J. Physiol. (1979), **289**, *pp.* 219–240
With 12 *text-figures*
Printed in Great Britain

THE PHYSIOLOGY, PHARMACOLOGY, AND TROPHIC EFFECTIVENESS OF SYNAPSES FORMED BY AUTONOMIC PREGANGLIONIC NERVES ON FROG SKELETAL MUSCLE

BY A. D. GRINNELL AND MARY B. RHEUBEN*

From the Department of Biology and the Jerry Lewis Neuromuscular Research Center, University of California, Los Angeles, California 90024, U.S.A.

(*Received 17 January 1978*)

SUMMARY

1. Frog sartorius muscles, newly denervated and transplanted to the lymph sac of the back, are reinnervated by implanted cholinergic nerves (spinal somatic motor nerves or the preganglionic sympathetic splanchnic nerve), but not by nerves that do not release ACh (skin sensory or sympathetic post-ganglionic nerves).

2. Foreign somatic motor nerves (s.m.n.s) form synapses that resemble normal sartorius neuromuscular junctions electrophysiologically.

3. Axons of the sympathetic preganglionic splanchnic nerve (s.p.n.) grow throughout the muscle, but only a small percentage of fibres form synapses. Most e.p.p.s are of low quantal content, generally subthreshold. Long onset latencies and multiple post-synaptic responses indicate that innervation is multiple, multi-terminal, and by unmyelinated axons.

4. Spontaneous miniature e.p.p.s at splanchnic junctions occur at an average rate under 0·1/sec. Their average amplitude and time course are about the same as for control muscles, but the variability of amplitudes is greater than for control muscles.

5. The amount of facilitation shown by s.p.n.-evoked e.p.p.s is the same as by s.m.n. e.p.p.s, but the time course is almost twice as long.

6. S.p.n.-reinnervated fibres show dramatic post-tetanic potentiation preceded by depression, following as few as 20–50 stimuli.

7. As judged by standard physiological and histochemical criteria, AChEsterase is absent at s.p.n. junctions.

8. The pharmacological responses of the s.p.n. junctions are similar to those of normal or foreign s.m.n. innervated neuromuscular junctions in their sensitivity to the cholinergic blocking agents D-tubocurarine and hexamethonium.

9. The s.p.n. is capable of restricting ACh sensitivity to the sites of nerve contacts, although this restriction occurs more slowly and less completely than with s.m.n. reinnervation. The loss of extrajunctional ACh sensitivity can be correlated with effectiveness of innervation; but significant restriction occurs even in s.p.n. reinnervated fibres that probably never contract to nerve stimulation.

* Present address and site of some of this work: Department of Biology, The Pennsylvania State University, University Park, Pennsylvania 16802.

0022-3751/79/2920-0232 $01.50

INTRODUCTION

It has long been known that denervated skeletal muscle can be reinnervated by nerves that release acetylcholine (ACh), even if they normally terminate on other tissues (Langley & Anderson, 1904; Beattie, Duel & Ballance, 1932; Hillarp, 1946; Brown & Satinsky, 1951; Guth & Frank, 1959; Landmesser, 1971, 1972; Ramirez & Luco, 1973; Bennett, McLachlan & Taylor, 1973). In addition, there have been reports of successful, apparently cholinergic, reinnervation by nerves whose normal transmitter is unknown, such as the central ends of spinal sensory nerves (Weiss, 1935) and the central ends of vagal sensory afferents (Vera & Luco, 1967).

The properties of synapses between cells that do not normally form functional contacts have been studied in only a few instances. Landmesser (1971, 1972) found that frog gastric vagus polyneuronally innervated fibres of transplanted sartorius muscles, apparently at the sites of the original end-plates. The junctional potentials were of low quantal content and showed very long-term facilitation. Most interestingly, the vagus nerve appeared unable to induce cholinesterase at its junctions and the ACh receptor was found to be abnormally sensitive to the preganglionic blocking agent, hexamethonium. Bennett *et al.* (1973) found that the vagus preferentially innervated the old end-plates of diaphragm muscle fibres in rabbits. Ramirez & Luco (1973) found that the cat cricothyroid muscle could be innervated by cervical sympathetic preganglionic fibres, and reported that the twitches elicited via stimulation of this nerve had a much slower time course than normal. They attributed the slowed time course to an alteration in the contractile biochemistry of the muscle.

In this paper, we discuss a series of experiments designed to test further the specificity and nature of the interaction between inappropriate nerves (particularly the preganglionic sympathetic splanchnic nerve) and skeletal muscle in frogs, and the trophic capabilities of autonomic preganglionic nerves innervating skeletal muscle. Subsequent papers will describe the distribution and the morphology of the synapses formed by the splanchnic nerve (A. D. Grinnell & M. S. Letinsky, in preparation) and the competitive interaction between foreign nerves reinnervating the same muscle (Grinnell, Letinsky & Rheuben, 1979).

METHODS

Bullfrogs (*Rana catesbeiana*) of 3–4 in. body length were maintained before and after operations in running water at room temperature (approx. 22 °C) and individually fed mealworms and canned cat food regularly. Frogs were anaesthetized for operations in 1:500 tricaine methanesulphonate (Ayerst). In most cases, one sartorius muscle was removed (usually from the left leg) and transplanted to the lymph space under the skin of the back. Here it was sutured at both ends and often along its lateral edges to the fascia of the back in order to hold the muscle at approximately its normal length in the body. In a few cases the thinner cutaneous pectoris muscle was used instead. Where muscle that was known to have no old end-plates was desired, a 1–2 mm segment ('pelvic end') of the sartorius near the pelvic tendon was used. For nerve implantation, an incision was made through the lateral body wall and the desired nerve (see below) located, sectioned distally and pushed through the musculature of the back, doing as little damage as possible. The nerve was then sutured into the muscle or affixed to it by a fibrin clot, and the skin sutured closed over the muscle. This preparation is very similar to that used by Landmesser (1971, 1972). The unoperated contralateral sartorius served as control in each experiment.

Four types of nerves were used to reinnervate the transplanted muscles; skin sensory nerves (always close to the muscle in the back and potentially a source of unwanted innervation), somatic motor nerves (usually the IV or V or occasionally the VI, spinal nerve, as numbered by Ecker & Weidersheim, 1899), a sympathetic post-ganglionic nerve (the posterior splanchnic), and the splanchnic nerve proximal to the coeliac ganglion where a large fraction of its component nerve fibres are preganglionic (Nichol, 1952).

After 2 weeks–2 years, the animals were pithed and, with the exception of a few experiments where the muscles were studied *in situ*, the contralateral unoperated control and experimental muscles were removed with at least 1–2 cm of their innervation intact. For electrophysiological tests, these muscles were pinned out on a layer of Sylgard in a plexiglass bath (*ca.* 10 ml. vol.) at the length giving maximal twitch tension.

Isometric tension was measured with a Statham UC 3 transducer or a more sensitive strain guage device built in our laboratory. Maximum sensitivity was 1·2 mV/mg. Muscle electrical potentials were recorded intracellularly by conventional 3 M-KCl micropipettes. Tension and electrical responses could be recorded with an oscilloscope camera or ink recorder (Brush 220); when desired, responses were averaged on a Hewlett–Packard 5480 A Signal Analyzer. Stimuli to the nerves (usually 1–10 V, 1 msec duration) were given via suction electrodes, while direct stimulation of the muscles (up to 100 V, 2–5 msec) was accomplished with platinum or chlorided silver wires applied to one end of the muscle.

For pharmacological tests, drugs were dissolved in either normal frog Ringer or high Mg^{2+}, low Ca^{2+} frog Ringer and administered by gravity superfusion through the bath. Drugs tested were acetylcholine chloride (ACh, Calbiochem), D-tubocurarine chloride (DTC, Abbott), hexamethonium chloride (C_6, K & K Laboratories), atropine sulphate (Sigma), and neostigmine methylsulphate (Prostigmine, Roche Laboratories). The normal frog Ringer (n.f.r.) consisted of 116 mM-NaCl, 2 mM-KCl, 1·8 mM-$CaCl_2$, buffered to a pH of 7·2 with approximately 2 mM-$NaHCO_3$. The temperature of the bath was maintained within 1–2° in the range 13–18 °C by thermo-electric cooling elements. When it was necessary to partially block excitatory junctional potentials (e.p.p.s), the Ca^{2+} concentration of the Ringer was lowered to 0·5–1 mM and $MgCl_2$ raised to 4–8 mM. ACh sensitivity of junctional and extrajunctional membranes was studied using conventional iontophoretic techniques, sampling at several spots along 1–2 cm of muscle fibre length. Alternatively, ACh was bath applied in different concentrations, and the concentration necessary to first evoke twitching was determined.

RESULTS

A. Success of innervation

Nerves that are known to release ACh from their terminals were able to successfully reinnervate the transplanted frog sartorius, others did not. Implanted post-ganglionic sympathetic fibres grew into the muscle and may have innervated smooth muscle cells of the blood vessels, but did not elicit skeletal muscle contraction or produce detectable e.p.p.s. Skin sensory nerves, if implanted into the muscle, became closely adherent to the muscle, but did not grow into the muscle and never evoked contractions or e.p.p.s when stimulated. On the other hand, s.m.n.s from the IV or V spinal roots showed successful innervation of the muscle within approximately 5 weeks after denervation and implantation, and by 3 months the muscle appeared to be completely (100 %) innervated. Degree of reinnervation in this context is arbitrarily taken to be the ratio of the tetanic tension produced by supramaximal stimulation of the nerve compared with the tetanic tension produced by supramaximal stimulation of the muscle directly.

