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# Orienting axon growth: spinal nerve segmentation and surround-repulsion

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**ABSTRACT The study of spinal nerve trajectories in higher vertebrate embryos has revealed an inherent polarity within somites along the antero-posterior axis, and provides a simple system in which to study the factors that influence axon pathfinding. We argue that the orientation of spinal axons is determined by the simultaneous operation of two distinct guidance mechanisms, contact repulsion and chemorepulsion. Motor and sensory axons traverse the anterior half of each somite because they are excluded by contact repulsion from the posterior half-somite, and the molecular nature of several candidate contact repellents is reviewed. In contrast, we find that the dorsoventral trajectory of primary sensory axons is oriented by diffusible repellents originating from the notochord medially and dermamyotome laterally. In this system, therefore, repulsion by surrounding tissues ('surround-repulsion') is the main force directing axon growth in three dimensions.**

KEY WORDS: *axon guidance, chemorepulsion, contact repulsion, segmentation, somite*

## Introduction

The construction of the nervous system requires that nerve cells become connected to their appropriate synaptic targets in the brain and periphery, and growing axons travel often enormous distances from their cell bodies and negotiate variable terrains in the embryo. This constitutes a remarkable feat of navigation. Axons extend along predetermined pathways by using their growth cones to interpret attractive or repulsive signals in the environment. These cues can be presented on cell surfaces or secreted by neighbouring tissues (Keynes and Cook, 1995; Goodman, 1996; Tessier-Lavigne and Goodman, 1996). In our laboratories, we are trying to elucidate in detail the mechanisms that guide axons. We need not only to understand how a growth cone senses and responds to its environment, but also to appreciate how appropriate guidance cues are laid down at the right time and in the right place in the embryo and how these cues are integrated in the three dimensions to determine axon trajectories. Here we focus on the anatomically simple and readily manipulable spinal nerves of higher vertebrate embryos, and discuss how factors secreted by the midline orient the dorsoventral trajectories of DRG axons. We also describe how contact repulsion is the major mechanism responsible for generating the segmental arrangement of spinal nerves, and place this in the context of the earlier process of antero-posterior somite patterning in the segmental mesoderm. Overall, our model of spinal axon guidance accounts for the earliest axon trajectories in the three axes of the embryo and emphasizes the pre-eminence of repulsion as the major guiding force.

## Somite and peripheral nerve development

During gastrulation, presumptive somites are laid down with the formation of unsegmented paraxial mesoderm (known as the segmental plate or presomitic mesoderm) on either side of the developing neural tube (reviewed in Keynes and Stern, 1988; Christ and Ordahl, 1995). As the neural tube closes, individual somites emerge from the anterior end of each segmental plate as epithelial spheres of radially-arranged cells surrounding a central cavity containing loosely-arranged luminal cells. The overall size of the segmental plate remains constant as mesenchymal cells are added to the posterior end through continued gastrulation. In chick embryos, a somite is formed every 90 min or so, and as each newly formed somite differentiates, the next somite buds off the segmental plate. Thus, in any one embryo there is an antero-posterior time gradient in somite differentiation, i.e., more anterior somites differentiate earlier.

The next stage of somite development involves an epithelial-to-mesenchymal transformation in which the ventromedial part of the

*Abbreviations used in this paper:* CSPG, chondroitin sulphate proteoglycan; DRG, dorsal root ganglion; GPI, glycosylphosphatidylinositol; PNA, peanut agglutinin; Shh, sonic hedgehog.

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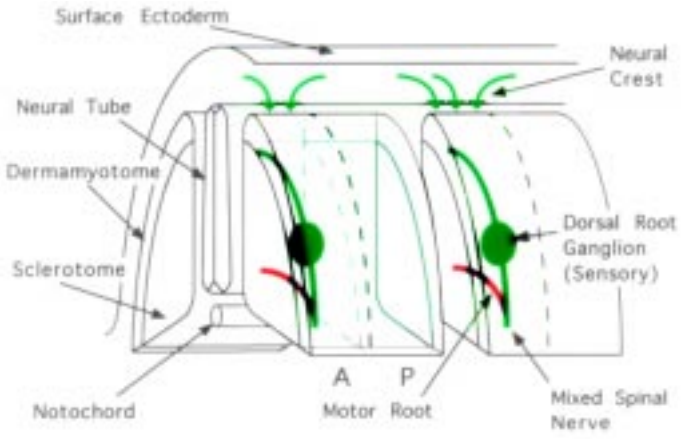


**Fig. 1. Summary diagram of spinal nerve segmentation in the vertebrate trunk.** *The main relations between the developing peripheral nervous system and the somite components are shown. A, anterior (rostral/cranial); P, posterior (caudal). Von Ebner's fissure, at the A/P boundary within the somite, is indicated by a dashed line.*

epithelial somite dissociates to generate the mesenchymal sclerotome (with an additional contribution from the luminal cells). Overlying the sclerotome is the dorsolateral part of the somite constituting the dermamyotome. This subdivides into the dermatome and myotome and these ultimately give rise, respectively, to all skeletal muscle of the trunk, limbs and tail, and the dermis. By contrast, the sclerotome/notochord complex generates the vertebral column and ribs.

