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Specificity of Early Motoneuron Growth Cone Outgrowth in the Chick Embryo[1]

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Abstract

During development, chick lumbosacral motoneurons have been reported to form precise topographic projections within the limb from the time of initial outgrowth. This observation implies, first, that motoneurons select the appropriate muscle nerve pathway and, second, that they restrict their ramification within the primary uncleaved muscle masses to appropriate regions. Several reports based on electrophysiology and orthograde horseradish peroxidase (HRP) labeling have shown muscle nerve pathway selection to be fairly precise. However, studies based on retrograde labeling with HRP have produced conflicting reports on the extent to which vertebrate motoneurons make projection errors.

Since it is difficult to distinguish between true projection errors and HRP leakage when using retrograde labeling, we decided to assess the distribution of labeled growth cones in 25-μm serial plastic sections, following orthograde labeling of identifiable subpopulations of motoneurons during the period of initial axon outgrowth. Examination of a large number of muscle nerves revealed no segmentally inappropriate axons, confirming earlier reports that muscle nerve pathway selection is very accurate. In addition, we observed that growth cones take widely divergent trajectories into the same muscle nerve, suggesting that growth cones are responding independently to some specific environmental cue rather than being passively channeled at this point.

The distribution of labeled growth cones within the muscle masses provided direct evidence that motoneurons did not at any time project to obviously inappropriate muscle regions. In fact, motoneuron growth cones remained together as a compact nerve in the central region of their target muscle and only ramified widely within the muscle primordium after the completion of muscle cleavage and the onset of motoneuron cell death, a delay of several days. We conclude that if projection errors occur, they must be of a minor spatial extent.

Earlier studies on the development of motor projections in the chick hindlimb showed that, although axons destined for particular muscles are widely dispersed within individual spinal nerves, they (*1*) sort out in the plexus region to take up characteristic positions (Lance-Jones and Landmesser, 1981a) and (*2*) are found, with only minor exceptions, within segmentally appropriate muscle nerves from the onset of muscle nerve formation (stage (st) 26) (Landmesser and Morris, 1975; Landmesser, 1978; Lance-Jones and Landmesser, 1981a). Since this is prior to normally occurring motoneuron cell death (Hamburger, 1975) and muscle cleavage (Romer, 1927), this was strong suggestive evidence that the basic motor pattern in this system is first laid down by motoneuron growth cones choosing the appropriate projection pathways, rather than by regressive events involving retraction of erroneously projecting axon collaterals and/or cell death. Generally similar conclusions have been made for motoneurons innervating the chick wing (Hollyday, 1983a, b), mouse facial (Ashwell and Watson, 1983) and limb (Lance-Jones, 1982a) muscles, and bullfrog hindlimbs (Farel and Bemelmans, 1985).

In contrast to these studies, several laboratories have reported substantial initial projection errors that appear to be removed by motoneuron cell death. These range from Lamb's (1976) observation that a moderate number of the earliest outgrowing *Xenopus* motoneurons project to a clearly inappropriate site in the limb to the description of Pettigrew et al. (1979) of widespread projection errors in the chick wing. Since these observations were based primarily on the retrograde labeling of motoneurons following horseradish peroxidase (HRP) injections into the uncleaved primary muscle masses, it was not possible to determine whether they resulted from errors in pathway selection or from growth of motoneurons over potential boundaries between muscles within the limb. In addition, they are subject to interpretive difficulties, since it is difficult to rule out leakage of HRP within the small (several hundreds of micrometers) uncleaved primary muscle masses of the vertebrate limb (Landmesser, 1980).

It is important to resolve these apparently contradictory observations, since they bear directly upon the mechanisms by which specific projections are formed in this system. We therefore decided to investigate the behavior of segmentally identified populations of chick lumbosacral motoneurons within the primary muscle masses from their time of entry (st 26) until the completion of muscle cleavage and the onset of motoneuron cell death (st 30). By injecting HRP into single segments of the lateral motor column (Lance-Jones and Landmesser, 1981a), it is possible to completely label a number of motoneurons in which the segment of origin can be unambiguously identified and in which growth cones can be clearly visualized within the limb. It is thus possible to characterize in some detail the behavior of segmentally identified growth cones at different stages of outgrowth into the limb. Our observations relating to the specific projection of motoneuron growth cones within muscle nerves and their target muscles are presented here. The subsequent paper (Tosney and Landmesser, 1985a) presents more detailed quantitative observations on the morphology of growth cones during their outgrowth into the limb.

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Materials and Methods

Ten percent HRP in Tyrode's solution was pressure injected into the lateral motor column or spinal nerves of specific lumbosacral (LS) segments in 13 isolated spinal cord-hindlimb preparations prepared by decapitating, eviscerating, and performing a ventral laminectomy on st 27 to 30 (Hamburger and Hamilton, 1951) chick embryos. The segments which we designate as LS 1 to 8 correspond to segments 22 to 30. Following 5 hr of incubation in oxygenated Tyrode's solution at 31 to 34°C, the embryos were fixed overnight in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, washed for 7 to 10 days in TRIS buffer, and then processed for visualization of the HRP reaction product with diaminobenzidine as described previously (Landmesser, 1978).

After rinsing in 0.1 M cacodylate buffer and postfixing for 1 hr in 1% osmium tetroxide in 0.1 M cacodylate, the embryos were dehydrated in ethanol and propylene oxide and infiltrated with Epon/Araldite. The blocks were polymerized at 60°C until they were no longer flexible and each embryo was then serially sectioned at 25 μm transverse to the long axis of the limb on a standard rotary microtome.

This form of HRP injection intensely labels a proportion of the motoneurons at the injection site. Transmission electron microscopy revealed that the reaction product was diffusely distributed throughout the cytoplasm of the labeled neurons including the fine lamellepodia and filopodia of their growth cone (Tosney and Landmesser, 1985b). We were thus reasonably confident that this technique allowed us to adequately characterize the termination sites of motoneurons at the stages studied. (See also Tosney and Landmesser, 1985a).

For analysis of growth cone projections, camera lucida drawings were made of each 25-μm section through the thigh; the boundaries of the dorsal and ventral muscle masses (or individual muscles at stages where muscle cleavage was complete) and the positions of the femur and major nerves were drawn, as was each HRP-labeled profile. This was carried out for 25 limbs where the HRP labeling was judged to be adequate.

The distribution of labeled profiles in the various muscle nerves was made directly from the camera lucida drawings. Since the mature segmental projection to each of the major thigh muscles was well characterized (Landmesser, 1978; Hollyday, 1981), we were able to determine whether there were any segmentally inappropriate projections at these early stages prior to motoneuron cell death (Hamburger, 1975) and muscle cleavage.

To determine the distribution of labeled growth cones within the embryonic muscle masses, it was necessary to normalize for slight differences in limb size between embryos. Therefore, the camera lucida drawings for each section were projected onto a screen in which the major landmarks at mid-thigh level had been drawn from a single embryo (i.e., the boundaries of the dorsal and ventral muscle masses, the femur, and the major nerve trunks). The size of the projected image was adjusted to align the boundaries of the muscle masses and the position of the femur. Then, all of the labeled growth cones and profiles were drawn. Color coding indicated whether individual growth cones were within nerve trunks and major muscle nerves or had penetrated the muscle tissue as individuals or small groups of axons. In this way, all of the projections resulting from labeling a single spinal segment were combined. An example of such a combined projection for LS 1 and LS 7/8 is shown in Figure 4*A*.

