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Different Subsets of Axonal Guidance Cues Are Essential for Sensory Neurite Outgrowth to Cutaneous and Muscle Targets in the Dorsal Ramus of the Embryonic Chick

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ABSTRACT The dorsal ramus nerve diverges dorsally from each spinal nerve to innervate the epaxial muscle and dermis that are derived in situ from each dermamyotome. The outgrowth of both the sensory and motor components of this nerve are sensitive to the proximity of the dermamyotome. Motoneurons display a direct target response that is not dependent upon the concurrent outgrowth of sensory neurites (Tosney: Dev. Biol. 122:540–588, 1987). Likewise, the outgrowth of sensory neurites could be directly dependent on the dermamyotome. Alternatively, sensory neurites could be dependent on motor axons that in turn require the dermamyotome for outgrowth. To distinguish between these possibilities, motor outgrowth was abolished by unilateral ventral neural tube deletion and the patterns of subsequent sensory neurite outgrowth were assessed. The cutaneous nerve branch formed in all cases. In contrast, neither of the epaxial muscle nerves formed in the absence of epaxial motoneuron outgrowth. Furthermore, sensory neurites could not be detected diverging into muscle from the cutaneous nerve or entering muscle via other novel routes. We conclude that motoneurons are essential for sensory outgrowth to epaxial muscle but not to cutaneous targets. It is clear that different subsets of navigational cues guide sensory afferents to muscle and to cutaneous destinations.

Neurites commonly project with a remarkable degree of precision to the appropriate targets during development (cf. Lance-Jones and Landmesser, '81; Honig, '82; Tosney and Landmesser, '85b; Landmesser, '87). As John Paul Trinkaus (1984) has said in another context, "This hitherto mysterious and elusive phenomenon really is understandable, but only if we break it down into its component parts so that discrete, answerable questions can be asked."* A central task in developmental neurobiology is to define each of the navigational cues that guide axons to their proper destinations and one strategy designed to do so proceeds in just this way: individual tissues are deleted one at a time and the patterns of nerve outgrowth that subsequently develop are assessed. This approach, which is continued in the current study, is capable of identifying tissues that provide essential and irreplaceable guidance cues. For instance, after Keynes and Stern ('84) had shown that the segmental pattern of axonal outgrowth was dependent upon somitic tissues, analysis using selective deletions established that the essential cues reside entirely within only one of the somitic tissues, the sclerotome. Segmentation of axon outgrowth in the chick embryo is altered only by deletion of the sclerotome (Tosney, '88a), not by deletion of the dermamyotome (Tosney, '87) or more distal tissues (Tosney and Landmesser, '84, reviewed by Tosney, '88b).

A deletion strategy has also shown that a second somitic tissue, the dermamyotome, provides a highly specific navigational cue that is active as neurites traverse and respond to the sclerotome. The dermamyotome is essential for outgrowth of the epaxial motoneurons but has no detectable influence on any of the other motor populations that traverse the same environment (Tosney, '87). The epaxial motoneurons are a major component of the dorsal ramus nerve that branches from the dorsal aspect of each spinal nerve. This nerve trifurcates to form a cutaneous branch that in-

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*K.W. Tosney is one of John Paul Trinkaus' "granddaughters," both on the undergraduate (via James Weston) and graduate (via Norman Wessells) sides of the lineage and was an honorary "Trink" lab member during her years at Yale. She is particularly delighted to contribute to this celebratory volume a paper that is aptly introduced and concluded by the master's own words.

vades dermis and two muscle branches that innervate the epaxial muscles (the longissimus and the intervertebral muscles). All three of these targets are derived in situ from the dermamyotome. When the dermamyotome is deleted from a segment and both immediately adjacent segments so that no myotubes develop in these segments, epaxial motoneurons do not grow out, even transiently. In contrast, epaxial motoneurons do extend within a segment deprived of target when epaxial muscle is present in an adjacent segment. These neurites reach a dorsal position within their segment a day or two after initial outgrowth and then extend only toward target in the closest adjacent segment (Tosney, '87). Nearby epaxial muscle target is thus essential for the outgrowth of epaxial motoneurons. In addition, the epaxial motoneurons exhibit their dependence on nearby epaxial muscle even in the absence of dorsal root ganglia, showing that they do not require sensory neurites for this response (Tosney, '87). Furthermore, even though the epaxial motoneurons normally become segmented as they interact with the sclerotome, this tissue provides neither essential nor sufficient cues for outgrowth. Epaxial motoneurons exhibit the same target-dependent outgrowth in the absence of the sclerotome (Tosney, '88a) and do not grow out when the sclerotome is undisturbed but the targets are removed (Tosney, '87). Thus, even though the gross patterns of epaxial motoneuron outgrowth can be influenced by other cues, the target provides the only cues that are essential for outgrowth of these axons.

The present investigation focuses on the guidance of the sensory components of the dorsal ramus, the afferents of the muscle and cutaneous nerves, both of which are also sensitive to dermamyotome proximity. Sensory neurites do not form muscle nerves in the absence of the epaxial muscles following dermamyotome deletion (Tosney, '87). Two interpretations of this result are plausible: in order to reach epaxial muscle targets, sensory neurites either require muscle or they require motoneurons that in turn require muscle. The latter possibility is consistent with the finding that sensory neurites do not project to a large subset of the limb muscles when the corresponding motoneurons are absent (Landmesser and Honig, '86; Swanson and Lewis, '86; Scott, '88). Since sensory neurites consistently form nerves to certain limb muscles without the aid of motoneurons (Landmesser and Honig, '86), we can not a priori assume that motoneurons are essential in all cases.

