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NEW GROWTH ELICITED IN ADULT LEECH MECHANOSENSORY NEURONES BY PERIPHERAL AXON DAMAGE

BY B. A. BANNATYNE, S. E. BLACKSHAW* AND M. McGREGOR
Institute of Physiology, University of Glasgow, Glasgow G12 8QQ

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Summary

1. New growth in cutaneous mechanosensory neurones elicited by axotomy or axon crush was studied using intracellular injection of horseradish peroxidase at different times after the lesion, ranging from a few days to over a year.

2. Cutting or crushing major, large-calibre axon branches of mechanosensory neurones elicits sprouting of new processes, either centrally within the ganglion neuropile or at the site of the lesion in the peripheral nerve. In contrast, cutting or crushing fine-calibre axon branches supplying accessory parts of the receptive field does not elicit sprouting of the main arbor or main axon branches.

3. Different modalities of mechanosensory neurone respond differently to lesions of their axons. Cutting the axons of high-threshold units responding to noxious stimulation of the skin elicits sprouting of additional processes from the axon hillock region within the central nervous system (CNS), whereas cutting or crushing the axons of low-threshold cells responding to light touch of the skin elicits sprouting at the site of the lesion only, and not within the CNS.

4. In addition to the new growth directed into the peripheral nerve, damaged nociceptive neurones also form new processes that wrap the somata of particular cells within the ganglion.

5. Sprouted processes of axotomized neurones are retained for long periods after the lesion (up to 425 days).

6. The electrical properties of touch and nociceptive cells were studied between 1 and 60 days after axotomy, by intracellular recording from the centrally located cell bodies. The amplitude, width and maximum dV/dt of the action potential and after-hyperpolarization, as well as the resting potential and input resistance, did not change significantly after axotomy, despite the considerable process sprouting known to occur during this time.

Introduction

Following damage to a nervous system, the properties of neurones change.

* Present address and address for correspondence: Department of Cell Biology, University of Glasgow, G12 8QQ.

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Changes in both physiological and morphological properties may occur, in the injured neurones as well as in connected uninjured cells. The functional consequences of changes induced by injury are not always clear. For example, axotomy induces changes in membrane properties (Eccles *et al.* 1958; Kuno & Llinas, 1970; Kuwada & Wine, 1981; Spira *et al.* 1987) whose role is not understood. It is not known whether they are a consequence of disruption of the normal cellular processes or whether they are specifically connected with repair of the damage and regeneration of the axon. Sprouting of neurone processes may occur at the site of damage, as part of functional regeneration of the axon, but new growth may also occur at sites on the branching arbor distant from the damage, or on connected uninjured cells (Cotman, 1978; Anderson, 1982; Rotshenker, 1988). In the CNS of the leech repair processes can be studied at the cellular level by examining the effects of lesions on individually identified neurones whose properties in the normal adult nervous system have been extensively described (Muller *et al.* 1981). There are striking examples of the ability of the leech CNS to repair itself after injury. Successful regeneration has been shown to occur in the animal, in ganglia in culture, and between single neurones isolated in culture (Nicholls, 1987). Frequently, however, regeneration in the animal is incomplete, for example, following lesions to CNS tracts (Macagno *et al.* 1985) or to peripheral nerves (Van Essen & Jansen, 1977). Using physiological mapping techniques Van Essen & Jansen showed that different modalities of cutaneous mechanosensory cells differ in their ability to regenerate peripheral receptive fields following peripheral nerve damage. Touch cells regained more complete receptive fields with greater frequency than nociceptive cells and both modalities regenerated more successfully following axon crush than after axotomy. The questions we have addressed concern the changes in physiological and morphological properties of mechanosensory neurones following lesions to peripheral nerve roots. Does sprouting induced by peripheral axotomy resemble that induced by axon crush? Are there differences in the pattern of growth of touch and nociceptive cells which might account for their differing abilities to regenerate peripheral fields successfully? What is the long-term fate of new growth within the nervous system? We have injected horseradish peroxidase (HRP) into axotomized neurones to characterize new growth at different times after damage ranging from a few days to over a year. We have recorded intracellularly from the centrally located cell bodies to see whether the insertion of new membrane in regenerating neurones is reflected in changes in the somatic membrane properties of the damaged neurones. Some of this work has appeared in preliminary form (Bannatyne & Blackshaw, 1987; Blackshaw & McGregor, 1987).

Materials and methods

Experiments were carried out at room temperature (20–25°C) on adult leeches (*Hirudo medicinalis*) obtained from a commercial supplier (Biopharm, UK) and maintained in copper-free dechlorinated water at room temperature. Leeche

were anaesthetized by immersion in ice-cold 0·15% chlorobutanol (Sigma) for 10–15 min. A small incision 1–2 mm long was made along the ventral midline through the skin and body wall musculature at the level of the ninth segmental ganglion. One or both peripheral nerve roots on one side of the ganglion were either crushed using watchmaker's forceps or cut with irridectomy scissors. Leeches were subsequently placed in 10% Ringer's solution (115·0 mmol l^{-1} NaCl, 4·0 mmol l^{-1} KCl, 1·8 mmol l^{-1} $CaCl_2$ and 10 mmol l^{-1} Tris–maleate, pH 7·4), containing approximately 1·5 μg ml^{-1} gentamycin sulphate (Sigma) to prevent infection, for 1 week, and then returned to copper-free water. All leeches survived the operation and many lived subsequently for long periods, some breeding successfully the following season. After intervals ranging from 1 day to over a year, the leech was re-anaesthetized and chains of three ganglia (the lesioned ganglion and the ganglia on either side) were removed from the leech together with attached body wall and placed in L15 culture medium. Touch and nociceptive cells were identified and labelled with horseradish peroxidase by pressure injection from microelectrodes as previously described (Nicholls & Baylor, 1968; Muller & McMahan, 1976). After injection of cells, ganglia were kept overnight in culture medium, fixed the following day in 0·8% glutaraldehyde in 0·1 mol l^{-1} Tris buffer, incubated in diaminobenzidine (0·5 mg ml^{-1}) for 20 min (the final 5 min with the addition of 10 μl ml^{-1} cobalt chloride), reacted with hydrogen peroxide, dehydrated, cleared and mounted. Reconstructions of the new growth elicited by the damage were made with the aid of a *camera lucida* using a 100× oil-immersion lens. Membrane properties of normal and axotomized neurones were investigated using conventional intracellular recording techniques to measure amplitude, width and maximum dV/dt of the action potential and after-hyperpolarization, as well as resting potential and input resistance (Appendix C, Muller *et al.* 1981).

