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The effects of implantation of an extra nerve on axonal sprouting usually induced by botulinum toxin in skeletal muscle of the mouse

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INTRODUCTION

Botulinum toxin prevents the release of acetylcholine from motor nerve terminals (Burgen, Dickens & Zatman, 1949) and causes prolonged paralysis of skeletal muscle. For several weeks after the injection of sublethal quantities of the toxin into muscle, sprouting occurs from the affected axonal terminals which results in the formation of new motor end-plates (Duchen & Strich, 1968; Duchen, 1970*a*). The axonal sprouting probably facilitates the recovery of neuromuscular transmission, which is usually complete after 3 months (Tonge, 1974*a*).

It is known that if an extra nerve is implanted into a normally innervated muscle it will not establish new motor-end plates unless the original innervation is interrupted (Elsberg, 1917; Gwyn & Aitken, 1966). Fex, Sonesson, Thesleff & Zelena (1966) showed that a muscle paralysed by botulinum toxin will become innervated by an extra nerve implanted in it. It was of interest to know whether the implantation of an extra nerve would have any effect on the axonal sprouting usually induced by botulinum toxin at the original end-plates. To investigate this question the common peroneal nerve was implanted into the soleus in the mouse prior to the injection of toxin. Preliminary observations (Tonge, 1974*b*; Duchen, Rogers, Stolkin & Tonge, 1975) showed that both axonal sprouting and the recovery of transmission at the original nerve terminals are inhibited.

METHODS

Adult albino mice (weighing about 30 g) of both sexes were used. Anaesthesia was induced by Avertin (Bayer) given intraperitoneally (0·5 g/kg). The right common peroneal nerve (CPN) was divided as it crossed the fibula and the cut end implanted into the end-plate-free zone of soleus near the proximal tendon. The femoral nerve was crushed to immobilize temporarily the right leg in order to prevent the implanted nerve from being pulled out of the muscle. Incisions were closed with steel clips.

Two weeks were allowed to elapse after the implantation of CPN into soleus so that axons from CPN could grow into the muscle (see Fex & Thesleff, 1967). A sublethal dose of type A botulinum toxin dissolved in 0·05–0·01 ml sterile phosphate buffer containing 0·2 % gelatin was then injected into the calf muscles. About 100 mice were used. The dose of toxin was sufficient to cause paralysis of the right hind leg muscles for several weeks. In 6 animals CPN was implanted into soleus and then

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cut 1 cm proximally. In 20 mice implantation of CPN was not followed by any further treatment. Toxin was also injected into leg muscles of many normal unoperated mice. Animals were allowed to survive after the different experimental procedures for periods of time ranging from 1 day to 8 months.

Electrophysiological methods

Under anaesthesia, soleus together with short lengths of the two nerves were removed from about 30 mice of the various groups for study *in vitro*. Neuromuscular transmission was assessed by the tension developed in response to indirect stimulation under isometric conditions at 30 °C within 10 minutes of removal. During this period of time *in vitro* there is no deterioration of the nerve–muscle preparation and responses to nerve stimulation are well maintained. After the completion of the electrophysiological investigations the muscles were blotted dry and weighed on a torsion balance.

The mammalian Ringer solution had the following composition (in m-mole/l): NaCl, 115; KCl, 3·5; $CaCl_2$, 2; $MgSO_4$, 1; $NaHCO_3$, 25; KH_2PO_4, 1; glucose, 10. The solution was continuously gassed with 95 % O_2/5 % CO_2.

Morphological methods

Under anaesthesia, mice were perfused with 10 % formalin in 1 % calcium acetate at 4 °C through the heart and the soleus was dissected out with its nerves. In some cases the soleus was fixed after *in vitro* electrophysiological investigations. Serial longitudinal frozen sections of the whole muscle were cut at 20 μm and stained by a modification of the method of Koelle & Friedenwald (1949) for the localization of cholinesterase (pH of substrate 5·6; incubation 20 minutes) and then subsequently treated by the method of Namba, Nakamura & Grob (1967) for the demonstration of nerve fibres by silver impregnation.

