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Changes in motor nerve terminals during bupivacaine-induced postsynaptic deprivation

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INTRODUCTION

In addition to blocking nerve conduction for variable periods of time, many local anaesthetic agents are known to damage skeletal muscle following local injection (Pizzolato & Mannheimer, 1961). Bupivacaine (Marcaine) as well as some of its derivatives have been shown to have a specific myotoxic effect and its injection into the muscle tissue of both amphibia and mammals results in rapid degeneration of muscle cells followed by regeneration with no damage to intramuscular nerves (Benoit & Belt, 1970; Hall-Craggs, 1974; Jirmanová, 1975; Nonaka *et al.* 1983; Tal & Rotshenker, 1984; Carlson & Rainin, 1985; Sadeh, Stern & Czyzewsky, 1985 and Helm, Lee & Pho, 1987). A similar effect is produced by cardiotoxin (Duchen, Excell, Patel & Smith, 1974). In this context, terminal axons initiate synaptogenesis as soon as the first regenerating myotubes appear (Jirmanová & Thesleff, 1972). Nevertheless, in previous reports it is not clear whether axonal endings remain unmodified in the same endplate site at the regenerative stage or, alternatively, whether nerve ending retraction and/or sprouting could be induced in the terminal branches during the period of postsynaptic deprivation by necrotising lesion of the muscle cell. In electron microscopy studies the observed nerve terminals seem to keep their normal ultrastructure through out the entire period of muscle cell deprivation. In amphibia (Tal & Rotshenker, 1984) and mammalian preparations (Jirmanová, 1975; Jirmanová & Thesleff, 1976) of silver-impregnated muscles, changes described as nerve ending disappearance or growth have been reported (Sadeh *et al.* 1985).

It seems interesting to know precisely the fate of the nerve endings during the period of the bupivacaine-induced necrosis and absence of the postsynaptic cell because the secretion by the muscle target of a trophic factor or growth regulatory substance(s), can be the most plausible mechanism governing the maintenance and growth responses of the terminal axons and endings (Brown, Holland & Hopkins, 1981; Slack, Hopkins & Pockett, 1983; Bennett, 1983). In the present work we have used Bielschowsky–Gros silver impregnations and conventional electron microscopy for evaluating the possible existence of reformation or reorganisation phenomena in the motor terminal axons of the sternocleidomastoid muscle of the young adult rat during the first days of bupivacaine-induced muscle cell necrosis. Morphological findings that indicate precise nerve terminal remodelling changes are discussed in the context of the regulatory mechanisms governing the maintenance of the motor nerve terminals.

MATERIAL AND METHODS

In the present series of experiments the sternocleidomastoid muscles (SCM) of adult Sprague–Dawley rats (200 g) of both sexes were used. The animals were maintained in a controlled temperature environment (18–20 °C). Because of the existence of a central narrow band of innervation in this muscle, bupivacaine (0·3 ml, 0·75% in saline) was injected into right SCM on both sides of the neural band and allowed to spread to the bulk of the muscle mass. This procedure is generally followed by the complete necrosis of the muscle cells. Contralateral muscles injected in the same way with physiological saline or not injected at all, together with twenty one muscles of normal animals in connection with a parallel study, served as three control groups.

Animals were killed, under ether anaesthesia, 1–7 and 14 days after injection and the muscles pinned on Silgard in small Petri dishes. They were then fixed in 10% neutral formalin for 3–10 days for haematoxylin and eosin and the Bielschowsky–Gros silver method (4 muscles/day), and in 2·5% glutaraldehyde in 0·1 M cacodylate buffer for two hours for transmission electron microscopy (3 muscles/day, Days 1, 2 and 3).

In the silver impregnation series, at least 200 neuromuscular junctions per day were observed to provide data with which to calculate the percentage of endings undergoing retraction and growth. Retracted nerve terminals were defined as those possessing one or more irregularly thick branches endowed with hypertrophied neurofibrillar material and with a length less than the mean terminal branch length of the nerve endings in normal muscle ($13{\cdot}39 \pm 3{\cdot}05$ μm, range 8·13–21·76 and normally distributed). Length measurements were performed directly with a semi-automatic image analysis system connected to a colour TV monitor displaying the silver impregnated nerve endings (Tomas i Ferré, Mayayo & Fenoll i Brunet, 1987). Sprouts were defined as those thin nerve terminal branches with a growth cone-like structure at the tip. Nevertheless, because there is not always a growth cone in the sprouts (Letinsky, Fischbach & McMahan, 1976; Wernig & Herrera, 1986), very thin axonal or nerve ending projections, infrequently found in the normal animal, were also counted. For the electron microscopy series about 25 neuromuscular junctions/day were studied.