Results

Morphologies in the normal adult nervous system

Experiments were performed on low-threshold touch (T) and high-threshold nociceptive (N) cutaneous mechanosensory neurones. The branching patterns of T and N cells in the normal adult nervous system are well known (Muller & McMahan, 1976; Yau, 1976; Blackshaw, 1981; Blackshaw *et al.* 1982; Johansen *et al.* 1984). T and N neurones send major axon branches to ipsilateral nerve roots which innervate skin on the ipsilateral side of the body and finer-calibre branches to ipsilateral connectives. Normally only a single process follows each of these pathways (Fig. 1). Rarely, N cells send a fine process to a contralateral root or connective (Muller, 1979) but multiple axon branches of the same cell in a root or connective are never seen. The axon branches to anterior and posterior connectives arborize in adjacent ganglia and send fine-calibre branches out through corresponding roots to innervate accessory fields in the skin. Individual T and N cells therefore innervate skin *via* segmental roots in three adjacent body segments

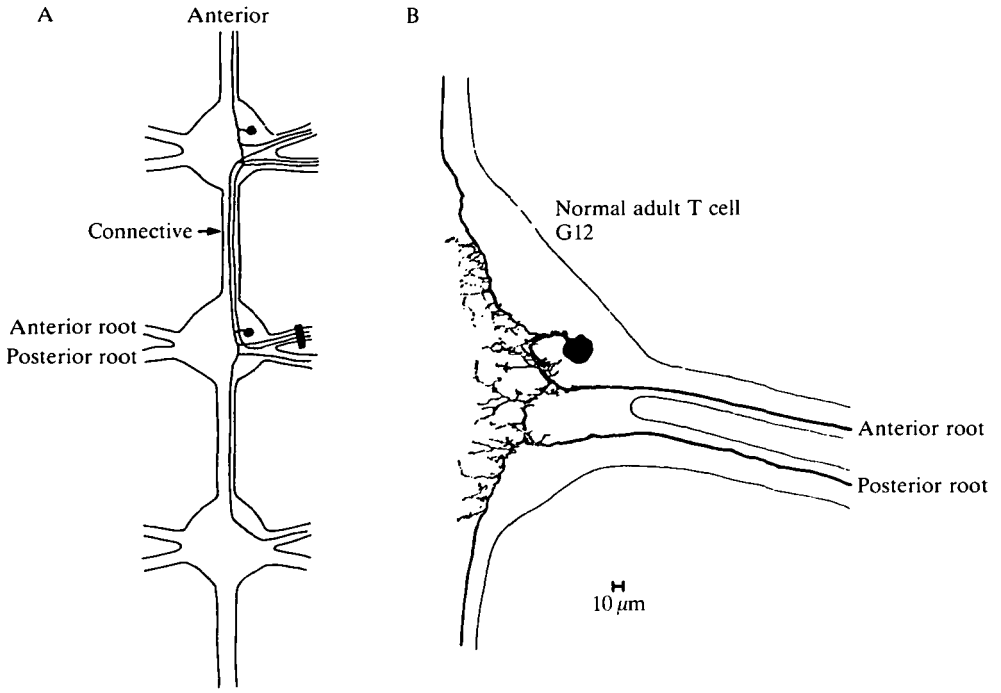

Fig. 1. The branching pattern of mechanosensory neurones in the normal adult ventral nerve cord. Both touch (T) and nociceptive (N) cells arborize over three ganglia, with large-calibre axon branches in the nerve roots of the cell's own ganglion and finer-calibre branches in the nerve roots of adjacent ganglia. Consequently, damage to a nerve root of one ganglion, for example (shown by the heavy bar in A), lesions major branches of the mechanosensory cells located in that ganglion as well as minor axon branches of the mechanosensory cells in adjacent ganglia. (B) A *camera lucida* drawing of the main arbor of a touch cell in ganglion 12. Both T and N cells normally send a single axon branch to each ipsilateral nerve root and connective. Axons in the connectives do not normally have side branches. Nociceptive cell processes in adjacent ganglia wrap the cell bodies of specific neurones (French & Muller, 1986). They do not normally wrap cell bodies in their own ganglion.

(Yau, 1976; shown diagrammatically in Fig. 1). In addition to conventional synapses made in the neuropiles of the three adjacent ganglia, N cells also have baskets of processes that wrap the somata of pressure (P) and N sensory cells in the adjacent segmental ganglia (Muller *et al.* 1978; Johansen *et al.* 1984; French & Muller, 1986). They do not normally wrap somata within their own ganglion.

