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TINKERING WITH SUCCESSFUL SYNAPSE REGENERATION IN THE LEECH: ADDING INSULT TO INJURY

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SUMMARY

In the leech, synapse regeneration in adults and synapse formation during embryonic development can be studied in single, identifiable cells that make precise connections with their targets. Certain cellular components, such as synaptic targets and glia, were selectively destroyed to study how the regenerating axons locate their targets, what triggers axons to start growing and what stops them. The results showed that glia and targets play only a limited role in synapse regeneration and in axon degeneration. For example, contact with the synaptic target may inhibit sprouting and availability of targets may promote it. Comparative studies on axon growth and synapse formation by interneurones in embryos showed that regeneration does not simply recapitulate embryonic development. There are clearly separate constraints on the two processes. Axon survival is a different problem. Although isolated axon segments can survive for up to a year in the leech, temperature is a major factor in survival. Axon segments in a tropical leech that regenerates synapses well at 31°C degenerated within 2–3 weeks at this elevated temperature, even when regeneration was prevented. In similar leeches at room temperature (22°C), segments survived for months. Overall, results in the leech support the idea that degeneration as well as regeneration share fundamental mechanisms with other invertebrates and the vertebrates, including mammals. Perhaps long-lived axon segments and other features of the leech that speed or encourage functional regeneration can now be made to operate in repair of the mammalian nervous system.

INTRODUCTION

Neurones in the leech send out axons to connect with their synaptic targets during regeneration as accurately as during embryonic development, although regeneration may be incomplete. Such precise reconnections between neurones was, in fact, first shown in the leech. Functional synapses regenerate between leech touch (T) sensory cells (Baylor & Nicholls, 1971) and between pressure (P) and nociceptive (N) sensory cells and particular motoneurone targets (Jansen & Nicholls, 1972). There is no physiological evidence that 'mistakes' are made by the regenerating axons. While

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regeneration cannot simply recapitulate development, the two share certain processes essential for successful synapse formation.

This paper discusses recent experiments in the leech on the role of particular cellular components in successful reconnection between neurones after injury and compares the results with new findings on axon growth and synaptogenesis in the embryo. The questions addressed include the following. How do regenerating axons in the leech find their normal synaptic targets and, importantly, is there similar regeneration (and degeneration) in mammals? Relevant related questions are as follows. What triggers and what limits axonal sprouting? As one might imagine, there are only a few definitive answers, but a number of intriguing results have been obtained.

The central nervous system of the leech is a chain of ganglia that runs the length of the animal. Each ganglion contains only about 400 neurones and eight large neuroglial cells. Axons emerging from the ganglia form bundles, two on each side, called roots, that innervate the periphery, and paired bundles, called connectives, that link the ganglion with each of its two neighbours. In adult leeches, ganglia are several millimetres apart, and several ganglia at the head and tail of the animal are fused to form 'brains' (for a complete description see, for example, Muller, Nicholls & Stent, 1981). One feature of the leech that has made it favourable for study is that sensory cells and other neurones normally and reliably extend axons towards adjacent ganglia and synapse with certain neurones there. Injections of horseradish peroxidase (HRP) into growing and regenerated neurones reveal what path the axons take as well as where they contact their targets. By a variety of manipulations, including selective deletions of targets, glia or other cellular elements, we have learned about the capabilities of regenerating neurones.

AXON PATHWAYS IN SENSORY NEURONE SYNAPSE REGENERATION

Leech neurones synapse with each other in the neuropile, where there are no obvious landmarks that we can use to chart the accuracy of growth. Fortunately, the axons and secondary branches of sensory cells of each modality lie near one another, so that axons of T cells from one ganglion, for example, extend along the axons of T cells in adjacent, target ganglia. Secondary branches of these cells, the sites of synapses, fork at similar points, nearly branch for branch in the neuropile. After a T cell axon has been severed, it can regenerate into the next ganglion, growing along the axon of the T cell in that ganglion, as shown by staining the regenerating cell with HRP and the T cell in the next ganglion with Lucifer Yellow dye (Macagno *et al.* 1981). The Lucifer-Yellow-stained cell is also the target of the regenerating T cell, and may be able to guide the growing T cell axon. After the axons have been severed by crushing the connectives, T cells successfully regenerate connections in approximately 20% of cases. Not all involve growth along the target axon. In some cases growth is obviously unusual, yet the cells reconnect with targets at apparently normal sites of synapse (Macagno, Muller & DeRiemer, 1985).

In about 5% of cases, the growing T cells reconnect directly with the pieces of axon cut away from the cell bodies, the severed distal stumps (DeRiemer, Elliott, Macagno & Muller, 1983). In this process, apparently a fusion, it is not known whether the growing cell finds its own axon or instead finds the axon of another sensory cell of the same modality, but the result is a rapid and complete reconnection with normal targets in the adjacent ganglion. In the leech this particular mechanism of repair – cell fusion – has only been seen for sensory neurones, but it has been reported as an axonal repair mechanism in several other systems, notably crayfish motor axons (Hoy, Bittner & Kennedy, 1967) and vertebrate neurones in tissue culture (Levi & Meyer, 1945). This contrasts with a different but functionally identical repair mechanism, the formation of an electrical synapse upon the severed distal stump, which occurs for an interneurone described below.