RESULTS

Bupivacaine-induced muscle cell degeneration and the regeneration that follows proceeds rapidly as described previously for mammalian preparations (Jirmanová & Thesleff, 1972; Jirmanová, 1975). Longitudinal control sections stained with haematoxylin and eosin and taken immediately after each section for the silver impregnation method showed that almost all fasciculi and muscle cells in the neural zone were completely affected after two days. Local necrotic changes and muscle cell disruption could be observed in the shortest period studied (Fig. 1) and their rapid and extensive progression could be followed during the first 72 hours along the whole length of the muscle fibres. Necrotic muscle cells at 24–72 hours, encircled by a morphologically intact basal lamina, were filled with variable amounts of cellular debris, interstitial fluid, mononuclear phagocytes and intact satellite cells showing 'blastic' transformation. Although blood vessels and connective interstitial elements show the structural variations that occur normally in an inflammatory environment, myelinated and non-myelinated intramuscular nerves and axons looked normal during the entire period of muscle cell necrosis and deprivation. Myotubes and small multinucleated cells within the original basal lamina were initially observed at the fourth day post-injection. Nevertheless, in one muscle of the silver impregnation series

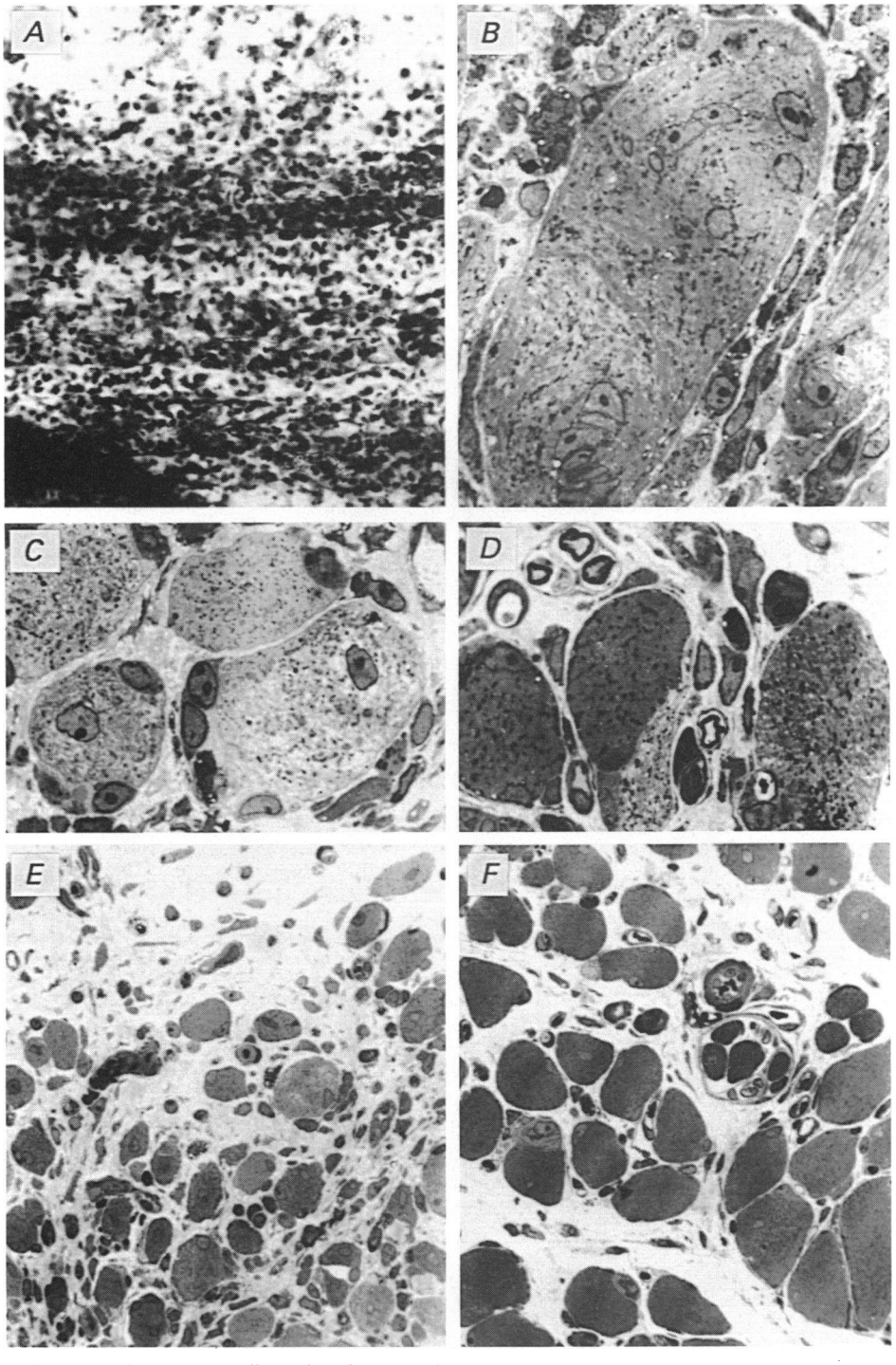

Fig. 1 (*A–F*). Haematoxylin and eosin (*A*) and toluidine blue-stained semithin sections (*B–F*) on bupivacaine-treated SCM muscle cells. One day after bupivacaine injection (*A–D*), necrotic changes affect all muscle cells, although well-preserved intramuscular axons can be seen in the necrotic environment at (*D*); five days after injection (*E*), myotubes and small multinucleated cells are present, and seven days after (*F*), small muscle cells with some internal nuclei can be observed. × 320 (*A*, *E*, *F*); × 640 (*B*, *C*, *D*).

