Independent Development of Sensory and Motor Innervation Patterns in Embryonic Chick Hindlimbs

Guoying Wang and Sheryl A. Scott
Department of Neurobiology and Anatomy, University of Utah School of Medicine, 50 North Medical Drive, Salt Lake City, Utah 84132

Previous studies suggest that sensory axon outgrowth is guided by motoneurons, which are specified to innervate particular target muscles. Here we present evidence that questions this conclusion. We have used a new approach to assess the pathfinding abilities of bona fide sensory neurons, first by eliminating motoneurons after neural crest cells have coalesced into dorsal root ganglia (DRG) and second by challenging sensory neurons to innervate muscles in a novel environment created by shifting a limb bud rostrally. The resulting sensory innervation patterns mapped with the lipophilic dyes DiI and DiA showed that sensory axons projected robustly to muscles in the absence of motoneurons, if motoneurons were eliminated after DRG formation. Moreover, sensory neurons projected appropriately to their usual target muscles under these conditions. In contrast, following limb shifts, muscle sensory innervation was often derived from inappropriate segments. In this novel environment, sensory neurons tended to make more “mistakes” than motoneurons. Whereas motoneurons tended to innervate their embryologically correct muscles, sensory innervation was more widespread and was generally from more rostral segments than normal. Similar results were obtained when motoneurons were eliminated in embryos with limb shifts. These findings show that sensory neurons are capable of navigating through their usual terrain without guidance from motor axons. However, unlike motor axons, sensory axons do not appear to actively seek out appropriate target muscles when confronted with a novel terrain. These findings suggest that sensory neuron identity with regard to pathway and target choice may be unspecified or quite plastic at the time of initial axon outgrowth.

Key Words: sensory neurons; chick embryo; muscle afferents; dorsal root ganglia; axon guidance.

INTRODUCTION

A central question in developmental neurobiology is how neurons grow to the correct targets and establish appropriate connections. Substantial progress has been made in elucidating the mechanisms that regulate motoneuron connectivity. It is generally accepted that prior to axon outgrowth motoneurons are specified with respect to their peripheral connectivity, allowing them to actively seek out and selectively innervate the appropriate muscle (Landmesser, 1992; Appel et al., 1995; Matise and Lance-Jones, 1996). Much less is known about the mechanisms that govern the development of sensory innervation patterns. Previous studies suggest that sensory neurons may simply follow motoneurons to their target tissues. Sensory axons grow out together with, but slightly later than, motor axons (Tosney and Landmesser, 1985; Landmesser and Honig, 1986), and muscle afferents travel to the periphery directly adjacent to motor axons serving the same muscle (Honig et al., 1998). Further, sensory projection patterns generally resemble motor patterns following a variety of experimental manipulations (Honig et al., 1986; Wang and Scott, 1997a). If motoneurons are removed before axon outgrowth, few sensory axons project to muscles (Landmesser and Honig, 1986; Swanson and Lewis, 1986; Scott, 1988; Tosney and Hageman, 1989). Together these results suggest that sensory neurons (at least the muscle afferents) are guided to their targets by motoneurons, rather than navigating independently through the periphery based on their own inherent specificity.

One caveat in this interpretation, however, is that sensory neurons are derived from neural crest cells, which migrate through the somites and coalesce into dorsal root ganglia (DRG) adjacent to the neural tube. Most experimental manipulations designed to test sensory neuron specification were done prior to neural crest migration and thus
assayed specification of neural crest cells rather than sensory neurons per se (Honig et al., 1986; Landmesser and Honig, 1986; Scott, 1986, 1988; Wang and Scott, 1997a). Neuronal crest cells have broad developmental potentials, and the differentially characterized features of neural crest derivatives are determined largely by the environment into which they migrate (Le Douarin, 1982). Thus, sensory neurons derived from manipulated neural crest (Honig et al., 1986; Scott, 1986; Wang and Scott, 1997a) most likely differentiate in accord with their new position, essentially negating the effects of the experimental perturbation.

