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THE INTERACTION BETWEEN FOREIGN AND ORIGINAL MOTOR NERVES INNERVATING THE SOLEUS MUSCLE OF RATS

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SUMMARY

1. The fibular nerve was transplanted on to the soleus muscle of rats. Interruption of the original soleus nerve then permitted cross-innervation, and subsequently, over a period of weeks, re-innervation by the original nerve.

2. Individual muscle fibres were often innervated by both the original and the foreign nerve. The original and foreign end-plates were located in separate regions of the muscle. There were no indications that the original nerve could displace or repress the foreign innervation.

3. The extent of re-innervation by the original nerve depended upon the method of denervation. A single crush of the nerve was followed by virtually complete re-innervation, even of muscle fibres already innervated by the foreign nerve. When re-innervation was delayed by resection of a segment of the nerve only muscle fibres without foreign nerve innervation were re-innervated. Denervation by a simple nerve cut gave an intermediate result.

4. Re-innervation by the original nerve can take place without measurable extrajunctional sensitivity to ACh.

5. The original end-plate region could retain high and localized sensitivity to ACh for several months despite degeneration of its motor nerve terminal and activity of the muscle fibre.

6. Established foreign end-plates were re-innervated by the foreign nerve on muscle fibres with intact original innervation.

7. The factors controlling synapse formation in skeletal muscles are discussed.

INTRODUCTION

The factors that control the formation of synaptic connexions are still largely unknown. Some progress, however, has been made in studying the establishment of synapses between nerve and muscle. Mammalian skeletal muscle fibres are normally innervated by a single nerve axon, and the presence of the original innervation prevents synapse formation by an implanted foreign nerve. If the original nerve is cut (Ellsberg, 1917), poisoned with botulinum toxin (Fex, Sonesson, Thesleff & Zelená, 1966), or reversibly blocked (Jansen, Lømo, Nicolaysen & Westgaard, 1973), however, the foreign axons will innervate the muscle. Gutman & Hanslikowa (1967) made the interesting observation that foreign nerve innervation of a muscle did not prevent re-innervation by the original nerve. This was extended by the finding that although direct electric stimulation of a denervated muscle prevented foreign synapse formation, re-innervation by the original nerve was virtually unaffected (Jansen *et al.* 1973).

In the present study we were interested in this difference between the original and foreign motor nerves in their ability to prevent hyperinnervation of muscle fibres. In addition there is considerable interest in the long-term fate of foreign synapses on muscle fibres re-innervated by their original nerve. Experiments in lower vertebrates suggest that the original nerve can suppress inappropriate foreign innervation (Marotte & Mark, 1970). The technique for producing hyperinnervated skeletal muscle fibres in the rat made it possible to look for such synaptic suppression in this preparation as well. Some of the results have been published in preliminary reports (Frank, Jansen, Lømo & Westgaard, 1974*a*, *b*).

METHODS

Soleus muscles from white rats initially weighing approximately 250 g were used. The rats were anaesthetized with Nembutal during transplantation and *in vivo* tension measurements, and with ether when muscles were excised.

Cross-innervation. The superficial branch of the fibular nerve was dissected, cut distally and transplanted on to the proximal dorsal surface of the soleus muscle. A fibrin clot was used to hold the nerve in position. After 10–14 days the tibial nerve, which contains the soleus motor axons, was crushed, cut or resected in the popliteal fossa, about 15 mm from the soleus muscle. In some rats, the tibial nerve was crushed again after an additional 2 weeks. All animals were treated with penicillin.

In vivo tension measurements. The Achilles tendon was freed from surrounding connective tissue and connected to an isometric tension transducer with a stout thread. It was not cut. The fibular and tibial nerves were exposed for stimulation *en passant* in the popliteal fossa. The deep branches of the fibular nerve were cut acutely to avoid interference from flexor contractions. Steel pins held the knee rigidly fixed in position. After measurement of soleus twitch tension to fibular nerve stimulation, the wounds were closed with sutures and the animals recovered with-

out complications. At the time of the final experiment, we confirmed that the transplanted fibular nerve innervated only the soleus muscle. In two animals the lateral gastrocnemius had also been cross-innervated, and results from these animals are not included here.

Acute experiments. 3–14 months after the original denervation the soleus muscle and sciatic nerve were excised and placed in a bath perfused with mammalian Ringer solution (~ 2 ml./min) at room temperature. The Ringer contained: Na^+, 162 mM; K^+, 5 mM; Ca^{2+}, 2 mM; Mg^{2+}, 1 mM; $H_2PO_4^-$, 1 mM; HCO_3^-, 24 mM; Cl^-, 148 mM; glucose, 11 mM. The pH was maintained at 7·3 by bubbling a mixture of 95% O_2 and 5% CO_2 through the solution.

Tension measurements were made *in vitro* by recording the isometric tension elicited by single shocks applied to the tibial and fibular nerves. For tension occlusion measurements the relative timing of stimulation of the two nerves was carefully adjusted until the minimum twitch tension was observed on an oscilloscope.

Intracellular membrane potentials were recorded with conventional micropipettes filled with 4M-KAc having resistances of 20–60 MΩ. Sensitivity to iontophoretic applications of ACh was measured with micropipettes filled with 3M-ACh chloride. A steady reverse current prevented leakage of ACh. Pulses were usually about 10^{-8} A. Sensitivity is expressed in mV of depolarization/nC of charge ejected from the pipette (Miledi, 1960).

Histology. At the end of the experiment most muscles were stained for ACh-esterase according to the method described by Buckley & Heaton (1968) and then fixed in 25% formalin. After fixation they were cut in two; the proximal end which contained the foreign end-plates was dehydrated, cleared and mounted as a whole mount in benzyl benzoate, while the distal end was dehydrated, mounted in paraffin, and sectioned at 10 μm for muscle fibre counts.