The segmental projection boundaries shown in Figure 4*B* were made by drawing a circumferential line which enclosed all labeled profiles. This analysis was carried out for 14 st 28 limbs, and one st 27.5 limb. Similar analyses were carried out for 10 st 29.5 to 30 limbs; however, since this was after the period of muscle cleavage (Romer, 1927), it was possible to assay the projection to individual muscles at these stages without resorting to the normalization procedure described above.

Results

Our main aim was to examine the projection pattern of segmentally identified motoneurons within their muscle targets prior to the period of motoneuron cell death. As previously described, motoneurons enter the limb at st 24 after having collected into an anterior crural and posterior sciatic plexus (Hamburger, 1975; Lance-Jones and Landmesser, 1981a). From these, major dorsal and ventral nerve trunks emerge to penetrate the premuscle mesenchyme (Tosney and Landmesser, 1985b). By st 26, muscle nerves are first recognizable, diverging from the main nerve trunks at characteristic positions and presenting a frayed appearance at their tip (Lance-Jones and Landmesser, 1981a; see also Al-Ghaith and Lewis, 1982, for chick wing). The basic anatomical pattern of nerve trunks and muscle

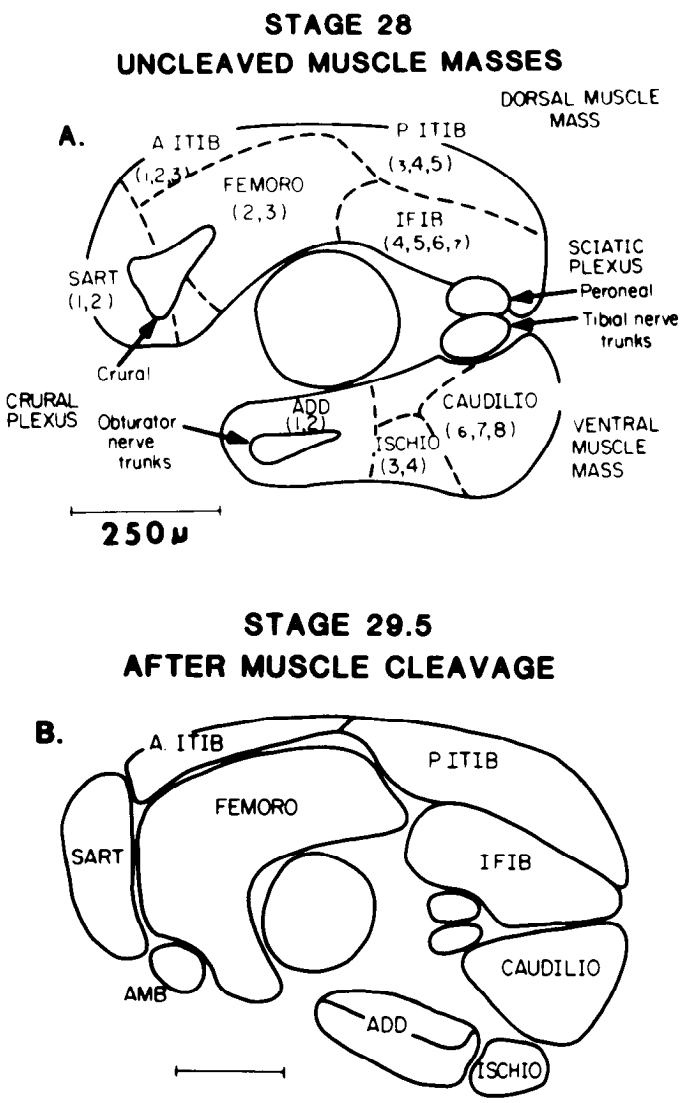


Figure 1. Positions of muscle and muscle precursors in the chick thigh at early embryonic stages. The approximate regions of the uncleaved dorsal and ventral muscle masses that contribute to the major thigh muscles (*B*) are shown in a cross-section of the limb in *A*. The major nerve trunks are also indicated. The major segmental innervation source of each muscle is indicated by *large numerals*; segments making only a minor contribution are indicated by *small numerals*. *SART*, sartorius; *A ITIB*, anterior iliotibialis; *FEMORO*, femorotibialis; *P ITIB*, posterior iliotibialis; *IFIB*, iliofibularis; *ADD*, adductors; *ISCHIO*, ischioflexorius; *CAUDILIO*, caudilioflexorius. In *B* the ambiens (*AMB*) is also indicated. *Calibration bar*, 250 μm.

nerves is limb imposed (Hamburger, 1939; Hollyday, 1981; Lance-Jones and Landmesser, 1981b; Tosney and Landmesser, 1984) and seems to constitute a highway system (Lewis et al., 1981) down which all lumbosacral motoneurons, even foreign ones, will preferentially elongate (Morris, 1978; Hollyday, 1981; Lance-Jones and Landmesser, 1981b; see also Landmesser, 1984).

A cross-section through the primary dorsal and ventral muscle masses at mid-thigh level is shown for a st 28 embryo in Figure 1*A*. The approximate boundaries of the regions that will contribute to the various muscles are indicated, as is their mature segmental source of innervation. Cleavage of these primary masses begins at st 28 and is largely complete by late st 29 to 30, producing the muscle pattern shown in Figure 1*B*.

In a previous study, Landmesser (1978), using extracellular recording from, and HRP injection into, the st 28 primary muscle masses, showed that most axons were projecting to and synapsing within segmentally appropriate regions even prior to muscle cleav-

