Development and Survival of Thoracic Motoneurons and Hindlimb Musculature Following Transplantation of the Thoracic Neural Tube to the Lumbar Region in the Chick Embryo: Functional Aspects

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SUMMARY

Following heterotopic transplantation of the thoracic neural tube to the lumbar region on embryonic day (E) 2, the transplanted cord differentiates normally and establishes neuroanatomical connections with the host central nervous system and hindlimb muscles. Beginning on about E12, however, the neuromuscular system begins to undergo regressive changes resulting in motoneuron degeneration and muscle atrophy (O'Brien and Oppenheim, 1990). In the present paper, we have examined the development of neuromuscular function in thoracic transplant embryos from E6 to the time of hatching on E20–21. The onset of hindlimb movements and reflexes occurred at the same time (E6–E8) in both control and thoracic transplant embryos. Further, both the nature (pattern) and frequency of these movements appeared normal in the thoracic transplants up to E10–E12, after which there was a gradual and marked reduction in the frequency, and an alteration in the pattern, of both spontaneous and reflex-evoked hindlimb movements. After E16 normal movements were virtually absent in many of the thoracic transplant cases. By contrast, movements of the head, trunk and wings were normal in these embryos throughout the observation period. Hindlimbs innervated partly by the thoracic transplant and partly by remaining host lumbar cord did not exhibit the regressive changes in function after E10 that occurred in hindlimbs innervated exclusively by the thoracic transplant. EMG recordings from specific hindlimb muscles innervated solely by thoracic motoneurons demonstrated that the activation pattern of both flexors and extensors was similar to the repetitive pattern observed in normal thoracically innervated intercostal muscles (i.e., extensor-like). Muscles did not show distinguishable EMG burst patterns with inhibitory periods as do control lumbar innervated muscles. We conclude that the development of the pattern generating circuitry in the transplanted thoracic cord was similar to normal thoracic cord and thus appeared to be uninfluenced by having contacted the foreign hindlimb muscle targets early in development. Activity blockade with curare from E6 to E14 suppressed the loss of motoneurons that occurs in the thoracic transplant after E10. Thus, the abnormal thoracic-like activation pattern of thoracically innervated hindlimbs may be a critical signal in the initiation of the neuromuscular regression that occurs after E10 in these preparations. Finally, although the innervation and formation of neuromuscular endplates in thoracic transplants appeared normal up to E12, by E14 both the intramuscular nerves and the endplates exhibited signs of degeneration and regression. Thoracic motoneurons are initially able to innervate and functionally activate hindlimb muscles in a manner similar to that of thoracically innervated intercostal muscles. Eventually, however, even this abnormal activation pattern disappears and the entire neuromuscular system breaks down.

As described in more detail in the preceding companion paper (O'Brien and Oppenheim, 1990), we have used heterotopic transplantation of the embryonic neural tube as a means for studying the mechanisms involved in the emergence of region specific differences in the structure and function of the spinal cord. The functional studies described here corroborate and extend the anatomical studies reported in the companion paper. We have

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chosen to focus on the activity of motoneurons in the nonlimb innervating thoracic spinal cord following transplantation to the lumbo-sacral, hindlimb innervating, region. This experimental paradigm allows us to examine in detail the earliest hindlimb movements (motility) in embryos whose musculature received completely inappropriate innervation. In addition, individual muscle activation patterns of inappropriately innervated embryonic hindlimb muscles were assessed using electromyographic (EMG) recordings (Landmesser and O'Donovan, 1984a). This provided a more detailed analysis of the activation patterns of specific hindlimb muscles than could be attained from the motility studies alone. Finally, by examining the function of thoracically-innervated hindlimb muscles throughout most of the embryonic period, we were able to reveal developmental changes in the ability of thoracic motoneurons to activate foreign muscles in the hindlimb.

MATERIALS AND METHODS

Motility

Movements (motility) and reflexes of hindlimbs that were wholly or partially innervated by thoracic cord were examined at varying developmental stages in ovo. All operations were done as previously described (O'Brien and Oppenheim, 1990).

Following surgery, all embryos were incubated, unturned, in a hatching incubator for varying periods of time. Hindlimb motility was recorded repeatedly in individual embryos at various stages in development from embryonic day (E) 6 to E16, as well as at the same developmental stage in different experimental embryos.

Motility recordings of spontaneous movements were made as previously described (Pittman and Oppenheim, 1979). Occasionally, the embryo required gentle manipulation to bring the hindlimbs into view. The majority of the recordings were of spontaneous movements and induced reflexes of the right hindlimb. Reflexes were evoked by gentle stroking of the hindlimb with a calibrated hair sufficient to induce a cutaneous tactile reflex. After the completion of recordings, the embryos were either sacrificed and used for anatomical studies or the windows were resealed with Parafilm (Sigma) and the embryos were returned to the incubator for later observation. Notes were also made concerning qualitative aspects of the limb movement patterns and reflexes.

All embryos used in the motility studies were eventually sacrificed and the thoraco-lumbar region was dissected, fixed with Carnoys solution, processed, embedded in paraffin, sectioned, and stained with thionin (O'Brien and Oppenheim, 1990). The histology from each of the cases was examined in detail and only those embryos which had had the entire extent of the lumbar cord removed and contained a thoracic transplant which had reasonably normal morphology were used for further analysis. The spinal cords from these same experimental or control cases were then used for the analysis of neuronal survival (O'Brien and Oppenheim, 1990). Those cases which had large portions of residual host lumbar cord remaining or with abnormal morphology of the transplant were discarded and the motility data were not used.

In many of those cases that were found to have successful transplants, motility recordings had been taken daily starting on E7. Recordings were done at approximately the same time each day from E7 to E16.

Electromyographic Recordings

The analysis of spontaneous EMG activity in the chick embryo has been carried out previously both in ovo and in an isolated spinal cord preparation maintained in vitro (Bekoff, 1976; Landmesser and O'Donovan, 1984a). In the present study, an *in vitro* method was used to compare the spontaneous EMG activity in hindlimb muscles innervated by thoracic transplant with control EMG activity. Following surgery, embryos were incubated until stage (St) 33 to 38 (E7.5 to E11). Experimental embryos were removed from the egg and rapidly decapitated and eviscerated. The embryo was pinned in a Sylgard-lined petri dish and superfused with oxygenated Tyrode's solution at room temperature (20° to 22°C). A ventral laminectomy was performed, the skin was removed, and individual muscles were exposed and cleaned of connective tissue. After the dissection was completed, the bath temperature was raised to 30°C and the embryo was left for several hours, after which time spontaneous motility reappeared (Landmesser and O'Donovan, 1984). The EMG signals associated with limb activity was recorded with suction electrodes, differentially amplified with a band width of 3Hz to 3kHz, and displayed on an oscilloscope and pen recorder. The activity was also stored on magnetic tape for further analysis.

Activity Blockade

In one group of embryos, activity blockade was induced by daily treatment from E6 to E10 with the neuromuscular blocker d-tubocurarine (Sigma) at a dose of 1.5–2.0 mg in 200 μL of saline (Pittman and Oppenheim, 1979). Because curare administered in this way is only very slowly metabolized, the embryos remained immobilized for several days after the cessation of treatment on E10 (Oppenheim, Pittman, Gray, and Maderdrut, 1978). Embryos were sacrificed on E14 and the thoraco-lumbar spinal cord was processed as described above. Motoneuron cell counts were performed as previously described (O'Brien and Oppenheim, 1990). The goal of this experiment was to determine whether the suppression of neuromuscular activity in embryos with

thoracic transplants altered the regression of motoneurons and muscle that occurs in these preparations. Although we attempted to maintain these embryos until E16–E18, when these regressive events are greatest, we were never successful in getting activity blocked embryos with thoracic transplants to survive beyond E14.