Fifty animals were lesioned. Of these, eight had the anterior root crushed on one side of the ganglion, usually the leech's left, 12 had both anterior and posterior roots crushed, eight had the anterior root cut on one side and 22 had both anterior and posterior roots cut. Successful lesions were identified by the observation that the body wall bulged out on the lesioned side of the segment owing to loss of muscle tone. The bulge gradually disappeared over the following 1–3 weeks in animals with crushed roots, longer in those with cut roots. The disappearance o

the bulge and the restoration of reflex contractions on the operated side gave clues as to the extent of the damage and the time course of repair. On re-exposing the lesioned ganglion in those animals whose roots were crushed rather than cut, it was often difficult to distinguish the site of the lesion, even after short recovery periods. In animals whose roots were cut rather than crushed, after short recovery periods (less than 5 days) the cut peripheral and distal stumps were still obvious. After longer recovery periods, in some preparations there appeared to be fine processes connecting the two ends; in others the presence of scar tissue around the ganglion and nerve roots made it difficult to visualize the course of the regenerating roots. Often the cut proximal end of the nerve appeared to enter the ventral body wall rather than rejoin the distal cut end. In all cases, the non-lesioned side of the ganglion appeared quite normal, as did adjacent anterior and posterior ganglia and nerve roots. 98 lesioned and 15 control N and T cells were labelled with HRP, 55 with damage to major axon branches and 38 with damage to accessory axons. Of these approximately 30 % were incompletely labelled and discarded. Those neurones whose processes were labelled continuously over long distances within the ganglion of origin and into the nerve roots and connectives were reconstructed.

Characteristics of new growth elicited by peripheral nerve cut or crush

Early stages after injury (less than 14 days)

The results of single- and double-root lesions were qualitatively similar and will be described together. The response to nerve crush was more variable than that to nerve cut. In some crush preparations the nerve root and the lesioned axon appeared normal even after recovery periods of less than a week and it was frequently difficult to distinguish the actual site of the lesion. In other crush preparations, presumably where the damage had been more severe, sprouting occurred at the site of injury and in these preparations the new growth elicited by crush resembled that seen in response to nerve cut. The characteristic pattern of growth in the damaged nerve at early stages after injury is shown in Fig. 2. Numerous fine beaded processes arose at the site of the lesion. Some took a parallel course back towards the cell's own ganglion, although the majority were directed peripherally. In some crush preparations, such as that shown in Fig. 2B, the fine axons were accompanied by a process of greater calibre, as might be expected if fusion of the proximal and distal ends of the original axon had occurred. Damaging touch cell axons elicited new growth at the site of injury only, and not from other regions of the arbor. The responses of N cells to peripheral axon damage differed from those of touch cells. Cutting or crushing the axons of N cells elicited sprouting of additional processes from the axon hillock region in the ganglion, from around the branch points where the major axons to the roots arise, and from the major axons within the nerve root proximal to the site of the lesion as well as at the site of damage (Figs 2C, 3–7). The sprouted processes were extremely fine and a photograph taken at any one focal level (Fig. 3) shows only a

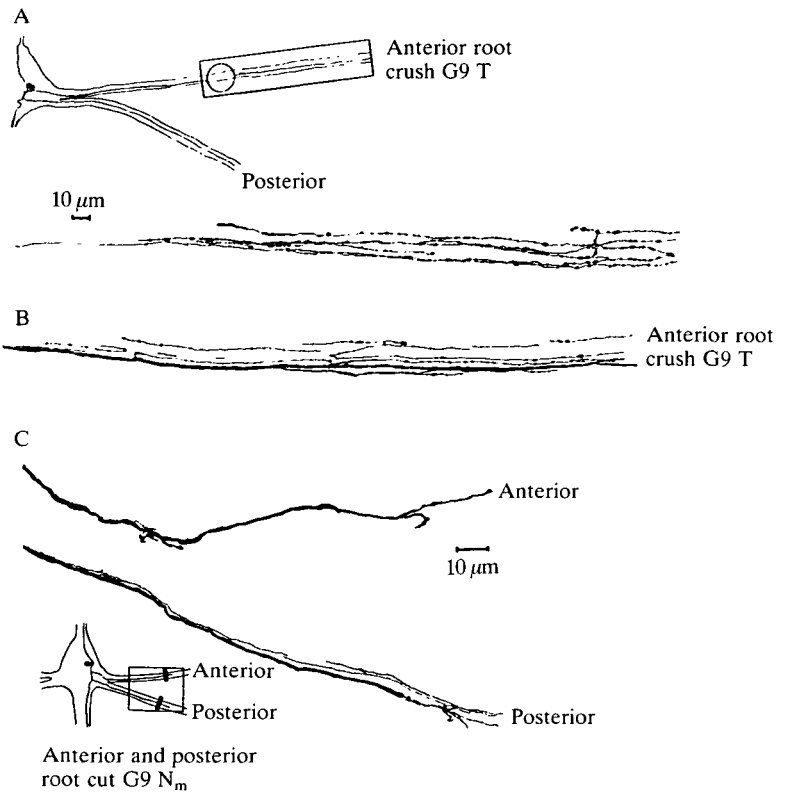

Fig. 2. Characteristic new growth at the site of damage at short times after injury. (A) Multiple fine processes from the damaged end of a T cell axon 8 days after anterior root crush. (B) A second preparation, also 8 days after crushing, in which the T cell main axon remained distinguishable after the damage. (C) Growth cones of N cell axons in anterior and posterior nerve roots 9 days after cutting the roots.

fraction of the total new growth induced in N cells by peripheral axon damage. The majority of new processes that arose centrally grew preferentially along the normal pathway, i.e. towards the periphery (Fig. 4), although they did not always enter the severed root. In some preparations where only the anterior root had been lesioned the new growth was directed into the non-damaged posterior root and ran parallel with the intact axon branch in the posterior root, rather than into the damaged anterior root (see Fig. 6). Occasionally additional processes were seen in the ipsilateral connectives. These were either long neurites running for hundreds of micrometres or shorter varicose processes. Thus, supernumerary processes did not generally have anomalous projections but duplicated existing routes.

