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DEVELOPMENT OF SENSORY PROCESSES DURING LIMB REGENERATION IN ADULT CRAYFISH

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Summary

The capacity of the crayfish *Procambarus clarkii* to regenerate its walking legs provides a system for studying the mechanisms of neural regeneration and repair. A set number of excitatory and inhibitory motor neurons innervate all the limb musculature throughout the normal development and regeneration of a limb. The cell bodies of the motor neurons reside within the segmental ganglion and, upon loss of the limb, their axons regrow from their severed distal ends. The cell bodies of the sensory neurons, in contrast, are located close to their sensory endings within the limb, and they are therefore lost, along with the limb, upon autotomy, leaving the severed, distal axonal stumps of the sensory neurons within the ganglionic root. During the regeneration of a limb, new sensory neurons develop within the limb, and their axons must then grow into the ganglionic root to make the appropriate connections for the new limb to become functional. Evidence is presented in the present paper that the sensory axonal stumps do not degenerate before the new sensory neurons appear within the root as the limb regenerates. These results also indicate a progressive advance of growth cones, presumably sensory in origin, towards the neuropil within the ganglion over time.

Key words: sensory, axonal, synapse, synaptotagmin, regeneration, development, autotomy, crayfish, *Procambarus clarkii*.

Introduction

Many crustaceans can autotomize and regenerate their limbs from juvenile to adult stages. The rate at which the limbs regenerate is dependent on the molt cycle (Morgan, 1900; Zeleny, 1908; Bliss, 1960; Skinner 1962, 1985). Self-induced autotomy occurs at a particular site at the base of the limb, the fracture plane of the basi-ischium segment (Bliss, 1960; Adiyodi, 1972; Moffet, 1975), where a new limb can form. However, the motor cell bodies are located in central nervous system (CNS) ganglia, so that autotomy removes only the distal processes of motor axons, which can regenerate from the proximal stumps. Since the cell bodies of sensory neurons are located in the limbs near their sensory dendrites, autotomy removes sensory nerve cell bodies and their proximal axons. Regenerating limbs must therefore develop new sensory cell bodies, which must grow axonal processes.

Relatively few motor neurons innervate limb muscles in crustaceans. For example, the entire opener muscle in crabs, lobsters and crayfish is innervated by only three motor neurons (Wiens, 1989). In contrast, the types and numbers of sensory neurons within an adult limb are much greater. For example, different sensory neurons monitor hair displacement (Laverack, 1987), cuticular stress (Libersat *et al.* 1987), muscle tension (Cooper and Hartman, 1994) and joint position and movement (Whitear, 1962; Cooper and Govind, 1991; Hartman and Cooper, 1994).

Following autotomy, the distal axon stumps of the sensory neurons and the proximal regions of the motor axons remain in the nerve trunk proximal to the fracture plane. During limb regeneration, sensory neurons must grow and re-establish the appropriate connections with the neural network of the ventral nerve cord if the new limb is to exhibit its original function. Newly forming sensory neurons might reconnect to the appropriate surviving sensory axon stumps in the original nerve trunk. Alternatively, the original axons may act as guidance cues to direct the growth cones into appropriate locations within the ganglion, allowing for the formation of entirely new ganglionic connections. Determining whether the proximal stumps of original sensory axons degenerate before the new sensory processes grow into the nerve trunk can help to discriminate between these possibilities. In this study, the antibody to synaptotagmin, a molecule associated with neurotransmitter-containing vesicles, provided a marker for new sensory neurons projecting into the original nerve stump (Cooper *et al.* 1996).

Previous studies have reported that, after repeated autotomy of a lobster limb through several molt cycles, there is a substantial reduction in the number of axons in the remaining ganglionic root (Govind *et al.* 1988), suggesting that the original sensory axons within the root degenerate over time when they lose their cell bodies in the autotomized limb. This

process does not appear to be rapid enough, however, to occur within a single molt cycle during limb regeneration. Possible mechanisms of distal stump survival for large motor axons have been discussed (Bittner, 1973), some of which may apply to sensory axons. The fusion of regenerating sensory neurons to the sensory stumps does not appear to be advantageous, as mentioned by Bittner and Johnson (1974), since the appropriate central connections would not be established. Both the results of the study of Bittner and Johnson (1974), in which the main nerve within the limb was severed distally, and the present results suggest that the sensory neuron regenerates to the CNS to re-make synapses with the appropriate targets so that functional use of the limb is restored.

