Ectoderm removal prevents cutaneous nerve formation and perturbs sensory axon growth in the chick hindlimb

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Abstract

Target tissues are thought to provide important cues for growing axons, yet there is little direct evidence that they are essential for axonal pathfinding. Here we examined whether target ectoderm is necessary for the formation of cutaneous nerves, and for the normal growth and guidance of cutaneous axons as they first enter the limb plexus. To do this, we removed a patch of ectoderm from the chick hindlimb at various times during early axon outgrowth. We find there is a critical period when cutaneous nerve formation requires target ectoderm. When the ectoderm is absent during this time, axons progress into the limb more slowly and, although a few sensory axons occasionally diverge a short distance from the plexus, they do not form a discrete nerve that travels to the skin. A few days later, when the nerve pattern is mature, axons normally destined for the ‘deprived’ cutaneous nerve are not segregated appropriately within the plexus. Some cutaneous axons are instead misdirected along an inappropriate cutaneous nerve, while others have seemingly failed to reach their correct target, or a suitable alternative, and died. These results demonstrate that the target ectoderm is necessary for normal sensory axon growth and guidance in the hindlimb.

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Introduction

Appropriate axon pathfinding during development is crucial for the establishment of normal synaptic connections. A prevailing notion is that target tissues provide important cues for growing axons. Indeed, a wealth of tissue culture studies show that target tissues can attract the axons that normally project to them. For example, for the mouse whisker pad, the elegant work of Lumsden and Davies (1983, 1986) has demonstrated that trigeminal axons extend specifically toward the maxillary and mandibular processes. There is, however, surprisingly little in vivo evidence that supports this idea. In only a few cases, has the target been shown to be necessary for normal axon outgrowth. For example, when the dermamyotome, which gives rise to epaxial muscles and the overlying dermis, is removed from three adjacent segments, the dorsal ramus and associated muscle and cutaneous nerves do not form (Tosney, 1987; see also Eisen and Pike, 1991; Zeller et al., 2002). In other systems, target tissues appear not to be necessary. Sympathetic preganglionic axons project correctly when the sympathetic chain is absent following neural crest deletions (Yip, 1987). Quite strikingly, individual muscle nerves form in vertebrate limbs when muscles are absent, for example, in chick embryos after the somites have been removed (Phelan and Hollyday, 1990), and in mice that are null for hepatocyte growth factor (Banerjee et al., 1998; Ebens et al., 1996).

In contrast, the skin appears to be essential for the normal development of cutaneous innervation of the limb. Some years ago, Martin et al. (1989) showed that following ultraviolet irradiation of the wing at St. 23–24, the ectoderm gradually degenerates, and the cutaneous nerve normally projecting to the denuded area is later absent. We sought to determine whether the ectoderm is indeed necessary for initial cutaneous nerve formation, and if so, whether it is also required for the normal growth and guidance of those cutaneous axons to the point where they would normally diverge from the limb plexus. The surgical approach we chose to use, in contrast to ultraviolet irradiation, does not directly damage underlying tissues and allowed us to
precisely control the embryonic stage at which the ectoderm was removed and the size and position of the excised area. Furthermore, by using sensitive immunofluorescent labeling techniques, we could detect growing axons at the earliest stages and could also distinguish sensory from motoneuron axons. In the experiments reported here, we removed the ectoderm at any of three different times during early axon outgrowth and examined the consequences at two times, just after normal cutaneous nerve formation and 2–3 days later, when the nerve pattern in the hindlimb is considered mature. Our results suggest that there is a critical period, during which a cutaneous nerve requires the presence of its target ectoderm to form. When the ectoderm is absent during this critical period, the sensory axons normally destined for the ‘deprived’ cutaneous nerve appear to not become segregated appropriately in the plexus, and some instead become misrouted along an inappropriate cutaneous nerve. Deficits in axonal projections into the limb, which appear to be a consequence of both decreased outgrowth and increased dorsal root ganglion (DRG) cell death, were also observed.

**Materials and methods**

**Embryonic surgeries**

White Leghorn chick embryos were windowed at 3 days of incubation and subsequently staged according to Hamburger and Hamilton (1951). The dorsal surface of the right hindlimb was exposed and Nile blue (1.5% in water) was applied, using a 50-μm-diameter micropipette tip, to the antero-proximal region (Fig. 1). Nile blue causes the ectoderm to blister, so that it can be peeled off, without damage to underlying tissue (Fig. 2; Yang and Niswander, 1995; see also Amthor et al., 1998; Araujo et al., 1998). This particular region of the skin was chosen because it is later supplied by one of the major cutaneous nerves in the limb, the lateral femoral cutaneous (LFCt) nerve, and it is easily accessible. Importantly, the excised region did not include the apical epidermal ridge or the ectoderm overlying the zone of polarizing activity, which are crucial for patterning the limb along its proximal–distal axis and anterior–posterior axis, respectively. The excised region will henceforth be referred to as the ‘target’ ectoderm.

The target ectoderm was removed at one of several different stages: (1) at St. 20–22, when the oldest sensory neurons are beginning to extend axons into the spinal nerves; (2) at early St. 24, just before the LFCt nerve normally begins to form; or (3) at late St. 24, just after the LFCt nerve first forms (Fig. 1). For most of the experiments, we took special measures to try to prevent the ectoderm from regenerating until St. 25–26, after the LFCt nerve would normally have formed. This was accomplished by placing a small piece of Kimwipe soaked in 1% fetuin in Tyrode solution onto the denuded surface and allowing the Kimwipe to adhere. Fetuin is one of several proteins shown to inhibit the regeneration of newt epidermis (Donaldson and Mahan, 1984). The adherent Kimwipe served as a physical barrier that impeded the migration of the epithelial cell sheet from the cut surface onto the denuded mesoderm, thereby slowing regeneration. Even with this approach, the rate of regeneration, as determined by visual inspection of the operated limb (Fig. 2D), varied considerably, depending in part on how tightly the Kimwipe adhered to the limb. Henceforth, regeneration will be referred to as ‘extensive’ whenever the remaining bare area was less than one-third the size of the original patch. For the St. 20–22 surgeries, regeneration was rapid and extensive enough that it was necessary to take off the Kimwipe and peel off the regenerating ectoderm, at least once but sometimes twice, before the embryos reached St. 26. In some experiments, we applied Nile blue to the ectoderm but did not physically remove it. Embryos were allowed to survive for varying times, up until St. 30, before they were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB). It should be noted that St. 30 was chosen as an endpoint for many of our analyses, because it is before the peak of cell death in the DRGs (Caldero et al., 1998) while also being the earliest stage at which we can routinely and easily retrogradely label individual peripheral nerves (see below).