Likewise, the cutaneous nerve branch of the dorsal ramus is dependent upon the presence of the dermamyotome but is sensitive to only a portion of it (Tosney, '87). The cutaneous nerve branch does not form when the dorsal dermamyotome is deleted but the ventral dermamyotome remains in place. This is the case even when the dermis appears to be substantially normal, suggesting that the developing dorsal muscle tissue contributes to the formation of the cutaneous nerve. This is somewhat paradoxical, since the muscle is clearly not a cutaneous target. In addition, the dorsal muscle tissue can be replaced by other cues. When *all* the dermamyotome is absent from a segment, epaxial motoneurons grow dorsally within their own segment and then turn along the anterior-posterior axis to innervate muscle in an adjacent segment; a cutaneous nerve branches from this nerve once it has reached its most dorsal position. The simplest explanation of these observations is that the sensory neurites are obligatorily associated with the axons of the longissimus motoneurons, which are in turn dependent on dorsal muscle tissue for outgrowth. Once the sensory neurites have been conveyed to a dorsal position, they could then respond to local cues and form the cutaneous nerve branch. However, while plausible, this explanation is not consonant with the finding that sensory neurites form cutaneous nerves in the hindlimb in the absence of motor axons (Landmesser and Honig, '86; Swanson and Lewis, '86; Scott, '88).

The present study directly addresses whether motor axon outgrowth is essential to the outgrowth of either the cutaneous or the muscle sensory afferents of the dorsal ramus by examining nerve patterns that develop following unilateral deletion of the ventral neural tube.

MATERIALS AND METHODS

Embryonic surgeries

Surgeries were performed on stage 16–17 (approximately 2 days of incubation, see Hamburger and Hamilton, '51) White Leghorn chick embryos that had been prepared for surgery as in Tosney ('88c). To prevent the development of motoneurons but preserve the development of dorsal root ganglia (DRG), the ventral neural tube was deleted as in Landmesser and Honig ('86) or one entire half of the neural tube was removed after neural crest migration had commenced as in Tosney et al. ('88). The neural tube was opened dorsally with a fine tungsten needle and part or all of

the neural tube over 1–4 segments was unilaterally deleted with a micropipette. Deletions were performed from thoracic level 4 through lumbosacral level 5, corresponding approximately to somities 21 through 30. In a few additional embryos the DRG were prevented from developing by opening the neural tube and removing its dorsal portion unilaterally. Examination of these embryos confirmed that motor axons do not enter the cutaneous nerve pathway, even at early stages, and that outgrowth of epaxial motor axons was not dependent on the outgrowth of sensory neurites (not shown). Embryos usually had spina bifida since the neural tube did not reclose after the operation.

Fixation and morphological analysis

Embryos were fixed for examination at stages 26–31 in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, embedded in paraffin, serially sectioned at 12 μm, and stained in cresyl violet. The presence or absence of motor axons and the gross anatomical pattern of the dorsal ramus and its branches were characterized in each segment by examining serial sections with fluorescein epifluorescent optics. Under these conditions, individual myotubes and neurites fluoresce bright yellow and are clearly visible against the brownish cells of the dermis and sclerotome (see Tosney, '87). We excluded from the analysis all segments in which the DRG was greatly reduced in size and all embryos with limb abnormalities. We analyzed in detail 48 operated segments from 24 embryos. Motoneuron outgrowth was abolished in 18 of these segments and was greatly reduced in the remaining 30 segments.

We identified individual nerve branches using gross anatomical criteria. To maximize our ability to identify these branches, we fixed most of the embryos after stage 26.5 (corresponding to 5 days of incubation, when all three nerve branches are first distinct) and before stage 32 (7 days of incubation, when the intervertebral muscle nerve branch has regressed in the lumbosacral segments). Two embryos with total motoneuron deletions in single segments were fixed early in stage 26 (approximately $4\frac{1}{2}$ days of incubation), before the cutaneous nerve has normally branched from the dorsal ramus nerve trunk. During earlier stages (17–25) few sensory neurites are detectable within the dorsal ramus when the DRG in unoperated segments are labeled with horseradish peroxidase (Tosney, in preparation). Although normal degeneration of sensory cells can be detected as early as stage 25, the bulk of these cells die after stage 30 and death continues through stage 37 (Hamburger et al., '81).

We identified sensory neurites in two segments without motor axon outgrowth and in five segments in which outgrowth was greatly reduced by injecting horseradish peroxidase into the DRG of partially dissected embryos and processing them according to previously published procedures (Lance-Jones and Landmesser, '81). It was not practical to determine whether sensory neurites had entered the muscle branches in operated segments by injecting HRP into the muscle targets since no barriers to diffusion of label exist at these stages. Likewise, it was not possible to inject HRP into the dorsal ramus branches because of their small caliber. The results of similar injections in unoperated segments have confirmed that sensory but not motor neurites enter the cutaneous nerve and that sensory neurites are a component of the muscle nerves at these stages (Tosney, in preparation).

RESULTS

Normal anatomy and development of the dorsal ramus

The normal development of the dorsal ramus and the criteria for identifying particular nerve branches are first briefly described to provide a context for the analysis of the experimental results. The branches of the dorsal ramus form in a stereotyped order and position during development. During early stages (18–24) the dorsal ramus is represented by a diffuse array of individual neurites and small fascicles that project from the proximal spinal nerve toward the myotome (Cf. Tosney and Landmesser, '85a). Motoneurons precede sensory neurites into the dorsal ramus; few if any sensory neurites emerge from the DRG prior to stage 22 (cf. Landmesser and Honig, '86). The axonal fascicles gradually become consolidated during stages 25–26 and form a compact dorsal ramus nerve trunk and two muscle branches, the ventrolateral intervertebral nerve and the dorsal longissimus nerve. Sensory neurites reach a dorsal position and form the first detectable cutaneous nerve branch at stage 26.5.

During stages 27–31 when the majority of the operated embryos were examined, all three branches of the dorsal ramus are consistently identifiable by positional criteria. The intervertebral muscle nerve is the most ventral and generally the most posterior branch; it extends laterally from the dorsal ramus nerve trunk (Fig. 1a).