New axosomatic contacts by N cell axons

In addition to the new growth directed into the nerve roots and connectives, sprouted processes arising near the origin of the main anterior root axon of N cells wrapped the cell body of a neurone situated laterally within the ganglion betwee

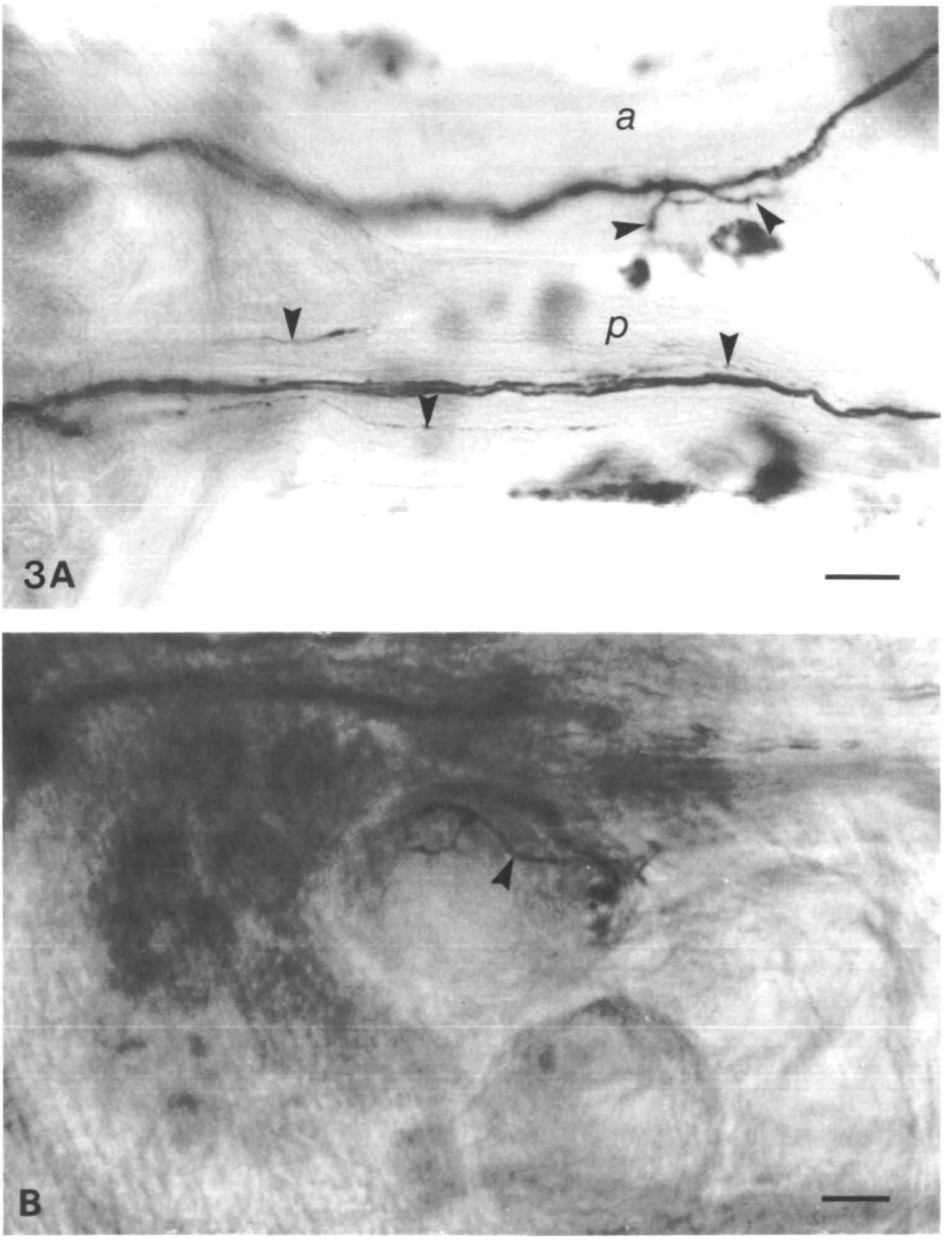

Fig. 3. (A) Photograph of a whole mount of anterior (*a*) and posterior (*p*) nerve roots emerging from a ganglion (to left of picture) in a preparation in which the roots were cut 9 days previously (site of damage outside frame of photograph to right). Sprouted processes (arrowheads) emerge from the anterior root axon of the injected N cell. At this focal level a few of the additional fine-calibre processes in the posterior root are visible. Scale bar, 10 μm. (B) New axosomatic contacts made by axotomized N cells. Photograph of the whole mount shown in the *camera lucida* drawing in Fig. 5, focused on N cell processes (arrowhead), wrapping the soma of a neurone situated laterally in the ganglion. Scale bar, 10 μm.

