A critical period for the specification of motor pools in the chick lumbosacral spinal cord

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SUMMARY

When 3-4 segments of the chick lumbosacral neural tube are reversed in the anterior-posterior axis at stage 15 (embryonic day 2.5), the spinal cord develops with a reversed organization of motoneurons projecting to individual muscles in the limb (C. Lance-Jones and L. Landmesser (1980) J. Physiol. 302, 581-602). This finding indicated that motoneuron precursors or components of their local environment were specified with respect to target by stage 15. To identify the timing of this event, we have now assessed motoneuron projections after equivalent neural tube reversals at earlier stages of development.

Lumbosacral neural tube segments 1-3 (± one segment cranial or caudal) were reversed in the anterior-posterior axis at stages 13 and 14 (embryonic day 2). The locations of motoneurons innervating two thigh muscles, the sartorius and femorotibialis, were mapped via retrograde horseradish peroxidase labeling at stages 35-36 (embryonic days 9-10). In a sample of embryos, counts were made of the total number of motoneurons in the lateral motor columns of reversed segments. The majority of motoneurons projecting to the sartorius and femorotibialis were in a normal position within the spinal cord. Segmental differences in motor column size were also similar to normal. These observations indicate that positional cues external to the LS neural tube can affect motoneuron commitment and number at stages 13-14. Since these observations stand in contrast to results following stage 15 reversals, we conclude that regional differences related to motoneuron target identity are normally specified or stabilized within the anterior LS neural tube between stages 14 and 15.

To examine the role of the notochord in this process, neural tube reversals were performed at stages 13-14 as described above, except that the underlying notochord was also reversed. Projections to the sartorius and femorotibialis muscles did not differ significantly from those in embryos with neural tube reversals alone, indicating that the notochord is not the source of cues for target identity at stages 13-14.

Key words: motoneuron, neural tube, specification, notochord, chick embryo

INTRODUCTION

The individual muscles of the chick hindlimb are innervated by motoneuron clusters or pools that occupy discrete positions within the lateral motor column (LMC) of eight lumbosacral (LS) spinal cord segments (Landmesser, 1978a, Hollyday, 1980). Detailed observations of normal axon outgrowth suggest that motoneurons are guided specifically to target muscles during early embryogenesis. Motoneuron axons grow out of the spinal cord via the adjacent spinal nerve and to their appropriate muscles in the hindlimb making few, if any, errors (Landmesser, 1978b; Lance-Jones and Landmesser, 1981a; Tosney and Landmesser, 1985). That this guidance involves an active process of recognition by predetermined motoneuron axons was demonstrated by experiments that challenged these axons with a novel environment at a time prior to their outgrowth into the limb bud (Lance-Jones and Landmesser, 1980). 3-4 segments of anterior LS neural tube were reversed in the anterior-posterior (AP) axis at stage 15 of Hamburger and Hamilton (1951). When connectivity was assessed at later stages (stages 35-36), motoneuron projection patterns from the reversed LS cord segments were reversed. Motoneuron axons from individual segments altered their pathways in the limb to project to their originally appropriate target muscles. These results indicated that prospective anterior LS cord segments differ from one another at stage 15. Factors that will determine motoneuron pathway choice appear to be encoded within the stage 15 neural tube. The studies reported here deal with the question of how and when this regionalization is achieved in the developing LS cord.

Gross regional determination of the vertebrate central nervous system (i.e. brain versus spinal cord) occurs during gastrulation and early neurulation (Mangold, 1933; see for review, Storey et al., 1992; Doniach, 1993; Ruiz i Altaba, 1993). At the same time or shortly thereafter, the spinal cord becomes patterned in the dorsoventral axis with different
neuronal cell types (i.e. motor versus sensory) being specified (Placzek et al., 1990; Artinger and Bronner-Fraser, 1993; Yamada et al., 1993). When regionalization occurs in the AP axis of the spinal cord is not known, but analyses of connectivity patterns following transplants of thoracic segments in place of limb-innervating segments in the chick (O’Brien and Oppenheim, 1990) suggest that differences between limb and non-limb regions exist near the time of neural tube closure.

In the anterior LS region, neural tube closure occurs several hours before stage 15, at stages 12-13 (Schoenwolf, 1979). This prompted us to ask if regional differences that result in motoneuron target specification are present at the time of tube closure or if they are acquired later, perhaps just before stage 15. We used the same cord reversal paradigm as Lance-Jones and Landmesser (1980) but performed our reversals at stages 13-14. Projection patterns at stage 35-36 were quite different from those seen after stage 15 reversals, suggesting that different AP regions of the LS neural tube obtain a stable identity with respect to motoneuron target only just before stage 15.

