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Surround Repulsion of Spinal Sensory Axons in Higher Vertebrate Embryos

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Summary

We have tested whether the orientation of axons sprouting from bipolar dorsal root ganglion neurons is influenced by diffusible cues from surrounding tissues. Surface ectoderm, dermomyotome, and notochord exert strong chemorepulsion on axons growing in collagen gels, operating at separations beyond those found in vivo and active in cocultures of chick and mouse tissues. Basal and alar plates of the neural tube are devoid of activity, as is the posterior-half-sclerotome, which repels in a contact-dependent manner. When ganglia are sandwiched between dermomyotome and notochord placed at a distance, axon growth is channeled in a bipolar trajectory. These results show that gradients of diffusible repulsion molecules flanking axon pathways can generate linear patterns of axon growth. We suggest that such "surround repulsion" may function generally, in concert with contact-dependent guidance mechanisms, to guide axons in the developing nervous system.

Introduction

There is now good evidence that axons are guided to their targets during development by a combination of diffusible and membrane-bound cues that are attractive or repulsive to growth cones (Keynes and Cook, 1995; Goodman, 1996; Tessier-Lavigne and Goodman, 1996). For individual axon populations, examples of each mechanism have been identified in both vertebrate and invertebrate model systems, and in vivo, it seems likely that growth cones integrate contact and diffusible guidance mechanisms to direct their axons.

Guidance mechanisms determine both linear and nonlinear axon trajectories. In the case of linear growth, axons may fasciculate with each other, and this process is controlled by expression of signaling molecules that regulate attraction and repulsion (Tessier-Lavigne and Goodman, 1996; Rutishauser and Landmesser, 1996). In three dimensions, the overall vector of growth is likely to be determined by the balance of attractive and repulsive forces operating on the earliest growth cones, but for individual systems, their relative contributions are unclear. Here, we address this question using the developing dorsal root ganglion (DRG) of higher vertebrate embryos as an experimental model for investigating the mechanisms that control linear growth.

DRGs develop from aggregates of neural crest cells that remain in the anterior half-sclerotomes following the earlier ventral migration of trunk neural crest cells into the somites (Le Douarin, 1982; Keynes and Stern, 1984; Rickmann et al., 1985; Bronner-Fraser, 1993). During differentiation, they adopt a characteristic position adjacent to the neural tube, medial to the dermomyotome, dorsolateral to the notochord, and sandwiched between adjacent posterior half-sclerotomes (Figure 1A). Individual neurons sprout axons within the dorsoventral plane of the anterior half-somite in a highly stereotyped manner, well described by Ramon y Cajal (1909; see also Levi-Montalcini and Levi, 1943; Tello, 1947). Following a transient phase (termed "stellate" by Cajal), when short extensions are generated in random orientations by the differentiating neurons, the definitive growth cones are produced at opposite poles of the cell (Cajal's "bipolar" phase). One growth cone orients and grows in a dorsomedial direction toward the dorsal root entry zone of the spinal cord, where it enters the central nervous system, while the other is directed ventrolaterally toward the ventral edge of the dermomyotome. In both cases, growth takes place within the anterior half of each somite-derived sclerotome, where the parent ganglia develop (Keynes and Stern, 1984).

Outgrowing spinal sensory and motor axons are known to be confined to the anterior (A) half-sclerotomes by differences they detect between anterior- and posterior (P) half-sclerotome cells (Keynes and Stern, 1984). A detergent-soluble glycoprotein fraction (M_r 48 K and 55 K) derived from posterior cells has been shown to cause collapse of sensory axon growth cones and inhibition of neurite extension in vitro (Davies et al., 1990). In vivo, axons may be contact repelled by this material if they encounter posterior sclerotome cells at the A/P boundaries within and between somites, so confining their growth to the anterior half-sclerotomes (Keynes et al., 1996; see also Discussion). Contact repulsion by posterior cells may prevent axons from growing inappropriately along the A/P embryonic axis, generating spinal nerve segmentation, but it is unlikely to be responsible simultaneously for determining the dorsoventral orientation of axons.

We have therefore investigated whether other cell populations in the immediate vicinity of the sprouting DRG neurons influence axon trajectories. Using collagen gel cocultures of explanted DRGs and neighboring tissues from both chick and mouse embryos, we find that tissues both immediately lateral (dermomyotome, surface ectoderm) and medial (notochord) to the DRGs exert strong chemorepulsion on sensory axons. In contrast, posterior half-sclerotome does not exert long-distance repulsion on axons. The bulk of the epithelium of the early spinal cord is devoid of either chemoattractive (alar plate) or chemorepulsive (basal plate) activity, although the floor plate may exert some chemorepulsion.

