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ACTIVITY-DEPENDENT AND -INDEPENDENT SYNAPTIC INTERACTIONS DURING REINNERVATION OF PARTIALLY DENERVATED RAT MUSCLE

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SUMMARY

1. Reinnervation of adult rat fourth deep lumbrical muscles was studied, following extensive partial denervation of the hindfoot by crushing the lateral plantar nerve (LPN). Most muscles remained innervated by between one and five motor axons supplied by the sural nerve (SN). Intact SN motor units expanded as a result of collateral sprouting. Virtually complete collateral reinnervation occurred in muscles containing more than two SN motor units. Twitch tension measurements from isolated muscles suggested that most of the sprouts evoked suprathreshold responses from the muscle fibres they innervated. Intracellular recordings suggested that only a small percentage of sprouts evoked subthreshold end-plate potentials.

2. Lateral plantar nerve motor axons returned to the lumbrical muscles within 15–18 days and subsequently reinnervated muscle fibres already innervated by SN motor nerve terminals. Nerve conduction in the regenerating axons was then blocked for 7–15 days by chronic superfusion with tetrodotoxin. In both LPN-blocked and control (LPN crushed but not blocked) animals, isometric tetanic tension overlap and intracellular recordings showed that some lumbrical muscle fibres became innervated exclusively by regenerating LPN motor axons.

3. With time, the tension evoked by stimulating regenerating motor axons increased and there was a parallel fall in the tension produced by stimulating the intact motor units. The extent of reinnervation by LPN motor axons was inversely related to the number of remaining SN motor units. In comparable muscles, regenerated LPN-blocked motor units produced only about half the tension of the controls. Selective glycogen depletion of motor units and intracellular recordings of end-plate potentials indicated that this was due to reduced numbers of muscle fibres innervated by the blocked motor axons.

4. Nerve conduction block prolonged the time course of the isometric twitch in regenerated motor units, and increased the duration of the end-plate potential in muscle fibres innervated only by the regenerating axons. LPN block did not affect the recovery of the latency of the end-plate potential. The regenerated motor units were more resistant to fatigue caused by continuous 4 Hz nerve stimulation than intact SN units, but the resistance to fatigue of LPN-blocked motor units was no different from the controls.

5. The regression of motor innervation provided by intact motor axons, the recovery of regenerated motor unit tension and the transient dual innervation of muscle fibres is described quantitatively by a system in which muscle fibres can exist in one of three reversible innervated states, and in which the rate of transition between states is governed by first-order rate constants. The relative magnitude of the rate constants (between 0·05 and 0·65 $days^{-1}$) suggests that regenerating motor nerve terminals are more labile with respect to both synapse formation *and* synapse elimination than intact terminals. Activity in motoneurones is only one of a number of factors which determine the outcome of the synaptic competition.

6. The results and analysis are consistent with the notion that the pattern of motor innervation in reinnervated lumbrical muscles is determined by an interaction between antagonistic neurite growth-promoting and growth-repressing stimuli at the motor end-plate.

INTRODUCTION

Changes in connectivity normally occur in many parts of the nervous system during late fetal and early post-natal development. At the cellular level, immature neurones make excessive numbers of connections with post synaptic cells. Later in development, some of the connections are withdrawn. Analogous processes occur during regeneration, when axons must retread developmental paths for normal function to be restored. Synapse elimination reduces neuronal divergence from the viewpoint of the presynaptic neurone, and it reduces neuronal convergence from the viewpoint of the postsynaptic cell (Purves & Lichtman, 1985). In many instances, during development or repair, selective use or disuse of connections can influence their stabilization: misused or inactive connections are selectively displaced in favour of ones expressing an appropriate or enhanced pattern of activity (Wiesel, 1982; Jackson, 1983; Ribchester & Taxt, 1983; Ridge & Betz, 1984; Chapman, Jacobson, Reiter & Stryker, 1986).

When motor axons return to skeletal muscle following a crush injury to the muscle nerve, the number of motor units (groups of muscle fibres responding to activity in single motor axons) is usually restored. But the number and distribution of muscle fibres reinnervated by each axon may be quite different from normal. Interaction between motor axons takes place on individual muscle fibres, which express a transient polyneuronal innervation (Boeke, 1921; McArdle, 1975). The disparities in motor unit size which may result are especially conspicuous following reinnervation of partially denervated muscle. Motor units which have expanded through motor nerve sprouting regress to a variable extent when regenerating axons return, but sprouted motor units usually remain larger than normal and regenerating axons rarely recover the same numbers of muscle fibres they once innervated (Guth, 1962; Brown & Ironton, 1978; Thompson, 1978).

In a previous study, it was shown that greater regression of expanded motor units occurs upon regeneration of injured axons when the intact units are paralysed (Ribchester & Taxt, 1984). The present experiments were undertaken to address the relative importance of disparities in the size of competing motor units and disparities in their activity, by allowing inactive motor axons to reinnervate a muscle inner-

vated by expanded, but active motor units. The analysis suggests that intact and regenerating motor nerve terminals are in dynamic equilibrium, and the persistence or withdrawal of terminals depends on antagonistic, local stimuli acting at the motor end-plates. The data suggest that differences in the activity of convergent motor axons may contribute an important, but not overriding, influence to the processes of synapse formation and synapse elimination.

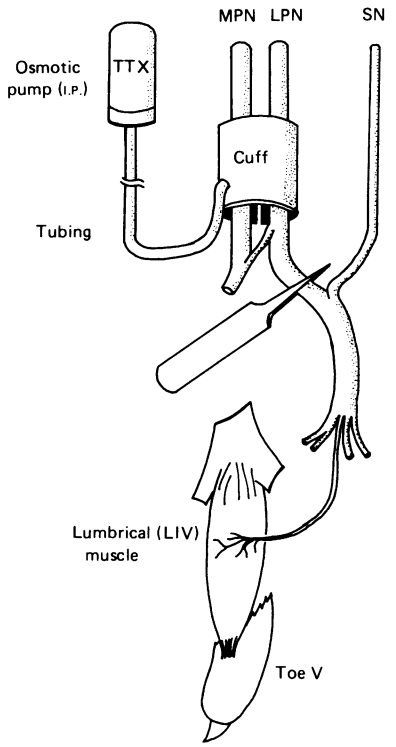

Fig. 1. Diagram of the experimental design. Fourth deep lumbrical muscles were partially denervated by crushing the lateral plantar nerve (LPN). After sprouting of motor axons in the intact sural nerve (SN), and upon return of injured LPN motor axons, nerve conduction in the LPN was blocked by superfusion with tetrodotoxin (TTX). The TTX was contained in osmotic minipumps (Alzet 2002) implanted intraperitoneally.

METHODS

Experiments were carried out using the fourth deep lumbrical muscle of adult rats (Fig. 1). In rodents, this muscle receives its motor innervation from the lateral plantar nerve (LPN) and a branch of the sural nerve (SN). The SN normally supplies between one and five of the lumbrical motor units and the LPN more than six motor units (Betz, Caldwell & Ribchester, 1979).

Adult female rats weighing 120–180 g were anaesthetized with sodium pentobarbitone (Sagatal, 60 mg/kg; I.P.) and the hindfoot was partially denervated by crushing the LPN and the medial plantar nerve with fine forceps, proximal to the SN anastomosis. The wounds were closed with 7/0 silk suture. In some animals, chronic LPN block was effected by superfusion of the nerve with tetrodotoxin (TTX; Sigma) according to methods described previously (Ribchester & Taxt, 1983). Briefly, 16–21 days after crushing the LPN, the animals were re-anaesthetized with pentobarbitone and osmotic minipumps containing TTX (500 μg/ml in 0·9% sterile saline) and ampicillin

(200 μg/ml) were implanted intraperitoneally. Silicone rubber tubing ending in a moulded cuff was connected to the pump, threaded under the skin and the cuff was placed round the tibial nerve in the right shank. Animals were tested daily for the security of the block by pinching the medial toe pads. Animals giving withdrawal reflexes before 1 week of continuous nerve block were not studied further. The security of nerve block was confirmed in the remaining animals on the day of the acute experiment. The animals were anaesthetized with pentobarbitone and the tibial nerve was cut. The distal stump was stimulated above the cuff (which produced no contraction in the foot) and below it (which caused vigorous contractions of plantar musculature).