At the time that the sclerotome begins to dissociate, neural crest cells migrating from the dorsal part of the neural tube start to migrate into the anterior half of each sclerotome, avoiding the posterior half-sclerotome (Rickmann *et al.,* 1985; Bronner-Fraser, 1986). Within the anterior half-sclerotome, crest cells that are fated to form the DRG coalesce (Teillet *et al.,* 1987) and then differentiate into bipolar sensory neurons as described below. Soon after neural crest migration has begun, motor axons emerging from the ventral neural tube also confine themselves to the nearest available anterior half-sclerotome (Keynes and Stern, 1984; Fig. 1).

The segmental organisation of the peripheral nervous system in the trunk is not inherent but is imparted by the segmentation of somites (Keynes and Stern, 1984, 1988). Axon outgrowth from the neural tube is not intrinsically segmented, since axons are seen to grow out from all positions along the length of a piece of explanted neural tube (Keynes *et al.,* 1996). This conclusion is also supported by *in ovo* experiments, where it is found that motor axon growth through multiple compound anterior half-somites remains unsegmented (Stern and Keynes, 1987; Kalcheim and Teillet, 1989). Moreover, if a portion of neural tube or 2-4 presumptive somites is reversed along antero-posterior axis, motor axons and neural crest cells always grow through the originally anterior half-somite (Keynes and Stern, 1984; Bronner-Fraser and Stern, 1991). Exclusion from posterior half-somite therefore applies both to axons and to migrating neural crest cells (Rickmann *et al.,* 1985; Teillet *et al.,* 1987; Krull *et al.,* 1995). Although it is possible that anterior half-somites attract peripheral nerve elements (see below), most work has concentrated on the repulsive nature of posterior half-somites. Repulsion has been clearly demonstrated using an assay that mimics the contact inhibition of axon growth (Raper and Kapfhammer, 1990), in which somite detergent extracts are found to induce growth cone collapse of cultured sensory axons (Davies *et al.,* 1990). In keeping with this, motor axons turn away or branch after encounters with posterior sclerotome cells, whereas anterior sclerotome cells stimulate motile activity (Oakley and Tosney, 1993).

## Molecular basis for the antero-posterior subdivision of the somite

The marked difference in permissiveness for peripheral nerve elements between the anterior and posterior somite-halves is reflected morphologically in chick embryos by the presence of a visible boundary (von Ebner's fissure) separating the two halves. This boundary is never breached by neural crest, sensory or motor axons, which always migrate through the anterior half-sclerotomes (Stern and Keynes, 1986; Christ and Ordahl, 1995). The antero-posterior subdivision of the somite is likely to be a necessary accompaniment to the formation and/or maintenance of somite boundaries along the antero-posterior axis (Keynes and Stern, 1988), and may also reflect the requirement of outgrowing spinal nerves to adopt the correct anatomical relationship with the developing vertebral column. Lastly, it could be important for the development of the vertebral column itself, since each half-sclerotome appears to contribute to distinct vertebral lineages (Verbout, 1985; Bagnall *et al.,* 1988, Goldstein and Kalcheim, 1992, Huang *et al.,* 1994).

Some clues to the genetic mechanisms underlying the development of somite polarity are beginning to emerge with the identification of several transcriptional regulators that show restricted expression to the anterior or posterior half-somite. Such factors could be involved in several facets of somitogenesis, ranging from the induction of anterior and posterior cell fates and the establishment of boundaries between them, to the elaboration and maintenance of the identities of the two cell populations. Genes that appear to be involved in the development of antero-posterior somite polarity are summarised in Figure 2, and some individual examples are discussed below.

Recently, an important insight into the molecular basis of somite development has come from the analysis of c-*hairy 1*, an avian homologue of the bHLH transcription factor *hairy,* a *Drosophila* pair- rule gene (Palmeirim *et al.,* 1997). *c-hairy1* mRNA is expressed in cyclical waves in the segmental plate, travelling from posterior to anterior, each wave having a periodicity corresponding to the formation time of one somite. An individual cell in the segmental plate alternates between expressing and non-expressing states, and is finally incorporated into the posterior half-somite after 12 cycles. The pulsatile nature of *c-hairy1* expression makes it a candidate component of a clock- and-wavefront mechanism, as suggested previously for somite formation (Cooke and Zeeman, 1976; Keynes and Stern, 1988), and its posterior-specific expression in the newly-formed somite suggests an accompanying role in the development of somite polarity. It will be interesting to determine whether this cyclical expression pattern is unique to *c-hairy1*, and whether similar genes are required to generate the future anterior half-somite.

