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The spatial relationships among cutaneous, muscle sensory and motoneuron axons during development of the chick hindlimb

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SUMMARY

Previous studies have suggested that interactions with other axons are important in sensory axon pathfinding in the developing chick hindlimb. Yet the nature of these interactions remains unknown, in part because information about the spatial relationships among the different kinds of axons is lacking. To obtain this information, we combined retrograde axonal tracing with an immunofluorescent labelling approach that distinguishes between sensory and motoneuron axons. This allowed us to follow the trajectories of sensory axons having a known destination, while also identifying their neighbors.

We found that as sensory and motoneuron axons meet in the spinal nerves and travel into the limb, sensory axons remain bundled together. The large bundles that are present proximally gradually split into smaller bundles as the axons course distally in the spinal nerves; more distally, some bundles join to again form large bundles. Younger, later-growing sensory axons appear to grow primarily along bundles of older sensory axons that grew out earlier.

Starting from very proximal levels, axons projecting along an individual cutaneous nerve are found together in bundles that are situated in characteristic regions of each spinal nerve. Some of these bundles are initially interspersed with bundles of axons projecting along other nerves, thereby indicating that the initial position of a cutaneous axon in the spinal nerves does not strictly determine its subsequent trajectory. As they travel distally, bundles of axons projecting along one cutaneous nerve gradually join one another, becoming increasingly separated from axons having different destinations.

In contrast, muscle sensory axons are situated adjacent to motoneuron axons innervating the same muscle for much of their course. This suggests that muscle sensory axons may be guided to the appropriate muscles by fasciculating along motoneuron axons.

Taken together, the results show that sensory axons projecting along different nerves are different from one another and respond to cues in their environment to navigate through the spinal nerves and plexus. Thus, sensory neurons must be intrinsically specified with respect to their peripheral targets. Sensory axons appear to respond differentially to the axons they encounter, segregating from axons that project along different nerves and often growing with axons destined for the same nerve, suggesting that fasciculation may aid pathfinding.

Key words: Pathfinding, Neuronal specification, Axonal guidance, Fasciculation, Chick

INTRODUCTION

In the chick hindlimb, axons of motoneurons from a single lumbosacral (LS) spinal cord segment and of sensory neurons in the corresponding dorsal root ganglion (DRG) meet and intermix as they travel distally in a given spinal nerve. Axons from several spinal nerves converge at the base of the limb in one of two plexuses and later diverge to grow along different peripheral nerves to reach their targets. During development, both motoneuron and sensory axons choose the correct peripheral nerves from the outset (Landmesser, 1978; Lance-Jones and Landmesser, 1981a; Honig, 1982). Embryonic manipulations have revealed that motoneuron axons are able to grow to their correct targets because they respond to specific limb-associated cues (Lance-Jones and Landmesser, 1980, 1981b; Lance-Jones and Dias, 1991). Sensory axons may similarly be responsive to limb-associated cues and, in addition, may be affected by nearby motoneuron axons. When motoneurons are removed before axonal outgrowth into the limb, sensory axons that normally project to muscles do not do so, and instead project along cutaneous nerves (Landmesser and Honig, 1986). Further, after anterior-posterior reversals of the neural crest alone (Scott, 1986; Wang and Scott, 1997) or together with the underlying neural tube (Honig et al., 1986), the segmental pattern of sensory projections tends to mimic that of the underlying motoneurons, as it normally does. Thus, motoneuron axons appear to influence the pathfinding decisions of sensory axons.

Although axonal interactions contribute to sensory axon pathfinding, the nature of these interactions and their cellular and molecular bases are not known. In fact, it has been difficult to determine what types of interactions could conceivably occur because our knowledge of the precise spatial relationships among growing axons has come primarily from

single labelling studies and has therefore been very limited. For example, we do not know if muscle sensory axons could be guided through the plexus by fasciculating along motoneuron axons because we do not know if these axons travel separately or together. To address these kinds of issues, a means of simultaneously identifying the multiple types of axons is required. In the studies described here, we employed a novel approach in which we combined a retrograde axonal tracing approach with immunofluorescence procedures that allowed us to distinguish sensory axons from motoneuron axons. This approach, in conjunction with the high resolution provided by confocal laser scanning microscopy, enabled us to follow axons having a known destination, while also identifying their neighbors, and thus to determine whether cutaneous, muscle sensory and motoneuron axons travel separately or together. The results provide new insights about sensory neuron specification and sensory axon pathfinding.

MATERIALS AND METHODS

Retrograde labelling

White Leghorn chick embryos were incubated, typically until stage 30 (Hamburger and Hamilton, 1951), removed from the egg and placed into a bath of oxygenated Tyrode solution. Each embryo was decapitated and eviscerated, and a ventral laminectomy was performed to expose the spinal cord. Axons projecting along either the lateral femoral cutaneous (LFCt), the medial femoral cutaneous (MFCt), or the sartorius muscle nerve were retrogradely labelled using fluorescent dextran amines, as described previously (Honig and Rutishauser, 1996). When only one nerve was labelled in a particular limb, either rhodamine dextran amine (RDA) or Texas red dextran amine (TRDA) was used. When two nerves were labelled, the second dye used was fluorescein dextran amine (FDA). In a few embryos, the LFCt nerve was labelled at stage 25, by removing a piece of skin from the anterior-lateral part of the thigh, so that the forming LFCt nerve was cut; RDA was immediately puffed onto the area and allowed to remain for several minutes. All dextran amines were 10×10^3 M_r and lysinated (Molecular Probes). After incubating for 6-15 hours at 28-32°C to allow for dye transport, embryos were fixed in 4% paraformaldehyde, 10% sucrose, 0.1 M phosphate buffer and subsequently cryostat-sectioned at 20 μm, transverse to the long axis of the thigh, so that the plexus and spinal nerves were cut in cross section. The sections were mounted directly, in serial order, onto Superfrost/Plus slides (Fisher). Prior to immune staining, sections were washed in phosphate buffer and then permeabilized with 1% digitonin in phosphate buffer for 30 minutes.

Immunofluorescence procedures

As we are unaware of any antibodies that specifically label developing motoneuron axons, in order to distinguish between sensory and motoneuron axons we double-labelled sections using one antibody that stains all axons and a second antibody that labels only sensory axons. Axons that were not labelled with the sensory-specific antibody were considered to be motoneuron axons.

To label all axons, we used R020, a rabbit polyclonal antibody that recognizes chick G4/L1 (provided by Urs Rutishauser). In preliminary experiments, we verified that G4/L1 is expressed on all peripheral axons from early stages (Thiery et al., 1985) by double-labelling sections with R020 and an anti-neurofilament antibody. With both antibodies, all peripheral nerves were labelled. Within the nerves, there were small, unlabelled spaces (Fig. 1), which were shown, by counterstaining the sections with bisbenzimide or propidium iodide, to be occupied by non-neuronal cells (not shown). With R020, there was occasionally some additional labelling around the periphery of the nerve trunks, probably because G4/L1 is also expressed on some glial cells (Dahm and Landmesser, 1988). However, because these differences were minor and because R020 penetrated through the tissue sections better than the neurofilament antibody, we used R020 for these studies.