When sensory fibres grow in typical fashion to the target ganglion without reconnecting with the stump, the time it takes to regenerate fully depends on the distance from the site of the lesion to the target ganglion. Thus, when axons are severed near the target ganglion, synapses may regenerate within 2 or 3 weeks, while recovery may take over a month after more distal lesions. Lesions that are closer to the target also seem to produce a greater number of successful reconnections, even when time is taken into account (Elliott & Muller, 1983*a*). The type of lesion is also important for success, just as it is in other nervous systems, including the human peripheral nervous system. Regeneration is far more successful after the nerve has been crushed than after it has been cut, despite apparently complete disruption of axons and glia at the crush and subsequent invasion of the crush by microglia. Accurate regeneration in the periphery by sensory axons is reduced even more by evulsion of segments of nerve; under these conditions sensory endings may innervate scars in the skin (Van Essen & Jansen, 1977).

ACCURACY OF SENSORY NEURONE RECONNECTION

We know that sensory neurones regenerate chemical and electrical synapses with normal targets, but limitations of sampling make it difficult to say with certainty that mistakes are not made. Morphologically, synapses can be confidently identified only with the electron microscope. Sensory neurones make synapses in the neuropile among hundreds or thousands of other cells' axons, thus it is difficult to determine if only the normal complement of connections is made in the neuropile. However, the N sensory cells make a distinctive set of contacts with targets in adjacent ganglia that can be readily identified with the light microscope. These contacts are axonal baskets wrapping the cell bodies of P cells, other N cells and some Leydig cells. Physiological experiments indicate that the wrappings are excitatory.

After N cell axons have been severed by crushing the connective, the axons regenerate baskets around the normal complement of targets and do not wrap other cell bodies in the same vicinity as the normal targets (French & Muller, 1986). In a large fraction of cases (more than 40% of regenerates), it has been found that the regenerating axons also wrap a highly unusual target in a different part of the

ganglion, near where the growing N cell axon leaves the target ganglion on its route to the periphery. This is perhaps an example of relaxed specificity; in hundreds of observations on normal animals, only once has a cell in a similar position displayed such a basket. It is tempting to consider that this might have been the vestige of a basket that arose transiently during embryonic development, and that regeneration might recapitulate development. However, preliminary evidence indicates that baskets arise late in development, forming a normal complement of wrappings, albeit simple ones, at the outset (E. McGlade-McCulloh & K. J. Muller, unpublished results).

REGENERATION IN TISSUE CULTURE MEDIUM

Sensory–motor connections regenerate in chains of leech ganglia isolated in tissue culture medium, as shown by Wallace, Adal & Nicholls (1977). Although in tissue culture some cells regenerate connections more readily than others, this is also true *in vivo*. Significantly, precise regeneration in tissue culture succeeds without functional validation of the connections. In this respect regeneration in the leech resembles most developing nervous systems that have been studied. It is not known whether functional activity can refine regenerated connections *in vivo*.

Sensory neurones and other neurones in the leech that have been removed singly from the animal will also form chemical and electrical synapses with each other when placed together in tissue culture (Fuchs, Nicholls & Ready, 1981; Fuchs, Henderson & Nicholls, 1982), providing perhaps the ultimate system for examining the basis of synaptic specificity. Experiments with small groups of neurones reveal that a reliable set of connections forms, in almost all cases resembling connections found in the animal, despite a dramatically different environment and configuration of cells (see paper by M. Chiquet & J. G. Nicholls in this volume).

PRECISE GROWTH AND REGENERATION BY S INTERNEURONES

The single S interneurone in each ganglion has been particularly favourable for studies of synapse regeneration because its axon, identifiable by its distinctive location, is also the largest in the nerve cord and it synapses with a single target, the tip of the axon of another S cell, in the connectives midway between the ganglia of the two cells. Thus, the S cells have an electrical synapse in a region that is less complex than the neuropile and is essentially free of other synapses.

In more than 80 % of cases, the injured S cell regenerates its axon along nearly half the length of the connective in about 1 month, synapsing with its target but not other cells (Muller & Carbonetto, 1979). In half the regenerates, the cell becomes functionally connected within 2 or 3 weeks when the growing axon forms an electrical synapse with its severed distal stump, which remains connected to the target and continues to conduct action potentials. The stump, which survives for months when regeneration is prevented, degenerates during the month after regeneration, its

demise somehow triggered by the regenerating axon's connection with its target. Experiments in which the target was selectively and completely destroyed by intracellular injection of protease have shown that it is not simply disconnection from the target that kills the stump, for the isolated axon stump survives without the target for months (Scott & Muller, 1980). Incidentally, this also shows that the stump does not depend on its electrical link with the target to survive. It has been hypothesized that glia maintain isolated axon segments (e.g. Lasek, Gainer & Barker, 1977; Meyer & Bittner, 1978), but in the leech the ensheathing glia can be destroyed with no effect on stump survival (or accuracy of regeneration) (Elliott & Muller, 1983*a*,*b*).

