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THE EFFECTS OF MICROENVIRONMENT ON THE REDIFFERENTIATION OF REGENERATING NEURONES: NEURITE ARCHITECTURE, ACETYLCHOLINE RECEPTORS AND Ca^{2+} CHANNEL DISTRIBUTION

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SUMMARY

Severed adult neurones, which are capable of regrowth, encounter different microenvironments from those encountered during development. Moreover, adult neurones may respond in a different manner from developing neurones to the same environmental cues. Thus, the recovery of the integrative and transmission capabilities (which depend on the neuronal architecture, passive and active membrane properties, and synaptic receptor distribution) by a regenerating adult neurone may not be complete. In the present review, we examine several aspects of the outcome of the interaction between the microenvironment and regrowing neurones using the cockroach giant interneurones (GINs) as a model system. We demonstrate that whereas extrinsic cues govern the morphological redifferentiation and distribution of synaptic receptors, the distribution of voltage-dependent Ca^{2+} channels is to a large extent determined by intrinsic factors.

The pathway of regrowth and the architecture of regenerating GINs were studied by examination of intracellularly stained fibres. The environments provided by the connectives and ganglia are different. The elongating sprouts in the connective appeared as smooth cylinders. Within the ganglionic domain, the main longitudinal sprouts emitted neurites which extended and branched into the neuropile. The local cues for branching of neurites were eliminated by freezing and thawing of the ganglia prior to the arrival of the growing tips. The failure to extend neurites under these conditions is attributed to the elimination of extrinsic signals for morphological redifferentiation of the fibres, since the same fibres emit neurites in anterior ganglia which have not been subjected to freezing and thawing.

The distribution of acetylcholine receptors (AChRs) on the GINs was mapped by ionophoretic application of ACh. In both the intact and regenerating GINs receptors were located only on the neurites. Freezing and thawing of a ganglion eliminated the local signals for insertion and/or activation of AChRs on the neurites. Thus, both the morphological redifferentiation and the distribution of AChRs are affected by the microenvironment.

Voltage-dependent Ca^{2+} channels were detected after intracellular injection of tetraethylammonium into the GIN and in the presence of tetrodotoxin (TTX) and

Key words: regeneration, acetylcholine receptors, calcium channels, neurite geometry, microenvironment, cockroach.

Ba^{2+} in the extracellular space. The regrowing axon tips always revealed large barium action potentials independent of the CNS microenvironment. This observation is consistent with the hypothesis that Ca^{2+} plays an important role in the growth process. However, increased Ba^{2+} responsiveness was also observed in axonal segments proximal to the region of neuronal extension.

The ability of severed adult neurones to recover their functional properties, in addition to regrowing an axon and forming presynaptic terminals and dendrites, is discussed. Our findings suggest that regenerating neurones which regrow through complex adult CNS microenvironments may respond by regrowing in an atypical way. The atypical morphology and slight changes in membrane properties may lead to abnormal functioning of the regenerating neurone.

INTRODUCTION

The characteristic morphological and physiological properties of a neurone are the outcome of a developmental programme involving interaction between the intrinsic properties of the developing neurone and extrinsic cues provided by the milieu through which it grows. The importance of the extrinsic factors in moulding a developing neurone into its adult form is manifested in a variety of ways. For example, factors provided by the environment promote extension of axons. Growth cones use surface recognition molecules to distinguish among the different surfaces of axons, glial cells and extracellular matrices. The pathways selected by the growing neurone and their branching pattern are, to a large extent, determined by extrinsic signals provided by the environment in both invertebrates (Bastiani & Goodman, 1984*a*,*b*; Bastiani, Doe, Helfand & Goodman, 1985; Bentley & Caudy, 1983; Ghysen & Janson, 1980; Goodman, Raper, Ho & Chang, 1982) and vertebrates (see for example, Lance-Jones & Landmesser, 1981; Tosney & Landmesser, 1984).

Local environmental cues also participate in the determination of some of the physiological properties of developing neurones. For example, the neurotransmitter phenotype of autonomic neurones is influenced by environmental factors (Coulombe & Bronner-Fraser, 1986; Patterson & Chun, 1974, 1977*a*,*b*; Potter, Landis, Matsumoto & Furshpan, 1986). Presynaptic neuronal elements provide factors for aggregation of ACh receptors and postsynaptic specializations in muscle fibres (Cohen, 1980; Fambrough, 1979; Fischbach *et al.* 1979; Steinbach & Bloch, 1986). Factors released from neurones also seem to regulate the distribution and density of receptor molecules of other neurones (O'Brien & Fischbach, 1986).

Regenerating neurones encounter different microenvironments from those encountered by developing neurones. Whereas regenerating peripheral axons encounter a relatively simple environment similar to that encountered by the developing neurone, regenerating neurones within the CNS have to regrow through potentially complex environments. Furthermore, adult neurones may respond in a different manner from developing neurones to the same environmental cues. Therefore, the morphological pattern and the physiological properties of a regenerating neurone may be very different from those of normally developed neurones.

In the present report we use identifiable giant interneurones (GINs) from the cockroach CNS to examine three aspects of the outcome of the interactions between the regenerating adult interneurone and its microenvironment. First, we studied the effects of various microenvironments on the architectural redifferentiation of the interneurone. Next, we examined the extent of the control exerted by the microenvironment on the distribution of acetylcholine receptors along the regenerating segment of the neurone. Finally, we describe the distribution of voltage-dependent Ca^{2+} channels (VDCCs) in the normal and regenerating neurone. Our findings indicate that, although the microenvironments provide information which controls the morphological differentiation of the regenerating neurone, the precise architecture of the regenerating segments is not re-established. External cues which define the sites where AChRs are expressed are present in the adult microenvironment. However, the distribution of VDCCs of the regenerating neurones is very different from that of the normal axon, and does not seem to be controlled by extrinsic factors.

We conclude that the regenerating giant interneurones are capable of regrowing and responding to extrinsic microenvironmental cues. However, because of differences in the structure and composition of the adult and embryonic microenvironments, the morphophysiological properties of the regenerating segment are not identical to those of normal GINs. In fact, the architecture of the regenerating segments and the distribution of VDCCs are sufficiently different from normal, that it is unlikely that the neurones will be able to serve their normal functions.

THE PREPARATION

The giant interneurones (GINs) have long axons which extend throughout the entire ventral nerve cord of the cockroach, from the last abdominal ganglion (A_6), to the supraoesophageal ganglion (Farley & Milburn, 1969; Spira, Parnas & Bergmann, 1969; D. Zeldes & M. E. Spira, unpublished observations). A_6 is the ganglion where the GIN cell bodies are located and where their dendrites receive cholinergic synaptic inputs from afferents originating in the cerci (Callec, 1974). In each of the ganglia the giant axons (GAXs) send out several neurites which ramify and branch into the neuropile (Fig. 1). These neurites serve as presynaptic terminals as

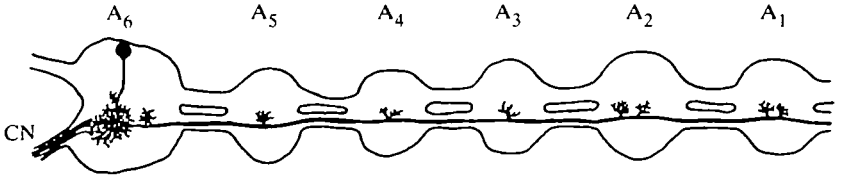

Fig. 1. Schematic drawing of the abdominal section of the cockroach ventral nerve cord and a giant interneurone. The cell body of the giant interneurone is in the last abdominal ganglion (A_6). Its axon extends throughout the cord and in each ganglion (A_6–A_1) emits neurites which branch into the neuropile. For the experiments described here the connectives were crushed between ganglia A_4 and A_3 and the regenerating segment of the interneurone was studied in the A_4–A_2 region. CN, cercal nerve.

well as postsynaptic elements (Castel, Spira, Parnas & Yarom, 1976; Ritzmann & Pollack, 1986; Spira & Yarom, 1983). The number of neurites, their distribution along the GAX within a ganglion, and their major branching pattern is constant among analogous adult GINs (Yarom & Spira, 1983). The characteristic architecture of the GINs is established during embryogenesis. Retracing of whole mounts of GINs intracellularly injected with cobalt ions and precipitated as cobalt sulphide reveals that GINs of early nymphs (stages Ad–5; Spira & Yarom, 1983) display the typical adult neuronal architecture. The relative position of the neurites within the ganglia is maintained throughout the postembryonic growth period.

