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Proximal Tissues and Patterned Neurite Outgrowth at the Lumbosacral Level of the Chick Embryo: Partial and Complete Deletion of the Somite

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The development of patterned axon outgrowth and dorsal root ganglion (DRG) formation was examined after partially or totally removing chick somitic mesoderm. Since the dermamyotome is not essential and a full complement of limb muscles developed, alterations in neural patterns could be ascribed to deletion of sclerotome. When somitic tissue was completely removed, axons extended and DRG formed, but in an unsegmented pattern. Therefore the somite does not elicit outgrowth of axons or migration of DRG precursors, it is not a manditory substratum and it is not required for DRG condensation. These results suggest that posterior sclerotome is relatively inhibitory to invasion, an inhibition that is released when sclerotome is absent. When somites were partially deleted, axonal segmentation was not lost proportionally with the amount of sclerotome removed, suggesting that properties that may vary with sclerotome volume (such as diffusible cues) do not play a primary role. Instead, spinal nerves lost segmentation only when ventral sclerotome was deleted, regardless of whether dorsal sclerotome was or was not removed. This strongly suggests that axonal segmentation is imposed by direct interactions between growth cones and extracellular matrices or surfaces of sclerotome cells. While DRG tended to be normally segmented when ventral sclerotome was deleted and to lose segmentation when dorsomedial sclerotome was absent, a coordinate loss of DRG segmentation with sclerotome volume could not be ruled out. However it is clear that axonal and DRG segmentation are independent. Observations on a subset of embryos in which the notochord was displaced relative to the spinal cord suggest that the ventromedial sclerotome surrounding the notochord inhibits axon advance. Posterior and ventromedial sclerotome are hypothesized to act as barriers to axon outgrowth due to some feature of their common cartilaginous development. Specific innervation patterns were also examined. When the notochord was displaced toward the control limb, axons on this side made and corrected projection errors, suggesting that the notochord can influence the precision of axonal pathway selection. In contrast, motor axons that entered the limb on all operated sides innervated muscle with their normal precision despite the absence of the somite and axonal segmentation. Therefore, the somite and the process of spinal nerve segmentation are largely irrelevant to the specificity of motoneuron projection. © 1988 Academic Press, Inc.

INTRODUCTION

The somites, which arise from the segmental plate in anterior to posterior sequence shortly after neurulation, have recently become a focus of research in developmental neurobiology, largely due to the pivotal work of Keynes and Stern (1984). These researchers showed that axons normally traverse only the anterior portion of each somite and will do so even when the anterior has been surgically displaced to a posterior position. This means that axonal segmentation is not the result of a relatively uninteresting passive process in which growth cones are physically channeled between somites that act as barriers or in which axons fasciculate to form a single bundle within a homogeneous somite. Similarly, neural crest cells prefer anterior over posterior somite for migration and become segmented accordingly (cf. Rickmann *et al.*, 1985; Bronner-Fraser, 1986; Loring and Erickson, 1987). Because growth cones and neural crest cells show a clear preference for invading a particular population of cells rather than the equally accessible adjacent population, the interactions between the somites and the populations that invade them have become worthy of study.

Each somite is composed of three different tissues by the time axons invade it. Only one of these is vital to segmentation. When the dermatome and myotome (the tissues that develop along the dorsal-lateral edge of each somite) are deleted, spinal nerves and dorsal root ganglia (DRG) are normally segmented (Tosney, 1987a). Since segmentation is abolished when the entire somite is removed (cf. Detwiler, 1934; Lewis *et al.*, 1981), this means the sclerotome (the ventrolateral portion of the somite) is essential to segmentation. In addition, there are no differences in the organization of anterior and posterior halves of each sclerotome that could physically channel axons or neural crest cells: barriers or channels are absent and the distribution of extracellular matrix, the cell density, and the cell orientation are similar in the anterior and posterior sclerotome halves of each somite before and during the invasion of neural crest cells and axons (Tosney, 1987b, 1988). Differences in the composition of extracellular matrix, the cell surfaces, or diffusible products of anterior and posterior

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sclerotome can be postulated to underlie the segmental patterning of the peripheral nervous system in the trunk of the embryo.

Two classes of cellular mechanisms, involving either short or longer range navigational cues, have been proposed to explain how the sclerotome imposes segmentation (reviewed by Tosney, 1988). Both are based on the capabilities of cultured cells and neurons. For instance, cells and growth cones in culture can make pathway choices based on the adhesivity of the substratum (Harris, 1973; Letourneau, 1975); likewise, growth cones and neural crest cells may prefer to advance upon the cell surfaces or extracellular matrix of anterior sclerotome. This type of guidance relies on short range interactions between cellular processes and the substratum. Another theoretical framework for guidance is provided by the ability of growth cones to respond in culture to distant sources of a diffusible substance such as NGF (Gunderson and Barrett, 1980). Populations could become segmented because they respond to either an attractive substance from anterior sclerotome or a repulsive substance from posterior sclerotome. If this is the case, the local environment of the growth cone or neural crest cell would be less important than the mass of the sclerotome, since the amount of a diffusible substance available is likely to be proportional to the number of cells in the source population (an assumption with some limitations).

I approached this issue by determining whether the local environment or the volume of the sclerotome was most important to the pattern of segmentation of spinal nerves and DRG. Somitic tissue was deleted to varying degrees and from different sites along the dorsal-ventral axis. If DRG and spinal nerve formation were somehow interdependent or if both depended on the physical mass of the sclerotome, the segmental pattern of DRG and spinal nerves should be similar in each segment and loss of segmentation should be proportional to the physical loss of sclerotome, regardless of the site of deletion along the dorsal-ventral axis. If the local environment were more important, DRG and spinal nerves should lose segmentation independently and their loss of segmentation should depend on the site of the deletion; e.g., spinal nerves would be unsegmented whenever the ventral sclerotome, through which axons normally grow, was deleted, but would be normally segmented when only dorsal sclerotome was removed. My results strongly suggest that the local environment is of primary importance to the development of the axonal segmentation but they do not rule out a role for volume-dependent cues in DRG segmentation.

Although it is clear that the tissues lying between the spinal cord and the limb base are responsible for the gross anatomical pattern of the local elements of the peripheral nervous system, these tissues have also been suggested to provide navigational cues that are essential for specific axonal pathfinding. For instance, at the base of the limb, axonal pathways diverge to form alternative dorsal and ventral paths. Growth cones evidently use specific cues to select the proper alternative (Ferguson, 1983; Whitelaw and Hollyday, 1983) and they can do so correctly even in the absence of the limb (Tosney and Landmesser, 1984) suggesting that the essential navigational cues lie within tissues proximal to the limb. Does the somite, which is physically the most prominent proximal tissue, provide essential information for this pathway decision? Do growth cones garner information when traversing the sclerotome that is essential for them to select their proper target within the limb? Is the process of segmentation itself essential to the specific innervation of targets?

I addressed these questions by deleting several lumbosacral somites and assessing the precision with which motoneurons selected pathways at the limb base and targets within the limb. One aspect of development that could have complicated the analysis of specificity in embryos deprived of somites is the fact that the limb muscles are derived from the somites (cf. Chevallier, 1977). For instance, when the brachial somites are removed by irradiation, the irradiated somite is replaced by loose mesenchyme of unknown origin (Chevallier *et al.*, 1978), axon outgrowth is unsegmented, and the wing is largely devoid of muscle (Lewis *et al.*, 1981). Because limb muscles provide important axonal guidance cues within the limb (cf. Landmesser, 1984) and their absence could invalidate the analysis, I used a strategy in which the development of the limb was substantially normal following somite deletion. This was possible because the hindlimb differs from the wing in one important respect: eight rather than five dermamyotomes contribute to the muscles of the hindlimb (Lance-Jones, 1988a). Since the limb muscle precursors have an exceptional ability to regulate (cf. Chevallier, 1977) and since some precursors migrate very early (Jacob *et al.*, 1979), it was not surprising that a sufficient number of precursors had entered the limbs for the normal complement of muscles to form in the present study, despite the substantial deletion of somitic tissue. In addition, the fact that limb muscles often arose from foreign segmental levels in these limbs did not pose a problem because the somitic level of origin of the muscle precursors is not important to the specificity of muscle innervation (Keynes *et al.*, 1987; Lance-Jones, 1988b). The present results show that neither the somite nor the process of segmentation are essential to the precise innervation of muscle targets.

A brief report of some of these results has appeared elsewhere (Tosney, 1986).

MATERIALS AND METHODS

Embryonic surgeries. White leghorn eggs (Michigan State University) of 2–4 days incubation were candled, 2 ml of albumin were removed with a sterile syringe and needle, and the eggs were returned to the incubator for 2–4 hr to allow the embryo to settle. One cm^2 of shell was removed from above the embryo with a dental drill, 2 drops of Tyrodes solution containing 100 units/ml of penicillin-streptomycin (GIBCO) were added, the window was sealed with surgical tape, and the embryos were incubated for 1–4 hr. The vitelline membrane was opened, and the embryos were stained with a drop of 0.2% neutral red in distilled water and staged using Hamburger and Hamilton (1951) criteria. A small cut was made in the ectoderm posterior to the operation site using a fine tungsten needle and somitic tissue was removed by aspiration using a small micropipet (tip size 20–50 μm) attached to polyethylene tubing. The sites of deletion ranged from thoracic level 5 (T5) through lumbosacral level 8 (LS8), corresponding approximately to somites 22 through 33. Eggs were sealed with surgical tape and incubated until the desired stage.

Somitic tissue was removed from different sites and to different degrees in individual segments, as illustrated in Fig. 1. The dorsal third of a segment was removed in class D operations (see Fig. 2A), the ventral half in class V operations, and three-quarters of the segment including both dorsal and ventral portions in class B operations, and the somitic tissue was completely removed in class C operations (see Fig. 2B). To avoid damaging dorsal somitic tissue in V operations, one or more somites were substantially removed to give access to the ventral portion of more anterior or posterior somites.

Operations were performed on 174 embryos; 114 survived the operation and 52 of these were discarded due to abnormalities associated with faulty amnion closure (N = 20), overstaining with neutral red (N = 17), or dehydration during the operation (N = 15). Five embryos were fixed early, leaving 57 in which development was assessed. Most operations were done before axon outgrowth, during the period before, during, and shortly after formation of the lumbosacral somites (stages 16–18). Single segments were removed from four embryos. In all the embryos included in the general analysis, 3–11 segments (average, 4.8 segments) were removed to minimize complications caused by migration of tissue from adjacent segments.

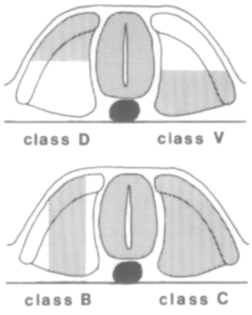


FIG. 1. Operation classes. Schematic diagram illustrates the position and maximum extent of deletion of somitic tissue in each operation class (stippling). The dermamyotome (d) and sclerotome (s) are shown to clarify what tissues would generally remain after the operation, even though these tissues had not separated before the operation in many of the segments. In class B, the medial–lateral position of the deletion varied. Horizontal lines, neural tube; Black, notochord.

Embryonic tissues mature in anterior to posterior order. Consequently, in each embryo, the maturity of the somites and the phase of crest migration varied over the deleted segments. The maturity of each operated segment was noted (N = 281 segments deleted). Unsegmented mesoderm was removed from 96 segments and the segmental position of each deletion was confirmed by comparison to the control side after fixation. Somites were removed one to three somites anterior to the unsegmented plate before neural crest had begun to migrate (N = 96), four to six somites anterior to the unsegmented plate as neural crest began to migrate ventrally and the very first crest cells entered the somite (N = 55), seven to nine somites anterior to the unsegmented plate when many crest cells were entering the somite (N = 22), and at more mature levels after crest had begun to accumulate within the sclerotome and the first growth cones had exited from the spinal cord (N = 12). Since the results did not correlate with the maturity of the somite, for most purposes the results were pooled.

