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An evaluation of myelomeres and segmentation of the chick embryo spinal cord

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Summary

We have investigated whether the neuromeres of the developing chick spinal cord (myelomeres) are manifestations of intrinsic segmentation of the CNS by studying the patterns of cell proliferation and neuronal differentiation. Treatment of 2-day embryos with colchicine does produce exaggerated myelomeres, in confirmation of Källén (Z. Anat. Entwickl.-Gesch. 123, 309–319, 1962). However, this does not imply that myelomeres are segmental proliferation centres: the undulations caused by colchicine are irregular alongside the unsegmented mesoderm, and another mitotic inhibitor, bromodeoxyuridine, has no such effects. In contrast to lower vertebrate embryos, there is no evidence for segmental groups of primary motor neurons in the chick: the earliest motor neurons express cholinesterase, and project their axons into the adjacent sclerotome, at random positions in relation to the somite boundaries. The population of motor neurons projecting HRP-labelled axons into a single somite lies out of phase with both myelomere and somite, and is placed symmetrically about the anterior half-sclerotome. The earliest intrinsic spinal cord neurons, as stained with zinc iodide–osmium tetroxide or anti-68×M_r neurofilament antibody, show no segmental patterns of differentiation. We conclude that, in contrast to the rhombomeres of the developing hindbrain, myelomeres are not matched by segmental groupings of differentiating nerve cells, and result from mechanical moulding of the neuroepithelium by the neighbouring somites.

Key words: segmentation, myelomeres, spinal cord, chick embryo, motor neurons.

Introduction

The mechanisms that generate the diversity of neuronal pattern along the vertebrate neuraxis are poorly understood. During the last century, it was suggested that subdivision of the neural epithelium by segmentation may provide an important basis for such variation. Early studies described the transient appearance within the neural epithelium of periodic swellings or undulations, first christened 'neuromeres' by Orr (1887). Since then, two contrasting views of the developmental significance of neuromeres have been taken. The first (Neal, 1918) holds that neuromeres are a consequence of mechanical interactions between the neural epithelium and the adjacent segmented mesoderm, and that they have no important, enduring influence on CNS morphogenesis. The second regards them as intrinsic repeat-units representing essential elements of CNS construction which, once established, have independent patterns of cell lineage and differentiation (see Keynes and Lumsden, 1990, for review).

Recent studies of neuronal development in the chick embryo hindbrain (Lumsden and Keynes, 1989) have shown that this region of the neural tube is segmented: the hindbrain neuromeres, or rhombomeres, bear a constant, well-defined relationship to the cranial branchiomotor nuclei and the adjacent branchial arches. The boundaries between neighbouring rhombomeres represent lineage restriction boundaries across which cells do not move (Fraser *et al.* 1990). These boundaries are matched, moreover, by the boundaries of expression of genes such as Krox-20 (Wilkinson *et al.* 1989*a*) and some of the Hox-2 genes (Wilkinson *et al.* 1989*b*) which, by analogy with their homologues in *Drosophila*, have been suggested to play important roles during the development and diversification of the segmental pattern (Holland and Hogan, 1988; Akam, 1989; Wilkinson, 1989).

In this study, we have investigated whether segmentation is involved in the development of the chick spinal cord. Although segmentation of the peripheral spinal nerves is governed by segmentation in the adjacent somite mesoderm (Lehmann, 1927; Detwiler, 1934), and by the further subdivision of each somite into

anterior and posterior halves (Keynes and Stern, 1984, 1988), the possibility remains that the spinal neural epithelium is also segmented intrinsically. Using a variety of techniques to analyse neuronal development, we conclude that the epithelial swellings of the spinal neural tube ('myelomeres') are, in distinct contrast to the hindbrain rhombomeres, a passive outcome of segmentation in the neighbouring mesoderm.

Materials and methods

Observations in ovo

Fertile hens' eggs were incubated at 38°C to stages 6–17 (Hamburger and Hamilton, 1951). Each egg was then windowed, and the embryo raised to the level of the window with calcium- and magnesium-free Tyrode's solution (CMF). The embryo was then viewed *in ovo* under the dissecting microscope using fibre-optic illumination, after the injection of 0.1 ml of Indian ink (Pelikan Fount India, 1:10 in CMF) into the sub-blastodermic space to provide a dark background.

Light microscopy

After dissection, embryos were washed in phosphate-buffered saline (PBS) and fixed in buffered formol saline for at least 2 h. They were then washed in PBS, stained with 1 % light green for 15 min, dehydrated in alcohols, cleared in xylene and whole-mounted in Permount (Fisher).

Scanning electron microscopy

Embryos were explanted and fixed overnight at 4°C in modified Karnovsky's fixative containing 1.25 % glutaraldehyde, 1 % paraformaldehyde and sucrose to a total of 310 mosmol kg^{-1} in 0.1 M sodium cacodylate buffer, pH 7.2. Embryos were then washed in buffer for 2 h, post-fixed in 1 % osmium tetroxide for 45 min, bisected along the midline, washed again in buffer and dehydrated with acetone. They were then critical-point dried, glued onto aluminium studs, coated with a 10 to 20 nm layer of gold (Polaron E5000) and viewed under the scanning electron microscope (Cambridge Stereoscan 600).

Heat shock

After 30–36 h at 38°C, eggs were incubated at 55°C for 50 min (Primmett *et al.* 1988), after which normal incubation was resumed until the embryos reached stages 13–15. Embryos were then stained with light green and whole-mounted as above.

Colchicine and bromodeoxyuridine treatment

Eggs were windowed after 48 h incubation, and 1 ml of 0.01 mg ml^{-1} colchicine (Sigma) in Tyrode's solution was placed onto the embryo, following the procedure of Källén (1962). Alternatively, 100 μl of 5-bromo-2'-deoxyuridine solution (BrdU, Sigma; either 5 μg ml^{-1} or 0.5 μg ml^{-1} in PBS) was injected into the sub-blastodermal space, following the procedure of Primmett *et al.* (1989). The window was sealed with PVC tape and the embryos incubated for up to 4 h at 38°C before examination as whole mounts.

BrdU labelling of mitotic neuroepithelial cells

Eggs were windowed and 50 μl of BrdU (1 mg ml^{-1}) were injected into the sub-blastodermal space. Eggs were then incubated for 1 h, after which embryos were fixed in 4 % formal-saline at 4°C for 12 h. After washing in PBS, they were immersed in 5 % sucrose in PBS for 2 h and then in 15 % sucrose in PBS overnight, both at 4°C. Embryos were then infiltrated with 10 % gelatin (Bloom 300, Sigma) containing 15 % sucrose in PBS at 37°C for 4 to 6 h, after which the gelatin was allowed to set. Specimen blocks were snap-frozen for cryostat sectioning in isopentane cooled with liquid nitrogen, and sectioned longitudinally at 12 μm at −25°C. Serial sections were mounted on gelatin-subbed slides.

