

Effects of delayed amputation on denervated forelimbs of adult newt

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SUMMARY

Left forelimbs of adult newts were repeatedly denervated prior to and following amputation. Limb amputations were performed at 7-, 14-, 21-, 27- and 45-day intervals after the initial denervation. Regeneration was found in the sham-denervated and control animals but did not occur in any of the experimental cases; instead cicatrix formation and dermal wound healing ensued. Soft-tissue dedifferentiation was evident, however. We conclude from these results that forelimb regeneration in the adult newt is completely nerve dependent (for growth).

INTRODUCTION

Numerous investigations have demonstrated the importance of nerves in amphibian limb and tail regeneration (Schotté, 1926; Butler & Schotté, 1941; Schotté & Liversage, 1959; Globus, 1978; see also reviews, Singer, 1952, 1974; Wallace, 1981).

Once 'addicted to nerves', amphibian limbs will not regenerate in their absence (Singer, 1952). However, limb regeneration in some urodeles becomes nerve independent if a dependence never forms ('aneurogenic limbs', Yntema, 1959; Wallace, 1980) or has been overridden ('chronically denervated limbs', Wallace, Watson & Egar, 1981; see also Thornton & Thornton, 1970). Wallace *et al.* (1981) amputated the forearms of juvenile axolotls (*A. mexicanum*) and found that they regenerated normally even though their innervation had been depleted for several weeks prior to amputation. They suggest that the amputated limb tissues can adapt to nerve deprivation and that such results are in accord with the 'addictive' version of the neurotrophic theory (Singer & Mutterperl, 1963) rather than its quantitative or 'threshold aspects' (Singer, 1946). Larval *Ambystoma* do not form a blastema in concomitantly denervated, amputated forelimbs, and if the peripheral nerves are then repeatedly denervated (resectioned) 'unchecked dedifferentiation' results, beginning at the amputation surface and proceeding proximally (Butler & Schotté, 1941).

Schotté & Liversage (1959) studied the initiation of regenerative activity in

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forelimb regenerates of the adult newt following concomitant denervation and re-amputation of the limb through the regenerate. They found that regenerates of 15–75 days are nerve dependent for the initiation of 'new regeneration'. Regenerates 15–60 days old exhibited the 'regression effect', whereas 75-day-old regenerates neither regenerated nor showed signs of regression. Liversage (1959) showed that, although limb regeneration in the adult urodele is nerve dependent, nervous connections between the regenerating limb and the central nervous system are unessential, the nerves exerting their influence solely at the local level, possibly providing some type of neurosecretion to the regeneration area.

The present experiments were designed to test the effects of delayed forelimb amputation on prolonged (repeatedly) denervated forelimbs in the adult newt.

MATERIALS AND METHODS

One hundred and seven adult newts (*Notophthalmus viridescens*), 1.8–2.2 g body weight, from Tennessee, U.S.A. were utilized in this study. They were kept in dechlorinated tap water at $22 \pm 1^\circ\text{C}$ on a 12 light/12 dark photocycle. Animals were fed minced beef heart every 5 or 6 days.

Operations were performed upon animals anaesthetized in tricaine methane sulfonate (MS 222) 0.1% w/v, immersed approximately 10–12 min prior to surgery. Somatic brachial denervations were performed on nerves No. 3, 4 and 5 according to the method of Schotté & Liversage (1959) and were repeated progressively dorsally toward the origin of the nerve trunks at 10- to 14-day intervals until fixation. Sham-denervation involved the same procedure as denervation with the omission of actually severing the brachial nerves. Sham procedures were repeated at the same intervals as denervations. Left forelimb amputations were performed through the distal upper arm (see Liversage & Scadding, 1969).

Newts were divided into four Groups: A. control animals, non-operated other than left forelimb amputation; B. newts with left forelimbs sham-denervated prior to and following amputation; C. cases with left forelimbs denervated repeatedly prior to and following amputation; and D. newts with left forelimbs denervated repeatedly prior to and following amputation of the left limb as well as that of the right unoperated limb. The concomitant bilateral forelimb study of Group D was an attempt to observe the effect of denervation of the left forelimb upon the rate and degree of regeneration of the right forelimb (the 'Tweedle Effect', see Tweedle, 1971).