Isometric twitch and tetanic tension measurements and intracellular recordings of end-plate potentials (EPPs) were made from isolated nerve–muscle preparations 14–55 days after nerve crush, as described previously (Ribchester & Taxt, 1983). In addition, glycogen depletion of motor units was produced in some muscles by stimulating either the LPN, SN or both together at 5 Hz continuously for 1 h. Perfusion of the recording chamber with glucose-free, oxygenated physiological saline was continued throughout the period of stimulation (Jones, Ridge & Rowlerson, 1987). The muscles were then fixed in cold (4 °C) 4% formaldehyde, 1% calcium chloride, sectioned in paraffin and stained with periodic acid and Schiff's reagent (PAS). Glycogen-depleted fibres were identified from their complete or partial failure to stain with PAS (see for example Fig. 11). Muscle fibre diameters and cross-sectional areas were measured in software, by tracing fibre profiles on a digitizing tablet (Summagraphics Bitpad-1) connected to a microcomputer.

Two muscles were stained with a combined silver/cholinesterase method (Namba, Nakamura & Grob, 1967; Hopkins, 1981).

RESULTS

Expansion of SN motor units

One aim of the experiments was to determine whether inactivity in motor axons would impair their capacity to reinnervate muscle, even when intact motor units were expanded to their maximum extent. First it was important to establish the extent of collateral reinnervation by sprouting of intact motor axons. A motor unit supplied by the SN normally contributes 3–20% of the total muscle tension (Betz, Caldwell & Ribchester, 1979, 1980*a*). A previous histological study showed that SN motor axons sprout extensively within 7 days of LPN section (Betz, Caldwell & Ribchester, 1980*b*). In the present experiments, the extent of sprouting was assessed from tension measurements and intracellular recordings 14–18 days after crushing the LPN, that is coincident with the return of regenerating LPN motor axons (see below).

Examples of isometric tension and intracellular EPPs are shown in Fig. 2. For instance, Fig. 2*A* and *B* show recordings from a muscle containing four SN motor units and in which sural nerve stimulation (0·1 ms pulse) and direct muscle stimulation with silver electrodes (nominally 100 V for 1 ms) produced the same twitch tension. Figure 3 shows the amount of isometric twitch tension produced by SN stimulation in muscles with different numbers of remaining SN units. Muscles containing either one or two SN units produced variable amounts of tension. These data were somewhat difficult to interpret because the 'direct' tension was sometimes equal to the indirect tension even though this was relatively small. The tension produced by muscles containing three or more SN units was more uniform, however, and in each muscle was within 10% of the total tension evoked by direct muscle stimulation. The conclusion is that muscles containing three or more units were virtually completely reinnervated by collateral nerve sprouts.

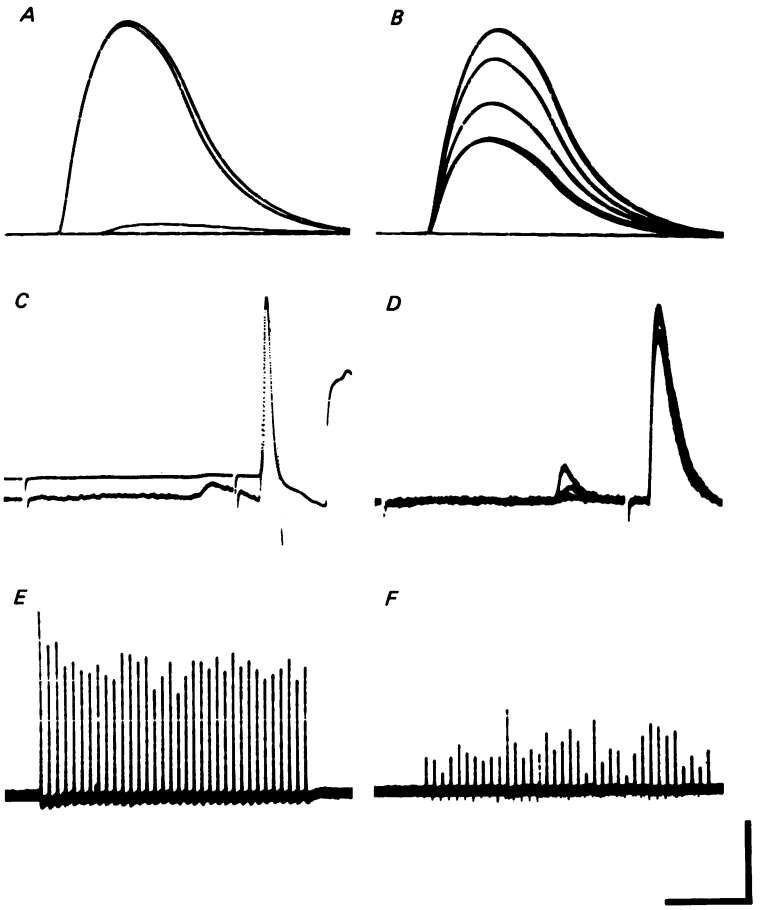

Fig. 2. Isometric twitch (*A* and *B*) and intracellular recordings (*C–F*) from muscles 15–18 days after crushing the LPN. *A–C* are recordings from intact muscles; *D–F* are from cut muscle fibre preparations. *A*, responses to supramaximal stimulation of the LPN (lowest trace), the SN (middle trace) and direct muscle stimulation (uppermost trace). The SN contributes almost all the muscle tension. *B*, graded stimulation of the SN in this muscle shows four large SN motor units. *C*, intracellular recording showing a long-latency, subthreshold EPP from the regenerating LPN (first stimulus) and a brief-latency action potential from the SN (second stimulus). *D*, weak, variable synaptic potentials from the LPN (first stimulus) in a dual-innervated muscle fibre, followed by a stronger synaptic potential from the SN. Three sweeps superimposed. *E* and *F*, trains of EPPs evoked at 10 Hz in two different muscle fibres in response to SN stimulation. The EPPs in *E* have a higher quantal content than those in *F*, based on the differences in their coefficients of variation. Vertical calibrations: *A* and *B*, 5 mN; *C*, 40 mV upper trace, 4 mV lower trace; *D–F*, 4 mV. Horizontal calibrations: *A* and *B*, 40 ms; *C* and *D*, 10 ms; *E* and *F*, 1 s.

Intracellular recordings made from cut muscle–fibre preparations (Ribchester & Taxt, 1983) of the partially denervated muscles supported the conclusions from the tension measurements (Fig. 2*C–F*). The resting membrane potentials of muscle fibres in these preparations were usually more negative than −30 mV, and miniature end-plate potentials were sometimes detectable. In muscles with three or more SN units,

121 of 137 fibres impaled from five muscles responded with end-plate potentials when the SN was stimulated. No systematic quantal analysis was made but the coefficient of variation of the EPP amplitudes in most fibres was sufficiently small (less than 15%) to suggest that they were the suprathreshold inputs that produced muscle contraction in intact preparations (Fig. 2*E*). In eleven fibres repetitive SN stimulation produced small, more variable responses however (Fig. 2*F*), and failed to evoke EPPs in a significant fraction of trials in some of them.

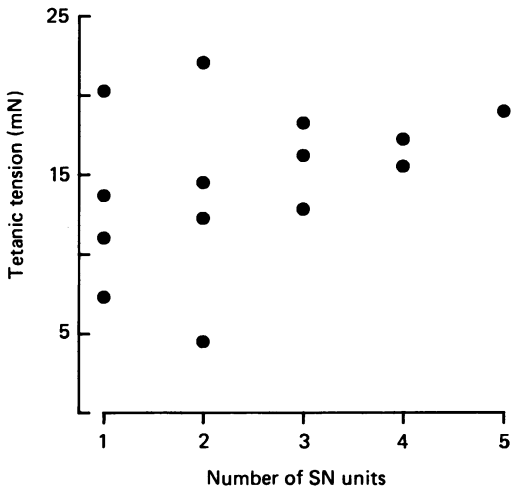

Fig. 3. Force of indirect twitch contractions evoked by SN stimulation in muscles containing different numbers of SN motor units 14–18 days after crushing the LPN.

