The pattern of innervation in serially duplicated axolotl limbs: further evidence for the existence of local pathway cues?

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Summary
The innervation of the biceps muscle was examined in regenerated and vitamin A-induced serially duplicated axolotl forelimbs using retrograde transport of horseradish peroxidase. The regenerated biceps muscle becomes innervated by motor neurones in the same position in the spinal cord as the normal biceps motor pool. In previous experiments in which the innervation of a second copy of a proximal limb muscle was examined in serially duplicated limbs (Stephens, Holder & Maden, 1985), the duplicate muscle was found to become innervated by motor neurones that would normally have innervated distal limb muscles. In the present study, the innervation of the second copy of biceps was examined under conditions designed to encourage nerve sprouting from 'correct' biceps axons. Following either partial limb denervation or denervation coupled with removal of the proximal biceps, the second copy of the muscle was still innervated by inappropriate motor neurones, which again would normally innervate distal limb muscles. These results are interpreted as evidence for the necessity for an appropriate local environment for axonal growth to allow reformation of a correct pattern of motor innervation in the regenerated limb.

Key words: axon, axolotl, specificity, reinnervation, vitamin A, motor neurone, regeneration.

Introduction
The motor innervation of axolotl limbs shows a remarkable capacity for functional recovery following limb amputation and regeneration or local nerve damage (Stephens & Holder, 1987; Holder, Mills & Tonge, 1982; Holder, Tonge & Jesani, 1984). After cutting nerves in the intact limb, neuromuscular connections are re-established by a combination of local pathway cues guiding regrowing axons to their correct target muscles (Holder et al. 1982, 1984; Grimm, 1971) and competitive interactions between correct and incorrect nerves at the target site (Cass & Mark, 1975; Bennett & Raftos, 1977; Genat & Mark, 1977; Dennis & Yip, 1978; Wigston, 1980; Holder et al. 1982).

In an alternative experimental approach to examining the mechanisms of specific neuromuscular regeneration, we have produced serially duplicated limbs with vitamin A treatment as described by Maden (1982, 1983). Such duplicated limbs have a second complete set of limb muscles (Stephens et al. 1985). Using HRP as a marker of motor neurone position in the spinal cord, the second copies of duplicate proximal muscles were shown to be innervated by motor neurones that would normally innervate distal limb muscles. This incorrect innervation was interpreted as being derived from nerves transected at the distal amputation site. In this experiment, therefore, motor neurone axons innervating proximal muscles were unable to sprout and grow through the foreign limb territory to innervate a second copy of the proximal muscles.

There are several possible explanations of this experimental result. The first is that in these duplicated limbs, the axons innervating the first copy of the biceps muscle are undisturbed; they are never stimulated to sprout and grow towards the second copy of the biceps muscle by a nonspecific growth signal such as transection. Sprouting of axons in urodele limbs has been demonstrated after a number of experimental procedures including those in which nerves have been cut and misrouted (Holder et al. 1982, 1984) and within muscles that have been denervated (Bennett & Raftos, 1977; Genat & Mark, 1977), a phenomenon which has also been extensively studied in mammals.
(see review by Brown, Holland & Hopkins, 1981). A second explanation is that even if these axons were stimulated to grow, they would be attracted to the first copy of the biceps muscle, which is in close proximity, rather than grow towards any attractive signal from the distant duplicate muscle. A third possibility is that appropriate axons are unable to grow towards and innervate the duplicate biceps muscle because this would require traversing limb territory that would contain no appropriate pathway cues to guide their growth. It is also possible that the duplicate biceps muscle is qualitatively different from although morphologically identical to (Stephens et al. 1985) the original biceps. If this is so it may not produce any putative chemoattractant or it may produce a different one. Since the only criterion for assignment of a name to a muscle is its anatomy, this is a possibility we cannot explore further. We also cannot rule out the possibility that the character of the duplicate biceps muscle is irrevocably altered by its initial innervation by inappropriate fibres (Stephens et al. 1985). However, to our knowledge, there is no evidence for this proposition in the motor system.

In order to control for transection as a nonspecific growth stimulus and a ‘flooding’ effect from the proximal biceps muscle, we have transected the proximal biceps motor innervation in one experiment and, in a second experiment, surgically removed the proximal biceps as well as transecting its innervation. In both experiments, the duplicate copy of biceps was innervated by motor neurones from incorrect, distal motor neurone pools, as it was in the original study (Stephens et al. 1985). This result provides further evidence for the importance of the axon’s immediate environment in the control of its growth.

Materials and methods

General

White and black axolotls spawned in the colony at King’s College were used for these experiments. Animals used for vitamin A treatments were all siblings from the same spawning, control animals were from different spawnings. All were reared separately from the time of limb outgrowth to ensure that the limbs were not regenerates. Animals were fed two or three times a week with raw heart. All surgical procedures were carried out with the animals anaesthetized in MS222 (Sigma).