A few muscles were prefixed in 1% glutaraldehyde in 0·1 M Tris buffer (pH 7·4) and stained for ACh-esterase using the method described by Karnovsky (1964). After several hours in a fixative solution of 2% formaldehyde and 2½% glutaraldehyde in 0·1 M-Na cacodylate (pH 7·4), small groups of muscle fibres were teased out, stained in a 1:10,000 solution of methylene blue in Ringer for 1 hr, dehydrated and destained in ethanol, cleared, and mounted between two coverslips. This method, suggested to us by Dr U. J. McMahon, resulted in a more delicate staining of the end-plate than that obtained with the other procedure.

RESULTS

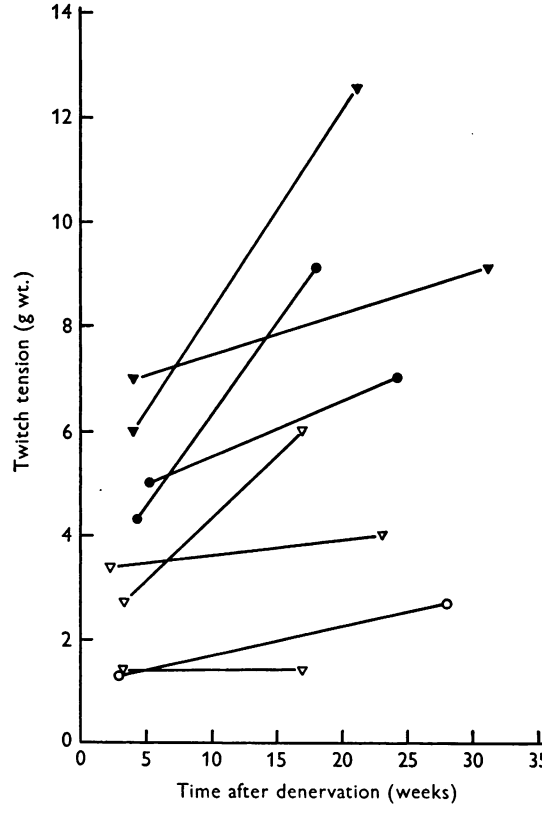
Long-term survival of foreign synapses

Transplantation of the superficial branch of the fibular nerve on to a normally innervated soleus muscle results in an extensive outgrowth of nerve fibres from the cut end of the nerve across the proximal, dorsal surface of the muscle. Virtually no new synapses are formed, however, until the original soleus innervation is blocked or destroyed (Jansen *et al.* 1973). After denervation, new synapses begin to appear and in 3–4 weeks the fibular nerve has innervated a substantial portion of the muscle.

If the soleus muscle is denervated by cutting the tibial nerve high in the thigh, re-innervation by the original nerve supply has already begun after one month. Since re-innervation by the original nerve presumably limits the formation of new synapses by the foreign nerve, we assessed the degree

of foreign innervation at this time by measuring *in vivo* the soleus twitch tension in the Achilles tendon in response to single stimuli applied to the fibular nerve.

To test for the possibility that foreign synapses had been suppressed or displaced by the regenerated original nerve supply we made a similar *in vivo* measurement of soleus twitch tension 3–6 months later. The results are shown in Text-fig. 1. We found no evidence for suppression of foreign innervation; on the contrary there was a clear *increase* in the tension measured at the later times.



Text-fig. 1. *In vivo* assessment of extent of foreign innervation. Soleus muscle tension was measured at two times in the Achilles tendon of each rat. Single shocks were applied to the foreign (fibular) nerve *en passant*. See Methods for details. Each line segment connects the interim and final tension measurements for one rat. Different symbols represent different methods of interrupting the original (soleus) innervation: ○, crush tibial nerve once in thigh; ●, crush tibial nerve twice in thigh at 2 week interval; △, cut only soleus nerve near soleus muscle; ▲, cut tibial nerve in thigh. Foreign innervation increased moderately during re-innervation by the original nerve and was not suppressed.

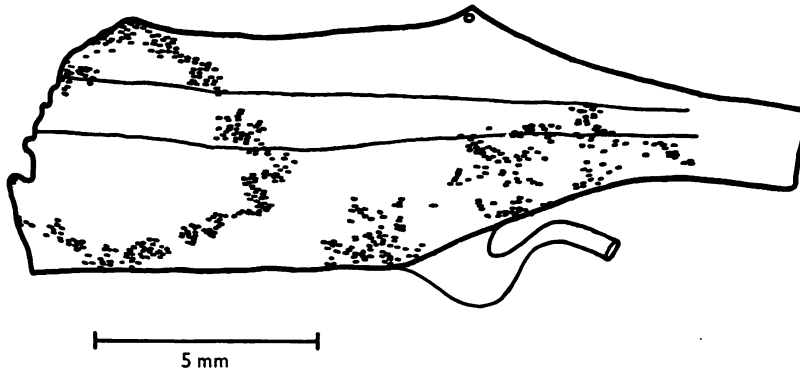
Many of the nerve fibres that grew back to the soleus muscle after complete transection of the tibial nerve may not be original soleus motor axons. The contraction speed of the re-innervated soleus muscle is faster than normal (Miledi & Stephani, 1969) and the ACh-esterase at the myotendon junction, characteristic of mammalian slow muscle, disappears. In three animals we denervated the muscle by cutting only the soleus nerve, between the lateral gastrocnemius and soleus muscles, to ensure that the soleus muscle would become re-innervated only by the original soleus motor axons. Both the slower contraction speeds and the presence of myotendon-esterase suggested that soleus motor axons had re-innervated the muscle. The distribution (see below) and persistence of the foreign innervation of these three muscles, however, was indistinguishable from that of the other muscles (Text-fig. 1). It appears that soleus and random tibial nerve motor axons are equally ineffective in suppressing or displacing fibular nerve innervation of soleus muscles.