Previous studies suggest that the notochord or midline mesoderm is involved in providing AP cues to the early neurroectoderm (see Doniach, 1993; Ruiz i Altaba, 1993). Several genes coding for putative signalling factors are expressed in the notochord (Parr et al., 1993; Echelard et al., 1993; Roelink et al., 1994). Further, inductive signals from the notochord (van Straaten et al., 1989; Placzek et al., 1990) or its precursors (Halpern et al., 1993) are involved in the differentiation of floor plate cells and motoneurons. These observations raise the question of whether the notochord is the source of AP patterning signals that influence LS motoneuron target specification between stages 13-14 and stage 15. We addressed this question by reversing the notochord along with the overlying neural tube at stages 13 and 14. We did not find a reversal of motor projection patterns when the notochord was included in a reversal, suggesting that the notochord is not the source of specification signals between stages 13 and 15.

**MATERIALS AND METHODS**

**Surgery**

We incubated White Leghorn chicken eggs (from SPAFAS or Penn State University) in a forced-draft incubator at 38°C, 60-70% relative humidity, until embryonic day (E) 2.0-2.5. Each egg was candled and a window made in the shell over the area of the embryonic disc. A 1-2% neutral red/avian saline solution was applied to the LS region to aid in the visualization of embryonic structures. The embryo was then staged according to Hamburger and Hamilton (1951), using the number of somites as the primary guide.

As done previously (Lance-Jones and Landmesser, 1980), neural tube segments LS1-3 and the last thoracic segment (T7) were chosen for manipulation. The spinal nerves from these segments will form the crural plexus, the more cranial of the two nerve plexuses in the limb, and contain motor axons that innervate anterior thigh musculature. Somites 26-29 ultimately lie adjacent to neural tube segments T7-LS3 but, at the stages of our surgeries (stages 13-15), segmentation has not progressed through this level (Fig. 1). The region of the neural tube corresponding to T7-LS3 was thus approximated by counting somite-equivalent lengths from the last segmented somite.

Neuronal tube excisions and reversals were performed using flame-sharpened tungsten needles as described by Lance-Jones and Landmesser (1980). In a first experiment, the neural tube alone was isolated, excised and reversed in the AP axis at stages 13-15. In a second experiment, the underlying notochord was included in the reversal (Fig. 1A). To control for the effects of surgical manipulation, the same neural tube segments, with and without the underlying notochord, were removed and placed back in a normal orientation. These sham operations were performed only at stages 13 and 14. To aid in the subsequent identification of segment boundaries, carbon particles (charcoal, animal bone black) were placed in the gaps at the cranial and caudal boundaries of the excised tissue (Fig. 1B). After all surgeries, the windows were sealed with tape and the eggs returned to the incubator.

**Determination of motoneuron projection patterns**

Motoneuron projection patterns were assessed at E9-10 or stages 35-36 because there is an extensive literature on motor projections at these stages (see Lance-Jones and Dias, 1991). While stages 35-36 are after the peak of motoneuron cell death (Hamburger, 1975; Hamburger and Oppenheim, 1982), prior studies indicate that motoneuron cell death does not alter motor projection patterns in both normal (Landmesser, 1978b) and experimental situations (see Lance-Jones and Landmesser, 1980; Oppenheim, 1981). Retrograde horse-radish peroxidase (HRP) labeling was used to identify the spinal cord position of motoneurons projecting to two representative anterior thigh muscles, the sartorius and femorotibialis. In a normal embryo, the motoneurons innervating each of these muscles (their motor pools) are located in distinct but partially overlapping positions within the LMC of spinal cord segments T7-LS3.

Protocols for retrograde HRP labeling were those described in detail in Landmesser (1977a) and Lance-Jones (1988). In brief, embryos were removed from the egg to an oxygenated bath of Tyrode’s solution, staged, decapitated and eviscerated. After a ventral laminectomy, a 10% solution of HRP (Sigma Type VI) was pressure-injected into the sartorius muscle of one limb and the femorotibialis muscle of the contralateral limb. The hindlimbs and attached trunk regions were incubated for 5-6 hours at 30-32°C with a constant circulation of fresh, oxygenated Tyrode’s solution. Trunk tissues were fixed in glutaraldehyde and processing for an HRP reaction product using diaminobenzidine (DAB). Following dehydration and paraffin embedding, 10 μm serial sections were cut transverse to the cranio-caudal axis and stained with cresyl violet.

For each embryo, the identification of individual segments was made by reference to the ribs and limb-projecting spinal nerves. In
most cases, the segment containing the last rib preceded the segment containing the first of three large spinal nerves contributing to the crural plexus. The former was identified as T7, the latter as LS1. Occasionally, this relationship was not found. In these cases, the first large spinal nerve to contribute to the crural plexus was designated LS1. The positions of the glycogen bodies and the caudal end of the LMC provided additional criteria for segment identification in sectioned material. Segment boundaries were defined as the midpoint between adjacent dorsal root ganglia.

The following criteria were met by all operated embryos: (1) the excised (sham) or reversed region included LS1-3, (2) the majority of segments LS1 and LS3 were within operation boundaries, (3) the majority of the spinal cord within the LS1-3 regions appeared undisturbed by the surgery and (4) the limbs were well formed. In most experimental embryos, the operated region included part or all of T7 (n=36), while in some only LS1-LS3 were included (n=15). Occasionally, a part of LS4 was included with LS1-3 (n=11) or T7-LS3 (n=1). Embryos with different reversal or excision boundaries were pooled for analysis as no consistent correlations were noted between the length of each spinal segment to obtain the total number of motoneurons labeled throughout that side of the cord. Because all analyses were comparative, corrections for double counting were not made. The Mann-Whitney U-Test was used to test the significance of results in all experiments.

