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Segmentation and Neuronal Development in Vertebrate Embryos

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Understanding the mechanisms of neuronal development and axon guidance necessarily entails the study of suitably simple and accessible experimental models. One such system concerns the generation of segmental patterns during early neuronal development in higher vertebrate (chick) embryos. We describe here the progress which has been made recently in this direction: first, at a molecular level, the factors involved in producing the segmented arrangement of the peripheral spinal nerves; and second, at a cellular level, the importance of segmentation as an influence shaping the neuronal pattern in the developing central nervous system.

Axon Guidance during Segmentation in the Peripheral Nervous System

Since the pioneering studies of Lehmann (1927) and Detwiler (1934) it has been known that the segmented arrangement of the spinal nerves is governed by the pattern of segmentation in the somitic mesoderm. In higher vertebrate embryos, spinal nerve segmentation is further determined by a subdivision of the somite mesoderm into anterior (A, cranial) and posterior (P, caudal) parts. After leaving the neural tube region, neural crest cells, motor and sensory axons are restricted to the anterior half of each somite-derived sclerotome as they traverse the adjacent somitic mesoderm (Keynes and Stern, 1984; Rickmann et al., 1985; Bronner-Fraser, 1986; Teillet et al., 1987; Loring and Erickson, 1987; Tosney, 1988; Kalcheim and Teillet, 1989). This restriction arises because peripheral nerve cells can detect molecular differences between A-and P-sclerotome cells, rather than resulting from

an intrinsic, segmentally-distributed property of the neural tube: in chick embryos, reversal of a strip of presumptive somite mesoderm along the A-P axis causes axons to grow through the posterior (original anterior) parts of the reversed sclerotomes (Keynes and Stern, 1984).

The sub-division of the sclerotome into anterior and posterior halves has important implications for the development of the segmental pattern in both embryonic paraxial mesoderm (the somites) and adult axial skeleton (the vertebral column), which have been reviewed elsewhere (Keynes and Stern, 1988). In this article we focus on the value of the system as an experimental model for the molecular analysis of axon guidance.

Molecular Differences between Anterior and Posterior Cells

The apparently simple, binary nature of the pathway choice made by crest cells and by axons suggested that the system might be suitable for the investigation of nerve guidance at the molecular level, and several approaches have now been taken in this direction. The most obvious is to look for differences between anterior and posterior sclerotome cells using probes, such as monoclonal antibodies, which recognise molecules already known to influence nerve growth in vitro. It might be expected, for example, that cell or substrate adhesion molecules for neural crest cells and/or axonal growth cones would be localised to anterior half-sclerotome. Immunohistochemical studies using antibodies to laminin, fibronectin, N-CAM and N-cadherin have, however, failed to reveal any differential distribution of these molecules within the sclerotome (Rickmann et al., 1985; Krotoski et al., 1986; Duband et al., 1987; Hatta et al., 1987; Mackie et al., 1988). Nevertheless, a number of molecules are distributed asymmetrically within the sclerotome at the stage of motor axon invasion. The posterior half of the sclerotome binds peanut agglutinin (Stern et al., 1986) and antibodies to a cytotactin-binding proteoglycan (Tan et al., 1987), while the anterior half contains the (probably identical) glycoproteins cytotactin (Tan et al., 1987) and tenascin (Mackie et al., 1988), as well as butyrylcholinesterase activity (Layer et al., 1988). Tanaka et al. (1989) have also described a 70kD membrane-associated macromolecule which is expressed in the anterior halves of the chick embryo trunk sclerotomes and, additionally, in the posterior halves of the upper cervical sclerotomes. Indeed, by two-dimensional gel electrophoresis, more than 20 macromolecules have been

found to be expressed differentially in the two sclerotome halves (Norris et al., 1989). The spatio-temporal expression patterns of these macromolecules are dynamic; some, for example, are present in one half of the sclerotome at an early stage of development but shift later to the opposite half. Such patterns imply an overall degree of complexity rather greater than the overt A/P subdivision might suggest at first sight. Of the various molecular differences noted above, only those which have been ascribed some functional role in the establishment of neural segmentation will now be considered.

