

The response of denervated axolotl arms to delayed amputation

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SUMMARY

Forearms of juvenile axolotls can be kept denervated for up to 4 weeks by deflecting brachial nerves to the flank. A more orthodox second denervation prolongs this state up to a total of six weeks. The denervated arms are unable to regenerate for the whole period, but eventually become reinnervated and then regenerate normally. These results and analogous experiments on adult newts prompt a partial retraction and reinterpretation of my previous report on regeneration after prolonged denervation.

INTRODUCTION

The neurotrophic theory stipulates that a certain threshold quantity or density of nerves in the amphibian limb is required to support regeneration. It is well established in adult newts and probably true of larval salamanders and anurans, but cannot apply to aneurogenic arms of very young larvae (Singer, 1952). An addictive version of the theory could accommodate all these cases, but also allows the possibility that chronically denervated limbs might eventually recover their ability to regenerate (Wallace, 1981). Amputating axolotl arms after 4 weeks of denervation seemed to support this possibility, as the denervated arms regenerated more rapidly than innervated control arms although they had become partly reinnervated by the end of the experiment (Wallace, Watson & Egar, 1981). Two repetitions of this experiment on adult newts did not yield any case of regeneration by limbs kept denervated for up to 45 days before amputation (Liversage & McLaughlin, 1983; Scadding, 1984). Either a relatively rapid reinnervation or a particularly low threshold requirement for axolotl arms could explain the discrepancy between these results. All three experiments employed the traditional operation of cutting spinal nerves 3–5 in the brachial plexus every 10–14 days. That should reduce innervation to a minor autonomic supply and collateral sprouts from adjacent spinal nerves, but later operations are less reliable as scar tissue increasingly obscures the brachial plexus. I have devised an alternative scheme of denervation involving fewer operations and now find that axolotl limb regeneration remains dependent on nerves for a tested period of 6 weeks.

MATERIAL AND METHODS

70 juvenile axolotls (*Ambystoma mexicanum*) about 80–90 mm long were subjected to the following initial operation on their left brachial nerves under anaesthesia with MS 222 (tricane methane sulphonate). The brachial plexus was exposed by a skin slit reaching the armpit. Major nerves emerging from the plexus were pulled out of the arm until they broke distally, often close to the elbow. Spinal nerves 3–5 were then separated from the brachial vein and deflected under the flank skin just ventral to the main lateral line. The ends of the deflected nerves were anchored by threading them through a separate hole in the skin, to emerge at least 3 mm posterior to the plexus. Reinnervation of the arm was monitored by recording movement at the shoulder and elbow of 50 specimens during the following 5 weeks, as described by Maden (1977). Groups of 10 specimens were amputated through both forearms at weekly intervals. Other groups were subjected to a second denervation when they were amputated, 3–5 weeks after the initial operation. All operations were carried out under MS 222 anaesthesia. The second denervation involved liberating the deflected nerves from scar tissue and pulling them until they broke proximally, sometimes extracting the third or fourth dorsal root ganglion. Regeneration was observed for at least 3 weeks after amputation at 18–20 °C, until control arm regenerates had grown two or three digits. A

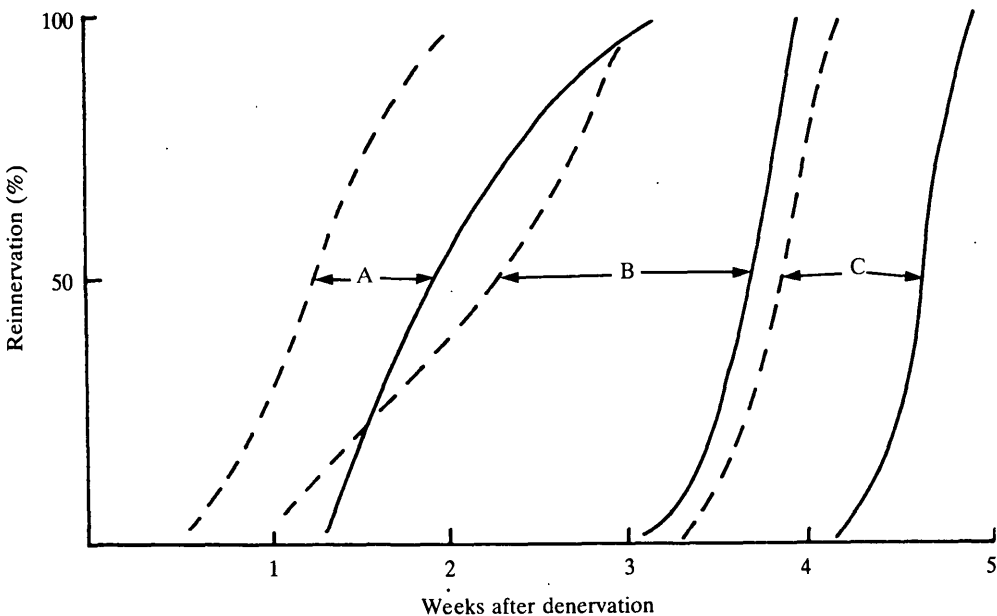


Fig. 1. Time of reinnervation estimated by recovery of movement at the shoulder (broken lines) and elbow (solid lines). The frequency of responding specimens is plotted against time after denervation by crushing (A), cutting (B), or deflecting (C) brachial nerves. A and B are derived from previous data on X-rayed arms of 70–90 specimens kept at a slightly lower temperature than C (cf. Maden, 1977).

marked delay in regeneration by a left arm relative to its contralateral control thus indicated nerve dependency at the time of amputation.