Here we have taken a novel approach to test the path-finding capabilities and specification of sensory neurons, rather than neural crest cells. In the present study we allowed neural crest cells to coalesce into DRG in their usual location, permitting DRG neurons to differentiate in response to their normal environmental cues. We then removed the motoneurons and assessed the resulting sensory innervation patterns. We report here that sensory neurons project more robustly to muscles in the absence of motoneurons if the motoneurons are removed after the coalescence of DRG rather than during neural crest migration. Moreover, sensory neurons project appropriately to their usual target muscles under these conditions. We further show that the correct innervation that is established in the absence of motoneurons most likely reflects the passive channeling of sensory axons to peripheral targets rather than active target selection by specified sensory neurons. We found, for example, that sensory neurons make more “mistakes” than motoneurons when limbs are shifted rostrally to cause sensory and motor axons to grow into a novel region of the limb. This finding suggests that at the stages when innervation is being established, sensory neurons are not as rigidly specified as motoneurons. Outgrowing sensory neurons appear, therefore, to have more flexibility in their pathway and target choices than do motoneurons. Preliminary reports of some of these results have been presented elsewhere (Wang and Scott, 1997b).

MATERIALS AND METHODS

Embryos

Fertile eggs of White Leghorn chick embryos were incubated in a forced-draft incubator at 38 ± 1°C. Embryos were staged according to Hamburger and Hamilton (1951) at the time of surgery and at sacrifice.

Surgery

Each egg was candled and a window was cut in the shell over the area of the embryonic disc. The embryo was stained with 0.5% neutral red in phosphate-buffered saline (PBS), the vitelline membrane was opened, and one of the following surgeries was performed.

Neural tube deletions. To eliminate motoneurons from the last thoracic through the third lumbar spinal segment (T7–L3) after neural crest migration was complete, we removed the entire neural tube opposite somites 24–30 at Stage 21 (E3.5). First we freed the neural tube from the adjacent somites with sharpened tungsten needles and then split the neural tube down the midline to allow better visualization. The whole neural tube was then removed by applying gentle suction through a micropipet, which had been broken to a suitable tip diameter. The eggs were sealed with a coverslip and returned to the incubator until the desired stage.

Motoneuron deletions. In order to remove motoneurons at Stage 17–18, when neural crest migration was still under way, we removed the ventral two-thirds of the neural tube by suction and left the dorsal one-third of the neural tube (including neural crest) intact, as described previously (Landmesser and Honig, 1986; Scott, 1988). To assess the success of motoneuron deletions, some operated embryos were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and embedded in paraffin. The spinal cords and DRG were sectioned transversely at 10 μm and stained with cresyl violet.

Limb shifts with spinal cord deletion. In approximately 450 embryos, the left limb bud was shifted forward by one to four segments at Stage 17–18, when significant sensory and motor axon outgrowth. The limb bud was severed next to the lateral boundary of the somites, using sharpened tungsten needles. The cut was then extended anteriorly by several segments and the limb bud was slid forward into the slit. After the limb bud had adhered to the trunk, the egg was sealed and returned to the incubator. In some embryos with limb shifts, the neural tube was subsequently removed at Stage 21, as described above.

Retrograde and Orthograde Labeling

Embryos were incubated until Stage 29–38 (E6–E11), when they were removed from the eggs, staged, and placed in a chamber containing oxygenated Ringer’s (Scott, 1984) at room temperature (20–22°C). They were quickly decapitated and eviscerated and a ventral laminectomy was performed. DRG or muscles (the sartorius, femorotibialis, and adductor muscles) were then exposed and injected with DiI (Molecular Probes, Eugene, OR; 2.5 mg/ml in dimethylformamide (DMF)) or DiA (Molecular Probes; 3 mg/ml in DMF). In some embryos DiI was injected into one or more DRG from T5 to LS8. Following injections, embryos were maintained in oxygenated Ringer’s solution at 28°C for 6 h, when they were fixed in 4% paraformaldehyde. Embryos were then stained in paraformaldehyde at 37°C for another 2–3 weeks to allow thorough orthograde labeling. In other embryos sensory projections to individual sartorius, femorotibialis, or adductor muscles were retrogradely labeled by injecting the muscle with DiI or DiA. In most embryos two muscles were injected on each side. After injections embryos were treated as described above.

All injected embryos were first observed as whole mounts with standard fluorescence optics to assess the overall pattern of transported dyes. Subsequently, most embryos were embedded and sectioned, or the DiI was photoconverted.

Photoconversion of DiI in DRG axons. To facilitate viewing of sensory axon trajectories in the limb, DiI was injected into DRG and allowed to diffuse, as described above. The DiI was then photoconverted to a stable diaminobenzidine (DAB) reaction product (Sretavan, 1990). Embryos with DiI-labeled axons were transferred to 0.1 M Tris buffer (pH 8.2, 4°C) containing 1.5 mg/ml DAB and exposed to a fluorescent light source equipped with a rhodamine filter set. The Tris/DAB solution was replaced with fresh, cold Tris/DAB every 15–20 min for approximately 2 h, until axons were clearly visible with bright-field optics.

