Axon repulsion during peripheral nerve segmentation

ROGER J. KEYNES, KAREN F. JAQUES and GEOFFREY M. W. COOK

Department of Anatomy, Downing Street, Cambridge CB2 3DY, UK

Summary

The guidance of axons during embryonic development is likely to involve both adhesive and repulsive interactions between growth cones and their environment. We are characterising the role and mechanism of repulsion during the segmental outgrowth of motor and sensory axons in the somite mesoderm of chick embryos. Axons are confined to the anterior half of each somite by the expression in the posterior half of a glycoconjugate system (48×10^3 M_r and 55×10^3 M_r) that causes the collapse of dorsal root ganglion growth cones when applied in vitro. Enzymatic cleavage of this fraction with specific combinations of endo- and exoglycosidases removes collapse activity, suggesting that carbohydrate residues are involved in the execution of collapse. A similar activity is also detectable in normal adult grey matter, suggesting roles for repulsion beyond the development of spinal nerve segmentation.

Key words: growth cone, contact inhibition, neural segmentation.

Introduction

Although differential adhesion has been recognised for some time as an important mechanism in neural development, a parallel role for contact inhibition or repulsion has been proposed only recently (for reviews see Patterson, 1988; Walter et al. 1990a; Keynes et al. 1991). Contact inhibition has been well known as a phenomenon in vitro since its original description by Abercrombie and Heaysman (1954). On mutual contact, chick heart fibroblasts were seen to undergo a sequence of reactions comprising adhesion, local paralysis of motility in the region of contact, and finally a cytoskeletal contraction and withdrawal. Growth cones can display a remarkably similar response in vitro: in cocultures of retinal and sympathetic explants, when a retinal growth cone contacts a sympathetic axon, or vice versa, the growth cone stops moving and collapses within a few minutes (Kapfhammer et al. 1986; Kapfhammer and Raper, 1987). As for interacting fibroblasts, fine strands of cytoplasm remain adherent to the axon during the collapse, and normal movement is then restored with the elaboration of another growth cone.

The full growth cone collapse seen in vitro has not been described in vivo (and would not necessarily be expected, see below), but such observations do suggest that analogous mechanisms may be involved in normal developmental processes such as axon guidance. The development of an in vitro assay for growth cone collapse (Raper and Kapfhammer, 1990) has also allowed a molecular analysis of the phenomenon to be undertaken. Candidate molecules have been isolated by the application of the assay to two vertebrate systems, the retinotectal projection (Stahl et al. 1990) and spinal nerve segmentation (Davies et al. 1990), and the latter system will form the subject of this review.

Spinal nerve segmentation

The generation of a segmented pattern of spinal nerves in higher vertebrate (chick) embryos provides a good model system for the study of axon guidance, being both phenomenologically simple and experimentally accessible. The peripheral nervous system of the trunk develops alongside the mesodermal somites, which appear in a segmented chain adjacent to the neural tube (Fig. 1). The somites first cleave from the unsegmented mesoderm as epithelial rosettes, and subsequently differentiate into dermomyotome and sclerotome (Keynes and Stern, 1988). The earliest components of the peripheral nervous system, migrating neural crest cells and outgrowing motor and sensory axons, are confined to each anterior (cranial) half-sclerotome when they encounter the somite mesoderm (Fig. 1; Keynes and Stern, 1984; Keynes and Stern, 1988). With the exception of a few presumptive sensory neurons that later migrate or degenerate, they appear to avoid posterior half-sclerotome, and do so also after its surgical displacement or enlargement in ovo (Keynes and Stern, 1984; Stern and Keynes, 1987; Stern et al. 1991).

This positional restriction produces a segmented pattern of spinal nerves along the anterior–posterior axis, and probably serves to ensure a correct anatomical relationship between the axial peripheral nerves and the flexible vertebral column (Keynes and Stern, 1988).
In principle, it could result from attractive influences in anterior half-sclerotome and/or repulsive influences in posterior half-sclerotome (Keynes and Stern, 1984). Prior ablation of the somites (Lewis et al. 1981) or sclerotomes (Tosney, 1988) abolishes axonal segmentation but does not prevent axonal growth, suggesting a prominent role for repulsion by posterior half-sclerotome in generating the segmental pattern.