RESULTS

Physiological observations

When CPN was implanted into the soleus and no further treatment was given, the nerve grew into the muscle; but when stimulated, only a very few fibres close to the site of implantation contracted, with negligible tension development. When a contraction was measurable in this group of animals it did not exceed 4 % of the response to stimulation of the original nerve. Stimulation of the original nerve to soleus always evoked a normal response. After botulinum toxin, tension developed by the soleus in response to stimulation of CPN increased rapidly during the first week and then more slowly during the next few weeks (Fig. 1). The muscle remained paralysed to stimulation of its original nerve for 2 weeks, after which there was slow but progressive return of transmission. Generally the rate of recovery of transmission at the original nerve terminals was considerably delayed when compared with recovery in unoperated muscles paralysed by botulinum toxin (Tonge, 1974*a*). Normally there is complete recovery of transmission within 3 months of the administration of toxin, whereas after implantation of CPN recovery at a varying proportion (up to 25 %) of the original end-plates had not occurred even at 8 months in some cases. In animals surviving 3 months or more after the injection of botulinum toxin the sum of the tensions developed by soleus in response to stimulation of each nerve separately was greater than the tension developed in response to stimulation of both

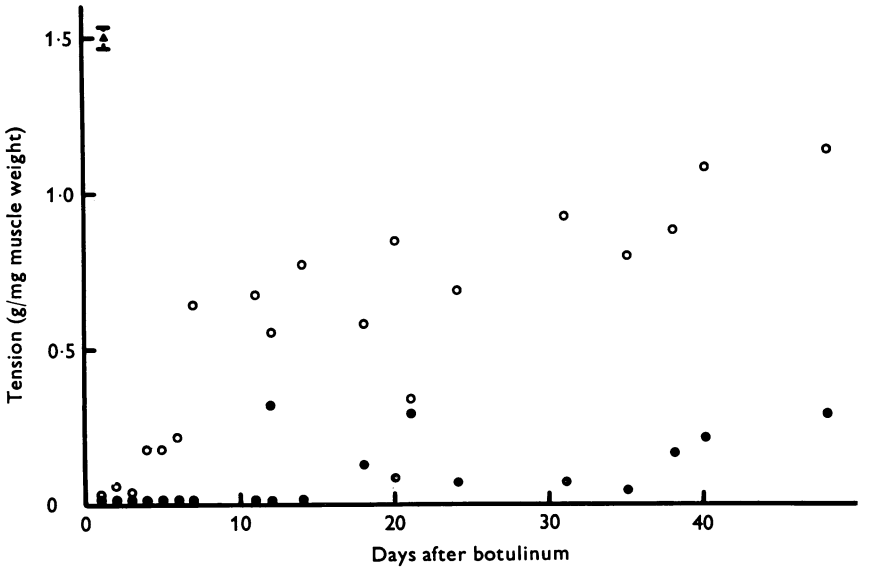

Fig. 1. Tension developed by soleus in response to indirect stimulation at 100/sec for 2 seconds. The mean response (±S.D.) to stimulation of the soleus nerve of four muscles 2 weeks after implantation of CPN is shown (▲). After botulinum toxin stimulation of the soleus nerve (●) elicits no contraction for 14 days, whereas stimulation of CPN (○) in the same muscles causes a strong contraction within a week.

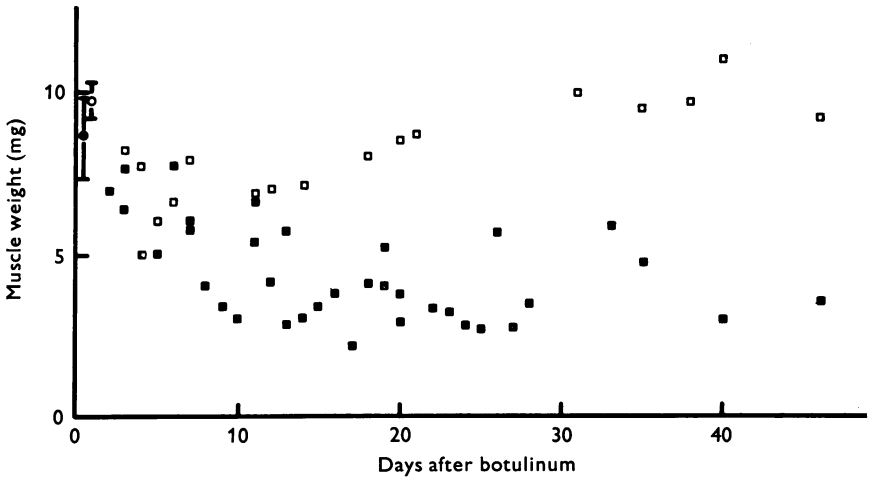

Fig. 2. Weight of soleus. The means (±S.D.) of 8 normal (●) and four muscles into which CPN was implanted (○) are shown. After botulinum toxin the weight of the unoperated soleus (■) fall to one third or less of normal and the muscle remains atrophied for more than 40 days. The soleus with the implanted CPN (□) loses little weight and is restored to about normal levels in 20 days.

nerves simultaneously. This indicates that muscle fibres had become functionally innervated by both nerves.