Fixation and analysis of neurite projection patterns. Embryos were examined after different survival periods to assess different aspects of patterned outgrowth: (1) within 1–6 hr of the operation (N = 5) to verify the accuracy of deletion, (2) during the period in which growth cones traverse the proximal environment and gather at the leg base (stages 21–24, N = 16) to examine outgrowth during the stages when segmentation develops, (3) shortly after motoneuron axons have sorted out along the dorsal–ventral axis at the leg base and muscle nerves have begun to form (stage 25–26, N = 16) to examine the precision of pathway choice at the limb base and at branch points to muscles in the limb, and (4) following invasion of muscles (stages 27–34, N = 25) to examine the precision of muscle innervation.

Operated embryos were removed to a Tyrode's bath, the viscera were removed, and the position of the notochord was noted. To visualize individual axons during outgrowth and to assess the specificity of axonal projection, the vertebral cartilage immediately dorsal or ventral to the spinal cord was carefully removed and se-

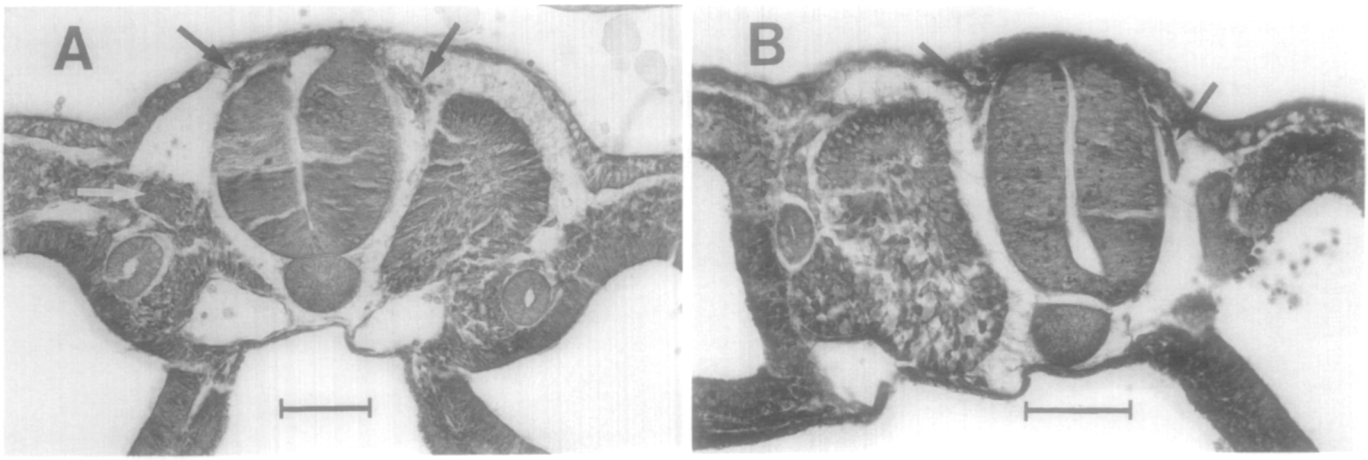


FIG. 2. Embryos fixed shortly after the operation. (A) In this class D deletion, the dorsal half of the somitic tissue was removed (left) and the embryo was fixed 3 hr later. Since the somite is wedge-shaped, lateral portions of the dermamyotome (white arrow) usually remained. Neural crest cells (black arrows) have begun to migrate but have yet begun to colonize the sclerotome. (B) In this class C deletion, 100% of the somitic tissue was removed (right) and the embryo was fixed 6 hr later. Neural crest (black arrows) remains on the operated side in this embryo. Lateral tissues are closer to the neural tube on the operated side and the notochord has shifted slightly relative to the neural tube. In all embryos, tissue was removed through a slit in the ectoderm posterior to the deletion site, leaving the local ectoderm undisturbed. 12-μm sections, Alcian blue and eosin stain. Calibration bars = 50 μm.

lected nerves or muscles were pressure injected with 10% horseradish peroxidase (HRP) in avian Tyrode's solution and processed by previously published procedures (Landmesser, 1978; Tosney and Landmesser, 1984). Injections were successful for six limb muscle pools (stages 30–36), 14 injections into the ventral crural nerve trunk (stages 26–31), and 34 injections into the spinal cord or ventral roots (stages 21–32).

Analysis of the anatomy of operated embryos. The degree of the deletion in each segment was confirmed from examination of serial sections. Criteria for inclusion in each class are detailed under Results and examples are illustrated in Fig. 3.

Derivatives of somitic tissues were identified using the following criteria. Remnants of even a few myotome cells could be easily detected since they are the only somitic tissue to autofluoresce at the stages examined (see Tosney, 1987a), they retain an epithelial organization during early development and form identifiable myotubes later. The dermis, derived from the dermatome, is more loosely organized then sclerotomal mesenchyme at later stages, but could be distinguished from other mesenchyme in early embryos only by position. During early stages, sclerotome is a loose mesenchyme and could be identified by position; during later stages, it forms vertebrae which could be identified by morphology and blue-to-violet staining with cresyl violet.

Axons and dorsal root ganglion cells could be distinguished in three ways. The entire gross anatomical nerve pattern can be reconstructed since the nerves appear white or translucent in cresyl violet-stained sections. Individual axons could be traced after horseradish peroxidase injections into the nerves or spinal cord. In addition, neurons and neurites autofluoresce bright yellow when viewed with fluoroscein epifluorescent optics and are clearly visible against a background of brownish cells (see Tosney, 1987a). Even a few axons could be reliably visualized with this method.

Reconstructions and quantitation of the segmental patterns of outgrowth. Complete reconstructions of the gross anatomical nerve pattern and the distribution of dorsal root ganglia (DRG), epaxial muscle (or myotome), and labeled somata and axons were made from serial sections using a camera lucida. Particular attention was paid to accurately depicting the extent of axon outgrowth and the DRG distribution along the anterior–posterior (A–P) axis, in order to obtain a measure of the degree of segmentation. Since somitic boundaries are not visible at later stages, a segment was designated as the region from the most anterior border of one spinal nerve to the beginning of the spinal nerve in the next posterior segment on the control side. This method was used because the DRG expand during development and the customary mode of assigning segmental boundaries between DRG (as is done for determination of motoneuron pool position) obscures the former somitic boundaries (cf. Tosney, 1988). Another method of designating a segment, by individual vertebrae, also does not correspond to the original somitic segment because each vertebrae is formed from A and P halves of an adjacent somite; this "resegmentation" does not com-

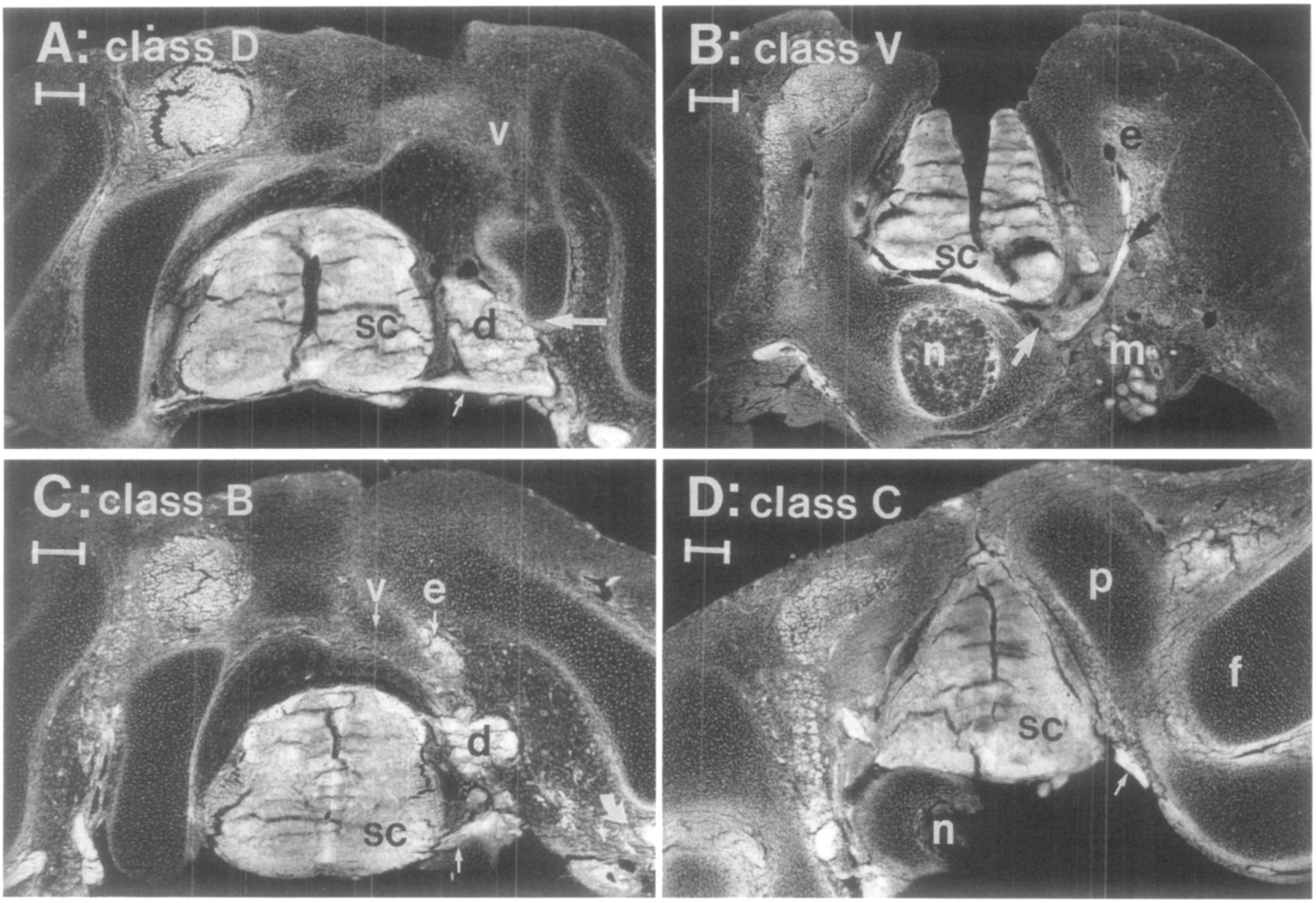


FIG. 3. Operation classes. The operated side is to the right in each figure. Some of the ventral cartilage was removed from embryos in (A), (C), and (D); dorsal cartilage was removed from the embryo in (B) and the dorsal spinal cord was opened. Epifluorescent micrographs of 12-μm sections. Calibration bars = 10 μm. (A) In a class D deletion, the dorsal vertebral condensation (v) was reduced in size, abnormal in morphology, or absent. A small space was occasionally found adjacent to the dorsolateral spinal cord (sc). DRG (d) and spinal nerves (small white arrow) have formed normally. This section is typical of the D class; the white arrow indicates the most extreme ventral extent of these deletions. Stage 30 embryo. (B) In a class V deletion, ventral sclerotome was absent or greatly reduced. The mesonephros (m) was often found in a more dorsal position; in extreme cases, it extended dorsal to the spinal nerve and axons traversed it. The dorsal ramus (black arrow) extends into epaxial muscle (e) which is slightly reduced in this segment. This section also illustrates a shift in the relative positions of the notochord (n) and spinal cord (sc). In the most extreme cases, the notochord was entirely toward one side of the spinal cord. This shift was commonly seen when somitic tissue was substantially deleted over a large number of segments (mean = 6.8 segments per embryo). In these cases the ventral roots emerged from a more ventral position than normal (white arrow) but spinal nerves retained their normal relationship to the perinotochordal mesenchyme. Stage 28. (C) In a class B deletion, 50–80% of both dorsal and ventral somitic tissues had been removed. All segments had one or more small vertebral condensations (v) and usually contained a fragment of epaxial muscle (e), but the position of these remnants varied. In this embryo, the spinal nerve (small white arrow) extends toward the plexus region where axons from adjacent segments can be seen (curved arrow). The DRG (d) were reduced on both sides in this segment. sc, spinal cord. Stage 30. (D) In a class C deletion, little or no somitic tissue could be detected and the pelvic girdle (p) lay close to the spinal cord (sc). The experimental leg was often tilted dorsally, as can be seen from the angle of the femur (f) on the right (see also Fig. 7A). The notochord (n), partially preserved in this embryo, has shifted in position relative to the spinal cord and the ventral roots have emerged more ventrally than normal on both sides. Small arrow, spinal nerve. Stage 30 embryo.

plicate the present analysis since it apparently does not involve movement of sclerotome cells along the A–P axis (cf. Verbout, 1976; Trelstad, 1977).