**Tissue processing**

Embryos were processed for immunofluorescence as whole mounts, as 100- to 200-μm-thick vibratome sections, or as 20- to 30-μm-thick cryostat sections. To enhance antibody penetration, the tissue was permeabilized using either freeze–thawing, 1% digitonin, or 0.1–1% Triton X-100 in 0.1 M PB, as described in Honig et al. (1998, 2002). Primary antibodies used included a monoclonal antibody against axonin-1 [23.4-5 at 1:100, from the Developmental Studies Hybridoma Bank (DSHB)] to label sensory axons, and antibodies against neurofilaments (3A10 at 1:200 from the DSHB, AHP246 at 1:500 from Serotec, Raleigh, NC), or against chick L1 (8D9 at 1:10 from the DSHB, R020 at 1:100 from Urs Rutishauser, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, New York, NY) to label all axons. To visualize limb morphology, some sections were counterstained with propidium iodide (5 μg/ml in 0.1 M PB, to which 100 μg/ml RNase was sometimes added), a dye that labels nucleic acids.

Most St. 30 embryos were dissected for retrograde labeling, and Texas red dextran amine (TRDA from Molecular Probes, Eugene, OR) was injected directly into the medial femoral cutaneous (MFCt) nerve, as previously described (Honig et al., 1998, 2002). The embryos were subsequently fixed and cryostat-sectioned transverse to the long axis of the thigh, so that the plexus and spinal nerves were cut in cross section. Sections from these embryos were processed as follows: (1) Sections through the DRGs were coverslipped and retrogradely labeled cell bodies were later counted in every section using conventional fluorescence
microscopy. (2) Sections in the region between the DRGs and the crural plexus were processed for double-immuno-
fluorescent labeling, using mouse anti-axonin-1 (23.4-5) and anti-mouse Alexa 488 (Molecular Probes) to label sensory axons, and rabbit anti-L1 (R020) and anti-rabbit Cy5 (Jackson ImmunoResearch Laboratories, West Grove, PA) to visualize all axons. It should be noted that this type of double-labeling strategy was not successful with either the whole mounts or the vibratome sections, because axo-
nin-1, which is linked to the plasma membrane by glyco-
sylphosphatidylinositol, tended to be extracted when Triton was used to permeabilize the tissue to achieve adequate neurofilament and L1 labeling. (3) Sections through the distal parts of the thigh were immune-labeled using one of the neurofilament antibodies and counterstained with propi-
dium iodide.

Confocal microscopy and analysis

Whole mounts and tissue sections were viewed with a Bio-Rad confocal laser scanning microscope, as previously described (Honig et al., 1998, 2002). Selected confocal images were subsequently processed using Adobe Photosh
op and quantification was done using NIH Image.

Fig. 1. The normal temporal sequence of sensory neuron development in relation to the design of the experiments for the early stage ectoderm removals described in the first part of the Results. The stippled area on the drawing of the hindlimb bud indicates the region where the ectoderm was removed.

Fig. 2. The effectiveness of ectoderm removal is readily assessed. The target ectoderm was removed at St. 21. (A–C) High-magnification views of propidium iodide-stained sections showing the dorsal margin of the thigh of an experimental embryo fixed 2 h after the surgery. (A) In the normal, left limb, the border between the ectoderm and the underlying mesenchyme can be discerned (arrows). (B) In the operated, right limb, the target ectoderm is missing. While the underlying mesenchyme is now exposed, it does not appear to have been damaged by the surgery. (C) The boundary of the excised ectoderm is marked with an arrowhead. To the left, the ectoderm is intact; the border between ectoderm and mesenchyme is indicated with arrows. On the right, the ectoderm has been removed and the mesenchyme is exposed. (D) The dorsal surface of the operated, right hindlimb of another embryo, viewed through a dissecting microscope, 16 h after the surgery. The ectoderm, whose borders are indicated by arrows, has partly regenerated such that the denuded area is ~40% its original size. The exposed mesenchyme can be distinguished from intact ectoderm. Scale bar, 50 µm for A–C.
Whole-mount preparations

The majority of experimental embryos sacrificed between St. 21 and St. 27 were prepared as whole mounts. After immunofluorescent staining, we mounted the whole-mount preparations between two coverslips, to allow viewing from both the dorsal and the ventral surface. Images were captured with a 4× objective and an iris setting of 5 to reveal the entire nerve pattern, and at higher magnification and smaller iris settings, as needed, to more clearly visualize the LFCt nerve. To quantify the extent of axonal growth, we measured the length of the longest axon extending from the crural plexus and the width of the crural plexus 200 μm distal to the juncture of spinal nerves lumbosacral (LS)1–3 (as shown in Fig. 9A). The length of the longest axon extending from the sciatic plexus and the length of the limb were also measured.

Vibratome sections

A few experimental embryos sacrificed between St. 25 and St. 27 were vibratome-sectioned, transverse to the spinal cord, and immunofluorescently stained. Images captured with a 4× objective and an iris setting of 5, and if needed, at higher magnification, were later scored as to whether or not the LFCt nerve was present.

