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Brachial muscles of dystrophic chick embryos atypically sustain interaction with thoracic nerves

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Summary

Previous analyses of experimental chick embryos of normal lineage demonstrate the inability of brachial muscles to sustain a successful union with foreign nerves derived from a thoracic neural tube segment transplanted to the brachial region at day 2 *in ovo* (day 2E). The present experiments were performed to determine if mutant chick embryos afflicted with hereditary muscular dystrophy would respond similarly to this experimental manipulation. Using the same criteria applied to our analysis of experimental normal embryos, our results demonstrated that dystrophic brachial muscles were capable of maintaining a compatible union with foreign thoracic nerves throughout the experimental period analysed. Significant muscle growth occurred, intramuscular nerve branches were maintained, motor endplates formed and wing motility was equivalent to that of unoperated dystrophic embryos. Thus, foreign nerves rejected by normal brachial muscles were accepted by brachial muscles of the mutant dystrophic embryo.

Key words: muscular dystrophy, chick embryo, foreign innervation.

Introduction

The mutant chick with hereditary muscular dystrophy, first described in 1956 by Asmundson & Julian, has provided an unique opportunity to investigate the etiology of avian muscular dystrophy throughout development both *ex ovo* and *in ovo* (for review see Harris, 1979). Although our previous research with this mutant stressed an analysis of both unoperated and experimentally manipulated chicks from the last week of embryogenesis through maturity *ex ovo* (for review, Cosmos, Butler, Allard & Mazliah, 1979; Cosmos, Butler, Mazliah & Allard, 1980; Gandy, 1986), more recently we have focused on identifying factors concerned with the phenotypic expression of the dystrophic gene during the early embryonic period (Cauwenbergs, Butler & Cosmos, 1986*a*,*b*). To date, our hypothesis states that the dystrophic process reflects impaired muscle–nerve interaction, *i.e.* the abnormal muscle is incapable of responding properly to neuronal influences associated with proper growth and differentiation of muscle, measured histochemically, biochemically and physiologically (for review, Cosmos *et al.* 1980).

Recently, we reported our analysis of an experimental embryonic model in which brachial muscles of normal lineage were coupled to foreign nerves derived from a thoracic neural tube segment transplanted to replace the extirpated brachial cord (Thor–Br procedure) at day 2 *in ovo* (day 2E) (Butler, Cauwenbergs & Cosmos, 1986). Although the brachial muscles initially accept and respond to neuronal influences derived from the foreign innervation, eventually this union uncouples. This uncoupling occurs coincident with the failure of brachial muscles and thoracic nerves to form neuromuscular junctions. Subsequently, intramuscular nerve branches withdraw and the experimental brachial muscles deteriorate to the state where they resemble aneurogenic muscles. Since unoperated brachial muscles of the dystrophic mutant express an impaired ability to interact with brachial nerves (Cauwenbergs *et al.* 1986*a*,*b*), we questioned whether they could recognize a foreign nerve as inappropriate. Therefore, we performed the Thor–Br surgical procedure on dystrophic embryos. Based on the same criteria applied to normal Thor–Br embryos, namely, the extent of muscle growth, the ability to maintain

innervation and form motor endplates and the ability to function properly (wing motility analyses), our results demonstrated that Thor–Br muscles of dystrophic genotype sustained functional and structural interaction with the foreign thoracic nerves throughout the embryonic period analysed, i.e. stage 29 (day 6E) through stage 40 (day 14E).

A preliminary report of these experiments has been presented (Butler & Cosmos, 1986).

Materials and methods

Surgical procedures

Experimental embryos

Fertile eggs homozygous for the dystrophic (am^{++}) gene (line S_1), obtained from the University of Connecticut (Storrs), were incubated at 37°C and 56% humidity for approximately 2 days. Embryos were staged according to the morphological criteria of Hamburger & Hamilton (1951). To achieve heterotopic innervation of brachial muscles by nerves derived from a thoracic neural tube segment, the brachial neural tube segment of a *host* embryo was removed at stage 13 (48–52 h) and replaced by the thoracic neural tube segment of a *donor* embryo at stage 14 (50–53 h), as described previously (Butler *et al.* 1986). Following surgery, the embryo was returned to the incubator to develop to the desired postoperative stage.

A total of 177 embryos of dystrophic genotype was subjected to this experimental manipulation with a postoperative mortality rate of 84·2%. The latter was significantly higher than the 74% mortality rate previously recorded for similarly manipulated embryos of normal genotype (Butler *et al.* 1986). 28 experimental thoracic-to-brachial (Thor–Br) dystrophic embryos survived for analysis between stage 29 (day 6E) through stage 40 (day 14E).

Control embryos

As a comparison for the Thor–Br embryos, both unoperated dystrophic embryos (n = 45) and dystrophic embryos subjected to the surgical removal of the brachial segment of the neural tube either without replacement (n = 103) or with replacement by the brachial neural tube of a donor embryo (Br–Br, n = 24), as described previously (Butler, Cosmos & Brierley, 1982*a*; Butler *et al.* 1986), were examined. Twelve embryos survived for an analysis of aneurogenic (An) brachial muscles and six for Br–Br muscles.

Histochemical and quantitative analyses

Experimental (Thor–Br) and control (An, Br–Br, unoperated) embryos were removed from the egg, decapitated, eviscerated and frozen for cryostat sectioning. 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 first described by Butler & Cosmos (1981*a*,*b*). In addition to the silver–cholinesterase method, both the Karnovsky & Roots (1964) and Koelle (Silver, 1963) procedures were utilized for endplate cholinesterase activity whereas the Oil Red O reaction (Cosmos, 1970) was employed to monitor any replacement of muscle fibres by lipid.

To assess the ability of experimental (Thor–Br) muscles to respond to neuronal factors associated with growth of embryonic muscles, quantitative volumetric analyses of representative experimental and control brachial muscles were compared by use of a Zeiss computerized Image Analysis System (Bloom, Butler, Brierley & Cosmos, 1985).