Innervation

Combined silver stain and cholinesterase histochemistry (Toop, 1976) was used to examine the innervation and neuromuscular junctions of thoracically-innervated hindlimb muscles on E14–E16. This technique allowed visualization of the neuromuscular junction with cholinesterase and the motor nerve terminal with silver impregnation. Embryos utilized in this part of the study always had all host lumbar cord removed so that limb innervation was derived solely from the transplanted thoracic cord. In a few embryos (E8 and E10), the innervation of individual muscles was examined immunocytochemically using a neurofilament antibody as described by Dahm and Landmesser (1988). In all cases used for innervation studies the host lumbar cord was completely absent and the donor neural tube was positioned so that the rostral portion of the thoracic transplant was closely apposed to the host thoracic cord, thereby often leaving a "gap" of 1–2 segments in the caudal portions of the lumbo-sacral region. Hindlimbs from embryos of varying embryonic ages (E8, E10, E12, and E14) were removed at the time of sacrifice and placed into ice cold saline. Skin was removed from the thigh and shank, the hindlimb was oriented flat, straightened in OCT compound on a cork block, and then frozen in isopentane that had been chilled with liquid nitrogen or with dry ice. The frozen tissue was then sectioned at 18 μm on the cryostat, mounted on 3% EDTA-coated slides and stained for silver and cholinesterase. Muscles examined immunohistochemically were stained as whole mounts as described by Dahm and Landmesser (1988).

RESULTS

Motility

Only those embryos with successful thoracic transplants and which lacked gross, nonspecific, defects were utilized in the motility study. Transplant success was determined histologically following completion of the motility observations, at which time a number of the same cases were also utilized in the cell death study reported in the previous companion paper (O'Brien and Oppenheim, 1990). Similar to the development of control embryos, the first signs of motility in the hindlimbs of experimental embryos innervated either wholly or partially by thoracic motoneurons occurred at approximately E6.0. Slight abductions or adductions of the hip followed by slight waving-like movements of the ankle were observed at this time. Experimental groups included embryos which had either the entire lumbar cord removed and five to six segments of thoracic cord transplanted to the lumbar region (Experimental Groups 1 and 2), or embryos which had only the rostral half of the lumbar cord removed (caudal half remained intact) and two to four segments of thoracic cord transplanted to this site. The control group consisted of unoperated stage matched embryos and embryos with excision and replacement *in situ* of the lumbar neural tube. The stage of development at which hindlimb motility was initiated was the same in experimental and control embryos. Taken together with the observation that cell death also begins at the same time in control and transplant thoracic motoneurons (O'Brien & Oppenheim, 1990), these data indicate that despite the one-half day difference in age of host and donor embryos at the time of operation on E2, the transplanted cord has differentiated according to the host age. Hindlimbs innervated wholly by host lumbar motoneurons (control), partially by thoracic motoneurons, or solely by thoracic motoneurons, all demonstrated their first movements on the sixth day of development. Since the earliest signs of hindlimb motility were observed at E6.0–7.0 in all experimental and control groups, quantitative recordings of hindlimb movements were recorded systematically from E7 and throughout development.

Data on the frequency of hindlimb movements are summarized in Figures 1 and 2. The data are expressed as the mean number of movements per three minute observation period. During early stages of development (E6 to E10), the spontaneous movements and tactile-evoked reflexes of hindlimbs that were innervated either wholly or partially by thoracic motoneurons appeared normal. There were no significant differences in the number of movements between experimental and control embryos from E7 to E10. Qualitative aspects of the spontaneous motility and reflexes also appeared normal in both experimental groups. Spontaneous movements involved the entire limb and consisted of short bouts of apparent muscle extension and flexion. Reflexes consisted of brief withdrawal movements of the limb away from the site of stimulation. The frequency of movement increased significantly from just under approximately 20 movements per three minutes on E7 to slightly over 40 movements per three

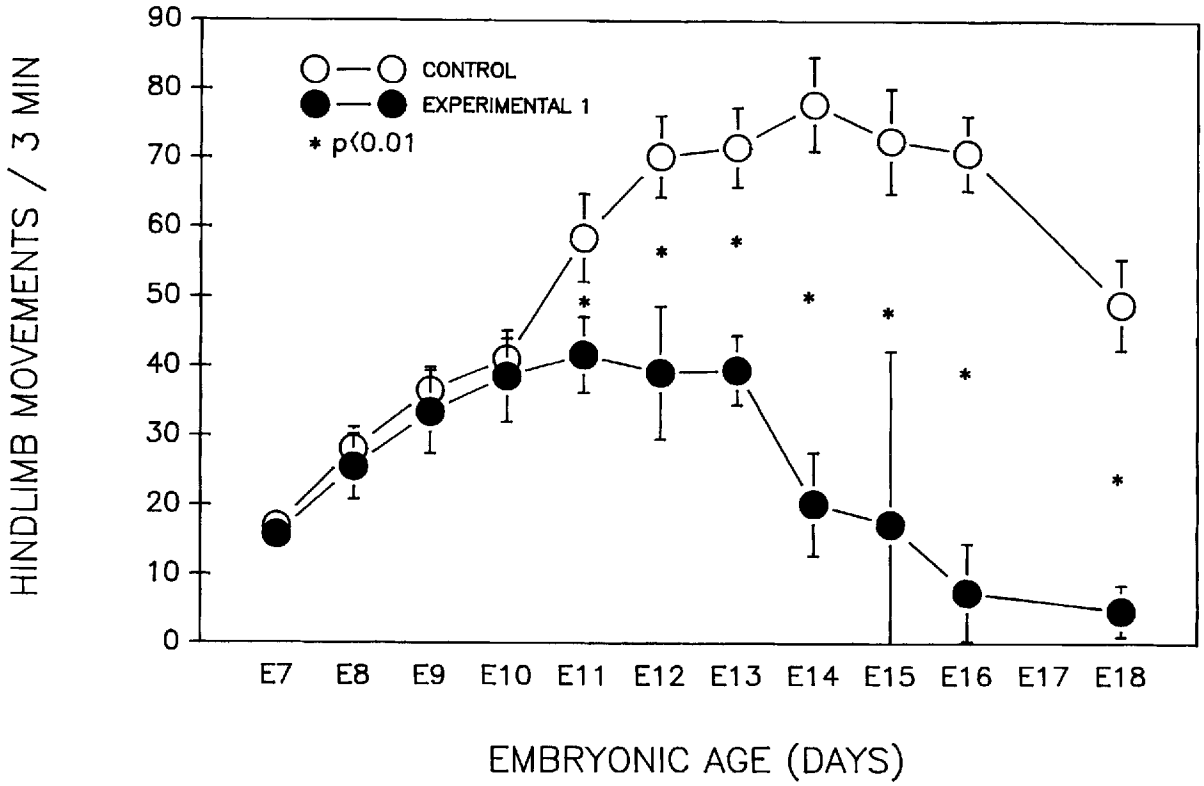


Figure 1 Movements (Mean ± S.D.) of hindlimbs in control and thoracic transplant embryos from E7 through E18. Statistical comparisons are between control and experimental embryos at each age.

minutes on E10 in both the experimental and control groups.

However, in experimental embryos in which the sole innervation of the hindlimbs was by thoracic motoneurons, both the quantitative and qualitative aspects of leg activity began to deteriorate by E11–E12 (Fig. 1) such that there were significantly fewer movements in the hindlimbs of experimental embryos compared to controls and the movements that did occur (spontaneous and evoked) consisted of brief, low amplitude jerks or twitches. Both the amount and quality of hindlimb movements continued to deteriorate at later stages of development, such that only a residual amount of low amplitude twitch-like activity was present by E15. In many of the E15 experimental cases, little or no activity was recorded throughout the entire three minute observation period. This was also true at later stages in development (data not shown); on E16 and E18 approximately 1/3 of the embryos demonstrated no activity while the remaining 2/3 demonstrated only very slight tremor-like movements of the legs. By contrast, although *hindlimb* motility was virtually absent in E15, E16, and E18 experimental embryos, frequent movements of the wing, head, and trunk were observed in these same cases. Therefore, the motility deficit was restricted to those regions receiving innervation from the transplant. Many of the thoracically innervated hindlimbs were markedly atrophied at these later stages of development (O'Brien and Oppenheim, 1990). Although neither neuron counts nor motility recordings were evaluated after E18, six experimental embryos were observed attempting to hatch. However, none of these actually completed hatching and all eventually died while still in the shell.