The A–P lengths of each segment, spinal nerves, and DRG on both control and operated sides in the reconstructions were measured. Since segments vary in length the extent of axon outgrowth or DRG condensation on the A–P axis was expressed as a percentage of the length of each segment. Axon outgrowth was measured along a parasagittal line immediately adjacent to the spinal cord. When a DRG extended throughout an entire segment and into an adjacent one, the additional portion was assigned to the adjacent segment to avoid having values greater than 100%. For instance, in Fig.

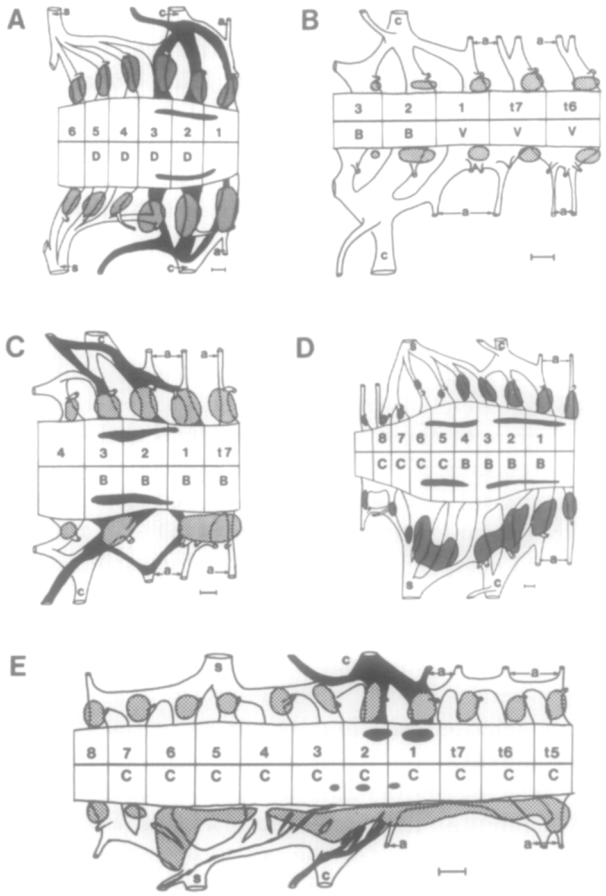


FIG. 4. Reconstructions of the proximal nerve pattern. The operated side is to the bottom in each figure. Segmental boundaries in the spinal cord were defined as beginning in the most anterior section in which ventral roots were seen on the control side. Reconstructions were corrected for section angle. The thoracic (t) or lumbosacral level of each segment and the operation class are indicated within each cord segment; segments without a letter are unoperated. In the more extensive operations, the lateral tissues were closer to the spinal cord; when axons entered these tissues, they formed an appropriate gross anatomical nerve pattern in all cases. For instance, axial nerves appear segmented because they are growing through segmentally repeated lateral tissues whose development was seldom affected by somite deletion. Likewise, the nerve trunks in the limb have formed just distal to the developing pelvic girdle. Stippling, DRG condensation: black, HRP label; c, crural nerve trunk; s, sciatic nerve trunk; a, axial thoracic nerves. The dorsal rami emerge immediately lateral to the DRG. Calibration bars = 100 μm. (A) Following a dorsal deletion of four consecutive segments, spinal nerves have formed in their normal segmental pattern. DRG are slightly enlarged along the A-P axis in segments 2 and 3 in which the ventral extent of the deletion was more extensive. HRP injected into the ventral crural nerve trunk on each side has labeled only the appropriate motoneurons in the spinal cord. Stage 30 embryo. (B) In contrast, following deletion of ventral somitic tissue in three consecutive segments, DRG are normally segmented but spinal nerve segmentation is abolished. In addition, dorsal rami in segments t6 and LS1 also appear unsegmented: multiple branches emerge from a more extensive region of the spinal nerve. In embryos with class V operations, adjoining segments were substantially deleted to give access to the ventral somite without disrupting its dorsal components, as in the two class B segments (LS2 and 3) shown here. In segment 2, the DRG and spinal nerves are enlarged and in segment 3 the spinal nerve is displaced posteriorly and DRG on both sides are reduced. Stage 28 embryo. (C) Following substantial deletion of both dorsal and ventral somitic tissue in four consecutive segments, the segmentation of spinal nerves and DRG is altered. The dorsal ramus is also more extensive and branches into LS1. Bilateral HRP injections into the ventral crural nerve trunks labeled only appropriate motoneurons on both sides of the spinal cord. Stage 28 embryo. (D) Following class B and C deletions in eight consecutive segments, the segmentation of both spinal nerves and DRG is altered. HRP injected into the sartorius and ventral shank muscles has labeled motoneurons in a similar distribution on both sides of the spinal cord, even though all the axons entering the hindlimb on the operated side had traversed regions in which the somite was greatly reduced (segments 1-4) or absent (segments 5-8). The dorsal ramus in segment 3 is broad and branches. DRG on both sides are reduced in the posterior. A quasi-segmental pattern of spinal nerves that has emerged distally is probably due to remnants of lateral sclerotome. Stage 34 embryo. (E) Following complete deletion of 10 consecutive somites, the segmentation of spinal nerves and DRG is abolished. Axon outgrowth could not be detected from segment 5. Small injections into the experimental side of the spinal cord labeled a few axons that could be traced to their appropriate muscle destinations (the ischioflexorius from segment 3 and the adductor from segments 1 and 2).

4E, the DRG that lies as a continuous column opposite segments 4, 5, and 6 was divided with respect to segment borders and assigned to each adjacent segment. Significant differences between means and 95% confidence levels were determined using Student's t test.

Analysis of the A-P distribution of DRG was complicated by two factors. First, in a subset of the segments in 29 embryos, DRG were obviously reduced or were absent on both sides of the embryo, probably because neural crest was inadvertantly depleted. Second, the normal extent of DRG varies with stage as the DRG alter in size during development. The extent in operated embryos was standardized by comparison with the normal range which was derived from examination of 70 segments in normal embryos. The normal range was arbitrarily designated as the mean percentage of the segment occupied by the DRG $\pm$ 1 SD about the mean for that stage, as follows: stage 21-23, 63 $\pm$ 15%; stage 24-26, 54 $\pm$ 9%; stage 28-29, 65 $\pm$ 10%; stage 30-34, 68 $\pm$ 10%. DRG that extended through a larger proportion of the A-P axis were classified as enlarged, DRG below these values were classified as reduced. Each operation class included a similar range of stages. The volume of each DRG was also estimated from its A-P, medial-lateral, and dorsal-ventral extents. Since many neurons have yet to differentiate during the stages studied and reliable cell counts cannot be done, only obvious alterations in ganglion volume were noted.

RESULTS

General mophology of operated embryos. The role of the somite in patterned neurite outgrowth and DRG

segmentation was investigated by removing all or selected portions of the somitic mesenchyme, as diagramed in Fig. 1. Since the segmentation of axons and DRG and the precision of limb innervation is not altered when the dermamyotome is deleted (Tosney, 1987a), the present study focused on the sclerotome. The degree and position of each deletion was confirmed by observation of serial sections and each segment was assigned a classification based primarily on the presence and morphology of sclerotome derivatives. Although the extent of the deletion varied about the average extent within a class, the criteria were definite enough for each segment to be confidently assigned to a particular class. The volume of tissue removed also varied somewhat along the A–P axis within a segment but in no case did A and P halves of a segment fall into separate classes. In all operations the ectoderm dorsal to the removed tissue was undamaged and in many segments the neural crest was substantially undisturbed. Despite the fact that limb muscles were occasionally reduced in size, the normal complement of thigh muscles formed in all but three embryos.

The physical relationship among the remaining tissues was often altered by the loss of a sizable chunk of somitic tissue. For instance, depending on the severity of the deletion, the limb might be tilted dorsally, the mesonephros might be found closer or adjacent to the spinal cord, and the notochord might be shifted in position relative to the spinal cord. In no case was there a cell-free gap the size of a deleted somite; adjacent tissue always filled in. Encroachment of somitic tissue into the operation site from adjacent segments would have been an unwelcome complication. When only a single somite was deleted, tissues had obviously shifted along the A–P axis: the somites to the anterior and posterior were juxtaposed and the segmental pattern was not in register with the control side. However, when three or more somites were removed, encroachment of sclerotome from adjacent segments was not detectable and the lateral tissues were closer to the spinal cord, even shortly after the operation (Fig. 2B). The tendency for lateral rather than somitic tissues to fill the gap was maximized by deleting several consecutive segments in all embryos analyzed. There was no consistent relation between the loss of segmentation and the maturity of the somitic mesenchyme at the time it was deleted or the stage at fixation (not shown).

Classification of operated segments. The criteria for a *dorsal* deletion (class D, $N = 85$ segments, see Fig. 3A) were that the dorsal portion of the vertebrae was reduced in size, abnormal in morphology, or absent, but derivatives of the ventral sclerotome were substantially normal. Ventrolateral portions of the dermamyotome usually remained (Fig. 2A). The estimated average volume of sclerotome deleted was 25%; in the most extensive operations ($N = 12$ segments) the deletion extended ventrally into the region where DRG normally condense.

In segments classified as *ventral* deletions (class V, $N = 21$ segments, see Fig. 3B), ventral sclerotome derivatives were absent or greatly reduced but the dorsal somitic tissue was normal or, in five segments, was slightly reduced. The mesonephros was in a more dorsal position in 14% of these segments; whenever it was adjacent to the ventrolateral spinal cord, axons extended through it. The estimated average volume of sclerotome deleted was 40%.

In segments classified as *both dorsal* and *ventral* deletions (class B, $N = 78$ segments), both dorsal and ventral vertebral condensations were reduced, often to a variable extent, but in all cases 50–80% of the sclerotome had been removed (Fig. 3C). The estimated average volume of sclerotome deleted was 65%. The mesonephros was adjacent to the cord in 14% of these segments. This was the most variable class since the site of deletion varied on the medial–lateral axis and the extent of deletion occasionally varied in the A and P of each segment.