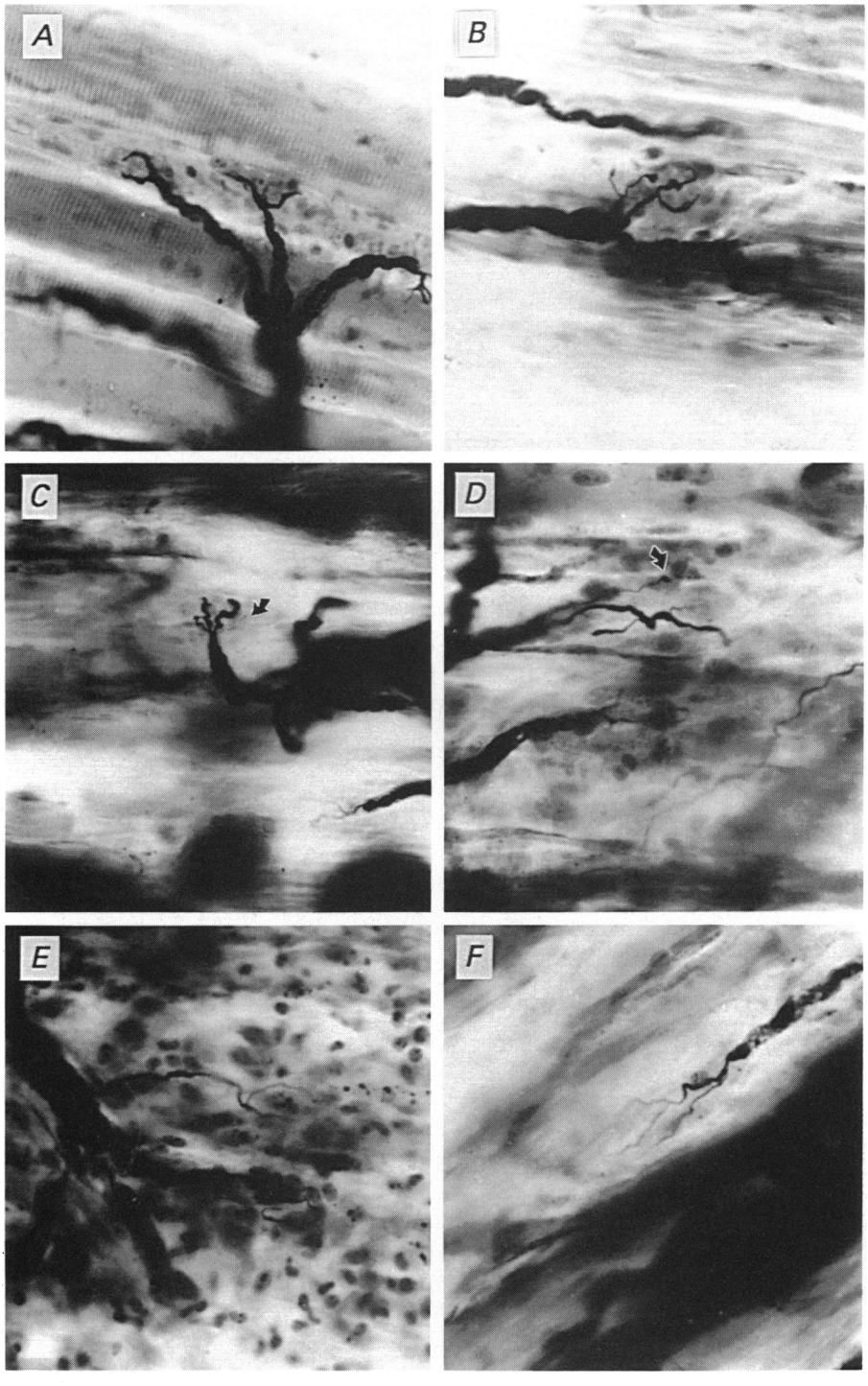

Fig. 2 (*A–F*). Examples of silver-impregnated motor nerve endings on bupivacaine-treated SCM muscles. One day after injection (*A*, *B*), some terminals show signs of retraction such as thickened terminal branches with hypertrophied argyrophilic neurofibrillar material. Two to three days after injection (*C–D*), most terminal arborisations show one or more thin sprouts usually endowed with a growth cone-like structure at the tip (arrows). In (*E*) and (*F*), poorly branched nerve terminals four and five days respectively after bupivacaine treatment. × 640.