RESULTS AND DISCUSSION

All left arms remained immobile for 3 weeks after nerve deflection, began to move as a rigid unit during the fourth week and showed slight elbow flexures during the fifth week. Since previous observations indicate that reinnervation is much more rapid after pinching or cutting the brachial plexus (Fig. 1), nerve deflection is evidently the most suitable single operation for prolonging forearm denervation up to about 4 weeks. The second denervation employed here was often more stringent than merely cutting nerves high in the plexus, especially when it permanently depleted the sensory supply. It delayed forearm reinnervation by an additional 2–3 weeks. The second operation or later autopsy usually revealed that the nerves remained deflected, but had produced a series of collateral branches which returned to the base of the limb.

Control blastemata usually became obvious 10 days after amputation and grew to a cone by 14 days. Successive stages were recorded as a flattened palette, notch and three-digit hand at intervals of about 3 days. The denervated arms showed an identical sequence and rate of regeneration after an initial delay which could be estimated to within 2 or 3 days relative to the control arm (Table 1). When amputated 2 weeks after denervation, regeneration was delayed by a further 2 weeks. Amputation after 3 weeks usually resulted in 1–2 weeks delay. Amputation after 4 weeks produced no delay on average: eight of the experimental arms regenerated a few days earlier than control arms, presumably the Tweedle effect reported previously; both arms of 11 specimens regenerated almost simultaneously and only one experimental regenerate was clearly retarded. Either the forearm became sufficiently reinnervated 4 weeks after nerve deflection or regeneration then no longer required the presence of nerves. Redenervation at the time of amputation always delayed regeneration for 1–3 weeks, however, demonstrating that regeneration remained dependent on a nerve supply for at least 6 weeks after the initial operation (Table 1).

Table 1. Numbers of regenerates on denervated arms classified by their delay relative to control regenerates

Second operation	Delay (days)	Weeks between deflection & amputation			
		2	3	4	5
Amputation only	0–3	0	1	19	–
	3–6	0	2	1	–
	7–13	0	5	0	–
	14–20	10	2	0	–
Amputation & redenervation	0–6	–	0	0	0
	7–13	–	1	6	0
	14–20	–	9	4	10

The prompt regeneration of arms amputated 4 weeks after nerve deflection could be predicted better by a recovery of movement at the shoulder than by elbow flexure (Fig. 1). That is partly attributable to the unknown interval while sufficient synaptic connections are made with more proximal muscles to allow detectable movement at a joint, so that nerves may have penetrated beyond the joint. It is also partly attributable to the initial phases of regeneration (wound healing and dedifferentiation) being independent of a nervous control, so that amputation may precede local reinnervation by several days without causing an appreciable delay of regeneration.

The present results support the concept that limb regeneration is permanently dependent on innervation in juvenile axolotls, confirming two recent experiments on adult newts. Surgery cannot entirely eliminate the limb nerves in either case, nor can reinnervation be prevented indefinitely, but adequate denervation prevents regeneration for at least 6 weeks. That is long enough to require a reinterpretation of the previous observation (Wallace *et al.*, 1981) of regeneration after a 4-week regime of repeatedly cutting the brachial plexus. Counts at the end of that experiment showed a considerable reinnervation had occurred, amounting to almost 50% of the axons present in a control arm. Although surely an overestimate of the nerve supply at the time of amputation, this figure illustrates the unreliability of later attempts at denervation. The most apparent difficulty and hence likely fault of the later operations was to identify the small fifth spinal nerve. I assumed at the time that a failure to eliminate this nerve could only have a minor influence, such as allowing a few cases of greatly retarded regeneration. Deck (1961) found that the fifth spinal nerve frequently supported regeneration of larval *Ambystoma maculatum* arms amputated one week after partial denervation, without much delay in some cases. If Deck was correct in attributing this regeneration to collateral sprouting or accidental reinnervation, then any further postponement of amputation might well allow partially denervated (or partially reinnervated) limbs to regenerate promptly.

The main tenets of the neurotrophic theory thus find as much support from juvenile axolotls as from adult newts, in that limb regeneration demands a certain quantity of nerves. The requirement persists during prolonged denervation and can be satisfied by regrowing unmyelinated axons. Counts of the latter (Wallace *et al.*, 1981) suggest the density of all axons in the amputation plane provides the most reasonable expression of a threshold value, but do not provide an estimate of the value. The concept of a threshold seems inescapable, however, as a residual innervation is present even in non-regenerating limbs.

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