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MOTONEURONE PROJECTION PATTERNS IN THE CHICK HIND LIMB FOLLOWING EARLY PARTIAL REVERSALS OF THE SPINAL CORD

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SUMMARY

1. The development of motoneurone projection patterns in the chick hind limb from reversed spinal cord segments was studied from the onset of axonal outgrowth (St. 24) to the establishment of mature connectivity patterns (St. 36). Approximately the first three lumbosacral cord segments were reversed along the anterior–posterior axis at St. 15–16.

2. Projection patterns from reversed cord segments were assessed electrophysiologically by direct spinal cord and spinal nerve stimulation and anatomically by retrograde horseradish peroxidase (HRP) labelling of motoneurones in St. 30–36 embryos. In younger embryos, paths taken by reversed axons were characterized by orthograde HRP labelling of motoneurones in specific reversed cord segments.

3. Lumbosacral motoneurones formed appropriate functional connexions with individual limb muscles in spite of anterior-posterior shifts in their spinal cord position and consequent shifts in their spinal nerve entry point into the limb bud. Reversed motoneurones supplying individual hind limb muscles formed discrete nuclei in the transverse plane of the cord. Each nucleus and the lateral motor column as a whole showed reversed topographical characteristics when compared to control embryos. These observations were made both before (St. 30) and after (St. 35–36) the major period of motoneurone cell death.

4. Correct connectivity resulted from specific alterations in axonal pathways within the plexus or major nerve trunks proximal to the branching of individual muscle nerves. Further such directed outgrowth was present from the earliest times that axons could be traced into the limb which is before the onset of motoneurone cell death and muscle cleavage.

5. It is concluded that motoneurones are specified to project to individual muscles or to follow particular pathways prior to motoneurone birthdays and limb bud formation. The establishment of specific motoneurone connectivity can not be accounted for by passive or mechanical guidance models alone. Rather, motoneurones must also actively respond to cues within the limb or interact among themselves on the basis of an early central specification.

INTRODUCTION

Anatomical and electrophysiological studies have shown that the motoneurones which innervate individual limb muscles are located in discrete pools within the spinal cord (Romanes, 1964; Cruce, 1974; Landmesser, 1978*a*), exit the cord in

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specific spinal nerves (Landmesser & Morris, 1975) and seem to form compact bundles within a specific region of the plexus and major nerve trunks (Ueyama, 1978; Stirling & Summerbell, 1979). Further, a general correlation exists between a neurone's anterior–posterior position in the cord and its target muscle's position in a normal limb (Landmesser, 1978*b*). These discrete spatial relationships may reflect an orderly axonal outgrowth pattern during development. Motoneurones may be passively guided to a particular target determined by their cord or plexus position (Horder, 1978). Motoneurones might also grow out in an orderly temporal sequence and form connexions with the nearest uninnervated muscle (Jacobson, 1978). Experimental evidence suggests that a spatio-temporal model for axonal outgrowth and synapse formation can account for the formation of specific optic fibre connexions in vertebrates (Bunt & Horder, 1978; Gaze, 1978) and invertebrates (Anderson, 1978; Macagno, 1978). While these models are economical in not requiring that each motoneurone and muscle have a specific identity, other characteristics of limb motoneurone projection patterns in the chick are not as compatible with a strictly passive contact guidance model for the establishment of specific connexions.

Motoneurone pools overlap extensively along the anterior–posterior axis of the cord and as a result their axons must cross one another in the periphery. It has been suggested that these discontinuities result from secondary events during development such as competition for targets or limb morphogenetic movements (Horder, 1978). However, the experimental evidence presented in the previous paper (Lance-Jones & Landmesser, 1980) excludes competition as necessary for the establishment of normal motoneurone projection patterns. In these deletion experiments, motoneurones appeared to recognize appropriate targets and did not innervate foreign but uninnervated tissue. Further, in the chick limb motoneurone projection patterns are correct and specific at early developmental stages prior to limb muscle morphogenesis (Landmesser & Morris, 1975; Landmesser, 1978*b*). These results are compatible with a hypothesis that motoneurones are specified to innervate a particular target or recognize correct pathways upon outgrowth. Such specificity might be imparted by the limb or develop independently. In either case, axonal outgrowth might be actively guided by cues within the limb (Morris, 1978). Studies *in vitro* (Letourneau, 1975, 1978; Ebendal, 1977) and *in vivo* (Hibbard, 1965) in other systems clearly demonstrate that axonal growth cones are active structures capable of responding differentially to environmental cues.

In order to address the question of whether motoneurones are specified prior to outgrowth an experiment was designed to provide lumbosacral motoneurones with a choice of pathways and targets within an unaltered and localized limb region. Specific lumbosacral cord segments were reversed prior to limb bud formation and lumbosacral motoneurone differentiation. Thus, axons from reversed segments were offered a choice between originally appropriate and spatially appropriate targets. Motoneurone projection patterns from the reversed cord segments were characterized from the time of initial axonal outgrowth to the establishment of the mature pattern using electrophysiological and anatomical horseradish peroxidase labelling techniques. A preliminary report of the results has been presented elsewhere (Lance-Jones & Landmesser, 1978).

METHODS

Surgical procedure

White Leghorn chick embryos were incubated until Stage (St.) 15–16 (2½–3 days) of Hamburger & Hamilton (1951). A window was made in the egg above the embryonic area with a dental drill and the embryo stained over the lumbosacral region with a small drop of a 1–2% neutral red saline solution. After opening the vitelline membrane, the part of the spinal cord to be transposed was identified by reference to adjacent somites.

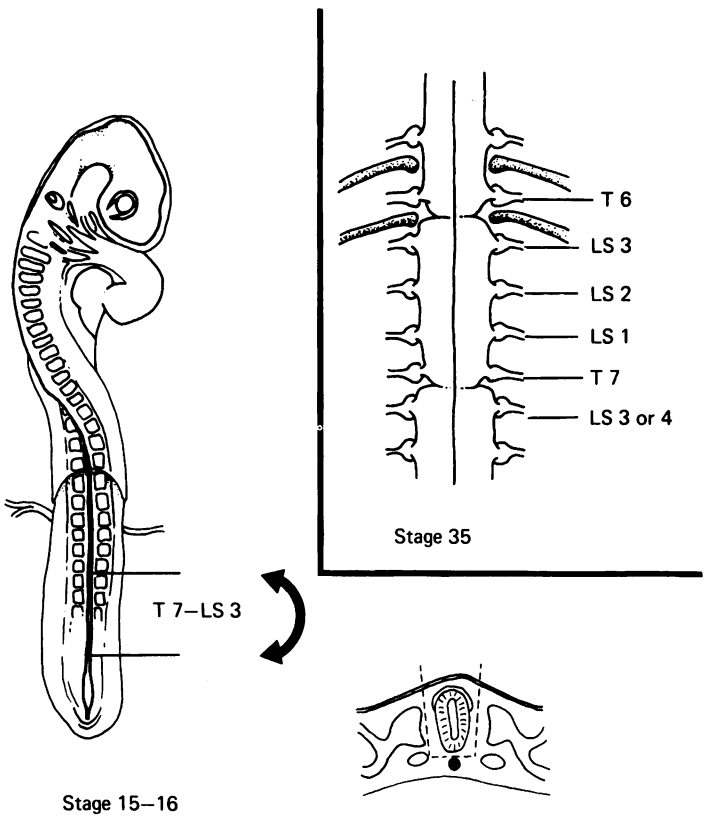

Fig. 1. Diagrams of the position and morphology of the cord segments reversed at the time of the operation (left) and at a representative time of examination (top right). The structures removed and reversed in the operation are shown in cross-section at the bottom right (within dashed lines). Not drawn to scale.

The anterior part of the lumbosacral enlargement, including cord segments 1 and 2 or 1–3 (segments 23–25 of Hamburger, 1975) were chosen for manipulation. In order to reverse these segments it was necessary to isolate that part of the cord opposite somites 26–29 (see Fig. 1). Variability in the actual extent of the reversal resulted from (1) slight changes in the developmental relationship between somite and cord segment number (see Wenger, 1951), (2) the necessity of estimating the position of the more posterior somites which have not formed at St. 15–16 and (3) the observation that between a quarter and one segment was often lost or damaged during the operation.