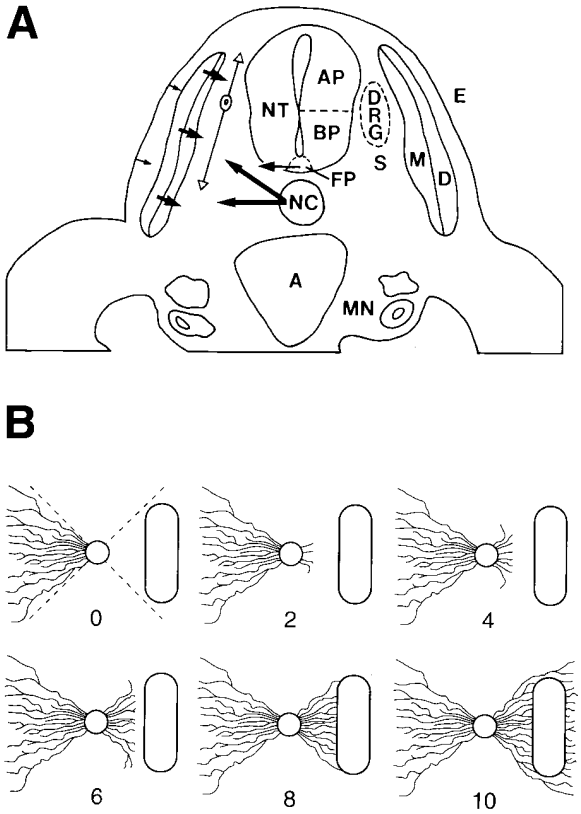

Figure 1. Chemorepulsion of Dorsal Root Ganglion Axons by Surrounding Tissues

(A) The diagram shows a chick embryo in transverse section at the level of the anterior half-sclerotome. DRG, dorsal root ganglion; E, ectoderm; D, dermatome; M, myotome (D and M together comprise the dermomyotome); S, sclerotome; NC, notochord; NT, neural tube; AP, alar plate; BP, basal plate; FP, floor plate; MN, mesonephros; and A, aorta. The left half of the figure illustrates the model for chemorepulsion of sprouting sensory neurons by surrounding tissues (see Discussion). Chemorepellents are secreted into the anterior half-sclerotome by the dermomyotome and notochord (thick arrows), with contributions from the ectoderm and floor plate. Bipolar DRG neurons sprout axons in dorsomedial and ventrolateral directions (open arrowheads).

(B) Scheme for quantitation of chemorepulsion. Cultures were assessed under phase contrast optics, and each confrontation between tissues was scored between 0 and 10 as follows: 0—no or very little axon outgrowth in the proximal quadrant (right) compared with the distal quadrant (left; dotted lines delineate quadrants); 2—few axon outgrowths in the proximal quadrant, strong asymmetry compared with the distal quadrant; 4—greater outgrowth in the proximal quadrant, but axons are still >50 μm distant from the target and strong asymmetry comparing proximal and distal quadrants; 6—growth in the proximal quadrant approaches target (<50 μm) but does not contact it, asymmetry persists; 8—axons contact target, asymmetry is still detectable; and 10—axons have grown beyond the target, no asymmetry. Scores of 8 and above indicate no chemorepulsion. The minimum distance separating the edge of the DRG from that of the coexplant was measured using a calibrated eyepiece graticule. In some experiments, the minimum distance separating the front of axons in the proximal quadrant from the edge of the coexplant was also measured. In the case of the "sandwich" experiments (Figure 4D), axon growth in both lateral quadrants was also assessed.

We suggest that, while the A/P pattern of axon growth is determined by contact repulsion, the early dorsoventral orientation of DRG axons is established predominantly by chemorepulsion from the dermomyotome/ectoderm and notochord. Repulsion by surrounding tissues ("surround repulsion") may therefore be an important determinant of linear axon trajectories in the three axes of the embryo.

Results

Unless stated otherwise, cultures were assessed using the criteria shown in Figure 1B.

Chemorepulsion of DRG Axons by Dermomyotome and Ectoderm

When a stage 28 chick DRG is placed in a collagen gel matrix and cultured in the presence of the neurotrophins NGF, BDNF, and NT-3, axons extend radially in three dimensions (Figure 2A). In contrast, when a DRG is coexplanted with the tissue that lies immediately lateral to it in the trunk region of the early embryo, the dermomyotome/ectoderm, axons are strongly repelled in the region of gel between the DRG and dermomyotome/ectoderm (Figures 2B, 2C, and 2E). Repulsion is maintained by both dermomyotome and ectoderm when these are physically separated from each other before explantation and tested individually (Figure 2E). It is detectable at separations of up to 500 μm and is seen clearly with dermomyotome/ectoderm explants dissected from single somites (Figures 2C and 2E). Specificity of the effect with respect to axon type is shown by the finding that repulsion by dermomyotome/ectoderm is not exerted on stage 36 (E10) chick olfactory axons (Figures 2D and 2E).

In these cultures, the DRG and dermomyotome/ectoderm explants were placed within 225 ± 15 μm ($n = 47$) of each other (see Figure 1B legend), and the density of axon growth from the DRG in the proximal quadrant, adjacent to the dermomyotome/ectoderm, was markedly less than that in the distal quadrant (Figures 2B and 2C). This raises the possibility that, rather than repelling axon growth, the dermomyotome/ectoderm inhibits it. We therefore tested for repulsion by placing the DRGs at a greater distance (mean 856 ± 27 μm, $n = 16$) from the dermomyotome/ectoderm explants, beyond the range of repulsion and allowing approximately equal densities of axon growth adjacent to the DRG in the proximal and distal quadrants. Under these conditions, axons grew to within 253 ± 29 μm of the target, at which point they turned within the gel and grew circumferentially, creating a "halo" region devoid of axons around the target (Figures 3A and 3B). This turning behavior is more consistent with repulsion than with growth inhibition, although these are not necessarily mutually exclusive mechanisms (Goodman, 1996; Wang et al., 1996).