Materials and methods

All experiments were performed on the first and second walking legs of crayfish *Procambarus clarkii* measuring 6–10 cm in body length (Atchafalaya Biological Supply Co., Raceland, LA, USA). The animals were housed in an aquatic facility and fed fish food pellets. Dissected preparations were maintained in crayfish saline, a modified van Harreveld's solution (containing, in mmol l^{-1}: 205 NaCl; 5.3 KCl; 13.5 $CaCl_2 \cdot 2H_2O$; 2.45 $MgCl_2 \cdot 6H_2O$; 0.5 Hepes adjusted to pH 7.4). During dissection of the thoracic nerve cord, the bathing medium was exchanged every 20 min with saline at 14 °C.

Individually tagged crayfish in their intermolt period were induced to autotomize a walking leg by pinching the merus segment and returning the crayfish to individual holding tanks for monitoring. The thoracic ventral nerve cord was removed by dissecting it from a dorsal approach. The ganglionic roots to the limbs were dissected to the breakage plane in both the regenerating and the control limbs, as illustrated in Fig. 1. The roots were dissected free, as far as possible, to the coxal base of the limbs to obtain an intact root very close to the breakage plane. In cases where a limb was developing within the papilla at the coxopodite–basipodite segment, a soft-bodied tissue resembled the folded distal segment of a regenerating limb. The developmental stage of the forming limb bud in the papilla stage is difficult to determine because it is enclosed within the base of the limb and, therefore, the degree of limb regeneration was measured in relative terms among identified limb buds. Measurements were made of limb bud structures from calibrated photographic prints.

Immunocytochemistry

The isolated thoracic nerve cord and its roots were pinned in a Sylgard dish as shown in Fig. 1. When the nerve cord was in place, the preparation was fixed [in 2.5 % (v/v) glutaraldehyde and 0.5 % (v/v) formaldehyde, dissolved in a buffer containing 0.1 mol l^{-1} sodium cacodylate, 0.022 % (w/v) $CaCl_2$ and 4 % (w/v) sucrose, adjusted to pH 7.4] for 1 h with two changes of solution. The preparations were then placed into vials and washed in buffer containing 0.2 % (v/v) Triton X-100 and 1 % (v/v) normal goat serum for 1 h with three changes at room temperature (19–21 °C). Antibody had previously been raised against the synaptic vesicle protein synaptotagmin from *Drosophila melanogaster* (Littleton *et al.* 1993). The tissue was then placed into the primary antibody (1:1000 of DSYT-2 in phosphate-buffered saline, PBS) and placed on a shaker at 4 °C for 12 h. The vesicle locations can be observed by immunocytochemistry, as previously shown in nerve terminals (Cooper *et al.* 1996). The tissue was washed three times and incubated in secondary antibody (goat anti-rabbit FITC-conjugated IgG) diluted 1:200 with PBS at room temperature for 2 h followed by two washes in buffer with two changes.

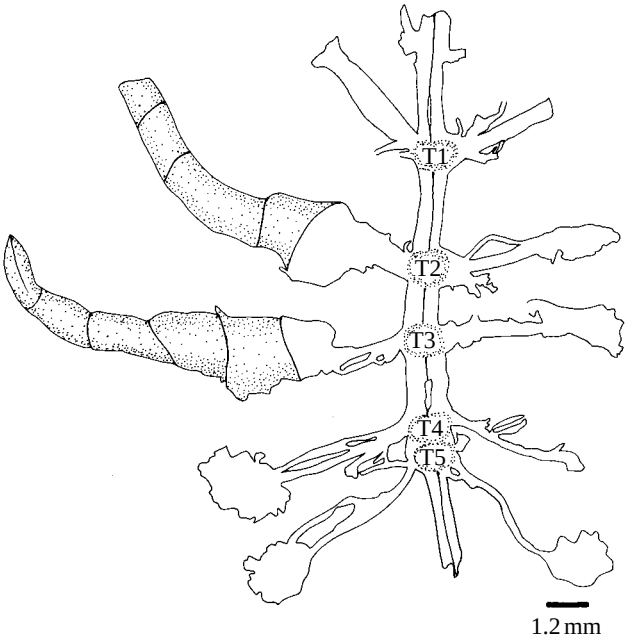

Fig. 1. A dorsal view of the thoracic nerve cord dissected out of a crayfish but with two limbs still attached. The third left limb (second walking leg; ganglion T3) is a regenerated limb after one molt, at stage B. The second left limb (first walking leg; ganglion T2) is a regenerated limb after two molts. The roots were dissected free up to the autotomy plane. Note that the nerve bundles spread out and intermingle in a network of connective tissue at the autotomy plane.