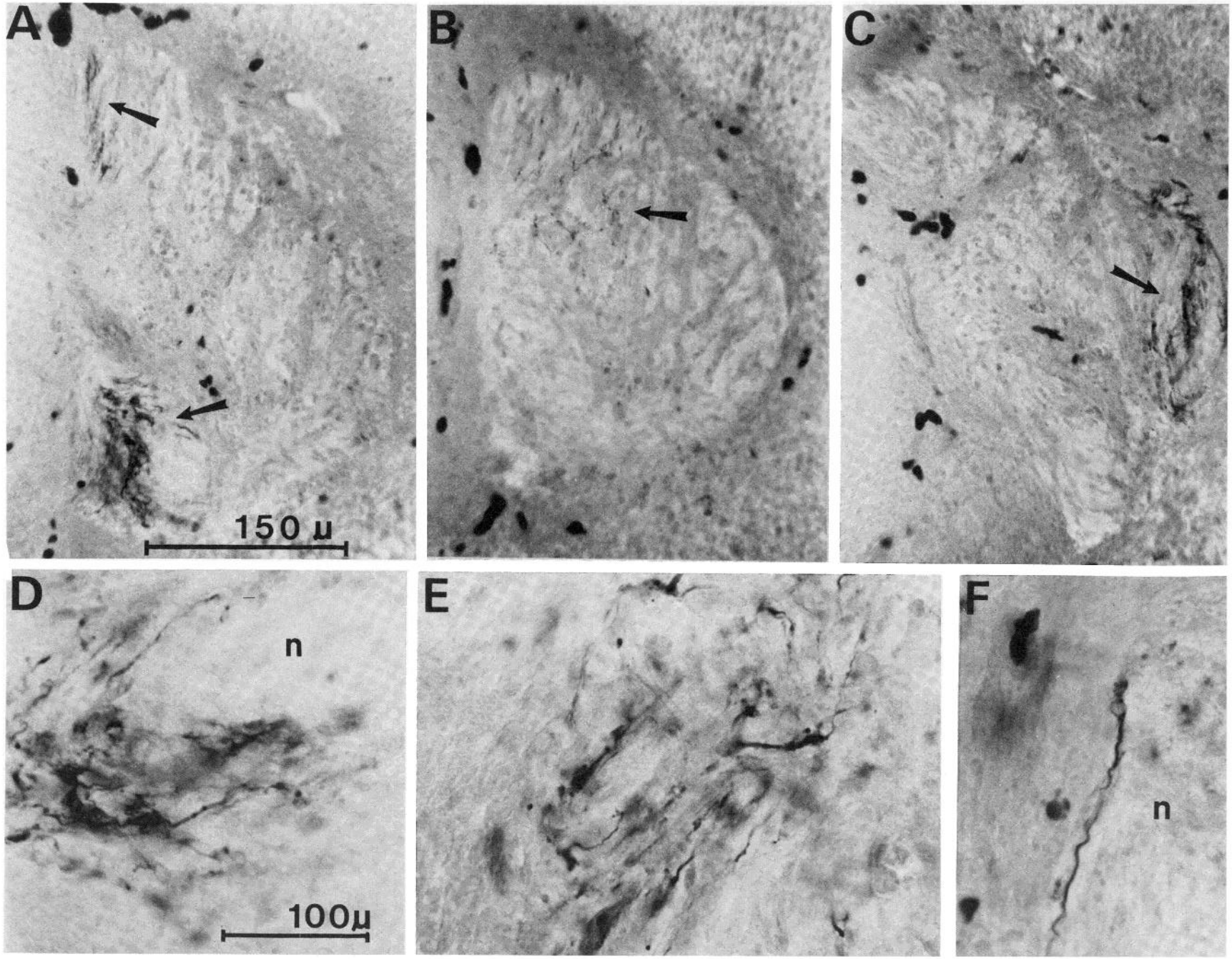


Figure 2. The appearance of HRP-labeled profiles in the plexus and muscle masses at st 28. The segregation of axons from different segments into characteristic positions within the sciatic plexus is shown in *A* to *C*, labeled axons and growth cones being noted by *arrows*. LS 3 was injected in *A*, LS 4, 5, and 6 in *B*, and LS 7 and 8 in *C*. *B* is from the proximal plexus region where spinal nerves LS 3 to 8 have come together to form the sciatic plexus. *A* and *C* are approximately 50 μm more distal where the most proximal muscle nerves are beginning to diverge. Dorsal is *up*; anterior is to the *left*. *Calibration bar*, 150 μm. *D* to *F* are higher magnification pictures of labeled growth cones and nerve endings in the primary muscle masses. In *D*, growth cones are penetrating the ischioflexorius region just ventral to the major nerve trunk (*n*). In *E*, a number of profiles in the femorotibialis region are shown. *F* shows a labeled LS 3 growth cone at the edge of the peroneal nerve trunk (*n*). *Calibration bar*, 100 μm.

TABLE I

The occurrence of labeled profiles in the major thigh muscle nerve as a proportion of the total number of cases studied, following HRP injection into the designated lumbrosacral spinal nerve or cord segment

Muscle (spinal nerve contribution)	Lumbosacral Spinal Nerve				
	1	2	3	4–6	7–8
SART[a] (1,2)[b]	6/6	2/3	0/6	0/3	0/7
A. ITIB (1,2,3)	1/6	1/3	2/6	0/3	0/7
FEMORO (2,3)	0/6	3/3	6/6	0/3	0/7
P. ITIB-IFIB (3–7)	0/6	0/3	5/9	2/3	0/7
ADD (1,2)	4/6	2/3	0/6	0/3	0/7
ISCHIO (3,4)	0/6	0/3	7/9	2/3	0/7
CAUDILIO (7,8)	0/6	0/3	0/9	0/3	7/7
Deep dorsal	0/6	0/3	3/9	0/3	0/7
Deep ventral	0/3	0/3	3/9	0/3	0/7

[a] SART, sartorius; A. ITIB, anterior iliotibialis; FEMORO, femorotibialis; P. ITIB-IFIB, posterior iliotibialis-iliofibularis; ADD, adductors; ISCHIO, ischioflexorius; CAUDILIO, caudilioflexorius.

[b] Numbers in parentheses, the adult segmental projections to each muscle.

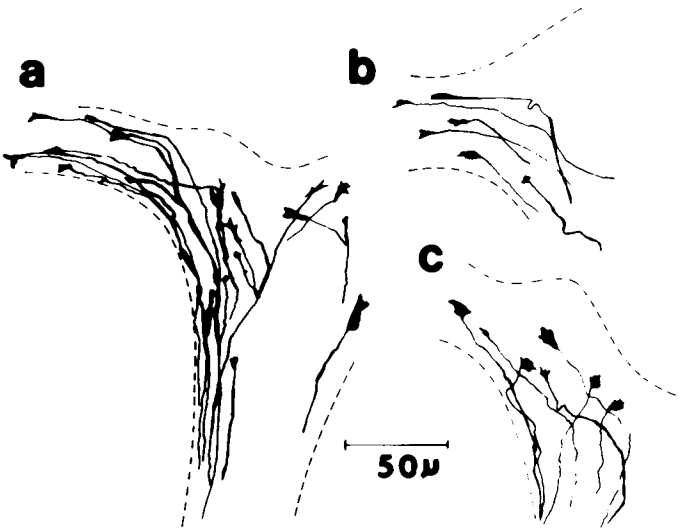


Figure 3. Trajectories of labeled axons in the region where muscle nerves diverge from main nerve trunks. In each of the three examples, which are camera lucida drawings made from 25-μm plastic sections, a number of labeled growth cones leave a common nerve trunk to project toward or actually enter a muscle nerve pathway, emerging to the left. The trajectory for each of the axons that has actually entered the muscle nerve in *a* and *b* is highly individualistic and in many cases involves axons crossing over each other. The complex trajectories and crossing of axons are also apparent in *c*. However, in this case, an earlier stage of muscle nerve formation, it is not possible to predict which growth cones will actually enter the nerve and which will turn to the right as several appear to be doing. *Calibration bar*, 250 μm.

age. However, these techniques did not allow a detailed characterization of axons within smaller regions of the muscle masses, especially their behavior at presumptive boundaries between muscles. For example, if one considers the ventral muscle mass (Fig. 1*A*), do "adductor" neurites from segments 1 and 2 grow into the ischioflexorius region and overlap with axons from segments 3 and 4 to any great extent? This is the sort of projection error that might be inferred from the work of Lamb (1976, 1979) and Pettigrew et al. (1979) described above.