The anatomical distribution of muscle fibres innervated by the fibular nerve also provides evidence against suppressed or displaced foreign innervation. The synapses formed by the fibular nerve were always in the proximal part of the muscle outside the zone of normal soleus end-plates which form a characteristic elliptical band near the centre of the muscle (Text-fig. 2). Usually the fibular nerve innervated a sharply defined region covering the lateral one third to two thirds of the muscle. The boundary between those fibres that it innervated and those it did not could be easily seen by observing muscle contractions through a dissecting microscope. Intracellular recordings from superficial muscle fibres showed that virtually every fibre within the fibular zone was innervated by the fibular nerve (average 92 % in the seven muscles examined). Outside this zone no fibres were innervated by it. After staining the muscle for ACh-esterase we could show the distribution of foreign and original end-plates (Text-fig. 2). We never saw foreign end-plates outside the zone of functional foreign innervation, nor could we see fibular nerve branches outside this zone. This evidence suggests that once a muscle fibre was innervated by the fibular nerve it remained so and that fibres outside the zone of functional foreign innervation had never been innervated by it.

The innervation of individual muscle fibres

Many muscle fibres were re-innervated by the soleus nerve. A large number received only soleus innervation. This is not surprising because the soleus nerve usually regenerated before the fibular branch had sufficient time to innervate the entire muscle. Even when re-innervation by the original nerve was completely prevented the fibular nerve did not fully innervate the soleus muscle. In four such control muscles the tensions

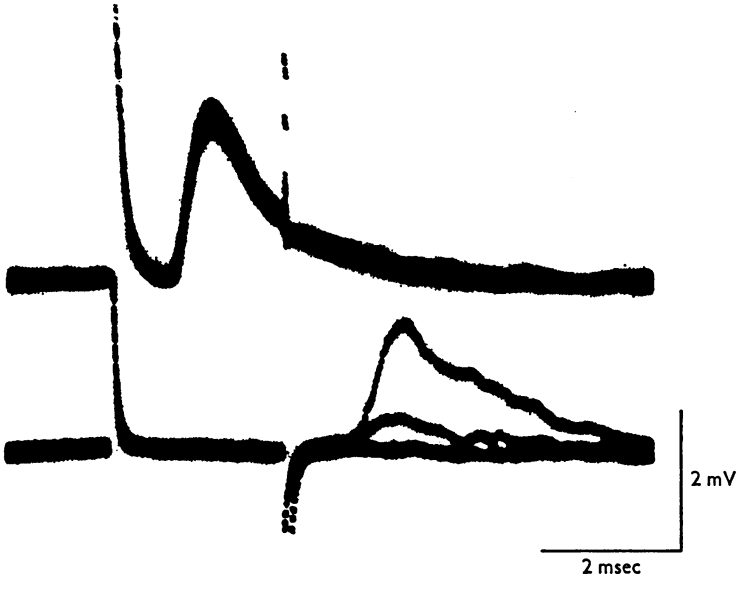
to nerve stimulation were between 6 and 10 g, less than 50 % of normal. Muscle fibre counts (see below) were 40–60 % below normal. In these muscles we saw groups of very small atrophic muscle fibres which were not included in the count. These were fibres that the implanted nerve had not innervated.



Text-fig. 2. Tracing of photograph of soleus muscle that had been hyper-innervated 23 weeks earlier. The foreign nerve (lower right) had been transplanted on to the muscle surface and two weeks later the normal innervation was interrupted by cutting the tibial nerve in the thigh. Original soleus end-plates (stained here for ACh-esterase) form an elliptical band near the centre of the muscle (left) and foreign end-plates are grouped near the proximal tendon (right). Thin longitudinal lines are borders between three zones: 1, fibres exclusively innervated by foreign nerve (closest to implanted nerve) 2, doubly innervated fibres (central region) 3, fibres exclusively innervated by the original nerve (medial region of muscle). The denervated original end-plates of region 1 still stained well for ACh esterase.

A second class of fibres was cross-innervated by the fibular nerve in addition to having soleus innervation. This dual innervation was shown by recording from individual muscle fibres that could be activated by stimulation of both the fibular and the soleus nerve. Doubly innervated fibres were found in nearly all experimental muscles. To demonstrate separate synaptic regions on single muscle fibres, we blocked neuromuscular transmission by adding Mg (6 mM) to the bathing fluid. As expected from the esterase-stained preparations fibular end-plate potentials were found in the proximal part of the muscle, and the synaptic activity of the regenerated soleus nerve fibres was found in its original zone of innervation. Usually these two regions were too far apart to observe the activity of both synaptic regions at one intracellular recording site. Therefore, two electrodes which could be used both for recording and for passing current were employed. By successive penetrations we could

follow a particular muscle fibre over an extended region and observe widely separated synaptic potentials. An example is shown in Text-fig. 3. This fibre had its fibular synaptic region about 1 mm from the proximal tendon end and a soleus synaptic region 12 mm further distally. The fibular synaptic region was innervated by two different fibular nerve fibres. Their individual end-plate potentials could be demonstrated by small changes in the stimulus intensity. Multiple foreign nerve innervation was found in several muscle fibres, but was not systematically examined. Frequently, however, we found more than one fibular end-plate on a single muscle fibre after staining for ACh-esterase.

