

## SELECTIVE AND NON-SELECTIVE REINNERVATION OF FAST-TWITCH AND SLOW-TWITCH RAT SKELETAL MUSCLE

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### SUMMARY

1. The problem of selectivity during reinnervation of skeletal muscle fibres was investigated in the rat using the fast-twitch extensor digitorum longus (EDL) and the slow-twitch soleus muscles and their nerves.

2. After an operation on these nerves permitting them to compete for reinnervation of one or the other muscle (hereafter called Y-union), virtually the total isometric tetanic tension of EDL muscle could be elicited by stimulating the EDL nerve, while stimulating the soleus nerve yielded little or no tension. In the case of the soleus muscle, stimulation of either nerve elicited about half of the total isometric tetanic tension.

3. During the course of reinnervation of these muscles in non-competitive situations, the time course of increase in the ratio of tension elicited by nerve stimulation to that by direct stimulation was slower in the case of soleus nerve reinnervating EDL muscle, compared with cross-reinnervation in the reverse direction or reinnervation of each muscle by its own nerve.

4. Crushing the common peroneal nerve 12 days after a Y-union in an attempt to retard the EDL nerve did not favour reinnervation of the EDL by soleus nerve, but crushing the nerve again or just once at 1 month after the original operation produced substantial partial reinnervation of the EDL by the soleus nerve.

5. It is concluded that soleus nerve fibres form functioning neuromuscular synapses on EDL muscle fibres only with difficulty. The pattern of reinnervation reveals characteristic differences between fast-twitch and slow-twitch muscles on the one hand and between their respective nerves on the other.

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## INTRODUCTION

When two or more mammalian muscles are allowed to be reinnervated after their nerves have been severed, functional neuromuscular synapses eventually reform. Certain factors which may affect the specificity of the neuromuscular reconnections have been investigated. Elsborg (1917) produced evidence to suggest that a given muscle preferred its original nerve to a foreign nerve. Work by subsequent authors (Weiss & Hoag, 1946; Bernstein & Guth, 1961) appeared to dispute his findings. Recent work has emphasized that mammalian muscles are composed of fibres of fast-twitch and slow-twitch types which are innervated by two types of nerve fibres (hereafter referred to as fast and slow nerve fibres respectively), which have characteristic properties. In the chick (Feng, Wu & Yang, 1965) and toad (Hoh, 1971), where differences between fast-twitch and slow-tonus muscle fibres and their nerve fibres are qualitatively different, type specific matching between nerve and muscle fibres has been shown to occur. The possibility that mammalian nerve and muscle fibres of matching types may preferentially reconnect has been investigated by Miledi & Stephani (1969). These authors produced evidence suggesting a lack of preferential matching.

In cross-reinnervation studies on rat fast-twitch and slow-twitch muscles, it was found that the fast-twitch extensor digitorum longus (hereafter called EDL) muscle reinnervated by the nerve to the slow-twitch soleus muscle was frequently reinnervated also by fast nerve fibres which could be excited by the stimulation of the common peroneal nerve (Close, 1969; Close & Hoh, 1969). These nerve fibres were presumably collaterals from the regenerating EDL nerve which had been used for reinnervating the soleus muscle. Special surgical techniques were found necessary to circumvent such adventitious self-reinnervation of the fast-twitch muscle cross-reinnervated with the soleus nerve (Báranáy & Close, 1971; Hoh & Salafsky, 1971). Cross-reinnervation of the soleus muscle did not present such a self-reinnervation problem. These observations suggested that the fast-twitch muscle might strongly prefer reinnervation by its original nerve. The problem of selectivity during reinnervation of rat muscle fibres has therefore been re-investigated using EDL and soleus muscles and their nerves. It will be shown that when the two nerves are given approximately equal chances to reinnervate one of these muscles, EDL is selectively reinnervated by its original nerve whereas the soleus is not, and that the selectivity is due to the fact that soleus nerve fibres form functioning neuromuscular junctions on EDL muscle fibres much less readily than EDL nerve fibres. A preliminary report on this work has appeared (Hoh, 1969).

