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COMPETITIVE INTERACTION BETWEEN FOREIGN NERVES INNERVATING FROG SKELETAL MUSCLE

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SUMMARY

1. Competition between two foreign nerves innervating frog skeletal muscle has been studied by using pairs of somatic motor nerves (s.m.n.s) or one s.m.n. and the preganglionic splanchnic nerve (s.p.n.) implanted into a denervated sartorius muscle that has been transplanted to the lymph sac of the back.

2. A single s.m.n. implanted into the muscle succeeded in innervating essentially every fibre within 2–3 months; tetanic stimulation of the nerve elicited 90–100% of the maximal direct tetanus tension. Most of the e.p.p.s were suprathreshold, since a single indirect stimulus evoked a twitch 60–100% as large as that to a direct stimulus.

3. If two s.m.n.s were implanted simultaneously, tetanic stimulation of either elicited 80–100% of the maximal tension to direct stimulation. If one nerve was implanted 2–3 months before the other, the second, although usually less effective than the first, normally innervated 50–100% of the fibres, with approximately the same time course of innervation as a single s.m.n.

4. Mutual synaptic repression was seen on examination of twitch tensions. With either simultaneous or staggered innervation, stimulation of each s.m.n. resulted in a twitch of 30–50% of the total direct twitch tension, with little overlap between the fields driven by the two nerves. Intracellular recordings showed that the distribution of subthreshold and spike-producing e.p.p.s reflected the existence of separate twitch fields. Even if one s.m.n. was implanted several months before the other and had time to establish suprathreshold junctions on most muscle fibres, an s.m.n. implanted later was able to reduce sharply the effectiveness of many junctions from the earlier nerve while itself innervating most muscle fibres.

5. The subthreshold e.p.p.s had low quantal content, typically ten or fewer quanta/e.p.p. The min e.p.p. frequency was very low, while min e.p.p. amplitude appeared to be normal.

6. In the vast majority of muscle fibres, junctions from the two nerves were not within recording distance of each other. Hence, we infer that the competitive interaction was mediated somehow via the muscle fibre.

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7. The preganglionic splanchnic nerve, which also successfully reinnervated frog skeletal muscle, competed with a foreign s.m.n. in ways which differ qualitatively from the competition by a second s.m.n. In the presence of a s.m.n., synapses of the s.p.n. were almost universally subthreshold. However, if the s.p.n. was implanted 2–3 months before the s.m.n., the s.m.n. was prevented for several months from innervating fibres driven by the s.p.n. This delay in s.m.n. reinnervation was greater than if the first nerve implanted was also an s.m.n.

8. After 6–8 months of dual innervation by s.m.n. and s.p.n., the s.m.n. became almost totally dominant. However, if the s.m.n. was then sectioned, the s.p.n. became as effective, within approximately 1 week, as it would have been in the absence of the s.m.n.

INTRODUCTION

Although denervated muscle in lower vertebrates can be reinnervated successfully by any cholinergic nerve, there is evidence of specificity in these connexions. The original or more appropriate nerve is preferred to others. This was first recognized in the ability of the appropriate fish extraocular muscle nerves to regenerate into their respective muscles, behaviourally displacing a previously functional foreign nerve (Sperry & Arora, 1965). It seems probable that the extensive work of Weiss (see review, 1955) on myotypic responses in supposedly inappropriately innervated amphibian muscle can best be explained by specific regrowth of nerve fibres to their correct muscles (Grimm, 1971). Miledi and his co-workers (Elul, Miledi & Stefani, 1970; Miledi, Stefani & Steinbach, 1971; Stefani & Schmidt, 1972) have reported that in frogs denervated slow muscle fibres of the iliofibularis muscle are first reinnervated by fast motor nerve fibres which in time are displaced by the more slowly regenerating motor fibres. Similar but less clear-cut specificity has been reported for the regeneration of fast and slow nerve fibres to mixed avian muscles (Feng, Wu & Yang, 1965; Hnik, Jirmanova, Vyklicky & Zalena, 1967; Bennett, Pettigrew & Taylor, 1973).

Thus, there is increasing evidence for competition between nerves and selectivity in the reinnervation of fish and amphibian muscle. The nature of this competition and the mechanism of functional displacement of one nerve by the other are unknown. In numerous studies on lower vertebrates Mark and his associates (Marotte & Mark, 1970*a*, *b*; Mark & Marotte, 1972; Mark, Marotte & Mart, 1972; Cass, Sutton & Mark, 1973; Mark, 1975), suggest that there is a reversible repression or inactivation of one population of synapses, in which failure is presynaptic, seen as a sharp reduction in quantal content. Other experiments on fish extraocular muscle reinnervation (Scott, 1975, 1977) and doubly reinnervated fish gill muscle (Frank & Jansen, 1976) have failed to reveal physiological evidence of the behavioural displacement of connexions reported by Mark. However, Yip & Dennis (1976); Dennis & Yip (1978) and Bennett & Raftos (1977) have confirmed the existence of synaptic displacement or repression (with reduced presynaptic release) in salamander limb muscle preparations. Bennett & Raftos (1977), on the basis of quantal content measurements, suggest that the sprouted terminals innervate the original synaptic sites, but with less affinity than the original nerve, which, when it regenerates, also reinnervates the old synaptic sites and causes the inappropriate nerve terminals to regress. In another recent study,

Haimann, Mallart & Zilber-Gachelin (1976), working with the doubly innervated pectoralis muscle in *Xenopus laevis*, where end-plates are more focal, have concluded that fibres can be reinnervated by both nerves, with competition favouring the original nerve if the two end-plates are very close to one another on the same fibre.

The present experiments provide evidence for competition between pairs of foreign nerves reinnervating frog skeletal muscle, when both nerves are foreign somatic motor nerves or when one is a somatic motor nerve, the other an autonomic preganglionic nerve (Grinnell & Rheuben, 1979). Some of these results have been reported in preliminary form elsewhere (Grinnell, Rheuben & Letinsky, 1977*a*).

METHODS

The experimental techniques used for operations, removal of the experimental muscle, and for electrophysiological recordings and tension measurements were the same as in the previous paper (Grinnell & Rheuben, 1979). The only major modification was the implantation into the transplanted frog sartorius muscle of a second nerve, either during the original operation or at a later time, normally after 60–90 days. The major combinations tested were: two somatic motor nerves (s.m.n., normally using spinal nerves IV and V and sometimes VI) or combinations of an s.m.n. and a sympathetic preganglionic nerve (s.p.n., the splanchnic nerve). Normally the nerves were implanted into the middle of the muscle *ca.* 0·5–1 cm from each other. No effort was made to attach either nerve near the point of entry of the original sartorius nerve, but care was taken to cause as little damage to muscle fibres as possible.

The success of reinnervation by a given nerve was judged by the ability of that nerve to elicit muscle fibre contraction on supramaximal stimulation with a single stimulus or a train of stimuli at 30–50/sec. The muscle was judged to be 100 % reinnervated when the tension developed due to nerve stimulation was as great as that produced by supramaximal direct stimulation of the muscle. Each nerve was independently stimulated by a capillary suction electrode. With this method the nerves were activated independently and no current spread was observed. Direct stimulation was accomplished by passing current between a bath electrode and an electrode located across the surface of the muscle near the pelvic end. In a few intances when there were excessive amounts of connective tissue, indirect stimulation via the nerve produced slightly greater tension than direct stimulation. In these cases, the maximum elicitable tension was taken as 100 %. It must be realized, of course, that subthreshold innervation is not measured by this technique. All values are expressed as mean ± S.D.

Tension was measured isometrically with either a Statham UC-3 transducer (sensitivity 1·4 mV/g) or a strain gauge built in the laboratory (sensitivity 1·2 mV/mg). The latter device was capable of recording the tension developed by a twitch in as few as five to ten fibres, and even single fibre contractions could be visualized with our dissecting microscope. The muscle length was set to yield optimum tension. Many of the values cited for relative tensions are based upon averages (Hewlett–Packard 5480 Signal Analyzer) of eight to thirty-two stimulation presentations. When tetani were measured, several minutes were allowed between stimulus trains to ensure complete recovery of the preparation.