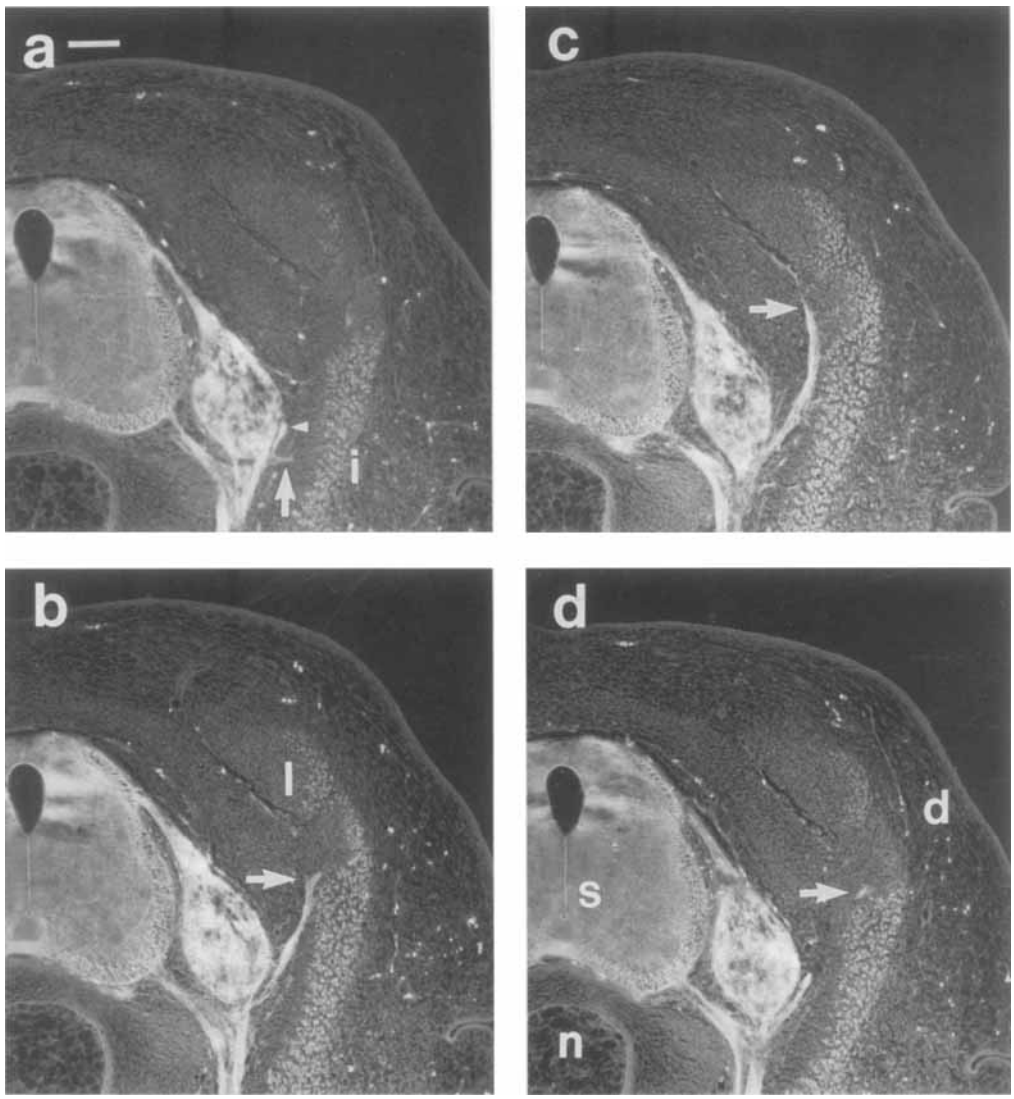

Fig. 1. Typical positions of dorsal ramus nerve branches in unoperated segments. **a:** The intervertebral muscle nerve (arrow) is the most ventral branch; it projects laterally from the dorsal ramus nerve trunk (arrowhead) toward the ventral portion of the myotome that will form the intervertebral muscle (i). **b:** The point of divergence (arrow) of the longissimus and cutaneous nerve branches is typically 100 μm ventral to the developing longissimus muscle (l). **c:** The longissimus muscle nerve (arrow) is the culmination of the medial branch of the dorsal ramus nerve trunk. It curves slightly toward the posterior and its distal portions are out of this plane of section. **d:** The cutaneous nerve curves toward the anterior and only short portions (arrow) are typically visible in cross sections. s, spinal cord; n, notochord; d, dermis. These figures represent, in posterior (a) to anterior (d) order, every second or third section through the anterior portion of a segment. Stage 29 embryo. Dorsal is toward the top. Calibration bar = 100 μm.

The nerve trunk projects dorsally and diverges to form two branches at a point approximately 100 μm ventral to the longissimus muscle (Fig. 1c). The longissimus muscle nerve branch projects dorsomedially, curving along the inner surface of the longissimus muscle (Fig. 1b). The cutaneous nerve branch projects dorsolaterally and anteriorly toward the dermis, passing through the most dorsal portion of the prospective intervertebral muscle or within the region where the two muscles separate by stage 29 (Fig. 1d). Because the nerve branches diverge at different positions along the anterior-posterior axis, all three are seldom visible in the same 12 μm cross section. A more detailed description of the normal development of the dorsal ramus and its targets is in preparation and has appeared in abstract form (Tosney, '88d).