## METHODS

*Operations.* Three series of operations were performed on 3-week-old female Wistar rats using pentobarbitone sodium (50 mg/kg body wt., injected intraperitoneally) as anaesthetic. In the first series, nerves to EDL and soleus muscles were transected and one of these muscles was completely excised. The ends of the proximal stumps of the transected nerves were tied together with fine silk and the distal stump of the nerve to the remaining muscle was united with the two proximal stumps. This type of operation is referred to as a Y-union. Extreme care was taken during the operation to avoid injury to the nerves proximal to the site of union. In order to avoid collateral nerve regeneration from the terminals of any intact motor axons (Edds, 1953), care was taken to ensure that all nerve branches entering each muscle were transected. As a further precaution, soleus muscles were carefully dissected at the end of the experiments to look for accessory nerves (Bárány & Close, 1971) which might not have been transected at the operation. One soleus muscle was found to have such an accessory nerve from the nerve to the plantaris muscle, and data from this muscle were rejected.

In the second series of operations, EDL and soleus muscles were cross-reinnervated or allowed to be reinnervated by their own nerves. In all cases, nerve stumps were approximated using fine silk. To ensure reinnervation of EDL muscle by the soleus nerve uncomplicated by competition from stray nerve fibres from the common peroneal nerve, this nerve was cut, reflected back and embedded in the biceps femoris muscle. In order to avoid the possible diversion of soleus nerve fibres towards the denervated soleus muscle, this muscle was excised. A simple nerve cross-union was sufficient to ensure exclusive reinnervation of the soleus by EDL nerve fibres. To ensure exclusive self-reinnervation of EDL or soleus muscle, only one experimental muscle was reinnervated in each hind limb.

In the last series of operations, a Y-union was performed to allow EDL and soleus nerves to reinnervate the EDL muscle as in the first series, but at various times afterwards the common peroneal nerve was crushed with fine forceps at about 3 mm proximal to the level of transection of EDL nerves.

*Experimental procedure.* The animals were anaesthetized with pentobarbitone sodium and the experimental muscles and nerves were very carefully dissected and set up for *in situ* isometric tension recording. The tibial and common peroneal nerves were dissected and all branches of these nerves except the soleus and EDL nerves respectively, were cut. The dissected leg was placed in a Perspex muscle bath containing about 120 ml. Ringer solution (NaCl, 137 mM; KCl, 5 mM; CaCl<sub>2</sub>, 2 mM; MgCl<sub>2</sub>, 1 mM; NaH<sub>2</sub>PO<sub>4</sub>, 1 mM; NaHCO<sub>3</sub>, 24 mM; glucose, 11 mM) which was aerated with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The solution in the bath was replaced at the rate of about 2-3 ml./min. The temperature of the bath was maintained at 35° C by an electronic circuit.

Isometric contractions in response to indirect stimulation of the muscles were recorded using a strain gauge (Statham G1-8-350) which was activated by a carrier amplifier (Tektronics, Q), the output of which was displayed on an oscilloscope (Tektronics, 565). The responses were also displayed on a slave oscilloscope and photographed with a Grass C4 camera. Nerves were stimulated in Ringer solution using platinum wire electrodes and a Grass stimulator. For each muscle, the optimal length for isometric twitch contractions in response to stimulation of its original nerve was found and all subsequent measurements on this muscle were made at this length. A series of twitches and responses to repetitive stimulation at various frequencies were recorded for each muscle from the stimulation of each of the two nerves.

Responses to direct stimulation were also recorded from some muscles. Prior to this procedure, the Ringer solution in the muscle bath was replaced by paraffin oil. The stimulator used for nerve stimulation was followed by a transistorized current amplifier, and stimulus pulses delivered to muscles were of 0.2–0.3 msec duration and of supramaximal strength (about 24 V). Platinum wire electrodes were placed on opposite sides of the muscle. In order to avoid mechanical interference from neighbouring muscles, the plantaris, tibialis anterior, the peroneal and part of the gastrocnemius muscle were excised during the initial dissection for the experiment.