The distribution of BrdU-labelled cells was assessed immunohistochemically using monoclonal anti-BrdU antibody (Amersham). Sections were treated with a solution containing 1 % BSA and 0.1 % Triton X-100 in PBS for 2 h at room temperature, followed by undiluted primary antibody overnight at 4°C. Sections were then washed in a solution containing 1 % goat serum and 0.1 % Triton X-100 in PBS, and stained with rhodamine-conjugated goat anti-mouse IgG (1:100, Sigma) for 1 h at 37°C. After 3 subsequent washes in PBS, nuclei were counterstained with Hoechst dye (1:800, Sigma) for 10 min at room temperature. Sections were then washed 3 times in PBS, mounted in Citifluor (Citifluor Ltd, London) and viewed with fluorescence optics.

Cholinesterase staining

Stage 10–18 embryos were fixed in formol saline at 4°C overnight, embedded in sucrose–gelatin and cryostat-sectioned as above. The enzyme reaction medium was prepared according to Karnovsky and Roots (1964). 5 mg of acetylthiocholine iodide were dissolved in 6.5 ml of 0.1 M sodium phosphate buffer, pH 6.0. The following were added in order, with stirring in between each addition: 0.5 ml of 0.1 M sodium citrate, 1.0 ml of 30 mM copper sulphate, 1.0 ml of water and 1.0 ml of 5 mM potassium ferricyanide. Slides were incubated in this solution for 2 h at 37°C. They were then washed in distilled water, air-dried, and mounted in Hydramount (Gurr) or Entellan (Merck). Control sections were incubated in the absence of the substrate acetylthiocholine iodide.

Zinc iodide–osmium tetroxide staining

Chick embryos were stained according to a modification of the zinc iodide–osmium tetroxide method (Keynes and Stern, 1984). Embryos were pinned out on Sylgard dishes in Tyrode's solution and bisected along the anterior–posterior axis. They were then immersed in a freshly prepared mixture of 6 ml zinc iodide and 1.75 ml of 2 % osmium tetroxide; the zinc iodide was made by combining 5 g iodine with 15 g powdered zinc in 200 ml distilled water. Specimens were then incubated at 55°C for 1 h 40 min, washed with distilled water, dehydrated in alcohols, cleared in xylene and whole-mounted in Permount between two coverslips.

Retrograde labelling with horseradish peroxidase

Chick embryos at stages 17–19 were used to analyse the early outgrowth of motor axons from the neural tube. Embryos were pinned out on clean Sylgard dishes and immersed in fresh oxygenated Tyrode's solution. Horseradish peroxidase (HRP, Sigma Type VI) was applied to the anterior half of one somite, using an insect pin with the enzyme dried to a crust on its tip. After HRP application, embryos were transferred to small Petri dishes containing oxygenated Tyrode's solution, and the dishes were placed in a sealed plastic chamber (Flow Labs) gassed immediately previously with 95 % oxygen and 5 % carbon dioxide. Embryos were then incubated at 37°C for 3 to 6 h, followed by fixation in 2 % glutaraldehyde (in 0.1 M sodium phosphate buffer, pH 7.4, 4°C) for 15 min. They were then embedded in sucrose–gelatin and sectioned as described above for cholinesterase staining. Serial cryostat sections

(20–40 μM) were mounted on gelatin-subbed slides and reacted with diaminobenzidine according to the method of Adams (1981), using cobalt chloride and nickel ammonium sulphate to enhance the enzyme reaction, or by a modification of the method of Straus (1982), using imidazole to intensify the reaction product. In the first method, the incubation mixture was a solution of 0.05% 3,3′-diaminobenzidine (Sigma), 0.025% cobalt chloride and 0.2% nickel ammonium sulphate in 0.1 M sodium phosphate buffer, pH 7.4. In the second method, 0.01 M imidazole was used instead of the cobalt/nickel mixture. Sections were incubated in the respective solutions for 15–20 min. Fresh hydrogen peroxide was then added to the incubation mixture to a final concentration of 0.015%, and the sections reacted for a further 10 min. Finally, sections were washed in distilled water and mounted directly in Hydramount, or air-dried overnight and then mounted in Entellan (Merck).

Whole-mount staining with neurofilament antibody

The procedure of Lumsden and Keynes (1989) was followed. Embryos were immersion-fixed in 4% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.2 at 4°C) for 24 h. Endogenous peroxidase was then blocked by overnight immersion in PBS+0.05% hydrogen peroxide at 4°C, and embryos were incubated in a monoclonal antibody (3A10; Developmental Studies Hybridoma Bank) recognising an epitope associated with neurofilaments, with 1.0% Triton X-100 for 4 days at 4°C. After overnight incubation in peroxidase-conjugated donkey anti-rabbit immunoglobulin (1:100, Amersham), embryos were washed and immersed in diaminobenzidine (500 μg in 1 ml Tris pH 7.3) for 3 h at room temperature in the dark. Hydrogen peroxide was added and the embryos reincubated at room temperature, after which the reaction was stopped by immersion in PBS. The spinal cords were then dissected from the embryos, cut down the dorsal midline, opened out and whole-mounted in cavity slides with 90% glycerol+10% PBS+0.2% sodium azide.

Results

Morphology of myelomeres

Myelomeres are periodic undulations of the neural epithelium of the presumptive spinal cord, visible in the living embryo *in ovo*. The convexity of each myelomere lies directly opposite an inter-somite boundary (Fig. 1). In agreement with Vaage (1969), the most anterior (A, rostral) pair of myelomeres becomes visible when the first inter-somite boundary forms, between somites 1 and 2, posterior (P, caudal) to the otic vesicle. Thereafter, myelomeres appear in parallel with the formation of successive pairs of somites; they are never

