SPROUTING OF ACTIVE NERVE TERMINALS IN PARTIALLY INACTIVE MUSCLES OF THE RAT

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SUMMARY

1. Certain muscles in the hind foot of rats were partially paralysed by applying tetrodotoxin to part of their motor innervation. In these muscles motor nerve sprouting occurred from the terminals of the unblocked axons. The extent of sprouting was compared with that seen in totally paralysed and in partially denervated muscles.

2. Action potentials were blocked in the medial and lateral plantar nerves of adult rats for 5–13 days by continuous superfusion with a solution containing tetrodotoxin. The drug was delivered through a tube and nerve cuff from an osmotic pump placed intraperitoneally. Control experiments showed that nerve block was complete and that signs of nerve damage were absent in the animals included in the study.

3. Two muscles (the second lumbrical and *flexor digitorum brevis*), which received innervation only from the medial plantar nerve, were totally paralysed by the nerve block. Two different muscles (the fourth lumbrical and *flexor digitorum quinti brevis*) were only partially paralysed, since they received their innervation from the lateral plantar nerve and, in addition, from the sural nerve which was not blocked. One day before the final experiment, the lateral plantar nerve was cut, and its terminals degenerated. Thus in the partially paralysed muscles only the unblocked terminals from the sural nerve remained. These terminals were observed after staining with zinc iodide and osmium tetroxide. Similarly, terminals from the medial plantar nerve were examined in the totally blocked muscles from the same animal.

4. In other experiments, muscles were partially denervated by cutting the lateral plantar nerve in order to compare effects of nerve block and nerve section.

5. Sprouting occurred under all three conditions. Active terminals in the muscles partially paralysed for 5–7 days sprouted to the same extent as terminals in muscles totally blocked during the same period: about 35 % of the terminals had sprouts, and their average length was about 13 μm. Sprouting was more pronounced in partially denervated muscles: about 65 % of the terminals had sprouts and they averaged 24 μm in length. Collateral (preterminal) sprouts were seen only after partial denervation.

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6. Physiological and histological observations suggested that sprouts in paralysed muscles, unlike those in partially denervated muscles, seldom if ever made new synapses on neighbouring muscle fibres, even after 12–13 days of nerve block.

7. The results show that inactive muscle fibres cause active nerve terminals on neighbouring fibres to sprout, perhaps by releasing a diffusible, sprout-promoting factor, which is part of the stimulus for motor nerve sprouting in partially denervated muscles.

INTRODUCTION

Motor nerve terminals in adult mammalian skeletal muscle maintain precise and stable anatomical relationships with the muscle fibres they innervate, covering all of the specialized post-synaptic membrane and seldom straying beyond it. This normal pattern is not immutable, however, since the nerve terminals, when given the proper stimulus, can sprout, grow, and innervate neighbouring muscle fibres, while maintaining their original synaptic connexions. The stimuli for motor nerve sprouting have been investigated in several kinds of experiments, in particular after partial denervation and after pharmacological block of nerve-muscle activity.

After partial motor denervation, the remaining nerve terminals sprout and innervate adjacent denervated muscle fibres (Hoffmann, 1950; Edds, 1953; Brown & Ironton, 1978). Evidence suggests that one stimulus for sprouting in such muscles comes from degenerating nerves: injection of lipid extracts of peripheral nerves into normal muscles causes sprouting of motor nerve terminals (Hoffmann, 1950), as does dorsal root section, which produces degenerating sensory nerves without motor denervation (Brown, Holland & Ironton, 1978).

While products of nerve degeneration apparently provide a sufficient stimulus for sprouting, they are not necessary; sprouting can occur in the absence of degenerating nerves. Thus, motor terminals sprout if muscles are completely paralysed by blocking neuromuscular transmission with botulinum toxin (Duchen, 1970) or by blocking nerve activity with tetrodotoxin (Brown & Ironton, 1977). In these cases, the sprouting stimulus must come from a different source. Further experiments have shown that the source is not intrinsic to the blocked motor nerves, since the sprouting can be blocked by stimulation of the paralysed muscles, through implanted electrodes (Brown, Goodwin & Ironton, 1977) or foreign motor nerves (Duchen & Tonge, 1977). Thus the sprouting stimulus must come from the muscle fibres, as a result of inactivity.

It is not known whether an inactive muscle fibre stimulates sprouting only of its own nerve terminal, or whether it is capable of causing terminals on neighbouring muscle fibres to sprout, perhaps by releasing a diffusible sprout-promoting factor. Such a diffusible factor, in addition to the products of nerve degeneration, would operate in partially denervated muscles.

The present experiments were designed to determine whether inactive muscle fibres can cause sprouting of active terminals on neighbouring muscle fibres, in the absence of nerve degeneration products. The experiments involved blocking activity in some, but not all, motor axons to a muscle for 1–2 weeks. Subsequent examination of the active terminals revealed that sprouting had occurred. Thus inactive muscle fibres communicate with nerve terminals on neighbouring muscle fibres, causing them

to sprout. The degree of sprouting in the partially blocked muscle was compared to that seen in totally blocked and partially denervated muscle.

METHODS

Experiments were carried out on male and female Sprague Dawley rats bred from stock obtained from Charles River Laboratories. At the time of initial surgery the rats weighed 150–200 g. Operations were carried out using ether or sodium pentobarbitone (60 mg/kg) anaesthesia.

Chronic block of nerve conduction. Prolonged block of nerve action potentials was achieved in the medial plantar nerve and lateral plantar nerve by chronic superfusion with tetrodotoxin (TTX; Sankyo). This drug was used because it blocks action potentials without affecting axoplasmic transport (Lavoie, Collier & Tenenhouse, 1977). The TTX, dissolved in 0·9 % saline was contained in osmotic mini-pumps (Alza Corp., Palo Alto, California U.S.A.) which were implanted intraperitoneally. Two types of pump were used: Model 1701, which delivered its contents in 5–7 days, was filled with 250 µg TTX/ml.; Model 1702, which delivered its contents in 12–13 days, was filled with 500 µg TTX/ml. Both types of pump supplied TTX at a rate of about 0·3 µg/hr. Silastic tubing led from the osmotic pump to a moulded silastic cuff (length 3–4 mm; o.d., 2·2 mm; i.d., 2·0 mm). The tubing was threaded under the skin and the cuff was placed around the plantar nerves in the mid-calf region (Fig. 1).

The cuffs fitted quite loosely, minimizing the possibility of damage to the nerves through compression. The tubing was filled initially with the TTX solution. The cuff served to hold the opening of the tubing in the vicinity of the nerves and to keep the TTX from rapidly diffusing away. In eleven rats studied in earlier experiments, relatively thick walled cuffs of much smaller internal diameter were used. These cuffs often damaged the nerves. Those experiments are not included here.

The plantar muscles receive innervation through the medial and lateral plantar and the sural nerves. The fourth deep lumbrical muscle and flexor digitorum quinti brevis receive innervation from the lateral plantar nerve and often, in addition, from an anastomotic branch of the sural nerve. The flexor digitorum brevis receives innervation from the medial nerve and also often from an anastomotic branch of the lateral (Betz, 1977; Betz, Caldwell & Ribchester, 1979). The second deep lumbrical muscle receives innervation from the medial plantar nerve only. Chronic superfusion of TTX around the plantar nerves therefore caused the fourth deep lumbrical and the flexor digitorum quinti brevis to become partially inactive and the second lumbrical and the flexor digitorum brevis to become totally inactive (Fig. 1).

The degree of nerve conduction block was assessed daily by pinching the toe pads of the foot with forceps (see Results). Briefly, light pinching of the most medial toe pads on the contralateral (unblocked) foot caused a brisk leg withdrawal response, while the animal ignored strong pinches made to the medial toe pads on the blocked foot.