During the first stages of somite formation in chick embryos, cytotactin is localised to the basal lamina surrounding the epithelial somite (Crossin et al., 1986). By the 30 somite stage, however, it is also detectable in the anterior halves of the newly-formed sclerotomes (Tan et al., 1987), correlating with the simultaneous appearance here of neural crest cells. Tenascin has a similar distribution in quail embryos (Mackie et al., 1988), raising the possibility that the spatial expression of cytotactin/tenascin may determine the segmented neural pattern. However, a number of observations argue against the notion that cytotactin/tenascin plays a critical role in directing the passage of neural crest cells or motor axons through the anterior half-sclerotome (Stern, Norris, Bronner-Fraser, Carlson, Faissner, Keynes and Schachner, submitted). First, the localisation of cytotactin/tenascin to the anterior half of the sclerotome occurs well after the neural crest begins its migration, at which stage cytotactin/tenascin is distributed throughout the sclerotome. Second, surgical removal of the neural crest prevents the localisation of cytotactin/tenascin immunoreactivity to the anterior half; and third, the molecular forms of the molecules expressed in the presence and absence of neural crest cells differ, the "native" high molecular weight form being expressed only if the neural crest is present. Finally, although removal of the neural crest alters considerably the A/P distribution of cytotactin/tenascin immunoreactivity, it does not prevent the segmental pattern of outgrowth of motor nerves into the anterior half-sclerotome (Rickmann et al., 1985).

It must be concluded, then, that cytotactin/tenascin-related glycoproteins are not directly responsible for the pattern of neural crest migration or motor axon outgrowth through the somites. It is possible that cytotactin/tenascin plays a subsidiary role in determining the neural pattern, for example by modulating adhesive interactions between crest cells and extracellular matrix within anterior half-sclerotome. The

observation of Tan et al. (1987) and Mackie at al. (1988), that neural crest cells round up when cultured on cytotactin/tenascin substrates, certainly suggests that cytotactin/tenascin does not simply provide an adhesive substrate for crest cell migration.

The properties of the cytotactin-binding proteoglycan described by Tan et al. (1987) are also relevant. The molecule becomes concentrated in the posterior half-sclerotome, and provides a poor substrate for crest migration in vitro. The proteoglycan may, therefore, be inhibitory for crest migration in vivo. Like its ligand cytotactin/tenascin, however, this molecule is evenly distributed within the sclerotome during the earliest stages of neural crest migration within anterior half-sclerotome, and so cannot dictate the segmented pattern of crest migration.

Peanut Agglutinin Receptors

In a study using peroxidase-conjugated lectins to stain sections of chick somites, it was found that peanut agglutinin (PNA) recognises P-sclerotome cells and not A-cells (Stern et al., 1986). The differences detected with PNA are related to qualitative changes in the surface glycoprotein composition of A-and P-cells (Davies, Cook, Keynes and Stern, in preparation). Histochemical studies using fluorescein-conjugated PNA (which recognises non-sialylated Gal β1-3 GalNAc residues) or jacalin (which recognises the same disaccharide whether sialylated or not) show lectin binding to P-cells but not A-cells. Staining is inhibited competitively by lactose, indicating specific binding; moreover, P-cell suspensions treated with FITC-PNA show a distinctive ring reaction at the cell surface, which evolves into fluorescent patches and caps. By affinity chromatography on immobilised PNA or jacalin, the PNA-binding glycoprotein fraction has been found to comprise components of apparent M_r 48kD and 55kD. Furthermore, examination of separated A- and P-sclerotome halves by SDS-PAGE shows also that the major differences detectable after silver staining are bands at 48kD and 55kD, being exclusively from P-sclerotome (Davies et al., in preparation).

The localisation of the PNA-binding material to P-sclerotome raises the possibility that this glycoprotein fraction may be inhibitory to axon outgrowth in vivo, thereby channelling axons into A-sclerotome. In order to assess this, we have established an assay system based on a method devised by Dr J. Raper (University of Pennsylvania). Detergent-

solubilised molecules derived from sclerotomes are incorporated into liposomes, which are then added to cultures of chick dorsal root ganglia growing on a laminin substrate (Fig. 1). Abrupt collapse of growth cones is observed (cf. Kapfhammer and Raper, 1987), which reverses after liposome removal. Untreated liposomes are devoid of collapsing activity, as are liposomes prepared after pre-treating the sclerotome extract with immobilised PNA. These observations support the possibility that the PNA-binding glycoprotein fraction may prevent nerve cells from entering P-sclerotome in vivo.