Fluorescent measurements of the nerve roots and ganglia were obtained with a Nikon Optiphot-2 upright fluorescence microscope using a 40× (numerical aperture 0.55) Nikon water-immersion objective (as shown in Fig. 3A). Photographs were taken with either a 2.5× or a 5× tube lens. Since many of the fluorescing neural processes traverse the ganglionic roots, photographs were taken at various focal planes to follow particular neurons that appeared to contain strings of varicosities separated from adjacent varicosities by less brightly stained regions.

The areas of the ganglionic roots containing immunoreactive varicosities were marked with fluorescent beads. This was accomplished by repeatedly dipping the tip of a fire-polished, sealed electrode into a slurry of fluorescent polystyrene beads (0.5 μm diameter, Duke Scientific Co.) allowing the tip to dry in air between dips. The electrode was gently washed with van

Harreveld's solution, to ensure that any loosely affixed beads were removed, and was then applied to the tissue to deposit beads at the site of interest. The location was then rephotographed with the beads in place (see Fig. 3). The tissue was subsequently processed for electron microscopy (Jahromi and Atwood, 1974; Cooper *et al.* 1995) (see Fig. 3A–C).

Electron microscopy

Tissues were fixed for 15 min, as described above, and then washed in PBS for 2 h with two changes. Post-fixation was performed for 1 h in 2 % osmium tetroxide in buffer, followed by three rinses in PBS. The tissue was then dehydrated using a graded ethanol series (50 %, 70 %, 80 %, 90 %, 95 %, three times 100 %) and embedded in an Araldite/Epon resin. The fluorescence of the beads persisted even after fixation, osmium staining and resin embedding of the specimen, and the beads were identified in thin sections. The bead-marked area was serially sectioned and then viewed using a Hitachi H7000 electron microscope.

Axonal measurements

Thin sections of the ganglionic roots were photographed at 1500× and printed at 2.5× to form montages. The maximum and minimum diameters of axons were measured. The mean diameter of an axon was calculated as the square root of the product of the maximum and minimum diameters.

Results

Anatomy

Regenerating limbs at various stages were used in this study (Fig. 2). The earliest limbs (A) were in the papilla stage (Fig. 2A), in which a swelling of the flexible cuticle contains the delicate limb bud until the animal molts. Most of the distal segments, carpus–propus–dactyl, can be resolved in the next stage, B (Fig. 2B). Regeneration proceeded in two further stages, stages C and D (Fig. 2C,D), as shown by the fact that it was possible to elicit reflexes in the distal chelated segment and by the clear anatomical correspondence with the segments of the control leg (Fig. 2E). The limb shown in Fig. 2C was fixed 5 days after the first molt following autotomy, whereas the limb shown in Fig. 2D was fixed 5 days after the second molt.

Development of the sensory axons in the root

Counts of axon profiles (marked as shown in Fig. 3A–C; see Materials and methods) in the sectioned roots of both control (Fig. 3D, upper section) and newly regenerating (Fig. 3D, lower section) limbs showed no reduction in the number of sensory axons prior to new sensory processes invading the root. Mitochondrial profiles could be seen around the inside periphery of the larger axons in both regenerating and control limbs. The distributions of the axon diameters were measured to determine whether differences could be seen between the normal (Fig. 4A) and regenerating (Fig. 4B) limbs. To resolve the distribution of the smallest axon diameters, these were plotted with smaller bin sizes (Fig. 4, insets).

Fig. 3D shows axon profiles in cross sections taken near the ganglion, from a root innervating a normal limb (top) and from a regenerating limb at stage B (bottom). At a somewhat later stage of regeneration, stage C, the total number of axon profiles in the regenerating limb (5417) did not differ from that in a control limb (5362). Separating large axon profiles into groups of axons either larger or smaller than 8 μm in diameter showed little difference between a control limb (140 larger than 8 μm; 5222 smaller than 8 μm) and a regenerating limb (163 larger than 8 μm; 5417 less than 8 μm).

Immunocytochemistry

The segmental ganglion, the connectives joining it to adjacent CNS ganglia and the regions of the ganglionic roots from the point at which they exit the ganglion to the coxal region of pristine or regenerating limbs were examined for immunoreactivity to the antibody to synaptotagmin (see Materials and methods). Special attention was given to structures that appeared as positively stained varicosities (swellings in the nerve terminals) with a gross morphology similar to that found at tonic excitatory crayfish neuromuscular junctions (Cooper *et al.* 1995, 1996). The neuropil within the ganglia stained intensely (data not shown). Interneurons and sensory axons have their terminal synaptic contacts in this region.

