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ELECTROPHYSIOLOGICAL RESPONSES IN THE RAT TAIL ARTERY DURING REINNERVATION FOLLOWING LESIONS OF THE SYMPATHETIC SUPPLY

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SUMMARY

1. Responses to perivascular stimuli have been recorded with intracellular microelectrodes from the smooth muscle of isolated segments of the main caudal artery of rats at various times between 7 and 128 days after all four collector nerve trunks had been lesioned near the base of the tail at 21 days of age.

2. In proximal segments (< 40 mm distal to the lesions), excitatory junction potentials (EJPs) and neurogenic α-depolarizations (NADs) evoked by stimuli presented via a proximally located suction electrode were similar to those in the same segments of unoperated control animals of the same age. Supramaximal EJPs in these segments decreased in amplitude with age.

3. Stimuli just supramaximal for EJPs in innervated preparations failed to evoke responses in segments farther than 30–40 mm distal to the lesions at any time after the nerves had been cut and 1 cm excised. Higher voltages evoked slow depolarizing potentials (SDPs) which were of longer time course than EJPs. Similar responses occurred in segments over 60 mm distal to the lesions at 20–50 days after the nerves had been frozen, and in all segments sampled over 100 mm distal to nerve lesions.

4. Spontaneous transient depolarizations (STDs) were recorded at all depths of the media in denervated segments. These occurred at frequencies similar to those of spontaneous events (including attenuated spontaneous EJPs) in innervated segments.

5. The earliest signs of reinnervation (24–42 days after freeze lesions) consisted of very small amplitude EJPs of normal time course which facilitated markedly during a short train of stimuli (5–10 Hz); these were followed by NADs which were large relative to the amplitudes of the EJPs. Less commonly, small focal EJPs of brief time course (resembling spontaneous EJPs in superficial cells of innervated arteries) were evoked in very restricted regions of the vessel wall.

6. At later times (57–128 days postoperative), six of eight segments located

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40–70 mm distal to freeze lesions showed EJPs of nearly control amplitude, but NADs that were larger than in equivalent segments from control animals. In the remaining two cases, reinnervation at this level was similar to that seen at the earliest postoperative times. High stimulus voltages prolonged the decay of EJPs in both control and reinnervated arteries.

7. Sensitivity to exogenous noradrenaline, assessed in terms of membrane depolarization, was increased in both denervated and reinnervated segments.

8. Catecholamine fluorescence disappeared from the arteries at a distance greater than 30–40 mm distal to the site of the nerve lesions. In segments adjacent to those studied electrophysiologically, regenerated fluorescent axons were first visualized at the same postoperative time as the first EJPs were detected. Reinnervation was often patchy in the early stages. When EJPs had regained control amplitude, the density of the perivascular plexus was subjectively assessed as $\sim 80\%$ of control. No regenerated perivascular plexus was detected on the distal parts of the artery, even at 150 days post operation.

9. The data show that postganglionic vasoconstrictor axons rapidly regenerate and restore functional contact with their arterial target within a few centimetres of a lesion of a major nerve trunk. EJPs have normal amplitudes but NADs are enhanced compared with controls, even after many months; this may reflect postjunctional supersensitivity. In contrast, distal arterial targets remain denervated for prolonged periods.

INTRODUCTION

Postganglionic sympathetic axons have been known for many years to be able to regenerate and regain functional control of their target organs after nerve lesions (Langley, 1897; see Sunderland, 1978). However, functional reinnervation of the vasculature has rarely been quantified or correlated directly with the appearance of the perivascular nerves. Regrowth of axons supplying vascular targets is rapid after chemical sympathectomy, which destroys only terminals and preterminal axons (Finch, Haeusler, Kuhn & Thoenen, 1973), although it has been reported that in non-vascular targets (iris and heart) reinnervation may be incomplete after many months (Lorez, Kuhn & Bartholini, 1975). Reinnervation of mesenteric arteries was found to be incomplete both functionally and anatomically six months after freeze lesions of the mesenteric nerves in young rats (Hill, Hirst, Ngu & Van Helden, 1985). There have, however, been few studies of the regeneration of perivascular axons after proximal lesions of the mixed nerve trunks via which they reach the periphery. This has important clinical implications for function, particularly in the limbs, after recovery from trauma.

The main ventral artery of the rat tail provides a unique opportunity to study the sequential stages of reinnervation, because it enables both quantal events (indicating the presence of neuromuscular junctions) and α-adrenoceptor activation (in response to neuronally released noradrenaline) evoked by perivascular nerve stimulation to be studied at the same time. Transmural stimuli presented via a proximal suction electrode to isolated segments of the artery evoke excitatory junction potentials (EJPs) followed by slow neurogenic α-depolarizations (NADs) (Cassell, McLachlan & Sittiracha, 1988; Hirst & Edwards, 1989; Jobling & McLachlan, 1992). These events

can be used to indicate the earliest arrival of a functioning innervation *in vitro* (see e.g. Bennett, McLachlan & Taylor, 1973), with much greater sensitivity than the detection of arterial contractions. The longitudinal nature of the artery allows a sequence of events to be studied in tissue from the same individual. It should be noted that it is not currently clear how either of these electrophysiological phenomena contribute under *in vivo* conditions to neurogenic vasoconstriction. Most of the constriction is sensitive to α_1-antagonists (Itoh, Kitamura & Kuriyama, 1983; Hirst & Edwards, 1989) which do not block either EJPs or NADs; in this artery, at least in Wistar rats, NADs are abolished by α_2-antagonists (see Cassell *et al.* 1988; Jobling & McLachlan, 1992).

In the present experiments, we have examined these neuromuscular events using conventional intracellular recording techniques at various stages during reinnervation of the rat tail artery. In order to minimize disruption to the nerve trunks and in an attempt to facilitate regeneration (Mira, 1971, 1979), the artery was denervated by freezing all four collector nerves near the base of the tail. In other experiments, the same four nerves were cut and short lengths extirpated in order to ensure that the artery remained denervated. The data revealed that reinnervation occurred rapidly within a few centimetres of the freeze lesions but, unexpectedly, that the postganglionic axons failed to reinnervate the distal parts of the artery even after several months.

METHODS

Experiments were performed on Wistar rats of both sexes at various ages up to 150 days postnatal. Approximately equal numbers of animals of each sex were used in all experiments and no differences were detected between data from different sexes. The animals were maintained as described previously (Jobling & McLachlan, 1992).

Denervation of tail arteries

Animals aged 19–26 days (55–75 g) were anaesthetized with a mixture of ketamine (60 mg kg^{-1}) and xylazine (10 mg kg^{-1}) (I.M.). Tourniquets were applied above and below the operation site which was at 5–7 mm from the hairy base of the tail (equivalent to 5% of the total tail length, see Fig. 1). The dorsal and ventral collector nerves were exposed via a single lateral incision on each side. Each nerve side was frozen for 1–2 s over about 1 mm using a small metal probe cooled in liquid nitrogen (Sittiracha, McLachlan, & Bell, 1987); in some other operations, the nerves were cut and a short length (2–5 mm) of each nerve trunk removed. The entire procedure usually took less than 15 min. Each wound was sutured after dusting the site with antibiotic powder. No structural or functional changes could be detected along the length of the artery 1–2 weeks following application of the tourniquets alone ($n = 3$).

In the postoperative period, some of the animals autotomized the tips of their tails. We were able to reduce this habit and limit the damage by painting the tails daily with 'anti-nailbiting' lotion. There was no detectable difference between the results from the animals which did or did not autotomize their tails (or which were or were not treated with lotion), and in no case did the 10–40 mm of tail lost encroach near the limit of the reinnervated region.

Animals were killed at various postoperative times and three or four segments from each tail artery studied electrophysiologically. Adjacent tissue was taken and processed for the demonstration of catecholamine fluorescence.