Members of the *Notch* family of transmembrane receptors, their ligands *(Delta and Serrate),* and associated signalling molecules

such as *Suppressor of Hairless* and *fringe*, have important functions in early cell fate decisions in *Drosophila*. Members of the same signalling pathway have been implicated similarly in vertebrates (Artavanis-Tsakonas *et al.,* 1995; Lewis, 1996), and recent studies have revealed a role for Notch signalling in somite development. In mice, null mutations for *Notch1* (Conlon *et al.,* 1995), *Delta-like 1 (Dll1)* (Harbe de Angelis *et al.,* 1997) and *RBP-Jκ*, a mouse homologue of *Drosophila Suppressor of Hairless* (Oka *et al.,* 1995), show delayed or perturbed segmentation. In Notch1 and *RBP-Jκ* mutants, irregularly produced somites appear to be morphologically normal after their formation, and show typical antero-posterior gene expression patterns. By contrast, somites in *Dll1* null mutants are irregularly formed, and cells fail to condense in the posterior half-sclerotome, remaining loosely packed with a concomitant loss of posterior-specific markers. *Dll1* expression is restricted to the posterior halves of normal somites, and its knock-out phenotype is consistent with a conversion of posterior half-sclerotome to an anterior fate, resulting in loss of boundaries within and between somites, and fusion of adjacent DRGs. A similar phenotype is seen in null mutant mice for the familial Alzheimer's Disease gene, *presenilin 1 (PS1)* (Wong *et al.,* 1997). PS1 is a homologue of *C. elegans* SEL12, a protein that facilitates Notch/LIN12 signalling (Levitan and Greenwald, 1995). The further finding that *Dll1* and *Notch1* expression are severely reduced in these mice suggests that *PS1* may function upstream in a pathway regulating these molecules.

The complexity of genetic interactions in somite formation is underlined by studies of other genes related to the Notch signalling pathway. *Dll3*, a divergent *Delta* gene, shows a complementary pattern of expression to *Dll1* in the anterior part of the segmental plate (Dunwoodie *et al.,* 1997). *Dll3* is expressed throughout the bulk of the plate, but only in the anterior portion of the newly-forming somite as it buds off at the anterior end of the plate, whereas *Dll1* is expressed in the posterior part of the newly-forming somite. Another gene expressed in the posterior part of the newly-forming somite is *Lunatic Fringe* (Johnston *et al.,* 1997), in this case being turned off once the somite has individuated. The interactions between Notch signalling pathway genes in somite formation remain to be elucidated in detail, but it is striking that they are expressed in patterns that prefigure the antero-posterior somite polarity, supporting the view that this polarity is necessary for segmentation. The antero-posterior polarity may also be an ancient feature in vertebrates, as suggested by the posterior-restricted expression of zebrafish homologues of *Notch* (Bierkamp and Campos Ortega, 1993) and *Groucho* (Wülbeck and Campos-Ortega, 1997), and of the *Amphioxus* homologue of *engrailed* (De Robertis, 1997; Holland *et al.,* 1997).

## Segmentation and contact repulsion

The demonstration that the posterior half-sclerotome excludes growing spinal axons has contributed significantly to the concept of contact repulsion as an important mechanism in axon guidance (see Keynes and Cook, 1990). Molecular differences between the two halves of the sclerotome that may underlie this phenomenon have been identified and investigated in our laboratories. Using a panel of lectins as histochemical reagents it was originally shown that peanut agglutinin (PNA) specifically binds to posterior half-sclerotome cells (Stern *et al.,* 1986; Fig. 3). This work was extended by Davies *et al.* (1990), who found that the lectin jacalin,