In those segments with *complete* deletions (Class C, $N = 96$ segments), little or no somitic tissue could be detected and the pelvic girdle lay close to the spinal cord (Fig. 3D). Included in this class are segments with a very small vertebral condensation and segments with only loose mesenchyme (which is probably plexus mesenchyme) between the spinal cord and pelvic girdle. The estimated average volume of sclerotome deleted was 90%. A few myotubes were present in seven segments. The experimental leg was often tilted dorsally and the mesonephros was closer to the spinal cord in 37% of the segments. Although neural crest was probably depleted in some segments, Fig. 2B shows it was possible to completely delete a somite without deleting neural crest.

Segmentation of spinal nerves. Reconstructions of individual embryos in each class illustrate the general response to site-specific deletions of sclerotome. Spinal nerves are normally segmented when dorsal sclerotome is deleted (class D; Fig. 4A). In contrast, segmentation is lost when the ventral sclerotome alone (class V; Fig. 4B) or the entire somite (class C; Fig. 4E) is deleted. This suggests that only the ventral sclerotome is essential to patterned neurite outgrowth. In addition, the simple fact that axons grew out in all segments tells us two things: (1) The somite is not an essential substratum for axon advance. (2) The somite or the late segmental plate does not elicit axon outgrowth.

When the ventral sclerotome was only partially removed (class B), spinal nerves were generally broader, particularly adjacent to the spinal cord (Figs. 4B–4D).

As the nerves extended more distally, they tended to fasciculate into groups that were, on occasion, organized in a quasi-segmental pattern (e.g., Fig. 4D). This tendency became more prominent at older stages when spinal nerves normally change from a sheet-like to a rounded morphology as they become packaged by sheath cells and are separated by the growth of surrounding tissues (Tosney and Landmesser, 1985a). In addition, a segmental pattern consistently emerged distally when lateral portions of sclerotome remained intact.

To obtain a more quantitative measure of the loss of segmentation, the degree of segmentation was defined by the extent of axon outgrowth along the A–P axis, standardized as the percentage of a segment with outgrowth. This measure somewhat underestimates the disruption of segmentation since in a few cases spinal nerves were normal in extent but were abnormally positioned along the A–P axis (see Fig. 4B and 4D). The mean extent of the spinal nerves on the A–P axis was significantly different from that of the controls following class V, B, and C deletions, but was not significantly different from that of controls following class D deletions (Fig. 5). In addition, the mean extent of spinal nerves did not significantly differ among classes V, B, and C. Therefore, segmentation was lost to statistically similar extents in all classes in which ventral sclerotome was deleted, regardless of whether dorsal sclerotome was substantially or completely deleted as well. These results are consistent with the proposal that the sclerotome that lies directly in the path of advancing growth cones dictates the segmental pattern of outgrowth.

It is clear that axonal segmentation is not lost in proportion to the average volume of sclerotome deleted in each class. In Fig. 5 the values to be expected if there is a linear relationship between loss of sclerotome volume and loss of segmentation are shown by a line extending from the control values (at 0% sclerotome deletion) to complete loss of segmentation (at 100% sclerotome deletion). The 95% confidence limits about the mean values of spinal nerve extent in each class, plotted as a function of the estimated average volume of sclerotome deleted, do not intersect this line. The absence of an obvious correlation cannot be explained by the uncertainties in estimating the volume of sclerotome deleted. The 95% confidence limits can be made to intersect the line only if equivalent volumes of 50–60% were deleted in class V, B, and C operations. This was clearly not the case. In addition, the median value (the most common case) was for complete loss of segmentation following class V operations in which no more than 50% of the sclerotome was removed. Furthermore, the distribution of values in class V and C operations is inconsistent with the possibility that segmentation is disrupted only when more than some threshold amount of sclerotome is deleted. For instance, segmentation was completely abolished in 50% of the segments when no more than half of the sclerotome was removed (class V; Fig. 6C), but was within the normal range in 25% of the segments when more than three-quarters of the sclerotome was absent (class C; Fig. 6E).

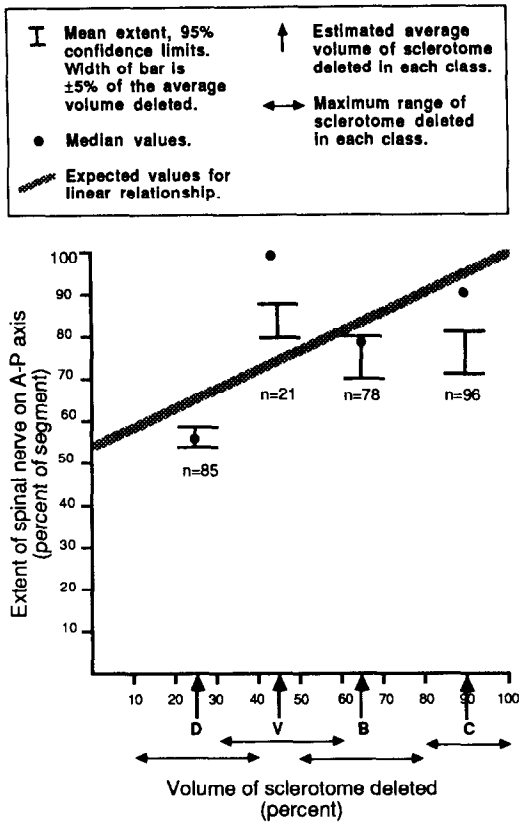
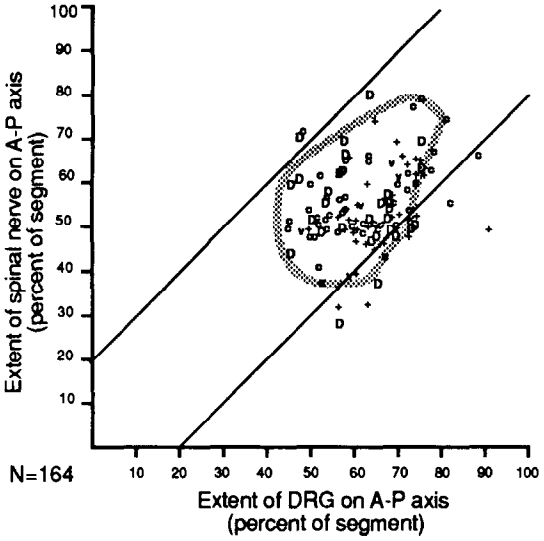


FIG. 5. Relation between loss of spinal nerve segmentation, operation class, and volume of sclerotome deleted. The mean extent of axon outgrowth on the A–P axis in each operation class is plotted as a function of the volume of sclerotome deleted. The average volume deleted within each class is an estimate; however, the average clearly did not fall at the extremes of the maximum range of the deletion in any class. The grey line extends from the mean control values at 0% sclerotome deletion to complete loss of segmentation with 100% sclerotome deletion; the width of this line indicates 95% confidence limits about the mean for controls. If there were a linear relation between loss of segmentation and loss of sclerotome, mean values would be expected to fall on this line. There is, however, no obvious relationship between loss of segmentation and loss of sclerotome volume. Instead, the means fall into two classes. The mean extent of spinal nerves is not significantly different from that of controls in class D operations. Classes V, B, and C are significantly different from controls ($\ll 0.01\%$ level) but are not significantly different from one another. These results suggest that segmentation is lost when ventral sclerotome is removed, regardless of whether or not more dorsal sclerotome is also removed.

The variation in the extent of spinal nerves on the A–P axis within each operation class can be explained if

A. Control sides



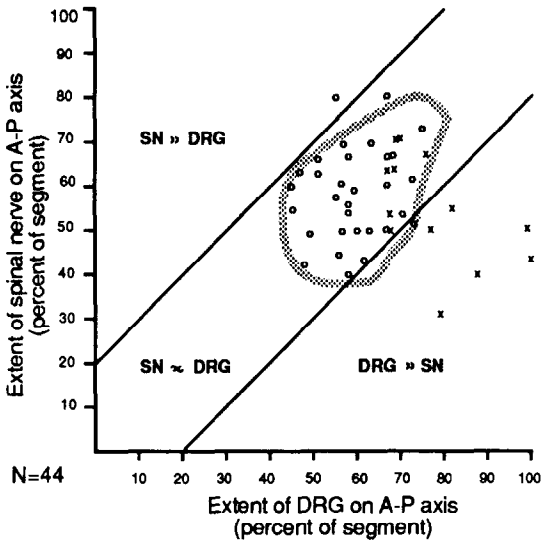
KEY:

Graph A: D: dorsal +: both
v: ventral c: complete

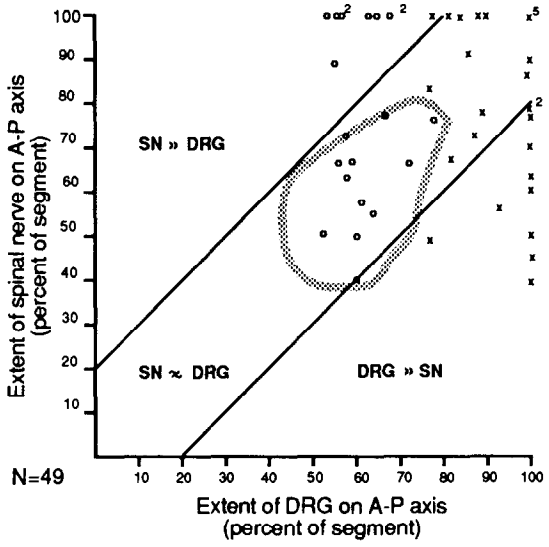
Graphs B-E: o: DRG within normal size range for embryonic stage
x: DRG larger than normal size range for embryonic stage
superscript: number of cases larger than one

All graphs: 95% of the control values lie within this boundary

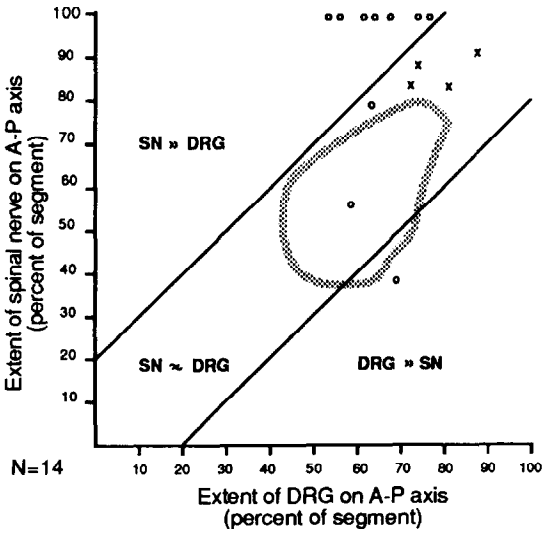
B: Class D, dorsal deleted



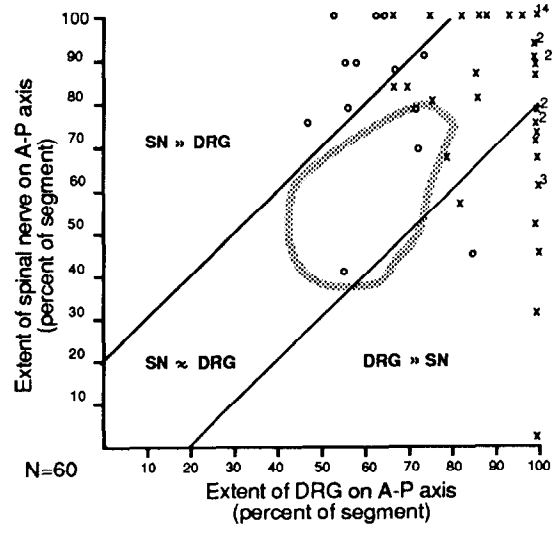
D: Class B, both dorsal and ventral



C: Class V, ventral deleted



E: Class C, complete deletion



the sclerotome that lies within the path of outgrowing axons is most important to segmentation and if posterior sclerotome inhibits axon advance. For instance, the more normal values in class V operations (Fig. 6C) are most easily explained if sclerotome was removed only ventral to the normal pathway of outgrowth, beyond the reach of questing filipodia. In class B operations, the site of deletion varied along the medial-lateral axis of the somite and in many segments the environment immediately adjacent to the spinal cord was undisturbed. Since the extent of axon outgrowth along the A-P axis is measured adjacent to the cord, it is not surprising that many segments fell within the normal range (Fig. 6D). In class C (Fig. 6E), only small amounts of sclerotome remained and the values within the normal range are explicable only if inhibitory sclerotome lay directly in the path of the extending growth cones. These results suggest that the critical environment for segmentation is very local.