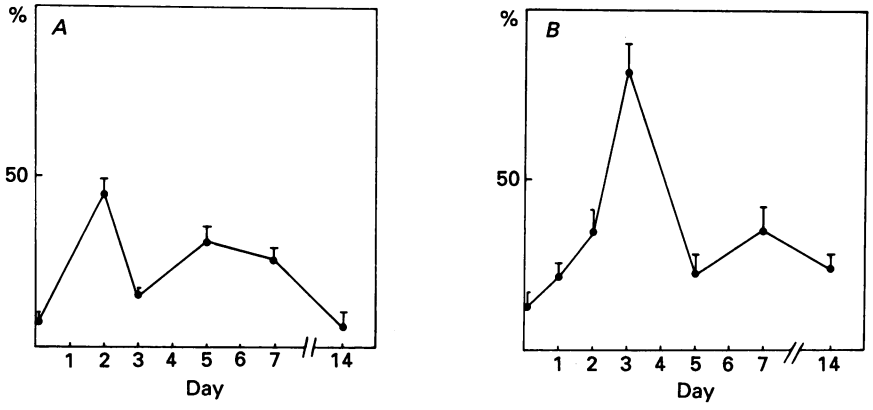

Fig. 3 (*A–B*). Evolution of nerve endings showing retraction (*A*) and sprouting (*B*) after bupivacaine treatment. Percentage values in the *y*-axis are expressed as mean ± S.D. of the results obtained in three muscles each day. At least 200 junctions/day were studied.

three days after the drug treatment, we observed the existence of some groups of small basophilic multinucleated tubes. This muscle was therefore discarded. Small muscle cells with some internal nuclei are seen initially at the seventh day.