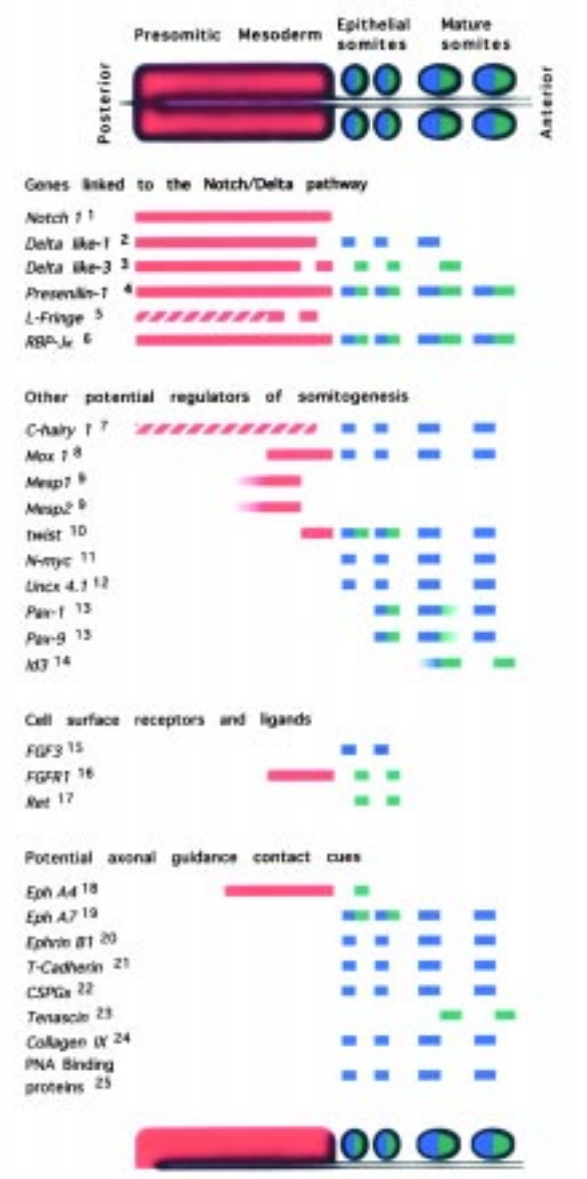


**Fig. 2. Summary of molecules showing restricted antero-posterior expression in somites.** *A variety of molecules show antero-posterior restricted expression in somites, and these are categorized into 4 main groups. A schematic diagram of somites budding from the presomitic mesoderm (segmental plate, red) is shown above; expression restricted to the posterior half-somite is shown in blue, and anterior expression in green. Genes with dynamic expression patterns in the presomitic mesoderm (L-Fringe, C-hairy1) are shown by diagonal hatching. Data from the following sources: 1) Reaume et al., 1992; Franco del Amo et al., 1992; 2) Bettenhausen et al., 1995; 3) Dunwoodie et al., 1997; 4) Wong et al., 1997; 5) Johnston et al., 1997; 6) Oka et al., 1995; 7) Palmeirim et al., 1997; 8) Candia et al., 1992; 9) Saga et al., 1997; 10) Füchtbauer, 1995; 11) Mansouri et al., 1997; 12) Conlon et al., 1995; 1 3) Müller et al., 1996; 14) Ellmeier and Weith, 1995; 15) Mahmood et al., 1995; 16) Yamaguchi et al., 1992; 17) Robertson and Mason, 1995; 18) Nieto et al., 1992; 19) Araujo and Nieto, 1997; 20) Wang and Anderson, 1997; 21) Ranscht and Bronner-Fraser, 1991; 22) Tan et al., 1987; Newgreen et al., 1990; Perris et al., 1991; Landolt et al., 1995; 23) Yip et al., 1995; 24) Ring et al., 1995; 25) Stern et al., 1986.*

which has the same carbohydrate specificity as PNA but whose binding is not impeded by the sialylation of the relevant Galβ1-3 GalNAc residues, shows the same specificity. This indicates that

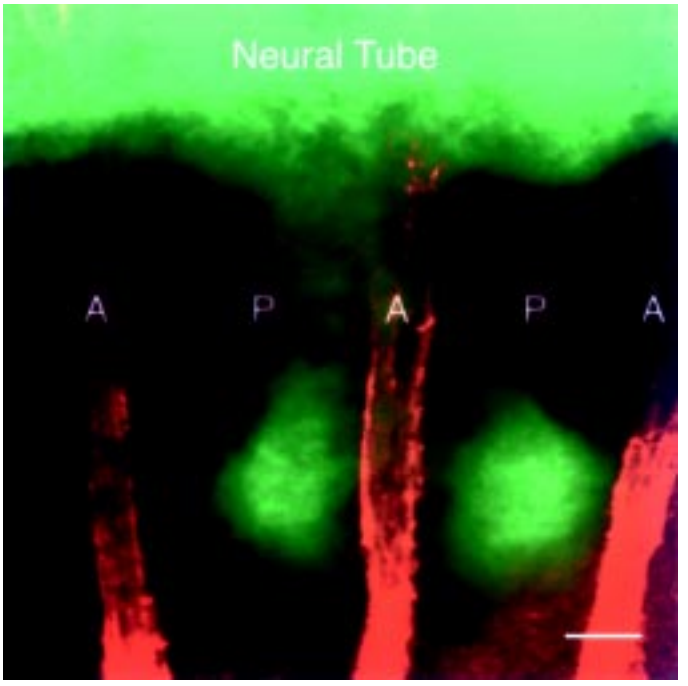


**Fig. 3. Motor axons avoid posterior half-sclerotome.** *A whole-mounted stage 19 chick embryo trunk viewed by confocal microscopy. Motor axons extending from the neural tube are labelled with anti-neurofilament antibody (red), and traverse the anterior (A) half-sclerotome. Axons avoid the posterior half-sclerotome (P), which is labelled with fluorescent peanut lectin (green). Bar, 50 μm.*