Reduction of axonal projection in a minority of segments. In all cases, the gross anatomy of nerves that form in distal, nonsomitic tissue was normal. Axial nerves that form in the lateral body wall assumed a segmental pattern coordinate with that of the surrounding muscles and ribs which had, in most cases, developed normally; nerve trunks and muscle nerves in the limb were also normally distributed. However, in a small subset of embryos the amount of hindlimb innervation was reduced: spinal nerves were absent in 3 segments and small ($\ll$40% of a segment) in 19 segments and the nerves within the limb that normally receive large contributions from these segments were smaller. This reduction in innervation was associated with reduction of limb muscles, plexus mesenchyme, or ventrolateral spinal cord. For instance, Fig. 4C illustrates an embryo in which fewer axons from segment LS1 contribute to the plexus region. The sartorius portion of the dorsal muscle mass was reduced and the nerves that normally receive a large contribution from LS1 (the sartorius muscle nerve and the adjacent anterior cutaneous nerve) were noticeably smaller than normal as though some axons failed to enter the limb or did so and regressed. In the most severe case, a stage 28 embryo, the dorsal muscle mass was absent in the anterior thigh; the limb was correspondingly smaller; plexus mesenchyme, the dorsal crural nerve trunk, and spinal nerves from segments 1 and 2 were absent; and spinal nerve 3 was small.

There are a number of possible explanations for the reduction in limb innervation. For instance, spinal nerves have been reported to stop short of the limb following complete deletion of neural crest (Carpenter and Holliday, 1986). However, in the present embryos neural crest and spinal nerves were not reduced concordantly. For example, spinal nerves were not reduced and entered the limb in 34 segments in which neural crest was probably substantially deleted since DRG were absent on both sides. In addition, while DRG were absent in two of the three segments without spinal nerves, the contralateral DRG was normal in A-P extent in both cases, suggesting that the crest had not been totally deleted. A subset of the reductions could be explained by the smaller size of some limb muscles in embryos that had reached the stage when nerves depend on muscle for survival. Small muscle size alone does not explain reduced outgrowth in younger embryos since axons can enter the wing even when muscle is substantially reduced (Lewis *et al.*, 1981). However, in these embryos, the limbs were often correspondingly smaller. Since axons in the most anterior and posterior segments often fail to enter a leg that is reduced in size (Lance-Jones and Landmesser, 1981), smaller limbs could explain reduced innervation in all but the more central lumbosacral segments. It is also possible that the neural tube was directly damaged during some operations. A final possibility, that lateral plate mesenchyme was inadvertantly removed, could explain both altered muscle development (see Dias and Lance-Jones,

FIG. 6. Extent of DRG and spinal nerves on the A-P axis on control (A) and operated (B-E) sides. The extent of axon outgrowth and DRG condensation was expressed as a percentage of each segment length. The graphs include only those segments in which the DRG was not reduced on the operated side. The grey elipse includes 95% of the control values (A) and is reproduced on all graphs for comparison. One important issue was whether spinal nerves and DRG responded independently and in a site-specific manner to the loss of sclerotome. The regions to the upper left and lower right of the diagonal lines include values that are predominantly outside the control range and in which the extent of the spinal nerve and DRG differs by more than 20% of the segment. These values are clearly cases in which the DRG and spinal nerves did not respond equivalently to the operation. (B) Most of the values lie within the control range following dorsal deletions. However, in those segments where there was an effect, the DRG rather than the spinal nerves tended to lose segmentation. For instance, the A-P extent of DRG was larger than normal (values indicated by $\times$) in 30% of the segments. Moreover, DRG extended farther than spinal nerves in 16% of the segments, while spinal nerves extended farther than DRG in only 2% of the segments. (C) In contrast, spinal nerves were completely unsegmented and corresponding DRG were within the normal range in 50% of the segments following ventral deletions. DRG and spinal nerves were enlarged concurrently in another 29% of the segments; in these segments, the deletion generally extended farther dorsally into the region where DRG normally form. (D) When both dorsal and ventral tissues were substantially but not completely deleted, 76% of the segments lay outside the normal range and showed some loss of segmentation. In 43% of the segments, DRG and spinal nerves did not lose segmentation to equivalent extents. (E) Following complete deletion, 95% of the values lay outside the control boundaries. DRG were more extensive than normal in 80% of the segments. Spinal nerves were more extensive than DRG in 15% of the segments and DRG were more extensive in 25% of the segments.

1987) and loss of the plexus mesenchyme, which may provide the only permissive pathway for outgrowth in the absence of the somite.

The number of noticeable reductions in innervation is small (8% of the segments) and these can be adequately explained by coincident reductions in the limb muscle, limb size, plexus mesenchyme, or the spinal cord. In addition, similar reduction in innervation is found following embryonic surgeries that leave the somite intact (cf. Lance-Jones and Landmesser, 1981; Lance-Jones, 1986). These results are consistent with the conclusion that the somite is not essential for axon outgrowth.

Specificity of limb innervation. The specificity of limb innervation on the operated side was normal in all but four embryos (discussed below) in which the notochord had hypertophied. The projection into dorsal and ventral pathways at the base of the limb was of particular interest, since important navigational cues for this pathway decision lie proximal to the limb. The precision of pathway selection was assessed following injections of HRP into the ventral crural nerve trunk in 14 embryos that included instances of all classes. In all cases projection into this pathway was normal in all respects (see Figs. 4A and 4C).

Target selection in the limb was evaluated using two methods. In the first, axons labeled by segmental injections into the spinal cord or ventral roots were traced from the somata to their destinations in the limb ($N = 14$). This allowed the specificity of innervation of a number of limb muscles to be assessed in the same embryo. No projection errors were found. For instance, in Fig. 4E, axons from the ischioflexorius and adductor pools innervate only their respective muscles in a representative embryo.

HRP injected into an individual muscle also showed that the specificity of innervation was normal; these injections labeled only motoneurons that lay in the appropriate position in the spinal cord (see Fig. 4D). However, in one embryo the number of motoneurons was obviously reduced; in two others, the total number was similar, but the contribution from one segment was larger and from an adjacent segment was smaller than normal. Since only appropriate neurons innervated the muscles, these anomalies are not relevant to the specificity of innervation. They can be explained if fewer axons from a particular segment entered the limb, so that neurons in the same pool but in adjacent segments would survive disproportionately. Similar redistributions have been seen when a part of the motor pool was physically deleted (Lance-Jones and Landmesser, 1980). Despite the evidence that innervation was reduced in a minority of cases, it is clear that all the axons that made it into the limb selected the proper pathways and targets. Therefore, interaction with somitic tissue and the process of segmentation are not essential to pathfinding or target selection in the limb.

Relative displacement of the notochord. In 20 of the embryos, the spinal cord and notochord were displaced relative to one another so that the notochord appeared to lie closer to the control leg (Figs. 3B and 3D); in another four embryos, the notochord had hypertrophied (Fig. 7). This relative shift may result from bulging or displacement of the neural tube toward the side where it is no longer supported by the somite, particularly since these anomalies were most common when tissue was substantially removed over a larger number of segments than average. However, operation trauma alone may sometimes cause this defect since the shift was also seen in an embryo in which only dorsal tissue was removed from two segments and in another embryo (not included in the analysis of segmentation) in which the somite had regulated following dorsal deletion and the embryo appeared otherwise normal. The relative displacement of the notochord was subtle and somitic tissues that can be reduced by a close interaction with the notochord, such as the dermamyotome (cf. Vassan, 1986), were only occasionally depleted and the gross anatomy of more distal nerves was always normal. DRG were occasionally reduced or absent, but the proportion of these was similar to that found in the total population.

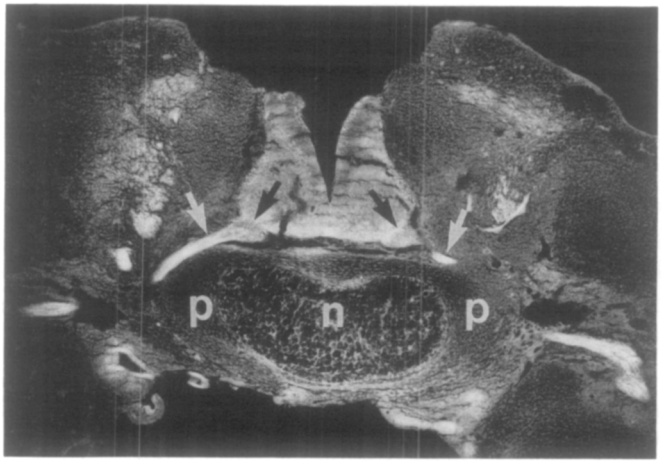


FIG. 7. Notochord hypertrophy and the relation of axon outgrowth to perinotochordal tissues. In this stage 26 embryo, the notochord (n) is bizzarely hypertrophied, a situation found in four embryos and not related to any particular operation class. In fact, this segment illustrates the least amount of tissue removed in any class B operation. Myotomes on both sides seem slightly reduced, probably because their development can be inhibited by the notochord (cf. Vassan, 1986). The ventral roots (black arrows) exited from a more ventral position than normal. Despite this, the spinal nerves (white arrows) retained their normal relationship to the dense perinotochordal tissues (p), growing out in the plane between this tissue and the more dorsal sclerotome. Calibration bar = 10 μm.

The ventral roots exited the spinal cord from a more ventral position then normal in segments with displaced or hypertrophied notochords, even though the spinal cord looked relatively normal (see Figs. 3B, 3D, and 7). The notochord may indirectly dictate the position where axons exit from the cord since, when an ectopic notochord is implanted, motoneurons are born and extend axons from dorsal as well as ventral regions of the spinal cord (VanStraaten *et al.*, 1985). It is, however, difficult to see how this phenomenon could explain the more ventral position of the ventral roots in the present results, since the motoneurons normally lie at the farthest ventral aspect of the cord. The notochord may affect the position of axon outgrowth by factors other than or in addition to its effect on motoneuron birth.

It is particularly interesting that, despite the initial more ventral trajectory of axons in these cases, the position of the spinal nerves relative to the perinotochordal tissue was conserved on both sides of the embryo. In no case did axons penetrate the mesenchyme that lay immediately ventrally, directly in advance of emerging growth cones. Instead, axons altered their trajectory and moved, as they normally do, along a plane between this mesenchyme and the dorsolateral sclerotome. This observation suggests that ventromedial sclerotome that surrounds the notochord may act as a barrier to axon advance.

