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ROLE OF DEGENERATING AXON PATHWAYS IN REGENERATION OF MOUSE SOLEUS MOTOR AXONS

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SUMMARY

1. The recovery of tension in mouse soleus was assayed 1–5 days after crushing the extramuscular nerve in muscles which had been previously either denervated by nerve crush, partly denervated by spinal nerve root section, or paralysed by I.M. injection of botulinum toxin. Recovery of tension following nerve crush in contralateral control muscles from the same mice was also measured. The muscles were then stained with zinc iodide-osmium and examined in the light microscope.

2. Recovery in control muscles began at about 50 hr after crush and was nearly complete by 5 days. Recovery began about 10 hr earlier and was more rapid in muscles denervated by crushing the muscle nerve 4 days before recrushing at the same site.

3. Paralysis 12 days earlier by intramuscular injection of botulinum toxin did not enhance recovery after nerve crush. The axons remaining following partial denervation 6 days before nerve crush also regenerated at a rate similar to controls.

4. It is concluded that (1) nerves regenerate more quickly down a pre-degenerated pathway, (2) chromatolysis does not significantly enhance reinnervation, and (3) each motor axon regenerating after a crush is constrained to follow its own denervated pathway back into the muscle.

5. Histology was consistent with these conclusions, and also showed that end-plates in control muscles reinnervated after short periods of denervation were normal in appearance and possessed little 'escaped' nerve growth. This was in contrast to end-plates which had been regenerated in muscle after a preceding nerve crush, botulinum toxin paralysis or partial denervation. This suggests that growth from nerve terminals is controlled locally within a muscle.

INTRODUCTION

It is not clear whether nerve growth rate is controlled primarily by availability of centrally supplied materials or locally by conditions around the growing axon tip. Enhancement of nerve growth following a conditioning lesion has been demonstrated clearly by several groups in diverse systems (McQuarrie & Grafstein, 1973; Agranoff, Field & Gaze, 1976; Scheff, Benardo & Cotman, 1977), and evidence has been presented that the nerve cell body plays the key role in this enhancement (Grafstein

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& McQuarrie, 1978). However prior axotomy which leads to chromatolysis does not stimulate peripheral nerve regeneration to any great extent (Gutman, 1942; Hall-Craggs & Brand, 1977; McQuarrie, 1978; McQuarrie, Grafstein, Dreyfus & Gershon, 1978), which suggests that in peripheral nerves axon growth may be modulated primarily by local factors rather than by the cell body.

One elegant study of the effect of such a local factor, the state of the denervated pathway, on nerve regeneration in frogs was reported by Lubinska (1952). She found that while the regeneration rate was unchanged when the sciatic nerve was recrushed 5 days after a conditioning crush, the delay in starting regeneration was shorter after the second crush. This delay was increased if the frogs were kept at lower temperatures between the first and second crush, and since degeneration of the nerve distal to the crush was visibly slower at lower temperatures, Lubinska attributed the delay to a requirement for degeneration to proceed to a certain level before regeneration could begin.

Lubinska's experiments still do not exclude the possible effect on regeneration of temperature-dependent changes in the state of the nerve cell body. Moreover, comparable experiments to demonstrate the requirement for predegeneration of the distal nerve stump have not been performed in mammals. We report here measurements on regeneration of the mouse soleus nerve crushed, and also recrushed, close to the point of its insertion into the muscle. The possible influence of the metabolic state of the cell body on regeneration has been investigated by subjecting the muscle before nerve crush to a period of paralysis induced by I.M. injection of botulinum toxin, which is known to cause chromatolysis of the nerve cell body (Watson, 1969) and to induce growth from intact nerve terminals (Duchen & Strich, 1968) and nodes of Ranvier (W. G. Hopkins & M. C. Brown, in preparation).