the difference in the surface glycans between anterior and posterior half-sclerotome cells is not due to differential sialylation in the two populations, but rather to the presence of exposed Galβ1-3 GalNAc residues in the surface glycoproteins of posterior cells. This latter observation has allowed us to develop a biochemical purification procedure for these molecules. Using immobilized PNA as an affinity chromatography absorbent, two glycoproteins of M 48 and 55K have been identified in the posterior but not anterior half-sclerotome (Davies *et al.,* 1990). Their relevance to the generation of spinal nerve segmentation by contact repulsion has been confirmed by the ability of immobilized antibodies raised against these components to deplete growth cone collapse-inducing activity from detergent extracts of somites. Further support comes from two separate studies. First, Krull *et al.* (1995) have found that treatment of chick embryo trunk explants with PNA destroys the ability of migrating neural crest cells to distinguish between anterior and posterior half-sclerotome, crest cells now being able to migrate through both somite halves. Second, work in our laboratory, using glycosidases known to release O-linked glycans that act as receptors for PNA, has shown that treatment of detergent-solubilized somite material with a mixture of sialidase and O-glycanase destroys growth cone collapse-inducing activity, while neither enzyme alone has any effect (Keynes *et al.,* 1996). This observation also suggests that the exposed Galβ1-3 GalNAc residues that bind to the lectin are not themselves directly responsible for biological activity, but nonetheless form a convenient chemical handle for isolation of the relevant molecules.

In addition to PNA-binding glycoproteins, several other molecules have been proposed as candidate contact repellents in the posterior half-sclerotome. The glycosylphosphatidylinositol (GPI)-linked calcium-dependent cell adhesion molecule, T-cadherin, is localised to the posterior but not anterior half-sclerotome, and to motor axon growth cones (Ranscht and Bronner-Fraser, 1991; Fredette and Ranscht, 1994). T-cadherin mediates homophilic binding *in vitro,* and can act as an inhibitory substrate for the extension of motor axons at the stages when they enter the anterior half-sclerotome *in vivo* (Fredette *et al.,* 1996). Versican, a large aggregating chondroitin sulphate proteoglycan (CSPG) that prevents attachment of fibroblasts to extracellular matrix molecules such as laminin and fibronectin (Yamagata *et al.,* 1989), is also localized to the posterior half-sclerotome (Landolt *et al.,* 1995), raising the possibility that contact repulsion of crest cells and growth cones might arise indirectly by the disruption of adhesive/attractive interactions between them and the extracellular matrix. Other chondroitin sulphate proteoglycans also show posterior half-sclerotome-specific localization, and are therefore further candidate repellents (Tan *et al.,* 1987; Newgreen *et al.,* 1990; Perris *et al.,* 1991), and one, collagen type IX, has been shown to inhibit spinal axon and neural crest cell migration in an *in vitro* outgrowth assay (Ring *et al.,* 1995). In each case, however, direct evidence that the molecule mediates spinal nerve segmentation *in vivo* is lacking.

More recently, following their identification as repulsive cues for retinal axons targeting the midbrain tectum, Eph receptors and their ephrin ligands have been implicated as short range contact repellents in a number of sites during neural development, including somites (Gale *et al.,* 1996). The ephrins can be subdivided into two classes on the basis of their mode of attachment to the plasma membrane, being tethered by a GPI anchor (ephrin-A ligands), or possessing a transmembrane domain and cytoplasmic tail enabling them to span the plasma membrane (ephrin-B ligands). Their receptors fall into two separate classes, EphA and EphB, binding ephrin-A and ephrin-B ligands respectively. Interestingly, there appears to be little or no cross talk between the two subclasses of receptors and ligands, while within each subclass a receptor can bind to more than one ligand and ligands can activate more than one receptor (Orioli and Klein, 1997). Attachment to the membrane appears to be essential for ephrins to activate their receptors, indeed only membrane-bound or artificially clustered ligands can trigger receptor autophosphorylation (Davis *et al.,* 1994).

Recently, Wang and Anderson (1997) have implicated this system in spinal nerve segmentation by showing that two Eph receptor transmembrane ligands, Lerk2 and HtkL (respectively ephrins-B1 and -B2 under the new nomenclature) are expressed in the posterior but not anterior halves of, respectively, chick and rat somites. Preclustered ligand-Fc-fusion proteins can repel both crest cell migration and motor axon outgrowth using several independent *in vitro* assays, and repulsion requires the ligands to be presented in a discontinuous or graded manner when tested in the context of a permissive substrate such as laminin or fibronectin. The expression of the cognate receptor (Nuk/EphB2) for these transmembrane ligands has been demonstrated in the early stages of motor axon outgrowth and during trunk crest cell migration (Henkemeyer *et al.,* 1994; Wang and Anderson, 1997). In a separate study, Krull *et al.* (1997) find that the application of unclustered ephrin-B2 as a competitive antagonist of endogenous ligands disrupts the metameric pattern of neural crest migration in whole trunk explants of chick embryos, allowing the entry of neural crest cells into the posterior halves of the sclerotome.

