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Altered Sensory Projections in the Chick Hind Limb following the Early Removal of Motoneurons

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Chick sensory neurons grow to their correct targets in the hindlimb from the outset during normal development and following various experimental manipulations. This may result not because sensory neurons respond to specific limb-derived cues, but because they interact in some way with motoneurons which are responsive to such cues. To test this possibility, we removed the ventral part of the neural tube, which contains motoneurons and their precursors, at stages 16½-20½ and later examined the pathways sensory neurons had taken within the limb. Muscle nerves generally were missing or were reduced in diameter beyond the extent expected simply from the absence of motoneuron axons. In many cases, cutaneous nerves were enlarged, presumably due to the addition of other sensory axons. This result suggests that, in the absence of motoneurons, sensory neurons that normally project to muscles are unable to do so and may instead project along cutaneous pathways. Sensory axons from different segments also crossed less extensively in the plexus region than they did in control embryos, suggesting that alterations in their trajectories may normally be facilitated by similar changes in motoneuron pathways. Thus, motoneurons greatly enhance sensory neuron growth to muscles and contribute significantly toward the achievement of the normal sensory projection pattern. Sensory axons may fasciculate with motoneuron axons, or motoneuron axons may provide an aligned substrate for sensory neurons to grow along. Alternatively, motoneuron axons may alter the environment, thereby making certain pathways in the limb permissive for sensory neuron growth.

INTRODUCTION

An important question in developmental neurobiology is how neurons are able to grow to the correct targets and establish appropriate connections. In some systems, for example, the chick hindlimb, appropriate pathway selection plays a major role and considerable attention has been directed at trying to understand how motoneurons choose among the available pathways (see Landmesser, 1984, for a recent review).

The chick hindlimb is innervated by motoneurons and sensory neurons originating from the eight lumbosacral segments. Each target site (muscle or region of skin) is innervated by a peripheral nerve that contains the processes of neurons from primarily two or three contiguous segments. The same pattern of segmental projections found in mature embryos is also found in young embryos from the time that axons first grow into the limb. Thus, sensory neurons (Honig, 1982; Scott, 1982) as well as motoneurons (Landmesser and Morris, 1975; Landmesser, 1978b; Lance-Jones and Landmesser, 1981a; Tosney and Landmesser, 1985b) appear to select the correct pathways from the outset.

A variety of embryonic manipulations of either the neural tube or the limb bud have been carried out to elucidate the mechanisms underlying appropriate motoneuron outgrowth (Lance-Jones and Landmesser, 1980a,b, 1981b; Ferguson, 1983; Whitelaw and Hollyday, 1983; Laing, 1984; Stirling and Summerbell, 1985). The results indicate that motoneuron growth cones make use of cues within the developing limb that enable them to grow to their correct targets even when the point at which they enter the limb has been experimentally altered. The evidence also strongly suggests that these cues are relatively local or short range (Lance-Jones and Landmesser, 1981b; see also Tosney and Landmesser, 1984; Lance-Jones, 1986). Much less attention has been directed toward understanding how sensory neurons select appropriate pathways. Recently we described the sensory projections following several of these operations which also altered the positions of sensory neurons with respect to the limb (Honig, 1979, 1980; Honig *et al.*, 1986). In situations in which motoneurons were able to grow to their correct targets, sensory neurons also tended to project along the segmentally appropriate pathways. In situations in which motoneurons were displaced greater distances from their normal point of entry into the limb and made wrong connections, sensory neurons also projected incorrectly. As always found in normal embryos (Honig, 1982), the segmental patterns of sensory projections to muscles were frequently similar to the motoneuron projections. Moreover, sensory neurons pro-

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jecting to skin showed similar types of responses to these manipulations. That is, they also frequently tended to project along the segmentally appropriate pathways. These results, which are discussed in detail in a preceding paper (Honig *et al.*, 1986) can be interpreted as evidence that sensory neurons are specified for spatially defined limb regions and possess the capacity to respond to specific guidance cues within the limb. They are, however, also consistent with another rather novel hypothesis which we have previously proposed (Honig, 1980, 1982; Honig *et al.*, 1986) and which we now examine directly.

It seemed that during normal development some sensory neuron axons might associate with motoneuron axons and be guided to muscles; the remaining afferents would then project along cutaneous nerves. Thus, both in normal embryos and following various manipulations, sensory axons might project to their appropriate targets, not because they were capable of responding to specific limb-derived guidance cues, but because they were associating with or following motoneurons which possessed this capacity. Here we have tested this possibility by examining the outgrowth of sensory neurons in the absence of motoneuron axons. The results from these experiments, as well as from a further examination of some aspects of normal development, support this hypothesis. Preliminary reports of some of these results have been presented elsewhere (Landmesser and Honig, 1982; Landmesser *et al.*, 1983).

MATERIALS AND METHODS

Embryonic Surgery

In an earlier study in which several segments of the neural tube had been removed at stage (st) 15–16 (Lance-Jones and Landmesser, 1980a) the adjacent dorsal root ganglia (DRGs) usually did not form (see also Honig 1980; Honig *et al.*, 1986), presumably due to removal of the neural crest and/or damage to crest migration pathways. Therefore, in the first series of experiments, we attempted to minimize damage to the developing sensory neurons by performing the operations relatively late (st $18\frac{1}{2}$–$20\frac{1}{2}$), when crest migration is virtually complete (Weston and Butler, 1966). In addition, we developed a technique that minimized direct surgical damage to the dorsal neural tube and adjacent regions where DRGs were condensing, but still allowed us to remove the ventral part of the neural tube, which contains motoneurons and their precursors (Wenger, 1950).

White Leghorn chick embryos were incubated at 38°C until st $18\frac{1}{2}$–$20\frac{1}{2}$ (Hamburger and Hamilton, 1951), at which time the eggs were opened. As shown in Fig. 1, the neural tube was split down the midline, allowing its entire dorsal-ventral extent on each side to be visualized from the surface bordering the central canal. We removed approximately $\frac{1}{2}$ to $\frac{2}{3}$ of the neural tube beginning at the ventral midline. This region was first isolated with sharpened tungsten needles, and then removed by applying suction through a micropipet, the tip of which had been broken off to a suitable diameter (approximately 50–100 μm). In this first series of experiments, the deletion was restricted to lumbosacral segments (LS) 1–3 which give rise to the anterior or crural plexus (see Fig. 4a), and was unilateral in some embryos, bilateral in others. This operation was performed on 105 embryos. The eggs were sealed with a coverslip and returned to the incubator until the embryos were st 26–36.

Although motoneuron axons do not enter the limb bud until st 23, as early as st 18 some motoneuron axons have grown out of the neural tube and by st 20 many have reached the plexus region at the base of the limb (Tosney and Landmesser, 1985a). Thus, in the experiments described above, motoneurons that had reached the plexus region by the time of neural tube removal, may already have either interacted with the first sensory neurons to grow out or they may have modified the local environment. A second set of surgeries (N = 52) was therefore performed at st $16\frac{1}{2}$–$17\frac{1}{2}$. In addition, these ventral neural tube deletions were always bilateral and were more extensive along the anterior-posterior axis and included the posterior segments (LS 4–8) that give rise to the ischiadic plexus (see Fig. 4a) as well as anterior segments.

Assessment of the Extent of Motoneuron Removal

At the time of sacrifice, the embryo was removed from the egg, placed in oxygenated Tyrodes solution, and decapitated and eviscerated. The neural tube was exposed by removing the vertebral cartilage ventrally. Individual spinal cord segments and/or spinal nerves were then sequentially stimulated with a suction electrode, and movement of limb muscles scored visually (Lance-Jones and Landmesser, 1980a). We found that even small numbers of motoneurons could be detected by this method; usually their presence could later be confirmed histologically.

Embryos were subsequently fixed in either 2% glutaraldehyde or Bouin's and embedded in paraffin. The spinal cords and DRGs were sectioned transversely at 10–20 μm and stained with either cresyl violet or hematoxylin-eosin orange G as previously described (Landmesser, 1978a,b). In a number of cases, prior to fixation, horseradish peroxidase (HRP) was injected into one or more spinal nerves or DRGs. These embryos were later processed for visualization of the reaction product using previously published procedures (Landmesser, 1978a,b). The limbs were similarly processed and were

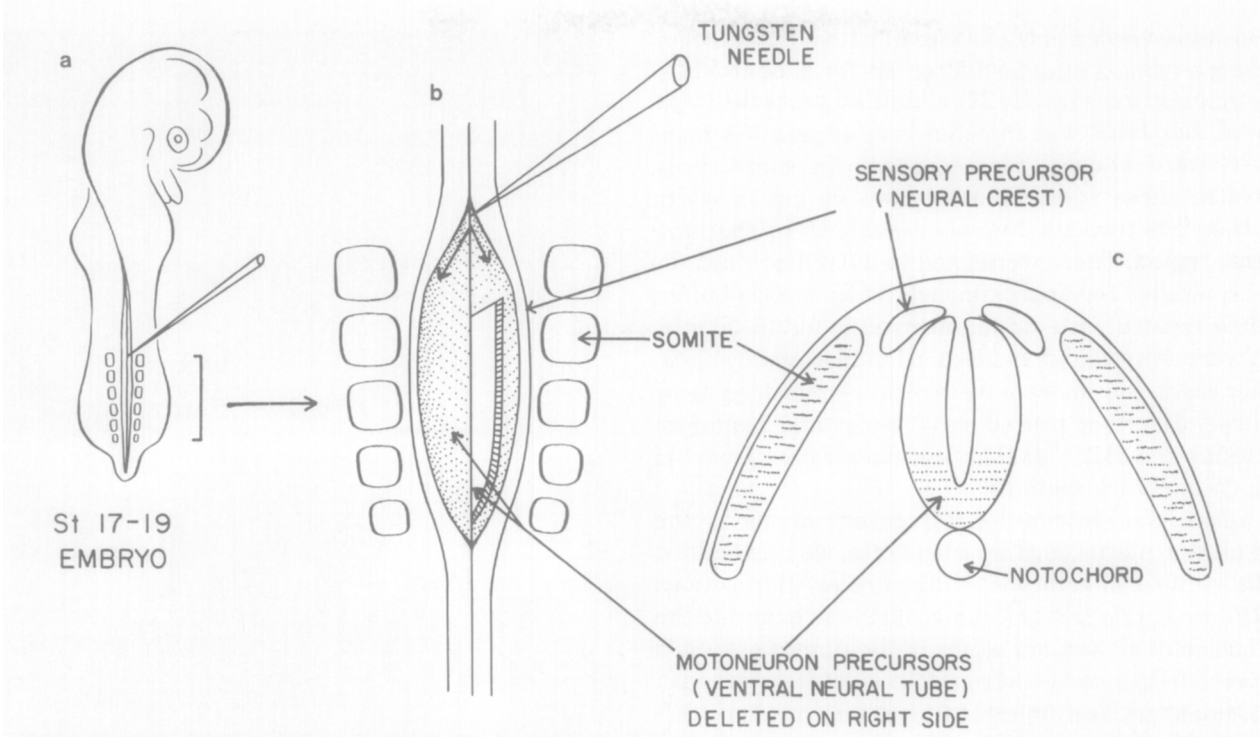

FIG. 1. Schematic of embryonic surgery. (a) The neural tube was opened by making an incision along the dorsal midline. (b) The ventral neural tube from which the motoneurons arise was removed. Sensory neurons arise from the neural crest, but to avoid damaging them the dorsal neural tube was also left undisturbed. (c) A cross section through the embryo at this stage to illustrate the spatial separation of motoneuron and sensory neuron components.

also sectioned transversely. In some embryos, HRP was injected into muscles which appeared to be lacking innervation, and the location of any retrogradely labeled motoneurons or sensory neurons was determined from serial 10-μm sections of the spinal cord and DRGs. In all embryos it was possible to confirm the extent of neural tube that did not contain any motoneurons, and also to note regions where the lateral motor column was depleted but where some motoneurons remained. Motoneurons could be easily recognized by their larger size and characteristic shape and staining properties (see Figs. 2 and 3).

Determination of Nerve Pathways and Projection Patterns in Motoneuron-Depleted Embryos

Only embryos with extensive motoneuron depletion in the desired segments and in which visual inspection of the dissected embryo and/or tissue sections indicated that the DRGs were not markedly depleted were used for further study; this amounted to 48 embryos. Nerve patterns within the limb were reconstructed from camera lucida drawings of serial transverse limb sections 10-20 μm in thickness (for further details, see Lance-Jones and Landmesser, 1980a, 1981a). Cross-sectional areas of individual peripheral nerves were determined. If the plane of section through a particular nerve was oblique, we did not measure the area of that nerve. In addition, each nerve was measured at a characteristic level shortly after it had branched off the main nerve trunk and before it had given off any collateral branches. The volume of DRGs was also determined in some cases by drawing the outline of individual ganglia from alternate serial sections with the aid of a camera lucida, measuring the cross-sectional area of the outline with the aid of a digitizing tablet, summing the individual areas, and then multiplying by two and the section thickness.