The sympathetic preganglionic s.p.n., which releases ACh at the coeliac ganglion, also grew profusely into the muscle and innervated it successfully. However, the degree of reinnervation was slight compared with innervation by a s.m.n. In forty-one out of forty-three experiments in which the s.p.n. had grown into the muscle

successfully, nerve-evoked contraction could be seen. The average degree of innervation (indirect/direct tetanus tension) was only 12·4 ± 16·8 % (S.D.) (range 1–76 %). Twitch tensions were much weaker, with some muscles showing no visually perceptible response to a single stimulus. As in the case of s.m.n. innervation, the first evidence of nerve-evoked contraction appeared about 5–6 weeks after innervation. At this time, muscles could usually be induced to twitch a few times and then could no longer be driven. In longer duration preparations, s.p.n. evoked twitches were stronger and did not show such severe synaptic depression during repetitive stimulation. In the case of the s.p.n., however, the ability of the nerve to evoke contraction was greatest at about 3–6 months, followed by an apparent decline to a lower plateau level of tension development in older preparations (Grinnell *et al.* 1979).

The number of motor units was assessed by slowly changing stimulus strength and observing the number of abrupt changes in twitch tension. In twenty-eight preparations, the mean was six motor units (range 1–15). Fifteen motor units is comparable to the number found in s.m.n. reinnervated muscles, or normal and control muscles. There was no clear correlation between number of motor units and the time since innervation. Motor units tended to be highly variable in size. Some included fibres throughout the muscle; others contained only a narrow band of fibres. The latter was generally the case. There was no apparent correlation between an axon's threshold and its motor unit size. These measurements do not exclude the possibility that each increment in tension could represent the contribution of many axons having approximately the same threshold.

B. Physiological properties of the axons and synaptic terminals

Latency and time course of s.p.n.-evoked e.p.p.s

S.p.n.-evoked e.p.p. onset latencies were long and highly variable compared with those of s.m.n.s. Values ranged from 6 to 250 msec, with an average value of 57 ± 32 (S.D.) msec (n = 200) (see Fig. 1) compared with about 2·5 msec for normal frog sartorius muscles. In muscles reinnervated by s.m.n., latencies ranged from 2 to 20 msec, with an average value of 7·7 ± 3·8 msec (n = 150). Changing the position of stimulating electrodes along the s.p.n. indicated conduction velocities of from 0·3 to 1·4 m/sec, with an average of 0·5 m/sec. In the s.p.n., the refractory period was normally about 5–7 msec, and extended up to as much as 50 msec in preparations innervated only a short time. By comparison, the refractory period of s.m.n.-reinnervated muscles was uniformly 1–2 msec. Preliminary electron microscope analysis of a portion of a splanchnic nerve innervating an experimental muscle showed, of 331 fibres counted, 97 % unmyelinated and 3 % myelinated. This large population of unmyelinated axons is consistent with the generally higher thresholds and long refractory periods seen in these experiments.

As Fig. 1*D–H* shows, s.p.n.-elicited e.p.p.s were often of longer duration than s.m.n.-driven e.p.p.s and had multiple components, either from multiple termination by the same nerve fibre, or from terminals of several nerve fibres. These characteristics were more common in the first 3–6 months following reinnervation than at greater intervals, when most e.p.p.s had only a single component (see Grinnell

et al. 1979). The latencies of the different e.p.p. components often differed by as much as 50–80 msec, and as many as four or five separate components, from as many different nerve fibres, could be detected, all with similar rise times (see Fig. 10). Movement of the stimulating electrode along the nerve in most cases produced parallel shifts in the latencies of all components, implying that the differences in

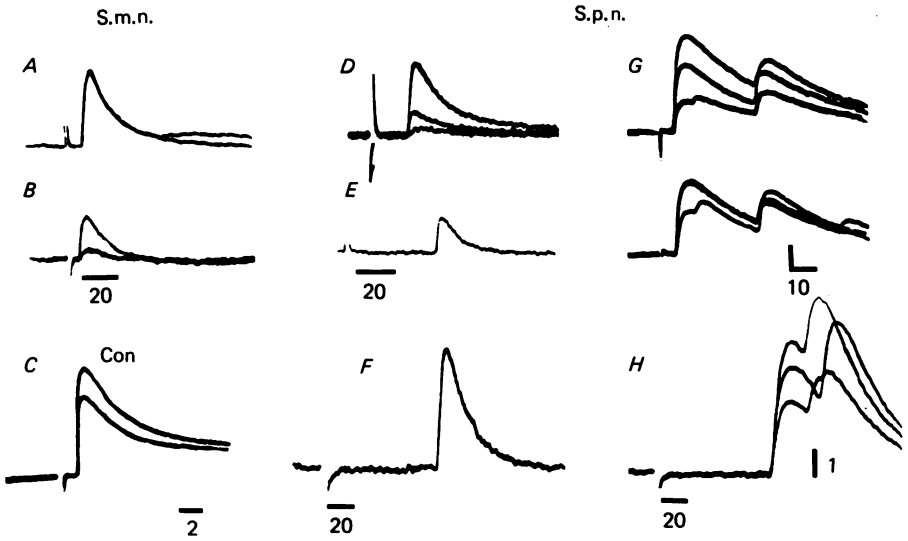

Fig. 1. Characteristic e.p.p.s recorded post-synaptically in frog sartorius muscle fibres reinnervated by a foreign spinal somatic motor nerve (s.m.n.: *B*, *A*), by the sartorius nerve in a control muscle (con.: *C*), and by a preganglionic sympathetic nerve (s.p.n.: *D–H*). S.m.n. and con. recordings were in Mg^{2+} Ringer; the s.p.n. recordings were made in normal frog Ringer. Note the generally shorter onset latencies of s.m.n. and con. e.p.p.s and the quantal fluctuations at s.p.n. junctions in normal frog Ringer. Note also the multiple components of the s.p.n. responses (*G* and *H*). The lower record of *G* shows a min e.p.p. near the end of one trace. Time calibration in msec is shown. Amplitude calibration (record *H*) applied to all traces except *G*, where the vertical bar represents 2 mV. Implantation times: *A*, *B* 102 days; *D*, 120 days; *E*, 175 days; *F*, *H*, 71 days; *G*, 88 days.

latency were not due to differences in conduction velocity of fibres within the nerve. Additionally, the latency of e.p.p.s with low quantal content and the same stimulus threshold sometimes varied by as much as 10–15 msec (Fig. 2), and the larger s.p.n.-evoked e.p.p.s typically showed a longer time course than the low quantal content responses. These observations could be explained if a s.p.n. fibre made multiple synaptic contacts, either clustered or spaced along the muscle fibre, with slow conduction velocity between points of contact.

These synaptic properties are reflected in the tension records (Figs. 3 and 4). The average s.p.n.-evoked twitch contraction latency was approximately 45 msec, with a rise time of 50–60 msec and a half the fall time of about 80 msec. This contrasts with values of 13 msec (latency), 48 msec (rise time) and 40 msec (half-fall) for s.m.n.-reinnervated muscles. At the temperatures used here (13–18 °C), normal sartorius muscles contracted to indirect stimulation with an average latency of

9 msec, rise time of 30 msec and half-fall time of 30 msec. The slower time course of the s.p.n.-induced twitch was not the result of any change in excitation–contraction coupling or muscle contraction characteristics, for direct stimulation produced indistinguishable twitches in s.p.n.-innervated and control muscles. Instead, the time

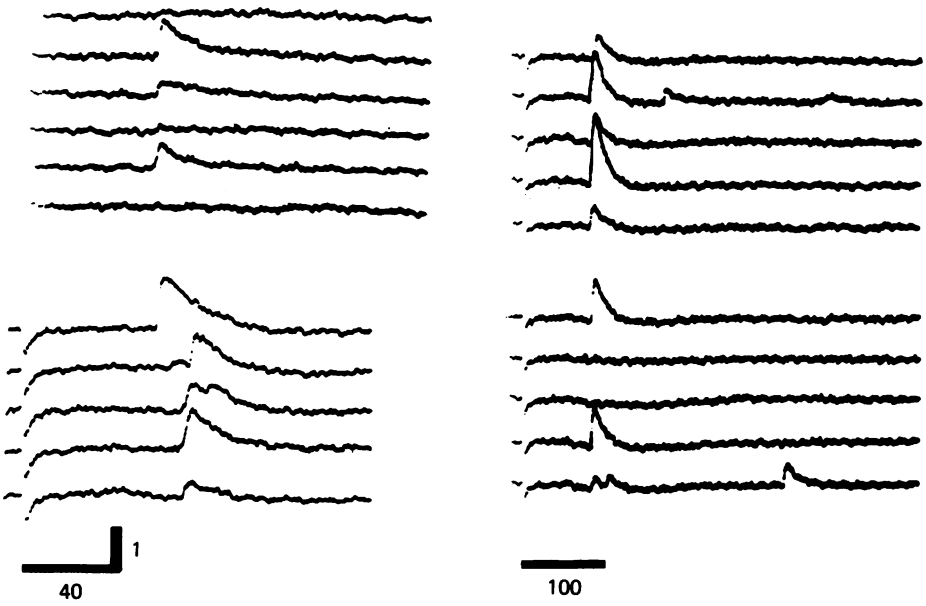

Fig. 2. S.p.n.-evoked e.p.p.s from preparations implanted 90 days (left) and 71 days (right). The records on the right show occasional min e.p.p.s as well. Both sets of records were made in normal frog Ringer. Note the failures, the large fluctuation in amplitude, and the dispersion in latencies of e.p.p.s.