DRGs first sprout axons in vivo when newly differentiated sensory neurons coalesce in the anterior half-sclerotomes. We tested whether repulsion is exerted on these early axons by coculturing anterior half-sclerotomes, dissected from stage 17–19 embryos, with dermomyotome/ectoderm. Repulsion in this case was significantly stronger than that seen using stage 28 DRGs,

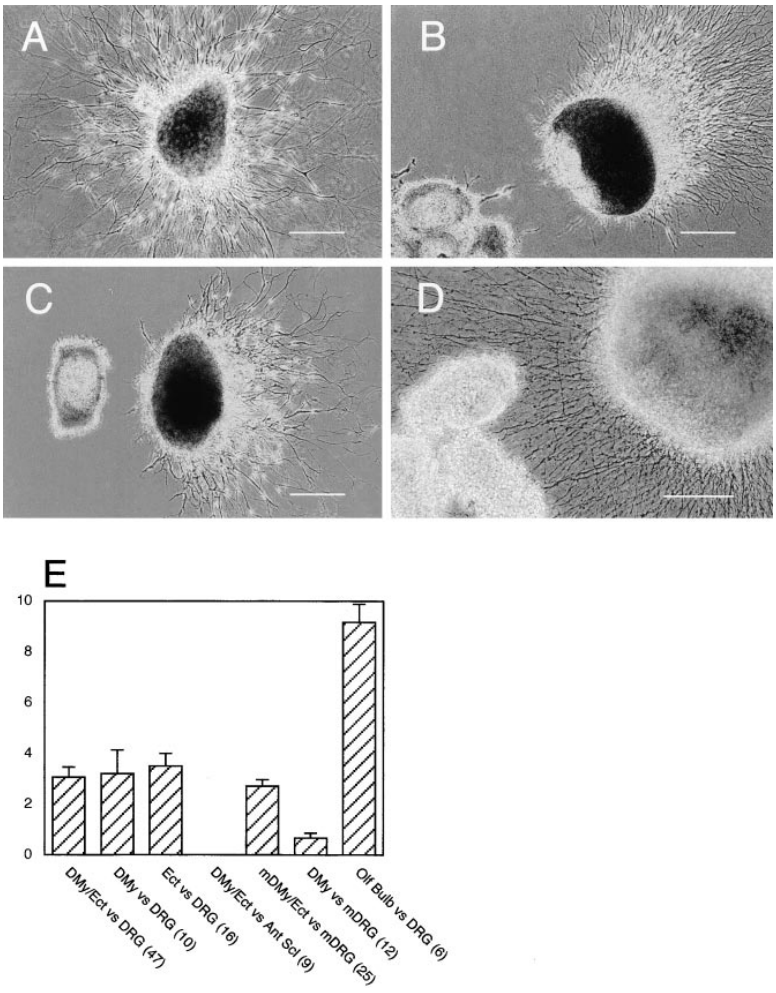

Figure 2. Chemorepulsion of Chick DRG Axons, But Not Olfactory Bulb Axons, by Dermomyotome/Ectoderm

(A) Stage 28 chick DRG cultured for 24 hr in collagen in the presence of 50 ng/ml NGF, BDNF, and NT-3. Axons extend radially in three dimensions, in and out of the focal plane.

(B) Stage 28 chick DRG (right) cocultured with a group of four dermomyotomes and attached ectoderm. Strong chemorepulsion of DRG axons is seen.

(C) Stage 28 chick DRG (right) cocultured with dermomyotome dissected from a single somite together with overlying ectoderm. Strong chemorepulsion is still visible.

(D) Stage 36 olfactory bulb (right) cocultured with a group of three dermomyotomes and attached ectoderm. Scale bar = 200 μm (A–D).

(E) Quantification of axon growth in cocultures, according to the scheme shown in Figure 1B. Each histogram shows the mean score + SEM (ordinate) for a set of cocultures between tissues indicated as follows: DMy, chick dermomyotome; Ect, chick ectoderm; DRG, chick dorsal root ganglion; Ant Scl, chick anterior half-sclerotome; mDMy/Ect, mouse dermomyotome/ectoderm; mDRG, mouse DRG; and Olf bulb, chick olfactory bulb. Single dermomyotome/ectoderm explants were used. The number in parentheses indicates the number of separate confrontations between tissues.

axon outgrowth being absent in the quadrant facing the target (Figure 2E). Repulsion was also seen when DRGs from E14 mouse embryos were cultured with dermomyotome/ectoderm from E11 mouse embryos (Figure 2E). Finally, E14 mouse DRG axons were repelled by chick dermomyotome/ectoderm explants (Figure 2E), showing conservation of the mechanisms underlying this repulsion between birds and mammals.

Chemorepulsion of DRG Axons by Notochord

To test further the possibility that DRG axon growth may be influenced by tissues that surround the DRG in vivo, stage 28 DRGs were cocultured with lengths (400–600 μm) of notochord from stage 17–19 chick embryos. Like dermomyotome/ectoderm, a striking chemorepulsive effect of the notochord was seen (Figure 4), detectable at the maximum separation tested of 360 μm. In principle, this activity could derive from the notochord cells, and/or residual sclerotome-derived perinotochordal cells, or notochordal sheath components. To test whether notochord cells are chemorepulsive in isolation, they were extruded from the cut end of the notochordal sheath by gentle mechanical pressure prior to explantation; chemorepulsion was found to be maintained (Figure 4B). Also like dermomyotome/ectoderm, chick notochord generated a halo region devoid of axons when DRGs were placed beyond range of its chemorepulsive activity (Figure 4C), consistent with a repulsive rather than inhibitory mechanism. Finally, chick notochord exerted strong repulsion on axons growing from newly formed DRGs/anterior half-sclerotome dissected from stage 17–19 chick embryos, and on those growing from E14 mouse DRGs (Figure 4E).

If DRG axons can be oriented by the combined action of chemorepulsion from the dermomyotome/ectoderm and notochord in vivo, it should be possible to replicate such directed growth by sandwiching a DRG between a single dermomyotome/ectoderm explant and a length of notochord. As predicted, under these conditions, axons projected from opposite poles of the DRG in a direction parallel to the two sources of repellent (Figure 4D). Using the scoring system shown in Figure 1B, in eight experiments, we found values of 2.2 ± 0.7 for the quadrant facing the notochord, 3.1 ± 0.6 for the quadrant facing the dermomyotome/ectoderm, and 10.0 for all lateral quadrants not facing either source of repellent.