Cryostat sections

St. 30 embryos, most of which had received TRDA injections, were cryostat-sectioned transverse to the long axis of the hindlimb. As described above, sections in the region between the DRGs and the crural plexus were double-immunofluorescently stained for axonin-1 and L1. Axons that were not labeled with the sensory-specific antibody were considered to be motoneuron axons. Images of the crural plexus and the spinal nerves were captured from serial cross sections at high magnification and high resolution, using a 40× oil immersion objective with a numerical aperture of 1.4. To determine the relative abundance of sensory axons within the spinal nerve LS1, we combined thresholding with the use of drawing tools to delineate the immunofluorescently labeled sensory axon bundles, because thresholding alone was insufficient due to variations in labeling intensity (see also Honig et al., 2002). The area the sensory axons occupied was then measured and this value was expressed relative to the area of the entire spinal nerve. Because spinal nerve LS1 was not cut in perfect cross section, and because axonal area varies depending on the angle of sectioning, normalizing in this way allowed values from different limbs to be compared. However, because sensory axons are smaller and more tightly packed than are motoneuron axons (Xue and Honig, 1999), the statement that sensory axons occupy 30% of a nerve, for example, does not imply that 30% of the axons are sensory.

Counts of dying neurons

Embryos were fixed in 4% paraformaldehyde and cryostat-sectioned at 30 μm. To identify dying neurons, sections were processed using the ApopTag Peroxidase kit (Intergen, Purchase, NY). Dying cells were counted in every section through DRGs LS1–LS5.

Results

Removal of the ectoderm at St. 20–22

Our initial goal was to confirm the observation of Martin et al. (1989) that when the ectoderm is removed, the cutaneous nerve that normally projects to the denuded region is later absent. Instead of using ultraviolet irradiation to destroy the ectoderm, as Martin and his colleagues had, we used a surgical approach employed by previous investigators (Amthor et al., 1998; Araujo et al., 1998; Yang and Niswander, 1995), which makes it possible to avoid directly damaging the underlying tissue (Fig. 2). However, whereas the ectoderm heals slowly, if at all, after irradiation, it regenerated very rapidly following surgical removal. Thus, in our initial experiments, when a patch of ectoderm was removed at St. 20–22, it usually had regenerated completely by the next day, when the embryos were ~ St. 25.

To determine if the temporary absence of the ectoderm affected cutaneous nerve formation, the embryos were allowed to survive two more days, until St. 30, at which time they were fixed, sectioned, and subsequently immunostained for axonin-1 to reveal sensory axons. The LFCt nerve typically was present, although sometimes it was smaller in diameter than normal and presumably contained a reduced number of axons (data not shown). Thus, the temporary absence of the target ectoderm did not prevent LFCt nerve formation.

We then took special measures to limit the extent to which the ectoderm regenerated until St. 25 (see the Materials and methods section), and again sacrificed the embryos at St. 30. The LFCt nerve was absent in 18 of 20 such limbs and was very small in the remaining two limbs, probably due to the partial regeneration of the ectoderm. As typified by the cross sections through the distal thigh in Fig. 3, in the region where the LFCt nerve normally reaches the skin and can easily be identified, no nerve was present in the operated limb. Thus, when the target ectoderm was absent from St. 20–22 through St. 25–26, the cutaneous nerve normally supplying it was later missing. In contrast, the MFCt nerve, which also arises from the crural plexus and projects to skin on the ventral surface of the limb, was normal in appearance. Moreover, the region of skin normally innervated by the LFCt nerve had not obviously been invaded by axons from other sources, suggesting that the sprouting from nearby cutaneous nerves into the denervated territory reported by Martin et al. (1989) for St. 35–36 embryos probably takes several days to occur.

Fig. 3 also shows that the overall structure of the denuded thigh was normal and that the major thigh
muscles were present. Nonetheless, the operated limbs were slightly smaller than normal, as noted by previous investigators (Yang and Niswander, 1995), and the subectodermal mesenchyme and some dorsal thigh muscles sometimes appeared depleted, presumably because signals derived from the ectoderm that normally promote the growth and differentiation of the underlying mesenchyme had been lost (e.g., see Amthor et al., 1998; Araujo et al., 1998; Yang and Niswander, 1995). Ascertaining the extent of these deficits and their possible association with LFCt nerve formation will require further studies. It is, however, important to note that these surgeries were performed after the fate of proximal regions of the limb had been determined (e.g., Akita, 1996; Riddle et al., 1995), and so, although the ectoderm is essential for normal dorsal–ventral patterning of the limb (see Niswander, 2002 for a recent review), we did not observe any obvious ventral transformation of dorsal structures.

Initial LFCt nerve formation

The absence of the LFCt nerve at St. 30 could, in theory, have been due to the failure of that nerve to form. Alternatively, the axons normally destined to project along the LFCt nerve might have initially diverged from the plexus, grown toward the skin, contacted the denuded dermis, and then retracted or degenerated. To distinguish between these possibilities, we examined embryos at short time intervals (3–43 h) after removing the ectoderm at St. 20–22.

At the time of the surgery, sensory and motoneuron axons were beginning to converge in the crural and sciatic plexuses (Landmesser and Honig, 1986; Tosney and Landmesser, 1985a; Wang and Scott, 2000). Immune-staining of sensory axons in whole-mount preparations showed that, a few hours after the surgery, the pattern of sensory axon growth was very similar on the two sides of experimental embryos (Fig. 4A). No obvious differences were found through St. 24 (not shown), as might be expected because St. 21–24 correspond to the waiting period before axons grow into the limb proper (Tosney and Landmesser, 1985a; Wang and Scott, 2000). However, the LFCt nerve was absent in the operated limbs, both at St. 25, as it was beginning to ramify within the skin of the normal contralateral limb (Fig. 4B), and several hours later, at St. 25 1/2–26, when it ramified even more extensively (Fig. 4C). Altogether, the LFCt nerve was absent from 34 of 35 operated limbs examined between St. 25 and St. 27, in which the ectoderm had been prevented from regenerating. In the one exception, in the region where the LFCt nerve normally diverges, two small sensory axon bundles extended a short distance from the plexus (Fig. 4C). Interestingly, examination of the whole-mount preparations at high magnifications revealed that, in about half the operated limbs, a few axons projected very short distances from the plexus, sometimes at a variety of angles, but did not form a discrete nerve (Fig. 4E). Thus, when the target ectoderm was absent from St. 20–22 through St. 25, axons did not extend along the LFCt nerve pathway to the skin.