We therefore assessed the position of labeled growth cones and axons from identified spinal segments in 22 limbs from st 27.5 to 30. Although our main goal was to determine the behavior of growth cones within the muscle masses, we made observations on the behavior of axons along their entire trajectories, including the plexus and muscle nerves. These latter observations, which in general confirm earlier work on this system, will be briefly presented first.

Behavior of identified axons in the plexus region. As noted in an earlier report (Lance-Jones and Landmesser, 1981b), we found that axons from different segments take up characteristic positions within the plexus. Axons from LS 3 rapidly diverge into two groups, one dorsal and destined to innervate the iliofibularis and posterior iliotibialis of the dorsal muscle mass, and one ventral, destined to innervate the ischioflexorius of the ventral muscle mass (Fig. 2*A*). Injection of LS 4, LS 5, and LS 6 labeled groups of axons in more intermediate positions (Fig. 2*B*), whereas axons from LS 7 and 8 were always confined to the most posterior part of the plexus (Fig. 2*C*). The plexus region is a site of complex interweaving of axons as they cross over one another in the process of achieving their characteristic positions (note the ventral LS 3 group in Fig. 2*A*).

Selection of muscle nerve pathways. Muscle nerves begin to emerge from the major nerve trunks at st 26 (Lance-Jones and Landmesser, 1981a; Tosney and Landmesser, 1985b), and by st 27.5 most have diverged sufficiently so that nerves can be unambiguously identified. In each of 11 embryos (predominantly from st 28, although they ranged from st 27.5 to 30), a specific subset of spinal nerves or a specific spinal cord segment was injected with HRP and the labeled axons were traced in serial sections. Often LS 1 and LS 7 and/or LS 8 were labeled on one side and LS 3 was labeled on the contralateral side.

We found no cases in which axons projected down segmentally inappropriate muscle nerves. For example, when LS 1 was injected, labeled axons were found in the sartorius nerve in every case (6 of 6) but never in the femorotibialis nerve (0 of 6). This is in accord with the mature projections to these muscles, as are the other data summarized in Table I. One may note that a given muscle nerve did not always receive a projection from a segment known to contribute to that muscle in the adult. This is because the injections were sometimes confined to less than an entire segment. For example, in the two cases where LS 1 did not contribute to the adductor, the injection site was in the anterior part of LS 1, whereas the adductor pool only begins in the posterior part of this segment. Also in Table I, the iliofibularis and posterior iliotibialis data have been combined because we were unable to unambiguously distinguish these nerves from each other at st 27 to 28.

In summary, none of the 238 muscle nerves assayed in this series contained any segmentally inappropriate axons. Although, as noted above, our HRP injections only labeled a subpopulation of the injected segment, the data are clearly in accord with earlier electrophysiological (Landmesser, 1978) and anatomical (Landmesser, 1978; Lance-Jones and Landmesser, 1981a; Hollyday, 1983ab) data, indicating that few if any errors were made in the process of muscle nerve pathway selection.

We also analyzed the trajectories taken by labeled axons in 18 sites of muscle nerve formation, in the hope that this might reveal something about the mechanism which allows axons to leave the nerve trunk at the appropriate position. We found that, although axons took up roughly characteristic positions in the plexus, those projecting to a single muscle were not tightly clustered at the site of muscle nerve formation. Thus, many axons leaving a common nerve trunk to project to an individual muscle often cross over axons continuing in the nerve trunk. This is apparent in the examples shown in Figure 3. Axons were also not tightly fasciculated with one another and often took independent trajectories to reach the muscle nerve (Fig. 3). Thus, although sorting out in the plexus region puts axons into roughly correct positions, subsequent shifts in their