Examination of the Outgrowth and Distribution of Motoneuron and Sensory Neuron Axons in Normal Embryos

Several procedures were used to compare the relative timing of initial sensory outgrowth with motoneuron outgrowth into the limb. In some embryos (st 18-24), the ventral surface of the spinal cord was exposed and a fluorescent marker (either rhodamine isothiocyanate (Thanos and Bonhoeffer, 1983) or a carbocyanine dye, diI-C_{18}-(3) (Honig and Hume, 1985, 1986) was injected into several lumbosacral segments of the spinal cord on one side, and into the corresponding DRGs on the contralateral side. After allowing 3-5 hr in oxygenated Tyrodes at 30°C for orthograde transport of the dye, the

embryos were viewed in whole mount with a fluorescence microscope using a standard filter set for rhodamine.

In other embryos (st 19–24) a similar procedure was followed, but HRP was injected instead and 3–5 hr at 32–34°C were allowed for transport. In some cases, transverse slices approximately 300–500 μm in width were then cut through the whole embryo in the lumbosacral region. These were fixed in 2% glutaraldehyde for 1 hr, washed for approximately 12 hr in Tris buffer, and then reacted with diaminobenzidine for 15–30 min. Slices were dehydrated and cleared in methylsalycilate. In other cases, the embryos were plastic-embedded using previously described procedures (Tosney and Landmesser, 1985a) and HRP-labeled processes were viewed in serial, 25-μm-thick sections.

To assess the distribution of sensory axons in the spinal nerves, plexus, and muscle nerves, we reexamined paraffin sections of embryos that had received injections of HRP into single DRGs (Honig, 1982). To examine the distribution of all sensory axons in the limb, we stained transverse frozen sections from four st 32 embryos with a monoclonal antibody specific for sensory neurons (SC-2, Tanaka and Obata, 1984). Alternate sections were stained with an antibody to a cytoskeletal component (H. Tanaka, unpublished observations) which labeled both sensory neuron and motor axons. Sections were then treated with a secondary antibody conjugated to fluorescein and viewed with the appropriate optics.

RESULTS

Morphology of the Spinal Cord following Motoneuron Removal

The morphology of the spinal cord and DRGs following motoneuron removal is shown in Figs. 2 and 3d–k, where it can be compared to that of a normal embryo, (Figs. 3a–c). Rostral and caudal to the operated region (not shown), the morphology of the spinal cord was normal. In the operated region, the spinal cord was distorted in shape, the two sides were often not fused dorsally (Figs. 2a and 3g), and usually the gray matter was reduced in size (Fig. 3g). At the boundary of the deletion (Fig. 3g) and on the unoperated side of embryos with unilateral deletions (Figs. 2a, b) motoneurons were present, as shown on the left side in Figs. 2a, b, where they were labeled by injection of HRP into a spinal nerve. Within the deleted region, motoneurons appeared to be lacking from some segments, and in confirmation of this HRP injection into spinal nerves did not result in any labeling in the cord (Fig. 2c). In other segments, motoneurons, distinguished by their large size and characteristic staining properties (Figs. 3g, h), were present but were greatly depleted in number (compare, for example, Figs.

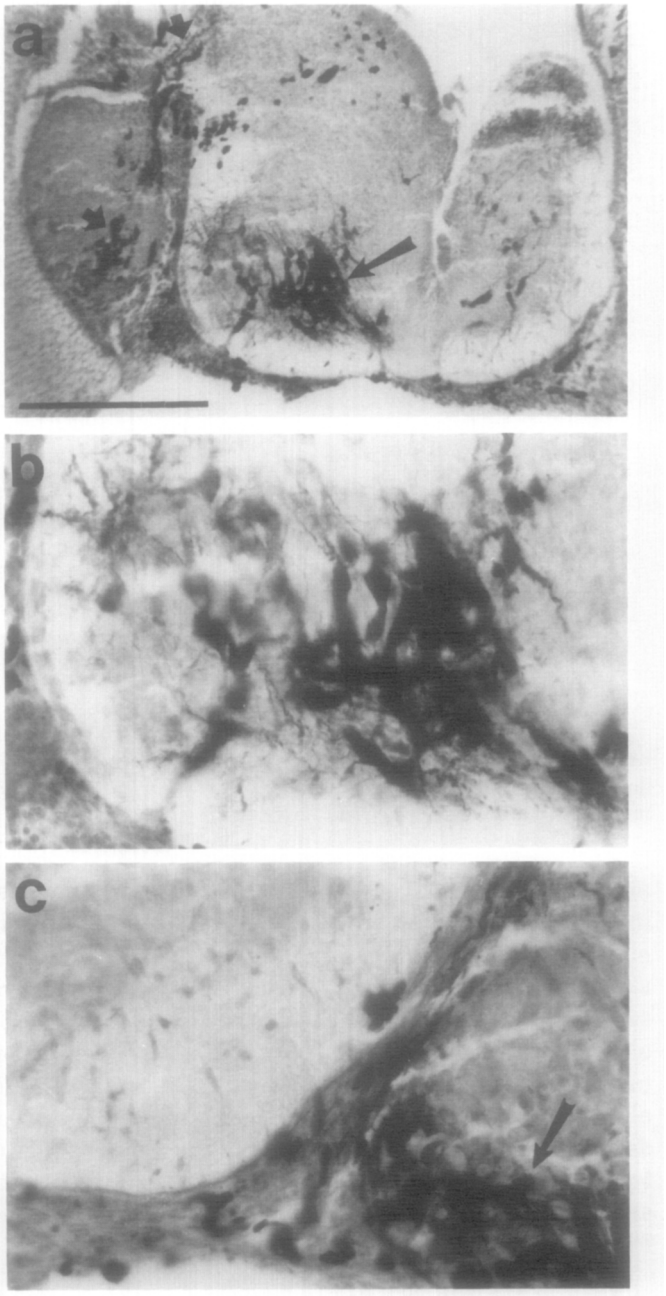

FIG. 2. Appearance of the spinal cord at st 30 following unilateral motoneuron deletion in crural segments. (a) At the level of LS1, the beginning of the deletion, large numbers of motoneurons are present on the left, unoperated side (large arrow) and are labeled by injection of HRP into spinal nerves. Some DRG neurons are also labeled and project into the dorsal cord (small arrows). On the right, operated side, the lateral motor column is nearly absent although two labeled motoneurons are present. Other dark-staining profiles in the spinal cord are red blood cells which exhibit endogenous peroxidase activity. (b) Higher magnification views of lateral motor column shown in (a), with many darkly stained motoneurons. (c) Right side of the same embryo at LS2. Although HRP injection into the spinal nerve labeled many DRG cells (arrow), no labeled cells were seen in the spinal cord, throughout LS2. Calibration bar = 200 μm for (a) and 70 μm for (b, c).

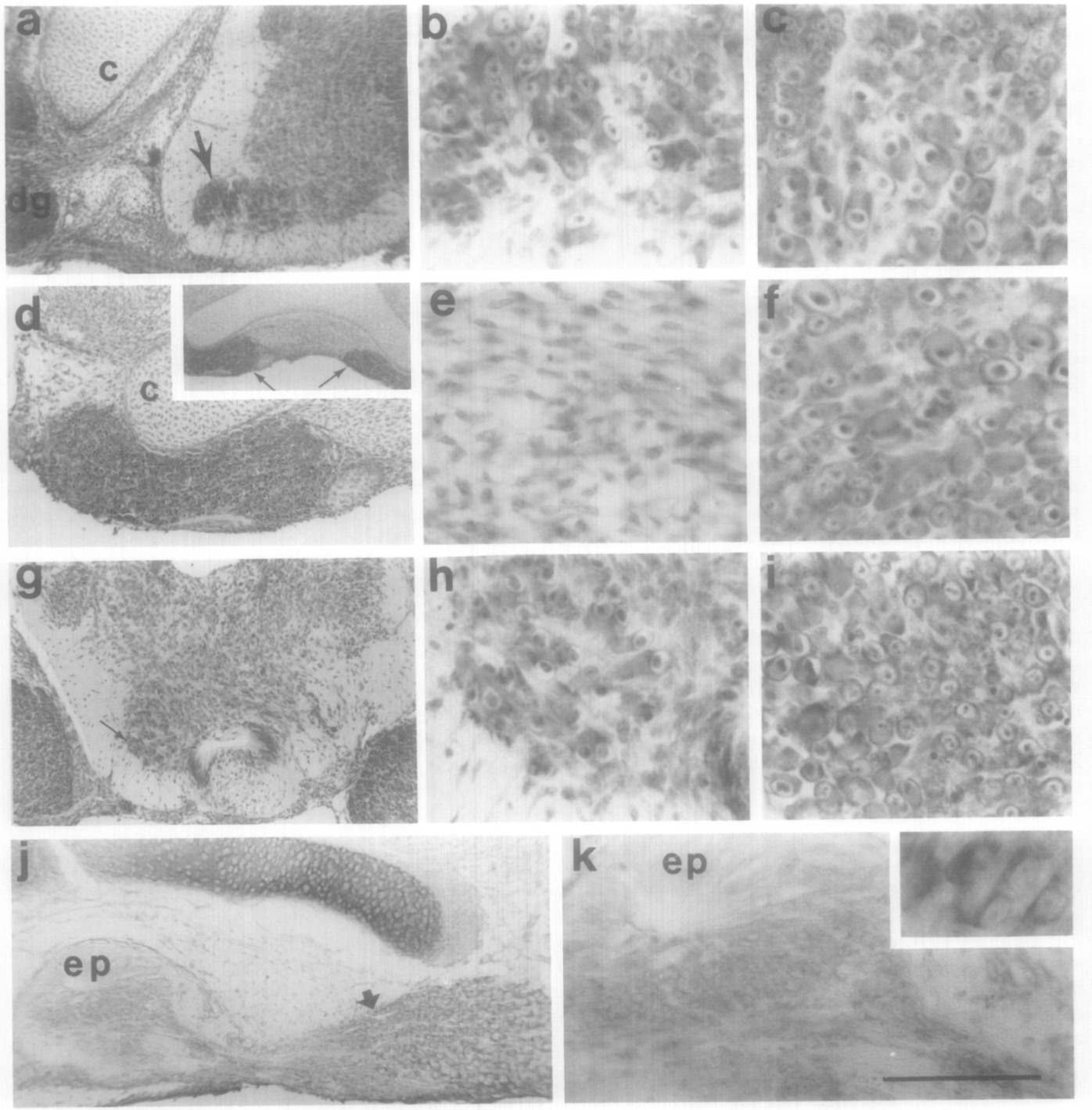

FIG. 3. Appearance of the spinal cord following more extensive removal of motoneurons. (a) Cross section of control spinal cord at LS4 at st 36, showing lateral motor column (arrow) and DRG (dg). (b and c) Show higher magnification views of the motoneurons and sensory neurons, respectively. (d) A comparable level from a motoneuron-deleted embryo showing an elongated DRG adjacent to dorsal vertebral cartilage (c). Inset shows the whole vertebral canal at this level, indicating the presence of DRGs bilaterally (arrows) but the complete absence of the spinal cord. This is confirmed in (e), which is a higher magnification view of the area between the ganglia, showing only connective tissue. (f) The sensory neurons were normal in size and appearance (compare with control in (c). (g) Caudal end of deletion from the same embryo, where motoneurons (arrow) first appear on the left side and are easily recognizable by their size and staining properties. (h and i) Show these motoneurons and sensory neurons in the adjacent DRG, respectively, at higher magnification. (j) Similar embryo at st 30, where a normal-sized but elongated DRG (arrow) projects into the remaining spinal cord consisting of only a small piece of ependyma (ep) and adjacent white matter. The remaining spinal cord, shown at higher magnification in (k), contains no motoneurons. The appearance and size of motoneurons at this stage is shown in the inset which is from the thoracic level anterior to the deletion. Calibration bar = 200 μm for (a, d, g); 160 μm for (j); 50 μm for (b, c, e, f, h, i); and 35 μm for (k).

3a, b with g, h). Following the more complete removals, no motoneurons remained and only white matter or connective tissue was present in the space within the vertebral cartilage (Figs. 3d, e, j, k). We found that it was difficult to remove all the motoneurons, presumably because small pieces of germinal epithelium could generate a substantial number of motoneurons and were inadvertantly left at the time of the operation. However, in the embryos used for further study, motoneurons were absent from some segments and severely depleted in the remaining segments.