Counts of labeled cells within the motor columns of the spinal cord were made on every section at x400-630. Only profiles with a distinct nucleus surrounded by labeling were scored. Counts from each side of a single embryo were plotted in histogram form as the number of labeled cells/30 μm of cord and the two histograms superimposed by aligning cord segment boundaries. For all motor pools analyzed, the total numbers of labeled profiles in identified segments were determined and expressed as percentages of the total number of cells labeled throughout that side of the cord. Because all analyses were comparative, corrections for double counting were not made. The Mann-Whitney U-Test was used to test the significance of results in all experiments.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Mean number of segments excised/ reversed ± s.e.m.</th>
<th>Mean number of labeled cells in the motor pool ± s.e.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3.5±0.2 (11)*</td>
<td>402±87 (5)†</td>
</tr>
<tr>
<td>Stage 15 reversals ± notochord</td>
<td>4.2±0.2 (12)</td>
<td>400±62 (11)</td>
</tr>
<tr>
<td>Stage 13-14 sham-operated</td>
<td>3.6±0.2 (11)</td>
<td>590±71 (10)</td>
</tr>
<tr>
<td>Stage 13 reversals + notochord</td>
<td>4.1±0.2 (9)</td>
<td>571±110 (9)</td>
</tr>
<tr>
<td>Stage 14 reversals + notochord</td>
<td>3.9±0.2 (9)</td>
<td>656±89 (9)</td>
</tr>
<tr>
<td>Stage 14 reversals + notochord</td>
<td>3.8±0.2 (11)</td>
<td>532±93 (10)</td>
</tr>
</tbody>
</table>

*Number of embryos. †Number of motor pools.

Table 1. Average reversal and motor pool sizes

Determination of motoneuron density

In a normal stage 35-36 embryo, there are marked differences in the overall size of the LMC in different LS segments. We used a motoneuron density measurement to assess the effect of manipulations on motoneuron numbers in individual segments. In 3-8 embryos from each experimental series and 5 normal embryos, all LMC motoneurons within every fifth section of LS1-3 were counted. Similar counts were made for T7 because it was often included in the reversal. In T7, a clear separation of lateral and medial motor columns is often not present. We counted only those motoneurons that occupied a lateral position and appeared to form a spatially discrete cluster. Motoneurons were identified by their large cell bodies and prominent nucleoli. After counting every fifth section of a single segment, the average motoneuron count per section was determined and multiplied by the number of sections in that segment. Counts were made on both sides in each embryo and the values averaged. This average was divided by the length of each spinal segment to obtain the total number of motoneurons per 10 μm. Density values were averaged together in each experiment for each spinal segment.

Identification of reversal or sham boundaries

The locations of transverse excision sites were identified by the presence of carbon particles or a morphological disruption in the cord at the time of killing and in histological sections. In most cases, carbon particles appeared embedded within the cord or vertebra and their position coincided with a disrupted region. If no carbon particles were present, the midpoint of the disrupted region was designated as a boundary. Cases in which neither carbon particles nor a disrupted region were evident were excluded.

RESULTS

Motor pool organization in normal, stage 15 reversal and sham embryos

The motor pools to the sartorius and femorotibialis muscles were characterized in normal and stage 15 reversal embryos to provide material for direct comparison to data from embryos operated on at earlier stages. As observed by Landmesser (1978a), motoneurons projecting to the normal sartorius (n=5) were located predominantly in LS1 and LS2, the peak concentration in LS1. Motoneurons projecting to the normal femorotibialis (n=6) were located predominantly in LS2 and LS3, with a peak concentration at the LS2-LS3 border (Fig. 2).

In agreement with Lance-Jones and Landmesser (1980), we found the pool positions for the sartorius (n=8) and femorotibialis (n=9) to be reversed in the AP axis following reversals of T7 and anterior LS segments at stage 15 (Fig. 2). Pools were mapped in embryos in which neural tube segments alone had been reversed (n=5) and embryos in which the underlying

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Motor pool organization in stage 13 neural tube reversal embryos

Motor pools were mapped in 11 stage 35-36 embryos in which anterior LS neural tube segments had been reversed at stage 13 (19-21 somites present). The AP positions of sartorius and femorotibialis pools from a single experimental embryo are shown in Fig. 3A. As in a normal or sham embryo, the sartorius motor pool lies anterior or cranial to the femorotibialis pool. For the sartorius pool, the peak number of labeled motoneurons are found in LS1; for the femorotibialis pool, the peak number are found at the LS2-3 border. Similar results were obtained in the other stage 13 reversal embryos (Fig. 3C). In no case did we find a reversal of sartorius and femorotibialis pool positions like that found after stage 15 neural tube reversals. Following stage 13 neural tube reversals, motoneurons appear to project in accord with their new rather than original AP position. This conclusion, in turn, suggests that factors determining motoneuron identity are not fixed in the LS neural tube at stage 13.

