **113:** 429–435.
52. RUDGE, J. S., G. M. SMITH, AND J. SILVER. 1989. An *in vitro* model of wound healing in the CNS: Analysis of cell reaction and interaction at different ages. *Exp. Neurol.* **103:** 1–16.
53. SCHWAB, M. E., AND P. CARONI. 1988. Oligodendrocytes and CNS myelin are nonpermissive substrates for neurite growth and fibroblast spreading in vitro. *J. Neurosci.* **8:** 2381–2393.
54. SILVER, J., M. POSTON, AND U. RUTISHAUSER. 1987. Axon pathway boundries in the developing brain. I. Cellular and molecular determinants that separate the optic and olfactory projections. *J. Neurosci.* **7:** 2264–2272.
55. SNOW, D. M., V. LEMMON, D. A. CARRINO, A. I. CAPLAN, AND J. SILVER. 1990. Sulfated proteoglycans in astroglial barriers inhibit neurite outgrowth *in vitro*. *Exp. Neurol.* **109:** 111–130.
56. STEINDLER, D. A., AND N. G. F. COOPER. 1987. Glial and glycoconjugate boundaries during postnatal development of the central nervous system. *Dev. Brain Res.* **36:** 27–38.
57. STERN, C. D., S. M. SISODIYA, AND R. J. KEYNES. 1986. Interactions between neurites and somite cells: Inhibition and stimulation of nerve growth in the chick embryo. *J. Embryol. Exp. Morphol.* **91:** 209–226.
58. STERN, C. D., W. E. NORRIS, M. BRONNER-FRASER, G. J. CARLSON, A. FAISSNER, R. J. KEYNES, AND M. SCHACHNER. 1989. J1/tenascin-related molecules are not responsible for the segmented pattern of neural crest cells or motor axons in the chick embryo. *Development* **107:** 309–319.
59. STIRPE, N. A., AND P. F. GOETINCK. 1989. Gene regulation during cartilage differentiation: Temporal and spatial expression of link protein and cartilage matrix protein in the developing limb. *Development* **107:** 23–33.
60. SWALLA, B. J., W. B. UPHOLT, AND M. SOLURSH. 1988. Analysis of type II collagen RNA localization in chick wing buds by *in situ* hybridization. *Dev. Biol.* **125:** 51–58.
61. TAN, S. S., K. L. CROSSIN, S. HOFFMAN, AND G. M. EDELMAN. 1987. Asymmetric expression in somites of cytotactin and its proteoglycan ligand is correlated with neural crest distribution. *Proc. Natl. Acad. Sci. USA* **84:** 7977–7981.
62. TANAKA, H., A. AGATA, AND K. OBATA. 1989. A new membrane antigen revealed by monoclonal antibodies is associated with motoneuron axonal pathways. *Dev. Biol.* **132:** 419–435.
63. TEILLET, M-A., C. KALCHEIM, AND N. LE DOUARIN. 1987. Formation of the dorsal root ganglia in the avian embryo: Segmental origin and migratory behavior of neural crest progenitor cells. *Dev. Biol.* **120:** 329–347.
64. THIERY, J. P., J. L. DUBAND, AND A. DELOUVEE. 1982. Pathways and mechanisms of avian trunk neural crest cell migration and localization. *Dev. Biol.* **93:** 324–434.
65. THOMASEK, J. J., J. E. MAZURKIEWICZ, AND S. A. NEWMAN. 1982. Nonuniform distribution of fibronectin during avian limb development. *Dev. Biol.* **90:** 118–126.
66. TOSNEY, K. W. 1987. Proximal tissues and patterned neurite outgrowth at the lumbosacral level of the chick embryo: Deletion of the dermamyotome. *Dev. Biol.* **122:** 540–588.
67. TOSNEY, K. W. 1987. Growth cone interactions with anterior and posterior sclerotome. *Soc. Neurosci. Abstr.* **13:** 253.
68. TOSNEY, K. W. 1988. Proximal tissues and patterned neurite outgrowth at the lumbosacral level of the chick embryo: Partial and complete deletion of the somite. *Dev. Biol.* **127:** 266–286.
69. TOSNEY, K. W. 1988. Somites and axon guidance. *Scan. Microsc.* **2**(88): 427–442.
70. TOSNEY, K. W. 1988. How to dissect an egg. Or, a beginners' guide to embryonic surgery. *Fine Sci. Points* **2:** 1–4.
71. TOSNEY, K. W. 1989. Experimental evidence that target maturity can influence axon outgrowth in vivo. *Soc. Neurosci. Abstr.* **15:** 1029.
72. TOSNEY, K. W., AND M. HAGEMAN. 1989. Different subsets of axonal guidance cues are essential for sensory neurite outgrowth to cutaneous and muscle targets in the dorsal ramus of the embryonic chick. *J. Exp. Zool.* **251:** 232–244.
73. TOSNEY, K. W., AND L. T. LANDMESSER. 1984. Pattern and specificity of axonal outgrowth following varying degrees of chick limb bud ablation. *J. Neurosci.* **4:** 2158–2527.
74. TOSNEY, K. W., AND L. T. LANDMESSER. 1985. Development of the major pathways for neurite outgrowth in the chick hindlimb. *Dev. Biol.* **109:** 193–214.
75. TOSNEY, K. W., AND L. T. LANDMESSER. 1985. Specificity of motoneuron growth cone outgrowth in the chick hindlimb. *J. Neurosci.* **5:** 2336–2344.
76. TOSNEY, K. W., AND L. T. LANDMESSER. 1985. Growth cone morphology and trajectory during outgrowth into the chick limb. *J. Neurosci.* **4:** 2345–2358.
77. TOSNEY, K. W., AND L. T. LANDMESSER. 1986. Neurites and growth cones in the chick embryo: Enhanced tissue preservation and visualization of HRP-labeled subpopulations in serial 25 μm plastic sections cut on a rotary microtome. *J. Histochem. Cytochem.* **34:** 953–957.
78. TOSNEY, K. W., S. SCHROETER, AND J. A. POKRZYWINSKI. 1988. Cell death delineates axon pathways in the hindlimb and does so independently of neurite outgrowth. *Dev. Biol.* **130:** 558–572.
79. TOSNEY, K. W., M. WATANABE, L. LANDMESSER, AND U. RUTISHAUSER. 1986. The distribution of NCAM in the chick hind limb during axon outgrowth and synaptogenesis. *Dev. Biol.* **114:** 437–452.
80. TRELSTAD, R. L. 1977. Mesenchymal cell polarity and morphogenesis of chick cartilage. *Dev. Biol.* **59:** 153–163.
81. TUCKER, R. P., AND C. A. ERICKSON. 1984. Morphological behavior of quail crest cells in artificial three-dimensional extracellular matrices. *Dev. Biol.* **104:** 390–405.
82. VASAN, N., K. M. LAMB, AND O. LA MANNA. 1986. Somite chondrogenesis in vitro. 1. Alterations in proteoglycan synthesis. *Cell Differ.* **18:** 79–90.
83. ZIMMERMANN, B., AND M. THIES. 1984. Alterations of lectin binding during chondrogenesis of mouse limb buds. *Histochemistry* **81:** 353–361.