In theory, the absence of the LFCt nerve could be attributable to an increase in DRG cell death. In normal embryos, DRG cell death starts at St. 28. However, when the entire limb bud is ablated, some neurons in the lumbo-sacral DRGs begin to die as early as St. 25 (Caldero et al., 1998). To examine the possibility that the LFCt nerve may have been absent because those neurons had already died, a series of experimental embryos were processed for TUNEL labeling. As shown in Fig. 5, very few apoptotic cells were present in DRGs LS1 and LS2 at St. 25 and there was no significant difference between the numbers of apoptotic cells on the two sides. However, slightly later, at St. 25 1/2, more apoptotic cells were found on the operated side than were found contralaterally. Thus, while ectoderm removal may ultimately lead to increased DRG cell death, the initial absence of the LFCt nerve preceded this increase and thus could not be a consequence of it.
The segregation of ‘LFCt’ axons in the crural plexus

The results described above indicate that when the target ectoderm was absent from St. 20–22 through St. 25, axons normally destined for the LFCt nerve did not grow along their normal nerve pathway toward the denuded skin. (In the rest of this paper, the term ‘LFCt’ axons is used to refer to those axons destined for the LFCt nerve, even if they did not actually project along the LFCt nerve.) It is possible that ‘LFCt’ axons may have nonetheless grown normally up to the point where the LFCt nerve usually diverges from...
the plexus, and then either simply failed to extend any further or altered their trajectory to grow to an alternative peripheral target. To address this issue, cross sections through the crural plexus of the St. 30 experimental embryos described earlier in this section were examined. We have previously shown, by combining immune-staining of sensory axons with retrograde labeling of individual peripheral nerves, that sensory axons that ultimately project along different peripheral nerves become progressively separated from one another as they extend along the spinal nerves and into the plexus (Honig et al., 1998). Thus, by St. 30, LFCt axons are normally grouped together in very large bundles at the anterior pole of the plexus, axons that project along the MFCt nerve are situated posteriorly, while small bundles of muscle sensory axons, intermixed with motorneuron axons, occupy the central part of the plexus. In contrast, in all 18 of the operated limbs lacking a LFCt nerve, the large bundles of anteriorly situated sensory axons were missing from the plexus (Fig. 6). These results suggest that after early target ectoderm removal, either axons normally destined for the LFCt nerve had not become appropriately segregated within the plexus or they had initially become segregated but had already died or retracted. In addition, the bundles of sensory axons in the plexus were not more numerous or larger than normal, arguing against the possibility that the entire population of ‘LFCt’ axons had entered the plexus and grown along an inappropriate peripheral pathway or pathways.

While the crural plexus shown in Fig. 6C appeared completely normal except for the absence of ‘LFCt’ axons at its anterior pole, in most of the operated limbs, the plexus was smaller and rounder than normal. These changes could be due to decreased axon growth and/or to increased DRG cell death (see Discussion), but in any case, there was never any indication that large numbers of ‘LFCt’ axons were present in the plexus.

Misdirection along the MFCt nerve

Given the absence of large bundles of ‘LFCt’ axons in the plexus, we sought to determine the fate of those axons that normally project along the LFCt nerve. One possibility was that some ‘LFCt’ axons had been misrouted along an alternative peripheral nerve pathway. Because independent markers of nerve-specific populations of sensory neurons do not yet exist, to examine this issue, we focused on LS1. Our reasoning was that, because DRG LS1 normally contributes many axons to the LFCt nerve (Honig, 1982), any changes observed in LS1 were likely to be attributable to changes in the behavior of ‘LFCt’ axons. Tracing the course of sensory axon bundles through sequential sections from the experimental limbs revealed that many sensory axons from LS1 traveled posteriorly upon entering the crural plexus and then diverged along the MFCt nerve, which typically receives just a small contribution from LS1. To examine this directly, in subsequent operated animals, the MFCt nerve was
retrogradely labeled with TRDA. Unlike normal embryos, in which the segmental patterns of sensory projections on opposite sides are typically very similar (Honig and Rutishauser, 1996), in the experimental embryos, there were more retrogradely labeled MFCt neurons in DRG LS1 for the operated limbs than contralaterally (Fig. 7). The number of retrogradely labeled MFCt neurons in DRG LS2, but not in DRG LS3, was also greater for the operated limbs. As 60% and 30% of LFCt neurons typically originate from DRGs LS1 and LS2, respectively (Honig, 1982; Honig et al., 2002), these results are consistent with the view that some ‘LFCt’ axons had projected along the MFCt nerve. Importantly, however, the number of retrogradely labeled MFCt neurons (mean = 2129) was far fewer than what would be expected if the full complement of LFCt neurons (>2000) as well as the normal complement of MFCt neurons (>2000) had extended along the MFCt nerve (Honig et al., 2002). Together, these results suggest that a significant, albeit limited, number of sensory axons normally destined for the LFCt nerve had been misdirected along the MFCt nerve. We did not determine if the remaining ‘LFCt’ axons had been misdirected along other peripheral pathways because, as previously discussed, the number and size of sensory axon bundles in the plexus suggested that massive misdirection was unlikely.