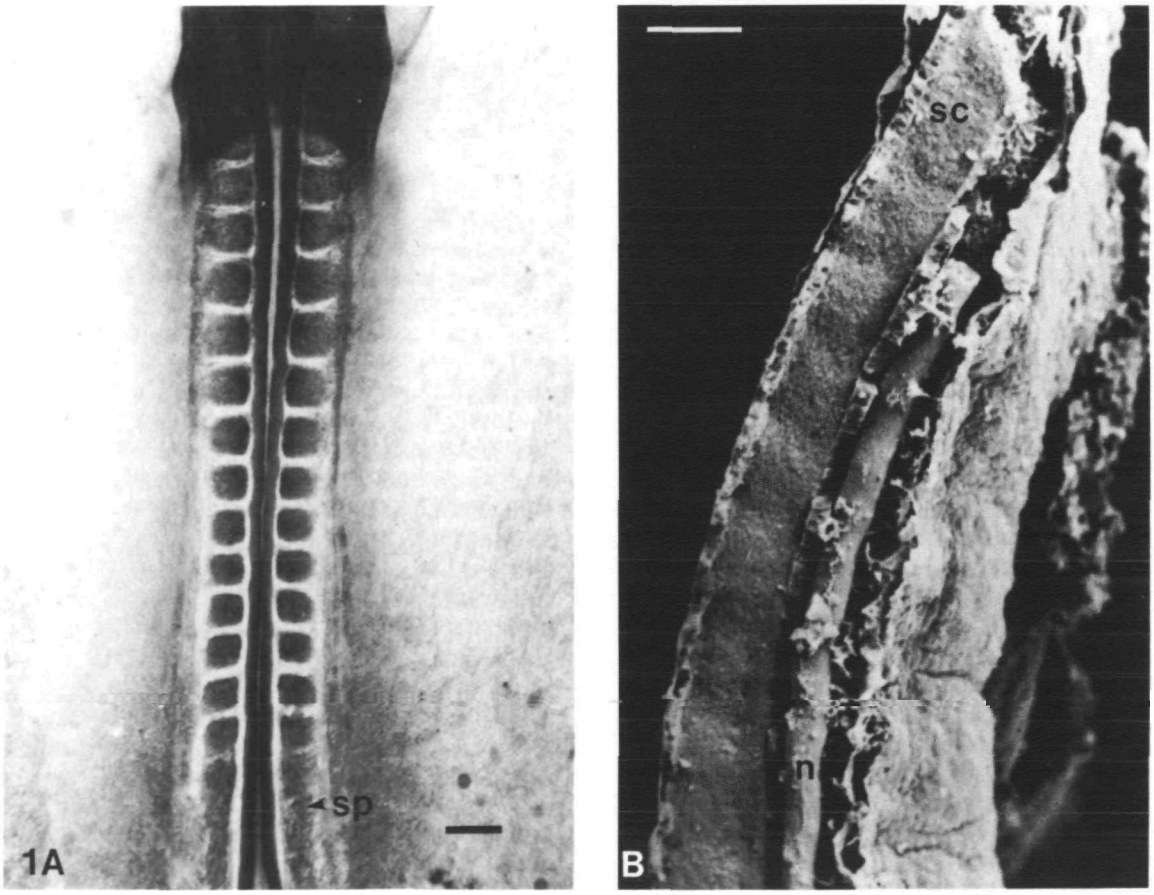


Fig. 1. Myelomeres in the developing chick spinal cord. (A) Whole-mounted 16-somite embryo (stage 12) stained with light green, viewed from dorsal side. Undulations of the neural epithelium are visible alongside the somites but not opposite the segmental plate (sp). (B) Scanning electron micrograph of the ventricular surface of a stage 13 spinal cord (sc), viewed after bisection of the embryo along the midline. Periodic undulations are visible. Anterior to the top, dorsal to the left; n, notochord. Scale bar 150 μm.

visible at the level of the non-segmented, presumptive somitic mesoderm, the segmental plate (Fig. 1A). At each A–P level they remain conspicuous until their motor neurons have begun to extend axons into the anterior halves of the adjacent sclerotomes, approximately 16 (trunk level) to 24 (occipital level) hours later.

Effects of heat shock

One explanation for the morphological appearance of myelomeres, first raised by Neal (1918), is that they arise simply as a consequence of mechanical moulding of the neural tube by the contours of the neighbouring somites, and do not represent a manifestation of intrinsic segmentation of the neural epithelium. If so, an experimental manipulation that changes the position and size of somites should, correspondingly, alter the myelomeres. We therefore used heat shock, which produces local disruptions in the segmental pattern by altering the number of cells that segment together (Elsdale and Davidson, 1986; Veini and Bellairs, 1986; Primmett *et al.* 1988, 1989). Ten embryos of a total of 110 subjected to heat shock showed somite abnormalities. Of these, 3 showed a loss of A–P registration of the somites between the left and right sides of the embryo. In each case, the registration of the myelomeres followed precisely the somite pattern, so that the myelomeres on the left and right halves of the neural