The number of terminals (or strings of varicosities) present in the outer layers of the roots correlated with the stage (A–D) of the regenerating limbs. To examine the staining along the root in relation to limb formation, various regions were viewed

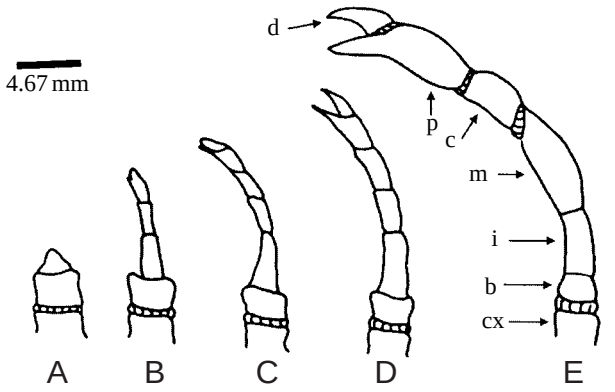

Fig. 2. The stages of limb regeneration used in this study. (A) Papilla stage (stage A), which occurs approximately 1–2 weeks after limb loss. (B) Stage B: in this early stage of limb formation, the limb bud is very soft and is just beginning to take on some of the most distal features of the new limb. (C) Stage C: the most distal features of the limb are well differentiated, although the chelated end cannot open or close. The more proximal segments can be discerned. (D) Stage D: all the leg segments are discernible and the muscles have some function since movements can be elicited. The chelated end has tactile reflexes that result in opening and closing. (E) Stage E: a representation of a typical pristine limb on an animal of the size used throughout this study. d, dactylopodite; p, propodite; c, carpopodite; m, meropodite; i, ischiopodite; b, basipodite; cx, coxopodite.

as shown in Fig. 5A. In the papilla stage (stage A), immunohistochemical staining revealed few terminals, except in the region of the ganglionic root lying within the coxal segment and nearest to the autotomy plane (Fig. 5B). In the next stage of regeneration (stage B), there were more varicosities within the roots near the base of the regenerating limb (Fig. 5C) and more isolated varicosities closer to the ganglion along the root, but not in the connectives. At even later stages, immunoreactive varicosities were observed along the entire length of the root (Fig. 5D), and a few strings of varicosities occurred in the cord connectives of the ganglion innervating the regenerating limb (Fig. 5E). Furthermore, the number of varicosities increased in the mid-portion of the root, and many processes showed extensive branching (Fig. 5F). Processing the tissue for immunohistochemistry with Triton X-100, followed by a second fixation and processing for electron microscopy, caused a reduction in contrast and in the sharpness of membrane staining (Fig. 6) compared with tissues processed directly for electron microscopy (note the synapse-like structures with pre- and postsynaptic thickenings; Fig. 6, arrows). The tenuous conformation of the postsynaptic structure shown in Fig. 6 suggests that it is probably a glial cell.

Discussion

By the time reflexive movements are observed in a regenerating limb, new sensory processes must have made contact with the original sensory neurons or with motor neurons in the ganglionic neuropil. Our results show that these connections are established before the original sensory neurons are degraded. The synaptic structures, such as those shown in Fig. 6, are similar to those observed in the growth cones of developing