The spinal cord segments were freed laterally from the somitic tissue with tungsten needles. Transverse cuts were then made and the piece of cord separated from the underlying tissue. It was then rotated 180° along the anterior–posterior axis and placed back in line with the rest of the

cord. In most cases the notochord was rotated with the spinal cord in order to minimize damage. Controls in which the notochord was not rotated gave similar results. Following the operation the embryo and vitelline membrane were moistened with Tyrode solution. The window was covered with a glass coverslip and sealed with paraffin, and the egg returned to the incubator.

Dissection

Experimental embryos were incubated until stages ranging from 24 to 36. The embryos were removed from the egg, placed into oxygenated Tyrode solution at 20–22 °C, staged, and dissected as described in a previous study by Landmesser (1978*a*). Following a ventral laminectomy of the thoracic and lumbosacral spinal cord, the extent of the reversal could be characterized visually (see Fig. 1). Small indentations on the ventrolateral edges of the cord were visible at the cranial and caudal ends of the transposed region. The lateral outline of the spinal cord usually expanded abruptly at the cranial end of the reversal, tapered down gradually, and expanded again just beyond the end of the reversal.

Electrophysiology

Techniques used to assess the innervation pattern of the embryonic limb have been described in earlier studies of normal projection patterns (Landmesser & Morris, 1975; Landmesser, 1978*b*). In St. 30 and 35–36 embryos muscle contraction was elicited by direct cord stimulation and/or by spinal nerve stimulation which were found to give equivalent results. Direct cord stimulation involved the use of suction electrodes at selected sites along the ventrolateral margin of the cord or just over ventral root exit regions. Stimulation points were mapped on a drawing of the cord. In order to examine spinal nerve patterns, the cord was removed and individual nerves isolated and stimulated with suction electrodes. Muscle contraction was scored for each cord point or nerve either visually or by recording electromyograms. Suction or bipolar silver electrodes were used to record electrical activity from the medial surface of individual muscles.

Retrograde labelling with horseradish peroxidase

Three muscles, the sartorius, femorotibialis and adductor have motoneurone pools which are located in specific regions of the first three segments of the lumbosacral lateral motor column (Landmesser, 1978*a*). In order to examine the topography of these pools muscles were injected with horseradish peroxidase (HRP) in St. 30 or St. 35–36 experimental embryos, after ascertaining the extent of the reversal by direct cord stimulation. The procedures for HRP injection, histological processing, and the counting and mapping of labelled motoneurones were described by Landmesser (1978*a*). Limbs were also histologically processed for visualization of the injection site.

Orthograde labelling with HRP

In order to characterize the pathways of limb innervating motor axons during the outgrowth period, an orthograde HRP labelling technique was designed (see Lance-Jones & Landmesser, 1980). The segment at the anterior end of the reversal region was filled directly with HRP on one side of an experimental embryo, the segment at the posterior end on the other. These segments corresponded in most cases to the 3rd and 1st lumbosacral segments, respectively. Camera lucida drawings and graphic reconstructions were made of the nerve patterns within the limbs of embryos ranging in age from St. 24 to 30. Control injections were also made into normal embryonic cords at an equivalent stage of development.

RESULTS

Anatomy of the reversed segments and spinal nerves

At St. 30 and St. 35–36 individual lumbosacral segments can be identified on the basis of the size and concomitant lateral expansion of the lateral motor column. The number of lateral motoneurones increases as one progresses caudally through the lumbosacral enlargement, reaching a maximum number per segment around LS 6

and then declining through LS 8 (see Hamburger, 1975). In LS 1 and LS 8 the lateral motor column projects very little beyond the external contours of the ventrolateral grey matter. Between these segments the lateral motor column is large and extends well beyond the rest of the grey matter. Within this area, the glycogen body aids further in segment identification as it usually is found in segments 4–7.

In embryos in which the first three segments have been reversed, the characteristic morphology of the individual cord segment is maintained despite 180° rotation. A series of representative sections taken from progressively more posterior segments through the posterior thoracic and lumbosacral spinal cord of a control and experimental St. 36 embryo is shown in Pl. 1. The reversed LS 3 has a large and laterally expanded motor column even though it is now in a more anterior position adjacent to thoracic segments. The reversed LS 1, now posterior to LS 3, nevertheless has a very small lateral motor column like its counterpart in the control. Sections taken from segments beyond the reversal region appeared anatomically normal. These morphological characteristics account for the abrupt expansion and then tapering of the reversed cord seen after the ventral laminectomy (see Fig. 1). The development of normal segmental morphology despite an altered anterior–posterior orientation suggests that the form of the lateral motor column is determined prior to St. 15–16. It should be noted that a disordered or abnormal cord region equivalent to about a quarter to half a segment was usually found at each end of the reversed cord. These regions were frequently characterized by whorls of commonly oriented cells projecting beyond the normal contours of the grey matter, a displaced central canal and poor development of the dorsal white matter. As a result, in small reversals (less than two segments) a morphological reversal was not as evident.

Spinal nerves emanating from the region of a reversal appeared anatomically discrete and could be identified as specific spinal nerves corresponding in number to the number of segments reversed. In a few cases, small extra spinal nerves or fibre groups projected from the junctional regions but usually these could be identified as parts of the major spinal nerves. Further, spinal nerve patterns within the limb and plexus formation appeared normal despite the fact that these nerves were projecting from transposed segments. Thus, spinal nerves appeared to be taking routes anatomically appropriate to their new position.

Segmental and spinal nerve projection patterns from the reversed cord region to individual muscles in the St. 35–36 embryonic limb.

The normal motoneurone and spinal nerve projection patterns from the first three segments of the lumbosacral cord to the limb of a St. 36 embryo are schematically shown in Fig. 2. This diagram represents a summary of data from the studies of Landmesser & Morris (1975) and Landmesser (1978*a*). Four muscles, the sartorius, femorotibialis, adductor and ischio-flexorius receive all or most of their motor innervation from these segments. Of these muscles, the sartorius and femorotibialis were chosen for close electrophysiological examination. These two muscles have motoneurone pools which overlap only slightly in the anterior half of segment two. The sartorius, one of the most anterior muscles of the thigh normally contracts in response to stimulation of spinal nerves 1 and 2. The femorotibialis, a slightly more posterior muscle, contracts in response to stimulation of spinal nerves 2 and 3.

E.m.g.s recorded from these muscles following spinal nerve stimulation in a normal and an experimental St. 36 embryo are shown in Fig. 3. When the first three spinal segments were reversed, the spinal nerve projection pattern was reversed. The sartorius was innervated by the last two spinal nerves of the reversal. These nerves projected from the reversed first and second segments indicating that they had established appropriate muscle connexions. Similarly, the femorotibialis contracted in response to stimulation of anterior spinal nerves which projected from the displaced posterior segments.

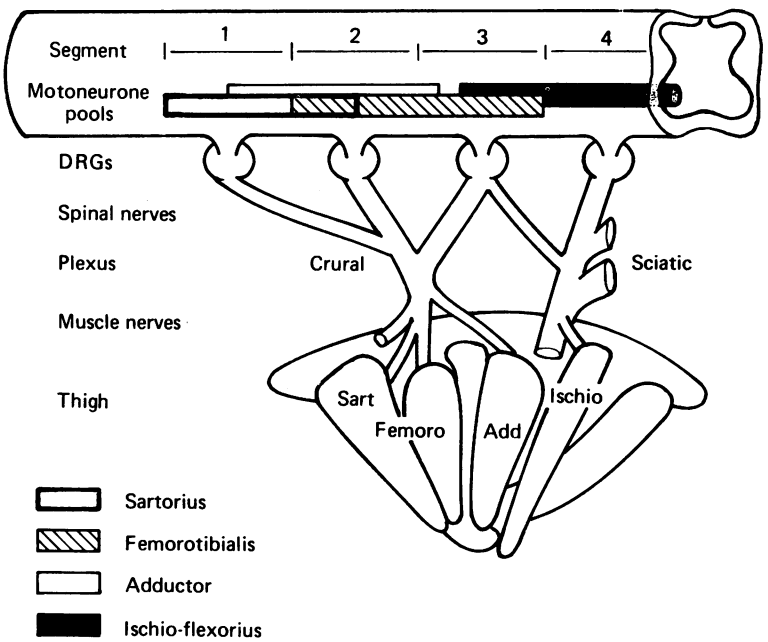

Fig. 2. Schematic diagram of the spinal cord and hind limb illustrating the motoneurone projection patterns from the first four lumbosacral segments to the sartorius, femorotibialis, adductor and ischio-flexorius muscles. The cranio-caudal extents of motoneurone pools innervating each muscle are graphically represented. While pools overlap on this axis, they are discrete in the transverse plane. DRG, dorsal root ganglion.