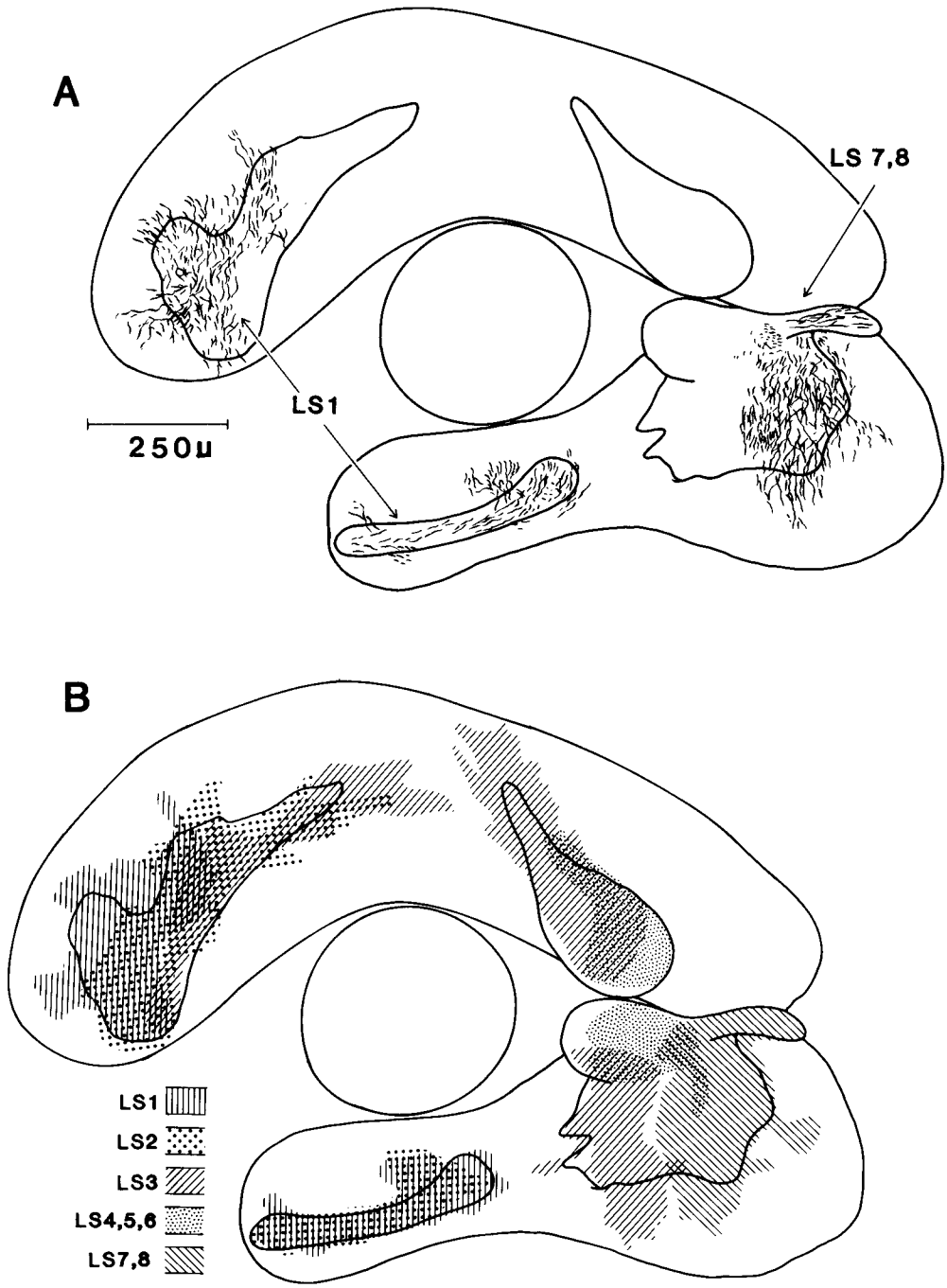
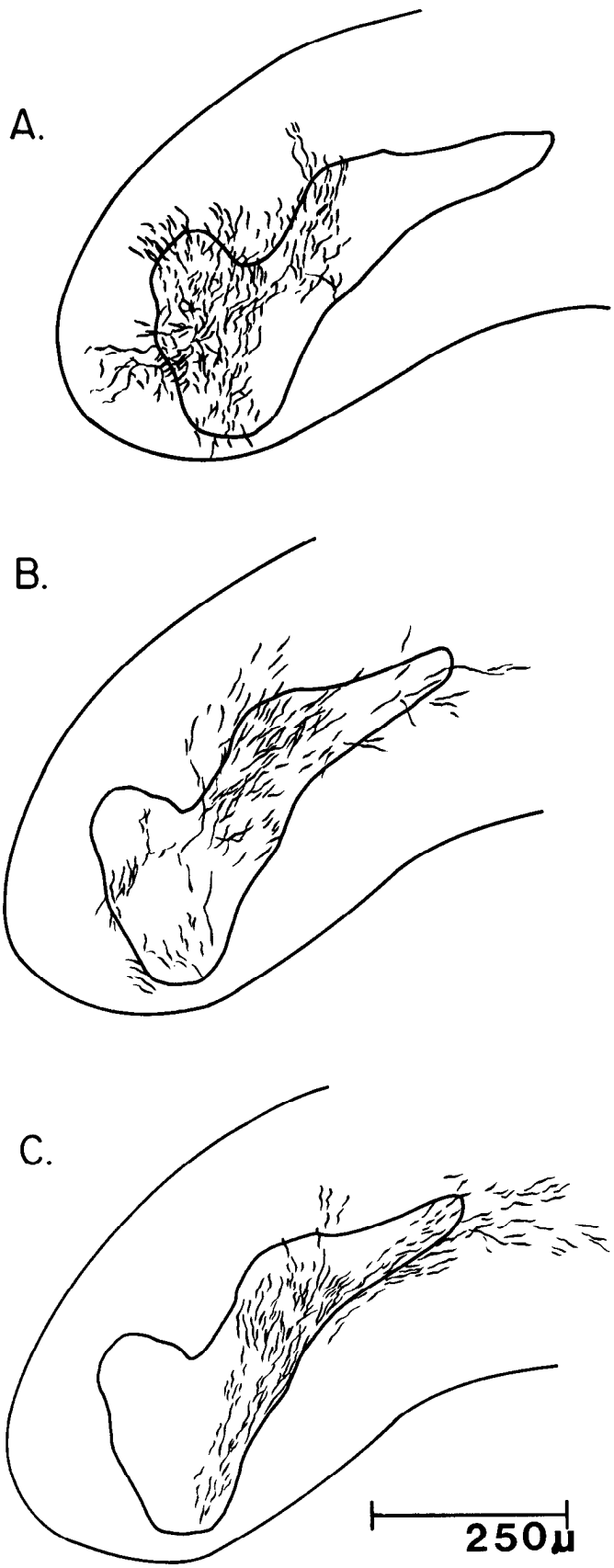


Figure 4. The projection of segmentally identified axons into the uncleaved muscle masses at st 28. In *A*, the distribution of labeled LS 1 growth cones and neurites in the dorsal and ventral muscle masses and the LS 7 and 8 projection to the ventral muscle masses can be seen to lie within regions appropriate for their mature projection (compare with Fig. 1*A*). The position of the major nerve trunks within the muscle masses is also shown. One should note that incipient muscle nerves projecting into the muscle mass from the major nerve trunks usually extend over only 75 to 100 μm of the proximodistal axis of the muscle mass. For example, the LS 7/8 and LS 3 sciatic projections to the ventral muscle mass occur more proximally than the sciatic projections of LS 3 to the dorsal muscle mass. For simplicity in this figure, the greatest extent of each of the muscle nerve projections has been drawn on the same normalized section from the mid-thigh level. More distal in the limb, after the emergence of these thigh muscle nerves, the major nerve trunks appear more compact as in Figure 1*A*. *B*, The boundaries of each of the segmental projections are summarized here as indicated by the different forms of shading. For further details, see the text.

relative positions take place more distally in the limb where muscle nerves emerge. These regions, like the plexus region itself, are sites of complex axonal rearrangements and must contain whatever environmental cues are needed for axons to choose the appropriate pathway.

Projection of axons within primary muscle masses. For 13 st 28 limbs used in the previous section, the position of labeled growth cones and axons within the dorsal and ventral muscle masses of the thigh were also characterized. Many of the labeled profiles were growth cones (see Fig. 2, *D* to *F*) and form part of the data base for the subsequent paper (Tosney and Landmesser, 1985a). However, since it was often difficult to be sure that a given profile was actually a growth cone, particularly in heavily labeled sections or in the sections transverse to the limb from which most of the data were obtained for this paper, all labeled profiles were included in the following analysis. For each limb, camera lucida drawings were made of every section through the thigh and the position of all labeled profiles noted (for examples, see Fig. 2, *C* to *E*). The limbs were normalized for slight differences in size as described under "Materials and Methods," and all of the projections for each labeled spinal nerve were superimposed. The spinal nerve projections characterized in this way were LS 1 (N = 5), LS 2 (N = 2), LS 3 (N = 6), LS 5 and 6 or LS 4, 5, and 6 (N = 3), and LS 7 or 8 or LS 7 and 8 (N = 5).

The combined projections of LS 1 and LS 7/8 are shown in the camera lucida cross-section of Figure 4*A*, in which the approximate boundaries of the major nerve trunks and muscle nerves at mid-thigh are also indicated. Both LS 1 and LS 7/8 only project to regions appropriate for their mature projection (cf. Fig. 1). In addition, it was apparent that most labeled profiles including growth cones are confined to the major nerve trunks and muscle nerves or their immediate surroundings. This leaves large regions of the primary



muscle mass devoid of axonal contacts. Although consistent with our observation that axons tend to fasciculate with each other from the time of earliest outgrowth rather than diverging widely within the mesenchyme through which they are growing (Tosney and Landmesser, 1985b), this is a rather novel observation. Since most of the muscle fibers run transverse to the plane of section shown in Figure 4, it would appear that many muscle fibers are not innervated at this stage.

One consequence of the failure of nerves to ramify widely within the primary muscle masses is that many growth cones will not encounter presumptive boundaries between muscles until st 30 or later. By this time a cleavage plane will have physically separated the two muscles, presumably restricting a growth cone's access to foreign targets (see for example, the adductor-ischioflexorius, femorotibialis-iliofibularis, and anterior iliotibialis-posterior iliotibialis boundary regions).