In now classic experiments, McMahan and his co-workers (Sanes, Marshall & McMahan, 1978) showed in the frog that motoneurones will regenerate to old endplate regions and elaborate a presynaptic terminal even when the muscle has been destroyed, so long as the surrounding basement membrane remains. Although we do not know what signals guide and stop growth in the leech, in somewhat similar experiments in which the S cell target was selectively destroyed, regeneration followed a normal course, including the stopping of axon growth at the normal site of synapse midway between ganglia. In this case no synapses were ever made.

COMPARISON OF REGENERATION AND DEVELOPMENT

Recently the S interneurones have been studied during embryonic development, beginning with the outgrowth from the soma of S cell axons at 10 days of embryonic development at 20 °C (McGlade-McCulloh & Muller, 1986). In the ensuing week the axons grow more than halfway down the medial connective (Faivre's nerve) to a point about 85 % of the way to the adjacent ganglion. Axons from adjacent ganglia meet at the halfway point at about day 12, and a day or two later they become electrically coupled, as judged by the passage of fluorescent dye from the injected cell into its S cell neighbours (Figs 1, 2). Unlike the situation for growing adult S cell axons, when the embryonic axon contacts its target (the S cell from the adjacent ganglion) the axon continues to grow, moving closer to the next ganglion. This difference suggests that the stop signal may change during maturation, and leaves open the possibility that the signal lies in the extracellular matrix, despite the lack of basal lamina in the leech CNS. Certainly experiments in the adult in which the regenerating axon stops midway between ganglia even after the target has been selectively destroyed (Scott & Muller, 1980) are consistent with this hypothesis.

After the S cell axon has grown to within 15 % of the connective length towards the next ganglion by about 16 days, the differential growth of the S cell axon and the connectives, which extend more rapidly, causes the tip of the S cell axon apparently to slip back to approximately the midpoint of the connectives, a point halfway between ganglia. At the same time, those axons that synapse within the adjacent ganglion extend at least at the same rate as the connectives, thus growing along their length and not simply at the tip.

SEVERED AXONS AND AXON SEGMENTS CAN FORM NOVEL SYNAPSES

Sprouting is a typical response to axonal injury, even in the CNS of mammals, where regeneration of most axons is abortive. For those neurones that do successfully regenerate, in the leech as well as in vertebrates, many of the misdirected sprouts are reabsorbed. Some 'misdirected' sprouts of sensory cells and other neurones in the leech form synapses near the site of lesion within the connective, a region where synapses are rarely made (Fernandez & Fernandez, 1974; Muller, 1979). Little is known about the nature of the synaptic targets, whether some synapses are transient,