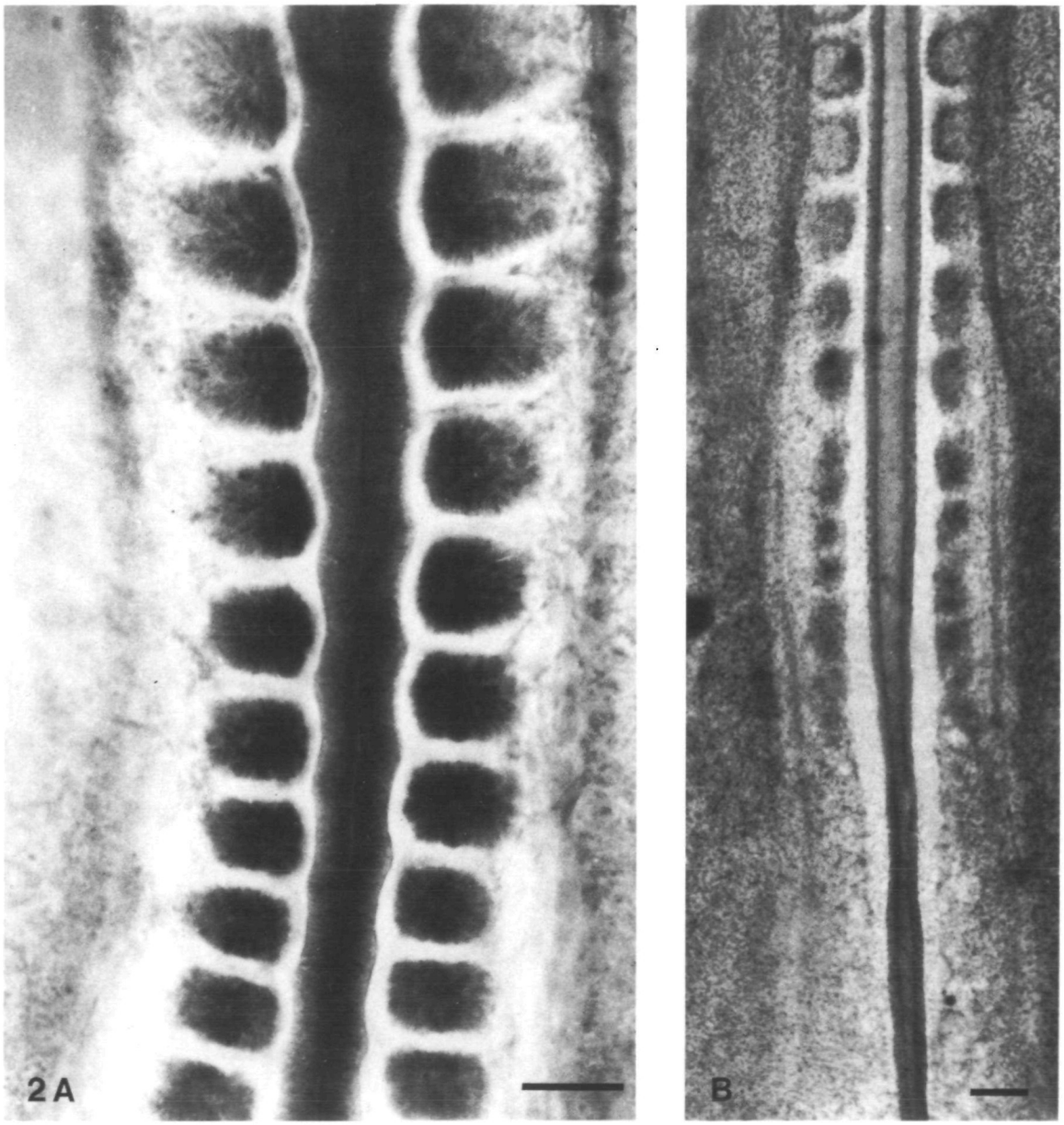


Fig. 2. Effects of heat shock on the myelomere pattern. (A) After disruption of the A–P registration of the somite pairs flanking the neural tube (compare Fig. 1A), the myelomere registration is altered in parallel. (B) Loss of myelomeres when the somites are abnormally small and the space between the somite epithelium and the neural epithelium is increased. Scale bar 100 μm.

tube were not aligned (Fig. 2A). In 7 embryos the epithelial somites immediately anterior to the segmental plate were abnormally small, the space between them and the neural epithelium was correspondingly increased, and the myelomeres were absent (Fig. 2B). These findings are consistent with a mechanical explanation for myelomere formation.

Colchicine and bromodeoxyuridine treatment

Källén (1962) found that the amplitude (i.e. mediolateral excursion) of the myelomeres was increased substantially by the application *in ovo* of colchicine for 4 h. In the present study, 7 embryos were treated with colchicine and examined 4 h later, when all showed exaggerated myelomeres, confirming Källén's observation (Fig. 3). In each case, however, abnormal undulations of the neural epithelium also appeared in the neural tube opposite the segmental plate, and these were irregularly spaced (Fig. 3). Moreover, examination of 2 embryos 45 min after drug application showed that the effect of colchicine was already visible by this stage. In contrast to colchicine treatment, 4 h after application of BrdU at a dose shown previously to inhibit the cell cycle in chick embryos (100 μl of 0.5 μg ml^{-1} solution; Primmett *et al.* 1989), myelomeres were unchanged (3 embryos). They were also unchanged 2 h after BrdU application at 10× this dose (3 embryos).

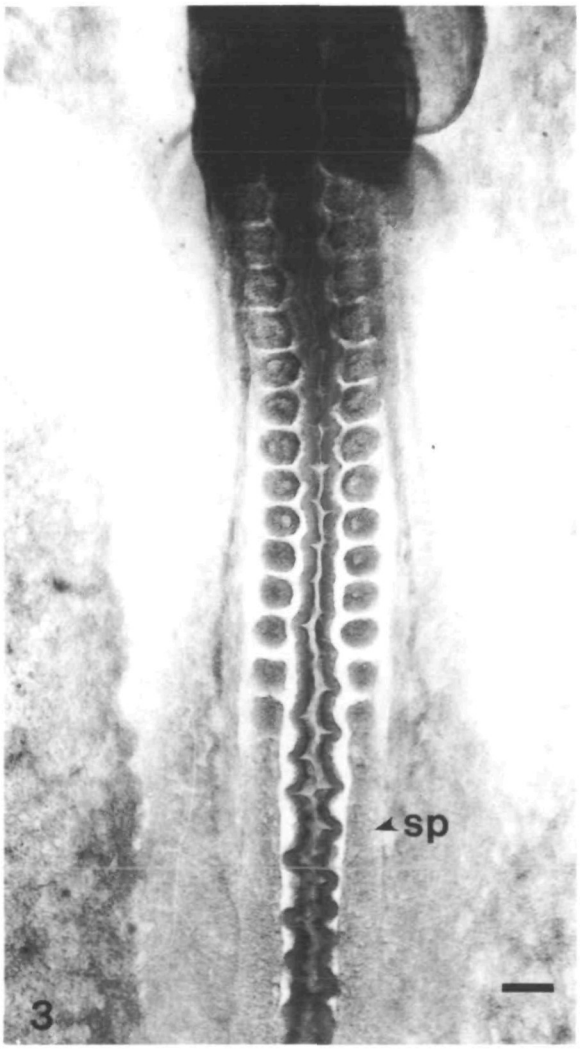


Fig. 3. Effects of colchicine. A stage 12 chick embryo after 4 h exposure to colchicine, whole-mounted and stained with light green. The myelomere amplitude is increased, and irregular undulations of the neural epithelium are now visible opposite the segmental plate (sp). Scale bar 150 μm.

S-phase labelling of neuroepithelial cells

In 6 stage-17 embryos, monoclonal anti-BrdU antibody was used to label proliferating neuroepithelial cells in S-phase. In contrast to the hindbrain (see Discussion), in the developing spinal cord no segmental groupings of nuclei were visible.

Motor neuron development in the trunk neural tube

(a) *Cholinesterase staining*

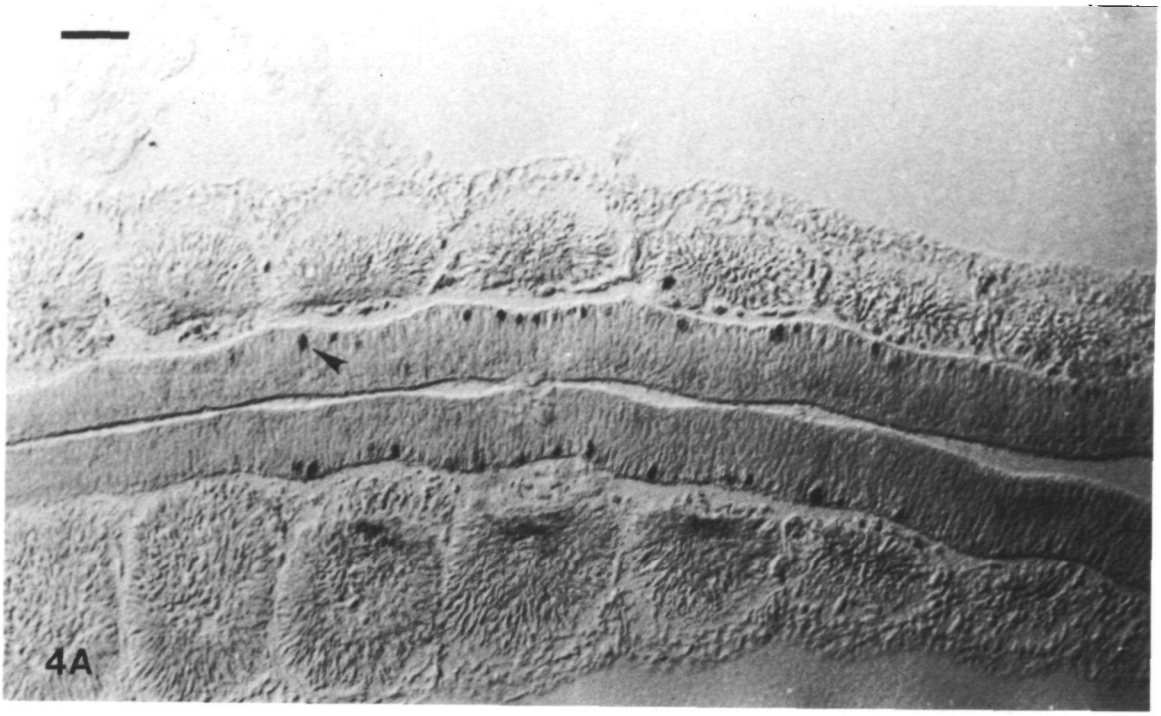
Cholinesterase-positive cells were first detected in the occipital region, at stage 13 (19 somites). They were located in the ventrolateral part of the neural tube, adjacent to the basal lamina, consistent with their designation as motor neurons. With increasing embryonic age, the appearance of cholinesterase activity in the neural tube progressed in a posterior direction, in parallel with the anterior–posterior development of axial structures, but not in a segmented manner: new cholinesterase-positive cells appeared at random positions along the A–P axis, and bore no periodic positional relationships with the neighbouring somites (Fig. 4A).