Reinnervation of specific target tissues is generally quite precise following a simple crush to peripheral nerves, which has been taken as evidence that individual axons regenerate inside their own endoneurium (Sunderland, 1978, p. 108). However, axons must be able to leave their endoneurium within a muscle when they produce nodal sprouts (Hoffman, 1950) and also when multiple innervation of muscle fibres occurs on reinnervation (McCardle, 1975). We therefore also investigated whether a simple extramuscular nerve crush would permit axons to leave their endoneurium if they were presented with an adjacent predegenerated pathway produced by prior partial denervation.

METHODS

All operations were performed on young female mice (15–25 g) of the Olac strain under chloral hydrate anaesthesia (0·5 mg/g body wt. injected I.P. as 3·5 % solution in water). The right soleus muscle of each mouse was subjected to a period of either denervation, partial denervation or paralysis. Left and right soleus nerves were subsequently crushed, and the recovery of muscle tension was then assayed *in vitro* up to 5 days later.

Denervation

The right soleus nerve was exposed at its point of insertion into the muscle. The nerve was crushed within 1 mm of the muscle surface with no. 5 watchmaker's forceps, filed so that the last 2 mm of the tips had a uniform width of 0·2 mm. Two different crushing methods were used. In the first, nerves were gripped firmly at one site for three 10 sec intervals and three 1 sec intervals. A second series of animals had a single crush for 10 sec. None of the muscles examined subsequently had

intact myelinated axons distal to the crush. Care was taken not to damage the blood vessels which enter the muscle beside the nerve. The animals were allowed to recover for 4 days to allow nerves to degenerate. The left soleus nerve was then crushed and the right soleus nerve was recrushed at the same site and in the same manner.

Partial denervation

The right soleus nerve and muscle were partly denervated by using a ventral approach to expose and cut the L4 spinal nerve. The animals were then left to recover for 6 days to allow degeneration of the cut nerves. Animals were then anaesthetized, the right sciatic nerve was exposed above the knee and all nerves to the lower leg muscles except soleus were cut. The soleus muscle was exposed, the muscle surface flooded with Ringer solution, and a suction electrode (normally used for nerve stimulation) brought into contact with the muscle. The electromyogram of the soleus muscle was then recorded on a storage oscilloscope as graded stimuli (0–10 V, 100 μsec duration) were applied to the sciatic nerve at the point of its exposure through a pair of stainless-steel wires separated at their tips by 1 mm. Discrete increments in the e.m.g. were counted as motor units remaining after the partial denervation operation. The right solei of several mice were found to be either completely denervated or completely innervated and these were discarded. The remaining mice had between three and fourteen intact units, and in these animals the right and left soleus nerves were crushed as described above and the animals allowed to recover from the anaesthesia.

Paralysis

The right leg in the region of the soleus was injected with 2 μl. of crude type A botulinum toxin (0·01 mg/ml. in phosphate buffer). Paralysis of the whole lower leg was obvious one day later. The animals were left for 12 days to allow development of nerve terminal growth (Duchen & Strich, 1968) and chromatolysis (Watson, 1969). The right and left soleus nerves were then crushed as described above.

Assay of recovery of tension

One to five days after the bilateral soleus nerve crush operation the mice were killed by decapitation and their soleus nerves and muscles dissected out in oxygenated, Hepes-buffered Krebs solution. Direct and indirect muscle twitch tensions were recorded as previously described (Brown & Ironton, 1978) and the recovery of tension represented by expressing the indirect tension as a percentage fraction of the direct tension. Discrete steps in the indirect twitch tension occurring as the nerve stimulus was graded were counted as regenerated motor units; for each muscle the number of motor units was expressed as a percentage fraction of the normal number by assuming that normal mouse soleus has twenty-two units (Brown, Holland & Ironton, 1980). In expressing recovery in partly denervated muscles allowance was made for the number of motor units remaining at the time of the crush: the indirect tension was expressed as a fraction of the direct tension expected with the remaining motor units (direct tension × number of units × $\frac{1}{22}$); the number of units regenerated was expressed simply as a fraction of the number remaining after partial denervation.

Histology

The muscles were pinned out and stained for 6 hr with zinc iodide-osmium (ZIO, Maillet, 1959). The stained muscles were then teased, dehydrated and mounted to display end-plates and also the muscle nerve in the region of the crush.