Muscle weights

The mean weight of the soleus in 8 normal male mice was $8{\cdot}6 \pm 1{\cdot}3$ mg (Fig. 2). In 4 animals the mean weight of soleus 2 weeks after implantation of CPN was $9{\cdot}7 \pm 0{\cdot}6$ mg. After the injection of botulinum toxin the weight of soleus in unoperated animals fell to about one third of normal within 2 weeks and had not returned to normal even at 6 weeks (Fig. 2). In contrast, in the group of animals in which CPN had been implanted into the soleus prior to the injection of botulinum toxin the weight of the muscle fell only slightly and was back to normal within about 3 weeks.

Morphological observations

In the normal mouse, innervation of the soleus is confined to a narrow band of end-plates which is situated in the middle of the muscle at right angles to its long axis. Apart from muscle spindles, which lie outside this zone of innervation, there are no motor nerve terminals between the transverse band of end-plates and the proximal tendon of the muscle, giving about 5 mm of end-plate-free muscle in which the cut end of the CPN could be implanted. In the soleus, as in mammalian spinal muscles generally, each extrafusal muscle fibre is innervated by a single preterminal myelinated axon which terminates in an arborization of a single motor end-plate. The characteristics of the normal appearance of end-plates in mouse skeletal muscle have been described with light and electron microscopy by Andersson-Cedergren (1959) and Duchen (1970*a*, *b*; 1971*a*) among others. Axonal extensions beyond the end-plate (i.e. ultraterminal axons) are very rarely found in normal circumstances. Cholinesterase activity is most concentrated within the synaptic space, and extends into the subneural folds.

Normally innervated muscles into which CPN was implanted showed no disturbance of the arrangement of the original end-plates, nor of their individual morphology, and only very few small end-plates could be demonstrated in the vicinity of the implanted nerve (Fig. 3A). At 1 week after botulinum toxin (i.e. 3 weeks after CPN implantation) there were no clearly localized patches of cholinesterase activity, even though stimulation of CPN elicited a definite response. However, within 2 weeks after toxin administration there were numerous patches of cholinesterase near the site of implantation, and these became more clearly defined with the passage of time. By 6 weeks many end-plates, some quite large and complex, were readily demonstrable near the implanted nerve (Fig. 3B). The effects of botulinum toxin on the morphology of motor end-plates has been described with light and electron microscopy by Duchen & Strich (1968) and Duchen (1970*a*, *b*; 1971*b*). In the present series of experiments the appearances seen in those muscles without an implanted CPN were as expected from the previous studies. In the initial stages, after the onset of paralysis, the end-plates were normal in appearance. Axonal sprouting began after 7 days and within 2–4 weeks extended from almost all end-plates in the muscle (Fig. 4A). Later (Fig. 5A) these sprouts spread widely on either side of the zone of innervation, and eventually end-plates were scattered over a wide band across the belly of the muscle (see Fig. 6A) instead of only in a narrow band as in the normal. These end-plates varied widely in their morphology, many being abnormally small. In contrast with the effects of botulinum toxin alone, those muscles into which CPN had been implanted showed little or no axonal sprouting at the original end-