RESULTS

Reinnervation of muscle with a single nerve

Muscle fibres in transplanted frog sartorius muscles can survive indefinitely even if permanently deprived of innervation (see Grinnell & Rheuben, 1979; Landmesser, 1971). When either a somatic motor nerve (s.m.n.) or sympathetic preganglionic nerve (s.p.n.) was implanted into a transplanted sartorius muscle, it grew profusely into the muscle and formed functional synapses. The first evidence of suprathreshold excitation of muscle fibres and tension development was seen 30–40 days after

implantation. Fig. 1 shows the time course of innervation by either a foreign s.m.n. or the s.p.n. Subthreshold reinnervation probably occurred earlier than this, but was not studied systematically.

A single s.m.n. quickly innervated virtually every fibre in the muscle and was capable of driving each above threshold. There was $98 \pm 5\%$ ($n = 13$) reinnervation

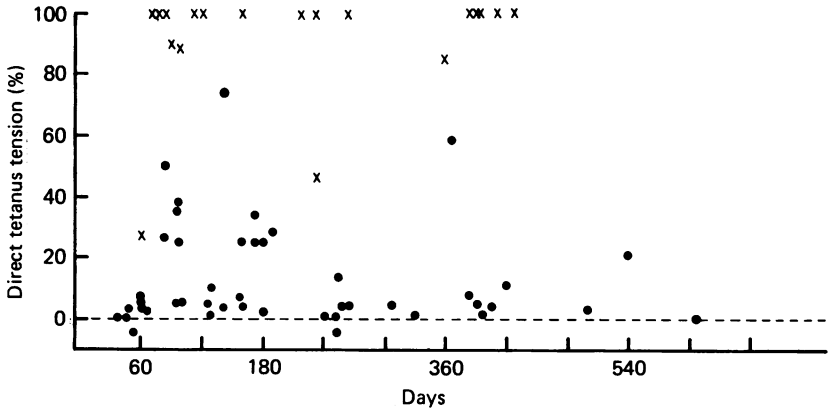

Fig. 1. Degree of innervation of muscles by s.m.n. (×) and s.p.n. (●) as a function of duration of implantation (degree of innervation is defined as indirect tetanus tension/direct tetanus tension × 100). Each point represents a different experiment. Points below the dashed line were cases in which stimulation of the nerve elicited no detectable tension.

of muscles at times longer than 90 days (× s in Fig. 1). Synaptic transmission in such muscles was indistinguishable from those of normally reinnervated control muscles.

With a splanchnic nerve, the degree of reinnervation was much less; it reached a peak at 90–180 days, followed by an apparent decline thereafter (Fig. 1, and Grinnell & Rheuben, 1979). In a few instances, especially at periods of 180 days or more, there was either no detectable tension (no visible twitch), or it was visible but too weak to be measured by our transducing apparatus ($< 0{\cdot}2$ mg). The average tetanus tension for s.p.n.-reinnervated muscles was $12{\cdot}4 \pm 16{\cdot}8\%$ of the directly elicited tension. As was pointed out in the previous paper (Grinnell & Rheuben, 1979), the contractile properties of the muscle are not changed by the s.p.n. reinnervation; the lack of tension lies in the failure of the s.p.n.-evoked e.p.p.s to reach threshold and thereby cause muscle contractions. The properties of junctional potentials produced by the s.p.n. are described in detail in the previous paper (Grinnell & Rheuben, 1979).

Reinnervation by two somatic motor nerves

(*a*) *Success of double-reinnervation measured with tetanic tension*

The presence of one s.m.n. in a muscle did not exclude reinnervation by another. In most cases, when two spinal nerves (usually IV and V) were implanted into a muscle simultaneously, tetanic stimulation (eliciting maximum tetanus tension at a stimulus frequency of 30–50/sec) indicated that each nerve was able to evoke contraction in most of the fibres (Figs. 2, 3*A* and Tables 1 and 2). The time course of development of functional reinnervation, in these dually reinnervated muscles, as judged by tetanus tension, may be slightly slower than when only one s.m.n. was implanted into a

muscle (×s in Fig. 1), but the difference, if real, was slight. Ninety days or more after implantation each nerve produced, on the average, 86 ± 15% (n = 13) of the tetanus tension that could be evoked by direct stimulation, implying that approximately 86% of the fibres were being driven above threshold (Fig. 3, Table 1).

As is evident in Fig. 1, when a single s.m.n. had been implanted into a muscle for 60–90 days, it was usually able to elicit contractions in virtually all fibres. If, at this time, a second s.m.n. was implanted into this already substantially reinnervated

TABLE 1. Degree of reinnervation and overlap of innervation in muscle implanted simultaneously by pairs of foreign s.m.n.s

Preparation	Duration of implantation (days)	Degree of reinnervation, twitch (% of control twitch)		% Overlap (twitch)	Degree of reinnervation, tetanus (% of control tetanus)		% Overlap (tetanus)
		S.m.n.$_{I}$	S.m.n.$_{II}$		S.m.n.$_{I}$	S.m.n.$_{II}$	
1	64	40	41	16	36	22	30
2	70	50	12	0	86	21	90
3	121	11	11	0	100	52	100
4	123	32	28	15	90	88	85
5	127	40	35	10	95	77	95
6	156	3	63	0	70	100	100
7	167	30*	—	—	69	—	—
8	346	31	45	12	100	88	100
9	524	31	31	28	98	86	85
		31 ± 15†		10 ± 10	75 ± 27†		86 ± 23

* The second nerve in this preparation was damaged during dissection, so data are available for only one nerve.

† Means (± S.D.) are for both sets of nerves, since all nerves were treated identically and showed comparable success of reinnervation.

TABLE 2. Degree of reinnervaton and overlap of innervation in muscles implanted sequentially by pairs of foreign s.m.n.s

Preparation	Duration of implantation (days)		Degreee of reinnervation (% direct twitch tension)		% Overlap (twitch)	Degree of reinnervation (% direct tetanus tension)		% Overlap (tetanus)
	1st	2nd	1st	2nd		1st	2nd	
A	125	65	50	33	100	100	92	100
B	118	72	18	22	0	100	56	100
C	132	72	94	12	50	100	22	100
D	166	79	53	38	13	95	71	90
E	150	88	70	7	0	100	21	100
F	218	139	24	20	8	100	100	100
G	287	196	68	52	62	100	71	100
H	304	217	49	7	0	97	62	88
I	313	237	38	6	0	85	32	73
J	317	259	24	85	38	56	93	83
K	390	237	26	1	0	100	7	100
M	620	458	66	25	6	93	56	85
			48 ± 23	26 ± 24	23 ± 33	94 ± 13	57 ± 31	93 ± 9

muscle, it also was able to establish functional junctions on most of the muscle fibres. The first of the two nerves, with staggered innervation, produced an average tetanus tension of $94 \pm 13\%$ ($n = 12$) of the direct tension, compared with $57 \pm 31\%$ ($n = 12$) for the second nerve. (The latter value includes data from some preparations in which the second nerve had been implanted less than 90 days, and hence might

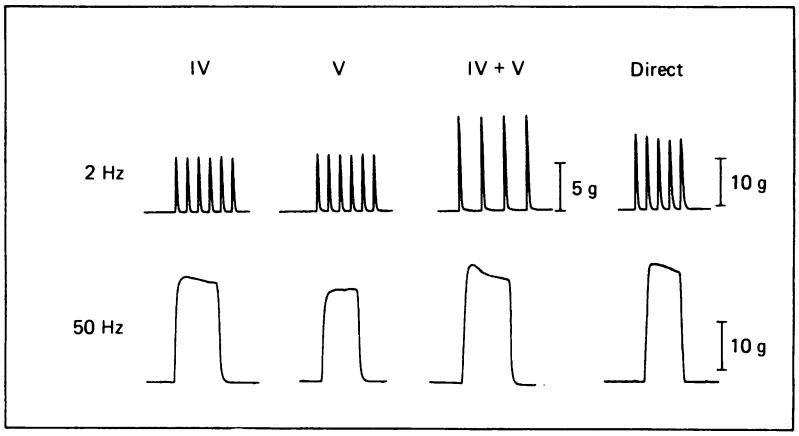

Fig. 2. Twitch and tetanus tension records from a sartorius muscle implanted 524 days earlier with two foreign nerves (spinal nerves IV and V). The responses to stimulation of IV alone, V alone and both together are compared with the response to direct stimulation. The twitch response to either nerve alone was much less than to direct stimulation (unlike the typical condition when one nerve is implanted), and there was considerable summation of twitch tensions with simultaneous stimulation (IV + V), implying that each drive many fibres that the other did not drive. Each nerve, when stimulated tetanically, elicited about as much tension as direct tetanic stimulation and there was much less summation of tetanus tensions.

still have been increasing in effectiveness). Thus, despite the presumed presence of effective innervation on virtually all fibres at the time a second s.m.n. was implanted, the second was still able to innervate effectively a large fraction of the fibres, in some cases all of them. The tetanus data does not provide evidence for strong competitive interaction between pairs of reinnervating s.m.n.s.