In twenty-five experimental St. 35 or 36 embryos, projection patterns were mapped by direct cord stimulation and/or spinal nerve stimulation. Visual observation of muscle contraction in all cases revealed that appropriate connexions were established in spite of the altered anterior-posterior position of cord segments. In twenty-two of these embryonic limbs, e.m.g.s monitored in the femorotibialis and sartorius muscles confirmed visual observations. A schematic tabulation of muscle responses is presented in Fig. 4. In individual embryos, stimulation of a given spinal nerve or the cord adjacent to it produced the same response. Therefore, axons did not take aberrant courses of exit from the cord; rather, they projected from individual cord segments via the corresponding spinal nerve.

The length of cord which was reversed without damage varied from 1 to $2\frac{1}{2}$ segments, the largest reversal encompassing LS 1, LS 2 and the anterior part of LS 3. The sequence of muscle contractions in response to sequential stimulations is a

mirror image of the normal innervation pattern. In all reversals contraction of the femorotibialis was always elicited by stimulation at the anterior end of the transposed region. In large reversals, a mid-reversal stimulation elicited both sartorius and femorotibialis responses and more posterior stimulation elicited only sartorius contraction. In some embryos only small sections of the reversed cord gave rise to muscle contractions presumably as a result of damage to the ends of the reversed segments. The projection patterns from these small areas matched discrete and identifiable parts of the control in mirror image. As can be seen in Fig. 4, these areas usually corresponded to a reversed LS 2. The undamaged sections of the cord thus seemed to behave as a mosaic showing no indication of compression of the whole projection pattern.

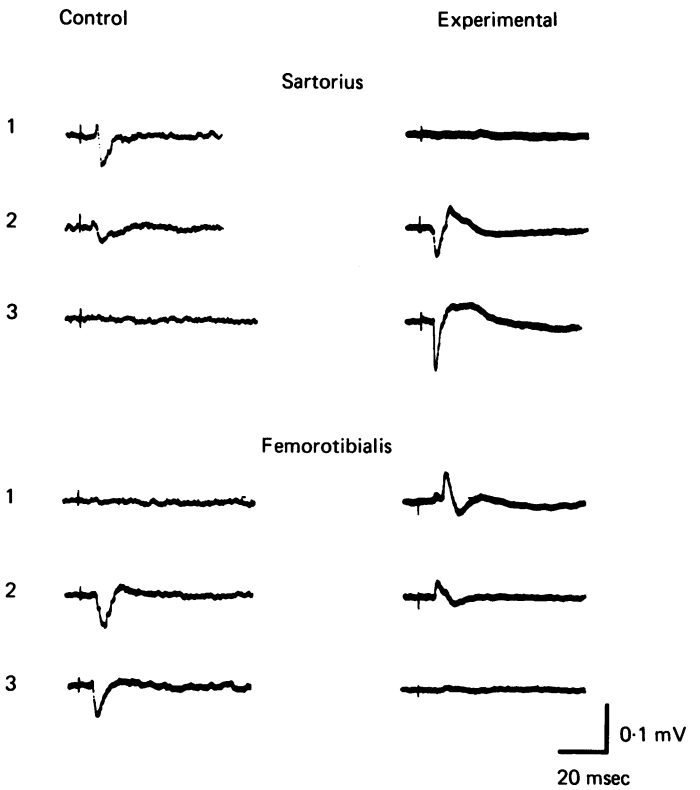

Fig. 3. E.m.g. recordings from the sartorius and femorotibialis muscles at St. 36 in a control embryo (left) and in an experimental embryo in which the first three cord segments had been reversed (right). Stimulation of spinal nerves 1 and 2 elicited responses from the sartorius in the control embryo. In the experimental embryo, spinal nerves 2 and 3 (numbered according to their order of projection from the cord after the reversal, not according to their origin prior to reversal) caused sartorius contraction. The femorotibialis contracted in response to stimulation of LS 2 and 3 in the control but LS 1 and 2 in the reversal embryo.

In some cases, electrophysiological evidence indicated that motoneurone pools to individual muscles had been split and parts of the pools reversed, giving rise to separation along the anterior–posterior axis of the cord. Two examples of spatially separated projections to the femorotibialis are illustrated in Fig. 4. Stimulation at

the anterior end of the reversed cord and at the beginning of the normal cord posterior to the operated area elicited femorotibialis contraction. The femorotibialis was thus appropriately innervated by the reversed second segment and by segment 3 which was not included in the reversal. The segmental projection to the sartorius muscle was also split in the first example. Here a part of segment 1 had not been reversed. Stimulation just anterior to the reversal region and at its posterior end gave rise to sartorius contraction. Thus the topography of individual projection patterns as well as the relationship between them are altered by a reversal.

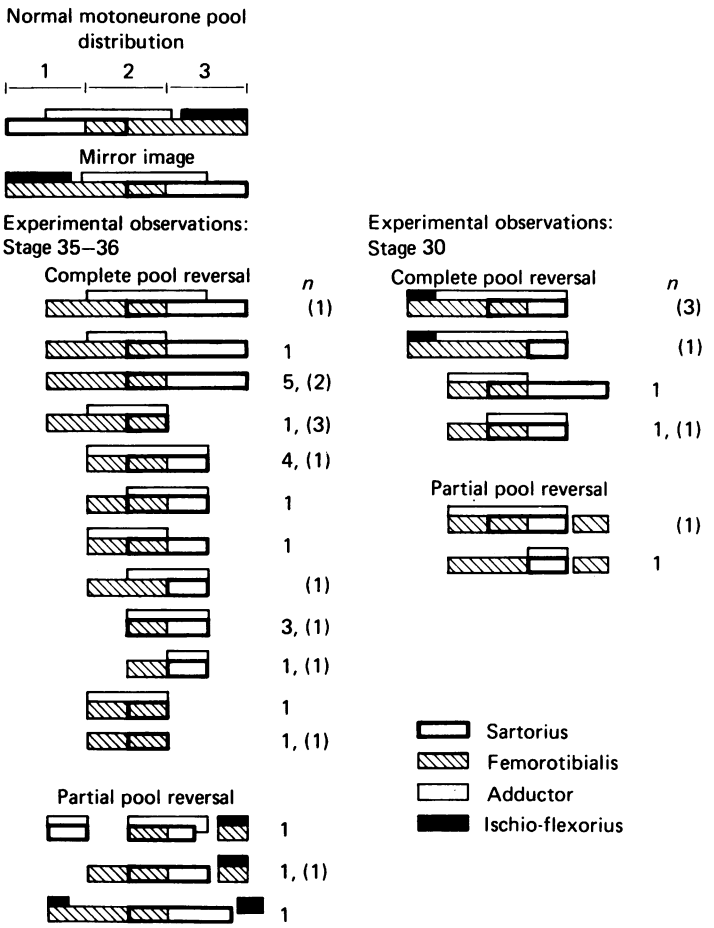

Fig. 4. Summary of the anterior–posterior position of motoneurone pools, following partial spinal cord reversals in St. 35–36 (left) and St. 30 (right) embryos as determined electrophysiologically. The normal motoneurone pool distribution and the mirror image appearance expected following complete reversal are shown at the top. Each change in muscle response along the horizontal axes marks a shift to a new recording site on the cord or to the next spinal nerve. In the partial pool reversals, gaps along the horizontal axis represent transitions between non-reversed and reversed cord regions. The complete pool reversal diagrams are vertically organized according to the size of the reversal discounting disorganized cord regions. In a few cases only sartorius and femorotibialis contractions were scored. Reversed motoneurone pools generally are similar to all or part of the mirror image control. n = number of limbs in which e.m.g.s were recorded. (n) = additional limbs in which muscle contraction was scored visually.