In the SCM of the young adult rat some motor endings show qualitative signs of partial retraction in silver-impregnated preparations (6 ± 0·15% S.D., n = 1680 nerve terminals). This finding has been interpreted in the frame of the remodelling concept of the motor nerve terminals in the normal adult (Tomas i Ferré *et al.* 1987; but see Lichtman *et al.* 1987). In the first two days after bupivacaine injection we observed an increasing percentage of motor nerve terminals showing abnormal signs in their terminal branches (Fig. 2*A–C*). Morphological abnormalities included retraction and thickening in the terminal branches, granular swellings, and pyriform and bulbous branches with hypertrophied argyrophilic neurofibrillar material. In the more affected endings only the thickened stumps of the terminal branches could be seen, and in these endings on the second day, the mean number of terminal branches (2·75 ± 0·96 S.D., n = 87) was roughly 50% reduced with respect to the normal controls (normal endings in control animals give the value of 5·22 ± 1·95 S.D., n = 516; range 2–10, normally distributed; see also Tomas i Ferré *et al.* 1987). Occasionally, some terminal axons were seen completely retracted within distal intramuscular glial sheets some micrometres inside, but complete degeneration and fragmentation of axons and endings was never observed. The percentage of retracted endings attained a maximum (45%) at two days post-injection (Fig. 3*A*), and then declined abruptly, although a small increase could be seen at the fifth day. In the course of the following days the percentage of retracted endings was similar to that observed in the control groups.

The simultaneous finding of very thin sprouts in some endings together with abnormal terminal branches (see Fig. 2*C*), precluded the existence of major faults in the quality of the silver preparations. Furthermore, 32 motor end-plates out of 47 observed during the first two days showed some abnormality at the ultrastructural level. The most frequent finding was the existence of total or partly vacant postsynaptic primary gutters in necrotic muscle cells. When nerve terminals were present, axonal profiles showed central clustering of the cytoskeletal elements and mitochondria. It is interesting to note that in most axonal profiles at 1–2 days post-injection, small coated vesicles were frequently seen (Figs. 4, 5*B*). Teloglial cells generally showed a moderate increase in the number of Golgi and granular reticulum