Removal of inactive nerve terminals before final experiment. When nerve block began to wear off after 5–7 days, the lateral plantar nerve was cut in order to allow its nerve terminals to degenerate. Control experiments (see Results) showed that motor nerve terminals degenerated and no longer stained with zinc iodide and osmium tetroxide 12–24 hr after nerve section. Rats were therefore sacrificed 24 hr after section of the lateral plantar. This procedure ensured that only the nerve terminals belonging to motor axons in the unblocked sural nerve were stained.

In early experiments the lateral plantar nerve was cut above the anastomotic branch to the medial as this was technically the simplest operation. However, this procedure restricted the use of other muscles in the foot as controls for nerve damage. In later experiments the lateral nerve was cut just proximal to the anastomotic branch of the sural nerve (Fig. 1). In those rats electrophysiological recordings and tension measurements (see below) were made from the flexor digitorum brevis and second deep lumbrical muscles respectively, to test for the possibility of nerve damage caused by initial surgery or by the cuff. As an additional control a piece of the lateral plantar nerve was removed at the time of denervation and processed for histological counts of the number of myelinated axons. The nerves were fixed in phosphate buffered 2·5 % glutaraldehyde and embedded in Epon. Transverse sections were taken at 1 µm and the sections were stained with toluidine blue and photographed. Myelinated axons were counted from × 2000 photomicrographs of the sections.

Partial denervation. In a separate group of rats the fourth deep lumbrical and flexor digitorum quinti brevis muscles were partially denervated by cutting the lateral plantar nerve above the anastomotic branch to the medial plantar nerve. This operation left the fourth lumbrical
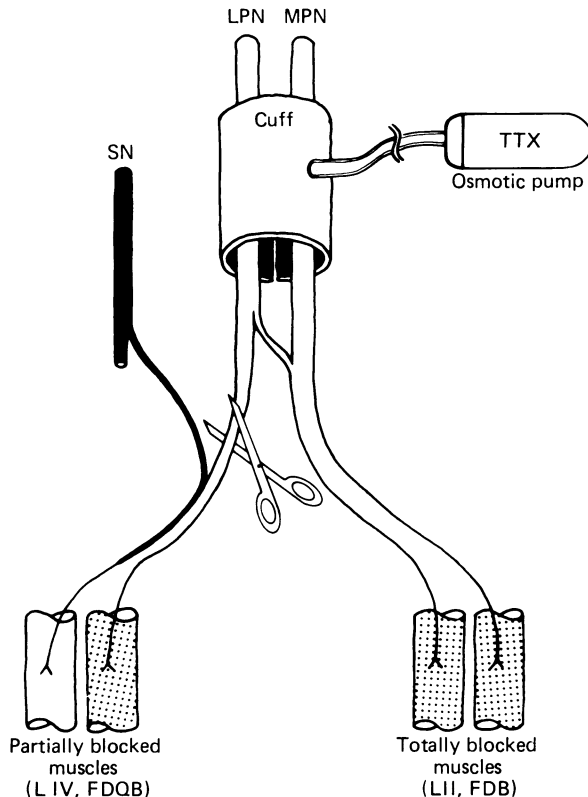

Fig. 1. Schematic diagram illustrating anatomy and experimental design. The osmotic pump, filled with TTX, was implanted intraperitoneally. Action potentials were completely blocked in two nerves (lateral and medial plantar, LPN and MPN) by the TTX, which was delivered from the pump through tubing to a cuff around the nerves in the lower leg. The sural nerve (SN), which lies on the other side of the leg, was not blocked. Thus, two muscles (fourth deep lumbrical and flexor digitorum quinti brevis, LIV and FDQB), which received inputs from both blocked (lateral plantar) and unblocked (sural) motor axons, were partially paralysed (note that the partial paralysis was due to *complete* block of *part* of the nerve supply). Two other muscles (second lumbrical and flexor digitorum brevis) were totally paralysed. In the diagram, paralysed muscle fibres are stippled. Nerve block lasted 4–13 days (see text). One day before the final experiment, the lateral plantar was cut (scissors in diagram). This operation had the effect that only the blocked axons to the partially paralysed muscles were severed. Their terminals degenerated, leaving only the active, unblocked terminals to be examined in the final experiment. The totally blocked muscles were unaffected by this operation.

muscles innervated by 1–5 sural motor axons (Betz, Caldwell & Ribchester, 1980). The animals were killed 5–6 days later.

Zinc iodide/osmium tetroxide staining. In the final experiments, muscles were stained with zinc iodide and osmium tetroxide (ZIO). The staining medium was made by a modification of the method of Akert & Sandri (1968). Zinc iodide was made freshly on each occasion by reacting 3 g powdered zinc with 1 g iodine in 40 ml. deionized water. The reaction proceeded until the

supernatant was colourless. Excess zinc was rapidly filtered off. To the filtrate, 8·5 ml. of an aqueous 2 % solution of osmium tetroxide was added and the mixture allowed to stand for 45–60 min. This mixture was then filtered and the muscles were pinned to dental wax and stained with the filtrate for 5 hr. The muscles were rinsed in deionized water, teased into bundles of up to ten fibres, and mounted in glycerol on microscope slides. Sprouts were identified as fine beaded outgrowths from the nerve terminal arborization. Measurements were made only on those sprouts which could be identified unambiguously, i.e. where the myelinated pre-terminal axon was visible or where multiple sprouts were observed. The length of nerve terminals (excluding sprouts) and the length of the longest terminal sprouts were measured at 500 × using an eyepiece graticule.

Intracellular recording and tension measurements. Physiological experiments were carried out to check for nerve damage and to search for signs of polyneuronal innervation of muscle fibres. Intracellular recordings were made using 4 M-potassium acetate-filled micro-electrodes of 40–80 MΩ resistance. In muscles with innervation from two nerves, each nerve was stimulated separately. In other muscles D-tubocurarine chloride was added to the bathing fluid and stimuli to the muscle nerves were graded in order to recruit synaptic inputs of different thresholds (Redfern, 1970).

Isometric tension recordings were made by attaching one tendon of a muscle to a transducer (Grass FTO3). The other tendon was pinned to the bottom of a Sylgard-lined dish. The resting length of the muscle was adjusted for maximal twitch responses. Motor units were counted from tension increments by grading the strength of stimuli applied to the nerve. Pulses of 1–10 V and 100 μsec duration were applied using suction electrodes. Maximal direct tension was evoked by pulses of 100 V amplitude and 1 msec duration from a suction electrode placed directly on the surface of the muscle. Polyneuronal innervation was assessed by recording the tetanic tension produced in response to separate stimulation of each of the two nerves innervating the muscle, compared with the total tetanic tension (Brown & Matthews, 1960). The muscles were stimulated at 50 Hz for 0·5–1 sec. In addition, the amount of polyneuronal innervation was assessed from the twitch tension seen on delaying by 10 msec, single stimuli applied to each nerve (Brown & Matthews, 1960). Specifically, the percentage of fibres innervated by axons in both the lateral plantar and sural nerves was given by:

$$\% \text{ dual} = \frac{(LS'/LS) - 1}{(S'/S) - 1} \times 100, \tag{1}$$

where LS is the twitch tension seen on stimulating both nerves simultaneously and LS' is the tension seen on delaying the stimulus applied to the sural nerve by 10 msec. S and S' are the tensions seen on applying two stimuli to the sural nerve simultaneously (S) or separated by a 10 msec delay (S').

RESULTS

Control experiments

The present experiments were done to determine whether partial inactivity in a muscle could elicit sprouting of motor nerve terminals on normal, active muscle fibres. It was especially important that three conditions be satisfied. First, the nerve block had to be complete in order to compare quantitatively the sprouting in partially blocked muscles with that in partially denervated muscles. Secondly, there had to be no nerve damage produced by the cuffs. Thirdly, a method had to be found to identify histologically the active nerve terminals in partially blocked muscles. These are described below.