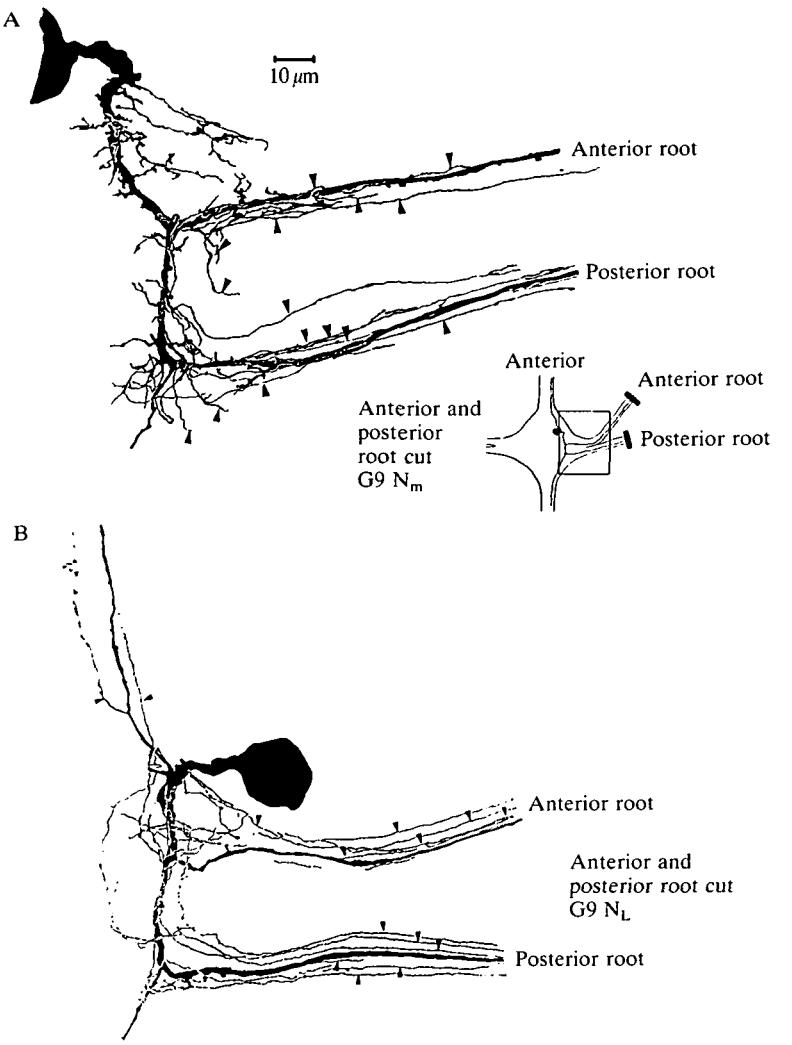

Fig. 4. Multiple additional fine-calibre branches in N cells (arrowheads) arising far from the site of the lesion take normal routes to the periphery parallel with the damaged major axon branches. (A) 5 days post lesion; (B) 4 months post lesion.

the emerging nerve roots (Figs 3, 5). Frequently two cell bodies lie in this position, only one of which is wrapped. The clusters of processes resembled the wrappings made in the normal undamaged nervous system by nociceptive cells onto N, P and other unidentified cells in *adjacent* ganglia (Muller *et al.* 1978; French & Muller, 1986). French & Muller showed that after damage to connective axons, injured N cells regenerate somatic contacts similar to those in intact preparations and also wrap novel cells. In the present experiments no somatic wrappings were seen on any neurone other than that situated between the nerve roots. The location of this

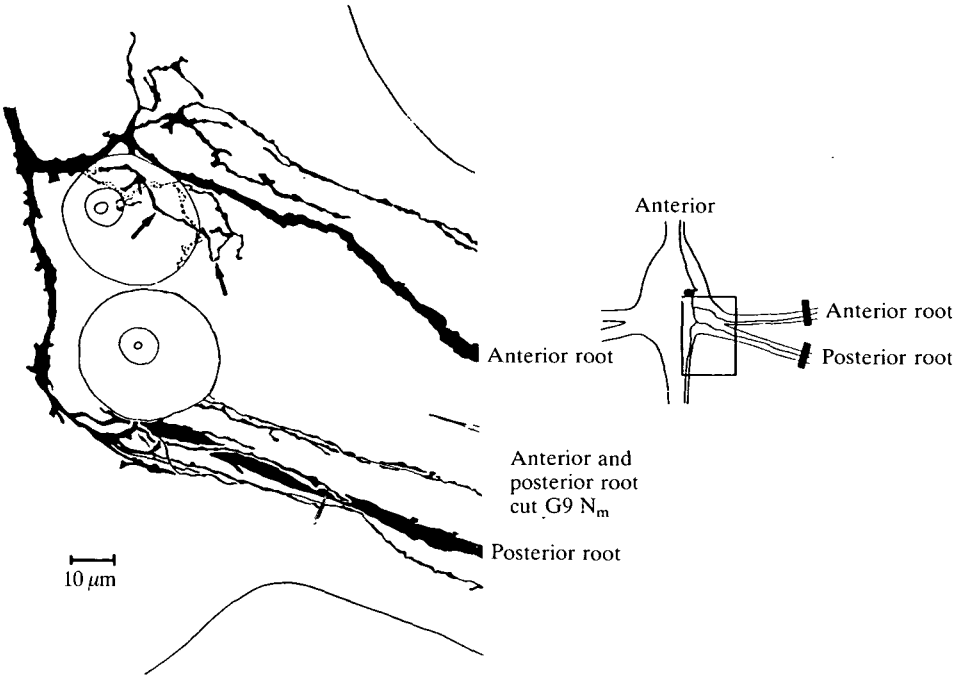

Fig. 5. New axosomatic contacts by axotomized N cells 9 days after damage. Sprouted processes arising in the ganglion wrap the soma of a neurone situated laterally within the ganglion. Same cell as that in Fig. 3B.

neurone corresponded to that of the lateral P cell, although its identity has not been confirmed.

Lesioning accessory axons

Since individual mechanosensory neurones project axons to the periphery *via* the nerve roots of three adjacent ganglia, lesioning the nerve roots of a mid-body ganglion, such as ganglion 9, damages both the major axon branches of T and N cells located in ganglion 9 and the minor axon branches of T and N cells located in ganglia 8 and 10 (see Fig. 1). Thirty-eight N and T cells whose accessory axons were damaged by lesions to the nerve roots of adjacent ganglia were labelled with HRP. All these cells had normal arbors within their own ganglion. Thus, in contrast to cutting or crushing major-calibre axon branches which elicits new growth in that segment, cutting or crushing fine-calibre axons in adjacent ganglia that supply accessory parts of the receptive field does not elicit sprouting of the main arbor, main axon branches or connective axons (see below).