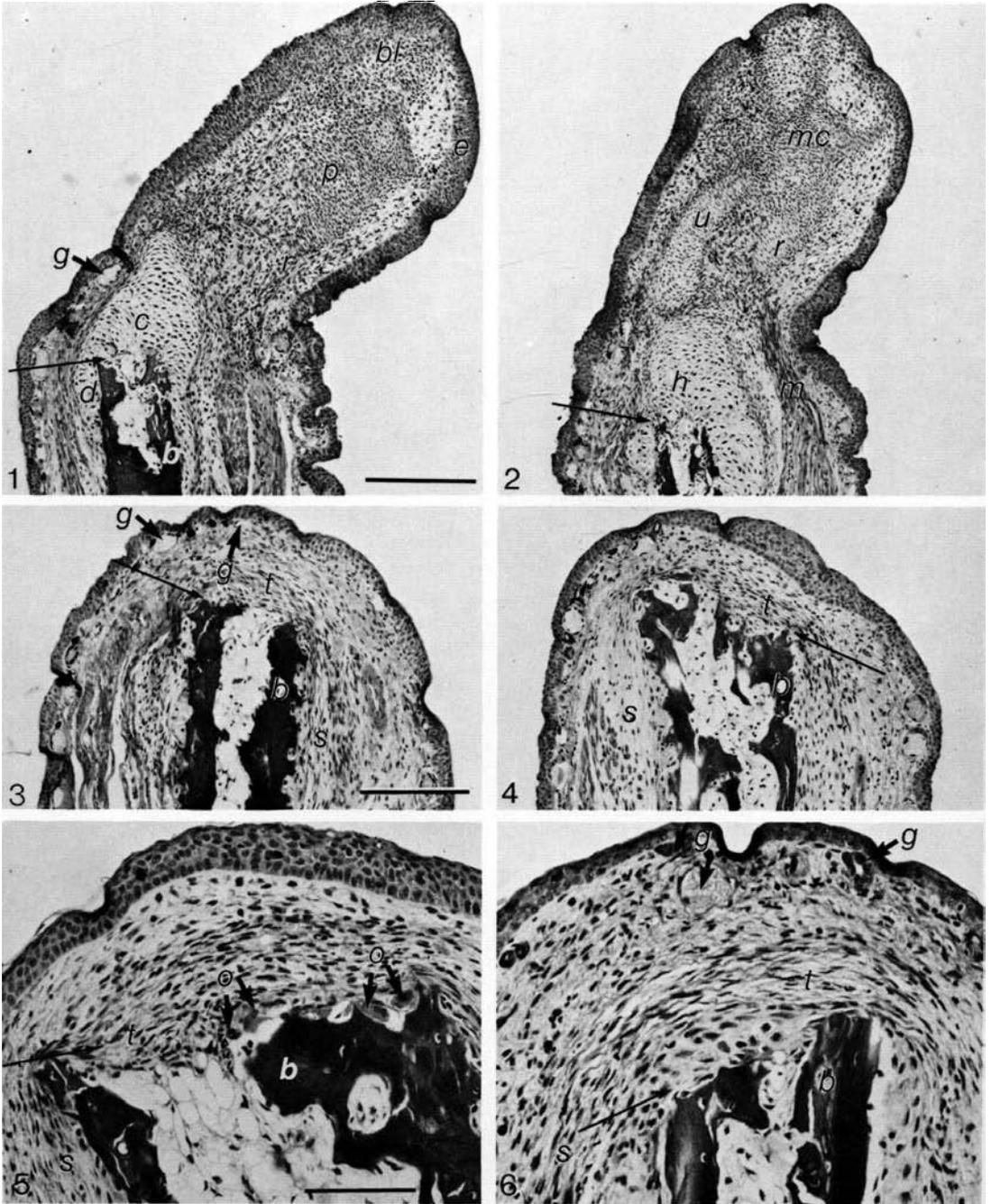
Groups were divided into 15 series according to the surgical procedure employed, the number of days between the initial operation (denervation or sham-denervation) and the time of delayed amputation, and amputation and fixation (see Table 1). Fixations were performed 20–25 days after amputation except for the series 13 cases, fixed at 7 days, and also the series 12 newts which were

