J. Embryol. exp. Morph. 95, 147–168 (1986)
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Fate of brachial muscles of the chick embryo innervated by inappropriate nerves: structural, functional and histochemical analyses

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SUMMARY

The extent of interaction between brachial muscles and foreign (thoracic) nerves of the chick embryo was determined during an extended period of development *in ovo* from the perspectives of innervation pattern, function (motility analyses), muscle growth (quantitative analyses of muscle volume) and fibre-type expression (myosin–ATPase profiles). Results indicated that according to all parameters analysed, *initially* a compatible union existed between the foreign nerves and their muscle targets. During *subsequent* stages of development, deterioration of the once compatible relationship emerged, until eventually denervation of muscles, i.e. an actual loss of intramuscular nerve branches, was observed. The process of denervation, which proceeded at a differential rate among individual muscles, however was restricted to brachial muscles derived from the premuscle masses of the wing bud. In contrast, brachial muscles of myotomal origin were spared the fate of wing-bud-derived muscles and maintained a successful union with the foreign nerves.

INTRODUCTION

During normal embryogenesis of the chick, specificity of nerves for appropriate muscles has been documented (for review, Landmesser, 1980); however, whether this specificity is achieved by active and, or, passive guidance of axons is still under investigation by the use of various experimental manipulations designed to determine whether inappropriate innervation can be forced to occur (for review, Stirling & Summerbell, 1985). Whereas previous investigations have focused primarily on the projection pathways of developing peripheral motoneurones, our interest is in the consequences of this observed specificity as related to the sequential expression of phenotypes characteristic of individual muscle targets during embryogenesis.

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Key words: inappropriate innervation, muscle–nerve interaction, chick embryo, myosin–ATPase profiles.

Previous reports from our laboratory have indicated that the initial differentiation of distinct muscle fibre types (myosin–ATPase profiles) occurs during the first week of development *in ovo*, temporally coincident with the entry of motor nerves (Butler & Cosmos, 1981*a*,*b*; Butler, Cosmos & Brierley, 1982*a*). Examination of aneurogenic brachial muscles, however, reveals that this event proceeds on schedule in the absence of peripheral neuronal influences (Butler *et al.* 1982*a*; Phillips & Bennett, 1984). In contrast, we have demonstrated that neuronal influences, specifically impulse-mediated activity, do determine the growth rate and, indeed, the survival of developing brachial muscles (Butler *et al.* 1982*a*; Bloom, Butler, Brierley & Cosmos, 1985). Thus, specific phenotypes of differentiating embryonic muscles are independent of neuronal input while others are either totally or partially nerve dependent.

For the present experiments, we sought to further an understanding of nerve–muscle specificity by determining the degree of interaction between foreign (inappropriate) nerves and muscles during an extended period of development *in ovo* from the perspectives of innervation patterns, function, muscle growth and fibre-type expression. Using these parameters, we questioned to what extent can inappropriate innervation substitute for *in situ* appropriate innervation.

Using an experimental model previously examined by Straznicky (1963, 1967), foreign innervation of brachial muscles was accomplished by replacing the brachial segment of the neural tube of a host embryo with the thoracic segment of a donor embryo. By performing the surgery between 48–52 h *in ovo*, prior to the outgrowth of peripheral motoneurones (Castro, 1963; Butler & Cosmos, 1981*a*), we ensured that the brachial musculature received innervation exclusively from the foreign nerves; this procedure constituted an embryonic *cross-innervation*.

Straznicky (1967) observed that the early formation of neuromuscular primordia was normal in experimental embryos, but that the wing muscles degenerated eventually 'despite the presence of motor fibres'. Although his work entailed a detailed microscopic analysis of the structural appearance of wing muscles during the initial 'normal' phase and the subsequent degenerative phase, evidence was lacking concerning functional interaction between foreign nerves and their targets, fibre-type expression or the possibility that all brachial muscles did not respond similarly. Furthermore, in contrast to his observations, our analyses of this model indicated that the subsequent deterioration of muscle fibres was associated with an actual withdrawal of intramuscular nerve branches. Prior to this event, however, thoracic nerves and brachial muscles interacted successfully according to the parameters analysed in our experiments. Furthermore, the denervation phenomenon was restricted to brachial muscles originally derived from myogenic stem cells of primary somites (Christ, Jacob & Jacob, 1977), which migrated to form the premuscle masses of the wing bud. Indeed, brachial muscles of myotomal origin maintained a compatible union with thoracic nerves throughout embryogenesis.

Preliminary reports of these experiments have been presented (Butler, Cosmos & Brierley, 1982*b*; Cauwenbergs, Cosmos & Butler, 1983).

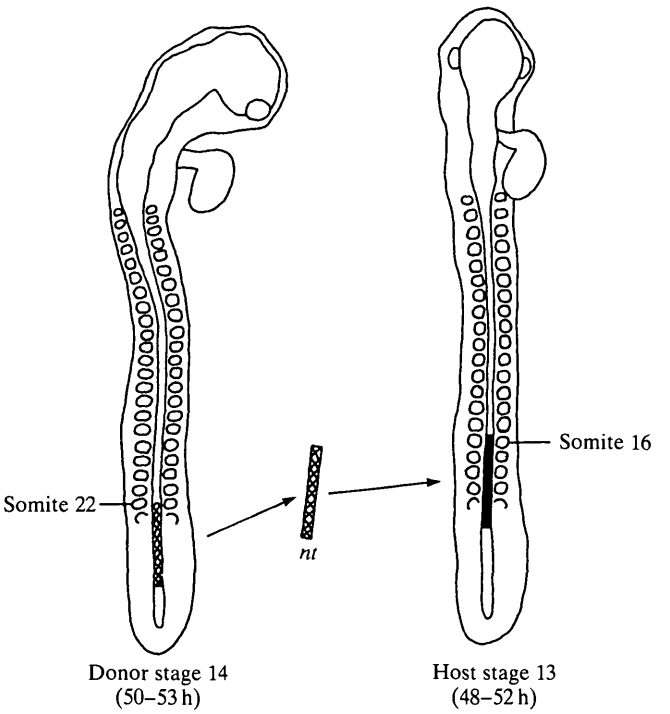


Fig. 1. Diagram to illustrate heterotopic innervation, i.e. replacement of host brachial neural tube segment by thoracic neural tube (*nt*) segment of donor embryo.

MATERIALS AND METHODS

Surgical procedures

Experimental embryos

To prepare embryos for innervation of brachial muscles by nerves derived from a thoracic neural tube segment (heterotopic innervation), the following protocol was employed. Fertile White Leghorn eggs were incubated at 37·4 °C and 56 % humidity for approximately two days. Embryos were staged according to the morphological criteria of Hamburger & Hamilton (1951). For host embryos, the brachial neural tube segment, i.e. the segment opposite somites 16–21 inclusive, was removed from embryos at stage 13 (48–52 h) utilizing the surgical technique previously employed for the preparation of aneurogenic brachial muscles (Butler *et al.* 1982*a*). The neural tube segment to be deleted was determined by counting the number of somites already formed and by estimating the region opposite presumptive somites caudally. Care was taken to leave the notochord, somitic mesoderm and endoderm intact. The extirpated brachial segment of the neural tube was then replaced by the thoracic neural tube segment, i.e. the segment opposite somites 22–26 inclusive, from a donor embryo at stage 14 (50–53 h) *in ovo* (Fig. 1). To facilitate maintenance of the proper craniocaudal and dorsoventral orientation of the graft, the caudal end of the graft was cut at a slight angle when excised from the donor embryo. Following surgery, the shell window was either sealed with cellophane tape or fitted with a glass coverslip which was secured to the shell with dental wax. The latter method provided visualization of the embryo for subsequent motility analyses. The embryo was returned to the incubator to develop to the desired postoperative stage.

A total of 360 embryos was subjected to this experimental manipulation and a postoperative mortality rate of 74 % was observed. Ninety experimental thoracic-to-brachial (Thor–Br) embryos survived for analysis between stage 29 (day 6E) through stage 45 (day 19E). In addition, two experimental (Thor–Br) embryos hatched and were examined *ex ovo*.