There were notable differences in segmental motoneuron distributions in embryos with stage 13 neural tube reversals when compared to normal or shams. Experimental pools appeared to be more widespread in the AP axis. In a normal or sham embryo, about 90% of sartorius motoneurons are located in LS1 and LS2 (Fig. 2C). In experimental embryos, only about 72% of sartorius motoneurons were located in these segments. In conjunction, we found a significant reduction in the percentage of sartorius motoneurons in LS1 (comparison to shams, $P \leq 0.008$) and a significant increase in LS3 ($P \leq 0.003$) (Fig. 3C). Similar findings apply to the femorotibialis motor pools. In a normal or sham embryo, about 97% of femorotibialis motoneurons are found in LS2 and LS3. In experimental embryos, only about 73% were located in these segments, the percentage in LS2 being significantly lower than the sham value ($P \leq 0.024$) and the percentage in LS1 being significantly higher ($P \leq 0.035$) (Fig. 3D). In addition, small numbers of labeled cells were occasionally found in LS4.

To further characterize the specificity of projections from reversed cord segments, we examined the positions of the sartorius and femorotibialis pools in the transverse plane of the spinal cord in LS1-3. To compare individual cases, the LMC was divided into 6 divisions and an average measure of the number of cells in each division determined (Fig. 4). In a normal embryo, sartorius motoneurons are found predominantly in lateral and middle ventral divisions of the LMC,
while femorotibialis motoneurons are found predominantly in middle dorsal divisions (Figs 4A,B, 5A,B). In experimental embryos, labeled motoneurons arising from positionally correct segments were normally placed in the transverse plane. For example, sartorius motoneurons were most prevalent in lateral divisions of the LMC in LS1. In contrast, femorotibialis motoneurons were most prevalent in middle divisions of LS3 (Figs 4B, 5C,D).

As described above, some labeled motoneurons were found in positionally incorrect segments in stage 13 reversal embryos. For example, approximately 16% of labeled sartorius motoneurons were located in LS3. In a normal embryo, few if any sartorius motoneurons are located in LS3. In stage 13 reversal embryos, the process of specification may have been interrupted. These cells may represent a population that was specified prior to the reversal and thus projected in accord with their segmental origin (T7-LS1). If true, one might expect these cells to be placed in originally correct positions in the transverse plane as well. This did not prove to be the case. Labeled motoneurons in originally correct segments were not positioned normally in the transverse plane. For example, sartorius motoneurons in LS3 (originally T7 or LS1) were highest in number in middle dorsal divisions of the LMC, a position normally occupied by femorotibialis motoneurons (Fig. 4B). Thus, in LS3, there is a mismatch between motor column position in the transverse plane and target site in the limb. A similar mismatch is found in LS1 for cords in which the femorotibialis pool was labeled. These cells in originally correct segments are not positioned in the transverse plane in a manner that would suggest that they have been specified prior to the reversal. While small in number, these projections as well as those from LS4 may have resulted from some disruption of the specification process and a tendency for motoneurons to project to the nearest available target.
than that of the normal or stage 13 neural tube reversal embryos (see also Fig. 7). Calibration bar=100 positions. In the section from the stage 13 neural tube + notochord reversal embryo (F), the LS3 LMC is smaller through LS3 following injections of the femorotibialis. Most labeled motoneurons occupy middle dorsal divisions. In stage 13 and 14 reversal embryos, femorotibialis motoneurons in LS2 be described as an originally correct segment for the sartorius pool. In a normal embryo (B), the highest numbers of femorotibialis (femoro) motoneurons are found in middle dorsal divisions of LS2 and LS3. In stage 13 and 14 reversal embryos, femorotibialis motoneurons in LS2 and LS3 occupy similar transverse positions, but those in LS1 are more widespread in the transverse plane.

Fig. 4. The transverse distribution of HRP-labeled motoneurons in the LMC of LS1-3 in normal embryos and embryos in which the neural tube has been reversed at stages 13-14. (A) The distribution of labeled cells in a single normal embryo, following HRP injection of the sartorius on one side and the femorotibialis on the other. The positions of labeled cells were mapped within camera lucida drawings. Here, every tenth section is shown, however every fifth section was actually mapped. The LMC is outlined. (B) Summaries of the transverse positions of labeled cells. Numbers represent average number of labeled cells in every fifth section within each division for each spinal segment, ±s.e.m (see Materials and Methods). The highest value in each segment, and values within 5%, are in bold. In a normal embryo (B, left), the highest number of labeled sartorius (sart) motoneurons are found in lateral divisions of the LMC in LS1 and in lateral and middle ventral divisions of the LMC in LS2. In stage 13 (B, middle) and stage 14 (B, right) reversal embryos, the highest number of sartorius motoneurons in LS1 and LS2 are located in the same divisions. In contrast, many sartorius motoneurons are incorrectly positioned in LS3. This segment would have become T7 or LS1 if it had not been reversed and, thus, can be described as an originally correct segment for the sartorius pool. In a normal embryo (B), the highest numbers of femorotibialis (femoro) motoneurons are found in middle dorsal divisions of LS2 and LS3. In stage 13 and 14 reversal embryos, femorotibialis motoneurons in LS2 and LS3 occupy similar transverse positions, but those in LS1 are more widespread in the transverse plane.

