DEVELOPMENT OF CONNECTIONS BY AXONS GROWING THROUGH INJURED SPINAL CORD OF NEONATAL OPOSSUM IN CULTURE

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Summary

The ability of neurites to grow through a lesion and form synaptic connections has been analyzed in a developing mammalian spinal cord *in vitro*. After isolation of the entire central nervous system (CNS) of the newly born South American opossum (*Monodelphis domestica*) the spinal cord was crushed. Outgrowth through and beyond the lesion was observed in living preparations for 2–5 days by staining axons with carbocyanine dyes. The structure of the acute crush and the growing neurites was examined by light and electron microscopy in tissue fixed immediately after the crush had been made. All axons had been severed and the site was filled with debris and amorphous vesicular structures. By 3 days after injury, numerous labelled neurites had grown into the lesion; by 4 days, many had extended several millimetres beyond it. At this time normal axonal profiles were apparent in electron micrographs of the crush site. Although fewer axons grew across the lesion than had been severed by the crush, the amplitudes of compound action potential volleys conducted across the crush in injured preparations were comparable with those recorded from uninjured spinal cords. Physiological experiments made with raised concentrations of extracellular magnesium in the culture fluid indicated that growing axons had formed synaptic connections. Thus, delayed major peaks of the response were abolished while the small component corresponding to through conduction remained unaffected by magnesium. These experiments demonstrate the development of synaptic interactions by the growing neurites and confirm the far greater powers of repair in neonatal mammals compared to adults. They set the stage for comparing molecular mechanisms involved in development and regeneration of the mammalian CNS.

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Introduction

The central nervous system (CNS) of adult mammals has limited capacity for repair (Ramon y Cajal, 1928). Axons can grow beyond a lesion to form synapses only after a conduit has been provided by peripheral nerve grafts (David and Aguayo, 1981; Vidal-Sanz *et al.* 1987; Aguayo *et al.* 1991) or after inhibitory growth molecules have been neutralised (Savio and Schwab, 1990; Schnell and Schwab, 1990; Schwab, 1991). In contrast, we have recently shown that neurones within the CNS of the neonatal opossum, *Monodelphis domestica*, are capable of growing rapidly and reliably across a lesion in the spinal cord *in vitro* (Treherne *et al.* 1992).

In common with all marsupials, *M. domestica* is extremely immature at birth. The newly born animal has only rudimentary eyes, ears and hindlimbs and is unable to walk or to right itself. Its CNS is still developing and corresponds to that of a 14-day rat embryo, having a poorly developed cerebellum and only an elementary forebrain (Saunders *et al.* 1989). Yet, this neonatal animal is able to suck, breathe and perform selected vital functions. In previous experiments (Nicholls *et al.* 1990; Stewart *et al.* 1991; Zou *et al.* 1991) it has been shown that the CNS can be removed in its entirety from 3-day-old pups and maintained in tissue culture for over a week. Cells continue to divide and migrate within the CNS and spontaneous rhythmical respiratory activity as well as reflex responses to electrical stimulation are maintained *in vitro*. The fine structure of the nervous system, revealed by electron microscopy, appears normal during culture, with good preservation of axons, dendrites, cell bodies and radial glia; a striking feature is the absence of myelin and the small number of glial cells that have differentiated at this stage.

The sparsity of glia within the neonatal CNS of *M. domestica* may explain why spinal cord repair occurs (Treherne *et al.* 1992), since oligodendrocytes have been shown to produce molecules that inhibit neurite outgrowth (Savio and Schwab, 1990; Schnell and Schwab, 1990; Schwab, 1991). In our previous study (Treherne *et al.* 1992), the spinal cord was crushed with forceps immediately after isolation. Thereafter, preparations were left in oxygenated culture medium for periods of up to 7 days. To test for restoration of conduction through the crush, stimuli were applied above while recordings were made below and *vice versa*. To our surprise, restoration of through conduction was observed within 3–5 days. Spinal cord preparations were then fixed and individual growing axons were stained with a carbocyanine dye and with horseradish peroxidase (HRP) to observe axonal outgrowth across the crush.

In the present study, we have confirmed these results and examined axons that grow through the crush by electron microscopy. We show that with the carbocyanine dye DiI the fibres can be observed as they traverse the lesion in *living* preparations maintained in culture. In addition, we present electrophysiological evidence that the repairing neurites form synaptic connections after growing through the lesion.