However, even in regions supplied by a common nerve trunk where two projections abut, they do not overlap to any great extent. The LS 3 projection to the ischioflexorius overlaps only slightly with the LS 7 and 8 projections to the adjacent caudilioflexorius region (Fig. 4*B*). Similarly, outside of the common nerve trunk in which they both run, the LS 1 and LS 3 projections to the crural are not overlapping, LS 1 projecting heavily to the sartorius and anterior iliotibialis regions and LS 3 to the femorotibialis region, both in accord with their mature projections (see also Fig. 5, *A* and *C*).

Another feature apparent from the data in Figure 4*B* is the lack of a strict segmental topography within the early muscle masses. For example, although both LS 1 and LS 2 project to the adductor region, there is no tendency for LS 1 to project more anteriorly and LS 2 more posteriorly within this region. On the contrary, their projections are almost completely overlapping. A similar lack of strict topography can be seen in the LS 1, 2, and 3 projections to the dorsal muscle mass (Fig. 5). Although each spinal nerve projects most heavily to a different region, there is considerable overlap between spinal nerve projections that contribute to the same muscle in the adult. For example, LS 2 overlaps extensively with LS 1 in both the sartorius and anterior iliotibialis regions (both segments projecting to both muscles in the adult). LS 2 also overlaps extensively with the LS 3 femorotibialis projection. In fact, some LS 2 growth cones extend almost as far posteriorly as do those from LS 3.

Finally, it is only at st 30, after muscle cleavage and the onset of motoneuron cell death, that axons ramify widely within muscles. For example, at st 28 (Fig. 6*A*), axons are confined for the most part to the region of the muscle immediately adjacent to the peroneal nerve trunk and major muscle nerve branches. In contrast, by st 30 (Fig. 6*B*), many iliofibularis growth cones and axons penetrate the muscle, forming small intramuscular nerve branches.

Discussion

The results reported here confirm earlier studies which showed that avian limb innervating motoneurons project to segmentally appropriate muscle nerves (Landmesser and Morris, 1975; Landmesser, 1978; Lance-Jones and Landmesser, 1981a; Hollyday, 1983a) and to regions of the uncleaved primary muscle masses (Landmesser, 1978) prior to the period of motoneuron cell death (Hamburger, 1975). Although occasional errors in muscle nerve pathway selection were detected by Landmesser (1978) and Hollyday (1983a) using electrophysiological and anatomical techniques, respectively, these were quite rare. This evidence, together with the absence of errors in the large number of muscle nerves assayed in

Figure 5. Topography of the crural segmental projections within the dorsal muscle mass. The projections of the crural segments LS 1 (*A*), LS 2 (*B*), and LS 3 (*C*) into the anterior half of the st 28 dorsal muscle mass are indicated. Each projects most heavily to a different region, although there is considerable overlap between LS 1 and 2 and between LS 2 and 3. *Calibration bar*, 250 μm.

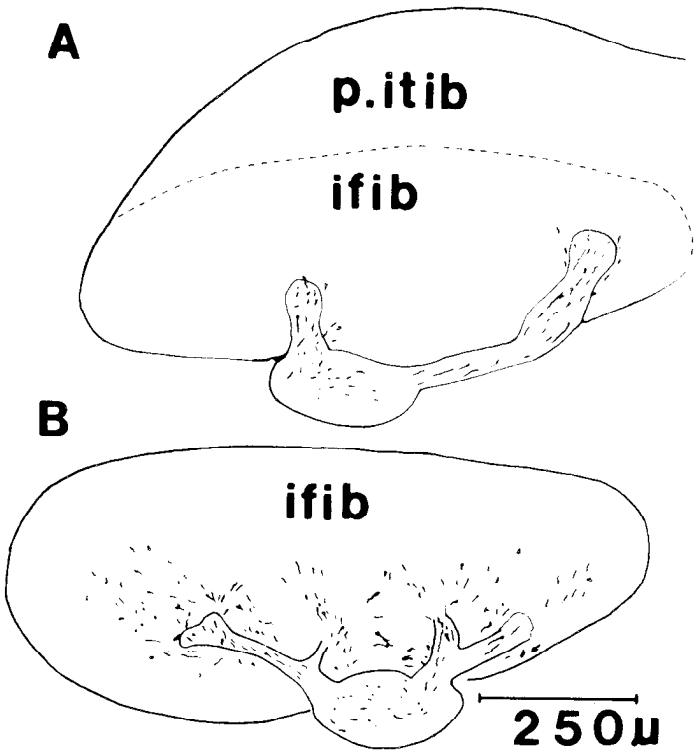


Figure 6. Penetration of axons into the iliofibularis muscle. In a camera lucida reconstruction from a single embryo at st 28, labeled axons can be seen to lie primarily within the major muscle nerve trunks to the iliofibularis (*ifib*) region. The approximate boundaries of this muscle within the uncleaved dorsal muscle mass are indicated by the *dashed line*. By st 29.5, when muscle cleavage is complete, axons have ramified much more widely within the muscle. Dorsal is *up*; anterior is to the *right*. *Calibration bar*, 250 μm.

the present series, leads us to conclude that muscle nerve pathway selection is very accurate.

As for the mechanism by which motoneuron growth cones are able to select the appropriate pathway, Summerbell and Stirling (1981) have suggested that passive deployment of axons based on their spatial position within the major nerve trunks might suffice. This is consistent with observations showing that axons destined to exit in specific muscle nerves take up characteristic spatial positions within the plexus (Lance-Jones and Landmesser, 1981a, and the present results) and that the position at which muscle nerves diverge from the main nerve trunks is imposed by the limb (Hamburger, 1939; Hollyday, 1981; Lance-Jones and Landmesser, 1981b).

However, other studies showing that experimentally displaced axons are able to alter their trajectories to select the appropriate muscle nerve (Lance-Jones and Landmesser, 1980b, 1981b; see also Ferguson, 1983) are not consistent with a passive deployment model. Our observations showing that axons take highly individualistic trajectories at the site of muscle nerve emergence, often crossing over other axons to exit from the common nerve trunk, are also not consistent with such a model. We therefore favor a dual model in which some features of the environment cause muscle nerves to form at certain points. These features appear to be nonspecific, acting on all neurons equally since even foreign axons can be induced to form muscle nerves at these points (Hamburger, 1939; Hollyday, 1981; Lance-Jones and Landmesser, 1981a; Summerbell and Stirling, 1981; Lance-Jones, 1982b). However, these sites must also contain specific cues which can be recognized by growth cones, since only a specific subset of axons normally leaves the nerve trunk at this point.

An analysis at both the light and electron microscopic level (Tosney and Landmesser, 1985b) has not revealed any obvious structural correlates of guidance cues at such sites (i.e., similar to the guidepost cells of invertebrates; see Goodman et al., 1982; Ho et al., 1983; Bentley and Caudy, 1984). However, from the work of Lewis et al. (1981) it can be inferred that cues for muscle nerve formation require the somitic component of the limb bud mesenchyme, because when these "premuscle" cells are destroyed prior to their migration into the limb, muscle nerves fail to form.