Some DRGs appeared normal in size and probably contained their normal complement of neurons since we did not observe any obvious reduction in the size of individual cells (compare, for example, the control in Fig. 3c with f, i). Other DRGs were clearly reduced in size and contained fewer neurons than those in normal embryos, presumably due to damage to the neural crest during the operation. Although we did not count the number of neurons in these ganglia, for a small number of embryos we calculated the volumes of the three crurally projecting ganglia (LS 1, 2, and 3) and compared them with controls. In four control limbs the combined volume of these ganglia was 3.5 ± 0.79 (mean ± SD) $\times 10^7$ μm^3. Of the three experimental limbs analyzed, one did not differ from the control value and the other two were 66 and 70% of that value. Based on this data and our estimates of ganglion size from visual inspection of the histological sections, the embryos for which data is presented in Fig. 5 had DRGs ranging from approximately 60–100% of their normal size. Furthermore, animals with ganglia reduced by more than 50% were excluded from further analysis at the time of dissection.

The differentiation of these sensory neurons also appeared to progress normally. HRP labeling indicated that at least some sensory neurons, even those in DRGs that were situated more ventrally than normal (as in Fig. 3d and j) still sent processes into the spinal cord as well as into the limb. In addition, HRP injections into spinal nerves showed that neurons in the spinal cord (other than motoneurons) did not appear to send processes into the limb (Fig. 2).

Sensory Projections following Removal of Motoneurons in Anterior Segments at St 18½–20½

Nerve patterns were first studied in a series of embryos at st 30–31½ because individual peripheral nerves, which initially form at st 26–27 (Lance-Jones and Landmesser, 1981a; Honig, 1982; Tosney and Landmesser, 1985a), are readily discernible at this time even without HRP labeling. In addition, although st 30 is after the onset of normal sensory cell death (Hamburger *et al.*, 1981), it is prior to the bulk of cell death, which continues until st 37.

The peripheral nerve patterns in three motoneuron-depleted embryos are shown in Fig. 4, where they can be compared with the normal pattern. These illustrate the variation from embryo to embryo, but also several common findings. The ischiadic plexus and the related peripheral nerves were normal, since the segments giving rise to these were not affected by the operation. In contrast, the posteriorly directed ramus, which contains the contribution of LS3 to the ischiadic plexus, was usually missing and there were dramatic changes in the formation of peripheral nerves which arose from the anterior motoneuron-depleted segments. In all limbs, one or more muscle nerves were absent, and almost all of the remainder were reduced in size, some quite markedly. These observations are summarized and presented quantitatively in Fig. 5.

Since motoneurons were substantially depleted in all experimental embryos, some reduction in muscle nerve diameter would have been expected. However, the absence of some muscle nerves, and the substantial reduction in the diameters of many others cannot be accounted for only by the removal of motoneuron axons, assuming that the cross-sectional areas of these nerve branches are roughly proportional to the number of axons in them. This assumption appears reasonable, since, at these early stages, axons in the limb are still fairly uniform in diameter (based on conduction velocity data—Landmesser, 1978b; Honig, 1982; and unpublished observations—see also Saxod and Verna, 1978).

Three lines of evidence show that sensory neuron axons make a major contribution to muscle nerves in the chick. First, retrograde HRP labeling indicates that roughly equal numbers of sensory neurons and motoneurons project along the sartorius muscle nerve at stages 30–32 (Honig, 1980, 1982, and unpublished observations). Second, following HRP injections into DRGs, the level of labeling in muscle nerves frequently is similar to the level seen in purely sensory cutaneous nerves. For example, injections of DRG1 labeled an average of two-thirds as many axons in the sartorius nerve as in the lateral femoral cutaneous nerve in the seven st 28–31½ embryos examined (compare also Figs. 13d–f with c). Third, labeling with a monoclonal antibody specific for DRG neurons (Tanaka and Obata, 1984) shows a major contribution of sensory axons to the crural nerve in the thigh distal to the divergence of all cutaneous nerves and at a point where only axons that will project to muscles remain (Fig. 13h). Therefore, the reduction in cross-sectional area of many muscle nerves in the operated embryos to 10% or less of the control values (Fig. 5) must result from a decrease in their sensory component as well as from the absence of motoneurons.

Further evidence that the sensory innervation of muscles was reduced comes from nine operated embryos

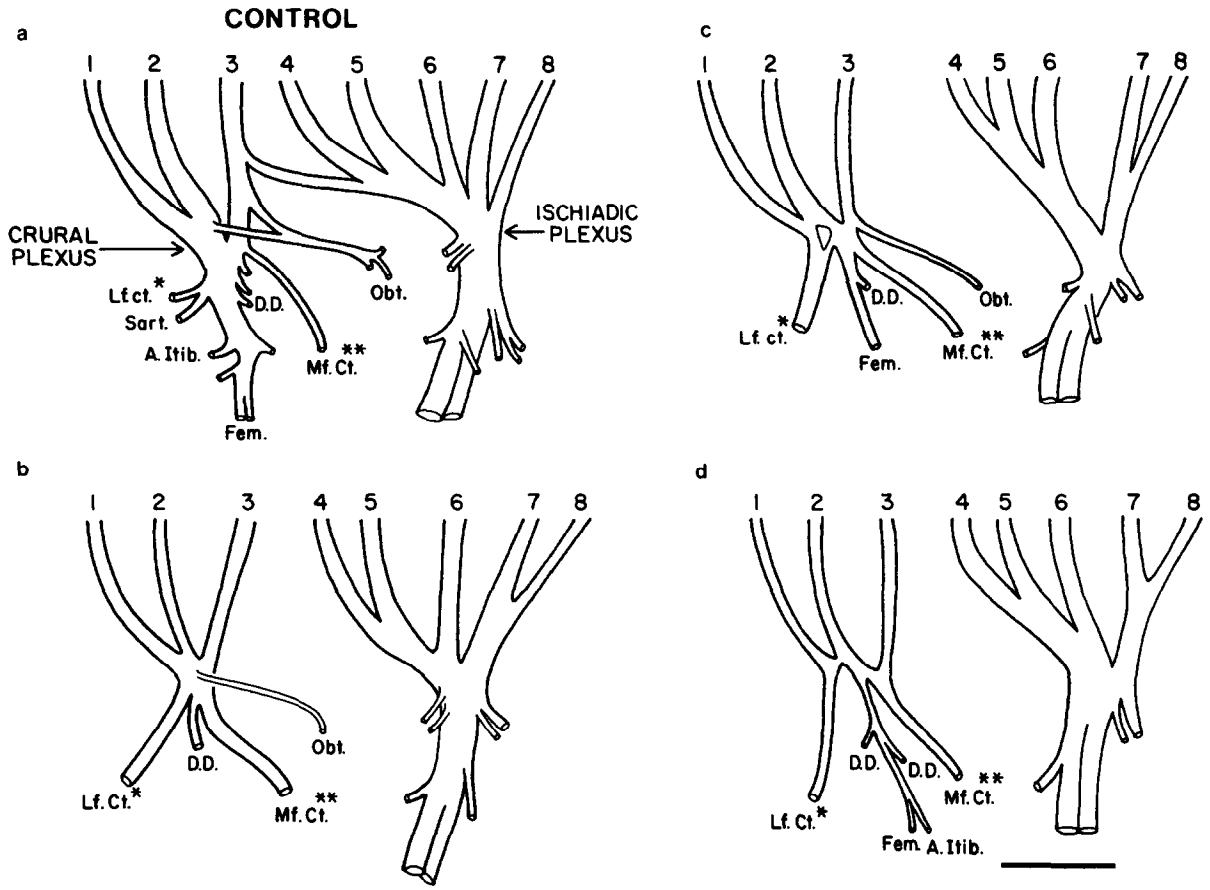

FIG. 4. Peripheral nerve patterns at st 30 following the deletion of motoneurons in LS1–3. Reconstructions were made from camera lucida drawings of individual serial transverse sections. The control nerve pattern in (a) can be compared with three experimental embryos in (b–d). The numbers 1–8 denote the lumbosacral spinal nerves. See text for further descriptions. Nerve abbreviations: Lf. Ct., lateral femoral cutaneous; Sart., sartorius; A. Itib., anterior iliotibialis; Fem., femorotibialis; D.D., deep dorsals; Mf. Ct., medial femoral cutaneous; Obt., obturator. Asterisks denote pure sensory nerves. Calibration bar = 200 μm.

in which we observed the absence of orthogradely labeled axons in some muscles following HRP injections into spinal nerves. For example, the lateral femoral cutaneous nerve in Fig. 4c contained numerous HRP-labeled profiles, yet no labeled profiles projected into the immediately adjacent sartorius. In addition, HRP injection into six muscles, which by electrophysiological criteria appeared to be uninnervated, resulted in the absence of retrogradely labeled sensory neurons in four cases and in only a few labeled cells in the remaining two. In these two cases, a few motoneurons were also labeled. It seemed likely that incomplete motoneuron removal accounted for many of the muscle nerves that still formed in our experimental embryos.

Although sensory innervation of muscles was reduced or absent, the sensory ganglia were, nevertheless, often of approximately normal size as discussed above. Even in cases where DRGs were reduced in size, it seems unlikely that our operative procedure selectively depleted muscle afferents since no clear differential loss of "muscle" afferents compared to "cutaneous" afferents was found following partial neural crest deletions performed at similar stages (Honig, 1980). In addition, many DRGs in the cord reversal experiments reported on in the preceding paper (Honig *et al.*, 1986) were depleted to a greater extent than in these motoneuron-depleted embryos, yet in that case we never observed failure of neurons to project to muscle. Finally, Swanson and Lewis (1986), who recently carried out experiments on chick wings similar to those described here, also observed depletion of DRGs but found no correlation between the amount of DRG depletion and the number of muscle nerves which formed. Therefore, taken together, these results suggest that it is the absence of motoneurons which results in the failure of sensory neurons to project to muscles.

In contrast to the reduction in muscle nerves, the cutaneous nerves (indicated by asterisks in Fig. 4) were usually of normal size or in some cases actually enlarged. In 8 of 20 of the limbs examined (Fig. 5), the cross-sec-

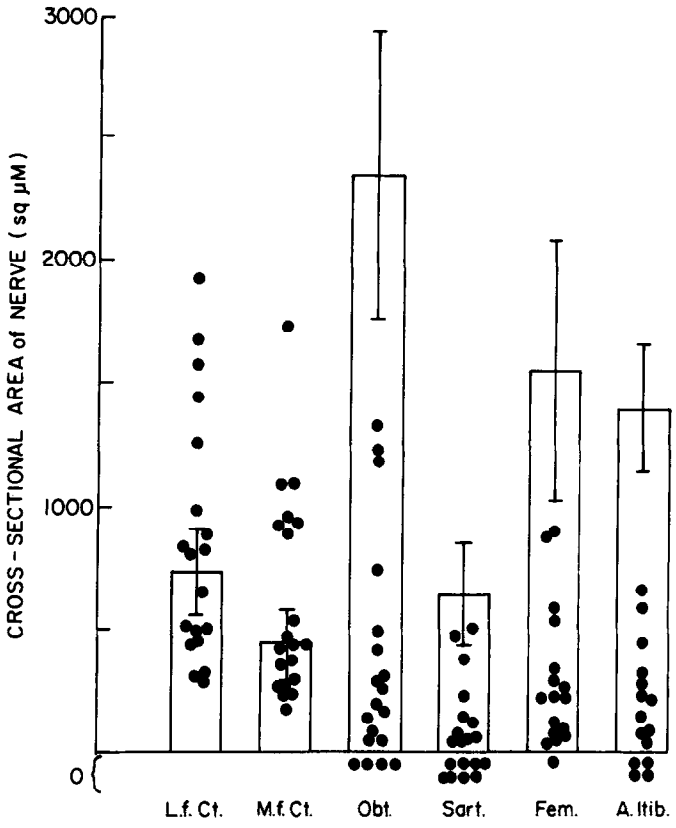

FIG. 5. Bar graph showing cross-sectional areas of cutaneous and muscle nerves at st 30. The means ± standard deviation for 13 control embryos are indicated by the columns and the error bars. Individual values from experimental embryos are plotted as filled circles. Muscle nerves are reduced or absent, while cutaneous nerves (Lf. Ct. and Mf. Ct.) are often enlarged. Same abbreviations as in Fig. 4.

tional area of the lateral and/or medial femoral cutaneous nerves was increased substantially over control values. Further, the conduction velocities of these nerves (assessed by whole nerve stimulation and recording with suction electrodes—Landmesser, unpublished observations) did not obviously differ from those in normal embryos, suggesting that there was not a significant change in axonal diameters. Therefore, the increase in the diameters of the cutaneous nerves probably results from an increase in the number of axons they contain. This change, taken together with the accompanying decrease in muscle nerve diameters suggests that, in the absence of motoneurons, at least some sensory neurons which normally project out muscle nerves instead project along cutaneous nerves.