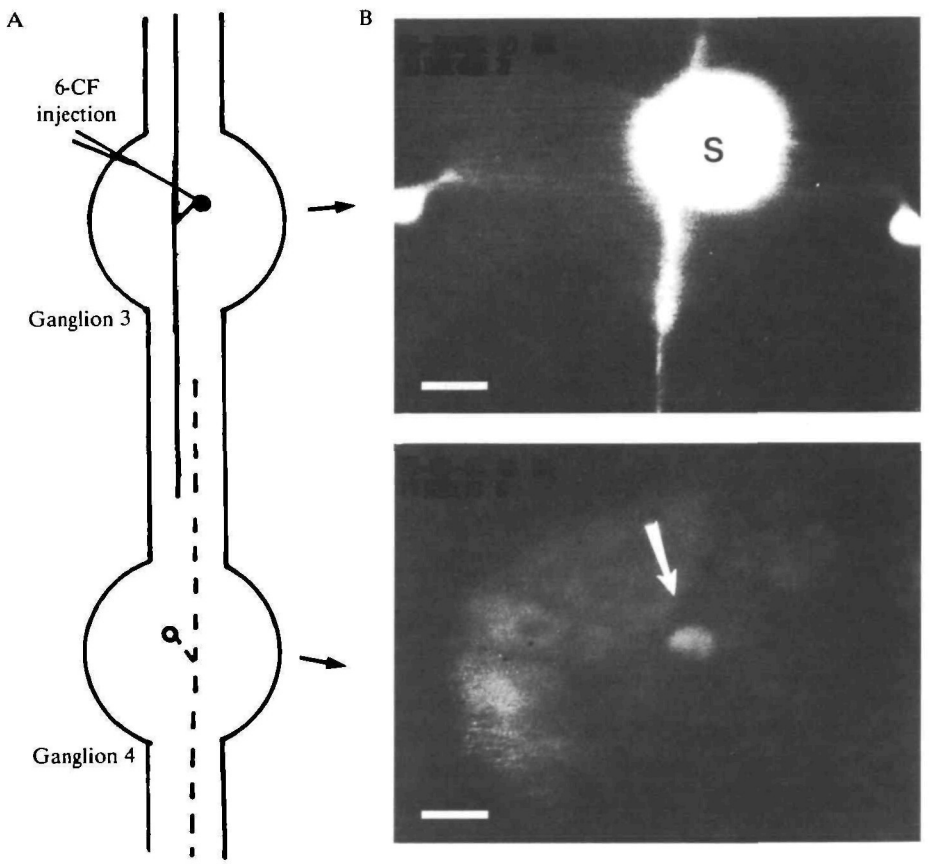


Fig. 1. Dye moves selectively between embryonic S interneurones. The dye 6-carboxyfluorescein (6-CF), injected into a single S cell soma in ganglion 3 at day 14, travelled into the coupling interneurones in the same ganglion and into the S cell in the adjacent ganglion 4 (arrow). The junctions between cells passed only small dye molecules and not larger molecules such as horseradish peroxidase, just as in the adult. (A) Diagram showing the injected S cell (solid line) and the neighbouring S cell (broken line) into which 6-CF spreads. (B) Photomicrographs recorded through a silicon-intensified target camera showing cells containing 6-CF in ganglia 3 (upper) and 4 (lower). The 10 μm diameter soma of the S cell in ganglion 3 (S) is out of focus and therefore appears large. Two small lateral cells in ganglion 4, the coupling interneurones, appeared when 6-CF was subsequently injected into the S cell soma (arrow) in ganglion 4 (not shown). Scale bars, 10 μm.

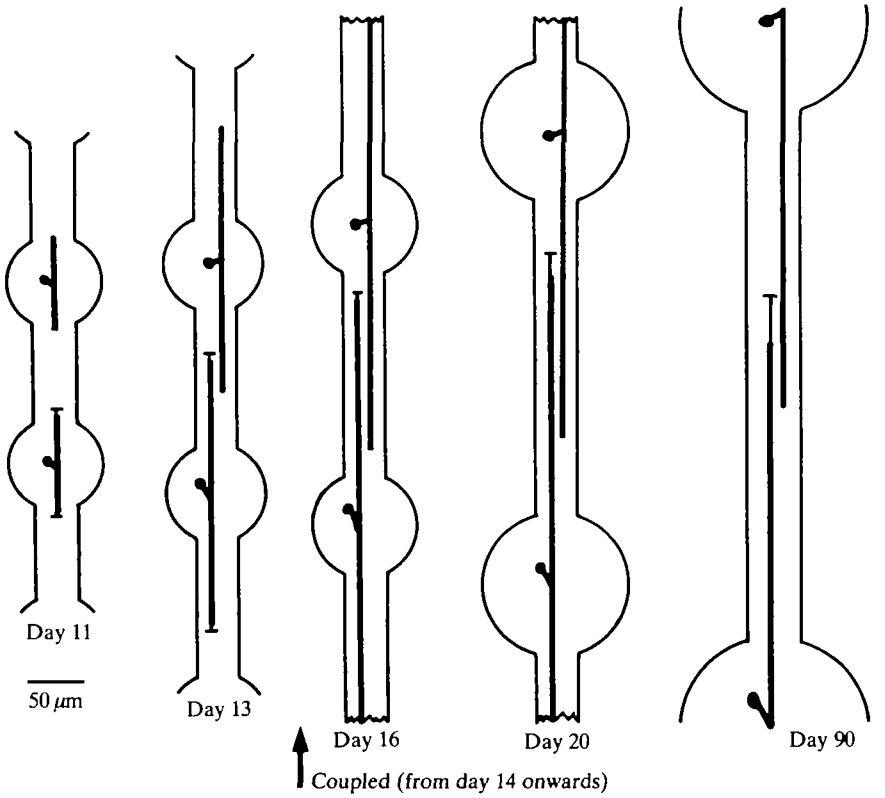
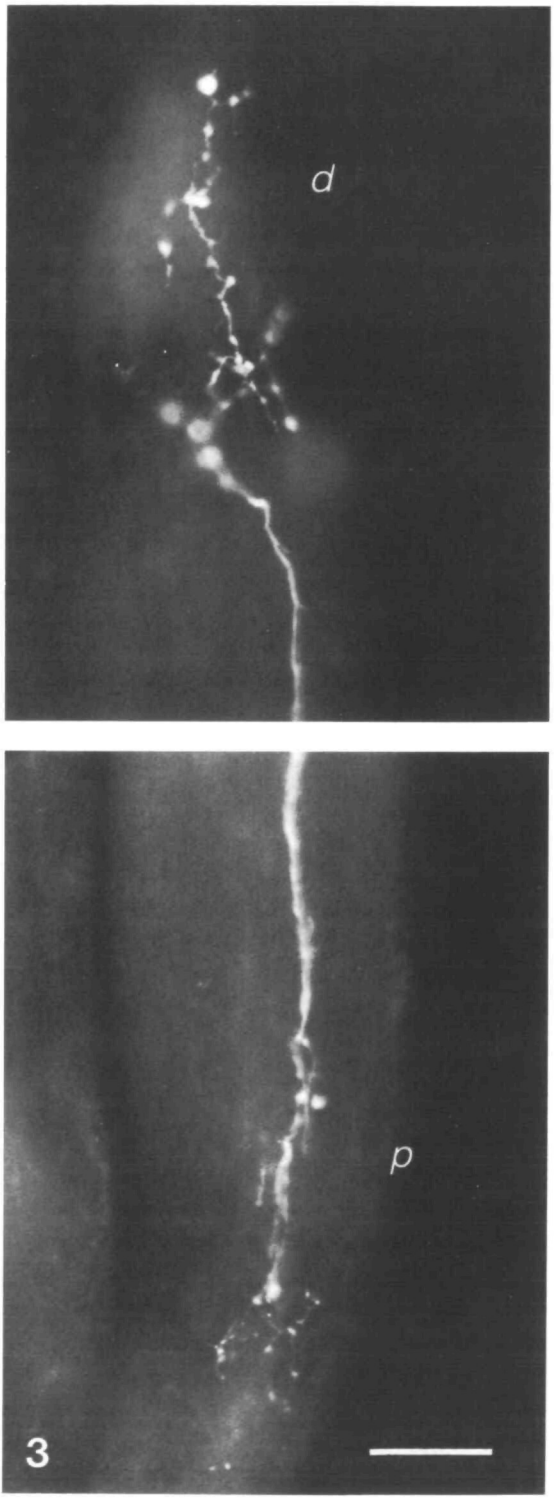