Additional insight into why large bundles of ‘LFCt’ axons might be absent from the plexus was provided by examining confocal images of the spinal nerves. As shown in Fig. 8, spinal nerve LS1 on the operated side appeared to have a smaller sensory component than on the contralateral side. Indeed, the relative cross-sectional area occupied by retrogradely labeled TRDA. Unlike normal embryos, in which the segmental patterns of sensory projections on opposite sides are typically very similar (Honig and Rutishauser, 1996), in the experimental embryos, there were more retrogradely labeled MFCt neurons in DRG LS1 for the operated limbs than contralaterally (Fig. 7). The number of retrogradely labeled MFCt neurons in DRG LS2, but not in DRG LS3, was also greater for the operated limbs. As 60% and 30% of LFCt neurons typically originate from DRGs LS1 and LS2, respectively (Honig, 1982; Honig et al., 2002), these results are consistent with the view that some ‘LFCt’ axons had projected along the MFCt nerve. Importantly, however, the number of retrogradely labeled MFCt neurons (mean = 2129) was far fewer than what would be expected if the full complement of LFCt neurons (>2000) as well as the normal complement of MFCt neurons (>2000) had extended along the MFCt nerve (Honig et al., 2002). Together, these results suggest that a significant, albeit limited, number of sensory axons normally destined for the LFCt nerve had been misdirected along the MFCt nerve. We did not determine if the remaining ‘LFCt’ axons had been misdirected along other peripheral pathways because, as previously discussed, the number and size of sensory axon bundles in the plexus suggested that massive misdirection was unlikely.

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sensory axons in spinal nerve LS1 of the operated limbs was less than for their normal, contralateral counterparts. Thus, following ectoderm removal, many sensory axons were seemingly absent from spinal nerve LS1. This paucity of sensory axons could in theory be explained by decreases in sensory neuron proliferation or axon extension, or by increases in axon retraction or neuronal cell death, as will be discussed later.

Early axon outgrowth

As shown in Fig. 4, axons arising from DRGs LS1–3 and traversing the crural plexus appeared to be less numerous and to extend shorter distances on the operated side than on the normal, contralateral side of the whole-mounted St. 25–27 embryos. Thus, ectoderm removal somehow altered the growth not only of ‘LFCt’ axons but also of sensory axons destined to project to targets not directly affected by the surgery. This raised the intriguing possibility that motoneuron axon outgrowth might also be perturbed. To examine this issue, one of several pan-axonal antibodies was used to immune-stain the whole-mount preparations, due to the unavailability of motoneuron axon-specific markers. We reasoned that if motoneuron axon growth was normal, any nerve deficits revealed with the pan-axonal labeling should be less than when only sensory axons were labeled. Surprisingly, the results were similar regardless of the antibody used for the immune labeling (data not shown). Thus, ectoderm removal appeared to decrease both motoneuron and sensory axon growth into the hindlimb.

Quantification of these results is shown in Fig. 9B. The length of the longest crurally derived axons and the width of the crural nerve trunk in the operated limbs were 74% and 64%, respectively, compared to the contralateral side. The length of the sciatic nerve and of the operated limbs themselves were slightly decreased, presumably due to a small deleterious effect of ectoderm removal on overall limb growth. To remove any influence of varying limb size, each nerve measurement was divided by the length of the corresponding limb. When normalized in this way, the length of the longest crurally derived axons and the width of the crural nerve trunk still showed statistically significant decreases, to 81% and 70%, respectively, whereas the length of the longest sciatic axons did not. These results suggest that axons traversing the crural nerve trunk were shorter than normal and also were less abundant, because a narrow nerve would contain fewer axons than a wider one, all other things being equal. Thus, removal of the ectoderm appears to specifically affect the growth of crurally derived axons, which underlie the ablated area, and not the more distant sciatic axons.

A greater difference was evident between the two limbs shown in Fig. 4B than between those in Fig. 4C, suggesting that the crural nerve deficits may have decreased as the embryos matured. To examine this possibility, because of the inexactness in staging embryos this precisely, we used the length of the normal, contralateral limb as an indicator of developmental stage, and divided the experimental embryos into two groups based on this measurement. The data in Fig. 9C indicate that normalized crural nerve width was reduced more in the smaller embryos than in the larger ones (to 58% vs. 87%). Thus, the extent of the overall nerve deficits decreased as the limbs grew larger, and so presumably as the embryos grew older. One possible reason for this would be if axons progressed into the operated limbs at a slower than normal pace. Importantly, these deficits in sensory axon projections were obvious before, and therefore cannot be explained by, any increase in DRG cell death.

We next wanted to know if the temporary absence of the ectoderm resulted in a decrease in overall crural nerve growth, even when the ectoderm regenerated rapidly.
enough for the LFCt nerve to form. To examine this possibility, we either simply applied the Nile blue solution to the target ectoderm, which was gradually sloughed off, or we removed the Nile blue-stained patch of target ectoderm but did not apply a Kimwipe to the denuded surface. The extent of crural deficits was similar to those found when the ectoderm was prevented from regenerating and sizable deficits were observed even in operated limbs in which the LFCt nerve had formed (Fig. 9C). Thus, even the brief absence of the ectoderm slows crural axon growth, and this effect of the ectoderm seems to be separable from its effects on LFCt nerve formation. These results also indicate that neither fetuin nor the Kimwipe is responsible for decreased axonal outgrowth.

Removal of the ectoderm at St. 24

Between St. 20 and 24, motoneuron and sensory axons normally extend along the spinal nerves and into the plexus region (Lance-Jones and Landmesser, 1981; Landmesser and Honig, 1986; Tosney and Landmesser, 1985a; Wang and Scott, 2000). By St. 24, all motoneuron axons and the axons of the oldest sensory neurons are positioned at the base of the limb, segregated into nerve-specific bundles, awaiting a signal or signals from maturing limb tissues before growing into the limb proper (Tosney and Landmesser, 1985a; Wang and Scott, 2000). A few hours later, these axons have exited the plexus, forming individual muscle and cutaneous nerves. In the case of the LFCt nerve, ~200 axons appear to rapidly grow 150–200 μm to the skin (Honig et al., 1998; Honig, unpublished observations). Over the next few days, as additional sensory neurons are born, their axons progressively extend along the spinal nerves, into the plexus, and ultimately to their targets, appearing to fasciculate along their predecessors as they grow (Honig et al., 1998; Xue and Honig, 1999). Given this temporal sequence of axonal outgrowth, we wanted to determine whether the ectoderm is necessary for LFCt nerve formation when the oldest ‘LFCt’ axons have already become appropriately segregated in, and are about to diverge from, the plexus. To do this, in a second group of embryos, the target ectoderm was removed at St. 24, special measures were again taken to limit the regeneration of the ectoderm until after the LFCt nerve would have normally formed.