Control embryos

Both unoperated embryos (n = 123) and embryos that survived the homotopic innervation procedure (n = 41) served as the primary control groups. Homotopic innervation was achieved by replacing the extirpated brachial segment of the host neural tube by the brachial neural tube from a donor embryo at the same stage (Br–Br control). In addition, a third control group was employed specifically for motility analyses, namely, the prebrachial removal (PBR) series. To determine if a possible interruption of appropriate supraspinal or propriospinal (intersegmental) input might affect motility, a small portion of the prebrachial neural tube segment, specifically the area opposite somites 11 to 13 inclusive, was removed without replacement. Thirty-three embryos subjected to the PBR procedure survived.

Motility analyses

To monitor functional interaction between brachial muscles and nerves derived from the heterotopically transplanted thoracic neural tube, daily motility observations were performed on experimental (Thor–Br) embryos and compared to similar analyses of control (Br–Br, PBR and unoperated) embryos from day 6 *in ovo* (day 6E), the period when discrete movements of the wing are first observed (Hamburger & Balaban, 1963), until day 16E. Prior to each motility observation, the embryos were allowed to equilibrate for 10–15 min in a temperature- and humidity-controlled chamber. Individual embryos, illuminated with a fibre-optic light source, were observed through a low-power magnifying glass and the total number of all spontaneous right wing movements (total frequency) that occurred in a 10 min period (M/10) was recorded. Any wing movements in relation to the body, including movements at the shoulder, elbow and carpus were regarded as a single count. In addition, if the wing was moving in one direction and then abruptly changed direction, this combination was counted as two separate movements. All passive movements of the wing that resulted from either contractions of the amnion or motility of other body parts were excluded.

Data obtained from daily motility analyses of experimental (Thor–Br, n = 36) and control (Br–Br, n = 33; PBR, n = 33; unoperated, n = 74) embryos were stored in a computer and were analysed by the Mann-Whitney U-test for statistical significance at the 95 % confidence level.

Histochemical, histological and quantitative analyses

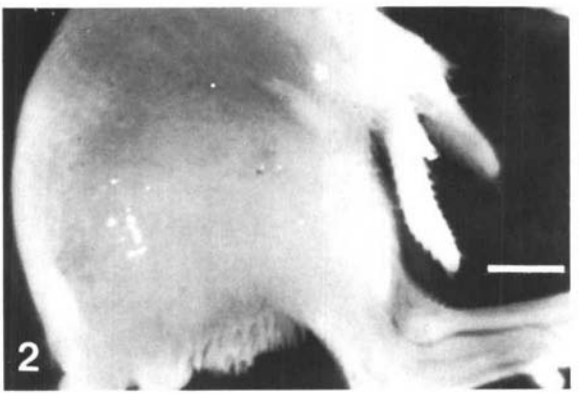
Between stage 29 (day 6E) through stage 45 (day 19E), experimental (Thor–Br) and control (Br–Br, unoperated) embryos were removed from the egg, decapitated, eviscerated and either frozen for cryostat sectioning (histochemical analyses) or fixed in glutaraldehyde (2 % in 0·1 M-phosphate buffer) for histological examination. The exact developmental stage of each embryo was assessed according to the morphological criteria of Hamburger & Hamilton (1951). Serial longitudinal sections of frozen embryos were analysed by the myosin–ATPase reaction, following alkali (pH 10·0) or acid (pH 4·35) preincubation, and by a silver–cholinesterase reaction to monitor differentiation of muscle fibre types and innervation respectively, as described (Butler & Cosmos, 1981*a*,*b*). Fixed embryos were cut in cross section and stained with 0·25 % aqueous thionin. In addition, the Oil Red O histochemical reaction (Cosmos, 1970) was employed to demonstrate the replacement of muscle fibres by lipid as observed in older Thor–Br embryos.

To assess the ability of experimental (Thor–Br) muscles to respond to neuronal factors associated with proper growth of embryonic muscles, quantitative volumetric analyses of selected experimental and control brachial muscles were compared by use of a computerized Image Analysis System (Zeiss), as described (Butler *et al.* 1982*a*).

RESULTS

Gross examination of experimental (Thor–Br) and control (Br–Br) surgically manipulated embryos between stage 29 (day 6E) to stage 37 (day 11E) indicated

that these embryos were morphologically similar to unoperated embryos and that their chronological age correlated with the morphological criteria of Hamburger & Hamilton (1951). Thus, by these criteria, the surgery *per se* did not interfere with normal development. Beyond stage 37 (day 11E), however, a marked abnormality of the wings was noted in the Thor–Br embryos exclusively. Normally, embryonic wings are held close to the body in a flexed position; in the Thor–Br embryos, the wings were in a downward position (Fig. 2). This abnormal wing position characterized all Thor–Br embryos from stage 38 (day 12E) onward and, in fact, was maintained after hatching (Fig. 3A,B).



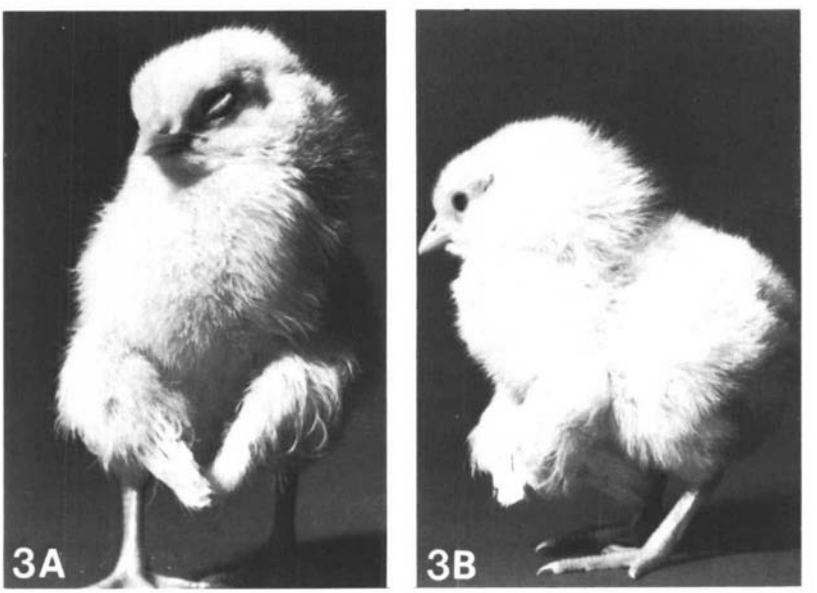


Fig. 2. Photograph of a stage 38 (day 12E) experimental embryo to illustrate the abnormal wing position characteristic of Thor–Br embryos from this stage onward. Bar, 0·8 mm.

Fig. 3. A,B. Photographs of a hatched (1 week *ex ovo*) Thor–Br chick to illustrate the abnormal wing position (cf. Fig. 2).

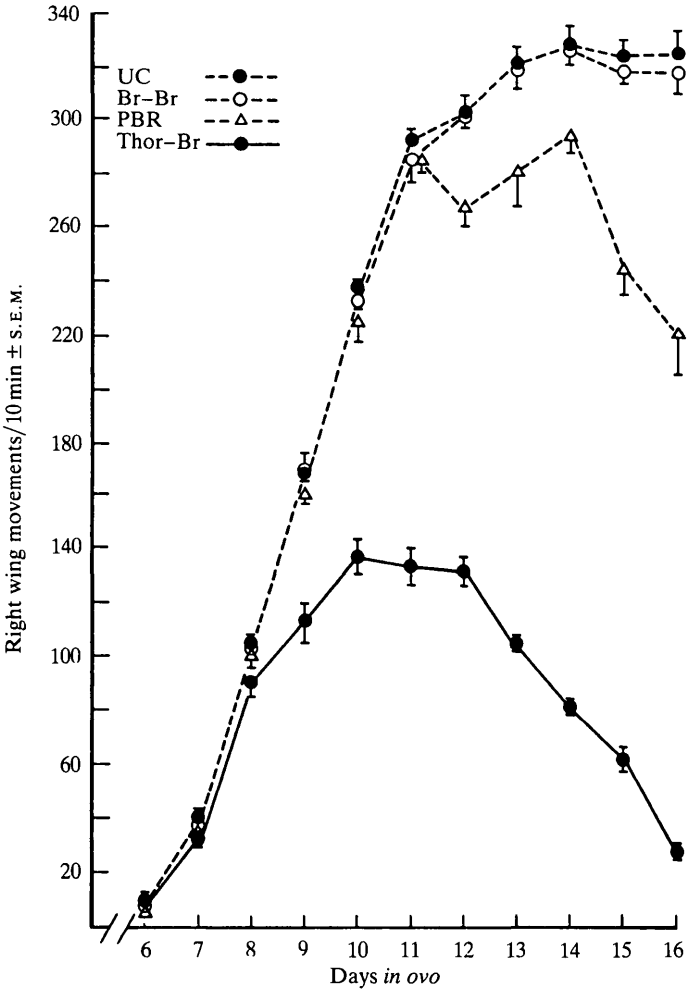


Fig. 4. Mean frequency ± S.E.M. of wing movements characteristic of experimental (Thor–Br) and control (UC, Br–Br, PBR) embryos from day 6E through day 16E.