Fig. 5. Photomicrographs of transverse sections through the LMC’s of control embryos (A,B), stage 13 neural tube reversal embryos (C,D), and stage 13 neural tube + notochord reversal embryos (E,F). Section orientation is shown in the schematic. The upper row of photomicrographs (A,C,E) show sections through LS1 following injection of the sartorius. HRP-labeled motoneurons (arrows) occupy a lateral position in all cases. (Arrowheads indicate erythrocytes that have endogenous peroxidase activity.) The LMCs in the sections from experimental embryos (C,E) are small, like the normal LMC (A). The lower row of photomicrographs (B,D,F) show sections through LS3 following injections of the femorotibialis. Most labeled motoneurons occupy middle dorsal positions. In the section from the stage 13 neural tube + notochord reversal embryo (F), the LS3 LMC is smaller than that of the normal or stage 13 neural tube reversal embryos (see also Fig. 7). Calibration bar=100 μm.
Motor pool organization in stage 14 neural tube reversal embryos

The patterns of motor projections in embryos following stage 13 neural tube reversal suggest that motoneuron target identity is acquired or fixed between stage 13 and 15. We assessed projections in embryos (n=9) following neural tube reversals at stage 14 to look for evidence of a transition between labile and determined states. The AP distributions of sartorius and femorotibialis pools in a representative stage 14 reversal embryo are shown in Fig. 3B, and summaries of the segmental distribution of all pools studied are shown in Fig. 3C and D. AP motoneuron distributions following stage 14 reversals were found to be very similar to those in stage 13 reversal embryos; neither sartorius nor femorotibialis motoneuron distributions were significantly different in any segment.

As following stage 13 reversals, most motoneurons located in positionally correct segments were correctly positioned in the transverse plane (Fig. 4B). Moreover, the minority of motoneuron located in originally correct segments were often incorrectly positioned in the transverse plane, as in stage 13 reversal embryos. These similarities in motor pool organization between stage 13 and 14 reversal embryos suggest that mechanisms governing the acquisition of target identity are the same at the two stages and, thus, that target identity is likely to be acquired or fixed in the anterior LS neural tube between late stage 14 and early 15.

Motor pool organization in neural tube + notochord reversals at stages 13 and 14

To test whether the notochord is a source of cues that influences the axial specification of the spinal cord, the underlying notochord was reversed together with the neural tube at stage 13 and 14. Motor pools to the sartorius and femorotibialis muscles were characterized at stage 35-36 in 9 embryos following stage 13 reversals of the neural tube and notochord and in 11 embryos following stage 14 reversals.

The AP distribution of sartorius and femorotibialis motoneurons in spinal segment T7-LS3 did not differ significantly from reversals without the notochord at either stage 13 or stage 14 (Fig. 3C,D). The transverse positions of sartorius and femorotibialis pools in stage 13 and 14 reversals including the notochord is shown in summary in Fig. 6, and in an individual stage 13 embryo in Fig. 5E,F. No major differences were noted between reversals with and without the notochord. These findings suggest that cues from the notochord do not provide target identity between stages 13 and 15.

LMC size

We used a motoneuron density measurement to determine if segmental differences in LMC size were altered by the reversal of the neural tube ± notochord. All LMC motoneurons in LS1-3 and laterally positioned motoneurons in T7 were counted in sections from a sample of experimental and normal embryos (n=3-8). To correct for differences in spinal cord length, the average number of motoneurons per 10 μm in a segment was calculated. In normal (n=5) and sham (n=6) embryos, average motoneuron density values increase between T7 and LS3. For example, in normal embryos the average numbers of motoneurons per 10 μm in T7-LS3, were 10±2, 19±2, 35±2, 45±2, respectively (Fig. 7A). Following reversals of the neural tube ± notochord at stage 15 (n=6), values for LS1-3 appeared to be reversed, LS1 containing more motoneurons than LS3 (Fig. 7B). These results are in agreement with the findings of Lance-Jones and Landmesser (1980). In stage 15 reversal embryos, T7 values were similar to normal. No particular significance is ascribed to this finding because most stage 15 reversals included only a small portion of T7 (see Table 1).

Following reversals of the neural tube at stage 13, segmental motoneuron densities were similar to normal. Motoneuron numbers increased from an average of 12±2 motoneurons per 10 μm in T7 to 43±1 motoneurons in LS3 (n=7). The average number of motoneurons per 10 μm did not differ significantly from those in normal or sham embryos in any segment (compare Fig. 7A,C). Thus, like motoneuron projection patterns from reversed segments, total motoneuron numbers reflect segment position rather than origin. Since many stage 13 reversals included all or most of T7, these findings suggest that cells within a single thoracic segment have responded to environmental influences in the same manner as cells within LS segments.