Taken together, the tension measurements and intracellular recordings suggest that SN motor units could expand by a factor of three to five following partial denervation by LPN crush. Almost all the intact and sprouted terminals probably released transmitter with a quantal content sufficient to induce muscle contraction, but a small proportion may have produced subthreshold responses.

Dual innervation of muscle fibres

As the regenerating motor axons in the LPN returned to muscles in which sprouting was completely effective, all the fibres they confronted would be innervated by SN motor nerve terminals. Intracellular recordings and histological examination of the muscles confirmed that the SN and LPN motor axon terminals subsequently converged on the same muscle fibres, and that SN terminals were eliminated from some of the fibres.

Intracellular recording. Reinnervated muscles contained some fibres innervated only by SN motor nerve terminals, some receiving dual innervation by regenerating LPN axons and SN axons, and some innervated exclusively by the regenerating axons. This was found even in LPN-blocked muscles containing three or more intact SN motor units (Fig. 4). Thus inactive LPN axons evidently displaced some of the active SN terminals from muscle fibres. As in a previous study (Ribchester & Taxt, 1983), LPN block significantly prolonged the time course of the EPP in muscle fibres innervated exclusively or mainly by LPN axons (Fig. 4*D*).

Relatively few muscle fibres were dual innervated at any stage, and the proportion of dual innervated fibres varied little with time, in spite of the progressive increase in the proportion of fibres innervated only by the LPN and the decrease in the numbers of SN innervated fibres. Figure 5 shows data from muscles containing comparable numbers (either three or four) SN motor units. The numbers of motor

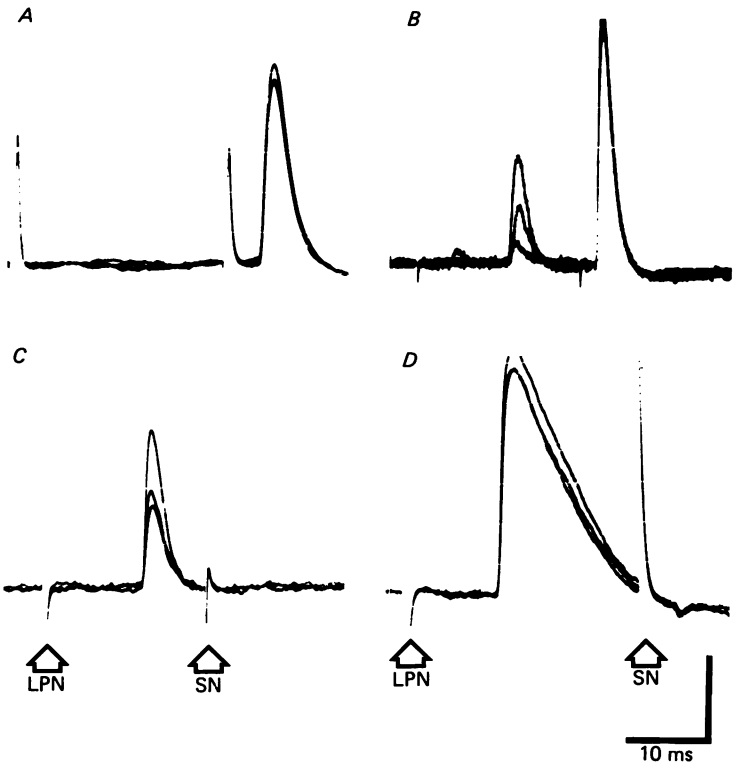

Fig. 4. Intracellular recordings from reinnervated muscle fibres taken 25–35 days after LPN crush. *A–C*, control muscles, *D*, LPN-blocked muscle, responses evoked by stimulating below the site of the cuff and after washing away the TTX in the recording chamber. In each case, the LPN was stimulated first and the SN a few milliseconds later. These records show examples of fibres innervated only by the SN (*A*), receiving dual innervation (*B*), and exclusive innervation by the LPN (*C* and *D*). Note the prolonged time course of the EPP in the LPN-blocked muscle fibre innervated only by an LPN motor axon. Vertical calibration: 10 mV (*A* and *D*); 4 mV (*B* and *C*).

units were determined from tension measurements prior to intracellular recording. In LPN-blocked muscles studied at 25–35 days, $61 \pm 10\%$ ($n = 5$ muscles) of the fibres innervated by the LPN were also innervated by SN motor axons compared with 47 ± 33 ($n = 5$) in reinnervated controls. At this stage, there were significantly more fibres innervated by the SN and significantly fewer fibres innervated by the LPN in these muscles compared with control muscles ($P < 0{\cdot}001$, χ^2 test).

EPP latency. Differences in the conduction time in LPN motor axons compared with SN axons were evident in the latency of the isometric twitch in all muscles (e.g. Fig. 2*A*), and these differences decreased with time. In intracellular recordings,

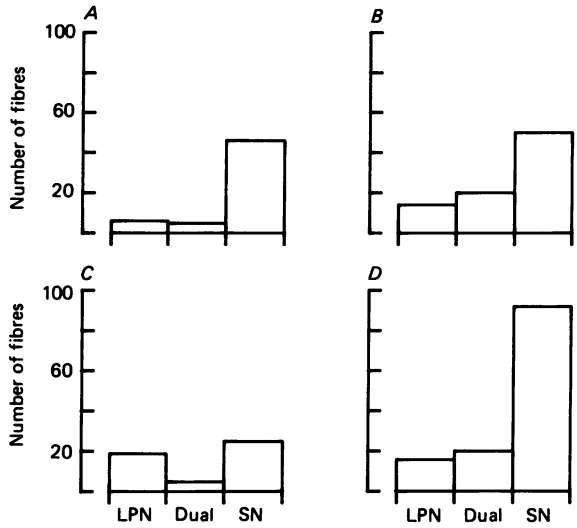

Fig. 5. Cumulative distribution of muscle fibres receiving innervation by the SN, receiving dual innervation, and exclusive LPN innervation, obtained in intracellular recordings of EPPs from cut muscle fibre preparations. *A*, 15–24 days after LPN crush ($N = 4$ muscles); *B*, 25–35 days ($N = 4$); *C*, 36–50 days ($N = 2$); *D*, 25–35 days after nerve crush and 9–15 days of LPN block ($N = 5$). All these data were from muscles containing either three or four SN motor units.

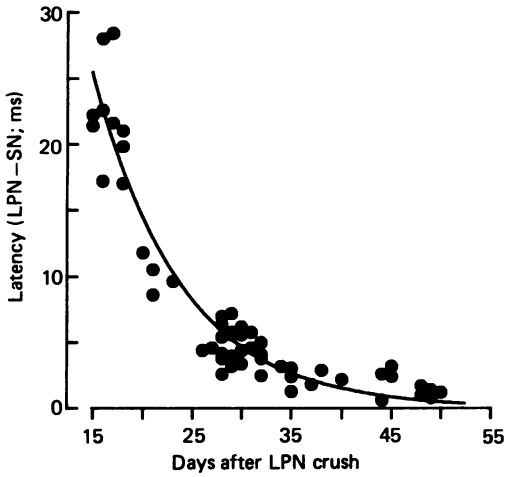

Fig. 6. Recovery of nerve conduction in the LPN following crush. Each point is the mean difference between the latency of the LPN-evoked and SN-evoked EPPs measured from intracellular recordings from ten to sixty muscle fibres in one muscle. The curve is a single exponential with a time constant of 8·18 days.

examples of subthreshold EPPs evoked by LPN stimulation were seen during early stages of reinnervation, and these EPPs had a much longer latency than those evoked by SN stimulation (see Fig. 2*C* and *D*). At later stages, the EPPs in most fibres varied little in amplitude upon low-frequency, repetitive stimulation of the motor axons and the latency of the LPN-evoked EPPs had decreased (see Fig. 4). To