To label sensory axons, we used 23.4-5, a mouse monoclonal antibody that recognizes axonin-1 (made by Jessell and Dodd and obtained from the Developmental Studies Hybridoma Bank). Axonin-1 is expressed transiently by motoneuron axons, being down-regulated by stage 25, but is expressed by sensory axons throughout embryonic development (Halfter et al., 1994). In preliminary experiments, we verified that 23.4-5 specifically labels sensory axons by showing that: (1) cutaneous nerves were labelled in their entirety with 23.4-5, (2) if we anterogradely labelled sensory axons, by injecting TRDA into DRGs, those axons were also labelled with 23.4-5, and (3) if we anterogradely labelled motoneuron axons, by injecting TRDA into ventral roots, those axons were not labelled with 23.4-5. We also found that 23.4-5 labels muscle sensory axons (see below) and is not specific for only cutaneous axons as claimed by Halfter et al. (1994).

When we retrogradely labelled one peripheral nerve, we visualized 23.4-5 with an anti-mouse secondary antibody conjugated to fluorescein and R020 with an anti-rabbit secondary antibody conjugated to Cy5. When we retrogradely labelled two peripheral nerves, the 23.4-5 labelling was visualized with a Cy5-conjugated anti-mouse secondary antibody. In this situation, only those motoneuron axons that were retrogradely labelled were visualizable, but the general outlines of the plexus and the spinal nerves were still discernable.

Microscopy

Sections were mounted in glycerol in carbonate buffer and viewed using a BioRad confocal laser scanning microscope. We routinely used a 40× oil immersion objective with a numerical aperature of 1.30 or 1.35, or, occasionally, a 60× or 100× objective with a numerical aperature of 1.4. For every field of view, three images were captured, each showing the labelling with one fluorophore, and saved to an optical disk. Images from selected levels were photographed. These images were then contrast-enhanced and merged using either Comos or Lasersharp software, and the merges were photographed. Analysis was done by examining the photographs and additional images on the computer monitor. For publication, images were processed using Adobe Photoshop. Although modifications were done to optimize color distinctions and contrast, we always compared the final merged images with the initial, unmodified single images to verify that the original distributions of labelled axons were accurately represented.

A potential concern is that isolated axons that were labelled either retrogradely or with the sensory marker might go undetected with our standard viewing conditions using the 40x objective. However, we do not think this occurred, because we did not see more small labelled profiles when we examined sections at higher magnification and higher resolution, and yet we have observed small labelled profiles in embryos subjected to certain experimental perturbations (Honig et al., 1997). Further, our recent EM observations confirm the confocal results described later in this paper in showing that sensory axons are typically grouped together in bundles (Xue and Honig, 1997). Thus, we believe that with the methodology used, if isolated labelled axons were present in significant numbers, we would have detected them (see also Results).

It was also crucial for these experiments that the injected axonal tracer did not spread significantly to other nerves (see Honig and Rutishauser, 1996 for a description of how this was assessed) and that the retrograde labelling of individual peripheral nerves was highly effective. We verified the latter for each limb that we included in our analysis using two criteria. First, the number of retrogradely labelled cells (for counting methods, see Honig and Rutishauser, 1996) had to be within the range that we have found typical for that particular nerve at stage 30. Second, the injected nerve had to appear completely

labelled when viewed in tissue sections with the confocal microscope. Based on these criteria, we are certain that the vast majority of axons were labelled in each case, but we cannot be certain that every axon was labelled. Indeed, the axons of those sensory neurons that are born at stage 28-29 (McPheeters, 1982) could not have reached the injection site, and so could not be labelled. To overcome this problem, we carried out some experiments on stage 32-34 embryos and came to conclusions similar to those for stage 30 embryos. However, labelling with the sensory-specific antibody was considerably dimmer at stage 32-34, and so we carried out most experiments at stage 30. The results are based on successful labelling of 21 limbs at stage 30, 5 at stage 32-34, and 4 at stage 25.

Limb deletions

After 2 days of incubation, a window was made in the eggshell and the embryo was stained with neutral red. At stage 17, the right hindlimb bud was removed using fine iridectomy scissors. The eggs were sealed with Scotch transparent tape and returned to the incubator. At stage 27-29, embryos were removed from the egg, placed into oxygenated Tyrode, partly dissected, fixed, sectioned, and processed for double-immunofluorescence as described above. To better reveal the defasciculation exhibited by sensory axons in this situation (see Results), images from these embryos were captured so that most of the depth of each section could be visualized. We did this by using an iris setting of 3 or 4, and using either a 10× objective, or the 40× objective and capturing a z-series through each section. The results are based on 13 embryos for which the limb deletion was complete.

RESULTS

Overall organization of sensory axons

As a first step in assessing the spatial relationships among the different types of limb-innervating axons, we double-labelled sections from stage 30 embryos with the ‘pan-axonal’ marker, R020, and the sensory-specific marker, 23.4-5. Sensory and motoneuron axons enter the spinal nerves from a dorsal and a ventral position, respectively, and soon become intermixed. As shown in Fig. 1, we found that, even when motoneuron axons (red) and sensory axons (yellow) intermix, the sensory axons stay bundled together. At very proximal levels, sensory axons are grouped together in large bundles that gradually split into smaller bundles as the axons extend distally in the spinal nerves. Within the plexus, some bundles join together to form very large bundles while other bundles remain separate and small.

Information about the numbers of axons provides additional insight into their organization. Based on counts of motoneurons in the spinal cord (Hamburger, 1975) and of sensory neurons in individual DRGs (Oakley et al., 1997; Honig, unpubl. observ.), we estimate that spinal nerves LS1-LS3 each contain ~2000 motoneuron axons and about three times as many sensory axons. However, the areas occupied by sensory and motoneuron axons are roughly equivalent, and so sensory axons must be smaller in diameter than motoneuron axons and/or more densely packed (see also below). Further, for the section shown in Fig. 1E, for example, we would estimate that the largest sensory bundles probably contain about 500 axons and the smallest about 10 axons. Thus, although we cannot be certain that single sensory axons are never present and escape detection, it is clear that, throughout