Motility analyses

Frequency of wing movement

To monitor functional interaction between thoracic nerves derived from the transplanted thoracic neural tube and brachial muscles, the frequency of wing movements per 10 min observation period (M/10) was compared between experimental (Thor–Br) and control (Br–Br, PBR and unoperated) embryos from day 6E to day 16E (Fig. 4). All values represent the mean frequency ± S.E.M. Characteristically, the frequency of wing movements of unoperated embryos increased from a level of 9 M/10 at day 6E to a peak value of 328 M/10 at day 14E; the latter was maintained to day 16E. During a similar developmental period, the activity of Br–Br embryos was statistically equivalent to that of unoperated embryos, reaching a peak level of 326 M/10 at day 14E. Although between day 6E

to day 11E the motility pattern of PBR control embryos also paralleled that of unoperated embryos, the PBR embryos demonstrated a lower frequency from day 12E to day 16E, which was only statistically significant at days 15E and 16E.

Analyses of the motility pattern of Thor–Br embryos demonstrated a marked deviation from control embryos (Fig. 4). Although initially, i.e. between day 6E to day 8E, the frequency of movements was similar for all embryos, significant differences from control values were observed in Thor–Br embryos from day 9E onward. Peak values of only 135 M/10 were recorded between day 10E through day 12E. Beyond day 12E, the frequency of wing movements declined precipitously until by day 16E only 30 M/10 was recorded. Furthermore, it should be emphasized that although visually Thor–Br wings demonstrated a limited range of movement throughout the developmental period analysed, movement was observed at all joints, namely, the shoulder, elbow and carpus.

Innervation patterns of experimental versus *control embryos*

Microscopic examination of both the Thor–Br and Br–Br embryos revealed that in all cases the neural tube graft was viable (Fig. 5A) and that bilaterally a brachial plexus had formed from both the transplanted thoracic and brachial segments, as noted by others (Wenger, 1951; Straznicky, 1963, 1967). Serial reconstruction of individual embryos demonstrated that for the vast majority of cases, the graft was anatomically continuous with both the cranial and thoracic neural tube segments of the host embryo. The transplanted thoracic graft could be easily identified since it retained characteristics of an *in situ* thoracic neural tube segment, namely, a well-defined medial motor column (MMC), a small, intermittent lateral motor column (LMC) and the column of Terni. In contrast, the brachial region of Br–Br embryos, as for unoperated embryos, demonstrated a large, continuous LMC and the absence of the column of Terni. In all cases, the brachial plexus derived from the graft always entered the wing at the appropriate site, i.e. immediately anterior to the first (false) rib (Fig. 5B).

Although intramuscular nerve branches and axons derived from the brachial plexus of the transplanted thoracic neural tube were noted in *all* brachial muscles of the Thor–Br embryos examined from stage 30 (day 6·5E) through stage 35 (day 8–9E), e.g. Fig. 6A–D, during subsequent stages of development the intramuscular branches began to disappear. The impression was one of a 'withdrawal' of nerves from their target, a phenomenon occurring at different stages of development in specific brachial muscles. Analyses of Thor–Br embryos with the silver–cholinesterase reaction demonstrated that the anterior latissimus dorsi (ALD) and scapulohumeralis anterior (SHA) muscles were the first to show loss of innervation, i.e. beyond stage 35 (day 8–9E), axons were never observed within these muscles. Eventually, by stage 42 (day 16E) the ALD muscles of Thor–Br embryos were not maintained but, instead, were replaced by lipid. In contrast, at stage 40 (day 14E) axons and distinct endplates were observed in the ALD (Fig. 7) and SHA muscles of Br–Br embryos. Between stage 37 (day 11E) and stage 38 (day 12E), the phenomenon of denervation had extended to other

brachial muscles of Thor–Br embryos, including the posterior latissimus dorsi (PLD) and deltoideus muscles. Ultimately, the PLD muscle was not present in experimental embryos from stage 40 (day 14E) onward whereas fascicles of lipid were observed in the position of the deltoideus muscle. Fig. 8A illustrates the formation of a well-formed deltoideus muscle at stage 34 (day 8E) compared to the subsequent replacement of this muscle by adipocytes (Fig. 8B,C) by stage 40 (day 14E) associated with the loss of innervation.

Histochemical analyses of experimental muscles indicated that the extent of anatomical connectivity between developing muscle fibres and axons was limited to primitive nerve–muscle contacts *only* that did not mature to form discrete endplates or neuromuscular junctions. These observations applied to all wing-derived experimental muscles examined, including certain muscles such as the scapulohumeralis posterior (SHP), triceps brachii and biceps brachii muscles

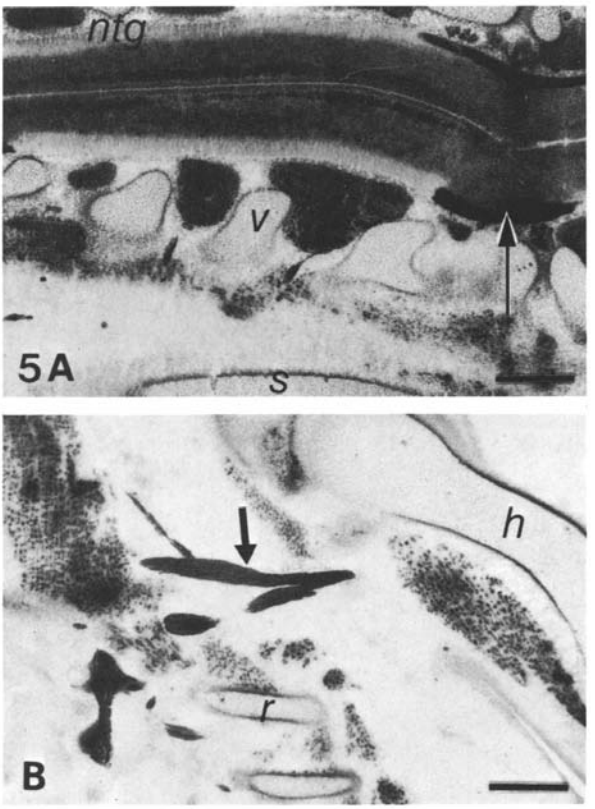


Fig. 5. (A) Photomicrograph from a stage 34 (day 8E) Thor–Br embryo to illustrate viability of the thoracic neural tube graft (*ntg*) which spans four vertebrae at this level. Note the continuity between the graft and the host *in situ* thoracic neural tube (arrow) and the dorsal root ganglia derived from the graft. *s*, scapula; *v*, vertebrae. Silver–cholinesterase. Bar, 0·5 mm. (B) Photomicrograph from a more ventral section of embryo shown in (A) to illustrate branches of the brachial plexus (arrow) derived from the thoracic neural tube graft entering the wing anterior to the first rib (*r*). *h*, humerus. Silver–cholinesterase. Bar, 0·5 mm.