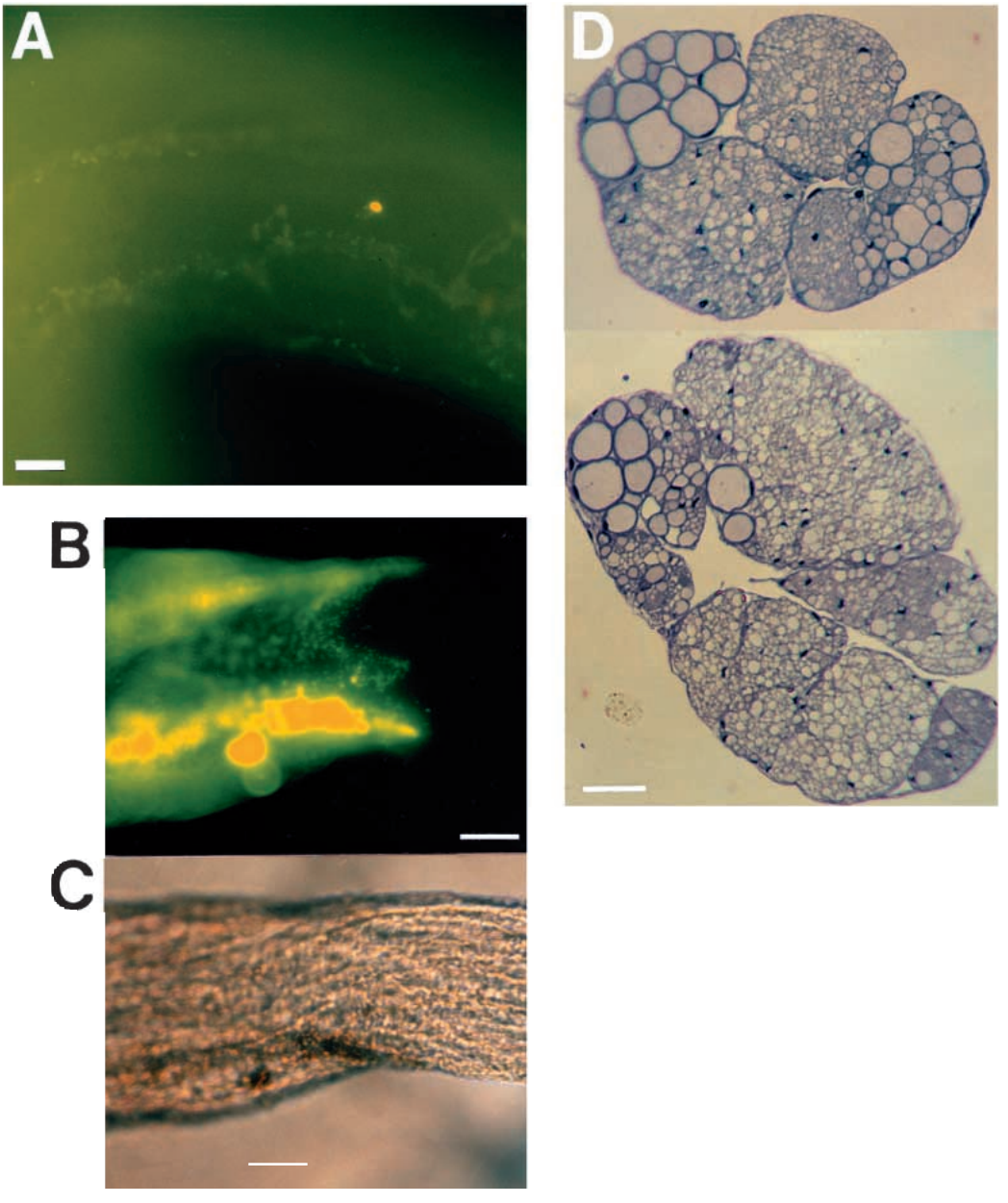

Fig. 3. Photographs of limb roots close to the breakage plane. (A) Immunohistochemical staining with an antibody raised against synaptotagmin. This fluorescence micrograph shows many processes stained within the root in this advanced stage (stage D). The varicose processes appear as the faint yellow beaded structures seen throughout the tissue. The very bright yellow spot is a fluorescent bead. (B) A region containing varicosities, as shown in A, marked with fluorescent beads located around the root. (C) A transmitted light view of the marked root shown in B, which was then processed for immunohistochemistry. (D) Thick sections taken close to the breakage plane showing the main leg nerve for a control limb (top) and for a regenerating limb at stage B (bottom). Note the large variation in axon diameter within each section. Scale bars: A, 50 μm; B, C, 350 μm; D, 50 μm.