(b) Success of double reinnervation measured with twitch tensions

If one examines twitch tensions, however, some form of interaction is apparent (Fig. 3*B* and Tables 1 and 2). If only one s.m.n. was implanted ($\times$s in Fig. 3*B*), within 60–90 days it generated 50–100% of the twitch elicited by a single supramaximal direct stimulus to the muscle. The average ratio of indirect to direct twitch, at periods from 60 to 800 days in singly reinnervated muscles, was $88 \pm 16\%$ ($n = 25$). The average twitch tension elicited by each of the two s.m.n.s in simultaneously innervated muscles was $31 \pm 15\%$ ($n = 17$) (Table 1). With staggered reinnervation (Table 2), the average was $37 \pm 26\%$ ($n = 24$) and together the mean was $35 \pm 22\%$ ($n = 41$). In preparations with staggered reinnervation, the first nerve implanted retained some advantage over the second ($48 \pm 23\%$, $n = 12$ for the earlier, $26 \pm 24\%$, $n = 12$ for the latter nerve); but both were conspicuously less effective than

a single s.m.n. implanted for the same length of time. The sum of the tensions independently produced by both of the paired nerves was less, on the average, than that produced by the nerve in a singly reinnervated muscle. It is significant that the

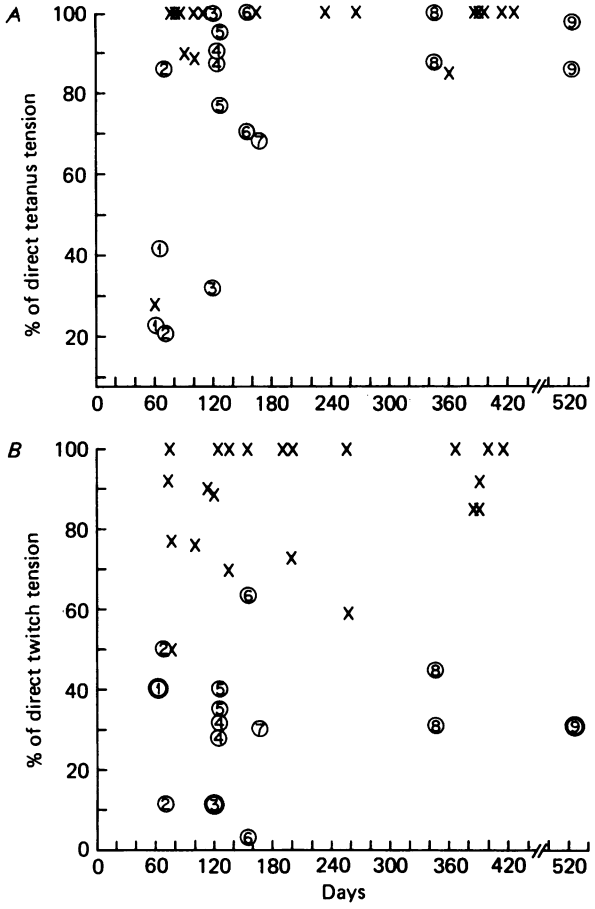

Fig. 3. Degree of reinnervation of experimental muscles by a single foreign nerve (× s) and by pairs of foreign nerves (encircled numbers) implanted simultaneously for the period shown. The tetanus (*A*) and twitch (*B*) tensions evoked by stimulation of each nerve separately are plotted as a percentage of the tension to direct muscle stimulation. The tetanus frequency was 30–50/sec (giving the maximum tension elicitable) and was the same for nerve and direct stimulation. The numbers inside the circles identify the experimental preparation, for each of which (except 7) there were two implanted nerves (see also Table 1). Note that the tetanus tensions were only slightly less than those elicited by single s.m.n.s at the same intervals, while twitch tensions in the doubly reinnervated muscles were much smaller.

earlier of two implanted s.m.n.s, which presumably was capable of producing twitches in close to 90% of the fibres at the time the second was implanted, must have been reduced in its effectiveness to being able to elicit a twitch in less than half of the fibres.

The high proportion of junctions producing subthreshold e.p.p.s in doubly reinnervated muscles was clearly reflected in tetanus/twitch ratios. In single s.m.n.-

reinnervated muscles, the tetanus/twitch ratio was $2{\cdot}5 \pm 0{\cdot}8$ ($n = 15$). This is approximately the same as that seen in control sartorius muscles and is not significantly different to the tetanus/twitch ratio observed with direct stimulation ($2{\cdot}4 \pm 1$, $n = 21$). In doubly s.m.n.-reinnervated muscles, however, the tetanus/twitch ratio (excluding two extreme cases where it was between 300 and 500) was $6{\cdot}3 \pm 4{\cdot}5$ ($n = 22$). This suggests that many terminals were causing subthreshold responses in the muscle in response to a single stimulus and only after facilitation or summation resulting from the tetanic stimulation did the e.p.p.s become suprathreshold.

(c) *Overlap of innervation in doubly reinnervated muscles*

In doubly reinnervated preparations, there was almost linear summation of twitch tensions (Fig. 2), suggesting very little overlap of the populations of fibres ('twitch fields') driven by each nerve. Tables 1 and 2 show the percentage overlap obtained for twitch and tetanus tensions produced by double s.m.n.s.

Percentage overlap is calculated as the difference between the sum of the individual tensions ($A+B$) and the tension produced by simultaneous stimulation of both nerves (AB), divided by the smaller of the two individual tensions (which can be called A), i.e. $((A+B)-AB)/A$. This corresponds to the 'twitch deficit' or overlap determined by Hunt & Kuffler (1954) and Brown & Matthews (1960). In similar measurements, Bagust, Lewis & Westerman (1973) divided by the mean combined tension, which gives smaller but proportional values for overlap. There are several potential sources of uncertainty inherent in such calculations. A systematic error arises from the 'series elastic component' of the muscle, which, as was shown by Brown & Matthews (1960), can lead to an over-estimate of the percentage overlap. More specific to individual preparations are the effects of double innervation of muscle fibres. The measurement AB will be misleading if on some fibres there are suprathreshold e.p.p.s from both nerves occurring at an interval greater than the refractory period of the muscle membrane, thus causing a double contraction of those fibres. Similarly, if there are a significant number of muscle fibres having adjacent subthreshold endings from axons of the two nerves, so that tension is developed only when both are stimulated approximately simultaneously, AB will again be disproportionately large. This latter condition probably does occur in some fibres in our preparations. Both cases would lead to an underestimate of the percentage overlap of innervation. Finally, if strong contraction of some motor units, especially during a tetanus, had the effect of mechanically stimulating the endings of other nerves, causing antidromic firing in them and thereby driving other motor units, the degree of reinnervation by any one nerve and the overlap of innervation would appear larger than the actual value. In several attempts to record antidromic activity on one nerve while tetanically stimulating the other, no such activity could be seen. Moreover, as will be described below, intracellular recording did show that a large fraction of the fibres were doubly innervated.

We feel, on consideration of these possible sources of error, that they do not affect the qualitative nature of our conclusions.

In the present experiments, as Tables 1 and 2 show, fifteen of twenty preparations showed twitch overlaps of less than 20%. In preparation A, an apparent exception, the twitch field of the earlier s.m.n. totally overlapped that of a second that had been in place only 65 days; the second nerve had perhaps not yet had a chance to exert its influence. On the other hand, all but the youngest preparation (preparation 1) showed 80–100% overlap of tetanus tensions, reflecting the fact that both nerves appeared to reinnervate almost all of the fibres as judged by their tetanus tensions.

