Regeneration in Crustacean Motoneurons: Evidence for Axonal Fusion

Abstract. *Crayfish motor axons remain excitable for over 100 days after severance from their central cell bodies, and continue to store and release normal amounts of transmitter substance. Evidence indicates that regeneration occurs by fusion of the central process with its surviving peripheral segment.*

When a vertebrate motor nerve is crushed or cut, the axon distal to the interruption degenerates within a few days and the portion still connected to the cell body later grows through the unoccupied sheath to reinnervate the muscle. Because reports have indicated that the first event requires a comparatively long period in arthropods (*1*, *2*), we have sectioned crayfish motor axons and followed the electrophysiological consequences in the muscle fibers they innervate. The results suggest a mechanism quite different from that in vertebrates, involving repair of the break rather than a regrowth of the distal apparatus.

The opener muscle of the crayfish claw is innervated by a single motor axon, which was experimentally interrupted by opening the meropodite of *Procambarus clarkii* and cutting the nerve trunk or crushing it between fine forceps (Fig. 1). Operated animals were marked, kept in tap water in large plastic trays at approximately 16°C, and tested regularly for recovery of reflexive claw opening, a reliable behavioral test for regeneration that utilizes visual and tactile inputs outside the claw itself. Animals that did not show regeneration were tested at various intervals to determine the course of degeneration in the severed peripheral axons. Claws were removed at a proximal joint and the entire meropodite was opened in van Harreveld's solution to expose the nerve bundle for electrical stimulation, as shown in Fig. 1. Junctional potentials from opener and stretcher muscle fibers were recorded with 3*M* KCl-filled microelectrodes connected to a neutralized-capacity preamplifier and thence to conventional oscillographic equipment. The original operative procedures were as follows. In cutting, both thick and thin nerve bundles in the meropodite were severed with scissors. After the cuts were made, peripheral and central ends of these nerves could be seen to pull apart; they were usually separated by several millimeters after the operation. Crushes were made by sharply pinching both bundles with fine forceps, usually under 40× magnification. The fibers within each bundle immediately drew apart, leaving approximately 1 mm of translucent empty sheath at the site of the crush. Thirteen such crushed preparations were subsequently tested before regeneration had occurred to ensure that the opener axon is indeed interrupted by the standard crushing procedure. Electrical stimuli were applied to the thin bundle, which always contains the opener axon, at a point distal to the lesion site and at another point proximal to it. In each of these cases, distal stimulation was effective in evoking excitatory junctional potentials in opener muscle fibers, and proximal stimulation was ineffective. This test was applied to all of the crushed preparations described below to insure that the opener axon had been severed in the original procedure.

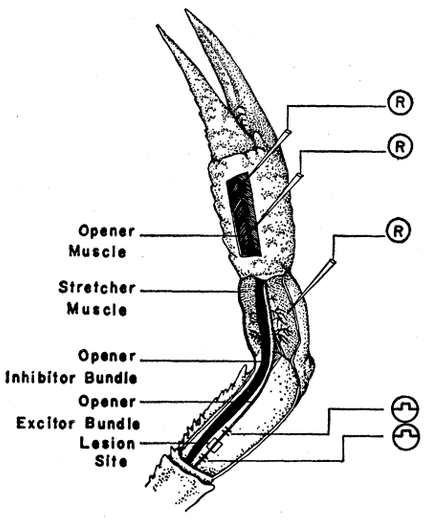

Fig. 1. The experimental arrangement for electrical stimulation of the opener axon in the meropodite; the right claw of *Procambarus clarkii* is shown. *R*, recording sites for intracellular microelectrodes in opener muscle fibers; square-wave symbols, stimulation sites in the meropodite.