Tests for total nerve block. In previous studies (e.g. Brown & Ironton, 1977; Lavoie et al. 1977; Thompson, Kuffler & Jansen, 1979) the absence of a toe spreading reflex was used to indicate completeness of motor nerve block. In those studies, TTX was applied to the whole sciatic nerve causing extensive paralysis of the lower hind limb. In the present experiments the TTX was applied to only a part of the nerve supply

innervating the plantar muscles and skin of the hind foot, and the toe spreading reflex was not a useful test of nerve block. An alternative behavioural test, which proved to be reliable and sensitive, involved observing leg withdrawal in response to pinching (with fine forceps) certain toe pads of the hind foot. Control experiments on seven rats, in which the three nerves, medial and lateral plantar and sural were cut in every possible combination shortly before behavioural testing, showed that the
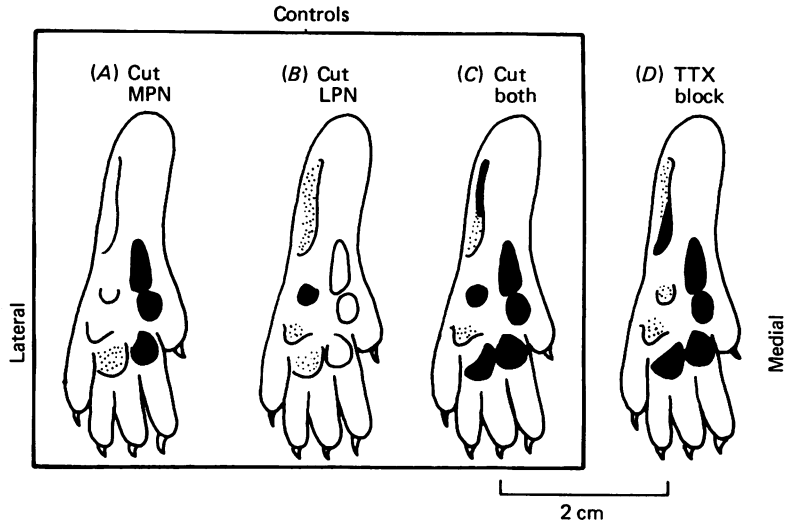

Fig. 2. Drawings of rat left hind feet showing foot pads on plantar surface that were pinched with forceps during daily tests of nerve block. Filled pads, total anaesthesia; stippled pads, partial anaesthesia; open pads, normal sensitivity. *A*, *B* and *C* from control animals in which the normal innervation pattern was mapped. Observations were made 1–2 days after cutting the medial plantar (*A*), the lateral plantar (*B*) or both nerves (*C*). The results show that the medial foot pads were innervated exclusively by the medial plantar nerve, and the lateral foot pads received innervation from the lateral plantar nerve and another source (shown in other experiments to be the sural nerve). Shown in *D* are typical results from an animal with 7 day *TTX* block of both medial and lateral plantar nerves. The pattern of anaesthesia is very similar to that seen after nerve section (*C*).

medial nerve always innervated the medial foot pads, and that the lateral plantar and sural shared innervation of the lateral foot pads. Typical results are illustrated in Fig. 2. In experimental animals, before the supply of TTX contained in the osmotic pump was exhausted, the behavioural responses (Fig. 2*D*) were very similar to control animals in which both plantar nerves had been cut (Fig. 2*C*). Thus the animals ignored strong pinches of the medial foot pads, and withdrew variably to pinches of the lateral foot pads, owing to innervation by the unaffected sural nerve. Light pinching or mere touching of the toe pads on the contralateral foot always evoked a brisk leg withdrawal.

This profound sensory anaesthesia reflected a concomitant motor nerve block, as demonstrated in three experimental animals. Three days (two rats) or 13 days (one rat) after pumps were implanted, these animals were tested behaviourally and showed signs of complete block, as described above. Then they were anaesthetized

and the plantar nerves were exposed and stimulated electrically above and below the cuff. Stimulation above the cuff caused no contraction while stimulation below the cuff caused a strong contraction of plantar muscles in each animal.

In nerve-blocked animals, the pattern of foot pad anaesthesia remained constant throughout the period of nerve block (up to 13 days). In the partially denervated animals, however, the areas of anaesthesia shrunk over a period of several days after the plantar nerves were cut. This partial recovery was observed long before reinnervation could have occurred. Devor, Schonfeld, Seltzer & Wall (1979) investigated this phenomenon more thoroughly, and showed that, after section of the plantar nerves, saphenous nerve terminals in adjacent areas of skin sprout and innervate the denervated plantar skin. Since the animals with plantar nerve block showed no behavioural signs of sprouting, it seems that nerve block alone is not a sufficient stimulus to provoke sprouting and formation of functional receptors by adjacent, active sensory terminals.

Tests for motor nerve damage. It was of critical importance that experimental animals which suffered any nerve damage be excluded from the study, since sprouting in these animals might be attributed to the partial denervation resulting from the nerve damage. Signs of nerve damage were sought at the time of the final experiment, by studying properties of the two totally blocked muscles (flexor digitorum brevis and second deep lumbrical). Several tests were used, as follows (see Table 2).

1. Evoked end-plate potentials were recorded with intracellular recording electrodes, ordinarily from flexor muscle fibres. In some animals, recordings were made from more than one muscle. Of the animals included in the study, every muscle fibre impaled ($n = 220$ in eight muscles from five rats) produced an evoked synaptic response. Although this is suggestive of lack of nerve damage, it is not a severe test since sprouts might have innervated fibres denervated by nerve damage. Thus other tests were also performed.

2. Motor units in second deep lumbrical muscles were counted by observing the number of increments of twitch tension in response to graded nerve stimulation (Fig. 3). The numbers were compared to contralateral control muscles. None of the experimental muscles showed signs of a reduced number of motor units (Table 2).

3. The numbers of myelinated axons were counted in histological sections of the nerves from the lateral plantar region. The nerve sample was taken when the nerve was sectioned, 1 day before the terminal experiment. In each case, the number of axons was about the same as in a similar control nerve (Table 2). In addition there were no obvious signs of damage to the nerves and the axon profiles were uniformly packed in the nerve (Pl. 1).

4. In four rats, totally blocked second lumbrical muscles were stained with ZIO and small bundles of fibres teased apart. All fibres examined received a nerve terminal, and while terminal sprouts were visible (Table 1), no *collateral* (pre-terminal) sprouts, which are characteristic of partially denervated muscle (Brown & Ironton, 1978) were observed.

In summary, the combined results of these tests suggest that no damage had been produced by the cuffs in the animals included in the study.