The stereotypic morphology of the GINs, their ability to regrow after injury (Meiri, Spira & Parnas, 1981), their large diameter and accessibility to experimental manipulations make them a suitable preparation to study morphological, biophysical and physiological aspects of the outcome of the interaction between the regenerating neurones and the complex CNS milieu through which they regrow.

MORPHOLOGICAL REDIFFERENTIATION OF THE REGENERATING NEURONES

The characteristic regrowth patterns of the severed GINs was studied by examination of whole-mounts of intracellularly stained GINs. The initial response of the proximal segment of the injured axons is a retraction with respect to the boundary of the crush. In some cases the retraction stops only at the ganglion caudal to the point of crushing. Regrowth by tip sprouting can be observed as early as 8 days after crushing. Typically, several small sprouts emerge from the enlarged tip of the axon. The largest sprouts extend rostrally; however, some short sprouts also transiently extend perpendicular to the long axis of the GAXs. In most cases the sprouts which extend longitudinally continue to grow, whereas the others fail to elongate. The number of sprouts which successfully elongate for several millimetres (i.e. the length of the connective between ganglia A_4 and A_3 or even further to ganglion A_2) varies. In most cases 3–6 long sprouts extend from the point of crushing. The sprouts which regrow along the connectives become smooth cylinders very similar to the intact GAXs. However, their diameter is always smaller.

The linear growth pattern of the sprouts in the connective is altered as the sprouts encounter the ganglionic microenvironment. Within ganglion A_3, the main longitudinally oriented sprouts send out neurites which ramify and branch into the neuropile (Fig. 2B). These neurites form neuritic trees. The number of neurites, their precise location and their branching pattern differ from the normal (compare Fig. 2A and Fig. 2B). In most cases the number of neurites of the regenerating GAX is greater than that of the normal GAX. Whereas in the intact GINs the neurites occupy the central field of the abdominal ganglia (Fig. 2A), the regenerating neurites occupy larger fields (Fig. 2B). In spite of these differences, as in the intact GINs, the regenerated neuritic tree almost never extends contralaterally across the mid-line. The variability in the morphological patterns of the neurites and the fields occupied by them is greater in the regenerating neurones than in the normally developed one. It is interesting to note that, while neurite outgrowth is restricted, the sprout

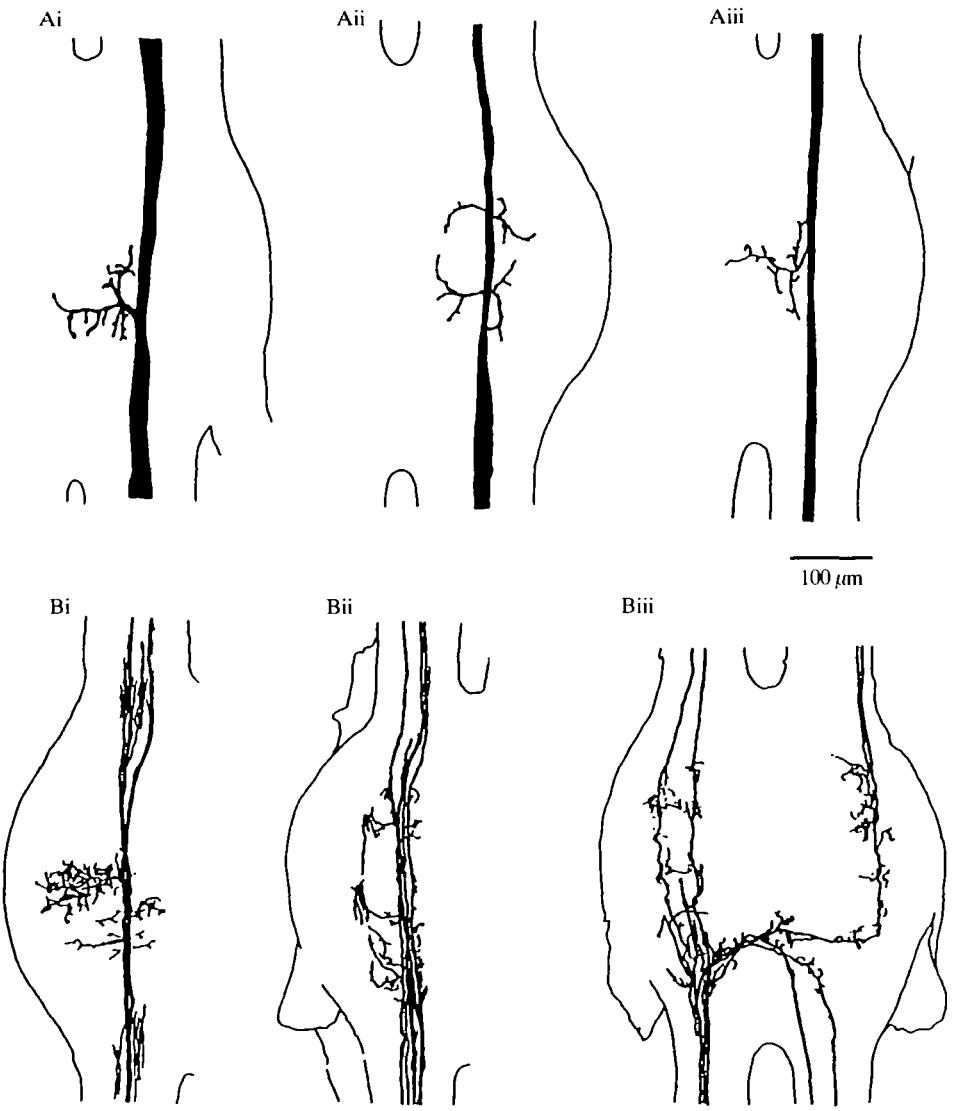

Fig. 2. *Camera lucida* retracing of cobalt-sulphide-filled giant interneurones in ganglion A_3. (Ai–iii) Control; (Bi–iii) regenerating segment of the giant interneurones. In the control, a single cylindrical giant axon sends out one (Ai, Aiii) or two (Aii) neurites which branch in the centre of the ganglion into the neuropile. Few regenerating sprouts enter ganglion A_3 after the connectives have been crushed between ganglia A_4 and A_3. (The bottom of the drawing corresponds to the posterior region of the nerve cord.) The elongating sprouts send out several neurites which extend into the neuropile. The regenerating neurites occupy a larger field than normal. Even when major errors in direction of growth are observed (Biii), the elongating sprouts regenerate as smooth cylinders within the connective and central path in the ganglion, while sending out neurites into the neuropile. 103 (Bi), 136 (Bii) and 92 (Biii) days after crushing of the A_4–A_3 connectives.

occupying the original position of the GAX within the ganglion continues to elongate to the more distal connective. The characteristic growth in the connective and the ganglia repeats itself, i.e. linear growth in the connectives and the reformation of neuritic trees within the ganglionic microenvironment.

On several occasions we observed major errors in the direction of growth. An example is illustrated in Fig. 2Biii. Three major sprouts cross ganglion A_3 at its base to the contralateral side; one of these grows rostrally, whereas the other two elongate caudally. Such crossing of the GIN was never observed in intact preparations. It is interesting to note that the contralateral sprouts, too, maintain the characteristic pattern of growth, i.e. the sprouts occupying the original position of the GAXs grow linearly as smooth cylinders. However, within the ganglion limited neuritic outgrowth perpendicular to the main axonal pathway takes place.