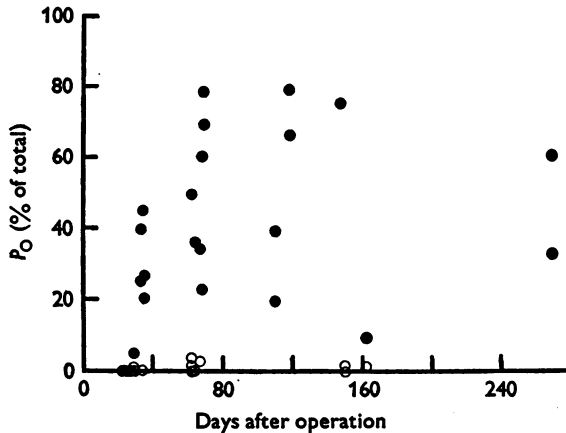


Fig. 1. Reinnervation by the soleus nerve of soleus (● filled circle) and extensor digitorum longus (○) muscles receiving nerves to both these muscles. The maximum isometric tetanic tension ( $P_o$ ) obtained from the stimulation of the soleus nerve is expressed as a percentage of the total  $P_o$  (i.e. sum of the  $P_o$  from the stimulation of each of the two nerves). Values for individual muscles are plotted against days after the operation.

## RESULTS

### *Reinnervation of EDL and soleus muscles by competing EDL and soleus nerves*

The Y-union of EDL and soleus nerves permitted them to compete for the reinnervation of the same muscle. Fig. 1 shows the extent of reinnervation of the soleus muscle (●) and the EDL muscle (○) by the soleus nerve, at various times after the operation. For each muscle, the maximum isometric tetanic tension ( $P_o$ ) obtained from the stimulation of the soleus nerve is expressed as a percentage of the total  $P_o$  (sum of the  $P_o$  from the stimulation of each of the two nerves), i.e.  $P_o$  (% total), and plotted against days after operation. The  $P_o$  (% total) gives a measure of the extent of functional reinnervation by the soleus nerve. For soleus muscles, the values ranged widely from 4.7% to 79% with a mean of  $42.0 \pm 4.9$  (s.e. of mean) % ( $n = 21$ ) and showed no correlation with time after

operation. For EDL muscles, on the other hand, the functional reinnervation by the soleus nerve was undetectable in five out of fifteen preparations, the average  $P_o$  (% total) being  $0.37 \pm 0.15$  (s.e. of mean) %.

The possibility that some muscle fibres after Y-innervation may be reinnervated by both types of nerve fibres was examined in fourteen out of the twenty-one soleus preparations. The ratio of the total  $P_o$  from the stimulation of the two nerves separately to the  $P_o$  obtained from the simultaneous stimulation of both nerves, at the same frequency, was calculated for these muscles. Overlap of innervation would lead to a ratio in excess of unity. The mean ratio for these muscles was  $1.029 \pm 0.006$  (s.e. of mean,  $n = 14$ ) and was significantly different from unity ( $P < 0.001$ ). These observations suggest that less than 3% of soleus muscle fibres were reinnervated by fibres from both EDL and soleus nerves. This degree of overlap in innervation would raise the mean value of  $P_o$  (% total) for soleus nerve to about 43% if total tension were taken to mean that obtained from simultaneous stimulation of the two nerves. Reinnervation of EDL by soleus nerve was so small that overlap in innervation, if present, could not be detected by this technique.

*Time course of self-reinnervation and cross-reinnervation of EDL and soleus muscles*

In the second series of experiments, the time course of functional reinnervation of EDL and of soleus muscles by each of the two types of nerves were compared in situations which precluded competition between them. At various times after the operation, the  $P_o$  from direct and indirect stimulation of the muscle were measured. In Fig. 2 the ratios of  $P_o$  from indirect to  $P_o$  from direct stimulation, for each of the four nerve-muscles combinations, are plotted against days after operation. It can be seen that the time course of functional reinnervation of the soleus muscle by either nerve, and of the EDL muscle by its own nerve, was very rapid. Virtual complete reinnervation was attained by 28 days, whereas the EDL muscle was only about 50% functionally reinnervated by the soleus nerve at this time. The curve for the reinnervation of EDL by soleus nerve appears to be flattening out at 28 days after operation but further work to cover the complete time course of reinnervation is necessary to determine whether this is statistically significant.