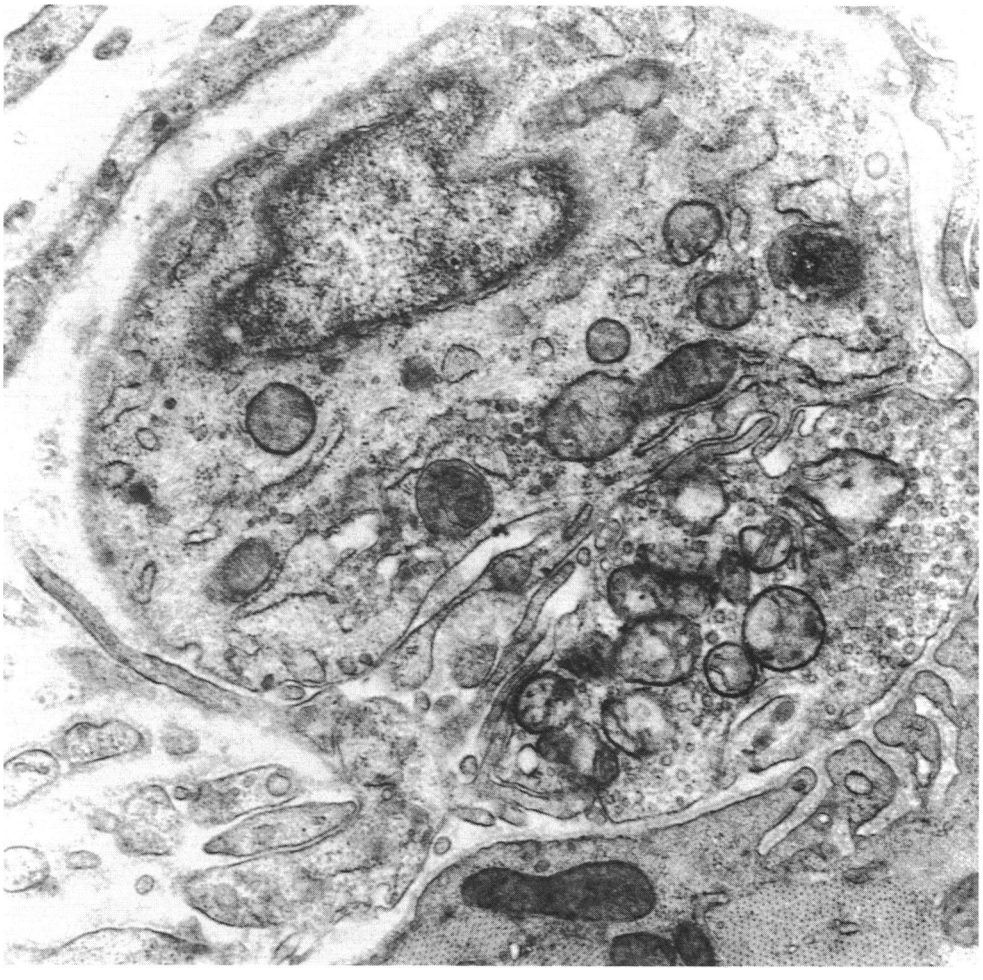

Fig. 4. Neuromuscular junction one day after injection. Note the increase in lysosomal and cytoskeletal elements in a teloglial cell. × 10000.

profiles, lysosomes and cytoskeletal elements (Fig. 4) but the most striking change affecting these cells was the increase in filopodia and cytoplasmic extensions which total or partly encircled axon terminals (Fig. 5*A–C*).

In the normal animal there were also some nerve terminals (11·8 ± 1·5% S.D., n = 1680) showing a preterminal sprout that resulted in a small accessory ending close to the original one (Tomas i Ferré *et al.* 1987). In bupivacaine-treated muscles the sprouting phenomena without accessory ending formation was notably increased during the period of the muscle cell degeneration. Some nerve endings affected by retraction during the first two days post-injection also showed simultaneous terminal sprouting (3% at 24 hours and 5·5% at two days, see Fig. 2*C*). Nevertheless, the bulk of the sprouting occurred at the third day when it passed from 30% at 48 hours to more than 80% of endings showing at least one sprout (Fig. 3*B*). Then the sprouting phenomena reduced abruptly to a moderate but higher than normal level which was maintained thereafter.