Sensory Projections following More Extensive Motoneuron Deletions at St 16½–17½

In another series of embryos, the ventral neural tube was removed at st 16½–17½, prior to the outgrowth of motoneurons (Hollyday, 1983; Tosney and Landmesser, 1985a) and even before most motoneurons are born (Hollyday and Hamburger, 1977). These deletions extended through most of the lumbosacral cord although motoneurons were usually present in the most posterior segments, LS7 and 8. It was difficult to completely remove motoneurons without extensively depleting DRGs in embryos operated on at this stage. However, for four limbs examined at st 30 and eight limbs examined at st 35–36, this goal was met (see for example the spinal cords pictured in Figs. 3d, j). As in the case of the less extensive motoneuron deletions just discussed, the cutaneous nerves formed normally and tended to be either of normal or larger than normal size (Figs. 6; 7; 8b, c; Table 1). While we rarely saw (2 out of 32 cases) an enlargement of these nerves in the limbs examined at st 35–36, most lumbosacral DRGs appeared to be somewhat depleted, possibly accounting for this finding. In contrast, in all experimental embryos, many of the muscle nerves were absent, regardless of the stage at which the embryos were examined (Table 1). In some embryos, the muscle nerves that formed seemed to be correlated with a small number of motoneurons remaining at the appropriate segmental level for those motoneuron pools (e.g., Fig. 6). In other embryos, although motoneurons were not detected in the cross-sections of the lumbosacral cord (e.g., Fig. 3d), small nerves were found projecting to the femorotibialis, the ischioflexorius, and the deep dorsal muscles (e.g., Fig. 7). These three nerves formed fairly consistently in the apparent absence of motoneurons. However, since motoneuron cell death begins at st 29–30 (Hamburger, 1975), we cannot exclude the possibility that a few motoneurons had originally projected to these muscles and had subsequently died.

A second possibility is that, although the presence of motoneurons may normally facilitate the entry of sensory neurons into muscles, alternative structures within the limb, for example blood vessels, may also serve this role. An analysis of motoneuron growth cones at the time axons invade the limb bud has indicated that motoneurons do not slavishly follow blood vessels throughout their course (Tosney and Landmesser, 1985a). Confirming this, we noticed in both normal and experimental limbs that many nerves took characteristic turns and diverged from common nerve trunks some distance before becoming associated with blood vessels. Nevertheless, several muscle and cutaneous nerves had a tendency to be closely associated with blood vessels during the final portion of their trajectory to muscle or skin; these were the lateral femoral and medial femoral cutaneous nerves, one branch of the femorotibialis, and the ischioflexorius (see Figs. 6, 7). This association was even more obvious in some of the motoneuron-depleted embryos where most of the muscle nerves that formed did not penetrate deeply into the muscle as in controls (Fig. 8e),

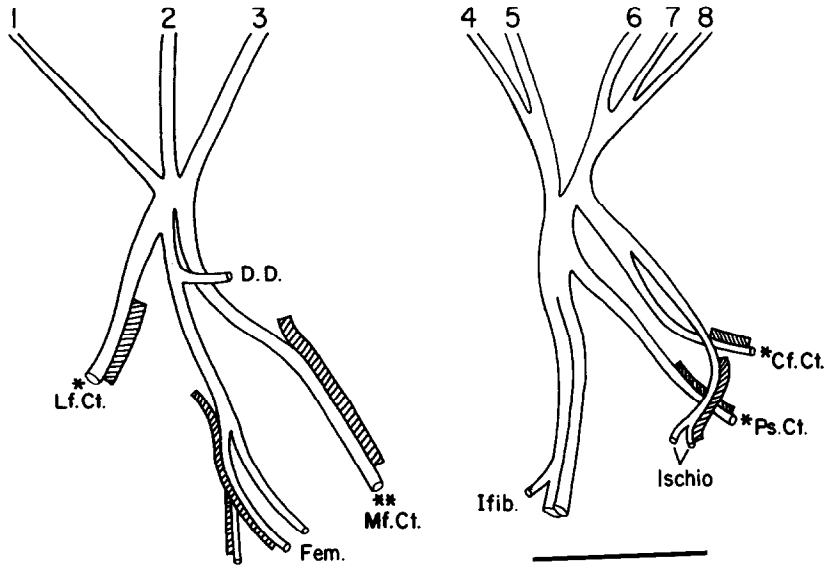

FIG. 6. Nerve pattern in a st 30 embryo after extensive motoneuron deletion from LS1–7 at st 17. In this embryo only a few motoneurons remained and these were located in LS 3–4. This reconstruction was made as described in the legend to Fig. 4. All cutaneous nerves (asterisks) are present and the Mf. Ct. and Lf. Ct. are enlarged. Most muscle nerves are lacking (sartorius, anterior iliotibialis, obturator, posterior iliotibialis, caudilioflexorius) or reduced in size (ischioflexorius, iliofibularis). Some nerves are closely associated with blood vessels (indicated by hatched profiles) during the final portion of their trajectory. Nerve abbreviations as in Fig. 4 and: Ifib., iliofibularis; Ischio., ischioflexorius; Cf. Ct., caudal femoral cutaneous; Ps. Ct., plantar sural cutaneous. Calibration bar = 200 μm.

but instead associated with blood vessels and ran along the muscle surface for some distance (Fig. 8d) before diminishing gradually to a point where they could no longer be seen.

The only nerve branch that consistently formed in the apparent absence of motoneurons, and which was not associated with a blood vessel, was the nerve to the deep dorsal muscles (Figs. 4, 6, and 7). It is possible that some alternative guidance feature in this proximal limb region enhanced the growth of sensory axons into this muscle.

Finally, an additional peculiar feature of these older embryos was the extensive clustering of neuronal cell bodies at aberrant positions within the spinal nerves, plexus, and major nerve trunks (Fig. 7b). These neurons were similar in size and appearance to typical DRG cells (see Figs. 9b–d) and were presumably DRG cells that had migrated out along the nerves. In some cases this distance was quite considerable; a few cell clusters were even found in the sciatic nerve just proximal to the knee. While we have not investigated this phenomenon in detail, it did not occur in one st 35½ embryo in which many motoneurons remained, and therefore may be associated with extreme depletion of the motoneurons, and subsequent failure of most muscle nerves to form. It also was not observed in any of the st 30 embryos with extensive motoneuron deletions, and therefore appears to be a secondary migration occurring between st 30–35, long after DRG neurons form ganglia and send out axons. It is interesting to note that a similar secondary migration of ciliary ganglion cells has been described following ablation of their peripheral targets (Landmesser and Pilar, 1974).

Nerve Patterns at St 26–28

It is possible that some sensory neurons might, prior to st 30, send processes along muscle nerve pathways in these experimental embryos. If this were true, either retraction of axons or neuronal degeneration rather than an inability of sensory neurons to project along motor pathways could explain our results. We therefore examined nine st 26–28 limbs from embryos operated on between st 16½ and 19. In all embryos, some muscle nerves were absent. The proportion of muscle nerves present was not different from that in the embryos analyzed at st 30. Examination of the spinal cords indicated that motoneuron removal was never complete; clusters of motoneurons remained in some segments and probably were responsible for the muscle nerves that formed.

In three additional embryos examined at these stages a different result was obtained. In these, spinal nerves from the motoneuron-depleted segments grew toward but did not actually enter the limb. We occasionally observed similar behavior from individual spinal nerves in embryos examined at other stages as well. It is therefore not clear whether the nerves' failure to grow into the limb is a consequence of complete motoneuron removal or to the alteration of some other required limb component as a result of the surgery.

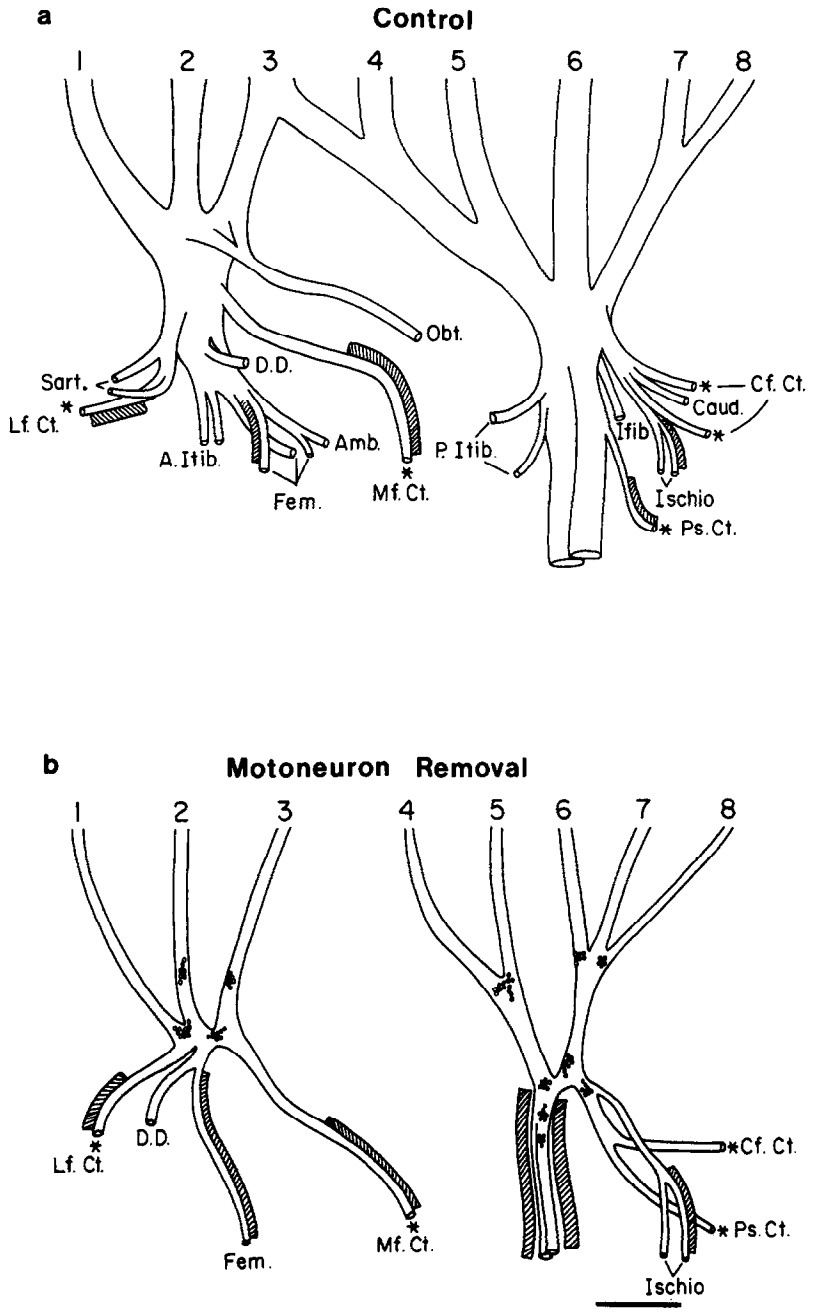

FIG. 7. Peripheral nerve pattern in control and in a motoneuron-deleted embryo at st 35½. These reconstructions were made as described in the legend to Fig. 4. (a) The control embryo has a full complement of muscle nerves and cutaneous nerves (asterisks). (b) In a st 35½ motoneuron-deleted embryo, most muscle nerves are lacking. Exceptions are the ischioflexorius, femorotibialis, and deep dorsal muscles. All cutaneous nerves are present, but none are obviously enlarged. Some nerves are closely associated with blood vessels (indicated by hatched profiles) during the final portion of their trajectory. In addition, the peroneal and tibial nerve branches of the sciatic nerve are considerably separated from one another, each running in close apposition to the femoral artery or vein. Clusters of neuronal cell bodies within nerves are indicated by small circles. Nerve abbreviations as in Figs. 4 and 6 and: Amb., ambiens; P. Itib., posterior iliotibialis; Caud., caudilioflexorius. Calibration bar = 500 μm.