Table 1. *Results of delayed amputation on denervated forelimbs of adult newt*

Series No. and operation	No. days between operation and amputation	No. days between amputation and fixation	Total No. days between operation and fixation	Fixed	Regenerated	No. Cases
A. 1-3 Non-denervated Controls	-	21,25	-	21	21	21
B. 4-5 Sham-denervated	14-21	25	39,46	11	11	11
C. 6-11 Repeatedly denervated	7-27	20-25	27-48	48	0	0
12.* Repeatedly denervated and Re-amputated	21 and 45	24 and 31	76	9	0	0
D. 13-15 Repeatedly denervated and bilaterally amp.	14-21	7-21	21-42	18	18	left denerv. 0 right non-denerv. 18
					Total No. Cases	107

* See Fig. legend (Fig. 6) for details of series 12.

amputated a second time through the distal left forelimb stump. Left forelimbs of this latter series were fixed 31 days after the second amputation, denervation being repeated (as described above) throughout the 76-day experimental period. Stumped tips of series 12 animals were fixed at the time of the more proximal



second amputation. In all cases, forelimbs were removed at the shoulder and placed into vials containing G-Bouin's solution (Liversage, 1967). Tissues were decalcified in Jenkins' fluid, embedded in paraplast and serially sectioned at 8–10 μm , then stained with haematoxylin and counterstained with orange-G eosin (Humason, 1979).

RESULTS

This investigation is based upon morphological and histological observations of amputated forelimbs of the following: 21 non-denervated control animals; 11

In all Figures the level of limb amputation is indicated by a long arrow(s).

Fig. 1. Longitudinal section through the palette-shaped forelimb regenerate of a typical case from Control series 1, 25 days postamputation. Normal forelimb regeneration is evident. Note the presence of developing procartilage (p), including development of digits distally and radius (r) and ulna in the midregion of the regenerate (seen more clearly in adjacent sections). More advanced cartilage (c) differentiation proximally includes the cartilage bone collar (d) derived from the chondrogenic layer of the periosteum surrounding the amputated bone (b) tip. Notice the population of blastema cells (bl) forming an arc at the tip of the regenerate, and the thickened, darkly stained epidermis (e) covering the entire regenerate. There is little evidence of dermis (dermal glands = g) in the regenerate. Bar = 500 μm .

Fig. 2. Longitudinal section through a palette-shaped forelimb regenerate from Sham-denervated series 5, 25 days postamputation. The rate and degree of normal forelimb regeneration is comparable to that observed in Fig. 1. In addition to the observations described in Fig. 1, a more complete cartilaginous skeletal pattern can be seen due to the angle of section. Note new cartilage in the pitted lateral regions of the stump bone, the extensive bone collar, the newly formed distal humerus tip (h), the presence of ulna (u) and radius (r) cartilages, as well as the differentiation of new muscle bundles (m), and the beginning formation of the carpal–metacarpals (mc) and digital pattern. There is thickened, darkly stained epidermis covering the entire regenerate, as well as the absence of dermal skin glands in the regeneration area. Magnification as in Fig. 1.

Figs 3–6. Longitudinal sections through the forelimbs of cases from series 7, 9, 11 and 12 respectively, all 25 days postamputation. Series 7 animals were denervated for 14 days prior to forelimb amputation, series 9 newts 21 days prior to limb amputation, and series 11 cases 27 days prior to amputation. These forelimbs display typical stumping characteristics. Connective tissue elements (stellate-shaped fibroblast cells) (t) are present beyond the amputated end of the humerus (b). Soft tissue dedifferentiation (s) has occurred, nevertheless, only a few small accumulations of loosely packed blastema cells immediately beneath the epidermis are present. Following denervation, there was no continuous proliferation of de-differentiated cells, and a blastema did not form. There is evidence of continued presence of osteoclasts ((o) – Fig. 5), cells involved in bone dedifferentiation which normally disappear by 22 days postamputation. Neither cartilage nor pro-cartilage is seen to be developing, nor are bone collars overtly evident. Fig. 6 is typical of the series 12 cases whose forelimbs were denervated for 21 days prior to first amputation, then re-amputated after another 24 days for a total of 55 days of amputation, 76 days under continuous denervation at final fixation. The characteristics of stumped forelimbs, as described above, are in evidence in this Figure as well. Note extent of dermis invasion (g) of the wound areas in Figs 3 and 6. Figs 3 & 4: bar = 250 μm . Figs 5 & 6: bar = 166 μm .