Visual observation of twitches showed that the two nerves in a doubly reinnervated muscle tended to have segregated twitch fields. Often one side of a muscle was driven predominantly by one nerve, the other side by the other, with only small patches of

fibres that twitched to both nerves or to the one whose 'territory' was on the opposite side of the muscle. Intracellular recording verified this distribution. Action potentials or large e.p.p.s were seen in response to the nerve that appeared to be most effective

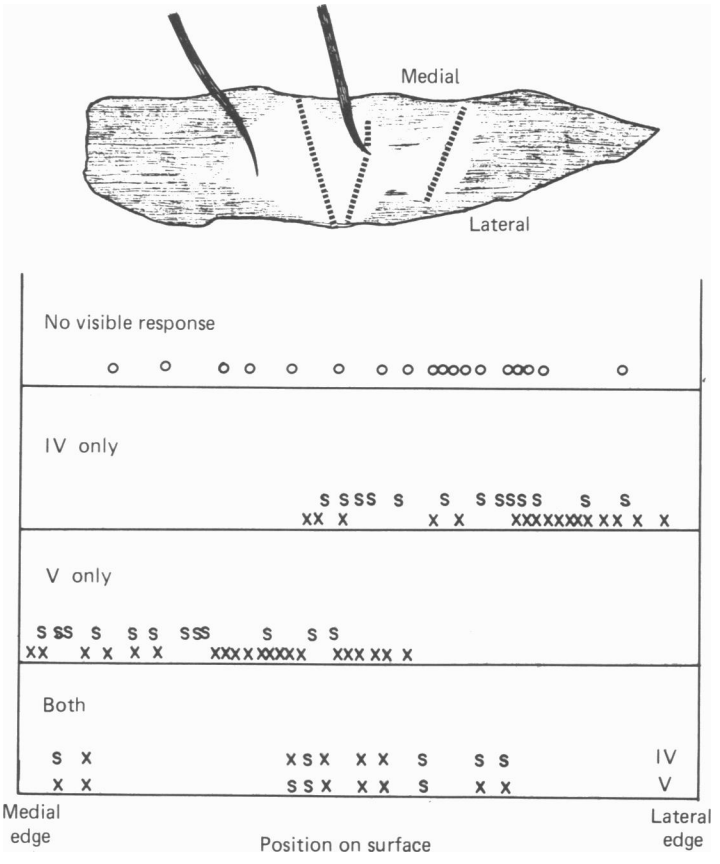

Fig. 4. Map of effectiveness of s.m.n. IV (287 days) and s.m.n. V (196 days) on ninety-one fibres tested with intracellular micro-electrodes across the surface from medial to lateral edges, in most instances between the points of entry of the two nerves (approximately along the dashed lines shown). Some of the ninety-one might represent second penetrations of fibres already recorded from another location. Plotted are the relative positions of fibres which gave no responses to single stimulation of either nerve (○), those that produced spikes (*S*) or e.p.p.s (×) to only nerve IV or only V, and those that showed either a spike or e.p.p. to stimulation of both nerves. In this preparation, nerve IV produced 100% of the direct tetanus tension and nerve V produced 77%. There was 100% overlap of tetanus tensions.

in driving a given population of fibres, whereas the other nerve produced only a small e.p.p. or no apparent response. Fig. 4 shows a characteristic map of functional innervation by each nerve in a doubly reinnervated muscle. Where two populations existed on opposite sides of the muscle, the fibres at the common border of the two populations often did not twitch to either nerve, although they contracted to tetanic stimulation of either nerve. In these fibres subthreshold e.p.p.s were frequently produced by stimulation of both nerves. They were sometimes recorded at the same site.

(d) Evidence for mutual repression as detected with intracellular micro-electrodes

Intracellular recording confirmed that a large fraction of the fibres had been innervated by axons from both nerves. Although e.p.p.s from both nerves could be recorded at the same site in only about 10% of the fibres from which e.p.p.s were recorded (47 of 469 in the muscles studied in this report), many other fibres (16%)

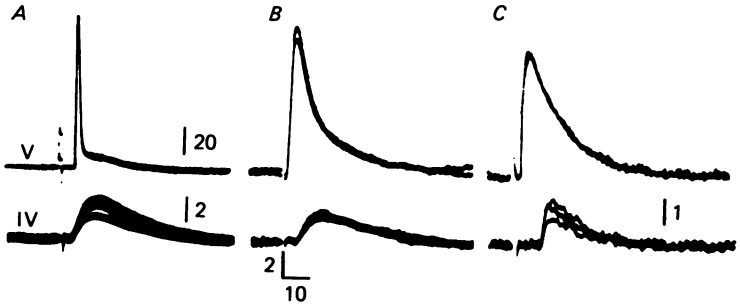

Fig. 5. Responses from three different fibres to stimulation of both s.m.n.s in doubly reinnervated muscles. In each case, both responses were recorded at a single electrode location. *A*, the most characteristic response, in which stimulation of one nerve elicited a spike at some distant point, stimulation of the other elicited a small e.p.p. The two nerves were implanted simultaneously 119 days before. *B*, same preparation, a fibre with both inputs subthreshold. Nerve V elicited a large e.p.p., nerve IV a small one. *C*, more nearly equal, sharply rising e.p.p.s in a preparation implanted 149 days (IV) and 87 days (V).

showed an e.p.p. to stimulation of one nerve and a spike which arose at some distant point in response to single, double or triple stimulation of the other nerve. Approximately 2% of the muscle fibres showed spikes to both, although usually only when one or both nerves were multiply stimulated. Fig. 5 shows characteristic examples of double innervation. Frequently, an e.p.p. showed multiple components indicating innervation by more than one axon of the same nerve, sometimes within recording distance of an ending from the other nerve. However, the frequency of such multiple innervation was not studied systematically. When e.p.p.s could be elicited by both nerves near one recording site, usually they had different rise times, suggesting that they arose at sites separated by appreciable distances along the muscle fibre. In less than 3% (12 of 469) of the fibres studied, the time course of the e.p.p.s elicited by both nerves were similar, which suggests that they arose from the same site or at sites equidistant from the recording electrode. We conclude that on most fibres, the nerves tend to end at considerable distance from each other (at least greater than two length constants) and only rarely could they interact directly at their terminals.

The subthreshold e.p.p.s in doubly reinnervated muscle typically had quantal contents of ten or less. Fig. 6 shows four examples of low quantal content e.p.p.s recorded in normal frog Ringer. In Fig. 6*A* and *B*, stimulation of one nerve produced small, low quantal content e.p.p.s; stimulation of the other elicited abruptly rising action potentials, indicating the existence of suprathreshold e.p.p.s at junctions formed by that nerve at considerable distance from the recording site in each fibre. In Fig. 6*C* and *D*, responses to both nerves could be recorded at the same site, with

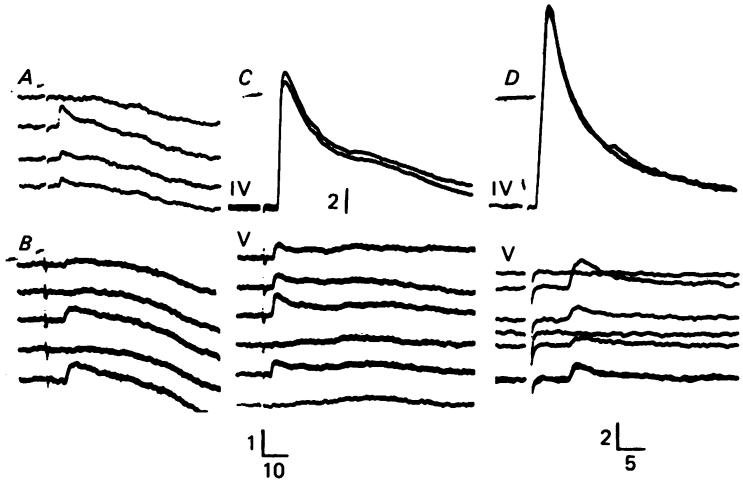

Fig. 6. Examples of low quantal content e.p.p.s in muscles reinnervated by two s.m.n.s. All were recorded in normal frog Ringer. *A*, *B*, responses to nerve V at two junctions (nerve implanted 317 days). Nerve IV (259 days) evoked spikes in both fibres (not shown). *C*, from the same preparation, a large e.p.p. evoked by stimulation of nerve IV and a series of low quantal content e.p.p.s from nerve V (note the two failures). *D*, a similar site in a muscle implanted 307 days by nerve IV, 217 days by nerve V. The deflexion on the falling phase of one superimposed trace of the nerve IV e.p.p. is a min e.p.p. Traces in records *A*, *B*, and *C* show movement artifacts due to contraction of fibres elsewhere in the muscle, following the e.p.p.s.