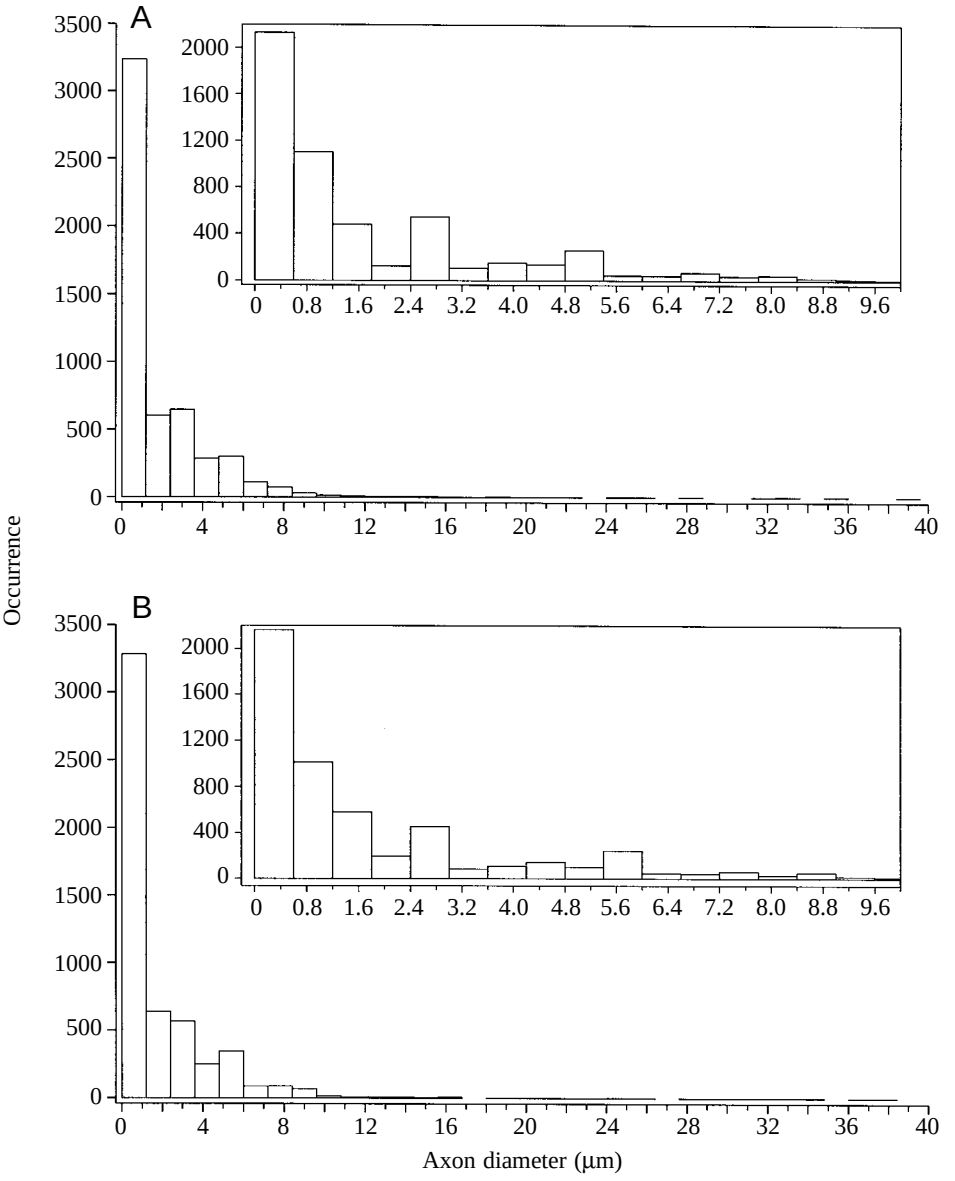

Fig. 4. Distribution of axon diameters in the ganglionic root (T2) of a pristine control limb (A) and of a contralateral regenerating (B) first walking leg at the stage at which a small limb bud is present (stage B). The samples were taken from the root close to the breakage plane just proximal to where it becomes wrapped in the mass of connective tissue associated with this region. Measurements were made from montages of electron micrographs at a final magnification of 3750 times. The bin size is 1.2 μm (0.6 μm for the insets).

neurites (Letourneau, 1979) and to varicosities observed at the neuromuscular junctions, which contain patches of synapses. In addition, the string-like varicosities observed by fluorescence microscopy may, in fact, be growth cones of multiple sensory neurons in close apposition, appearing at the light microscopic level as a single terminal with repeated swellings along its length.

Since the number of immunoreactive varicosities decreased in the distal part of the ganglionic roots, but increased in the more proximal regions, as the regenerating limb approached the size of the pristine limb, the terminals appeared to continue growing until they reached the ganglion instead of fusing with the sensory stumps in the root. In addition, in regenerating limbs that showed reflexes (e.g. advanced regenerative stage), immunoreactive regions of terminals were present within the ventral nerve cord connectives closest to the ganglion, possibly associated with the regenerating limb. These results indicate that the developing sensory neurons in the regenerating limb continue to grow towards the central connectives.