LFCt nerve formation

To start this analysis, embryos were sacrificed at St. 25–26, a day after the surgery, and either vibratome-sectioned or
prepared as whole mounts. The LFCt nerve was absent in 20 of 21 operated limbs. This result suggests that the ectoderm must be present when cutaneous axons normally start diverging from the plexus, for the LFCt nerve to form.

Given the absence of the LFCt nerve, what was the fate of those axons that normally project along it? To gain insight into this question, cross sections through the plexus and spinal nerves of a series of operated embryos sacrificed at St. 30 were examined, using the same kinds of analyses previously described. In 10 of the 11 experimental limbs, the size, number, and distribution of sensory axon bundles within the plexus were normal, except for the clear absence of large, anteriorly situated ‘LFCt’ bundles. This might be taken to suggest that axons normally destined for the LFCt nerve did not become appropriately segregated within the plexus. However, this seemed implausible because the axons of the older ‘LFCt’ neurons would have navigated to the anterior part of the plexus by the time of the surgery. Indeed, in the one remaining limb, large bundles of anteriorly positioned sensory axons were present at a proximal level of the plexus but were not evident more distally, suggesting that ‘LFCt’ axons began to segregate, but then stopped growing (Figs. 10A,B). To further investigate this possibility, five additional experimental embryos were examined half a day earlier, at St. 29. In each of these, one or two bundles of sensory axons were situated in the anterior part of the crural plexus at a proximal level, but not distally, seemingly terminating within the plexus itself. On the basis of their size and position, these bundles seemed to be composed of ‘LFCt’ axons although they were not as large as on the contralateral side (Figs. 10C,D). While markers for specific subtypes of axons are needed to reach firm conclusions, these observations are consistent with the view that the axons of the older ‘LFCt’ neurons segregate appropriately in the plexus, but cannot reach their normal target or change their trajectory to find another target. Being unable to contact a suitable, sustaining peripheral target, these ‘LFCt’ axons may subsequently retract and/or degenerate, so that by St. 30, there is little or no evidence of their previous existence. That the complement of ‘LFCt’ axons on the experimental side was relatively small further suggests that the axons of the younger ‘LFCt’ neurons, which were just beginning to extend at the time of the surgery, were not becoming bundled together. Similarly, when the ectoderm was removed at St. 20–22, LFCt axon bundles were not evident at any level in the 18 St. 30 embryos or in an additional 3 embryos examined at St. 29 (data not shown).

Together, these observations raise the intriguing possibility that the target ectoderm normally provides cues responsible for ‘LFCt’ axons becoming segregated as they grow into the plexus.

We next measured the cross-sectional area occupied by sensory axons in spinal nerve LS1. The results in Fig. 11A show that spinal nerve LS1 on the operated side had a smaller sensory component than spinal nerve LS1 contralaterally, as was found for the St. 20–22 surgeries. Because

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![Fig. 10.](image-url) When the ectoderm is removed at St. 24, some axons normally destined for the LFCt nerve initially segregate appropriately within the plexus. Cross sections of the crural plexus immune-stained using an axonin-1 antibody to reveal sensory axons. (A and B) The plexus from the same operated limb at a distal level (A) and a proximal level (B). Distally, large bundles of sensory axons, corresponding to those that would normally project along the LFCt nerve, are absent from the anterior pole of the plexus (see Fig. 6B). Proximally, large bundles of anteriorly positioned sensory axons (shown outlined in white and marked by arrowheads) are present, although they are stained very lightly for axonin-1, suggesting that they were perhaps degenerating. (C and D) The plexus on the operated side (C) and the normal, contralateral side (D) of an experimental embryo, sacrificed at St. 29. In D, LFCt axons are grouped together in three bundles near the anterior edge of the plexus, which are smaller than at St. 30 (see Fig. 6B), because sensory axons continue growing into the limb until ~ St. 32. On the operated side, an intermediate-sized bundle of sensory axons (circled and marked with an arrowhead) is situated anteriorly. On the basis of its size and position, it seems likely that this bundle is composed of ‘LFCt’ axons. This bundle was not present more distally, seemingly terminating within the plexus itself. Note that the plexus is at a more proximal level in all these images than in Fig. 6B and so MFCt axons are located spread along the ventral edge of the plexus, on their way toward the posterior pole, where they will diverge to form the MFCt nerve. Scale bar, 50 μm.
spinal nerve LS1 normally contributes many sensory axons to the LFct nerve, this finding suggests that many ‘LFct’ axons may be absent from spinal nerve LS1 following the removal of the ectoderm at St. 24.

To determine whether some ‘LFct’ axons had become misrouted along the MFCt nerve, as occurred after removing the ectoderm at St. 20–22, we retrogradely labeled the MFCt nerve with TRDA, and again focused on DRG LS1 because of its normally robust projection along the LFct nerve. The results in Fig. 11B show that there were more retrogradely labeled MFCt neurons in DRG LS1 on the operated side than there were contralaterally. Moreover, immediately upon entering the crural plexus, TRDA-labeled axons from spinal nerve LS1 coursed in a posterior direction to diverge along the MFCt nerve. These results suggest that some sensory axons normally destined for the LFct nerve, presumably some of the later-growing ‘LFct’ axons, become misdirected at fairly proximal levels in the absence of their target ectoderm and subsequently project inappropriately in the limb.