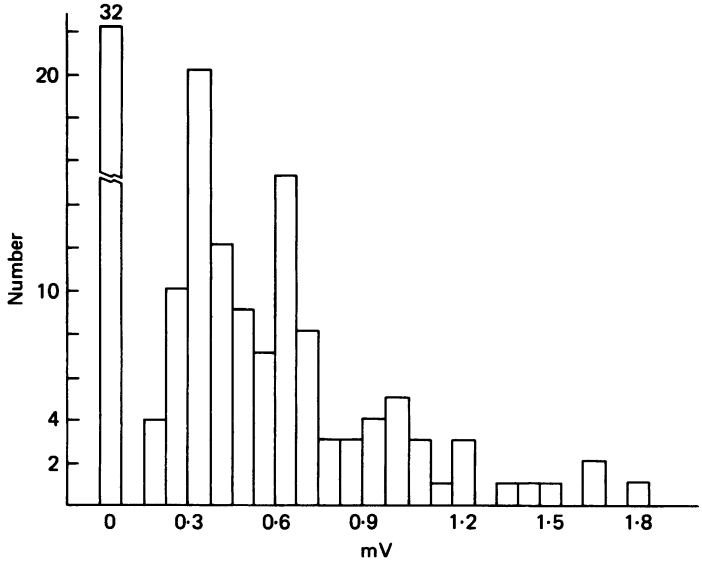

Fig. 7. E.p.p. amplitude histogram for the low quantal content junction shown in Fig. 6*A* (preparation K, Table 2). The junction had thirty-two failures in 146 trials. The quantal content calculated by the method of failures was 1·52, by the variance method, 1·29. Three min e.p.p.s were seen at this junction, having amplitudes of 0·3, 0·3 and 0·4 mV.

one input much stronger than the other, although both were subthreshold. In all four cases, the weaker synapses showed many failures and had mean quantal contents of only one to two. Fig. 7 shows an e.p.p. amplitude histogram for the junction of a fibre with low quantal content e.p.p.s, in this case the same fibre from which the data of Fig. 6*A* were obtained. As indicated above, this fibre also received suprathreshold excitation from an axon of the other nerve, at a distant point.

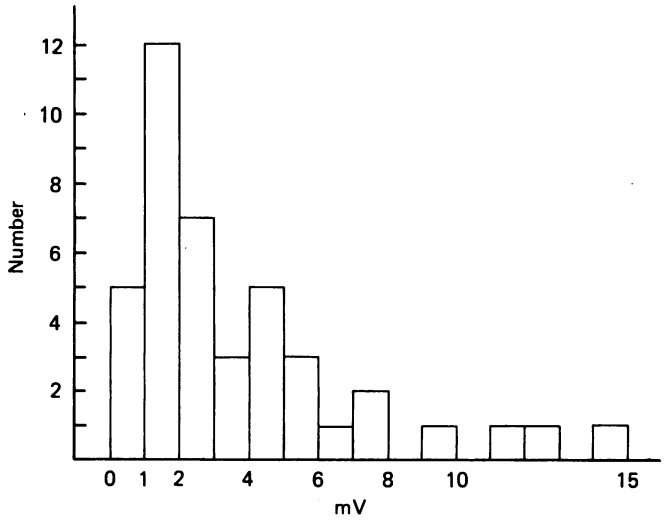

Fig. 8. Plot showing the distribution of mean subthreshold e.p.p. amplitudes having rise times of under 3 msec for forty-two junctions in two doubly reinnervated muscles (preparations K and M, Table 2). The majority of e.p.p.s were below 3 mV.

To measure the average e.p.p. size in these preparations, two muscles in which both nerves had been implanted 6 months or more were carefully assayed for e.p.p.s in superficial fibres. Of 183 fibres studied in these two muscles, forty-two showed subthreshold e.p.p.s with fast-rise times within recording distance of the electrodes (sixty-six had action potential responses, in four cases to both nerves, twenty-six had slow-rising e.p.p.s, and forty-nine showed no evidence of functional innervation). The mean amplitude of the forty-two with sharp e.p.p.s was $4{\cdot}2 \pm 3{\cdot}4$ mV. Fig. 8 shows a histogram of the distribution of the mean e.p.p. amplitudes from these forty-two fibres. Thirteen of these forty-two fibres responded with small e.p.p.s to stimulation of one nerve, and with spikes arising at distant locations to stimulation of the other. Three had sharply-rising e.p.p.s to stimulation of either nerve: in these fibres the mean amplitudes of the e.p.p.s elicited by the two nerves (root IV *vs.* root V) were 10 mV *vs.* 0·5 mV, 0·5 mV *vs.* 9 mV, and 1 mV *vs.* 3 mV.

Miniature e.p.p.s at junctions with subthreshold e.p.p.s were less frequent ($0{\cdot}2/\text{sec} \pm 0{\cdot}22$, $n = 22$) than at normal sartorius junctions or those resulting from reinnervation of a muscle with a single s.m.n. ($2{\cdot}25/\text{sec} \pm 3{\cdot}8$, $n = 42$). The min e.p.p.s and evoked single quanta observed at junctions with low quantal content e.p.p.s nevertheless had rise times, mean amplitudes (0·3–1 mV), and amplitude distributions that did not differ significantly from those of min e.p.p.s seen at control neuromuscular junctions. If one assumes there is no difference in quantal size, then there is no indication of loss of post-synaptic ACh sensitivity.

In double s.m.n.-preparations, when one or both nerves were stimulated tetanically the tension often fell sharply with time (Fig. 9), reminiscent of the decline in tetanus tension characteristic of s.p.n.-driven muscles (Grinnell & Rheuben, 1979). This was not seen in nerves with tetanus/twitch ratios below 4, was never seen in normally reinnervated control or single s.m.n.-preparations of the same age even when their tetanus/twitch ratios were enhanced by partial Mg block, and was not seen during comparable direct muscle stimulation (Fig. 9). Hence, the ability of a junction to become easily fatigued may represent a significant release property of s.m.n.-terminals resulting from competitive interactions.

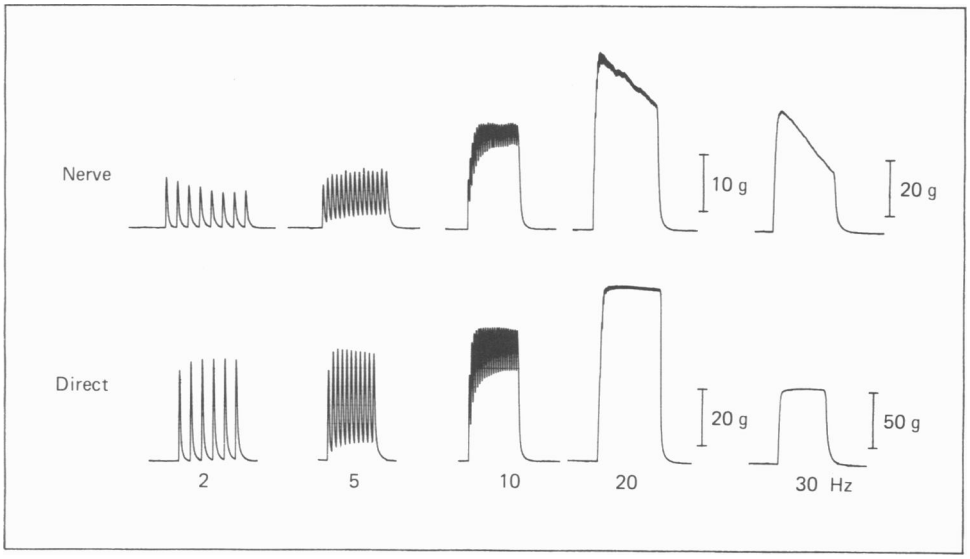

Fig. 9. Tensions records produced by stimulation of one nerve in a doubly reinnervated preparation (167 days) compared with the muscle's response to direct stimulation. This preparation was like several others in which the tetanus tension evoked by one or both nerves showed a rapid decrease with time at stimulation frequencies of 20/sec or higher. This decrease was not observed with direct stimulation of the same muscle, or with direct or indirect stimulation in single-reinnervated muscles.