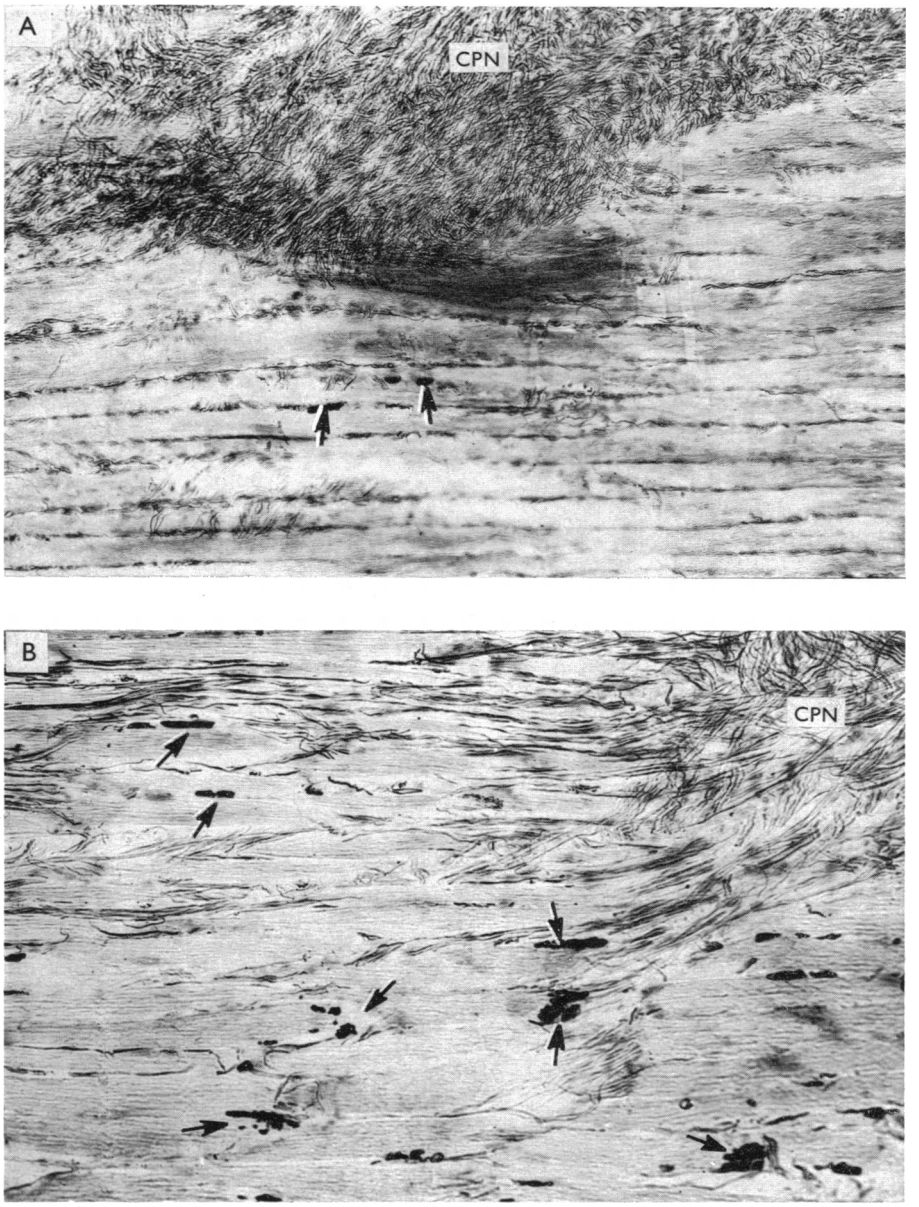

Fig. 3. Longitudinal frozen sections of soleus stained to show cholinesterase activity and nerve fibres by the method of Namba *et al.* (1967). The region of the muscle in which CPN had been implanted 8 weeks previously is shown. (A). This muscle received no further treatment and very few small end-plates (arrows) are seen in the vicinity of the implanted nerve (CPN). (B). Two weeks after nerve implantation botulinum toxin was injected. Numerous end-plates (arrows to some) are seen near CPN. × 100.