Sectioning and cell counting. Most embryos with DiI or DiA injections were sectioned on a Vibratome to analyze further either
axon trajectories or the distribution of labeled sensory and motoneurons. Blocks containing either the spinal cord and DRG from T5 to L5B or a hindlimb together with the ipsilateral DRG and spinal cord were cut out and embedded in gelatin–albumin. Blocks were hardened overnight with 1% glutaraldehyde and serially sectioned at 80 μm (for general observation) or 60 μm (for cell counting). Most embryos with motoneuron deletions were cut transversely, although a few such embryos were cut sagittally. Embryos with limb shifts were cut longitudinally. Sections were mounted in glycerol containing 0.1% p-phenylenediamine (Johnson and Araujo, 1981), to retard fading, and were viewed with a Zeiss Axioskop microscope and/or a Bio-Rad MRC-1024 confocal scanning laser microscope.

To chart the segmental distribution of DRG neurons serving different muscles, DRG neurons were retrogradely labeled by injecting DiI or DiA into the muscle, as described above. Labeled neurons were then counted in DRG T7–LS4. Serial 60-μm Vibratome sections were imaged at 10-μm intervals with the confocal microscope. Labeled cells were counted at 30-μm intervals through each DRG by counting cells in every third image. For each muscle the numbers of labeled neurons in all the DRGs were summed, and the percentage of labeled cells in each DRG was calculated. The distributions of labeled cells in control and operated embryos were compared for significance with a t test.

Cartilaginous Skeleton Preparations

To view the morphology of the pelvic girdle in embryos with limb shifts, the embryos were stained with Alcian blue (modified from Jegalian and DeRobertis, 1992). Embryos (Stage 23–31) were fixed in 4% paraformaldehyde, washed in 0.1 M PBS, followed by 35% methanol in PBS, and stained with Alcian blue BGX (Sigma, St. Louis, MO; 0.02% in staining buffer (70% methanol, 5% glacial acetic acid, 25% H2O)) overnight at 4°C. Embryos were then washed and destained overnight in the same buffer and cleared in 2:1 benzyl benzoate:benzyl alcohol for approximately 2 days.

RESULTS

The goal of the studies presented here was to assess further the specification and pathfinding capabilities of sensory neurons. As described above, earlier studies designed to test sensory neuron specification were carried out prior to coalescence of neural crest cells into DRG and thus tested the specification of neural crest cells rather than testing the pathfinding capabilities of sensory neurons.

Sensory innervation in the absence of motoneurons

Late removal of motoneurons. As a first approach, we asked whether muscle sensory neurons could grow to muscle without guidance by motor axons, if motoneurons were eliminated after DRG coalescence. To test this possibility we eliminated motoneurons at Stage 21 (E3.5) by removing the entire neural tube from segments T7–LS3. At this stage some motor axons have grown to the plexus region at the base of the limb, where they wait for approximately 24 h before invading the limb itself (Tosney and Landmesser, 1985; Landmesser and Honig, 1986). Thus, removing motoneurons at Stage 21 may leave behind a pathway that sensory axons can follow to the plexus, but should eliminate all motoneuron-derived guidance cues from the plexus to individual muscles.

Of the 175 operated embryos, 18 survived, were missing motoneurons for at least three consecutive spinal segments but had DRG throughout the operated region, and were successfully labeled with dye on at least one side (see below). In most embryos, such as the one illustrated in Fig. 1, the entire spinal cord was missing from LS1 through LS3, whereas the DRG developed normally. In a few embryos the spinal cord was deleted from T6 to LS2 or LS3 or from LS1 to LS4.

Surprisingly, orthograde labeling of DRG neurons showed that sensory innervation of muscle was robust in the absence of motoneurons (Figs. 2B and 2D), although somewhat reduced in size compared to sensory innervation in unoperated control embryos (Fig. 2A). Indeed, nearly all muscle nerves, which were populated exclusively by sensory axons in the absence of motoneurons, were present in these embryos; however, occasionally a muscle could not be scored in an embryo because the DRG that normally serve the muscle were not injected. Of the muscle nerves that we could analyze, the sartorius was present in seven of eight embryos, the femorotibialis in all six embryos, and the adductor in all seven embryos. As in previous studies (Landmesser and Honig, 1986; Scott, 1988) the two cutaneous nerves, the cutaneous femoralis lateralis (CFL) and cutaneous femoralis medialis, which are derived from DRG in the operated region, appeared to develop normally. Since cutaneous innervation following motoneuron deletion has been studied extensively (Scott, 1988), these nerves were not analyzed further.