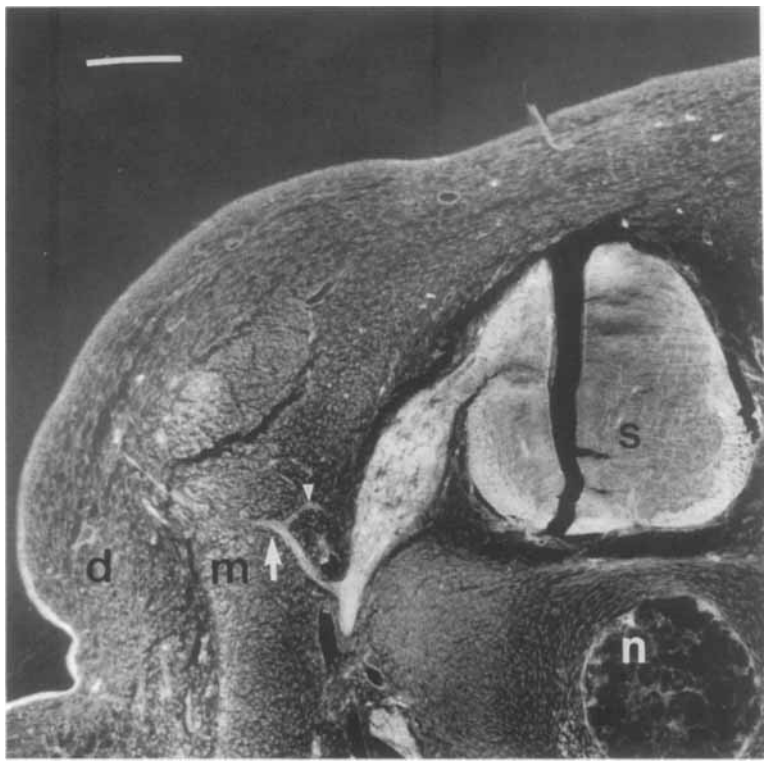

Fig. 2. Dorsal ramus morphology in the absence of motor axon outgrowth. This segment represents our best case in that the spinal cord was relatively intact except for the total absence of the lateral motor column on the operated side. As in all other segments in this class, no motor axons exited from the operated side of the spinal cord. The dorsal ramus is represented solely by a cutaneous nerve (arrow) that is particularly robust in this specimen. In more anterior segments, this nerve penetrates through the myotome into the dermis (d). No neurites could be detected in the intervertebral or longissimus muscle nerve pathways and none ramified from the cutaneous nerve into muscle. A blood vessel (arrowhead) joins and courses in concert with the cutaneous nerve as it projects distally. s, control side of spinal cord; m, myotome; n, notochord. Stage 28 embryo. Dorsal is toward the top. Calibration bar = 100 μm.

Anatomy of operated embryos

The ventral spinal cord was unilaterally reduced or absent in operated segments (see Fig. 2). Spina bifida was common in the operated segments and often extended into the immediately adjacent segments where the spinal cord was also occasionally reduced in size. In about half of the segments, the dermis appeared to be somewhat reduced on one or both sides of the embryo. In nine segments with total motoneuron deletion the myotome was also slightly reduced on the operated side, but in no case was the dorsal myotome absent. This is important since a distinct cutaneous nerve does not form when this tissue is totally removed (Tosney, '87).

The criterion for a successful deletion was the absence of axonal outgrowth from the ventral spinal cord. Our assay appears to be reasonably sensitive for use of this criterion, since we could detect fascicles that contained very few neurites and in many instances could detect what appeared to be single neurites (see Figs. 4a, 5, 6). For example, motor outgrowth was detected in one segment when it was totally confined to one 12 μm section and the nerve was less than 5 μm in diameter (see Fig. 6). By this criterion, motoneurons had been successfully deleted in 18 segments and outgrowth was greatly reduced in 30 segments.

Outgrowth to cutaneous targets in the absence of motor axons

The cutaneous nerve had obviously formed in all the segments that were totally deprived of motor axon outgrowth, as illustrated in Figure 2. In the majority of the cases the nerve was normal or slightly enlarged in caliber. In a few cases the nerve was diminished in diameter and this could be ascribed to a reduction in the size of the DRG or to the immaturity of the embryo. Despite the variation in the diameter of some of these nerves, the results support the conclusion that outgrowth

of motor axons is not essential for sensory neurites to reach cutaneous targets.

Although the cutaneous nerve was obvious in all operated segments, in some of these it did not penetrate into the dermis to its usual extent. The abbreviated length could not be ascribed to a diminution in motor axon outgrowth; for instance, in three segments this nerve was also shorter on the control side where motor outgrowth was not detectably disturbed. We suspected that the dermis might be important for the full ramification of this nerve, particularly since the volume of the dermis varied in the operated segments. However, we did not detect a straightforward relationship between the length of the cutaneous nerve and the amount of dermis that had developed. The cutaneous nerve was often of normal extent when the dermis was greatly reduced and was short when little reduction in dermis was visible. While we do not have a ready explanation for the variation in the length of this nerve, it remains plausible that the dermis provides the substratum or a short-range cue for the more distal outgrowth or that the ectodermal epithelium plays some role (cf. Verna, '85). We are currently investigating these possibilities more directly.

During normal development the cutaneous nerve is usually associated with a blood vessel that it first intersects just distal to the point where it branches from the dorsal ramus nerve trunk (Tosney, unpublished observations). Likewise, the cutaneous nerve was often associated with a prominent blood vessel in the operated segments. For instance, in Figure 2 a blood vessel (arrowhead) and the cutaneous nerve (arrow) converge and traverse the myotome in concert (see also Fig. 4b,c). While motor axons do not slavishly follow blood vessels (Tosney and Landmesser, '85a), sensory neurites have been suggested to associate with blood vessels both under normal and experimental conditions (Landmesser and Honig, '86; Tosney, '87). Since the present operations did little to disturb the developing vascular pattern, these experiments do not disclose the role, if any, of blood vessels in the outgrowth of neurites to cutaneous targets.

Outgrowth of sensory afferents to muscle targets in the absence of motor axon outgrowth

In contrast to the invariant presence of the cutaneous nerve, neither of the muscle nerves was represented in the absence of motor axons (Fig. 2), despite the fact that the epaxial muscle targets were fully normal in half of the segments. While the muscles were reduced in other segments, in no case were they greatly reduced. The absence of the muscle nerves contrasts dramatically with the outgrowth of epaxial motoneurons to muscle remnants composed of only a few myotubes (Tosney, '87). The presence of epaxial muscle is clearly not sufficient to elicit muscle afferent outgrowth in the absence of motoneurons.