It has been shown that axons separated from their cell body, but in close contact with the proximal intact axon, survive for years (Bittner, 1988). This survival occurs by the exchange of nutrients through exocytotic and endocytotic processes with other neurons and possibly with glial cells (Bittner, 1988, 1991). On the basis of the tracing of labeled proteins in neighboring cells, it has even been postulated that there might be an exchange of proteins. In addition, radioactively labeled amino acids injected into one axon were shown to be present in the targeted, coupled axon, thus providing strong evidence of nutrient support for the isolated axonal process (Bittner, 1988). The survival of the distal stumps probably occurs by an endocytotic process in which cellular nutrients are gained from surrounding glial cells, as has been shown to occur in the abdominal nerve cord of crayfish (Bittner, 1988, 1991). Following limb autotomy, the isolated sensory axons survived long enough for the initial sensory neurons in the regenerating limb to reach their central connections, but over a longer period

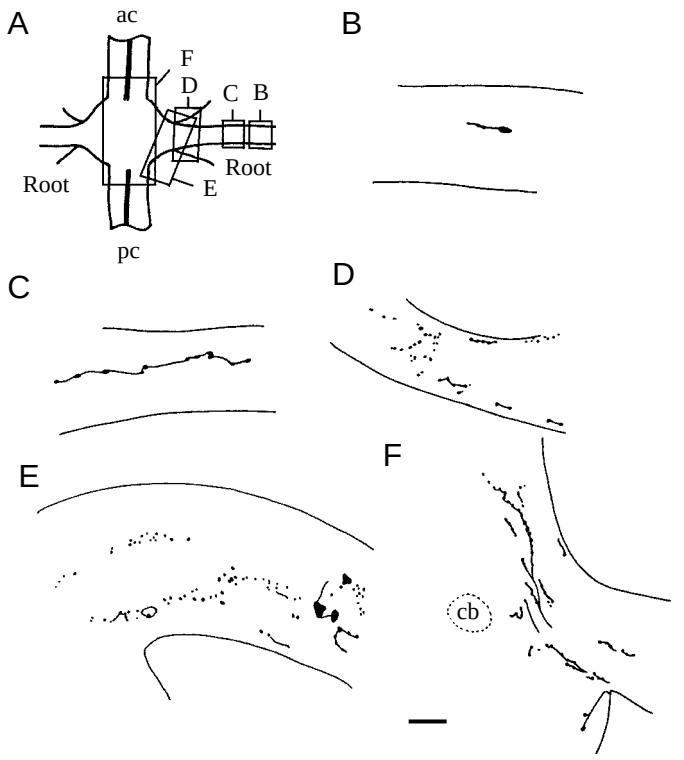

Fig. 5. Tracings of immunohistochemically stained preparations of sensory processes of the ganglionic roots and connectives of the nerve cord at various stages in limb regeneration. All traces were made directly from photographs. (A) A schematic of a ganglion and root to illustrate the region of each view. (B) Few processes are stained within the root in the papilla stage (stage A) and, when staining is present, it is restricted to the most distal regions. (C) In stage B, there is an increase in the presence of varicosities along the middle length of the root. (D) In a regenerating preparation after one molt, immunoreactive processes can be seen in the most proximal region of the root next to the base of the ganglion. (E,F) At the most advanced stage (stage D), large numbers of varicosities can be seen within the root and in the connectives between ganglia. The abbreviation 'cb' denotes a cell body of a motor neuron close to the base of the root at a stage (stage D) when the limb appears to be fully functional. The root was photographed from a dorsal view in each region. ac, anterior connective; pc, posterior connective. Scale bar: A, 700 μm; B–E, 100 μm.