Early elimination of motoneurons. The present finding that sensory neurons consistently innervated muscle in the absence of motoneurons stands in contrast to earlier studies.
which reported that sensory neurons were unable to grow to their target muscles when motoneurons were deleted (Landmesser and Honig, 1986; Scott, 1988; Tosney and Hageman, 1989). To determine whether this difference was due to differences in the stage at which motoneurons were eliminated (after neural crest migration in the present studies vs during neural crest migration in earlier studies) or instead was a result of the greater sensitivity of our labeling method (DiI vs HRP), we eliminated motoneurons at Stage 17–18 and labeled sensory projections by injecting DRG with DiI at Stage 31. Of the 46 operated embryos, 21 survived until Stage 31, developed a full complement of DRG, and were missing motoneurons throughout the operated region (Fig. 3B); 11 of these had adequate dye transport in one or both limbs. Sensory projections were clearly reduced in these embryos in comparison to projections in

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embryos with late motoneuron removal. The sartorius nerve was missing entirely from 11 limbs and consisted of only a few axons in 4 other limbs. Some sensory axons projected to the femorotibialis and adductor muscles in all embryos, but these projections were clearly less extensive than following late motoneuron deletion, as illustrated in Figs. 2C and 2D. Thus, eliminating motoneurons during neural crest migration has much more profound effects on sensory innervation of muscle than eliminating motoneurons after neural crest migration is complete.

The segmental pattern of sensory projections in the absence of motoneurons. The ability to produce muscle sensory innervation in the absence of motoneurons allowed us to examine the ability of sensory neurons to navigate through the periphery without guidance from motor axons. To test sensory neuron capabilities we compared the segmental distribution of DRG neurons innervating the sartorius and femorotibialis muscles in the presence or absence of motoneurons, as described under Materials and Methods. Briefly, DRG neurons were retrogradely labeled by injecting DiI or DiA into a muscle, labeled DRG cells were counted in confocal images, and the percentage of labeled cells in each DRG was calculated. Dye injections confirmed that muscles were well innervated by sensory neurons in the absence of motoneurons. Overall, dye injection of sartorius and femorotibialis muscles in operated embryos resulted in 67 and 100%, respectively, as many labeled DRG neurons as in control embryos. Moreover, sensory neurons innervated both the sartorius and the femorotibialis muscles accurately in the absence of motoneurons, as illustrated in Fig. 4. Although there appeared to be a larger than normal contribution from LS1 to the sartorius muscle in operated embryos, this difference was not significant (P > 0.5). Further, the apparent increase was due primarily to a single embryo, in which sensory innervation of the sartorius was derived exclusively from DRG LS1. Thus, in the absence of motoneurons, sensory neurons navigate accurately to their appropriate target muscles, consistent with their ability to make correct cutaneous projections (Scott, 1988).

Sensory Innervation Following Limb Shifts

The accuracy of muscle sensory innervation could be taken as evidence that sensory neurons, like motoneurons, are specified to innervate particular muscles. Alternatively, sensory neurons may be guided to muscles by other, possibly nonspecific, cues in the limb which remain intact following motoneuron removal. To distinguish between these possibilities we challenged sensory neurons to innervate their appropriate target muscles in the novel environment created by shifting the limb bud rostrally at Stage 17-18 (Lance-Jones and Landmesser, 1981; Honig et al., 1986), first in the presence of motoneurons and subsequently with motoneurons eliminated.

Of the 450 operated embryos, 190 survived to the desired stages and had morphologically normal limbs that were shifted rostrally by one to four segments (see below). The pelvic girdle, which plays an important role in channeling axons into the two limb plexuses (Tosney and Landmesser, 1984, 1985), developed normally, albeit in a more rostral location, following limb shifts, with the exception that the caudal portion was shortened somewhat (not shown). Thus, in the anterior region of the limb, the pelvic girdle, a major source of passive guidance, was apparently unaltered.