Posterior Half-Sclerotome Does Not Chemorepel DRG Axons

In contrast to the repulsive action of dermomyotome/ectoderm and notochord, explants of chick posterior half-sclerotome exerted no significant long-distance

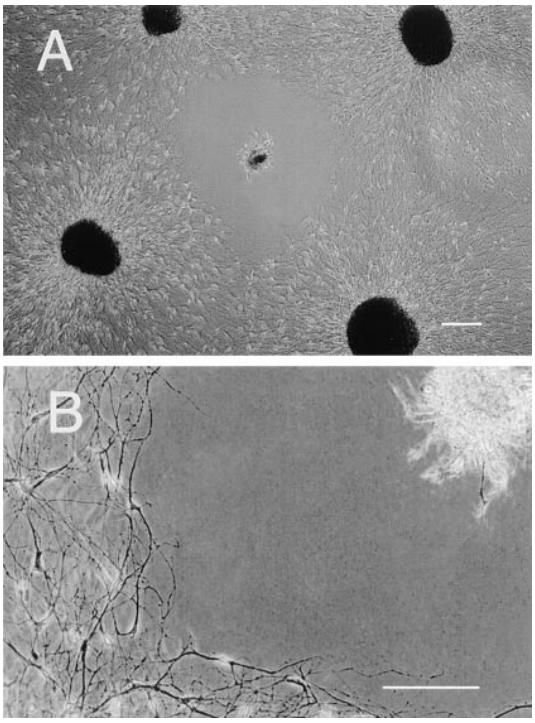

Figure 3. Axons Turn within the Collagen Gel to Avoid Dermomyotome/Ectoderm at a Distance

(A) Four stage 28 DRGs, placed beyond the range of chemorepulsion, extend normal numbers of axons toward the dermomyotome/ectoderm (centre), but avoid it when within range of chemorepulsion, generating a "halo" region devoid of axons around the target.

(B) Axons have turned within the collagen gel and are growing circumferentially. Scale bar = 200 μm ([A] and [B]).

repulsion on axons growing from stage 28 DRGs or from stage 17–19 anterior half-sclerotomes (Figure 5). Axons were seen to grow both toward and over the sclerotome explants and often contacted them, even when cocultured with several half-sclerotomes. Mouse posterior half-sclerotome was likewise devoid of repulsive activity, as were fragments of chick stage 29 thoracic vertebral column, a tissue derived from posterior half-sclerotome (Figure 5B). We also sought possible chemorepulsive activity from the mesonephros, a tissue that sensory axons avoid as they project in a ventrolateral direction from the DRG (see Figure 1A), but none was seen (Figure 5B).

DRG Cocultures with Neural Tube

In view of the known chemorepulsion of chick DRG axons by stage 28 basal plate (Shepherd et al., 1997), it was important to test whether basal plate from earlier embryos, coinciding with the initial period of DRG axon growth outside the neural tube, is also repulsive for DRG axons. Lengths of stage 17–19 neural tube were dissected into pieces comprised of two (left and right) basal plates joined ventrally by the floor plate, and were found to be devoid of chemorepulsive activity when the DRGs were placed near the basal plate epithelium (Figure 6A). In most cases, it was possible to distinguish outgrowing DRG axons from those of the basal plate using phase contrast optics, but the result was confirmed using orthograde labeling of DRG axons by DiA in several coexplants (Figures 6B and 6C). When DRGs were placed opposite the floor plate, a repulsive effect was detectable but was significantly weaker than the dermomyotome/ectoderm/notochord activities (Figure 6D).

Centrally projecting DRG axons target the dorsal root entry zone of the dorsal spinal cord (alar plate) before entering the central nervous system. The possibility that the alar plate epithelium might be chemoattractive for DRG axons was therefore tested. Axon outgrowth from stage 17–19 anterior half-sclerotomes, in the presence of the neurotrophins NGF, BDNF, and NT-3, was diminished overall compared with that from stage 28 DRGs, but was unaffected by the presence nearby of alar plate epithelium from embryos of the same stage and from the same A/P position (Figure 6A). In the absence of neurotrophins, anterior half-sclerotomes in 13 coexplants failed to sprout axons whether or not the alar plate was cocultured with them, while stage 28 DRGs produced axons but showed no show chemoattraction toward the alar plate (13 coexplants). These experiments did not, therefore, reveal the presence of chemoattractants for DRG axons derived from the dorsal neural tube.

Analysis of Candidate Chemorepellents

A number of molecules have been identified previously that are candidates for mediating the chemorepulsive activities derived from dermomyotome/ectoderm and notochord. These are chondroitin sulfate proteoglycans (CSPGs), the secreted glycoprotein sonic hedgehog (Shh), and collapsin-1/sema III/semD (see Discussion). The contribution of CSPGs to the repulsion of DRG axons in the present study was tested using both a neutralizing monoclonal anti-CSPG antibody and the glycosylation inhibitor β-D-xyloside (Fichard et al., 1991). Neither reagent affected repulsion by dermomyotome/ectoderm or notochord (data not shown). Shh was tested as a candidate notochord-derived chemorepellent, and COS cells expressing the N-terminal domain of Shh were found to be devoid of chemorepulsive activity (data not shown).