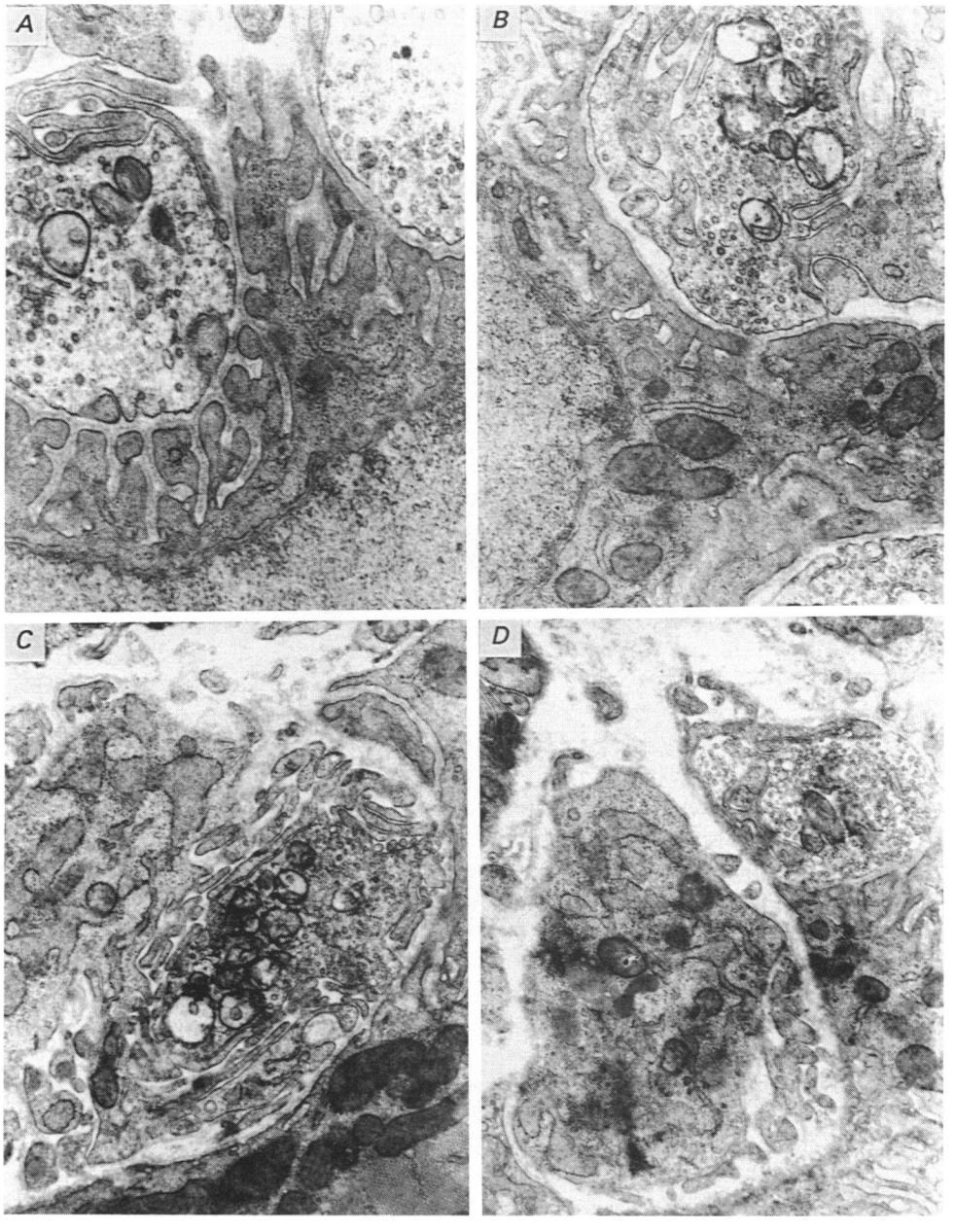

Fig. 5 (*A–D*). Electron microscopy of the motor nerve terminals undergoing retraction one day after bupivacaine injection. The most conspicuous change is the increase in filopodia and cytoplasmic extensions of the teloglial cells which partly encircle axon terminals. × 12500.

Most of the sprouts were of the preterminal and ultraterminal type although nodal sprouts represented roughly 30% of all growth configurations seen at the third day. Growth cone-like enlargements were frequently but not necessarily observed at the tip of the sprouts.

DISCUSSION

Intramuscular axons, glial cells and their myelin, and vascular and connective tissue elements do not degenerate in this bupivacaine-destroyed muscle. Nevertheless, our findings show that most nerve terminals partly retract their terminal branches and initiate growth by sprouting at a time when, within the structurally intact sarcolemmal tube, myoblastic cells are the only viable muscle element and muscle fibres themselves are in a progressively necrotic condition and finally become eliminated. A direct effect of bupivacaine in causing the initial retraction of the nerve endings could not be definitely excluded, especially if certain ultrastructural changes such as the frequent swelling and clustering of the axon terminal mitochondria and the formation of the Schwann cell cytoplasmic extensions encircling axon terminals are taken into account. Nevertheless, the absence of other signs of nerve terminal damage, like complete mitochondrial degeneration, formation of myelin-like structures and membrane whorls surrounding terminal structures and nerve ending fragmentation followed by Schwann cell phagocytotic activity (Manolov, 1974; Winlow & Usherwood, 1975), seems to indicate that nerve terminal damage induced by bupivacaine was not the cause of the observed retraction. It is likely that changes in motor endings were a consequence of the muscle cell degeneration. This interpretation is supported by the finding of the apparent accelerated retrieval of the axolemma by the normal physiological mechanism of coated vesicle formation and the rapid sprouting reaction of the retracted terminals.