Motility analyses

To monitor functional interaction between dystrophic brachial muscles and nerves derived from the transplanted thoracic neural tube, wing motility analyses were performed on windowed eggs, as described previously (Butler *et al.* 1986). The frequency or number of spontaneous wing movements per 10 min observation period (M/10) characteristic of dystrophic Thor–Br embryos from day 6E onward was compared to previously determined values characteristic of normal Thor–Br embryos as well as unoperated normal and dystrophic controls (Butler *et al.* 1986; Cauwenbergs *et al.* 1986*a*,*b*).

Statistical analyses

Data obtained from quantitative volumetric analyses of muscle growth and motility values were stored in a computer and were analysed by the Student's *t*-test and Mann-Whitney U-test for statistical significance at the 95% confidence level.

Results

Comparative development of unoperated dystrophic and normal brachial muscles

Unlike other mutant chick embryos, such as the *wingless* (Waters & Bywaters, 1943) or *crooked neck dwarf* (Asmundson, 1945) mutants, embryos homozygous for the dystrophic gene are morphologically indistinguishable from normal embryos and can be classified by the Hamburger & Hamilton (1951) staging criteria. To provide a baseline necessary for the proper assessment of dystrophic Thor–Br muscles, the internal differentiation of unoperated dystrophic brachial muscles was compared with that of the brachial musculature of unoperated normal embryos from stage 18 (65–69 h) onward (see Butler & Cosmos, 1981*a*; Butler *et al.* 1982*a*). This comparison emphasized (1) the chronological individualization of muscles from the primary dorsal and ventral muscle masses of the wing bud, (2) the differentiation of muscle fibre types, (3) muscle growth and (4) the onset of innervation.

Analyses of chick/quail chimaeras (Christ, Jacob & Jacob, 1977) demonstrate that all brachial muscles are somitic in origin and that premyogenic cells migrate

from brachial somites to form the primary dorsal and ventral muscle masses of the wing bud. Subsequently, the latter undergo a series of divisions or cleavages, according to a precise chronological schedule, to form the intrinsic muscles of the wing as well as muscles associated with the pectoral girdle (Sullivan, 1962; Butler & Cosmos, 1981*a*). Henceforth, the term brachial muscles will refer specifically to those muscles derived from the primary muscle masses of the wing bud. Comparative histochemical analyses indicated that the process of cleavage proceeded on schedule in dystrophic embryos with one minor exception. The scapulohumeralis anterior (SHA) muscle of dystrophic genotype did not separate completely from the subscapular division until stage 35 (day 8–9E), an event that occurs one stage earlier in normal embryos. Thus, the presence of the dystrophic gene did not interfere significantly with the chronological sequence associated with the individualization of brachial muscles.

Likewise, the differentiation of muscle fibre types, a process initiated during the first week *in ovo* (Butler & Cosmos, 1981*a*; Butler *et al.* 1982*a*), proceeded normally in dystrophic brachial muscles, as judged by alkali- and acid-stable myosin–ATPase reactivities. The fibre type composition of *all* brachial muscles of dystrophic lineage was qualitatively similar to their normal counterparts at all stages analysed (see table in Butler *et al.* 1982*a*). To illustrate, at stage 35 (day 8–9E), the anterior latissimus dorsi (ALD) muscle of both genotypes exhibited dual acid- and alkali-stable myosin–ATPase activity whereas the deltoideus, triceps brachii and scapulohumeralis posterior (SHP) muscles expressed alkali stability only (Fig. 1A,B). These observations concur with our previous finding that throughout development *ex ovo* the myosin–ATPase profiles of dystrophic muscles are equivalent to those of normal muscles (Cosmos *et al.* 1979).

Thus, specific developmental events previously demonstrated to occur independent of peripheral neuronal influences in normal brachial muscles (Butler *et al.* 1982*a*) proceeded on schedule within the brachial musculature of dystrophic embryos. One parameter, however, that has been identified as being neurally dependent, namely, the proper growth of individual muscles (Bloom *et al.* 1985), was impaired to varying degrees among individual brachial muscles of the mutant embryos analysed quantitatively. Although both potentially slow tonic (ALD) and fast twitch (posterior latissimus dorsi, PLD; SHP) unoperated dystrophic muscles continued to grow throughout the developmental period analysed, individual muscle volumes were significantly lower than those recorded for similarly aged unoperated normal muscles (Figs 2–4). From stage 31 (day 7E) through stage 40 (day 14E), growth curves depicting total

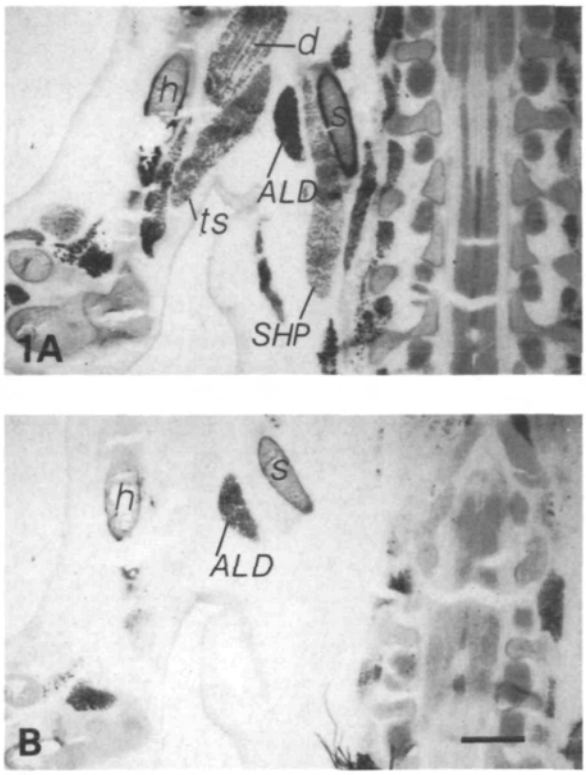

Fig. 1. A,B. Photomicrographs of serial sections from a stage 35 (day 8–9E) unoperated dystrophic embryo to illustrate myosin–ATPase profiles. The ALD muscle exhibits dual alkali (A) and acid (B) stability whereas the deltoideus (*d*), the scapular head of the triceps brachii (*ts*), and the SHP muscles exhibit alkali stability only (compare A and B). Similar profiles are expressed by unoperated normal embryos at this stage. *s*, scapula; *h*, humerus. Myosin–ATPase. Bar, 1·0 mm.