(b) *Zinc iodide-osmium tetroxide staining*

Cervical motor neurons began to extend axons into the anterior half-sclerotome at stage 15, while axons from thoracic motor neurons started to emerge at stage 17. We saw no stereotyped pattern of axonal outgrowth in relation to the somites. In a series of several consecutive segments, any somite could receive the first outgrowing axon in advance of its more anterior neighbours, and the earliest axons showed no preference for growth within any particular region of the anterior half-sclerotome (Fig. 4B).

(c) *Retrograde labelling of motoneurones with horseradish peroxidase*

HRP was applied to the anterior halves of 20 thoracic somites in 15 stage-17 embryos, to label retrogradely the cell bodies of the earliest outgrowing motor neurons. Labelled cell bodies were unipolar and were located near the ventrolateral margin of the neural tube (Fig. 5A). One to three cells were labelled per segment at stage 17. These cell bodies did not bear any consistent positional relationship with respect to the boundaries between adjacent sclerotomes. Thus, in 13 cases the earliest axon came from a cell placed opposite anterior half-sclerotome (Fig. 5B), and in 4 cases it came from a cell opposite posterior half-sclerotome; in



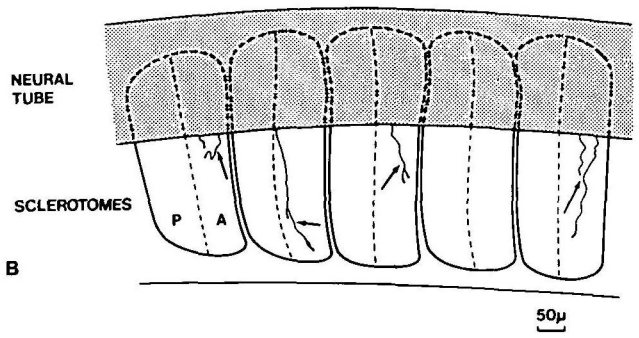


Fig. 4. (A) Longitudinal section through the ventral part of a stage 13 chick embryo neural tube, showing cholinesterase-positive cells (e.g. arrowhead) adjacent to the lateral margin of the epithelium, and their relation to the myelomeres. No segmental arrangement of cells is visible. Scale bar 50 μm. (B) Drawing of a zinc iodide–osmium tetroxide stained, whole-mounted stage 17 chick embryo, showing the earliest motor axons (arrows) growing into the neighbouring somites at the thoracic level. Anterior to the right, dorsal uppermost. The contours of the sclerotomes are outlined, as are the positions of the A–P boundaries within each sclerotome. Axons appear at random positions within each anterior half-segment.

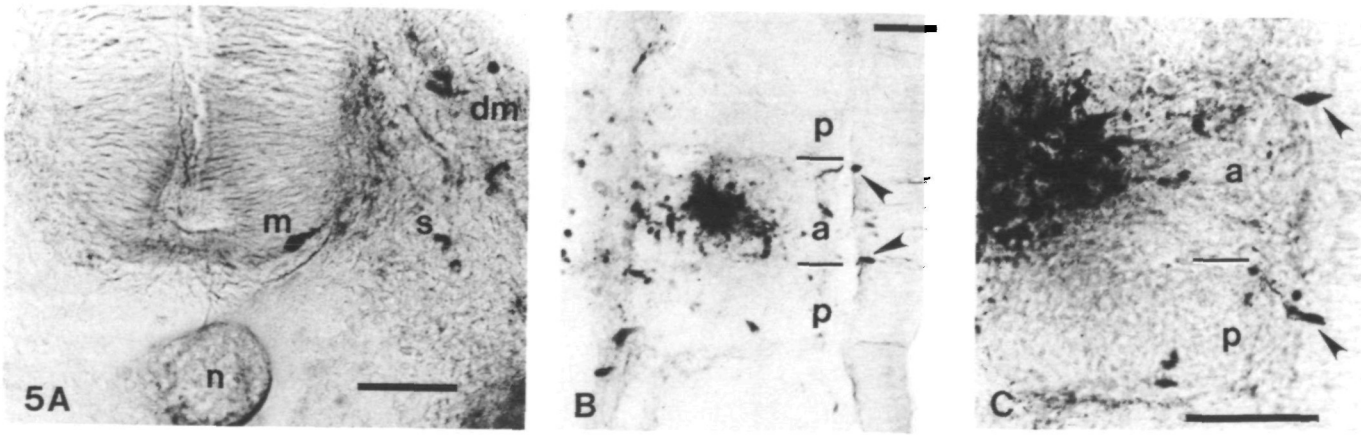


Fig. 5. Sections through stage 17 chick embryos after application of HRP to the outgrowing motor axons in the anterior half-sclerotome, thoracic region. Scale bar 50 μm. (A) Transverse section; a single HRP-filled motor neuron (m) is visible in the ventrolateral part of the neural epithelium. n, notochord; dm, dermomyotome; s, sclerotome. (B) Longitudinal section; two HRP-labelled motor neurons (arrowheads) lie opposite the anterior half-sclerotome (a). The upper and lower thin horizontal lines indicate, respectively, the positions of the a–p intersomite boundary and the a–p intrasclerotomal boundary; p, posterior half-sclerotome. (C) Longitudinal section; HRP-labelled motor neurons (arrowheads) lie opposite anterior (a) and posterior (p) half-sclerotome; thin horizontal line denotes the a–p intrasclerotomal boundary.