Hindlimb motility was also recorded in a second group of experimental embryos that underwent surgical procedures identical to embryos described above. However, because these embryos died prior to the intended day of sacrifice, it was not possible to process their spinal cords for histology. Consequently, we were unable to determine either the success of the transplant or the extent of thoracic innervation of the hindlimb. Nonetheless, the frequency of motility in this group of embryos was similar to that seen in those embryos in which hindlimb innervation was determined histologically to be solely from the thoracic transplant (Fig.

2). Thus, although the spinal cords were not processed and examined for the success of the surgery, these data support the original findings in demonstrating a deterioration in the frequency of hindlimb motility in the later stages of development in embryonic hindlimbs innervated solely by thoracic motoneurons.

By contrast, the loss of motility at late stages did not occur when the transplant was composed of only two to four segments of thoracic cord located in the rostral lumbar region with the caudal segments of adjacent host lumbar cord remaining intact (Figure 3). In these cases, there were no significant differences in the number of limb movements (motility) between experimental and control embryos at any of the developmental stages examined. However, because we were mainly interested in hindlimbs wholly innervated by thoracic motoneurons, these experimental embryos were not observed beyond E13.

Electromyographic Recordings

Although the isolated cord-transplant/muscle preparation did not exhibit *spontaneous* activity when maintained in oxygenated Tyrode solution at room Temperature (20° to 22°C), the spinal cord and peripheral nerves remained electrically excitable at this temperature. Therefore, spinal cord regions innervating specific hindlimb muscles could still be distinguished in such preparations by stimulating individual spinal cord segments, one segment at a time, and recording EMG activity from specific hindlimb muscles (Landmesser and O'Donovan, 1984a).

The diameter of chick myotubes at these stages and the size of the electrode tip (~100 μm) make it likely that several muscle fibers were being recorded from simultaneously. Moreover, since action potentials do not develop in chick myotubes until between E16 and E19 (Kano, 1975), it is also likely that the muscle activity recorded at St 34–36 (E8–E10) was generated by junctional potentials resulting from a low level of activity in the motor terminals (Landmesser and O'Donovan, 1984). By recording EMG activity in this manner, it was possible to identify specific hindlimb muscles wholly innervated by thoracic transplants in eight separate experimental embryos.

All of the isolated cord/muscle preparations

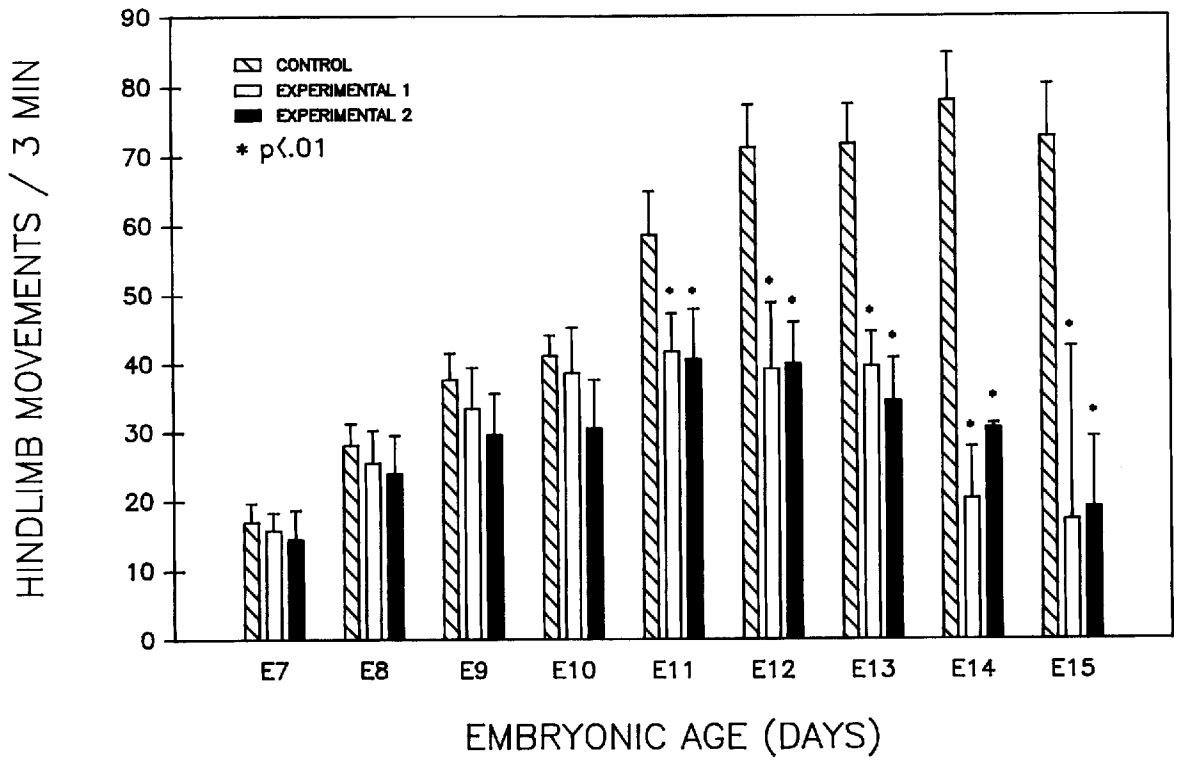


Figure 2 Total number (Mean ± S.D.) of hindlimb movements per three-minute observation period in control embryos compared to Experimental Group 1 (histology on spinal cord), and Experimental Group 2 (no histology on spinal cord) from E7 through E15 (see text for details).