mean volumes for both the unoperated dystrophic ALD (Fig. 2) and PLD (Fig. 3) muscles are shifted to the right of the corresponding normal curves. Analyses of unoperated dystrophic and normal SHP muscles from stage 34 (day 8E) onward revealed a similar differential pattern with one exception; at stage 36 (day 10E), the dystrophic SHP value was not significantly different from the normal one (Fig. 4). To investigate the response of dystrophic muscles to the absence of innervation, the growth of aneural dystrophic muscles was compared to previous analyses of aneural normal muscles. Volumetric analyses of aneural ALD and SHP muscles of both lineages exhibited a limited capacity for growth between stage 34 (day 8E) to stage 38 (day 12E) (Figs 2, 4). Although the volume of the stage 38 (day 12E) dystrophic aneural ALD appears to be lower than that of the normal aneural ALD (Fig. 2), it was not significantly different ($P > 0{\cdot}05$). Similar to the normal aneural PLD (Butler *et al.* 1982*a*), the dystrophic aneural PLD muscle demonstrated limited survival.

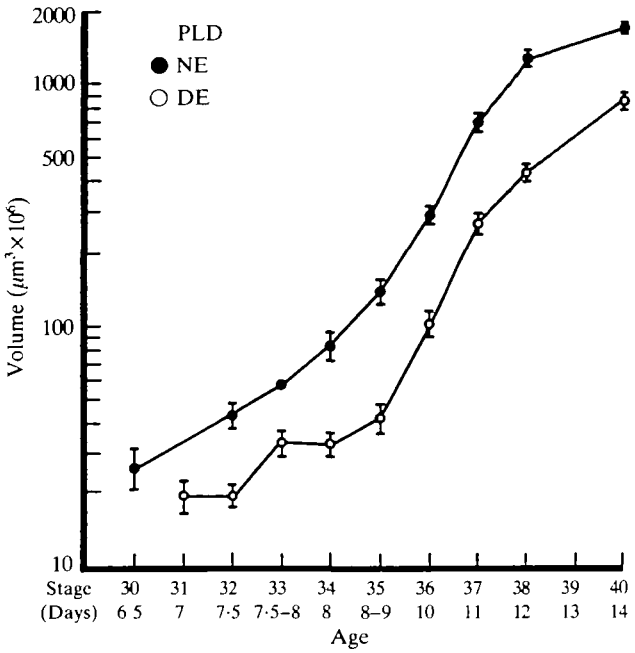

Fig. 2. Comparative growth of unoperated (Uc) and aneurogenic (An) ALD muscles of normal *versus* dystrophic genotype from stage 30 (day 6·5E) through stage 40 (day 14E). Values represent the mean muscle volume ($\mu m^3 \times 10^6$) ± S.E.M. Note semilog plot. NE, normal embryo; DE, dystrophic embryo. [Data for normal (Uc, An) muscles derived from Butler *et al.* 1986.]

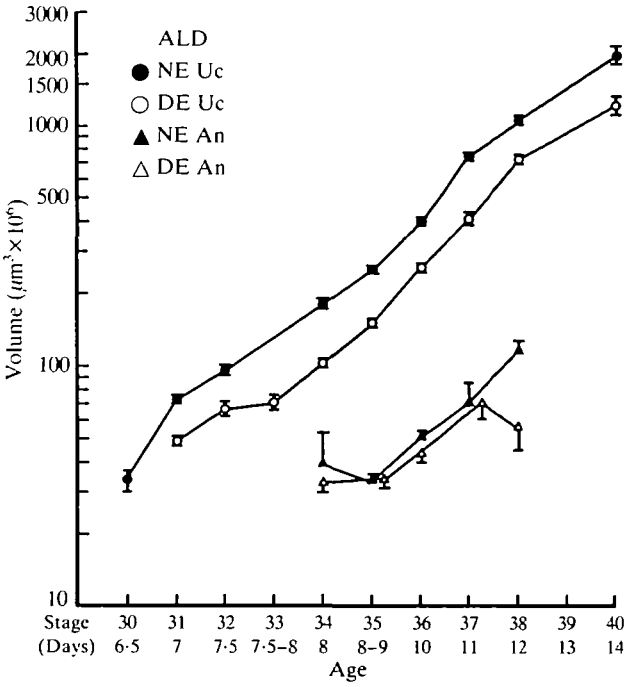

Fig. 3. Comparative growth of unoperated PLD muscles of normal *versus* dystrophic genotype from stage 30 (day 6·5E) through stage 40 (day 14E). Values represent the mean muscle volume ($\mu m^3 \times 10^6$) ± S.E.M. [Data for normal muscles derived from Butler *et al.* 1986.]