These observations indicate that the adult microenvironment through which the GAXs regrow provides cues to support linear growth of the main sprouts, branching of neurites into the neuropile to form a neuritic tree and, finally, local signals to terminate the growth of the neurites. Nevertheless, the architecture of the GAXs within ganglion A_3 is not identical to that of the normally developed neurone. Furthermore, the variability of the morphology of the regenerating neurites is greater than that observed in the normally developed GAXs.

ALTERATIONS OF THE GANGLIONIC MICROENVIRONMENT AND THEIR EFFECTS ON THE MORPHOLOGICAL REDIFFERENTIATION OF THE REGENERATING AXON

As a first step towards understanding the sources and nature of the cues provided by the ganglionic microenvironment to the regenerating GAXs, we destroyed the cellular elements within A_3 by freezing and thawing the ganglion, prior to the arrival of the growing tips. This was achieved by freezing the exposed ganglion (A_3) with a metal rod cooled in liquid nitrogen. The ganglion was then placed back into the abdomen. The effects of this procedure on the structural organization of the ganglion are illustrated in Figs 3–6. (For a description of the structural organization of a normal ganglion see Lane, 1985.) Most of the neuronal cell bodies which normally occupy the cortex of the ganglion are destroyed (compare Fig. 3A with Fig. 4A,B). In a few experiments a small number of cell bodies survive the treatment. The typical arrangement of the glial layer separating the cortex from the neuropile is no longer identifiable (compare Fig. 3A,B with Fig. 4A,B). The characteristic organization of the neuropile, which includes axonal profiles oriented in various directions, and the presynaptic and postsynaptic elements disappear (Figs 3–5). Cross-sections of the neuropile prepared for electron microscopy reveal that 24–48 h after freezing and thawing, the neuropile contains fragmented membranes, swollen mitochondria, myelinated bodies but no identifiable intact neuronal elements (Fig. 5).

20–30 days after connective crushing, and freezing and thawing of A_3, regenerating fibres regrow through the core of the ganglion (Figs 4, 5). However, the

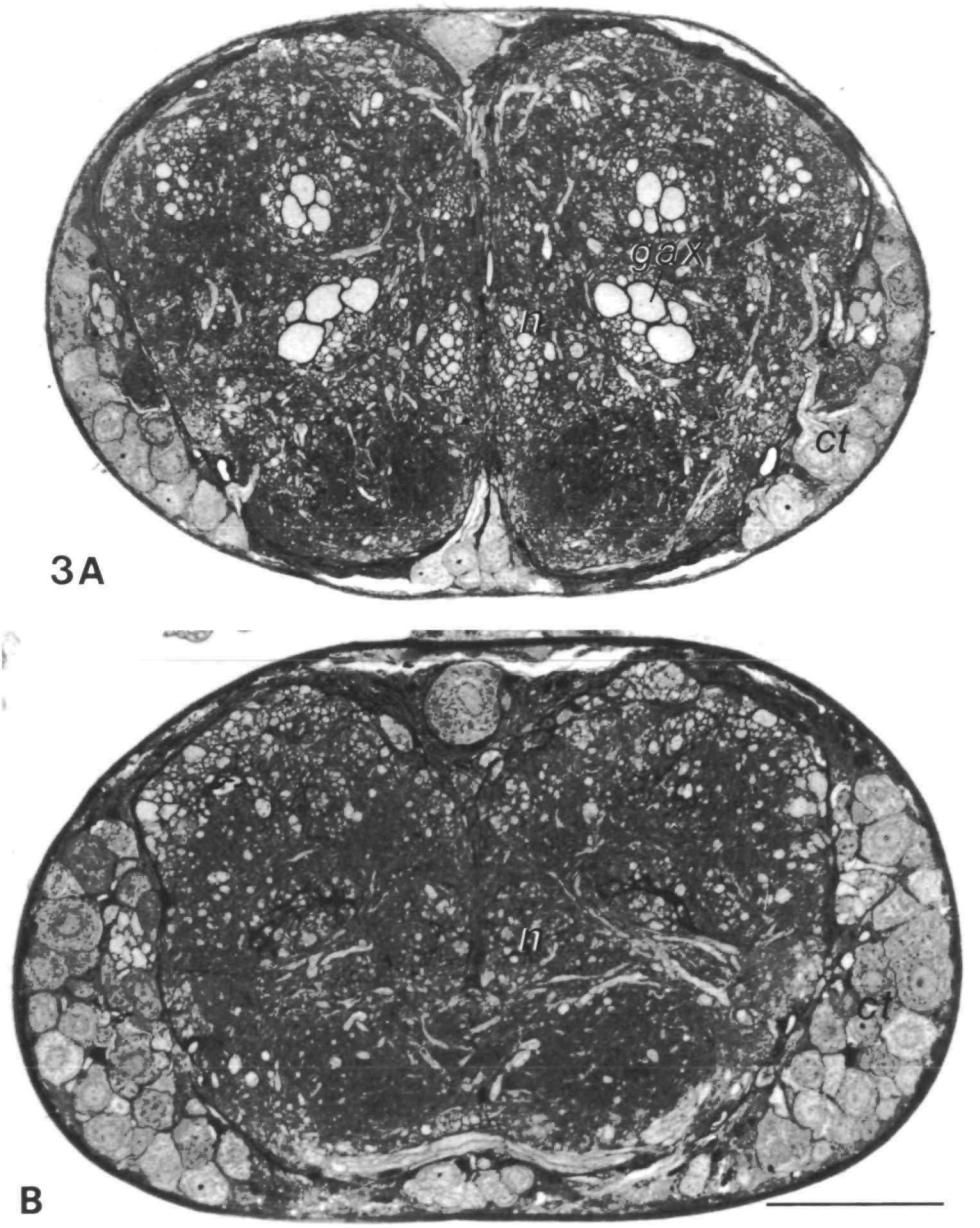

Fig. 3. Structural organization of ganglion A_3, in the control (A) and 58 days after crushing of connectives A_4–A_3 (B). The general structural organization of the ganglion was not altered by the crush. However, the profiles of the giant axons seen in A are no longer recognizable after crushing of the connectives. *gax*, giant axons; *n*, neuropile; *ct*, cortex. Scale bar, 100 μm.

Structural organization of the ganglion never recovers. The cortex remains almost devoid of cell bodies. The glial layer separating the cortex and neuropile is not reformed (Fig. 4). The neuropile is occupied by a large number of neuronal profiles,