*Effect of crushing the common peroneal nerve on the reinnervation of EDL muscle by the EDL and soleus nerves*

In the third series of experiments, EDL and soleus nerves were allowed to reinnervate the EDL muscle, but attempts were made to favour reinnervation by the soleus nerve by crushing the common peroneal nerve

at various times after the original operation. In nine preparations, the nerve crush was done 12 days after the original operation and the extent of functional reinnervation by the soleus nerve was determined at various times thereafter by comparing the  $P_o$  from indirect and direct stimulation. On the day of nerve crush, functional reinnervation by the soleus nerve in two preparations was 0% and 4%, compared with 22% and 33% by the EDL nerve. Muscles examined from 8 to 39 days after the nerve crush showed a functional reinnervation of  $0.8 \pm 0.6$  (s.e. of mean) % ( $n = 7$ ) by

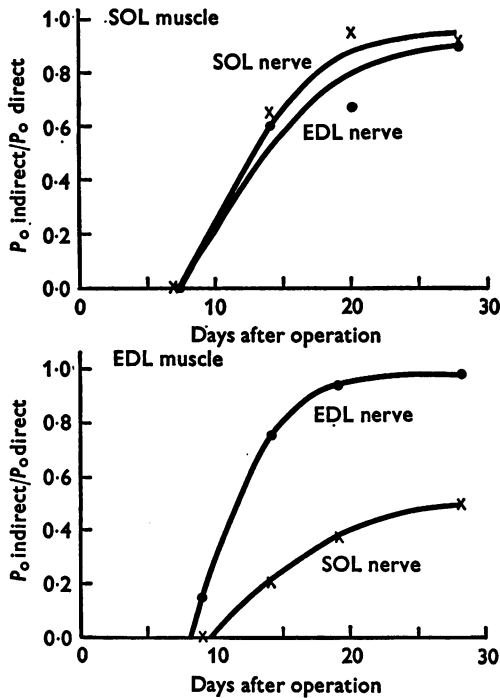


Fig. 2. Rate of functional reinnervation of soleus (SOL) muscle and extensor digitorum longus (EDL) muscle by the soleus nerve (x) and the EDL nerve (●) in preparations in which only one nerve was permitted to reinnervate the muscle. The ratio of maximum isometric tetanic tension from indirect stimulation to that from direct stimulation is plotted against days after operation.

the soleus nerve. When the nerve crush was done 1 month and muscles examined 68–74 days after the original operation (two preparations), there was a marked improvement in functional reinnervation by the soleus nerve (8.4 and 17.3%). In four preparations, the common peroneal nerve was crushed twice, at 12 days and at about 1 month after the original

operation. Functional reinnervation by the soleus nerve averaged 37% (range: 8.4–68%) when examined 46–48 days after the original operation.

*Contractile properties of fibres in EDL and soleus muscles reinnervated by EDL and soleus nerves*

Denervation of mammalian fast-twitch and slow-twitch muscles results in an increase in both the contraction time and the twitch-tetanus ratio of these muscles (Kean, Lewis & McGarrick, 1974). The time course of changes in contraction time and twitch-tetanus ratio of fibres in EDL and