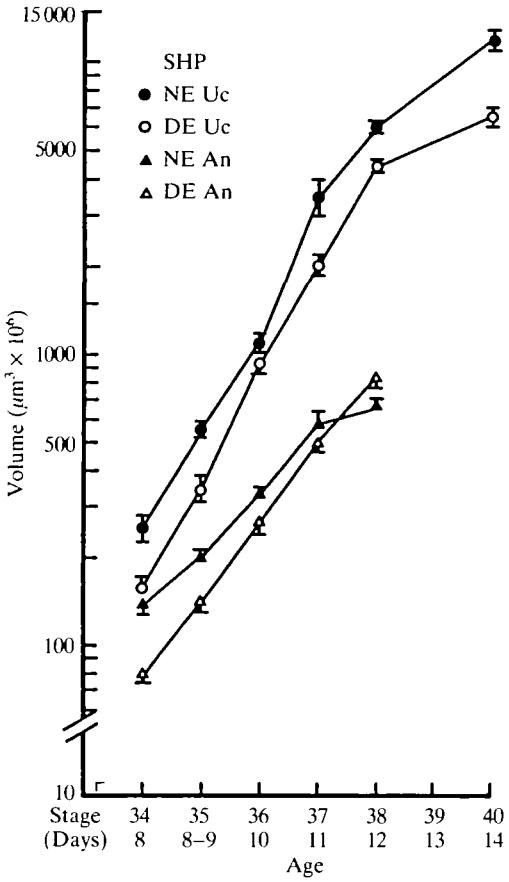

Fig. 4. Comparative growth of unoperated (Uc) and aneurogenic (An) SHP muscles of normal *versus* dystrophic genotype from stage 34 (day 8E) through stage 40 (day 14E). Values represent the mean muscle volume ($\mu m^3 \times 10^6$) ± S.E.M.

The observed impaired growth characteristic of unoperated dystrophic muscles does not represent either a delay in the onset of innervation or a size differential between dystrophic *versus* normal embryos. Comparative histochemical analyses (silver–cholinesterase reaction) demonstrated that both the formation of the brachial plexus at the base of the wing bud and the subsequent branching of intramuscular nerves within the brachial musculature occurred according to the same time schedule in embryos of both genotypes. The possibility that a size differential was responsible for the impaired growth of unoperated dystrophic muscles was eliminated since previous analyses indicated that the body weights of both strains of embryos are statistically equivalent from day 6E through day 16E (Cauwenbergs *et al.* 1986*a*). Likewise, volumetric analyses of the scapula, an internal, nonmuscular structure, at stage 36 (day 10E) also indicated statistically equivalent values for dystrophic (451·7 $\mu m^3 \times 10^6 \pm 23{\cdot}4$ S.E.M.) and normal (396·5 $\mu m^3 \times 10^6 \pm 16{\cdot}2$ S.E.M.) embryos.

Thus, impaired growth appeared to be restricted to muscular elements.

Response of dystrophic muscles to the Thor–Br transplant

Previous analyses of normal Thor–Br embryos reveal that nerves derived from the foreign thoracic neural tube initially interact successfully with brachial muscles, both functionally and structurally; eventually, this union deteriorates, distinct motor endplates are not observed and intramuscular nerve branches appear to withdraw. Consequently, Thor–Br muscles of normal embryos degenerate to the state where they resemble aneurogenic muscles (Butler, Cosmos & Brierley, 1982*b*; Cauwenbergs, Cosmos & Butler, 1983; Butler *et al.* 1986) and the transplanted thoracic neural tube exhibits massive neurothanasia (Cauwenbergs, Cosmos & Butler, 1986). Externally, normal Thor–Br embryos reflected the eventual loss of innervation and muscle degeneration observed microscopically. From stage 38 (day 12E) onward their wings were positioned abnormally, i.e. they hung downward instead of being held in the flexed position typical of unoperated wings (Butler *et al.* 1986). In contrast, all dystrophic Thor–Br embryos examined from stage 29 (day 6E) onward demonstrated a proper positioning of their wings. This observation represented a gross manifestation of the differential response of dystrophic *versus* normal embryos to the Thor–Br procedure, a phenomenon confirmed by subsequent quantitative and microscopic analyses of dystrophic brachial muscles innervated by nerves derived from the transplanted thoracic neural tube.

Figs 5 and 6 compare the growth (mean muscle volume) of representative Thor–Br muscles of dystrophic and normal lineage. Prior to stage 36 (day 10E), the Thor–Br ALD muscle of normal embryos exhibited continual increments in growth that exceeded values recorded for the dystrophic Thor–Br ALD muscle (Fig. 5), as noted with unoperated muscles of both lineages. Beyond this period, however, further growth of the normal Thor–Br ALD muscles did not occur and by stage 40 (day 14E) this muscle was atrophied, similar to aneural ALD muscles. In contrast, the dystrophic Thor–Br ALD muscle demonstrated significant growth between stage 36 (day 10E) and stage 40 (day 14E). At the latter stage, the dystrophic Thor–Br ALD muscle was only 21% smaller than its unoperated counterpart whereas the normal Thor–Br ALD muscle was 98% smaller than the control normal ALD value.

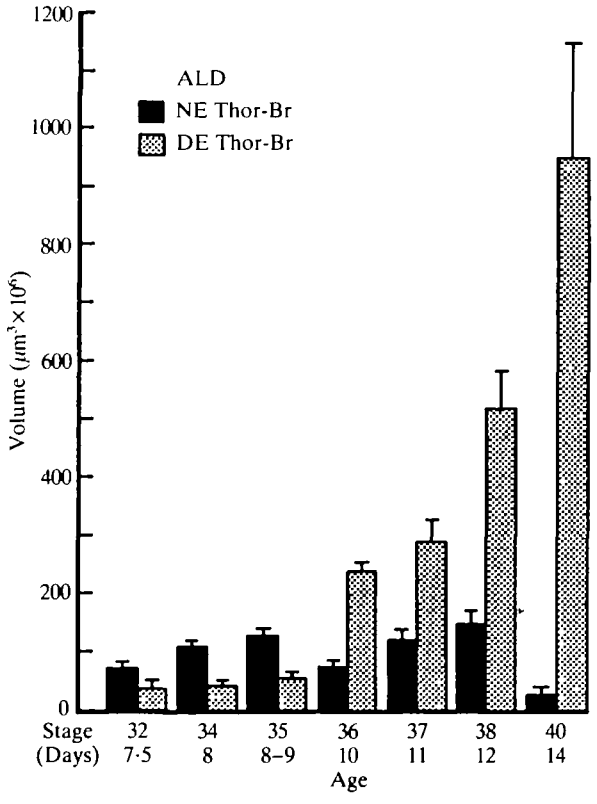

Fig. 5. Comparative growth of the Thor–Br ALD muscle of normal and dystrophic genotype from stage 32 (day 7·5E) through stage 40 (day 14E). Values represent the mean muscle volume ($\mu m^3 \times 10^6$) ± S.E.M. [Data for the normal Thor–Br ALD muscle derived from Butler *et al.* 1986.]