Alterations in Nerve Pathways in the Plexuses

We also observed less extensive joining together of spinal nerves in the plexus region of motoneuron-depleted embryos. Normally, the individual spinal nerves converge in the plexus region and run together for several hundred microns or more with a common epineurium (see Figs. 10d–f). HRP labeling has shown that the plexus is a site for extensive crossing of both sensory and motoneuron axons (i.e., a site where axons which

TABLE 1

Nerve		Absent	Reduced	Normal	Enlarged
Muscle nerves					
D.D.	St 30	0/4	0/4	4/4	0/4
	St 36	0/8	3/8	5/8	0/8
A. Itib.	St 30	3/4	1/4	0/4	0/4
	St 36	5/8	3/8	0/8	0/8
Sart.	St 30	3/4	1/4	0/4	0/4
	St 36	5/8	3/8	0/8	0/8
Fem.	St 30	1/4	3/4	0/4	0/4
	St 36	1/8	7/8	0/8	0/8
Ifib.	St 30	3/4	1/4	0/4	0/4
	St 36	5/8	3/8	0/8	0/8
P. Itib.	St 30	4/4	0/4	0/4	0/4
	St 36	7/8	1/8	0/8	0/8
Add.	St 30	3/4	1/4	0/4	0/4
	St 36	6/8	2/8	0/8	0/8
Ischio.	St 30	1/4	2/4	1/4	0/4
	St 36	1/8	5/8	2/8	0/8
Caud.	St 30	4/4	0/4	0/4	0/4
	St 36	6/8	2/8	0/8	0/8
Total		58/108	38/108	12/108	0/108
Cutaneous nerves					
Lf. Ct.	St 30	0/4	0/4	1/4	3/4
	St 36	0/8	0/8	7/8	1/8
Mf. Ct.	St 30	0/4	0/4	2/4	2/4
	St 36	0/8	0/8	8/8	0/8
Cf. Ct.	St 30	0/4	0/4	3/4	1/4
	St 36	0/8	0/8	7/8	1/8
Ps. Ct.	St 30	0/4	0/4	2/4	2/4
	St 36	0/8	0/8	8/8	0/8
Total		0/48	0/48	38/48	10/48

Note. To be classified as enlarged, nerves had to differ from controls by more than 25%; to be classified as reduced, they had to be less than 50% of controls. Abbreviations are as defined in the legends for Figs. 4, 6, and 7, and Add., adductor.

will project to common targets, segregate and sort out from axons projecting to different targets—Lance-Jones and Landmesser, 1981a, Honig, 1982). In most of the motoneuron-depleted embryos, the spinal nerves physically converged, but stayed in contact for only a short distance (40–60 μm) before diverging into individual nerves (compare Figs. 4d, 6, and 7b with controls in Figs. 4a and 7a—see also Figs. 10a–c). This apparent failure to exchange fibers was greatest in preparations where motoneuron removal was most complete.

This reduction in fiber exchange between spinal nerves might be expected to result in alterations in the spinal nerve contributions to the cutaneous nerves. We tested for this by recording from the lateral femoral cutaneous and medial femoral cutaneous nerves in seven motoneuron-depleted embryos during sequential stimulation of the relevant spinal nerves. Although we encountered more variation than normal and some cases that were clearly outside the normal range (for example, in one case the lateral femoral cutaneous nerve received all of its innervation from LS1 which is never seen in controls; Honig, 1980, 1982, and unpublished observations), more data are required to substantiate this point.

Growth Cone Morphology

We had previously found that the growth cones of sensory axons were smaller and less elaborate than those of motoneuron axons (Landmesser and Honig, 1982; Tosney and Landmesser, 1985c). This might occur because of interactions between sensory and motoneuron axons (i.e., sensory axons appear to be guided by, and to some extent grow out in an environment dominated by, motor axons; see below). However, in the present experiments where most motoneurons were absent when sensory axons grew into the limb, sensory neuron growth cones were not obviously larger or more complex when compared to controls (Fig. 11).

Additional Observations of Normal Development

To gain insight into the mechanisms by which motoneurons might influence sensory neuron growth, we reexamined several aspects of normal development. Our previous studies using orthograde HRP labeling in different but similarly staged embryos (Lance-Jones and Landmesser, 1981a; Honig, 1982) suggested that the earliest sensory neurons to grow into the limb and along distinct peripheral nerves do so concurrently with motoneurons. Further, it was evident that many DRG neurons must grow out later since they are not even born when the peripheral nerves first form. To examine these time courses in more detail and at earlier stages, we made large HRP injections into many of the lumbosacral DRGs on one side and into many segments of the lateral motor column on the opposite side of the same embryo. Six embryos receiving such injections were examined between stages 19–23½. By st 19–20 numerous motoneuron axons had exited from the spinal cord and reached the plexus area (Fig. 12f) but only an occasional sensory neuron axon had emerged from the dorsal root ganglia and these projected only a short distance (Figs. 12f–h). By st 22–23 some sensory neuron axons had reached the plexus, but again their numbers were considerably fewer than for motoneurons (Figs. 12a–e). Thus, from st 20–23½ during the "waiting period" prior to neurite entry into the limb bud proper (Hollyday, 1983; Tosney and Landmesser, 1985a), the plexus region is dominated by motoneurons. As axons begin to invade the limb bud at st 23½–24, sensory growth cones are present in the plexus

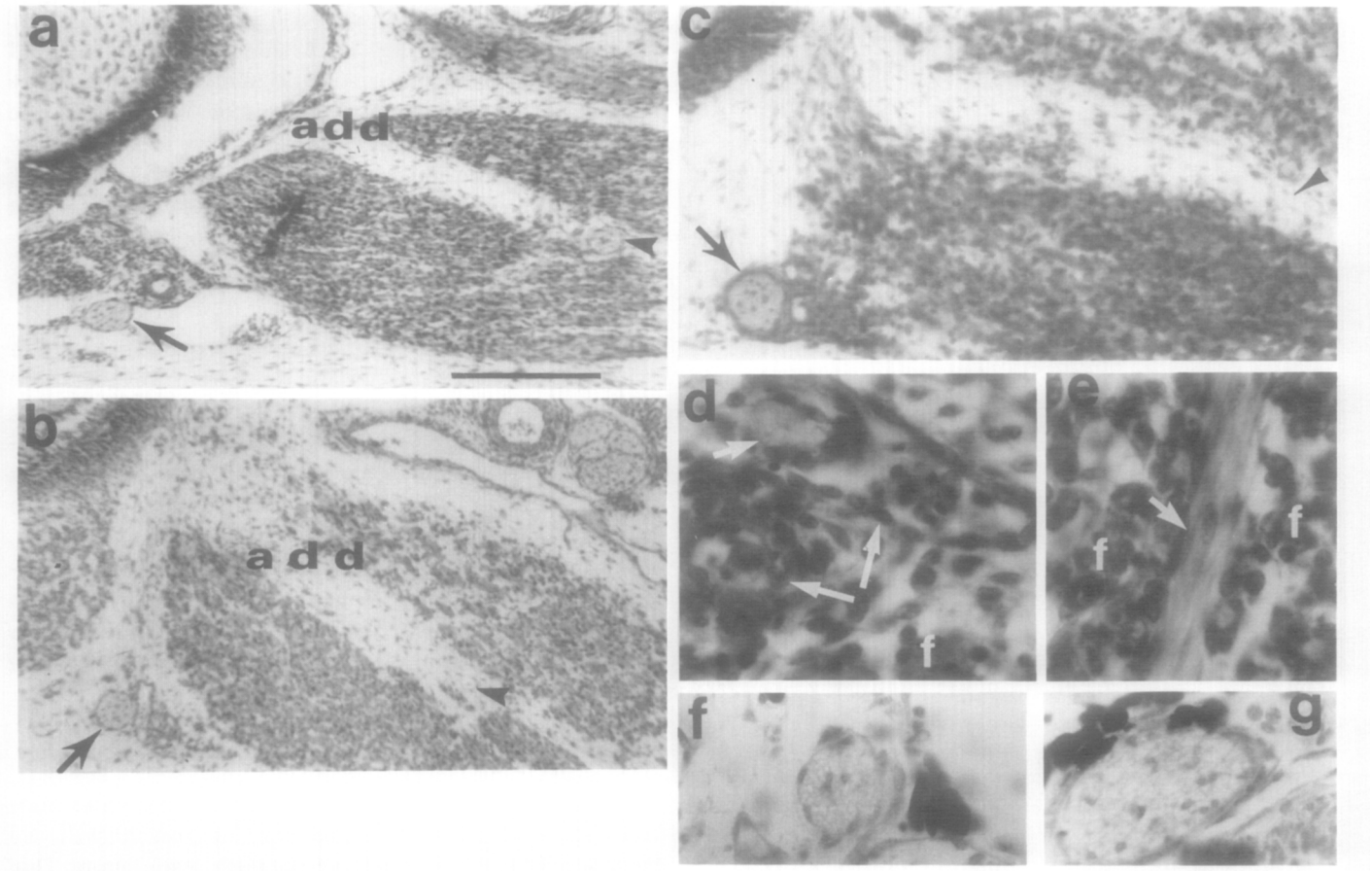

FIG. 8. Nerves within the limbs of motoneuron-deleted embryos. (a) Cross section through the thigh of a control st 35 embryo showing the Mf. Ct. nerve (arrow) adjacent to a blood vessel and the adductor muscle nerve (arrowhead) lying between the two heads of the adductor muscle (add). This nerve normally is easily discernible in this position for a long distance throughout most of the thigh. (b) A section through a similar level of a st 35 motoneuron-deleted embryo, showing a prominent Mf. Ct. nerve (arrow). The nerve in the upper right hand corner of this photomicrograph is the sciatic nerve trunk. The adductor nerve is not present in its usual position (arrowhead). (c) A cross section at higher magnification of a st 30 embryo, showing a prominent Mf. Ct. nerve (arrow) and again the absence of the adductor nerve, even at this early stage (arrowhead denotes its usual position). (d) Many nerves that projected to muscles in motoneuron-deleted embryos did not seem to penetrate deeply into those muscles. Small arrow indicates a small nerve branch which was closely associated with blood vessels (large arrows) and which ran for many millimeters along the surface of this st 36 femorotibialis muscle (f). (e) In contrast, a femorotibialis nerve branch (arrow) in this st 36 control embryo dives deeply into the muscle. This is apparent in all normal embryos, even at earlier stages, as shown by the femorotibialis nerve branch in Fig. 13f. The muscle tissue appears less darkly stained in Fig. 13, because it is stained with cresyl violet in contrast to the hematoxylin-eosin-stained sections shown here (8a–e). (f) A cross section of a control st 30 Mf. Ct. nerve. (g) The Mf. Ct. nerve from a st 30 motoneuron-deleted embryo is greatly enlarged in comparison to the nerve shown in (f). Dark-staining profiles are red blood cells in a blood vessel adjacent to the nerve. Calibration bar = 200 μm for (a, b); 100 μm for (c); 45 μm for (d, e); and 80 μm for (f, g).

region close to the growth front of the nerve. Our general impression from these embryos, as well as from others in which the axons were labeled with fluorescent dyes, is that some sensory growth cones are always present near the growth front, but that many grow out at later stages along nerve pathways where motoneuron axons are already abundant. In contrast, motoneuron axons grow out more synchronously as a relatively broad growth front.

To determine the spatial relationships between sensory neuron and motoneuron axons during outgrowth, we examined the distributions of axons labeled retrogradely or orthogradely with HRP in transverse sections through the limb. Although sensory neuron and motoneuron axons are initially separate in the most proximal parts of the spinal nerves (Fig. 13a), more distally, in the spinal nerves and in the plexus, sensory neuron axons and motoneuron axons each assume a widespread distribution (Figs. 13b–d). In the plexus region, the sensory and motoneuron axons that project to the same muscle sort out together in a single cluster, segregating from axons projecting to other muscles and from the clusters of axons that will project to the skin (Lance-Jones and Landmesser, 1981a; Honig, 1982). The widespread dis-