Electrophysiological recording

Segments of the main ventral artery (10–15 mm long) were studied electrophysiologically using methods described previously (Jobling & McLachlan, 1992). The segments were taken from proximal (5–25 mm), medial (50–70 mm) and distal (80–130 mm) sites depending on tail length at

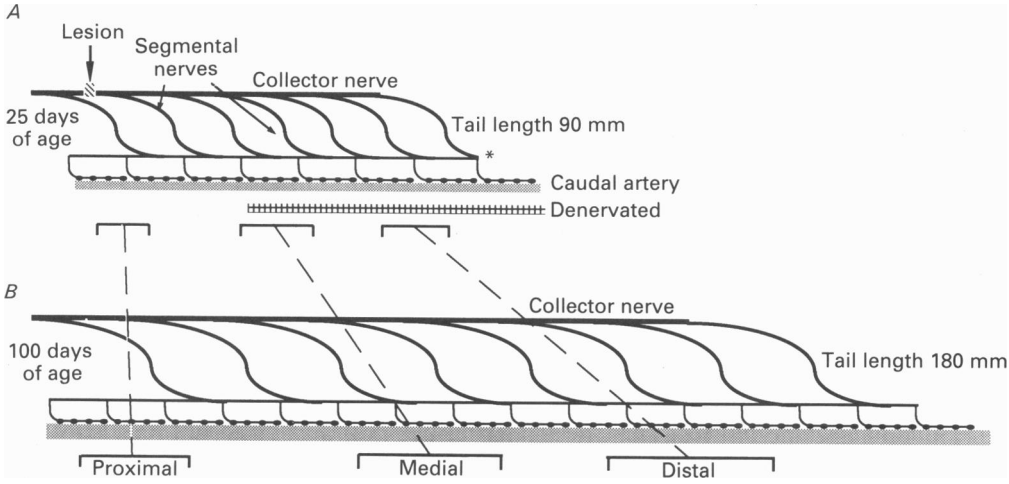


Fig. 1. Diagram of the innervation of the extracorporeal part of the ventral caudal artery of the rat, showing the site of the nerve lesions and the changing dimensions of the tail during growth after the operation. *A*, at 21 days of age, lesions were made at 5% of tail length, denervating the artery from about 35 mm distally. *B*, by 100 days of age, the tail was nearly its maximum length. The location of segments isolated from proximal, medial and distal parts of the artery in electrophysiological experiments is indicated. The asterisk indicates the paravascular nerve bundle that runs with the artery in the ventral vascular groove.

the time of the experiment. These levels corresponded to 10–15%, 35–50%, and 60–80% of the length of the extracorporeal tail (see Fig. 1).

Nerve-evoked responses

Excitatory junction potentials (EJPs) and neurogenic α-depolarizations (NADs) were examined following single stimuli at less than one per minute as well as after trains of five stimuli at 1 and 10 Hz. Quantitative comparisons between control and reinnervated preparations always involved only data in response to just supramaximal stimuli from cells lying between 1 and 2 mm from the suction electrode. Other criteria were the same as described previously (Jobling & McLachlan, 1992).

Depolarization by exogenous noradrenaline

After examining the responses to perivascular stimulation, membrane potential was recorded in each segment during the application of noradrenaline at concentrations in the range 5×10^{-8} to 10^{-5} M. Noradrenaline was added by transferring the perfusion inlet for a period of 2 min into the stated concentration freshly made up in physiological saline. This has been found to be sufficient time to achieve the stated concentration in the recording chamber (volume 0·8 ml) for a period of about 10 s before wash-out commences; longer exposures were avoided to limit desensitization. Peak depolarizations were measured if contractions did not dislodge the microelectrode.

Fluorescence histochemistry

Segments of artery (about 10 mm in length) immediately proximal and distal to the segments taken for electrophysiology were prepared for the demonstration of catecholamines using the technique of de la Torre (1980), as described previously (Jobling & McLachlan, 1992). Arteries were sectioned longitudinally in order to visualize areas of the perivascular plexus within single sections.

Statistical analyses

All data are expressed as means $\pm$ S.E.M. unless specified. Significant differences were determined using Student's *t* test.

RESULTS

These experiments were conducted on material from animals which were growing. The tail was denervated at 22·2±0·6 days of age ($n = 16$) so that intracellular recordings could be obtained from tail arteries over many months before they became too difficult to impale. Experiments on age-matched control material revealed changes in the nerve-evoked responses during normal growth (Jobling & McLachlan, 1992), and so all data presented here have been compared with those from age-matched control animals.

Proximal arterial segments

The most proximal segments of the artery (down to 35–40 mm from the base of the tail) were not denervated by the lesions (see Fig. 1; also Sittiracha *et al.* 1987). This was confirmed by fluorescence microscopy (see below). In electrophysiological studies of these segments, resting membrane potential (RMP) was −67·2±0·6 mV ($n = 57$). EJPs were evoked by stimuli presented via a suction electrode; the time constant of decay of the EJP (τ_{EJP}) ranged between 190 and 450 ms, with mean values similar to those in proximal segments from unoperated control animals (see Table 1).

TABLE 1. Properties of smooth muscle and responses to nerve stimulation at various sites along the tail following nerve lesion

Age (days)	RMP (mV)	τ_{EJP} (ms)	EJP amplitude (mV)	NAD amplitude (mV)
Proximal				
45–100	−67·3±0·9^{ab} (35)	287·7±12·0 (33)	9·9±0·8^{e} (35)	3·9±0·4^{i} (21)
107–150	−66·9±0·7^{cd} (31)	282·2±18·5 (9)	8·7±1·0^{f} (20)	4·8±0·4^{h}* (13)
Medial				
45–100	−62·9±0·7^{b} (38)	273·8±21·2 (8)	2·1±0·5^{eg}** (13)	2·5±0·5^{i} (12)
107–150	−63·6±1·4^{c} (24)	336·5±24·5 (12)	5·4±0·8^{fg} (10)	3·8±0·3^{h} (9)
Distal				
45–100	−61·9±1·3^{a} (21)	—	—	—
107–150	−59·1±1·9^{d} (9)	—	—	—

Values are means±S.E.M. with the numbers (n) in parentheses.

Statistical significance between pairs of values with the same letter: $^{chi}P < 0·05$, $^{f}P < 0·02$, $^{a}P < 0·01$, $^{deg}P < 0·0001$. Asterisks denote significant differences from age-matched, innervated controls: $*P < 0·02$, $**P < 0·001$.

The amplitude and time course of EJPs and NADs were not significantly different from those in age-matched controls (Table 1, Fig. 3*Ba*), except that, in cells from one of the oldest animals, mean NAD amplitude was significantly larger ($P < 0·05$) than in the corresponding controls (see Fig. 8*b*, Table 1). (These data came from only a small number of cells, because we concentrated on obtaining satisfactory impalements in the reinnervated segments, thus limiting the time available to study the proximal (intact) segments while they were in good condition.) Peak amplitude of the NAD was on average 14·7±2·7% larger after a train at 10 Hz than at 1 Hz ($P < 0·001$, paired t test, $n = 69$). NADs were abolished by idazoxan (10^{-6} M). Slow depolarizing potentials (SDPs) were evoked in some cases when the stimulating

voltage was increased above 30 V; these prolonged the decay phase of the EJPs (see Jobling & McLachlan, 1992). Spontaneous events included spontaneous transient depolarizations (STDs) identified by their large amplitude and prolonged time course relative to the attenuated spontaneous EJPs. The mean amplitude of all spontaneous events was $1{\cdot}25 \pm 0{\cdot}11$ mV and half-width was 123 ± 11 ms ($n = 51$); they occurred at a frequency of $0{\cdot}05 \pm 0{\cdot}02$ Hz ($n = 6$). In every way, these features were indistinguishable from the same events in tissue from control animals (see Jobling & McLachlan, 1992).