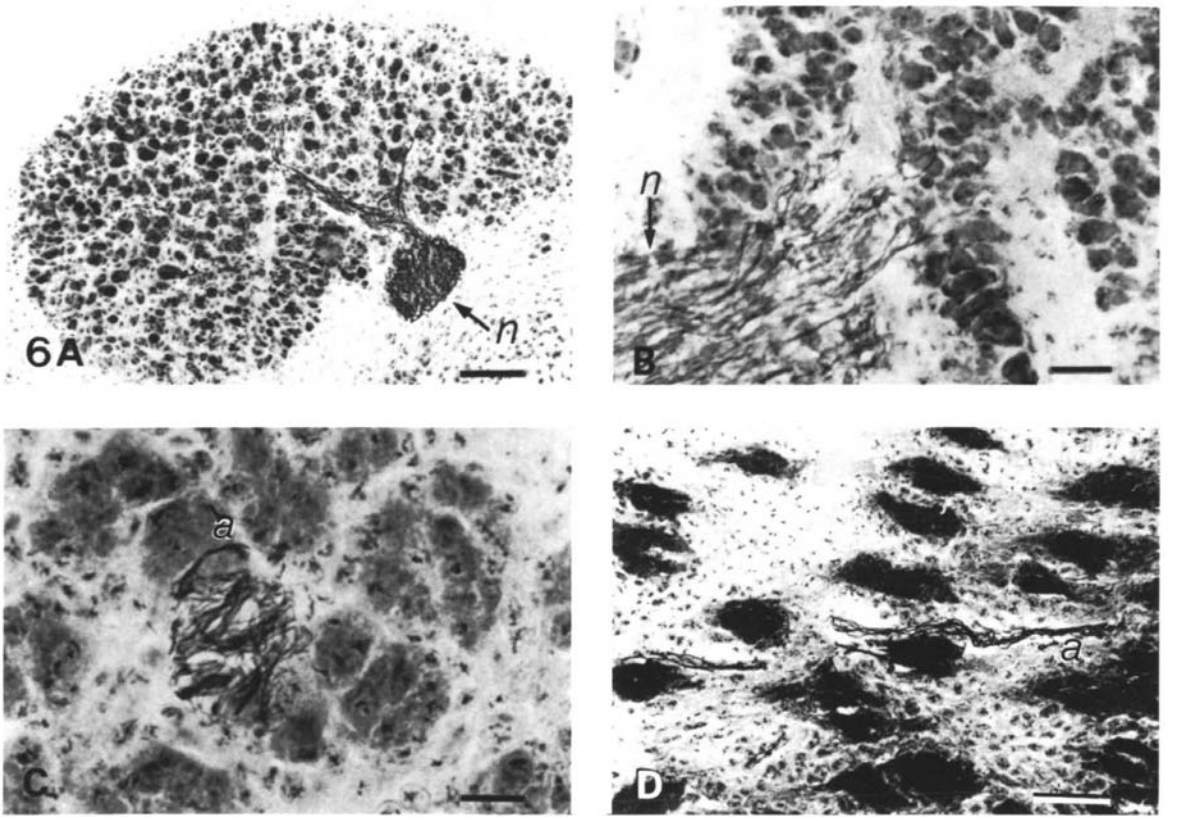


Fig. 6. A–D. Photomicrographs from stage 34 (day 8E) (A) and stage 35 (day 8–9E) (B–D) Thor–Br embryos to illustrate innervation of individual muscles. Intramuscular nerve branches (*n*) are noted entering the biceps brachii (A) and triceps brachii (B) muscles respectively and individual axons (*a*) are observed in the ALD (C) and triceps brachii (D) muscles during this period of development. Silver–cholinesterase. Bar in A, 0·06 mm; bar in B, 0·025 mm; bar in C, 0·02 mm; bar in D, 0·05 mm.

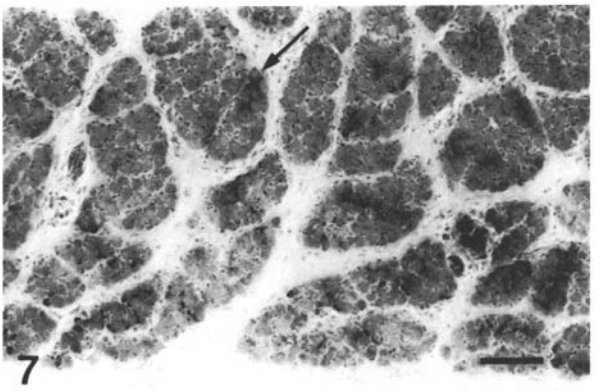


Fig. 7. Photomicrograph from a stage 40 (day 14E) Br–Br embryo to illustrate the formation of distinct endplates (arrow) in the ALD muscle. Silver–cholinesterase. Bar, 0·06 mm.

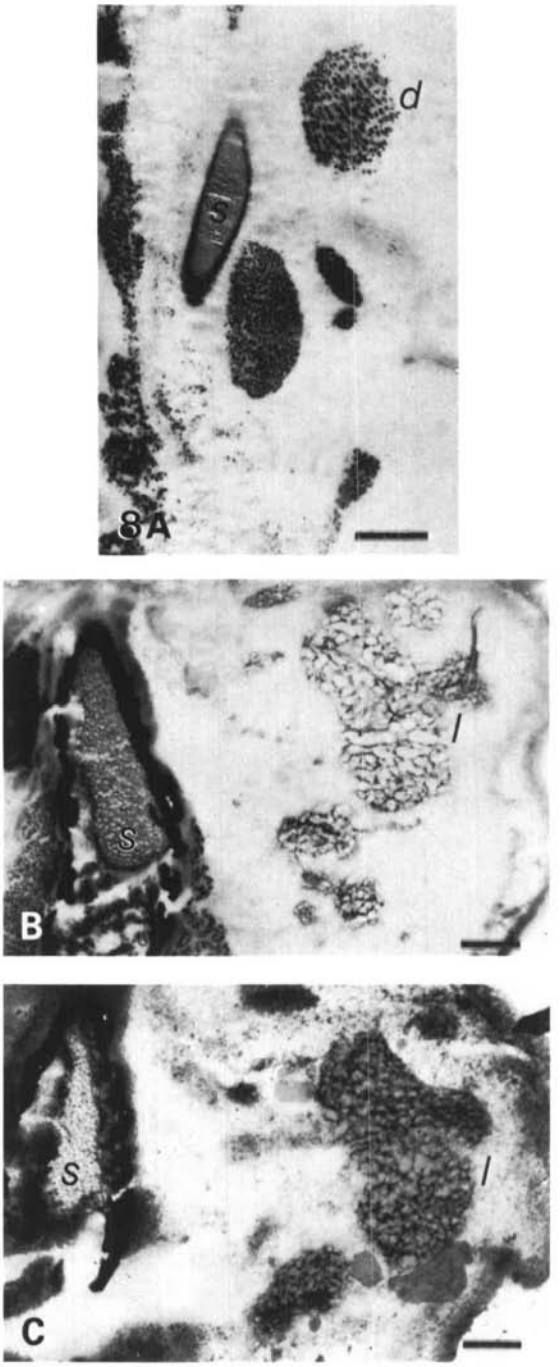


Fig. 8. A–C. Photomicrographs from stage 34 (day 8E) (A) and stage 40 (day 14E) (B,C) Thor–Br embryos. The deltoideus (*d*) muscle identified at stage 34 by a positive alkali-stable myosin–ATPase reaction (A) has been completely replaced by fascicles of lipid (*l*) at stage 40. Compare the negative alkali-stable reaction at the site of the deltoideus muscle (B) with the positive reaction observed with the Oil Red O lipid reaction (C). *s*, scapula. Bar in A, 0·5 mm; bars in B,C, 0·25 mm.

which demonstrated the persistence of intramuscular axons even beyond stage 40 (day 14E). In contrast to the fate of wing-derived appendicular muscles, experimental axial muscles of myotomal origin, such as the intervertebrals, did grow, survive and exhibit distinct endplates similar to unoperated and control embryos.

Innervation patterns of all control Br–Br embryos were indistinguishable from those of unoperated embryos, indicating that the surgical procedure *per se* performed at day 2E did not interfere with the formation of appropriate neuromuscular junctions.