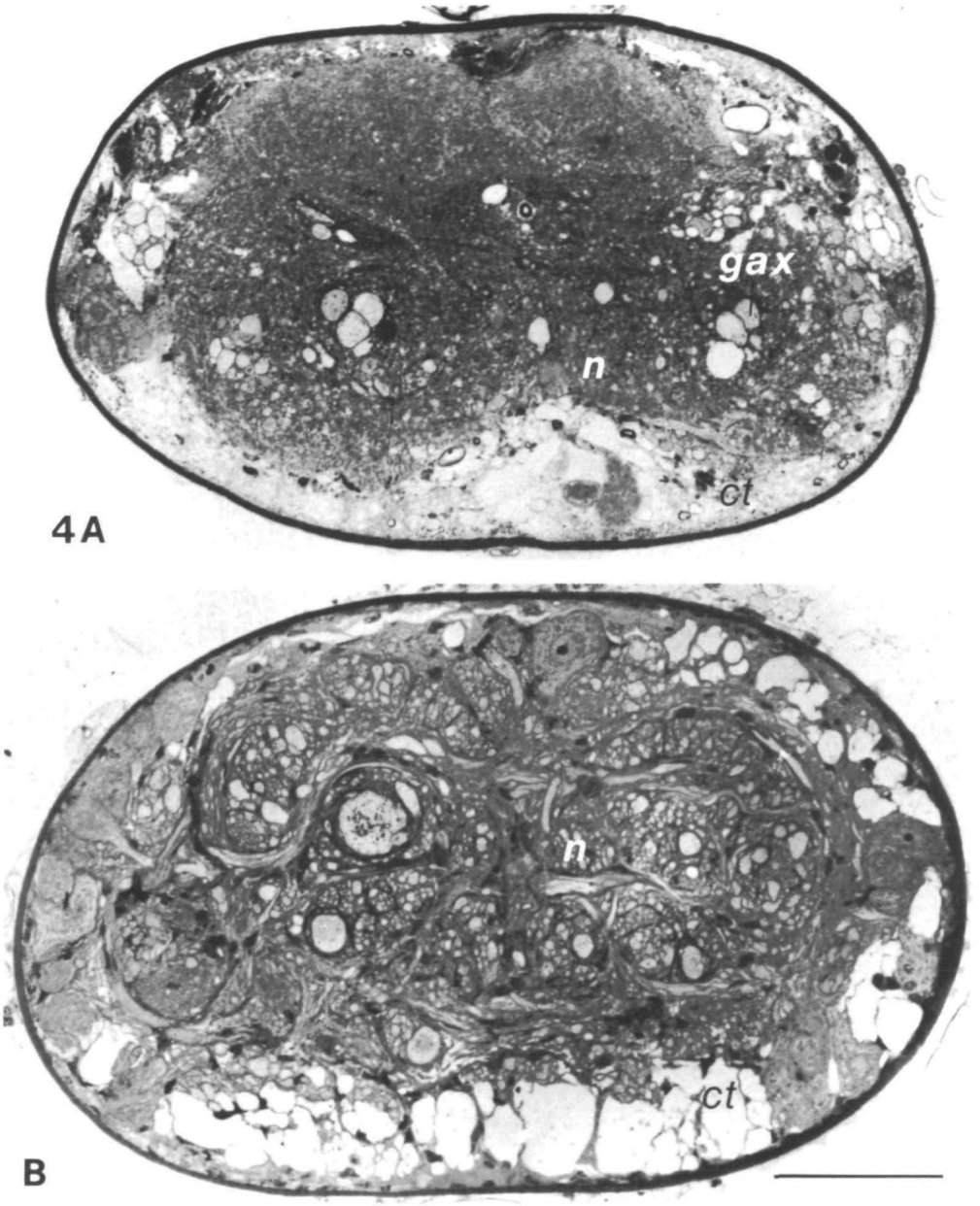

Fig. 4. Structural organization of ganglion A_3 after crushing of A_4–A_3 connectives and freezing and thawing of ganglion A_3. (A) 24 h and (B) 58 days after the crush, freeze and thaw procedure. (A) The cell bodies and most of the axonal profiles within the ganglion are destroyed. Profiles of giant axons (*gax*) can still be recognized. (B) The core of the ganglion is occupied by regenerating axons. Apart from the very few cell bodies that survive the freezing procedure, the cortex (*ct*) is devoid of cell bodies. *n*, neuropile. Scale bar, 100 μm.

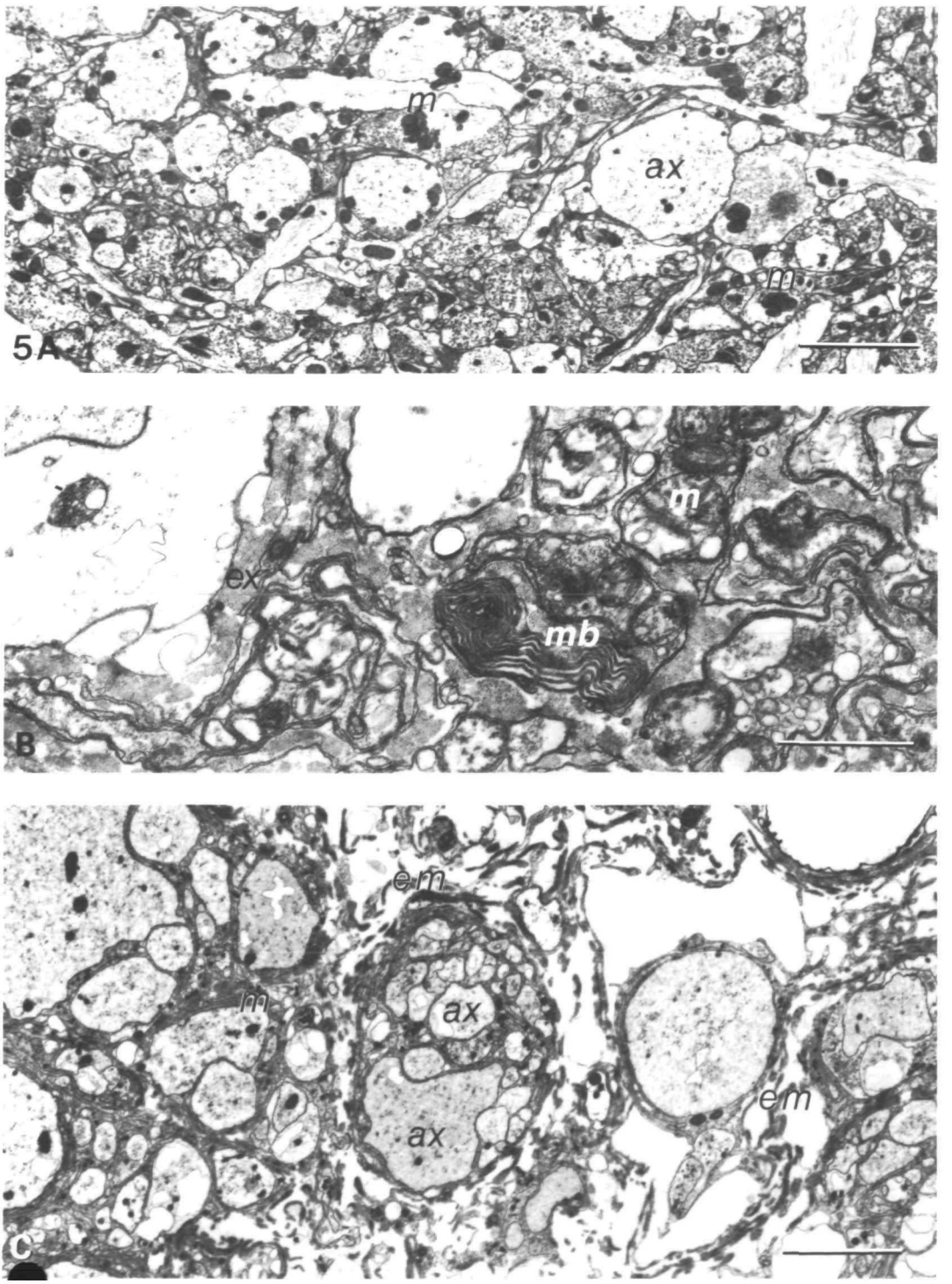

Fig. 5. Electron micrograph of the neuropile. (A) Control, (B) 24 h and (C) 30 days after crushing the connectives (A_4–A_3) and freezing and thawing ganglion A_3. *ax*, axons; *m*, mitochondria; *mb*, myelinated bodies; *em*, extracellular matrix. Scale bars; A,C, 5 μm; B, 2 μm.

some of which traverse the core in various directions (Fig. 4B). However, very few neuronal elements contain synaptic vesicles and reveal synaptic specializations.

Retracing of whole-mounts of cobalt-filled GAXs reveals that GAXs regrowing through the 'freeze–thawed' A_3 extend as smooth axial cylinders, and fail to send out neurites into the core of the ganglion (Fig. 6). The failure to extend a typical neuritic tree cannot be attributed to a mechanical impedance to regrowth into the neuropile. Electron microscopic observations clearly revealed large spaces within the core of the ganglion. Furthermore, even in cases where the GAX regrows through the ganglion in various directions, the axon never extends typical neurites (Fig. 6C). The failure of the GAXs to extend neurites under these conditions is attributed to elimination of extrinsic signals for branching within A_3 rather than to loss of the ability of the axon to respond to external cues. This is evident from the fact that the same axons that failed to respond to the altered environment extend neurites into the neuropile of anterior ganglia which have not been subjected to freezing and thawing.