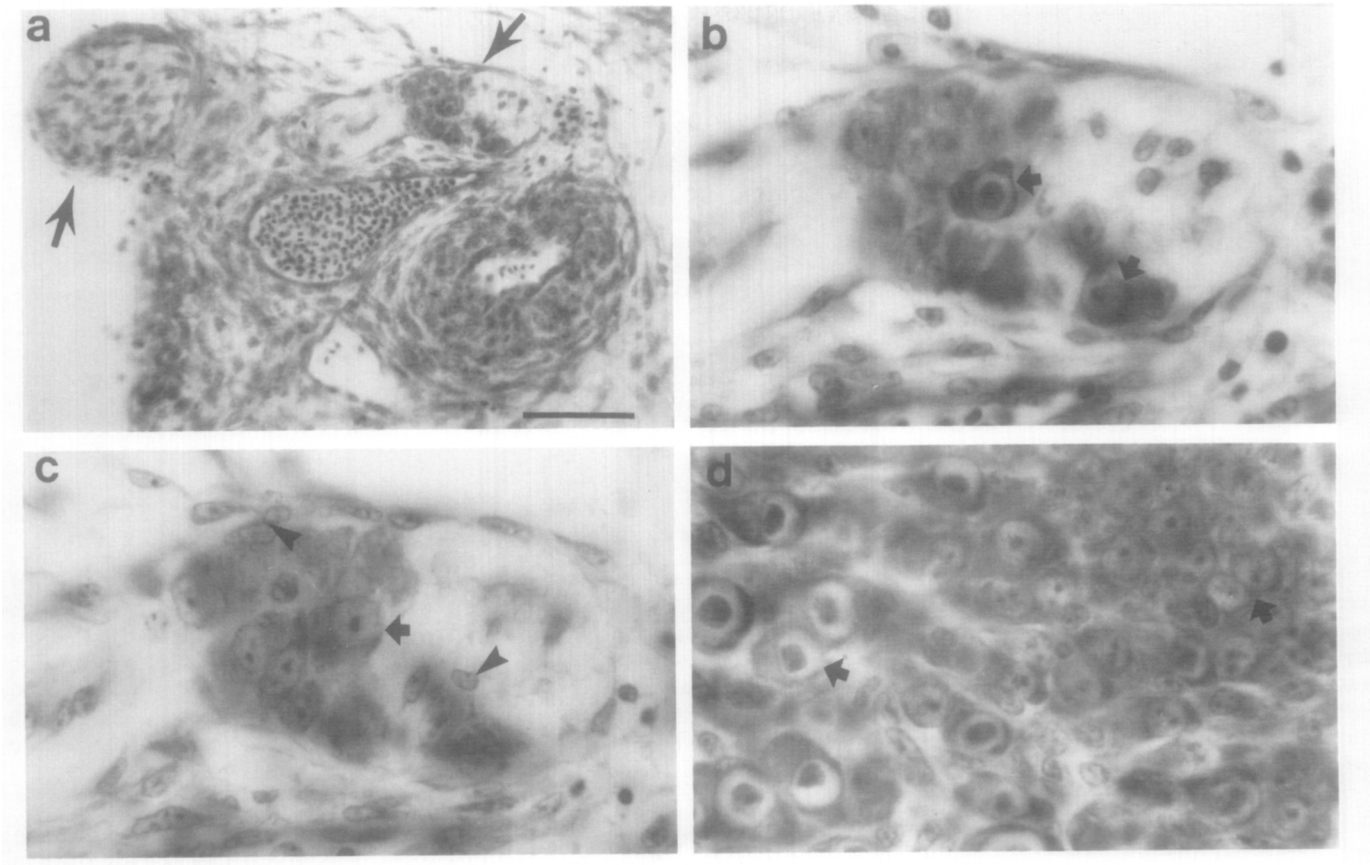

FIG. 9. Neuronal cell bodies within peripheral nerves in st 35–36 motoneuron-deleted embryos. (a) Low-magnification view of spinal nerves (arrows) converging in the ischiadic plexus. The nerve trunk on the right contains a cluster of neuronal cell bodies. (b) Higher magnification view of (a) showing both large (arrows) and small cell bodies. (c) The same nerve trunk several sections more distally contains additional somata (arrow) with large nuclei and relatively dark-staining cytoplasm. Glial and endoneurial cells have much smaller nuclei (arrowheads) and their cytoplasm is not darkly stained. (d) A section through a DRG from the same embryo, showing neuronal cell bodies (arrows) of similar appearance and size. Calibration bar 100 μm for (a) and 20 μm for (b–d).

tribution of sensory neuron and motoneuron axons in the distal spinal nerves and the plexus, and the sorting out of those axons projecting to a single muscle suggest, as we have previously noted (Honig, 1982), that there is a considerable degree of intermingling of sensory neuron and motoneuron axons. However, a closer examination of these embryos and of others that received injections of diI-C_{18}-(3) into the DRGs revealed that there is also some clustering of sensory neuron axons even in the distal spinal nerves and plexus (Figs. 13b–e; see also Honig, 1982).

To better examine the extent of intermingling and of clustering, we determined the distribution of all sensory neuron axons in the nerve trunks. Alternate frozen sections were stained with an antibody directed against a sensory neuron-specific cell surface antigen (Tanaka and Obata, 1984) or with an antibody to a cytoskeletal component which labels all axons. Labeling with the sensory antibody was not uniform in the spinal nerves, plexus, and nerve trunks, and areas of intense fluorescence indicative of some clustering of sensory axons (see Fig. 13g) were apparent at levels proximal to the emergence of cutaneous nerves. Regions of dimmer fluorescence were nevertheless present throughout the spinal nerves, plexus, and muscle nerves (Fig. 13h), indicating, since labeling with the other antibody was fairly uniform and intense, a considerable degree of admixture of sensory and motoneuron axons. However, since we were unable to visualize individual axons, we could not determine how fine grained this admixture was.

DISCUSSION

The experiments described here show that motoneurons have a profound effect on sensory neuron outgrowth. When motoneurons were deleted, muscle nerves either did not form or were greatly reduced in diameter, beyond the extent expected from the removal of motoneurons alone. Further, the small muscle nerves that did occasionally form in the apparent absence of any

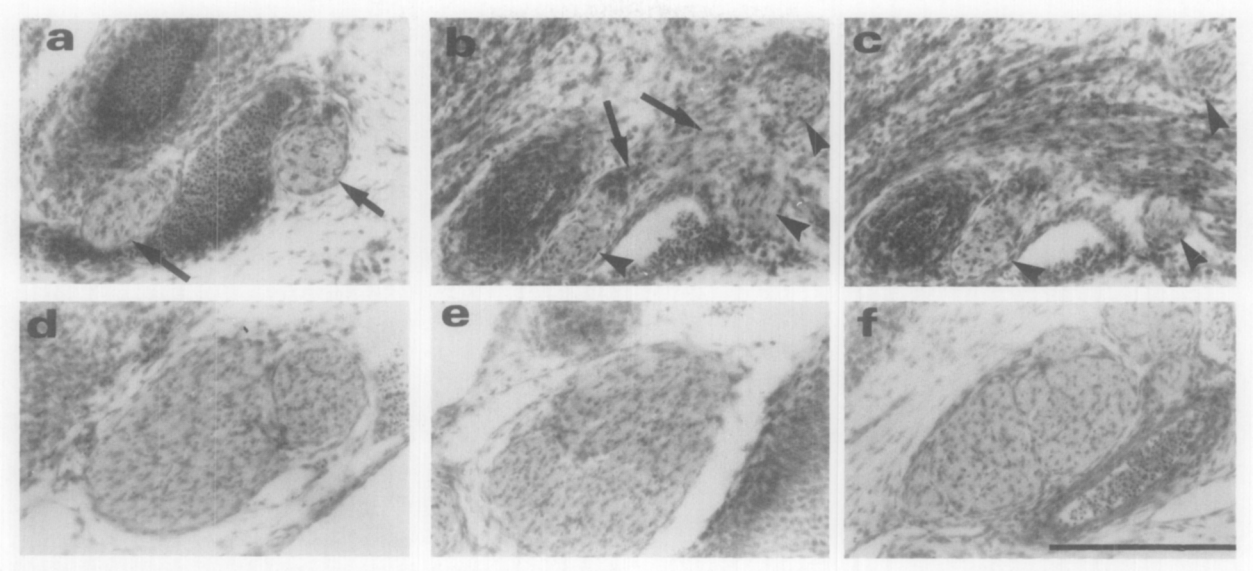

FIG. 10. Convergence of spinal nerves in the ischiadic plexus region of a st 36 motoneuron-deleted (a–c) and control (d–f) embryo. In (a) a trunk consisting of LS spinal nerves 4 and 5 (arrow, left) is separated from the trunk arising from LS spinal nerves 6, 7, and 8 (arrow, right) by a blood vessel. At a comparable level in the control (d) these spinal nerves have already converged. (b) The only exchange of axons (indicated by arrows) between the main sciatic trunks in the deleted embryo occurred within two 15-μm-thick sections and by one section more distal (c), the sciatic had diverged into three separate nerves (indicated by arrowheads in both (b) and (c)). (Note: it is difficult to visualize the exchange of axons in the single section shown in (b), but observations of sequential serial sections allowed us to confirm such exchange.) (e) In contrast, the spinal nerves converged completely in the control and ran together for 500 μm before beginning to diverge into separate nerves as shown in (f). Calibration bar = 100 μm.

motoneurons did not penetrate deeply into the muscles but rather ran along their surface for long distances. In contrast, cutaneous nerves were often considerably increased in diameter, presumably due to the addition of other sensory axons. These results suggest that sensory neurons that normally project along muscle nerves are unable to do so in the absence of motoneurons and may instead project along cutaneous nerves. We also observed less exchange of axons originating from different spinal nerves than occurs normally in the plexus region, suggesting that alterations in the trajectories of sensory neurons may be facilitated by the presence of motoneurons which show similar changes in their pathways. Therefore, motoneurons seem to be required for the growth of sensory neurons into muscle and contribute significantly toward the formation of the normal sensory neuron projection pattern.

Taylor (1944) obtained results in frogs similar to ours (i.e., the formation of cutaneous nerves and the absence of muscle nerves following removal of the larval spinal cord). However, he interpreted this as indicating selective innervation by sensory neurons of their normal targets. Apparently the sensory component to muscle nerves in frogs is normally sufficiently small that the absence of muscle nerves was not judged to represent a significant deviation from the normal sensory projection pattern. More recently, Swanson and Lewis (1986) have obtained similar results on the chick wing, and like us, have interpreted these to indicate a dependence of sensory neurons on motoneurons to execute at least the final part of their trajectory into muscle.

The results of both Taylor (1944) and Swanson and Lewis (1986) differ from ours in their failure to observe any obvious enlargement of cutaneous nerves. In the frog, this might be expected due to the apparently small proportion of sensory fibers that normally innervate muscle. Also, a moderate increase in cutaneous nerve diameter might not have been detectable with the whole mount silver-staining procedure used by Swanson and Lewis (1986). The increase in cutaneous nerve diameter that we observed and which led us to suggest that sensory neurons which would have projected to muscle were now projecting out cutaneous nerves, was not observed in all cases. It is our impression that we failed to obtain enlargement in many cases due to depletion of DRGs as a result of the experimental surgery. In addition, we would expect a greater loss of sensory neurons in experimental embryos during the cell death period due to an absence of central targets (Yip and Johnson, 1984), mismatch in the nature of available peripheral targets, increased peripheral competition, or some combination of these factors. This would explain why enlarged cutaneous nerves were less frequently observed in embryos examined at st 35–36, which is toward the end of the cell death period.

In summary, therefore, while it is clear that sensory neurons are capable of growing into the limb and out cutaneous pathways in the absence of motoneurons, the

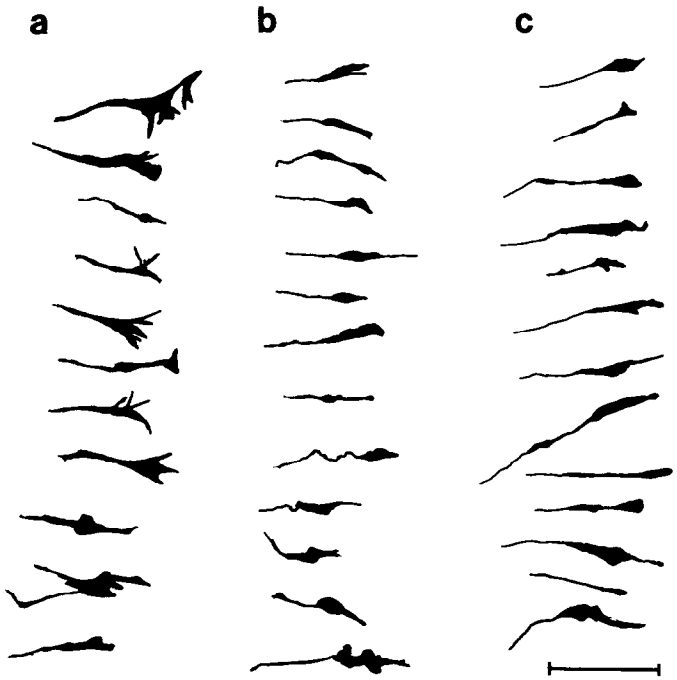

FIG. 11. Growth cone morphology of motor and sensory neurons. Camera lucida tracings of HRP-labeled growth cones of (a) motoneurons from control embryos at st 24-27, and (b) sensory neurons compared with (c) sensory growth cones in motoneuron-depleted embryos at similar stages (st 23-28). All growth cones are from the plexus region except the last growth cone in row (b), which is from a cutaneous nerve. Although one occasionally encounters relatively elaborate sensory growth cones (i.e., the last in row (b), which is from a st $26\frac{1}{2}$ cutaneous nerve), most end in simple bulbs and lack the lamellipodia and filopodia characteristic of motoneuron growth cones; they are also smaller. Sensory neuron growth cones in motoneuron-deleted embryos also tended to be smaller than motoneurons and to end in simple bulbs. Calibration bar = 20 μm.

observations described above, as well as the observed alterations in the degree of spinal nerve convergence in the plexus, lead us to suggest that motoneurons affect sensory neurons to some extent along their entire trajectory, and not just at the point where they leave the main nerve trunk to project to muscle.

Possible Mechanisms for Motoneuron Guidance of Sensory Neurons

We consider it most probable that the altered projection patterns we observed resulted from changes in pathway selection. However, there are several other explanations which we cannot exclude, but consider less likely.