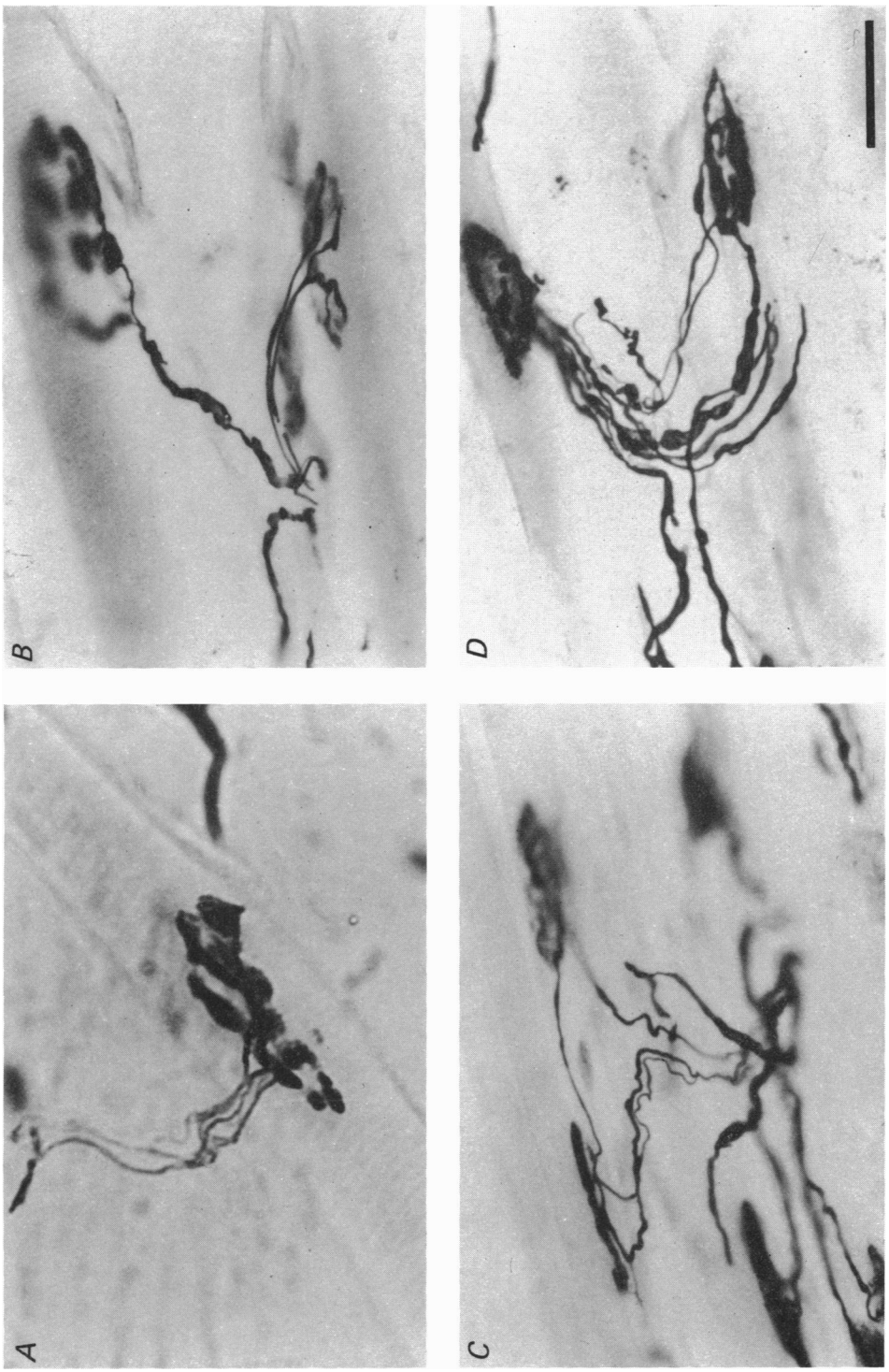

Fig. 7. Examples of polyneuronally innervated muscle fibres in a lumbrical muscle, 24 days after crushing the lateral plantar nerve. Combined silver and cholinesterase stain. Calibrations: *A* and *B*, 10 μm; *C* and *D*, 20 μm.

assess this recovery and to compare LPN-blocked with control muscles, the differences between the latency of SN-evoked EPPs and LPN-evoked EPPs were plotted against time after return of the LPN axons. The conduction distance (about 3 cm) was similar for both nerves and similar between preparations. Rectangular hyperbolic and exponential curve fits were attempted for the data. A single exponential with a time constant of 8·18 days provided a better fit than hyperbolic functions (Fig. 6). This is perhaps surprising, since the recovery of conduction

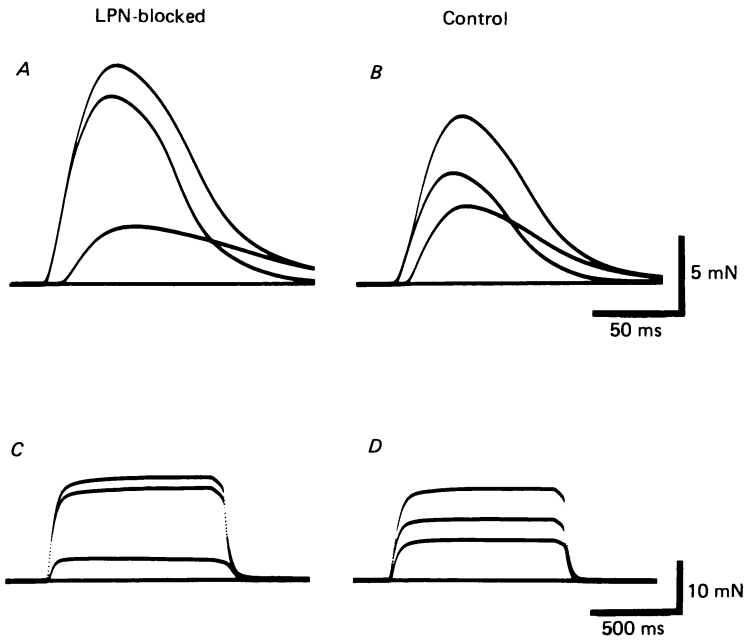

Fig. 8. Isometric twitch (*A* and *B*) and tetanic (*C* and *D*) tension responses obtained from a pair of lumbrical muscles 29 days after crushing the LPN. *A* and *C*, LPN-blocked, starting 19 days post-LPN crush and maintained for 10 days. *B* and *D*, contralateral control. Both muscles contained three SN motor units. In each case, the lowest trace is the response to LPN stimulation; the middle trace is the SN response and the top trace the response to combined nerve stimulation. Note the incomplete tension overlap; the prolonged time course of the LPN-evoked twitch, and the relatively small contribution of the LPN to the total tension in the LPN-blocked muscle.

velocity in regenerating motor axons must depend on many factors, including the rate of increase in girth of the axons and the reformation of myelin.

LPN conduction block had no effect on the recovery of EPP latency. By 29–32 days, that is after 11–13 days of LPN block, the mean difference (LPN−SN) was $5{\cdot}57 \pm 1{\cdot}29$ ms ($n = 6$ muscles), which was not significantly different from the controls ($5{\cdot}31 \pm 1{\cdot}01$ ms, $n = 7$; $P > 0{\cdot}05$, Mann–Whitney test).

Histology. Two reinnervated muscles were stained with silver and histochemically for cholinesterase, 24 days after crushing the lateral plantar nerve. Examples of stained motor end-plates are shown in Fig. 7. Of 234 end-plates examined in these muscles, thirty-seven appeared to receive a convergent input from more than one

motor axon, confirming that the dual innervation of muscle fibres occurred over a restricted area of the muscle fibre surface – namely, the original motor end-plates.