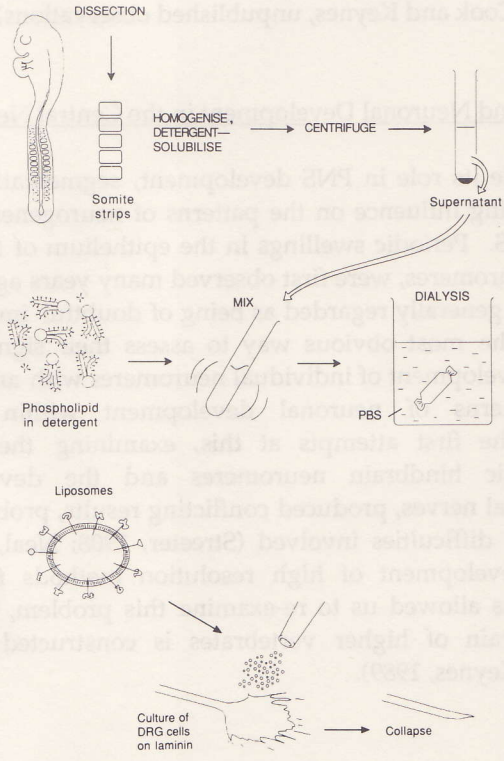

Figure 1. Diagram summarising the assay for growth cone inhibitory activity. Detergent-solubilised somite material is mixed with phospholipid and the detergent is then removed by dialysis. The resulting liposomes, incorporating the somite material, are added to cultures of sensory axons growing on laminin.

Whether similar inhibitory mechanisms are operative elsewhere during embryonic neural development, or whether they might contribute to the failure of regeneration in the adult central nervous system, are interesting questions for future research (see Patterson, 1988). The observation that CNS growth cones also respond to the A/P sclerotome difference is at least consistent with these possibilities: when fragments of 4 day chick embryo telencephalon are grafted in place of spinal cord in 2 day host embryos, telencephalic axons grow selectively into anterior half-sclerotome (Keynes, unpublished observations). In addition, immunoblotting techniques have demonstrated that the somite-derived glycoprotein fraction described above is also present in adult chicken brain (Davies, Cook and Keynes, unpublished observations).

Segmentation and Neuronal Development in the Central Nervous System

Alongside its role in PNS development, segmentation is also an important shaping influence on the patterns of neurogenesis within the developing CNS. Periodic swellings in the epithelium of the vertebrate neural tube, neuromeres, were first observed many years ago (see Vaage, 1969), but were generally regarded as being of doubtful importance (e.g. Neal, 1918). The most obvious way to assess their significance is to correlate the development of individual neuromeres with any underlying segmental patterns of neuronal development within the neural epithelium. The first attempts at this, examining the relationship between specific hindbrain neuromeres and the development of individual cranial nerves, produced conflicting results, probably because of the technical difficulties involved (Streeter, 1908; Neal, 1918). The more recent development of high resolution methods for following neurogenesis has allowed us to re-examine this problem, and to show that the hindbrain of higher vertebrates is constructed segmentally (Lumsden and Keynes, 1989).

Rhombomeres

In the developing hindbrain, the neuromeres ("rhombomeres") lie on either side of the midline floorplate, being visible macroscopically in the chick embryo between days 2 and 4 of incubation. Soon after their first appearance in the chick, the boundaries between adjacent rhombomeres are colonised by axons growing laterally in the marginal

zone of the epithelium. The local application of lipid-soluble dyes, DiI and DiO, to individual cranial nerve roots has allowed the relation between the pattern of neurogenesis of the cranial branchiomotor nerves and the rhombomere series to be examined; the fluorescent dye diffuses retrogradely in the neuronal membranes, allowing the early motor nuclei to be identified (Lumsden and Keynes, 1989). The results are illustrated schematically in Fig. 2. Each motor nucleus in the sequence of cranial branchiomotor nerves V (trigeminal), VII (facial) and IX (glossopharyngeal) originates from a specific, sequential pair of rhombomeres; in turn, each rhombomere pair lies in register with an adjacent branchial arch, which is innervated by the appropriate cranial nerve. Thus, the trigeminal nucleus originates from rhombomeres 2 and 3 (r2,3) and innervates the 1st arch; the facial nucleus originates from r4,5 and innervates the 2nd arch; and the glossopharyngeal from r6,7, innervating the 3rd arch. Neurones differentiate in a two-segment repeat pattern: reticular neurones and motor axons arise in the anterior member of each rhombomere pair before the posterior member, and the anterior member contains the cranial nerve root. The boundaries between rhombomeres also represent lineage restriction boundaries (Plate 2; Fraser, Keynes and Lumsden, in preparation).