Long-term changes in neuronal morphology

Nociceptive neurones labelled after relatively long recovery periods are shown in Figs 4B, 6 and 7. Multiple additional processes were still present in the roots, although it was our impression that there was no longer the profusion of fine

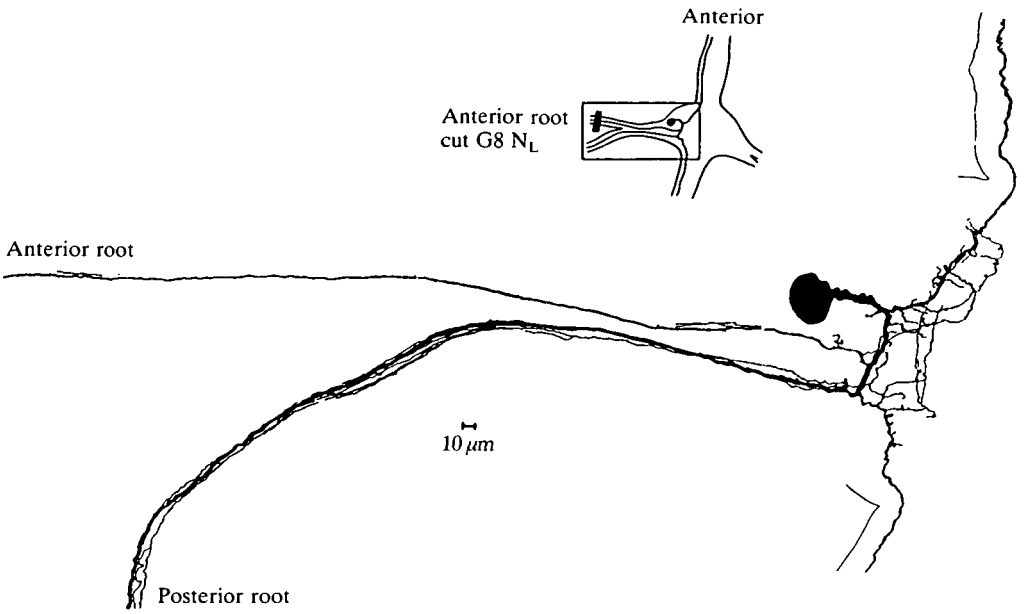

Fig. 6. Additional growth present in an N cell 425 days after damage. In this preparation as a result of damage to the anterior root of G8, the additional processes entered the undamaged posterior root and projected peripherally in parallel with the undamaged posterior root axon.

processes seen after recovery periods of a few days. In the preparation illustrated in Fig. 6, only the anterior root was lesioned yet the greater part of the new growth was directed into the posterior root. The continued presence of multiple axons in the peripheral nerves was not the only long-term change in morphology. Regenerated axons after long recovery periods, as after short recovery periods, were often of a smaller calibre than normal. Changes were also seen in axon branches of the lesioned cell that run within the CNS. Fig. 7 shows lengths of the connective axon of the lesioned N cell illustrated in Fig. 6 and a comparable length of the connective axon of the homologous N cell in the anterior adjacent ganglion whose accessory axon was damaged by the lesion. In the N cell whose major axon was damaged, instead of the smooth contour normally seen in adult N cells, the connective axon was encrusted with short varicose outgrowths. Such outgrowths were not seen in N cells whose accessory axons had been damaged.

No change in electrical properties during process sprouting

The electrical properties of T and N cells in the normal adult nervous system were first characterized by Nicholls & Baylor (1968). In the present experiments two types of control were used, neurones from unoperated animals and homologous neurones contralateral in the lesioned ganglion to the axotomized cell. Typical intracellular recordings of T and N cell responses to depolarizing current in normal and axotomized neurones are shown in Fig. 8. Normal T cells will fir

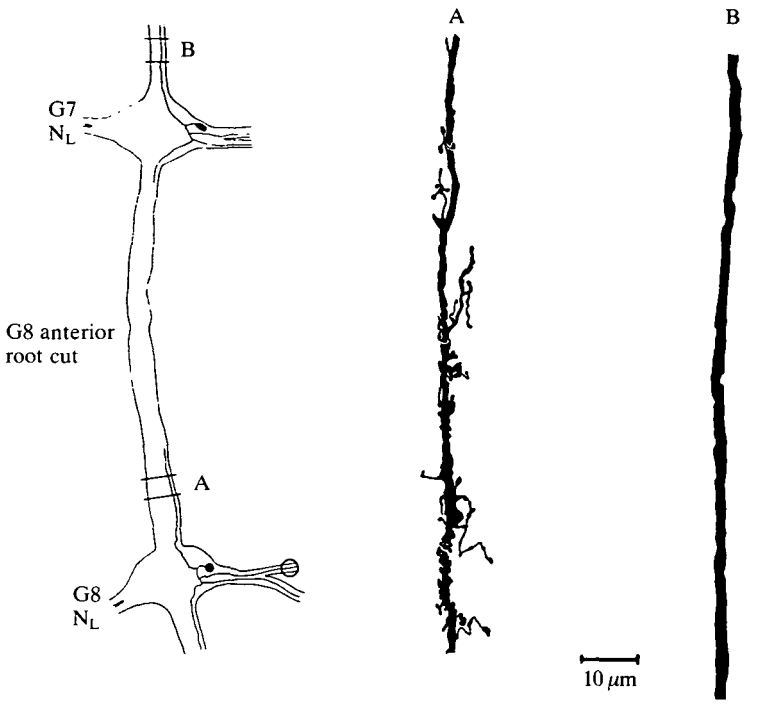

Fig. 7. Sprouted processes from connective axons of N cells at long periods after damage. (A) High-power drawing of a 100 μm length of the connective axon of an N cell situated in ganglion 8 whose major axon branch had been cut 425 days previously. (B) Cutting the accessory axon of the N cell in ganglion 7 did not elicit sprouting of its connective axon: comparable length of the connective axon of the N cell situated in G7.