Fig. 2. S cell axons made contact near the connective midpoint, but continued to grow after synapsing with each other. The extent of S cell axon outgrowth is depicted at various stages (days) of development. The percent interganglionic distance was determined for four cells injected with horseradish peroxidase at each of the following developmental days: day 11, $7 \pm 3{\cdot}5\%$; day 13, $60 \pm 4{\cdot}9\%$; day 16, $86 \pm 2{\cdot}9\%$; day 20, $75 \pm 8{\cdot}1\%$ (mean $\pm$ S.E., $N = 4$). Day 90 leeches (juveniles) were similar to adults and therefore were combined with adult data to generate an axonal extension of $55 \pm 10\%$. For clarity, the standard error bars are drawn only on the lower S cell axons. Adjacent S cells became coupled, as judged by selective passage of dye between them, by day 14, while the percent axonal overlap peaked later, at day 16. After this the axons grew less rapidly than the distances between ganglia.

or whether synapse formation in the connectives impedes regeneration to the normal sites of synapse within the ganglion. In the mammalian spinal cord, injured axons are also known to form novel synapses that may be related to partial recovery (Bernstein & Bernstein, 1971; Goldberger & Murray, 1974). We know that electrical synapses can form transiently with axon segments, and it is possible that chemical synapses can too, as they can in crayfish (Krasne & Lee, 1977).

The ability of isolated axon segments to form synapses may be related to their ability to sprout and grow for days (Fig. 3; Mason & Muller, 1982). Experiments in which glia ensheathing isolated axon segments were selectively destroyed with protease showed that the segments survived for weeks or months (Elliott & Muller, 1983*a*,*b*). Preliminary experiments with the same system indicate that segments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without glia can, in fact, sprout and grow for at least a week (K. French & K. Muller, in preparation), just as isolated glial-ensheathed axons can. This result casts added doubt on the assertion that isolated stumps survive by virtue of their ensheathing glial cells.

TRIGGERS FOR SPROUTING

It is well known that uninjured neurones sprout in the peripheral nervous systems of most species, and limited sprouting also occurs in the CNS (for a review see, for example, Sanes, 1982). Partial denervation typically causes uninjured axons to sprout and innervate the vacated sites. In the CNS and at the neuromuscular junction, the sprouted axons form synapses. For many peripheral sensory terminals, particularly in the leech (Blackshaw, Nicholls & Parnas, 1982), the sprouted axons probably do not form synapses, but rather occupy territory previously occupied by the injured axon(s) (however, see Scott, Cooper & Diamond, 1981). Which feature of denervation signals the intact axon to sprout may vary with the preparation.

For some cells, during embryonic development, axons sprout or grow an additional distance if their synaptic targets have been removed (Schneider & Jhaveri, 1974). This contrasts with the S cell in the adult leech after its synaptic contact, another S cell, has been selectively destroyed with intracellularly injected protease. In this case the intact axon simply projects, targetless, along the connective and does not form alternative synapses (Scott & Muller, 1980). In vertebrates a similar phenomenon is seen in the cerebellum of *nervous* mutant mice, where the granule cell axons continue to project among other parallel fibres but do not form new synapses after their Purkinje cell targets degenerate post-embryonically (Sotelo & Triller, 1979).

The adult S cell, unlike embryonic neurones, is not actively growing. Significantly, when the cell is triggered to grow by injuring one of its two axons, the other axon (which remains intact) sprouts within weeks of killing the cell that is its synaptic target. These and related experiments indicate that the synaptic contact inhibits axon sprouting, but the inhibition is manifest only when the inhibited cell is otherwise stimulated to grow, as by direct injury. It is not yet known whether sprouting of the S cell axon can occur in the absence of injury, simply when new targets become available, as in the leech peripheral nervous system (Bowling, Nicholls & Parnas, 1978; Blackshaw *et al.* 1982).