Denervated arterial segments

In medial and distal segments of the tail artery from three animals in which the collector nerves had been cut and several millimetres resected, and in distal segments

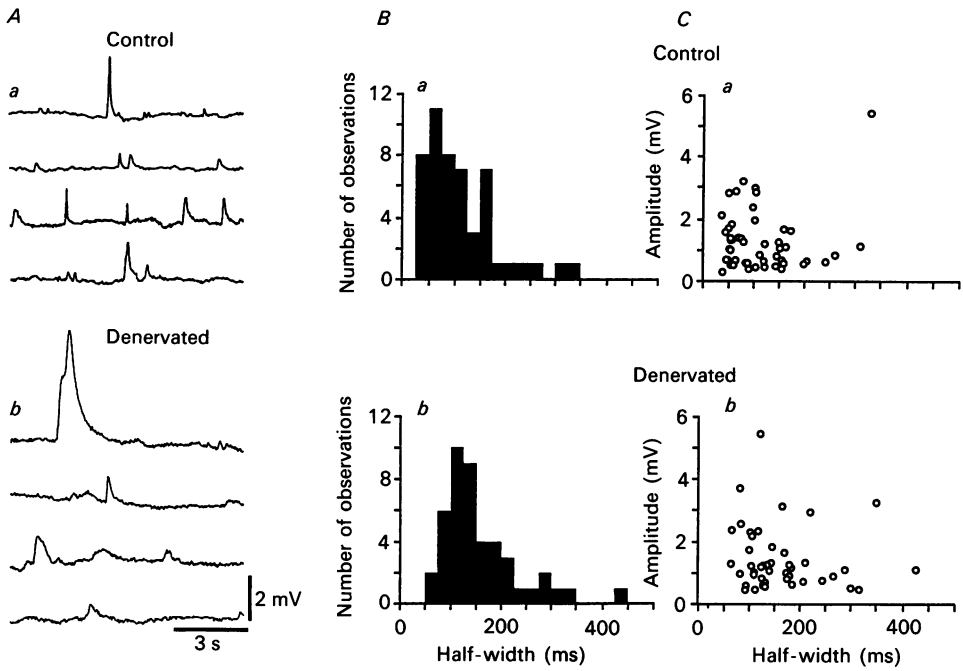


Fig. 2. Spontaneous depolarizations in control and denervated arteries. *A*, selected records from cells from (*a*) medial segment, control animal (72 days of age) and (*b*) distal segment, denervated animal (42 days p.o.). Upper trace in *b* shows the summation of two spontaneous transient depolarizations (STDs). Calibrations apply throughout. *B*, frequency distributions of half-widths of spontaneous events in cells from (*a*) medial segments from control animals ($n = 50$) and (*b*) distal and medial segments of denervated arteries ($n = 46$). *C*, relation between amplitude and half-width of spontaneous events shown in *B*.

from all but 2 of 16 animals in which the four nerves had been frozen, no EJPs or NADs were evoked by stimuli up to 70 V amplitude applied via the suction electrode, or via platinum wires placed on either side of the arterial segment. It was noted that these denervated arterial segments, unlike innervated segments, were prone to spasm during the dissection procedure, and sometimes remained constricted for up to 30 min following pinning.

RMPs in denervated preparations ($-61{\cdot}5 \pm 0{\cdot}9$ mV, $n = 50$) were not different from control values, except in distal segments which had spasmed in which the cells were depolarized by about 10 mV (see Table 1).

Spontaneous transient depolarizations (STDs) of amplitude 0·5 to 10 mV occurred at frequencies between 0·5 and 5 per minute, with higher frequency bursts in some cells (e.g. Fig. 2*B*). Mean frequency of STDs in denervated segments was $0{\cdot}05 \pm 0{\cdot}04$ Hz ($n = 6$), i.e. equivalent to the frequency of all spontaneous events in

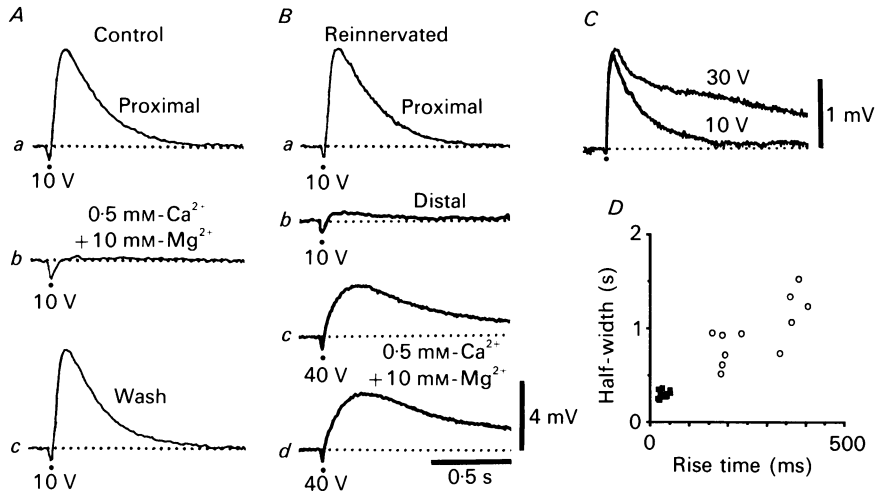


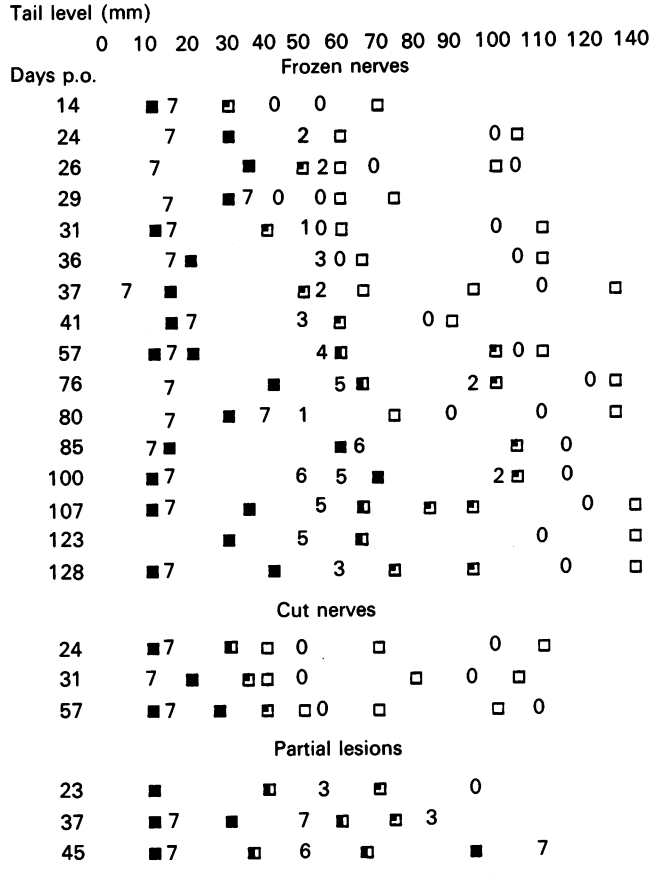
Fig. 3. Slow depolarizing potentials (SDPs). *A*, responses recorded in cell of control proximal segment (96 days of age): *a*, EJP evoked by single 10 V stimulus in normal saline; *b*, responses to same stimulus after changing to solution containing 0·5 mM-Ca^{2+}–10 mM-Mg^{2+}; *c*, after return to control saline. *B*, responses evoked in cells from a nerve-lesioned animal (123 days p.o.): *a*, EJP evoked in proximal segment by 10 V stimulus; *b*, response to 10 V in denervated distal segment; *c*, SDP evoked by 40 V stimulus in the same cell as *b*; *d*, SDP in same cell as *b* and *c* evoked by 40 V in solution containing 0·5 mM-Ca^{2+}–10 mM-Mg^{2+}. *C*, mixed response during early reinnervation (medial segment, 80 days p.o.). Superimposed traces show responses to single stimuli at 10 V and 30 V; the latter gave a complex response with rising phase identical to the EJP but a prolonged decay. Calibration in *B* applies also to *A*; time calibration in *B* to all traces. *D*, relation between rise time (10–90%) and half-width of SDPs in denervated segments (○) and EJPs in proximal innervated segments from the same animals (■) ($n = 6$ animals).

innervated arterial segments (see Jobling & McLachlan, 1992). However, half-widths of $170 \pm 14{\cdot}9$ ms ($n = 46$) were significantly longer than those of the spontaneous events in equivalent control segments ($P < 0{\cdot}001$; Fig. 2), which included some attenuated spontaneous EJPs. This confirms that STDs occur independently of the innervation, as they are present in both innervated and denervated arteries, and suggests that their frequency may be slightly increased in some cells after denervation.