Growth of experimental versus *control muscles*

To examine the influence of thoracic nerves on the growth and survival of brachial muscles, parameters known to be neurally dependent (Butler *et al.* 1982*a*; Bloom *et al.* 1985), the growth of individual Thor–Br and control (Br–Br and unoperated muscles) was compared quantitatively. Furthermore, to verify that any observed growth of experimental Thor–Br muscles was actually due to interaction with foreign thoracic nerves and not merely an expression of the endogenous, albeit limited, growth observed with aneural muscles, previously published values of aneural muscles (Bloom *et al.* 1985) were compared to those calculated for the present experiments. The ALD and PLD muscles were selected for volumetric analyses since the former represents a muscle which microscopically showed the earliest loss of innervation in Thor–Br embryos whereas innervation persisted longer in the PLD muscle.

Growth curves characteristic of ALD muscles from aneural, Thor–Br, Br–Br, and unoperated embryos are presented in Fig. 9. Each point represents the mean total muscle volume ($\mu m^3 \times 10^6$) ± S.E.M. from a minimum of four to a maximum of 10 muscles analysed per age group. Continuous increments in muscle volume typify ALD muscles of both Br–Br and unoperated control (UC) embryos throughout the developmental period analysed. Indeed, the values for the Br–Br ALD muscle are not only significantly greater than either the Thor–Br or the aneural ALD muscle values, but they approach those characteristic of the unoperated ALD muscle. In contrast, the growth curve for the ALD muscle of the Thor–Br embryos reveals two distinct phases: (1) an initial phase between stage 31 (day 7E) and stage 35 (day 8–9E) when experimental values more closely approximate those of innervated Br–Br and unoperated ALD muscles; and, (2) a phase subsequent to stage 35 when the experimental ALD values more closely approximate those of aneural ALD muscles (Fig. 9, arrow). The time span for the initial phase coincides temporally with the period when microscopically Thor–Br ALD muscles demonstrated intramuscular nerve contacts and when experimental embryos exhibited a normal frequency of wing movements. The positive effect of nerves derived from the transplanted thoracic neural tube segment on the growth of the Thor–Br ALD muscle during this initial period is emphasized when experimental values are contrasted to those obtained previously for aneural muscles. At stage 31 (day 7E) the aneural value is 72 % less than the unoperated one whereas the Thor–Br ALD muscle is only 14 % less; by stage 36

(day 10E) when the second phase is apparent, values for the aneural and Thor–Br ALD muscles are similar, i.e. 87% and 80% lower than unoperated values, respectively. The biphasic growth curve of the Thor–Br ALD muscle is a striking demonstration of the dependency of muscle growth on neuronal input since coincident with the sudden decline in muscle volume, i.e. the change in the growth pattern of the Thor–Br ALD muscle, there were both an abrupt loss of intramuscular nerve branches and a decrease in wing motility.

Similar to the ALD muscle, the PLD muscle of control (Br–Br; unoperated) embryos exhibited continual increments in growth throughout the developmental period analysed (Fig. 10). Volumetric analyses of the PLD muscles of Thor–Br embryos very strikingly illustrate the ability of brachial muscles to respond *initially* to foreign thoracic nerves and, likewise, the ability of thoracic nerves to assume

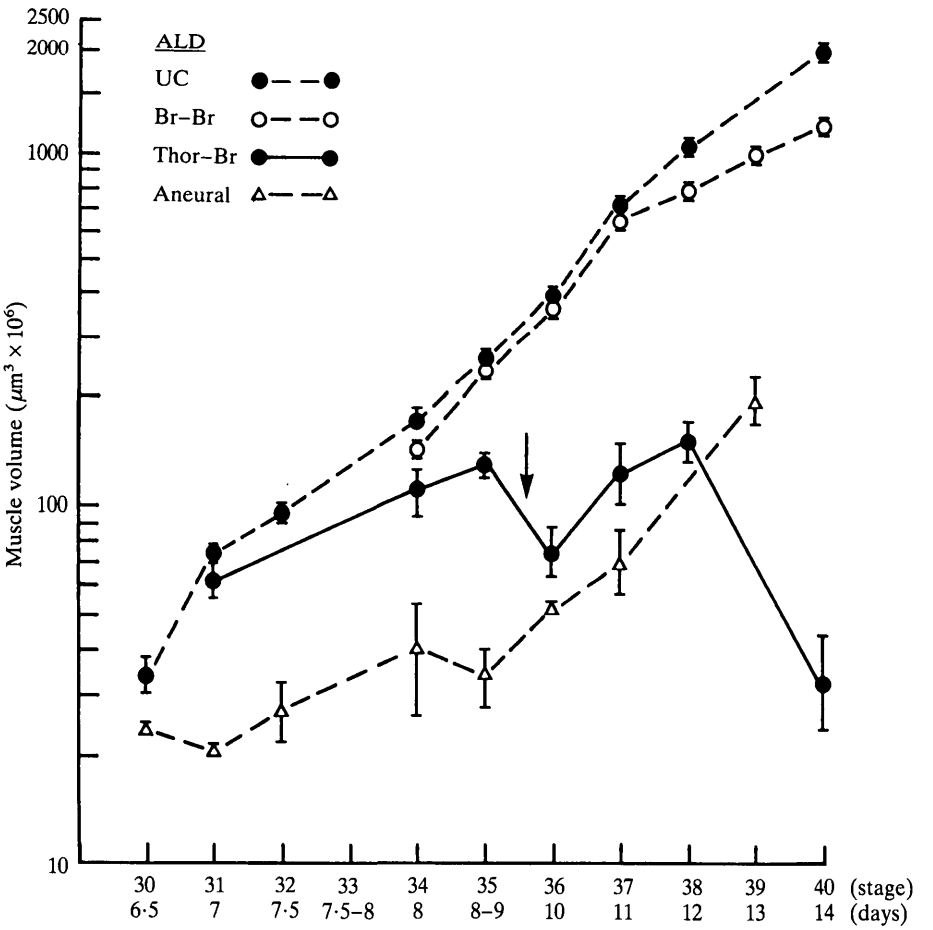


Fig. 9. Comparative growth of experimental (Thor–Br) and control (UC, Br–Br, aneural) ALD muscles from stage 30 (day 6·5E) through stage 40 (day 14E). Values represent the mean muscle volume ($\mu m^3 \times 10^6$) ± S.E.M. Arrow indicates shift in growth curve of Thor–Br ALD muscle towards aneural values between stage 35 (day 8–9E) and stage 36 (day 10E). [Data for aneural muscles derived from Bloom *et al.* 1985.]