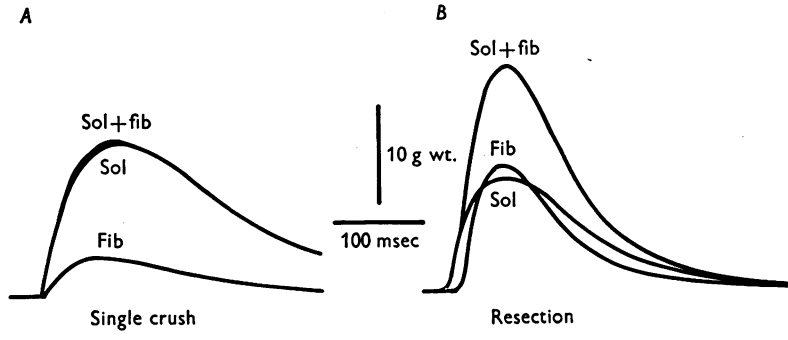


Text-fig. 3. Synaptic potentials in a dually innervated soleus muscle fibre 13 weeks after interruption of original innervation (single crush). The bathing fluid contained 6 mM-Mg^{2+}. Upper trace, record from region of original end-plates showing response to stimulation of soleus nerve; lower trace, record from foreign end-plate region showing multiple innervation by foreign nerve (three different stimulus intensities). The electrodes were shown to be recording from the same muscle fibre by electrical coupling.

The third class of muscle fibres consisted of those exclusively innervated by the transplanted fibular nerve. We were surprised at the failure of the soleus nerve to re-innervate its old end-plates because earlier experiments had shown that electrical stimulation of denervated muscle did not prevent re-innervation by the original nerve (Jansen *et al.* 1973). Therefore several independent checks were made of this result. The region of the muscle

normally innervated by the soleus nerve was explored for spontaneous miniature synaptic potentials. After the experiment the end-plates were stained for ACh-esterase and small groups of soleus end-plates on fibres exclusively innervated by the fibular nerve were examined with Nomarski optics for myelinated axons. These tests all verified that the end-plates were denervated.

The particular method for disruption of the original innervation (crushing, cutting, or resecting the tibial nerve) had a pronounced effect on the proportion of fibres with single or double innervation. One demonstration of this is presented in Text-fig. 4, which shows the twitch tensions recorded *in vitro* from two hyperinnervated soleus muscles when each



Text-fig. 4. Effect of denervation procedures on amount of dual innervation shown by tension occlusion measurements. A. After a single tibial nerve crush (26 weeks earlier) stimulation of both nerves together (timed for maximum occlusion of tension) produced no more tension than stimulation of the soleus nerve alone, suggesting that nearly all fibres with foreign (fibular) innervation had been reinnervated by the original nerve. B. After resection of a 3 mm length of tibial nerve (32 weeks earlier) there was nearly linear summation of the individual tensions, suggesting that few, if any, muscle fibres were dually innervated.

nerve was stimulated alone and when they were both stimulated together timed to produce maximum occlusion of tension. In Text-fig. 4*A* the muscle had been originally denervated by a single tibial nerve crush. When the fibular nerve was stimulated together with the soleus nerve there was no more tension than when the soleus nerve was stimulated alone. This suggested that nearly all fibres innervated by the fibular nerve were also innervated by the soleus nerve. In contrast, for muscles denervated by resection of the tibial nerve (Text-fig. 4*B*), the tension produced by stimulating either one of the nerves alone was much smaller than when both were stimulated together. The sum of the tensions in response to each nerve was approximately equal to the total tension produced when both were stimulated; few, if any, fibres had dual innervation.

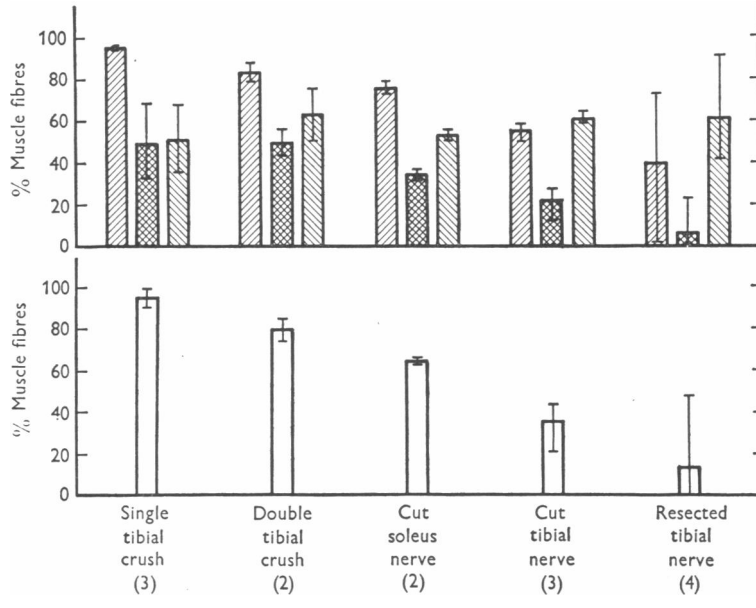
Intracellular recordings from individual muscle fibres made it possible to determine more precisely the proportion and distribution of individual muscle fibres with single and double innervation. From forty to seventy fibres uniformly distributed across the dorsal surface of the muscle were impaled and examined for functional innervation from each of the two nerves. Text-fig. 2 shows the observed distribution in one dually innervated muscle which was denervated by cutting the tibial nerve. Muscle fibres nearest the point of entry of the transplanted fibular nerve were innervated exclusively by it, fibres in the middle regions were innervated by both nerves, and fibres on the edge of the muscle farthest from the foreign nerve were innervated exclusively by the soleus nerve. Although the anatomical distribution was somewhat variable in different muscles, the basic pattern shown in Text-fig. 2 was by far the most common.

The observed proportions of single muscle fibres with the various kinds of innervation are presented in Text-fig. 5. We have included the muscles presented in Text-fig. 1 and six others in which the interim *in vivo* experiment was not done. The muscles have been grouped according to the type of denervation performed after transplantation of the foreign nerve. The proportion of fibres innervated by both nerves varies systematically with the method of original denervation and is progressively less for the more severe methods of denervation.

The total amount of foreign and regenerated original innervation is also indicated in the upper part of Text-fig. 5. Foreign innervation increased somewhat with more severe denervations of the original nerve, while the amount of original re-innervation decreased. The proportion of dually innervated fibres (double hatching) decreased with more severe original nerve lesions, but this effect was most easily seen when the amount of dual innervation was expressed as a percentage of the total foreign innervation (Text-fig. 5, lower).