Projection patterns from reversed cord segments to the adductor and ischio-flexorius muscles were less easily interpreted than those to the sartorius or femorotibialis. In only one St. 35–36 embryo was there a clear indication of a reversal of a part of the ischio-flexorius motoneurone pool, which normally lies in LS 3 and 4. As shown in Fig. 4, stimulation at the anterior end of the reversed cord segments and just posterior to them, gave rise to contraction of the ischio-flexorius. While the low frequency of this occurrence might suggest that motoneurones are only capable of establishing appropriate connexions, if displaced only limited distances, it appears more likely that the motoneurones to the ischio-flexorius were often damaged as a result of the reversal. In most cases where part or all of segment 3 had been reversed, no contraction of the ischio-flexorius could be elicited from any part of the cord at all. The extent of cord contributing to the innervation of the femorotibialis also appeared shorter than normal in these operations (see Fig. 4). Further, segment 4 which also innervates the ischio-flexorius, was frequently damaged. Projection of cord segments posterior to the reversal region normally corresponded to those of normal segments 5–8. It thus appears that the disorganized cord areas usually included the motoneurone pool of the ischio-flexorius. Identification of segments on the basis of cord morphology supported this. However, even larger cord reversals are necessary before this can be definitely determined.

The adductor muscle of a normal embryo is primarily innervated by spinal nerves 1 and 2. However, most of the motoneurones which project to the adductor are located in segment 2 (see Landmesser, 1978*a*). Thus, a reversal of LS 1, 2 and part of 3 alters the position of the pool, as a whole, only slightly, and little information about the appropriateness of projection patterns to the adductor can be gained from the type of analysis presented in Fig. 4. However, in some muscles including the adductor, segmental and spinal nerve projection patterns vary depending on the region of the muscle from which one is recording. In a normal embryo, stimulation at the cranial end of segment 1 consistently elicits contraction at the posterior border of the adductor and little or no contraction at the anterior border. Further posteriorly in segments 1 and 2 stimulation gives rise to contraction at both borders of the adductor. Following the reversal of segments 1 and 2, this innervation pattern was observed to be reversed as well.

Topography of motoneurone pools in a St. 35–36 embryo

The electrophysiological data indicate that motoneurones within a particular segment establish appropriate limb muscle connexions in spite of the alterations in their position along the anterior–posterior axis of the cord. However, these data provide little information about the topographical relationships of motoneurone pools in the transverse plane of the cord or about the normality of the spatial distribution of motoneurones to an individual muscle. In order to localize motoneurones in the reversed and adjacent segments of the lumbosacral cord which project to an individual muscle, the retrograde transport of HRP was utilized. HRP was injected into either the sartorius, femorotibialis or adductor in each limb of ten St. 35–36 experimental embryos after the extent of the reversal had been characterized electrophysiologically.

In all cases, reversed motoneurones which were labelled by injection of a single

muscle were found in a discrete nucleus positioned normally in the transverse plane. In segments where two or more affected motoneurone pools overlapped along the anterior–posterior axis, the individual pools were separated in the dorso-ventral and medio-lateral axes. Individual motoneurone pools could thus be identified by their location in transverse cord sections, as in the normal condition (see Landmesser, 1978*a*).

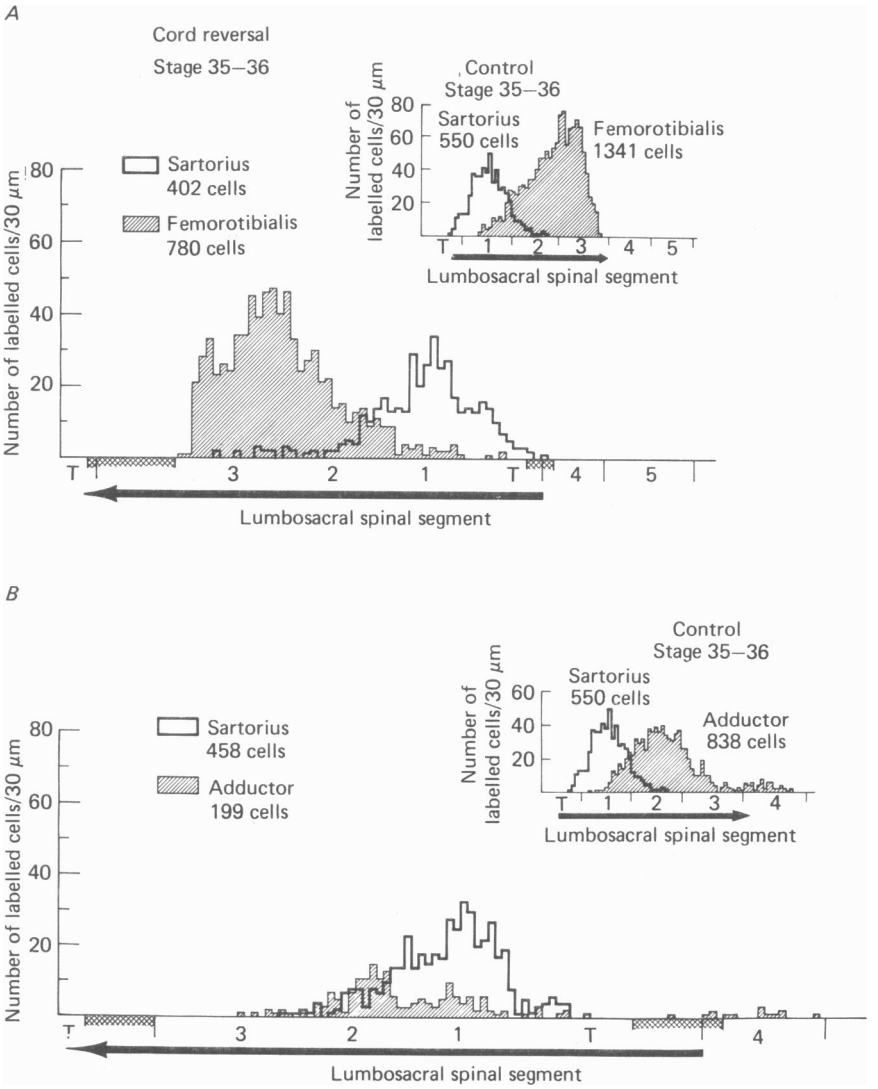

Fig. 5. Anterior–posterior positions of motoneurones projecting from reversed cord segments to individual limb muscles determined anatomically. *A*, histogram of labelled motoneurones in a St. 35–36 experimental embryo in which the femorotibialis had been injected on one side, the sartorius on the other. Inset at top shows a similar injection in a control embryo. *B*, histogram of St. 35–36 experimental and control (inset) embryos in which the adductor and sartorius had been similarly injected. Note that the adductor motoneurone pool has been split into two parts as a result of the operation in the experimental embryo. The arrows indicate cord segments reversed and the corresponding region in a control. Cross-hatched areas represent damaged cord sections.

In order to characterize the distribution of motoneurones within a nucleus, histograms of the number of labelled motoneurones per 30 μm increment of lumbosacral cord were constructed and compared to histograms of normal motoneurone pools compiled from the original data of Landmesser (1978*a*). It should be noted that while motoneurone distribution could be compared in the control and experimental embryos, the actual number of motoneurones could not be readily compared due to segmental damage following a reversal and variability in the number of motoneurones normally projecting to individual muscles.

As is clearly evident in Figs. 5 and 6, the spatial distribution of the reversed motoneurones to an individual muscle confirms the electrophysiological data. In the case illustrated in Fig. 5*A*, the sartorius and femorotibialis motoneurone pools were totally reversed by the reversal of segments LS 1–3. Both nuclei have peaks in reversed normal positions; the sartorius in LS 1, the femorotibialis in LS 3. Injections into both muscles gave rise to at least a small number of labelled cells in all segments. While this is often the case following femorotibialis injections in normal embryos (see Fig. 6), labelled cells are not normally found in segment 3 as a result of HRP injection into the sartorius. Although these might represent a few aberrant connexions, some leakage of HRP into the adjacent femorotibialis is a more likely possibility. In normal and most experimental St. 35–36 embryos, the sartorius motoneurone nucleus is distinctly more lateral than that of the femorotibialis. In the experimental embryo illustrated in Fig. 5*A*, the bulk of labelled cells in the reversed segments 1 and 2 were positioned quite laterally in the cord and formed a nucleus of cells unseparated by unlabelled cells. The few labelled cells in segment 3 were isolated from one another and in a position in the transverse plane characteristic of femorotibialis motoneurones.