repeatedly during a maintained depolarization. The action potentials elicited by brief current pulses were typically overshooting with an amplitude of $67{\cdot}8 \pm 11{\cdot}0$ mV ($x \pm$ S.D., $N = 11$), a width at half amplitude of $1{\cdot}5 \pm 0{\cdot}2$ ms, a maximum rate of rise (dV/dt_{max}) of $27{\cdot}1 \pm 4{\cdot}4$ V s^{-1} in the depolarizing direction, and a similar dV/dt_{max} in the repolarizing direction ($22{\cdot}2 \pm 3{\cdot}2$ V s^{-1}). The input resistance (R_{in}) of T cells derived from the linear portion of the current–voltage relationship was $48{\cdot}8 \pm 25{\cdot}1$ MΩ ($N = 8$) and the cells showed rectification with depolarizing current. Often this could not be seen as the cells would fire repeatedly with low levels of injected current. N cells had an action potential larger ($75{\cdot}4 \pm 11{\cdot}5$ mV, $N = 18$) and wider ($4{\cdot}6 \pm 1{\cdot}4$ ms) than that of T cells. Their striking after-hyperpolarization had an amplitude of $-19{\cdot}8 \pm 6{\cdot}2$ mV and a duration of 1450 ± 230 ms. The dV/dt_{max} of the depolarization ($25{\cdot}0 \pm 5{\cdot}4$ V s^{-1}) was approximately the same as that of T cells, whereas that of the repolarization was $12{\cdot}1 \pm 3{\cdot}7$ V s^{-1}. The R_{in} of N cells was $39{\cdot}6 \pm 31{\cdot}3$ MΩ ($N = 11$). After axotomy, no systematic changes were seen in lesioned T or N cells in any of the parameters examined (Fig. 9). The duration of the after-hyperpolarization

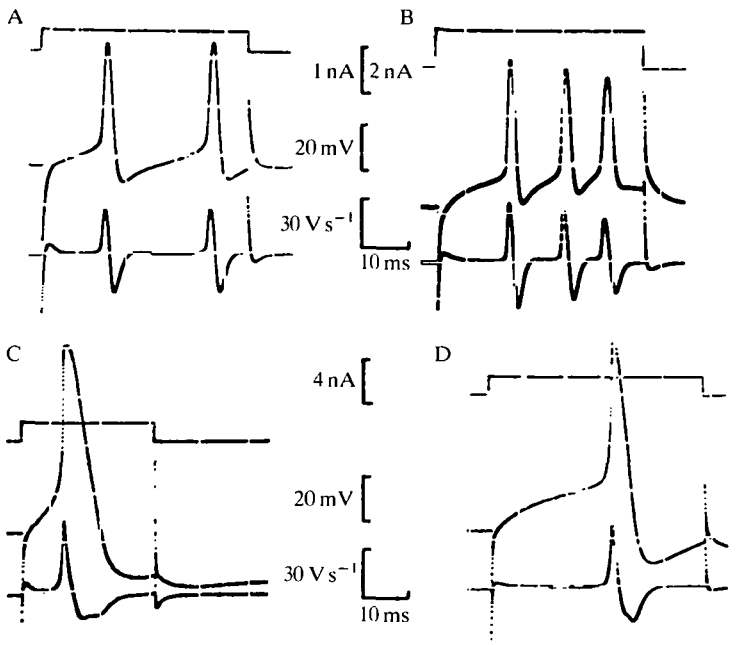

Fig. 8. Representative intracellular recordings of N and T cell responses to depolarizing current used to construct the graphs in Fig. 9. In each panel current is monitored on the uppermost trace, action potentials on the middle trace and the differentiated signals on the lowest trace. (A) Medial T cell of an unoperated animal; (B) medial T cell 15 days after lesioning the ipsilateral nerve roots; (C) medial N cell of an unoperated animal; (D) lateral N cell, 15 days after lesioning the ipsilateral nerve roots.

appeared to have decreased slightly in the axotomized N cells, but this was insignificant.

Discussion

Different sprouting responses to different lesions

The new growth elicited in adult mechanosensory neurones *in vivo* by damage to axon branches in peripheral nerves differs from that seen in the same cells following lesions to central connectives (Wallace *et al.* 1977; French & Muller, 1986). Although the pattern of growth at the site of the lesion is generally similar to that seen at central connective lesions in chains of ganglia maintained in culture (Wallace *et al.* 1977), the pattern of new growth elicited in the ganglion is different. Damage to fine-calibre accessory axon branches in peripheral nerves of adjacent segments did not elicit sprouting of the main arbor and axon branches, unlike damage to accessory branches in central connectives (Wallace *et al.* 1977). Damage to major axon branches in the peripheral nerves of the cell's own ganglion elicited new axosomatic contacts by N cells on specific neurones located in tha