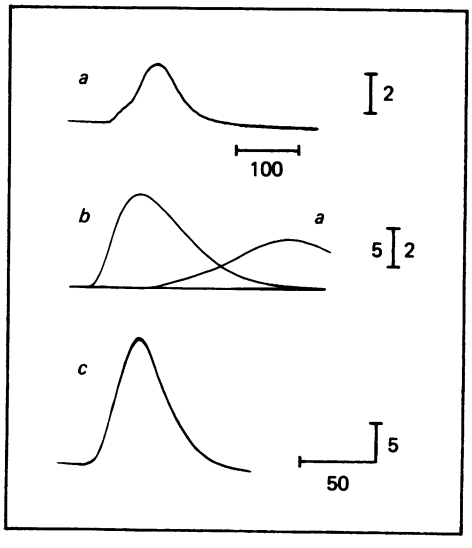

Fig. 3. Twitch tension records from a sartorius muscle implanted for 75 days with both the s.p.n. and an s.m.n., when stimulated via the s.p.n. (*a*), the s.m.n. (*b*), or directly (*c*). Calibrations in msec and g.

course presumably reflects asynchrony of activation of different motor units. This asynchrony also results in a much lower fusion frequency, between 10 and 15/sec in s.p.n. preparations, between 20 and 30/sec in s.m.n.-reinnervated muscles (Fig. 4).

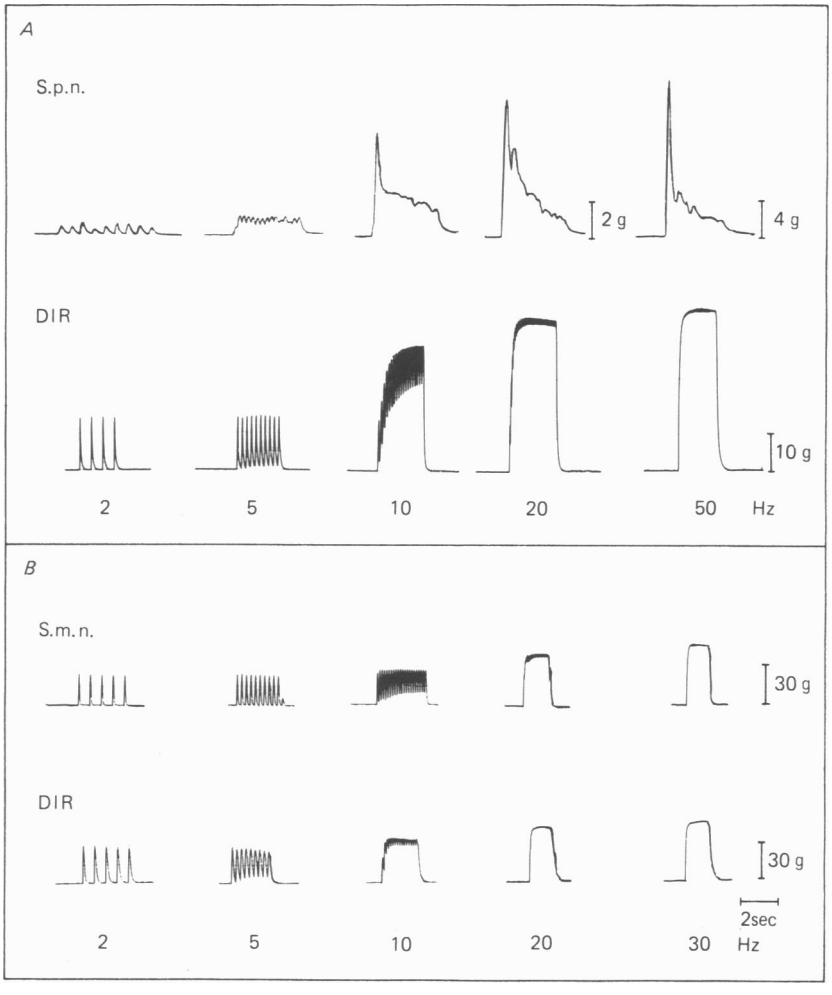

Fig. 4. Tension records for two sartorius muscles, one (*A*) innervated by the s.p.n. implanted 141 days, the other (*B*) by an s.m.n. implanted 140 days. The s.p.n. reached a maximum tetanus tension of about 40% of that evoked by direct stimulation, which was unusually high. The s.m.n., as was usually the case, had reinnervated its muscle completely. Note the low fusion frequency and the steep decline in tension during a tetanus in the s.p.n.-reinnervated muscle, even though the responses to direct stimulation were indistinguishable from those of s.m.n.-reinnervated or normal muscles.

It seems quite possible that the longer time course of contraction reported in cat cricothyroid muscle reinnervated by s.p.n. fibres is also a result of asynchronous driving rather than a change in the contractile biochemistry as proposed by Ramirez & Luco (1973).

Transmitter release properties at s.p.n. junctions

The mean miniature frequency, in eighteen fibres where miniatures were counted over a period of several minutes, was 0·06 ± 0·02/sec. Since this does not include the vast majority of fibres with e.p.p.s in which no minatures were seen in a period of at least 30 sec of examination, this is an over-estimate. S.m.n.-reinnervated muscle fibres had an average miniature frequency of 2·3 ± 3·8/sec (n = 42), comparable to

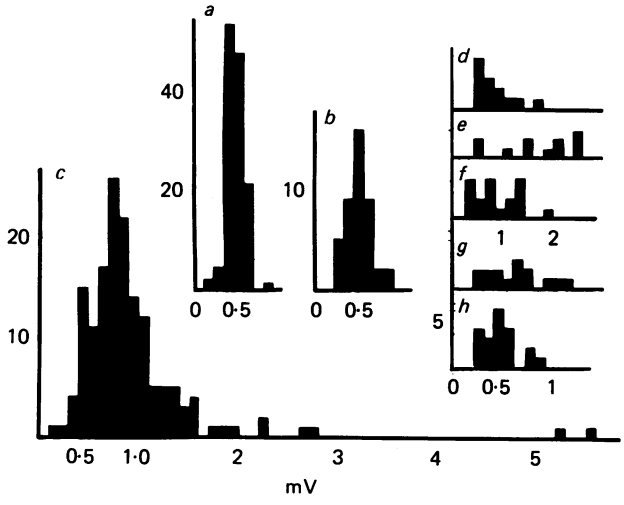

Fig. 5. Amplitude histograms of min e.p.p.s recorded from control (*a*), s.m.n.-reinnervated (*b*) and s.p.n.-innervated muscle fibres (*c–h*). Implantation times: *b*, s.m.n., 174 days; *c*, 114 days; *d–f*, 216 days; *g*, 98 days; *h*, 102 days. All s.p.n. junctions had been formed *de novo* on the pelvic end of a sartorius muscle.

the frequency in normal sartorius muscle, where the average is approximately 2/sec. Where a sufficient number of miniatures occurred in one s.p.n.-innervated junction, it was shown that the interval between events was random. There was no apparent correlation between the age of the preparation and miniature potential frequency. In both s.p.n.- and s.m.n.-reinnervated fibres, stimulation at a frequency of 1/sec caused more than a doubling in minature release frequency. Similarly, 100 mM-sucrose or 15 mM-KCl added to the Ringer caused a sharp increase in spontaneous release frequency, by 2·5–15 times the control rate for both (n = 7).

The mean amplitude of s.p.n. miniature e.p.p.s was not significantly different from those of s.m.n.-reinnervated or control muscles; the average in each of the three types of preparation was between 0·5 and 0·8 mV with overlapping ranges of 0·2–1·6 mV. On the other hand, there was much greater variability in the amplitudes of the s.p.n. miniatures than in s.m.n.-released miniatures. The coefficient of variation was 0·47 ± 0·13 (S.D.) for twelve s.p.n. terminals studied, compared with 0·29 ± 0·06 in seventeen representative s.m.n.-reinnervated junctions. Fig. 5 shows characteristic miniature potential amplitude histograms for a control sartorius junction, an s.m.n.-reinnervated fibre, and a number of s.p.n.-reinnervated fibres. There was considerable variability in the shapes of the s.p.n. histograms, some being broad and flat, others skewed to the small amplitudes. In a high percentage of cases there were

a few larger miniatures that extended well beyond the expected edge of the distribution, assuming gaussian statistics.

One of the most conspicuous features of the s.p.n.-junctional potentials was their low quantal content. E.p.p.s could often be examined in n.f.r. because they were subthreshold. In several instances whole muscles failed to show any twitching to a single stimulus, although pairs, triplets or tetani could produce strong contraction. It seems probable, in fact, that there were innervated fibres that could not be induced to contract even to tetanic stimulation, i.e. that many fibres were innervated by junctions with such low releases rates that they never exceeded threshold. In extreme cases, the average quantal content for individual junctions was less than

TABLE 1. Quantal contents of representative s.p.n. junctions

Duration of s.p.n. implantation (months)	Condition	$m = \frac{\bar{v}}{q}$	$m = \ln \frac{1}{f/n}$	$m = \frac{\bar{v}^2}{\text{var. } v}.(1-\bar{v}c)$
3·2	NFR*	1·2	1·2	1·0
3·3	NFR	0·39	0·41	0·36
4·75	Mg^{2+} block†	2·0	3·5	3·2
5·3	NFR	—	3·89	4·94
5·7	NFR	—	4·55	3·42
7·5	NFR	4·2	2·67	2·23

m = mean quantal content; q = mean quantal amplitude; f/n = number of failures/number of times stimulated; $\bar{v}$ = average e.p.p. amplitude; var. v = variance of v; c = reciprocal of the voltage difference between the resting potential and the equilibrium potential of the transmitter, assumed to be -10 mV.