In no case could we detect individual neurites emerging from the dorsal ramus nerve trunk in the region where the intervertebral nerve formed. Likewise, neurites were never observed within the longissimus nerve pathway; all neurites that reached the dorsal position where the cutaneous branch normally diverges then projected laterally and formed an unremarkable cutaneous nerve. Since individual neurites were detectable in this preparation, the absence of sensory neurites in the muscle nerve pathways strongly suggests that sensory neurites do not respond directly to muscle in the absence of motoneurons and are dependent upon motor outgrowth to reach muscle targets.

Sensory neurites could require motoneurons to reach muscle targets because motor axons in some way aid their pathfinding. Alternatively, the sensory neurites might reach muscle targets without the aid of motor axons but regress in the absence of motor innervation. If this were the case, then we should be able to detect sensory neurites in contact with muscle at an earlier stage. However, muscle nerves were not detected in two segments that were examined at early stage 26, before the bulk of sensory neurite outgrowth and before the cutaneous nerve branch is normally first detectable. In these two cases neurite outgrowth was very sparse (as was to be expected since only a subset of the sensory neurites would normally have grown out at this stage) and the neurites were confined entirely to a cutaneous nerve trajectory (see Fig. 4a). Neurites did not extend dorsally beyond the cutaneous nerve branch point into the longissimus nerve pathway and did not diverge laterally toward the intervertebral muscle in either segment. While it remains possible that a few neurites might have colonized the muscle at even earlier stages, it is quite clear that the entire sensory component does not enter muscle and then regress in the absence of motor outgrowth.

The possibility that the sensory neurons die before sending out neurites can not be rigorously excluded. However, death of sensory neurons appears to be target dependent (Hamburger et al.,

'81), and it should be pointed out that the muscle targets were normal in the majority of the segments analyzed. Moreover, since sensory cells generally begin to die only after contact with the target (see Hamburger and Oppenheim, '82), we would not expect to find trophic sensitivity before these neurites had extended. These considerations suggest that regressive events such as neurite retraction or sensory cell death are unlikely to explain the absence of sensory afferents to epaxial muscle in the segments that lacked motor axons.

Sensory neurites might enter muscle via novel pathways in the absence of motor axons. For instance, fasciculation with any neurites, motor or sensory, that pass close to muscle might suffice to bring sensory neurites within range of a short-range muscle cue. If this were the case, then the most likely place that a novel nerve to muscle might emerge is from the cutaneous nerve as it passes through the myotome. To determine if this were so, we carefully examined all cutaneous nerves formed in the absence of motor axons. In no case did we detect neurites exiting from the cutaneous nerve and ramifying within the muscle. The continued coherence of this nerve in the vicinity of muscle was particularly obvious in embryos examined after stage 28, when the longissimus and intervertebral muscles have become separated by a space that coincides with the cutaneous nerve (Fig. 3). In addition, we did not detect neurites leaving the cutaneous nerve from other sites or traversing the sclerotome individually and ending in either muscle. To the best of our ability to detect outgrowth, sensory neurites do not enter epaxial muscle via normal or abnormal routes in the absence of motor axons. This suggests that the motor axons are an obligate substratum for these neurites, rather than serving as a preferred, but replaceable, navigational aid.

The observation that, in the limb, sensory neurites that form nerves to muscles in the absence of motoneurons often do so in close contact with blood vessels (Landmesser and Honig, '86) led us to closely examine the vascular pattern in our operated segments. A prominent blood vessel often penetrates the ventral myotome in the region where the intervertebral muscle nerve normally forms and another lies subjacent to the inner surface of the dorsal myotome along the longissimus nerve pathway (see Figs. 3, 4c). Despite the availability of this potential substratum, we did not detect neurites in contact with either blood vessel. However, the blood vessels also fluoresce lightly and it would be difficult to identify single neurites against this higher background fluorescence; a small number of neurites might have reached muscle via an association with blood vessels. Nevertheless, since these blood vessels seldom formed a continuous strand from the DRG, we should have been able to detect neurites as they traversed sclerotome in their approach to blood vessels and we did not. While we can not rule out the possibility that a few sensory neurites can reach epaxial muscles using blood vessels as substratum in lieu of motoneurons, we do not find compelling evidence that blood vessels play a major role in sensory afferent outgrowth to muscle targets.

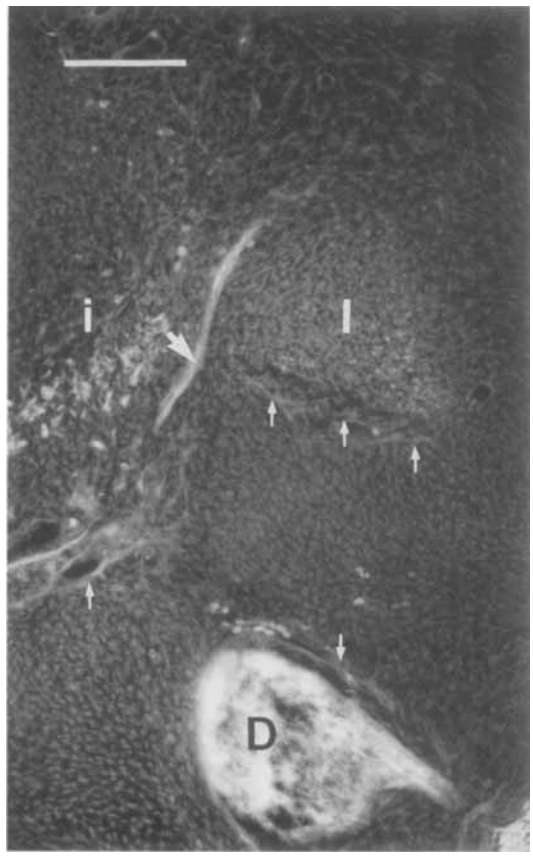

Fig. 3. This cutaneous nerve (large arrow) has formed in the absence of motor axon outgrowth. It is shown at the point where it passes between the intervertebral (i) and the longissimus (l) muscles. Neurites could not be detected ramifying from the nerve into either muscle. D, DRG; small arrows, blood vessels. Stage 29 embryo. Dorsal is toward the right. Calibration bar = 100 μm.