Discussion

The main findings of this study are that the dermomyotome/ectoderm and notochord, tissues that lie respectively dorsolateral and ventromedial to the DRG in the embryo, exert strong chemorepulsion on DRG axons cultured in the presence of NGF, BDNF, and NT-3. The effect is strong in the sense that it is easily detected in coexplants of DRGs with single pieces of dermomyotome/ectoderm and notochord, and operates over distances considerably greater than those separating DRGs from the dermomyotome and notochord in the embryo. Repulsion by the dermomyotome/ectoderm, for example, can be detected in vitro at distances up to 500 μm, the maximum tested, compared with a maximum distance in the dorsolateral–ventromedial axis in vivo (stage 18 chick, midthoracic) of ~75 μm between the far boundary of the early developing DRG and the dermatome/myotome boundary; equivalent figures for

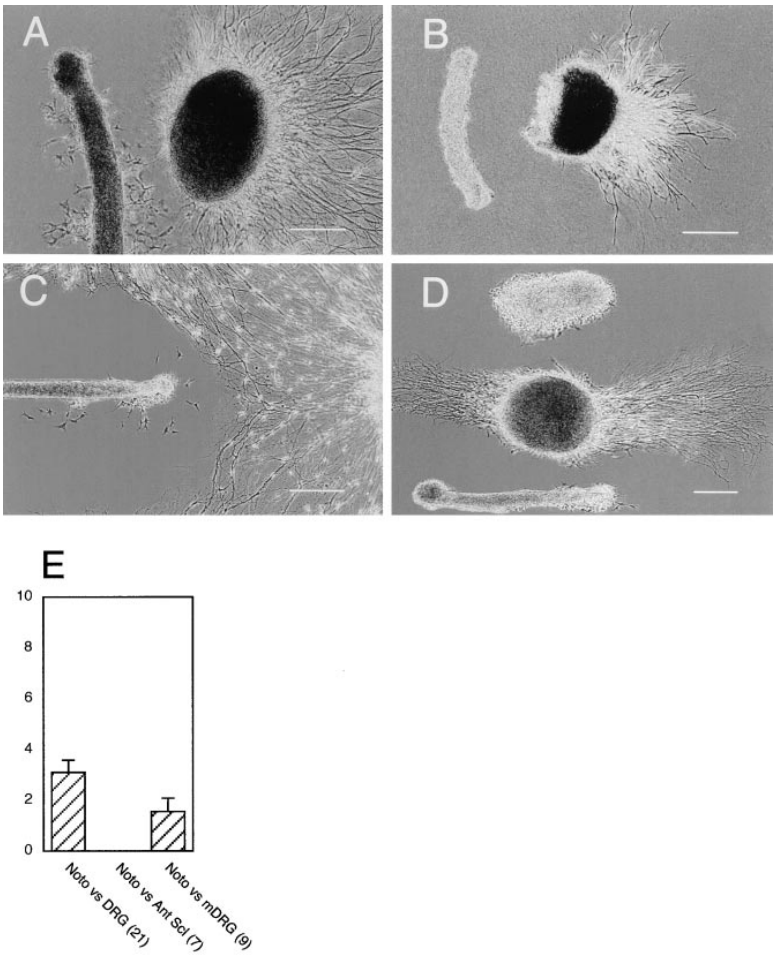

Figure 4. Chemorepulsion of DRG Axons by the Notochord

(A) Stage 28 DRG cultured with a piece of stage 17 notochord. Residual perinotochordal mesenchyme cells are visible.

(B) Stage 28 DRG cultured with extruded notochord cells.

(C) "Halo" experiment of the type shown in Figure 3, using stage 16–17 notochord. Mean separation between DRG and notochord, 780 ± 48 μm, n = 4; mean distance between front of axons and notochord, 285 ± 40 μm. As before, axons avoid the target when within range of chemorepulsion, turning in the gel to grow circumferentially (compare with Figure 3B).

(D) "Sandwich" experiment, with a stage 28 DRG placed between stage 17 dermomyotome and notochord (from the same embryo). Axons project in a bipolar manner parallel to the two sources of repellent. Scale bar = 200 μm (A–D).

(E) Quantification of axon growth in the presence of notochord (ordinate, mean score). Noto, notochord.

the DRG and notochord are 360 μm in vitro (maximum tested) and 190 μm in vivo.

Although most experiments have been carried out using chick DRGs at stage 28, when axon growth under neurotrophin stimulation is vigorous in vitro, we have also shown that repulsion is active, and indeed significantly stronger, on axons at the earliest stages of DRG sprouting in the anterior half-sclerotome. It is therefore plausible that the dermomyotome/ectoderm and notochord exert a similar action on DRG neurons during normal development, secreting chemorepellent molecules into the anterior half-sclerotome that confine DRG axons of all subtypes to their characteristic dorsomedial–ventrolateral trajectory by a surround-repulsion mechanism (Figure 1A). If, in the simplest model, the repellents originate from these two sources to establish two concentration gradients within the sclerotome, these gradients would be presumed to decrease in dorsomedial and ventrolateral directions from the DRG, and axons would grow down them. This model predicts that when DRGs are explanted between dermomyotome/ectoderm and notochord in the same experiment, axons will grow parallel to the two repellent sources, as was seen (Figure 4D). We also detected a weaker repulsion by the floor plate, which may contribute to the repulsion derived from the ventral midline. No evidence was found for repulsion from the basal plate of the early chick spinal cord neuroepithelium, although the ventral spinal cord becomes chemorepulsive for NGF-dependent DRG axons at later stages of development, correlating with the confinement of nociceptive afferents to the dorsal spinal cord (Fitzgerald et al., 1993; Messersmith et al., 1995; Shepherd et al., 1997).