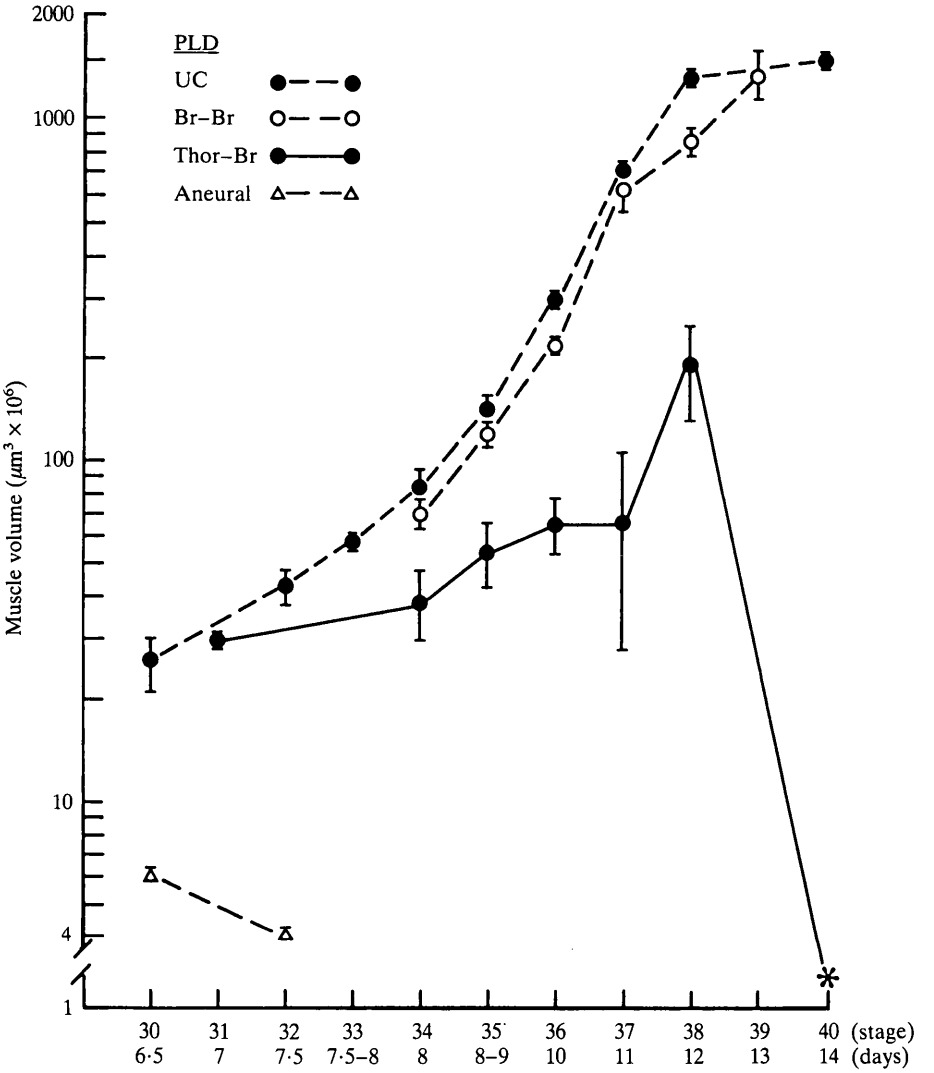


Fig. 10. Comparative growth of experimental (Thor–Br) and control (UC, Br–Br, aneural) PLD muscles from stage 30 (day 6·5E) through stage 40 (day 14E). Values represent the mean muscle volume ($\mu m^3 \times 10^6$) ± S.E.M. Although the aneural PLD muscle did not survive beyond stage 32 (day 7·5E), the Thor–Br PLD muscle survived through stage 38 (day 12E). By stage 40 (day 14E), the Thor–Br PLD muscle was absent (*) in all preparations. [Data for aneural PLD muscles derived from Bloom *et al.* 1985.]

the role of appropriate brachial nerves concerning both the initial growth and survival of individual brachial muscles. Fig. 10 demonstrates that the PLD muscle of Thor–Br embryos was not only rescued by foreign thoracic nerves but that it exhibited nearly a seven-fold increase in volume between stage 31 (day 7E) to stage 38 (day 12E). The comparatively large S.E.M. values for the Thor–Br PLD

muscle at stage 37 (day 11E) and stage 38 (day 12E) reflect the fact that the loss of innervation in different preparations did not always occur at the same time. Thus, the higher values at stage 37 (day 11E), for example, were associated with PLD muscles in which intramuscular axons could still be detected. By stage 40 (day 14E), however, the PLD muscle was absent (Fig. 10, *) in all Thor–Br embryos examined (n = 7). These observations emphasize the neuronal dependency of the PLD muscle, i.e. without innervation the PLD muscle shows very limited survival but when given foreign thoracic innervation, it responds dramatically. However, when the foreign nerve is withdrawn, again the PLD muscle cannot survive.

In contrast to the impaired growth and limited survival of the eventually denervated ALD and PLD muscles, experimental muscles of myotomal origin remained innervated, survived and grew normally. At stage 40 (day 14E), a Thor–Br intervertebral muscle located in the brachial region exhibited a total muscle volume of $337{\cdot}95\,\mu m^3 \times 10^6 \pm 31$ (n = 4) whereas the value for the same Br–Br intervertebral muscle was $328{\cdot}05\,\mu m^3 \times 10^6 \pm 16$ (n = 4).

Fibre-type composition

In the adult chicken, muscles of myotomal origin, such as the intervertebral and intercostal muscles, exhibit a myosin–ATPase profile distinct from those characteristic of muscles ultimately derived from premuscle masses of the wing bud. Fig. 11A,B illustrates the mosaic distribution of fibre-types within a brachial intervertebral muscle. In contrast, although the profiles of various brachial muscles of wing origin do differ among individual muscles (see Butler *et al.* 1982*a*), the patterns are more homogenous, i.e. composed predominantly of one type of fibre. Typically, all ALD fibres express dual alkali and acid myosin–ATPase stability whereas the PLD muscle exhibits alkali stability only (Fig. 11C,D). For the present experiments we examined the fibre-type composition of brachial muscles innervated by foreign thoracic nerves. Although the initial differentiation of muscle fibre types is *independent* of neuronal influences (Butler *et al.* 1982*a*; Phillips & Bennett, 1984), we queried the ability of the foreign nerve to alter previously established profiles, a phenomenon observed experimentally in post-hatched muscles (Cosmos, Butler, Allard & Mazliah, 1979). Histochemical analyses, however, indicated that foreign thoracic nerves did not influence muscle fibre-type expression. Instead, a comparison of the alkali- and acid-stable myosin–ATPase reactivity of all brachial muscles, experimental and control, demonstrated profiles that were appropriate for each individual muscle (see table 1, Butler *et al.* 1982*a*) throughout the developmental period analysed. Fig. 12A,B illustrates the distinctive myosin–ATPase profiles characteristic of the ALD, SHP and triceps brachii muscles of a Thor–Br embryo at stage 35 (day 8–9E), a period when these muscles are still innervated by foreign thoracic nerves. Similar to unoperated muscles, the Thor–Br ALD muscle exhibits dual acid and alkali stability; the Thor–Br SHP muscle exhibits predominantly alkali stability at this period; and the scapular portion of the triceps brachii muscle expresses alkali activity only.

DISCUSSION

Foreign innervation of brachial muscles was accomplished by transplanting the thoracic segment of the neural tube from a donor embryo to the extirpated brachial region of a host embryo at stage 13 (48–52 h). Since the surgical procedure was performed prior to the outgrowth of peripheral motoneurones (Castro, 1963; Butler & Cosmos, 1981*a*), the subsequent innervation of brachial muscles by nerves derived from the transplanted thoracic neural tube constituted an embryonic cross-innervation experiment. Unlike cross-reinnervation experiments performed *ex ovo* that involve denervation of a muscle prior to reinnervation by a foreign nerve, in our model the embryonic experimental muscles had never experienced their own appropriate innervation. Although *ex ovo* foreign nerves can substitute adequately for appropriate nerves and, indeed, alter the phenotype of their new partner (Cosmos *et al.* 1979), during embryogenesis muscle–nerve specificity is expressed and inappropriate innervation occurs only after extreme