First, motoneuron removal may affect the generation of neurons in the DRGs such that only cutaneous neurons are produced and hence only cutaneous nerves are formed. However, embryonic manipulations, at least of peripheral targets, do not alter neuronal proliferation (Hamburger and Oppenheim, 1982; see also Carr and Simpson, 1978b). Second, motoneurons may normally have an inductive effect on sensory neurons which influences the course of their subsequent differentiation. Motoneurons could, for example, be necessary for the determination or differentiation of muscle afferents but not of cutaneous afferents, although it is difficult for us to envision how this would occur. Third, the operation may result in the selective cell death of muscle afferents. However, since muscle projections were not any more extensive at early stages, at the start of the normal period of cell death, this mechanism would require that most muscle afferents die before forming muscle nerves. Yet in most cases cell death starts after neurons contact their targets (for a review see Hamburger and Oppenheim, 1982; but see Carr and Simpson, 1982; Tanaka and Landmesser, 1986). Finally, none of these mechanisms can easily explain why the cutaneous nerves were enlarged, or why there were also changes in the plexus region.

Previous studies have shown that at the lumbar level sensory neurons grow along the segmentally correct peripheral nerves (starting at st 26) before they penetrate the grey matter (starting at approximately st 30–31) to form connections in the spinal cord (Honig, 1982, and unpublished observations; see also Romanoff, 1960). Thus it is unlikely that sensory neurons are instructed by their central connections to make specific peripheral pathway choices. Further, the lack of central targets is unlikely to have influenced the extent of sensory cell death in embryos examined at or before st 30–31, since sensory neurons would not have normally established central connections in the lumbar spinal cord by this time.

It seems most probable that the presence of motoneuron axons is somehow necessary for sensory neurons to grow along muscle nerve pathways. Motoneuron axons may have this effect either by interacting with sensory neuron axons directly or by altering the environment through which they grow. More specifically, we can envision three different ways in which motoneurons may affect sensory neuron growth.

First, the interaction between motoneuron axons and sensory neuron axons may be one of fasciculation. All (or some—see section below on specificity) sensory axons may find that motoneuron axons provide a more adhesive surface than other sensory axons provide; specific "recognition" molecules identifying neurons destined to project to the same muscle may, but need not, be involved. Alternatively, the clustering we see of sensory axons raises the possibility that only some sensory axons are in a position that allows them to fasciculate with motoneuron axons. Other experiments have shown that axons growing out into the limb generally do so in fascicles (Al-Ghaith and Lewis; 1982; Tosney and Landmesser, 1985a) although the identities of the axons in those fascicles were not determined. Moreover, fascic-

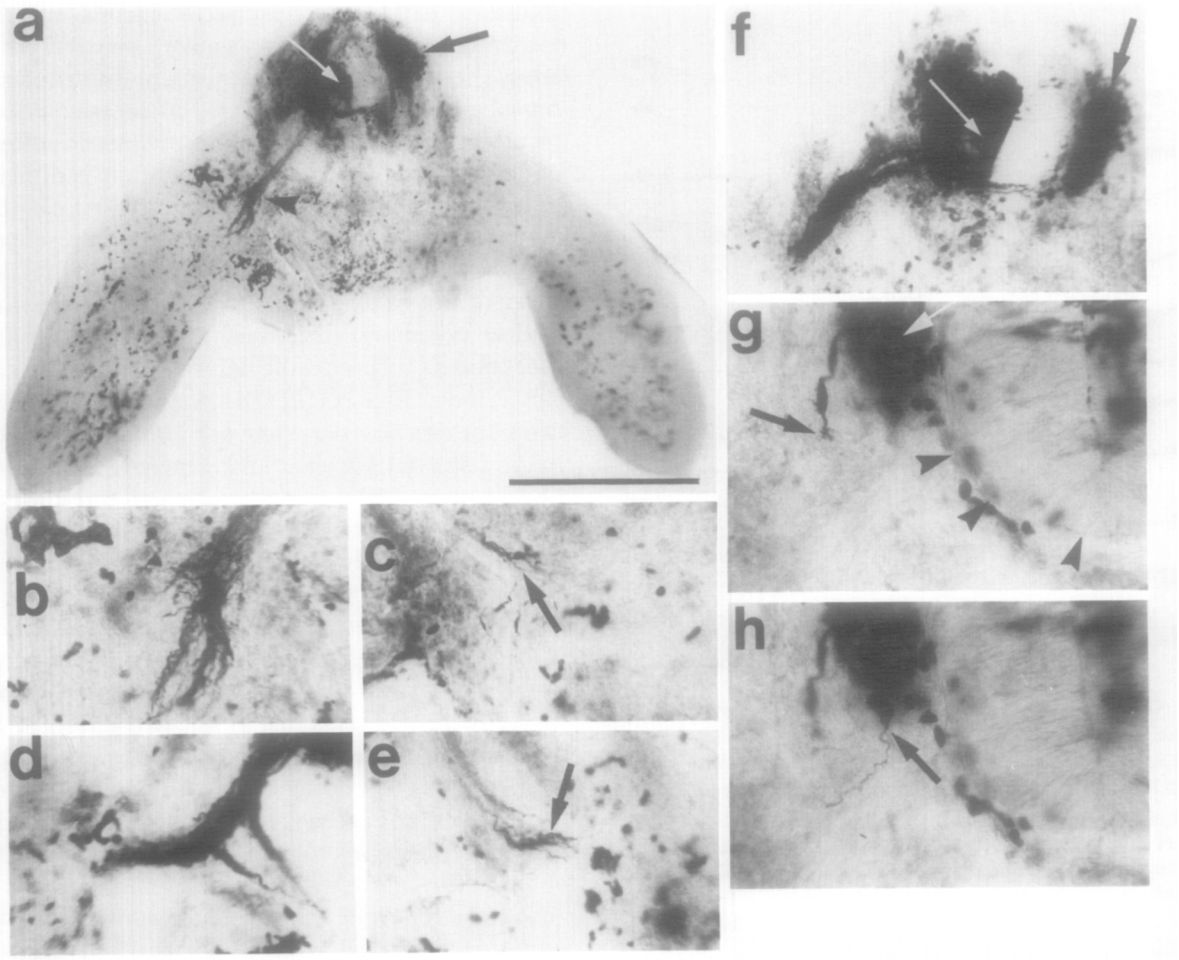

FIG. 12. The extent of sensory and motoneuron axon growth into limb buds in normal st 20–23½ embryos. These micrographs are of 300-μm-thick transverse slices through embryos, reacted for HRP labeling. (a) In this st 23 embryo, HRP injections had been made into the lateral motor column on the left side (white arrow) and into the DRG on the right side (black arrow). Numerous motoneuron axons (arrowhead) have reached the plexus on the left and some have begun to invade the limb bud. (b) The growth front of the motoneurons is shown at higher magnification. (c) Although many DRG neurons were labeled, only a few sensory axons (arrow) have reached the plexus region. These are not visible in (a) because they are at a different focal plane within the slice. (d and e) Show the motoneuron and sensory neuron growth fronts, respectively, from a st 22½ embryo. In (d) many motoneuron axons have reached the plexus. In (e), several sensory growth cones (arrow) are seen in the plexus, but again sensory axons are much less abundant than motoneurons. (f) The spinal cord and plexus region of a slice from a st 20 embryo in which the spinal cord had been injected on the left (white arrow), the DRG on the right (black arrow). Numerous motoneuron axons project to the plexus region but, although many cell bodies were labeled in the DRG, no axons left the ganglion. (g and h) Two different focal planes through a slice from another st 20 embryo in which the DRG on the left (white arrow) was heavily labeled. The spinal cord, whose ventrolateral circumference is indicated by arrowheads, is unlabeled. In (g), a single DRG cell located at the periphery of the DRG and presumably labeled from its central process projecting into the region of HRP injection has a growth cone (black arrow) projecting just outside of the ganglion. (h) At another focal plane, a second cell (arrow) has an axon which has just reached the unlabeled ventral roots and has not yet reached the plexus. In this embryo seven dorsal root ganglia were heavily labeled with HRP and only one additional axon was observed to project out of any of the DRGs. The dark-staining oval profiles scattered throughout the tissue are red blood cells which have endogenous peroxidase activity. Calibration bar = 500 μm for (a); 170 μm for (b–e); 250 μm for (f); and 110 μm for (g, h).

ulation is frequently a feature of developing systems (e.g., Rusoff and Easter, 1980; Bodick and Levinthal, 1980; Erzurumlu and Killackey, 1983; Roberts and Taylor, 1983; Goodman *et al.*, 1984) and may be important in formation of appropriate projections (Raper *et al.*, 1984; Thanos *et al.*, 1984). Second, motoneuron axons may simply provide an aligned substrate along which sensory neuron axons more readily grow. The possibility that contact guidance (Weiss, 1934) may play a role receives support from the observation that sensory neurons, particularly in motoneuron-depleted embryos, were frequently associated with blood vessels, which may similarly serve as an aligned substrate for axon elongation. Alternatively, blood vessels may be preferred because they contain certain molecules (i.e., laminin) which are particularly adhesive for growth cones. Third, motoneu-

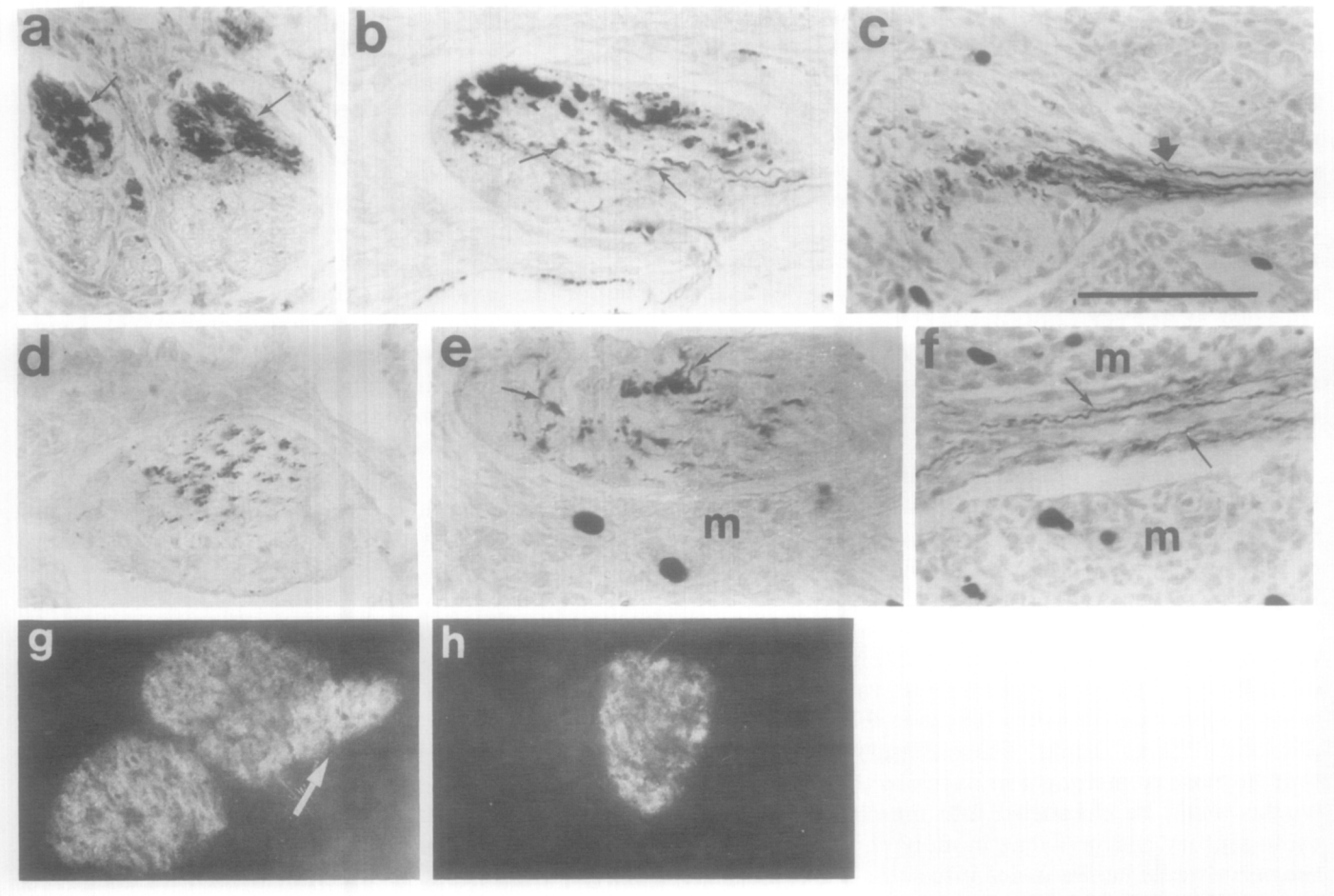

FIG. 13. Distribution of sensory axons within nerve pathways in normal embryos. (a–f) Distribution of HRP-labeled axons within nerves following injection of DRG3 at st 29; Successively more distal levels are shown as one progresses from (a) to (f). (a) In the proximal part of the spinal nerve, just after sensory axons join the ventral root, the DRG axons which are still situated dorsally (arrows) are heavily labeled. There is no label in the ventral parts of the nerve, indicating that all labeled axons are in fact sensory. (b) In the plexus, sensory axons (arrows) begin to redistribute and intermingle with unlabeled axons. (c) The Mf. Ct. nerve (arrow) as it branches from the crural nerve trunk contains numerous labeled axons. (d) A large number of labeled axons remain in the crural nerve trunk after the cutaneous nerves diverge. All axons at this level will innervate muscle. (e and f) Sensory axons (arrows) within the femorotibialis muscle nerve, deep within the developing femorotibialis muscle (m). (g and h) Distribution of sensory axons labeled with a sensory neuron-specific monoclonal antibody and visualized using a fluorescent secondary antibody in a section from a st 32 embryo. (g) Sensory axons are distributed throughout the sciatic nerve trunk but are most dense in the region where a cutaneous nerve (arrow) is beginning to diverge. (h) All the axons in the crural nerve trunk at this level, from the same section as (g), will innervate muscle. (Note the fairly high density of sensory axons in this nerve, by comparing the relative amount of labeling with that in the cutaneous nerve branch in (g). Calibration bar = 100 μm for (a–f); 200 μm for (g, h).

