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RE-INNERVATION OF FAST AND SLOW TWITCH MUSCLE FOLLOWING NERVE CRUSH AT BIRTH

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SUMMARY

1. The frequency of miniature end-plate potentials (m.e.p.p.s) was significantly greater in the fast twitch extensor digitorum longus muscle (extensor) than in the slow twitch soleus, even though end-plate surface area was greater for fibres in the latter muscle.

2. Crush of the sciatic nerve at birth did not prevent the appearance of this difference in m.e.p.p. frequency. However, the frequency of the potentials in the re-innervated muscles was less than normal, even though the regenerated neuromuscular junction was qualitatively normal in morphology.

3. Though the re-innervated muscles were differentiated with respect to twitch time course, the extensor muscle was more responsive than normal to the contracture-inducing action of caffeine.

4. The Z line of the re-innervated extensor muscle was similar to that of the normal soleus in thickness.

5. Resting potential, passive electrical properties and action potential generating mechanism of the sarcolemma were normal.

6. Since the re-innervated muscles lacked muscle spindles, a role of sensory feed-back in the function of the neuromuscular junction as well as the neurotrophic regulation of muscle is discussed.

INTRODUCTION

The development and maintenance of adult muscle types is dependent upon the presence of intact innervation. For instance, neonatal neurectomy prevents the differentiation of fast and slow twitch muscles (Engel & Karpati, 1968; Shafiq, Asiedu & Milhorat, 1972) and denervation in the

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adult causes alterations of many properties of the differentiated muscles (Guth, 1968; Gutmann, 1976).

In the adult, re-innervation restores many of the muscle properties to normal (Miledi, 1960*a*,*b*; McArdle & Albuquerque, 1973). The primary objective of this study was to evaluate the effects of nerve crush at birth upon the development of the re-innervated muscles. In particular, spontaneous transmitter release, some electrical properties of the sarcolemma, and the morphology of the fast twitch extensor digitorum longus and the slow twitch soleus muscles of the rat were examined.

A secondary objective of this work was related to the observation that these two muscles contained significantly different numbers of muscle spindles (Zelená & Hník, 1962). Since denervation at birth precludes the differentiation of muscle spindles (Zelená & Hník, 1960), this study also presented an opportunity to determine the properties of fast and slow muscles containing no spindles.

Preliminary reports of this work have been presented (McArdle & Shramowiat, 1974; McArdle & Sansone, 1975; Sansone & McArdle, 1975).

METHODS

Preparation

All experiments were performed *in vitro* upon the extensor digitorum longus (extensor) and soleus muscles of male and female rats of the Wistar strain. These muscles were denervated within 8 hours after birth (NC-B) or at 14 (NC-14) or 35 (NC-35) days after birth. To do this, the animals were anaesthetized with diethyl ether and watchmaker forceps were used to crush a 0·2 mm segment of the left sciatic nerve at the mid-thigh region. The circulation to the experimental hindlimb was not damaged during this procedure. The wound was closed and the animals were allowed to recover for varying intervals. Weaning occurred at 25–28 days after birth. On the selected day after nerve crush, the extensor and soleus muscles were excised under a continuous flow of Krebs–Ringer solution and prepared for electrophysiological, morphological or mechanical analysis as described below.

Electrophysiological measurements

The isolated muscles were stretched to less than 5% beyond their resting length (Fatt & Katz, 1952) and secured by means of stainless steel pins inserted through the tendons, to a paraffin-lined Plexiglass plate having a plano-convex lens at its centre. This plate was then inserted into an insulated chamber having a volume of 15 ml. and perfused continuously at a rate of 2–3 ml./min with an oxygenated (95% O_2–5% CO_2, v/v) solution containing (mM); NaCl, 135·0; KCl, 5·0; $MgCl_2$, 1·0; Na_2HPO_4, 1·0; $NaHCO_3$, 15·0; $CaCl_2$, 2·0; dextrose, 11·0. All experiments were made at a room temperature of 20–23 °C.

Glass micro-electrodes filled with 3 M-KCl and having resistance of 5–15 MΩ were used to make intracellular recordings from single fibres on the dorsal surface of the muscles. Micro-electrodes with tip potentials in excess of 3 mV were discarded (Adrian, 1956). Membrane potentials were amplified (W. P. Instruments, M701), displayed on an oscilloscope (Tektronix 565) and photographed (Grass C4 Kymograph Camera). The entire recording circuit, including an electrode with a resistance of 15 MΩ, had

a time constant of approximately 40 μsec. The micro-electrodes were positioned with Jena micromanipulators and observed under a Zeiss binocular dissection microscope. For those techniques involving insertion of two micro-electrodes into the same muscle fibre, an eyepiece micrometer was used to measure interelectrode distance.

To record miniature end-plate potentials (m.e.p.p.s), the recording micro-electrode was inserted at the apparent terminal portion of each axon arborization visible in the transilluminated muscle. Recordings of m.e.p.p.s were regarded as focal when their rise time, or the time required for the potential to reach its summit, was less than 1·0 msec. After locating the end-plate region, the recording micro-electrode was left in position for at least 1 min in order to evaluate the frequency of spontaneous transmitter release.

The cable theory of Hodgkin & Rushton (1946) and the method of 'square pulse analysis' (Fatt & Katz, 1951) was used to determine the passive electrical properties of the muscle membranes.

To determine the time and voltage characteristics of the action potential generating mechanism, two micro-electrodes, one for passage of current and one for recording, were inserted into the same muscle fibre at about 50 μm apart. The influence of resting membrane potential upon generation of action potential was standardized by hyperpolarizing the muscle fibres to -90 ± 2 mV with anodal current delivered through the stimulating electrode prior to excitation with a cathodal pulse of approximately 10 msec duration. An operational amplifier (Burr-Brown, Model 3500 B; RXC = 4×10^{-6} sec) was used to determine the maximum rate of rise (dV/dt) of the action potentials. The threshold (E_{crit}) was taken as that level of membrane potential at which the beginning of the active response appears. The amplitude of the 'overshoot', as well as the duration at zero membrane potential, was also measured (Thesleff, Vyskočil & Ward, 1974). The sensitivity of the action potential-generating mechanism to tetrodotoxin (TTX; Sankyo Co.) was determined by exposing the muscles to Krebs–Ringer solution containing 1 μg/ml. TTX for 30 min prior to electrophysiological analysis. Those responses which failed to exceed the zero level of membrane potential were regarded as TTX-sensitive.

Morphological analysis

After completion of electrophysiological recording, the muscles were blotted and weighed. Some muscles were then fixed in Bouin's and dehydrated in ethanol–xylene prior to embedding in paraffin. The entire muscle was then cut into sections of 10 μm thickness and stained with haematoxylin–eosin. The total number of muscle spindles was counted for each muscle by examining every fourth section.

Four rats were sacrificed at 8 months after nerve crush at birth and experimental and control extensor and soleus muscles were removed. All four muscles were pinned to a common paraffin-lined dish and fixed in cold (5 °C) buffered neutral formalin for 3·5 hr. The muscles were then rinsed in distilled water and stained for cholinesterase (Koelle & Friedenwald, 1949). Individual surface fibres were isolated and mounted in glycerine jelly with the motor end-plate region up. Each end-plate was photographed along with a calibration scale. Prints of these photographs were made and fibre diameter and end-plate surface area were determined. The latter measurement was made by cutting out the end-plate region and comparing the weight with the weight of a photograph of a known surface area (Kuno, Turkanis & Weakly, 1971). Some muscle fibres had several separate regions which stained for cholinesterase. The total end-plate surface area was determined for such fibres having multiple end-plate regions. A minimum of twenty-five surface fibres was evaluated in each muscle.