There are several reasons why severe nerve lesions could reduce the proportion of fibres with soleus innervation. For example, there might be a reduction in the number of nerve fibres reaching the muscle, or the fibres that arrive might have a reduced ability to make synapses. Alternatively the regenerating nerve might be normal but there might be a progressive decrease in the ability of muscle fibres with foreign innervation to accept re-innervation at their original end-plates. In an attempt to distinguish between these possibilities we counted the number of nerve fibres in the regenerated soleus nerve just as it entered the muscle. The results, presented in Table 1, show that regenerated soleus nerves contained at least as many and usually more axons than normal. The increased number of nerve fibres was particularly associated with the more severe types of denervation procedures and is probably explained by nerve branching at the site of injury. This makes it difficult to assess the significance of the

nerve fibre counts, but at least they help rule out the explanation that a simple reduction in number of nerve fibres was responsible for the deficient re-innervation of the muscle.



Text-fig. 5. Effect of various denervation procedures on cross and re-innervation of soleus muscle fibres. Type of innervation was determined by recording from individual fibres on the dorsal surface of the muscle while stimulating the two nerves. The upper graph shows the percentage of all sampled fibres that were re-innervated by the original nerve (hatching with positive slope), cross-innervated (hatching with negative slope), and innervated by both nerves (cross-hatching). The lower graph shows the percentage of all fibres innervated by the foreign nerve which were also re-innervated by the original nerve. Increasingly severe original nerve disruption decreased the proportion of dually innervated fibres. The number of animals in each group is indicated in brackets. The error markers show the maximum and minimum values in each group.

We also counted the number of normal sized muscle fibres in cross-sections of the central region of the soleus. All muscles with a regenerated soleus nerve had muscle fibres of a uniform and approximately normal diameter. This is in contrast to muscle fibres that have been denervated for comparable periods of time. These are greatly atrophic with diameters many times smaller than normal. The muscle fibre counts therefore give a measure of the number of muscle fibres that had been innervated and active in the different types of experimental material. The results are given in Table 1. It appears that there is a 10–20 % reduction in the number of muscle fibres in all muscles with a regenerated original nerve

supply, and that the various denervation procedures do not have a pronounced effect on the total number of innervated muscle fibres.

TABLE 1. Counts of soleus axons and muscle fibres in experimental material. Twitch tensions were measured *in vitro* (*, *in vivo*) at room temperature (18–23° C) in response to single shocks applied to the foreign (fibular) nerve. The axon counts of soleus nerves include sensory axons. Muscle fibre counts do not include very small fibres that had not been re-innervated

Experiment	Denervation procedure	Twitch tension to foreign nerve g-wt.	Number of soleus nerve fibres	Number of muscle fibres
1	single tibial crush	1·8	157	2818
2		2·7	134	2569
3		4·1	130	2252
4	double tibial crush	2·7	171	—
5		2·7	107	2305
6		5·0	203	2265
7		7·0*	104	2078
8	cut soleus nerve	1·4	323	2398
9		2·9	81	2296
10		3·0	171	
11	cut tibial nerve	7·8	149	2324
12		10·7	216	2484
13	resected tibial nerve	0·9	118	2192
14		2·0	—	2272
15		2·3	—	2721
16		3·1	156	2440
17		9·9	70	2120
18		10·3	143	1964
19		12·0	210	2502
20		13·6	282	1891
21		13·7	182	2432
22	normal soleus muscle	—	128	2630
23		—	132	2802

Re-innervation and extrajunctional ACh sensitivity

One way in which the foreign synapses might reduce the extent of original nerve re-innervation is by reducing the extra-junctional ACh sensitivity. Re-innervation by the original nerve begins during the period of expanding foreign innervation, especially after a single crush of the original nerve. Muscle fibres only recently cross-innervated might be re-innervated by the original nerve because they were still supersensitive. Fibres cross-innervated for longer times would be insensitive and hence not re-innervated.

To test this possibility we looked for re-innervation in muscles where extra-junctional sensitivity was blocked by direct electrical stimulation.

The soleus muscle was denervated in each leg by a single crush of the lateral gastrocnemius–soleus nerve in the popliteal fossa. From the time of denervation one muscle was stimulated continuously with implanted electrodes at 100 Hz for 1 sec every 10 sec. This pattern of stimulation eliminates supersensitivity in denervated soleus muscles (Lømo & Westgaard, unpublished).

Two rats were examined after 9 days. A few fibre (less than 1 % had been re-innervated in the control muscles while no re-innervation had yet occurred in the stimulated muscles. As expected all the fibres of the non-stimulated muscles were supersensitive to ACh. We found sensitivities to iontophoretic application of ACh of 175–500 mV/nC in nine fibres examined 2 mm from the tendon (myotendinous junction). In the stimulated muscles there was no extrajunctional sensitivity ($\leqq$ 0·1 mV/nC in thirty-three fibres examined) and the end-plate region of nine fibres showed localized ACh sensitivity indistinguishable from that of normal fibres.

After 14 days both stimulated and control muscles were re-innervated approximately equally (60–90 % of surface fibres were activated by nerve stimulation) in the two pairs of muscles examined. In the stimulated muscles both the re-innervated and the denervated fibres were sensitive to ACh only in the end-plate region. The peak and extent of the ACh sensitivity were indistinguishable from those in normal muscles (four innervated, five denervated end-plate regions were examined). In the two non-stimulated muscles the re-innervated fibres showed varying degrees of extrajunctional sensitivity. Finally, two pairs of muscles were examined after 16 days. These were completely re-innervated (96–98 % of surface fibres) on both the control and the stimulated side and we found no extra-junctional sensitivity in any of the four muscles. From this we conclude that re-innervation of the original end-plate region can take place in the absence of measurable extrajunctional ACh sensitivity.