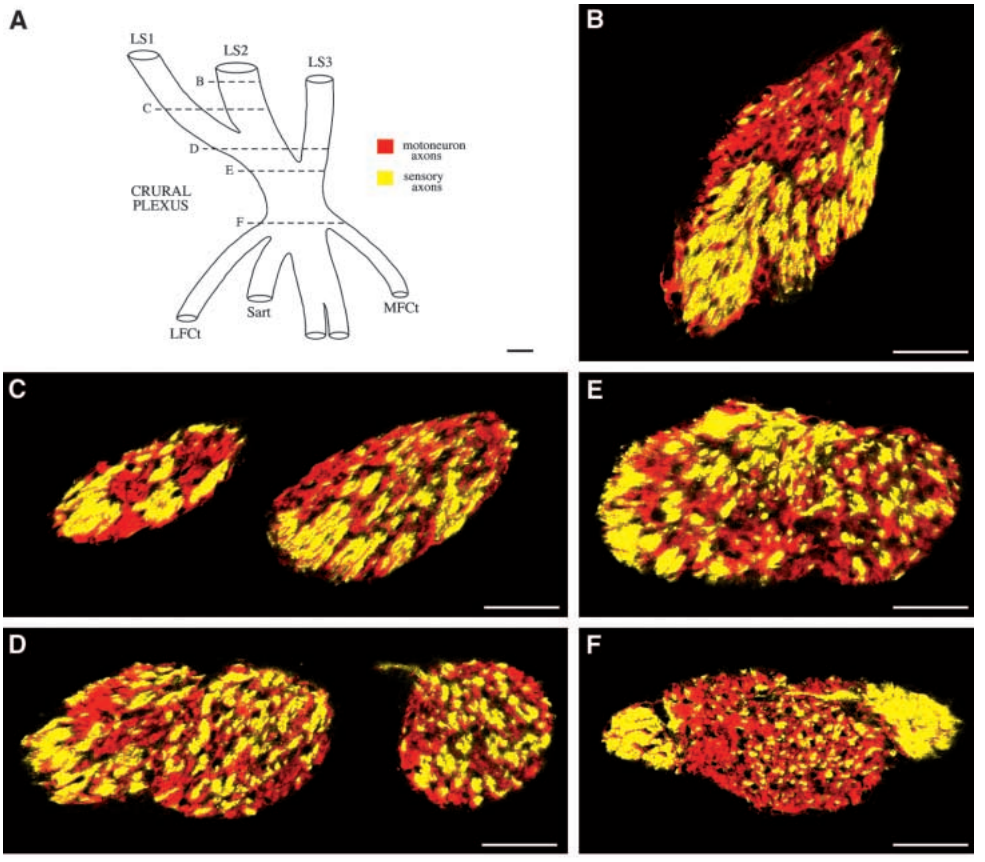

Fig. 1. The spatial relationships between sensory and motoneuron axons. Sections from a stage 30 embryo were processed for double-immunofluorescence as described in the Materials and Methods. (A) The nerve pattern in the anterior part of the hindlimb, drawn to scale. The first three lumbosacral spinal nerves (LS1-3) converge in the crural plexus. Only the dorsal component of the crural plexus is shown. Several peripheral nerves diverge from the crural plexus; those discussed in the text are shown: the lateral femoral cutaneous (LFCt), medial femoral cutaneous (MFCt), and the sartorius nerve (Sart). Anterior is to the left, proximal toward the top. (B-F) Cross-sections through the plexus and spinal nerves at the levels indicated in A. Sensory axons are found in bundles that are large proximally and gradually split into smaller bundles as the axons travel in the spinal nerves and intermix with motoneuron axons. Within the plexus, some bundles join together to form large bundles while others remain separate and small. The small sensory bundles in the ventral part of spinal nerve LS2 in B may be visceral afferents. For B-F, anterior is to the left, ventral toward the top. Scale bars, 100 μm for A, 50 μm for B-F.

the spinal nerves and plexus at stage 30, sensory axons are primarily found bundled together.

Retrograde tracing of cutaneous axons

To visualize the trajectories of axons that project along specific cutaneous nerves, we initially combined retrograde labelling with the double-immunofluorescent labelling described above. In later experiments, we retrogradely labelled both the LFCt and the MFCt nerves and used single-immunofluorescent labelling to visualize sensory axons. An example of the latter type of experiment is shown in Fig. 2.

As they exit the DRGs, LFCt axons are spread through much of the anterior-posterior extent of individual spinal nerves (not shown). Within 100 μm, LFCt axons (yellow) are more localized, with the vast majority being situated either in bundles that appear to contain only other LFCt axons or in restricted parts of bundles that also contain sensory axons projecting to other targets (Fig. 2B-D). This bundling is best appreciated if one considers the numbers of retrogradely labelled DRG neurons. For the embryo shown in Fig. 2A-G, 167 neurons were retrogradely labelled in DRG T7, 1560 in DRG LS1, 392 in DRG LS2, and 22 in DRG LS3. Accordingly, even the smallest of the yellow profiles in Fig. 2B-D are likely to represent bundles of 5 to 10 retrogradely labelled axons, rather than single, isolated retrogradely labelled axons. At progressively more distal levels of the spinal nerves (Fig. 2E), an increasing proportion of LFCt axons are found in bundles that are composed solely of LFCt axons, and a decreasing proportion in bundles that also contain sensory axons projecting to other targets. At progressively more distal levels of the plexus, bundles of LFCt axons are increasingly larger, more frequently contain only LFCt axons and are more separated from other kinds of axons (Fig. 2F-G). Before the LFCt nerve diverges, LFCt axons are grouped in several very large bundles in the anterior-most part of the crural plexus, completely segregated from other axons (Fig. 2G).

In the proximal spinal nerves, MFCt axons (light blue) are restricted in their locations and are found primarily in bundles with other MFCt axons (Fig. 2B-D). Given the numbers of retrogradely labelled MFCt neurons (550 in DRG LS1, 1198 in DRG LS2, and 167 in DRG LS3), most of these bundles probably contain at least 50 MFCt axons. At progressively more distal levels, bundles containing MFCt axons are increasingly larger, more homogeneous in their axonal composition, more separated from other axons, and gradually situated more ventrally and posteriorly (Fig. 2E-G). Before they diverge along the MFCt nerve, MFCt axons are grouped together in the posterior part of the plexus (Fig. 2G).

These observations indicate that LFCt axons tend to travel with other LFCt axons, while MFCt axons tend to travel with other MFCt axons. As the axons course distally toward the limb, bundles of axons destined for the same cutaneous nerve gradually join together, forming progressively larger bundles,