Ultrastructural analysis was performed at 60, 90 and 180 days after nerve crush

at birth. The left extensor and soleus muscles were excised, rinsed in physiological solution and pinned to a paraffin-lined petri dish at resting length prior to fixation for 24–48 hr at room temperature in a combined paraformaldehyde–glutaraldehyde solution (Karnovsky, 1965). Control muscles, obtained from the contralateral hindlimb, were similarly treated. The motor end-plate regions of surface fibres were dissected into 2 × 3 mm strips in 0·1 M-cacodylate buffer (pH 7·4) and post-fixed for 1 hr in a 1 % osmium tetroxide–cacodylate buffered solution (pH 7·4) at room temperature. After an acetate buffer rinse, the tissues were block stained for 16–24 hr in the dark with a 1 % uranyl acetate solution, buffered to a pH of 5·2 with acetate. Following routine dehydration, the tissues were embedded in TAAB and 80–90 nm sections were cut, mounted on copper grids, stained with uranyl acetate and lead citrate (Reynolds, 1963) and examined in a JEOL-6C electron microscope.

Analysis of twitch and contracture properties

The time characteristics of the muscle twitch, as well as the contracture tension in response to 20 mM-caffeine (Gutmann & Sandow, 1965) were measured for the re-innervated muscles. The distal tendon was tied to the bottom of an insulated glass chamber while the proximal tendon was connected to a force transducer (Grass Inst. Co., Model FT 0·03) with surgical silk or a gold chain in the twitch studies. The compliance and maximal frequency response of the transducer used to measure twitches was 0·5 mm/100 g of force and 170 Hz, respectively. Twitches were elicited by indirect stimulation of the muscle nerves with square waves having a duration of 0·02–0·08 msec and supramaximal amplitude. The resting length of the muscles was adjusted to allow maximal twitch tension. The signal from the force transducer was amplified and displayed on a polygraph (Grass Inst. Co., Model 5 or 79) or an oscilloscope and photographed. Caffeine-induced contractures were measured at 20–23 °C while twitches were examined at 32 °C. The latter temperature was maintained by circulating a heated fluid (Neslab Inst., Inc.) through a jacket surrounding the glass muscle chamber.

RESULTS

Gross morphology

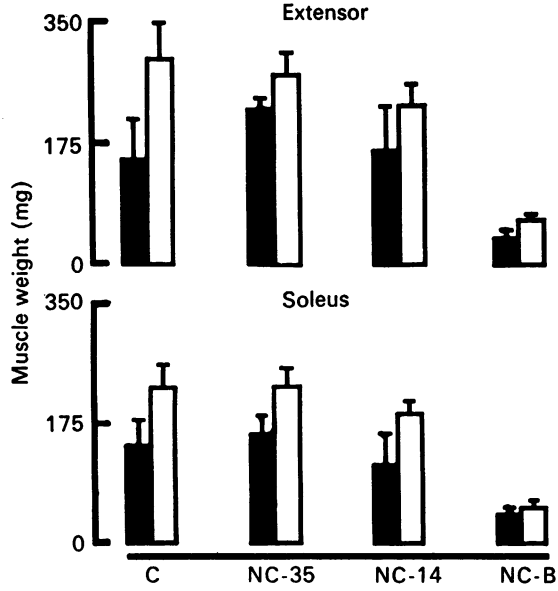
The weight of the extensor and soleus muscles whose nerves were crushed at birth never reached control levels. In contrast, nerve crush at 14 or 35 days after birth did not prevent the muscles from attaining weights which were equivalent to control (Text-fig. 1).

This decrease in mass of the nerve crush at birth muscles was due to a reduction in the number of mature extrafusal muscle fibres (Table 1) which also were small in diameter (Text-fig. 2). The diameter of fibres in the re-innervated extensor and soleus muscles was (mean ± S.E. of mean) $32{\cdot}9 \pm 0{\cdot}9\ \mu m$ ($n = 104$) and $50{\cdot}9 \pm 1{\cdot}9\ \mu m$ ($n = 107$), respectively, while the corresponding control values were $45{\cdot}6 \pm 1{\cdot}2\ \mu m$ ($n = 107$) and $64{\cdot}3 \pm 1{\cdot}8\ \mu m$ ($n = 104$) 8 months after birth.

The number of muscle spindles counted in three control extensor muscles was thirty-one, twenty-nine and fifty while the ipsilateral soleus muscles contained nineteen, fourteen and twenty-one of these structures. This difference between the fast twitch extensor and the slow twitch soleus

muscles did not appear following nerve crush at birth. No spindles were found in the three pairs of experimental muscles examined.

One feature of the nerve crush at birth extensor muscle which distinguished it from the ipsilateral soleus muscle was the presence of myotubes along one surface of the extensor (Pl. 1). Such immature cells were



Text-fig. 1. Effect of crushing the sciatic nerve at 35 (NC-35) or 14 (NC-14) days after birth or within 8 hr (NC-B) after birth upon the weight of the re-innervated extensor digitorum longus (extensor) and soleus muscles from 3- (filled bars) and 6- (open bars) month-old rats. Each bar represents the mean ± S.D. of mean for at least three muscles.

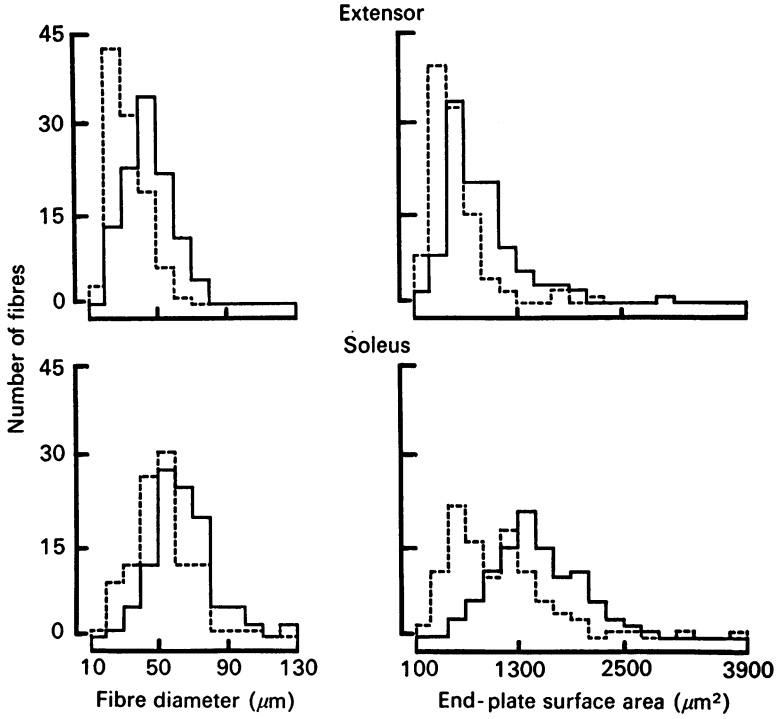
TABLE 1. Effect of nerve crush at birth upon the number of muscle spindles and extrafusal fibres in the extensor digitorum longus (extensor) and soleus muscles from a 3-month-old rat

Muscle	Spindles	Extrafusals
Extensor Control	31	2484
NC-B	0	336
Soleus Control	19	2708
NC-B	0	430

found in every experimental extensor muscle subjected to histological analysis (n = 30). At the mid-belly region of these muscles, the myotubes formed up to 20% of the cross-sectional area.