Identification of active nerve terminals. In partially blocked muscles, the blocked axons (in the lateral plantar nerve) were cut one day before the terminal experiment (see Fig. 1). Their terminals degenerated, and only the active terminals (from the sural nerve) stained with ZIO. Control experiments showed that the 1 day period

allowed complete degeneration of terminals of cut axons, without permitting sprouting from neighbouring intact terminals. First, five muscles were totally denervated one day before being stained with ZIO; no stained terminals were observed (cf. Slater & Wolfe, 1977). Secondly, three normal fourth deep lumbrical muscles were partially denervated by lateral plantar nerve section for 1 day, and then stained with ZIO. Of ninety-nine teased muscle fibres examined, four had terminal sprouts. This frequency
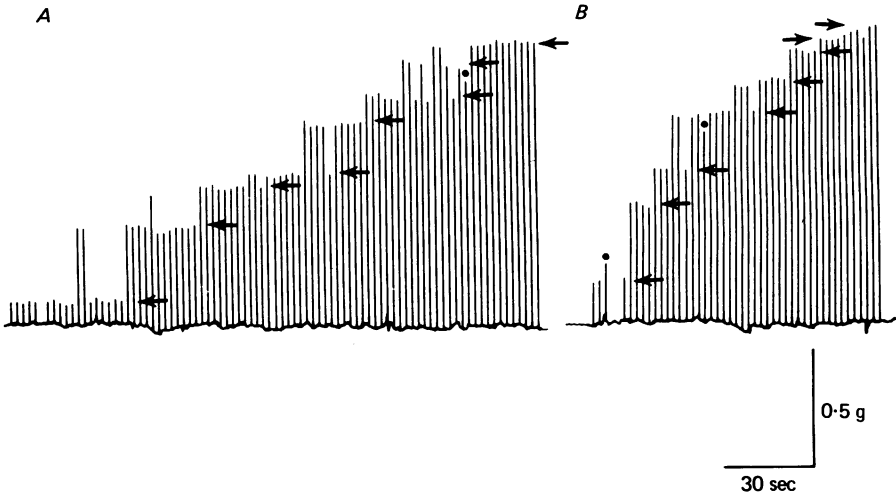

Fig. 3. Continuous pen recordings of isometric twitch tensions produced by second deep lumbrical muscles in response to increasingly strong nerve stimulation. *A*, from a muscle totally paralysed for 7 days. *B*, from the contralateral control muscle. The number of reproducible, discrete increments in tension indicates the number of motor units present. In each muscle, eight motor units can be counted (arrows), suggesting that the blocked nerve had not been damaged. Dots mark unique responses, which were not counted.

of sprouting is not significantly different from that seen in normal muscles (about 5%, cf. Barker & Ip, 1966; Tuffery, 1971), illustrated in Fig. 4 ($P > 0.05$, t test). Thus a 1 day period of partial denervation is not sufficiently long to cause sprouting of remaining terminals.

In summary, the osmotic pump assemblies were capable of maintaining a stable prolonged block of nerve conduction for 5–13 days without damaging the axons in the plantar nerves. A single day of partial denervation preceding the final experiment allowed degeneration of the nerve terminals of the severed axons without causing sprouting of the remaining terminals.

Experimental muscles

Plantar nerve block produced total paralysis of some muscles (second lumbrical and flexor digitorum brevis) and partial paralysis of others (fourth lumbrical and flexor digitorum quinti brevis), which also received innervation via the (unblocked) sural nerve. One aim of this study was to compare sprouting in these two types of muscles. In addition, some muscles were partially denervated for 5–6 days, to

compare effects of denervation and nerve block. The results show that sprouting occurred under all three conditions, but was most pronounced following partial denervation, as shown in Fig. 4.

Sprouting in partially inactive muscles. The morphology of active motor nerve terminals in partially inactive muscles (fourth lumbrical and flexor digitorum quinti brevis) was visualized by ZIO staining 1 day after cutting the lateral plantar nerve
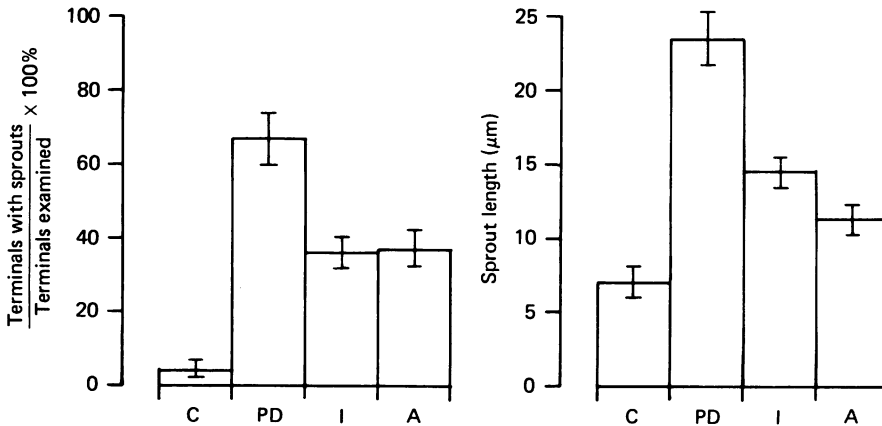

Fig. 4. Results of measurements of terminal sprouts in muscles under four different conditions. The left-hand graph shows the frequency at which sprouts were observed at end-plates, and the right-hand graph shows the mean length of the longest sprout at end-plates that showed any sprouting. *C*, control (normal) muscles; *PD*, partially denervated muscles; *I*, inactive muscles with nerve supply totally blocked by TTX; *A*, active terminals in muscles in which other axons were totally blocked by TTX (partially paralysed muscles). Results of *t* tests comparing different columns were the same for each graph, columns *I* and *A* are not significantly different; all other comparisons are significantly different at the $P < 0.01$ level. Vertical bars = S.E. of mean.

which had been blocked for the preceding 5–7 days. Sprouting of the active (sural) nerve terminals had clearly taken place (Fig. 5; Pl. 2). Terminal sprouts were seen in about 35 % of the terminals examined and the average length of the sprouts was about 11 μm (Fig. 4). No collateral (pre-terminal) sprouts were seen and only eight out of 104 of the terminal sprouts extended to adjacent muscle fibres (Table 1), and even these sprouts terminated only in small varicosities, which did not resemble mature terminal arborizations (Fig. 5).

Sprouting in totally inactive muscles. The second deep lumbrical muscle is innervated solely by the medial plantar nerve, and therefore was totally paralysed by the TTX. Also, cutting the lateral plantar nerve the day before the terminal experiment did not affect second lumbrical innervation. The extent of sprouting observed in ZIO these stained muscles was very similar to that seen in the fourth lumbrical (partially inactive) muscles described above. About 35 % of the terminals examined in second lumbrical muscles possessed sprouts and the sprouts were about 14 μm long (Fig. 4). Examples are shown in Fig. 5 and Pl. 2. Less than 5 % of the sprouts projected to adjacent muscle fibres, and no collateral sprouts were seen in the totally inactive muscles. These observations are very similar to those of Brown & Ironton (1977*b*),

who studied sprouting in mouse soleus and peroneus tertius muscles which had been totally paralysed by application of TTX impregnated cuffs to the sciatic nerve.

Longterm nerve block. It was of considerable interest to know whether sprouts in the paralysed muscles were capable of forming functional synaptic connexions with

TABLE 1. Nerve terminal sprouts

Rat	Duration (days)	Terminals with sprouts/ total terminals	Sprout length (μm) mean $\pm$ S.D.	Sprouts forming new terminals/ total sprouts
		(A) Partially blocked muscles		
1	5	12/30	7·4 ± 5·0	0/12
2	4	14/31	19·6 ± 8·1	3/14
3	4	15/35	16·2 ± 1·4	0/15
3a	4	11/29	12·0 ± 4·3	0/11
4	5	11/26	13·4 ± 4·5	1/11
5	7	21/55	12·3 ± 7·5	2/21
6	6	14/51	11·5 ± 12·5	2/14
7	7	6/30	9·8 ± 7·6	0/6
		(B) Totally blocked muscles		
1	5	15/43	16·9 ± 9·9	0/15
3	4	10/28	14·0 ± 5·8	0/10
5	7	17/39	7·8 ± 5·5	1/17
7	7	9/30	6·1 ± 2·3	0/9
		(C) Partially denervated muscles		
8	5	23/38	27·1 ± 23·0	7/23
9*	5	22/26	22·5 ± 12·3	12/22
10*	6	18/33	20·1 ± 8·2	11/18
11*	6	18/27	24·6 ± 12·3	12/18

* Single remaining motor unit

Summary of measurements of nerve terminals and sprouts stained with ZIO. Nerves (lateral and medial plantar) were blocked by TTX (*A*, *B*) or the lateral plantar was cut to produce partial denervation (*C*). *A*, partially blocked muscles were the flexor digitorum quinti brevis (rats 3a and 6) or fourth deep lumbrical (all others). The lateral plantar nerve was cut 1 day before staining the muscles, so that only active terminals were measured (see Methods). *B*, totally blocked muscles were the second deep lumbrical. *C*, partially denervated muscles were the fourth deep lumbrical.

neighbouring fibres, thereby producing muscle fibres with polyneuronal innervation. However, as noted above, after 5–7 days of nerve block, the sprouts were quite short. Thus, attempts were made to produce a nerve block of longer duration, using a long lasting osmotic pump (see Methods). In two animals, plantar nerve block was achieved for 12–13 days, without signs of nerve damage. In these animals the lateral plantar nerve was not sectioned one day before the final experiment. All four muscles with their nerve supplies were removed from each animal and examined for signs of polyneuronal innervation with intracellular and tension recording techniques, before staining with ZIO. Results were very similar to those observed after nerve block lasting only 5–7 days.