RESULTS

Reinnervation in control muscles

The pooled data for recovery in all the left soleus muscles is shown in Fig. 1. The considerable scatter in these data and in the data for right soleus muscles is probably a result of variations in length of nerve distal to the site of crush. However it is quite clear that recovery in these control muscles is independent of the treatment sustained by the right leg. Recovery begins at about 50 hr after nerve crush; nerve-evoked tension has reached approximately 50 % of the direct tension by 4–5 days after crush,

and the full complement of motor units has returned in some muscles by this time. The two methods of crushing produced no consistent differences in recovery in control muscles (or in the muscles from the right legs), and therefore the points for the two series are not differentiated on the graphs.

Degenerating axons distal to the site of crush (Pl. 1 *B–F*) are easily distinguished from intact axons proximal to the crush (Pl. 1 *A*). Regeneration is first seen as black

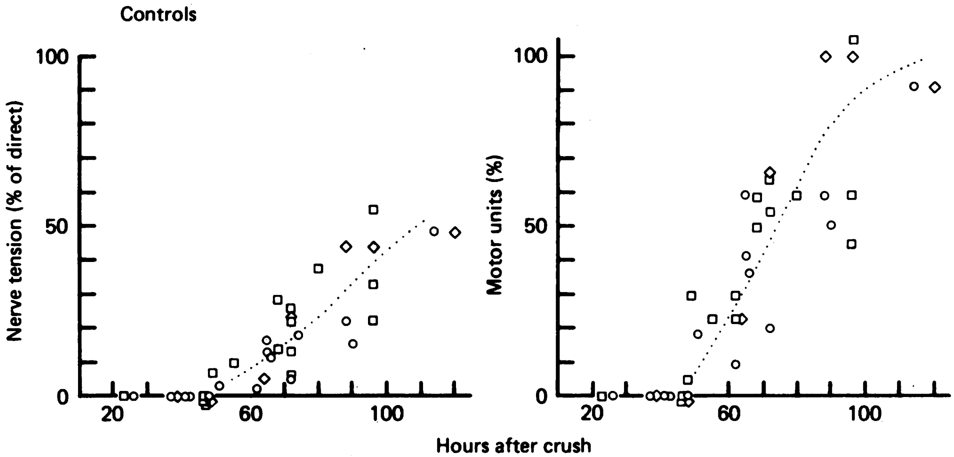

Fig. 1. Reinnervation of left (control) soleus muscles following soleus nerve crush in mice in which the right soleus had been previously denervated (○), partly denervated (□) or paralysed (◇). Curves fitted by eye. In Figs. 1–4 abscissa represents time in hours after nerve crush; left-hand graphs show recovery of tension expressed as a percentage of direct tension, right-hand graphs show number of motor units present expressed as a percentage of the normal number (see Methods).

processes in the muscle nerve distal to the crush at 48 hr after crush. These are not visible at 42 hr (Pl. 1 *B*) but are well developed by 72 hr after crush (Pl. 1 *C*). Reinnervated endplates are first seen in the muscles at 48 hr after crush. The first reinnervated endplates (Pl. 1 *H*) are remarkably similar to control endplates (Pl. 1 *G*). Later the endplates show more 'escaped' (Gutmann & Young, 1944) terminal growths

Reinnervation following previous denervation

Tension begins to develop in soleus muscles 41 hr after the nerve is recrushed, 10 hr earlier than in controls from these animals and 7 hr earlier than in combined controls. The recovery thereafter is also more rapid than in contralateral control muscles (Fig. 2): 50 % of the units have returned to the right soleus muscles at 50–60 hr while it takes 70–80 hr for combined controls to reach this value; the indirect tension reaches 50 % by 70–80 hr in the right solei, compared with 90–100 hr in combined controls.

Regenerating axons can be seen distal to the site of nerve recrush at 27 and at 38 hr, and the first reinnervated end-plates are seen at 41 hr after recrush. These endplates show more escaped terminal growth than the first reinnervated control endplates (compare Pl. 1 *J* and *H*).