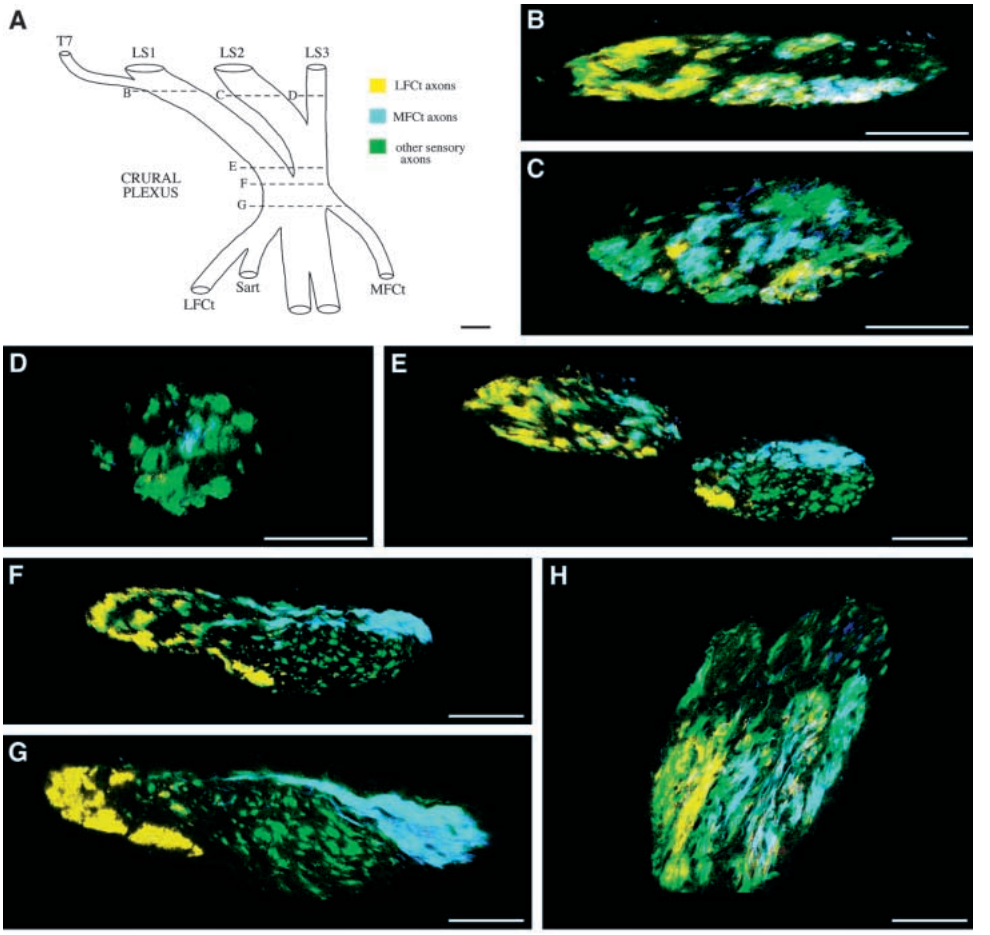

Fig. 2. The trajectories of cutaneous axons. The LFCt and MFCt nerves were retrogradely labelled with FDA and TRDA, respectively, and sections were processed for immunofluorescence to label sensory axons. (A) The nerve pattern in the anterior part of the hindlimb, as in Fig. 1A. In this embryo, spinal nerve T7 contributed to the crural plexus. (B-G) Cross sections at the levels indicated in A. LFCt and MFCt axons travel separately for most of their course. Axons projecting along each cutaneous nerve are bundled together and occupy characteristic regions of each spinal nerve. In B and C, some small bundles of LFCt axons are intermixed with small bundles of MFCt axons; these areas appear white. That these axons later segregate from one another means that their initial position in the spinal nerves does not strictly determine their subsequent trajectory. (H) Cross section through spinal nerve LS2, from a different embryo, only 40 μm from the DRG. LFCt and MFCt axons are each restricted in their locations. For B-H, anterior is to the left, ventral toward the top. The few dark blue spots barely visible in B-D and H represent motoneuron axons that were labelled by spread of the TRDA. Scale bars, 100 μm for A, 50 μm for B-H.

which eventually come to occupy a characteristic, discrete position in the plexus.

Two additional features of the organization of cutaneous axons in the proximal spinal nerves are notable. First, LFCt axons and MFCt axons each occupy characteristic positions within each spinal nerve. LFCt axons are concentrated in the anterior part of spinal nerve LS1, whereas MFCt axons are typically found posteriorly (Fig. 2B). In spinal nerve LS2, most LFCt axons are situated either anteriorly or dorsally, whereas most MFCt axons are located more centrally (Fig. 2C). While there is some normal variation in segmental projection patterns among embryos (Tyrrell et al., 1990; Honig and Rutishauser, 1996), the different types of cutaneous axons occupy characteristic positions relative to one another, despite this variation. For example, when the LS2 contribution to the LFCt is especially large, the bundles of LFCt axons in spinal nerve LS2 are larger and/or more numerous than shown in Fig. 2C, but they are still located predominantly anteriorly or dorsally. Similarly, when the LS1 contribution to the MFCt is especially large, although some MFCt axons are situated more anteriorly in spinal nerve LS1 than shown in Fig. 2B, they are still posterior to the majority of LFCt axons. Surprisingly, this spatial organization of cutaneous axons is sometimes evident very proximally, within 50 μm of the DRGs (Fig. 2H).

Second, the extent to which axons that project along the two cutaneous nerves initially intermix with one another is very limited. In Fig. 2, regions where LFCt and MFCt axons overlap, for example, the posterior part of spinal nerve LS1 (Fig. 2B), appear white. However, careful examination of the labelling with the individual fluorophores, together with the counts of retrogradely labelled neurons, suggest that in this area of overlap the retrogradely labelled axons travel in bundles of at least 3 axons. It is our impression that these small bundles lie in close proximity to one another but do not appear separated in the merged images because the resolution with the 40× objective (about 0.4 μm in the x-y axis, and about 0.9 μm in the z axis) is greater than the diameter of the sensory axons (typically 0.2-0.5 μm; Xue and Honig, 1997). This viewpoint is supported by the finding that when we sectioned some embryos so that spinal nerves LS1 and LS2 were more in cross section, to minimize the overlapping of axons in the z axis, and/or when we used a 60× objective to provide increased resolution (to 0.27 μm in the x-y axis and to 0.42 μm in the z axis), there was very little apparent intermixing of the two kinds of cutaneous axons (not shown).

Retrograde tracing of muscle nerve axons

To visualize the spatial relationships between the muscle sensory and motoneuron axons projecting to individual muscles, we combined retrograde labelling of the sartorius