Staining for the whole muscle cholinesterase provided an indication of the distribution of end-plates along the surface of muscles, re-innervated

after nerve crush at birth. Normally, end-plates are distributed over the proximal width of the extensor muscle in a V-shaped pattern. In contrast, end-plates were scattered randomly over the entire surface of the innervated extensor muscle, although a large number of end-plates were clustered across the muscle surface at the point of nerve entry. Likewise, end-plates were randomly distributed over the surface of the re-innervated soleus muscle (Pl. 2, *A*) rather than forming the normal W pattern across the mid-belly of the muscle (Pl. 2, *B*).



Text-fig. 2. Distribution of diameters and end-plate surface areas for fibres in the normal (continuous lines) and re-innervated (dashed lines) extensor digitorum longus (extensor) and soleus muscles at 8 months after nerve crush at birth.

It is interesting to note that isolated fibres which had been stained for cholinesterase occasionally had several distinct end-plate regions (Pl. 3) which might be indicative of multi-innervation (Brown, Jansen & Van Essen, 1976). The incidence of multiple end-plates was the same in the normal (eight out of 104 fibres) and re-innervated (eight out of 107 fibres) soleus muscles. However, their incidence in the re-innervated extensor (thirty out of 104 fibres) was greater than in normal extensor muscles (four out of 107 fibres). These multiple end-plates were usually found within

17 μm of each other. However, three fibres had multiple end-plates which were 25–45 μm apart. Occasionally, these multi-end-plates appeared to be innervated by a nerve running along the surface of the muscle fibre.

Miniature end-plate potential (m.e.p.p.) frequency and the morphology of the neuromuscular junction

During maturation of the normal extensor and soleus muscles the frequency of miniature end-plate potentials (m.e.p.p.s) progressively increased in both muscles up to 35 days after birth. The m.e.p.p. frequency of the adult soleus muscle did not significantly exceed the level seen at this time. In contrast, the frequency of m.e.p.p.s continued to increase in the extensor muscle so that at 60, 180 (Table 2) and 365 (Table 3) days after birth, m.e.p.p. frequency was significantly greater for the fast twitch extensor muscle.

TABLE 2. Frequency of miniature end-plate potentials (sec^{-1}) in control (C) and re-innervated (NC-B) extensor digitorum longus (extensor) and soleus muscles from rats of varying ages

Days after birth	Extensor		Soleus	
	C	NC-B	C	NC-B
10	0·07 ± 0·01* (26)	0·02 ± 0·01 (4)	0·09 ± 0·01 (36)	—
17	0·23 ± 0·17 (73)	0·20 ± 0·08 (8)	0·22 ± 0·02 (67)	0·14 ± 0·07 (12)
25	1·09 ± 0·05 (94)	0·47 ± 0·07 (32)	0·80 ± 0·04 (5)	0·13 ± 0·03 (9)
35	1·56 ± 0·09 (59)	0·59 ± 0·07 (20)	1·24 ± 0·07 (59)	0·35 ± 0·07 (13)
60	2·10 ± 0·19 (27)	0·76 ± 0·09 (46)	1·36 ± 0·12 (34)	0·26 ± 0·06 (19)
180	2·71 ± 0·15 (32)	0·89 ± 0·06 (89)	1·36 ± 0·13 (34)	0·17 ± 0·02 (48)

* Mean ± S.E. of mean. Numbers in parentheses indicate number of fibres examined.

Re-innervation of the nerve crush at birth extensor and soleus muscles occurred within 17 days after birth. At this time, all of the muscles twitched when their nerves were pinched and m.e.p.p.s were present. The frequency of the spontaneous potentials progressively increased only in the extensor muscle, so that beginning at 25 days after birth their mean frequency in this muscle always exceeded values obtained from the re-innervated soleus. Despite the development of different m.e.p.p. frequencies in re-innervated extensor and soleus muscles, these values were always less than control (Table 2). This low frequency of m.e.p.p.s persisted for as long as 1 year

after nerve crush at birth and was not due simply to denervation and re-innervation, since muscles whose nerves were crushed at 14 or 35 days after birth had m.e.p.p. frequencies equivalent to control (Table 3).

TABLE 3. Frequency of miniature end-plate potentials (sec^{-1}) in re-innervated extensor digitorum longus (extensor) and soleus muscles from 1-year-old rats whose sciatic nerve was crushed at birth (NC-B) or at 14 (NC-14) or 35 (NC-35) days after birth

	Extensor	Soleus
NC-B	$0{\cdot}76 \pm 0{\cdot}07$* (49)	$0{\cdot}20 \pm 0{\cdot}03$ (14)
NC-14	$2{\cdot}61 \pm 0{\cdot}10$ (82)	$1{\cdot}05 \pm 0{\cdot}05$ (59)
NC-35	$2{\cdot}96 \pm 0{\cdot}13$ (53)	$1{\cdot}14 \pm 0{\cdot}08$ (52)

* Mean ± S.E. of mean. Numbers in parentheses indicate the number of fibres examined.

Kuno *et al.* (1971) have demonstrated that the frequency of m.e.p.p.s is directly related to the diameter of muscle fibres and the surface area of the cholinesterase-stained end-plate membrane. Therefore, the differences in m.e.p.p. frequency between the normal extensor and soleus muscles, as well as between normal and nerve crush at birth muscles, could be due to morphological differences at the neuromuscular junction. This possibility was investigated by measuring the area of the end-plate membrane and qualitatively examining the ultrastructure of the neuromuscular junction.

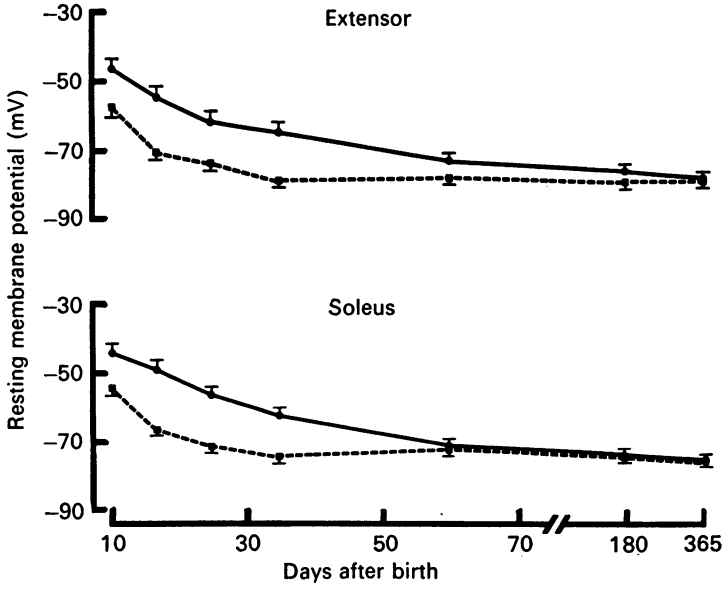
The area (mean ± S.E. of mean) of the end-plate region was $1516 \pm 45\ \mu m^2$ ($n = 104$) and $1059 \pm 58\ \mu m^2$ ($n = 107$) in the normal and re-innervated soleus muscles, respectively. These values were significantly greater than those obtained from the corresponding extensor muscles. End-plate surface area was $890 \pm 40\ \mu m^2$ ($n = 107$) in the normal and $509 \pm 31\ \mu m^2$ ($n = 104$) in the re-innervated extensor muscle. Since fibre diameter was also greater in the soleus muscles (see above; Text-fig. 2), the data indicates that in the rat, as in the frog (Kuno *et al.* 1971), larger muscle fibres have larger end-plate regions. It is important to note, however, that m.e.p.p. frequency was consistently greater in the extensor muscle in spite of the smaller surface area of the end-plate.

Ultrastructural investigation revealed qualitatively normal neuromuscular junctions in the re-innervated extensor (Pl. 4 *A*, *C*) and soleus (Pl. 4 *B*, *D*) muscles.