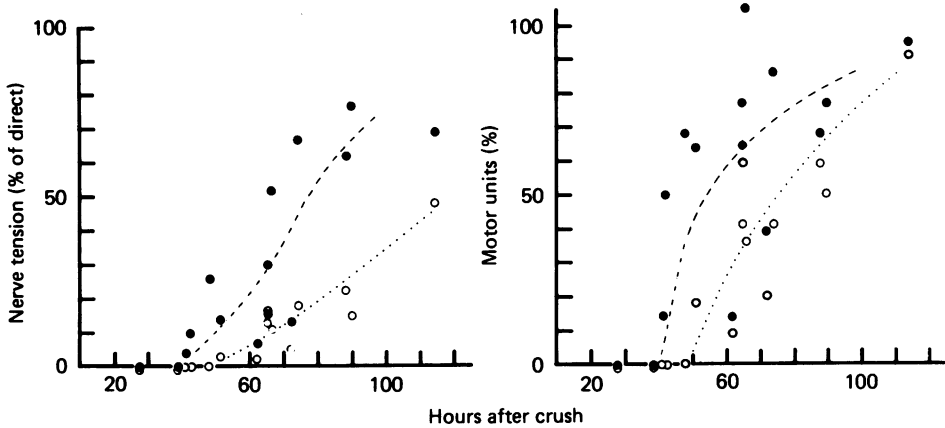

Fig. 2. Reinnervation of right soleus muscles denervated 4 days before nerve crush by a similar crush (●), and of contralateral control muscles from the same mice (○). The differences between extents of return of tension and units are significant ($P = 0{\cdot}0001$, sign test). Curves fitted by eye.

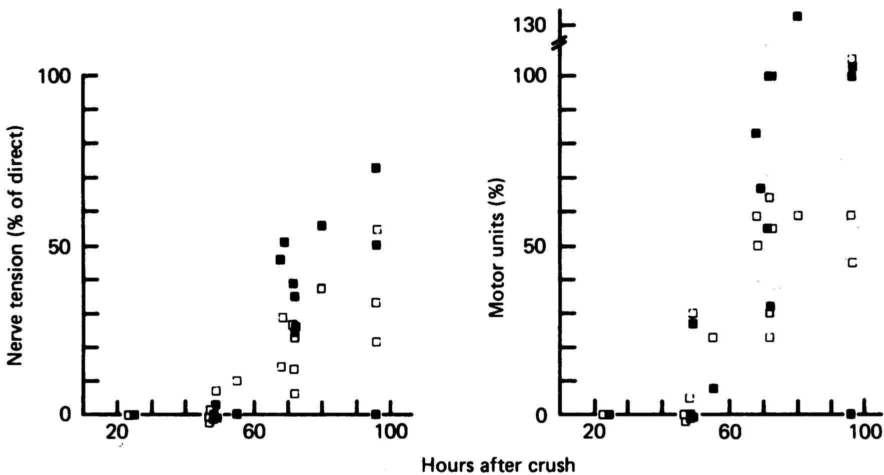

Fig. 3. Reinnervation of right soleus muscles partly denervated 6 days before nerve crush (■), and of contralateral control muscles from the same mice (□). The absence of reinnervation of one of the muscles at 96 hr (which had four remaining units) was confirmed by histology. There is no significant difference between extents of return of tension or units in left and right muscles ($P > 0{\cdot}05$, sign test).

Reinnervation following partial denervation

Three partly denervated muscles with ten, thirteen and fourteen remaining motor units were examined 48 hr after nerve crush. There was no detectable indirect tension, and no endplates were visible in the stained muscles. Regenerating axons could be seen in the muscle nerve distal to the crush from two of these muscles, and it was clear that these axons were growing down the endoneurium newly denervated by the nerve crush rather than down endoneurium previously denervated by the partial

denervation (Pl. 1 *E*). Subsequent development of tension and return of units were not significantly different from controls (Fig. 3).