Since muscle nerve pathway selection appears to be highly selective, the projection errors observed by others (Lamb, 1976, 1979; Pettigrew et al., 1979; Laing, 1982) would presumably result from axons growing over premuscle boundaries within the limb. The extent to which such errors occur is still controversial. Both Lamb (1976) and Pettigrew et al. (1979) have presented evidence for obvious misprojection of varying numbers of limb motoneurons. However, since these studies were based primarily on retrograde labeling with HRP, the possibility of HRP leakage cannot be excluded (see Ashwell and Watson, 1983, and Farel and Bemelmans, 1985, for further discussion of this possibility). In contrast, a recent report by Laing (1982), who prevented motoneuron cell death with neuromuscular blocking agents, has shown that the extent of motoneuron cell death is not uniform throughout the chick lumbosacral motor column. Using indirect evidence based on the distribution of surviving motoneurons following limb ablations at the knee in normal and paralyzed embryos, he has concluded that projection errors do occur in the chick hindlimb. Contrary to this, Oppenheim (1981), using similar paralyzed embryos, found that the motoneurons rescued from cell death were localized appropriately in the spinal cord for the muscle to which they were projecting.

Although it may not be possible to fully resolve these conflicting reports, our present results concerning the distribution of labeled growth cones provides very direct evidence that hindlimb motoneurons are confined to segmentally appropriate regions of the primary muscle masses. This evidence is not subject to the errors in interpretation which might result from possible HRP leakage. We are also certain that we did not overlook errors which occurred at earlier stages, since in the larger experimental series from which these data were taken, we assayed axonal outgrowth from st 19 when axons first leave the spinal cord. Axons do not enter the limb until late st 24, and from this stage on their growth cones are confined to appropriate regions—if anything, more restricted in distribution than at later stages (Tosney and Landmesser, 1985a, b; see also Lance-Jones and Landmesser, 1981a).

In summary, these results, together with other studies where artifactual leakage of HRP has been excluded or minimized (Landmesser, 1978; Lance-Jones and Landmesser, 1981a; Ashwell and Watson, 1983; Hollyday, 1983b; Lance-Jones, 1982b) show that if projection errors occur, they must be minor in spatial extent. We must then explain how growth cones are confined to appropriate regions of the muscle masses.

One possibility is that they recognize regionally localized chemical cues. Data supporting the existence of localized cues within the developing chick limb have been obtained in several studies (Lance-Jones and Landmesser, 1981b; Summerbell and Stirling, 1981; Whitelaw and Hollyday, 1983). In addition, Wigston and Sanes (1982) have shown that rat preganglionic autonomic neurons exhibit a weak preference for intercostal muscles from the same segment. This implies the existence of segmentally based recognition markers on these muscle fibers. Whether the cues used by limb motoneurons to form specific projections are contained in the muscle or connective tissue components of the limb bud, and whether they too are based on the segmental origin of these components, remains to be determined.

Even if recognition molecules exist, it is not certain whether they would be sufficiently unique or localized with sufficient precision to create the observed projection patterns. For example, adductor muscle fibers up to the boundary with the ischioflexorius are innervated by LS 1 and 2 motoneurons. Yet none of these motoneurons innervate the immediately adjacent ischioflexorius muscle fibers, normally innervated by LS 3 and 4 (Landmesser, 1978). It is unlikely that weak segmental preferences similar to those demonstrated by

Wigston and Sanes (1982) could alone result in such precise boundaries. However, several other features of the developing limb may contribute to the formation of these patterns.

The first is the tendency we observed for most growth cones to remain for some time in the central parts of their prospective muscles close to or within major nerve branches. It is only at st 30 and later that axons ramify widely in the muscles and approach muscle boundaries. This delay probably results from growth cones' preference for other neurites over the limb bud mesenchyme at these stages, a preference which appears to be at least temporally correlated with changes in the level of muscle NCAM (Tosney et al., 1985). One important consequence of this delay is that many growth cones do not reach muscle boundaries until after cleavage, when a physical space containing collagen and other connective tissue components has created an effective barrier to their advancement.

Neurons may also be constrained by interactions with other neurons. We observed that neurons have a strong tendency to fasciculate with each other (see also Tosney and Landmesser, 1985b), and other studies have shown that motoneurons from the same segments and motoneuron pools tend to fasciculate with each other in the plexus (Lance-Jones and Landmesser, 1980a, b, 1981a) and run together in the major nerve trunks (Lance-Jones and Landmesser, 1980a, 1981a; Summerbell and Stirling, 1981). This would enable axons to sort out on the basis of segments or even muscles, and should enhance the probability of axons selecting the appropriate muscle nerve pathways. This, together with their delay in ramifying within the muscle mass, would help to create the observed projection patterns.

Neurons may also be constrained by competitive interactions with other neurons. Scott (1983) has recently shown that the projection of hindlimb sensory neurons can be shifted to adjacent nerves by depleting these nerves of their sensory component through neural crest ablations. Lance-Jones and Landmesser (1980a) did not observe similar shifts in the projections of motoneurons following deletion of adjacent spinal cord segments. For example, LS 7 and 8 axons did not spread into the adductor region when these were left uninnervated following removal of LS 1 to 4. However, the present results show that these two projections do not abut at these stages. Thus, the possibility that competitive interactions play some role in restricting immediately adjacent populations of neurons (i.e., LS 3 and 4 versus LS 7 and 8) remains (see also Hollyday, 1983b).

Finally, although we have shown that the basic pattern of matching between motoneuron pool and muscle in this system does not result from selective cell death, we do not wish to imply that cell death and other regressive events are not important in shaping neural circuits. There is evidence that cell death, competitive interactions between neurons, and even patterns of functional activity may play a role in refining circuits in the CNS (Meyer, 1983; Cowan et al., 1984).

More subtle matching of motoneurons with their targets may involve similar phenomena. There is evidence that the achievement of mature topographic projections within muscles may result from motoneuron cell death (Bennett and Lavidis, 1981) and the selective elimination of axon collaterals (Brown and Booth, 1983). The matching of fast and slow motoneurons with appropriately typed muscle fibers may involve similar mechanisms (McClennan, 1983; Laing and Lamb, 1983; Vogel and Landmesser, 1984). Nevertheless, the initial precise axonal projections of motoneurons into topographically appropriate places in the limb is apparently achieved primarily by the accurate guidance of growth cones. A detailed description of growth cone morphology and behavior during initial outgrowth is presented in the following paper (Tosney and Landmesser, 1985a).

References

Al-Ghaith, L. K., and J. H. Lewis (1982) Pioneer growth cones in virgin mesenchyme: An electron microscope study in the developing chick wing. J. Embryol. Exp. Morphol. *68:* 149–160.

Ashwell, K. W., and C. R. Watson (1983) The development of facial motoneurons in the mouse: Neuronal death and the innervation of the facial muscles. J. Embryol. Exp. Morphol. *77:* 117–141.

Bennett, M. R., and N. A. Lavidis (1981) Development of the topographical projection of motor neurons to an amphibian muscle accompanies motor neuron death. Brain Res. *254:* 448–452.