Projection errors in the control limb correlate with a relative displacement of the notochord. The most unexpected finding was that unusual patterns of axonal projection were seen on the *control* side of these embryos. A subset of axons appeared to make—and correct—projection errors. When HRP was injected bilaterally into the ventral nerve trunks, labeled somata were in their appropriate medial positions on both sides of the spinal cord ($N = 7$). However, some of the labeled axons on the control side coursed into the dorsal nerve trunk pathway, made a U-turn in the limb, returned to the plexus region, and projected correctly along the ventral nerve trunk pathway (Figs. 8A and 8B). It is unlikely that all the abnormal axonal projections were sensory; at least some of the projection errors were made by motoneurons, since in two of these embryos, DRG were absent in the crural region. The specificity of motoneuron outgrowth to limb targets was normal on the experimental side in all embryos except in those four in which the notochord had hypertrophied. These made and corrected projection errors on both sides. These results suggest that the notochord may be important in some way to the specificity of axon projection.

Outgrowth of epaxial motoneurons. The epaxial neurons are of particular interest because they grow out to form the dorsal ramus nerve which lies entirely within the somite and because their muscle target, derived from the myotome within each segment, is essential for outgrowth (Tosney, 1987a). The present results confirm that outgrowth of epaxial motoneurons is primarily dependent upon the proximity of the target. These neurons obeyed the same rules they did following dermamyotome deletions, even when the sclerotome that they normally traverse had been substantially reduced. In 33% of the segments, no fragment of the myotome remained in the segment, myotubes were few in number or absent in adjacent segments and the dorsal ramus did not emerge from the spinal nerve. In 11% of the segments no fragment was present and the dorsal ramus extended into a fragment in the closest adjacent segment. In 56% of the segments a small fragment remained and in all cases a dorsal ramus grew into the

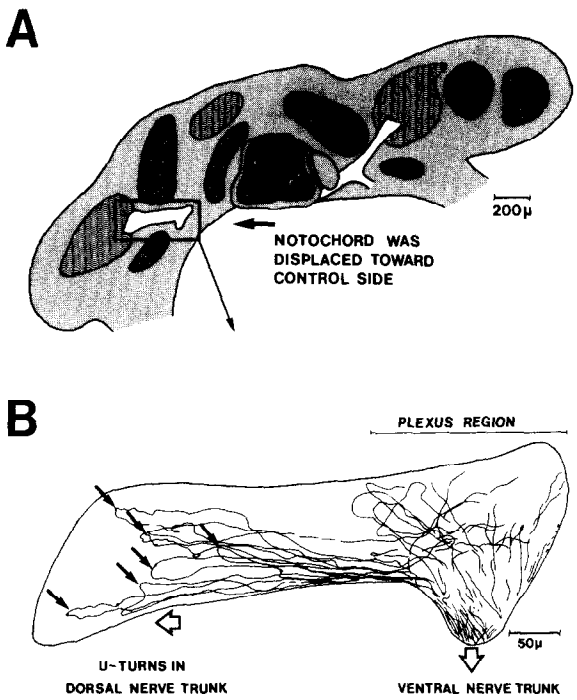


FIG. 8. Projection errors in the control limb. In a subset of the embryos, the notochord lay closer to the control leg (relative to the spinal cord) as illustrated in the camera lucida tracing in A (see also Figs. 4B and 4D). The specificity of axonal projection was normal on the operated side in these embryos. In particular, axons pursued a relatively straight trajectory in the spinal nerves, altered their trajectory within the plexus region, and then projected into the appropriate dorsal or ventral nerve trunk pathway. In contrast, some axons made—and corrected—projection errors on the control side in these embryos. (B) Camera lucida tracing of several sections through the plexus region and dorsal nerve trunk of the control side. Some of the axons labeled by an injection of HRP into the ventral nerve trunk have extend through the plexus region into the dorsal nerve trunk pathway. These invariably made a U-turn (arrows), returned to the plexus region, and projected into the ventral nerve trunk. Motoneurons labeled by the injection were always in their normal medial position in the spinal cord.

fragment. This includes seven segments in which at least 80% of the sclerotome was deleted. This supports the notion that sclerotome cells do not mediate the response of epaxial motoneurons to their target.

Previous evidence has suggested that, like the motoneurons that innervate the limb, epaxial motoneurons respond to the environmental constraints imposed by the somite. For instance, when they project into epaxial muscle in an adjacent segment, they do not extend along the A-P axis until they are dorsal to the bulk of the sclerotome (Tosney, 1987a). It is more difficult to assess the degree to which segmentation is lost in this population because the dorsal ramus is such a small nerve. However, in 13% of the segments in which a dorsal ramus was observed, it was clearly broader than normal or extended in multiple branches from the spinal nerve (Figs. 4B-4D). In these segments, the spinal nerve was often but not always unsegmented, suggesting that epaxial and limb motoneurons respond to the sclerotome independently. The sclerotome does not appear to be a manditory pathway for the outgrowth of either population.

Development of DRG. The analysis of DRG segmentation was complicated by an obvious reduction of DRG in some segments and by the normal growth of DRG during development. To obtain a standardized measure of segmentation, the extent of each DRG on the A-P axis was compared to the normal range for each stage and each DRG was assigned to a class, *enlarged, normal,* or *reduced.* DRG in the reduced class were not used in the analysis of segmentation.

DRG were reduced or absent on the experimental side in 41% of the segments. This reduction appears to be due to inadvertant depletion of neural crest rather than to the loss of somitic tissues, a view that is supported by several observations. First, none of the DRG were reduced in 53% of the embryos, even though these included all classes of operations. Second, the dorsal portion of the spinal cord was often noticeably smaller when DRG were reduced. Third, in 80% of the segments in which the DRG on the operated side was reduced or absent, the contralateral DRG was reduced or absent as well, despite the fact that the contralateral somitic tissue was normal (Fig. 9).

Assuming that neural crest is more likely to be removed from the operated side, there is a surprising discordance in the proportion of DRG that were reduced on experimental (41%) and contralateral (54%) sides. The most likely explanation is that more of the experimental DRG fell within the normal class, despite a reduction in the total number of cells, because they were more spread out along the A-P axis. While the loss of DRG segmentation may be somewhat underestimated

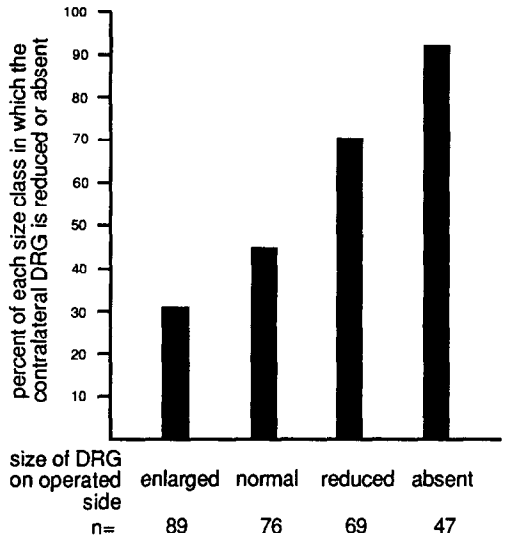


FIG. 9. Relation between the extent of DRG along the A-P axis on operated and contralateral sides. The A-P extent of each DRG on the operated side was classified as enlarged, normal, reduced, or absent (X axis), compared to the normal range for the stage (see Materials and Methods). The proportion of each size class in which the contralateral DRG was reduced or absent is shown. DRG on operated and contralateral sides appear to be reduced concurrently, suggesting that any reduction in the extent of DRG is due to depletion of neural crest. Since the lowest proportion of reduced contralateral DRG is found among those DRG that have extended farther than normal along the A-P axis, the enlargement of DRG on the experimental side is not necessarily due to recruitment of neural crest from the unoperated side.

because the neural crest was often depleted, the error must be relatively small, since it is clear that even DRG that are partially reduced in volume can extend throughout a segment. For instance, in 12 segments, DRG extended throughout a segment despite the fact that neural crest must have been somewhat depleted because the contralateral DRG was reduced.

In some of the operated segments DRG appeared to be larger in volume since a few were normal in extent along dorsal-ventral and medial-lateral axes throughout 80-100% of a segment. DRG could increase in volume if the environment supported the differentiation of more cells in the ganglion, if sympathetic precursors contributed to the ganglion, or if the initial population were larger because neural crest was recruited from the contralateral side. If the latter were the case, we might expect that contralateral DRG would be reduced when DRG are enlarged in volume or extend farther along the A-P axis on the operated side. However, fewer contralateral DRG were reduced when the DRG were enlarged than in any other size class of DRG (Fig. 9). Therefore, recruitment is not essential for DRG to extend farther along the A-P axis on the operated side. Presumably

neural crest that normally move anterior or posterior before entering the somite (as described by Teillet *et al.*, 1987), move directly ventrally when the adjacent posterior sclerotome is absent.

DRG were occasionally found in anomalous positions along the dorsal-ventral and medial-lateral axes in operated segments. For instance, one or more small ectopic DRG were found adjacent to the region where the dorsal roots normally enter the spinal cord in 10% of the segments that had DRG. In addition, DRG were closely apposed to nerves and extended into the plexus region in 12% of the segments that contained DRG. In some but not all of these segments, the notochord was displaced relative to the spinal cord. In a few embryos, labeled neural somata lay within the proximal nerves. These have also been seen following deletion of motoneurons (Landmesser and Honig, 1986). In two segments, DRG were absent, but a few cells with neurites were scattered in the dorsal sclerotome.

Despite the slightly anomalous positions of some ganglia, it is obvious that neural crest cells do not require the sclerotome to begin migration, since in many embryos somitic tissue was removed before migration commences (see Tosney, 1978). In addition, these cells can advance through the embryo and condense to form DRG without the aid of somitic tissues. Condensation may be mediated by alterations in surface moieties on crest cells (cf. Thiery *et al.*, 1982), by interaction with products of the notochord (Newgreen *et al.*, 1986) or spinal cord (Kalcheim and Le Dourain, 1986), or by interaction with the spinal nerves (Loring and Erickson, 1987). The present results are not, however, consistent with the hypothesis of Lim *et al.*, (1987) that trunk somites help to maintain DRG integrity. Their result that small DRG are maintained when occipital neural tube is implanted at trunk levels may be explained if tissues in the trunk other than the somite provide trophic support or if occipital tissues inhibit maintenance.

Segmentation of DRG. Reconstructions of individual embryos illustrate the differential loss of DRG segmentation with operation class: DRG were normal in A-P extent following the ventral deletion of three consecutive segments (Fig. 4B) but segmentation was usually lost in B and C operation classes (Figs. 4C and 4D). Following the most extensive deletions, DRG could form a column that was continuous over several segments (Fig. 4E). In contrast to spinal nerves, the mean extent of DRG along the A-P axis was significantly different from controls only when both dorsal and ventral sclerotome tissues were deleted (classes B and C; Fig. 10A). Following deletion of sclerotome dorsal or ventral to the normal site of DRG condensation, the mean extent of the DRG on the A-P axis was not different from that of controls (classes D and V; Fig. 10A). The dorsomedial portion of the sclerotome thus appears to be most important to segmentation.

The mean extent of DRG following different classes of operation bears no obvious relationship to the volume of sclerotome deleted (Fig. 10A). Depletion of neural crest might tend to artificially lower the measured mean extent of the DRG. To minimize this problem, Fig. 10B excludes those segments in which DRG were reduced on the contralateral side. Again, no relationship with volume is obvious. The uncertainty in estimating the volume of sclerotome deleted also does little to explain these results since the values for the V class would fall on the line only if 30% rather than 45% of the sclerotome were deleted. This is unlikely. Nevertheless, the conclusion that there is no proportional relationship between loss of DRG segmentation and sclerotome volume should be met with some skepticism, since it rests primarily on class V operations which were few in number. The possibility that the volume of the somite plays some role in neural crest segmentation should be tested further.