Overall nerve patterns following limb shifts. In initial experiments we assessed the extent of limb shift and overall pattern of sensory innervation by injecting DiI into DRG T7-LS8 and photoconverting the dye. Normally, axons from segments LS1-3 (and occasionally T7) come together in the crural plexus, before projecting to muscles in the anterior thigh, including the sartorius, femorotibialis, and adductor; a few axons from LS3 join the more posterior

FIG. 3. Removal of the ventral neural tube during neural crest migration eliminates motoneurons but allows DRG to develop normally. (A) Transverse sections through the spinal cord of one such embryo operated at Stage 17 (E2.5) shows that both motoneurons (arrows) and DRG developed normally in the region just rostral to the operated region. (B) More caudally in the operated region of the same embryo, there are no motoneurons but the DRG appear normal. Scale bar is 140 μm.
sciatic plexus. Following limb shifts, axons enter a more posterior region of the limb than normal. Thus, some axons that normally project into the crural plexus are channeled into the sciatic plexus. The shift in segmental contribution to the sciatic plexus was used to assess the extent of limb shift, as described by Lance-Jones and Landmesser (1981) and illustrated in Fig. 5. For example, if all LS3 axons projected to the sciatic plexus, the limb was considered to be shifted one segment rostral; if LS2 axons were channeled to the sciatic plexus as in Fig. 5A, the limb was considered to be shifted two segments rostral, and so on. This assessment showed that most limbs were shifted one to four segments (usually two to three) rostral, as in earlier studies (Lance-Jones and Landmesser, 1981; Honig et al., 1986).

Orthograde labeling revealed several consistent alterations in the overall pattern of sensory innervation. First, sensory innervation of the limb was derived from more rostral segments than normal following limb shifts. Axons from segment T7 usually projected into the crural plexus. Other more rostral thoracic DRG often contributed to the limb, but they usually grew straight into the limb without joining the crural plexus. Second, sensory axons that were channeled into the “wrong” (in this case, sciatic) plexus seldom “corrected” their mistake by growing to their embryologically appropriate muscle. Occasionally, some adductor sensory axons that were channeled into the sciatic plexus took a novel route to reach their appropriate muscle. In these rare instances, the adductor muscle received sensory innervation from both the crural and the sciaticplexes (Fig. 5B). Sensory axons, unlike motor axons (Lance-Jones and Landmesser, 1981; see also Lance-Jones and Landmesser, 1980) did not take novel routes from the sciatic plexus to innervate the sartorius or femorotibialis muscles. Third, the most anterior cutaneous nerve, the CFL, was missing in five of eight embryos analyzed by dye injection into rostral DRG. Finally, following limb shifts caudal DRG (LS5–8 or LS6–8) often failed to innervate posterior limb, coursing posteriorly toward the tail (Fig. 5A).

**Comparison of sensory and motor innervation following limb shifts.** To compare directly the precision with which sensory neurons and motoneurons innervated muscles in the novel environment created by limb shifts, we injected DiI or DiA into selected muscles on operated and control sides of individual embryos. Surprisingly, sensory innervation patterns following limb shifts consistently differed from motor patterns mapped in the same embryos. As reported previously (Lance-Jones and Landmesser, 1981), motoneurons tended to project to their embryologically appropriate muscles following limb shifts, as judged by the segmental location of retrogradely labeled motoneurons, although motor innervation occasionally arose from one segment more rostral than normal. In contrast, sensory innervation was consistently derived from more rostral segmental levels than normal and from more rostral segmental levels than the motor innervation, as shown in the examples in Fig. 6 and summarized in Fig. 7. The split sensory projection shown for the adductor muscle in Fig. 6C was rare and most likely represents the projections of adductor sensory axons through both the crural and the sciaticplexes (see above). Thus, following limb shifts muscles generally received sensory innervation from both embryologically appropriate as well as novel, embryologically inappropriate DRG. These results differ somewhat from those reported previously (Honig et al., 1986). Possible reasons for this apparent discrepancy are discussed below.

One potential explanation for the rostral and widespread patterns of sensory innervation in comparison to the motor innervation is presented in Fig. 4. Sensory neurons innervate muscles accurately in the absence of motoneurons. Histograms show the segmental contribution of DRG to sensory innervation of the sartorius and femorotibialis muscles in the presence (Control) and absence (MN-deleted) of motoneurons. For each DRG, cells retrogradely labeled with DiI were counted from confocal images and expressed as a percentage of the total labeled neurons in all DRG of that embryo. There were no significant differences (P > 0.5) in the distribution of labeled neurons between control and operated embryos. Control: sartorius, n = 5; femorotibialis, n = 7. MN-deleted: sartorius, n = 7; femorotibialis, n = 6.