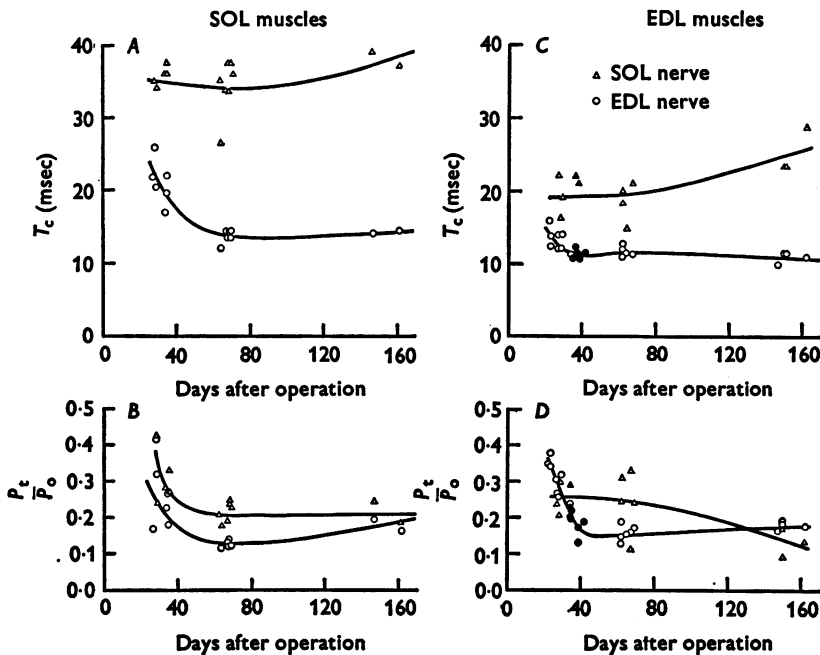


Fig. 3. Contraction times ( $T_c$ , msec) and twitch-tetanus ratios ( $P_t/P_o$ ) of soleus (SOL) muscles (A, B) and extensor digitorum longus (EDL) muscles (C, D) reinnervated by both nerves. Values obtained from the stimulation of soleus ( $\Delta$ ) and EDL ( $\circ$ ) nerves are plotted against days after operation. Results from EDL preparations in which the common peroneal nerves had been crushed are plotted (filled symbols) against days after the nerve crush. The lines were drawn by eye.

soleus muscles during the course of reinnervation by both EDL and soleus nerve after a Y-union is given in Fig. 3. It shows (i) the reversal of the effects of denervation on these properties in self-reinnervated muscle fibres and (ii) the reciprocal changes in the contraction time of cross-reinnervated fast-twitch and slow-twitch muscle fibres. The difference in contraction

time of fibres within the same muscle reinnervated by the two different nerves is already clearly seen about 30 days after operation. This change is attributable to the neural influence on the speed of muscle contraction (Close, 1969) and is associated with a change in the type of myosin synthesized in muscle (Bárány & Close, 1971; Hoh, 1975).

#### DISCUSSION

##### *Non-selective reinnervation of the soleus muscle*

Studies on contractile properties of motor units in rat EDL and soleus muscles have revealed that EDL contains about forty fast motor units with an average contraction time 11 msec while soleus contains about thirty motor units of which about twenty-seven are slow and three are intermediate with average contraction times 38 and 18 msec respectively (Close, 1967). Furthermore, average values for maximum isometric tetanic tension were 4.7 g for both fast and slow units and 5.85 g for intermediate units. Assuming the absence of any selective factors in reinnervation, one would expect that, after a Y-union, the average size of motor units associated with fast, slow or intermediate nerve fibres would be proportional to their respective sizes in normal EDL and soleus muscles. On this basis and using the above figures from Close, the soleus nerve is expected to reinnervate on the average 43.7% soleus muscle. The observed average value for the fraction of isometric tetanic tension for soleus muscle elicited via the soleus nerve is in excellent agreement with this prediction. It may thus be concluded that the soleus muscle showed no preference for fibres from either nerve, i.e. its reinnervation is non-selective. Furthermore, the extent of reinnervation of the soleus muscle by its own nerve showed no correlation with time after operation. This rules out the occurrence in this muscle of progressive repression or displacement of the foreign junctions by the muscle's original nerve such as that seen in lower vertebrates (Mark, 1974).