The time course of degeneration in severed distal segments was analyzed by using animals in which the nerves were cut instead of crushed. Eighteen such animals that failed to show regeneration were examined at various times after the cutting operation by electrically stimulating the opener axon distal to the lesion. In each case, stimulation proximal to the lesion had failed to produce junctional activity in opener muscle fibers, confirming the lack of continuity in the opener axon at the site of the original cut. Table 1 summarizes the results. All animals tested before 100 postoperative days showed normal junctional potentials in opener muscle fibers in response to stimulation of the distal thin bundle. The frequency and amplitude of spontaneous miniature junctional potentials and their facilitation ratios were all indistinguishable from those in intact preparations (Fig. 2). In one of the experiments, a nerve that had been severed 90 days earlier was continuously stimulated at 20 pulses per second for over an hour, without abnormal diminution in amplitude of junctional potentials. Our results thus indicate that even long disconnection from cell body and nucleus imposes no deficit upon the capacity of the nerve terminal to release transmitter quanta. Similar results were obtained from the distal segments of crushed axons that failed to show regeneration.

The degenerative changes observed in the three animals that appear in the right-hand column of Table 1 involved nearly complete electrical silence in the opener muscle fibers. In the 160-day-old preparation, the resting potentials of the muscle fibers were

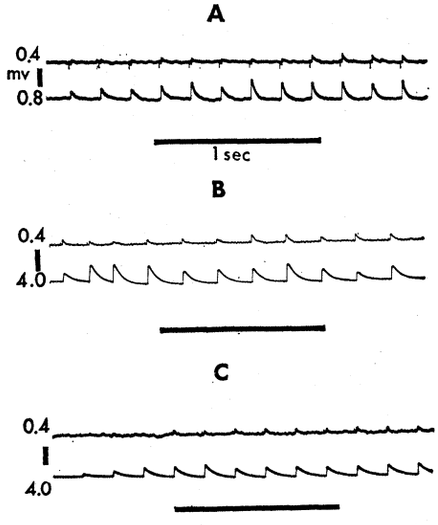

Fig. 2. Junctional potentials recorded intracellularly from opener muscle fibers in response to stimulation of the opener axon at 5 pulses per second. (A) Control preparation not previously operated. (B) Regenerated crushed preparation 31 days postoperative with the point of stimulation proximal to the lesion. (C) Nonregenerated cut preparation 51 days postoperative, with stimulation applied to the distal trunk. The central stump was also stimulated, and produced no electrical activity in the opener muscle.

Table 1. Survival of the opener motor axon in cut preparations.

Days post-operative	Number tested	Number degenerated
0 to 20	5	0
20 to 40	2	0
40 to 60	3	0
60 to 80	0	0
80 to 100	2	0
100 to 120	3	2
120 to 140	0	0
140 to 160	3	1

reduced by approximately 20 percent.

When it occurred, regeneration was completed in a substantially shorter time than that required for the severed peripheral axons to degenerate. Table 2 shows that cut nerves required, on the average, more time than crushed ones, and that their percentage of recovery was lower. The results of regeneration were, however, similar for both types of operations: though on the first few days of recovery opener contractions sometimes appeared weak, regenerated preparations were indistinguishable (Fig. 2) from intact ones by electrophysiological criteria, that is, normal and miniature junctional potentials as well as normal facilitation were observed. These data were obtained by applying electrical stimulation at a point *proximal* to the original lesion site. Successfully regenerated animals showed intact nerve trunks that appeared normal in the region of the meropodite where the lesion had been made. In failures examined 50 to 90 days after the operation, only limited outgrowth from the central stump was found.

These facts suggested the possibility that regeneration might result from fusion of the central axonal process with its surviving distal segment. Such an alternative to reinnervation by centrifugal outgrowth would lead to two expectations: (i) only a single excitatory axon should be found innervating the muscle at the time function is recovered; (ii) innervation should be reestablished simultaneously at proximal and distal points, rather than sequentially.