Counts made in stage 14 neural tube reversal embryos (n=7) were similar to those described above in that motoneuron numbers increased from T7 through LS3. However, in stage 14 reversal embryos, we found a small but significant increase in motoneuron density in T7 and LS1 when compared to normal and sham means (P<0.05) (Fig. 7D). In a normal embryo, these segments would have become LS3 or LS2. Since LS3 and LS2 are normally much larger than T7 or LS1, an increased motoneuron density after reversal might reflect some intrinsic control of motoneuron numbers.

In embryos in which the neural tube + notochord were reversed at stages 13-14, motoneuron numbers also increased from T7 to LS3. However, significantly fewer motoneurons
were found in LS3 than in a normal embryo or an embryo in which only the neural tube was reversed (P < 0.05, Fig. 7C,D). In stage 13 neural tube + notochord reversal embryos, the average number of motoneurons per 10 μm in LS3 was 33 ± 3 (n = 8). The corresponding averages for normal and stage 13 neural tube reversal embryos were 45 ± 2 (n = 5) and 43 ± 1 (n = 10). Similar small declines were found for LS3 in stage 14 neural tube + notochord reversal embryos (n = 7) and for LS2 in stage 13 neural tube + notochord reversal embryos (n = 8). These small declines may reflect damage done to the notochord and neural tube when they were removed and repositioned in reversed order. Previous studies (see van Straaten et al., 1989; Yamada et al., 1993) suggest that the notochord plays an important role in the promotion of motoneuron differentiation prior to stage 13, at neural plate stages. This process may normally continue into early neural tube stages and have been disrupted by our surgery. We do not know why the disruption was localized to the LS2-3 region of the reversed tissues.

**DISCUSSION**

An important step in the formation of specific neuromuscular connections is the early guidance of axons to correct muscle regions. Studies in the chick embryo indicate that two types of guidance mechanisms are used in the formation of stereotyped projections from somatic motoneurons of the spinal cord to individual limb muscles. Non-specific or permissive guidance cues dictate the positioning and morphology of the spinal nerves and the major nerve trunks of the plexuses (Lewis et al., 1983; Keynes and Stern, 1984). Specific guidance cues are responsible for the choice of pathways to an individual muscle or muscle region (Lance-Jones and Landmesser, 1980; Ferguson, 1983; Stirling and Summerbell, 1985; Tosney and Landmesser, 1985). All motor axons respect the ‘highways’ provided by permissive cues, however, a motor axon’s ability to recognize and/or respond to specific cues depends on a specification event that occurs prior to axon outgrowth. We have used a neural tube reversal paradigm to address the question of when this specification event occurs in the LS region of the embryonic chick neural tube.

During normal development, LS motoneurons exit the cord and make specific pathway choices between stages 18 and 27 (Tosney and Landmesser, 1985). When neural tube segments LS1-3 (± one segment cranial or caudal) are reversed in the AP axis at stage 15, motoneuron axons consistently alter their course to project to originally correct target muscles, indicating that the stage 15 neural tube is polarized in some manner that later permits specific target choice (Lance-Jones and Landmesser, 1980). We have now obtained evidence that stage 15 is the earliest time at which one can see this form of regionalization. After carrying out similar reversals of the neural tube at stages 13-14, we used retrograde HRP labeling to assess projections from LS1-3 to two prominent thigh muscles, the sartorius and the femorotibialis. The sartorius was innervated predominantly by motoneurons in LS1 and LS2, the positionally correct rather than originally correct, segments. Similarly, the femorotibialis was innervated mainly by motoneurons in LS2 and LS3, the positionally correct segments. Most motoneurons in both pools were also located in normal positions within the transverse plane of the LMC. These observations stand in marked contrast to those obtained following stage 15 reversals and suggest that factors governing motoneuron target identity are not fixed at stages 13-14. Until stage 15, environmental or positional factors external to the neural tube appear capable of influencing the future target identity of LS motoneurons.
The attainment of regional differences within the LS neural tube

Do environmental signals normally define regional differences relevant to motoneuron target identity in the period between stage 13 and 15? The fact that most projections were positionally correct after reversals at stages 13-14 is compatible with this hypothesis. However, motoneuron projections in stage 13 and 14 reversal embryos were not identical to normal. Sartorius and femorotibialis motoneurons were distributed over a wider AP area than normal and small but significant increases were found in segments that normally contain few if any motoneurons projecting to one or the other of these muscles. Additionally, sartorius and femorotibialis motoneurons in positionally incorrect segments were abnormally positioned in the transverse plane. These characteristics were not the result of cord excision alone as no such abnormalities were found in sham embryos where the neural tube was simply excised and replaced. The added manipulation of a reversal may be responsible. For example, tissue at reversal boundaries may have united poorly with surrounding neural tissue because of differences in level of maturity.