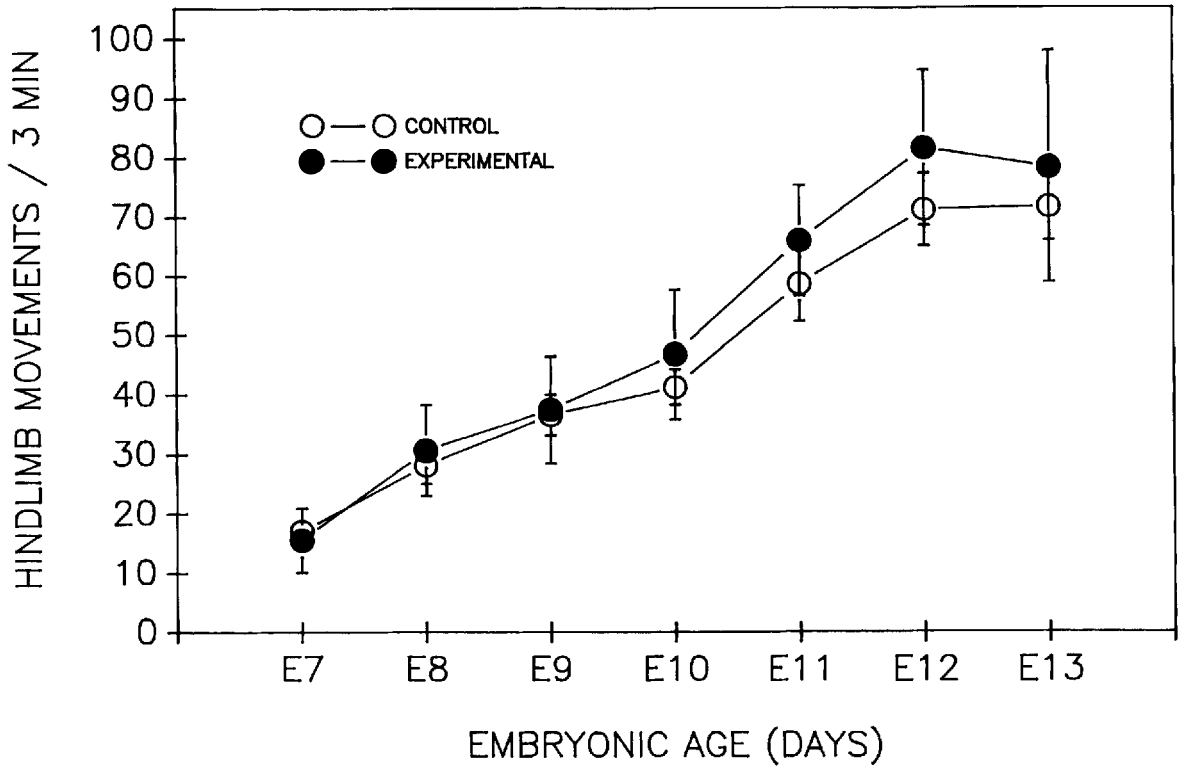


Figure 3 Movements (Mean ± S.D.) of hindlimbs in control and experimental embryos which had two to four segments of thoracic neural tube transplanted to the rostral lumbar region (L1–4), while the remaining (L5–8) host lumbar region remained intact. Motility recordings were only taken on E7 through E13.

also exhibited spontaneous neuromuscular activity after they had been warmed and maintained in oxygenated Tyrode's solution at 30°C for several hours. In each and every case, EMG burst sequences could also be initiated in hindlimb muscles receiving thoracic innervation by delivering a single electrical stimulus to the host cervical spinal cord, thereby further substantiating the anatomical evidence (O'Brien and Oppenheim, 1990) for the existence of continuity between the host cord and transplant. EMG recordings were obtained from a number of muscles, including both flexors (e.g., sartorius, anterior iliotibialis) and extensors (e.g., caudilioflexorius, adductor, gastrocnemius), in several of the eight experimental cases (Fig. 4).

The results from the EMG recordings indicate that the thoracic motoneurons were able to innervate the limb muscles and activate them with robust bursts with a repetitive rate similar to normal thoracically-innervated intercostal muscles. Thus, the basic pattern generating circuitry in thoracic transplants does not appear to have been altered by innervation of hindlimb muscles. Most muscles were activated with extensor-like bursts of somewhat variable duration. Muscles did not show distinguishable EMG burst characteristics with differing inhibitory periods as they do in control lumbar innervated muscles. As demonstrated in Figure 4(A), flexor and extensor muscles innervated by lumbar motoneurons (control) normally have an alternating pattern of activation. The onset of two cycles, indicated by arrowheads, shows that at the start of a cycle, the flexor (sartorius) muscle is inhibited, whereas the extensor (caudilioflexorius) is activated. In controls, each motoneuron pool has a characteristic and highly stereotyped activation pattern with specific burst durations and periods of inhibition (Landmesser and O'Donovan, 1984a). By contrast, the innervation pattern of thoracically innervated hindlimb musculature often differs markedly from the control activation pattern demonstrated in Figure 4(A). The sartorius muscle in Figure 4(B), which is innervated by thoracic motoneurons (top trace), does not exhibit its normal burst pattern with a typical long inhibitory period [compare with top trace of 4(A)]. Similarly, the adductor [4(B), bottom trace] did not exhibit a characteristic adductor burst pattern (which has a brief but consistent inhibitory period). However, both muscles were activated strongly during each cycle,

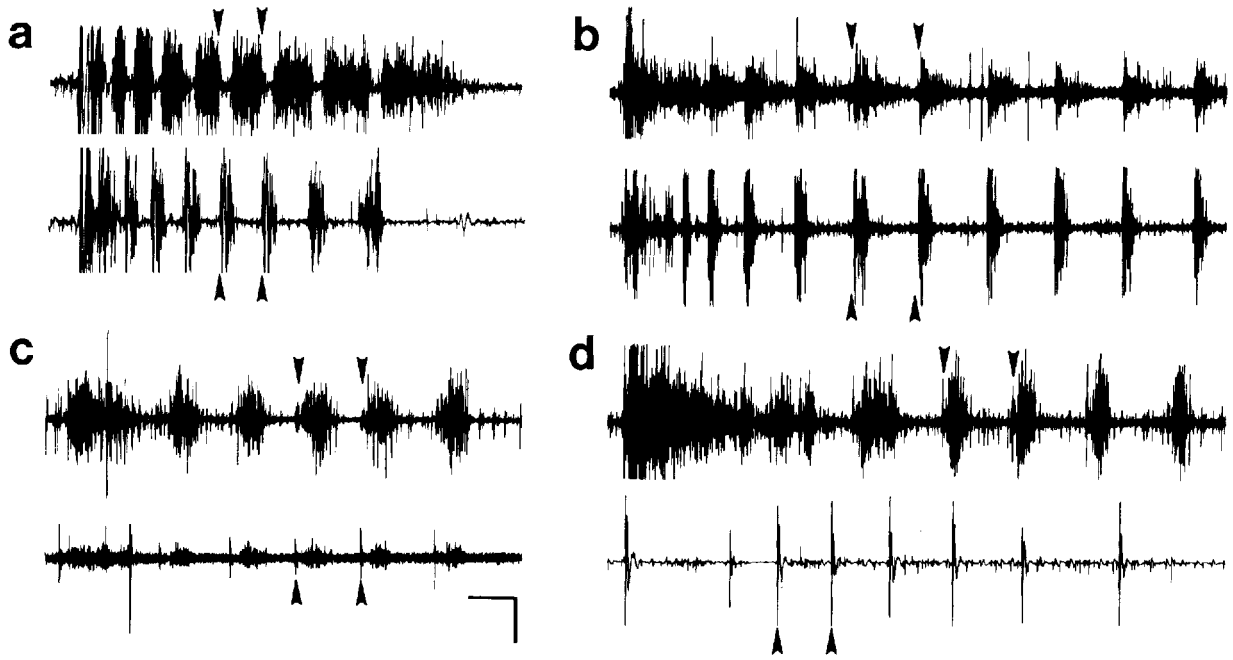


Figure 4 Activation patterns of thoracic motoneurons innervating limb muscles. EMG recordings from isolated cord preparations of control and experimental embryos (for more details, see Landmesser and O'Donovan, 1984a). (A) Control EMG pattern at the onset of two cycles is indicated by arrowheads. Top trace, sartorius, a flexor motoneuron pool; bottom trace, caudilioflexorius, an extensor motoneuron pool. (B) Top trace, sartorius, innervated by thoracic motoneurons. Bottom trace, adductor, innervated by thoracic motoneurons. (C) Top trace, ischioflexorius, innervated by thoracic motoneurons. Bottom trace, the intercostal muscle which is the normal target of thoracic motoneurons. (D) Top trace, thoracically innervated iliofibularis; bottom trace control iliofibularis EMG recording. The onset of several cycles is indicated in each case by arrowheads. In this case (D), the control recording is derived from a different embryo from that which the thoracically innervated data was obtained. Thus, the timing of individual cycles is not synchronized as was the case for (A)–(C) where recordings were made simultaneously from several muscles in the same limb. Calibration bar = 2 s. EMG amplitude varied from muscle to muscle, but was in general between 0.2 to 1 mV.

and although burst durations varied somewhat from muscle to muscle (Table 1), all thoracic-innervated muscles were activated with an extensor-like activation pattern. The differences which were observed in burst duration from muscle to muscle may reflect some heterogeneity in thoracic motoneurons, or may be due to differences in how effectively some muscle fibers are activated. For example, some sartorius fibers [Fig. 4(B), top trace] may have a lower threshold and so be activated by weaker synapses that are subthreshold in the adductor [Fig. 4(B), bottom trace] resulting in shorter bursts.