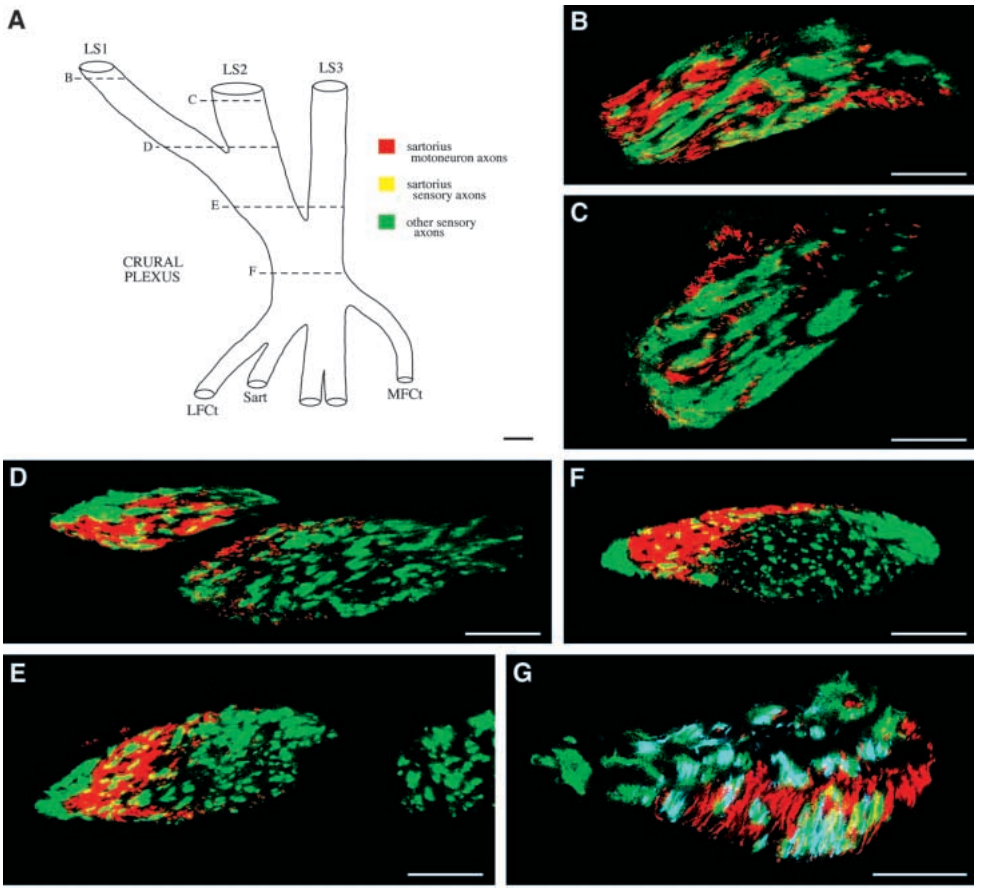

Fig. 3. Muscle sensory and motoneuron axons. The sartorius nerve was retrogradely labelled with RDA, and sections were processed for double-immunofluorescence. (A) The nerve pattern in the anterior part of the hindlimb, as in Fig. 1A. (B-F) Cross sections at the levels indicated in A. Merged images of the RDA and the sensory axon labelling are shown. Most sartorius sensory axons are located adjacent to sartorius motoneuron axons except for very proximal levels (B and C) where some sartorius sensory axons travel separately. Muscle sensory axons travel partway along the spinal nerves before contacting motoneuron axons, indicating that they are capable of some independent navigation, but after coming into contact, muscle sensory axons may follow motoneuron axons the rest of the way to their target muscle. In F, the green centers within the bundles of sartorius sensory axons may be sartorius sensory axons that were still growing toward, but had not reached, the site of RDA injection. (G) Cross section through spinal nerve LS2, from a different embryo, 100 μm from the DRG. The LFCt nerve and sartorius nerve were labelled with FDA and TRDA respectively, and sections were processed for immunofluorescence to reveal sensory axons. At this proximal level, a few LFCt axons (light blue) and some but not all sartorius sensory axons lie adjacent to sartorius motoneuron axons. Thus, a sensory axon's initial proximity to motoneuron axons does not strictly determine its subsequent trajectory. For B-G, anterior is to the left, ventral toward the top. Scale bars, 100 μm for A, 50 μm for B-G.

nerve with double-immunofluorescent labelling. An example of this type of experiment is shown in Fig. 3A-F.

In the proximal spinal nerves, some sartorius sensory axons (yellow) are located adjacent to sartorius motoneuron axons (red), and others are intermixed with sensory axons (green) innervating other targets (Fig. 3B,C). At progressively more distal levels of the spinal nerves, an increasing proportion of sartorius sensory axons are found adjacent to sartorius motoneuron axons (Fig. 3D,E). In the plexus, sartorius axons are grouped together and, at progressively more distal levels, are increasingly segregated in the anterior part of the crural plexus, adjacent to the region occupied by LFCt axons (Fig. 3F). Within this region, sartorius sensory axons lie adjacent to or are surrounded by sartorius motoneuron axons. The continued proximity of these axons suggests that, once muscle sensory axons are positioned next to motoneuron axons, they may subsequently grow along them and thus be guided the rest of the way to their target muscle.

Information about the numbers of sartorius axons provides additional insight into their organization. In the embryo for which sections are shown in Fig. 3B-F, 640 motoneurons and 540 sensory neurons (350 in DRG LS1, and 188 in DRG LS2) were retrogradely labelled. Given that the total area occupied by the sensory axons is much less than the total area occupied by sartorius motoneuron axons, muscle sensory axons must be smaller in diameter and/or more densely packed than motoneuron axons. Further, even the smallest (yellow) labelled profiles must represent groups of sartorius sensory axons rather than individual axons. Thus, muscle sensory axons, like cutaneous axons, appear to travel together in bundles.

We have previously suggested that a sensory axon's initial position could potentially determine its ultimate fate (Honig, 1982). To examine this possibility, we assessed whether muscle sensory axons are found in closer proximity to motoneuron axons than are cutaneous axons as they enter the spinal nerves. We retrogradely labelled both the sartorius nerve and the LFCt nerve, which each receive a major contribution from LS1, and sectioned these limbs so that spinal nerve LS1 was cut in cross section. An example of this type of experiment is shown in Fig. 3G. As just described, in proximal spinal nerve LS1, some, but not all, sartorius sensory axons (yellow) are adjacent to sartorius motoneuron axons (red). Although the vast majority of LFCt axons (light blue) travel separately from sartorius motoneuron axons, a few LFCt axons are close to sartorius motoneuron axons. Thus, there is not a strict relationship between a sensory axon's initial proximity to motoneuron axons and its subsequent choice of a muscle or a cutaneous nerve.

The organization of the plexus at earlier and later stages

We also examined the spatial relationships between sensory and motoneuron axons at stage 25, when individual peripheral nerves first form. By this time, all motoneuron axons, but only some sensory axons have reached the plexus (Tosney and Landmesser, 1985a; Landmesser and Honig, 1986). At stage 25, the plexus is more loosely fasciculated than at stage 30 (compare Fig. 4A and C), but sensory axons already form bundles. These bundles are smaller than at stage 30, but the number of bundles is similar, suggesting that later-growing sensory axons grow along sensory axons that grew out earlier.

To examine the trajectories of the earliest cutaneous axons, we retrogradely labelled the LFCt nerve at stage 25. As shown in Fig. 4B, the retrogradely labelled axons are restricted to the anterior part of the plexus, as they are at stage 30 (Fig. 2G), but within this region, they are scattered, indicating that sensory axons are not simply channelled into a cutaneous nerve from a single location in the plexus. Further, since 275 DRG neurons were retrogradely labelled in this embryo, each of the RDA-labelled profiles in Fig. 4B must represent a bundle of 3 or more LFCt axons. Thus, even by stage 25, sensory axons having a common destination appear to travel together in bundles. Although some retrogradely labelled LFCt axons are adjacent to sensory axons that were not retrogradely labelled

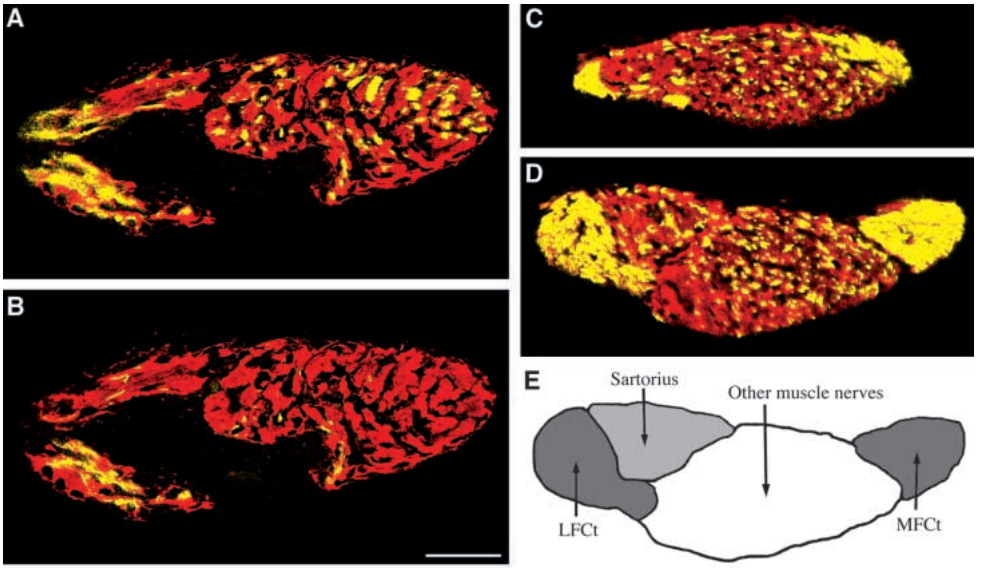

Fig. 4. The crural plexus at different stages. (A,B) A cross section through the plexus from a stage 25 embryo in which the LFCt nerve was retrogradely labelled. The sections were processed for double-immunofluorescence. (A) Sensory axons are yellow and motoneuron axons are red. Sensory axons are already found in bundles. (B) LFCt axons are yellow and all other axons are red. The first axons to grow along the LFCt nerve diverge from a variety of positions within the plexus. (C,D) Cross sections through the plexus at stage 30 and stage 32, respectively. Sensory axons are yellow and motoneuron axons are red. The section in C is from a more proximal level than D and so the bundles of cutaneous axons are somewhat more dispersed. While the general organization of the plexus is the same, areas occupied by LFCt and MFCt axons are larger at stage 32 than at stage 30. (E) Drawing of the plexus shown in D, indicating the regions occupied by axons projecting along different nerves. Anterior is to the left, ventral toward the top. Scale bar, 50 μm.