Materials and methods

Dissection, lesioning and culture of the CNS

The breeding colony of South American grey opossums (*Monodelphis domestica*) was

maintained as described previously (Saunders *et al.* 1989; Nicholls *et al.* 1990). Opossum pups were removed from their mothers 3 days after birth and anaesthetized by inhalation of methoxyflurane (Metofane®, Pitman-Moore, Arovet AG, Zollikon-Station, Switzerland) and then by cooling on ice. This procedure provides a highly effective method of anaesthesia for new-born marsupials, which cannot thermoregulate. The animals were then killed by rapid excision of the heart and lungs. The entire CNS was removed (Nicholls *et al.* 1990) while immersed in Basal Medium, Eagle's, which contained Earle's salts but not L-glutamine (BME, Gibco, Life Technologies Ltd., Scotland, UK) and was continuously gassed with O_2:CO_2 (95%:5%).

For physiological experiments, lesions were made with two crushes using watchmaker's forceps. Each crush extended well beyond the mid-line, at two adjacent segments (cervical 7 and thoracic 1) of the spinal cord of 3-day-old pups. The crush completely separated nervous tissue under the *pia mater* (see Figs 1A, 2A and Treherne *et al.* 1992). Paired, staggered crushes were made because a single lesion of the width produced by forceps extending all the way across the spinal cord would have led to disintegration of the preparation. For morphological studies, single as well as paired crushes were made.

The CNS was cultured in 15ml of BME, with the following additions: nerve growth factor 30ngml^{-1} (Sigma, St Louis, MO, USA); insulin 10μg ml^{-1} (Sigma); foetal calf serum 0.2% (Gibco); garamycin 0.1mgml^{-1} (Essex Chemicals, Lucerne, Switzerland) at 23°C, continuously bubbled with O_2:CO_2 (95%:5%).

Labelling of growing neurites with DiI

Neurites were stained with a fluorescent, lipophilic carbocyanine dye, DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, reviewed in Honig and Hulme, 1989). DiI was obtained from Molecular Probes (Junction City, OR, USA). A saturated solution of DiI in 100% ethanol or acetone was applied to the tip of a conventional glass microelectrode and the ethanol was allowed to evaporate, leaving the tip coated with DiI crystals. The spinal cord was then impaled with the electrode several millimetres from the lesion and the glass tip was broken off and left embedded in the cord. DiI was applied on either side of the crush. Preparations were then kept in culture for 2–5 days. Labelling was viewed in whole mounts with a standard Rhodamine filter set and a long working distance (25× or 10×) objective. DiI labelled axons within and well beyond the crush.

To test the ability of the crushing method to produce an effective lesion, some preparations were crushed and then immediately fixed overnight in 4% paraformaldehyde in 0.1moll^{-1} phosphate buffer (pH7.4) and washed in buffer. The CNS was pinned out in a dish coated with Sylgard® (Dow Corning) and a saturated filtered solution of DiI in 100% ethanol was used for injection into the preparation. DiI was injected dorsally into the right or left side of the fixed brain stem (to avoid the central spinal canal) with a microelectrode (tip diameter 20–30μm) attached to a mouth pipette. The fixed CNS was kept in phosphate buffer at 37°C and viewed as described above.

Electron microscopy

The entire isolated CNS was fixed for 2h in Karnovsky solution diluted to 50% with distilled water, rinsed for 2h in $0.1\,mol\,l^{-1}$ cacodylate buffer, postfixed in 1% OsO_4, dehydrated in graded ethanol and embedded in Epon 812. Thin sections were double stained with saturated uranyl acetate and lead citrate and viewed in a Zeiss 910 electron microscope.

Electrical recordings

Recordings were obtained from both acute and cultured preparations. The brain was separated from the spinal cord just caudal to the brain stem and the rostral and caudal ends of the spinal cord were drawn into suction electrodes (containing BME). Either electrode could be used for stimulation or for recording (chlorided silver wires in the bath acting as indifferent electrodes). Supramaximal electrical stimuli were applied to the preparation and electrical signals were amplified by a low-noise differential amplifier (Almost Perfect Electronics, Basel) and displayed on an oscilloscope and chart recorder. Signals were stored on magnetic tape (Hewlett-Packard 3964A, CA, USA). In some experiments, repaired preparations were placed in a recording chamber with a partition made of Sylgard, such that the position of the lesion was on one side of the partition (see Fig. 6). Fluid was perfused rapidly on both sides. The spinal cord fitted tightly inside the partition so that different solutions could be applied selectively to the cord on either side of the lesion. Dye introduced into one compartment did not spread into the other.