A candidate molecular system for mediating axon repulsion was provided by the observation that, in the chick embryo, the plant lectin, peanut agglutinin (PNA) binds the cells of posterior but not anterior half-sclerotome (Stern et al. 1986). Subsequent SDS–PAGE analysis of PNA-binding material from embryonic trunks showed two components (48×10^3 M_r and 55×10^3 M_r) which correspond to the major bands seen in posterior but not anterior half-sclerotome in one dimensional separations. The same PNA-binding glycoprotein fraction causes a three-fold reduction over control values for axon outgrowth when presented as a substrate in vitro, confirming that it might indeed repel growing axons (Davies et al. 1990). Using the growth cone collapse assay (Fig. 2), Davies et al. (1990) have also shown that detergent extracts of chick sclerotomes or trunks cause collapse of motor and sensory growth cones extending on a laminin substratum (Fig. 3). This activity is eliminated from the extracts by the use of immobilised PNA. Rabbit polyclonal antibodies, directed against the 48×10^3 M_r and 55×10^3 M_r components and applied to sections of chick embryo trunks, bind to posterior half-sclerotome in preference to anterior half-sclerotome. When immobilised on a suitable support, they can also be used to eliminate collapse activity from detergent extracts of somite material (Davies et al. 1990).

These observations strongly suggest that the PNA-binding material is responsible for excluding growing axons from the posterior half-sclerotome in vivo. Consistent with this possibility, the lectin receptor is found to be associated with the surface of isolated posterior half-sclerotome cells; on treatment with fluorescent PNA a characteristic ring reaction is seen, followed by patching and capping (Davies et al. 1990). Although axons are able to grow on substrates of posterior cells in vitro, under these circumstances they may avoid those regions of the cell surface bearing the lectin receptor (Stern et al. 1986). During normal development, such conditions for escape from the surface-associated contact repellant would not exist, and the entire posterior half-sclerotome would provide an exclusion zone for trespassing axons. If so, a critical test of this hypothesis will be to administer in ovo suitable antibodies against the 48×10^3 M_r and/or 55×10^3 M_r components (that block, for example, the
growth cone collapse in vitro), which should allow axon entry.

The full growth cone collapse detected in the assay does not necessarily imply that the same takes place in vivo. Nor has it been seen in cocultures of axons and posterior half-sclerotome cells (Tosney, 1987). When a motor axon exits the neural tube opposite a posterior half-sclerotome, a single filopodial contact with a posterior half-sclerotome cell may result in local collapse or paralysis of the growth cone and avoidance
of this region. The direction that the growth cone subsequently takes along the anterior–posterior axis is not random, however. Retrograde labelling of outgrowing motor axons, by the application of horseradish peroxidase to the anterior half-sclerotome, shows that such axons grow consistently towards the nearest-available anterior half-sclerotome (Lim, T. M., Jaques, K. F., Stern, C. D. and Keynes, R. J., 1991; Fig. 4). This directionality is most easily explained by the coexistence of a chemotropic cue for axon guidance, emitted by cells in the anterior half-sclerotome (Fig. 5).