Re-innervation of established foreign end-plates

Functional original innervation or direct electrical stimulation can prevent *de novo* formation of foreign nerve synapses in the muscle. It was of interest to determine whether re-innervation of established foreign end-plate regions could take place on muscle fibres with intact original innervation. This was done successfully in two muscles that had been hyperinnervated by the foreign nerve for several months. The original denervation of these muscles was by a crush of the tibial nerve so that the great majority of the muscle fibres were re-innervated by the original nerve (see Text-fig. 5). After several months, the foreign nerve was cut or crushed, and one month later, there was substantial re-innervation of

the foreign end-plate region by the foreign nerve. Most cross-innervated fibres were also innervated by the soleus nerve (80 and 90 % in the two cases). Thus, despite the active original innervation of these muscle fibres regenerating fibular nerve axons were able to re-innervate their old end-plates.

In a similar experiment on a third rat there was also substantial re-innervation by the crushed foreign nerve, but only three of seventeen surface fibres innervated by the fibular nerve were also activated by the soleus nerve. There are several plausible explanations for this result, but in any case it does not negate the positive results in the two cases described above.

Decline of ACh sensitivity at denervated end-plates

One feature of denervated end-plates on cross-innervated muscle fibres that might underlie their ability to become re-innervated by the original nerve is their sensitivity to ACh which lingers on after extra-junctional sensitivity is suppressed by muscle activity (Lømo & Rosenthal, 1972). We therefore measured the ACh sensitivity of denervated soleus end-plates on fibres that were innervated only by the fibular nerve.

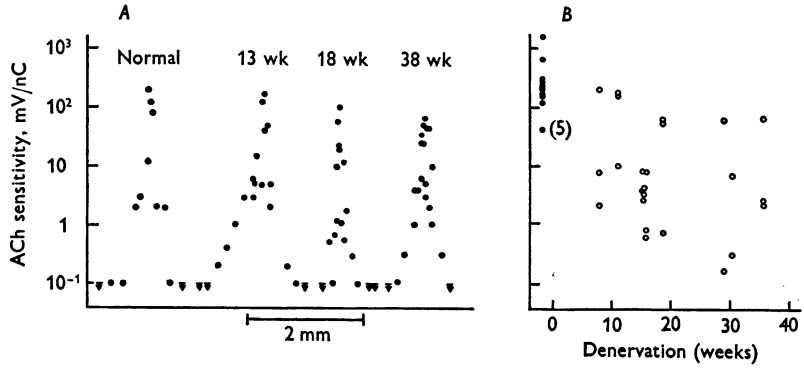
The experimental material consisted of soleus muscles innervated by the fibular nerve in which re-innervation by the original nerve was prevented by transecting the tibial nerve in the thigh and implanting its proximal end into adjacent biceps muscle with sutures. Lack of regeneration was confirmed anatomically and physiologically during the experiment.

Some measurements were also made on other material in which the resected tibial nerve had regenerated and re-innervated the soleus muscle, and in these cases we verified that the end-plates we were studying had not become re-innervated (see above). ACh sensitivity was measured by iontophoretic application of ACh from a micropipette. When a sensitive spot was found, a dye mark was made nearby and the muscle was subsequently stained for ACh-esterase. Because the synapses made by the fibular nerve always lay outside the band of original end-plates we could verify that the sensitivity corresponded to the presence of an old soleus end-plate.

Many end-plates remain sensitive to ACh for long periods of time following denervation. Text-fig. 6*A* illustrates the distribution of sensitivity at a normal soleus end-plate and at three other end-plates denervated 2–8 months earlier. Peak sensitivities of denervated end-plates with detectable levels of ACh sensitivity are plotted against the duration of denervation in Text-fig. 6*B*. It is clear that substantial sensitivity to ACh can remain for many months after denervation.

Foreign nerve terminals that innervate a region of muscle previously insensitive to ACh induce persistent changes in the muscle membrane.

In one dually innervated muscle we resected the fibular nerve 3·5 months after transplanting it on to the soleus muscle. Seventeen days later there were no signs of foreign re-innervation. All the muscle fibres we examined were innervated by the soleus nerve and none were sensitive to ACh outside the synaptic region. In three fibres we found three spots with relatively high and localized sensitivities (120, 39, and 20 mV/nC), which corresponded closely in position to fibular end-plates stained for ACh-esterase at the end of the experiment.



Text-fig. 6. Persistence of ACh sensitivity at denervated original end-plates on foreign-innervated muscle fibres. *A*, distribution of sensitivity at one normal and three denervated end-plates; the length of denervation is indicated above each distribution. *B*, peak ACh sensitivities of all denervated end-plates with detectable sensitivity plotted against time of denervation. Several normal end-plates are included at zero time for comparison. Many end-plates retain appreciable sensitivity for several months after denervation.

Not all denervated soleus end-plates appeared to be equally sensitive. Many of the peak sensitivities we recorded were quite low (see Text-fig. 6*B*). Undoubtedly some of these were the result of diffusion barriers to ACh (such as, for example, when the end-plate was on the deep side of a muscle fibre) but it is our impression that the low values were more frequent at denervated end-plates. Often a careful search for several millimetres along the length of a fibre without soleus innervation failed to reveal any sensitive area even though subsequent staining for esterase showed that we had covered the region of the old end-plate. Lack of any detectable sensitivity is quite uncommon in fibres with intact soleus innervation.

The ACh-esterase at denervated end-plates also seemed to stain with variable intensities, especially with the Karnovsky stain, even among superficial fibres in the same muscle. Often a denervated end-plate stained

almost normally. In other cases the stain was less dense and more filamentous or was barely detectable. Three examples of denervated soleus end-plates are shown in Pl. 1; a re-innervated end-plate is included for comparison. The differences in staining were usually obvious at a glance and were quite unlike the small variations seen in a normally innervated muscle.