It is also possible that LS regionalization begins before stage 13 and that the observed pattern of projections resulted from the interruption of an ongoing process. Neural tube reversal at stages 13-14 may have disrupted or prevented the process of motoneuron specification. Without a target identity, some or all motoneurons within reversed segments may have projected to individual muscles using nonspecific guidance cues. When motoneurons are forced to innervate foreign muscles by placing them far from their normal targets, they tend to form somatotopically arranged pools which are roughly similar to normal (Lance-Jones and Landmesser, 1981b; Hollyday, 1981; O’Brien and Oppenheim, 1990). In our reversal embryos, the grossly correct organization of motoneuron projections could have arisen because the sartorius is a more anterior muscle than the femorotibialis. Motoneurons in the most anterior LS segments may have been passively deployed to the sartorius; motoneurons in more posterior segments, to the femorotibialis.

Another possibility is that at stages 13-14 some motoneuron precursors or regions of the anterior LS were specified while others were not. We did not observe any clear patterns suggesting that this was the case. Motoneurons projecting to both the sartorius and femorotibialis muscles were found in originally appropriate segments but they were not discretely positioned in the transverse plane as might be expected were they specified prior to the reversal. We also did not see differences in the discreteness of the two pools that might reflect cranio-caudal or mediolateral gradients of spinal cord maturation (Hollyday and Hamburger, 1977). Nevertheless, it is important to point out that evidence of an ongoing process of specification may have been difficult to see because of the short time period involved and the possibility that factors governing a motoneuron’s position in the LMC may be separate from those specifying muscle target-fate.

In sum, while our results indicate that extrinsic signals can dictate motoneuron target choice at stages 13-14, they do not tell us whether this aspect of AP regionalization normally occurs between stages 13 and 15 or whether it is a gradual process that is initiated earlier and then fixed or stabilized just before stage 15. Studies of the development of the prosencephalon, mesencephalon and metencephalon in avian embryos suggest that the attainment of diversity in the AP axis may be a multistep process. Restricted patterns of expression of Wnt and Engrailed genes are apparent in these regions during neural fold stages (Gardener and Barald, 1992; Bally-Cuif and Wassef, 1994). However, studies of embryos where portions of the met-mesencephalic region have been grafted into the prosencephalon (Alvarado-Mallart et al., 1990; Martinez et al., 1991; Bally-Cuif and Wassef, 1994) indicate that region-specific gene patterns and cytoarchitecture are not fixed within the neuroepithelium until well after neural tube closure. Whether a parallel situation exists in the LS spinal cord may be addressed with the identification of early onset genes.

Regardless of when specification begins, the axial information leading to motoneuron target specification clearly becomes fixed within the anterior LS neural tube just before stage 15. Similar axial information appears to be fixed at early neural tube stages within the avian hindbrain. Following reversals (Guthrie and Lumsden, 1992) and transpositions (Guthrie et al., 1992) of hindbrain segments at stages 9-11, the form and position of branchiomotor nuclei and proximal axon trajectories develop in accord with segment origin. In the hindbrain region, stages 9-11 correspond to a time immediately after neural tube closure. In the anterior LS region, neural tube closure occurs at stage 12, about 5-6 hours before the time (stage 15) that regional differences within LS1-3 are fixed. These observations suggest that the attainment of regional motor diversity occurs slightly later in the spinal cord than in the hindbrain. As we do not know how early hindbrain diversity is set, the difference in timing might be even greater. This difference may reflect the fact that the limb is newer in an evolutionary sense than the targets of hindbrain motoneurons. The organization of motoneurons within the spinal cord and hindbrain are also quite different (see Guthrie and Lumsden, 1992). Unlike branchiomotor nuclei, LMC motoneurons are not arranged in a clear segmental pattern. A motoneuron pool to a single limb muscle may be located mainly in one LS segment but it may also span several segments.

The attainment of target identity by motoneurons

The results of our reversal experiments tell us that regional differences are established within the stage 15 LS neural tube, but do not tell us that they are specifically localized to motoneuron precursors. At stage 15, there are no postmitotic cells in the LS neural tube. Motoneurons will not begin to withdraw from the cell cycle in this region until stage 17 (Hollyday and Hamburger, 1977). Therefore, factors that lead to specification either act on motoneuron precursors, or become stably determined in the environment into which motoneurons will later be born and migrate.

Within two cell divisions of their birth, motoneuron precursors are probably not target-specified. Multiple motoneuron-containing clones derived from retroviral infections at stages 11-14 are not restricted to any particular transverse position in the LMC, suggesting that motoneuron siblings can belong to different motor pools (Leber et al., 1990). The target identities of some motoneuron precursors may become fixed during their last cell division, similar to the specification of laminar fate in the rodent cortex (McConnell and Kaznowski, 1991). Roughly 15% of the total final number of motoneurons in the LS region
are born at stage 17 (Hollyday and Hamburger, 1977). Since the cell-cycle time for ventral neuroepithelial cells is of the order of 10 hours (Stern et al., 1988), and stage 15 precedes stage 17 by 6-10.5 hours, it can be inferred that about 15% of the LS motoneurons are in their last cell cycle during stage 15. Recently, it has been shown that motoneurons within axial and limb-innervating motor columns and within dorsal and ventral regions of these columns express unique combinations of LIM homeobox genes (Tsuchida et al., 1994). Some LIM genes appear to be expressed in a unique manner prior to motor column formation, a finding compatible with the idea that motoneuron identity with respect to broad groups of targets is established early.