Although no EJPs were evoked in denervated preparations (Fig. 3*Bb*), a single transmural stimulus at high voltage (usually $\geqslant 40$ V) produced a slow depolarizing potential (SDP; Fig. 3*Bc*, see Jobling & McLachlan, 1992). This voltage was much

higher than that normally required to evoke EJPs (2–20 V, Fig. 3*Aa*, see Jobling & McLachlan, 1992). SDPs had rise times of $300 \pm 28{\cdot}6$ ms ($n = 13$) and slow non-exponential decay phases (Fig. 3*Bd*) lasting up to 5 s. SDPs persisted in the presence of TTX (10^{-6} M, see also Hill *et al.* 1985), or during perfusion with modified saline

TABLE 2. Reinnervation of tail following lesions to the sympathetic supply



Functional innervation is rated on a 0–7 scale: 0, no EJP, no NAD, only SDPs and STDs; 1, very rare patchy EJP, some fast spontaneous events, small NAD after train, some areas 0; 2, small amplitude EJP, relatively large NAD with train, occasional patches 0; 3, small amplitude EJP, no patches of 0; 4, EJP about half-normal, NAD about normal amplitude; 5, EJP almost normal, NAD slightly enhanced; 6, EJP normal, NAD relatively large; 7, indistinguishable from responses at that level in age-matched controls.

Density of catecholamine fluorescent perivascular plexus rated subjectively: ■ ⩾ 80% of plexus in age-matched controls at that level; ◧, 50%; ◩, < 20%; □, no perivascular axons present.

containing 0·5 mM-Ca^{2+} and 10 mM-Mg^{2+} (Fig. 3*Bd*, $n = 2$) which abolished EJPs (Fig. 3*Ab*), confirming their non-neuronal origin. SDPs often disappeared during repeated stimulation. All these properties were the same as those of SDPs evoked in innervated preparations (Jobling & McLachlan, 1992).

Early signs of reinnervation

The earliest time at which EJPs were observed at a level greater than 45 mm along the tail was 24 days after freezing the collector nerves, although EJPs could not be detected in medial segments from some other animals at later postoperative times (see Table 2).

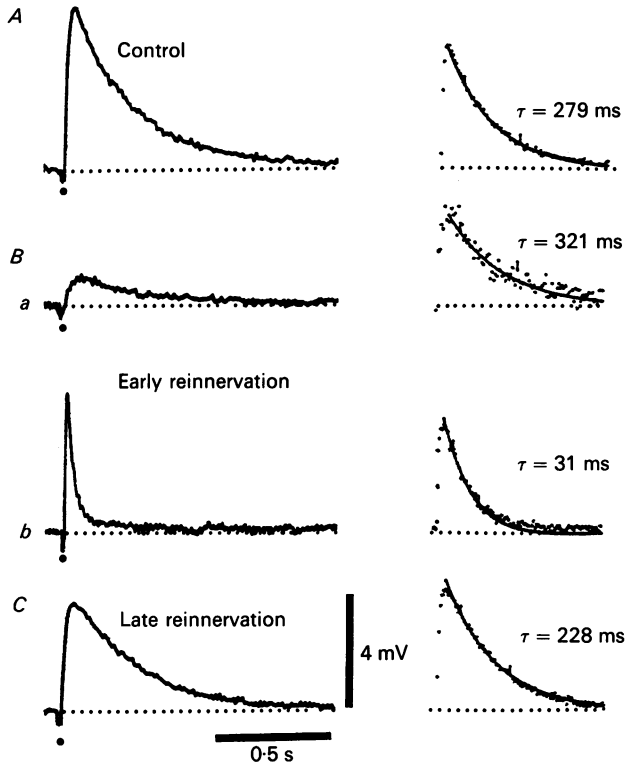


Fig. 4. Time course of EJPs evoked by single maximal stimuli at various stages during reinnervation. *A*, EJP in medial segment of control artery (96 days of age). *Ba*, EJP in medial segment (24 days p.o.) has reduced amplitude but normal time course. *Bb*, brief time course EJP in another medial segment (80 days p.o.). *C*, EJP in medial segment (79 days p.o.). Calibrations apply to all EJPs on left. Traces to the right are the same EJPs with amplitudes normalized and single exponential functions fitted to decays (with time constants indicated in ms).

Spontaneous activity consisted of both STDs and faster time course spontaneous EJPs (sometimes of large amplitude). The frequency of all spontaneous events ($0{\cdot}05 \pm 0{\cdot}02$ Hz, $n = 11$) was not on average significantly different from that in innervated control preparations ($0{\cdot}05 \pm 0{\cdot}03$ Hz, $n = 6$).

EJPs were evoked in all medial segments from animals greater than 40 days postoperation (p.o.), with a progressive increase at later postoperative times in the amplitude of the EJP in response to supramaximal stimulation (see Fig. 7*Ab*). At the early postoperative times, 10–20 V stimuli usually evoked small amplitude EJPs of normal time course (see Table 1, Figs 3*C* and 4*Ba*). Facilitation of EJPs during a

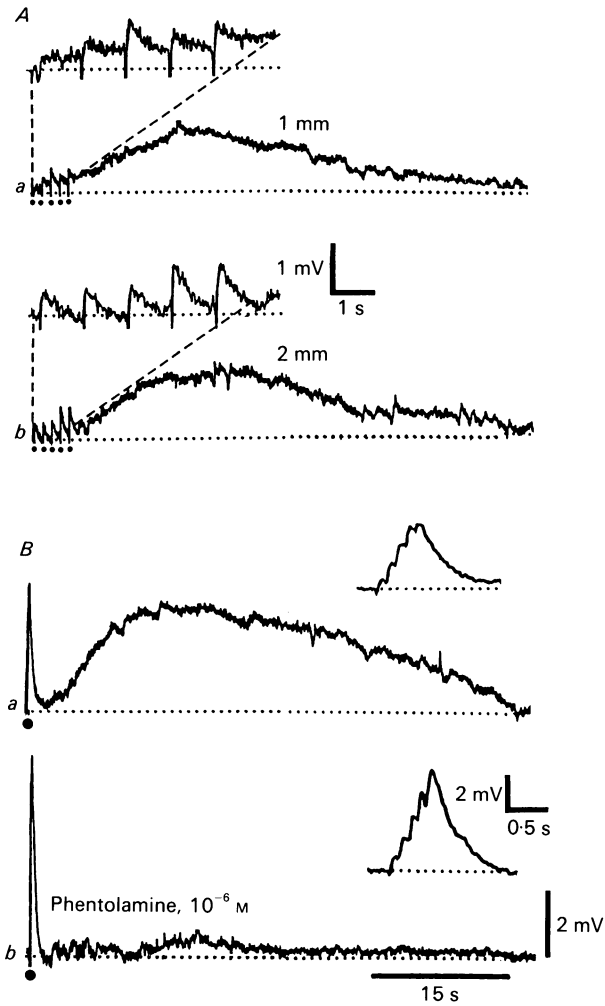


Fig. 5. Nerve-evoked responses early during reinnervation (medial segment, 24 days p.o.). *A*, responses to five stimuli at 1 Hz at two sites (*a*) 1 mm and (*b*) 2 mm from the proximal suction electrode. Upper traces show EJPs on an expanded time scale; calibrations in *b* apply only to these. *B*, effect of α-adrenoceptor blockade on response evoked by five stimuli at 10 Hz in another region of the same preparation: (*a*) in control solution, (*b*) in the presence of phentolamine (10^{-6} M). Insets show EJPs in train on expanded time scale, with increased facilitation in the presence of phentolamine. Calibrations at lower right in *Bb* apply to all long traces; those above only to insets.

train of stimuli was more marked than in age-matched control preparations (ratio of fifth to first EJP at 1 Hz: $1{\cdot}68 \pm 0{\cdot}10$, $n = 18$; c.f. $1{\cdot}22 \pm 0{\cdot}05$, $n = 44$, $P < 0{\cdot}001$; see Fig. 5).