Fig. 5*B* shows a cord reversal in which the adductor muscle had been injected on one side and the sartorius on the other. Again the spatial pattern of each motoneurone pool has been conserved. A small portion of the adductor motoneurone pool in segment 4 was not reversed in the operation and formed a separate small nucleus of labelled cells which projected to the adductor. Although the number of motoneurones was lower than normal (603 ± 43) in this embryo, injections into other embryonic adductor muscles following a reversal have given normal sized pools.

A more striking case of a split motoneurone pool is presented in Fig. 6. In the example illustrated, only one of the segments which normally projects to the femorotibialis, LS 2, was reversed. Following HRP injection into this muscle, labelled motoneurones were found in two separate pools along the cranio-caudal axis of the lateral motor column, whereas in the normal embryo a similar number of motoneurones are found in one pool in segments LS 2 and 3. Direct cord stimulation at four points confirmed that the femorotibialis muscle was mainly innervated by the reversed second segment and by segment 3 which was not included in the reversal.

Motoneurone projection patterns prior to the peak of cell death

It has been suggested that the large amount of cell death which occurs in the lateral motor column in the chick between St. 30 and 35 might be the result of the loss of cells which have formed inappropriate or an insufficient number of appropriate connexions in the limb (Hamburger, 1975). If such an event occurred one might

postulate that although inappropriate connexions are not seen in St. 35 or 36 embryos following a partial lumbosacral cord reversal, such connexions may have been present earlier in development. In order to examine this possibility, motoneurone projection patterns were characterized in experimental embryos at St. 30, preceding the peak of cell death in the lateral motor column. In nine embryos in which part or all of LS 1–3 had been reversed, electrophysiological and anatomical observations yielded results comparable to those obtained at St. 35–36. No evidence of incorrect connexions or connexions in accord with a new cord position was found.

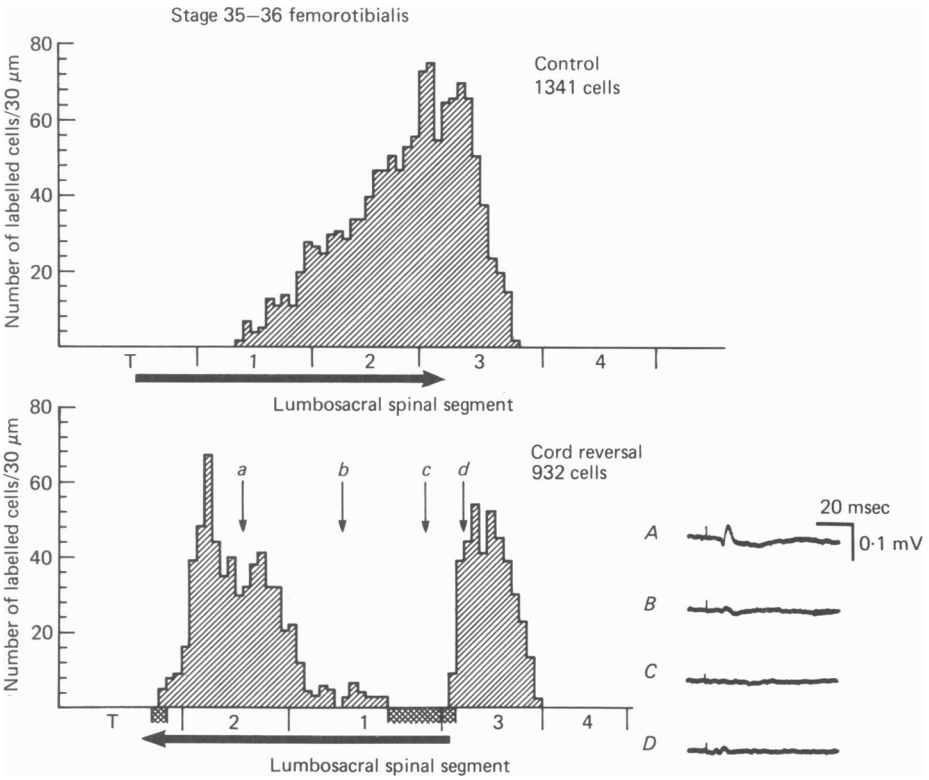

Fig. 6. Anterior–posterior position of motoneurones innervating the femorotibialis following a reversal that split the motoneurone pool. A control femorotibialis motor nucleus (top) extends from the end of segment 1–3. In a St. 35–36 experimental embryo (bottom) femorotibialis motoneurones are found in two nuclei, one in reversed segment 2, the other posterior to the reversal in segment 3. Inset shows e.m.g.s recorded from the femorotibialis in response to stimulation at four points (*a–d*) along the cord whose spatial distribution is indicated above the histogram.

Axonal pathways

The electrophysiological evidence presented above demonstrates that motoneurones in reversed cord segments projected via adjacent spinal nerves into the limb. Alterations in their pathways must have been made distal to this point in order to account for the establishment of appropriate connexions. One possibility is that motoneurones projected initially to targets in accord with their new anterior–posterior position, 'recognized' the incorrectness of the muscle, and then altered