The overt rhombomere pattern is therefore matched at the cellular level. It is also matched at the gene level: a two-segment repeat has been found recently in the transcription pattern of a mouse zinc finger gene, Krox-20, which is expressed only in r3 and r5 during early mouse development (Wilkinson et al., 1989). Many mouse homeobox genes, moreover, have A/P boundaries of expression that lie within the embryonic hindbrain (Holland and Hogan, 1988) and which may correspond with rhombomere boundaries in some cases [e.g. Hox-1.5, (Gaunt, 1987)]. Such patterns, being reminiscent of the spatial expression patterns of the Drosophila segmentation and homeotic genes (Nusslein-Volhard and Wieschaus, 1980; Akam, 1987), raise the possibility that some of the mechanisms of neural development operative in this region of the vertebrate brain may turn out to be similar to those of invertebrates.

Whether segmentation extends beyond the hindbrain within the higher vertebrate CNS remains to be established. Segmental arrangements of neurones are not present during the development of the chick spinal cord; the neuromeres visible in this part of the neural tube probably arise as a result of mechanical interactions between the

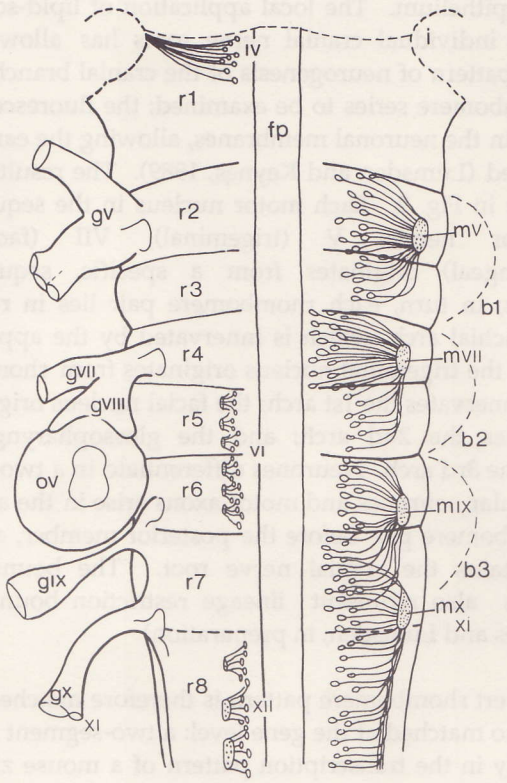

Figure 2. Schematic diagram of neuronal development in the 3 day chick embryo hindbrain. The cranial sensory ganglia (gV-gX), branchial motor nuclei, somatic motor nuclei (IV, VI, XII) and the combined roots of the sensory and branchial motor nerves (mV-mXI) are shown in relation to the rhombomeres (r1-r8) and branchial arches (b1-b3). ov, otic vesicle; fp, floorplate; i, isthmus/midbrain-hindbrain boundary. Reproduced by permission from Nature Vol. 337, p.428. Copyright (c) 1989, Macmillan Magazines Ltd.

neuroepithelium and the adjacent somites (Lim, 1987), as suggested originally by Neal (1918). Segmentally arranged neurones have been described, however, in the spinal cord and hindbrain of certain lower vertebrates (Bone, 1960; Whiting, 1948; Myers, 1985; Metcalfe et al., 1986; Hanneman et al., 1988). It seems probable that during the course of

vertebrate evolution, as brain centres for movement control came to dominate spinal neuromuscular circuits, intrinsic spinal cord segmentation disappeared. In the hindbrain region, on the other hand, the requirement for independent as well as integrated control of the branchial arch derivatives, such as the facial and jaw musculature, has caused segmentation to be conserved. The challenge now is to identify the molecular mechanisms which underlie such ordered patterns of neuronal development.