MAPPING OF ACETYLCHOLINE SENSITIVITY OF REGENERATING GIANT INTERNEURONES

The distribution of acetylcholine receptors (AChRs) in control and regenerating GINs was studied by recording intracellularly the membrane responses to ionophoretic application of ACh from a second micropipette positioned at various locations within A_3 (Fig. 7). To maximize the responses the experiments were performed after superfusion of the isolated CNS by a solution containing an acetylcholinesterase inhibitor (10^{-4} mol l^{-1} neostigmine). ACh sensitivity maps were generated also in the presence of a high-magnesium, low-calcium solution to avoid confusion between direct responses of GAXs to ACh and indirect depolarizations induced by activation of presynaptic neurones (Fig. 7A).

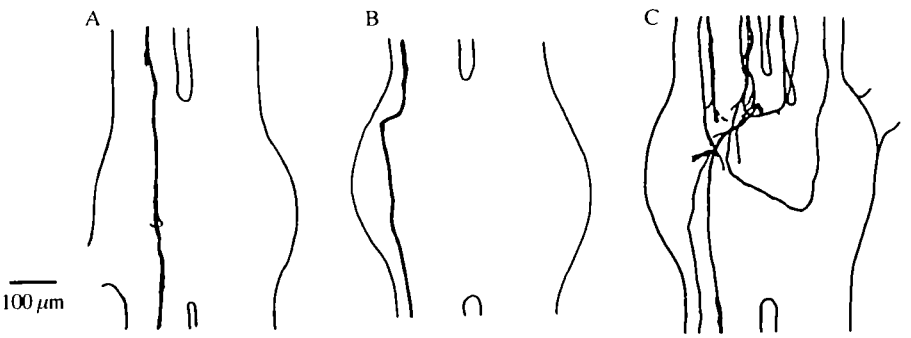

Fig. 6. Regrowth of the giant axon into the altered ganglionic microenvironment. *Camera lucida* retracing of cobalt-sulphide-filled regenerating giant interneurones growing into ganglion A_3 which had been frozen and thawed 93 (A), 52 (B) and 80 (C) days prior to the fixation. The regenerating neurone regrows as a smooth cylindrical sprout (A,B). Even when the regenerating sprout regrows in various directions within the ganglion, it never extends branching neurites (C).

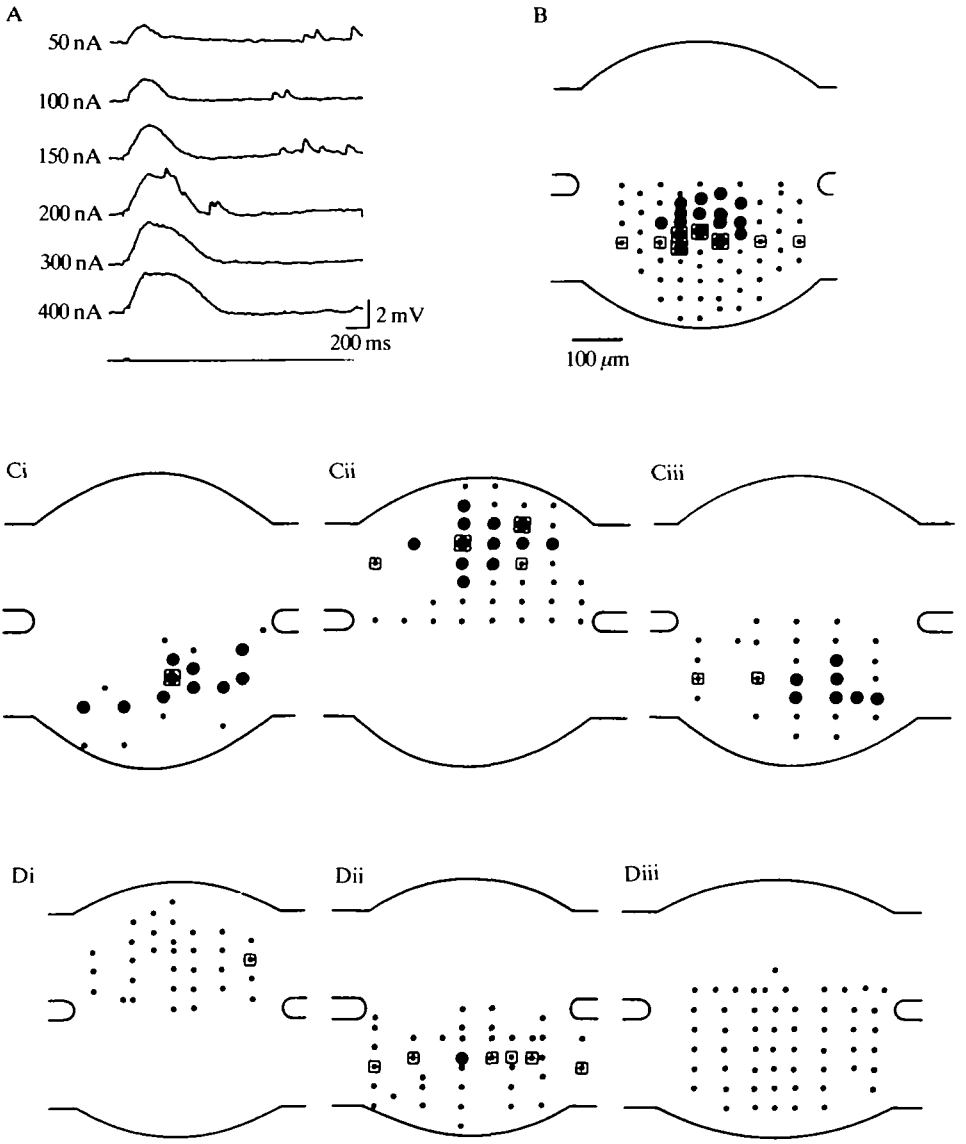

Fig. 7. Acetylcholine sensitivity maps of an intact giant axon (B) and regenerating giant axons after crushing of the connectives (C) and after crushing of the connectives and freezing and thawing of A_3 (D). Responses to ionophoretic application of acetylcholine at various locations within the ganglia were recorded intracellularly at the base of A_3 (A). In the control, the responses are restricted to the central field of the ganglion (B). Small circles, no responses; large circles, positive responses; squares, ionophoretic electrode penetrated the giant axon. The distribution of positive responses in regenerating axons, 60–90 days after crush, corresponds to the enlarged fields occupied by the neurites (C). Giant axons regenerating through the freezed–thawed A_3 rarely exhibit any acetylcholine sensitivity (D).

In the control preparation, positive responses were obtained from the central field of the ganglion, an area which corresponds to the sites occupied by the neurites (Fig. 7B). Ionophoretic application of ACh to the main axon within the ganglion, or

the connective, produced no depolarizations (Fig. 7B, small circles surrounded by squares). The failure to initiate ACh responses in the axonal region is not due to diffusional barriers or to problems with placement of the ionophoretic micropipette close enough to the fibre, since in many cases the ACh-containing micropipette penetrated the fibre while ionophoresing ACh (circles surrounded by squares).

Sensitivity maps were established for preparations in which the connectives had been crushed 40–95 days prior to the experiment (Fig. 7C). Positive responses were recorded from much larger regions within the ganglia. These enlarged sensitive fields correspond well to the larger field occupied by the neurites. The sprouts within the connectives do not reveal ACh sensitivity. Regenerating GAXs that regrow through a freezed–thawed ganglion A_3 show no responsiveness to the ionophoresis of ACh (Fig. 7D).