* Normal frog Ringer solution.

† Partial block by use of Ringer containing 4 mM-Mg^{2+}; 0·5 mM-Ca^{2+}.

one, with more failures than e.p.p.s. In these low quantal content junctions, or in others where the quantal content was reduced by exposure to high Mg^{2+} and low Ca^{2+}, the mean quantal content, m, was calculated in two ways, which usually produced similar values: (*a*) by the independent methods of failures ($m = \ln n/f$), where n was the number of stimuli, and f the number of times the stimuli did not elicit a response, and (*b*) by e.p.p. variations [$m = \bar{v}^2(1-\bar{v}c)/\text{var. } v$], where $\bar{v}$ is the average e.p.p. amplitude, var. v the variance of v, and o represents the reciprocal of the voltage difference between the muscle fibre resting potential and the equilibrium potential of the transmitter, assumed to be -10 mV. In rare cases there were enough miniature potentials to calculate m using $m = v/q$. Table 1 shows values of m calculated by the different methods for six apparently single-component s.p.n. junctions (five of them in n.f.r.). There was no clear correlation between m and the duration of innervation.

It was possible to approximately fit the observed e.p.p. amplitude histograms with the expected Poisson distribution, given a certain quantal content. One example is shown in Fig. 6. However, the fit was usually far from perfect. Commonly, there was an unexpectedly large number of high quantal content e.p.p.s. The explanation for this relatively poor fit is not clear; perhaps it is in part a function of the wider distribution of miniature amplitudes and of release along a significant length of nerve.

Synaptic facilitation during paired s.p.n. stimulation

The s.p.n. junctions showed a much prolonged time course of facilitation. Table 2 summarizes the data for experiments in which pairs of stimuli were given and e.p.p. amplitudes (R_1 and R_2) were measured at several s.m.n. and s.p.n. junctions. The

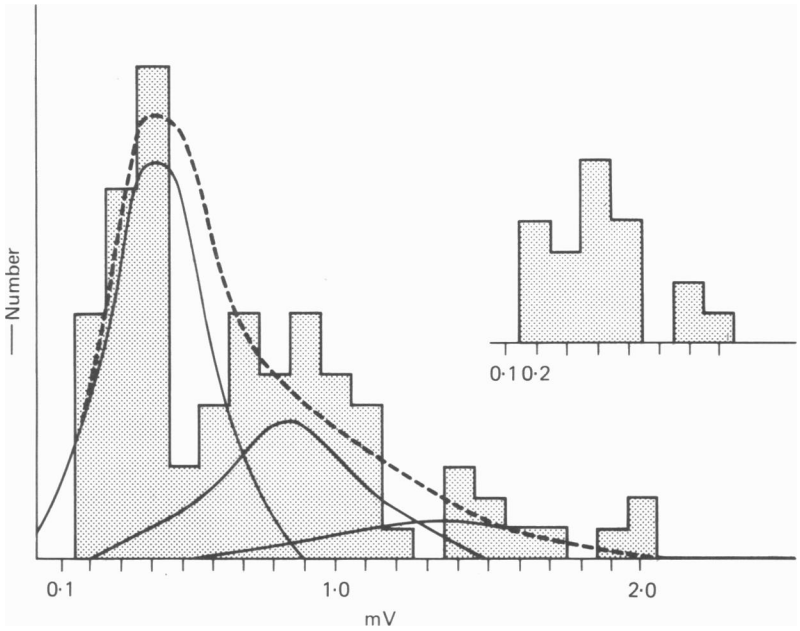

Fig. 6. E.p.p. amplitude histogram for a s.p.n. junction (102 days of implanatation). Also drawn in are the predicted single, double and triple quantum components and the composite curve, based on an m of 1 (estimates by different methods varied from 0·5 to 1·15) and an average min e.p.p. amplitude of $0{\cdot}43 \pm 0{\cdot}16$ mV. The min e.p.p. amplitude histogram is shown to the right. The calibration to the left represents a single event.

TABLE 2. Average values of e.p.p. facilitation parameters

	S.m.n. (n = 10)	S.p.n. (n = 7)
Maximal facilitation (R_2/R_1)	2·35 (range 1·6–4)	1·93 (1·6–2)
Interval which gave peak facilitation (msec)	10 (range 5–0·05)	17 (15–20)
Half-time of facilitation decay (msec)	38 (range 25–50)	70 (30–113)

degree of facilitation did not differ significantly in the two cases, and all values for the s.m.n. junctions are consistent with those reported by other workers for normally innervated muscles (Mallart & Martin, 1967). The time course of facilitation was distinctly different in the two cases, with slower onset and much slower decay in the s.p.n. junctions. Furthermore, about 40% of the s.p.n. junctions studied (not included in Table 2) showed depression instead of facilitation. The depression was maximal immediately after the nerve refractory period, and slowly declined over a period of 25–500 msec. Half-recovery usually required 10–15 msec.

The average tetanus–twitch ratio of s.p.n.-reinnervated muscles was greater than 10 ($12{\cdot}6 \pm 19{\cdot}4$ S.D., $n = 32$), compared with a ratio of approximately 2·5 in s.m.n.-reinnervated preparations. Since the degree of facilitation of s.m.n. and s.p.n. end-plate potentials was not significantly different, this can be attributed to the existence of a large number of junctions with e.p.p.s that did not exceed threshold to a single stimulus, but did exceed threshold with e.p.p. facilitation and temporal summation.

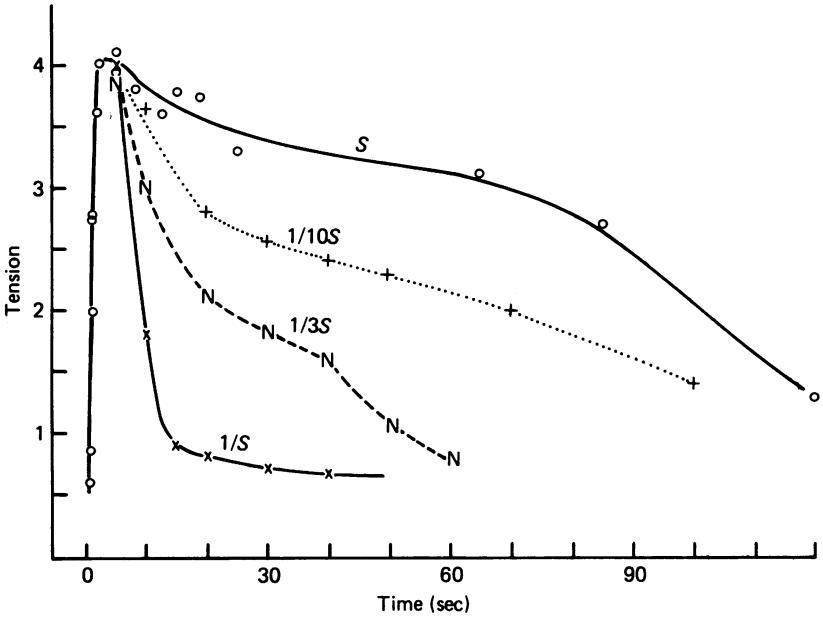

Fig. 7. Post-tetanic potentiation, judged by tension measurements, in a 266-day s.p.n.-implanted preparation. A 5 sec (30 stimuli/sec) tetanus was presented, ending at time 0. If a single stimulus was given at various times after such a tetanus, the time course of onset and decay of potentiation of release was as shown in the top line (*S*). If, beginning at the time of maximal potential, the nerve was stimulated at the rate 1/10 sec, 1/3 sec, or 1/sec, the three lower curves were obtained (not all points are plotted). Note the more rapid decline of potentiation at increased repetition rates of the test stimuli.