The preceding series of experiments showed that when the target ectoderm was absent from St. 24, just before normal LFct nerve formation, until at least St. 25, sensory axons did not grow along the LFct nerve. To determine if the brief absence of the ectoderm at this could prevent the LFct nerve from forming, we took advantage of the tendency of the embryonic ectoderm to regenerate rapidly. When we either simply applied Nile blue to the skin or applied the Nile blue and then removed the ectoderm, the LFct nerve was absent in 24 of 47 limbs (51%) examined the following day. Importantly, the LFct nerve was present in only one of nine limbs (11%) in which the denuded area was a third or more of its original size a day after the surgery, but in 22 of 38 limbs (58%) in which there had been more extensive regeneration. By comparison, when no special measures were taken to prevent the ectoderm from regenerating following removal in St. 20–22 embryos, the LFct nerve was present in the majority of limbs (17/23 or 74%). While we would need to continuously monitor the extent of ectoderm regeneration to draw firm conclusions, together these results suggest that a specific part and/or a minimal amount of the target ectoderm must be present during the period between St. 24 and St. 25, for the LFct nerve to form. These results also indicate that the failure of the LFct nerve to form when the ectoderm was prevented from regenerating was not simply due to an adverse effect of the fetuin and/or the Kimwipes.

To gain further insight into the possibility that there is a critical time period for LFct nerve formation, in a final series of embryos, the surgery was performed at late St. 24, when the LFct nerve would be in the actual process of forming, with no attempt being made to prevent regeneration. The LFct nerve was present in each of the 10 limbs treated this way. Interestingly, when the ectoderm had regenerated completely by time the embryos were sacrificed 19–20 h after the surgery, the LFct nerve tended to ramify

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**Fig. 11.** The results of ectoderm removal at St. 24. Data shown are mean ± S.E. (A) The area occupied by sensory axons within spinal nerve LS1 relative to the total nerve area at St. 30 is less on the operated side than contralaterally (seven experimental embryos, *P* = 0.05, one-tailed paired *T* test). Thus, following ectoderm removal, many sensory axons are seemingly absent from spinal nerve LS1. (B) The projection from DRG LS1 to the MFCt nerve at St. 30 is increased. There are more retrogradely labeled MFCt neurons in DRG LS1 on the operated side than contralaterally (seven experimental embryos, **P** = 0.0034, paired *T* test). As most LFct neurons normally originate from DRG LS1, this result is consistent with the view that some of them are misrouted along the MFCt nerve. (C) Normalized crural length and crural width measurements for different groups of experimental embryos at St. 25–26. Paired *T* tests were used to compare corresponding measurements from the two sides of each embryo (*P* < 0.05, **P** < 0.00005). Crural length and width are reduced when the ectoderm is removed at St. 24 and prevented from regenerating, although the decrease in crural width (to 87% of contralateral) is less than following the comparable earlier stage surgeries (to 70% of contralateral; *P* = 0.049, analysis of variance). Substantial nerve deficits are found when the ectoderm is not prevented from regenerating, and are similar whether or not the LFct nerve is present.
normally within the skin, whereas when a bare region remained, the LFCt nerve seemed to grow along its border. Thus, removing the target ectoderm when cutaneous axons had already begun to extend along the LFCt nerve did not prevent the nerve from forming.

Overall axon growth

We also wanted to know if the absence of the ectoderm from St. 24 through St. 25 caused overall crural nerve deficits, as was found when the ectoderm was absent from St. 20–22 through St. 25. As shown in Fig. 11C, following the later stage surgeries, normalized crural length and width were significantly less for the operated limbs than for the normal, contralateral limbs. Interestingly, the earlier stage surgeries resulted in a larger decrease in normalized crural width (to 70% of contralateral as compared to 87% for the St. 24 surgeries). This difference presumably reflects the fact that axons had further to grow to reach the distal plexus at St. 20–22 than they did at the time of the later stage surgery.

We next examined embryos in which we did not try to prevent the ectoderm from regenerating, so that it had healed completely or nearly completely within a day. The results in Fig. 11C show that crural nerve projections were decreased even in these limbs. Furthermore, the deficits in crural length and crural width in the operated limbs in which the LFCt nerve had formed were similar to those without LFCt nerves. Thus, as with the earlier stage surgeries, these results suggest that normal crural axon growth somehow depends on the continuous presence of the ectoderm overlying the dorsal–anterior part of the developing hindlimb, until at least St. 25.

Discussion

In the work described here, we have used embryonic surgeries to show that there is a critical period during which the ectoderm is necessary for cutaneous nerve formation in the chick hindlimb. The absence of the ectoderm during this time also affects axons at proximal levels in that some cutaneous axons do not segregate appropriately in the plexus and instead become misdirected along an alternative cutaneous nerve pathway. Furthermore, we observed deficits in peripheral projections that appear to result from slowed axonal outgrowth and increased DRG cell death.

LFCt nerve formation

When the ectoderm was removed before the formation of the LFCt nerve and prevented from regenerating until just after the LFCt nerve normally forms, the LFCt nerve was absent from nearly all the embryos (54/56) we examined between St. 25 and St. 27. In some operated limbs, a few sensory axons left the plexus, but extended very short distances and projected at unusual angles, as if perhaps searching aimlessly for some missing cue. Due to the sensitivity of our approach, it is unlikely that cutaneous axons could have initially projected to the skin, and later retracted and/or degenerated, without our having detected them. Thus, ‘LFCt’ axons are not able to diverge from the plexus along their normal pathway to the skin when the target ectoderm is absent.