In conclusion, the microenvironment within the adult ganglia provides localized cues which influence the morphological patterns produced by the regenerating GAXs and determine the sites for expression of ACh sensitivity. Freezing and thawing eliminates both the cues governing neurite formation and the signals for AChR expression.

DISTRIBUTION OF VOLTAGE-DEPENDENT Ca^{2+} CHANNELS IN THE INTACT AND REGENERATING GIANT AXONS: VOLTAGE-DEPENDENT Ca^{2+} CHANNELS ALONG THE INTACT GIANT AXONS

To establish the experimental procedures for detection of voltage-dependent calcium channels (VDCCs) in the GAXs, we first examined the axonal segment of the metathoracic ganglion (T_3). This axonal segment was selected since previous morphological and physiological studies had demonstrated that the neurites of the GAXs in T_3 serve as presynaptic terminals for neurones involved in the escape response of the cockroach as well as for other GAXs (Castel *et al.* 1976; Ritzmann & Pollack, 1986; Spira, Zeldes & Krasner, 1987; Yarom & Spira, 1982). Thus, it was expected that voltage-dependent Ca^{2+} responsiveness should be detected in this region.

The experiments were carried out by insertion of two microelectrodes close to the caudal base of T_3 (Fig. 8A). Brief stimulation of the connective evokes an action potential (Fig. 8B). Intracellular injection of tetraethylammonium ions (TEA^+) produces a large increase in spike duration due to blockade of potassium conductance (Fig. 8C) (Armstrong & Binstock, 1965; Hochner & Spira, 1986; Pelhate & Pichon, 1974; Pelhate & Sattelle, 1982; Pichon, 1976; Yawo, Kajima & Kuno, 1985; Yarom & Spira, 1983). The prolonged action potential produced under these conditions is abolished by TTX (10^{-5} mol l^{-1}, Fig. 8D), indicating that its initiation requires the activation of voltage-dependent sodium channels (Narahashi, 1966). Under these conditions, depolarization of the axon in physiological solutions containing up to 50 mmol l^{-1} Ca^{2+} does not produce any regenerative responses. However, when 100 mmol l^{-1} barium ions are substituted for sodium ions, a brief stimulation produces a long-lasting action potential (Fig. 8E). In most cases, the response is

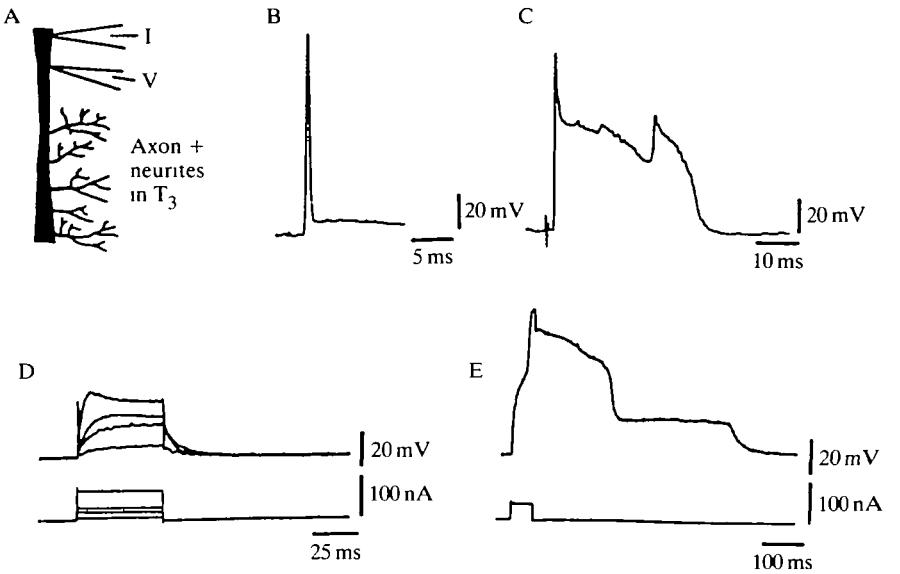

Fig. 8. The Ba^{2+} action potential in the intact giant interneurone at the level of the metathoracic ganglion. (A) Schematic drawing of the position of the electrodes. I, current-injecting electrode; V, voltage-recording electrode. (B) The action potential in control solution. (C) The action potential was prolonged after intracellular injection of tetraethylammonium ions. (D) The prolonged spike of C was blocked with tetrodotoxin (TTX) (10^{-5} mol l^{-1}). (E) When Ba^{2+} (100 mmol l^{-1}) was added and in the presence of TTX a regenerative Ba^{2+} action potential was generated (for further details see text).

composed of an early overshooting plateau and a later, smaller plateau. The two components of the spike may represent regenerative potentials initiated at two sites on the axon; for example, close to the recording electrode (at the base of the neurites) and remote from the electrodes (at the tip of the neurites). Alternatively, they may represent two populations of channels whose properties differ. The responses are blocked by the addition of 5 mmol l^{-1} cobalt or 2 mmol l^{-1} cadmium ions, and are not blocked in sodium-free solution. The potentials can be terminated by a brief hyperpolarizing pulse superimposed on the plateau. Thus, we may conclude that these regenerative potentials are produced by the flow of Ba^{2+} through the VDCCs.

We next examined whether such Ba^{2+} potentials could be recorded in the connectives between ganglia A_4 and A_3, the region of connective crushing and sprouting of the GAXs. In more than 10 experiments in which we have applied the same procedures described earlier for T_3, only once have we detected a small and prolonged regenerative Ba^{2+} response (Fig. 9). To further improve our ability to detect VDCCs in this region, we took advantage of the fact that the crushed end of the GAXs reseals rapidly (Yawo *et al.* 1985). By crushing the connectives *in vivo* at two sites (between connectives A_3–A_4 and A_3–A_2, 24 h prior to the experiment), an isolated short segment of the GAX containing A_3 was formed. This isolated segment revealed normal resting and action potentials, and had an input resistance of 6–10 times the normal value. Even under these favourable conditions, we have detected no

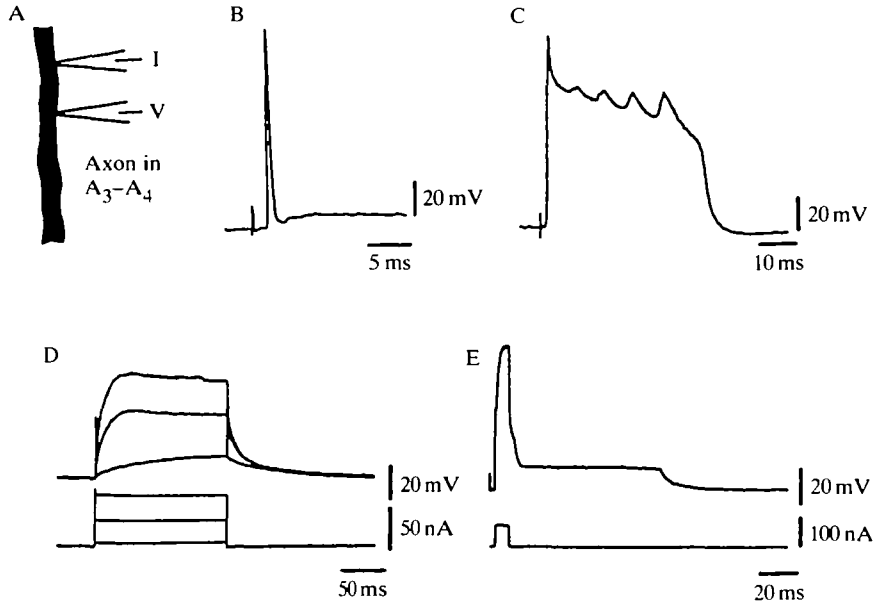

Fig. 9. The Ba^{2+} action potential in the giant axon at the connective between ganglia A_3 and A_5. (A) Electrode arrangement (I, V as in Fig. 8). (B) Action potential in control solution. (C) after intracellular injection of tetraethylammonium; (D) after addition of tetrodotoxin (TTX), 10^{-5} mol l^{-1}; (E) after substitution of 100 mmol l^{-1} Ba^{2+} for Na^{+}. This is the only case in which a small regenerative Ba^{2+} response was recorded. In no other experiments were regenerative Ba^{2+} action potentials detected in this region.