Tetanic potentiation and depression: tension measurements

After tetanic stimulation, the junctions in s.p.n.-reinnervated muscles showed dramatic depression followed by potentiation of release. This was seen in individual junctions, but it was most easily examined by recording tension in unblocked preparations where shifts in the number of e.p.p.s exceeding threshold were reflected in the tension generated. There was a pronounced drop in tension during a tetanus at any stimulation frequency above about 10/sec (see Fig. 4). As few as twenty stimuli in 1 sec could cause sharp depression followed by 20–50% potentiation in twitch tension. With more conditioning stimuli, the depression was only marginally greater, but recovery from depression and onset of potentiation occurred at progressively shorter delays after the tetanus. Stimulation at 30/sec for 3–5 sec caused depression of twitch amplitude to 20–30% of the amplitude of the initial single response for periods of a few seconds, followed by potentiation to a tension of ten or

more times the control amplitude after an average delay of 5 sec. Fig. 7 shows an example characteristic of the seven s.p.n.-reinnervated muscles studied in this way, after implantation times varying from 5 to 14 months. The time course of decline in potentiation depended on the number of stimuli in the initial train, but normally

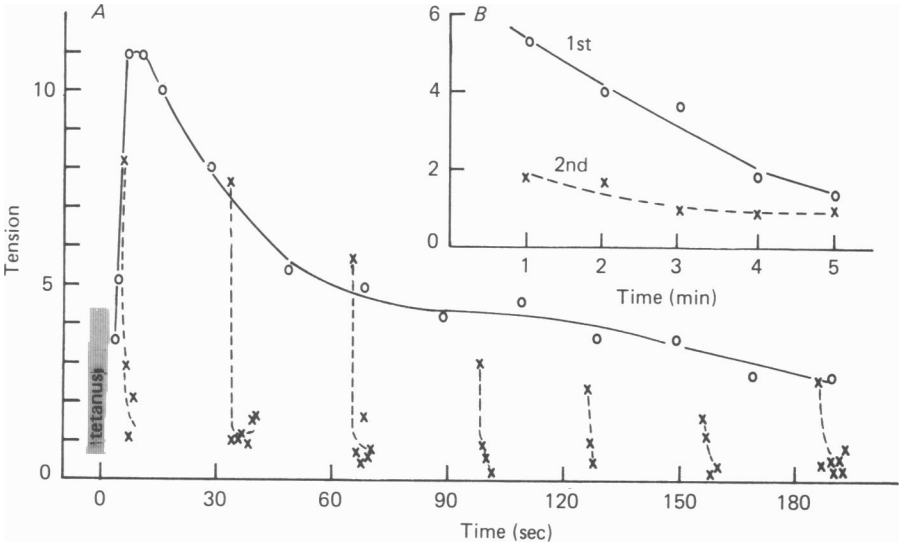

Fig. 8. *A*, post-tetanic potentiation following a 5 sec (30/sec) tetanus in a s.p.n.-reinnervated muscle (164 days after implantation). The continuous line shows the response to a single stimulus given at different intervals after identical tetani (the minimum interval between tetani was 10 min). The interrupted lines show the response amplitude to several brief trains of stimuli at 1/sec given at various times after a single tetanus. Note that each burst of test stimuli led to a rapid drop in tension, but that between bursts the state of potentiation recovered so that the first stimulus in the next burst resulted in essentially as much tension as it would have had there been no previous bursts. Note also the large decline in tension between the first and second stimulus in each burst, a drop that was larger the greater the potentiation. *B*, comparison of tension elicited by first and second stimuli in similar bursts of test stimuli in another preparation (s.p.n., 266 days), showing the same dependence of depletion within a burst on the amount of potentiation.

required several minutes, unless the junction was again stimulated, even at very low repetition rate. If, at any time during potentiation of release, test stimuli were given repetitively at frequencies of 0·1–1/sec, the drop in potentiation from that point could be made very steep (Fig. 8). If a rest period was given after such test stimuli, the post-tetanic potentiation showed recovery to approximately the level of decay without stimulation.

In contrast, similar short trains of stimuli applied to s.m.n.-reinnervated junctions partially blocked by Mg^{2+} resulted in no detectable tetanic depression or potentiation, nor did tetanic direct stimulation of s.p.n.-innervated (or control) muscles yield any depression or potentiation of release at these stimulus frequencies and durations.

C. Trophic effectiveness of the s.p.n.

Denervated muscles, transplanted to the lymph sac and left without innervation, quickly became revascularized and survived indefinitely. They atrophied to approxi-

mately 35% of their original weight within 6–12 months, but then remained at a relatively stable level, still capable of good contraction to direct electrical stimulation (Fig. 9). There was often considerable loss of fibres (sometimes up to 50%). However, the persistence of cholinesterase deposits at old end-plates, the ability of the muscle to contract to direct stimulation at all times after transplantation, and the histological finding that individual fibres continued to look normal throughout the period of transplantation indicates that the fibres that remained were the original fibres, not newly differentiated ones, as might occur in transplanted mammalian muscle (Carlson & Gutmann, 1975).

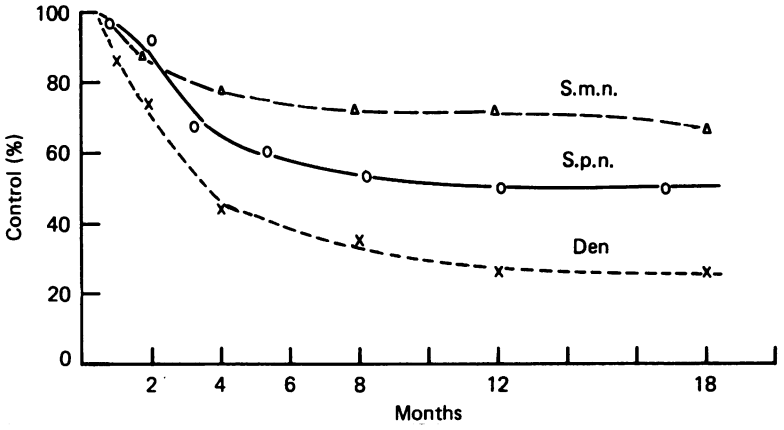

Fig. 9. Average curves showing the degree of weight loss of sartorius muscles transplanted to the lymph sac and either left denervated (den.) or reinnervated by either a somatic motor nerve (s.m.n.) or sympathetic preganglionic nerve (s.p.n.). Each point is the average of seven or more preparations within ±1 month of the time shown. The S.D. for each point was approximately ±15%.

The action potentials of long-term denervated muscle fibres, like those of control fibres, were quickly and completely blocked by 10^{-6} M-tetrodotoxin.

Muscles that were innervated by a s.p.n. atrophied less than completely denervated muscles, but nevertheless they were poorly maintained compared with muscles reinnervated by a s.m.n. Fig. 9 shows the change in weight of different muscles, as a percentage of the weight of the unoperated sartorius in that frog, when they were denervated or reinnervated by s.m.n. or s.p.n.s. Associated with this atrophy was an increase in input resistance. At reinnervation times greater than 3 months, the average input resistance of splanchnic innervated muscle fibres was $1{\cdot}41 \pm 0{\cdot}56$ MΩ ($n = 135$) (S.D.), compared with $0{\cdot}98 \pm 0{\cdot}28$ MΩ ($n = 116$) for fibres in s.m.n.-reinnervated muscles. The average input resistance of control sartorius innervated fibres was $0{\cdot}51 \pm 0{\cdot}3$ MΩ. Specific membrane resistances were not determined.

ACh sensitivity

It is well known that a denervated frog muscle develops high extrajunctional sensitivity to ACh, and that reinnervation by the normal nerve causes the restriction of ACh sensitivity to the region of the end-plate again (Miledi, 1960). We tested ACh sensitivity in two ways: (*a*) by immersing the muscle in different concentrations of ACh in the bathing medium and determining the concentration at which the most

sensitive fibres began to contract, and (*b*) by measurement of the responses of individual fibres to ionophoretically applied ACh. In long term experimental muscles, the build-up of connective tissue made it increasingly difficult to measure ACh sensitivity

TABLE 3. Sensitivity to bath-applied ACh: concentrations of ACh necessary to elicit twitching in fibres of muscles that had been denervated or reinnervated by a s.p.n. for 2 months or more, compared with the values for normally innervated muscles

	Denervated	S.p.n. reinnervated	Control
Threshold concentration (mean ± S.D.)	$1{\cdot}67 \pm 1{\cdot}9 \times 10^{-7}$ M	$9{\cdot}65 \pm 16 \times 10^{-7}$ M	$6{\cdot}6 \pm 5 \times 10^{-6}$ M
Number of muscles	13	17	20
Range	$0{\cdot}1$ to 5×10^{-7}	1 to 50×10^{-7}	10 to 100×10^{-7}
Sensitivity relative to control	38·6	5·7	1

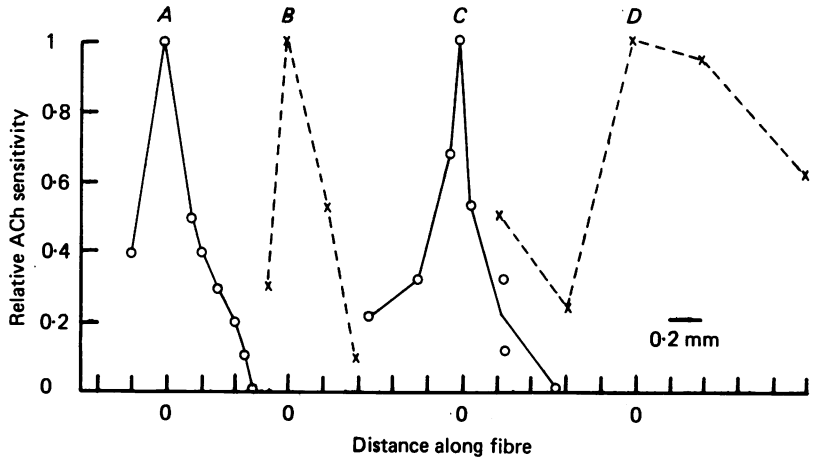

Fig. 10. Localization of sensitivity to ionophoretically applied ACh in s.p.n.-reinnervated muscles. Percentage of peak sensitivity is plotted as a function of distance from the most sensitive spot. The sensitive spots were correlated with the presence of subthreshold e.p.p. in *a*, *b*, and *d* (in normal frog Ringer). There was no e.p.p. at *c*. *a*, 6-week-old preparation, peak sensitivity 40 mV/nC. *b*, 3·3-month preparation, peak sensitivity 700 mV/nC. *c*, 5·6-month preparation, 10 mV/nC peak sensitivity. *d*, 3·3-month preparation (same as *b*), peak sensitivity 84 mV/nC. This profile may indicate the presence of two nearby spots of high sensitivity.

accurately by iontophoretic techniques, and even bath-applied ACh may have diffused more slowly due to connective tissue barriers; however, denervated muscles continued to be highly sensitive whenever tested. Table 3 summarizes the data on sensitivity to bath applied ACh in denervated, s.p.n.-reinnervated, and control muscles. The first twitching seen to bath-applied ACh in preparations innervated for more than 2 months was at concentrations intermediate between those needed for control and denervated muscles: approximately six times more sensitive than control muscles and one sixth as sensitive as denervated muscle. The s.p.n. was apparently capable of causing some loss of extrajunctional ACh sensitivity, but not as effectively as the appropriate s.m.n.