The thoracically innervated ischioflexorius [Fig. 4(C), top trace] also demonstrated an extensor activation pattern. In this case, a simultaneous

Table 1 Burst Duration (seconds)

Muscle	Control Mean ± S.E.	(*n*)	Experimental Mean ± S.E.	(*n*)
Sartorius	2.04 ± 0.60	(28)	1.52 ± 0.19	(21)
Adductor	0.80 ± 0.17	(8)	2.28 ± 0.58	(6)
Iliofibularis	0.36 ± 0.08	(12)	1.60 ± 0.40	(19)
P. iliotibialis	1.30 ± 0.18	(9)	1.88 ± 0.40	(10)
Intercostal	2.40 ± 0.72	(6)	—	—

recording was made from the intercostal muscles [Fig. 4(C), bottom trace], which are the normal target of these thoracic motoneurons. Both the intercostals and the thoracically innervated ischioflexorius are activated at each cycle and the bursts are of similar durations. Another example of a thoracically-innervated limb muscle, in this case the iliofibularis [Fig. 4(D), top trace], demonstrates that it too is activated with an extensor pattern not unlike the sartorius and ischioflexorius shown in Figure 4(A) and 4(C). This pattern differs markedly from the normal iliofibularis, which exhibits a brief synchronous activation at the onset of each cycle [Fig. 4(D), bottom trace].

Figure 5 shows the activity histograms for a variety of muscles in three experimental embryos. These data illustrate that while there is some variation, all muscles are activated like extensors. In addition, while there is variation in the burst duration of a given muscle from embryo to embryo (e.g., the sartorius), all of the thoracically-innervated muscles in an individual embryo tend to have similar activation patterns (e.g., in TC-6 there are long bursts with no inhibitory periods, TC-8 has short bursts with no inhibitory periods, and TC-7 has longer bursts all with some inhibitory periods). Intercostal recordings from different cases also differ from each other, but are similar to the recordings from thoracically-innervated limb muscles obtained from the same embryos. In both cases shown in Figure 5(A), the intercostal muscles were activated by motoneurons in host thoracic cord located just cranial to the thoracic transplant.

The activity histograms in Figure 5(B), emphasize that the thoracically innervated limb muscles are not activated like stage-matched homologous control limb muscles. For instance, the thoracically innervated sartorius does not exhibit its characteristic long inhibitory period at the start of a burst pattern, but rather it is activated at the start, much like an extensor muscle. Thus, muscle specific differences in motoneuron activation patterns characteristic for control embryos (Landmesser and O'Donovan, 1984a), were not observed in the thoracically innervated hindlimbs. In contrast, all muscles were activated in a manner very similar to that of the intercostal muscles from the same host embryo (i.e., extensor-like).

In those experimental cases where residual host lumbar cord remained caudal to the thoracic transplant, the lumbar motoneurons innervated only their appropriate muscles while the adjacent thoracic cord transplant innervated the remaining muscle not innervated by residual lumbar cord. For instance, as seen in Fig. 6, the most posterior limb muscles were innervated by residual lumbar cord, whereas all other limb muscles were innervated only by the thoracic cord transplant.

Curare Treatment

Experimental embryos with thoracic transplants were subjected to daily treatments of 1.5–2.0 mg of curare beginning on E6 and continuing throughout the major period of naturally occurring neuronal death (E6–E10). Movements were reduced by approximately 80 to 90% compared to untreated stage matched controls (data not shown). Results from previous investigations (Pittman and Oppenheim, 1979) indicated that following cessation of curare treatments on E9 or E10, motility levels continue to remain significantly reduced below control levels for five to six days. The last injection of curare in our thoracic transplant embryos was given on E10 and although the frequency of hindlimb movements was not systematically recorded after this time, there appeared to be only a minimal amount of limb activity present at the time of sacrifice on E14. There were approximately 38% more motoneurons in the thoracic transplant cord of embryos treated with curare throughout the cell death period compared to untreated thoracic transplant embryos (Fig. 7). Thus, the loss of motoneurons in thoracic transplant embryos that occurs between E10 and E14 (O'Brien and Oppenheim, 1990) was largely prevented by neuromuscular blockade.

Neuromuscular Innervation

As expected from the results of the motility and EMG recordings, hindlimb muscles of experimental embryos contained numerous nerve fibers derived solely from the thoracic transplant cord. The combination of acetylcholinesterase staining and silver impregnation of embryonic hindlimbs wholly innervated by thoracic transplant provided direct anatomical evidence for the presence of muscle nerves and motor end plates (Fig. 8). Furthermore, although more detailed analyses of the innervation pattern of individual muscles are in progress, preliminary results indicate that the distribution and branching pattern of thoracic nerves innervating hindlimb muscles is similar to control lumbar innervated muscles. By E14, many muscle nerves and motor end-plates in thoracically innervated hindlimb muscles appear to undergo regressive changes (Fig. 8). Studies are presently in progress to examine these changes in more detail at the ultrastructural level.