(e) *Competition between axons of the same nerve*

It is possible that the axons within one nerve also compete with one another for successful innervation of muscle fibres. To test for this, the nerves innervating control sartorius muscles and singly reinnervated experimental muscles were subdivided so that different subpopulations could be stimulated independently. In most cases, each portion contained one to three functioning motor axons as evidenced by repeatable, discrete jumps in the tension records with increase in stimulus strength, a small fraction of the normal fifteen to thirty present in the whole nerve (unpublished observation). It was found that there was normally no more than 5 % overlap using twitch tension as the criterion, but significant overlap of innervation did occur as judged by tetanus tension (mean 24·3 ± 9 %, n = 13, range 6–47 %). This suggests that there could be a significant number of 'subthreshold' junctions on doubly innervated fibres under normal conditions. However, the degree of overlap appears to be less than between two separate nerves, and there is little evidence for the very

low quantal content, labile junctions seen with two foreign nerves (Katz & Kuffler, 1941; Iwasaki, 1957; Vyskozil & Magazanik, 1977; Weakly, 1978; A. D. Grinnell & A. Herrera, unpublished). Hence the competition between two nerves, from different segments of the spinal cord, may be either quantitatively greater or qualitatively different from that occurring between axons of the same nerve (see Luff & Proske, 1976; Mark, 1977; Grinnell *et al.* 1977*b*).

Reinnervation by s.m.n. and s.p.n.

We next sought to discover whether another, totally abnormal source of cholinergic innervation, the autonomic preganglionic splanchnic nerve, which normally does not innervate skeletal muscle *in vivo*, could successfully reinnervate muscle fibres or retain effective synapses if a muscle was given the opportunity to accept reinnervation by a foreign somatic motor nerve at the same time.

(*a*) *Simultaneous reinnervation by s.m.n. and s.p.n.*

If both nerves were implanted simultaneously (Fig. 10*B*), the s.m.n. functionally reinnervated the muscle to essentially the same extent (usually 90–100% of direct tetanic tension) and with approximately the same time course as an s.m.n. implanted alone (Fig. 1). Similarly, twitch tension ($90{\cdot}0 \pm 11{\cdot}6$% of direct twitch) evoked by the s.m.n. was not significantly different.

On the other hand, the s.p.n. was very much less successful at reinnervating the muscle in the presence of an s.m.n. (cf. Fig. 10*A* and *B*). Of twenty-six such simultaneous preparations, at durations of 60–800 days after implantation, thirteen showed no apparent contraction on stimulation of the s.p.n., even with tetanic stimulation, and eight others showed tension of less than 1% of that elicited by direct tetanic stimulation of the muscle. Only three preparations showed functional s.p.n. reinnervation of more than 10% of the fibres, and in each case, this was associated with a reduced s.m.n. effect. The average s.p.n. tetanic tension, in all doubled preparations older than 60 days, was $3{\cdot}3 \pm 8{\cdot}2$% ($n = 28$). Excluding the three cases in which the s.m.n. was unusually ineffective and the s.p.n. abnormally strong, the average s.p.n. tetanic tension was $0{\cdot}6 \pm 1{\cdot}8$% ($n = 25$) compared with $12{\cdot}4 \pm 15{\cdot}8$% for s.p.n. alone.

Almost certainly the s.p.n. formed functional but subthreshold junctions over much more of the muscle than was revealed by twitch and tetanus measurement. The junctional potentials recorded throughout the muscle, with intracellular electrodes, were generally of very low quantal content (see Grinnell & Rheuben, 1979). Tetanic s.p.n. stimulation produced contraction throughout a much wider band of fibres than twitched to a single stimulus, and partial depolarization of the fibres with 10^{-6} M-ACh produced stronger and more widespread contractions to either single or tetanic stimulation. The fibres that did contract to s.p.n. stimulation tended to be in one or more localized bands, usually at one edge of the muscle where fibres are often smaller and probably have significantly higher input resistances. In most preparations there was no summation of tensions elicited by the two nerves, indicating that the s.m.n. had successfully innervated the fibres that were also driven by the s.p.n. However, in the preparations with abnormally strong s.p.n. and weak s.m.n. innervation, there was more than 50% summation of tetanus tensions, indicating

much less overlap of functional innervation. Similar results were obtained when the s.p.n. was implanted 60–90 days before the s.m.n. (see below).

Few cases were found in which the s.p.n. and s.m.n. endings were close enough so that junctional potentials evoked by both nerves could be recorded at one site on

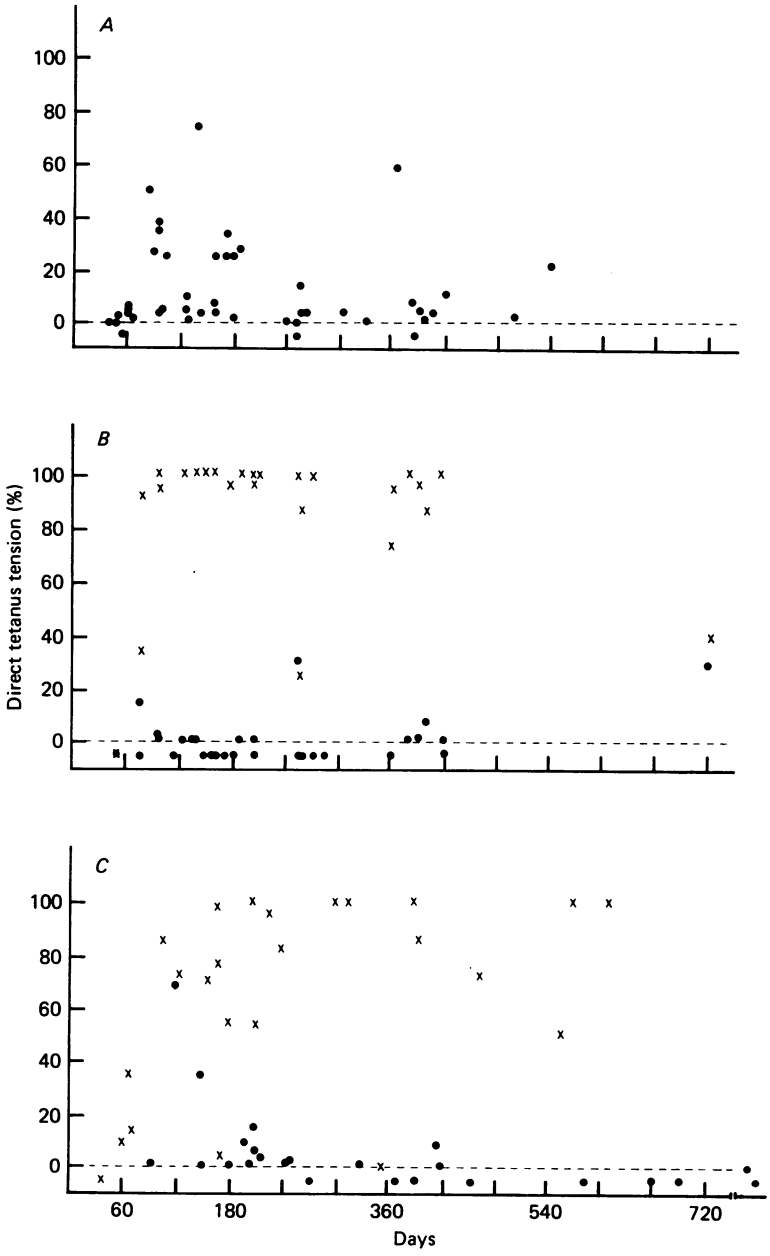

Fig. 10. Graphs showing the tetanus tension that can be evoked by stimulation of the nerve, as a percentage of that to tetanic direct muscle stimulation at different times after nerve implantation: *A*, s.p.n. nerve only; *B*, s.m.n. (×) and s.p.n. (○) implanted simultaneously; and *C*, s.p.n. implanted 60–90 days before the s.m.n. Each point represents one nerve innervating one muscle.

a muscle fibre. It was technically difficult to study responses from both nerves at the same time, since to reduce s.m.n. junctions below threshold requires Mg^{2+} or curare concentrations which abolish the s.p.n. junctional potentials. In unblocked preparations, fibres showing s.p.n. junctional potentials usually showed s.m.n.-evoked action potentials that apparently arose at some distance.