(compare Figs. 4A and B), some of the unlabelled axons may grow along the LFCt nerve at a later stage, and so we cannot conclude from this observation that sensory axons projecting along different nerves are more intermingled at stage 25 than at stage 30.

We also examined some embryos at stage 32-34. The plexus of a stage 32 embryo (Fig. 4D) reveals the same basic organization found at stage 30 (Fig. 4C). Interestingly, the areas occupied by cutaneous axons are larger than in stage 30 embryos, probably because the axons of younger sensory neurons, some of which are born as late as stage 29 (McPheeters, 1982), are still growing toward the plexus at stage 30.

Limb deletions

The observation that different types of sensory axons are highly organized in the proximal spinal nerves was unexpected. As a first step in elucidating the mechanisms responsible for this, we removed the limb bud. These embryos were killed at stage 27-29, rather than at stage 30 when peripheral nerves could easily be retrogradely labelled, because of the extensive neuronal cell death that occurs when axons cannot find their targets (Hamburger and Yip, 1984). When the limb is absent, spinal nerves form, join together and project toward the tail (Tosney and Landmesser, 1984). We found, as shown in Fig. 5, that for most of the length of the spinal nerves, sensory axons appear to be arranged in a normal manner in that they are grouped into several bundles that are interspersed with motoneuron axons. However, starting near the point where the spinal nerves join together, sensory axons defasciculate extensively, in striking contrast to the situation when the limb is present, where sensory axons are still bundled at these levels. Sensory axons remain defasciculated at more distal levels where sensory axons destined for different peripheral nerves normally are clearly segregated from one another and where cutaneous axons normally would have begun to aggregate into progressively larger bundles.

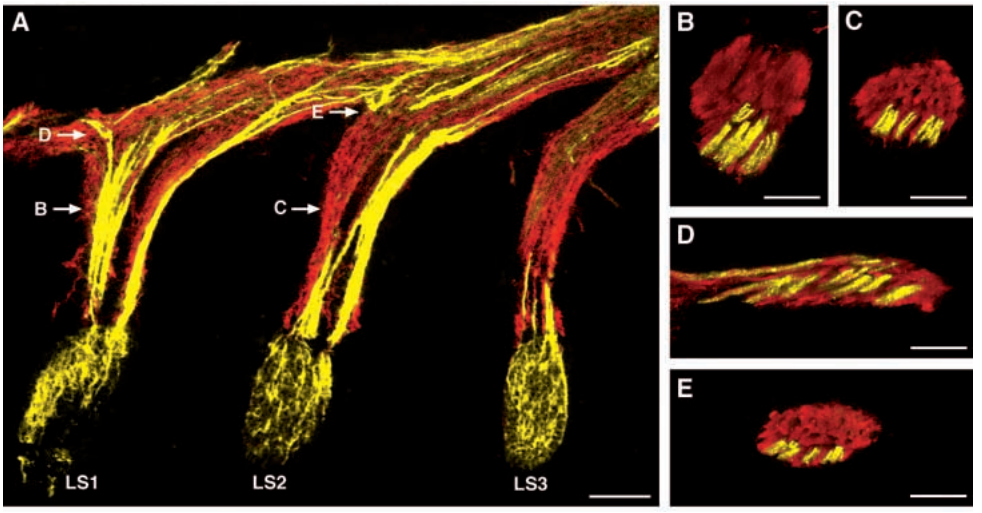

Fig. 5. Limb deletions. Sections from embryos with limb deletions were processed for double-immunofluorescence, as described in the Materials and Methods. Sensory axons appear yellow, motoneuron axons appear red. (A) Cross section from the right side of a stage 29 embryo from which the right hindlimb bud had been removed at stage 17. Although this embryo, like all others in this study, was sectioned transverse to the long axis of the left thigh, it was slightly tilted dorsoventrally such that nearly the entire nerve pattern can be visualized in this one section. Spinal nerves LS1, LS2 and LS3 are shown arising from their respective DRGs, coursing ventrally and distally, and then meeting and projecting posteriorly, toward the tail. In each spinal nerve, sensory axons are grouped into several bundles that are interspersed with motoneuron axons, as they normally are. However, unlike the situation when the limb is present, starting near the point where the spinal nerves join together, sensory axons defasciculate extensively. (B-E) Cross sections from the normal, left side of another similarly staged, experimental embryo, showing spinal nerves LS1 and LS2 at levels comparable to those indicated by the arrows in A. At a midpoint along either spinal nerve LS1 (B) or LS2 (C), sensory axons are grouped in several bundles. More distally (D,E), the bundles of sensory axons have rearranged and are more widely spaced, particularly in spinal nerve LS1 (D), but there is no indication of the extensive defasciculation found when the limb has been removed. For all images, anterior is to the left, ventral toward the top. Scale bars, 100 μm for A, 50 μm for B-E.

DISCUSSION

The studies reported here provide important new information on the positioning of and spatial relationships among cutaneous, muscle sensory, and motoneuron axons, and thereby help clarify the mechanisms underlying sensory axon pathfinding.

The results obtained by retrogradely labelling different peripheral nerves allow us to construct a ‘map’ of the crural plexus (Fig. 4E). Axons projecting along each cutaneous nerve are grouped together: LFCt axons are situated anteriorly, whereas MFCt axons are situated posteriorly. In the central part of the plexus, motoneuron axons surround small bundles of muscle sensory axons. The axons in this region innervate the sartorius, femorotibialis, anterior iliotibialis, and deep dorsal muscles. Sartorius axons occupy a characteristic, triangular area adjacent to the area occupied by LFCt axons. Presumably, the axons innervating the other muscles are similarly segregated within restricted parts of the plexus.

Sensory axon bundling

From earlier single labelling studies, we knew that after motoneuron and sensory axons meet in the spinal nerves they become intermixed, but how completely they intersperse could not be determined (Honig, 1982; Landmesser and Honig, 1986). One striking finding from the present study was that sensory axons are found in bundles along their entire course into the limb. Such bundles could form if growing sensory axons extend preferentially along other sensory axons, perhaps because they adhere more strongly to one another than to motoneuron axons. However, the extent of bundling is not constant. Sensory axon bundles are very large proximally and gradually split into smaller bundles as the

axons travel in the spinal nerves and intermix with motoneuron axons. Subsequently, in the distal spinal nerves and plexus, some bundles (those destined for a particular cutaneous nerve) join, to again form large bundles. The multiple changes in bundling suggest that the adhesiveness of growing sensory axons is modulated over space and time and/or that the adhesive contacts between some axons are weak enough that they can be broken when those axons sense specific environmental cues and diverge to follow different trajectories.