The sites at which EJPs could be recorded early during reinnervation were often very patchy. For example, in one preparation, EJPs of normal time course were recorded in several cells in a region 2 mm from the stimulating electrode when no response was detectable in many cells impaled only 0·5 mm from the same electrode.

In other preparations, EJPs were recorded on one side of the arterial segment but not at sites only 150–200 μm away across the vessel. In many medial segments, larger voltage stimuli (> 30 V) elicited mixed responses with both EJP and SDP components (Fig. 3*C*). This contrasts with the report that SDPs disappear upon the return of EJPs in denervated mesenteric arteries (Hill *et al.* 1985).

Evidence for an even sparser patchy innervation was seen in two other preparations. In each case, small EJPs (about 1–1·5 mV amplitude) of normal time course were detected at a level along the artery about 50 mm from the lesion. When

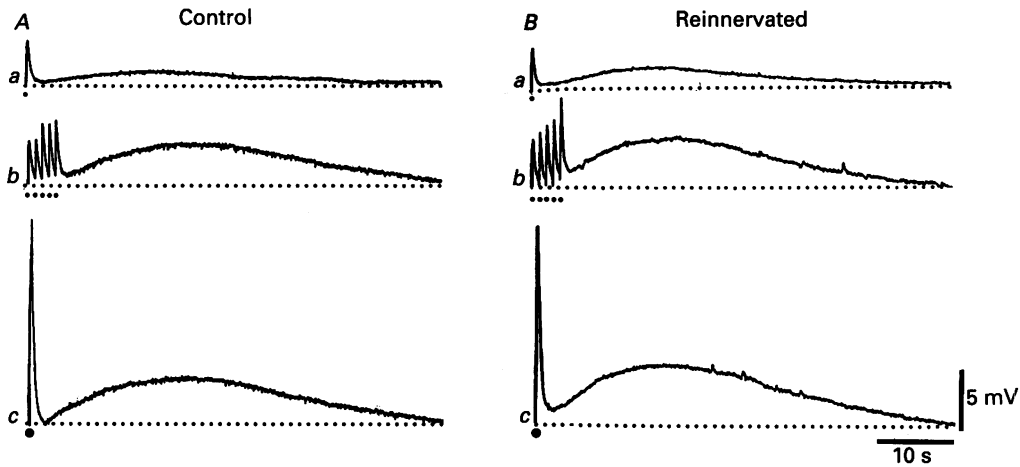


Fig. 6. Nerve-evoked responses at later stages of reinnervation. *A*, medial segment of control artery (115 days of age); *B*, medial segment of reinnervated artery (76 days p.o., i.e. 96 days of age). EJPs and NADs in response to single supramaximal stimuli (*a*), five stimuli at 1 Hz (*b*), five stimuli at 10 Hz (*c*). Note larger NADs in *B*. Calibrations apply throughout.

cells were impaled 1 mm more distally at various sites across the preparations, no EJPs were detected except in one small region (about 50 μm diameter). In three to four cells in that region, a low stimulus strength evoked an EJP with a very brief time course similar to that of a spontaneous EJP (Fig. 4*Bb*). These responses showed great variation in amplitude (1–6 mV) with successive stimuli at supramaximal voltage, compared to the relatively constant amplitude of EJPs in control preparations, suggesting that they arose from a few sites close to the recording electrode. In these regions, no NAD could be detected even following a train of stimuli.

Single stimuli rarely elicited a detectable NAD. However, short trains were followed by NADs sometimes even in cells in which no EJP could be detected in response to a single stimulus (Fig. 5*Aa*). Although these NADs were only a few millivolts in amplitude, they were large relative to the amplitudes of the EJPs (Fig. 5). The ratio of peak amplitude of the NAD following a 1 Hz train to the amplitude of the first EJP was $6{\cdot}6 \pm 2{\cdot}2$ ($n = 11$) compared with $0{\cdot}6 \pm 0{\cdot}2$ in age-matched controls ($n = 17$). NADs following trains at 10 Hz were even larger. The NADs were abolished after exposure to phentolamine (10^{-6} M, $n = 3$; Fig. 5*B*) or idazoxan

(10^{-6} M, $n = 1$). In the presence of these α-antagonists, facilitation of EJPs was further enhanced (Fig. 5*Bb*, inset; cf. Bennett & Middleton, 1975).

This range of functional responses was recorded in medial segments up to 50 days after freeze lesions. However, there was considerable variation between animals in the stage of reinnervation, even at later times (see Table 2). For example, responses

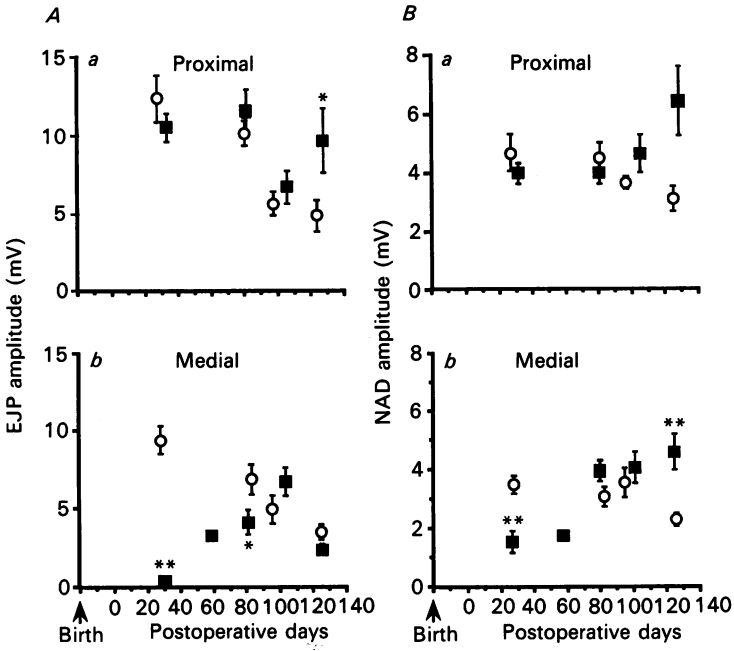


Fig. 7. Changes in amplitude of EJPs and NADs during the course of reinnervation. *A*, mean EJP amplitudes in response to single supramaximal stimuli; *B*, mean NAD amplitudes after five stimuli at 1 Hz. *a*, proximal intact segments; *b*, medial reinnervated segments. ○, mean data from control animals at different ages (from Jobling & McLachlan, 1992). ■, mean data from reinnervated animals (four to twenty cells from two to five animals (except those at 128 days p.o. in *a* and 57 days p.o. in *b* which are each from three to four cells from only one animal). Significant differences from age-matched controls indicated by * ($P < 0{\cdot}05$) and ** ($P < 0{\cdot}01$).

like those recorded after over 30 days were observed in medial segments of two animals aged 80 and 128 days. Further, although no signs of reinnervation were identified in the majority of distal segments, all of the features of early reinnervation were present in two distal segments after longer postoperative periods (76 and 100 days, see Table 2). The variable time course of reappearance of EJPs and NADs could not be related to variables such as gender, weight or incidence of autotomy.