The DRGs are flanked by the posterior half-sclerotomes of consecutive somites along the A/P embryonic axis. These have been shown to express at least six candidate repulsion molecules, any or all of which may restrict the migration of neural crest cells and outgrowth of axons to the anterior half-sclerotomes, generating the segmented pattern of spinal nerves (Davies et al., 1990; Krull et al., 1995; Landolt et al., 1995; Wright et al., 1995; Adams et al., 1996; Fredette et al., 1996; Ring et al., 1996; Wang and Anderson, 1997). The finding that posterior half-sclerotome does not cause chemorepulsion of DRG axons in vitro raises the question of how a DRG cell positioned in the middle of the anterior half-sclerotome, beyond filopodial contact with posterior half-sclerotome, is prevented from sprouting its initial axons along the A/P axis to contact the adjacent posterior half-sclerotomes. In principle, however, two gradients as outlined above would suffice to explain the trajectory of DRG axons, since the concentration of repellents in the anterior half-sclerotome at any single position in the dorsoventral and mediolateral axes would

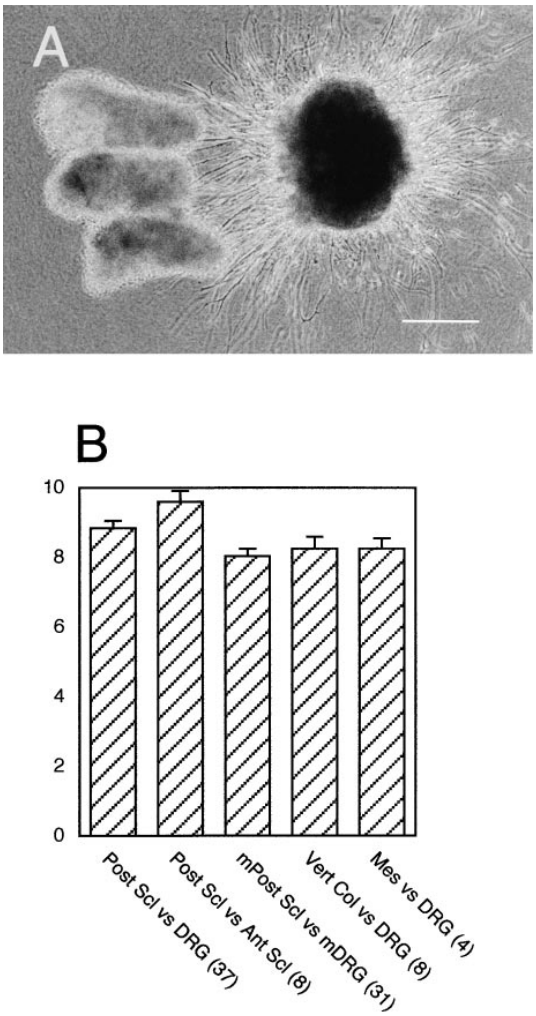

Figure 5. Absence of Chemorepulsion by Posterior Half-Sclerotome

(A) Stage 28 DRG cultured with three chick posterior half-sclerotomes. Scale bar = 200 μm.

(B) Quantification of axon growth (ordinate, mean score). Post Scl, chick posterior half-sclerotome; Ant Scl, chick anterior half-sclerotome; mPost Scl, mouse posterior half- sclerotome; Vert Col, vertebral column; and Mes, mesonephros.

not change along the A/P axis. The lack of sclerotome-based chemorepulsion is also relevant in considering whether the sclerotome-derived perinotochordal mesenchyme cells, as distinct from the notochord, may be inhibitory to crest cells and axons in vivo (Tosney and Oakley, 1990). We have not tested this tissue in isolation, and it remains a further candidate tissue for ventral midline-derived chemorepulsion, but it is worth noting that extruded notochord cells are chemorepellent in the absence of any residual mesenchyme cells.

A further prediction of the surround-repulsion model is that the trajectory of axon growth should be altered after deletion of the dermomyotome/ectoderm or notochord during early development, but the interpretation of such experiments is not straightforward. Following surgical deletion of the dermomyotome, the residual ectoderm regenerates in situ (Tosney, 1987), maintaining the source of repulsion. When the notochord and perinotochordal matrix are removed, migrating trunk neural crest cells cross the ventral midline of the embryo, and might also disturb axon trajectories (Stern et al., 1991). Moreover, although aggregates of DRG cell bodies can develop in normal positions following notochord deletion (Teillet and Le Douarin, 1983), the continued presence of the floor plate could suffice to maintain normal DRG axon trajectories under these conditions. It will be interesting to examine the mouse mutant Danforth's short-tail (*Sd*) to see whether DRG projections are altered in the combined absence of notochord and floor plate (Grüneberg, 1958; Boloventa and Dodd, 1991).

Alongside repulsion, it is also possible that chemoattraction from the presumptive dorsal root entry zone in the alar plate is involved in guiding axons in a dorsomedial direction within the sclerotome. We did not detect chemoattraction in cocultures of anterior half-sclerotome and alar plate, but the presence of neurotrophins in the culture medium may have masked it. In the absence of neurotrophins, however, axons failed to sprout in the presence of the alar plate. While chemoattraction remains possible, we have been unable to reveal it by these experiments.

We have also assessed molecular candidates for these chemorepellent activities. There was no evidence to implicate CSPGs, although Verna (1985) and Fichard et al. (1991) have shown that E7 chick epidermis releases a CSPG that repels DRG axons, while Newgreen et al. (1986) and Pettway et al. (1990) have also implicated CSPGs in the avoidance by migrating neural crest cells of the notochord and perinotochordal matrix. CSPGs have been localized to the chick posterior half-sclerotome in a number of studies (Tan et al., 1987; Newgreen et al., 1990; Perris et al., 1991), yet we find that this tissue is not chemorepulsive. Two proteins identified as secreted products of the notochord and floor plate, Shh (reviewed by Tanabe and Jessell, 1996) and netrin-1 (Serafini et al., 1994), should also be considered as candidates for the midline-derived repulsive activities. Shh is unlikely to be involved, since our transfection experiments show no chemorepulsion attributable to the diffusible N-terminal domain. Although netrin-1 has been shown to be chemorepulsive for trochlear motor axons in vitro (Colamarino and Tessier-Lavigne, 1995), it is not repulsive for sensory axons (K. H. Wang and M. Tessier-Lavigne, personal communication) and is not expressed by the notochord at the stages used in our experiments (Kennedy et al., 1994).