The properties of the s.p.n. junctional potentials were not distinguishably different from those seen in muscles reinnervated only by an s.p.n. (Grinnell & Rheuben, 1979).

(*b*) *Staggered innervation – s.p.n. implant followed by s.m.n. implant*

If the s.p.n. was implanted 60 days or more before the s.m.n., the s.m.n. nevertheless eventually caused contractions in most or all fibres (Fig. 10*C*). However, the s.p.n. was seldom completely ineffective until the s.m.n. had been present for 240–300 days. In preparations in which the s.m.n. had been present for between 60–240 days, the average s.p.n. tetanus tension was 11·6 % (n = 12) of the direct tension and there was only one case of a totally ineffective s.p.n. At greater innervation times, however, seven of eleven preparations showed no detectable contraction even to tetanic stimulation of the s.p.n. The over-all average s.p.n. tetanus tension was 6·7 ± 15·7 % (n = 23).

Interestingly, the s.p.n. retarded and reduced the amount of s.m.n. reinnervation. Not only was the time course of reinnervation somewhat slowed (cf. Figs. 3*A* and 10*C*); many preparations showed only partial (50–90 %) s.m.n. reinnervation even on tetanic stimulation. The average s.m.n. degree of reinnervation at 60 days or more, judged by tetanic tensions, was 68 ± 34 % (n = 22). The average s.m.n. twitch tension was 58 ± 27 % of that to direct stimulation, compared with approximately 90 % in muscles that were reinnervated by a s.m.n. alone or s.m.n. and s.p.n. simultaneously.

At least during the first 200–250 days, the s.p.n. apparently prevented functional reinnervation of many of the fibres itself had innervated. There was little overlap of fields, either judged visually or by tension measurement. Even with tetanic stimulation, the overlap was never more than 33 %. Again the s.p.n. usually was most successful in driving fibres near the edge of the muscle, but there were also motor units spread throughout the muscle.

(*c*) *Effect of cutting the s.m.n. in a s.m.n.–s.p.n. preparation*

We next sought to determine whether in an s.p.n.–s.m.n. reinnervated preparation, cutting the s.m.n. a second time might lead in a short time to activation of previously undetectable s.p.n. synpases. The results of several experiments are summarized in Fig. 11*C*. Three days after section of the s.m.n., the remaining s.p.n. was totally ineffective in the three animals tested. Two weeks after s.m.n. section, however, all seven preparations tested showed significant s.p.n.-elicited contractions (mean 6·2 ± 2·9 % of the direct tetanus tension). At 6–8 days between s.m.n. section and s.p.n. testing, the results were less clear-cut. Two preparations showed barely detectable contractions, one about 2·5 % and one about 10 % of the directly elicited tensions. Thus, it appears that within 2 weeks, and to a partial degree within 1 week of s.m.n. section, the s.p.n. has either formed new junctions or activated previously 'repressed'

ones to the point that they are nearly as effective as an s.p.n. implant alone. Fig. 11*A* and *B* show, for comparison, the effectiveness of the s.p.n. in singly reinnervated muscles and the effectiveness of s.p.n. in doubly reinnervated muscles with both

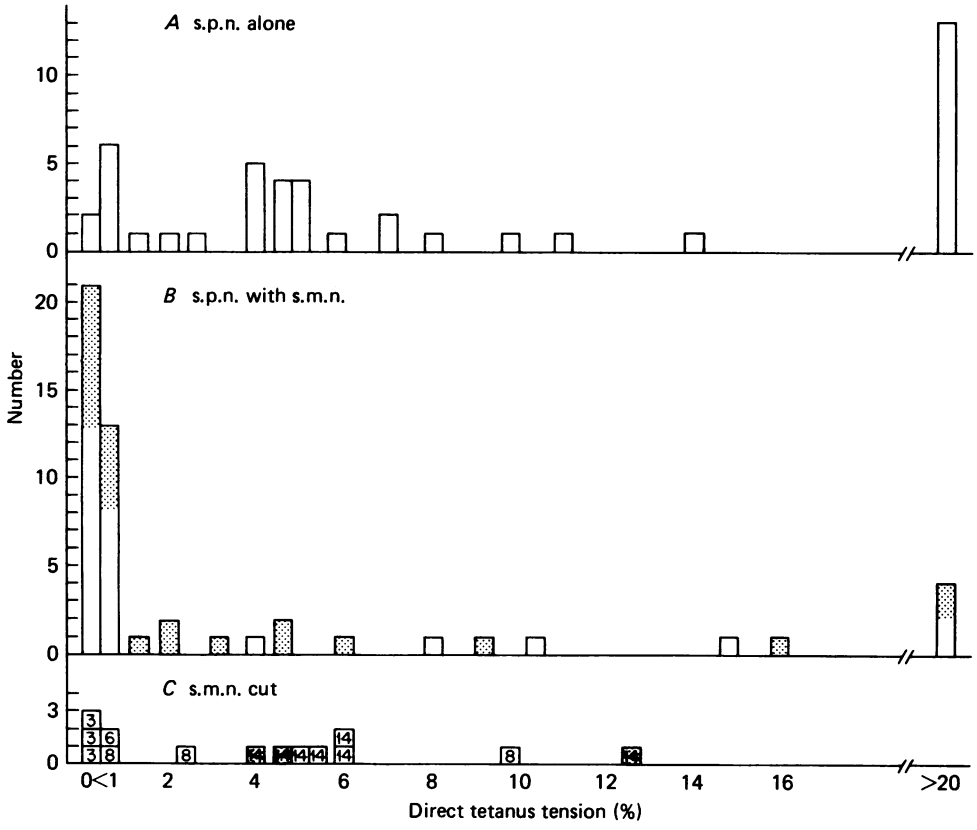

Fig. 11. Effect of sectioning the s.m.n. in muscle reinnervated by both a s.p.n. and s.m.n. on tetanus elicited by s.p.n. stimulation. *A*, effectiveness of s.p.n. when it was implanted alone; *B*, effectiveness of s.p.n. in doubly reinnervated muscles with s.m.n. intact; *C*, at 3, 6–8, and 14 days after section of the s.m.n., the s.p.n. evoked the tetanus tensions shown (shaded blocks refer to staggered innervations, with s.p.n. implanted 2 months or more before the s.m.n.; open blocks represent simultaneous innervation).

nerves intact. It is noteworthy that in the doubly reinnervated muscles, the vast majority of s.p.n.s elicited no tension at all. In the case of simultaneously reinnervated muscles, eleven of twenty-five showed no detectable tension, and only five showed more than 2% of the direct tetanus tension.

DISCUSSION

S.m.n.–s.m.n. interaction

We conclude that there is competitive interaction between pairs of s.m.n.s, or an s.m.n. and s.p.n., during reinnervation of denervated muscle fibres in the frog. In the case of pairs of s.m.n.s, although both nerves formed and maintained junctions on most or all fibres, usually one or the other, or both, inputs to any given fibre were subthreshold on single stimulation. Indeed, in contrast to the vast majority of

junctions formed by a single regenerating s.m.n., apparently most of the junctions formed by pairs of foreign nerves were subthreshold to single stimulation.

The fact that a second s.m.n. grew in and took over a large twitch territory of its own, even from an s.m.n. that was implanted as much as 180 days before, shows that this form of competition did not inhibit the formation of 'second' synapses on a fibre but did, in most cases, ensure that only the input from one nerve was suprathreshold. Furthermore, it caused a reduction in the e.p.p. amplitude of many well established synapses.