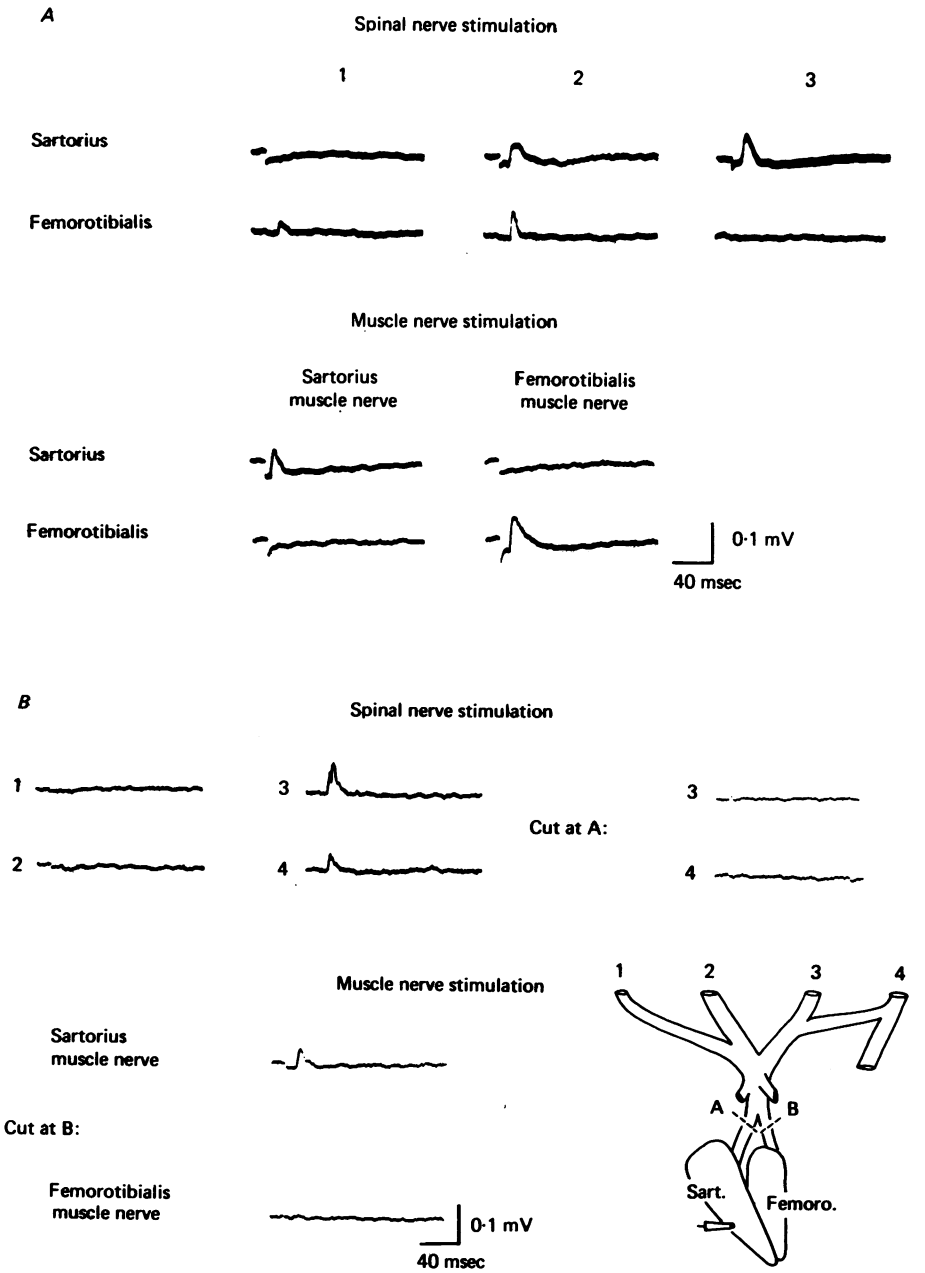

Fig. 7. Pathways taken by reversed sartorius and femorotibialis motoneurones determined electrophysiologically. *A*, e.m.g. recordings from both muscles in a St. 34 embryo in which the first three segments were reversed. Spinal nerve projection patterns are reversed (compare to Fig. 3, control), but muscle nerve pathways are normal, each muscle contracting only in response to stimulation of the appropriate muscle nerve. *B*, e.m.g. recordings from the sartorius muscle in a St. 34 embryo. Sartorius contraction was elicited by stimulation of the 3rd and 4th spinal nerves projecting from the reversed cord. These responses disappeared after transecting the sartorius muscle nerve (level *A* in the diagram). Stimulation of the cut distal stumps of the sartorius and femorotibialis muscle nerves (A and B) indicates that axons innervating the sartorius course through the sartorius muscle nerve only.

their course to establish connexions with the correct muscle. While the anatomical pattern of muscle nerves appeared to be normal, small branches might go undetected if they projected between muscles. In order to examine this possibility individual muscle nerves of St. 34–36 embryos were isolated and stimulated with suction electrodes and e.m.g.s recorded from muscles innervated by reversed motoneurones. The sartorius and femorotibialis were chosen for examination as they are directly apposed to one another in the limb. In a normal embryo, each muscle is innervated by discrete branches off the femoral trunk (Landmesser & Morris, 1975). Anatomically similar muscle nerves could be identified in experimental embryos.

These muscle nerves were cut and stimulated after an electrophysiological identification of spinal nerve projection patterns to the femorotibialis and sartorius. Two examples of the results obtained are shown in Fig. 7. In the first experimental embryo (Fig. 7*A*) sequential stimulation of spinal nerves projecting from the reversal region indicated that the sartorius and femorotibialis motoneurones exited the cord in a reversed order. However, muscle nerve stimulation showed that motoneurone axons coursed through appropriate muscle nerves. Thus, reversed motoneurone axons corrected their pathways between the level of the spinal nerves and the muscle nerves. In the second example (Fig. 7*B*), stimulation of spinal nerves 3 and 4 elicited responses in the sartorius which disappeared after transection of the sartorius muscle nerve. We did not detect any connexions which might have been made by axons taking aberrant pathways to the sartorius. Since the sartorius muscle was activated by stimulation of the sartorius muscle nerve, but not the femorotibialis muscle nerve, we can conclude that displaced sartorius motoneurones did not first grow down the femorotibialis muscle nerve and then cross over to innervate the sartorius muscle. These results suggest that axons can recognize or are directed to their target at some distance from its actual location.

However, it is possible that a period of pathway modification might occur between the time of initial axonal outgrowth and St. 35–36. Experimental evidence in the amphibian limb (Lamb, 1976; 1977; McGrath & Bennett, 1979) and the chick wing (Pettigrew, Lindeman & Bennett, 1979) suggests that some incorrect projections are made and subsequently removed during normal embryogenesis. Further, these events occur just prior to and at the onset of motoneurone cell death periods in the cord. Orthograde labelling of motoneurones with HRP (see Methods) lends itself particularly well to this question as it allows the visualization of regionally identified motoneurones and their axons from the time of initial axonal outgrowth into the chick hind limb (St. 23–24) to the time at which correct connectivity patterns are seen and muscle cleavage has occurred (St. 28½–30). Twenty-two cord reversal embryos ranging from St. 24–30 were examined utilizing this technique.

At all stages labelled axons projected into the limb in discrete bundles as they do in normal embryos (C. Lance-Jones & L. Landmesser, unpublished observations). No indications of random or diffuse outgrowth were found even in the youngest material. Axonal pathway differences between control and experimental embryos, were visible as early as St. 24 (Fig. 8). In a normal St. 23–24 embryo labelled axons from lumbosacral segment 1 maintain an anterior topographical position as they project via spinal nerve 1 to the base of the limb. Axons destined for axial musculature

branch off the anterior proximal border of the spinal nerve. In an experimental embryo in which T7–LS 3 had been reversed, labelled axons from T7 and LS 1 mainly project straight into the limb as normal LS 3 motoneurones do. However, a few labelled axons clearly cross in the plexus region and appear to be growing towards the anterior axial musculature. No such crossing is visible following the labelling of LS 3 motoneurones in a normal embryo.

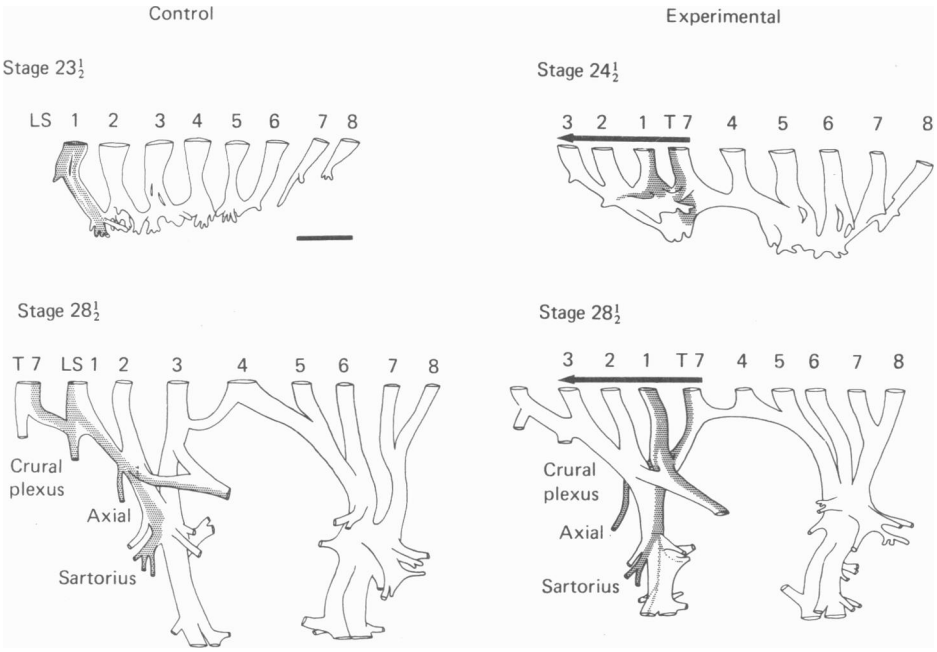

Fig. 8. Pathways taken by motoneurones projecting from segments T 7 and LS 1. Paths of axons labelled by direct HRP injection into the cord are stippled. Left: control embryos injected at stages before (top) and after (bottom) the onset of motoneurone cell death and muscle cleavage. Right: experimental embryos in which four segments had been reversed (T7–LS3) injected at similar stages. Calibration bar = 255 μm.

At later stages, labelled axons can be followed to individual muscle primordia (see Fig. 8). Labelled motoneurones projecting from reversed segments exited the cord in adjacent spinal nerves as they do in a normal embryo. Distally, however, they alter their course either within the plexus or proximal nerve trunks to project to correct targets. In the control St. 28½ embryo labelled T7 and LS 1 motoneurone axons projecting to the sartorius and the axial musculature maintain an anterior position within spinal nerves, the plexus, and nerve trunks. In the experimental embryo the labelled axons course from a posterior to anterior position just prior to the branching of the discrete muscle nerves. Thus, motor axons appeared to pass over inappropriate muscles or muscle regions directly in their path. A few labelled axons project to other muscles. While not seen in the control illustrated, such projections from segment 1 were found in other normal embryos. Similar results were

obtained following injections of a reversed LS 3. Labelled motoneurones crossed from the anterior to the posterior side of the femoral trunk and projected to the femorotibialis muscle via the femorotibialis muscle nerves. Such pathway alterations were visible as early as sartorius and femorotibialis muscle nerves could be identified (St. 25½–26) and before muscle cleavage. Thus, motoneurone axons do not seem to follow inappropriate pathways even transiently.