The hypothesis that segmentation is altered only when more than a threshold amount of sclerotome is removed seems consistent with Figs. 10A and 10B since in both graphs the mean extents clearly fall into two groups: segmentation is altered only when more than half the somite is removed (classes B and C). There is, however, no progressive loss of segmentation with volume above this apparent threshold, since the most common results in the B and C classes, represented by the median values, are complete loss of segmentation. The apparent necessity for deletion of a minimum volume may be an artifact of the experimental design in which the dorsomedial sclerotome was not removed unless at least half of the somite was deleted. It is also possible that, unless a minimum volume is removed, adjacent posterior sclerotome fills up the deletion site and inhibits invasion.

An examination of individual segments in which the deletion was more extensive than the average for each class provides support for a local interaction. For instance, a subset of the DRG extended beyond the normal range for their stage following both class D (Figs. 6B and 4A) and class V (Fig. 6C) deletions. In each case, the region in which DRG normally condense appeared to have been at least partially included in the deletion even though less than half of the sclerotome was removed. In addition, the extent of DRG lies within the normal range in 20% of the segments following class C deletions. Since at least 80% of the sclerotome is absent, these cannot be explained by the volume of tissue deleted. As with spinal nerves, the variation in results

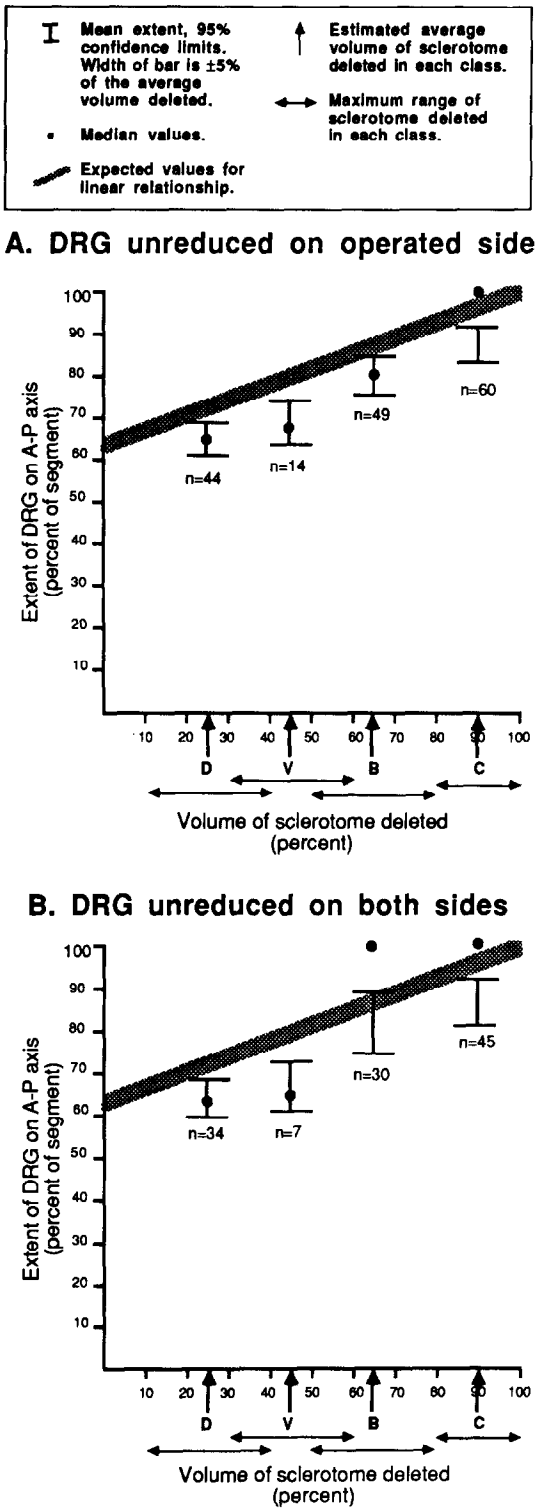


FIG. 10. Relation between loss of DRG segmentation, operation class, and volume of sclerotome deleted. The mean extent of DRG on the A–P axis in each operation class is plotted as a function of the volume of sclerotome deleted. (A) includes only those segments in which the DRG on the operated side was not reduced. In (B) the segments in which the contralateral DRG was reduced are excluded to further minimize the effect of neural crest depletion. If there were a linear relation between loss of segmentation and loss of sclerotome, mean values would be expected to fall on the grey line which extends from the mean control values at 0% sclerotome deletion to complete loss of segmentation with 100% sclerotome deletion as in Fig. 5. There is, however, no obvious relationship between loss of segmentation and loss of sclerotome volume. Instead, the mean extent of DRG along the A–P axis in the four operation classes falls into two groups. Means in classes D and V are not significantly different from each other or from those of controls. In contrast, means in classes B and C are not significantly different from one another but are significantly different ($\ll 0.01\%$ level) from those of controls. The two groups of results are significantly different from one another ($\ll 0.05\%$ level for V and B, $\ll 0.01$ level for D and B, for D and C, and for V and C).

within each class is explicable in terms of the sclerotome that remains within contact distance of neural crest cells.

Independence of DRG and spinal nerve segmentation. DRG formation and axon outgrowth have been suggested to be interdependent in an interesting way. Loring and Erickson (1987) have proposed that the outgrowing axons promote the formation of DRG by providing a barrier to the ventrad migration of neural crest. This hypothesis is engaging because it can explain why sympathetic precursors migrate farther ventrally than DRG precursors. Since the sympathetics apparently migrate first and move through a more lateral portion of the somite, they would not be trapped by the earliest axons; DRG precursors, in the more medial sclerotome, would be trapped. An alternative explanation for the limited ventral migration of DRG precursors is that these cells avoid perinotochordal materials, as hypothesized by Newgreen *et al.* (1986). If this is the case, crest migration is unlikely to depend on axon outgrowth.

The results in Figs. 6A and 6B convincingly show that axons and neural crest cells can respond independently to the sclerotome. In these graphs, regions to the upper left and lower right of the diagonal lines include values that are predominantly outside the control range and in which the extent of the spinal nerve and DRG differ by more than 20% of the segment. These regions include from 18 to 50% of the segments, depending on operation class. In fact DRG can be completely unsegmented when spinal nerves are normal in extent (Figs. 6B, 6D, and 6E) and vice versa (Figs. 6C, 6D, and 6E). It is unlikely that axons grew out and then retracted after trapping DRG precursors in all the cases in which DRG extended farther than spinal nerves. Nevertheless, these findings do not disprove Loring and Erickson's hypothesis since axons may not have to trap all the neural crest cells that ultimately contribute to the DRG. Neural crest may merely require a nucleation site that is provided in these operated embryos by a few

axons or by contiguous condensations of neural crest cells in adjacent segments. However, whether or not axons provide a "stop" signal of some sort for these neural crest cells, it is certain that axons and neural crest respond to the environmental constraints of the somite and become segmented independently.

DISCUSSION

Several novel findings are reported that extend our understanding of how neural patterns develop. (1) Local interactions with the sclerotome are of primary importance to the segmentation of spinal nerves. This suggests that growth cones respond directly to the cell surfaces or extracellular matrix of the sclerotome rather than to long-range diffusible cues. (2) The sclerotome is not essential for neural crest cells to migrate or axons to grow out: it merely imposes a pattern on the migration and outgrowth. If environmental cues elicit migration and outgrowth, they must be derived from nonsomitic tissues. (3) Neural crest cells need not interact with sclerotome to form DRG. The process of DRG condensation may be cell-autonomous or require an interaction with nonsomitic tissue. (4) Segmentation of spinal nerves and DRG are independent processes. (5) The sclerotome provides no essential cues for specific innervation. (6) The process of segmentation is not essential to the specific innervation of limb targets. (7) The ventromedial sclerotome that surrounds the notochord acts as a barrier to axon advance. (8) The notochord may play a heretofore unsuspected role in the development of specific patterns of innervation. The results are discussed in the context of specific and nonspecific guidance.

Specificity of motor axon outgrowth. The specificity of motor innervation is clearly not dependent on the sclerotome or on the development of a segmented pattern of outgrowth. Motoneurons innervate only the appropriate limb targets and the epaxial muscles despite deletion of the sclerotome. Similarly, limb innervation is normal following somite deletion (Phelan and Hollyday, 1986; Tosney, 1986) or transplantation to foreign axial levels (Keynes *et al.*, 1987; Lance-Jones, 1988b). Therefore motoneuron growth cones are not garnering vital information from the sclerotome with regard to their selection of pathways or their final destination. Conversely, specific navigational cues are independent of the cues that channel axons into a segmental pattern.

What tissues do provide the specific cues required for motoneuron outgrowth? The target is essential for outgrowth of epaxial motoneurons (Tosney, 1987a) and may provide a short-range signal for limb motoneurons (Lance-Jones and Landmesser, 1981; Lewis *et al.*, 1981; Lance-Jones, 1986). However, target attraction is clearly insufficient for the specific innervation of limb muscles. It does not, for instance, contribute to selection of the appropriate pathway in the plexus region at the limb base (Tosney and Landmesser, 1984). The specific cues in the plexus region and limb may have a common origin in the mesenchyme of the lateral plate. This gives rise to plexus mesenchyme and limb connective tissue (Dias and Lance-Jones, 1988), dictates the physical pattern of limb muscles (Chevallier and Kieney, 1982), and may also confer identity upon limb targets (Keynes *et al.*, 1987; Lance-Jones, 1988b).

The present study suggests that the notochord also plays a role in the specificity of motor innervation. When the notochord had shifted relative to the spinal cord and lay closer to the control leg, axons on the *control* side made projection errors at the limb base but corrected these errors after entering the limb. Similar projection errors have been seen in preliminary experiments in which an extra notocord was implanted but the somites were not deleted ($N = 10$), confirming the importance of the notochord. Projection errors corrected by U-turns are rather novel; to the best of my knowledge they have been reported only in insects (see Costello, 1986).

The notochord could influence specific projection in at least three ways. First, the position of the notochord can influence the birth of motoneurons in the ventral spinal cord (VanStraaten *et al.*, 1985). Since motoneurons appear to be specified about the time they are born (cf. Lance-Jones and Landmesser, 1980, 1981) and their birthdates correlate roughly with their identity (Hollyday and Hamburger, 1977), the specification of motoneurons may have been altered when the spatial relation between the spinal cord and notochord was changed. If this is the case, then the projection errors seen may have been made by motoneurons with altered or confused identities. A second possibility is that axons are normally inhibited from altering their trajectory until they are at some critical distance from the notochord. If so, some of the axons might not have been able to turn in response to specific cues until they were within the limb. A third possibility is that the notochord itself provides specific cues essential for pathfinding that extended farther into the control limb when the notochord is displaced toward it. I am currently testing these hypotheses.

The development of neural segmentation. It has long been known that the somites impose their own segmental pattern on the populations that invade them (Lehman, 1927; Detwiler, 1934; Weston, 1963). The present study confirms that the sclerotome is the essential tissue for this process: the intact dermamyotome is not important (Tosney, 1987a) and a role for limb muscle

precursors that arise from the dermamyotome is ruled out because segmentation is disrupted after somite deletion regardless of whether a full complement of limb muscles has (present results) or has not (Lewis *et al.*, 1981) developed. The identification of the sclerotome as the tissue of interest is not surprising, but it is nonetheless a prerequisite for investigations of the cellular and molecular bases of segmentation.