Together, these studies present compelling circumstantial evidence for Eph-receptor/ephrin interaction as a contact repulsive system in the posterior half-sclerotome, but a number of issues remain to be resolved. A direct test would be provided by receptor knockouts. Wang and Anderson (1997) have reported no obvious perturbations in spinal nerve segmentation in mouse embryos carrying homozygous mutations in two receptors for these ligands, Nuk/EphB2 and Sek4/EphB3. If the explanation is not that another system plays the dominant role in segmentation, the lack of a clear mutant phenotype could result from the expression by crest cells or motor axons of other EphB receptors that can interact with HtkL and Lerk 2, such as Elk/EphB1 and Myk1/EphB4. In our laboratory we are examining the role of the Eph-receptor/ephrin families in sensory axon guidance within the somites, and have been unable to find evidence for their involvement (M. Vermeren, unpublished results). In addition, we are examining whether the 48K and 55K glycoproteins previously isolated by us are ephrin-related. Krull *et al.* (1997), have found that competitive antagonists to endogenous ephrins disrupt the metameric pattern of crest migration in avian embryo trunk explants, but also suggest that there are likely to be multiple inhibitory mechanisms operating in the posterior half-sclerotome. It is possible, then, that motor and sensory axons are repelled by distinct molecular cues.

One further molecule that should be considered is collapsin-1/sema III/semD, originally identified as a secreted glycoprotein that causes collapse of chick DRG growth cones (Luo *et al.,* 1993), and as a homologue of grasshopper Sema I (Kolodkin *et al.,* 1993). Collapsin-1 is not expressed in the chick posterior half-sclerotome (although this is not the case for sema III/semD in rodent embryos), and neither chick or mouse posterior half-sclerotome provides a source of diffusible repulsive activity in collagen gels (Keynes *et al.,* 1997). No abnormalities of spinal nerve segmentation have been reported, moreover, in either the sema III/semD knockout mouse (Behar *et al.,* 1996; Taniguchi *et al.,* 1997), the knockout mouse for neuropilin-1, a candidate sema III receptor (Kitsukawa *et al.,* 1997), or following surgical ablation of the dermamyotome (Tosney, 1987), a site of collapsin-1/sem III/semD expression during axon and crest migration (Wright *et al.,* 1995; Adams *et al.,* 1996; Shepherd *et al.,* 1996). While these findings indicate that a contact-repulsive role for collapsin- 1/sema III/semD in the posterior half-sclerotome is unlikely, we argue below that this protein may be involved in mediating chemorepulsive spinal axon guidance in the dorsoventral axis.

## Chemoattraction of spinal axons

Although contact repulsion by posterior half-sclerotome appears sufficient to explain the antero-posterior pattern of spinal axon outgrowth, chemoattraction by secreted molecules derived from the anterior sclerotomes may also play a role. Both retrograde and orthograde labelling of chick motor axons (Lim *et al.,* 1991; R.J. Keynes and D. Tannahill, unpublished results) have shown that axons exiting the neural tube opposite posterior half-sclerotome project into the nearest available anterior half-sclerotome, and motor axons leaving basal plate explants in collagen gels can turn and orient toward DRGs (Keynes *et al.,* 1996). Hotary and Tosney (1996) have also shown that chick dorsal- anterior half-sclerotome, where DRGs develop, is chemoattractive for both sensory and motor axons. Lastly, Ebens *et al.* (1996) have found mouse