Results

Fine structure of spinal cord repair following injury

The crush site was examined in preparations that had been crushed and fixed immediately with no opportunity for regeneration. Longitudinal sections of spinal cord stained with Toluidine Blue showed that the tissue had been completely separated under the pia mater (Fig. 1A). In similar preparations that had been crushed and fixed immediately, the fluorescent lipophilic dye DiI was applied on one side of the lesion. Under these conditions DiI diffused up to the crush but no labelled fibres entered the lesion itself (Fig. 1B). Labelling was intense, so that stained fibres could be detected more than 3mm from the site of application, away from the crush. In living spinal cord labelled with DiI, an acute crush immediately disrupted every visible axon. Together, these experiments demonstrated that lesions made by crushing severed all axons, while leaving the pia mater largely intact.

This finding was confirmed by electron micrographs such as those shown in Fig. 2 in which the structures of normal and lesioned spinal cords are shown. The spinal cord of the neonatal opossum consists of an inner neuroepithelium with dividing stem cells, an intermediate mantle zone containing developing glial and nerve cells and a peripheral marginal zone with abundant nerve fibres and processes of radial glial cells (see Stewart *et al.* 1991). Axonal process in the marginal layer were $0.1–1\,\mu m$ in diameter, had irregular outlines and were closely packed (Fig. 2A,D). Microtubules, neurofilaments,
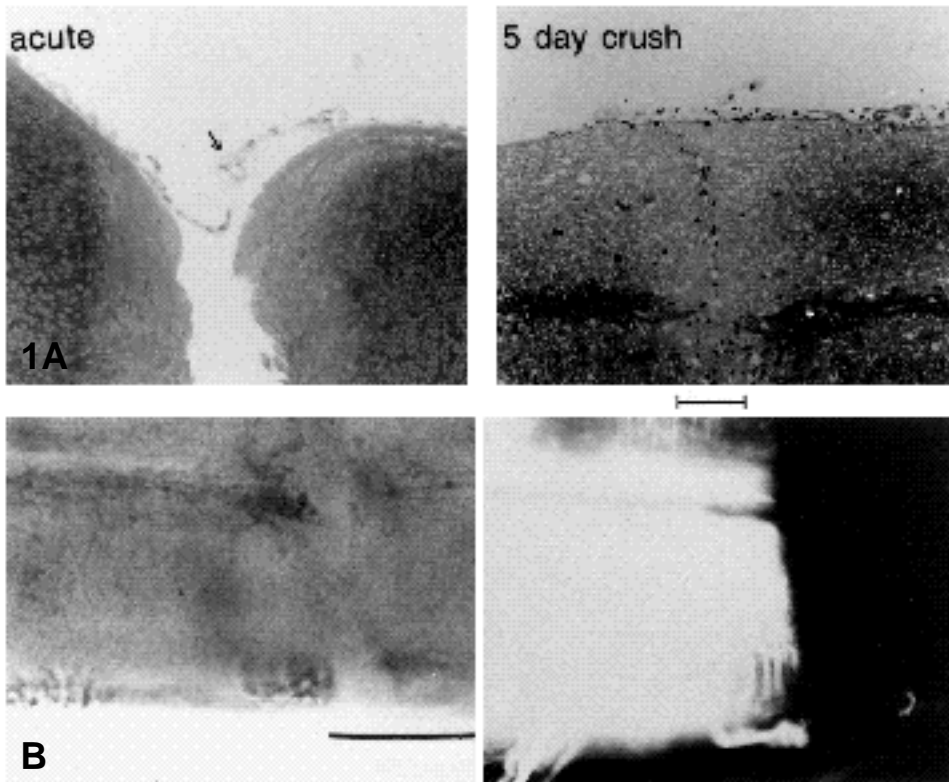
Fig. 1. Evidence for disruption of fibres by crushes to spinal cord. (A) 1 μm Toluidine-Blue-stained longitudinal section through a thoracic segment of neonatal opossum spinal cord fixed immediately after lesioning. After this acute crush, the two parts of the spinal cord were completely separated. The pia mater, indicated by the arrow, remained intact. Five days after the crush had been made in a similar preparation, the lesion had been repaired with material filling the gap. Scale bar, 100 μm. (B) Bright-field (left) and fluorescent (right) photographs of acutely crushed spinal cord, fixed immediately and then labelled with DiI. The preparation was left for 14 days to allow DiI to diffuse. Note fibres running medially but the complete absence of fibres in the region that had been crushed. The scale bar (0.2mm) indicates the site of the crush in the bright-field photograph. Although no labelled fibres could be seen within the crush, label was apparent in fibres more than 3mm in the rostral direction at a distance from the site of application of the dye.