DISCUSSION

Morphological polarity

Previous investigations have shown that, in general, regional determination along the anterior-posterior axis of the neural tube occurs between the neural plate stage and the time of neural fold closure. Anterior-posterior reversals or displacements of prospective brain tissue in the amphibian neural plate or tube indicate that individual regions behave as mosaics in spite of inappropriate surrounding tissue (Jacobson, 1964; Chung & Cooke, 1978). Similarly, chick brachial, thoracic and lumbosacral cord regions differentiate in accordance with their origin in spite of early alterations of their position along the anterior–posterior axis of the cord (Bueker, 1945; Wenger, 1951). Experiments involving limb ablations or additions confirm these observations and suggest that the initial pattern of motoneurone proliferation and differentiation is independent of the periphery (Hamburger, 1939, 1958; Hollyday, Hamburger & Farris, 1977).

Our study demonstrates that the morphology of the lateral motor column in individual segments of the lumbosacral cord is also determined prior to limb bud formation and axonal outgrowth. The size of the lateral motor column in reversed cord segments is characteristic of its origin before the reversal. That this is not a result of differential motoneurone survival following the establishment of appropriate peripheral connexions is demonstrated by segmental morphological differences at St. 30 prior to the major motoneurone cell death peak.

Active vs. passive mechanisms

The present study demonstrates that motoneurones from reversed cord segments establish functional connexions in accord with their original cord position. The size, form and topographical positions of individual motoneurone pools within the reversed segments are conserved in a mirror image of the normal condition. Reversed motoneurones exit the cord via adjacent spinal nerves and therefore are confronted with a foreign environment. Motoneurones then demonstrate a specific pathway or target preference by altering their course and establishing correct connexions. These pathway alterations are apparent from the onset of axonal penetration into specific limb regions. At no time in their development did reversed motoneurones project to incorrect muscle primordia.

These observations provide the first clear evidence that the establishment of specific motor connexions is an active process. Limb motoneurones do not form a homogeneous or unspecified pool prior to outgrowth. Rather, by St. 15 intrinsic differences between motoneurones exist such that upon outgrowth axons are capable of 'choosing' appropriate pathways or targets. The acquisition of these differences

or target specificities is independent of direct limb interactions. Cord segments were reversed before limb bud formation and the birth of lumbosacral motoneurones (see Hollyday & Hamburger, 1977). If the limb normally determines motoneurone identity, one would have expected the specification of reversed motoneurones in accord with their new cord position.

Two major hypotheses implicating passive contact guidance mechanisms alone in the establishment of specific connectivity in the limb are thus ruled out by our results. Horder (1978) has argued that at the time of initial fibre outgrowth parallel arrays of neurones and muscles exist and that motoneurone distribution to particular muscles is dictated solely by fibre arrangement and by morphogenetic events within the limb. Although there is an early general correspondence between motoneurone position along the anterior–posterior axis of the cord and muscle position in the limb (Landmesser, 1978*b*), the present experiments clearly refute Horder's hypothesis. In the cord reversed embryos, axons exit the cord in a disrupted topographical order and course through foreign tissue yet are able to find appropriate pathways to their respective targets.

The second hypothesis invokes a temporal as well as spatial gradient of axonal outgrowth into the limb (Jacobson, 1978). Motoneurones that grew as a result of general spatial constraints to a particular limb region would bypass muscles innervated by axons which had grown out earlier and would innervate the neighbouring uninnervated target. In our experimental embryos, axons from reversed cord segments clearly bypassed muscles which would have been innervated by motoneurones exiting from that cord level in a normal embryo. In order to account for these results in terms of this model, one would have to suggest that muscles are innervated in a disrupted temporal order in the cord reversal embryos. It is possible that motoneurones are prespecified only with respect to time of outgrowth. The observed correct patterns after a reversal might then be due to an unaltered time of axonal outgrowth. However, the temporal sequence of limb innervation in both the normal and cord reversed chick embryos does not show sufficient variability along the anterior–posterior axis to account for selective connectivity. As early as St. 24, all but the eighth spinal nerve contribute to the formation of the plexus and are at parallel levels along the proximo–distal limb axis. Thus observations of correct and specific motoneurone projection patterns in partial cord reversal and also deletion experiments (Lance-Jones & Landmesser, 1980) refute this hypothesis.

Limb directed processes

We do not mean to suggest that passive or mechanical factors are not involved in the establishment of connectivity. Although motoneurone axons clearly alter their pathways to reach appropriate targets in our experiments, the anatomical pattern of the plexus and major trunks is similar to that in a normal embryo. Further, the major muscle nerves affected, those to the sartorius and femorotibialis muscles, branch from the femoral trunk in a normal sequence and spatial pattern. These observations are compatible with results following other experimental manipulations of the cord or limb. Nerves projecting from foreign cord segments into a normal or supernumerary limb form a grossly normal pattern in chicks (Hamburger, 1939; Wenger, 1951; Straznicky, 1967; Hollyday, Hamburger & Farris, 1977; Morris,

1978) and amphibians (Detwiler, 1920, 1936; Piatt, 1956; Szekely, 1963). Following anterior–posterior and anterior–posterior, dorsal–ventral reversals of the chick limb (Narayanan, 1964; Morris, 1978; Stirling & Summerbell, 1979; Ferguson, unpublished observations) or complete brachial cord reversals in amphibians (Detwiler, 1923), the form of the plexus mirrors the orientation of the limb rather than that of the cord. Further, the pathways taken by nerves in chick wings following proximo-distal deletions, truncations (Stirling & Summerbell, 1977) and duplications (Lewis, 1978) appear to be appropriate for the limb region approached by the outgrowing axons. All of these experiments, including ours, suggest that limb directed processes can affect anatomical nerve branching patterns.

What then is the relationship between passive and active processes in the development of specific connectivity in the limb? When a disparity is experimentally created between the anterior–posterior position of the cord and limb, two conflicting results are obtained. In the cord reversal experiments, motoneurones appear to actively recognize the disparity and establish correct connexions. However, following other experimental manipulations, the polarity of the limb appears totally to govern axonal outgrowth and incorrect connexions are formed (Hollyday *et al.* 1977; Morris, 1978; Stirling & Summerbell, 1979). Several hypothesis may account for these differences and define conditions necessary for the formation of correct connexions.

The conditions for the establishment of specific connectivity patterns

Although our results indicate that motoneurones differ at St. 15, we know little of the nature of these differences. One possibility is that each motoneurone bears a specific label corresponding to a particular muscle and that correct connexions are established as a result of differential chemo-affinities between axons and specific targets or pathways (see Sperry, 1963). An active target or pathway recognition could then account for the formation of correct connexions in the cord reversal experiments and also in deletion experiments (see Lance-Jones & Landmesser, 1980). The apparent absence of such recognition following other experimental manipulations might result because motoneurones have been radically displaced. Following an anterior–posterior limb reversal or the addition of a supernumerary limb, axons which would normally course through the anterior half of the limb in the crural plexus are confronted with posterior limb tissue and ischiadic plexus pathways. In a three or four segment cord reversal correct pathway alterations need only be made within the crural plexus. It might be hypothesized that motoneurones are only capable of responding to local cytochemical cues. Normally, passive or general limb-directed processes may ensure that axons get to muscle regions in which they can respond to such cues. Further displacements may give rise to connectivity determined by passive mechanisms which only generally approximates a normal spatially ordered pattern. Alternatively, a hierarchy of neuronal specificities may exist such that motoneurones chose the most appropriate muscle in a particular region (see Hollyday *et al.* 1977). It is not possible to distinguish between these possibilities with the data presently available. Although it is known that patterned connexions are formed in anterior–posterior reversed or supernumerary limbs and that their polarity corresponds to that of the limb (Hollyday *et al.* 1977; Morris, 1978), topographical relationships between motoneurone pools in the cord have not been

sufficiently characterized. Thus, the degree of selectivity shown by motoneurones in a foreign environment has yet to be determined.