Vitamin A treatment

20 animals between 4.5 and 5.5 cm in length (snout to tip of tail) had their right hand amputated through the carpals. 3 days later they were reanaesthetized and each given a single intraperitoneal injection of 3 μl of 0.1 g ml⁻¹ all-trans retinoinic acid (Sigma) dissolved in dimethyl sulphoxide. This dose has been reported to be the most effective in creating pattern duplication in animals of this size (Thoms & Stocum, 1984). The unoperated, left limbs were used to assess the normal motor pool positions for biceps in each animal.

Surgery

(1) Controls

To check that the biceps muscle is specifically reinnervated in the normal regenerated limb within the time scale used in these experiments (see also Stephens & Holder, 1985), three animals of length 13-5, 13-6 and 15-8 cm had their right arm amputated at the shoulder under MS222 anaesthesia. After 108 days (by which time they had grown to 15-8, 15-0 and 17-5 cm, respectively), the specificity of innervation of the regenerated biceps was assayed using HRP histochemistry (see below).

(2) Denervation of the proximal copy of biceps

In the normal axolotl forelimb, the biceps is innervated by the forearm flexor nerve (FFN – Francis, 1934; Holder et al. 1982; Stephens, 1985). In order to denervate the biceps muscle, 91 days after vitamin A treatment both divisions of the FNN were cut in the shoulder of the duplicated limb of seven animals. By this time, the animals had grown to 11.0–12.0 cm in length. The FNN was then allowed to regenerate for 101–115 days before analysis. During this period, the animals grew to 15.0–17.5 cm. Thus, the total limb regeneration time was 192–206 days.

(3) Transection of the FFN in combination with removal of the proximal copy of biceps

The first copy of the biceps muscle was removed 222–225 days after vitamin A treatment and both divisions of the FFN were cut in the duplicated limbs of seven animals. At operation, the animals in this group were 11.0–16.0 cm in length. After a survival time of a further 77–80 days the specificity of innervation of the second biceps was assayed. At the time of analysis, the animals had grown to 12.8–18.3 cm. The total limb regeneration was therefore 290–305 days.

To check whether the biceps muscle had regenerated after removal, the operated limbs were removed at the time of perfusion fixation for HRP analysis and fixed overnight in Bouin’s solution. They were then decalcified for 1 week in EDTA, dehydrated through alcohols, cleared in toluene and embedded in wax. Serial 10 μm sections were cut through the proximal segment of the limbs and mounted on gelatin–chrome alum subbed slides. Sections were hydrated, stained with Mayer’s haemalum and 1 % aqueous eosin, dehydrated, cleared in xylene and mounted in DPX. Serial sections from all seven operated limbs were carefully examined for regenerated biceps fibres.

HRP histochemistry

The techniques of fixation, histochemistry and analysis of HRP-treated animals were the same as those described previously (Stephens & Holder, 1985, 1987). The same schedule was used for all animals studied. Briefly, HRP was placed in the experimental (right) biceps and the distribution of labelled motor neurones compared with the
control (left) biceps motor neurone pool. In the rostrocaudal axis, the position of filled cells was recorded with respect to spinal roots 3, 4 and 5 and were counted with respect to bins 500 μm long, allowing presentation in the form of a histogram. In the transverse plane, the position of filled cells in the ventral horn was assessed with respect to a 3x3 grid (Stephens & Holder, 1987) and the mean coordinate position for each motor pool plotted.

For all control and experimental groups, the rostrocaudal distributions of filled cells were compared between unoperated (left) and operated (right) sides by superimposing the median position of the distribution of the unoperated biceps motor pool and comparing this with the contralateral distributions (Stephens & Holder, 1987).

Results

(A) The innervation of the control biceps muscles
The biceps motor neurone pools in the control unoperated limbs contralateral to those that were vitamin A-duplicated had the same characteristics as those previously described as the normal motor neurone pool position (Figs 1, 2, 3 and Stephens & Holder, 1985). The pools contained 23-6 cells (±2-2 S.E.M., n = 17) and extended from above root 3 to root 4. In the transverse plane, filled neurones were confined to the dorsolateral region of the ventral horn typical of flexor muscles in the axolotl.

In the three cases in which the motor pool position of biceps was examined in a regenerated limb not treated with vitamin A, a small proportion of errors was seen in the projection (Fig. 1). Similar numbers of cells were labelled in the regenerate motor pool (24-7 ± 2-3) as compared to the normal motor pool seen in the unamputated controls described above. A mean of 18-8 ± 5-8 % of motor neurones innervating the regenerated biceps was in an ectopic, caudal position in the spinal cord (Fig. 1); cells were arbitrarily classified as ectopic in the regenerate pools if they were greater than 500 μm rostral or caudal to the control distribution — see above and Stephens & Holder (1987). The small caudal extension in the regenerate motor pools was reflected by a mean caudal shift in the distribution of 417 ± 180 μm with respect to the control pools described above.