The first end-plates to become reinnervated in the partly denervated muscles have well-developed escaped terminal growths (Pl. 1 *K*).

Reinnervation following paralysis

Fig. 4 shows that there is no consistent difference between the rates of reinnervation of control muscles and of muscles previously blocked with botulinum toxin. At 48 hr

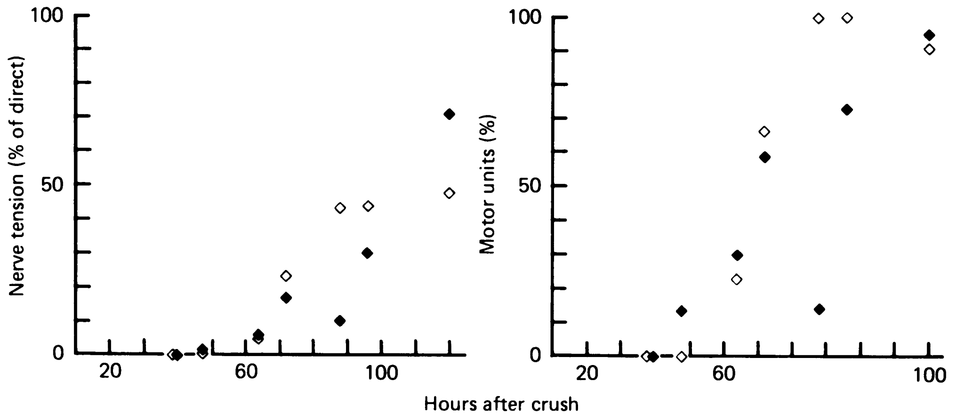

Fig. 4. Reinnervation of right soleus muscles paralysed by I.M. injection of botulinum toxin 12 days before nerve crush (◆), and of contraleteral control muscles from the same mice (◇). There is no significant difference between extents of return of tension or units in left or right muscles ($P > 0{\cdot}05$, sign test).

post-crush there were several units in the right soleus which, however, developed very little tension. Although the control muscle at this time produced no detectable tension, one reinnervated end-plate was found after staining. Neither left nor right solei showed reinnervating axons in the muscle nerve at 40 hr after crush. The onset of recovery in left and right solei must therefore be considered to be similar.

The first reinnervated endplates in the previously blocked muscles show such prolific terminal growth that their original boundaries cannot be defined reliably (Pl. 1 *L*).

DISCUSSION

The data presented here have shown that reinnervation following a crush to the soleus nerve occurs earlier and more rapidly if the nerve has been given a conditioning crush 4 days earlier. Reinnervation is not enhanced either when the muscle has been previously partly denervated for 6 days, or when the muscle has been previously paralysed for 12 days. These observations allow several conclusions to be drawn about the factors which might control nerve growth.

(1) *Chromatolysis has little or no effect on motor nerve regeneration.* Chromatolytic changes in the cell bodies of rat motor neurones are maximal within 5–10 days of injection of their muscles with botulinum toxin (Watson, 1969). Such changes are

likely to occur in mice after a similar delay. Mouse soleus nerve cell bodies may also be engaged in enhanced synthesis of materials for nerve growth, since after 12 or more days of paralysis there is prolific nerve terminal sprouting in this muscle (Holland & Brown, 1981). Nevertheless neither the return of nerve evoked tension nor the return of motor units were enhanced by prior muscle paralysis. This confirms a previous attempt to alter the rate of nerve regeneration by altering the state of the cell body with a 'conditioning' nerve crush 14 days earlier (McQuarrie, 1978); the resulting increase in maximum rate of growth over an 8 day period after a more proximal crush was only 11 %, and at least part of this increase can be attributed to higher uptake in the experimental nerve of radioactivity used to assay growth.

(2) *Nerve growth is enhanced along a predenervated pathway.* Chromatolytic changes in the cell body four days after a crush of the muscle nerve are unlikely to be more pronounced than following twelve days of paralysis (see Discussion in Watson, 1969). Since paralysis did not enhance regrowth, then the enhancement that occurs following a second crush implies that axons grow more easily down a predenervated pathway. This confirms and extends to mammals Lubinska's (1952) earlier observations on frogs.