Nearly all of the regions with high ACh sensitivity corresponded to spots of intense esterase stain. Conversely, regions of the muscle where we failed to detect any sensitivity often corresponded to areas of weakly stained end-plates. The absence of an ACh response is not, however, a particularly strong indication of the absence of sensitivity, and we did not explore the correlation between ACh sensitivity and esterase further.

DISCUSSION

There was no indication of suppression of foreign synapses after re-innervation of the soleus by its original nerve supply. Ideally this should have been studied by following the synaptic activity of individual muscle fibres over prolonged periods of time in different stages of cross- and re-innervation. This was impossible for technical reasons, however, and our evidence against suppression is largely based on cross-innervated muscles examined long after suppression should have occurred. Two points are quite clear. First, foreign and original innervation can coexist and remain active for long periods, perhaps indefinitely, on individual muscle fibres in the rat. Second, if suppression of foreign synapses occurs at all under the present experimental conditions it happens only to a very limited extent. Our strongest evidence in this connexion was the absence of any sign of foreign innervation (nerve fibres, esterase stained end-plates) outside the region of functional innervation (Text-fig. 2).

Suppression of supernumary end-plates can, however, occur in immature mammalian skeletal muscles. Both in the rat (Redfern, 1970) and the cat (Bagust, Lewis & Westerman, 1973) many muscle fibres initially have polyneuronal innervation. This additional motor innervation is apparently inactivated and degenerates during the first few weeks of life. Mature skeletal muscles appear to lose their ability to suppress inappropriate synapses.

Our observations are in contrast to those reported by Mark and collaborators for fish and salamanders. In these lower vertebrates re-innervation by the original nerve apparently suppresses the function of foreign synapses while leaving them structurally intact (see Mark, 1974). Although a conclusive demonstration of a repressed foreign end-plate has not been presented by Mark and his group our strikingly different

results may well be due to species differences. In lower vertebrates there seems to be a special mechanism by which a muscle is preferentially re-innervated by its original nerve (Grimm, 1971; Cass, Sutton & Mark, 1973). Furthermore, the reported examples of suppression are all from muscles with polyneuronal innervation and distributed synapses, and the muscle fibres do not generate action potentials. Mammalian skeletal muscle fibres normally have single, focal end-plates and during regeneration a particular nerve seems to have no preference for its old muscle (Weiss & Hoag, 1946). Although the bases for such differences between the different types of muscles are not understood, they might be associated with the ability of the foreign nerve to suppress foreign innervation.

The effect of different types of interruption of original innervation on the proportion of hyperinnervated muscle fibres was a striking result in the present experiments (Text-fig. 5). Increasingly severe denervation procedures probably progressively delayed the arrival of regenerating axons. A single crush of the tibial nerve caused a shorter delay before re-innervation than repeated crushes, and a complete cut resulted in additional delays while the growing axons bridged the gap between proximal and distal ends of the nerve. Resection of a segment of the nerve increased this delay still further. A reasonable possibility is that the differences in time of re-innervation are causally related to the degree of hyperinnervation.

One way in which these longer delays before re-innervation could lead to decreased hyperinnervation is if there were some progressive change in cross-innervated muscle fibres that eventually prevented re-innervation by the original nerve. Another possibility is that the more severe denervation procedures resulted in a decreased ability of the original nerve to fully re-innervate an otherwise comparable muscle. The strongest evidence against this second view comes from preparations that had been originally denervated by resection of a short length of the tibial nerve. In three of these muscles (Expts. 19, 20 and 21 in Table 1) over 50 % of the fibres were innervated exclusively by the fibular nerve yet the number of soleus nerve fibres was within the normal range. Moreover, the total number of muscle fibres was comparable to that in similar muscles with very little fibular innervation (Expts. 13, 14 and 15 in Table 1). The absence of complete re-innervation of cross-innervated muscles is therefore unlikely to be caused by a defect in the regenerating original nerve.

The anatomical distribution of muscle fibres with different types of innervation is consistent with the idea that absence of hyperinnervation might result from progressive changes in the cross-innervated muscle fibres. Fibres innervated by the foreign nerve were localized to the lateral segment of the muscle. This was the site of implantation of the foreign

nerve, and these muscle fibres were the first to become cross-innervated. After a single crush and early re-innervation all these muscle fibres were re-innervated by the original nerve. With moderately delayed re-innervation the original nerve only hyperinnervated a central group of muscle fibres which presumably had been cross-innervated relatively late. Finally, with extreme delays in re-innervation the original nerve innervated only the deeper medial parts of the muscle which are never cross-innervated by the foreign nerve.

The reasons for the progressive decrease in the re-innervation of the original end-plates is not clear. It has been suggested that ACh receptors may be required for synapse formation in muscle (Katz & Miledi, 1964; Fex *et al.* 1966). But the reduction in re-innervation took place while the denervated end-plates were still highly sensitive to ACh (Text-fig. 6). Possibly synapse formation is controlled by some other property of the muscle fibres. The recent report of normal re-innervation of the rat diaphragm in spite of complete block of ACh receptors with α bungarotoxin (Van Essen & Jansen, 1974) can be interpreted similarly.

The complete re-innervation of the electrically stimulated muscle was important to demonstrate that re-innervation could take place on muscle fibres that were not supersensitive. Consequently, the progressive loss of supersensitivity in cross-innervated fibres could not be used to explain why some fibres were not re-innervated by the original nerve. But these observations raise the possibility that the foreign nerve innervation has some direct effect, independent of activity, on the distant denervated end-plates. However, the denervation of the electrically stimulated muscles was performed by a single nerve crush, and such muscles are completely re-innervated even when cross-innervated. It remains to be seen whether re-innervation of electrically stimulated muscles is complete also after more severe types of denervation.