Severe lesions in the leech CNS, such as transection of the connectives, produce profound and persistent changes in particular connections (Jansen, Muller &

Fig. 3. Isolated axon segments sprout at both ends and grow for at least a week. In this photomicrograph, a P sensory cell was injected with a fluorescein-labelled dodecapeptide consisting of D-amino acids (kindly supplied by Dr David Weisblat) and its anterior axon was isolated by cutting the connectives in two places approximately 1 mm apart. A week later the preparation was fixed in 2% paraformaldehyde, mounted in basic glycerol (pH 9) and photographed with epifluorescence optics, revealing distal (*d*) and proximal (*p*) ends of the millimetre-long segment that were growing at the time of fixation. Anterior at the top; posterior axon segments grew equally well. Scale bar, 30 μm.

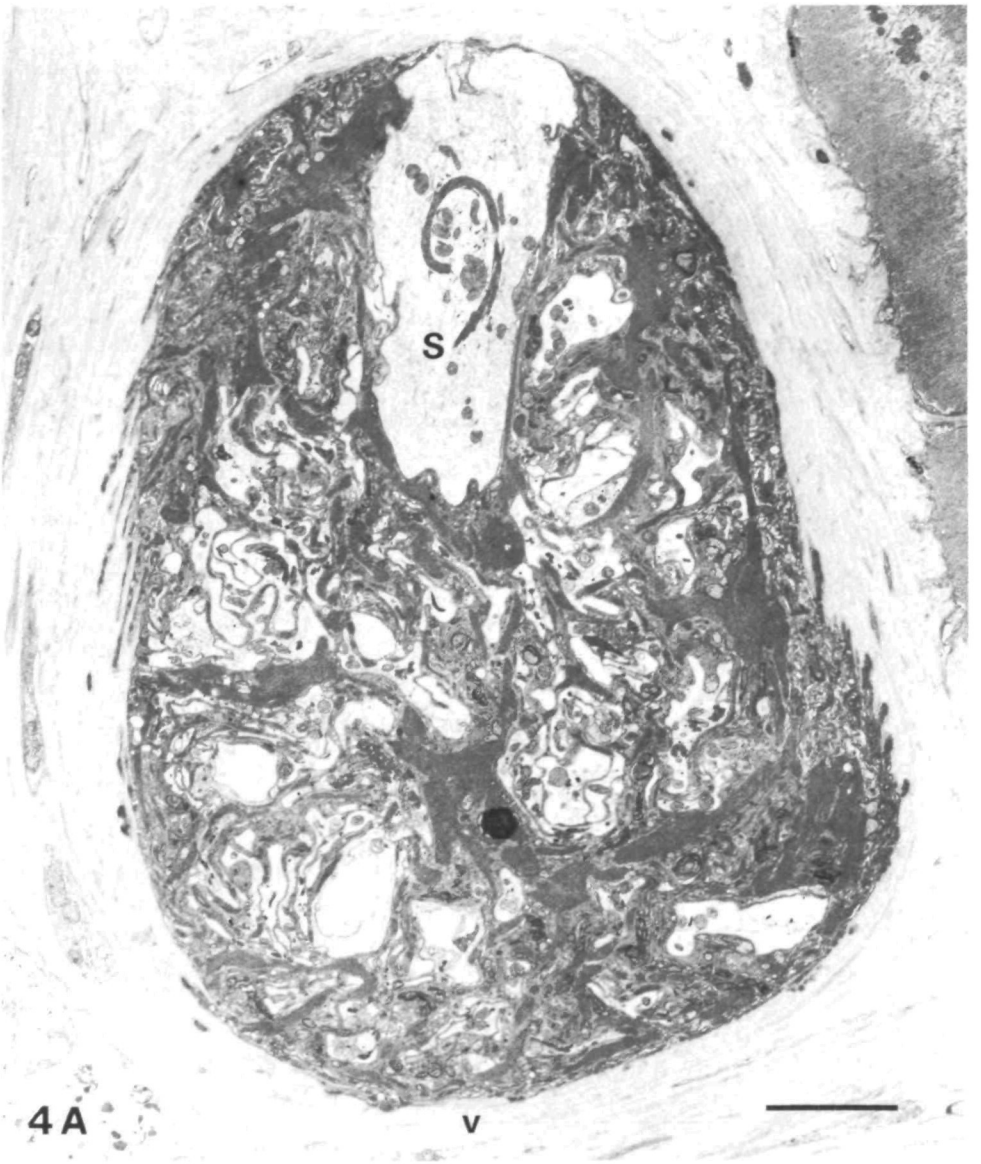
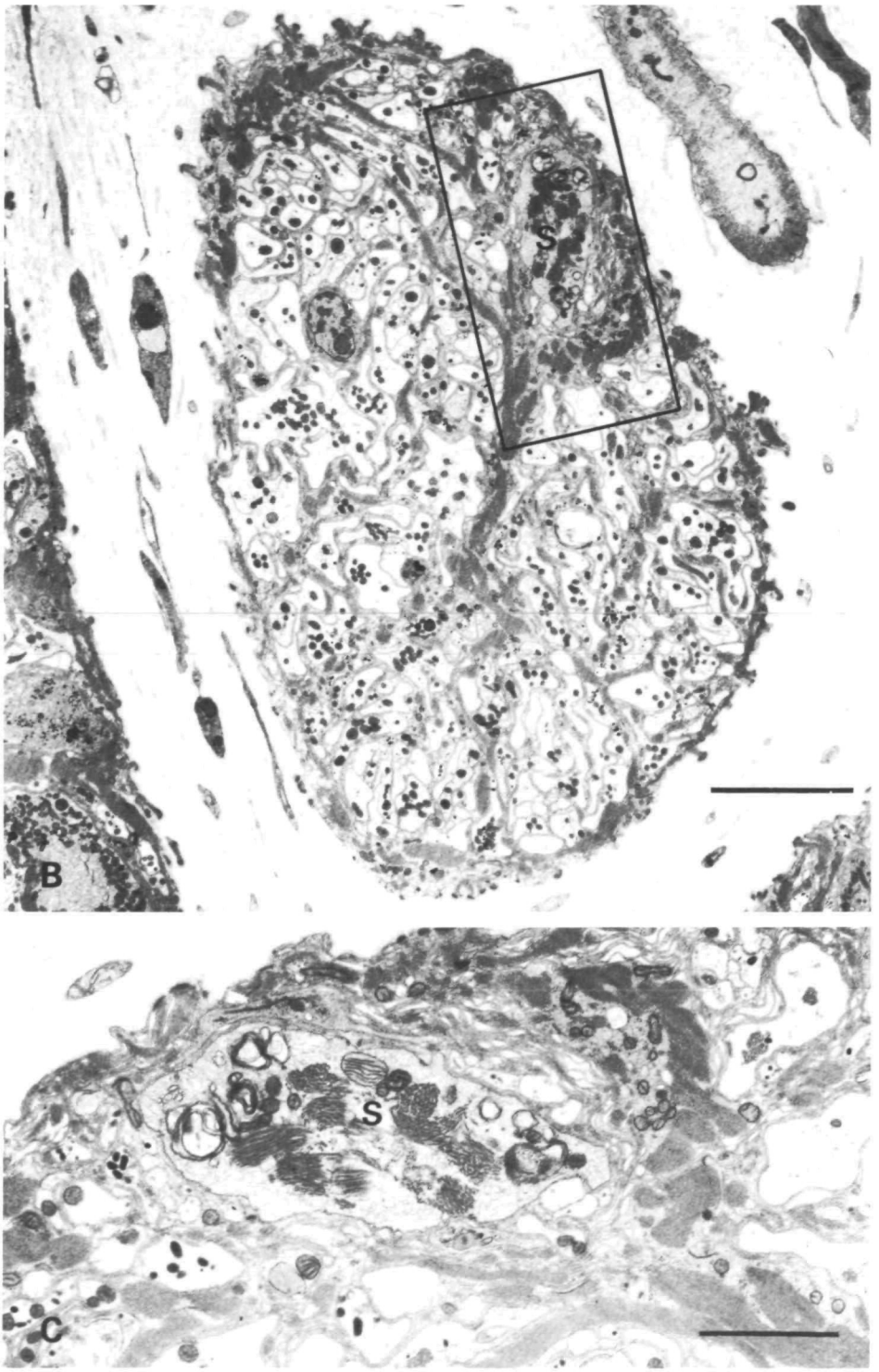