It is likely that regrowth does not begin immediately following the second crush. Assuming a maximum regeneration rate of 4 mm/day (McQuarrie, 1978) and 3 mm of denervated pathway to the nearest end-plates, regrowth would take 20 hr, leaving a delay of 20 hr before regrowth begins after recrush. The delay after a single crush would probably be longer than this. Indeed, in controls regenerating axons are not seen in the nerve distal to the crush even 42 hr after crush, but this may be an overestimate of the delay because of failure to visualise the first regenerating axons.

(3) *Axons are confined to their own endoneurium in the extramuscular nerve following nerve crush.* Guidance of regenerating axons by the endoneurium distal to a crush is a well known phenomenon, but it has not been clear whether axons could escape from their endoneurium at the site of a crush. Since there is a shorter delay and more rapid growth with regeneration down a predenervated pathway it might have been expected that axons escaping from their endoneurium at the site of a crush in a partly denervated nerve would grow along the predenervated pathway. There would then have been nerve-evoked tension at 48 hr after crush, and possibly also significantly lower tension than in controls thereafter, since some motor axon growth might have been diverted along predenervated sensory axons. However, reinnervation in partly denervated muscles was not different from that in controls, which is consistent with the retention of each regenerating axon within its own endoneurium. This was confirmed by the histological observation that the first visible regenerating axons distal to the crush were in the newly denervated Schwann cell pathways.

The fact that axons are able to leave their own endoneurium in nerves within a muscle implies that there is a structural gradient in the endoneurium which permits penetration by axons in the more distal branches of motor nerves. Indeed, finer myelinated axons have less well developed collagenous sheaths (Thomas, 1963). This gradient could help to explain why nodal sprouts are localized to the more distal segments of nerves within a muscle (Hoffman, 1950; Slack, Hopkins & Williams, 1979).

Finally, the histological observations have some bearing on the stimulus for terminal sprouting. The first end-plates reinnervated two days after crush in control

muscles are remarkably normal in appearance: the old end-plate site often appears to be fully reinnervated, yet there is little 'escaped' terminal growth. Endplates reinnervated after a recrush or after partial denervation are less compact and have more nerve growths from their terminals, and reinnervation after 12 days of paralysis produces prolific nerve terminal growth. Thus there is sufficient material available from the cell body in the first regenerating axons to fill end-plates after a single crush, yet escaped growths occur later and end-plates appear less fully reinnervated after longer periods of denervation. This suggests that a stimulus from denervated muscle rather than a growth impetus from a chromatolytic cell body is the cause of escaped terminal growth and of terminal sprouting, which develops structurally and temporally in a similar manner (Holland & Brown, 1981).