We have tested for the presence of "extra" innervation on the first and subsequent days of reflex recovery. Since all these animals were used on or before the 45th postoperative day, degeneration of the peripheral portion of the original axon would not have been expected. The bundle containing the opener axon was electrically stimulated distal to the point of the original lesion in one cut and 11 crushed preparations; in no case could two sizes of junctional potentials, indicative of activity in two motor axons, be produced. Methylene blue staining of eight newly regenerated crushed preparations, moreover, always revealed only the two axons (excitor and peripheral inhibitor) characteristic of normal animals.

The opener motor axon also innervates the stretcher muscle. The proximal stretcher fibers are about 20 mm from the proximal opener fibers, which in turn are 10 to 20 mm from the distal-most opener muscle fibers (Fig. 1). Regrowth of the vertebrate pattern should produce a proximal-to-distal sequence of reinnervation. In a series of eight animals that had undergone the crushing operation and had recovered weak opener reflexes on the day of the experiment, activity in distal and proximal opener fibers was recorded simultaneously while stimulating distal to the lesion. In all cases, junctional potentials appeared in both distal and proximal fibers at the same threshold. Animals (16 cut, 15 crushed) were also examined for return of stretcher innervation before opener reflexes had appeared. In no case was there electrical activity in stretcher muscle fibers when the nerve bundle was stimulated proximal to the lesion site. Behavioral observations made at weekly or biweekly intervals on large numbers of animals (59 cut, 77 crushed) failed to reveal a single instance in which functional recovery of the stretcher muscle preceded that in the opener.

The results indicate that the developmental events underlying axonal regeneration are quite different in crayfish and vertebrates. Studies on cockroach motor nerve regeneration (*3*), however, suggest that regeneration by fusion is not universal among arthropods. When cockroach motor nerves are cut the separated distal segment degenerates rapidly, resulting in complete junctional silence within 3 to 5 days. Regeneration occurs by sprouting from the intact central stump; its time course is quite similar to that for crayfish (*4*). Crushed motor axons regenerate faster (within 15 days) and in a higher percentage of cases (100 percent) than cut motor nerves (about 36 percent regenerate in 30 or more days). Locusts are intermediate in degeneration rate (*2*); normal junctional transmission persists for about 1 week after motor nerves are severed, and abnormal junctional responses can be demonstrated for about 3 weeks before ceasing altogether.

Table 2. Regeneration of crushed and cut axons.

Days post-operative	Regeneration ratio	
	Crushes	Cuts
0 to 10	1/69	0/37
10 to 20	18/46	0/36
20 to 30	35/58	0/27
30 to 40	37/58	2/23
40 to 50	32/48	3/20
50 to 70	33/46	4/16
70 to 90	36/45	4/16
90 +	35/44	5/16

That a severed peripheral axon should survive for 3 months raises questions about the source of metabolic maintenance, especially in view of the doctrine that the cell body is the trophic center of the neuron. Crayfish axons, like many other invertebrate axons, are invested with a sheath of glial cells, which could be the source of metabolic support for the distal segment. There is evidence (*5*) that in vertebrates glial cells are capable of transporting metabolites from the surrounding environment into axons, and biosynthetic support may be provided from exogenous sources in the present case as well.

It is uncertain why two kinds of regenerative mechanisms appear in arthropod nervous systems. Both are capable of restoring normal function, and both require equivalent specificity of cell recognition. Repair by fusion clearly offers two advantages: it is less expensive metabolically, since so much less resynthesis is required; and it demands only a single correct reconnection rather than the reassembly of an entire set of peripheral terminals.

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References and Notes

1. C. A. G. Wiersma, in *Physiology of Crustacea*, T. H. Waterman, Ed. (Academic Press, New York, 1961), p. 258.
2. P. N. R. Usherwood, *J. Insect Physiol.* **9**, 811 (1963).
3. J. W. Jacklet and M. J. Cohen, *Am. Zoologist* **6**, 526 (1966).
4. D. Bodenstein, *J. Exptl. Zool.* **136**, 89 (1957); J. F. Case, *J. Insect Physiol.* **1**, 85 (1957).
5. M. Singer and M. M. Salpeter, *Nature* **210**, 1225 (1966).
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