sham-denervated newts; and 75 denervated cases. Our findings were compared with the normal stages of forelimb regeneration in adult *Notophthalmus viridescens* (with consideration given to temperature differences) as described and illustrated by Liversage & Scadding (1969), Iten & Bryant (1973), and Schotté & Liversage (1959).

Non-denervated Controls, Group A

By 21 days the cone blastema became flattened dorsoventrally, giving rise to a palette-shaped regenerate 25 days after amputation. At this time, an alignment of procartilage cells extending distally and contiguously with the end of the newly regenerated distal epiphysis of the humerus was formed. As well, the two zeugopodial cartilaginous bones and the beginning of the digital pattern became evident (Fig. 1).

Sham-denervated Controls, Group B

Left forelimbs of these cases were sham-denervated prior to and following limb amputation 14 (series 4) or 21 (series 5) days after the initial surgery. Histological and morphological observations demonstrated that the regenerates of sham-denervated animals were identical in their rate and degree of regeneration with normal control regenerates (Figs 1 and 2).

Denervated Groups C and D

In all cases in these two groups, forelimbs denervated repeatedly prior to and following amputation remained unresponsive and immobile during frequent tactile stimulation.

Group C. Denervation and left forelimb amputation

In the six cases of series 6, amputated 7 days following initial denervation, epidermal wound healing of the amputation surface was evident at 20 days postamputation. No apparent differences were observed histologically among the amputated, denervated forelimbs in this series; all animals showed typical non-regenerating (stumped) left forelimbs. Our results correlate well with other investigations concerned with the lack of regeneration in concomitantly denervated, amputated adult newt limbs (Singer, 1942; Schotté & Liversage, 1959). The findings in this series are corroborated by those of series 7–15, as described below.

In absence of sensory and motor innervation from the brachial plexus for 14, 21, 27 and 45 days prior to amputation, forelimb regeneration was inhibited. Successive nerve resections were easily performed due to the continued presence of the myelinated nerve sheaths proximal to the initial cut.

At fixation, denervated forelimbs displayed an epidermal layer of uniform thickness which was continuous with the lateral edges of the wound and covered

the amputation surface. The dermis, as indicated by the presence of skin glands, partially covered the wound in some cases (Fig. 3), and completely in others. Soft tissue dedifferentiation is evident in the denervated stumps (Figs 3, 4, 5, 6). Due to this cellular activity a fibrocellular cicatrix always formed. In a few cases, limited osteoclast activity was observed (Fig. 5). The cicatrix and dermis tend to seal the wound, preventing cell-cell contact between epidermis and cut mesodermal stump tissues, essential to normal regeneration.

Group D. Denervation and bilateral forelimb amputation

In 1915 Herrick and Coghill discovered that in *Ambystoma* many nerve fibres cross the spinal cord via the ventral commissure. Tweedle (1971) showed that denervation or amputation of one adult newt forelimb hinders regeneration of the opposite limb by perturbing transneuronal connections, inducing chromatolysis of the contralateral nerves (see also Maden, 1977; McLaughlin, Rathbone, Liversage & McLaughlin, 1983). Following bilateral amputation of the 18 cases in this Group, all left denervated forelimbs formed stumps identical to those observed in our other denervated cases (series 6-12, Table 1, extreme right column). The non-denervated right forelimbs which were amputated concomitantly, regenerated at a rate delayed by as much as 3-4 days compared with the regeneration rate observed in our control cases (Group A, Table 1). Although the rate of regeneration was retarded in the non-denervated contralateral forelimbs the degree of differentiation was normal. These results are meaningful and corroborate those of Tweedle; however, the primary interest of the present study lies elsewhere.