In order to differentiate between responses to junctional and extrajunctional

receptors, iontophoretic application of ACh was used to map surface fibres. In s.p.n. reinnervated fibres there was considerable variation from fibre to fibre in the distribution of ACh sensitivity. Three examples in which ACh sensitivity was high and closely localized to the region of an identified end-plate are shown in Fig. 10. Fibres that showed e.p.p.s or contraction exhibited lower extrajunctional sensitivity than neighbouring groups of fibres that showed no signs of innervation. In one 4·8 month preparation, eight out of ten fibres having e.p.p.s showed no extrajunctional sensitivity, the other two responded with 1 and 2 mV/nC. Twelve out of thirteen fibres having no indication of being innervated showed non-localized sensitivity, mean 6·8 mV/nC (mean ACh sensitivity of fibres denervated for this length of time was 50 ± 29 mV/nC). However, no strict correlation was observed between strength of innervation (ability to cause contraction) and the distribution of ACh sensitivity. There were preparations as old as 8–12 months in which demonstrably innervated fibres had non-localized ACh sensitivity and, conversely, ones in which weakly innervated muscles showing little or no contraction, even to tetanic stimulation, had low (bath applied) or restricted (ionophoresed) ACh sensitivity. Apparently extrajunctional sensitivity can be restricted, or at least maintained at low levels, even when the muscle fibres cannot be caused to contract. (The possibility cannot be eliminated, however, that at least some of the fibres in such muscles had been innervated with suprathreshold junctions at some point before the experiment.)

Cholinesterase

The s.p.n. differed from the s.m.n. in being unable to induce or maintain cholinesterase at its endings. In preparations tested more than 3–5 months after reinnervation, histochemically detectable cholinesterase (Karnovsky & Roots, 1964) had disappeared and no physiological effect of anticholinesterase (10^{-6} M-prostigmine) on e.p.p.s could be demonstrated. On the other hand, in shorter term transplants, it was sometimes possible to find physiological evidence for cholinesterase. E.p.p. enhancement by prostigmine was observed in the region of old end-plates, as judged by the diffuse AChEsterase still present histochemically. Fig. 11 shows an example in which the influence of prostigmine was shown to differ on two different e.p.p. components.

S.p.n. junctional pharmacology

Although the s.p.n. frequently innervated the sites of old end-plates, it was also able to induce the localization of ACh receptors to form functional post-synaptic sites *de novo*. S.p.n. nerves implanted on a thin (2 mm) fragment excised from the end-plate-free pelvic end of the muscle (Miledi, 1962) formed effective synapses with localized regions of high ACh sensitivity.

The properties of s.p.n.-induced receptors were tested pharmacologically to see if they were different from normal receptors, which are highly sensitive to DTC, poorly blocked by the ganglionic blocking agent, hexamethonium (C_6). Using tension as a criterion (Fig. 12*A*), it would appear that the s.p.n. innervated muscles were more sensitive both to DTC and hexamethonium (C_6) than were the s.m.n. innervated muscles. The same result was obtained with atropine. However, because of the lower safety factor at s.p.n. than at s.m.n. junctions, this is to be anticipated of any

blocking agent. When the effects of the drugs on subthreshold junctional potentials were examined (Fig. 12*B*), there was no indication that the susceptibility of the two types of endings differed to either DTC or C_6.

Interestingly, following hexamethonium block of either tension production or e.p.p.s in s.p.n. junctions, there was often an over-shooting recovery; responses were briefly as much as 20–50% bigger than they had been before the drug was applied. The explanation of this phenomenon is not known.

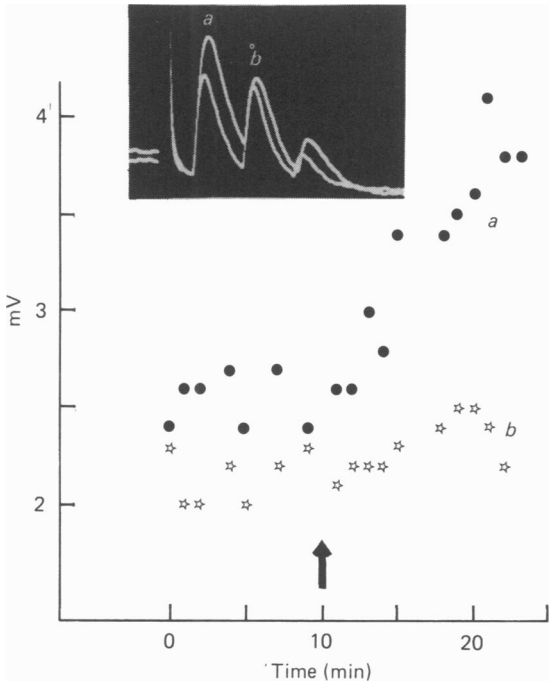

Fig. 11. Effect of 10^{-5} M-prostigmine on different components of a multiple e.p.p. in an 86-day s.p.n.-implanted muscle. The drug was introduced into the bath at the time indicated by the arrow. In the next 10 min, the first peak (*A*) increased markedly in amplitude and duration, while the second peak (*B*) was virtually unchanged. The third peak behaved much like the first. The inset shows averages of sixteen responses before (lower trace) and 10 min after prostigmine (upper trace). Possibly some release sites (first and third peaks) were near concentrations of cholinesterase at old end-plates, while one was not. The first peak was actually composed of two components, and all four components had different thresholds, indicating innervation by separate nerve fibres.

DISCUSSION

It was necessary to establish that the fibres in the splanchnic nerve that drive muscle fibres are true autonomic preganglionics, and not a small population of aberrant somatic motor nerves. We are satisfied that the latter is not the case for the following reasons. The splanchnic nerve fibres that innervate the transplanted skeletal muscle are relatively high threshold, slow conducting, and have long refractory periods compared with somatic motor nerves. Moreover, the properties of the synapses found are consistently different from those found in muscles reinnervated by an s.m.n.: e.p.p.s are longer, of lower quantal content, more often show multiple

components, show different depression and facilitation characteristics, and are not prolonged by anticholinesterases.

The conduction velocities and differences in latency between different e.p.p. components observed in our experiments are consistent with effective innervation by unmyelinated or very small diameter myelinated fibres. Assuming a constant conduction velocity of 0·5 m/sec, a latency difference of 50 msec would correspond to an extra 25 mm of nerve fibre. While this is possible in a 3–4 cm long muscle, it seems unlikely that there was a length difference of that magnitude. Probably the longer latency components were evoked by nerve fibres that had greatly slowed their conduction velocity within the muscle. Differences as great as 25 msec between

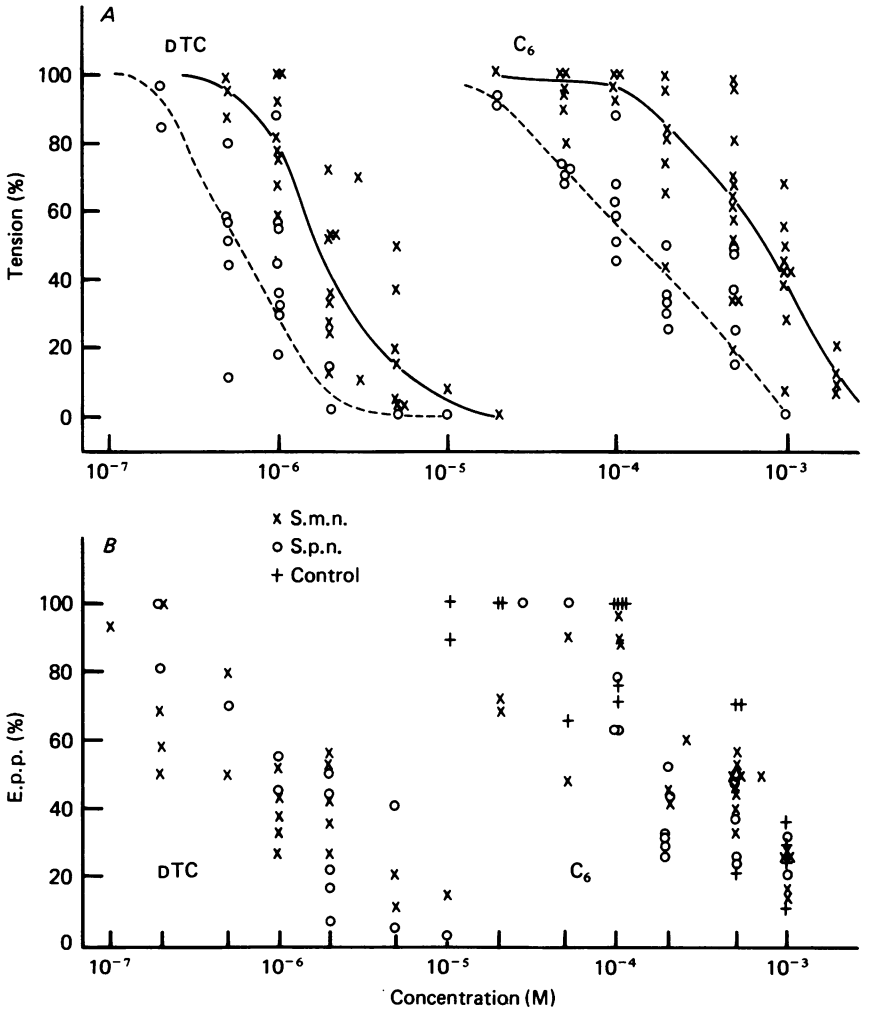

Fig. 12. *A*, dose–response curves for the blocking effect of D-tubocurarine chloride (DTC) and hexamethonium (C_6) on tension developed in s.p.n. (○) and s.m.n. (×) reinnervated muscles. The s.p.n. was much more easily blocked by both drugs. *B*, dose–response curves showing the blocking effects of DTC and C_6 on e.p.p.s in s.p.n. (○), s.m.n. (×), and control sartorius innervated muscles (+). There was no apparent difference in effect correlated with the type of innervation.