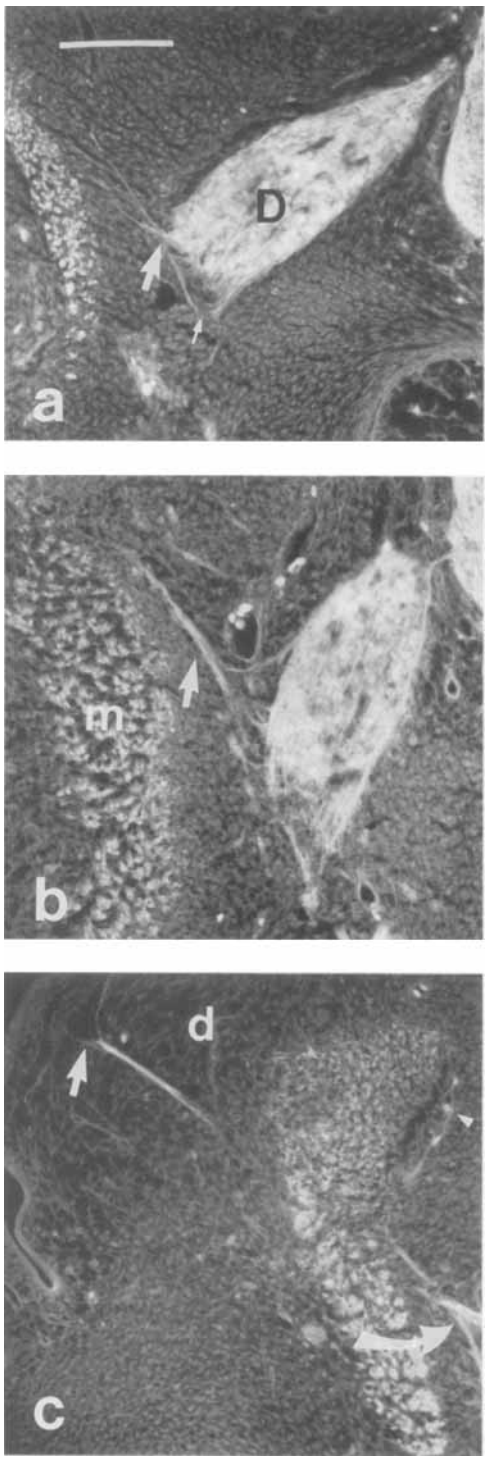

Fig. 4. Disordered dorsal ramus nerve trunk in the absence of motor axons. **a:** Sensory neurites (large arrow) often exit from the lateral surface of the DRG (D) and project along the cutaneous nerve pathway with independent trajectories. Others (small arrow) momentarily dip ventrally into the spinal nerve region before projecting dorsally. None of the neurites project into the intervertebral muscle or dorsal to the cutaneous branch point along the longissimus nerve pathway. Neurite outgrowth is still sparse in this young, stage 26 embryo. **b:** Neurites that exit from the lateral surface of the DRG form fascicles that gradually consolidate proximal to the myotome (m) to form a coherent cutaneous nerve (white arrow). A blood vessel can be seen just to the right of and running parallel to this nerve. **c:** Sensory neurites converge (curved arrow) proximal to the myotome. The coherent cutaneous nerve passes through the myotome in adjacent sections and can be seen in this section projecting into the dermis (d). A blood vessel parallels this nerve along much of its trajectory and can be most easily seen at the distal tip of the nerve (arrow). The blood vessel that lies along the longissimus nerve pathway can also be seen in this section (arrowhead). Dorsal is toward the top. b and c show stage 29 embryos. Calibration bar = 100 μm.

Disordered dorsal ramus nerve trunk in the absence of motor axons

We did note one apparent anatomical anomaly in the absence of motor axons. The proximal trunk of the dorsal ramus was less coherent than normal and often consisted of several widely separated fascicles. These neurites often originated from points on the lateral surface of the DRG and extended directly and with initially independent trajectories along the cutaneous pathway rather than joining the spinal nerve (Fig. 4a,b). The neurites gradually converged and formed a consolidated nerve proximal to the myotome (Fig. 4b,c). The exit of neurites directly from the lateral surface of the DRG is unlikely to be a response to the absence of motor axons since some sensory neurites appear to do this during normal development (Tosney, unpublished observations) and when a reduced number of epaxial motoneurons are present (see Fig. 5). However, the decreased coherence of this nerve is likely to reflect the loss of motor axons. In their absence the nerve trunk adjacent to the ganglion is not robust; consequently, there would be fewer opportunities for fasciculation and this could limit the ability of these sensory neurites to immediately form a coherent nerve. Nonetheless, the wide ramification of these neurites in the absence of motoneurons suggests that they do readily fasciculate with motor axons in the embryo during normal development.

Dorsal ramus formation following depletion of motor axons

We found that we had depleted but not totally prevented motor axon outgrowth in 30 additional segments. The cutaneous nerve was present in all