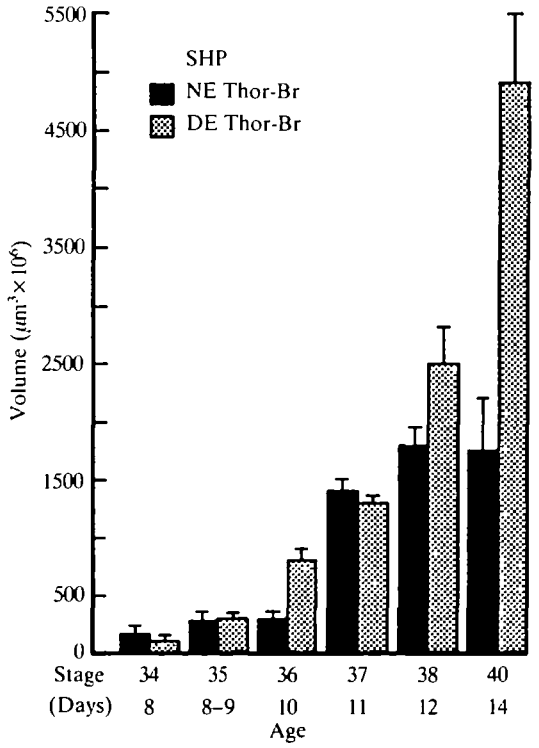

Fig. 6. Comparative growth of the Thor–Br SHP muscle of normal and dystrophic genotype from stage 34 (day 8E) through stage 40 (day 14E). Values represent the mean muscle volume ($\mu m^3 \times 10^6$) ± S.E.M.

Since both the ALD and PLD muscles are derived from the common latissimus dorsi primordium which begins to cleave at stage 29 (day 6E) (Butler & Cosmos, 1981*a*), our original intention was to compare the growth of the Thor–Br PLD muscle of dystrophic genotype with our previous analysis of the normal Thor–Br PLD muscle. However, we observed that the PLD muscle was not viable beyond stage 34 (day 8E) in the majority of dystrophic Thor–Br embryos analysed. Although the reason for this limited survival is unknown, we have noted that the unoperated dystrophic PLD exhibited the greatest impairment of growth when compared to unoperated ALD and SHP muscles (cf. Figs 2–4). Evidently, the dystrophic PLD muscle could not survive the Thor–Br experimental manipulation even though it did survive the Br–Br procedure. Therefore, the SHP muscle was selected to assess the differential growth of a fast twitch muscle in dystrophic *versus* normal Thor–Br embryos. Fig. 6 illustrates that the dystrophic Thor–Br SHP muscle grew continually from stage 34 (day 8E) through stage 40 (day 14E); in contrast, the normal Thor–Br SHP muscle ceased growing significantly beyond stage 37 (day 11E). By stage 40 (day 14E) the dystrophic Thor–Br SHP muscle was approximately three times larger than the corresponding normal Thor–Br muscle. Thus, as in the ALD, the overall growth of the dystrophic SHP experimental muscle exceeded that characteristic of the normal Thor–Br SHP. The fact that the normal Thor–Br SHP muscle continued to grow for periods beyond those observed with the normal Thor–Br ALD is related to previous histochemical observations that the withdrawal of nerve branches varied temporally among various muscles analysed (Butler *et al.* 1986). For the experimental ALD muscle, an abrupt, complete loss of innervation occurs at stage 36 (day 10E); therefore, subsequent growth resembled that of the aneural ALD. In contrast, histochemical analysis of the experimental normal SHP muscle demonstrates the persistence of foreign intramuscular nerve branches through stage 40 (day 14E). Evidently, despite the fact that discrete motor endplates were not observed in the normal Thor–Br SHP muscle, the intramuscular nerve axons present were capable of promoting growth of this experimental muscle for a significant period of development.

Histochemical analyses provided both a confirmation of, and a reason for, the divergent growth patterns characteristic of dystrophic and normal Thor–Br brachial muscles. Serial reconstruction of all dystrophic Thor–Br embryos revealed that the transplanted thoracic neural tube was viable and that a brachial plexus formed (Fig. 7). From stage 29 (day 6E) through stage 35 (day 8–9E), the development of dystrophic Thor–Br embryos was qualitatively indistinguishable from that of normal Thor–Br embryos except for the fate of the dystrophic PLD muscle. Thor–Br brachial muscles of both genotypes formed on schedule, exhibited appropriate myosin–ATPase profiles and received innervation from nerves derived from the transplanted thoracic neural tube. By stage 36 (day 10E), however, the emergence of a divergent