VDCCs in this axonal segment. Thus, we may conclude that, whereas the presence of VDCCs is easily detected in the region of T_3, the density of VDCCs in the region of A_3–A_4 is too low to be detected by our methods.

DISTRIBUTION OF VOLTAGE-DEPENDENT CALCIUM CHANNELS IN THE REGENERATING INTERNEURONE

To study whether the density and distribution of VDCCs is altered in the regenerating GAX, the axon was impaled with two microelectrodes just proximal to the region of crush (Fig. 10A). Using the same experimental procedures described earlier, it became evident that either intracellular stimulation of the axon (Fig. 10E) or extracellular stimulation of the sprouts (Fig. 10F) evoked a Ba^{2+} spike. Typically, intracellular stimulation evoked a spike with a large overshooting plateau and a delayed smaller component (Fig. 10E). The two components probably represent two regions of Ba^{2+} spike initiation. The overshooting plateau is probably generated by the axonal membrane and the smaller component by the sprouts. It is conceivable that the delayed smaller component is attenuated while propagating from the fin sprouts into the large-diameter axon. This interpretation is supported by the observation that a weak extracellular stimulation to the sprouts produces only a small

response (Fig. 10F). However, when the stimulus intensity is increased, a larger overshooting plateau is generated.

Experiments of this type clearly show that the density of VDCCs is increased in the region of regrowth with respect to the control. Nevertheless, the experiments do not allow us to define whether the increase in VDCCs is localized to the regrowing axonal segment, the sprouts or to non-regrowing regions of the injured axon. To address this question, we utilized several procedures, only one of which will be described here. The regenerating GAXs were recrushed between ganglia A_4 and A_3 to form an isolated segment containing mainly the proximal segment (Fig. 11A), or a regenerating segment including the sprouts (Fig. 12A). Using the same procedures, i.e. intracellular TEA^+ injection, superfusion with 10^{-5} mol l^{-1} TTX and 100 mmol l^{-1} Ba^{2+}, we recorded a large overshooting Ba^{2+} spike from the proximal segment (Fig. 11E) and a smaller Ba^{2+} spike from the sprout-containing segment (Fig. 12B,C). From these experiments, and others in which we microperfused the sprouts or segments of the axons with solutions containing high Ba^{2+} concentrations,

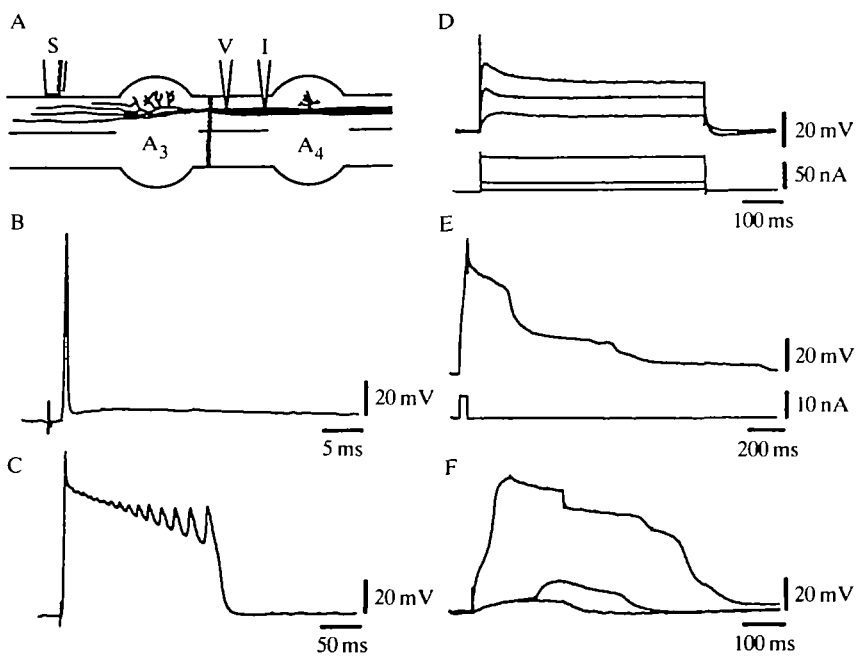

Fig. 10. The overshooting Ba^{2+} action potentials in the regenerating segment of the crushed giant axon. (A) Schematic drawing of the electrode arrangement. The two electrodes (I, V as in Fig. 8) were inserted close to the site of crushing, and an external stimulating electrode (S) was placed on the connectives between ganglia A_3 and A_2. (B) Action potential in control solution. (C) Prolonged action potential after intracellular injection of tetraethylammonium. (D) Application of tetrodotoxin (TTX, 10^{-5} mol l^{-1}) blocked the prolonged action potential. (E,F) The Ba^{2+} action potential after substitution of Ba^{2+} (100 mmol l^{-1}) for Na^+. Intracellular stimulation produced an overshooting action potential followed by a smaller potential (E). Intracellular stimulation of the sprouts produced a small response (F); with increased stimulus intensity an overshooting spike was initiated (see text for further details).

we conclude that the increased Ba^{2+} responsiveness in the regenerating axon is not restricted to the sprouts but also occurs in regions proximal to the original crush. Increased Ba^{2+} responsiveness was also observed in GAXs regrowing through the frozen and thawed ganglionic microenvironment. We conclude that the microenvironment through which the sprouts regrow does not modulate the distribution and density of the VDCCs as it does the morphological redifferentiation and the distribution of AChRs.

DISCUSSION

Morphological redifferentiation of GAXs – implication for functional repair at the cellular level

Our observations show: (1) that regenerating segments are capable of responding to a variety of local extrinsic cues and (2) that the basic signals which promote linear growth of the sprouts occupying the original region of the axon are present. Further, cues which promote branching of neurites into the neuropile are effective and factors which terminate dendritic growth are operational. However, in spite of the fact that

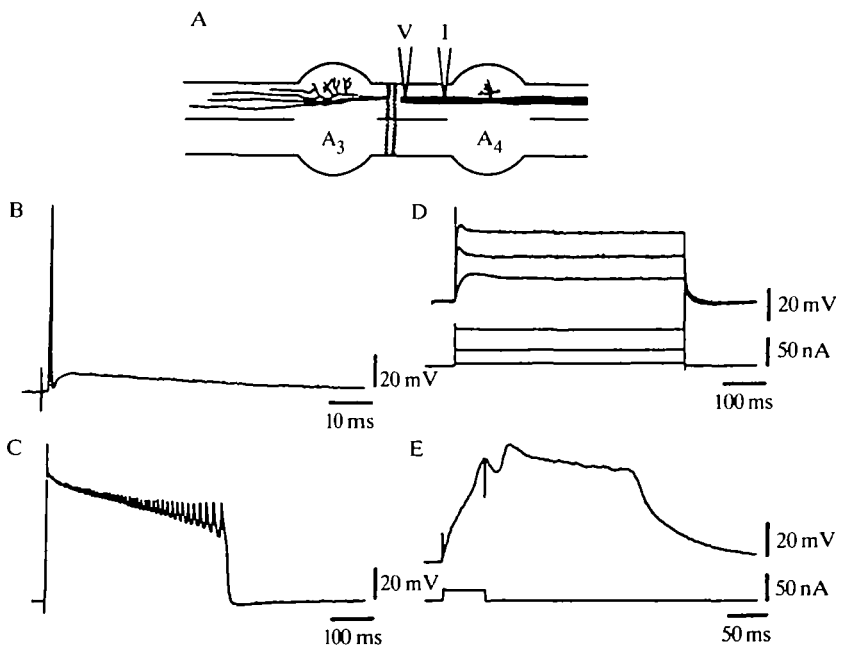

Fig. 11. The overshooting Ba^{2+} action potential generated by the giant axon membrane proximal to the region of sprouting. 24 h prior to the experiment the regenerating axon had been recrushed between ganglia A_4 and A_3. The crushed end resealed and the recording was made from the giant axon proximal to the region of sprouting (A). (B) Action potential in control solution. (C) Prolonged action potential after injection of tetraethylammonium. (D) The regenerative potential was blocked by tetrodotoxin (TTX, 10^{-5} mol l^{-1}). (E) After substitution of 100 mmol l^{-1} Ba^{2+} for Na^{+}, depolarization produced a prolonged overshooting Ba^{2+} spike. Note: the intact giant axon never revealed such a Ba^{2+} response in this region.