**Molecular mechanisms**

The relationship of the $48 \times 10^3 \, M_r$ and $55 \times 10^3 \, M_r$ components to one another in the native state has yet to be determined, and a full characterisation of the peptide moieties will await their molecular cloning. We have investigated, meanwhile, the role of carbohydrate groups in executing growth cone collapse. One possibility would be that the carbohydrate group that binds PNA directly, the disaccharide residue Galβ1–3GalNAc, might also be directly responsible for executing collapse, binding to an appropriate receptor on the growth cone. That this is not the case is shown by the observation (Davies, J. A., Cook, G. M. W. and Keynes, R. J., unpublished observations) that liposomes incorporating the ganglioside GM1 (at a concentration of $1\, \text{mg}\,\text{ml}^{-1}$), which bears the same disaccharide sequence at the non-reducing terminus (Fig. 6), do not elicit growth cone collapse, whether the ganglioside is in sialylated or non-sialylated form. On the other hand, pretreatment of liposomes incorporating detergent extracts of chick embryo trunks with specific combinations of endo- and exo-glycosidases does remove collapse activity (Wajed, S., Howells, R., Cook, G. M. W. and Keynes, R. J., unpublished observations). This indicates the involvement of glycan moieties in mediating biological activity, either directly (for example, providing a ligand for a growth cone receptor) or indirectly (by maintaining the optimal tertiary structure of the collapse-inducing molecule). The fact that collapse activity survives heat treatment (incubation of extracts at $100^\circ\text{C}$ for 3 min prior to incorporation into liposomes) may favour the former possibility.

Besides the PNA receptor, a variety of other molecular differences between the anterior and posterior half-sclerotome have been detected (see Table 1 for summary). In many cases it has been difficult to assign a critical functional role for these molecules, either as adhesion or repulsion systems for nerve cells, because their spatiotemporal expression patterns do not correlate precisely with neural crest migration and/or spinal axon outgrowth. To take one example, the $70 \times 10^3 \, M_r$ antigen described by Tanaka et al. (1989) is restricted to the anterior half-sclerotome below upper cervical segmental levels, a pattern consistent with a possible role as an adhesion system; but against this, it has no anterior–posterior polarity of expression in the upper cervical sclerotomes.

As regards possible repulsion systems in addition to the peanut lectin-binding glycoconjugate, both chondroitin sulphate proteoglycans and T-Cadherin are candidates in view of their selective expression in posterior half-sclerotome. Tan et al. (1987) have correlated the expression in the somite mesoderm of a
cytotactin-binding chondroitin sulphate proteoglycan (CTB, core protein $280 \times 10^3 M_r$) with neural crest migration. During the early stages of crest migration into the sclerotome the proteoglycan is evenly distributed in both sclerotome halves, and it later adopts a posterior location. Essentially similar expression patterns have been described using antibodies against native chondroitin sulphate and chondroitin-6-sulphate (Newgreen et al. 1990; Perris et al. 1991). The CTB proteoglycan also provides a poor substrate for crest attachment and migration in vitro. Because its polarised expression pattern arises comparatively late, it seems unlikely to play a dominant role in repulsion from posterior half-sclerotome, but (along with other chondroitin sulphate proteoglycans) it may contribute to this process, both in relation to the crest and also to axon growth cones.

T-Cadherin is expressed as soon as the sclerotome dissociates from the epithelial somite and, therefore, appears early enough to influence the pathway of neural crest cell migration from its initiation (Ranscht and Bronner-Fraser, 1991). Its functional effects are unknown, however, and it is equally plausible that it acts as an intercellular adhesion molecule for the cells of the posterior half-sclerotome themselves. As for the PNA-receptor, an important test for the function of any of these molecules will be to interfere with their action in vivo, either by the use of antibodies or with transgenic techniques.

**Cranial nerve segmentation**

If the segmented pattern of spinal nerves is generated by segmentation and repulsion in the adjacent mesoderm, is the same true for the segmentation of cranial nerves?
nerves? There are interesting differences between the manifestations of segmentation in the trunk and cranial regions. In contrast to the developing spinal cord (Lim et al. 1991) the neural epithelium of the developing hindbrain displays conspicuous segmentation (rhombomeres; Lumsden and Keynes, 1989), while the paraxial mesoderm is overtly segmented in the trunk (somites) but less obviously so in the cranial paraxial mesoderm (somitomeres; Anderson and Meier, 1981; Jacobson, 1988).