Chemotropism is commonly invoked as the mechanism by which target tissues attract growing axons. It is well known that the rodent whisker pad exerts tropic effects on trigeminal axons (Lumsden and Davies, 1983, 1986). The neurotrophins, NGF, BDNF, and NT3, are expressed by the skin (Cahoon-Metzger et al., 2001) and Tucker et al. (2001) have shown that sensory axons in slice preparations of the limb grow toward beads coated with these molecules. Moreover, work by the group of Patel et al. (2001) has suggested that NGF/TrkA signaling is essential for the formation of peripheral cutaneous innervation. Whether the absence of the LFCt nerve following ectoderm removal can be attributed to the loss of NGF and/or other neurotrophins requires further examination.

Chemotropism, at least by itself, is unlikely to explain the formation of the LFCt nerve, however. Cutaneous axons do not project to the epidermis in the chick, but to the dermis (Martin et al., 1989; Saxod, 1978), and sensory axons in tissue culture are repelled by epidermal explants (Cahoon and Scott, 1999; Honig and Zou, 1995; Verna, 1985; Verna et al, 1986) and attracted by the dermis (Honig and Zou, 1995). Furthermore, an expanse of undifferentiated skin seemingly could not serve as a point source of chemottractant, and so some additional means of confining cutaneous axons to a discrete pathway from the plexus to the skin would be required. Along a different line of thought, a contact-mediated mechanism for guiding cutaneous axons to the skin can be excluded because sensory growth cones are small and the skin would be beyond the reach of their filopodia (Tosney and Landmesser, 1985b).

It seems likely, therefore, that the effects of the ectoderm on cutaneous axons are not direct. Rather, the ectoderm may signal underlying tissues such that they in turn elicit LFCt nerve formation. Ectoderm–mesoderm interactions are known to play a crucial role in the development of the skin. Furthermore, at early stages of development, the dorsal ectoderm specifies the dorsal–ventral axis of the limb by inducing Lmx1 expression in the underlying mesoderm (Riddle et al., 1995; Vogel et al., 1995). In our experiments, there was not an obvious ventral transformation of dorsal thigh structures, although these were sometimes depleted. Interestingly, when the dorsal ectoderm is removed from part of the wing, the nerve trunk normally extending within the underlying mesoderm is later absent. Araujo et al. (1998) attributed this innervation defect to the down-regulation of EphA7 expression in adjacent regions. Whether a similar change in the repulsive properties of the mesoderm
The projection to the MFCt nerve from DRG LS1, which nonetheless provide some insight as to their fate. LFCt nerve to definitively address these issues, our experiments initially segregate but die before we are able to detect them. May indeed be required for appropriate axonal segregation, in sensory axon growth. Within a day of ectoderm removal, the earliest growing 'LFCt' axons have already been segregated at the base of the limb at the time of the surgery and later-growing sensory axons presumably fasciculate along them (Xue and Honig, 1999). A possible explanation, consistent with our observations of St. 29 embryos, is that following the St. 24 surgeries, the earlier-growing sensory axons do become segregated, but fail to reach their target and die; consequently, the route that the later-growing axons would normally follow is not available. In the case of the St. 20–22 surgeries, the apparent absence of 'LFCt' axon bundles suggests that the target ectoderm may indeed be required for appropriate axonal segregation, although we cannot exclude the possibility that 'LFCt' axons initially segregate but die before we are able to detect them.

While it would be necessary to specifically identify those sensory axons normally destined to project along the LFCt nerve to definitively address these issues, our experiments nonetheless provide some insight as to their fate. The projection to the MFCt nerve from DRG LS1, which normally contributes many sensory axons to the LFCt nerve, was greater than normal in the operated limbs. The simplest explanation for this is that some 'LFCt' axons became misdirected along the MFCt nerve. Interestingly, the "MFCt-projecting" LS1 axons turned posteriorly, rather than maintaining the anterior course typical of 'LFCt' axons, within the distal spinal nerve and immediately upon entering the plexus. Thus, the effects of ectoderm removal on sensory axons seemingly are not restricted to distal parts of their trajectories. Our results also suggest that large numbers of 'LFCt' axons are not misdirected along available muscle nerve pathways in that muscle sensory bundles in the plexus were not obviously wider or more numerous than normal. If 'LFCt' axons indeed selectively misproject along another cutaneous nerve, rather than more proximate muscle nerves, differences in cell surface molecules on cutaneous vs. muscle sensory axons may account for this specificity (Honig and Kueter, 1995). Along this same line of thought, it is possible that those 'LFCt' axons that are most closely associated with MFCt axons in the proximal part of the spinal nerve are the ones that become misdirected.

Misrouting along the MFCt nerve cannot account for the fate of all 'LFCt' axons in that far fewer neurons were retrogradely labeled from the MFCt nerve than would have been expected had the full complement of both LFCt and MFCt neurons extended along the MFCt nerve. A likely possibility is that many LFCt neurons die, because their axons do not reach their normal target nor do they find a suitable alternative soon enough to receive sufficient trophic factor to enable their survival. Indeed, we found an increase in DRG cell death, which occurs too late to account for the initial absence of the LFCt nerve, but which may help explain the low number of retrogradely labeled neurons. Increased cell death may also contribute to the apparent paucity of sensory axons in spinal nerve LS1 and in the plexus.

Another consequence of ectoderm removal is a decrease in sensory axon growth. Within a day of ectoderm removal, axons projecting into the limb were shorter and less numerous than normal. Because the generation and initial differentiation of sensory neurons proceed normally even when the entire limb is ablated (Caldero et al., 1998; Oakley et al., 1997), it seems likely that here either outgrowth was delayed or the overall growth rate was decreased. In either case, the youngest sensory axons would not have extended as far as normal at St. 30, and this would contribute to the apparent depletion of sensory axons and the relatively normal numbers of retrogradely labeled MFCt neurons. While the mechanism responsible for the ectoderm’s effects on axon growth requires further examination, work using knockout mice has suggested that NGF emanating from the skin promotes sensory axon elongation (Patel et al., 2001). Two additional observations are noteworthy with regard to elucidating mechanisms. First, axonal projections into the limb were decreased even when the ectoderm was absent...
References