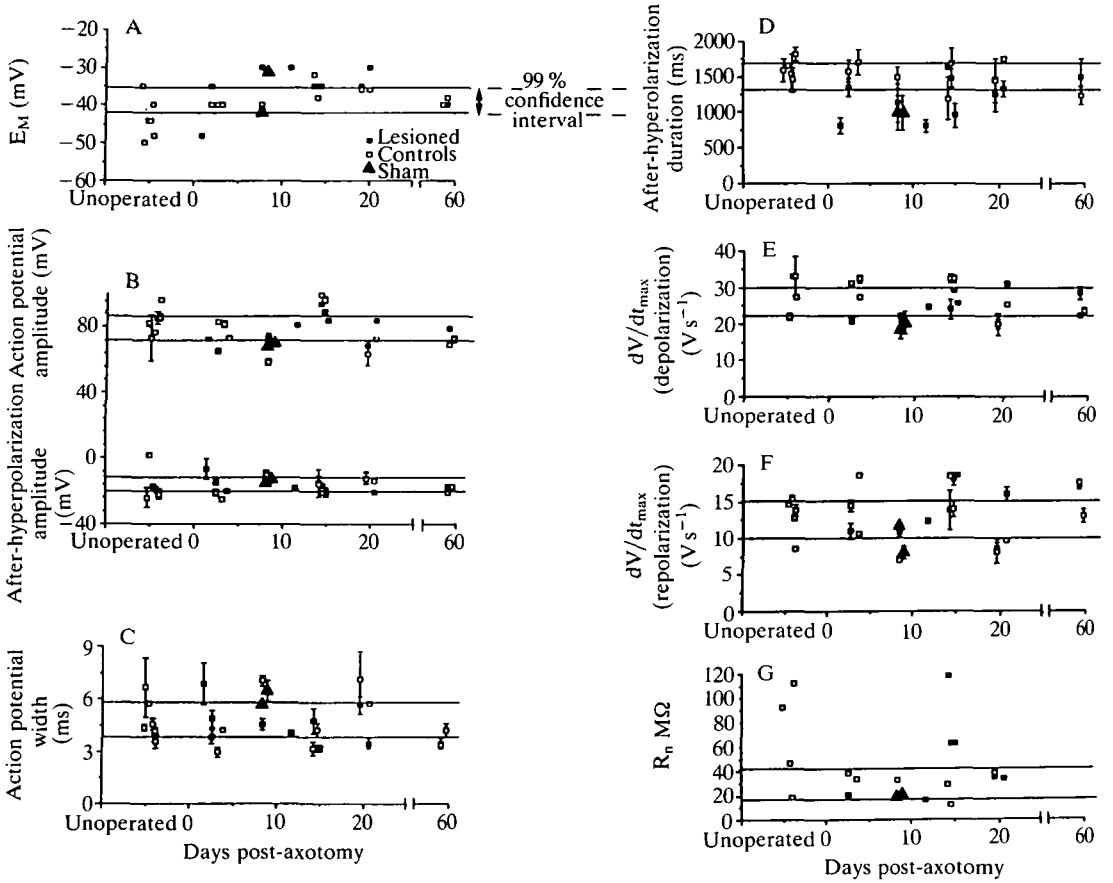

Fig. 9. The electrical properties of N cells: normal, sham-lesioned (▲), lesioned (■) and cells contralateral to the lesion (□). The axotomized cells show no significant trend in any of the parameters investigated. (B–F) Points represent the mean of 5–9 action potentials, A and G are single determinations. Confidence intervals were calculated assuming a normal distribution.

ganglion, whereas damage to axons in central tracts elicited new axosomatic contacts on different specific cells in adjacent ganglia but not in the same ganglion (French & Muller, 1986). Also, apart from the new axosomatic contacts and the supernumerary N cell branches in ipsilateral nerves, the main arbors of N and T cells were not significantly different from those of uninjured cells, whereas it is our impression that profuse sprouting of the main arbor follows lesions to central tracts (Wallace *et al.* 1977). In all types of lesions examined, however, axotomized cells develop supernumerary processes that enter tracts that have not themselves been damaged, as has been shown for axotomized frog motoneurones (Rotshenker, 1979, 1982), suggesting that local changes in the environment caused by scar tissue are not inducing or directing the new growth.

Different sprouting responses by different modalities of mechanosensory neurone

Differences in the abilities of various types of cells to grow have already been noted for neurones growing in chains of ganglia maintained in tissue culture (Wallace *et al.* 1977). In the present experiments, the low-threshold touch sensory cells produced new growth at the damaged end of the axon only, and not at ectopic sites, whereas the high-threshold nociceptive neurones produced new growth within the ganglion as well as at the site of the lesion. Physiological mapping of regenerated receptive fields following peripheral nerve lesions (Van Essen & Jansen, 1977) has shown relatively low rates of successful repair for N cells compared to T cells, even after long recovery periods. In the present experiments, there was no evidence for later retraction of the new growth that appears within days of damage to N cells, since supernumerary processes were present at the longest survival times examined. Thus, the ability of a neurone to produce new growth after injury does not necessarily contribute to successful restoration of its function. It may be that the ectopic growth in N cells represents a metabolic load on the cell that prevents successful direction of its main axon back into peripheral territory.

No change in somatic membrane properties following axotomy

Axotomy has been shown in both vertebrates and invertebrates to induce changes in the membrane properties of neurones. For example, peripheral nerve section in the cat modifies the rectification characteristics of sensory neurones in dorsal root ganglia (Czeh *et al.* 1977). In cat motoneurones the dendrites of the axotomized cell develop the ability to generate action potentials and the safety factor for antidromic invasion of the initial segment is altered (Eccles *et al.* 1958; Kuno & Llinas, 1970). Increased excitability after axotomy has also been reported for crayfish central neurones (Kuwada & Wine, 1981) and leech motoneurones (Fuchs *et al.* 1981; Pellegrino *et al.* 1984, 1985). The functional significance of such changes in membrane properties after axotomy is not clear. It is not known, for example, whether they are a consequence of disruption of normal cellular processes or whether they are specifically connected with repair of the damage and regeneration of the axon. For the formation of growth cones and the elongation of regenerating axons, new membrane must be added to growing processes. In the present experiments there were no obvious changes in *somatic* membrane properties during the time that these neurones were sprouting new processes. Thus, either the formation of new membrane for growth cones and sprouted processes is not reflected in changes occurring in the cell body or changes do occur in the cell body but are too small to be detectable by intracellular recording of whole-cell voltage responses. These experiments do not rule out the possibility of changes occurring at the site of new growth which might be too distant from the recording site to be detectable. It is clear from work on regenerating giant axons in cockroach, where recordings have been made from the axon close to the site of the lesion, that changes in membrane properties do occur and that the sequence o

induced changes (from inactivity to calcium-dependent activity to sodium-dependent activity; Meiri *et al.* 1981) resembles that seen in vertebrate neurone cell bodies during normal development (Spitzer, 1979; Fulton & Walton, 1986).

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