Collapsin-1/Sema III/SemD (Kolodkin et al., 1993; Luo et al., 1993) is a good candidate for the dermomyotome/ectoderm activity. It is chemorepulsive in vitro for both NT-3- and NGF-stimulated axons at early stages of DRG development (Luo et al., 1993; Messersmith et al., 1995; Püschel et al., 1995, 1996; Shepherd et al., 1997), and its mRNA is expressed in the dermomyotome/ectoderm (Wright et al., 1995; Adams et al., 1996; Shepherd et al., 1996). The finding that chick posterior half-sclerotome is devoid of chemorepulsive activity also correlates with the absence here of collapsin-1 mRNA expression (Shepherd et al., 1996). Sema III/SemD mRNA is expressed, however, in the non-chemorepulsive posterior half-sclerotome of rodent embryos (Wright et al., 1995;

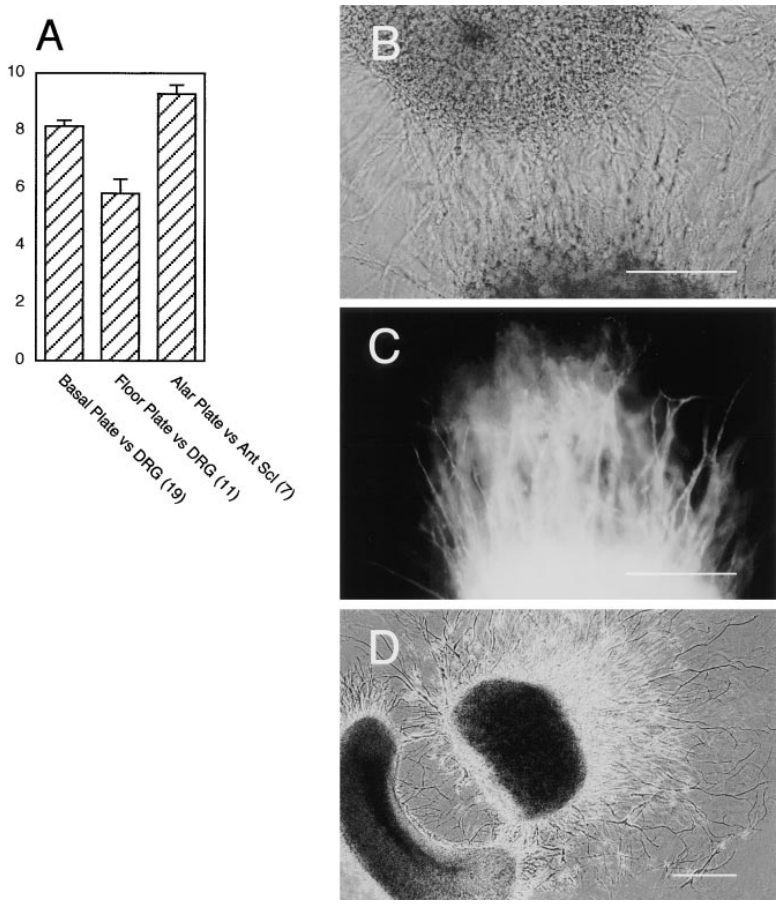

Figure 6. Coculture of DRGs with Neural Tube

(A) Quantification of axon growth in the presence of neural tube. All tissues were dissected from chick embryos. The mean score (ordinate) for floor plate cultures was significantly less than that for basal plate cultures ($P < 0.001$, Student's *t*-test), and significantly greater than that for dermomyotome/ectoderm (Figure 2E; $P < 0.001$).

(B) Stage 28 DRG (below) cultured with stage 18 basal plate (above). No chemorepulsion is seen.

(C) DiA labeling of the DRG seen in (B) to confirm the absence of chemorepulsion.

(D) Stage 28 DRG (center left) cultured with stage 18 basal plate/floor plate. The DRG lies adjacent to the floor plate, and some chemorepulsion is visible. Scale bar = 200 μm (B–D).

Adams et al., 1996), so its candidacy remains open. No abnormalities of DRG sprouting were reported in a *sema III/semD* knockout mouse (Behar et al., 1996), but residual mRNA expression was apparently still present in these animals, and an examination of DRG axon trajectories in the complete absence of Sema III/SemD would be valuable.

Previously suggested models for diffusible axon repellents have placed the source of repellent either ahead of axons, to prevent them from overshooting their target region (for example, in confining nociceptive axons to the dorsal spinal cord; Fitzgerald et al., 1993; Messersmith et al., 1995; Shepherd et al., 1997), or behind them so axons grow down the concentration gradient (for example, chemorepulsion of trochlear motor axons by the floor plate; Colamarino and Tessier-Lavigne, 1995). The present study shows that diffusible repellents may also channel axon growth linearly by establishing concentration gradients that flank axon pathways, a mechanism that may reinforce linear axon guidance by contact-repulsive and attractive cues in vivo.