Electrical properties of the sarcolemma

Denervated mammalian muscle fibres are characterized by a decreased resting membrane potential (RMP: Locke & Solomon, 1967; McArdle &

Albuquerque, 1975), increased passive electrical properties of the sarcolemma (Albuquerque & Thesleff, 1968) and altered time and voltage characteristics, as well as lowered sensitivity to tetrodotoxin, of the action potentials (Redfern & Thesleff, 1971*a*,*b*). Since these alterations persist for some time after the beginning of re-innervation in the adult (McArdle & Albuquerque, 1973; Sellin & McArdle, 1977*a*,*b*), the trophic competence of the regenerated nerves was evaluated by examining these properties at 6 months after nerve crush at birth.



Text-fig. 3. Resting membrane potential of the extensor digitorum longus (extensor) and soleus muscles at various times after birth. The squares and interrupted lines represent control muscles and the circles and continuous lines represent muscles whose nerves were crushed at birth. Each point represents the mean of at least sixty fibres from at least three muscles. Vertical lines indicate S.E. of means.

A detailed analysis of the RMP (Text-fig. 3) and the passive electrical properties (Table 4) of the sarcolemma revealed that these were normal in the re-innervated muscles. The high value for input resistance recorded in the re-innervated extensor muscle was probably due to the smaller diameter of the fibres examined.

The voltage and time characteristics of the action potentials recorded in the re-innervated muscles were also normal (Table 5). In addition, the action potentials in the re-innervated muscles were blocked by TTX (Text-fig. 4 and Table 5).

TABLE 4. Passive electrical properties of the membrane of normal and re-innervated (NC-B) extensor digitorum longus (extensor) and soleus muscles at 6 months after birth*

Muscle	R_{in} (MΩ)	λ (mm)	τ_m (msec)	r_{calc} (μm)	R_m (Ω-cm²)	C_m (μF/cm²)
			Extensor			
Control	0·14 ± 0·01 (40)	0·77 ± 0·03	1·4 ± 0·1	41·2 ± 1·3	513 ± 35	2·8 ± 0·1
NC-B	0·19 ± 0·01 (41)	0·75 ± 0·04	1·6 ± 0·1	36·2 ± 2·0	544 ± 32	3·0 ± 0·2
			Soleus			
Control	0·17 ± 0·01 (35)	0·79 ± 0·02	1·6 ± 0·1	38·2 ± 1·2	642 ± 20	2·6 ± 0·1
NC-B	0·18 ± 0·01 (48)	0·78 ± 0·03	1·7 ± 0·1	36·9 ± 1·4	609 ± 40	3·1 ± 0·2

* Mean ± S.E. of mean. The values in parentheses are the number of fibres studied in at least five muscles.

R_{in}, input resistance; λ, space constant; τ, time constant; r, fibre radius; R_m, transverse resistance of a unit area; C_m, membrane capacitance per unit area. The myoplasmic resistance (R_i) was assumed to be 180 Ω-cm.

TABLE 5. Characteristics of action potentials recorded in control and nerve crushed at birth (NC-B) extensor digitorum longus (extensor) and soleus muscles of 6-month-old rats

Muscle	Overshoot (mV)	E_{crit} (mV)	Duration (msec)	dV/dt (V/sec)	Action potentials in presence of TTX*
		Extensor			
Control	+41·4 ± 0·6† (65)	−47·3 ± 0·5	1·22 ± 0·18	606 ± 30	0/35‡
NC-B	+40·6 ± 0·6 (53)	−45·0 ± 0·8	1·44 ± 0·04	535 ± 37	0/84
		Soleus			
Control	+36·6 ± 0·7 (50)	−44·6 ± 0·5	1·41 ± 0·03	454 ± 30	0/22
NC-B	+37·9 ± 0·7 (51)	−44·2 ± 1·03	1·29 ± 0·03	520 ± 37	0/132

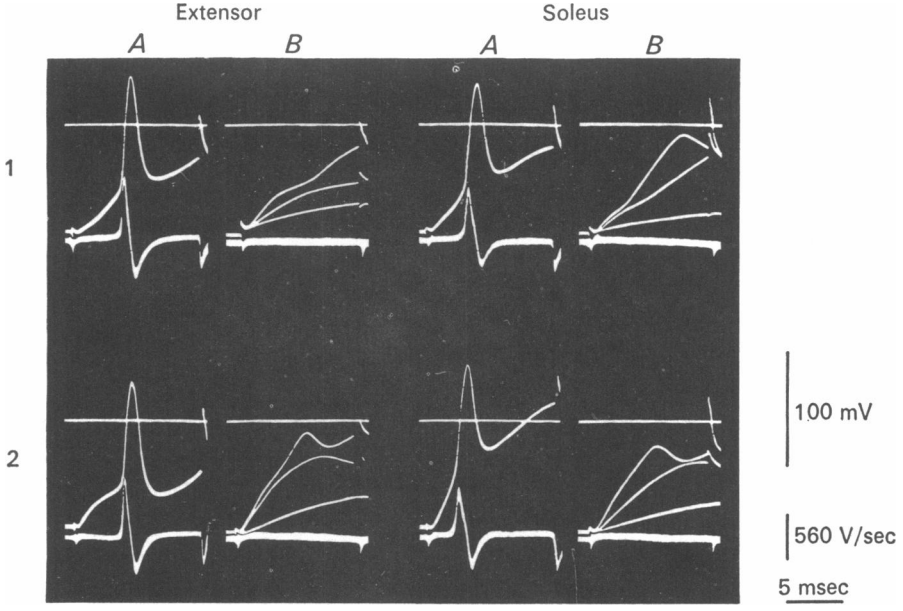
* Concentration of tetrodotoxin (TTX) was 1×10^{-6} g/ml.

† Mean ± S.E. of mean. Numbers in parentheses indicate the number of fibres examined.

‡ Number of fibres with TTX resistant action potentials/number of fibres examined.

Contracture and twitch characteristics and the morphology of the sarcomere

Fully differentiated fast and slow twitch muscle fibres can be distinguished on the basis of their response to caffeine (Gutmann & Sandow, 1965), twitch time course (Close, 1964) and the structure of the sarcomere (Padykula & Gauthier, 1970). These properties of re-innervated muscles were evaluated in 6 months old animals in order to determine the effects of nerve crush at birth upon the differentiation of distinct muscle types.

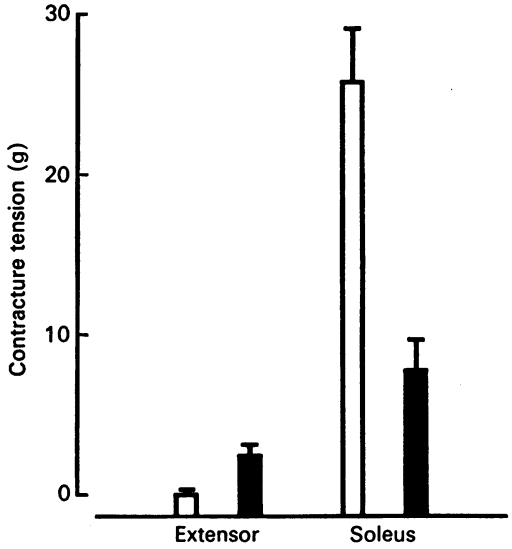


Text-fig. 4. Directly elicited action potentials in control (1) and nerve crush at birth (2) extensor digitorum longus (extensor) and soleus muscles before (*A*) and (*B*) exposure to tetrodotoxin (1×10^{-6} g/ml.). All records have been retouched.