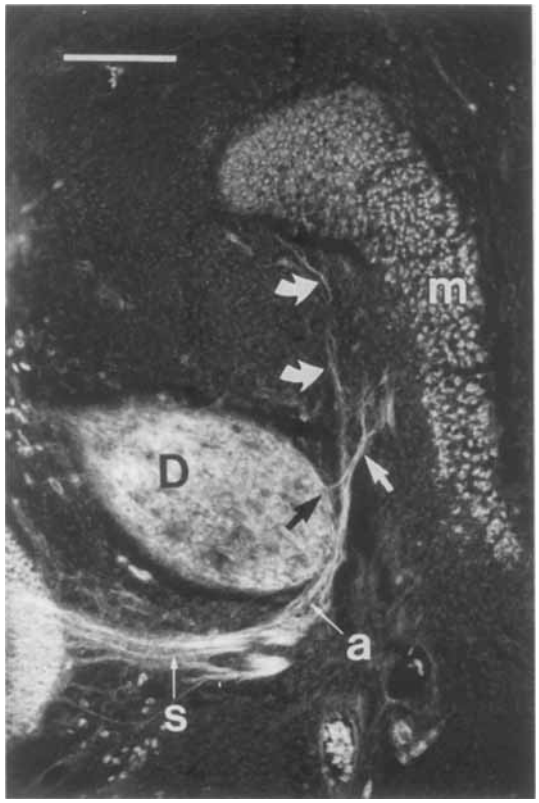

Fig. 5. Dorsal ramus morphology following reduction of motoneuron outgrowth. In this segment, epaxial motor axons (a) clearly diverge dorsally from the spinal nerve (s). Neurites can be seen in the longissimus nerve pathway (curved arrows) and along the cutaneous nerve pathway (white arrow) in this section; the intervertebral nerve was present in a more posterior section. At the black arrow, one or more neurites exit from the lateral surface of the DRG (D) and join the cutaneous nerve. m, myotome. Stage 27 embryo. Dorsal is toward the top. Calibration bar = 100 μm.

Fig. 6. Motor axon outgrowth (arrowhead) was very reduced in this segment and was detected only in the section shown here. None of the motor axons projected dorsally toward the epaxial muscle, presumably because epaxial motoneurons had been successfully deleted. A few sensory neurites (arrows) leave the lateral surface of the DRG in this and adjacent sections and project along the cutaneous nerve pathway. None of these projected along the muscle nerve pathways. D, DRG; i, intervertebral muscle. Dorsal is toward the right. Stage 26 embryo. Calibration bar = 100 μm.

cases. Muscle nerves were absent in two segments and all three nerve branches were represented in the rest. We could easily trace motor axons into the dorsal ramus in the cases in which muscle nerves formed (Fig. 5). While we could not unequivocally determine that sensory neurites contributed to each of these muscle nerves, in a few of these cases sensory neurites appeared to fasciculate with motor axons that entered muscle.

In two segments we were reasonably sure that epaxial motoneurons were absent since motor axons were few in number and none of them projected toward epaxial targets. A cutaneous nerve was obvious in both segments, but sensory neurites did not extend toward or enter muscle targets. Figure 6 illustrates one of these in which sensory neurites exit from the DRG and project directly along the cutaneous nerve pathway without diverging into the intervertebral muscle or progressing dorsomedially along the longissimus muscle nerve pathway. The results support the conclusion that elimination of the epaxial motoneurons is sufficient to prevent sensory afferents from innervating epaxial muscle.

DISCUSSION

We have shown that outgrowth of epaxial motor axons is essential for sensory neurites to innervate epaxial muscle but not cutaneous targets. These results are in accord with the growing consensus that different subsets of navigational cues guide neurites to different destinations (cf. Landmesser, '85, '87; Bentley and Caudy, '84; Lance-Jones, '86; Tosney, '87). However, we do not have as yet a full understanding of all the influences that act on any single population of neurites as they extend from their somata, traverse the complex embryonic environment, and enter appropriate target tissue. We discuss our results below in

the context of our current understanding of how sensory neurite outgrowth may be guided.

Guidance of sensory neurites to muscle targets

Sensory neurites normally project rather precisely to their segmentally correct targets (Honig, '82) and do so following various experimental manipulations (Honig et al., '86; Scott, '84, '86), suggesting that they are actively guided to appropriate destinations. The identity of the relevant guidance elements is much less clear for sensory neurites than it is for the motor axons that grow out in concert with them. One of the first insights was provided by Honig ('82), who proposed the then quite novel hypothesis that sensory neurites could be guided to their muscle targets by motoneurons. It was in fact found that when lumbosacral motoneurons are removed, sensory neurites invade few of the limb muscles (Landmesser and Honig, '86; Swanson and Lewis, '86; Scott, '88). The present results show that sensory innervation of epaxial muscles is also dependent upon motoneurons. Sensory neurites that invade the limb are not, therefore, unique in this regard. Motoneurons in some way play a primary role in the outgrowth of sensory neurites to muscle targets.

Landmesser and Honig ('86) have cogently discussed three possible mechanisms for motoneuron guidance of sensory neurons, all of which are consistent with the present results, and these will be only briefly reviewed here. The first possibility is that sensory neurites fasciculate with motor axons, either finding them more adhesive than other sensory neurites, specifically recognizing that certain motor axons are specified to project to the same muscle, or because they happen to come in contact with motor axons and thereafter tend to remain with them. The neural cell adhesion molecule NCAM, which has been shown to promote adhesion among neurites (see Rutishauser, '84, for review), is expressed at lower levels on DRG neurites than on motoneurons during their early outgrowth (Tosney et al., '86), suggesting that sensory neurites may indeed be less adhesive than motor axons. In addition, our present results suggest that sensory neurites do normally fasciculate with motor axons within the embryo. A second possibility is that motor axons and, in some cases, blood vessels provide an aligned substratum along which sensory neurites readily advance in response either to the molecular characteristics of such substrata, or to the mere fact that these are physically oriented and provide a medium for contact guidance (see Weiss, '34). A final possibility is that motoneurons alter the environment by secreting proteins or proteases (cf. Kryostosek and Seeds, '81; Pittman, '85) that render the environment more adhesive or penetrable by sensory neurites. The apparent inability of sensory neurites to penetrate limb muscles in the absence of motor axons, even when they have reached the muscle in association with a blood vessel (Landmesser and Honig, '86), is particularly consistent with the latter possibility.