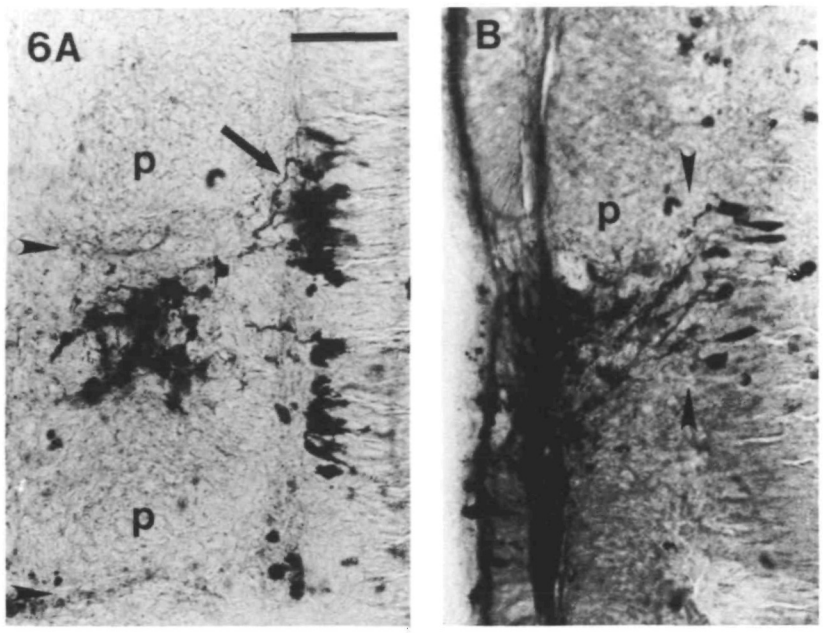


Fig. 6. Longitudinal sections through stage 19 chick embryos after application of HRP to anterior half-sclerotome. In each case the neural tube lies on the right. Scale bar 50 μm. (A) The population of labelled motor neurons is centred upon the anterior half-sclerotome, and lies out of phase with the somites. Motor axons (arrowed) originating from neurons placed opposite posterior half-sclerotome (p) turn posteriorly, outside the neural tube, towards the nearest anterior half-sclerotome. Arrowheads denote the somite boundaries. (B) In this case, axons from motor neurons lying opposite posterior half-sclerotome (p) turn posteriorly within the neural tube before entering the neighbouring anterior half-sclerotome. Arrowheads denote the position of neural tube–mesoderm boundary.

the remaining 3 cases axons appeared to extend simultaneously from cells opposite both sclerotome halves (Fig. 5C).

By stage 19, the population of cells contributing axons to one anterior half-somite occupied an A–P length of neural tube equivalent to approximately one somite (Fig. 6A). Although this length is also equivalent to that of one myelomere, each population of motor neurons, being centred on the adjacent anterior half-sclerotome, was seen to be out of register with the myelomeres by a distance equivalent to approximately one quarter-somite (Fig. 6A). One myelomere does not, therefore, contribute motor axons exclusively to one somite. Instead, all motor neurons opposite an anterior half-sclerotome project axons into that region, taking a direct lateral trajectory from the cell body, while motor neurons opposite posterior half-sclerotome extend axons into the nearest anterior half-sclerotome. In the latter case, the axons usually turn towards the anterior half-sclerotome immediately after penetrating the basal lamina of the neural tube (Fig. 6A); more rarely, axons turn within the neural epithelium itself (Fig. 6B).

Interneuron development

Segmental patterns of development were sought at the thoracic level in the earliest differentiating spinal cord interneurons, namely ascending and descending relay neurons, and commissural neurons. Both zinc iodide–osmium tetroxide and neurofilament antibody were used to stain whole mounts of chick embro spinal cord between stage 16 (when the first interneurons differentiate) and stage 22. For each staining method, at least 2 embryos were studied at each developmental stage. No segmental arrangements related to the myelomere/somite periodicity could be seen, as regards either cell body position or axon arborisation pattern (Figs 7, 8).

Discussion

Periodic undulations of the neural tube in the region of the developing spinal cord have been recognised for many years, and became known as myelomeres (a subclass of neuromeres) after McClure (1890). As noted by Minot (1892), Johnston (1916), Neal (1918) and Vaage (1969), myelomeres appear and disappear in parallel with the adjacent segments of the paraxial mesoderm, the somites, and the repeat-distance of the myelomeres matches precisely that of the somites. We have looked, therefore, for evidence of intrinsic segmentation in the spinal neural tube with a spatial frequency related to that of the myelomere pattern.

In principle, signs of intrinsic segmentation underlying the overt morphological periodicity can be sought in at least four different ways (Keynes and Lumsden, 1990). First, the overt pattern might be matched by a parallel, underlying pattern of mitotic proliferation in the neural epithelium; second, by a segmental pattern of cell (neuronal and/or glial) differentiation; third, by the existence within the epithelium of periodically distributed lineage restriction boundaries; and fourth, by a matching pattern of expression of genes or their products.

In the case of the chick hindbrain, evidence for segmentation is found according to all these criteria: there is a segmental arrangement of boundaries, cranial nerve nuclei and axonal pathways (Lumsden and Keynes, 1989), the pattern of cell proliferation mirrors the arrangement of rhombomeres (Lumsden, 1990), descendants of single labelled cells respect rhombomere boundaries (Fraser *et al.* 1990) and the patterns of expression of several genes also reflect the rhombomeric organisation (Wilkinson *et al.* 1989*a*,*b*). In the present study of the trunk neural tube, however, we have found no evidence for intrinsic segmentation with respect to cell proliferation or cell differentiation.