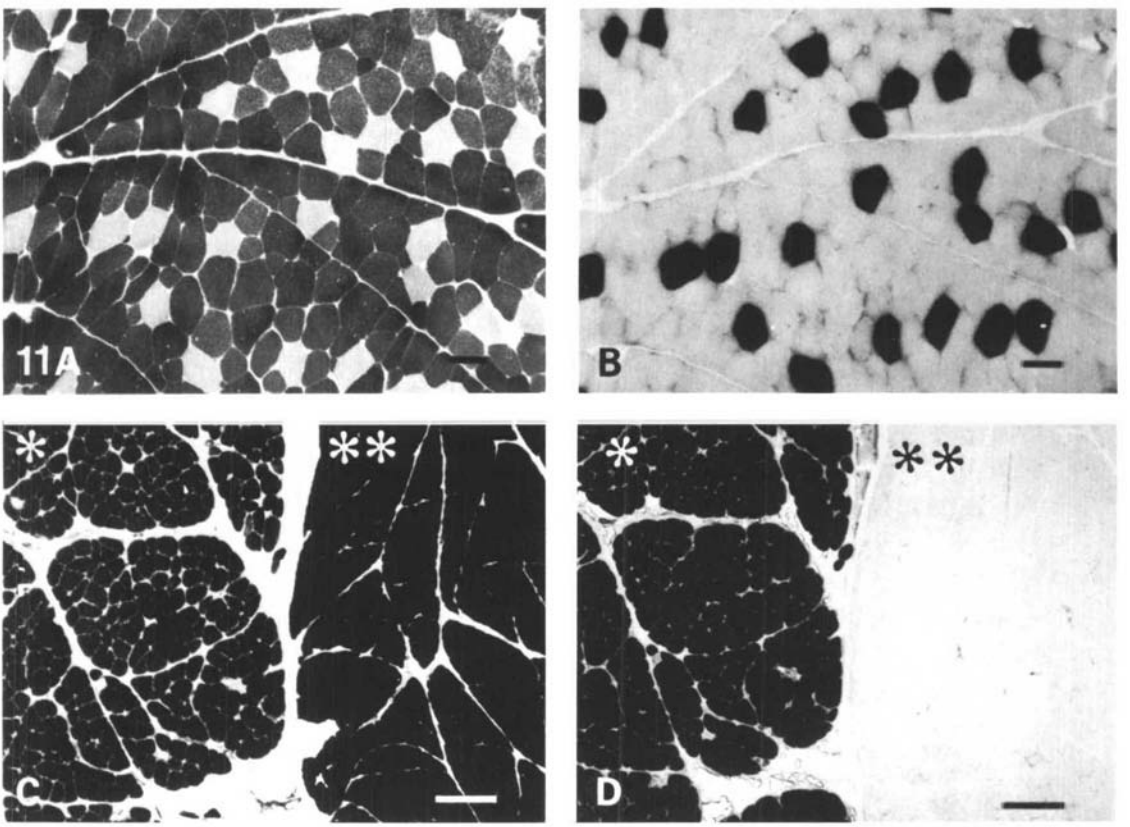


Fig. 11. A–D. Photomicrographs of serial sections from adult chicken muscles to demonstrate diverse myosin–ATPase profiles characteristic of brachial muscles. The intervertebral muscle following alkali (A) and acid (B) pre-incubation demonstrates a mosaic pattern. In contrast, individual fibres of the ALD muscle (*) exhibit dual alkali (C) and acid (D) stability whereas fibres of the PLD muscle (**) express alkali stability only. Myosin–ATPase. Bars in A,B, 0·06 mm; bars in C,D, 0·25 mm.

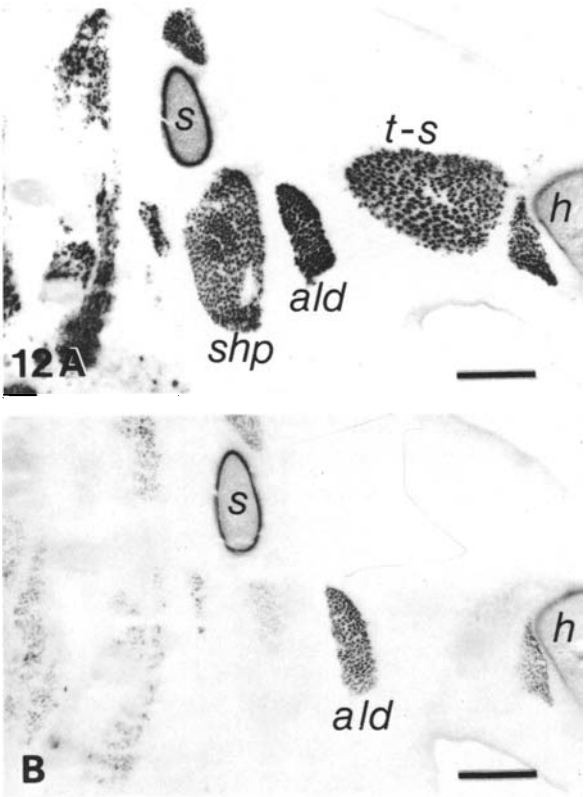


Fig. 12. A,B. Photomicrographs of serial sections from a stage 35 (day 8–9E) Thor–Br embryo to illustrate myosin–ATPase profiles. Similar to the adult ALD muscle shown in Fig. 11C,D, the Thor–Br ALD muscle exhibits dual alkali (A) and acid (B) stability whereas the SHP muscle is predominantly alkali-stable only and the scapular portion of the triceps brachii (t-s) muscle expresses alkali stability only. Similar profiles were expressed by unoperated and Br–Br embryos at this stage. *s*, scapula; *h*, humerus. Myosin–ATPase. Bars in A,B, 0·5 mm.

experimental manipulations (Lance-Jones & Landmesser, 1981; Stirling & Summerbell, 1985). Since in the latter experiments the fate of the target has been neglected, for the present experiments we queried the degree to which foreign thoracic embryonic nerves could interact with brachial muscles and whether or not they could modify characteristics of their new target.

Viability of the transplant

Microscopic examination of experimental Thor–Br embryos revealed that the transplanted neural tube was viable and that it developed morphological characteristics similar to those of the *in situ* thoracic cord, as first described by Wenger (1951). Since prior to 4·5 days *in ovo* the common motor column is morphologically uniform throughout the length of the spinal cord (Levi-Montalcini, 1950), the ability of the transplanted segment to express its own individuality in the brachial region attests to the autonomous nature of the development of specific

regions within the spinal cord. In contrast to the absence of plasticity within the cord, nerves derived from the foreign thoracic neural tube atypically formed a brachial plexus bilaterally and nerves comprising these plexi penetrated brachial muscles*, as noted also by others (Wenger, 1951; Straznicky, 1963, 1967). Thus, the gross pattern of peripheral nerve outgrowth is determined exclusively by environmental cues derived from the periphery and is unrelated to the segmental origin of the spinal nerves (see also Stirling & Summerbell, 1977; Straznicky, 1983).

Extent of interaction

Previous analyses of aneurogenic muscles have demonstrated that during initial stages of embryogenesis individual muscles form and express appropriate fibre-type profiles in the absence of nerves (Butler *et al.* 1982*a*; Phillips & Bennett, 1984); however, aneurogenic muscles remain non-functional, fail to grow properly and, eventually, fail to survive. Thus, for the present experiments, parameters associated with muscle function, growth and survival were utilized to determine the extent of interaction between foreign thoracic nerves and their targets.

Embryonic limb motility is a well-documented method to monitor functional interaction between developing limb muscles and their motor innervation. Starting on day 6E, chick embryos exhibit spontaneous, rhythmic limb movements which are non-reflexogenic and originate within the spinal cord (for review, Oppenheim, 1982). Previous investigators have indicated that avian limbs innervated by thoracic nerves are either immotile or exhibit limited motility. The studies of Straznicky (1963, 1967) who also replaced the brachial neural tube segment with a thoracic segment and Székely & Szentágothai (1962) who transplanted limb buds to the thoracic region, however, were limited to a detailed analysis of the postembryonic period only. More recently, Morris (1978), employing the model of Székely and Szentágothai, reported that direct stimulation of thoracic nerves elicited contractions of specific limb muscles during a limited period of embryogenesis. For the present experiments, daily wing motility analyses were used to monitor sequentially the degree of functional interaction between developing wing muscles and foreign thoracic nerves characteristic of an individual embryo from day 6E to day 16E inclusive. Similar to control embryos, wing motility of Thor–Br embryos was initiated at day 6E and the frequency of wing movements equalled control levels until day 9E. Thereafter, frequencies deviated from control values and actually declined precipitously during the last week of embryogenesis; some wing movement, however, was still observed at day 16E, indicating that specific wing muscles were still functionally contacted by the foreign nerves. Contrary to the suggestion of Mark (1980), a possible interruption of proper propriospinal (intersegmental) input within the spinal cord and, or,

* For the remainder of the discussion, the term brachial muscles will refer specifically to muscles derived from premuscle masses of the wing bud; brachial muscles of myotomal origin will be identified as such.

impaired supraspinal input do not appear to be factors associated with the eventually impaired motility of Thor–Br embryos. Motility analyses of PBR embryos indicated that their frequency of wing movements is only significantly reduced at days 15E and 16E (see also Provine & Rogers, 1977), a period well beyond the time when function of the Thor–Br embryos began to falter.