Bentley, D., and M. Caudy (1984) Navigational substrates for peripheral pioneer growth cones: Limb-axis polarity cues, limb-segment boundaries and guidepost neurons. Cold Spring Harbor Symp. Quant. Biol. *48:* 573–585.

Brown, M. C., and C. M. Booth (1983) Postnatal development of the adult pattern of motor axon distribution in rat muscle. Nature *304:* 741–742.

Cowan, M. W., J. W. Fawcett, D. D. M. O'Leary, and B. B. Stanfield (1984) Regressive events in neurogenesis. Science *225:* 1258–1265.

Farel, P. B. and S. E. Bemelmans (1985) Specificity of motoneuron projection patterns during development of the bullfrog tadpole (*Rana catesbeiana*). J. Comp. Neurol., in press.

Ferguson, B. (1983) Development of motor innervation of the chick following dorsal-ventral limb bud rotations. J. Neurosci. *3:* 1760–1772.

Goodman, C. S., J. A. Raper, R. Ho, and S. Change (1982) Pathfinding by neuronal growth cones in grasshopper embryos. In *Cytochemical Methods in Neuroanatomy: Proceedings*, V. Chan-Palay, ed., pp. 461–494, Alan R. Liss, Inc., New York.

Hamburger, V. (1939) The development and innervation of transplanted limb primordia in chick embryos. J. Exp. Zool. *80:* 347–389.

Hamburger, V. (1975) Cell death in the development of the lateral motor column of the chick embryo. J. Morphol. *88:* 535–546.

Hamburger, V., and H. L. Hamilton (1951) A series of normal stages in the development of the chick embryo. J. Morphol. *88:* 49–82.

Ho, R. K., E. E. Ball, and C. S. Goodman (1983) Muscle pioneers: Large mesodermal cells that erect a scaffold for developing muscles and motoneurons in grasshopper embryos. Nature *301:* 66–68.

Hollyday, M. (1981) Rules of motor innervation in chick embryos with supernumerary limbs. J. Comp. Neurol. *202:* 439–465.

Hollyday, M. (1983a) Development of motor innervation of chick limbs. In *Limb Development and Regeneration*, J. Fallon and A. Caplan, eds., Part A, pp. 183–193, Alan R. Liss, Inc., New York.

Hollyday, M. (1983b) Patterns of initial wing innervation in normal chick embryos and in embryos lacking some brachial spinal cord segments. Soc. Neurosci. Abstr. *9:* 210.

Laing, N. (1982) Motor projection patterns to the hindlimb of normal and paralyzed chick embryos. J. Embryol. Exp. Morphol. *72:* 269–286.

Laing, N., and A. Lamb (1983) The distribution of muscle fibre types in chick embryo wings transplanted to the pelvic region is normal. J. Embryol. Exp. Morphol. *78:* 67–82.

Lamb, A. (1976) The projection patterns of the ventral horn to the hindlimb during development. Dev. Biol. *54:* 82–99.

Lamb, A. (1979) Evidence that some developing limb motoneurons die for reasons other than peripheral competition. Dev. Biol. *71:* 8–21.

Lance-Jones, C. J. (1982a) Motoneuron cell death in the developing lumbar spinal cord of the mouse. Dev. Brain Res. *4:* 473–479

Lance-Jones, C. J. (1982b) Motoneuron connectivity in chick limbs with an altered complement of muscle. Soc. Neurosci. Abstr. *8:* 435

Lance-Jones, C. J., and L. T. Landmesser (1980a) Motoneuron projection patterns in embryonic chick limbs following partial deletions of the spinal cord. J. Physiol. (Lond.) *302:* 559–580.

Lance-Jones, C. J., and L. T. Landmesser (1980b) Motoneuron projection patterns in the chick hindlimb following early partial spinal cord reversals. J. Physiol. (Lond.) *302:* 581–602.

Lance-Jones, C. J., and L. T. Landmesser (1981a) Pathway selection by chick lumbosacral motoneurons during normal development. Proc. R. Soc. Lond. (Biol.) *214:* 1–18.

Lance-Jones, C. J., and L. T. Landmesser (1981b) Pathway selection by embryonic chick motoneurons in an experimentally altered environment. Proc. R. Soc. Lond. (Biol.) *214:* 19–52.

Landmesser, L. T. (1978) The development of motor projection patterns in the chick hindlimb. J. Physiol. (Lond.) *284:* 391–414.

Landmesser, L. T. (1980) The generation of neuromuscular specificity. Annu. Rev. Neurosci. *3:* 279–302.

Landmesser, L. T. (1984) The development of specific motor pathways in the chick embryo. Trends Neurosci. *7:* 336–339.

Landmesser, L. T., and D. G. Morris (1975) The development of functional innervation in the hindlimb of the chick embryo. J. Physiol. (Lond.) *249:* 301–326.

Lewis, J., A. Chevallier, M. Kieny, and L. Wolpert (1981) Muscle nerves do

not develop in chick wing devoid of muscle. J. Embryol. Exp. Morphol. *64:* 211–232.

McClennan, I. S. (1983) The development of the pattern of innervation in chick hindlimb muscles: Evidence for specification of nerve-muscle connections. Dev. Biol. *97:* 229–238.

Meyer, R. L. (1983) Tetrodotoxin inhibits the formation of refined topography in goldfish. Dev. Brain Res. *6:* 293–298.

Morris, D. (1978) Development of functional motor innervation in supernumerary hindlimbs of the chick embryo. J. Neurophysiol. *41:* 1450–1465.

Oppenheim, R. (1981) Cell death of motoneurons in the chick embryo spinal cord. Evidence on the role of cell death and neuromuscular function in the formation of specific connections. J. Neurosci. *1:* 141–151.

Pettigrew, A. G., R. Linderman, and M. R. Bennett (1979) Development of the segmental innervation of the chick forelimb. J. Embryol. Exp. Morphol. *49:* 115–137.

Romer, A. S. (1927) The development of the thigh musculature of the chick. J. Morphol. *43:* 347–385.

Scott, S. A. (1983) Effects of neural crest deletion on the segmental pattern of skin sensory innervation in embryonic chick limb. Soc. Neurosci. Abstr. *9:* 847.

Summerbell, D., and R. V. Stirling (1981) The innervation of dorsoventrally reversed chick wings: Evidence that motor axons do not actively seek out their appropriate targets. J. Embryol. Exp. Morphol. *61:* 233–247.

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

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

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

Tosney, K., M. Watanabe, L. Landmesser, and U. Rutishauser (1985) Distribution of NCAM in the chick hindlimb during axon outgrowth and the establishment of mature nerve-muscle realtionships. Soc. Neurosci. Abstr. *11:* in press.

Vogel, M., and L. T. Landmesser (1984) Patterns of fast and slow muscle fibers in embryonic chick muscles innervated by foreign motoneurons. Soc. Neurosci. Abstr. *10:* 62.

Whitelaw, V., and M. Hollyday (1983) Thigh and calf discrimination in the motor innervation of the chick hindlimb following deletions of limb segments. J. Neurosci. *3:* 1199–1215.

Wigston, D., and J. Sanes (1982) Selective reinnervation of adult mammalian muscles by axons from different segmental levels. Nature *299:* 464–467.