components with the same threshold and similar, fast rise times, apparently from the same nerve fibre, reinforced this conclusion. It is unlikely that the same nerve would terminate twice, close together on the same muscle fibre, with a 10–15 mm length of nerve between. It seems more likely that the s.p.n. fibres, near their terminations, might have a much reduced conduction velocity, no greater than approximately 0·08–0·10 m/sec, or that there can be large delays at axonal constrictions or branch points.

Generally, the properties displayed by the splanchnic e.p.p.s are consistent with what is known about release properties of endings in frog sympathetic ganglia, although different sympathetic and parasympathetic ganglia exhibit significant differences in properties. There are even major differences among cell populations in the same ganglion (e.g. the chick ciliary ganglion (Marwitt, Pilar & Weakly, 1971)). Since nothing specific is known about the population of cells which synapse in the coeliac ganglion, we can only compare in a general way the s.p.n. reinnervated sartorius with other autonomic preparations.

Min e.p.s.p.s are infrequent (under 1/sec) in frog sympathetic ganglia (Blackman, Ginsborg & Ray, 1963*b*; Nishi, Soeda & Koketsu, 1967) as at the s.p.n.-skeletal muscle synapses, and min e.p.s.p. frequency is sharply increased by either an increase in tonicity or external KCl (Blackman *et al.* 1963*b*). In contrast, the min e.p.s.p. frequency in parasympathetic ganglia is reported to not be detectably increased by changes in tonicity (Dennis, Harris & Kuffler, 1971).

The broad and skewed nature of min e.p.p. amplitude histograms that we observed is similar to that reported for other ganglia (Blackman *et al.* 1963*b*; Dennis *et al.* 1971; Martin & Pilar, 1964*a*; Blackman & Purves, 1969). This broad distribution is reflected in the larger coefficient of variation for min e.p.p. amplitudes: 0·47 for s.p.n., 0·29 for s.m.n.-reinnervated junctions. This compares with 0·32–0·71 (mean 0·48) for *Rana pipiens* sympathetic ganglia (calculated from data presented by Blackman *et al.* 1963*c*), and with values of 0·202 (our data) to 0·225 (Boyd & Martin, 1956) or 0·3 (Fatt & Katz, 1952) for normal neuromuscular junctions. The physiological explanation for this variability in ganglionic synapses is not known. It might represent a wider range in quantal sizes, or some simultaneous release of multiple packets of quanta (Martin & Pilar, 1964*a*). In the s.p.n.-reinnervated fibres it could also result from a dispersion of release sites relative to the recording electrode (which is less likely in ganglia, where most synapses appear to be on the cell body (Pick, 1963)), or the presence of AChEsterase at some release sites but not others.

A curious feature of the s.p.n. min e.p.p.s was their average amplitude, which did not differ significantly from that of the s.m.n.-reinnervated junctions. Both in amphibian sympathetic ganglia (Blackman *et al.* 1963*b*; Nishi *et al.* 1967; Hunt & Nelson, 1965) and parasympathetic ganglia (Dennis *et al.* 1971) most min e.p.s.p.s fall in the range of 0·5–2·6 mV, with maximum rates of rise of 0·1–3 V/sec, in the same range as normal neuromuscular min e.p.p.s. Since the input impedance of the ganglion cells is 100 or more times higher than that of muscle fibres (Hunt & Nelson, 1965; Harris, Kuffler & Dennis, 1971), the single quantum conductance change is estimated to be 10–100 times less than at the normal frog neuromuscular junction ($0{\cdot}5–1{\cdot}8 \times 10^{-8}$ mho (Blackman *et al.* 1963*b*) and $1{\cdot}7 \times 10^{-9}$ mho for sympathetic ganglion cell min e.p.s.p.s (Nelson & Hunt, 1965) compared with $1{\cdot}43 \times 10^{-7}$ mho

for the sartorius neuromuscular junction (Takeuchi & Takeuchi, 1960)). If these values are accurate, and if the number of ACh molecules in a packet released by a preganglionic terminal remains constant, it might be predicted that min e.p.p.s would not be detectable in our preparation. Yet they were approximately the same as those of s.m.n. e.p.p.s. These observations suggest that the smaller conductance change per quantum indicated for ganglion cells, compared with muscle, may be due to lower concentrations of ACh receptor (or reduced coupling to conductance changes) at post-synaptic sites in ganglion cells (Hunt & Nelson, 1965; Harris *et al.* 1971), rather than to smaller amounts of ACh in the preganglionic quanta (Hunt & Nelson, 1965). If this is the case, it can also be concluded that *de novo* junctions induced on muscle by an s.p.n. have receptor concentrations characteristic of muscle rather than the ganglionic post-synaptic sites.

Quantal content of ganglionic e.p.s.p.s is normally small, corresponding to no more than 1–10 quanta/impulse per synaptic knob (Blackman *et al.* 1963*c*; Nishi *et al.* 1967; see also Martin & Pilar, 1964*a*; McLachlan, 1975; and Blackman & Purves, 1969, for similar findings in avian and mammalian ganglia). This would be consistent with the existence of very few functional varicosities at any given s.p.n. axonal termination on a muscle fibre, since quantal contents of only 1–5/impulse were seen frequently in unblocked preparations.

The release properties of s.p.n. junctions differed from those of s.m.n. or control junctions in two respects which cannot be accounted for simply by the lower quantal content. S.p.n. junctions exhibited a much longer time course of facilitation, and much more prominent potentiation of release. The long time course of facilitation is similar to that observed in the chick ciliary ganglion at room temperature (20–25 °C) by Martin & Pilar (1964*b*), and in guinea-pig sympathetic ganglia by Blackman & Purves (1969). Interestingly, vagus-reinnervated frog muscles showed facilitation at 1/sec. (Landmesser, 1971), a rate that is significantly slower than that in almost all s.p.n.-reinnervated fibres studied here. McLachlan (1975), in a more recent analysis of guinea-pig ganglionic junctions, described two components of facilitation. One decayed exponentially with a time constant of approximately 200 msec the other with a time constant of 13 sec. The longer time course was not seen with single stimuli in our experiments, but a similar phenomenon may have contributed to the prominent potentiation of release observed following only a few impulses.

Such potentiation is seen at normal frog neuromuscular junctions only after several hundred impulses (Rosenthal, 1969). In the present experiments, s.m.n.-reinnervated muscles partially blocked by Mg^{2+} showed neither post-tetanic depression nor potentiation even with as many as 200–300 stimuli. S.p.n.-reinnervated muscles, on the other hand, showed prominent depression and potentiation following as few as twenty stimuli in 1 sec. Both depression and potentiation were dependent on the absolute number of conditioning stimuli rather than the stimulus frequency, at least between rates of 10/sec and 60/sec. Thus there appears to be much greater lability of release in the s.p.n. terminals than in s.m.n. terminals.

The reasons for the greater potentiation in s.p.n. than s.m.n. junctions is not clear. It is generally accepted that the potentiation of release at normal frog neuromuscular junctions is due to a build-up of intraterminal Ca^{2+}, which leads primarily to an increase in the probability of release rather than to an increase in available transmitter

stores (Rosenthal, 1969; Weinreich, 1971). Possibly the s.p.n. terminals are smaller, with a larger surface-to-volume ratio, and a few impulses produce a larger influx of Ca^{2+}. In this case, however, one might have expected a higher degree of facilitation, which was not observed. Alternatively, there might be fewer mitochondria in the terminals, resulting in reduced ability to sequester Ca^{2+} internally (Alnaes & Rahamimoff, 1975) or there might be less effective mechanisms of pumping Ca^{2+} out of the cell.

The properties described above all apply to transmitter release, and appear generally consistent with what is known about the preganglionic fibres. It is not evident that any of these properties is influenced by the fact that muscle is a totally abnormal post-synaptic cell.