The normal soleus muscle developed $26{\cdot}0 \pm 3{\cdot}2$ g (mean $\pm$ S.E. of mean, $n = 10$) of contracture tension after a 2 hr exposure to caffeine, while the ipsilateral extensor was much less affected. Only two of the ten extensor muscles tested responded to caffeine with 1 g and 2 g of tension. In contrast, nine of the re-innervated extensor muscles developed a caffeine-induced contracture. However, this response was significantly less than that of the re-innervated soleus muscle (Text-fig. 5).

While the time to peak tension was slightly prolonged for the re-innervated extensor, this value remained significantly less than that obtained from the normal and re-innervated soleus muscles (Table 6).

Though a quantitative ultrastructural evaluation was not performed, the T-tubules, triadic junctions as well as the longitudinal components of the sarcoplasmic reticulum of the re-innervated muscles had a normal appearance. There were, however, readily observable abnormalities of the fibrillar elements. The width of the Z line for all of the re-innervated ex-



Text-fig. 5. Caffeine-induced contracture tension developed by the normal (open bars) and re-innervated (filled bars) extensor digitorum longus (extensor) and soleus muscles at 6 months after birth. Each bar represents the mean + S.E. of mean for ten muscles.

TABLE 6. Time (T_c, msec) to peak tension (P, g) and time to half-relaxation ($T_{1/2}R$) of twitches produced by the normal and re-innerverted (NC-B) extensor digitorum longus (extensor) and soleus muscles at 6 months after birth

Muscle	T_c	$T_{1/2}R$	P
Extensor			
Control	14·9 ± 0·5 (8)	11·5 ± 1·0	18·1 ± 6·7
NC-B	17·6 ± 1·2 (10)	17·6 ± 2·0	5·3 ± 1·7
Soleus			
Control	33·8 ± 1·6 (9)	42·8 ± 2·1	8·9 ± 2·2
NC-B	36·4 ± 1·5 (11)	35·6 ± 1·6	3·2 ± 0·5

* Mean ± S.E. of mean. Numbers in parentheses indicate the number of muscles examined.

tensor muscles examined (Pl. 5, *B*) was more like that of the normal soleus (Pl. 6, *A*) rather than the normal extensor (Pl. 5, *A*). The width of the Z line was also somewhat enlarged for the re-innervated soleus muscle (Pl. 6, *B*). Streaming of the thickened Z line was more frequent and extensive than normal in both re-innervated muscles. This alteration was encountered in one or more sarcomeres placed deep within the fibre, but never was the entire Z line material involved. In some instances, streaming was associated with complete (Pl. 5, *C*) or partial (Pl. 6, *B*) dissolution of the Z line. In such areas of Z line breakdown, there also appeared to be a focal loss of mitochondria.

DISCUSSION

As reported in the other studies (McArdle & Albuquerque, 1973, 1975; McArdle, Garnes & Sellin, in the Press), m.e.p.p. frequency was significantly greater in the fast twitch extensor digitorum longus than in the slow twitch soleus muscle. Since end-plate surface area was larger for fibres in the soleus muscle, the difference in m.e.p.p. frequencies cannot be explained on the basis of this morphological parameter as proposed by Kuno, Turkanis & Weakly (1971). In fact, the present data suggests that their hypothesis can be applied only to synapses within the same muscle, and not to groups of synapses from different muscles. Since motor neurones to different muscle types have distinct electrophysiological (Eccles, Eccles & Lundberg, 1958; Ridge, 1967; Huizar, Kuno & Miyata, 1975) and morphological properties (Padykula & Gauthier, 1970; Ellisman, Rash, Staehelin & Porter, 1976), it is quite conceivable that there may be subtle differences in the process of transmitter release from these nerves.

The frequency of m.e.p.p.s was also greater in the re-innervated extensor muscles which were denervated at birth, despite the greater surface area of end-plates in the corresponding soleus muscles. Thus, crush of the sciatic nerve in the new-born rat does not prevent the differentiation of fast and slow motor neurones and re-innervation of the appropriate muscles (see below). However, m.e.p.p.s were significantly less frequent than normal in these re-innervated muscles, even though morphological analysis revealed a neuromuscular junction which was qualitatively normal in appearance (see also Teräväinen & Juntunen, 1968). There are several possible explanations for this reduction in m.e.p.p. frequency. For instance, it is known that post-junctional folds do not appear in rat skeletal muscle until 5 days after birth (Teräväinen, 1968) and denervation does not affect the longitudinal growth of muscle (Zelená & Hník, 1957). Thus, subtle abnormalities may exist in the apposition of the presynaptic and post-synaptic membranes of the regenerated neuromuscular junction. As shown in this study, denervation at 14 days after birth, a time at which post-junctional folds are

well developed (Terävainen & Juntunen, 1968; Kelley & Zachs, 1969), does not preclude the appearance of m.e.p.p. frequencies equivalent to control.

It is also conceivable that the processes involved in the release of transmitter are defective following nerve crush at birth. This hypothesis is supported by preliminary data which indicates a reduced sensitivity of the regenerated nerve terminal to the facilitatory action of elevated Ca^{2+} upon stimulus-evoked transmitter release (McArdle & Sansone, 1975). Crush of the muscle nerves at birth would profoundly alter transmission of peripheral information to the spinal cord, perhaps to the detriment of synaptogenesis (Changeux, Courège & Danchin, 1973). Antidromic, as well as orthodromic, axoplasmic flow would be discontinued during an early and rapid period of development. The re-innervated muscles would lack muscle spindles which may play a role in modulating synaptic processes. This is conceivable, since procedures known to alter muscle use also change spindle activity (Hník & Lessler, 1973) as well as transmission through the myotatic reflex arc. For instance, tenotomy facilitates (Kozak & Westerman, 1961; Robbins & Nelson, 1970) and peripheral nerve section reduces (Eccles & McIntyre, 1953; Eccles, Krnjević & Miledi, 1959) monosynaptic reflex activity. In turn, muscle disuse causes alterations of neuromuscular transmission (Robbins & Fischbach, 1971). Thus, changes in the nature of afferent impulse activity may play a role in the function and plasticity of central and peripheral synapses.

Neonatal muscle fibres are innervated by several functioning motor nerves. With time this multiple innervation is lost (Redfern, 1970). Benoit & Changeux (1975) have shown that tenotomy of neonatal muscle delays the elimination of multiple innervation and they attribute this to altered sensory feed-back. This may explain the high incidence of multiple end-plates found on the surface of fibres in the spindleless extensor muscle and the associated multiple innervation (Sansone & McArdle, unpublished observation). Thus, sensory input may influence the processes of synapse selection and formation of efficient motor units.

Since the disappearance of multiple innervation is believed to involve neurotrophic phenomena (McArdle, 1975; Frank, Jansen, Lømo & Westgaard, 1975) it is conceivable that sensory input may mediate this and other trophic effects of muscle activity in the intact animal.

The re-innervated extensor and soleus were differentiated not only with respect to m.e.p.p. frequency and end-plate surface area but also on the basis of their response to caffeine and the twitch course. Since the latter two properties are determined neurotrophically (Miledi & Stefani, 1969; Hoh, 1975) the data suggests that the muscles were re-innervated by the correct nerve (Mark, 1974). However, the half relaxation time and caffeine response of the re-innervated extensor muscle were greater than normal.

Also, the half relaxation time was shortened for the re-innervated soleus muscle. This could be due to some non-selective or cross-re-innervation. The extent of non-selective re-innervation may have been greater if the sciatic nerve was cut and a section removed rather than merely crushed.