Regression of SN motor units and recovery of LPN motor units

The first signs of recovery of tension in regenerating LPN motor units were seen 15 days after nerve crush (e.g. Fig. 2*A*). It was not possible to determine the numbers of LPN motor units at these early stages of reinnervation, because the tension

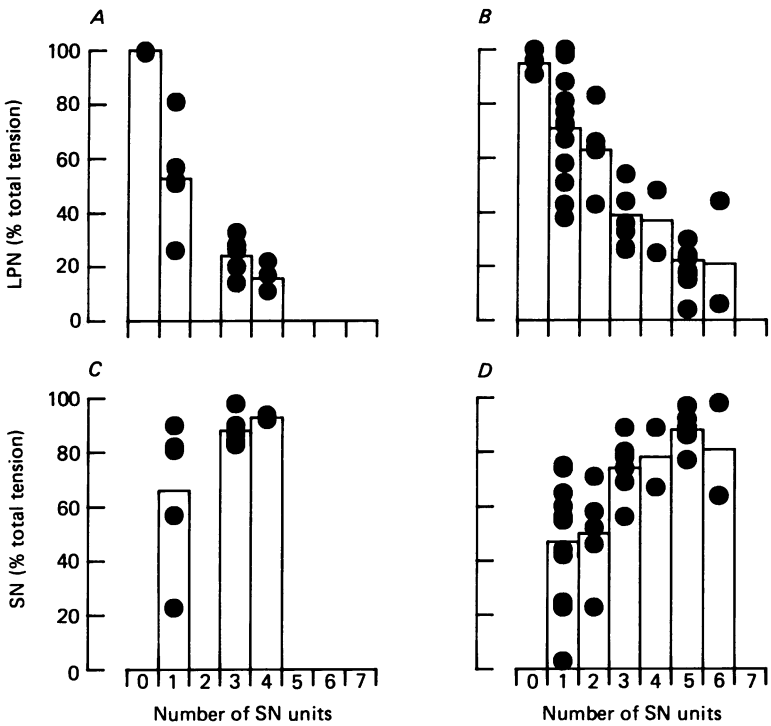

Fig. 9. Isometric tetanic tension data from reinnervated muscles containing different numbers of SN motor units 25–35 days after crushing the LPN. Circles show the tension in individual muscles and the bars represent the mean tension. *A* and *C*, data from LPN-blocked muscles; *B* and *D*, from contralateral controls. The only statistically significant differences comparing LPN-blocked and control muscles were in the ones containing three or four SN motor units. The SN tension was significantly larger and the LPN significantly smaller in LPN-blocked muscles.

responses to graded nerve stimulation were too weak and too variable. Within a further 7 days however, graded nerve stimulation became more reliable and showed that all the muscles were reinnervated by at least six LPN motor axons, as in normal muscles. One LPN-blocked muscle was discarded because it contained fewer than six LPN units. In the remaining LPN-blocked muscles, the lateral plantar nerve supplied 8 ± 1 units.

Control muscles. The tension generated by LPN stimulation returned rapidly after 20 days following nerve crush (Ribchester, 1984). By 25–35 days, the tetanic tension produced by stimulating the LPN axons was $52 \pm 28\%$ ($n = 34$) of the total (Fig. 8)

and by 40–50 days, it was 63±23% ($n = 15$, $P > 0.05$, t test). The large variation between muscles (coefficient of variation 30–60%) could at least partly be explained by differences in the numbers of remaining SN motor units. Thus, the tension evoked by stimulating the regenerated LPN axons in muscles containing three or four SN motor units was significantly larger than in muscles containing five or more SN units (Fig. 9; $P < 0.005$, t test). The tension produced by LPN stimulation was even larger in muscles containing only one or two SN units, but this was expected because these muscles probably contained many vacant denervated motor end-plates at the time the LPN axons returned (see Fig. 3).

The regeneration of LPN motor units was accompanied by a fall in the tension produced by SN motor units. In muscles containing three or more units, SN tension fell to 67±21% ($n = 17$) of total tension by 25–35 days, and only slightly more – to 59±20% ($n = 5$) – at 40–50 days. Less regression of SN motor units occurred in muscles with more than four SN motor units compared with those containing fewer than three (Fig. 9; $P < 0.03$, t test).

Single SN unit muscles. As in previous studies (e.g. Betz *et al.* 1980*a*), many lumbrical muscles contained only one motor unit supplied by the sural nerve. Tension recordings indicated that these motor units retained most of their connections with muscle fibres, and that the regenerating axons mainly reinnervated the denervated muscle fibres. An example of a reinnervated muscle containing only one SN unit is shown in Fig. 10. At 49 days after LPN crush, this SN unit produced almost as much tension as the nine motor units supplied by the regenerated LPN. Altogether, sural nerve-evoked tetanic tension was 32·8±12·7 mN at 16–21 days ($n = 6$ muscles) and 24·5±10·3 mN (50±20% of total tension, $n = 10$) at 25–35 days. At 40–50 days, single motor unit tension was 25±14 mN (42±22%, $n = 6$). None of these differences is statistically significant, but the total muscle tension increased during this period, along with the growth of the animals so the possibility that some sprouts were displaced cannot be ruled out. None the less, the percentage innervation of the muscles by these single motor axons should be compared with the mean motor unit size of about 9% in unoperated muscles (Betz *et al.* 1979).

The overall conclusion from these tension data is that the sprouted SN motor units retained control of a relatively expanded proportion of the muscles in spite of the greater numbers of regenerating motor axons supplied by the lateral plantar nerve. But it would also seem that the ability of the expanded SN motor units to withstand displacement by the reappearing LPN axons is related to the number of SN units. This could be because in muscles containing few SN units, the SN terminals were more fragile, compared with muscles containing more SN units, each of which supported fewer terminals. However, there is the additional factor that in muscles with fewer SN units there were likely to be more reappearing LPN units which, together, would have exerted a stronger competitive influence.

LPN-blocked muscles. Regenerated LPN motor axons produced less tension, and SN axons comparatively more tension, when LPN conduction was blocked (Fig. 8). In those muscles containing three or four SN units, the LPN tension was about half that of controls and the SN tension was correspondingly larger (Fig. 9; $P < 0.003$, t test). Like controls, it is impossible to make any firm conclusion about muscles containing one or two SN units because they probably contained many denervated

muscle fibres at the time the LPN axons returned (see Fig. 3). Thus, the SN-evoked tetanic tension in LPN-blocked muscles containing only a single SN motor unit was 27·9 ± 12·1 mN (57 ± 34 % of total tension; $n = 5$), not statistically different from single-SN unit controls. None of the LPN-blocked muscles in the present study contained more than four SN motor units.

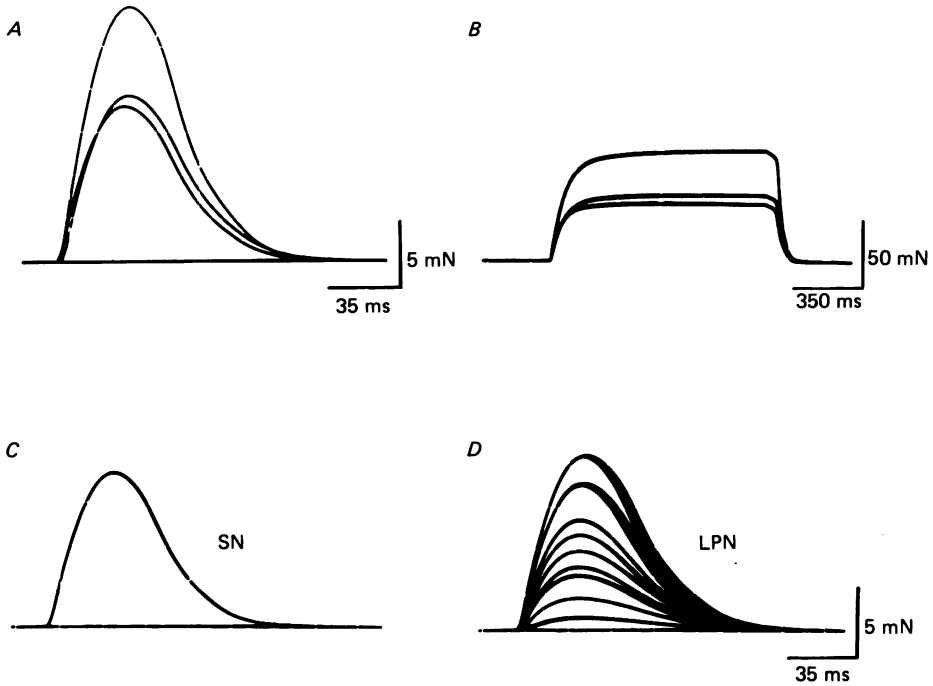

Fig. 10. Isometric tension responses from a control muscle 49 days after crushing the LPN. This muscle contained only a single SN motor unit. *A*, twitch tension obtained by supramaximal stimulation of the SN (lowest trace), LPN (middle trace) and combined nerve stimulation (uppermost trace). *B*, similar order of responses to supramaximal, tetanic stimulation at 70 Hz. *C* and *D*, motor unit tension responses to careful grading of the stimulus intensity applied to the SN (*C*) and LPN (*D*). The single SN unit produced almost as much tension as the nine regenerating LPN units.