![FIG. 4. Sensory neurons innervate muscles accurately in the absence of motoneurons. Histograms show the segmental contribution of DRG to sensory innervation of the sartorius and femorotibialis muscles in the presence (Control) and absence (MN-deleted) of motoneurons. For each DRG, cells retrogradely labeled with DiI were counted from confocal images and expressed as a percentage of the total labeled neurons in all DRG of that embryo. There were no significant differences (P > 0.5) in the distribution of labeled neurons between control and operated embryos. Control: sartorius, n = 5; femorotibialis, n = 7. MN-deleted: sartorius, n = 7; femorotibialis, n = 6.](image-url)
patterns is that sensory neurons are less rigidly specified than motoneurons and are rather nonselectively guided to target muscles by cues encountered when they enter the limb. This could account for the inappropriate innervation of muscles by rostral DRG. We suspected that appropriate sensory innervation might depend on guidance by axons of the neighboring motoneurons. Since motoneurons tend to innervate their appropriate muscles after limb shifts, some sensory neurons in DRG LS1–3 might be directed to their appropriate muscles by neighboring motor axons. To test this possibility we eliminated motoneurons by removing several segments of neural tube at Stage 21 in embryos with rostral limb shifts. Fifteen of the 153 operated embryos survived and met our criteria (see above) for analysis.

Contrary to our expectations, sensory innervation patterns in embryos with both limb shifts and neural tube removal were nearly identical to those in embryos with simple limb shifts. As summarized in Fig. 8, sensory projections were equally as widespread as when motoneurons were intact, and innervation arose from both appropriate and inappropriate segments, with a only few exceptions. Thus, the appropriate sensory innervation that is observed following limb shifts does not require the presence of motor axon guides. Moreover, these results provide further evidence (Scott, 1998; Adams and Scott, 1998) that sensory neurons appear to be less rigidly specified than motoneurons during their initial outgrowth to targets.

**DISCUSSION**

The experiments reported here reevaluate the apparent dependency of muscle afferents on motor axon guidance. Our results suggest that sensory neurons acquire independence from motoneurons after they coalesce into DRG and that sensory neurons do not appear to be as rigidly specified as motoneurons with respect to pathway and target choice.

**Requirement of Motoneurons?**

Previous studies designed to test specification and pathfinding capabilities of sensory neurons (Honig et al., 1986; Wang and Scott, 1997a) were carried out prior to neural crest cell migration and therefore tested specification of neural crest cells rather than sensory neurons per se. Elimination of motoneurons during neural crest migration greatly reduces sensory projections to muscles, suggesting that sensory neurons require guidance by motor axons to grow to target muscles (Landmesser and Honig, 1986; Swanson and Lewis, 1986; Scott, 1988; Tosney and Hageman, 1989). However, neural crest cells differentiate in response to signals in their environment (Le Douarin, 1982). An important source of such signals for sensory neurons may be the spinal cord (Le Douarin, 1986; Kalcheim and Le Douarin, 1986), perhaps the motoneurons themselves. Early deletion of ventral neural tube may, therefore, eliminate...
signals required for muscle afferent differentiation. Thus, the relative paucity of muscle afferents, rather than lack of guidance cues from motoneurons, may account for the failure of sensory neurons to innervate muscle in the absence of motoneurons.

To distinguish between these possibilities, in the present experiments we allowed neural crest cells to coalesce into DRG in their usual location and to differentiate in response to their normal environmental cues before removing motoneurons. Under these conditions, sensory neurons consistently project to target muscles, and these projections are segmentally appropriate. Thus, when motoneurons are removed early (Stage 17–18), while neural crest cells are still migrating, few afferents grow to muscle. However, when motoneurons are removed somewhat later (Stage 21), after coalescence of DRG, afferent innervation of muscle is robust. A similar trend is apparent in the data presented by Landmesser and Honig (1986).

Clearly something changes during this time window. One possibility is that by Stage 21 sensory neurons have matured and differentiated sufficiently to gain autonomy from motoneurons. Alternatively, by Stage 21 some motoneurons have already reached the plexus region where axon sorting occurs (Tosney and Landmesser, 1985). Thus, removal of motoneurons at Stage 21 leaves behind a pathway laid out by the early growing motor axons that may provide an essential substrate for sensory axon growth to the plexus. However, motor axons do not begin to exit the plexus region and grow to individual muscles until Stage 24 (Tosney and Landmesser, 1985; Landmesser and Honig, 1986; unpublished observations), approximately 24 h after we removed motoneurons. Thus, motor axons could not have physically guided sensory axons from the plexus through the limb to their target muscles in our experiments, since they were never present in this region. Currently we cannot distinguish whether the increased sensory innervation of muscle that we observed in the present experiments relative to earlier studies results from maturation of sensory neurons or from enhanced growth of these neurons to the plexus region or both. Regardless, our data suggest that after they coalesce into DRG sensory neurons can grow to their target muscles without motor axon guidance.