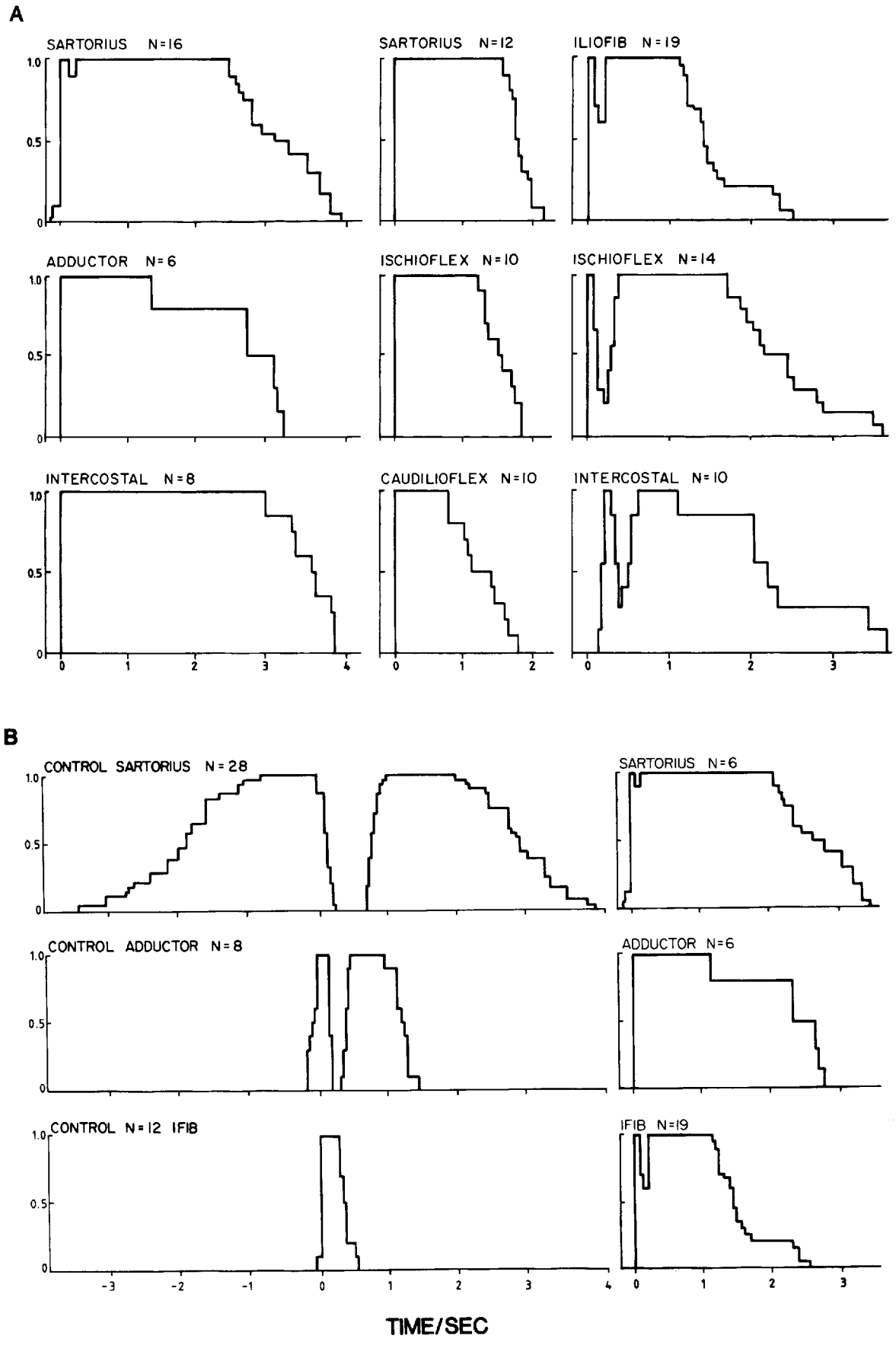
DISCUSSION

In the preceding companion paper (O'Brien and Oppenheim, 1990), we reported on the anatomical development of motoneurons and peripheral skeletal muscle following the translocation of the thoracic neural tube to the lumbar region at a stage (E2) prior to the normal establishment of nerve-muscle interactions. In the present paper we have been mainly concerned with neuromuscular function in these preparations, as indicated by spontaneous and reflex-evoked hindlimb movements (motility) and by EMG activity recorded from specific hind-limb muscles. Although previous studies of adult animals have demonstrated that within certain limits neurons can form functional contacts with foreign targets (Bennett, McLachlan, and Taylor, 1973; Guth and Frank, 1959; Landmesser, 1971, 1972; McLachlan, 1974; Purves, 1976), there have been only a few studies of the functional development of neurons following the formation of inappropriate connections during early stages of embryogenesis (Wenger, 1951; Straznicky, 1963, 1967, 1983; Szekely and Szentagothai, 1962; Morris, 1978; Butler, Cauwenbergs, and Cosmos, 1986; Narayanan and Hamburger, 1971; Landmesser and O'Donovan, 1984b; Vogel, 1987), and in most of these, the functional analysis was neither systematic nor detailed, or was only focused on restricted periods of development.

In the chick embryo, overt movements begin on E3.5 and are mediated by the nervous system (against being myogenic) from the time of their initiation (Hamburger, 1963, 1973; Oppenheim, 1982). The earliest movements are restricted to the head and trunk followed by the emergence of limb movements on E6 (Hamburger and Balaban, 1963; Landmesser and Morris, 1975). Both the early trunk movements and the limb movements are initiated by endogenously generated electrophysiological activity within the spinal cord (Provine, 1973). Within the limb, specific muscles are activated as flexors and extensors (or antagonists and synergists) at the earliest stages of movement initiation (Bekoff, 1976; Landmesser and O'Donovan, 1984a). Furthermore, this early patterned activation of muscles is initiated prior to the formation of functional reflex circuits (Bekoff, 1976), indicating that the earliest movements are generated by endogenous spinal pattern generating circuits.

In the present study, we have shown that hindlimbs receiving their sole innervation from thoracic transplants initiate motility at the same time (E6) as hindlimbs innervated by the normal lumbar spinal cord and that from E6 to E10 the frequency of hindlimb movement is comparable to that of control embryos. Furthermore, tactile induced cutaneous reflexes could be demonstrated in both control and experimental embryos beginning on E7.5 to E8 and the qualitative nature of the reflex movements appeared indistinguishable in the two groups. Accordingly, we conclude that both the motoneurons and sensory neurons in the thoracic transplants are able to initiate and sustain functional activation of hindlimb muscles at least up to E10. By contrast, after E10 both the spontaneous and reflex-induced movements of thoracically-innervated hindlimbs exhibited gradual regressive changes. The frequency of spontaneous movements progressively decreased and the qualitative aspects of both the spontaneous and reflex-induced movements became noticeably different from controls. By E16, spontaneous movements were virtually absent and reflex movements could only rarely be induced. Since head, trunk, and wing movements appeared normal throughout the period studied, we conclude that the progressive functional deficits are restricted to those parts of the embryo receiving innervation from the thoracic transplant and thus are not an indication of a general deterioration in the entire neuromuscular system. These results support and extend the earlier reports of Butler et al. (1986) and Morris (1978) in demonstrating that limb muscles innervated by nonlimb (thoracic) motoneurons can initiate and sustain normal motility during early stages of development but that eventually functional activity of the limbs is lost. However, it appears as if the decline in activity occurs a few days earlier in the thoracically innervated *wing* preparations of Butler et al. (1986) compared to our own thoracic-hindlimb preparations. Morris (1978) also observed a marked decrease in nerve-stimulation induced movements of supernumerary *hindlimbs* innervated by thoracic motoneurons between E12 and E16. Because this is the same period when we also observed a regression of *spontaneous* hindlimb motility, we conclude that these functional deficits reflect a real regression of the neuromuscular system and not merely a deficit in the generation of *spontaneously* generated movements.

Interestingly, the regression in hindlimb motility after E11 failed to occur if the leg musculature was dually innervated by both thoracic and lumbar motoneurons. That is, embryos in which only the rostral four segments of lumbar neural tube were replaced with thoracic cord displayed normal motility up to E13, which was the latest age examined. Because both EMG recordings and HRP back-fills (O'Brien and Oppenheim, 1990) from these preparations indicate that the rostral trans-


A
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ILIOFIB N=19
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CAUDILIOFLEX N=10
INTERCOSTAL N=10
1.0
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B
CONTROL SARTORIUS N=28
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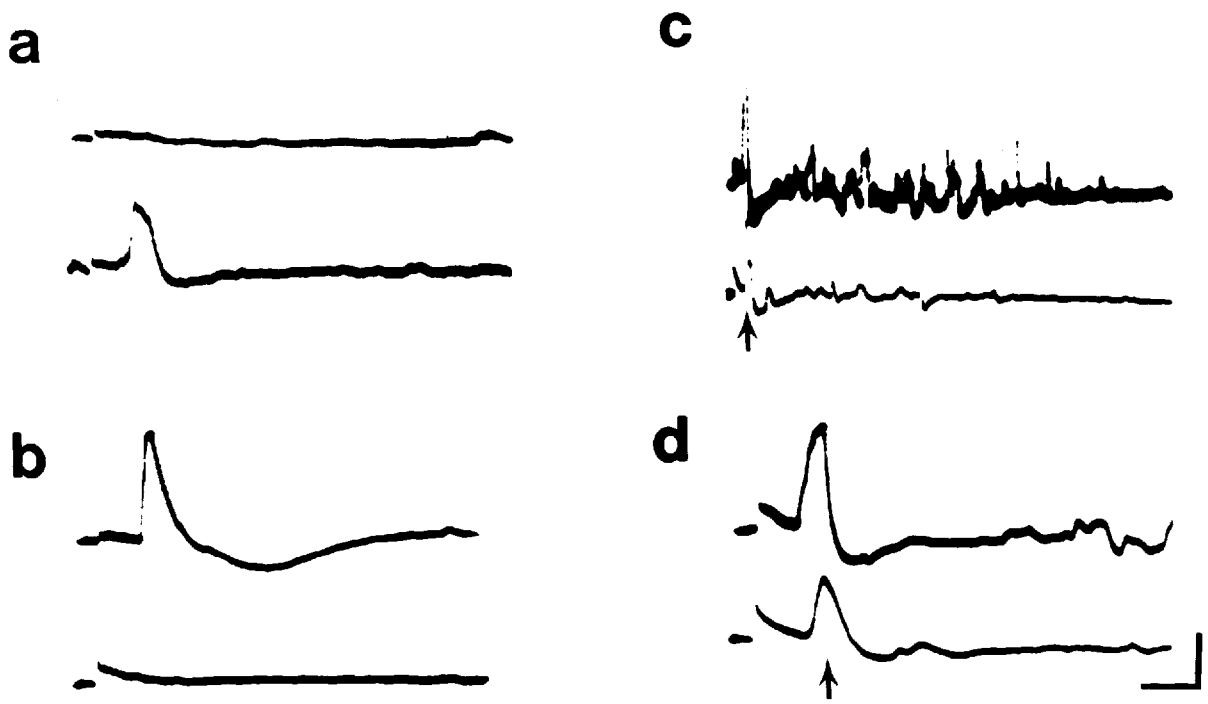