Signs of reinnervation at later postoperative times

In medial segments more than 80 days p.o., EJPs and NADs became similar to those in age-matched controls (Figs 4*C*, 6*B* and 7, Table 2), although the amplitude of NADs increased progressively until they were relatively greater than in age-matched controls (Figs 6 and 7*Bb*). The ratio of peak amplitude of the NAD following

a 1 Hz train to the amplitude of the first EJP was significantly larger ($1{\cdot}2 \pm 0{\cdot}2$, $n = 12$) than in age-matched controls ($0{\cdot}7 \pm 0{\cdot}1$, $n = 12$; $P < 0{\cdot}05$). The NAD was on average $8{\cdot}9 \pm 3{\cdot}7\%$ ($n = 32$) larger after a train at 10 Hz than at 1 Hz ($P < 0{\cdot}05$, paired t test, $n = 32$; cf. $14{\cdot}0 \pm 4{\cdot}3\%$, $n = 32$ in age-matched controls; Jobling & McLachlan, 1992).

Figure 7 summarizes the changes in mean EJP and mean NAD amplitude at various times after all collector nerves were frozen compared with age-matched control data (from Jobling & McLachlan, 1992), and also shows the data for the proximal (intact) segments from the same animals. It can be seen that during reinnervation NADs reached amplitudes equivalent to control earlier than did EJPs. Furthermore, as they continued to increase in amplitude, the NADs were significantly larger than those in age-matched controls at the latest stage we examined (Fig. 7*Bb*). At this stage, the effect of the NADs on τ_{EJP} was such that the values were $15{\cdot}2 \pm 4{\cdot}4\%$ longer ($P < 0{\cdot}005$, paired t test, $n = 13$; cf. $7{\cdot}8\%$ in age-matched controls, Jobling & McLachlan, 1992) during stimulation at 0·1 Hz than when stimuli were presented at one per minute or less frequently. Thus EJPs would be significantly prolonged during bursts of sympathetic activity in reinnervated arteries (see Discussion).

Sensitivity to exogenous noradrenaline

Superfusion with noradrenaline produced depolarization of the arterial smooth muscle cells which was graded with concentration (10^{-7}–10^{-5} M) until contraction dislodged the recording microelectrode. Depolarization was blocked by idazoxan. In the presence of idazoxan (10^{-6} M), noradrenaline (10^{-5} M) produced marked constriction of the arterial segment which could be abolished by adding prazosin (10^{-6} M).

Because it was common for contraction to precede depolarization (see Itoh *et al.* 1983), it was difficult to determine noradrenaline sensitivity. Average values for depolarization could be obtained at only two concentrations of applied noradrenaline. Cells in proximal segments of control animals (at all ages) depolarized by $3{\cdot}8 \pm 0{\cdot}8$ mV at 5×10^{-7} M ($n = 5$) and $5{\cdot}8 \pm 0{\cdot}7$ mV at 10^{-6} M ($n = 8$), whereas those in proximal segments of nerve-lesioned animals depolarized by $2{\cdot}5 \pm 0{\cdot}2$ mV at 5×10^{-7} M ($n = 3$) and $5{\cdot}1 \pm 0{\cdot}8$ mV at 10^{-6} M ($n = 4$). Most impalements in innervated segments were lost in 5×10^{-6} M-noradrenaline because of contraction.

Denervated segments usually constricted in 5×10^{-7} M-noradrenaline. Depolarization was $4{\cdot}46 \pm 1{\cdot}20$ mV ($n = 8$) at 10^{-7} M and $6{\cdot}02 \pm 0{\cdot}99$ mV ($n = 4$) at 5×10^{-7} M-noradrenaline, compared with $1{\cdot}46 \pm 0{\cdot}32$ mV ($n = 14$) and $3{\cdot}86 \pm 0{\cdot}46$ mV ($n = 26$) in innervated segments from control animals. Only the responses to the lower concentrations were significantly higher in controls ($P < 0{\cdot}01$).

In contrast, at late stages of reinnervation (101 ± 11 days p.o.), cells in medial segments depolarized at 5×10^{-7} M-noradrenaline by $6{\cdot}8 \pm 1{\cdot}1$ mV ($n = 5$) which was significantly greater ($P < 0{\cdot}05$) than in age-matched controls ($3{\cdot}0 \pm 0{\cdot}4$ mV, $n = 4$, 112 ± 7 days of age). Contractions dislodged the electrode at concentrations equal to or greater than 10^{-6} M-noradrenaline.

It was notable that concentrations of noradrenaline up to 5×10^{-6} M had no effect on either amplitude or time course of the EJPs in either normally innervated or

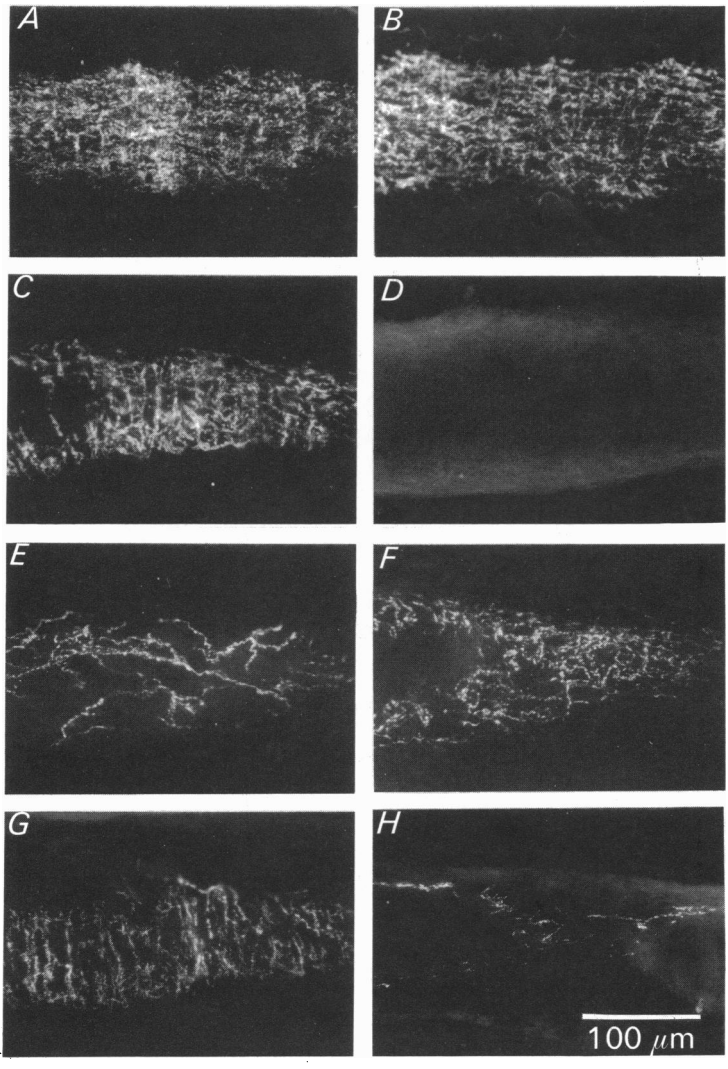


Fig. 8. Catecholamine fluorescence at the medio–adventitial border of the tail artery. Photomicrographs of longitudinal (tangential) sections (20 μm thick) from various sites along the artery in different animals: *A*, 10 mm, control 51 days of age. *B*, 10 mm, 31 days after freeze lesions. *C*, 60 mm, 47 days of age. *D*, 60 mm, 24 days after freeze lesions showing complete denervation (cf. *B*). *E*, 60 mm, 41 days after freeze lesions. *F*, 70 mm, 100 days after freeze lesions. *G*, 100 mm, control 90 days of age. *H*, 110 mm, 100 days after freeze lesions.

reinnervated preparations ($n = 3$). This suggests that presynaptic α_2-adrenoceptors have a lower affinity than postsynaptic α_2-adrenoceptors in this tissue.

Fluorescence histochemistry

Control caudal arteries bear an extremely dense noradrenergic plexus, with the density decreasing along the tail (Fig. 8*A* and *C*; see Sittiracha *et al.* 1987; Jobling

& McLachlan, 1992). At 21 days of age (at the time of the operation), the perivascular plexus was present along the entire length of the artery and on nearby arteriovenous anastomoses right to the tip of the tail (average tail length 89±2 mm, $n = 4$), although the plexus, particularly distally, had not developed to the same density as in the mature animal (see Anderson & McLachlan, 1991).