We do not yet know to what extent synapses physically disappear as a result of this competitive interaction; but it is clear that a large number remain, with low levels of release. In this respect the competition is unlike the process of elimination of multiple synapses in immature mammalian skeletal muscle, where supernumerary junctions are lost altogether (Redfern, 1970; Bagust *et al.* 1973; Brown, Jansen & Van Essen, 1976; Rosenthal & Taraskevich, 1977). On the other hand, mature mammalian skeletal muscle fibres can be multiply innervated with no evident displacement or repression of one nerve by the other (Aitken, 1950; Gutmann & Young, 1944; Bernstein & Guth, 1961; Guth, 1962; Gutmann & Hanzlikova, 1966; Frank, Jansen, Lømo & Westgaard, 1974, 1975). The same has been reported for double innervation of fish gill muscles (Frank & Jansen, 1976) and eye muscles (Scott, 1975, 1977), although in the latter case it is noteworthy that when an eye muscle is innervated by both the original and a foreign nerve, neither is as effective as it would be alone. Mark and his colleagues (Cas *et al.* 1973; Mark, 1975), working with salamander limb innervation, concluded that synapses formed by sprouted collateral branches of adjacent nerves were functionally respressed to subthreshold levels but not eliminated by the regenerating original nerve. Bennett & Raftos (1977) and Dennis & Yip (1978), however, report a severe to total loss of foreign nerve influence and physical regression of endings in similar salamander limb preparations. This would represent stronger suppression of inputs than we observe with pairs of foreign nerves in frogs. One obvious difference between our preparation and those showing selective dominance by the original nerve is that in our preparation both nerves are foreign, and both may lack some special feature of the original nerve. Another possible explanation for the incomplete suppression we observed is that in the salamander limb experiments the 'foreign' axons that sprout collaterals to innervate vacated synaptic sites maintain a full complement of normal synapses. It may be relatively easy to displace such 'extra' synapses, whereas the foreign nerve synapses found in our experiments, being the only synapses formed by the nerves, may be more resistant to total elimination. A similar explanation for selective synaptic displacement in the regeneration of competing connexions in the mammalian superior cervical ganglion has been suggested by Purves (1976).

Salamander limb muscle fibres are normally innervated repeatedly, at close intervals, along their length. When the original nerve regenerates and displaces synapses formed by collaterals of adjacent nerves, both nerves run together along the length of the fibre, probably with both competing for the original post-synaptic sites (Bennett & Raftos, 1977). The competitive interaction could involve direct influence of one nerve on another. This may also characterize the interaction between different axons of the sartorius nerve during development in frogs, but does not seem to apply in our preparations where both nerves are foreign. In our preparations, the

competitive suppression was clearly evident over considerable distances in many cases. We cannot rule out the possibility that there were sites of close apposition, and therefore direct interaction, between the terminals of axons from the two foreign nerves. However, this was rarely seen, while low quantal content junctions formed by one, with evidence for distant innervation by the other, were common. It seems likely that the mechanism of interaction involves some influence of one nerve on the muscle, which in turn influences the other synapses on it. Haimann *et al.* (1976), who studied the interaction between regenerating original nerve and sprouted collaterals in the normally doubly innervated *Xenopus* pectoralis muscle, concluded that the suppressive interaction was present only at close proximity. They reported that the collateral 'foreign' terminals were not reduced in effectiveness by the regenerating nerve in dually innervated end plates, but that foreign nerve terminals located at some distance from the regenerated original nerve tended to be stronger. This proximity effect, which was not clear in our findings, may have resulted from the fact that the foreign nerve was interacting with the original nerve rather than with another foreign nerve. Alternatively the sprouted terminals that were strongly affected might have been at the end of much longer collaterals, and hence more susceptable to competitive interaction.

It is important to emphasize that the competitive interaction between foreign s.m.n.s is apparently non-specific. Each nerve, regardless of the order of implantation into the muscle, reduces the influence of the other on a large proportion of the fibres. It seems probable that comparable interaction may occur normally during the development of innervation of the sartorius muscle, and we have reported that there is some evidence for competition between axons of the same nerve in normal adult sartorius muscles (Grinnell, Rheuben & Letinsky, 1977*b*). However, there are several features of the junctions observed in doubly reinnervated muscles which suggest that axons from different foreign nerves interact more strongly, or qualitatively differently, than do axons of the same nerve. The interaction was so strong that more than half of the synapses failed to drive their muscle fibres above threshold on single stimulation. The sum of the twitch tensions elicited by stimulation of both nerves was considerably less ($60 \pm 25\,\%$, $n = 20$) than the average twitch tension in singly reinnervated muscles ($99 \pm 16\,\%$, $n = 25$). Moreover, the synapses in doubly reinnervated muscles showed a lability not seen in singly reinnervated muscles. Often the former showed a sharp decline in tension during a 20–30/sec tetanus, unlike the responses to stimulation of single nerves, branches of nerves, or the muscle directly. These observations, when coupled with the absence of very low quantal content junctions in normal or singly reinnervated muscles, lead us to conclude that the interaction we are seeing goes beyond that occurring between different axons in the same nerve. This does not constitute evidence of specificity, but may reflect some difference between motoneurones in different segments of the spinal cord and mutual interaction between the axons of such unlike neurones to reduce the efficacy of synapses from both.

S.p.n. and s.p.n.–s.m.n. reinnervated muscles

One of the curious features of s.p.n. reinnervation of the sartorius was that there appeared to be a time of maximal effectiveness, at about 90–180 days after implantation, following which the amount of twitch or tetanus tension decreased. The

explanation for this phenomenon is not known, but there are several possible contributing factors. It may simply be that the s.p.n. endings decreased in effectiveness with time because some feed-back trophic influence supplied by normal muscle is lacking in slowly atrophying and relatively unused muscle fibres. Alternatively a gradual functional replacement of some endings by others, further away from the implanted nerve, perhaps due to mutual competition, may be responsible. At early reinnervation times many e.p.p.s from junctions that are close together may sum to exceed threshold. Later, without an obvious decrease in e.p.p. amplitude, the terminals appear to become dispersed, with fewer close enough to each other for their summed e.p.p.s to exceed threshold (Grinnell & Rheuben, 1979).

When a s.m.n. and s.p.n. were implanted simultaneously into a muscle, the s.m.n. was generally successful in almost totally suppressing the formation of functional (suprathreshold) synapses by the s.p.n. Surprisingly, if a s.p.n. was given a head-start of 60–90 days in the muscle, it seemed even more effective than a s.m.n. in slowing innervation by a subsequent s.m.n., and in excluding the s.m.n. from a certain population of fibres. Although the s.m.n. did quickly become dominant, it was slow in assuming complete control of the muscle and in suppressing the pre-existent s.p.n. junctions. Even though the s.p.n. usually drove only a small fraction of the muscle (often bands of edge fibres), it seemed to retain control over them, excluding reinnervation by the s.m.n., at least for a period of 200–250 days. This finding implies a qualitative difference in the competitive influence of s.m.n. and s.p.n.

The results of experiments on s.p.n.–s.m.n. preparations in which the s.m.n. was subsequently sectioned and the effectiveness of the s.p.n. tested 3–14 days later, showed that the effectiveness of the s.p.n. increased within 1–2 weeks to a level approximating that seen in muscles that had been reinnervated by a s.p.n. alone. Since the rate of formation of synapses is not known, it is not possible to conclude that intact but 'turned-off' s.p.n. synapses were activated following s.m.n. section, analagous to the 'derepression' of foreign synapses following resection of the original nerve in a salamander limb muscle, claimed by Mark and his colleagues (Cass *et al.* 1973). Nevertheless, it does appear safe to conclude that within 7–14 days, the effectiveness or number of the s.p.n. synapses had sharply increased.

At present we cannot say anything about the activity pattern of the different nerves during the time they have reinnervated the experimental muscle *in vivo*. It is possible to reflexly drive the s.m.n.-reinnervated muscles, and the s.m.n.s probably were active during the period they were implanted in the muscle. How this compares with the normal activity pattern of the sartorius is not known, and even less is known about the activity of the s.p.n. normally or in our preparations. If hyperinnervation or competition between nerves is a function of their activity, as seems likely, this could be a highly significant factor.

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