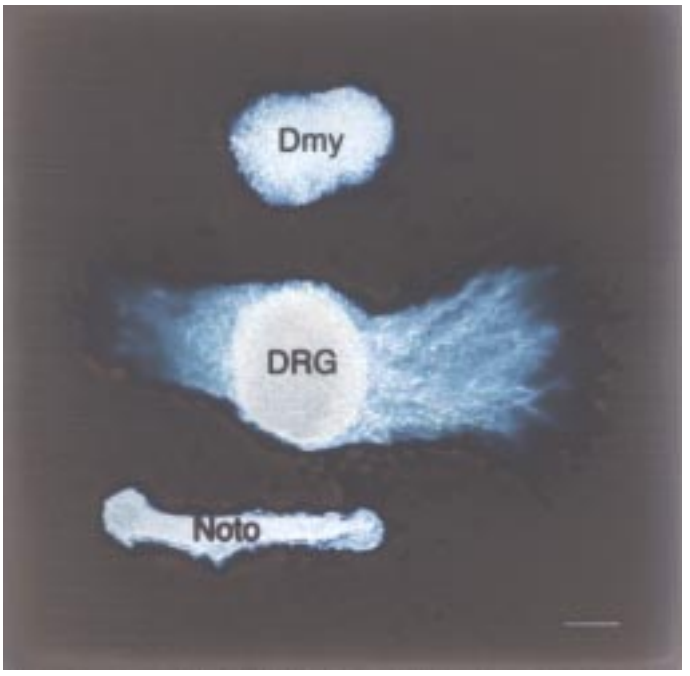


**Fig. 4. Chemorepulsion of DRG axons by dermamyotome and notochord.** *A chick DRG is sandwiched in a collagen gel between a single dermamyotome (Dmy, above) and a length of notochord (Noto, below). Axons extend from the DRG in a polarized trajectory rather than radially, a growth pattern resulting from chemorepulsive molecules secreted by dermamyotome and notochord. Bar, 200 μm.*

sclerotome to be chemoattractive for motor axons; part of this activity can be attributed to the upregulation of hepatocyte growth factor/scatter factor (HGF/SF) expression in isolated sclerotome, but the involvement of other factors is likely since chemoattraction is unaffected by a neutralizing antibody to HGF/SF and continues in sclerotome isolated from mice lacking HGF/SF. Several observations suggest, however, that somites are not essential for motor axon outgrowth. Explants of ventral neural tube in collagen gels extend axons profusely in the absence of serum or growth factors (Keynes *et al.,* 1996), and *in ovo* somite deletion does not prevent motor axon outgrowth (Lewis *et al.,* 1981; Tosney, 1988). It seems likely, therefore, that while chemoattraction of spinal axons by sclerotome may contribute to early pathfinding along the anterior-posterior axis, repulsion exerts the dominant influence on axon trajectories.

## Orientation of sensory axon trajectory by diffusible repellents

Alongside its segmental patterning along the antero-posterior axis, spinal nerve growth is also highly oriented within the dorsoventral axis. Dorsal root ganglia assume a characteristic position alongside the neural tube, medial to the dermamyotome and surface ectoderm, and dorsolateral to the notochord. As individual DRG neurons differentiate, they first generate short extensions in random directions, and then produce the definitive growth cones from the dorsal and ventral poles of the cell which sprout in dorsomedial and ventrolateral directions (Ramon y Cajal, 1909; Levi-Montalcini and Levi, 1943; Tello, 1947). To investigate how this trajectory is

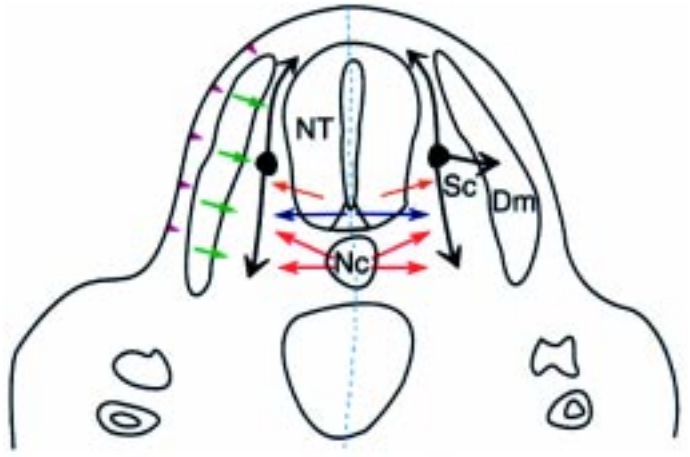


**Fig. 5. Model for chemorepulsion of primary sensory axons in the trunk.** *Schematic transverse section through the anterior half-sclerotome of a chick embryo, showing the direction of repulsive forces operating on sprouting DRG axons. Lateral repulsive signals derive from the ectoderm (purple arrowheads) and dermamyotome (Dm, green arrows); midline repellents derive from the notochord (Nc, red arrows), floor plate (blue arrows) and basal plate of the neural tube (NT, orange arrows). The diagram half to the left of the dotted line represents normal development. The diagram half to the right of the dotted line represents the phenotype of sema III/neuropilin knockout mice, in which loss of lateral repulsion causes some DRG axons to grow abnormally towards the dermamyotome due to the continued influence of midline-derived repulsion.*

established, we have examined the influence of the tissues surrounding the DRGs on axon outgrowth using collagen gel co-cultures (Keynes *et al.,* 1997). The dermamyotome, surface ectoderm and notochord secrete potent sensory axonal repellents, and the bipolar trajectory of DRG axon outgrowths can be mimicked *in vitro* by sandwiching a DRG between an explant of dermamyotome and one of notochord (Fig. 4). In marked contrast, the contact-repulsive posterior half-sclerotome, which flanks the DRG along the anterior-posterior axis, is devoid of long-distance diffusible repulsive activity. With the exception of the floor plate, the bulk of the neural tube is also devoid of chemorepulsive activity, although weak activity is detectable if early (E4) DRGs are used rather than more mature (E6) DRGs (K. Ohta, unpublished observations). The chemorepulsive activities are evolutionarily conserved, since chick and mouse DRGs are repelled by chick dermamyotome and notochord, and by mouse dermamyotome.