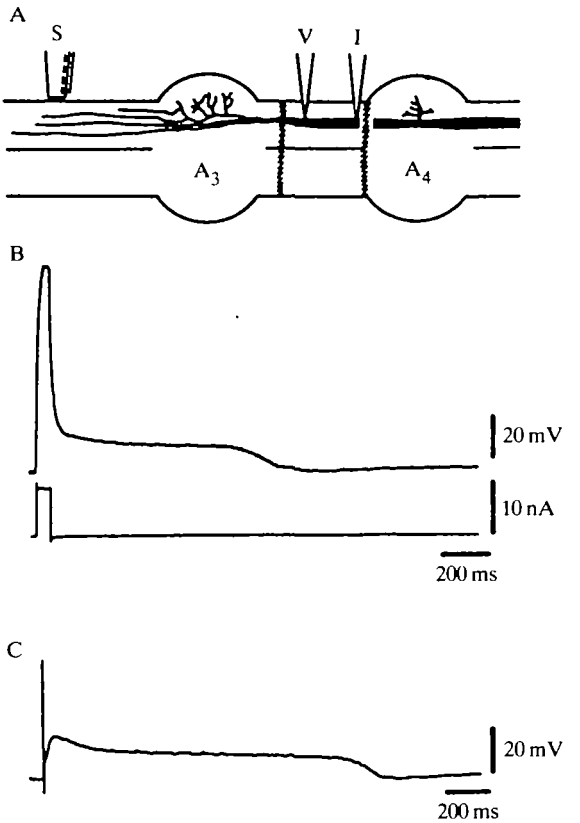

Fig. 12. The small regenerative Ba^{2+} spike generated by the giant axon sprouts (procedure as in Fig. 11). (A) Schematic drawing of electrode placements (labelled as in Fig. 10). (B) The action potential after injection of tetraethylammonium, in the presence of tetrodotoxin (10^{-5} mol l^{-1}) and high Ba^{2+} concentration. Depolarization of a distal segment (B) or external stimulation of the sprouts (C) produced a small regenerative action potential.

the basic morphological pattern of the GAXs is reformed, regenerating segments differ from normal ones in many details. It is reasonable to assume that, due to the multiple sprouting from the crushed end of the axon and the complex environment provided by the adult CNS, the overall architecture of the regenerating GAX is more complex. In addition, the dimensions of the regenerating segments are different. For example, the diameter of the regenerating sprouts in the connectives and ganglia is substantially smaller than in the normal axon. These differences imply that in spite of the elongation of the axon, which may provide a communication link between ganglia, the normal functions of the GAX cannot be restored. For example, the conduction velocity of action potentials along the intact GAXs is 3–6 m s^{-1} (Spira *et al.* 1969; Dagan & Parnas, 1970), whereas along the regenerating segments it is less than 2 m s^{-1}. In addition, our unpublished results show that an area of low safety factor for impulse propagation is formed at the points of branching of sprouts from the original proximal segments of the GAXs. This is attributed to impedance

mismatching due to the geometry of the axons and sprouts (Parnas, Spira, Werman & Bergmann, 1969; Parnas, Hochstein & Parnas, 1976; Parnas & Segev, 1979; Spira, Yarom & Parnas, 1976). Thus, trains of action potentials at moderate frequencies may fail to propagate in this region. In view of the fact that the electrotonic dimensions of the neurites are determined to a large extent by the precise geometries of parent and daughter branches (Burke, 1987), it is predicted that the efficacy of synaptic potentials generated on the neurites will be different from normal. Thus, we conclude that, in spite of the ability of the GAXs to regrow and to respond to environmental cues, the atypical architecture of the regenerating sprouts will result in abnormal functioning of the neurone.

Modulation of acetylcholine sensitivity of the regenerating neurone

The results clearly demonstrate that the microenvironment of the neuropile promotes the accumulation of ACh-activated ionic channels on the regenerating neurites. The spatial resolution of our experiments does not allow us to determine the precise distribution of the receptors. Nevertheless, as in the normal GINs, the regenerating GAXs express ACh sensitivity only on the neurites and not along the axon, indicating that the promoting signals are very localized. Since the ACh-sensitivity-promoting signals are eliminated by freezing and thawing of the ganglion, it is likely that they are generated by presynaptic terminals. It is conceivable that the ACh sensitivity of the regenerating neurone is induced in a similar way to that induced by motoneurones on a target myotube during embryonic development (Cohen, 1980; Fambrough, 1979; Fischbach *et al.* 1979; Steinbach & Bloch, 1986). That ACh sensitivity of regenerating GAXs is not confined to the original central region of the ganglion, suggests that the regenerating neurites come into contact with presynaptic terminals which are either not present during development or are not accessible for contact with the developing neurite. Alternatively, it is possible that the signal promoting neurite branching also directly or indirectly promotes the expression of AChRs on the neurites. Whatever the mechanism, the complex environments provided by the adult ganglion promote an atypical distribution of AChRs.

Distribution of voltage-dependent Ca^{2+} channels

The presence of VDCCs in the region of sprouts, as well as in proximal segments of the regenerating GAX, clearly shows that this variable is not controlled efficiently by the environment. Our findings of an increase in voltage-dependent Ba^{2+} responsiveness at the region of sprouting is consistent with the hypothesis that voltage-dependent Ca^{2+} channels and free intracellular Ca^{2+} level play an important role in the growth process (Anglister, Farber, Shahar & Grinvald, 1982; Connor, 1986; Llinas, 1979; Llinas & Sugimori, 1979; MacVicar & Llinas, 1985). However, since increased density of VDCCs is not restricted to the growing sprouts but also appears in the membrane of the GAX proximal to the crush, it is possible that th increase is an epiphenomenon of the injury. Elevation in density of Na^+ and Ca^{2+} channels in injured neurones has been documented in a number of systems: for

example, insect cell bodies which are not excitable become excitable after axotomy (Pitman, Tweedle & Cohen, 1972). Axotomy of the Mauthner cell of fishes causes a persistent change in voltage-gated sodium channel distribution in the region of the soma and axon hillock (Faber, 1984; Titmus & Faber, 1986; Titmus, Faber & Zottoli, 1986). Axotomy of cat motoneurones results in the appearance of sodium-dependent regenerative responses in the dendrites (Sernagor, Yarom & Werman, 1986). These alterations are thought to be a result of the alteration in the metabolism and reduced dimensions of the injured neurone (Sernagor *et al.* 1986).

The consequences of increased density of VDCCs over larger areas of the neuronal membrane for the functioning of the GAXs was not investigated. Nevertheless, such changes may result in alteration of a number of parameters that may alter the function of the neurone. For example, the threshold for the generation of action potentials may be changed, the typical pattern of firing may be altered and, finally, if the increase in VDCC density is also present in presynaptic terminals, the output of the neurone may be altered.

To conclude, our findings demonstrate that the regenerating GAX can regrow and provide a functional link between ganglia. Furthermore, the GAX re-establishes some of the basic morphological patterns and physiological properties of the normal neurone. In spite of this, differences in the detailed morphological pattern, distribution of receptors and of VDCCs along the regenerating segment may prevent the neurone from re-establishing normal physiological functions.

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