Figure 6 Oscilloscope traces of EMG recordings from the left limb of a St 36 1/2 (E10) embryo which had a thoracic transplant extending from LS1–LS5. In this embryo (TC-7), there was a gap of approximately two segments caudal to the transplant followed by residual lumbar cord consisting of several segments. The most posterior muscles (the caudilioflexorius, iliofibularis, and accessory on the right) were innervated by the residual lumbar cord. All the other muscles were innervated only by the thoracic cord transplant. (A) Direct electrical stimulation of the thoracic transplant produced in the ischioflexorius (bottom trace) a relatively synchronous compound EMG response, but failed to activate the caudilioflexorius (top trace). (B) The caudilioflexorius was instead activated by direct stimulation of the residual lumbar cord (top trace) which failed to activate the ischioflexorius (bottom trace). (C) Both muscles were activatable indirectly via descending input following electrical stimulation (a single suprathreshold pulse) to the cervical cord. Note that these traces are on a much slower time base to show that following a relatively synchronous response (arrow) this form of stimulation produces a prolonged burst. For ease of comparison a similar set of responses is shown in (D) at the same time base used in (A) and (B), the arrow again indicating the synchronous response. Calibration bar = 25 ms for (A), (B), and (D) and 250 ms for (C).

plant thoracic cord and the caudal lumbar cord innervate distinct muscles, it appears likely that the maintenance of normal motility levels in these cases reflects the sustained activity of only the lumbar innervated muscles. However, we cannot entirely eliminate the possibility that some individual muscles receive dual innervation (lumbar and thoracic) and thus that the lumbar contribution remains functional as the thoracic innervation regresses.

Figure 5 Activity histograms of thoracically innervated hindlimb muscles. (A) Thoracically innervated muscles from case TC-6 are in the column on the left, muscles from case TC-8 are in the center column, and muscles from case TC-7 are in the column on the right. (B) In each case thoracically innervated muscles were not activated in an appropriate manner. This can be seen by comparing the experimental activity histograms on the right with the controls on the left. The ordinate indicates the probability of a given muscle being active at various times preceding or following a synchronous initiating discharge (time 0). The number (n) of bursts contributing to each histogram is indicated. (For additional details, see Landmesser and O'Donovan, 1984a).

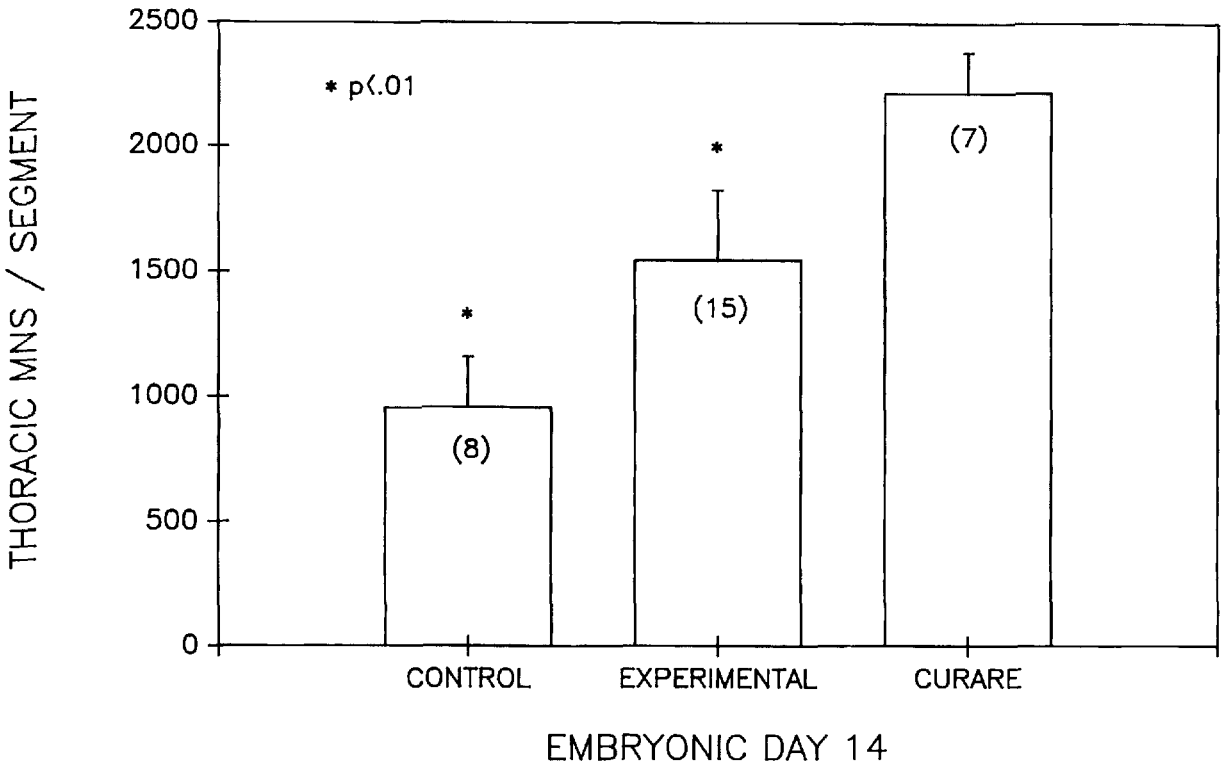

Figure 7 Number (Mean ± S.D.) of surviving thoracic motoneurons per segment on E14 in control embryos (Control), in untreated thoracic transplant embryos (Experimental), and in curare-treated thoracic transplant embryos (Curare). Control and Experimental groups were compared statistically to the Curare group. Sample sizes are indicated in the bars.

The observation that the function of thoracically innervated limb muscles regresses sometime after the mid-point of incubation in the chick raises the question of whether the normal development of limb muscles always requires innervation from appropriate motoneurons. In fact, the available evidence indicates that inappropriate *limb* motoneurons can sustain the anatomical and functional development of limb muscles. Hindlimb muscles innervated by the "wrong" lumbar motoneurons do not appear to undergo the same regressive changes observed in limb muscles innervated by thoracic motoneurons (Landmesser and O'Donovan, 1984b; Vogel, 1987). Furthermore, brachial motoneurons can innervate and sustain activity in hindlimb muscles throughout development and similarly lumbar motoneurons can substitute for brachial motoneurons in mediating wing innervation, development and function (Narayanan and Hamburger, 1971; Straznicky, 1963; Laing and Lamb, 1985). Consequently, the functional uncoupling observed in the present and related studies (Butler et al., 1986; Morris, 1978) appears to be restricted to the situation involving innervation of limb muscles by nonlimb (thoracic) innervating motoneurons and is not merely a result of foreign innervation per se. In what way might thoracic motoneurons be unable to substitute for limb motoneurons in sustaining the development and function of limb muscles? Although thoracic motoneurons can initially form functional contacts with limb muscles, at later stages a chemically-mediated mismatch between nerve and muscle involving cell–cell interactions and the loss of synaptic stability may result in an uncoupling that leads to the observed regression of both nerve and muscle. Alternatively, owing to differences in number or type, thoracic motoneurons may be unable to provide sufficient innervation or trophic support to sustain the development of the rapidly developing limb muscles during the last half of the incubation period (cf., O'Brien and Oppenheim, 1990). Another possibility that we do not favor, but cannot exclude, is that eventually limb muscles fail to provide appropriate trophic support for nonlimb motoneurons resulting in motoneuron regression which in turn leads to muscle degeneration. Against this is the fact that we have shown that even greater than normal numbers of thoracic motoneurons can be