Fig. 4. Effect of temperature on the survival of axon segments. (A) The S cell axon in the tropical leech *Haementeria ghiliani* resembles that in *Hirudo medicinalis*. The axon (S) is the largest in the nerve cord, is located in the dorsal quadrant of Faivre's nerve, shown here, and when cut from the cell body the axon segment survives for months at room temperature (22°C). The *Haementeria* axon segment depicted here has a normal appearance and yet had been cut from its cell body 3 months previously. *v*, ventral; scale bar, 5 μm. (B) At 31°C, S cell axon segments degenerate within a month. Shown is a segment, isolated 23 days earlier, in an advanced state of degeneration. Scale bar, 5 μm. Region in box, containing shrunken profile of S cell axon, is magnified in C. Scale bar, 2 μm.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Nicholls, 1974). For example, the excitatory synapses made by N sensory cells may become considerably stronger, even for chains of ganglia in tissue culture (Miyazaki & Nicholls, 1976). Typically the changes develop over weeks, with a time course similar to that seen for axon growth and regeneration of connections. It is not known why the connections change, but change is not simply a response to cellular injury, since in some cases changes occur between neurones not directly injured by the lesions. One hypothesis is that strengthening represents an increase in the number of synapses, due to sprouting within the ganglion, to substitute for inputs that have been lost or cut away. Recently we have obtained evidence to support this idea from morphological studies with N cells.