In the flexor digitorum brevis and fourth lumbrical muscles, evoked synaptic responses were recorded in response to stimulation of the three different nerves, the
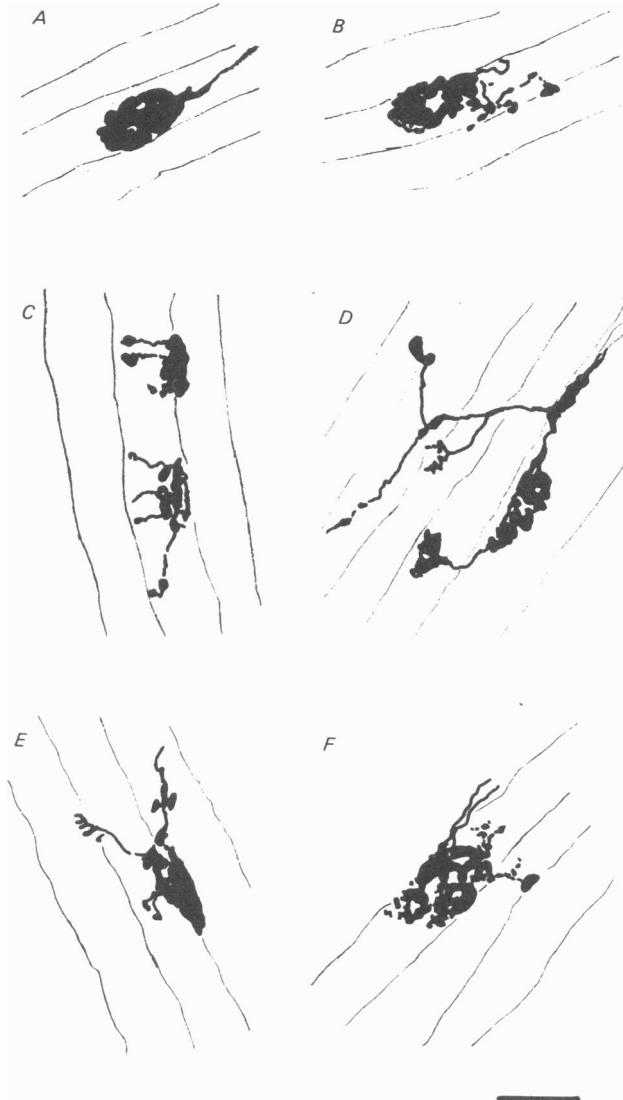
Fig. 5. Camera lucida drawings of motor nerve terminals stained with ZIO. Drawings were selected to show typical sprouting pattern, except *E* and *F*, which were rare occurrences. *A*, control showed no sprouts (fourth deep lumbrical muscle); *B*, muscle totally blocked by TTX for 5 days showed small varicose sprouts extending towards adjacent muscle fibre (second deep lumbrical muscle); *D*, muscle partially denervated for 5 days showed collateral (preterminal) sprouts which formed new terminal arborizations on adjacent muscle fibres (fourth lumbrical muscle); *C*, *E*, *F*, active terminals in muscles partially paralysed for 5 days (*C*), 7 days (*E*), and 13 days (*F*). Terminal sprouts are visible in each case, and in *E* and *F*, a sprout formed an ectopic terminal varicosity on a neighbouring fibre. Such contacts were rare in paralysed muscles. Drawings *A–D* correspond to photographs in Pl. 2*A–D*. Calibration bar = 25 *μ*m.

medial and lateral plantar and the sural. None of the fibres impaled received inputs from more than one nerve (twenty to fifty muscle fibres were sampled in each case).

In three of these muscles, resting membrane potentials were measured. The muscles were studied under identical conditions. In one totally blocked flexor digitorum brevis muscle, resting potentials averaged -40 ± 2 mV (mean $\pm$ S.E. of mean, $n = 10$ muscle fibres), while in the contralateral control, they averaged -67 ± 2 mV ($n = 12$). Similarly, in a partially blocked fourth

TABLE 2. Control experiments showing absence of damage to TTX-blocked nerves

Rat	Muscle†	A Muscle fibres (innervated/impaled)	B Number of motor units Blocked	Contralateral	C Number of LPN axons Blocked	Contralateral
5	FDB	55/55	—	—	633	599
	LII	—	9	8	—	—
6	FDB	22/22	—	—	847	811
	LII	—	8	8	—	—
7	FDB	30/30	—	—	962	943
	LII	—	8	9	—	—
12*	FDB	30/30	—	—	1472	—
	LII	—	8	9	—	—
	LIV	24/24	—	—	—	—
13*	FDB	27/27	—	—	1214	1050
	LII	10/10	7	7	—	—
	LIV	22/22	11	—	—	—

* LPN not cut in these animals, which had implanted 2 week pumps. See Text. Results were obtained from intracellular recordings (column *A*), tension measurements (column *B*), and histological counts of myelinated axons (column *C*).
† Abbreviations as in Fig. 1.

lumbrical muscle, paralysed fibres (innervated by the lateral plantar nerve) had lower resting potentials (mean $= 55 \pm 2$ mV, $n = 14$) than active fibres (innervated by the sural nerve) in the same muscle (70 ± 1 mV, $n = 8$). The differences between active and inactive muscle fibres in each case are highly significant ($P < 0.001$, t test). This suggests that inactivity causes a reduction in resting membrane potential, similar to that seen following denervation (McArdle & Albuquerque, 1973).

Tension recordings gave similar results. As illustrated in Fig. 6, the amount of polyneuronal innervation was estimated in two ways. First, tetanic stimulation of the two nerves to a muscle showed little overlap (Fig. 6*A*); that is, the tension generated by stimulating both nerves simultaneously was about the same as the sum of the tensions produced by stimulating each nerve alone. A second test for polyneuronal innervation, illustrated in Fig. 6*B*, was based on the twitch tensions seen when a short interval separated stimuli given to the lateral plantar or sural nerves or both (cf. Brown & Matthews, 1960). When one of the two nerves (sural) was given two stimuli separated by a 10 msec delay, a relatively large increment in the twitch tension was seen (Fig. 6*B*, left). Simultaneous stimulation of both lateral plantar and sural nerves caused a single synchronous contraction of every muscle fibre (Fig. 6*B* right, lower trace). When the stimulus to the sural nerve followed that to the lateral plantar by 10 msec, only fibres innervated by both nerves generated additional

tension. Only a very small increment in tension was seen on stimulating the two nerves in this way, further suggesting that few, if any, muscle fibres received poly-neuronal innervation.