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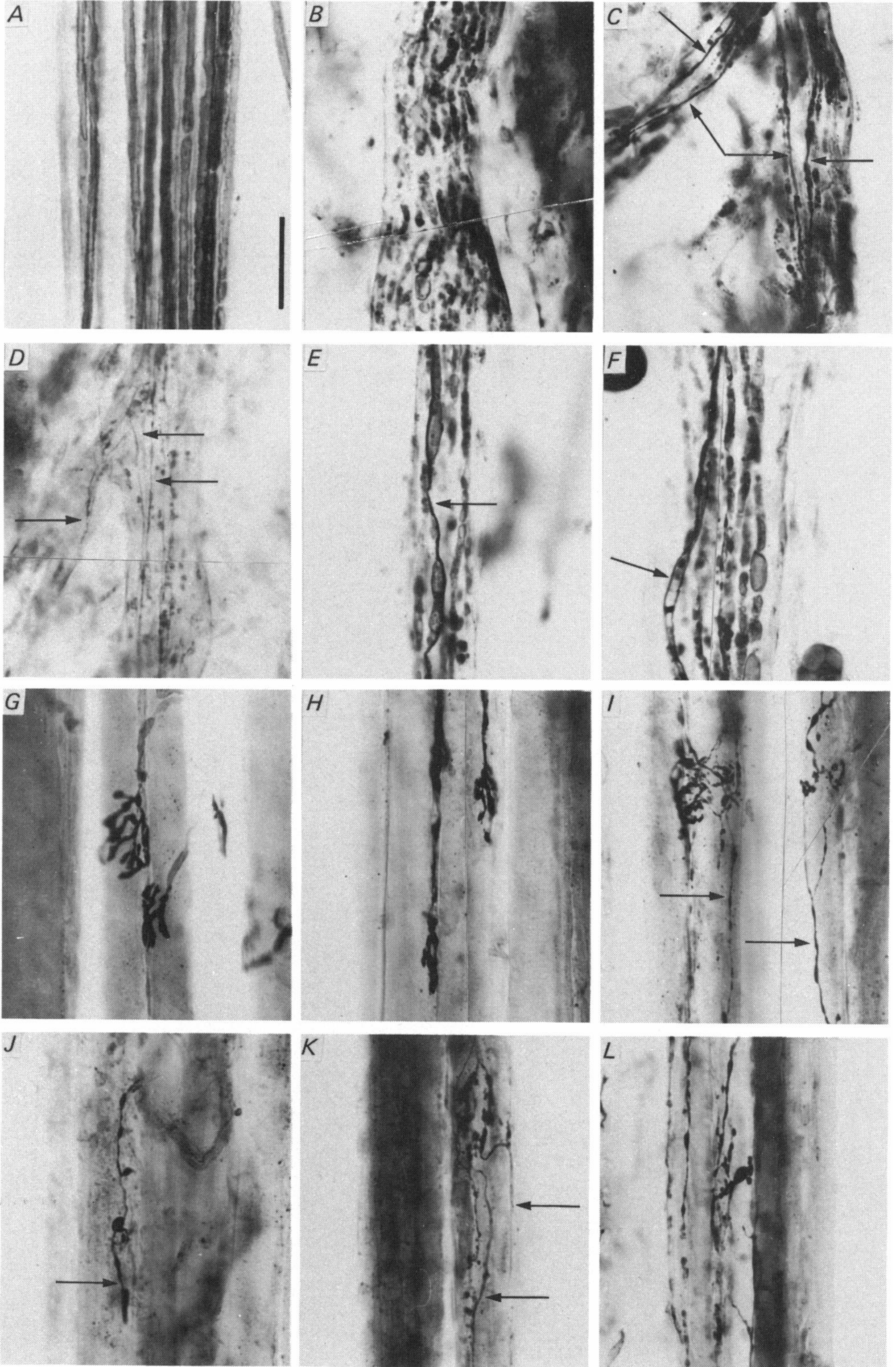
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EXPLANATION OF PLATE

Nerves (*A–F*) and end-plates (*G–L*) stained with zinc iodide-osmium. The bar in *A*. represents 50 μm, and all figures are to the same scale. *A*, myelinated axons in nerve proximal to site of crush (from muscle nerve, a portion of which distal to the crush is shown in Fig. *F*). *B*, control nerve 42 hr after crush showing degenerating Schwann cells and absence of reinnervating axons. *C*, control nerve 82 hr after crush showing regenerating axons (arrows). *D*, predenervated nerve 42 hr after crush showing regenerating axons (arrows). *E*, partly denervated nerve 48 hr after crush showing regenerating axon growing down newly denervated Schwann cell (arrow). *F*, previously blocked nerve 48 hr after crush showing regenerating axon (arrow). *G*, end-plates in normal mouse soleus. *H*, control 51 hr after crush; the end-plates are normal in appearance. *I*, control 96 hr after crush showing escaped growth (arrows). *J*, predenervated muscle 51 hr after crush. Arrow indicates escaped growth from terminal which does not appear to be fully reinnervated. *K*, partly denervated muscle 72 hr after crush. Escaped growth is quite extensive (arrows). *L*, blocked muscle 64 hr after crush, showing prolific nerve growth.