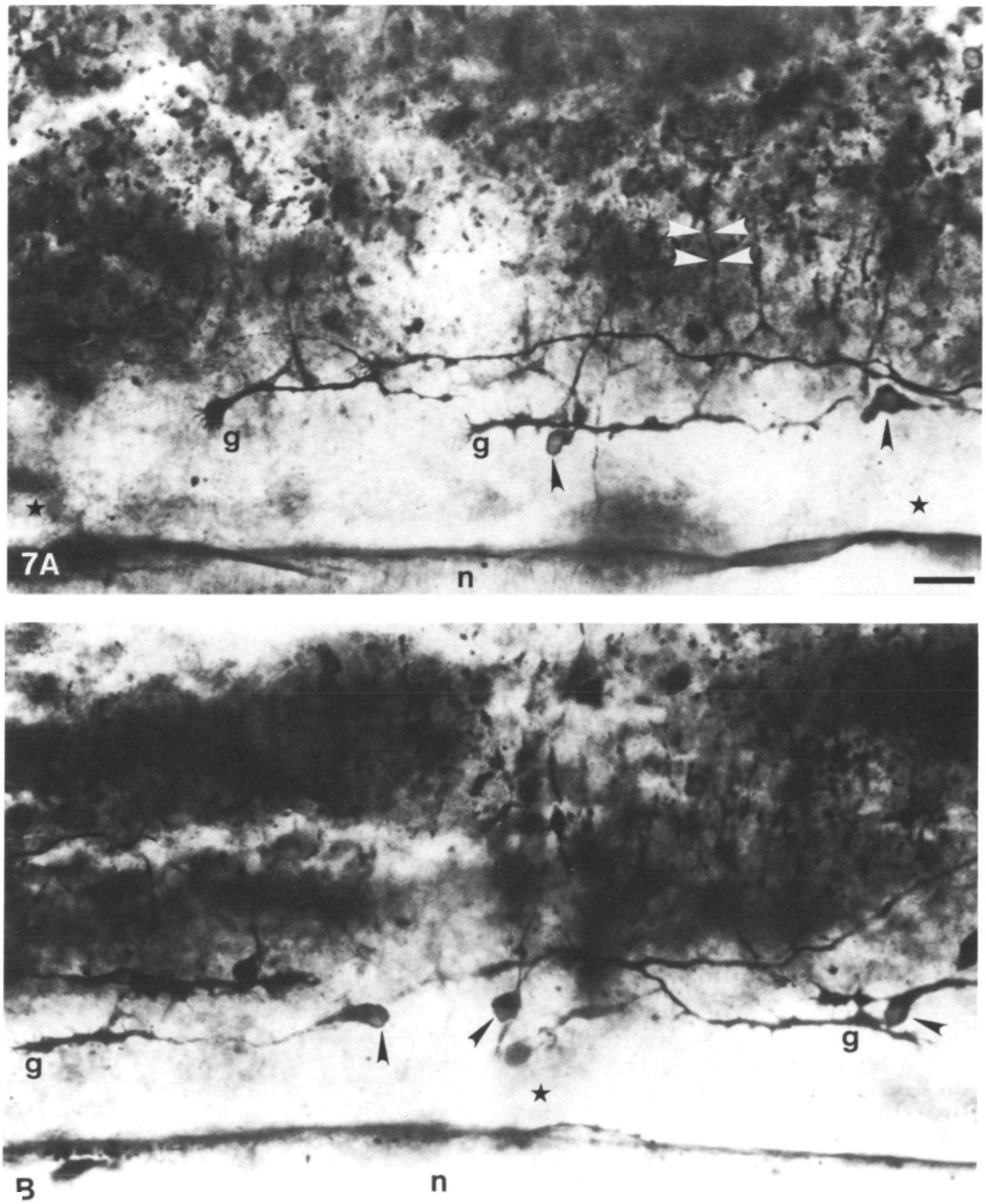


Fig. 7. (A,B) Whole-mounts of hemisected stage 17 chick embryo thoracic neural tube, stained with zinc iodide/osmium tetroxide; anterior to the left, dorsal uppermost. Black arrowheads indicate cell bodies of the earliest longitudinal projection neurons to differentiate in the region of the basal plate/floorplate boundary; g, growth cones with filopodia; n, notochord; white arrowheads indicate a commissural axon; asterisks denote positions of adjacent somite boundaries. Scale bar 10 μm.

The possibility that the myelomeres of the trunk correspond to segmentally distributed centres of neuroepithelial proliferation was raised by Källén (1952, 1953, 1962). In the case of the developing spinal cord, earlier studies in amphibian (Coghill, 1933) and chick (Hamburger, 1948) embryos had failed to show any clear evidence of periodic proliferation patterns along the A–P axis during normal development (likewise, the studies of Corliss and Robertson, 1963, and of Oppenheim *et al.* 1989). Källén (1962) argued, never-

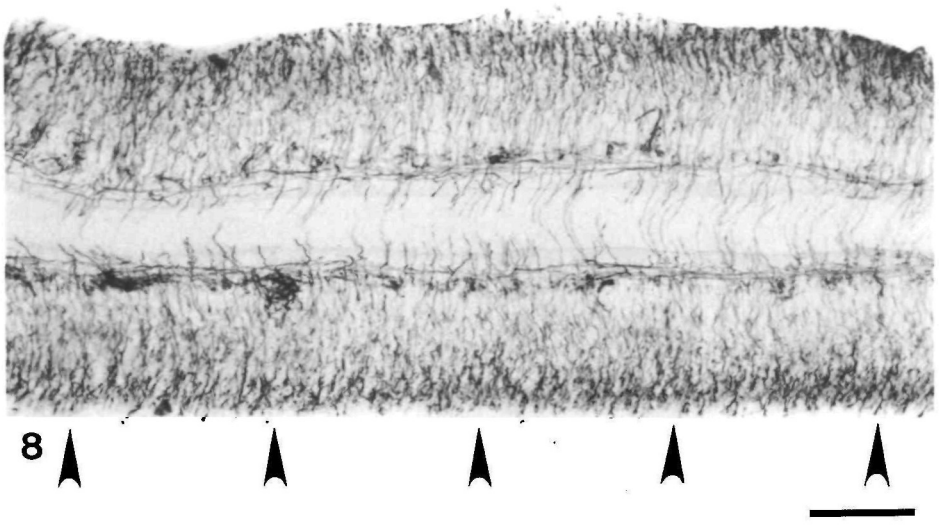


Fig. 8. Whole-mount stage 18 chick embryo stained with 3A10 monoclonal antibody. Anterior to the right. Arrowheads denote the positions of the adjacent somite boundaries. Commissural axons are seen growing in mediolateral plane, and some have entered the ventral midline floorplate region. Longitudinal projection neurons are also visible in the region of the basal plate–floorplate boundary. No segmental differentiation patterns are seen. Scale bar 70 μm.

theless, that the exaggerated myelomere undulations seen after colchicine treatment favour this possibility, and result from inhibition of neuroepithelial cell mitosis. He suggested that the centre of each myelomere (opposite the inter-somite boundaries) has a higher mitotic rate than the flanking regions.

Our observations that the effects of colchicine are visible within 45 min of drug application, that irregular undulations also appear opposite the segmental plate, and that another cell cycle inhibitor, BrdU, does not cause exaggerated neuromeres allow an alternative interpretation: namely, that the undulations are caused not by interference with the cell cycle, but by the disassembly of structural microtubules within each neuroepithelial cell, which may produce local alterations in the longitudinal rigidity of the neural tube. In the region of the somites, the undulations are in register with each intersomitic cleft, as would be expected if the somites restrict the excursions of the bulging neural tube; at the segmental plate, however, the undulations are not constrained by neighbouring structures and are randomly arranged. We cannot conclude, then, that the effects of colchicine provide evidence for periodic proliferation centres associated with the genesis of myelomeres.

A further observation is that in the spinal cord there are no segmental groupings of S-phase-labelled neuroepithelial cells. This contrasts with the hindbrain, where pulse-labelling with anti-BrdU reveals that the characteristic interkinetic nuclear migration within the neuroepithelium (Sauer, 1935) is reduced at the rhombomere boundaries (Lumsden, 1990).