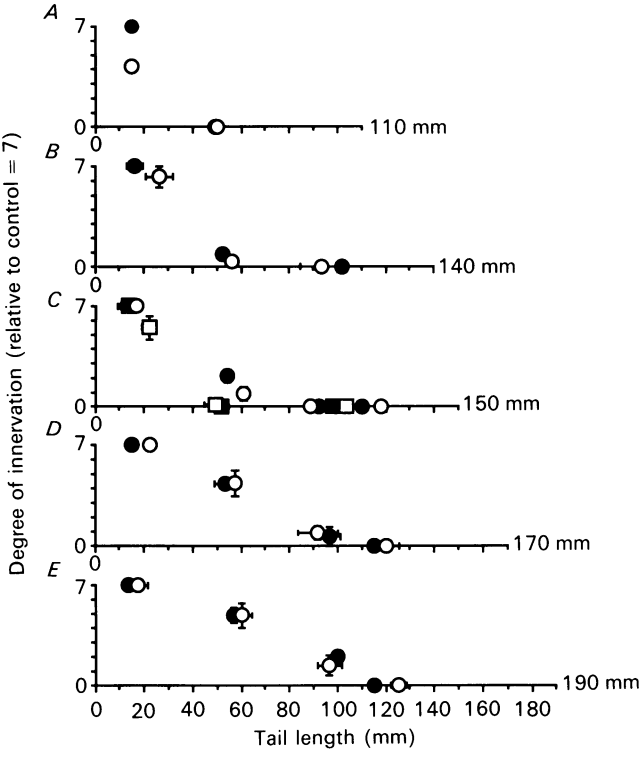


Fig. 9. Correlation between function and anatomy during reinnervation of tail artery. Data from Table 2 have been pooled at different stages using a scale of 0–7 (defined in Table 2) for function (filled symbols) as well as for density of perivascular plexus (open symbols, by conversion of percentages from Table 2 also to a scale of 7). Bars indicate ± S.E.M. *A*, 14 days p.o. ($n = 1$). *B*, 27±2 days p.o. ($n = 4$). *C*, circles show data 38±2 days after freeze lesions ($n = 3$); squares, data 37±10 days after nerve resection ($n = 3$). *D*, 71±7 days p.o. ($n = 3$). *E*, 109±8 days p.o. ($n = 5$). Length of the axis represents tail length for each age group.

Seven days after the nerve lesions, this plexus was absent at all levels along the artery at, or farther than, 35 mm from the base of the tail ($n = 3$, see Fig. 8*D*). In six animals in which the nerves were cut, no innervation was present further than 45 mm along the tail after periods as long as 161 days (Fig. 10).

After freeze lesions, the appearance of sparse fluorescent axons at sites below 50 mm along the tail was variable between animals, but followed exactly the return of EJPs and NADs in the electrophysiological studies of adjacent pieces of artery (Fig. 9, Table 2). At early stages, the innervation was very patchy, with some regions

of artery bearing a sparse plexus but with totally bare artery extending for hundreds of micrometres on either side. In other regions, there were only sparse but very brightly fluorescent axons (Fig. 8*E*).

At about 60 mm along the artery, once the density of the plexus was close to normal, so too was the functional innervation (Figs 8*F* and 9*E*), although reinnervation was incomplete in all animals (Fig. 9, Table 2). After about 60 days p.o., when sparse paravascular fibres had reached around 100 mm along the artery (Fig. 8*H*), the rate of longitudinal growth slowed. Although some paravascular axon bundles and individual axons within nerve trunks reached the distal parts of the tail, there was little or no evidence of reinnervation of the caudal artery itself at greater than 110 mm along the tail during the time course of these experiments (Figs 8*G* and *H*, and 10).

Observations on partially denervated arteries

In three experimental animals, there was some anatomical evidence of innervation below 50 mm, although in some regions this was patchy. EJPs were relatively large at levels which in other experiments were denervated (see Table 2), suggesting that all four collector nerves had not been completely lesioned. In all instances, some of the changes found in denervated and reinnervated arterial segments were observed. For example, in one preparation at 23 days p.o., the NAD was large and the EJP was relatively small at 55 mm, but some perivascular fluorescent axons were present on the artery at 70 mm. In another preparation, at 45 days p.o., EJPs in the medial segment had amplitudes in the range of controls but the NADs were relatively large and there were many large spotaneous EJPs; in contrast, the distal segment was normally innervated. The density of fluorescent perivascular axons was again correlated with the functional data (Table 2). Therefore it seems that some of the changes observed following denervation may occur when only part of the innervation is lost. This would be consistent with the maintenance of some abnormal features after reinnervation.

DISCUSSION

This study has examined the capacity of lesioned sympathetic postganglionic axons to regenerate and reinnervate a peripheral vascular effector, the main caudal artery of the rat. The sequence of electrophysiological events evoked by perivascular stimulation in the arterial smooth muscle cells over a period of months following disruption of the major nerve trunks by freezing was found to parallel closely the degree of restoration of the noradrenergic perivascular plexus (Table 2, Fig. 9). The data indicate that functional neuromuscular junctions are formed very soon after the arrival of the axon terminals. Initially rapid regeneration resulted in reinnervation of the most proximal regions of the denervated artery, with EJPs reaching control values about 60 mm distal to the lesion by about 100 days postoperatively. At this stage, the density of the perivascular plexus, subjectively assessed, was still below control levels, but the α-mediated response to neuronally released noradrenaline (the NAD) was enhanced. In contrast, regenerating postganglionic axons failed to regenerate to sites farther than 110 mm along the artery even after 5 months.

The sympathetic postganglionic axons supplying the tail artery travel in the main collector nerve trunks together with the sensory and motor innervation of the tail

(Sittiracha *et al.* 1987). Lesions of all collector nerves close to the base of the tails at 21 days of age totally denervated the artery from about 35 mm distally. When a segment of each nerve was removed, the artery remained denervated, indicating that sprouting of terminals along the artery was minimal. In the absence of the innervation, the tail grew as normal with no detectable deficiencies.

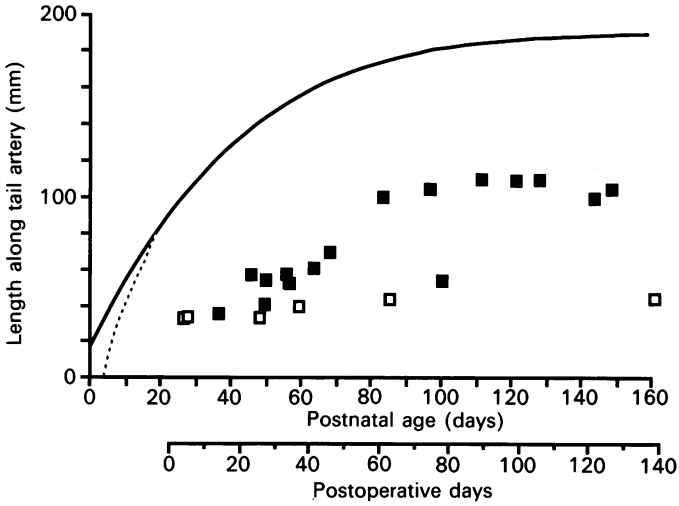


Fig. 10. Limited regeneration of perivascular axons along the tail artery at various times after lesions to the collector nerves. Continuous curve indicates the normal growth of the tail. Dashed line (largely superimposed) indicates the extent along the artery at which catecholamine fluorescent axons are normally present during postnatal growth. Symbols indicate the furthest distance at which perivascular axons or EJPs were identified after cutting (□) or freezing (■) the collector nerves.