The finding that DRGs are surrounded both medially and laterally by sources of potent chemorepulsion raises the possibility that the early dorsoventral trajectory of primary sensory axons is oriented by opposing chemorepulsive gradients derived from the dermamyotome/ectoderm and notochord (Fig. 5). According to the model, axons grow along the paths of least repulsion by navigating down concentration gradients of repellent in both dorsomedial and ventrolateral directions. While the molecular nature of these repellents is unknown at present, several potential candidates seem unlikely to be involved directly. Migrating neural crest cells are known to avoid the notochord and perinotochordal matrix, and CSPGs have been implicated in this avoidance (Newgreen *et al.,* 1986; Pettway *et al.,* 1990). CSPGs have also been suggested to be responsible for chemorepulsion by E7 chick embryo epidermis of DRG axons, since repulsion can be prevented by proteoglycan synthesis inhibitors or by neutralizing antibodies against CSPGs (Verna, 1985; Fichard *et al.,* 1991). Repulsion of DRG axons by dermamyotome/ectoderm or by notochord in collagen gels is unaffected, however, by these inhibitory reagents (Keynes *et al.,* 1997). Although a repulsive role for the core protein of a CSPG has not been excluded, the further finding that posterior half-sclerotome, which expresses several CSPGs (Perris *et al.,* 1991), is not chemorepulsive also argues against a non-specific repulsive effect for this class of molecule.

Two proteins secreted by the notochord and floor plate, sonic hedgehog (Shh; Tanabe and Jessell, 1996) and netrin-1 (Serafini *et al.,* 1994), are also candidates for the midline chemorepellents. Shh is unlikely to be involved since its diffusible N-terminal domain is not chemorepulsive in transfection experiments (Keynes *et al.,* 1997), while netrin-1 is not expressed by the midline at the stage of our experiments (Kennedy *et al.,* 1994). A further protein, collapsin-1/sema III/semD, is a good candidate for the dermamyotome/ectoderm activity, being expressed by the dermamyotome and overlying ectoderm at the appropriate stages of development (Wright *et al.,* 1995; Adams *et al.,* 1996; Shepherd *et al.,* 1996), and chemorepulsive for NGF- and early NT-3-stimulated DRG axons (Luo *et al.,* 1993; Messersmith *et al.,* 1995; Püschel *et al.,* 1996; Shepherd *et al.,* 1997). The finding that mouse posterior half-sclerotome expresses sema III yet is not chemorepulsive suggests that the correlation between collapsin-1 expression and chemorepulsion is not absolute (Keynes *et al.,* 1997). However, although no abnormalities of DRG sprouting were reported in one sema III/semD knockout mouse (Behar *et al.,* 1996), aberrant outgrowths extending laterally towards the dermamyotome have been described in another (Taniguchi *et al.,* 1997), as well as in mice deficient for neuropilin-1 (Kitsukawa *et al.,* 1997). According to the surround-repulsion model outlined above, this trajectory would result from the imbalance created by loss of lateral, sema III-derived chemorepulsion in the face of continued chemorepulsion from the midline (Fig. 5). It is clear, nevertheless, that many DRG axons do take a normal ventrolateral course in these mice, so other chemorepellents are likely also to be secreted by the dermamyotome/ectoderm. Our laboratory is now taking an expression cloning approach towards identifying the midline-derived chemorepellent(s), and it will be interesting to see whether more than one such molecule can be detected in this way.

## Conclusions

Early work on the guidance of spinal axons revealed the antero-posterior subdivision of the somite to be a fundamental feature of vertebrate mesoderm segmentation, a view now confirmed by molecular genetic studies. Our recent work on guidance has shown that the orientation of growing spinal axons is critically dependent on repulsion by surrounding tissues. In the antero-posterior axis, contact repulsion by posterior half-sclerotome cells dominates in creating the segmental pattern of spinal nerve outgrowth, although chemoattraction may also contribute to axon guidance. Several candidate contact repellents have been identified and an important question to resolve is whether these operate combinatorially, or whether one repellent plays the major role *in vivo*. In the dorsoventral axis we have identified opposing gradients of diffusible repellents acting on outgrowing axons to constrain their trajectories. Following molecular characterisation of the repellents, a challenge for the future will be to determine how growth cones integrate multiple, surround-repulsive cues simultaneously in three dimensions.

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