An essential function of the spinal cord is to activate the muscles derived from each segmental myotome. On this basis alone, some degree of segmental organisation within the developing spinal cord could be anticipated, with a period matching that of the myotomes. However, using a variety of morphological methods to delineate the early differentiation of neurons, segmental patterns are not detected within the spinal cord. This applies not only to the positions of neuronal cell bodies, but also to axon trajectories. There is also no evidence for the existence of primary motor neurons with stereotyped axon projection patterns outside the spinal cord (see below). At any particular segmental level, the motor axons contributing to one ventral root arise from a cell group whose A–P extent is equivalent to one myelomere, but which is not in register with the myelomeres (see also Hirano and Fuse, 1989). Instead, each group is centred upon the adjacent anterior half-sclerotome (Fig. 6A). Although such cell groupings retain a metameric pattern, they can be explained solely on the basis of mesodermal segmentation, without invoking additional segmentation within the neural epithelium. Only A-half-sclerotome is permissive for axon outgrowth, while P-half-sclerotome repels axons (Keynes and Stern, 1984; Davies *et al.* 1990). The groupings suggest that motor cell bodies, whether sited opposite A- or P-half-sclerotome, project their axons into the nearest available anterior half-sclerotome. Axons arising opposite P-half-sclerotome might be attracted to the nearest A-half-sclerotome by a short-range diffusible cue (Keynes *et al.* 1991).

Mesodermal segmentation also controls the formation of the dorsal root ganglia. Like motor axons, the dorsal root ganglia and their parent neural crest cells are confined to anterior rather than posterior half-sclerotome (Keynes and Stern, 1984; Rickmann *et al.* 1985; Bronner-Fraser, 1986), and Teillet *et al.* (1987) have shown that an individual ganglion is derived from a strip of crest centred upon the anterior half-sclerotome. Whether mesodermal segmentation also determines segmentation of the autonomic preganglionic neurons is unclear. The axons of these cells arise, like those of the somatic motor axons, from cell bodies at each corresponding spinal level (Rubin and Purves, 1980), and the projection patterns of thoracic preganglionic sympathetic neurons appear to have a segmental periodicity (Ezerman *et al.* 1990).

The absence of segmental differentiation patterns in

the developing chick spinal cord has also been noted by Layer *et al.* (1988) for acetylcholinesterase expression in developing motor neurons, and by Schlosser and Tosney (1988) for projection neurons that send axons longitudinally within the lumbar spinal cord (see also Oppenheim *et al.* 1988; Yaginuma *et al.* 1990). Previous anatomical studies of the adult higher vertebrate spinal cord have not reported any periodic neuronal arrangements, with the exception of certain groups of cells associated with the preganglionic autonomic motor columns. The marginal (Hoffman's) nuclei, first recorded by Gaskell (1885) in the alligator, have since been described in birds and mammals as well as reptiles (von Kölliker, 1902; Huber, 1936; Nieuwenhuys, 1964; Anderson *et al.* 1964). The cells of these nuclei appear after the extension of the first motor axons from the ventral neural tube, and aggregate preferentially in regions of the ventro-lateral white matter between the emerging ventral roots. There is no cell-free zone between one cluster and the next, however, and it seems likely that such segmentation could be secondary to that of the motor axons, determined in turn by segmentation in the mesoderm (Keynes and Stern, 1984).

Another group of cells with a periodic arrangement in the adult is the 'zone intermédiaire à cellules intercalées' described in a variety of mammals by Laruelle (1937) and named the 'nucleus intercalatus spinalis' by Petras and Cummings (1972) in a study of the rhesus monkey. This consists of a series of transverse bands of cells, which extend across the spinal cord in the region of the central canal, appearing to unite the intermedio-lateral (preganglionic sympathetic) cell columns on right and left sides of the spinal cord. Petras and Cummings (1972) note how the metameric arrangement of these neurons is 'a strikingly consistent characteristic'. In the cat, periodic clusters of neurons have also been described within the intermedio-lateral cell column itself (Oldfield and McLachlan, 1981; Morgan *et al.* 1986). In both cases, however, the A–P repeat length is less than that of the spinal nerves, and is not precisely regular. It may be related to the 'microsegmentation' of alternating ipsi- and contralateral projecting neurons, described by Altman and Bayer (1984) in the developing rat spinal cord. As suggested by the name, the A–P length of each 'microsegment' is less than that of the myelomeres and somites; the periodicity is also variable, and may arise from irregular fasciculation of axon bundles, in much the same way that each developing ventral and dorsal root subdivides into (irregularly sized) axon fascicles (Keynes and Stern, unpublished observations).

The absence of clear segmental patterns of neuronal development in the higher vertebrate spinal cord contrasts with the spinal cords of cephalochordates, agnathans and gnathostome fishes, for which there are several such descriptions. Bone (1960) described segmental arrangements of interneurons and motor neurons in the nerve cord of the adult *Amphioxus*. Whiting (1948) described a class of large interneurons in the larval brook lamprey matching the periodicity in the adjacent mesoderm (one neuron per myotome). Finally, both the primary motor neurons and certain intrinsic interneurons in the developing zebrafish spinal cord are segmentally organised (Myers, 1985; Eisen *et al.* 1986; Westerfield *et al.* 1986; Myers *et al.* 1986; Hanneman *et al.* 1988; Hanneman and Westerfield, 1989; Kuwada and Bernhardt, 1990), as they may be in other teleosts (Fetcho, 1987). In the zebrafish there are three primary motor neurons per myotome; each triad occupies a distinct position in relation to the myotome, and the three neurons bear defined positional relations both with respect to one another along the A–P axis and to the myotomes on which they arborise (Eisen *et al.* 1986). As noted above, we find no evidence for the existence of segmental primary motor neurons during chick development, and the same holds for the amphibian spinal cord (Coghill, 1913; Youngstrom, 1940; Silver, 1942; Blight, 1978; Forehand and Farel, 1982; Roberts and Clarke, 1982). The possibility remains, however, that in amphibians segmentation of motor neurons does exist very early in development, but becomes obscured as a result of the relative displacement of the myotomes (Westerfield and Eisen, 1985; Nordlander, 1986).

In the absence of segmental patterns of neuronal differentiation, mechanisms must nevertheless exist to specify broad regions along the A–P axis of the spinal cord. For example, groups of neurons such as the limb motor columns, autonomic motor columns and Clarke's column span A–P distances of several somites/myelomeres and, within these groupings, cells at different A–P positions have distinct target preferences (Lance-Jones and Landmesser, 1981; Purves *et al.* 1981; Wigston and Sanes, 1982). It seems possible that, during the course of vertebrate evolution, intrinsic spinal cord segmentation was lost in subservience to paraxial mesodermal segmentation, as higher motor centres exerted increasing control over spinal neuromuscular circuits. In parallel, regional specification of the spinal cord perhaps acquired mechanisms operating over multiples of the primitive segmentation period. The presence within the developing mouse spinal cord of A–P boundaries of expression of certain homeobox genes raises the possibility that these genes may be involved in such specification. It is interesting to speculate that the A–P boundaries of these multisegmental regions may correspond to ancestral segmental boundaries.

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