The finding that axons take a bipolar trajectory when DRG neurons are sandwiched between two repellent sources contrasts with a previous study of olfactory axon chemorepulsion, where axon growth was completely suppressed when neurons were placed between two septal explants (Pini, 1993). An interesting possibility is that the repulsive versus suppressive response simply reflects the exposure of neurons to different concentrations of different repellents in the two experiments, with higher concentrations suppressing rather than repelling axon growth. In the case of olfactory axons, the concentrations will sum, since identical tissues are producing repellent, while for sensory axons, they may not, if the repellents produced by dermomyotome/ectoderm and notochord are qualitatively different. Identifying new molecular candidates for chemorepulsion of sensory axons by dermomyotome/ectoderm and notochord, and testing their function, will be critical steps toward answering such questions. It will also be important to determine whether surround repulsion is involved more generally in axon guidance.

Experimental Procedures

Tissue Dissection

Chick embryos were removed from hens' eggs of Comet Hubbard strain. Mouse embryos were obtained from HSDOLA:MF1 mice (Harlan UK Ltd.). All embryonic tissues were dissected in HEPES-buffered Dulbecco's modified Eagle's medium (DMEM, Sigma) at 4°C, with no additives. Dorsal root ganglia were dissected from the thoracic region of stage 28 chick embryos (Hamburger and Hamilton, 1951). In some experiments, anterior half-sclerotomes containing the newly forming DRGs were dissected from stage 17–19 embryos, at levels from 15–20 somites anterior to the most recently formed somite. These levels correspond to the region where sensory axon outgrowth from the early DRGs into the anterior half-sclerotome is beginning (Keynes and Stern, 1984). Pieces of olfactory bulb epithelium were dissected from stage 36 embryos.

Dermomyotomes with overlying ectoderm were dissected from individual somites of stage 17–19 embryos, at segmental levels 15–20 somites anterior to the most recently formed somite. In some experiments, the dermomyotome and ectoderm were separated from each other by dissection before explantation as individual tissues. Posterior half-sclerotomes were also dissected from somites at these segmental levels. Notochords were removed from these levels and cleared of adherent perinotochordal mesenchyme cells by further dissection prior to explantation. In some experiments, notochord cells were separated from the notochordal sheath and residual mesenchyme by gentle mechanical pressure on the body of the notochord, resulting in extrusion of a column of cells from the cut end. Lengths (corresponding to ~4 somites) of neural tube were isolated from stage 17–19 embryos, also at segmental levels 15–20 somites anterior to the most recently formed somite, and cut along the dorsoventral midline as well as along the roof plate. The resulting pieces, which were cleared of adherent mesoderm, comprised two alar plates and a larger single fragment comprising two basal plates united in the ventral midline by the floor plate. Mouse DRGs were dissected from E14 embryos (thoracic level), and dermomyotome/ectoderm explants and posterior half-sclerotomes were dissected from E11 embryos (thoracolumbar levels).

Collagen Gel Cocultures

Tissues were placed in 35 mm plastic tissue culture dishes (Corning), and carefully positioned relative to each other in 30 μl collagen gel. The gel was prepared by combining 45 μl rat tail collagen (Fitzgerald et al., 1993) and 5 μl of 10× concentrated DMEM (Sigma), and set by the addition of 2 μl 7.5% sodium bicarbonate. Unless otherwise stated, explants of dermomyotome/ectoderm and posterior half-sclerotome were derived from a single somite. Tissues were positioned in the gels at a mean distance of 264 ± 5 μm from each other (389 total coexplants), except for the "halo" experiments illustrated in Figure 3 and 4C, where the mean separation was 841 ± 24 μm (20 coexplants). Explants were incubated at 37°C in a humidified atmosphere containing 7.5% CO_2. Cultures were fixed after 24 hr for at least 1 hr in 4% formaldehyde in phosphate-buffered saline, pH 7.4, containing 2% sucrose.

Growth Media

Culture medium comprised DMEM containing gentamycin (Sigma, 10 μg/ml) and neurotrophins (NT-3 and BDNF, 50 ng/ml; gift from Regeneron Pharmaceuticals; NGF, 50 ng/ml, Sigma). Medium for mouse tissues also contained a serum-free supplement (ITS, Stratech Scientific; diluted at 1/100) and LIF (murine leukemia inhibitory factor, ESGRO, 1000 U/ml).

Antibodies and Inhibitors

These were added to both the collagen gel mixture and culture medium as follows: α-D-xyloside and β-D-xyloside (Sigma), final concentration 2 mM; monoclonal antichondroitin sulfate antibody (Sigma, mouse IgM, clone CS-56; 0.1% sodium azide preservative was removed from the antibody by repeated exchange ultrafiltration with phosphate-buffered saline using a Centricon-100 [Amicon]); antibody was used at 1/10 dilution of the original concentration.

Evaluation of Axon Growth

Axon growth from individual DRGs in relation to coexplanted tissue was scored by an independent observer, unaware of the experimental conditions for each gel, who compared the degree of axon outgrowth in the quadrant of gel facing the coexplant (proximal quadrant) with that in the opposite (distal) quadrant using a scale of 0–10 (see Figure 1B).

Fluorescent Labeling

DRG axons were orthograde labeled in fixed cultures by pressure injection (Picospritzer II, General Valve Corporation) of a small drop of 0.5% DiA (4-Di-10-Asp, Molecular Probes) in 0.3 M sucrose. Axons were left to label overnight before examination under fluorescence optics.

COS Cell Transfection

COS-7 cells (gift of Sean Munro) were transfected with an Shh N-terminal expression construct (gift of Andy McMahon) using LipofectAMINE (GIBCO BRL). Aggregates of transfected COS cells were made by the hanging drop method described by Kennedy et al. (1994). Aggregates were then trimmed with tungsten needles and cocultured with DRGs in collagen gels.

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