Following the physiological experiments, muscles were examined histologically. Zinc iodide/osmium staining revealed motor nerve terminals with sprouts in these
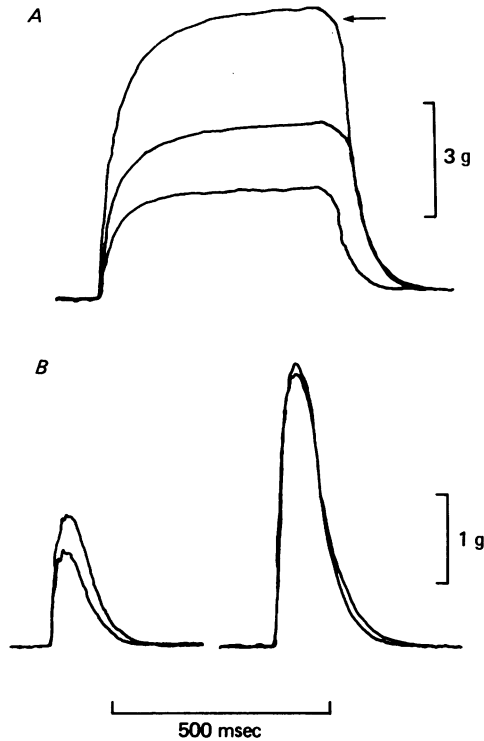

Fig. 6. Tracings of pen recordings of tension generated by a muscle (fourth deep lum-brical) that was partially blocked for 13 days, showing the lack of tension overlap, indicating that few, if any, muscle fibres were dually innervated. *A*, tetanic tensions produced by stimulation of the sural nerve (lower trace), lateral plantar (middle trace) and both nerves (upper trace). Stimulation frequency = 50/sec. Arrow shows sum of lower two traces, which is the same as that observed when both were stimulated, indi-cating a lack of dual innervation. *B*, twitch tensions from the same muscle. The amount of polyneuronal innervation was assessed by delaying stimuli applied to the separate nerves. Left traces: stimulation of sural nerve only; right traces: stimulation of lateral plantar and sural nerves simultaneously (lower trace) and delayed (the sural stimulated 10 msec after the lateral plantar, upper trace). Using eq. (1) in text, 7 % of the muscle fibres were calculated to be innervated by both nerves.

muscles, although, as in muscles partially blocked for only 5–7 days, the sprouts were generally short. Only one clear case of a long sprout terminating on an adjacent muscle fibre was seen. This unusual sprout is shown in Fig. 5 *F*. Thus, the histological picture was consistent with the physiological results: even after nearly 2 weeks of nerve block, few sprouts had formed functional synapses on neighbouring fibres.

Sprouting in partially denervated muscles. Four second deep lumbrical muscles were

partially denervated by lateral plantar nerve section for 5–6 days, and then examined for signs of sprouting. The tension produced by each remaining (sural) motor unit was $31{\cdot}5 \pm 5{\cdot}8\,\%$ (mean $\pm$ S.E. of means) of the total direct tension. This is nearly 4 times larger than in control muscles ($8{\cdot}6 \pm 0{\cdot}8\,\%$; Betz *et al.* 1979), and probably reflects sprouting and reinnervation of denervated fibres by the sural motor neurons (cf. Thompson, 1978).

This interpretation was supported by the histological appearance of motor nerve terminals. Two kinds of sprouts were identified in ZIO stained preparations. Terminal sprouts were fine axon outgrowths from the terminal arborization itself. In addition, pre-terminal sprouts (also called collateral sprouts) were identified as axon outgrowths arising from nodes of Ranvier of pre-terminal axons (cf. Edds, 1953; Brown & Ironton, 1978). Examples of both kinds of sprouts are shown in Fig. 5 *D* and Pl. 2 *D*). Quantitative results are given in Table 1 and Fig. 4. About 65 % of the terminals examined in partially denervated muscles possessed terminal sprouts and these sprouts were about 25 μm long (Fig. 4). Of all the sprouts examined, about 50 % extended to and formed terminal arborizations on adjacent, otherwise denervated muscle fibres (Table 1).

It was not possible to measure the frequency of collateral sprouting accurately, since the ZIO technique (which stained nerve terminals jet black) sometimes did not stain the pre-terminal axons at all. For this reason the number of muscle fibres innervated by sprouts might have been underestimated. If a terminal arborization could not be traced to a sprout, it was assumed to be an original nerve terminal.

Three of the partially denervated muscles were innervated by only a single motor axon after the lateral plantar nerve section. In these muscles all motor nerve terminals belonged to the same motor neurone. If sprouting is governed by a central command from the motor neurone cell body, one might expect that each nerve terminal would sprout to the same extent in such muscles. This was clearly not the case. In all three muscles, some motor nerve terminals had well defined sprouts, while other terminals, located on adjacent muscle fibres, possessed no sprouts at all.

In summary, two types of sprouting were observed in partially denervated muscles: one arising from terminals, and the other arising from pre-terminal nodes of Ranvier (the latter were not observed in the paralysed muscles described earlier). About two thirds of the end-plates had terminal sprouts, and many of these extended considerable distances to form contacts on neighbouring muscle fibres. This degree of terminal sprouting was significantly greater than in the paralysed muscles, where sprouts grew from about one third of the terminals, and very few reached other muscle fibres.

DISCUSSION

The results of this study show that paralysed muscle fibres were capable of causing active nerve terminals on adjacent active muscle fibres to sprout. The degree of sprouting was not as great, however, as that seen after partial denervation. Even after nearly two weeks of partial (or total) nerve block, few sprouts had grown to neighbouring fibres, and physiological signs of polyneuronal innervation were virtually absent. In similar experiments on the frog cardiac ganglion, Roper & Ko

(1978) also found no physiological signs of functional sprouting when they blocked some of the preganglionic axons with TTX.

A possible explanation for the difference between paralysed and denervated muscles is that, in partially denervated muscles, sprouts have ready access to vacant target sites, the denervated end-plates, for which growing axons show a high affinity (Sanes, Marshall & McMahan, 1978). In the blocked muscles, however, all end plates were occupied by motor nerve terminals. If sprouts, like cultured neurones, grow in a pulsatile way (Harrison, 1910), extending and withdrawing growth cones in search of vacant end plates, then it is perhaps not surprising that the sprouts were shorter in blocked muscles. By itself, however, this explanation seems inadequate, for the fraction of terminals showing any sprouts at all was significantly lower in blocked muscles (35 %) than in partially denervated muscles (65 %), and collateral sprouts were observed only in partially denervated preparations (cf. Brown & Ironton, 1978). This suggests the presence of a stronger or additional stimulus to sprouting in partially denervated muscles. As others have shown, the source of this additional stimulus is the degenerating nerves (Hoffman, 1950; Brown *et al.* 1978).

Thus, two independent sources of sprouting stimuli exist, whose effects are additive. It is perhaps simplest to envision the stimuli as diffusible sprout-promoting molecules, released by degenerating nerves and paralysed muscle fibres. However, different sprouting control mechanisms have been postulated in other preparations. For instance, amphibian skin sensory nerve terminals are thought to release an anti-sprouting factor normally, and the loss of this factor in denervated skin apparently allows intact terminals to sprout from adjacent regions (Diamond, Cooper, Turner & MacIntyre, 1976). Also, axotomized frog motor neurons can convey a sprouting stimulus to their contralateral homologues, producing sprouting in otherwise normal muscles (Rotshenker, 1978). This latter mechanism apparently does not operate in rats, since in the present study total denervation of a muscle did not cause sprouting in the contralateral muscle.