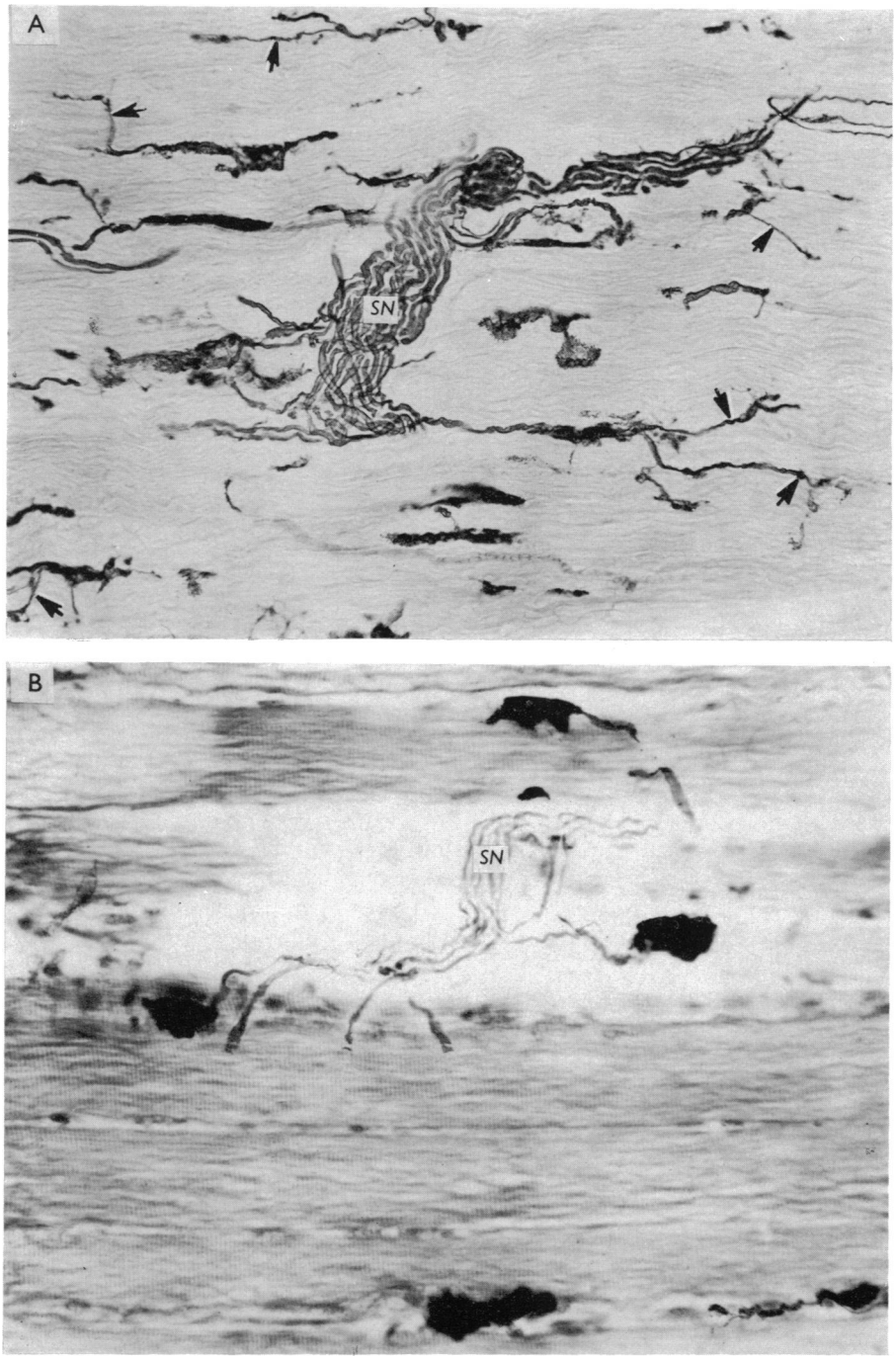

Fig. 4. Sections showing the original zone of end-plates of soleus 4 weeks after botulinum toxin. *SN*, intramuscular branches of soleus nerve. (A). An unoperated muscle. Many ultraterminal axonal sprouts (arrows) are seen. (B). A muscle into which CPN had been implanted. The end-plates are normal in appearance and no ultraterminal sprouts are present. Method of Namba *et al.* (1967). × 250.