The one muscle nerve that was consistently missing following early motoneuron deletions in the present and in previous studies was the sartorius. Its absence raises the possibility that sensory neurons may grow to muscle in part due to a diffusible chemotropic signal (Honig and Zou, 1995). If the amount of signal is proportional to the size of the developing muscle, then the sartorius, which is a smaller muscle than the adductor or femorotibialis, may simply not secrete a sufficient quantity to direct axons to it in the absence of guidance cues from motoneurons.

Pathfinding Capabilities of Sensory Neurons?

As described above, following late motoneuron removal muscle afferents appear to innervate their embryologically appropriate muscles. These results cannot be taken as evidence that sensory neurons, like motoneurons, are specified with respect to their peripheral connectivity, since all spatial and temporal guidance cues in the limb except motoneurons were still intact. To test for sensory specification directly, we challenged sensory neurons to find and

FIG. 6. Sensory innervation of muscles in shifted limbs arises from more rostral and more widespread segmental levels than the motor innervation. Uncleared whole-mount views of the ventral spinal cord and DRG in three embryos with limb shifts; individual muscles were injected with Dil or Dia at Stage 33 (E8). Left side of each: control limb. Right side of each: shifted limb. In A the sartorius muscle was injected, in B the femorotibialis muscle, and in C the adductor muscle.
innervate their correct targets in a novel environment, without potential guidance cues from motoneurons. To this end we created a mismatch between the location of DRG and their usual point of entrance into the limb by shifting a hindlimb bud several segments rostral at Stage 17–18, prior to axon outgrowth, and then removed motoneurons at Stage 21. Under these conditions, innervation of embryologically appropriate targets by sensory neurons would provide

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FIG. 7. Sensory neurons make more mistakes than motoneurons following limb shifts. Bar graphs summarizing sensory and motor projections to the sartorius muscle (A), the femorotibialis muscle (B), and the adductor muscle (C) following limb shifts. Individual muscles were injected with DiI or DiA, and distribution of labeled sensory and motoneurons was plotted. Narrower bars indicate that segments contained fewer labeled cells than normal. Note that following limb shifts sensory innervation of individual muscles arises from more rostral levels than the motor innervation and from more rostral and widespread segmental levels than normal.
strong evidence that the neurons were specified. As a control we simply shifted the limb, leaving motoneurons intact. We expected that after a simple limb shift, both sensory and motor axons would alter their trajectory through the limb to innervate their embryologically appropriate targets, as previously reported (Lance-Jones and Landmesser, 1981; Honig and Landmesser, 1986).

To our surprise, we found that after simple limb shifts, muscle sensory projections differed from motor projections to the same muscle. Whereas muscles were generally innervated by motoneurons from the appropriate segmental levels, sensory innervation consistently arose from more rostral segmental levels than normal and from more rostral segmental levels than the motor innervation. These findings clearly demonstrate that outgrowing sensory axons do not simply follow motor axons to muscles since sensory neurons appear to make more mistakes than motoneurons when confronted with the same altered periphery.

We noticed another difference between pathway selection by sensory and motor axons. Sensory axons that projected into the wrong plexus following a limb shift seldom left that plexus to take a novel route to their embryologically correct target, as motoneurons usually do (Lance-Jones and Landmesser, 1981; Honig and Landmesser, 1986; see also Lance-Jones and Landmesser, 1980). The only such aberrant sensory pathways we observed were projections to the adductor muscle, which is located quite close to the sciatic plexus. We never saw sensory afferents taking novel pathways through the sciatic plexus to innervate the femorotibialis or sartorius muscles, which lie much farther from the sciatic plexus. Since these aberrant pathways could only be observed with orthograde labeling, which delineated sensory but not motor axons, we could not determine whether sensory axons navigated these novel pathways on their own or followed aberrant pathways laid out by motor axons. Nevertheless, as discussed below, these observations suggest that the cues that sensory neurons use to navigate to muscle appear to be quite restricted in distribution, irrespective of whether the signals are specific.