In the trunk, anterior–posterior reversals of the neural tube, before axon outgrowth, do not alter the peripheral axon trajectories in the somite mesoderm (Keynes and Stern, 1984). We have recently tested whether this also holds for the cranial region. Fig. 7 shows the effects of anterior–posterior reversals of the early chick embryo hindbrain on the outgrowth pattern of the trigeminal and facial nerves. In normal embryos the trigeminal motor nerve exits the hindbrain only from the 2nd rhombomere, while it recruits axons from rhombomeres 2 and 3, and the trigeminal sensory ganglion also lies adjacent to the 2nd rhombomere; motor and sensory axons merge to grow directly into the adjacent mesenchyme of the 1st branchial arch. Similarly, the facial motor nerve exits from the 4th rhombomere, recruiting from rhombomeres 4 and 5, the facial ganglion lies opposite the 4th rhombomere, and motor and sensory axons grow into the 2nd branchial arch (Fig. 7, normal; Lumsden and Keynes, 1989). When the reversal is such that the positions of rhombomeres 2 and 4 relative to the adjacent mesoderm are directly transposed (Fig. 7, symmetrical rotation), the nerves (visualised by whole-mount neurofilament staining) grow separately into the arches immediately adjacent, as in normal embryos, and show little tendency to alter their trajectories outside the neural tube (17/28 embryos). As a result, the 1st arch is colonised inappropriately by axons from rhombomeres 4 and 5, while the 2nd arch is colonised by axons from rhombomeres 2 and 3. When, however, the reversal simultaneously shifts rhombomeres 2 and 4 one segment caudal to the normal positions of the nerve

---

Table 1. Distribution of molecules in avian sclerotome during the phase of neural crest migration and axon outgrowth. Those expressed directly by neural crest cells and axons are excluded from the table

<table>
<thead>
<tr>
<th>Molecular species</th>
<th>Location</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lectin receptors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peanut agglutinin</td>
<td>P(early stages)</td>
<td>1,2,3,4</td>
</tr>
<tr>
<td>Jacalin</td>
<td>P</td>
<td>3</td>
</tr>
<tr>
<td><strong>Extracellular matrix molecules</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytotactin/tenasin/J1</td>
<td>A</td>
<td>5,6</td>
</tr>
<tr>
<td>Chondroitin sulphate/CTB proteoglycan</td>
<td>A (later stages)</td>
<td>7,8</td>
</tr>
<tr>
<td>Uniform distribution at early stages</td>
<td>P (later stages)</td>
<td>5,8,9</td>
</tr>
<tr>
<td>Uniform distribution in both halves</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Variable, A/P distribution depending on</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>epitope</td>
<td>8,10,11</td>
<td></td>
</tr>
<tr>
<td>Uniform distribution in both halves</td>
<td>6,10,11,12</td>
<td></td>
</tr>
<tr>
<td><strong>Cell adhesion molecules</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-CAM</td>
<td>Uniform distribution in both halves</td>
<td>12,13</td>
</tr>
<tr>
<td><strong>Enzymes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butyrylcholinesterase</td>
<td>A</td>
<td>14</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>70×10³ M₁ antigen</td>
<td>A (lower cervical segments and below)</td>
<td>15</td>
</tr>
<tr>
<td>Uniform distribution in upper cervical</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>segments</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>T-Cadherin</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>HNK-1 epitope (quail)</td>
<td>Variable according to region and stage</td>
<td>17</td>
</tr>
<tr>
<td>Differences resolved on 2D gels</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

References:
roots in the paraxial mesoderm (Fig. 7, asymmetrical rotation), in only 1/26 embryos is the normal pattern observed. In the remainder, axons turn along the anterior-posterior axis immediately adjacent to the neural tube, so merging and fasciculating with axons of the adjacent cranial nerve, before entering the arch mesenchyme (Fig. 7).