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The Colour Plates

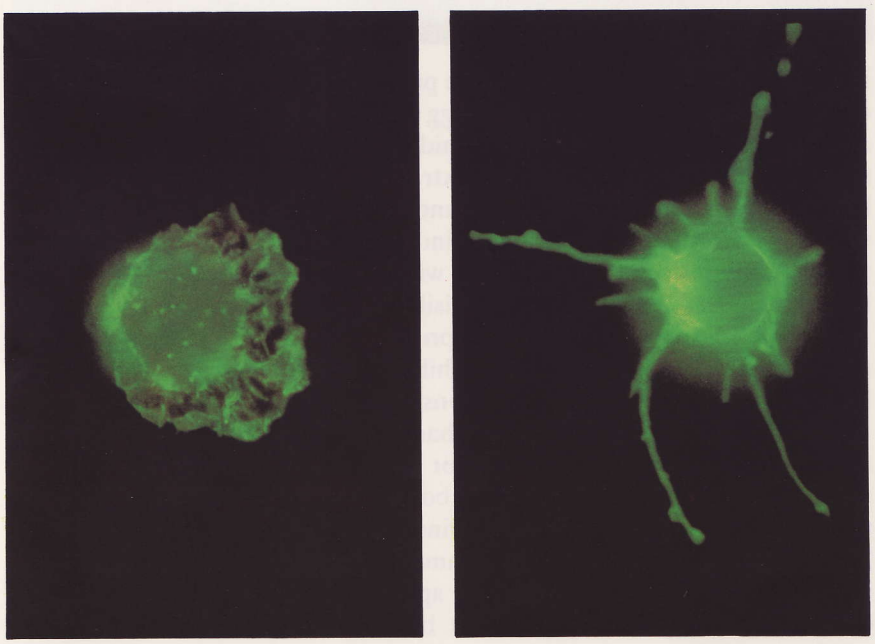

Plate 1 GAP-43 induces filopodia in COS cells. Cells transfected with control plasmids, in this case one which expresses the cell membrane protein CD8 (left panel), are generally round. Cells expressing large amounts of GAP-43 after transfection (right panel) tended to extend filopodia. Shown are immunofluorescent labelling of CD8 (left) and GAP-43 (right)

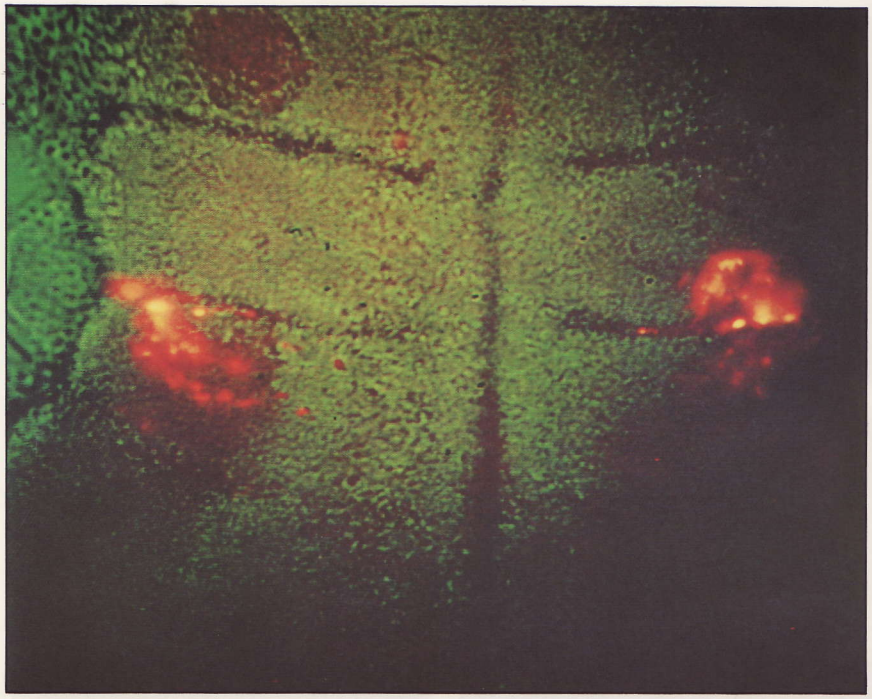

Plate 2 Clones derived from a single parent cell on both left and right sides of the chick embryo hindbrain. The egg was windowed at the 5-somite stage, and one cell on each side of the midline was labelled by iontophoretic injection of lysinated rhodamine dextran near the site of the presumptive boundary between rhombomeres 2 and 3. Two days later the embryo was fixed in paraformaldehyde, and the hindbrain viewed as a whole-mount on a fluorescence microscope equipped with an SIT camera. Rhombomere boundaries 1/2, 2/3 and 3/4 are visible on either side of the midline floorplate. Two expanded clones are present at the 2/3 boundary: on the left, the clone straddles the boundary, while the right-sided clone respects the boundary. In a series of such injections, clones were seen always to respect the boundaries when the parent cell had been labelled at or after boundary formation, whereas they respected or straddled the boundaries when the parent cell had been labelled before boundaries had appeared; boundaries therefore represent regions of cell lineage restriction (Fraser, Keynes & Lumsden, in preparation). In the example illustrated here, each parent cell was labelled several hours before the appearance of the 2/3 boundary. That on the right is presumed to have been further from the site of the presumptive boundary than that on the left; its progeny first reached, and thereby challenged, the 2/3 position only after the boundary had formed, and the clone was unable to cross the boundary. The clone on the left is presumed to have crossed the 2/3 position before boundary formation