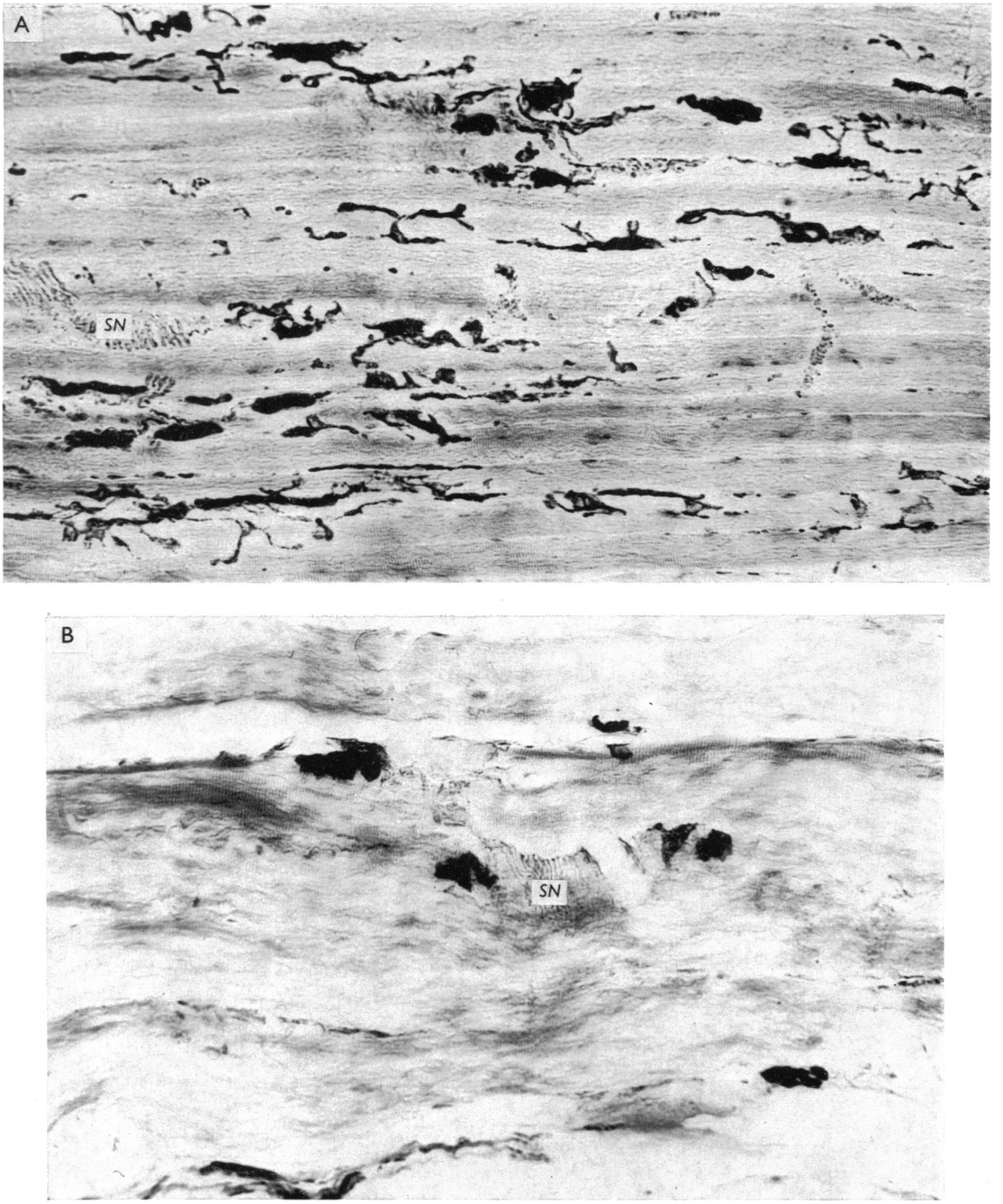

Fig. 5. The original zone of innervation of soleus 6 weeks after botulinum toxin. (A). Unoperated muscle. Note the abundant axonal sprouts and that the muscle fibres are atrophied, resulting in crowding together of the end-plates. (B). Soleus in which CPN was implanted 2 weeks before the injection of botulinum toxin. There is no axonal sprouting and muscle fibres are of normal size. The normal parallel axons of the intramuscular branches of the soleus nerve (*SN*) are seen. Method of Namba *et al.* (1967). × 250.

Fig. 6. (A). At 106 days after botulinum toxin there are many end-plates (arrows to some) of varying shape and size scattered over a wide region of the central innervation zone of soleus. (B). Soleus with CPN implanted at 106 days after botulinum toxin. End-plates in the original zone of innervation are not abnormal in appearance. *SN*, intramuscular branches of soleus nerve. Method of Namba *et al.* (1967). × 100.

plates (Figs. 4B, 5B, 6B). The original band of end-plates could be identified, even in the longest surviving animal (8 months), and their morphology was similar to that of the normal end-plates. It was possible to identify the intramuscular branches of the original nerve to soleus since their axons were arranged in an orderly parallel manner (Fig. 6B). In contrast, the axons of CPN had no regular arrangement (Fig. 3B). The pattern of innervation after botulinum toxin alone was very abnormal, with much branching within the muscle (see Duchen & Strich, 1968). This disturbance of the pattern of innervation of the soleus usually caused by botulinum toxin was not found in those muscles in which CPN had been implanted. Some axonal sprouting was seen at original soleus end-plates in the part of the muscle opposite to the site of the implant where CPN axons probably did not grow. In muscles in which the implanted CPN was cut before the toxin was injected the axonal sprouting was like that found after toxin alone.