One need not suggest that each muscle and neurone bears a specific biochemical label. Rather, motoneurones might be specified to respond differentially to a gradient of some sort distributed along the anterior–posterior axis in the periphery. An axial gradient might guide axons to a particular limb region where separate mechanisms would operate to define innervational boundaries and specific synaptic connectivity. Such secondary processes might include limits on the number of synapses a motoneurone can form, physical boundaries created by dividing muscles, or competition for a trophic factor in the periphery (see Feldman, 1977; Lance-Jones & Landmesser, 1980). A likely site for the axial gradient is within the tissue between the cord and limb proper. Preliminary evidence based on tracings of retrograde HRP labelled axons suggests that axons sort out into discrete bundles destined for a particular muscle within a short distance from the cord (Landmesser, 1978*b* and unpublished observations). Further, reconstructions of axonal pathways following the orthograde labelling of individual reversed cord segments demonstrate that the displaced axons alter their course by crossing through the plexus or femoral nerve trunk before entering specific muscle regions. Thus, motoneurones may be actively choosing pathways on the basis of cues emanating from tissue immediately adjacent to the cord, such as somitic or lateral mesenchyme. Such a proximal sorting out process might account for the differing results in anterior–posterior limb reversal and cord reversal experiments. In the former, motoneurones grow into an immediate periphery which is not reversed. Consequently, motoneurone axons would take spatially normal pathways which would lead them to incorrect muscles in the reversed limb. However, following a cord reversal, the outgrowing axons would immediately encounter tissue of reversed polarity and thus alter their course appropriately.

Alternatively or in addition to active interactions between neurones and peripheral structures, interactions between neurones may determine the pathways taken by limb motoneurones. Models implicating fibre sorting mechanisms based on a neurone's original position (Cook & Horder, 1977; Hope, Hammond & Gaze, 1976) and the maintenance of neighbour relationships between two structural arrays (von der Malsberg & Willshaw, 1977) have been proposed to account for the formation of retino-tectal connexions. It is possible that motoneurones acquire topological specificity on the basis of their position in the cord which is manifested in a subsequent sorting out process within the limb. For example, motoneurones regardless of their relative level in the cord (i.e. brachial, thoracic or lumbosacral) may distribute to particular peripheral paths or targets by interacting among themselves according to their relative early cranio-caudal axial position and that of their neighbours. This hypothesis is compatible with anterior–posterior limb and cord reversal results if one assumes that cues within the immediate periphery provide the polarity information necessary to orient the sorting out process. In addition, it can account for the orderly innervation of supernumerary and adjacent ipsilateral limbs by a less than normal complement of spinal nerves. It is possible that differences between motoneurones are quantitative or graded rather than qualitative. Thus, axons within four spinal nerves would sort out spatially in a manner similar to those in the normal eight spinal nerves innervating the limb on the basis of relative differences. However, a neuronal

interaction model cannot account for the results of our cord deletion experiments in which remaining motoneurones innervate only their normal targets, leaving some limb regions entirely devoid of innervation. If a fibre sorting mechanism does operate during normal development, one must suggest that additional factors determine the area over which neurones can interact.

It is not possible to fully explain the establishment of chick limb connectivity without invoking the operation of active as well as passive mechanisms. Our results clearly demonstrate that motoneurones possess specific identities prior to axonal outgrowth and direct limb interactions. However, several conditions may be necessary before specific and correct connexions can be established. If such conditions are not met, motoneurones may obey other laws governed by their origin within the cord, by cues emanating from the limb, or by a hierarchy of target or pathway preferences. Alternatively, only passive mechanisms may be operative. In order to examine these possibilities, precise characterizations of motoneurone projection patterns to individual muscles are necessary under various experimental conditions.

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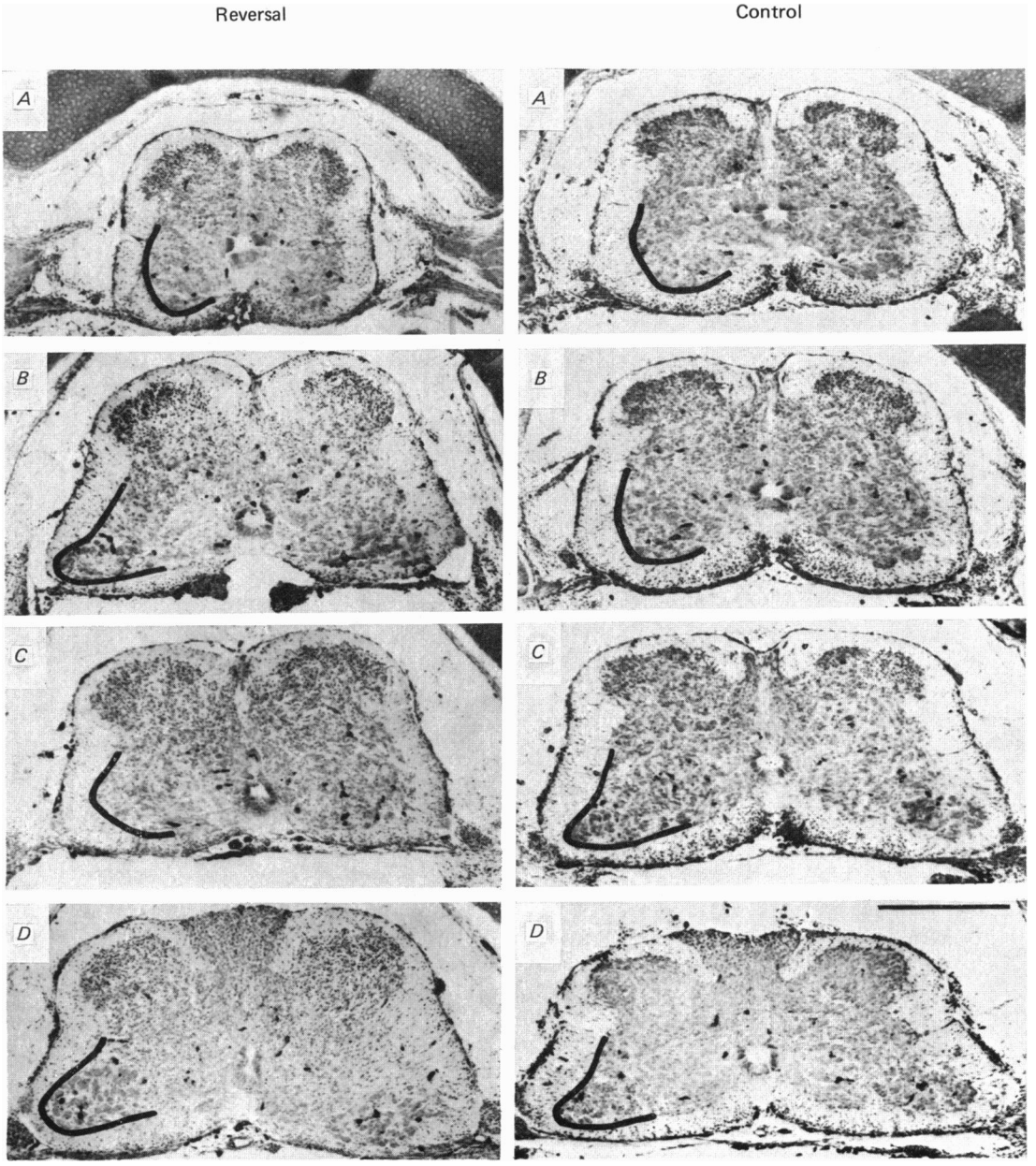
EXPLANATION OF PLATE

Transverse sections through the spinal cord in St. 36 control and experimental embryos. LS 1–3 were reversed at St. 16 in the latter. Section *A* in the control embryo was made through T7, the last thoracic segment. A slightly more anterior thoracic segment is illustrated in *A* of the reversal as T7 was lost in the operation. The size discrepancy is normal. Sections *B* and *C* were taken from LS 1 and LS 3 in the control and from similarly positioned segments within the reversal region of the experimental embryo. The segments in the reversal, which correspond to the reversed LS 1 and LS 3, show a morphology characteristic of their position prior to the operation. The lateral motor column (outlined in black) expands abruptly at the anterior end of the reversal (section *B*) in a manner similar to LS 3 (section *C*) in the control. Cord segments posterior to the reversed ones appear anatomically normal when compared to segments at an equivalent level in the control (sections *D*). Calibration bar = 250 μm.


Reversal
Control
A
A
B
B
C
C
D
D