Glycogen depletion. It is possible that the reinnervated muscle fibres produced less tension than those left with intact innervation following LPN crush. Previous studies suggested that denervation and inactivity in lumbrical motor units causes only slight atrophy, at least over the duration of the present experiments (Ribchester & Taxt, 1983). But to examine this point further motor units were selectively stimulated in an attempt to deplete their muscle fibres of glycogen, and the muscles were then examined histologically (Fig. 11). The results of these attempts, while generally supporting the previous findings, were not entirely conclusive. This was mainly because the amount of glycogen depletion was usually less than the proportion of the muscle tension evoked by stimulating the nerve used to produce it. For instance, in two muscles the entire motor nerve supply was stimulated at 5 Hz for 1 h to see whether glycogen could be depleted from all the muscle fibres. In one of these, an

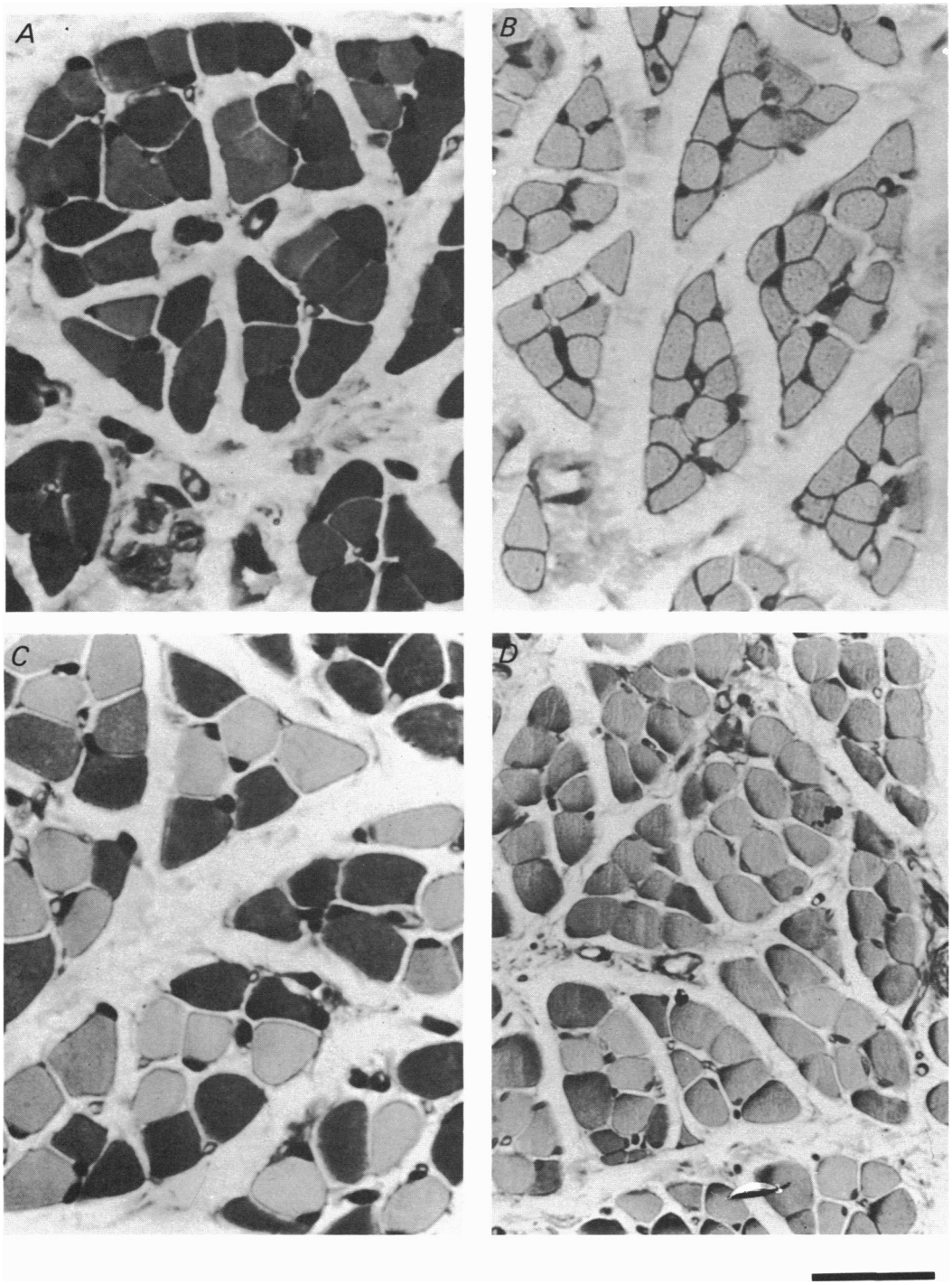

Fig. 11. Examples of PAS staining for glycogen in reinnervated lumbrical muscles. *A*, unstimulated control muscle, all fibres stain positive. *B*, complete depletion in an LPN-blocked muscle in which the entire nerve supply was stimulated. *C*, partial depletion produced by SN stimulation in a control muscle. *D*, partial depletion in an LPN-blocked muscle produced by SN stimulation. More fibres are depleted than in *C*. Calibration: 30 μm.

TABLE 1. Glycogen depletion of lumbrical motor units

Muscle	Days denervated/ blocked	Nerve stimulated	Number of SN units	Percentage tension	Percentage depletion	Total fibres	Diameter (μm)		Area (μm^2)	
							Depleted	Non-depleted	Depleted	Non-depleted
					LPN blocked					
1	31/10	LPN	0	100	93	1220	6·3	6·9	52·5	64·1
2	28/9	SN	1	57	46	756	—	—	—	—
3	33/12	SN	3	84	70	1042	11·3	9·9	183	145
4	24/10	SN	3	90	45	817	8·7	7·4	91	75
					Control					
5	28/—	LPN+SN	1	100	55	722	—	—	—	—
6	27/—	SN	1	45	28	831	9·4	8·4	117	90
7	28/—	SN	2	52	35	793	7·9	7·8	83	84
8	29/—	SN	3	69	52	714	10·0	9·3	108	103
9	33/—	SN	3	57	21	865	9·7	9·8	119	116
10	31/—	SN	4	67	30	1210	8·5	9·0	91	105
11	29/—	SN	5	88	74	868	9·0	9·0	103	109

Distribution of glycogen-depleted and non-depleted muscle fibres following stimulation of the LPN, SN or both nerves. Percentage tension refers to the tetanic tension produced by stimulating the nerve compared with the total muscle tension. Percentage depletion refers to the proportion of the total numbers of fibres in a transverse section that showed partial or negative staining with PAS. Diameters and areas are the mean for all the muscle fibres in each muscle.

LPN-blocked muscle, all but a ring of fibres (amounting to about 5% of the total) around the perimeter of the muscle were depleted (Table 1). In the other completely stimulated muscle (a control, muscle 5 in Table 1), however, only about 50% of the fibres were depleted. Perhaps this partial failure of the technique was due to the experimental conditions: in these isolated preparations, it may not have been possible to prevent block of neuromuscular transmission in some fibres before glycogen depletion was complete.

TABLE 2. Fatigue resistance of LPN-blocked and control muscles

	LPN-blocked		Control	
	LPN	SN	LPN	SN
T_p (ms)	35·8 ± 5·9*	31·7 ± 2·4	29·8 ± 1·1*	30·6 ± 0·7
T_d (ms)	54·0 ± 9·6†	36·7 ± 8·4	44·2 ± 6·3†	32·6 ± 6·0
FR (% initial)	43 ± 14†	23 ± 4†	49 ± 18*	23 ± 7*
n	4	4	4	4

Tension data from muscles subject to continuous fatiging stimulation at 4 Hz. Time to peak (T_p) and time from peak to half-decay (T_d) were measured at the start of the stimulus train. Fatigue resistance (FR) is the twitch tension after 10 min stimulation given as a percentage of the initial twitch tension. All the measurements were made at room temperature. *$P < 0·05$, †$P < 0·02$.