While it is clear that the sprouting stimuli in mammalian muscles arise from two different sources, it is not known whether the stimuli themselves are the same or different. Collateral sprouting is observed after partial denervation, but not after nerve block. This might suggest that nerve degeneration products are required for collateral sprouting. On the other hand, the two stimuli might be the same, but the response could be dose-dependent. That is, the nodes of Ranvier, from which collateral sprouts arise, are close to the source of the stimulus (degenerating nerves) in partially denervated muscles, but are somewhat isolated from the stimulus source (inactive muscle fibres) in paralysed muscles, by virtue of the diffusion barrier formed by the perineurium.

The site of action of the stimulus for terminal sprouting is also unclear. The stimulus might act directly on the nerve terminals, or it might act indirectly, for example by causing some change in the surface properties of the muscle fibre, that change being detected in turn by the nerve terminal. Experiments designed to detect changes in surface membrane properties (e.g. resistance to the blocking action of TTX) of innervated fibres in partially denervated muscles have produced contradictory results (Cangiano & Lutzemberger, 1977; Tiedt, Albuquerque & Guth, 1977). In other experiments, Pestronk & Drachman (1978) concluded that chronic block of

acetylcholine receptors with α-bungarotoxin prevented sprouting in botulinum poisoned muscles. This would suggest that the sprouting stimulus acts indirectly, by causing changes in acetylcholine receptors on the muscle fibre surface. This conclusion, however, seems inconsistent with an earlier study, in which sprouts were observed during reinnervation of muscles chronically paralysed with α-bungarotoxin (see Fig. 4 in Jansen & Van Essen, 1975). Thus it is not yet clear what role is played by the surface membrane of innervated fibres in causing terminal sprouting.

Regardless of how the stimulus is received by a motor neurone, its terminals do not respond very synchronously. That is, as the present results showed, in muscles with only a single remaining motor unit, some terminals developed well-defined sprouts, while others failed to sprout at all. This might reflect the ability of individual terminals to respond locally to the presence of sprout-promoting stimuli, although a non-uniform response to a central command from the cell body cannot be ruled out.

REFERENCES

AKERT, K. & SANDRI, C. (1968). An electron-microscopic study of zinc iodide/osmium impregnation of neurons. I. Staining of synaptic vesicles at cholinergic junctions. *Brain Res.* **7**, 286–295.

BARKER, D. & IP, M. C. (1966). Sprouting and degeneration of mammalian motor axons in normal and deafferented skeletal muscle. *Proc. R. Soc.* B **163**, 538–554.

BETZ, W. J. (1977). Motor nerve terminal sprouting in partially denervated muscle. *Proc. int. Union Physiol. Sci.* **13**, 73.

BETZ, W. J., CALDWELL, J. H. & RIBCHESTER, R. R. (1979). The size of motor units during postnatal development of rat lumbrical muscle. *J. Physiol.* **297**, 463–478.

BETZ, W. J., CALDWELL, J. H. & RIBCHESTER, R. R. (1980). The effects of partial denervation at birth on the development of muscle fibres and motor units in rat lumbrical muscle. *J. Physiol.* **303**, 265–279.

BROWN, M. C., GOODWIN, G. M. & IRONTON, R. (1977). Prevention of motor nerve sprouting in botulinum toxin poisoned mouse soleus muscles by direct stimulation of the muscle. *J. Physiol.* **267**, 42–43P.

BROWN, M. C., HOLLAND, R. L. & IRONTON, R. (1978). Degenerating nerve products affect innervated muscle fibres. *Nature, Lond.* **275**, 652–654.

BROWN, M. C. & IRONTON, R. (1977). Motor neurone sprouting induced by prolonged tetrodotoxin block of nerve action potentials. *Nature, Lond.* **265**, 459–461.

BROWN, M. C. & IRONTON, R. (1978). Sprouting and regression of neuromuscular synapses in partially denervated mammalian muscles. *J. Physiol.* **278**, 325–348.

BROWN, M. C. & MATTHEWS, P. B. C. (1960). An investigation into the possible existence of polyneuronal innervation of individual skeletal muscle in certain hindlimb muscles of the cat. *J. Physiol.* **151**, 436–457.

CANGIANO, A. & LUTZEMBERGER, L. (1977). Partial denervation affects both denervated and innervated fibers in the mammalian skeletal muscle. *Science, N.Y.* **196**, 542–545.

DEVOR, M., SCHONFELD, D., SELTZER, Z. & WALL, P. D. (1979). Two modes of cutaneous reinnervation following peripheral nerve injury. *J. comp. Neurol.* **185**, 211–220.

DIAMOND, J., COOPER, E., TURNER, C. & MacINTYRE, L. (1976). Trophic regulation of nerve sprouting. *Science, N.Y.* **193**, 371–377.

DUCHEN, L. W. (1970). Changes in motor innervation and cholinesterase localization induced by botulinum toxin in skeletal muscle of the mouse: differences between fast and slow muscle. *J. Neurol. Neurosurg. Psychiat.* **33**, 40–54.