(B) Reinnervation of the second biceps following transection of the FFN
After sectioning the nerve to biceps in the shoulder, the duplicate biceps muscle did not acquire a correct innervation in any of the seven cases studied (Fig. 2). In fact, the motor pools of these muscles were very similar to those described previously for the second copy of biceps in the undisturbed duplicated limb (Stephens et al. 1985). Fewer cells were labelled in the pool of the second biceps compared with the contralateral control distributions — a mean ± S.E.M. of 8-1 ± 1-7 (compared with 23-6 ± 2-2, P < 0-02; Wilcoxon test). The rostral and caudal limits of the experimental distributions were more variable than those of the control pools, but generally extended from halfway between roots 3 and 4 to root 5. The median rostrocaudal position was shifted over 2000 μm caudally although some filled cells were clearly within the bounds of the normal distribution of the biceps pool (Fig. 2). In the transverse plane, the mean positions of motor neurones that innervated the second biceps had the same overall distribution as controls (Fig. 2).

(C) Reinnervation of the second biceps following transections of FFN and removal of the proximal biceps copy
The purpose of this experiment was to eliminate any influence of the normal biceps muscle on the behaviour of the biceps motor axons. To ensure that this was so, the limb was carefully examined histologically at the end of the experiment. The biceps muscle is readily identifiable with respect to several landmarks in the normal limb; it is the smallest of the three muscles in the proximal limb segment, situated between the two divisions of the FFN and the brachial artery on one side and the extensor cranialis nerve and a large vein on the other. This region was examined for the presence of muscle fibres in the seven experimental cases. Fig. 4 shows the two types of result obtained. In four out of seven cases no trace of the biceps muscle remained. In the other three cases there was evidence of regeneration (or incomplete removal) of the biceps. In two of these three cases, a small number of fibres was found either associated with the FFN or the humerus, and in the remainder a small number of apparently immature fibres of small diameter with centrally located nuclei were found.

The complete absence or much-reduced volume of the first copy of the biceps muscle did not result in selective reinnervation of the second copy of the muscle (Fig. 3). In all seven cases studied, the duplicated biceps was innervated by inappropriate, distal limb muscle motor neurones located caudal to the normal biceps motor pool; the caudal shift of the median was over 3500 μm. In the transverse plane, the distribution of means in two cases was clearly more ventral and medial (Fig. 4) but, in two others, they fell within the control distribution. The duplicate biceps motor neurone pool contained around half the cells found in the normal biceps motor neurone pool, a mean ± S.E.M. of 12-0 ± 1-6 compared with 23-6 ± 2-2 (P < 0-05; Wilcoxon test). Again, the rostral and caudal limbs of these experimental distribution were more variable, but generally extended from halfway between roots 3 and 4 to root 5.
Fig. 1. The motor neurone pool of a regenerated biceps compared with a control biceps distribution. (A) An example of one case in which a plot of cell position is shown on outline drawings of grey matter every 1500 µm. Note the small number of caudal ectopic cells projecting to the regenerate muscle (left side). The number of cells every 500 µm is shown in the histogram in relation to the spinal roots. (B) Diagram summarizing pooled data in which the median rostrocaudal positions of the control biceps pools have been superimposed. The arrows show the medians of control and regenerate muscle pools. (C) Diagrammatic representation of the ventral horn showing the mean positions of motor pools of control (•) and regenerated (△) biceps muscles. D, dorsal; V, ventral; L, lateral; M, medial.
Discussion

The results of the experiments described in this paper and in a previous study (Stephens et al. 1985) indicate that axons from neurones that normally innervate a proximal limb muscle do not grow through foreign limb territory to reach a second, distal, copy of the same muscle. This is true whether or not the normal

Fig. 2. The locations of motor pools in the rostrocaudal (A,B) and transverse axes (C) for vitamin A serially duplicated limbs which had been partially denervated by cutting the FFN (left side) as compared to unoperated controls (right side). The arrangement of the figure is as shown for Fig. 1.
limb innervation has been cut to allow axonal sprouting or whether the normal proximal target is present or absent (Fig. 5). These results are consistent with the existence of defined pathways within the mature or regenerating limb (Holder et al. 1982, 1984; see also Katz, Lasek & Nauta, 1980 for a discussion of

Fig. 3. The location of biceps motor pools in the rostrocaudal (A,B) and transverse (C) axes for biceps muscles in vitamin A serially duplicated limbs in which the proximal biceps had been removed and the FFN cut (left side) as compared to unoperated controls (right side). The arrangement of the figure is as shown for Fig. 1.
Local axon pathway cues (in the central nervous system) and argue against the existence of long-range attraction of appropriate nerve fibres by a regenerating muscle.