Denervation of a skeletal muscle produces a series of striking changes in muscle properties. Several of these effects have traditionally been regarded as 'trophic' effects of nerve on muscle (Guth, 1968). This concept postulates an influence of nerve on muscle that acts independently of muscle activity. Recently several phenomena that have commonly been regarded as examples of trophic effects have been shown to depend largely on muscle activity *per se*. This applies to the development of supersensitivity after denervation (Lømo & Rosenthal, 1972), the inability of normal muscle to accept foreign innervation (Jansen *et al.* 1973) and the altered mechanical properties of cross-innervated muscle (see Lømo, Westgaard & Dahl, 1974).

The present experiments, on the other hand, demonstrate some neuromuscular interactions that are not easily explained as effects of muscle activity. Established foreign synapses stained for ACh-esterase as intensely

as normal soleus end-plates did. In addition, the extrajunctional ACh receptors that were present at a new foreign synapse were modified or replaced so that they remained functional despite muscle activity. Extrajunctional receptors either disappear or become non-functional within several days after electrical and mechanical activity of the muscle begins (Lømo & Rosenthal, 1972) whereas the receptors at the new foreign end-plates appear to become permanent features of the muscle membrane. These receptors persisted after denervation for at least several weeks on muscle fibres that were kept active by the regenerated original nerve. Furthermore, they were re-innervated by regenerating fibular nerve axons just as denervated soleus end-plates are re-innervated by the original nerve. Since existing information suggests that muscle activity directly reduces extrajunctional sensitivity and prevents the establishment of new synapses, it is appealing to regard the changes in the foreign end-plate region as possible examples of a neurotrophic effect.

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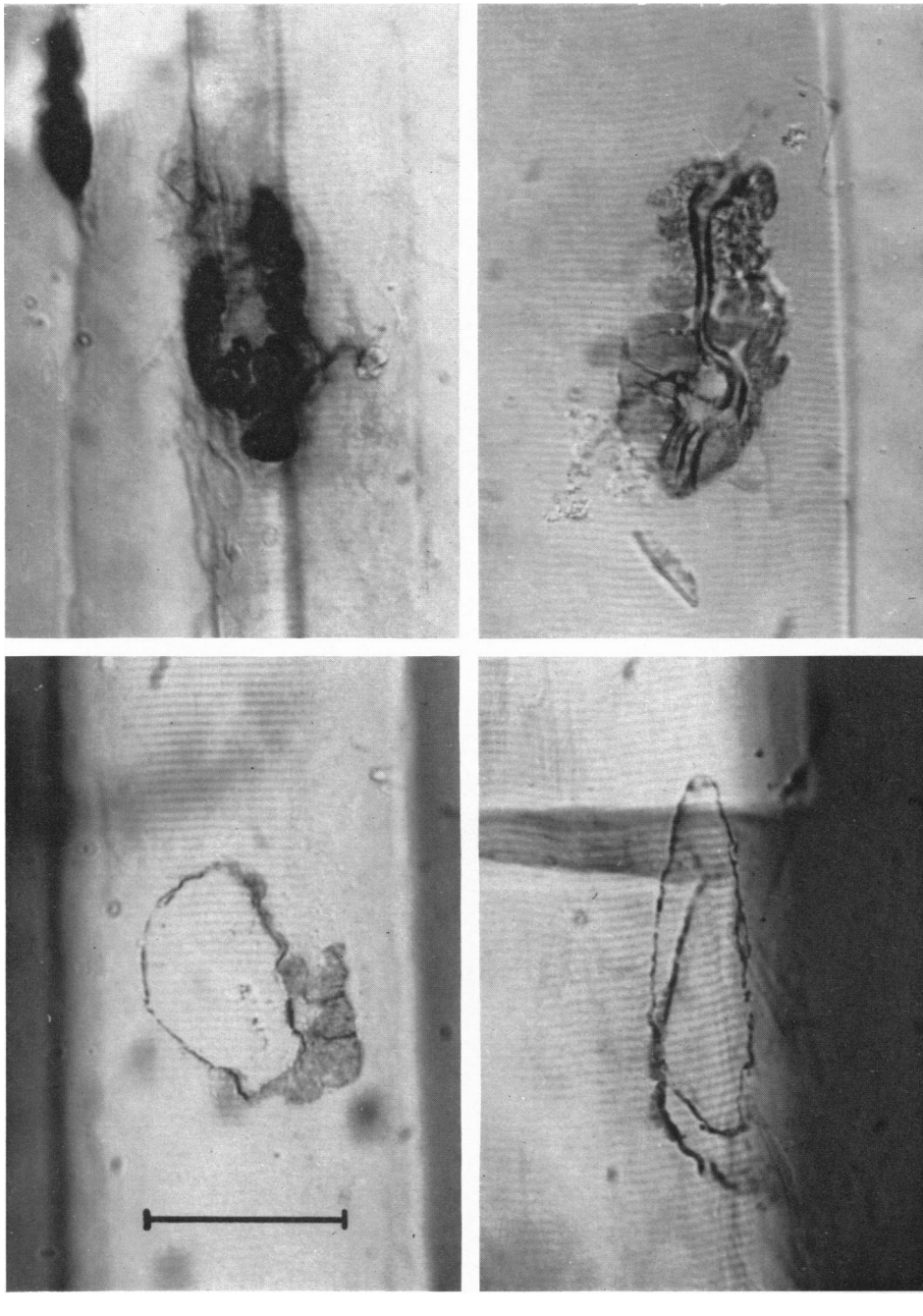

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EXPLANATION OF PLATE

Light micrographs of original end-plates. The end-plate in the upper left had been reinnervated by the original nerve and appears normal, while the other three were all denervated. All end-plates were on superficial fibres in one soleus muscle that had been denervated 56 weeks earlier by resection of the tibial nerve. Karnovsky ACh-esterase stain, Nomarski optics. Calibration, 50 μm.