There is direct evidence that blood vessels, like motoneurons, can facilitate the divergence of sensory neurites toward muscle in selected cases. In the hindlimb, the ischioflexorius nerve and one of the branches to the femorotibialis muscle normally form in close association with blood vessels and, in embryos without motoneurons, sensory neurites associate with these blood vessels to form somewhat abnormal muscle nerves (Landmesser and Honig, '86). Similarly, the epaxial muscle nerves are often associated with blood vessels during normal development (Tosney and Landmesser, '85a; Tosney, unpublished observations). Unlike the corresponding sites in the limb, our results suggest that these blood vessels are not sufficient for outgrowth. However, it remains possible that a few neurites associated with these blood vessels could have gone undetected in our embryos. The possibility that the vascular endothelium can facilitate at least some sensory neurite outgrowth to epaxial muscle and cutaneous targets remains open. It would be informative to learn how consistently blood vessels parallel certain sensory pathways and whether they precede or follow neurite outgrowth in time. We have begun to assess this possible relationship.

Specification of sensory neurons

Landmesser and Honig ('86) have suggested that sensory afferents that normally innervate muscle may instead project down cutaneous nerves in the absence of motoneurons. They have reported that cutaneous nerves were often enlarged in the absence of limb motoneurons, suggesting that sensory neurites have indeed altered their pathfinding behavior when motoneurons are not available. Taylor ('44) in the amphibian and Swanson and Lewis ('86) and Scott ('88) in the chick fore and hind limbs have not noted an increase in the caliber of cutaneous nerves under similar conditions. While the cutaneous nerve occasionally seemed unusually robust in the absence of epaxial motoneurons, this nerve is nor-

mally relatively small and a significant increase in the number of neurites present would be difficult to document in our preparations.

While the possibility that sensory neurites might project to alternative targets under experimental conditions is interesting, workers in this field have rightly emphasized that altered pathfinding is not necessarily evidence that sensory neurites were specified for a particular target before outgrowth (see Landmesser and Honig, '86; Scott, '86, '88). Sensory neurites may normally be channeled rather passively to various destinations and gain a target identity during or after pathfinding. It has in fact not been possible to determine whether sensory neurons are specified for particular targets before outgrowth since there are no markers that indicate their target specificity before the neurites reach their destinations and, unlike motoneurons, the target specificity cannot be deduced from the position of their somata (cf. Honig, '82). Whether or when sensory neurons are specified for particular targets remains unclear.

Guidance of sensory neurites to cutaneous targets

The results clearly show that motor axons are not essential for sensory neurites to form the cutaneous branch of the dorsal ramus. The cutaneous pathway was colonized in all cases and, despite the fact that the nerve branch was occasionally shorter and varied in diameter, it is clear that sensory neurites can form a normal cutaneous nerve despite the absence of motoneurons. This observation clearly refutes the hypothesis that longissimus motoneurons are essential for outgrowth to cutaneous targets. This was a plausible explanation of previous results following dermamyotome deletion in which it was found that a cutaneous nerve formed only if epaxial motoneurons had grown dorsally to innervate developing longissimus muscle in an operated or adacent segment (Tosney, '87).

It should be emphasized that the deletion strategy used in this study is only capable of identifying those cues that are *essential and irreplaceable*. Since motor axons are sufficient for sensory outgrowth to cutaneous targets in the total absence of the myotome, it is likely that motoneurons normally play at least a subsidiary role during outgrowth of this nerve. In addition, our results suggest that sensory and motor populations do normally fasciculate in the embryo. It should also be emphasized that the present results do not rule out the possibility that the dependence of the cutaneous nerve on the presence of dorsal dermamyotome is in part mediated by motoneurons. We are currently addressing this dependence by simultaneously deleting both the motoneurons and the dorsal dermamyotome. Our preliminary results indicate that cutaneous outgrowth up to the point where it normally branches from the dorsal ramus nerve trunk is strongly dependent on the dorsal extent of the myotome and that it retains this dependence even in the absence of motoneurons (Tosney and Hageman, in preparation). This suggests that, in spite of a possible facilitation of outgrowth by motoneurons and in spite of the apparent ability of sensory neurites to make do with motoneurons in the absence of muscle, the primary impetus for reaching a dorsal position is some interaction with developing muscle or muscle-associated tissue. However, the cutaneous neurites are obviously facultative in that they can make use of either muscle alone or motoneurons alone to reach the site where they normally branch from the nerve trunk.

It is reasonable to posit the existence of an additional and independent set of cues that guide sensory neurites from the point where they normally leave the nerve trunk to their final destination. The sensitivity of sensory neurites to myotome declines distal to the cutaneous branch point (Tosney and Hageman, unpublished observations) and motoneurons clearly do not convey sensory neurites through the dermis. Once sensory neurites have been guided to a dorsal position, they may respond to short range cues provided by the local environment. The dermis is an obvious substratum for further outgrowth and sensory neurites readily advance on dermal cells in culture (Verna, '85). Blood vessels are another likely substratum, as indicated by the commonplace association between the distal cutaneous nerve and a blood vessel during normal development and in the present experimental series. An additional set of cues may control the extent of distal outgrowth. The fact that sensory neurites in the chick seldom colonize the region directly beneath the epithelium (Verna, '85) may be explained by a repulsive force exerted by the ectodermal epithelium; DRG neurites in culture strongly avoid ectoderm and veer away from it before actually contacting it (Verna, '85; Verna et al., '86). The ectodermal epithelium could have an indirect effect as well since it inhibits adjacent vascular development (Feinberg et al., '83).

The evidence in favor of these disparate guidance elements suggests that sensory neurites reach cutaneous targets by sequentially using a number of different cues. The relative importance of each guidance element probably varies with the distal extent of outgrowth as neurites enter slightly different local environments. In addition, there appear to be a multiplicity of cues within each local region, each one of which may or may not be sufficient for outgrowth in the absence of the other cues. It is, therefore, abundantly clear that guidance of sensory neurites, and in particular of those that end in cutaneous targets, is likely to be complex. Further efforts are necessary to identify all of the relevant navigational cues and to determine their relative importance to guidance. The following (Trinkaus, '84) can serve to summarize our viewpoint. "And here again, we may say with some emphasis that in further research on the matter it will be well to keep in mind the probability that there may be several mechanisms at work and these various mechanisms may act in concert, at least to some degree, each by itself being inadequate for the task at hand."

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