W. J. BETZ AND OTHERS
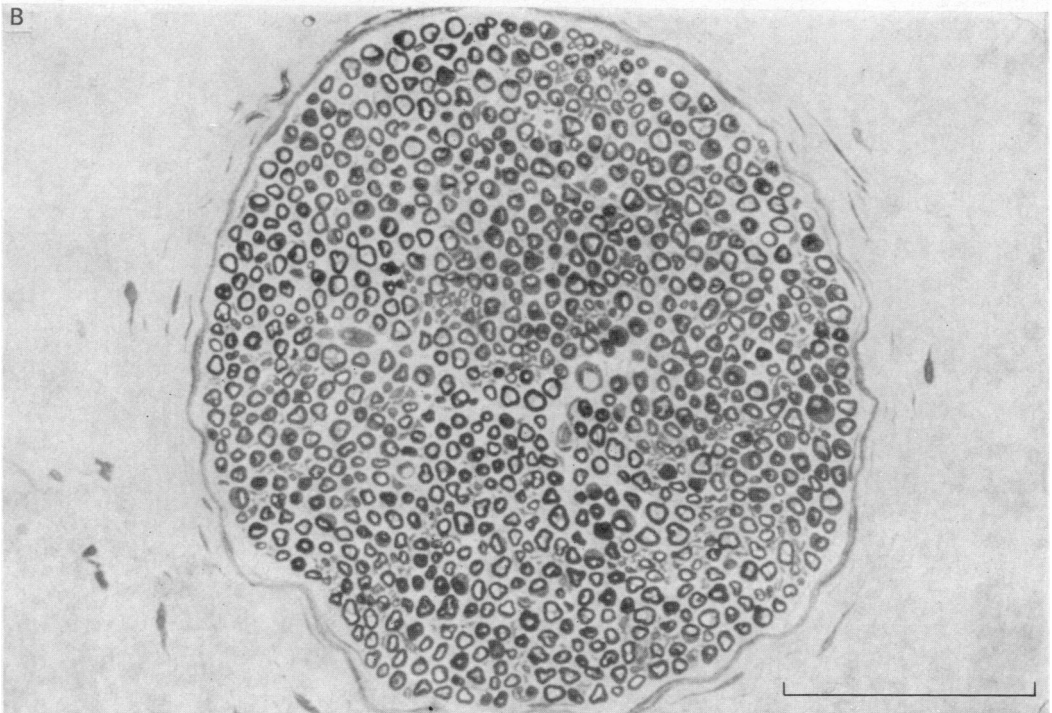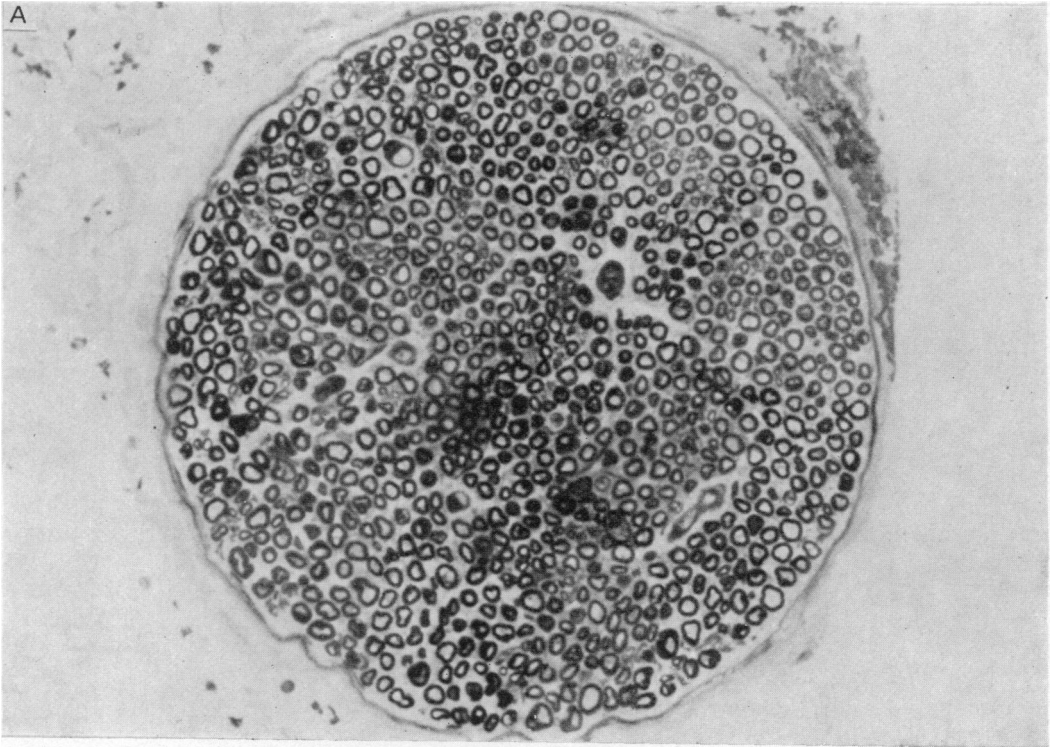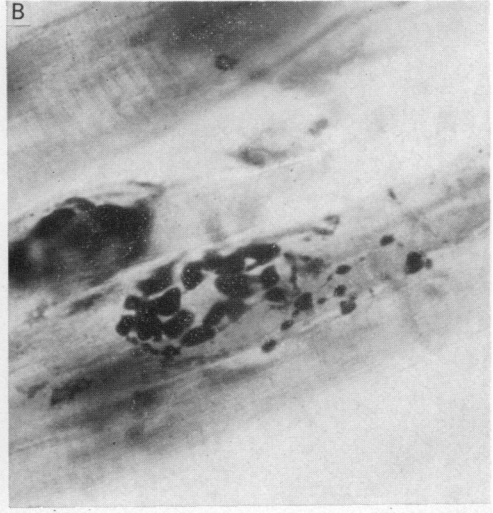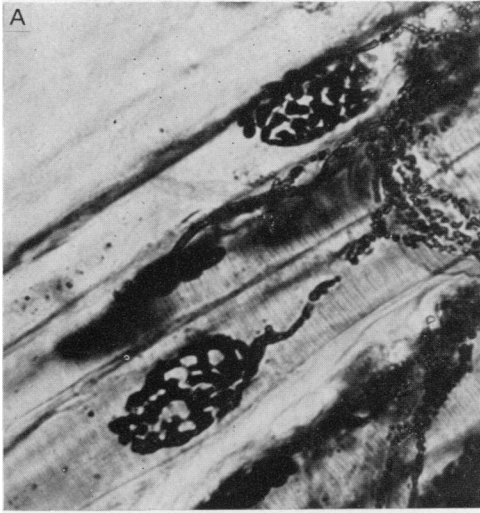
DUCHEN, L. W. & TONGE, D. A. (1977). The effects of implantation of an extra nerve on axonal sprouting usually induced by botulinum toxin in skeletal muscle of the mouse. *J. Anat.* **124,** 205–216.

EDDS, M. V., JR. (1953). Collateral nerve regeneration. *Q. Rev. Biol.* **28,** 260–276.

HARRISON, R. G. (1910). The outgrowth of the nerve fibre as a mode of protoplasmic movement. *J. exp. Zool.* **9,** 787–848.

HOFFMAN, H. (1950). Local re-innervation in partially denervated muscle: a histophysiological study. *Aust. J. exp. Biol. med. Sci.* **28,** 383–397.

JANSEN, J. K. S. & VAN ESSEN, D. C. (1975). Re-innervation of rat skeletal muscle in the presence of α-bungarotoxin. *J. Physiol.* **250,** 651–667.

LAVOIE, P.-A., COLLIER, B. & TENENHOUSE, A. (1977). Role of skeletal muscle activity in the control of muscle acetylcholine sensitivity. *Expl Neurol.* **54,** 148–171.

MCARDLE, J. J. & ALBUQUERQUE, E. X. (1973). A study of the reinnervation of fast and slow mammalian muscle. *J. gen. Physiol.* **61,** 1–23.

PESTRONK, A. & DRACHMAN, D. B. (1978). Motor nerve sprouting and acetylcholine receptors. *Science, N.Y.* **199,** 1223–1225.

REDFERN, P. A. (1970). Neuromuscular transmission in new-born rats. *J. Physiol.* **209,** 701–709.

ROPER, S. & KO, C.-P. (1978). Impulse blockade in frog cardiac ganglion does not resemble partial denervation in changing synaptic organization. *Science, N.Y.* **202,** 66–68.

ROTSHENKER, S. (1978). Sprouting of intact motor neurons induced by neuronal lesion in the absence of denervated muscle fibers and degenerating axons. *Brain Res.* **155,** 354–356.

SANES, J. R., MARSHALL, L. M. & MCMAHAN, U. J. (1978). Reinnervation of muscle fiber basal lamina after removal of myofibers. *J. cell Biol.* **78,** 176–198.

SLATER, C. R. & WOLFE, A. F. R. (1977). Abnormal reinnervation of muscles of dystrophic mice. *J. Physiol.* **275,** 73–74P.

TIEDT, T. N., ALBUQUERQUE, E. X. & GUTH, L. (1977). Degenerating nerve fibre products do not alter physiological properties of adjacent innervated skeletal muscle fibers. *Science, N.Y.* **198,** 839–841.

THOMPSON, W. (1978). Reinnervation of partially denervated rat soleus muscle. *Acta physiol. scand.* **103,** 81–91.

THOMPSON, W., KUFFLER, D. P. & JANSEN, J. K. S. (1979). The effect of prolonged, reversible block of nerve impulses on the elimination of polyneuronal innervation of newborn rat skeletal muscle fibers. *Neuroscience* **4,** 271–282.

TUFFERY, A. R. (1971). Growth and degeneration of motor end-plates in normal cat hindlimb muscle. *J. Anat.* **110,** 221-247.

EXPLANATION OF PLATES

PLATE 1

Transverse sections of the lateral plantar nerves from a rat in which one of the nerves (*A*) was superfused with tetrodotoxin for 7 days. No evidence, qualitative or quantitative, of axonal damage can be seen. *B*, contralateral control nerve. Calibration bar = 65 μm.

PLATE 2

Zinc iodide and osmium tetroxide staining of motor nerve terminals. Camera lucida drawings of the same fields are shown in Fig. 5. *A*, control muscle; *B*, muscle totally paralysed for 5 days showing terminal sprouts; *C*, muscle partially paralysed for 6 days (blocked axons were cut one day before staining) showing sprouts arising from formerly active nerve terminals; *D*, muscle partially denervated for 5 days showing collateral (preterminal) and terminal sprouts, one of which has contacted an adjacent muscle fibre. Calibration bar = 25 μm.