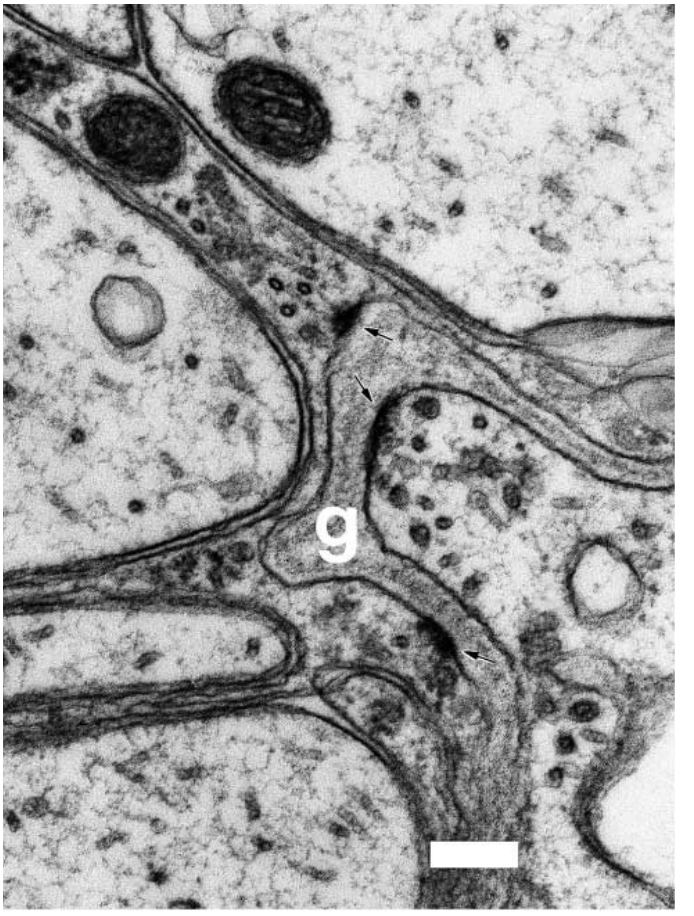

Fig. 6. A representative electron micrograph from a series to determine whether the varicosities observed immunohistochemically contain synaptic regions. The pre- and postsynaptic thickenings are labeled with arrows. The postsynaptic structure labeled g is probably a glial cell. Scale bar (shown as a blank space), 200 nm.

there may have been a loss of sensory stumps. A reduction in sensory neuronal number with repeated autotomy of the same limb in lobsters has been demonstrated. This resulted in a substantial loss of axon profiles, which appeared to be sensory neurons, in the associated ganglionic roots to the autotomized limb (Govind *et al.* 1988). This identification was supported by the fact that most of the limb muscles were innervated by very few motor neurons and that there was a set number of motor neurons to innervate all the limb musculature.

During the normal development of the limbs, there is an increase in the number of sensory neurons but not of motor neurons. In crabs, the number of sensory neurons associated with the homologous proprioceptive chordotonal organ continues to increase up to the late juvenile stages, but not into adult stages (Hartman and Cooper, 1994). In lobsters, as in the crab, the sensory neurons associated with the analogous chordotonal organ continue to increase in number throughout the entire life of the animal (Cooper and Govind, 1991).

The ability to regenerate entire limbs after autotomy depends on the presence of the stump of the ganglionic root at the base of the regenerating limb (Needham, 1945, 1947). Since it is now known that the cell bodies of the sensory neurons are lost with the autotomized limb, the implication is that the motor neurons may be influential in the early stages of limb regeneration, and it has been suggested that trophic factors released from the nerves affect regenerating limbs (Carlone and Mescher, 1985). The time at which the new sensory neurons become functional in regenerating limbs has not been well characterized. Their synaptic efficacy may be somewhat different from that of the previous pristine axons so, even though the terminals reach the central locations by regenerative stages, as shown in Fig. 2C, the degree of synaptic communication may not fully reproduce that found in an intact limb of the same developmental size.

The use of immunoreactivity to identify and locate regions where processes are growing is a relatively easy, but indirect, approach to assessing regeneration of sensory and motor neurons in a variety of systems. The antibodies to synaptotagmin used in this study have proved useful for locating sensory nerve terminals growing in the ganglionic roots of regenerating limbs. Although the staining pattern was similar to that seen at crustacean and *Drosophila melanogaster* neuromuscular junctions, in that the varicosities were highlighted, there was no punctate staining within the varicosities (Cooper *et al.* 1995, 1996). This was due to the presence of vesicles in regenerating axons, which added to the growth cone but were not necessarily used at defined synapses. Since synaptotagmin is a protein specifically associated with vesicles, immunoreactivity will indicate where vesicles are located during axonal regeneration and development. Synaptotagmin has been shown to be abundantly expressed in growth cones and axons before synapse formation has occurred (Littleton *et al.* 1995).

Synapses visualized with electron microscopy were only visible within the ganglionic roots associated with the regenerating limbs. Extensive viewing of serial sections of ganglionic roots on the contralateral side, which contained a normal limb, did not reveal any synapse-like structures among the axons. The histochemical results showing that stained varicose structures are present in the roots of regenerating limbs and are absent in the roots of pristine limbs were supported by extensive examinations of thin sections using electron microscopy. Although the commonly observed synaptic ultrastructure of the tissue processed for electron microscopy was not as clear after prior immunohistochemical processing, this sequential process allowed direct electron microscopic assessment of regions that had been shown to contain sensory processes. It is possible that, by modifying the incubation times and the amount of permeabilization of the tissue during antibody labeling, the membranous structures could be better preserved for electron microscopy.

In summary, the regeneration of sensory processes was followed using immunocytochemical procedures, and the finding that growth extended more proximally over time suggested that the sensory processes grew into the ganglionic neuropil to re-establish appropriate CNS connections.

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