Alterations of the Z line material similar to those shown for muscles re-innervated after nerve crush at birth have also been reported during various pathological states. Pellegrino & Franzini (1963) sectioned the sciatic nerve of adult rats and found an initial widening of the Z line which progressed to a generalized waviness and bending at 2 weeks post-denervation. This defect was found at several foci along the length of one fibre, but no more than two or three sarcomeres were affected. At later times after denervation, complete disorganization of the Z line was frequently observed. Hanzlíková & Schiaffino (1973) have further demonstrated the necessity of innervation in the development of the Z line. An altered Z line is also found during an early stage of progressive muscular dystrophy in man (Cullen & Fulthorpe, 1975) although it is not known whether that is due to denervation produced by the disease (McComas & Mrożek, 1967). It is important to note that, though Z line streaming is also encountered in normal muscle (Meltzer, Kuncl, Click & Yang, 1976), this occurs in approximately 2 % of the fibres, whereas the Z line was thickened in all of the re-innervated extensor muscles examined in the present study.

An intriguing explanation for the Z line abnormality in myopathic states derives from the studies of Busch, Stromer, Goll & Suzuki (1972). They have isolated a substance from rabbit skeletal muscle which causes the specific removal of Z lines from intact muscle fibres. It is postulated that the substance is normally inactive *in vivo*, due to compartmentalization and/or low levels of Ca^{2+}; the substance requires Ca^{2+} in excess of 0·1-mM for activity. Since the Ca^{2+} binding capacity of muscle is reduced in denervation (Stauber & Schottelius, 1975) and dystrophy (Sugita, Okomoto, Ebashi & Okimaka, 1967) intracellular Ca^{2+} could conceivably increase to levels sufficient to activate the process for Z line removal and subsequent myofibrillar breakdown. Likewise, the nerve crush at birth and re-innervated extensor muscle was apparently defective in the ability to store Ca^{2+}, as indicated by the excessive sensitivity to caffeine (Isaacson & Sandow, 1967). Thus, the widened Z line seen in this muscle may also be explained by the preceding hypothesis.

Of the post-synaptic properties examined in this study those related to the mechanical event were most severely affected by nerve crush at birth while membrane electrical properties were quite normal. Thus, the neurotrophic mechanisms regulating these two general aspects of muscle must be different. To summarize, the altered properties may be due to: (1) lack of a specific trophic influence during a critical stage of development;

(2) altered response of the muscles to neural regulation; (3) inadequate trophic function, or competence, of the regenerated motor neurone; (4) inadequate afferent information from the spindleless muscles. The latter two conditions may be interrelated. Further study of nerve crush at birth muscles, as well as pathological models (Duchen, 1975; Swash & Fox, 1975), will facilitate evaluation of such a neurotrophic mechanism.

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EXPLANATION OF PLATES

PLATE 1

A. Transverse section taken from the mid-belly region of a re-innervated extensor digitorum longus 6 months after nerve crush at birth. The arrows point to clusters of myotubes found in the re-innervated extensor muscle. Haematoxylin and eosin stained.

B. Transverse section taken from the mid-belly region of a re-innervated soleus muscle 6 months after nerve crush at birth. Clusters of myotubes are not found in the re-innervated soleus muscle. Haematoxylin and eosin stained.

C. Enlarged photomicrograph of the myotubular region of part *A*. Smaller, outlined arrows indicate obliquely-sectioned myotubes; black arrows point to clusters of transversely-sectioned myotubes. Note the prominent central nuclei in these cells.

PLATE 2

Whole muscle cholinesterase incubations used to demonstrate the location of motor end-plates.

A. The motor end-plates of this 8-month re-innervated soleus muscle are distributed throughout the length of the dorsal surface. There appears to be some clustering of the end-plates in the mid-belly region, the area of nerve entrance.

B. Motor end-plates of the control 8-month soleus muscle follow a wide W-shaped distribution across the dorsal mid-belly region.

PLATE 3

Single fibres of 8-month re-innervated extensor digitorum longus muscle that were incubated for cholinesterase.

A. This fibre has two motor end-plates of grossly uneven dimension. Their close proximity suggests that they are probably innervated by the same parent nerve.

B. These five discrete motor end-plates appear to be innervated by the common nerve indicated by the arrows.

PLATE 4

A. Control extensor digitorum longus motor end-plate from a 3-month-old rat. Longitudinal section.

B. Control soleus motor end-plate from a 6-month-old rat. Longitudinal section.

C. Motor end-plates of a re-innervated extensor digitorum longus muscle fibre at 3 months after nerve crush at birth. Tangential section. All cytoplasmic organelles of the nerve terminal are intact and a normal complement of synaptic vesicles is seen. At the arrows, the synaptic vesicles are gathered in nests opposite the post-junctional fold. The post-junctional folds are well formed and similar to control in depth.

D. Motor end-plate of a re-innervated soleus muscle fibre at 6 months after nerve crush at birth. Numerous synaptic vesicles are tightly and continuously packed adjacent to the presynaptic membrane. Post-junctional folds are normal in configuration.

PLATE 5

A. A longitudinally-sectioned control extensor digitorum longus muscle fibre from a 3-month-old rat. This fibre is characterized by a thin Z line, small mitochondria and an abundant sarcoplasmic reticulum. Z lines measure about 50 nm.

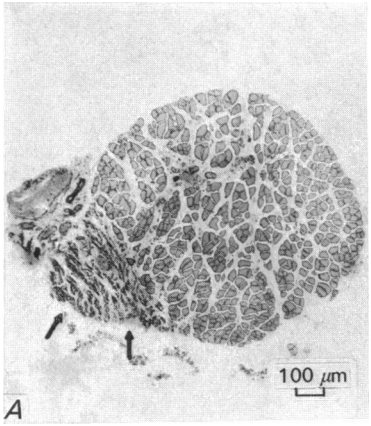
B. A longitudinally-sectioned fibre from a re-innervated extensor digitorum longus muscle at 3 months after nerve crush at birth. The thickened Z lines are seen to be quite wavy and the Z band material at the arrows is expanding and invading the entire I band region, as well as the A band areas. Sarcoplasmic reticular elements and T tubules are intact.

C. A longitudinally-sectioned fibre from a re-innervated extensor digitorum longus muscle at 3 months after nerve crush at birth. The Z band is dissolving and the A band elements are displaced into the I band region. Myosin appears to attach to the intact Z bands.