Comparative analyses of the growth of individual Thor–Br, control, and aneural muscles corroborated the motility data and verified that *initially* the foreign thoracic nerves supplied appropriate neuronal factors necessary for the growth and survival of embryonic muscles. Prior to the withdrawal of innervation, experimental ALD and PLD muscles exhibited increments in growth which exceeded aneural levels and which approached those characteristic of unoperated and Br–Br muscles. Coincident with the onset of denervation (silver–cholinesterase analyses), a sudden reduction in muscle volume towards aneurogenic values was observed. Eventually, the Thor–Br ALD muscle was replaced by lipid and the PLD muscle was absent. Similar fates are experienced by aneural ALD and PLD muscles (Butler *et al.* 1982*a*).

These data verified that, during a specific period of embryogenesis, the thoracic neural tube could substitute with impunity for the extirpated brachial neural tube. Despite the synaptic relationship established during initial stages of embryogenesis, however, the previously compatible union between brachial muscles and thoracic nerves uncoupled, as evidenced by eventual declines in motility and muscle volume coincident with the observed withdrawal of intramuscular nerve branches. We should emphasize that the uncoupling phenomenon did not occur simultaneously in all experimental brachial muscles. Although several muscles experienced a complete withdrawal of thoracic nerves between stage 36 (day 10E) and 38 (day 12E), a limited number of axons still persisted to stage 42 (day 16E) in other muscles, such as the triceps brachii, biceps brachii, and SHP. Instead, the denervation process temporally coincided with the period when normally permanent neuromuscular junctions are established in individual brachial muscles (Atsumi, 1977; Adachi, 1983; present observations).

Fibre-type expression

Even though the initial differentiation of appropriate myosin–ATPase profiles during embryogenesis is neurally independent (Butler *et al.* 1982*a*; Phillips & Bennett, 1984), postembryonic cross-reinnervation experiments demonstrate the ability of nerves to alter established muscle phenotypes (Cosmos *et al.* 1979). For the present experiments, we anticipated that once the initial differentiation of muscle fibre types had occurred, the foreign nerves could conceivably express the ability to alter this expression. At all developmental stages analysed, however, the myosin–ATPase profile of individual Thor–Br muscles remained unaltered compared to their control counterparts. It should be emphasized that unaltered profiles were observed at the time when the foreign thoracic nerves still formed functional and structural connections with the experimental brachial muscles, a period well beyond the stage when the incipient differentiation of fibre types

normally occurs (Butler & Cosmos, 1981*a*). Moreover, the completely mosaic distribution of fibre types unique to certain muscles normally innervated by thoracic nerves *in situ* (e.g. intervertebral, intercostal muscles) was never noted in the non-myotomal-derived brachial muscles coupled to foreign thoracic nerves. Since in the Thor–Br experiments thoracic nerves derived from the transplanted thoracic neural tube had never experienced contact with appropriate thoracic muscles, one might conclude that these naïve nerves lacked the memory of a previous partner and, thus, could not function to alter a new target. Alternatively, the formation of mature myoneural junctions or endplates may be a prerequisite for the expression of neuronal influences on fibre-type alteration. It has been established, however, that brachial muscles experimentally innervated by foreign lumbosacral nerves exhibit myosin–ATPase profiles appropriate for brachial, not hindlimb muscles. In these experiments, endplates are observed and the foreign nerves persist until at least stage 44 (day 18E) (Khaskiye, Toutant, Toutant, Renaud & Le Douarin, 1980; Laing & Lamb, 1983*b*; Cosmos & Butler, unpublished observations). Based on available evidence, *cross-innervation* experiments performed *in ovo* have not succeeded in altering fibre types. Such a conclusion may imply that the documented ablity of nerves to dictate fibre-type expression during development *ex ovo* is the result of a learning process derived from a previous association with an appropriate target. Alternatively, if indeed, nerves endogenously possess the potential to alter fibre types, this can only be expressed after a specific stage in their maturation process has been attained.

Fate of myotomal muscles

In direct contrast to the eventual incompatibility of thoracic nerves and brachial muscles derived from premuscle masses of the wing bud, a sustained compatibility existed between foreign thoracic nerves and brachial muscles of *myotomal* origin, such as the intervertebral muscles. This was an interesting observation since, *in situ*, thoracic nerves do innervate homologous muscles in the thoracic region. Furthermore, during embryogenesis intervertebral muscles, segmentally arranged along the entire length of the vertebral column, demonstrate a similar myosin–ATPase profile, regardless of their location (Butler & Cosmos, 1981*a*). Conversely, thoracic nerves *in situ* do not innervate limb-forming regions which comprise a large group of locomotor muscles with diverse myosin–ATPase profiles (Butler *et al.* 1982*a*; Laing & Lamb, 1983*a*; Phillips & Bennett, 1984). These observations imply that the presence or absence of a recognition factor may be responsible for the diametrically opposite responses of myotomal- and non-myotomal-derived brachial muscles of Thor–Br embryos.

Possible sources of incompatibility

Factors intrinsic to the transplanted spinal cord *per se* may contribute to the ultimate incompatibility observed between brachial muscles and foreign thoracic nerves. As indicated (Wenger, 1951; present observations), the transplanted

thoracic neural tube differentiated phenotypes characteristic of an *in situ* thoracic cord and not those typical of the brachial segment, namely, a greatly reduced LMC and the retention of the column of Terni. Innervation from the latter which contains visceral sympathetic preganglionic neurones (Oppenheim, Maderdrut & Wells, 1982) may, indeed, be unsuitable for the brachial musculature. To identify the origin of neurones innervating the periphery, we have recently initiated experiments using the technique of retrograde labelling with horseradish peroxidase (HRP) injected into the biceps brachii or triceps brachii muscles at stage 34 (day 8E). Although these muscles of Br–Br and unoperated embryos were innervated exclusively by motoneurones located within the well-formed LMC of the brachial region, analyses of the Thor–Br muscles indicated labelled cells either exclusively or predominantly within the MMC; very few labelled cells were noted in either the LMC or the column of Terni (Cauwenbergs, Butler & Cosmos, unpublished observations). Thus, based on a limited number of embryos, Thor–Br brachial muscles are innervated primarily by somatic motoneurones. The finding that labelling occurred within the MMC was unexpected since this column normally innervates myotomal-derived muscles exclusively (Smith & Hollyday, 1983). Since the LMC in the brachial region of the Thor–Br embryos is greatly reduced in size and, thus, contains far fewer motoneurones than does the LMC of the brachial area of control embryos, the HRP analyses indicated the compensatory ability of experimental brachial muscles to draw motor innervation from an atypical site within the transplanted cord, and, to interact functionally with the foreign nerves. To explain why available motoneurones derived from the transplanted cord interacted only temporarily, not permanently, with brachial muscles, we should consider an additional site for the expression of incompatibility.

Therefore, we suggest that the absence of an appropriate recognition factor may also contribute to the uncoupling of nerve–muscle contacts within experimental brachial muscles and that recognition exists at the interface of muscle fibres and individual axons. The nature of the proposed recognition factor is unknown. We do know, however, from previous experiments that individual myogenic cells possess distinct phenotypes during early phases of myogenesis and that these characteristics are expressed independent of neuronal factors (Butler *et al.* 1982*a*; Phillips & Bennett, 1984). Furthermore, the present Thor–Br experiments demonstrate that brachial muscles preserve their identity (myosin–ATPase profiles). Concerning the individuality of motoneurones, recently Sickles & Oblak (1984) have demonstrated metabolic variation of motoneurone cell bodies associated with the innervation of different muscle fibre types in the rat. Whether or not individual developing motoneurones also express unique phenotypes during embryogenesis is unknown although such a possibility has been suggested (Thompson, Sutton & Riley, 1984). If this is true, then it is conceivable that for the Thor–Br embryos the expression of the individuality of motoneurones has progressed to the point that they can recognize a foreign target as inappropriate but they have not developed far enough to alter the phenotypes of the foreign

target and to render it appropriate. Consequently, the partnership uncouples and the nerve withdraws.

The authors express their gratitude to Joyce Brierley for her expertise in embryonic surgery and to E. Paul Allard for technical assistance and for the final preparation of the photographic figures. This research was supported by grants awarded to E.C. by the Muscular Dystrophy Association, the Muscular Dystrophy Association of Canada, and the John A. Bauer Fund. P.C. was the recipient of a predoctoral fellowship from the Muscular Dystrophy Association of Canada.

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(Accepted 31 January 1986)