The segmental pattern of the peripheral nervous system in the trunk is a consequence of the serial repetition of tissues that are more (anterior) and less (posterior) permissive for axonal invasion (cf. Keynes and Stern, 1984). Deletion of the somite removes the dichotomy in the environment. However, this does not stop crest migration or axon outgrowth. These populations behave as though what is missing is something that normally inhibits outgrowth rather than something that is essential for outgrowth. This "something" is presumably the posterior sclerotome, which axons do not invade during normal development. This observation rules out one simple hypothesis, that the anterior sclerotome is the sole source of a substance that elicits axon outgrowth and neural crest migration. Although a very early interaction with the segmental plate could be essential for outgrowth, this does not account for segmental patterning, since the difference in anterior versus posterior somite arises later (Stern and Keynes, 1987). Viewing the posterior sclerotome as inhibitory does not necessarily mean that posterior rather than anterior sclerotome is the sole source of whatever molecular substance imposes segmentation; the important difference may be relative. For instance, anterior sclerotome could provide more outgrowth-enhancing properties than posterior sclerotome; in the absence of both populations, other tissues may elicit or maintain outgrowth, if an exterior signal is indeed required.

The results of partial somite deletions indicate that the local environment along the path of axon outgrowth is of primary importance; axonal segmentation is lost to a similar extent regardless of whether only the ventral sclerotome or the entire sclerotome is deleted. This observation is difficult to explain unless only the sclerotome that is directly in the path of outgrowing axons affects their pattern of outgrowth. In addition, there is no obvious relation between loss of axonal segmentation and the volume of sclerotome deleted. This suggests that characteristics of the sclerotome that may be related to the volume of the tissue (such as diffusible cues) are of secondary importance, if they contribute at all, and implicates short-range interactions.

Cellular mechanisms that depend on short-range interactions include contact paralysis, proteolytic alteration of the local environment, and substratum preference (see review by Tosney, 1988). The mechanism of substratum preference, in which growth cones select the path of greater adhesivity (Letourneau, 1975), has been implicated by studies in culture. Growth cones emerging from spinal cord explants (many of which should be motoneurons) are more frequently found on the surface of anterior rather than posterior sclerotome cells when cultured on collagen or polylysine substrata (Stern *et al.*, 1986). A second set of more preliminary experiments (Tosney, 1987b) identified motoneuron growth cones using a nontoxic fluorescent dye (di-I; see Honig and Hume, 1986) and videotaped their interactions with similarly identified anterior or posterior sclerotome cells. This time lapse study shows that motoneuron growth cones are not contact paralyzed by either population. However, when cultured on laminin, motoneuron growth cones traverse the surface of anterior sclerotome cells but do not advance onto the surface of posterior sclerotome cells. This suggests that motoneuron growth cones have a hierarchy of adhesive preferences in which laminin or the surface of anterior sclerotome cells are preferred to the surface of posterior sclerotome cells. This is what we would expect if segmentation arose because growth cones preferred anterior over posterior sclerotome as a substratum. It remains to be seen whether this mechanism plays a primary role within the embryo.

The local environment may also be important to the segmentation of neural crest cells that form DRG since, in all cases in which DRG lost segmentation, dorsomedial sclerotome had apparently been removed. The loss of segmentation in only these cases could be explained if neural crest cells are inhibited from invading posterior sclerotome upon contact. If this were the case, DRG could form whenever posterior sclerotome was removed adjacent to a site that contained neural crest cells, provided that adjacent posterior sclerotome did not move in and fill up the space. However, the data do not clearly rule out a factor that varies with sclerotome volume and the possibility that diffusible cues attract or repel neural crest cells should be tested further.

Even if both neural crest cells and axons primarily interact with the local environment, they clearly respond independently. They cannot, therefore, be assumed to respond to the same features on the molecular level. In fact, populations of neural crest cells that give rise to DRG and sympathetic ganglia may respond to different tissues within the somite. For instance, Loring and Erickson (1987) have proposed that the undersurface of the anterior dermamyotome is a prefered substratum for neural crest migration. While the dermamyotome is not essential for DRG formation (Tosney, 1987a), the possibility that it rather than the

sclerotome guides sympathetic ganglia precursors has not been ruled out.

Tissues that act as barriers to axonal outgrowth. The apparently divergent observations on anterior vs posterior and dorsolateral vs ventromedial sclerotome may be made sense of using a simplifying hypothesis: developing cartilaginous tissues act as barriers to axonal advance. This hypothesis was first proposed to explain descriptive and experimental studies on the role of the *pelvic girdle precursor* in axonal guidance; axons normally enter the limb through hiatuses in this tissue and when gaps are made, axons traverse the gaps to enter the limb (Tosney and Landmesser, 1984; 1985a). The *posterior sclerotome* also becomes cartilaginous and can reasonably be viewed as a barrier. These barrier tissues appear to contribute to the development of the gross anatomical nerve pattern and its correspondence with neighboring tissues. For instance, by interacting with the sclerotome, the axons form discrete bundles that are positioned in proper sequence with the vertebrae that develop from the sclerotome, thus assuring coordinated development, even during evolution. It seems improbable that the sole function of the relevant cues is to guide axons; they are more likely to be an essential part of the normal developmental program of the cartilaginous tissues.

The present study suggests that the *ventromedial sclerotome* is also a barrier to axon advance, even in the anterior of a segment: axons turned to avoid it and extended just beyond its outer borders, even when it was spatially displaced. A general barrier function is also consistent with the fact that neural crest cells never enter this mesenchyme (cf. Newgreen *et al.*, 1986; Loring and Erickson, 1987). Further, axons from both motor and preganglionic sympathetic populations do not penetrate it and form nerves only at its outer borders (Tosney, unpublished observations). In contrast, the more dorsolateral sclerotome readily supports an extensive ramification of epaxial motoneurons (Tosney and Landmesser, 1985a,b).

If the ventromedial sclerotome is inhibitory to axonal advance, how did it become so? It is unlikely to be intrinsically different from the dorsolateral sclerotome. For instance, no differences in cells from anterior sclerotome are detected by growth cones in culture (see Stern *et al.*, 1986). It seems likely that the notochord alters the mesenchyme that surrounds it. This is consistent with the formation of spinal nerves in a constant spatial relationship with displaced notochord and its investing mesenchyme. In addition, notochordal material inhibits neural crest motility in culture (Newgreen *et al.*, 1986). Moreover, the notochord is well known to stimulate sclerotome development and, in particular, to increase the synthesis of its cartilage components (cf. Cheney and Lash, 1981). The stimulus may be most effective in the adjacent mesenchyme. To emphasize the suggested role of the notochord, I refer to the ventromedial sclerotome as perinotochordal mesenchyme.

Do the three tissues proposed to act as barriers actually share a similar developmental fate? For the pelvic girdle precursor and the perinotochordal mesenchyme, the answer is a clear yes: both form dense cartilaginous tissues that subsequently become bone, the pelvic girdle, and the vertebral bodies, respectively (see Verbout, 1976; Trelstad, 1977). How anterior and posterior sclerotome fit into this scheme is less clear. There is evidence in the mouse that posterior sclerotome forms bone (the neural arch and transverse processes), while anterior sclerotome forms the connective tissue of the interarch space (Dalgleish, 1985). In the chick, however, it has been suggested that both anterior and posterior sclerotome cells contribute to bony structures (Stern and Keynes, 1987). Nevertheless, it is clear that even in the chick the bones receive a heavier contribution from posterior sclerotome (Lance-Jones, 1988a and personal communication). In addition, Trelstad (1977) reports distinct differences in the packing density and orientation of anterior and posterior sclerotome cells and suggests that they differ in extracellular materials, as do more mature cartilage and connective tissues (cf. Cheney and Lash, 1981). Posterior sclerotome, like the other barrier tissues and unlike adjacent pathway tissues, is densely packed and contains high levels of glycosaminoglycans (Tosney and Landmesser, 1985a). While the differences are most striking after axon outgrowth, it should be borne in mind that the important molecules may appear very early in the developmental program when we may be less able than growth cones to detect important differences.

An early molecular difference has been demonstrated between one set of path and barrier tissues, the anterior and posterior sclerotome. Peanut agglutinin lectin binds to posterior but not anterior sclerotome while a number of other lectins and adhesive molecules do not differ in distribution (Rogers *et al.*, 1986; Stern *et al.*, 1986; Tosney *et al.*, 1986). Preliminary results showing that peanut agglutinin lectin also binds to perinotochordal mesenchyme and to the pelvic girdle precursor during the critical stages (R. Oakley and K. Tosney, unpublished observations) suggest that the three barrier tissues do have interesting molecular features in common.

If an axonal pattern does not result from neuron-specific cues, is it a product of axon guidance? There has been a tendency to dismiss features of the environment that (merely) channel axon outgrowth since these are

clearly insufficient to account for specific innervation. On the other hand, it has become obvious that population-specific navigational cues are themselves insufficient to fully explain the stereotyped pattern of axon outgrowth, particularly in systems in which axons must traverse a great distance to reach their target. Even though general paths may be so nonspecific that they can channel axons from foreign populations (cf. Levi-Montalcini, 1979; Katz and Lasek, 1979; Lance-Jones and Landmesser, 1981), in many systems they are a prerequisite to specific innervation, if only because they channel growth cones into regions where population-specific cues are accessible (cf. Landmesser, 1984, 1987). In addition, the underlying mechanisms are likely to be those well-known ones that rely on properties common to many growth cones, such as substratum preference. This mechanism has been suggested to be a component of general axon guidance in the vertebrate CNS (cf. Silver and Rutishauser, 1984) as well as in insects (Nardi, 1983; Berlot and Goodman, 1984; Bentley and Caudy, 1984; Blair *et al.*, 1986) where the essential adhesive differences probably arose as a side effect of normal development and were not laid down primarily for axonal guidance (see Palka, 1987).

Since the nonspecific "channeling" of axons (1) is clearly important to at least the gross anatomy of the nerve pattern, (2) requires interactions between the growth cone and the environment, and (3) fits within our conceptual framework of guidance mechanisms, it seems most reasonable to call it a form of axonal guidance. Specific and nonspecific axonal guidance would have a common denominator if we defined a navigational cue as *any feature of the normal outgrowth environment that can alter the direction of axon outgrowth.* Nonspecific guidance would be a response to navigational cues that delineate common pathways, where the cues would most often play multiple roles in development. In contrast, specific guidance would result from navigational cues that mediate population-specific alterations in axon trajectories such as those seen within the plexus region (Tosney and Landmesser, 1985b,c), as motor axons approach a peripheral target (Tosney and Landmesser, 1985b,c; Tosney, 1987a), as trigeminal sensory neurites grow into a target field (Lumsden and Davies, 1986), as the axons of preganglionic sympathetic ganglia respond to segment-specific environments (Yip, 1987), as retinal axons deploy within the chiasm and tectum (Bovolenta and Mason, 1987; Bonhoeffer and Huf, 1982; Thanos and Bonhoeffer, 1987), and at guidepost cells and along labeled pathways in insect (Bentley and Caudy, 1983; Raper *et al.*, 1983), leech (Kuwada, 1985), and fish (Eisen *et al.*, 1986; Kuwada, 1986) nervous systems.

Specific and nonspecific guidance cues have often been treated as though they were separable conceptually (cf. Landmesser, 1984). The present study confirms that these two types of cues are indeed distinct for motoneurons traversing the proximal tissues and suggests that some of the nonspecific cues may be provided by a common feature of developing cartilaginous tissues. We need to understand how both types of navigational cues guide growth cones to satisfactorily explain the patterned development of the nervous system.

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