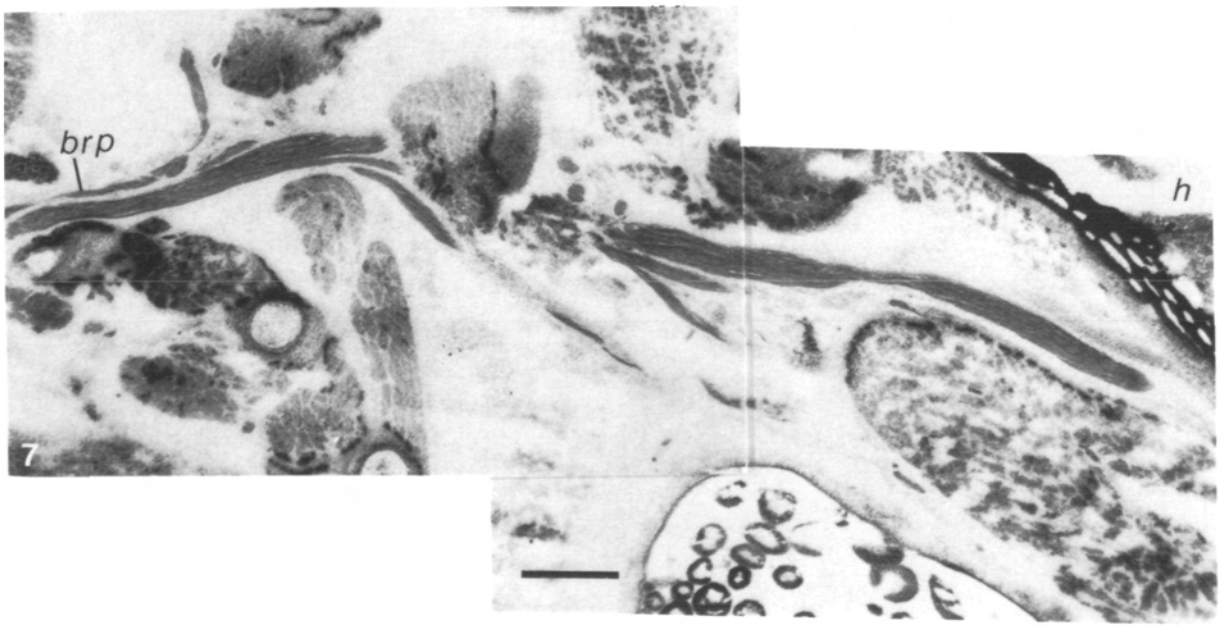

Fig. 7. Photomicrograph from a stage 40 (day 14E) Thor–Br dystrophic embryo to illustrate branches of the brachial plexus (*brp*) derived from the thoracic neural tube graft. *h*, humerus. Silver–cholinesterase. Bar, 0·5 mm.

response between dystrophic *versus* normal experimental muscles became evident. Although nerve axons abruptly disappeared at this stage in the normal Thor–Br ALD, intramuscular nerve branches persisted in the Thor–Br ALD muscle of dystrophic lineage and by stage 37 (day 11E) endplates were observed. Fig. 8A illustrates a segment of the ALD nerve entering the well-formed, compact dystrophic Thor–Br ALD muscle at stage 40 (day 14E); endplates are distributed throughout the muscle (Fig. 8A,B). In contrast, by stage 40 (day 14E), the normal Thor–Br ALD muscle was atrophied and/or replaced by lipid (Butler *et al.* 1986).

The atypical response of dystrophic Thor–Br brachial muscles was not limited to the ALD muscle. Examination of other experimental muscles also revealed maintenance of innervation and endplate formation. Consider the fate of two major muscles of the upper wing, namely, the deltoideus and triceps brachii muscles. At stage 40 (day 14E) both muscles consisted of well-formed fascicles (Fig. 9A) and at higher power the deltoid nerve is observed coursing throughout the deltoideus muscle (Fig. 9B). Serial sections reacted for cholinesterase activity demonstrate the formation of distinct motor endplates (Fig. 9C) in both muscles. The maintenance of synaptic contact between the dystrophic Thor–Br deltoideus muscle and nerves derived from the transplanted thoracic neural tube was significant since the Thor–Br deltoideus muscle of normal genotype becomes denervated by stage 37 (day 11E) and is completely replaced by lipid by stage 40 (day 14E) (Fig. 9D). (See also Butler *et al.* 1986.)

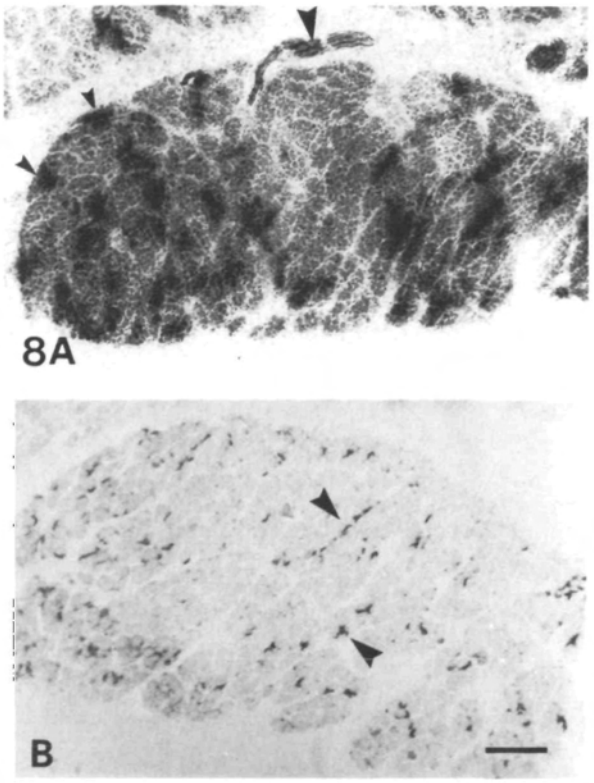

Fig. 8. A,B. Photomicrographs from a stage 40 (day 14E) Thor–Br dystrophic embryo to illustrate the maintenance of innervation within the ALD muscle. Both the ALD nerve (large arrow) entering the compact ALD muscle and the distribution of endplates (small arrows) are observed throughout the ALD muscle (A); discrete endplates within the ALD muscle are also demonstrated with the Koelle reaction (B, arrows). A, silver–cholinesterase; B, Koelle reaction. Bar, 0·1 mm.