We also found that the number of sensory axon bundles within the crural plexus is roughly constant between stage 25 and 34, and that some bundles get much larger during this time period. Given that sensory neurons are born between stage 19 and 29 (McPheeters, 1982), and thus many sensory axons must reach the plexus after stage 25, these observations suggest that the younger, later-growing sensory axons grow primarily along bundles of sensory axons that are older and grew out earlier. Bundles of cutaneous axons appear to increase more in size than do bundles of muscle sensory axons between stage 25 and stage 34, suggesting that the outgrowth of cutaneous axons is more prolonged than that of muscle sensory axons. This is consistent with the observations of others that cutaneous neurons tend to express TrkA and muscle sensory neurons TrkC, and that TrkA-expressing neurons appear to be generated later in development than those expressing TrkC (Oakley et al., 1997).

Cutaneous axons

These experiments revealed that a short distance after they exit the DRGs, axons that project along individual cutaneous nerves are already fairly restricted in their positions, with LFCt and MFCt axons tending to occupy different, characteristic regions of each spinal nerve. This result was unexpected for two reasons. First, our earlier HRP labelling experiments indicated that sensory axons projecting along any given peripheral nerve are widespread in the proximal spinal nerves (Honig, 1982). We now realize that we reached this incorrect conclusion previously because less than 10% of the axons projecting along each peripheral nerve had been labelled. Second, there is no apparent organization of the different types of neurons within the DRGs (Honig, 1982 and unpubl. observ.), and so how their axons become organized so proximally raises questions as to the mechanism responsible (see below).

The largely separate locations of cutaneous axons in the proximal spinal nerves suggest that LFCt and MFCt axons are intrinsically different from one another and hence, that the different types of cutaneous neurons are specified. Other observations also support this idea. First, small bundles of LFCt axons sometimes intermix with small bundles of MFCt axons in the proximal spinal nerves. That these axons are able to segregate from one another means that their position in the spinal nerves does not strictly determine their subsequent trajectory. Rather, they must respond differently to specific cues present on other axons or in the environment. Second, the first axons to grow along the LFCt nerve diverge from a variety of positions within the plexus, as if they are responding to a target-associated guidance cue, and are not directly channelled into the LFCt nerve based on their location in the plexus. Furthermore, both cutaneous and muscle sensory axons are in the plexus at stage 25, and so timing differences can not explain how sensory axons choose a particular peripheral pathway (see also Honig, 1982).

A striking observation from the experiments on stage 30 embryos was that, within 100 μm of the DRGs, many cutaneous axons are found in bundles that appear to contain only axons destined for that same nerve. Some of these bundles are large and probably contain hundreds of axons, and cutaneous axons become even more bundled as they progress distally. Moreover, even at stage 25, cutaneous axons appear to travel in groups of at least 3 axons. The extent of bundling seems greater than what would occur if the axons simply respond independently to cues in the local environment and/or emanating from the limb. Further, sensory axons appear to be very tightly packed. Together, these observations suggest that cutaneous axons may fasciculate along neighboring axons for much of their trajectory along the spinal nerves, through the plexus and to the skin. In light of our suggestion above that sensory neurons are intrinsically specified to project along particular cutaneous nerves, the observed bundling (and proposed fasciculation) of axons destined for the same cutaneous nerve may not be essential for, but may nonetheless facilitate appropriate pathfinding.

Muscle sensory axons

As they progress distally in the spinal nerves, muscle sensory axons become situated adjacent to motoneuron axons innervating the same muscle. Their continued, close proximity suggests that muscle sensory axons may fasciculate along motoneuron axons. Comparisons between stage 25 and stage 30 embryos further suggest that the earliest muscle sensory axons may grow along motoneuron axons and that later-growing muscle sensory axons may follow the earlier-growing muscle sensory axons. However, other observations indicate that fasciculation by itself cannot completely explain how muscle sensory axons are guided to the appropriate muscles. First, when they enter the spinal nerves, muscle sensory and motoneuron axons are separated and are not noticeably closer to one another than are some cutaneous axons. Second, some muscle sensory axons travel much of the length of the spinal nerves before they contact the appropriate motoneuron axons, and so are capable of at least some independent navigation. Together, these observations suggest that muscle sensory neurons are specified and that their axons, but not those of cutaneous neurons, are able to grow along motoneuron axons.

Specification of sensory neurons and pathfinding mechanisms

As explained above, our results suggest that sensory neurons are specified. Although this requires further testing, other recent studies are also consistent with the idea that rat sensory neurons expressing certain neuropeptides (Hall et al., 1997) and chick muscle spindle afferents (Oakley et al., 1997) are specified at early stages.

In at least some systems, neuronal precursors rather than the neurons themselves become specified (McConnell and Kaznowski, 1991). Indeed, retrogradely labelled sensory neurons are often found in small clusters (Honig, 1982) and in linear arrays (Honig, unpubl. observ.), as if they may be clonally related. In turn, their axons may travel together from the outset, forming the small bundles seen at proximal levels. However, if any spatial segregation of specified neuronal

precursors is initially present within DRGs, it appears to become degraded by subsequent cell movements, because neurons projecting along each peripheral nerve are widely distributed within individual DRGs (Honig, 1982 and unpubl. observ.). Yet some remnants of this organization may be reflected in the organization of axons observed a short distance from the DRGs.

Our results also suggest that sensory axons use at least two types of cues as they grow along the spinal nerves and into the plexus. First, they appear to respond differentially to different types of axons. For example, cutaneous axons projecting along the same nerve gradually associate, forming progressively larger bundles. Such bundling could occur if cutaneous growth cones preferentially adhere to axons of the same type. Differential adhesivity would be mediated, in turn, by cell adhesion molecules (CAMs). While several CAMs are expressed on sensory axons, we do not know of any that are expressed solely on cutaneous or on muscle sensory axons (Honig and Kueter, 1995) or on axons in one cutaneous nerve but not the other. Thus, any possible differential adhesivity may well be based on quantitative differences in the levels of expression of several CAMs. Alternatively, or in addition, repulsive interactions between axons may result in their segregation. For example, cutaneous growth cones may retract specifically from muscle sensory and motoneuron axons. However, specific patterns of repulsive responses between these subpopulations of neurons have not yet been demonstrated (Honig and Burden, 1993) and specific repulsive molecules whose patterns of expression fit the observed pattern of axonal segregation have not yet been identified.

The second type of cue appears to come from the environment. Sensory axons alter their trajectories such that the different types of axons come to occupy characteristic positions in the spinal nerves and plexus. It seems unlikely that this could be explained solely by the types of axonal interactions described above. Further, some of the changes in axonal trajectories occur so proximally that it is difficult to imagine how they could be in response to cues associated with specific limb targets, which are quite a distance away. Nonetheless, when the limb bud is ablated, sensory axons still group into several bundles, as they enter the spinal nerves, and still intermix with motoneuron axons. Thus, this process does not require the limb itself and may to due either to axonal interactions or to responses to local environmental cues. In contrast, the progressive segregation of axons projecting along each peripheral nerve that normally begins in the spinal nerves, requires that the limb be present, and may involve responses to signals emanating directly from the limb. However, the possibilities that axons respond to signals derived from proximal tissues but those signals somehow require the presence of the limb, or that later-growing sensory axons are able to interact normally only with earlier-growing axons that have successfully reached their targets in the limb cannot be excluded.