PLATE 6

A. A longitudinally-sectioned control soleus fibre with intact Z lines which measure 90–100 nm.

B. A longitudinally-sectioned re-innervated soleus muscle fibre at 6 months after nerve crush at birth. The Z band material, indicated by arrowheads, is quite thickened and invades the A band region. Thick filaments of myosin seem to attach themselves to the Z line.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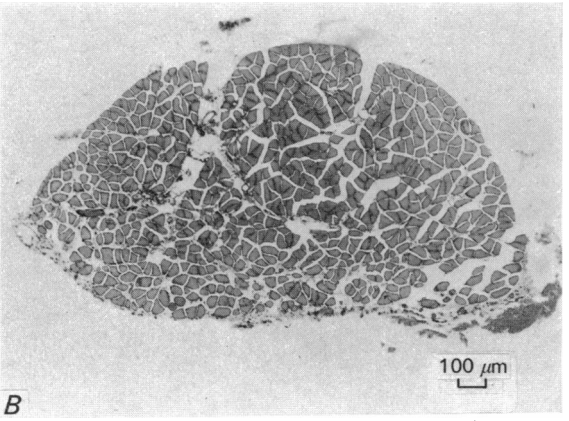
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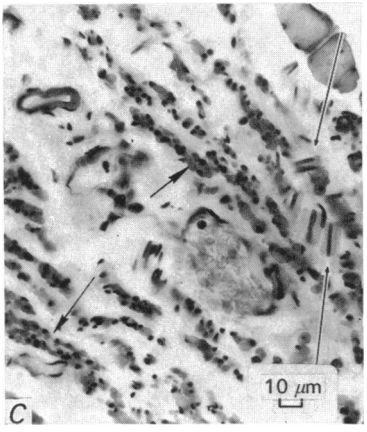
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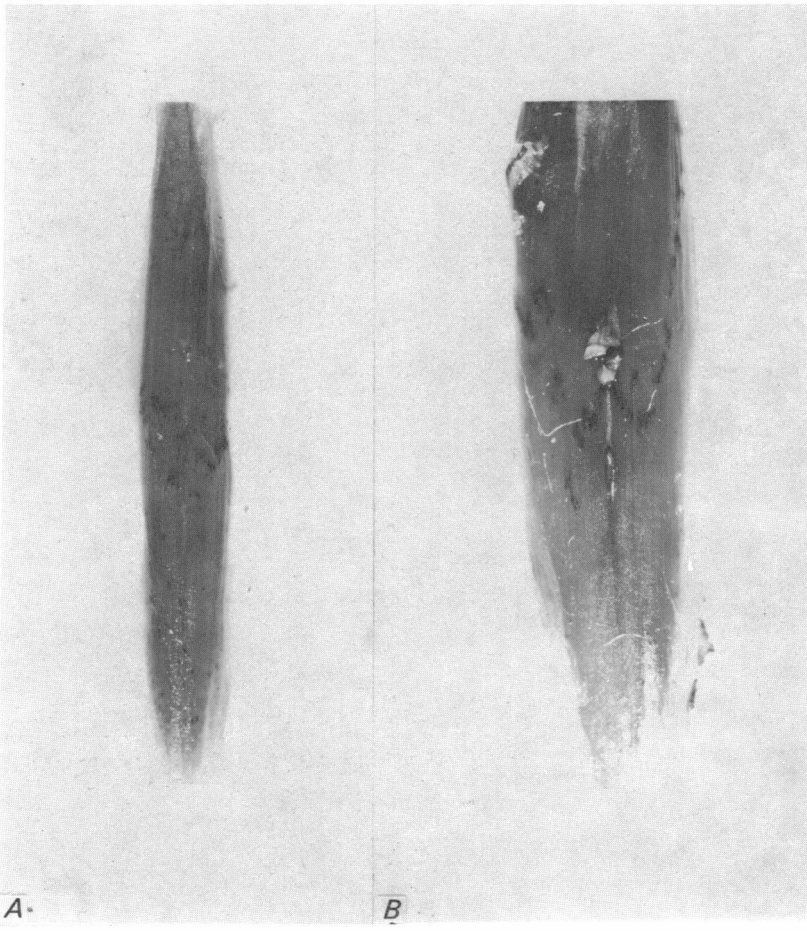
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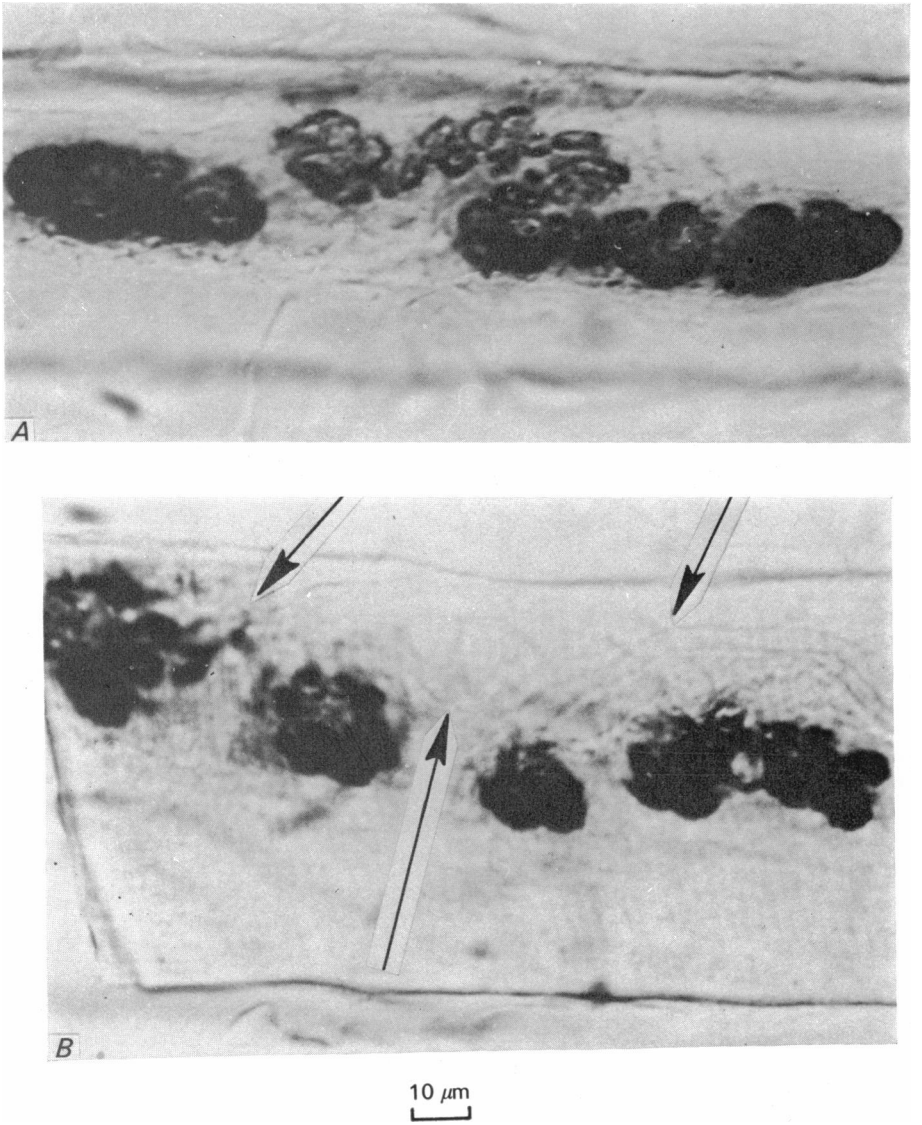
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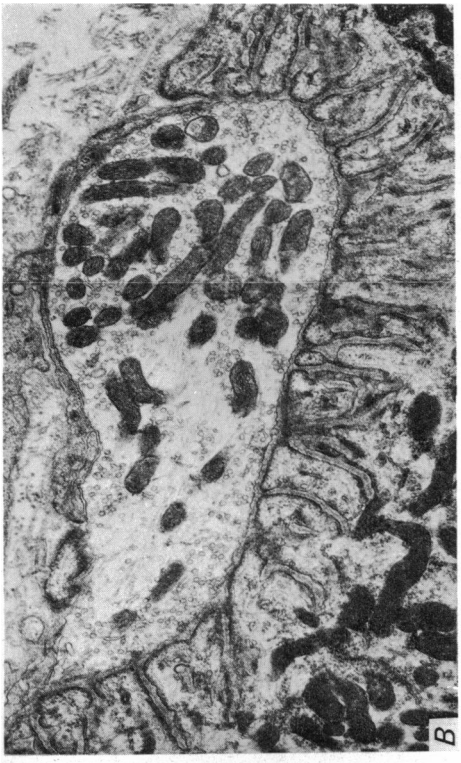