ron axons may alter the environment through which they grow so as to facilitate the growth of sensory neurons. Motoneuron axons could, for example, secrete a protein (or proteins) onto the substratum that sensory neuron axons grow along, thereby making the substratum more adhesive. Tissue culture studies have shown that different kinds of neurons selectively secrete different proteins (Sweadner, 1981; Pittman, 1985) and further, that at least some secreted proteins, when bound to artificial substrates, can direct the paths of neurite growth (Collins and Garrett, 1980). Neuronal growth cones may also alter pathways by releasing a variety of proteases (Krystosek and Seeds, 1981, 1984; Pittman, 1985) which either degrade or modify the protein substrate (Neurath, 1984) or which, perhaps, kill mesenchymal cells that lie in the region of the nerve pathways (Tosney and Landmesser, 1985a). Thus motoneurons may act by somehow making pathways in the limb permissive for sensory neuron outgrowth.

Observations on normal development are consistent with any of these mechanisms. The earliest afferents to grow into the limb (probably the oldest) do so at the same time as do motoneurons (see also Lance-Jones and Landmesser, 1981a; Honig, 1982; Tosney and Landmesser, 1985b,c). Most other sensory neuron axons grow out over subsequent stages, the asynchronous progression of outgrowth presumably reflecting the 3-day period during which these neurons are generated (Carr and

Simpson, 1978a; see also Honig, 1982). Whether the first afferents to grow into the limb play any role in guiding the growth of later-arriving afferents is not known. Clearly, however, motoneuron axons are present when sensory neurons grow into the limb and it is therefore conceivable that motoneurons act in any of the ways described above. The distributions of sensory neuron and motoneuron axons, which are both fairly widespread in the plexus, are also compatible with the possibility of an interaction between these different types of axons. However, it has not been possible to assess the spatial relationships between sensory neuron and motoneuron axons in sufficient detail to determine directly whether sensory axons actually fasciculate with motoneuron axons.

It is fairly easy to envision how a mechanism involving fasciculation could operate. Those sensory neuron axons that fasciculate with motoneuron axons would be guided to muscles; the remaining sensory neurons would grow instead along cutaneous pathways. Alternatively, if the underlying mechanism involves contact guidance or alterations in the substratum as just discussed, pathway selection could then be based on the proximity of some sensory axons to motoneuron axons in the spinal nerves, plexus and nerve trunks. Those sensory neuron axons that lie close to motoneuron axons in the major nerve trunks would be channeled into muscle nerves while those sensory neuron axons in more distant regions of the nerve would be channeled into cutaneous nerves. At more proximal levels, sensory neurons might tend to maintain their relative positions, since alterations in their trajectories may normally be enhanced by the changes in direction that motoneurons undergo in the plexus region. In all cases, sensory neurons would be unable to grow to muscles in the absence of motoneurons and would instead choose an alternative pathway, such as a cutaneous nerve or the surface of a blood vessel. Finally, these kinds of mechanisms can also explain how the specific segmental patterns of sensory innervation of muscle and skin are generated when motoneurons are present in both normal (Honig, 1982) and in experimental embryos (see Honig *et al.*, 1986).

Differences between Motoneurons and Sensory Neurons

Regardless of the exact nature of the mechanism involved, several lines of evidence indicate that motoneurons interact with limb tissue differently than do sensory neurons. First, as we have shown here, sensory neurons do not grow along muscle nerve pathways in the absence of motoneurons. This indicates that the growth cones of sensory axons are not responsive to the signals that cause motoneuron growth cones to diverge from the main nerve trunks and penetrate the differentiating muscle masses at points where muscle nerves normally form. Previous evidence suggests that this is a point where motoneuron growth cones make an active choice (Tosney and Landmesser, 1985b) and that this choice is not made in limbs devoid of muscle (Lewis *et al.*, 1981). In contrast, motoneuron outgrowth does not seen to be affected by the absence of sensory neurons (Hamburger *et al.*, 1966; Narayanan and Malloy, 1974). Second, motoneurons do not project along cutaneous pathways either at early stages of outgrowth (Lance-Jones and Landmesser, 1981a; Tosney and Landmesser, 1985a,b) or after a variety of experimental manipulations (Lance-Jones and Landmesser, 1980a,b, 1981b; Lance-Jones, 1986). Therefore, at least some sensory neurons are responsive to a set of cues in the limb that motoneurons do not sense or do not respond to. Third, after they have been experimentally displaced, motoneurons seem more able than sensory neurons to alter their courses in the limb in order to project to their embryologically correct targets. For example, following small neural tube (and neural crest) reversals, virtually all motoneurons, but only some sensory neurons, project correctly (Honig *et al.*, 1986). While this difference may simply reflect the later outgrowth of most sensory neurons (as discussed in the preceding paper), it is also possible that the difference is an intrinsic one.

A final difference is that motoneuron growth cones in the limb tend to be larger than sensory neuron growth cones. In several systems it has been noted that neurons that initially pioneer pathways tend to have large, complex growth cones with lamellipodia or with many long filopodia (e.g., Taghert *et al.*, 1982). In contrast, those neurons that grow out later and either fasciculate with more advanced axons (LoPresti *et al.*, 1973) or follow already established nerve tracts (Shankland, 1981a,b) tend to have simpler and smaller growth cones. These differences in growth cone morphology are thought to reflect the extent to which the neurons explore their environment. In the chick hindlimb, motoneuron growth cones tend to be largest when those growth cones are in what has been termed "decision regions" where they choose between alternative pathways (Tosney and Landmesser, 1985c). Sensory neuron growth cones are generally quite small, even those in decision regions (Fig. 11; Tosney and Landmesser, 1985c). Since sensory neuron growth cones and motoneuron growth cones traversing the same terrain differ in size, the nature of the environment alone cannot account for these differences. In addition, since we have shown that the growth cones of sensory neurons remain small, even in the absence of motoneurons, their small size cannot be due to constraints imposed by the large numbers of motoneuron axons normally present. Furthermore, and somewhat surprisingly, sensory neurons growing along cutaneous nerves do not appear to have large growth cones, even though in this case they are unaccompanied by motoneuron axons and must, at least in some cases, traverse

such pathways independent of all other nerve fibers. Our failure to detect large sensory neuron growth cones both in normal and in motoneuron-depleted embryos suggests that if any in fact exist, they may be few in number and transient in appearance. Taken together, these observations therefore suggest that the differences between sensory and motoneuron growth cones are probably intrinsic. The results further suggest that sensory growth cones may have a stronger tendency than motoneurons to follow other axons rather than to actively explore their environment. This is consistent with all three mechanisms previously proposed.

These differences between motor and sensory axons during normal development contrast with descriptions of their growth in tissue culture. The growth cones of cultured DRG neurons are frequently quite large (e.g., Letourneau, 1975, 1979; Honig, unpublished observations) and adhere very strongly to some substrates (Letourneau, 1975; 1979). In addition, both sensory neurons and spinal cord neurons (some of which are motoneurons) are able to extend neurites on many of the same substrates, including some extracellular matrix components present in embryonic tissues (e.g., Rogers *et al.*, 1983, 1984). While it has not yet been demonstrated that interactions with any of these molecules are essential for axonal outgrowth into the limb or along muscle nerves, it is interesting to note that the major difference in neurite extension that has been reported is the growth of DRG neurons, but not spinal cord neurons, on one substrate, fibronectin (Rogers *et al.*, 1983). Therefore it is apparent that the way in which sensory neurons and motoneurons interact with the tissue culture environment differs significantly from the way they interact with the obviously more complex *in vitro* environment, and further that the tissue culture experiments have not yet helped clarify the nature of the intrinsic differences between them.

A possible basis for at least some of the differences between motoneuron and sensory neuron axons is suggested by a recent study on the distribution of the neural cell adhesion molecule (NCAM) in chick embryos (Tosney *et al.*, 1986). NCAM has been shown to mediate adhesion between various types of neurons and between neuronal processes (see Rutishauser, 1984, for a review). NCAM disappears from neural crest cells during migration, reappears after the DRGs coalesce, and increases over time as the neurons differentiate (Thiery *et al.*, 1982; Duband *et al.*, 1985). At the stages during which axonal outgrowth occurs, NCAM levels in the DRGs are lower than in the motoneurons (Tosney *et al.*, 1986; see also Duband *et al.*, (1985). The difference in NCAM levels is maintained in the proximal parts of the spinal nerves (where the axons of DRG neurons and motoneurons are segregated from one another) and probably at more distal levels as well.

Thus, at the time of axonal outgrowth, sensory neurons appear to have less NCAM on their surfaces than do motoneurons. This in turn suggests that sensory neuron axons may be less adhesive than motoneuron axons. Other studies have shown that the adhesivity of a growth cone is reflected in its size, the least adhesive growth cones being among the smallest (e.g., Letourneau, 1979). Thus, low NCAM levels may explain why sensory neuron growth cones tend to be smaller than motoneuron growth cones. Differences in adhesivity might in turn explain some of the *in vivo* differences in behavior shown by sensory and motoneuron growth cones.

Specification of Sensory Neurons

These experiments leave unresolved the question of whether sensory neurons are specified with respect to their peripheral targets prior to outgrowth. During normal development, sensory neurons grow in an orderly fashion along the correct pathways in the limb from the outset (Honig, 1982; Scott, 1982). In many experimental situations, sensory neurons that have been experimentally displaced still form appropriate connections (Honig *et al.*, 1986). However, after other operations, some sensory neurons clearly project inappropriately (Honig *et al.*, 1986; see also Stirling and Summerbell, 1979). Similar behavior is also demonstrated by motoneurons which clearly are specified (Lance-Jones and Landmesser, 1980b, 1981b). Neurons displaced far from their normal targets may be unable to sense or respond to the local cues which could and normally do direct them to those targets (Lance-Jones and Landmesser, 1981b; Landmesser, 1984). In the situation described in the present paper, in the absence of motoneurons, many sensory neurons projected inappropriately. Wrong connections may have resulted in this case because the cues that sensory neurons normally use were absent, being imparted by the motoneurons which had been removed. Alternatively, sensory neurons may be responsive to other limb-derived cues but may still require the presence of motoneuron axons. In either case, the establishment of inappropriate connections here, as in the other situations, does not indicate that sensory neurons are not specified.

A surprising finding was that afferents that normally project to muscle seemed to project to skin following motoneuron removals. This raises the possibility that "cutaneous" and "muscle" afferents may not represent separate populations of neurons prior to outgrowth. The position of an individual sensory axon in the spinal nerves, plexus, or nerve trunks may instead determine its subsequent course of outgrowth and the final identity of its parent cell body. Alternatively, sensory neurons may be prespecified and only muscle afferents may be capable of interacting with motoneuron axons or growing along motoneuron-altered pathways such that they

project to muscles. Similarly, only cutaneous afferents may be able to establish pathways that go to skin. Unfortunately, we cannot distinguish between these two possibilities because at the present time "cutaneous" and "muscle" afferents can only be recognized by retrograde labeling following their outgrowth. Afferents projecting to skin and those projecting to muscle are not localized in separate regions in the DRGs as originally thought (Hamburger and Levi-Montalcini, 1949), but rather sensory neurons projecting along any given muscle or cutaneous nerve are scattered throughout individual DRGs (Honig, 1982). The use of biochemical or immunological markers for functional subpopulations of DRG neurons (e.g., see Dodd *et al.*, 1984) may ultimately help to resolve the issue of sensory neuron specification.

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