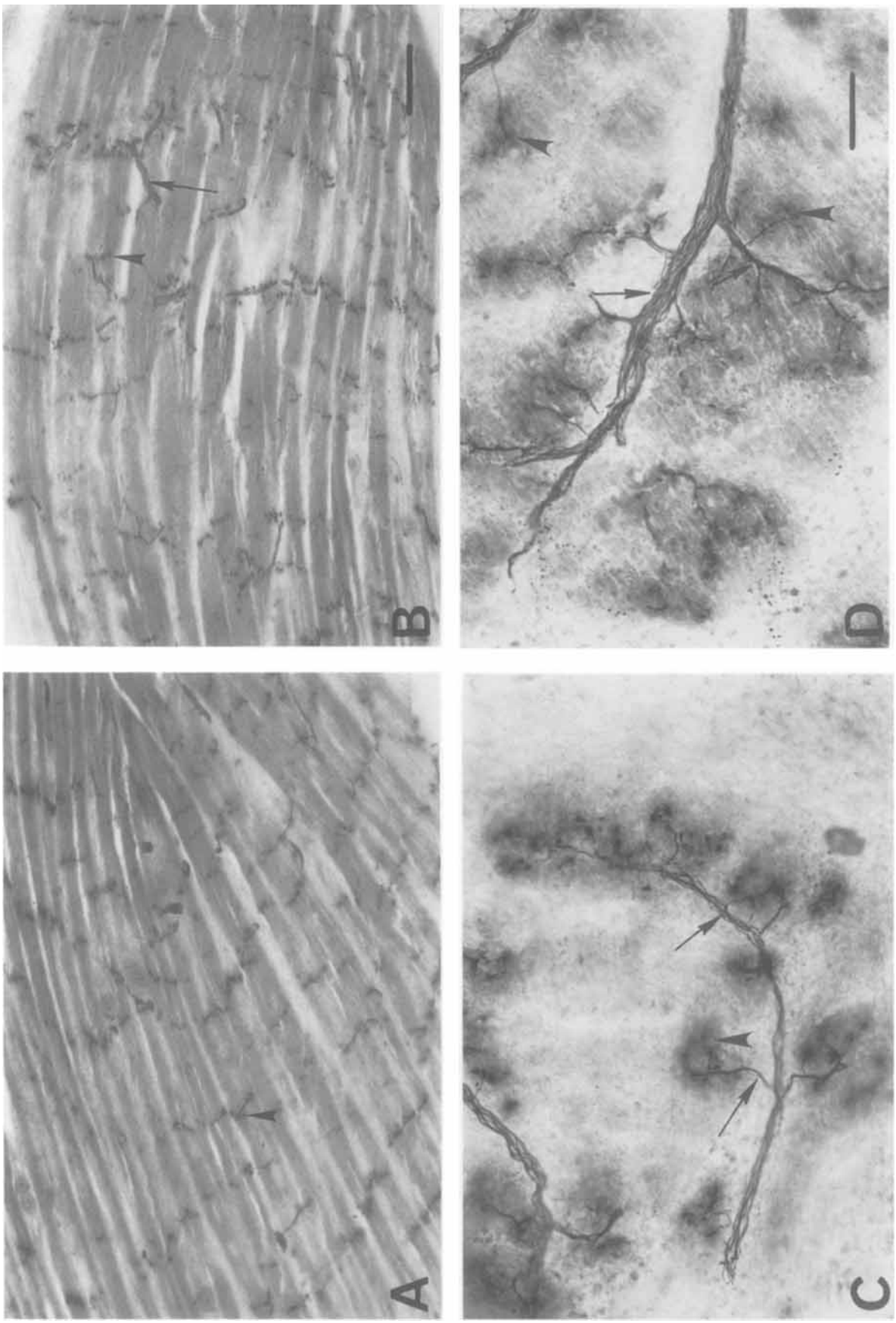

Figure 8 Staining of nerve fibers (arrows) and neuromuscular endplates (focal sites of AChE, arrowheads) in control (A,C) and thoracic transplant-innervated (B,D) peroneus muscle on E14. Note the appearance of regressive changes (apparent fragmentation) in the nerve fibers and endplates in (D). Scale bars = 50 μm (A,B) and 20 μm (C,D).

supported by limb muscles in thoracic transplants up to E14 (also see Oppenheim, Haverkamp, Prevette, McManaman, and Appel, 1988).

The results of the EMG analysis of thoracic transplants show that the activation pattern of thoracically innervated limb muscles is more similar to the activation of intercostal muscles than to that of limb muscles. Consequently, it is possible that the normal activation pattern of a muscle is somehow responsible for sustaining the development of normal nerve-muscle interactions. Interestingly, embryos with thoracic transplants in which this abnormal neuromuscular activity was blocked with curare did not exhibit the loss of motoneurons that normally occurs in these preparations after E10. Although activity blockade is known to prevent motoneuron loss in normal embryos (Pittman & Oppenheim, 1979), if an *activity-independent* mismatch between thoracic motoneurons and limb muscle is responsible for the regression of the transplant neuromuscular system (see above), one might have expected the regression to proceed even in the face of activity blockade. The fact that it did not suggests that the regression may, in fact, be initiated by the abnormal activation pattern of limb muscle by thoracic motoneurons. However, because EMG activity is abnormal in thoracically innervated hindlimbs already several days prior to the onset of regressive changes, in order for this explanation to be valid one must assume that the activation patterns only become effective in regulating nerve–muscle interactions at some critical period after E10. Furthermore, because *in ovo* curare treatment of chick embryos is also reported to alter the activation pattern of limb-innervating motoneurons, presumably by blocking acetylcholine receptors within the cord (Szente and Landmesser, 1986), activity blockade may act to reduce motoneuron loss in thoracic transplants by either (or both) neuromuscular blockade or by the suppression of putative cholinergic afferent input to motoneurons. In either case, the present results suggest that it may be the abnormal thoracic-like activation pattern of hindlimb muscles that provides a signal that initiates the cascade of regressive events in nerve and muscle in thoracically innervated hindlimbs. Considerable more work is needed, however, to prove that this is the case.

The EMG activation pattern of individual muscles reflects the organization of central pattern generators involving specific neuronal connections that are responsible for motor activity. Consequently, the data obtained from EMG recordings of thoracically innervated hindlimb muscles provide a more valid index of the development of spinal cord properties than the admittedly rather crude measures of hindlimb motility used here. According to the myotypic specification theory of Weiss (1941, 1955), one might expect that the pattern generating circuitry of thoracic spinal cord innervating limb muscles would develop properties resembling those of normal lumbar cord. However, the present results, as well as other related studies (Butler et al., 1986; Straznicky, 1963; Narayanan and Hamburger, 1971; Vogel, 1987; Landmesser and O'Donovan, 1984b), indicate that this is not the case. The EMG pattern of specific hindlimb muscles innervated by thoracic motoneurons closely resembled the patterns normally observed in thoracically-innervated intercostal muscles. From this we conclude that segmentally specific pattern generators in the chick spinal cord are not determined by target-derived cues. Rather, they develop according to intrinsic properties specific to their site of origin in the neural tube which are determined at least as early as the time of neural tube closure on E2. One major goal for future studies is to examine the mechanisms responsible for this early segment specific determination. A related goal is to determine how the subsequent differentiation of these properties influences the ability (or lack thereof) of specific motoneurons to sustain the development of neuromuscular interactions. Thoracic motoneurons that normally never innervate limb muscles nonetheless have the potential to innervate and functionally activate limb muscles during early stages of development. Eventually, however, the neuromuscular system in these preparations gradually breaks down and the limb muscles and motoneurons regress, degenerate, and die. Understanding the mechanisms involved in this regressive phenomenon can be expected to shed light on the broader issue of the development and maintenance of specific neuronal interactions in the central and peripheral nervous system.

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