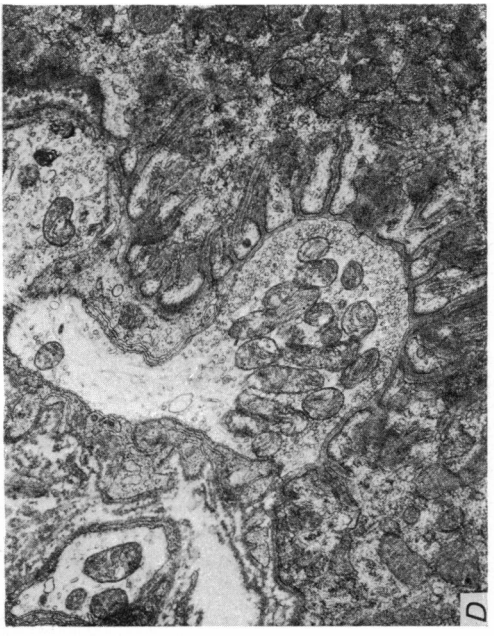

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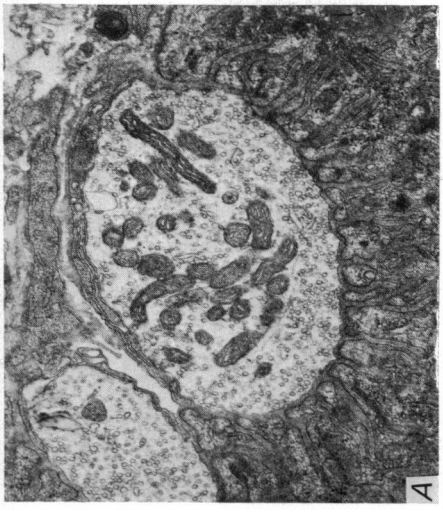
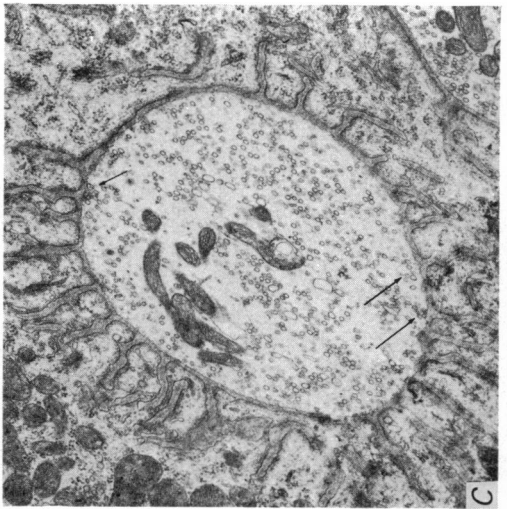
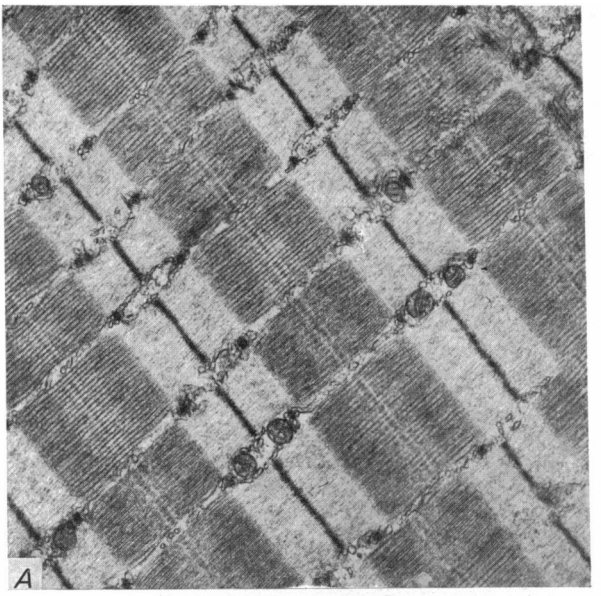
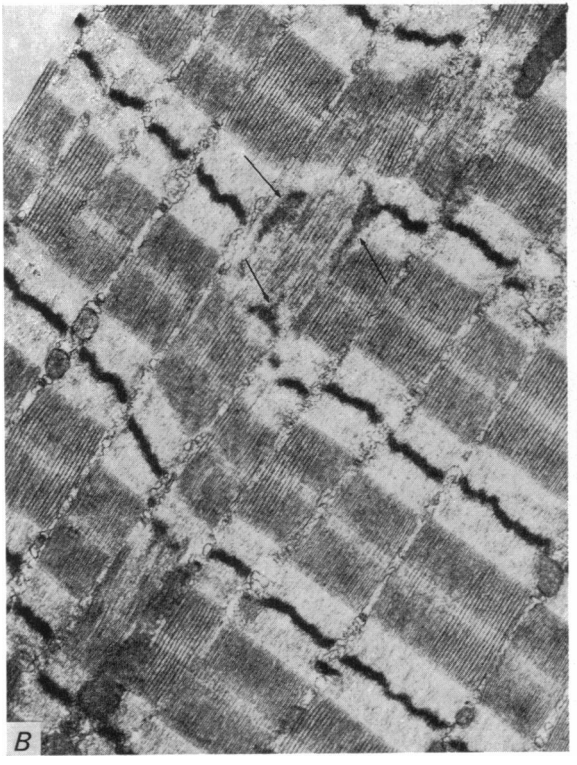
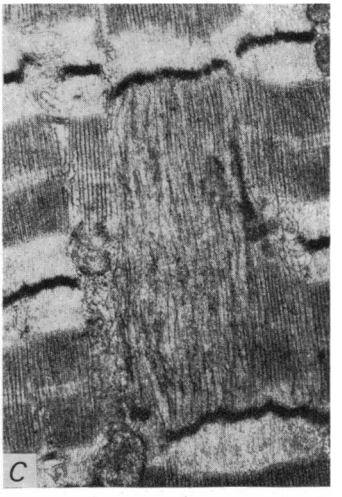


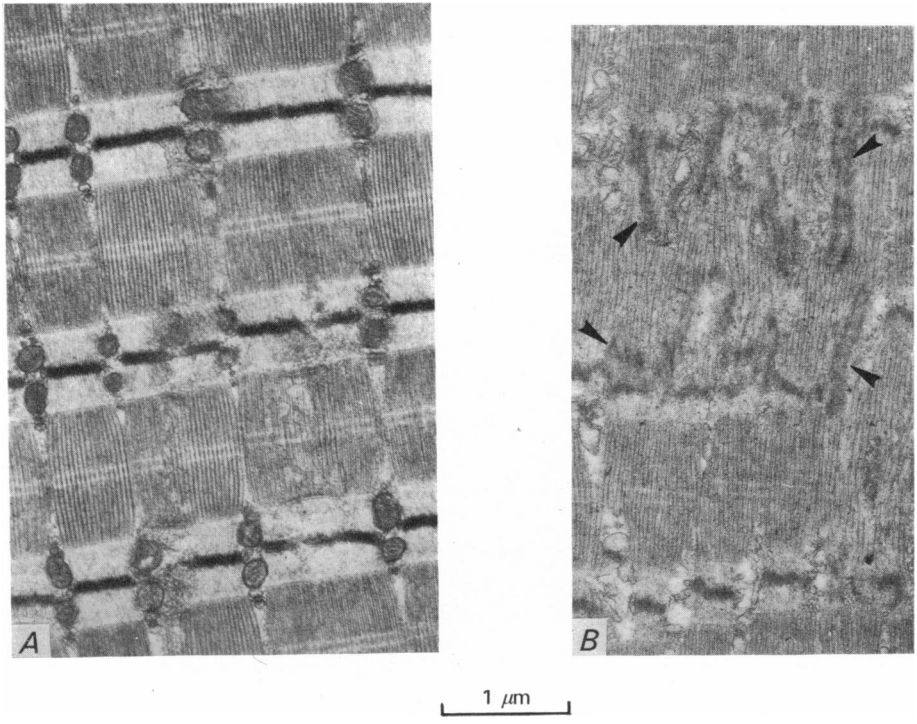








1 μm


A
B
1 μm