DISCUSSION

The mechanism by which botulinum toxin induces axonal sprouting at the intramuscular terminals of motor neurons is not clearly understood. The primary site of action of the toxin is generally accepted as being on the axonal terminal, preventing the release of acetylcholine, but leaving the structure intact. The muscle fibre remains responsive to direct electrical stimulation (Dickson & Shevky, 1923) as well as to close arterial injection of acetylcholine (Guyton & MacDonald, 1947). The axonal sprouting which is found in muscle paralysed by botulinum toxin (Duchen & Strich, 1968; Duchen, 1970*a*, 1971*a*, *b*) occurs as an extension of the intact motor neuron. It is not the result of axonal degeneration, and does not occur from the preterminal axon. The sprouts originate from axonal terminals in the motor end-plate and grow as ultraterminal sprouts. In mammalian skeletal muscles, particularly those innervated by spinal nerves, axons are not normally seen extending beyond the end-plate except in very young animals (Tello, 1917) and in muscle spindles (Boyd, 1962; Coërs, 1967). Axonal sprouting in muscles into which botulinum toxin has been injected directly is a phenomenon which we have now observed in many animals. The sprouting does not occur at the same time in all the muscles, being slower in onset (up to 4 weeks after botulinum) in white muscle, but always occurring within one week in the soleus (Duchen 1970*a*). Serial sections of the soleus have shown that we can expect axonal sprouting at virtually every end-plate at that time. The sprouts grow extensively for several weeks, and eventually new end-plates are formed, so that the pattern of innervation and distribution of end-plates remains permanently abnormal (Duchen & Strich, 1968). When botulinum toxin was injected after the implantation of CPN into the soleus the pattern of innervation made by the original soleus nerve was not disturbed. The normal band of end-plates could be recognized for many weeks, only occasional sprouting was seen, and the difference from those muscles which did not have an extra implanted nerve was very striking. The implanted nerve which effectively innervated the muscle also prevented the severe atrophy usually caused by the toxin.

Axonal sprouting has also been found in hereditary motor end-plate disease (*med*) in the mouse (Duchen, 1970*b*; Duchen & Stefani, 1971) and in muscles paralysed by tetanus toxin (Duchen & Tonge, 1973). In both these situations, however, there is a persistence of the spontaneous release of acetylcholine, whereas botulinum toxin almost totally abolishes spontaneous transmitter release (Spitzer, 1972; Tonge, 1974*a*). After botulinum and tetanus toxins, as well as in the *med*

mouse, the changes in the muscle fibres are those of functional denervation, i.e. atrophy, fibrillation and supersensitivity, although the presynaptic conditions are different. Innervation by the foreign nerve prevented the atrophy and reduced fibrillation and supersensitivity (Tonge, unpublished observations). Although the exact mechanism by which sprouting is suppressed is not yet understood it seems that the influence of the foreign nerve on the original nerve is mediated through the muscle fibre. The results indicate that the physiological state of the muscle is of importance in the initiation of axonal sprouting.

SUMMARY

The common peroneal nerve (CPN) of the mouse was divided and the proximal stump implanted into the end-plate-free upper third of soleus. Two weeks after the implantation of CPN, botulinum toxin was injected into the leg muscles and the physiological and morphological states of the innervation of soleus were studied in animals surviving for periods of time ranging from 1 day to 8 months. Changes were compared with the effects of botulinum toxin injected into unoperated normal mice.

After the injection of botulinum toxin, neuromuscular transmission was blocked for several weeks at the original end-plates in the soleus in both operated and unoperated animals. The muscles of the unoperated animals became severely atrophied and showed extensive axonal sprouting which produced a permanent alteration in the pattern of innervation of the original soleus nerve. However, muscles in which CPN had been implanted became innervated by that nerve within a few days of the injection of toxin, and numerous new end-plates were formed near the site of implantation. These muscles did not become much atrophied and little or no axonal sprouting took place from the original soleus nerve terminals. The finding that the innervation by a foreign nerve suppressed axonal sprouting from the original nerve indicates that extraneural factors, including the physiological state of the muscle fibre, are of importance in the control of axonal growth.

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