DISCUSSION

In the present study, forelimbs of adult newts were kept in a continuously denervated state prior to and following amputation, and regeneration was impeded. In these cases, dermal wound healing occurred, a fibrocellular cicatrix formed, and soft tissue dedifferentiation was evident. On the basis of our experimental results, we find that following denervation, regeneration is dependent upon the presence of brachial nerves, whether a limb remains denervated for 7, 14, 21, 27, or 45 days prior to amputation. In all instances, limbs failed to adapt to the nerveless state. There was initiation but not continuation of regeneration. Initiation and the continuation of regeneration was found in transplanted, aneurogenic forelimbs of newly innervated *Ambystoma* larvae which were later denervated (Thornton & Thornton, 1970; see also Yntema, 1959) and in forearms of juvenile axolotl which had been kept in a 'chronically denervated' state for many weeks prior to amputation (Wallace *et al.* 1981). It is apparent that *Ambystoma* larvae and juvenile axolotl, as developing organisms, retain the potential to adjust to the deprivation of brachial nerves (i.e. loss of neurotrophic

factor(s)) and maintain the ability to regenerate. Fully mature newts, however, cannot.

Singer (1942) and the present findings demonstrate that by concomitantly incising both the sensory and motor components of all three somatic brachial nerves, forelimb regeneration was prevented even when the sympathetic nerves were left intact. As well, adult newt forelimb regenerates, when amputated concomitantly with forelimb denervation, are dependent upon the brachial nerves for the initiation of a 'new regeneration' (Schotté & Liversage, 1959). Although spinal nerves 2 and 6 do not normally innervate the forelimb, after brachial plexus ablation they send regenerating fibres into the limb (Singer, 1946). If this occurred in our denervated newt forelimbs, it apparently had little or no effect upon regeneration.

The control and sham-denervated newts of Groups A and B regenerated normal cone blastemata by 21 days postamputation and early digit stage regenerates by 25 days at 22°C (Figs 1 and 2). The findings in Groups C and D show that forelimbs amputated after an extensive denervation period do not regenerate, but instead undergo stumping. Also, when a limb remained denervated for 3 weeks prior to amputation, was reamputated 24 days after the initial amputation, then fixed 31 days later, for a total of 76 days of denervation, at no time did regeneration ensue (series 12). The results of this series strongly support the findings of all series in Groups C and D, further emphasizing the nerve dependency of adult newt forelimb regeneration.

Only in areas where wound epidermis is present and interacting with the cut mesodermal stump tissues does regeneration occur (Mescher, 1976; Tassava & Loyd, 1977; Globus, 1978; Tassava & Garling, 1979). Tassava & Mescher (1975) and Globus (1978) have postulated and Globus, Vethamany-Globus & Lee (1980) have shown that the wound epidermis acts to keep the blastema cells in the cell cycle, thereby delaying differentiation. Following a prolonged state of denervation prior to forelimb amputation, epidermal wound healing, wound repair and dedifferentiation occur. However, the wound becomes invaded by dermis and a cicatrix forms between the stump mesodermal elements and the epidermis, resulting in the prevention of regeneration in adult newts.

It has been postulated that nerve cell bodies produce a trophic substance (i.e. peptide or protein) which is thought to initiate and/or control cellular activity (Singer, 1974; Wallace, 1981), and play a major role in regeneration by stimulating mesenchymatous (blastema) cell proliferation (Globus & Liversage, 1975; Globus & Vethamany-Globus, 1977). Denervation reduces and later prevents cell division and growth (Singer & Craven, 1948; Schotté & Liversage, 1959; Globus & Vethamany-Globus, 1977).

This work demonstrates that adult newt forelimbs are 'addicted' to the brachial nerve supply (i.e. neurotrophic influence) for regeneration, particularly the growth phase and as a consequence, differentiation. We assume that prolonged denervation of a forelimb by continual resection of the brachial plexus nerves

prior to and following forelimb amputation causes a marked reduction in whatever factors nerves contribute to the microenvironment of the regeneration area, and therefore inhibits regeneration and leads to the eventual stumping of the amputated forelimb.

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