Post-synaptic physiology does reveal differences attributable to the abnormal nerve supply. As Landmesser (1972) observed for vagal-reinnervated frog sartorius fibres, there is no histochemical or physiological evidence for cholinesterase at the new junctions. Cholinesterase has been found both histochemically and physiologically at frog ganglionic junctions, although its effect is variable and often slight (Giacobini, 1956; Blackman *et al.* 1963*a*; Nishi *et al.* 1967; Dennis *et al.* 1971; McMahan & Kuffler, 1971). In the present experiments, the only histochemically or physiologically detectable AChEsterase in s.p.n. preparations was that seen at the original endplates, which gradually became diffuse and disappeared over a period of several months, despite the apparent presence of s.p.n. terminals at or near the sites of old end-plates (Fig. 11). In contrast, AChEsterase can readily be demonstrated both histochemically and physiologically at *de novo* junctions formed by foreign s.m.n.s (unpublished observations).

Unlike the vagus (Landmesser, 1972), the s.p.n. formed junctions that were equally sensitive to DTC and hexamethonium. There is no obvious explanation for the difference, but consistency may not be expected in view of the variety of different properties exhibited by different autonomic neurones. Marwitt *et al.* (1971) found that different neuronal populations in the pigeon ciliary ganglion showed differential sensitivity to hexamethonium. Synaptic transmission was abolished in the choroid pathway with hexamethonium 5×10^{-4} g/ml. while that in the ciliary pathway was unaffected. On the other hand, DTC (2×10^{-6} g/ml.) was more effective on the ciliary response.

The s.p.n. is at least partially successful in reducing extrajunctional supersensitivity to ACh. Restriction of sensitivity to the sites of new and old junctions is not as rigid or complete as that effected by an s.m.n. but it was seen in some preparations even in the absence of any evoked contraction by the s.p.n. It may be concluded that the s.p.n. is weakly effective in inhibiting the synthesis of extrajunctional receptors. Since it is still not known what aspect of nerve (or muscle) activity is responsible for regulation of extrajunctional ACh receptor concentration, it is not possible to conclude what differs in the case of the s.p.n.

We gratefully acknowledge the participation of Dr K. Bloome in early phases of this research. We are also indebted to Drs M. Letinsky and P. O'Lague for valuable advice during the preparation of the manuscript. Essential histological assistance was provided by Bibbi Wolowske, Katherine Graves, and Claire Ling. This research was supported by U.S.P.H.S. research grant NS 06232 and training grant NS 05670.

REFERENCES

ALNAES, E. & RAHAMIMOFF, R. (1975). On the role of mitochondria in transmitter release from motor nerve terminals. *J. Physiol.* **248**, 285–306.

BEATTIE, J., DUEL, A. B. & BALLANCE, C. (1932). The effects of stimulation of the hypothalamic pupillo-dilator centre after successful anastomosis between the cervical sympathetic and certain motor nerves. *J. Anat.* **66**, 283–299.

BENNETT, M. R., MCLACHLAN, E. M. & TAYLOR, R. S. (1973). The formation of synapses in reinnervated mammalian striated muscle. *J. Physiol.* **233**, 481–500.

BLACKMAN, J. G., GINSBORG, B. L. & RAY, C. (1963*a*). Synaptic transmission in the sympathetic ganglion of the frog. *J. Physiol.* **167**, 355–373.

BLACKMAN, J. G., GINSBORG, B. L. & RAY, C. (1963*b*). Spontaneous synaptic activity in sympathetic ganglion cells. *J. Physiol.* **167**, 389–401.

BLACKMAN, J. G., GINSBORG, B. L. & RAY, C. (1963*c*). On the quantal release of the transmitter at a sympathetic synapse. *J. Physiol.* **167**, 402–415.

BLACKMAN, J. G. & PURVES, R. D. (1969). Intracellular recordings from ganglia of the thoracic sympathetic chain of the guinea-pig. *J. Physiol.* **203**, 173–198.

BOYD, I. A. & MARTIN, A. R. (1956). The end-plate potential in mammalian muscle. *J. Physiol.* **132**, 74–91.

BROWN, J. O. & SATINSKY, V. P. (1951). Functional restoration of the paralyzed diaphragm following cross-union of the vagus and phrenic nerves. *Am. J. med. Sci.* **222**, 613–622.

CARLSON, B. M. & GUTMANN, E. (1975). Regeneration in free grafts of normal and denervated muscles in the rat: morphology and histochemistry. *Anat. Rec.* **183**, 47–62.

DENNIS, M. J., HARRIS, A. J. & KUFFLER, S. W. (1971). Synaptic transmission and its duplication by focally applied acetylcholine in parasympathetic neurons in the heart of the frog. *Proc. R. Soc.* B. **177**, 509–539.

ECKER, A. & WEIDERSHEIM, R. (1899). *Anatomie des Frosches*, Part II, p. 548. Braunschweig: Vieweg.

FATT, P. & KATZ, B. (1952). Spontaneous subthreshold activity at motor nerve endings. *J. Physiol.* **117**, 109–128.

GIACOBINI, E. (1956). Histochemical demonstration of AChE activity in isolated nerve cells. *Acta physiol. scand.* **36**, 276–290.

GRINNELL, A. D., LETINSKY, M. S. & RHEUBEN, MARY B. (1979). Competitive interaction between foreign nerves innervating frog skeletal muscle. *J. Physiol.* **289**, 241–262.

GUTH, L. & FRANK, K. (1959). Restoration of diphragmatic function following vagophrenic anastomosis in the rat. *Expl Neurol.* **1**, 1–12.

HARRIS, A. J., KUFFLER, S. W. & DENNIS, M. J. (1971). Differential chemosensitivity of synaptic and extrasynaptic areas on the neuronal surface membrane in parasympathetic neurones of the frog, tested by microapplication of acetylcholine. *Proc. R. Soc.* B. **177**, 541–553.

HILLARP, N. A. (1946). Structure of the synapse and the peripheral innervation apparatus of the autonomic nervous system. *Acta anat.* suppl. IV, 153.

HUNT, C. C. & NELSON, P. G. (1965). Structural and functional changes in the frog sympathetic ganglion following cutting of the presynaptic nerve fibres. *J. Physiol.* **177**, 1–20.

KARNOVSKY, M. J. & ROOTS, L. (1964). A 'direct coloring' thiocholine method for cholinesterases. *J. Histochem. Cytochem.* **12**, 219–221.

LANDMESSER, L. (1971). Contractile and electrical responses of vagus-innervated frog sartorius muscles. *J. Physiol.* **213**, 707–725.

LANDMESSER, L. (1972). Pharmacological properties, cholinestrase activity and anatomy of nerve-muscle junctions in vagus-innervated frog sartorius. *J. Physiol.* **220**, 243–256.

LANGLEY, J. N. & ANDERSON, H. K. (1904). The union of different kinds of nerve fibres. *J. Physiol.* **31**, 365–391.

MALLART, A. & MARTIN, A. R. (1967). An analysis of facilitation of transmitter release at the neuromuscular junction of the frog. *J. Physiol.* **193**, 679–694.

MARTIN, A. R. & PILAR, G. (1964*a*). Quantal components of the synaptic potential in the ciliary ganglion of the chick. *J. Physiol.* **175**, 1–16.

MARTIN, A. R. & PILAR, G. (1964*b*). Presynaptic and post-synaptic events during post-tetanic potentiation and facilitation in the avian ciliary ganglion. *J. Physiol.* **175**, 17–30.

MARWITT, R., PILAR, G. & WEAKLY, J. N. (1971). Characterization of two ganglion cell populations in avian ciliary ganglia. *Brain Res.* **25**, 317–324.

MCLACHLAN, E. M. (1975). An analysis of the release of acetylcholine from preganglionic nerve terminals. *J. Physiol.* **245**, 447–466.

MCMAHAN, U. J. & KUFFLER, S. W. (1971). Visual identification of synaptic boutons on living ganglion cells and of varicosities in postganglionic axons in the heart of the frog. *Proc. R. Soc.* B. **177**, 485–508.

MILEDI, R. (1960). Properties of regenerating neuromuscular synapses in the frog. *J. Physiol.* **154**, 190–205.

MILEDI, R. (1962). Induced innervation of end-plate-free muscle segments. *Nature, Lond.* **193**, 281–282.

NICHOL, J. A. C. (1952). The autonomic nervous system in lower chordates. *Biol. Rev.* **27**, 1–49.

NISHI, S., SOEDA, H. & KOKETSU, K. (1967). Release of acetylcholine from sympathetic preganglionic nerve terminals. *J. Neurophysiol.* **30**, 114–134.

PICK, J. (1963). The submicroscopic organization of the sympathetic ganglion in the frog (*Rana pipiens*). *J. comp. Neurol.* **120**, 409–462.

RAMIREZ, B. & LUCO, J. V. (1973). Some physiological and biochemical features of striated muscles reinnervated by preganglionic sympathetic fibres. *J. Neurobiol.* **4**, 525–533.

ROSENTHAL, J. (1969). Post-tetanic potentiation at the neuromuscular junction of the frog. *J. Physiol.* **203**, 121–133.

TAKEUCHI, A. & TAKEUCHI, N. (1960). Further analysis of relationship between end-plate potential and end-plate current. *J. Neurophysiol.* **23**, 397–402.

VERA, C. L. & LUCO, J. V. (1967). Reinnervation of smooth and striated muscle by sensory nerve fibres. *J. Neurophysiol.* **30**, 620–627.

WEINREICH, D. (1971). Ionic mechanism of post-tetanic potentiation at the neuromuscular junction of the frog. *J. Physiol.* **212**, 431–446.

WEISS, P. (1935). Experimental innervation of muscles by the central ends of afferent nerves (establishment of one neuron connection between receptor and effector organ), with functional tests. *J. comp. Neurol.* **61**, 135–174.