##### *Selective reinnervation of EDL muscle by its original nerve*

On the assumption that reinnervation of EDL by EDL and soleus nerves after a Y-union is non-selective, the soleus nerve is expected to reinnervate on the average 43.7% (38.4% by slow fibres and 5.3% by intermediate fibres) of the EDL muscle. The observed fraction of isometric tetanic tension elicited by the stimulation of the EDL muscle is only  $0.37 \pm 0.15$  (S.E. of mean) %. Allowing for the fact that the mean diameter of EDL muscle fibres cross-reinnervated by the soleus nerve is only half of that reinnervated by its own nerve (Close, 1969), the extent of reinnervation by the soleus nerve would only be four times the observed value or



1.5% of the whole muscle. The conclusion is that reinnervation in this case is not random. The EDL muscle shows a preference for its original nerve fibres over slow or intermediate fibres of the soleus nerve.

*Mechanism of selective reinnervation of EDL muscle*

Reinnervation of muscle fibres by nerves may be considered to take place in two successive steps: (i) regeneration of severed nerve fibres to the region of synapse formation and (ii) the formation of transmitting neuromuscular synapses. A possible mechanism for the selective reinnervation of EDL muscle by its original nerve is that the rate of regeneration of the soleus nerve into the region of synapse formation in EDL muscle is significantly slower than that of the EDL nerve. Nerves reaching the muscle first could then dominate the muscle by rendering the muscle fibres refractory to further innervation (Elsberg, 1917; Aitken, 1950) by the foreign nerve. However, the following points argue in favour of the suggestion that the rate of regeneration of soleus nerve fibres do not differ from that of EDL nerve fibres: (i) following a similar Y-union, soleus muscle was not preferentially reinnervated by the EDL nerve; (ii) the rates of functional reinnervation of EDL and soleus muscles by their respective nerves were comparable; (iii) crushing the common peroneal nerve at 12 days after a Y-union, thus giving the soleus nerve the advantage of an early start in regenerating into the EDL muscle, did not improve the reinnervation of the EDL by the soleus nerve. The conclusion is that the slow soleus nerve fibres form transmitting neuromuscular synapses on fast EDL muscle fibres less readily than do fast EDL nerve fibres. This conclusion is corroborated by the observation that 28 days after the operation which precluded competition, reinnervation of EDL muscle by its own nerve was virtually complete while 50% muscle reinnervated with the soleus nerve remained functionally denervated. The difficulty in the formation of functioning synapses between soleus nerve and EDL muscle may be compounded during Y-reinnervation by the possibility that newly formed soleus nerve terminals on EDL end-plates may be functionally displaced by EDL nerve terminals. Further investigations using electrophysiological techniques should be helpful in clarifying the mechanism of selective reinnervation of EDL muscle.

*Nerve-muscle compatibility during reinnervation*

Y-reinnervation experiments show that EDL nerve is compatible with both EDL and soleus muscles with respect to the formation of functional synapses whereas soleus nerve is compatible with its own muscle but not with the EDL. There is therefore a difference in property between EDL and soleus nerves. Since this difference is recognizable by the EDL muscle

but not by the soleus muscle, there is a corresponding difference in property between these muscles. These properties of nerves and muscles may be termed reinnervation-compatibility states.

An important aspect of the results presented above was the eventual formation of functioning synapses between soleus nerve and EDL muscle fibres. This occurred when competition from EDL nerve fibres was greatly reduced by repeated or delayed crushing of the common peroneal nerve, or when competition was completely removed. One possible mechanism for eventual synapse formation is that reinnervation-compatibility states of fast-twitch and slow-twitch muscle fibres and their nerves remain unchanged, the slow rate of functional synapse formation simply reflect their intrinsic incompatibility. An alternative hypothesis is that reinnervation-compatibility state of fast-twitch muscle fibres changes under the trophic influence of the slow nerve into that of slow-twitch muscle fibres. This is then followed by functional reinnervation. This hypothesis has the advantage that it regards the difference in reinnervation-compatibility state of fast-twitch and slow-twitch muscle fibres as one of the many properties of mammalian muscle fibres which are subject to neural regulation (Guth, 1968; Close, 1972; Hoh, 1974, 1975).

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