Our results appear to differ from those of Honig and co-workers, who reported that both sensory and motor innervation arose from the appropriate segments following limb shifts (Honig et al., 1986). This difference is probably more apparent than real. Honig and co-workers analyzed sensory and motor innervation patterns with retrograde labeling in only three muscles in embryos with limb shifts. In one of these, sensory innervation was derived from one segment more rostral and from one segment more caudal than the motor, much like the innervation patterns we often observed. Moreover, we occasionally observed normal sensory and motor innervation patterns following a limb shift, similar to two of the three innervation patterns they described. Thus, the apparent discrepancy between the present and previous results most likely represents a differ-
ence in emphasis and interpretation. Honig et al. focused on the similarities between sensory and motor patterns and the large amount of correct innervation. We find more interesting, and therefore have emphasized, the differences between motor and sensory patterns, as well as the seemingly larger number of mistakes made by sensory vs motoneurons.

Specification of Sensory Neurons?

Motoneuron identity is specified prior to axon outgrowth (Landmesser, 1992; Appel et al., 1995; Matise and Lance-Jones, 1996) by cues derived from the paraxial mesoderm (Ensini et al., 1998). Our findings indicate that specification of sensory neuron identity must differ in some way from that of motoneurons. Sensory innervation of muscles in anterior limb arose from both thoracic (inappropriate) and lumbosacral (appropriate) segments, whereas motor innervation arose primarily from lumbosacral (appropriate) segments. Sensory neurons that innervated appropriate muscles were able to do so without guidance by motor axons, as some appropriate innervation persisted in the absence of motoneurons.

One possible explanation for these findings is that there may be regional differences in the pathfinding capabilities of DRG neurons, for example, between thoracic and lumbosacral DRG neurons. Differences between thoracic and lumbosacral motoneurons are well documented (Tsuchida et al., 1994; Daston and Koester, 1996). DRG in these two regions clearly differ in their trophic dependencies (Hory-Lee et al., 1993). Moreover, we found that following limb shifts thoracic DRG project to limb targets without joining the crural plexus, quite different from the behavior of lumbosacral DRG. Thus, thoracic and lumbosacral DRG could indeed be different. Perhaps sensory neurons in lumbosacral DRG are specified and have pathfinding capabilities similar to those of motoneurons (thereby accounting for the appropriate innervation in embryos with limb shifts), whereas thoracic sensory neurons simply grow straight into the limb and innervate the nearest available targets (thereby accounting for the mistakes that occur following limb shifts).

Another possible explanation for the large number of mistakes in sensory innervation is that sensory neurons may be less capable than motoneurons of detecting or responding to their usual guidance cues in the novel environment created by limb shifts. Growth cones of sensory axons are smaller than growth cones of motor axons (Tosney and Landmesser, 1985; Landmesser and Honig, 1986), thus they can sample a smaller area of their local environment. Therefore, even if both motor and sensory neurons are rigidly specified and use the same cues to find their targets, sensory neurons are clearly at a disadvantage. Conceivably the two types of neurons may use different guidance cues, with those of sensory neurons having more restricted distributions than those of motoneurons. The finding that rostral sensory neurons that are incorrectly channeled into the sciatic plexus take novel pathways to innervate the adductor muscle, which lies quite near the sciatic plexus, but never leave the sciatic plexus to project to the sartorius or femorotibialis muscles, which lie farther away, suggests that sensory neurons may detect cues from muscle over a very restricted distance.

A third possibility is that sensory neurons may generally be less rigidly specified, and therefore more flexible, in their pathway choices than motoneurons. Recently, evidence in support of this suggestion has been reported. When a limb bud is removed, both motor and sensory neurons are deprived of their targets. Under these conditions most motoneurons die, but most sensory neurons

FIG. 8. Sensory projections following limb shift and motoneuron removal are similar to projections following simple limb shifts. Each horizontal bar represents the location of sensory neurons retrogradely labeled with Dil or Dsl in the sartorius, femorotibialis, or adductor muscles in a single embryo following limb shift and subsequent motoneuron removal. Narrower bars indicate that the DRG contained fewer labeled cells than normal. Note that sensory projection patterns are indistinguishable from those in Fig. 7.
survive, apparently because they project to ectopic targets nearby (Calderó et al., 1998). Thus, sensory neurons appear to be much less selective than motoneurons in their pathway and target choices, suggesting that their identities with respect to these characteristics are indeed unspecified or quite plastic at the time of axon outgrowth.

In fact, it may not be necessary for outgrowing sensory neurons to be rigidly specified in order for appropriate sensory function to develop. Sensory neurons could be nonselectively guided to targets by local cues that they encounter in the limb, including cues from neighboring motor axons. Neuron identity, in particular a neuron’s central connections (Wenner and Frank, 1995), could then be determined by signals from the target. This type of developmental mechanism ensures sensory function is appropriate, irrespective of the initial degree of specification or precision of outgrowth of the neurons themselves.

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