Chick motoneurons within individual pools might become target-specified after motor column formation as appears to be the case for primary motoneurons innervating axial musculature in zebrafish embryos (Eisen, 1991). Single-cell transplantation experiments indicate that primary motoneurons become target specified postmitotically, at a time just prior to axogenesis, and that the local environment in which these neurons develop is an important determinant of target identity. If this is the case for the chick, then the specification of regional differences that we have identified at stage 15 must lie within non-motoneuron components of the spinal cord.

**Environmental cues for motoneuron target specification**

We also assessed motor pool organization after performing neural tube reversals that included the underlying notochord at stages 13 and 14. No significant differences in the positions of the sartorius and femorotibialis pools were found when reversals with and without the notochord were compared. This observation suggests that the notochord is not a primary source of cues governing motoneuron target specification at stages 13 and 14.

Another possible source of cues for motoneuron target specification is the segmental plate mesoderm, the precursor of the somites. Experiments by Stern et al. (1991) suggest that the segmentation of this mesoderm imposes lineage restriction boundaries on neural tube cells. In addition, analyses of the expression of a reporter transgene driven by a myosin light chain promoter suggest that somitic myoblasts carry heritable positional information (Donoghue et al., 1992; Grieshammer et al., 1992). When somitic tissues are reversed in the AP axis either at the LS (Lance-Jones, 1988) or brachial (Keynes et al., 1987) level, motoneuron projections from adjacent cord segments are not correspondingly altered. However, these manipulations have not been performed at stages likely to precede the stabilization of regional identity.

The presumptive limb, a thickening of LS somatopleural mesoderm covered by ectoderm, is also a candidate. This structure appears to be a primary source of cues for specific axon pathway choice (Stirling and Summerbell, 1988; Lance-Jones and Dias, 1991). While a limb bud is not visible until stage 18, LS somatopleural mesoderm is likely to be determined and polarized in the AP axis prior to stage 15 (see Hinchcliffe and Johnson, 1980).

Finally, signals propagating within the plane of the neuroepithelium may influence motoneuron target specification. Homeogenetic induction mechanisms are implicated not only in early neural induction (see Doniach, 1993; Ruiz i Altaba, 1993) but also in the rebuilding of a floorplate and motor columns after notochord removal (Artinger and Bronner-Fraser, 1993). Locally acting, diffusible molecules derived from the floorplate (Yamada et al., 1993) or other neural tube sources may play a similar role in motoneuron target specification. The more widespread distribution of motor pools after neural tube reversals may in fact reflect an interference with these signals in the stage 13 or 14 neural tube.

**Motor column size**

In a normal stage 35-36 embryo, there are clear segmental differences in the total numbers of motoneurons in T7-LS3, differences that may be determined by factors in the periphery as well as within the cord (Williams, et al., 1987; O’Brien and Oppenheim, 1990). Our results support the hypothesis that extrinsic cues determine motoneuron number within individual limb-innervating segments after stages 13-14. Following neural tube reversals at stages 13-14, segmental motoneuron densities at stage 35-36 were similar to normal, matching segment position rather than origin. Since motoneuron projections to the sartorius and femorotibialis muscles were also in accord with position, segmental motoneuron density may have been determined by axon-target interactions (Hamburger, 1975; O’Brien and Oppenheim, 1990). However, prior studies suggest that some region-specific characteristics may be established before axon outgrowth stages. When several segments of stage 14-15 thoracic neural tube are transplanted into the LS region of a stage 16-17 chick host, thoracic axons will innervate the limb, but a large, LS-like, LMC does not develop within transplanted thoracic segments (O’Brien and Oppenheim, 1990). In our stage 13 and 14 neural tube reversal embryos, we consistently found an LMC of normal size in the LS region, despite the fact that many of our reversals included T7. The difference between our results and those of O’Brien and Oppenheim (1990) may reflect the size of the transplant or the fact that we transplanted only the most posterior thoracic segment. Alternatively, thoracic segments may only begin to be determined at stages 13-14. Our operations may have been just early enough that T7 took on more LS characteristics when reversed to the position of LS3.

In embryos with reversals of both the neural tube and the notochord at stages 13-14, we found a small reduction in motoneuron numbers in LS2-3 when compared to embryos in which only the neural tube was reversed. The surgery involved in reversing both the neural tube and notochord may have been more damaging than the reversal of a single tissue. However, the finding of a reduction in motoneuron numbers in LS2-3, but not T7-LS1, raises the possibility that interactions between the notochord and neural tissue vary in the AP axis at early neural tube stages. This might be tested in the future by a reversal of the notochord alone.

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**REFERENCES**

Bally-Cuif, L. and Wassef, M. 


Grieshammer, U., Sassoon, D. and Rosenthal, N. 


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