How can previous experimental results be explained in light of our current view that sensory neurons are specified, their axons responding differentially to neighboring axons and other environmental cues? For some results, the explanation is straightforward. For example, muscle sensory axons may need to fasciculate along motoneuron axons in order to grow to muscles and may therefore fail to do so when motoneurons are removed (Landmesser and Honig, 1986). Other results can be explained if we also propose that changes in the trajectories of sensory axons may be either facilitated or impeded by nearby motoneuron axons depending on the trajectories of those motoneuron axons. This seems plausible, particularly because motoneuron axons grow into the plexus before most sensory axons do, and because sensory growth cones tend to be small, and therefore may explore the environment to only a limited extent (Tosney and Landmesser, 1985b). Accordingly, sensory axons may be able to cross the anterior-posterior axis of the plexus and choose the correct peripheral nerves when motoneuron axons also cross the plexus. For example, after several segments of neural tube and the overlying neural crest are reversed, the axons of both sensory neurons and motoneurons originally from LS1, now in the position normally occupied by LS3 neurons, cross from the posterior side of the crural plexus to the anterior part to project along the appropriate peripheral nerves (Honig et al., 1986). In contrast, sensory neurons, although specified, may be impeded from sending their axons along the correct peripheral nerves if this requires them to cross the anterior-posterior axis of the plexus in a situation where most motoneuron axons do not. For example, after the neural crest is reversed by itself, the axons of LS1 sensory neurons, now occupying the LS3 position, grow out with the axons of LS3 motoneurons and tend to maintain a posterior course through the plexus as do the motoneurons, even though this forces the sensory axons to project along the inappropriate peripheral nerves (Scott, 1986; Wang and Scott, 1997). In a similar way, sensory axons may make pathfinding errors because they are less able to travel across the plexus when CAM function is blocked and their ability to fasciculate is thereby reduced (Honig and Rutishauser, 1996). Future studies will focus on testing these ideas.

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REFERENCES

Dahm, L. and Landmesser, L. T. (1988). The regulation of intramuscular nerve branching during normal development and following activity blockade. *Dev. Biol.* **130**, 621-644

Halfter, W., Yip, Y. P. L. and Yip, J. L. (1994). Axonin-1 is expressed primarily in subclasses of avian sensory neurons during outgrowth. *Dev. Brain Res.* **78**, 87-101.

Hall, A. K., Ai, X., Hickman, G. E., MacPhedran, S. E., Nguaguba, C. O. and Robertson, C. P. (1997). The generation of neuronal heterogeneity in a rat sensory ganglion. *J. Neurosci.* **17**, 2775-2784.

Hamburger, V. (1975). Cell death in the development of the lateral motor column of the chick embryo. *J. Comp. Neurol.* **160**, 535-546.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92.

Hamburger, V. and Yip, J. L. (1984). Reduction of experimentally induced neuronal death in the spinal ganglia of the chick embryo by nerve growth factor. *J. Neurosci.* **4**, 767-774.

Honig, M. G. (1982). The development of sensory projection patterns in embryonic chick hindlimb. *J. Physiol.* **330**, 175-202.

Honig, M. G. and Burden, S. M. (1993). Growth cones respond in diverse ways upon encountering neurites in cultures of chick dorsal root ganglia. *Dev. Biol.* **156**, 454-472.

Honig, M. G., Camilli, S.J. and Kabir, N. (1997). The effect of function-blocking antibodies against DM-GRASP/SC1 on sensory projections in the chick hindlimb. *Neurosci. Abstr.* **23**, 1698.

Honig, M. G. and Kueter, J. (1995). The expression of cell adhesion molecules on the growth cones of chick cutaneous and muscle sensory neurons. *Dev. Biol.* **167**, 563-583.

Honig, M. G., Lance-Jones, C. and Landmesser, L. T. (1986). The development of sensory projection patterns in embryonic chick hindlimb under experimental conditions. *Dev. Biol.* **118**, 532-548.

Honig, M. G. and Rutishauser, U. S. (1996). Changes in the segmental pattern of sensory neuron projections in the chick hindlimb under conditions of altered cell adhesion molecule function. *Dev. Biol.* **175**, 325-337.

Lance-Jones, C. and Dias, M. (1991). The influence of presumptive limb connective tissue on motoneuron axon guidance. *Dev. Biol.* **143**, 93-110.

Lance-Jones, C. and Landmesser, L. T. (1980). Motoneurone projection patterns in the chick hind limb following early partial reversals of the spinal cord. *J. Physiol.* **302**, 581-602.

Lance-Jones, C. and Landmesser, L. T. (1981a). Pathway selection by chick lumbosacral motoneurons during normal development. *Proc. Roy. Soc. London* **214**, 1-18.

Lance-Jones, C. and Landmesser, L. T. (1981b). Pathway selection by embryonic chick motoneurons in an experimentally altered environment. *Proc. Roy. Soc. London* **214**, 19-52.

Landmesser, L. T. (1978). The development of motor projection patterns in the chick hind limb. *J. Physiol.* **284**, 391-414.

Landmesser, L. T. and Honig, M. G. (1986). Altered sensory projections in the chick hindlimb following the early removal of motoneurons. *Dev. Biol.* **118**, 511-531.

McConnell, S. and Kaznowski, C. (1991). Cell cycle dependence of laminar determination in developing neocortex. *Science* **254**, 282-285.

McPheeters, M. (1982). Autoradiographic and fluorescent labeling of neuron classes in chick embryo spinal cord and sensory ganglia: Isolation and culture of motoneurons. Ph.D. dissertation, University of Utah.

Oakley, R. A., Lefcort, F. B., Clary, D. O., Reichardt, L. F., Prevette, D., Oppenheim, R. W. and Frank, E. (1997). Neurotrophin-3 promotes the differentiation of muscle spindle afferents in the absence of peripheral targets. *J. Neurosci.* **17**, 4262-4274.

Scott, S. A. (1986). Skin sensory innervation patterns in embryonic chick hindlimb following dorsal root ganglion reversals. *J. Neurobiol.* **17**, 649-668.

Thiery, J.-P., Delouvée, A., Grumet, M. and Edelman, G. M. (1985). Initial appearance and regional distribution of the neuron-glia cell adhesion molecule in the chick embryo. *J. Cell Biol.* **100**, 442-456.

Tosney, K. W. and Landmesser, L. T. (1984). Pattern and specificity of axonal outgrowth folllowing varying degrees of chick limb bud ablation. *J. Neurosci.* **4**, 2518-2527.

Tosney, K. W. and Landmesser, L. T. (1985a). Development of the major pathways for neurite outgrowth in the chick hindlimb. *Dev. Biol.* **109**, 193-214.

Tosney, K. W. and Landmesser, L. T. (1985b). Growth cone morphology and trajectory in the lumbosacral regions of the chick embryo. *J. Neurosci.* **5**, 2345-2358.

Tyrrell, S., Schroeter, S., Coulter, L. and Tosney, K. W. (1990). Distribution and projection pattern of motoneurons that innervate hindlimb muscles in the quail. *J. Comp. Neurol.* **298**, 413-430.

Wang, G. and Scott, S. A. (1997). Muscle sensory innervation patterns in embryonic chick hindlimbs following dorsal root ganglion reversal. *Dev. Biol.* **186**, 27-35.

Xue, Y. and Honig, M. G. (1997). Fasciculation of sensory axons in the developing chick hindlimb. *Neurosci. Abstr.* **23**, 1970.