In the remaining muscles, sural nerve stimulation always produced a patterned depletion of muscle glycogen. SN units in LPN-blocked muscles also contained more muscle fibres than controls. Two of the LPN-blocked muscles contained three SN motor units. The SN tension in these was 84 and 90% of the total, and 70 and 45% of the fibres respectively were depleted of glycogen. Two of the controls contained three SN units and one contained four units. The mean SN tension was 64% and the mean glycogen depletion was 34% of the muscle fibres.

The mean diameter of glycogen-depleted fibres (innervated by the SN) was slightly, but significantly larger than non-depleted fibres in LPN-blocked muscles (mean difference 1·1 μm; $P < 0·02$, paired t test). There was no difference in the areas or diameters comparing depleted with non-depleted fibres in control muscles.

Fatigue resistance. The speed of contraction and fatigue resistance of a motor unit are labile properties which can be altered by muscle activity (Salmons & Sreter, 1976; Cotter & Phillips, 1986). It was therefore of interest to examine whether LPN-blocked motor units differed in their fatigue resistance from the other motor units.

Eight muscles were stimulated continously at 4 Hz for 10 min and the isometric twitch tension at the start of the period compared with that at the end. At the start, the times to peak and half-relaxation of LPN-blocked motor units were significantly longer than controls or SN motor units (Table 2), confirming the findings of a previous study (Ribchester & Taxt, 1983). Regenerated LPN motor units were more resistant to fatigue than intact SN motor units, but the fatigue resistance of LPN-blocked units was no different from LPN units in controls (Table 2). Evidently, inactivity in lumbrical motor units affects some of their properties – such as the time course of the twitch and motor unit size – but not others, such as strength of individual muscle fibre contractions or fatigue resistance.

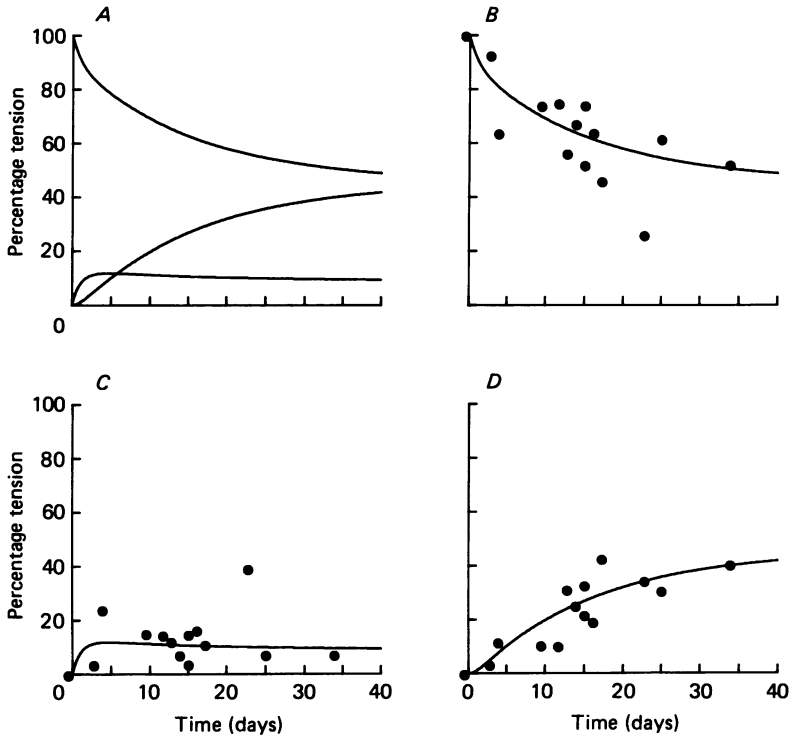

Fig. 12. Solutions to rate eqns (1)–(3) using rate constants given in the text, together with data from muscles containing three or four SN motor units. The amount of muscle innervated only by SN axons, only by LPN axons, or receiving dual innervation is plotted as a percentage against time after return of LPN motor axons to the lumbrical muscle. Time zero represents 17 days after LPN crush, the mean time found for the regenerating axons to arrive at the muscle. *A*, all three theoretical curves superimposed. Reading at 40 days: upper – SN only; middle – LPN only; lower – dual innervation. *B–D*, each curve presented separately together with the tension data. *B*, SN only. *C*, dual innervation. *D*, LPN only.

Empirical kinetic description

The proportion of the muscles innervated exclusively by SN motor axons, exclusively by LPN motor axons, and receiving convergent, dual innervation by axons in either nerve is plotted against time after return of the LPN axons in Fig. 12. These data were obtained from tetanic tension recordings by measuring the tension overlap on combined (LPN and SN) stimulation compared with separate LPN and SN stimulation (Fig. 8), viz.

$$\%\,\text{dual} = 100\ (\text{LPN}+\text{SN})/\text{combined}-1,$$
$$\%\,\text{LPN only} = 100\ (1-\text{SN}/\text{combined}),$$
$$\%\,\text{SN only} = 100-(\%\,\text{dual}+\%\,\text{LPN only}).$$

For instance, by 25–35 days after LPN crush, about two-thirds the LPN tension gave tension excess and about one-third tension overlap with the SN (Fig. 8). By comparison, the LPN tension was distributed about equally between tension overlap and tension excess in LPN-blocked muscles (Fig. 8).

The data shown in Fig. 12 are those obtained from control muscles containing either three or four SN motor units. The data on other muscles were excluded on the grounds that it probably required at least three units to effect complete collateral reinnervation by sprouting (Fig. 3). Muscles containing fewer SN units would contain denervated fibres on return of the LPN. Muscles containing five or more SN units had significantly different ratios of LPN to SN tension (Fig. 9).

Superimposed on the data in Fig. 12 are curves calculated assuming that the lumbrical muscle fibres can exist in one of three innervated states and these states represent a dynamic equilibrium, viz.

$$\mathrm{SN} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{dual} \underset{k_s}{\overset{k_{-s}}{\rightleftharpoons}} \mathrm{LPN}$$

In this scheme, k_1 determines the rate of formation of synapses by LPN axons on fibres already innervated by SN axons, and k_{-s} reflects the rate of elimination of SN sprouts. Similarly, k_{-1} would determine the rate of elimination of *regenerated* terminals from dual innervated fibres and k_s reflects the rate of formation of synapses by SN sprouts on fibres already innervated by LPN motor nerve terminals. Assuming the rate constants are first order, the formal rate equations are:

$$\frac{\mathrm{d}}{\mathrm{d}t}(\mathrm{SN}) = k_{-1}[\mathrm{dual}] - k_1[\mathrm{SN}],$$

$$\frac{\mathrm{d}}{\mathrm{d}t}(\mathrm{dual}) = k_1[\mathrm{SPN}] + k_s[\mathrm{LPN}] - (k_{-s} + k_{-1})[\mathrm{dual}],$$

$$\frac{\mathrm{d}}{\mathrm{d}t}(\mathrm{LPN}) = k_{-s}[\mathrm{dual}] - k_s[\mathrm{LPN}].$$

Two further assumptions were that the muscles were completely innervated by SN motor axons when the LPN first returned (Fig. 3), and at any given stage there were no denervated muscle fibres. Thus the total number of muscle fibres, M, is:

$$M = \mathrm{SN} + \mathrm{dual} + \mathrm{LPN} = 100\,\%.$$

Under these conditions, the amounts of SN, dual and LPN change with time according to the following equations:

$$\mathrm{SN}(t) = c_1 \exp(b_1 t) + c_2 \exp(b_2 t) + M(k_{-1} k_s)/D, \tag{1}$$

$$\mathrm{dual}(t) = c_3 \exp(b_1 t) + c_4 \exp(b_2 t) + M(k_1 k_s)/D, \tag{2}$$

$$\mathrm{LPN}(t) = c_5 \exp(b_1 t) + c_6 \exp(b_2 t) + M(k_1 k_{-s})/D, \tag{3}$$

where $$D = k_1 k_{-s} + k_{-1} k_s + k_1 k_s.$$

The constants b_i, c_i are entirely determined by the rate constants (there are no arbitrary constants) and their values were obtained from simultaneous equations given by Dawes (1972). In order to obtain a reasonable fit to the data, k_{-1} had to be made larger than the other rate constants. Otherwise, a much faster rate of regression of SN motor units would be predicted than was observed experimentally. Figure 12 shows the time course and outcome of an empirical fit using rate constants in the order $k_{-1} > k_{-s} > k_1 > k_s$; specifically, $k_{-1} = 0{\cdot}64$ days^{-1}, $k_{-s} = 0{\cdot}256$ days^{-1},

$k_l = 0{\cdot}128$ days^{-1} and $k_s = 0{\cdot}0512$ days^{-1}. Perhaps coincidentally, the ratios of rate constants in the direction away from dual innervation and in the directions towards dual innervation were about equal to the ratio of LPN to SN motor units in these muscles i.e.:

$$k_{-l}/k_{-s} = k_l/k_s = 2{\cdot}5.$$

It is noteworthy that this kinetic analysis accommodates three important, characteristic features of the reinnervated muscles. First, the regression of the sprouted units was incomplete even when relatively large numbers of regenerating axons returned. Second, the regression of the SN and expansion of the LPN innervation was not accompanied by extensive dual innervation at any stage in spite of the significant changes in relative SN and LPN tension. Third, small amounts of dual innervation persisted to the latest stages examined.