Further verification of sustained nerve–muscle interaction between nerves derived from the transplanted thoracic neural tube segment and dystrophic brachial muscles was derived from wing motility analyses. In direct contrast to the eventually impaired motility characteristic of normal Thor–Br wings (Cauwenbergs *et al.* 1983; Butler *et al.* 1986), experimental dystrophic embryos demonstrated wing motility frequencies comparable to those of unoperated dystrophic embryos. Although the number of dystrophic Thor–Br embryos available for motility analyses was limited, we were successful in determining the frequency of spontaneous wing movements (M/10 values) of eight individual embryos from day 6E through day 12E. These values were then compared to previously recorded values characteristic of experimental *versus* control normal embryos (Butler *et al.* 1986) and unoperated dystrophic embryos (Cauwenbergs *et al.* 1986*a*,*b*). From day 6E to day 8E, motility (M/10) values of both dystrophic and normal Thor–Br wings exhibited similar increments, i.e. from $10{\cdot}5 \pm 1$ to $90{\cdot}6 \pm 3$ and from $9{\cdot}9 \pm 1$ to $89{\cdot}5 \pm 4$, respectively. As development proceeded, however, the motility patterns of normal *versus* dystrophic experimental embryos changed. Whereas the frequency of wing movements characteristic of normal Thor–Br embryos declined significantly compared to normal unoperated values, e.g. at day 12E values of $130{\cdot}8 \pm 5$ *versus* $303{\cdot}4 \pm 6$ were recorded respectively, both experimental dystrophic Thor–Br and dystrophic unoperated wings were capable of an equivalent number of movements at day 12E, i.e. 252 ± 25 *versus* 243 ± 7 respectively. Thus, dystrophic Thor–Br wings analysed to day 12E failed to exhibit the impaired functional nerve–muscle interaction characteristic of normal Thor–Br forelimbs.

In contrast to the divergent response of dystrophic and normal embryos to the Thor–Br procedure, embryos of both genotypes exhibited a similar response to the Br–Br control manipulation. Br–Br brachial muscles of both genotypes were indistinguishable from their respective unoperated counterparts.

The salient features of the diverse response of dystrophic *versus* normal Thor–Br embryos, derived

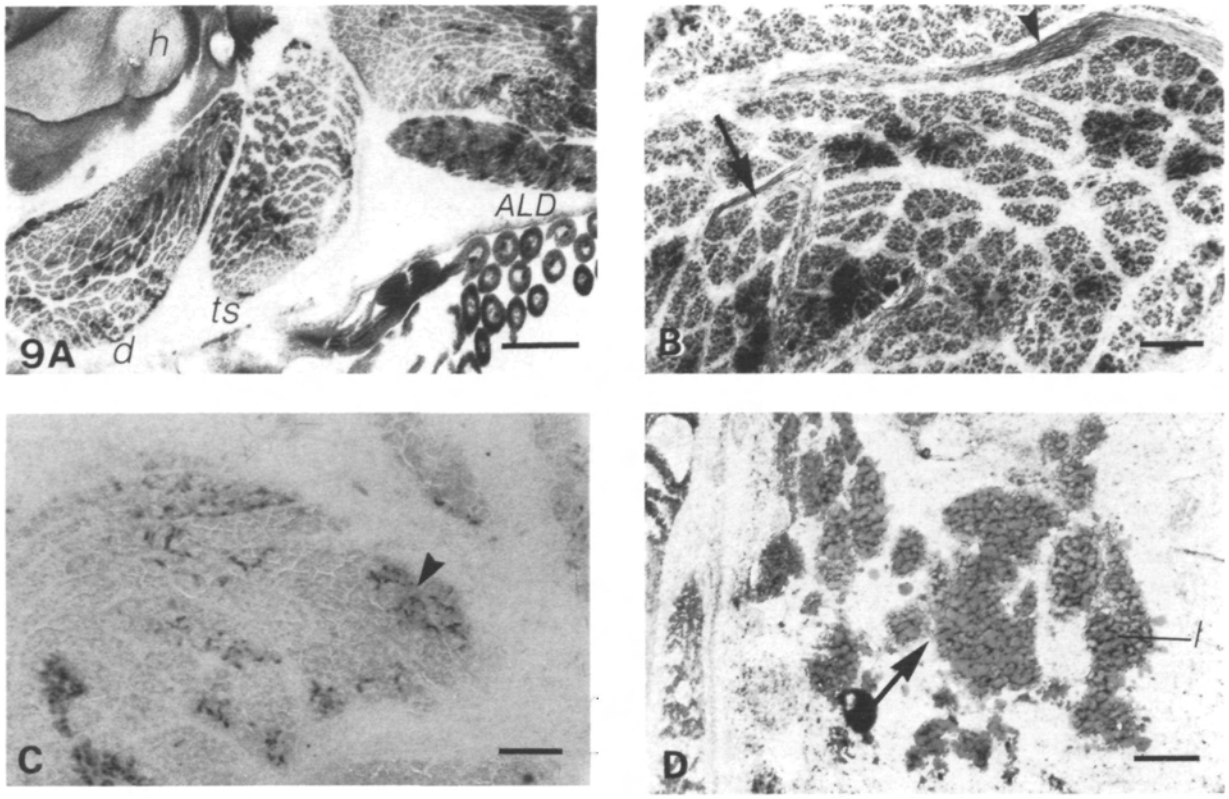

Fig. 9. (A–D). Photomicrographs from a stage 40 (day 14E) Thor–Br dystrophic (A–C) and Thor–Br normal (D) embryo. At this stage, Thor–Br dystrophic muscles, such as the deltoideus (*d*), the scapular head of the triceps brachii (*ts*), and the ALD, are compact muscles composed of muscle fibres (A). The maintenance of intramuscular nerve branches (arrows) within the deltoideus muscle is illustrated in B and the formation of distinct endplates within the triceps brachii muscle is shown in C (arrow). In contrast, by stage 40 (day 14E) in the normal Thor–Br embryo muscle fibres have degenerated and are replaced by fascicles of lipid (*l*, arrow) (D). (A,B) silver–cholinesterase; (C) Karnovsky and Roots reaction; (D) Oil Red O reaction. Bar in A, 0·5 mm; bar in B, 0·1 mm; bar in C, 0·06 mm; bar in D, 0·25 mm.

from quantitative, histochemical and functional parameters, are presented in Table 1. (Analysis of normal Thor–Br embryos derived from Butler *et al.* 1986.)