At light microscopy level the retracted endings described in this work are indistinguishable from certain terminals found in normal adult muscles of both amphibia (Haimann, Mallart, Tomas i Ferré & Zilber-Gachelin, 1981) and mammals (Barker & Ip, 1966; Tuffery, 1971; Tomas i Ferré *et al.* 1987), and this is also true for their ultrastructure (Cardasis & Padykula, 1981; Wernig & Herrera, 1986). In this context, retraction is currently interpreted as one of the aspects of the remodelling capacity of the nerve connections in the normal adult muscle because of local changes in the feedback mechanisms for the maintenance of synapses. It is well known that neurons need, for the maintenance of their connections, the existence of some trophic factor released by the tissues that they innervate (Brown *et al.* 1981; Bennett, 1983; Slack *et al.* 1983). This diffusible substance, linked to the activity of the neuromuscular junction and released locally by muscle cells (Slack *et al.* 1983), may be the regulatory agent missing in bupivacaine experiments on SCM muscle, because of the complete necrosis of all muscle cells. Therefore, nerve endings become partly retracted. A gradual disappearance due to interrupted turnover of basal lamina-linked nerve–muscle adhesion molecules like N-CAM (Rieger, Grumet & Edelman, 1985; Covault & Sanes, 1986), may be a complementary explanation for the observed retraction. The gradual disappearance of the basal lamina-linked end-plate cholinesterase activity over the second and third day after bupivacaine injection (Sadeh *et al.* 1985) supports this last hypothesis, although, on the other hand, empty basal lamina tubes with strong acetylcholinesterase reactivity persist after three weeks of the muscle cell disappearance (Sanes, Marshall & McMahan, 1978). An intense membrane retrieval because of an increase of coated vesicle formation from the plasma membrane of the axon terminal is the most apparent ultrastructural observation which could explain the cellular mechanism that mediates retraction.

It seems as if the initial brief period of nerve ending retraction is followed by an explosion of growth phenomena affecting most of the terminal axons in this muscle. This is not a regenerative growth reaction, because pre-existing endings do not

degenerate, but a sprouting reaction from retracted terminal branches and preterminal axons. In adult animals intramuscular axons and nerve endings grow by sprouting when the muscle cells they innervate are paralysed by different pre- and postsynaptic mechanisms and, conversely, artificially induced activity of the paralysed muscle cells generally suppress the generation of terminal sprouting (Brown *et al.* 1981). On the other hand, in untreated normal adult muscles there exist sprouting forms in certain nerve endings which have been interpreted as a manifestation of remodelling in the adult (Barker & Ip, 1966; Wernig, Pécot-Dechavassine & Stöver, 1980; Haimann *et al.* 1981; Wernig & Herrera, 1986), although direct observations of mouse motor terminals, which appear to be totally static, have been made recently (Lichtman, Magrassi & Purves, 1987). It seems that the only common denominator of the neuromuscular models showing sprouts is inactive muscle (Brown *et al.* 1981) or a diminution in muscle activity (Wernig *et al.* 1980). In this sense an activity-dependent motor nerve growth factor secretion from muscle cells is the most plausible regulatory mechanism in the control of sprouting (Slack *et al.* 1983). Nevertheless, precocious nodal and terminal sprouting is found when necrosis of the muscle fibre in the end-plate region is induced by the inhibition of the synaptic acetylcholinesterase with organophosphorus anticholinesterase derivatives (Keynes, Hopkins & Brown, 1983; Huang & Keynes, 1983), and the axon terminals probably increase in length after cardiotoxin-induced muscle cell necrosis (Duchen *et al.* 1974). In our material, there is not a viable differentiated muscle cell in the postsynaptic region at the time that the rate of sprouting increases rapidly (2–3 days). Furthermore, the active secretion of a growth-promoting substance from inactive or damaged but viable nearest muscle cells cannot be considered in this case because, in the bupivacaine injection procedure used, all muscle cells are necrotic during the period under consideration. Minimal damage of the nerve terminals, degradative products from the muscle cells, a teloglial effect or the absence of a growth inhibitory mechanism may be the sprouting stimulus acting in this case.

SUMMARY

The local anaesthetic agent bupivacaine induces a rapid degeneration of muscle cells when injected in the sternocleidomastoid muscle of the adult rat with no damage to intramuscular nerves. Nevertheless, silver impregnation and electron microscopy reveal a sequence of retraction and sprouting of the motor nerve endings during the period of the bupivacaine-induced muscle cell deprivation by necrosis. These morphological changes seem to be qualitatively similar to the retraction and growth phenomena described as forming part of the remodelling process affecting normal untreated neuromuscular junctions of the adult amphibia and mammals. The results are discussed in the context of the regulatory mechanisms governing the maintenance of motor nerve terminals.

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