The effects of selective LPN block on the outcome of the synaptic interactions could perhaps be predicted if one or more of the rate constants were to change, although the resolution of the data would have to be improved and the effects of various durations of nerve block would then have to be measured to settle this point.

DISCUSSION

The results and analysis extend previous findings that during reinnervation of partially denervated muscles, the probability that either intact or regenerating motor nerve terminals will persist depends on the number of interacting motor units (Brown & Ironton, 1978). But the kinetic analysis predicts that at the level of single muscle fibres, there is a finite probability that regenerating terminals are eliminated during competition with intact terminals and sprouts. Furthermore, this analysis predicts that the sprouted motor axons will have the competitive advantage, because when sprouted and regenerated motor axons converge on the same muscle fibres, the regenerated terminals are eliminated more rapidly ($k_{-l} > k_{-s}$). Perhaps less surprising, it may be inferred from the empirical rate constants that the rate of synapse formation by regenerating axons in the lateral plantar nerve was much greater than the rate of reformation of connections by sprouts from the sural nerve ($k_l > k_s$). Thus in general, regenerating motor axons appear to be more labile with respect to both synapse formation *and* synapse elimination, than motor nerve terminals belonging to intact axons, even when the intact motoneurones are compelled to support more terminals. In support of this, the form of intact neuromuscular junctions studied continuously *in vivo* changes little over periods in excess of 1 month, whereas regenerating terminals undergo radical shape changes over the same period (Lichtman & Rich, 1986; Lichtman, Magrani & Purves, 1987).

The exclusive innervation of muscle fibres by regenerating axons

The results also confirm that inactivity in regenerating axons does not impair their capacity to regenerate (Williams & Gilliatt, 1977) but it increases the probability of withdrawal of axon terminals during competition with active motor units (Ribchester & Taxt, 1983, 1984; but see also Callaway, Soha & Van Essen, 1987; Ribchester, 1988). Differences in activity may not be overriding, however, because inactive

motor nerve terminals made stable, functioning connections on fibres already innervated by active axons. In some fibres, inactive terminals evidently displaced active ones – rendering paralysed muscle fibres that were once mobile. This conclusion is based on the following evidence: first, the tension of the SN motor units decreased following the return of inactive LPN axons; second, stimulation of the LPN and SN together produced excess tension over that produced by SN stimulation alone; finally, it was quite easy to find muscle fibres giving EPPs exclusively to LPN stimulation in the LPN-blocked muscles. There is no contradiction here with the conclusion that sprouted nerve terminals had an overall advantage: merely, there was still a finite probability in these LPN-blocked muscles that SN terminals would be eliminated.

An alternative explanation is that the inactive axons selectively reinnervated the small population of denervated fibres that remained in the muscles after SN motor units had sprouted to their maximum extent. This seems unlikely in view of the regression of the SN tension but it cannot entirely be discounted because the tension measurements and intracellular recordings made prior to the return of the LPN axons showed that some muscle fibres remained denervated, even in muscles containing three or more SN motor units. Either way, muscles examined after more than 10 days of LPN block contained some fibres innervated only by inactive axons. If these connections were dynamic, then active terminals were eliminated: if they were not, then inactive axons must have made persistent stable connections on muscle fibres during expansion and regression of active motor units.

The mechanism of synaptic competition

Do the present results have any implication for the cellular mechanisms of motor unit interaction in skeletal muscle? One possibility is that the SN terminals may have been displaced by terminals which were functionally more appropriate for a particular muscle fibre type (Thompson, Sutton & Riley, 1984; Jones *et al.* 1987; Lichtman & Wilkinson, 1987). Alternatively, growth and regression of terminals may be controlled by factors independent of muscle fibre type. For instance, Gouzé, Lasry & Changeux (1983) have analysed a model based on the co-operative action of a muscle-derived growth promoting factor and a pre-synaptic 'stabilizing' factor; but this model does not adequately explain how stable connections persist in completely paralysed muscles (Brown, Hopkins & Keynes, 1982; Taxt, 1983).

Another possibility is that neurite-growth inhibitors might lead to displacement of nerve endings. Brown, Jansen & Van Essen (1976) argued against this, but a strong case was made for growth inhibitors by Diamond, Cooper, Turner & MacIntyre (1976), to explain the constraints on regenerating axons in amphibian skin. An interaction between growth-repressing molecules and antagonistic, anti-repressors was discussed as a possible model for plasticity in the central nervous system by Nelson & Brenneman (1982).

There is good evidence that skeletal muscles produce specific molecules which act on motoneurones to promote growth of their axons (Edgar, Timpl & Thoenen, 1984; Henderson, Huchet & Changeux, 1984; Dohrmann, Edgar, Sendtner & Thoenen, 1986; Gurney, Appattoff & Heinrich, 1986; Gurney, Heinrich & Lin, 1986). Evidence for endogenous neuronal growth inhibitors is more indirect. First, alcohol vinca

alkaloids, potent glucocorticoids, and tonic depolarization all inhibit growth of neurites when applied exogenously, either *in vivo* or *in vitro* (Speidel, 1941; Diamond *et al.* 1976; Unsicker, Krisch, Otter & Thoenen, 1978; Fishman & Nelson, 1981; Campenot, 1986). Second, neurite growth-inhibiting activity has been found associated with astrocytic neuroglia in the mammalian spinal cord (Liuzzi & Lasek, 1987). Third, in culture, the growth of neurites from different types of neurones is mutually inhibited when their growth cones contact one another (Kapfhammer & Raper, 1987). Finally, specific endogenous substances which inhibit growth of non-neuronal cells have been identified – for instance, a protein named transforming growth factor β which acts on epithelial cells (Silberstein & Daniel, 1987). O'Brien, Ostberg & Vrbová (1978) suggested that secretion of peptidases by muscle might lead to selective withdrawal of nerve terminals, and there is some evidence that inhibition of calcium-activated neutral peptidases inhibits synapse elimination (O'Brien, Ostberg & Vrbová, 1984; Connold, Evers & Vrbová, 1986).

The results of the present experiments, the analysis shown in Fig. 12 and data obtained by others, are perhaps compatible with the activity-dependent release of a muscle-derived promoting substance that stimulates neurite growth (as in the model of Gouzé *et al.* 1983), and the expression by growing *axons* of an antagonistic, growth-repressing substance that causes neurite withdrawal. The growth-promoting substance would drive the system towards polyneuronal innervation, while the growth repressor would act heterosynaptically to drive dual-innervated fibres towards mononeuronal innervation. The rate and end result of synaptic competition would then depend on the relative amounts and efficacy of both substances. This kind of model is consistent with the known effects of either complete or selective nerve conduction block. For instance, a normal active muscle (present experiments) might produce a paucity of growth factor and the rate of synapse formation by ingrowing axons and regression of intact nerve terminals would then depend on the amount of a synaptic repressor produced by the regenerating axons. Partly or completely inactive muscle, however (Ribchester & Taxt, 1983, 1984), might produce relatively large amounts of the growth factor in addition to the putative repressor produced by regenerating axons. In that case, a more rapid and profuse initial innervation by regenerating axons would be expected, but more intact terminals would then be exposed to the local effects of synaptic repressor.

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