The N sensory cells normally make synapses with other cells, including P sensory neurones and other N cells, in adjacent ganglia and in their own ganglia. As described above, the N cell synapses onto N and P cells include axonal wrappings of the target cell soma. These wrappings or baskets, which are easily seen with the light microscope after injecting the presynaptic neurone with HRP, are made by the lateral N cell only in adjacent ganglia and not in the cell's own ganglion. We have found that after cutting connectives on both sides of the ganglion, the lateral N cell will sprout and form baskets around N and P cells in its own ganglion (X.-N. Gu, unpublished observation). These are the very cells whose inputs are removed by disconnecting the ganglion from its two neighbours. Partially removing the inputs (cutting the connectives on only one side of the ganglion, anteriorly or posteriorly) is insufficient to prompt such sprouting. Thus it is not only injury to the sensory cell that causes it to sprout, but we have yet to determine whether sprouting occurs simply in response to lost inputs. As far as we can determine, the sprouting resembles that by motoneurones in vertebrate muscle (Brown, Holland & Hopkins, 1981) and some invertebrate muscle (Bowling *et al.* 1978).

AXON DEGENERATION AT HIGHER TEMPERATURES

The relationship between axon degeneration and regeneration is complex. It is plain that in the leech and some other animals neurones can repair their connections by linking with the severed distal segments of their axons, as cited above. Such reconnection is a particularly effective means of repair, but it obviously relies on the survival of the severed piece of axon.

In the leech, severed axon segments typically can survive for months after axotomy. This has been studied in most detail for the S interneurone, which has a large, easily recognizable axon. While not accounting for the mechanism of survival, one hypothesis is that temperature plays a critical role (for reviews see, for example, Cancalon, 1985; Carbonetto & Muller, 1982). In mammals such as the hibernating ground squirrel, for example, isolated segments of motor axons can survive for months and continue to excite muscle fibres so long as they are kept at reduced temperature. Moreover, in the frog optic nerve, which is notable for its powers of regeneration, segments of unmyelinated axons can survive for months at 20°C

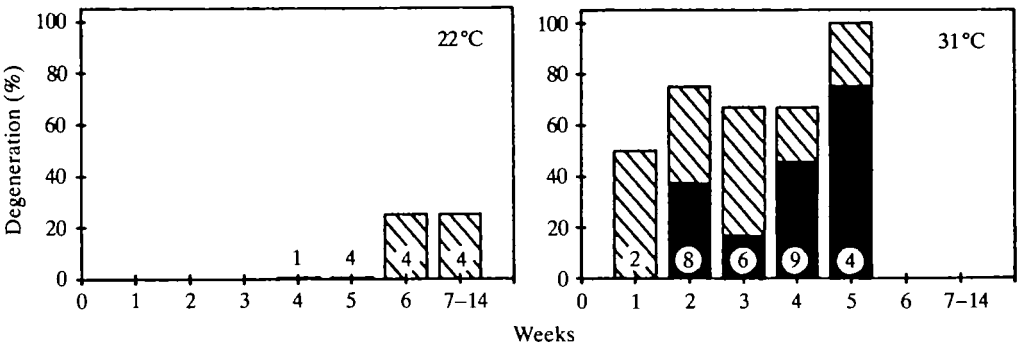

Fig. 5. Axon segments of S cells survive for months at 22°C, but degenerate at 31°C during the first month after cutting. The percentages of axons that have degenerated and disappeared are indicated by solid bars, those that have begun to degenerate are indicated by cross-hatching. Total numbers of preparations for each postoperative week are indicated in the clear circle within each vertical bar.

(Matsumoto & Scalia, 1981). Among workers on arthropods there is some disagreement about the role of temperature. The difficulty seems to be that in some animals, such as crayfish, elevated temperatures may be generally harmful to the animal. Still, it is interesting that degeneration seems to be most rapid in insects that normally regulate their temperature above ambient levels.

One leech that survives well at elevated temperatures (at least to 34 or 35°C) is *Haementeria ghilianii*, a giant tropical leech from South America. Features of the S cell in this animal are indistinguishable from those of the S cell in the commonly studied medicinal leech (*Hirudo medicinalis*), which unfortunately does not fare well above room temperature. Interestingly, when regeneration is prevented we have found that degeneration of the S cell's distal axon stump may take more than 3 months at room temperature (22°C) in both species (Muller & Carbonetto, 1979; Fig. 4A), but degeneration can occur within days and almost always occurs within a month in *Haementeria* maintained at 30 or 31°C (Figs 4B,C, 5). Regeneration is also speeded at higher temperatures, so it has not been possible to separate the two processes. It seems, then, that for the S cell, and probably for other neurones in the leech, elevated temperature causes axon segments in an otherwise healthy nervous system to resemble more closely degenerating axons in vertebrates.

CONCLUSION

Detailed knowledge of the projections and synaptic connections of identifiable neurones in the leech has permitted a similarly detailed study of the sprouting, regeneration, degeneration and embryonic development of axons. We have learned that certain cellular components, such as synaptic targets and glia, may play only a limited role in synapse regeneration and in axon degeneration. Yet contact with the synaptic target may inhibit sprouting and availability of targets may promote it. Continued growth by the developing S cell axon after contacting its target contrasts with regeneration in the mature animal, and suggests that the stop signal may be

different for embryonic and regenerative growth. Overall, the results support the idea that degeneration as well as regeneration share fundamental mechanisms in the leech, other invertebrates and the vertebrates, including mammals. We are encouraged that mechanisms that speed or promote functional recovery in leeches might be reproduced in the mammalian nervous system.

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