Table 1. *Differential response of dystrophic and normal Thor–Br embryos*

Parameter	Dystrophic	Normal
Morphology	Normal wing position (day 6E onward)	Abnormal wing position (day 12E onward)
Wing motility	Unaltered (day 6E onward)	Decreased (day 9E onward)
Myosin–ATPase	Unaltered profiles (day 6E onward)	Unaltered profiles (day 6E onward)
Innervation	Maintained (day 6E onward)	Withdrawn (day 10E onward)
Muscle growth	Increased (day 6E onward)	Declined (day 10E onward)
Motor endplates	Present	Absent
Muscle deterioration	Absent (day 6E onward)	Present (day 10E onward)

Discussion

Several investigators have demonstrated an incompatibility between the brachial musculature of normal embryos and innervation derived from the thoracic segment of the neural tube (Székely & Szentágothai, 1962; Straznicky, 1963, 1967; Morris, 1978; Butler *et al.* 1986). Thus, when subjected to the identical surgical manipulation, the response of the mutant embryo with hereditary muscular dystrophy to the Thor–Br experimental procedure described in the present experiments is atypical. Brachial muscles of dystrophic genotype sustained a successful interaction with foreign nerves derived from a transplanted thoracic neural tube throughout the experimental period examined, namely, from stage 29 (day 6E) through stage 40 (day 14E). Compatibility was evident at all levels of muscle–nerve interaction analysed: wing motility, muscle growth and endplate formation. Consequently, unlike Thor–Br muscles of normal embryos which eventually deteriorate to the state where they resemble aneurogenic muscles

(Butler *et al.* 1986), experimental dystrophic muscles were morphologically and functionally indistinguishable from their unoperated control counterparts. Perhaps the muscle that most graphically illustrated the atypical response of dystrophic experimental muscles was the SHP. Even though a limited number of axons persisted for a comparatively prolonged period in the normal Thor–Br SHP and, as a result, relative growth of this muscle exceeded that characteristic of other normal Thor–Br muscles, motor endplate formation was never observed. Conversely, motor endplate formation and continual growth characterized the SHP muscle of Thor–Br dystrophic embryos.

Our results suggest that the response of dystrophic Thor–Br embryos reflects the inability of dystrophic experimental muscles to recognize thoracic innervation as both foreign and inappropriate. Furthermore, this interpretation is consistent with our hypothesis that impaired peripheral muscle–nerve interaction is associated with an overt expression of the dystrophic gene. This hypothesis, originally based on an extensive analysis of the differentiation of both unoperated and experimentally manipulated dystrophic chicks during development *ex ovo* (for review see Cosmos *et al.* 1979, 1980), has now been extended to encompass the early development of dystrophic muscles *in ovo* (Cauwenbergs *et al.* 1986*a*,*b*).

A chronological analysis of the development of unoperated dystrophic muscles indicated that specific events that occur *independently* of neuronal influences proceeded according to the patterns characteristic of unoperated normal muscles, such as the division of the primary muscle masses of the wing bud and the expression of appropriate myosin–ATPase profiles (present experiments). Conversely, aspects of myogenesis that *depend* upon neuronal factors, such as muscle growth (Bloom *et al.* 1985) and wing motility (Butler *et al.* 1986), are impaired in the mutant embryo. Throughout the developmental period analysed, growth of unoperated dystrophic brachial muscles was impaired compared to their normal counterparts whereas aneurogenic brachial muscles of both genotypes exhibited a limited, endogenous capacity for growth. With innervation, unoperated dystrophic muscles failed to respond properly to neural factors responsible for proper muscle growth; without innervation, the responses of both normal and dystrophic muscles were similar. Recently, we reported that functional (wing motility) muscle–nerve interaction is also abnormal in unoperated dystrophic embryos (Cauwenbergs *et al.* 1986*a*,*b*). The latter exhibit decreased wing motility from day 10E through day 15E when compared to normal unoperated wings. Since this functional defect is not abolished when embryonic dystrophic muscles are coupled experimentally with nerves derived from a brachial neural tube of normal genotype (Cauwenbergs *et al.* 1986*b*), the impaired motility characteristic of dystrophic wings must reflect abnormal peripheral muscle–nerve interaction. Evidently, embryonic dystrophic brachial muscles are incapable of responding properly to either polyneuronal bursts of activity emanating from a normal brachial neural tube segment or contact with normal peripheral motoneurones. Augmenting our own findings, evidence exists in the literature that implicates a faulty interaction between dystrophic muscles and nerves. The *in vitro* studies of Johnson, Bailey & Wenger (1981) demonstrate that myogenic cells cultured from the pectoralis muscle of day 11E dystrophic embryos do not respond to neurotrophic influences derived from sheep sciatic nerve extract. More recently, Stewart, Hayakawa & Rathbone (1984) who performed brachial neural tube transplants between dystrophic and normal embryos have reported that their observed accelerated loss of motoneurones within the brachial lateral motor column of dystrophic embryos is related to faulty interactions at the periphery and does not reflect an intrinsic property of the spinal cord *per se*.

Combined, evidence to date points to the singular development of muscle–nerve interaction within the brachial musculature of dystrophic embryos. At one end of the spectrum, analyses of both unoperated and experimentally manipulated dystrophic embryos (Cauwenbergs *et al.* 1986*a*,*b*; Johnson *et al.* 1981; Stewart *et al.* 1984; present observations) provide evidence that dystrophic muscles are unable to respond properly to neural factors associated with several important developmental processes, including muscle growth and function. At the other end of the spectrum, experimental Thor–Br muscles exhibited an ability to maintain interaction with foreign nerves derived from the transplanted thoracic neural tube segment, nerves that are rejected by muscles of normal lineage.

At present we cannot offer an explanation for the diverse response of dystrophic *versus* normal embryos to the Thor–Br procedure. Evidence suggests that future investigations of this phenomenon should focus on an understanding of factors associated with the establishment of mature neuromuscular junctions. The mutant dystrophic embryo should serve as an excellent model to study such factors.

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