In these experiments, fewer neurones innervated the second copy of biceps; the inappropriate pool comprising approximately a third to a half of the normal number of neurones for this muscle (see Results and Stephens & Holder, 1985). In addition, the motor pool of the second copy of biceps had the characteristics of a distal muscle pool (see Stephens & Holder, 1985; Stephens et al. 1985). This was particularly clear in the experiment where the first copy of biceps was removed in the duplicated limb (Figs 3, 4). Interestingly, in the duplicated limbs where both biceps muscles were present, the median position of the distribution was closer to that of the control motor pools. In these cases, we can be less certain of the identity of the motor neurones that formed the innervation of the duplicate biceps muscle. In the majority of cases, the mean motor pool positions in the transverse plane of the cord of the motor neurones innervating the second biceps copy were in a dorsolateral position. In the normal spinal cord, motor neurones innervating flexor and extensor digitorum are found in this position in the caudal spinal cord (Stephens & Holder, 1985), thus neurones innervating the second biceps may be from one or other distal muscle motor pool or from a mixture of both.

The maintenance of these inappropriate distal motor neurone connections within the second biceps over the period of the experiment is to be expected if axons from the correct motor neurones are absent.

Fig. 4. (A) Cross section of a control normal limb cut through the midpoint of the humerus. Bar, 500 µm. (B) Cross section of a right limb, duplicated by vitamin A, in which the biceps had been removed 78 days previously. The section was cut through the midpoint of the proximal upper arm showing that the biceps is absent. The normal position of the biceps is arrowed. Bar, 500 µm. (C) Cross section at a similar position to that shown in B in a limb which had the same experimental history. The arrow indicated a small amount of biceps remaining. Bar, 500 µm. (D) High-power view of regenerated biceps muscle fibres in a biceps removal/vitamin A-duplicated limb. Note the immature muscle fibres with central nuclei. Bar, 50 µm. a, anconeus; cb, coracobrachialis; b, biceps; h, humerus.
Competitive interactions between correct and incorrect axons for the formation of synapses must occur if incorrect axons are to be excluded from the muscle (Bennett & Raftos, 1977; Genat & Mark, 1977; Dennis & Yip, 1978; Holder et al., 1982); in the absence of such competition inappropriate synapses are maintained (Slack, 1978). Furthermore, recent evidence from our laboratory (Wilson & Holder, unpublished data) shows that competitive interactions between appropriate and inappropriate axons are unlikely to control the reformation of specific neuromuscular connections during limb regeneration.

In the control operation in which the forelimb was amputated but not treated with vitamin A, the majority of neurones innervating the regenerated biceps were located in the normal biceps pool position. However, a minority of neurones innervating the regenerated muscle were in an inappropriate, caudal spinal cord position (Fig. 1). This pattern is also seen during forelimb regeneration, in proximal muscles in the mature hindlimb regenerate, and during reinnervation of the transplanted hindlimb iliotibialis muscle in the intact limb (Stephens & Holder, 1987; Wigston, 1986; Wilson & Holder, unpublished data). This result indicates that a limited number of motor neurones that previously innervated distal limb muscles are able to make stable synapses with proximal limb muscles. However, as yet, no comparable connections between distal limb muscles and motor neurones previously innervating proximal limb muscles have been recorded.

The conclusion that choices for directed axonal growth at proximal limb levels are not influenced by cues emanating from distal limb tissues is consistent with a number of results from experiments using the developing chick limb bud. For example, appropriate proximal sorting of growing axons occurs in the absence of distal tissue (Tosney & Landmesser, 1984) and serially duplicated chick limbs show a similar pattern of innervation to that reported here, i.e. with duplicated distal muscles being nonspecifically innervated (Whitelaw & Hollyday, 1983). The evidence from work in the chick limb indicates that major highways, which are nonspecific trunk routes for growing axons, coupled with specific cues to which a limited population of axons are responsive, combine to generate a pattern of neuromuscular connections (Tosney & Landmesser, 1985; see review by Landmesser, 1984). It appears that similar principles apply to regenerating axons in the amphibian limb.

One immediate objective is to establish, using motor neurone pools as an assay of specificity, whether axolotl limb muscles in supernumerary limbs that are duplicated in the transverse axes are specifically innervated. Evidence from electrophysiological analyses implies that they are (Stephenson, 1979; Bennett & McGrath, 1980), suggesting that motor neurones can negotiate a passage through normally positioned limb tissues provided that no break occurs in these nerve pathways in the proximodistal limb axis.

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References


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