The simplest interpretation of this result is that tissue adjacent to rhombomeres 2 and 4 in normal embryos is permissive for axon outgrowth, whereas that opposite rhombomeres 3 and 5 is non-permissive. Positive cues may be provided by the cranial neural crest cells (which had migrated from the neural tube before reversal in the majority of grafted embryos). It is also possible that a system of paraxial exclusion zones for nerve cells exists in the cranial mesoderm as well as the trunk mesoderm. If so, it will be important to establish the relationship of such regions to the somitomeres (Anderson and Meier, 1981; Jacobson, 1988), whose significance is somewhat uncertain (Keynes and Lumsden, 1990).

**Comparison with other systems**

Recent progress in the analysis of other developmental
systems where growth cone repulsion may be involved has been reviewed elsewhere (Keynes et al. 1991). One system, which bears particular comparison with peripheral nerve segmentation, concerns the development of an ordered retinotectal projection. In the normal avian projection, nasal and temporal retinal axons project respectively to posterior and anterior optic tectum, and their accurate targeting requires them to respond appropriately to directional cues on the tectal surface. The nature of at least one of these cues appears to be repulsive for a subset of retinal axons.

In an elegant analysis, Bonhoeffer and colleagues (Walter et al. 1987a,b) presented growing retinal axons with substrata of alternating stripes of anterior and posterior tectal membranes, and found that temporal retinal axons remain exclusively on anterior stripes while nasal axons show no anterior–posterior preference. The preference of temporal axons for anterior membranes was abolished by pretreatment of posterior membranes with heat or protease (Walter et al. 1987b), or phospholipase C (Walter et al. 1990a), suggesting that it arises because of repulsion by a component of posterior membranes rather than preferential attraction by anterior membranes. Consistent with this possibility, posterior tectal membranes also cause the collapse of temporal, but not nasal, retinal growth cones (Cox et al. 1990).

Stahl et al. (1990) have now identified a $33 \times 10^3 M_r$ glycoprotein as the candidate repulsion molecule. Liposomes incorporating detergent-solubilised material from embryonic chick brain membranes carry the $33 \times 10^3 M_r$ component and replicate the stripe-guiding and collapse-inducing properties of posterior tectal membranes. Only this component of the liposomes is also concentrated in posterior rather than anterior tectal membranes, shows sensitivity to phospholipase C and is subject to developmental regulation.

An interesting property of this system is that the responding (temporal) axons habituate on continued exposure to the repulsion stimulus: that is, temporal axons are able to grow normally on a substratum composed exclusively of posterior membranes (Walter et al. 1987a). Walter et al. (1990a) have recently proposed a model for axon guidance in the tectum in which habituation of the growth cone plays an important role. During spinal nerve segmentation, by contrast, habituation appears not to take place and, indeed, would not be anticipated. Unlike the tectum, the posterior half-sclerotome must exclude all axons. Consistent with this, axon extension in vitro is substantially reduced on a substrate of repellent material (see above).

Conclusions

Growth cone guidance mechanisms involving contact inhibition may prove to be widespread during vertebrate embryonic development. In the periphery, regions such as developing limb girdle mesoderm (Tosney and Landmesser, 1985), peri-notchochordal mesenchyme (Tosney and Oakley, 1990; Tosney, 1991) and epidermis (Verna, 1985) are also inhibitory to growing axons. In the CNS, midline systems such as the optic chiasm (Godement et al. 1990) and floor plate (Kuwada and Bernhardt, 1990) have been suggested to repel certain axons while allowing others to decussate, and growth cone collapsing activity is found in detergent extracts of embryonic chick brain (Raper and Kapfhammer, 1990). Lastly, growth cone-repelling activities are present in both the white matter (Schwab, 1990) and grey matter (Keynes et al. 1990) of the adult brain, and may contribute to the failure of axon regeneration following injury. To establish the extent to which these various systems are related, and to devise strategies for modifying their action, now depends on molecular cloning of the agents involved and, ultimately, detailed comparison of their structure and function.

We are grateful to T. M. Lim for Fig. 4. This work is supported by grants from Action Research for the Crippled Child, The Medical Research Council and The Wellcome Trust. G.M.W.C. is a Member of the External Scientific Staff of the Medical Research Council.

References