EJPs and NADs could not be elicited distal to 40 mm along the tail until about 3 weeks after freeze lesions. During this period, lesioned axons must have regenerated 35 mm ventromedially to reach the denervated artery, after which reinnervation of the most proximal 30–40 mm of the denervated artery occurred over the next 6–7 weeks. Thus the maximum rate of regeneration was initially about 1·5 mm day^{-1}, which is similar to that of sudomotor axons after freeze lesions (Kennedy, Navarro & Kaemi, 1988; cf. Olson, 1969). Perhaps the regenerating axons grow more slowly in the low temperature of the distal tail (Thorington, 1966), so that reinnervation might only be achieved after longer postoperative periods.

Denervated arterial segments were very reactive and tended to constrict during dissection. From the onset of reinnervation, NADs were large relative to EJPs, and this difference persisted after EJPs had regained control amplitudes. The NAD in this artery results from a decrease in conductance (probably to K^+ ions, Cassell *et al.* 1988) following activation of α_2-adrenoceptors. The relationship between the number of quanta of transmitter released and the amplitude of the NAD is not known, and postsynaptic events may be the primary determinant (see below). Noradrenaline-induced depolarization was enhanced in denervated segments (cf. Galligan, Jiang,

Shen & Suprenant, 1990), although probably not more than could be accounted for by the removal of the neuronal uptake mechanism ($Uptake_1$), as similar increases in sensitivity occur in innervated arteries treated with neuronal uptake blockers (Högestätt, Hammarström, Andersson & Holmin, 1986). Thus early in reinnervation noradrenaline (of either endogenous or exogenous origin) might have access to high affinity extrajunctional sites because of the low density of perivascular axons, leading to a relatively larger depolarization than in innervated controls. However, this would not explain the maintained increase in NAD amplitude at later stages of reinnervation, unless regenerated axons have a reduced catecholamine uptake capacity.

How the α_2-mediated conductance change in arterial smooth muscle comes about is not known, and there are no reported parallels. Currently, there is evidence in other tissues that α_2-activation is linked either to an increase in potassium conductance, g_K, or to inhibition of voltage-sensitive Ca^{2+} channels (Isom & Limbird, 1988; Brown & Birnbaumer, 1990). On the other hand, neuronal α_1-receptors have been shown to decrease g_K (Yoshimura & Nishi, 1985; Nicol, Malenka & Kauer, 1990). Enhancement of the conductance change in reinnervated vascular smooth muscle might result from changes in the number or affinity of α_2-receptors or from a postjunctional phenomenon associated with the non-specific 'denervation supersensitivity' (Fleming & Westfall, 1988). Functionally, the reduction in membrane conductance would potentiate responses to nerve activity *in vivo* by augmenting the duration of EJPs and increasing the likelihood of activation of voltage-sensitive calcium channels (see Cassell *et al.* 1988).

Such a mechanism might explain the increased reactivity of reinnervated vasculature recently described by Jänig & Koltzenburg (1991). Blood vessels in the central pad of the adult cat hindpaw became functionally reinnervated one year after connecting the proximal stump of the sural nerve to the distal stump of the tibial nerve about 120 mm proximal to the paw pad. Flow through the pad vessels was greatly reduced by stimulation of the lumbar sympathetic chain and by reflex activation of postganglionic vasoconstrictor neurones, and responses to systemic phenylephrine were greater than in controls. It is worth noting that only a third of the original number of postganglionic axons would have been available in the sural nerve to supply the tibial vascular bed. These findings indicate that hyperreactivity of reinnervated arteries to sympathetic activity can be maintained over a prolonged period. Similar functional potentiation at long times after postganglionic lesions was reported by Machida (1929).

Our second major observation was that regenerating sympathetic axons did not reinnervate the distal parts of the tail, either functionally or anatomically, for periods as long as four months. Growing sympathetic axons were present along nerve trunks at all levels of the tail (E. M. McLachlan, unpublished observations) but distally the axons did not contact the main ventral artery. Although previous studies have reported rapid reinnervation of denervated arteries up to a few centimetres distal to lesions (Lorez *et al.* 1975; Cowen, MacCormick, Toff, Burnstock & Lumley, 1982; Hill *et al.* 1985; Karanth, Dhital, Springall & Polak, 1990), reinnervation of distant vascular targets (as opposed to sweat glands, Navarro & Kennedy, 1989) after postganglionic lesions appears to be slow (Derom, 1948). As described above,

restoration of functional responses may not reflect the anatomical relationships, and responses may be enhanced despite a low innervation density.

It is not easy to conceive how freeze lesions *per se* could be responsible for the failure of distal reinnervation. We chose nerve freezing (Filogamo & Muti, 1968; Mira, 1971) in an attempt to facilitate axon regeneration by minimizing the local disruption to the Schwann cell sheaths, as well as to ensure reproducible experimental conditions between animals. Regeneration following freeze lesions is known to permit rapid recovery of function of all myelinated (Mira, 1979) and sudomotor axons (Navarro & Kennedy, 1989). In addition, the sympathetic innervation of the enteric plexuses was found to regenerate rapidly with complete restoration of function after freezing the rat mesenteric nerves; in contrast, reinnervation of the mesenteric arteries was poor (Hill *et al.* 1985). This latter observation led to the suggestion that vascular neurones might be particularly susceptible to axotomy. Death of some sympathetic neurones may follow distal axotomy even in adult animals (Jänig & McLachlan, 1984). However, a considerable length of the tail artery became effectively reinnervated within three months, and it was only the distal artery which the vascular neurones failed to reach. It should be noted that many animals responded to pinching the distal tail after 2 months (E. M. McLachlan, unpublished observations) indicating that at least some of the sensory axons must have regenerated to that level.

Some other factors should be considered in relation to these experiments. The sympathetic neurones were immature at the time of the operation. At 21 days of age, the perivascular plexus over the length of the tail artery is relatively sparse, and brightly fluorescent axons are present in the distal nerve trunks (Anderson & McLachlan, 1991). Thus many of the lesioned axons would never have been in contact with an effector. One possibility is that neurones which are destined to innervate the distal vascular targets (i.e. the most immature) may be selectively lost; this has been shown for developing motorneurones (Crews & Wigston, 1990). Alternatively, it is notable that the length of artery which failed to be reinnervated was that which grew during the postoperative period (see Fig. 1). Skeletal muscle fibres are preferentially reinnervated at the old sites of neuromuscular contact (Bennett *et al.* 1973) where basal lamina marker molecules remain after denervation (Sanes, Schachner & Covault, 1986). Vascular smooth muscle which has never been innervated may not bear appropriate recognition molecules to attract the ingrowing axon terminals. A third possibility is that only neurones whose axons had not yet projected into the collector nerves (and so were *not* lesioned at 21 days) survived to reinnervate the artery.

The extent of reinnervation was generally progressive with time and consistent between animals (Fig. 10), but in two cases very little regeneration occurred at all. In these cases, it is hard to conceive that all of four nerve trunks were inadvertently damaged during the operation. One conclusion is that some systemic factor prevented initiation of the regenerative process, or limited sprouting at the site of the lesions. Sympathetic and small sensory neurones have a particular requirement for nerve growth factor (NGF) throughout life (Thoenen & Barde, 1980). NGF is produced by Schwann cells in damaged nerve trunks following invasion by macrophages (Lindholm, Heumann, Meyer & Thoenen, 1987) and macrophage-

dependent regeneration of small sensory neurones has been attributed to NGF production (Brown, Perry, Lunn, Gordon & Heumann, 1991). Perhaps for some unknown reason macrophage invasion did not occur in these animals.

In conclusion, two unusual features of the behaviour of the sympathetic innervation of the rat tail artery have been identified during regeneration. When the nerve trunks in the tail were lesioned under conditions which should have favoured regeneration, reinnervation of the first few denervated centrimetres of artery proceeded rapidly. However, one postjunctional response to neuronally released noradrenaline – the NAD – was larger than control and remained enhanced when reinnervation (as judged by the EJP) was complete. This change may explain exaggerated responses to nerve activity in reinnervated vessels. Secondly, the distal part of the artery failed to become reinnervated. It seems possible that the lack of regeneration to distant targets for long periods after nerve lesions is a specific characteristic of vasoconstrictor axons.

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