mitochondria, smooth endoplasmic reticulum and vesicles were discernible. After acute lesions had been made in which one side of the cord had been completely crushed, all three layers of the spinal cord were disrupted with debris scattered across an amorphous matrix. Fig. 2B,E is representative of sections through the site of the lesion, which contained rounded and elongated vacuoles, approximately 0.1–1 μm in diameter. Unlike normal axons, the vacuoles were empty or enclosed granules and collapsed collections of membranes. In addition, fibrillar profiles were scattered singly and in the form of short bundles. Examination of the entire crush area at higher power (Fig. 2E) failed to reveal any axonal profiles similar to those seen in normal preparations.
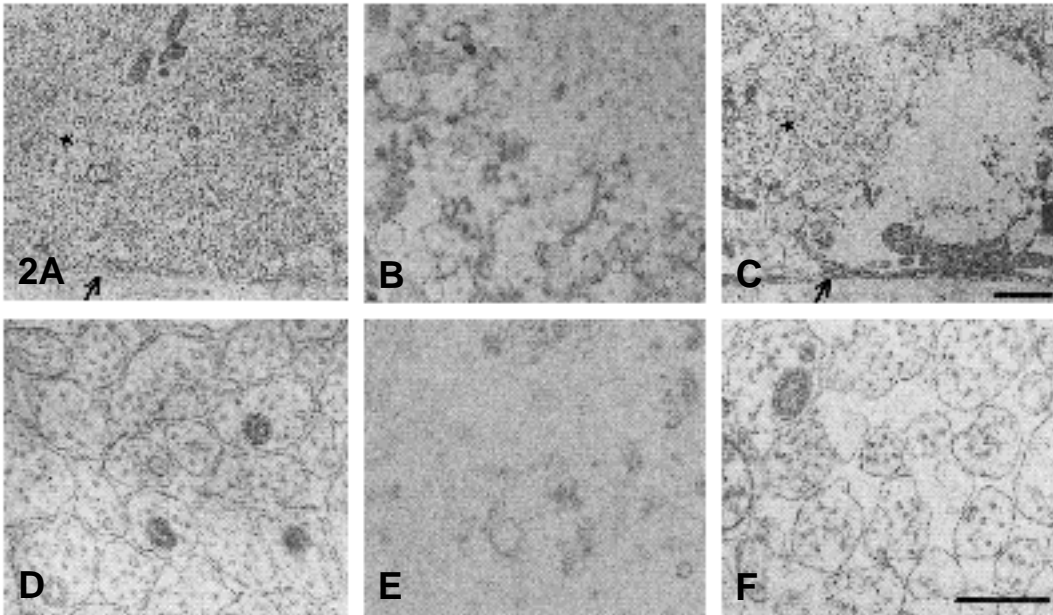
Fig. 2. Electron microscopical evidence for disruption of axons and for repair of neonatal spinal cord in culture. A and D show thin sections at low and high magnification through normal spinal cord. The pia mater (arrow at the bottom of A) is in contact with processes of radial glial cells. Numerous axonal profiles are present (star) and are seen at higher magnification in D. B and E are low- and high-power electron micrographs of the site of a crush made immediately before fixation. Axonal profiles, radial glia and cell bodies are no longer apparent. The tissue shows debris scattered across an amorphous matrix. Membranes and granular and fibrillar profiles, but no axons, are apparent. The vacuoles with dimensions similar to axons could be clearly distinguished from them by the absence of cytoplasm or organelles. The sections shown are representative of the entire crush through the one side of the spinal cord that was systematically scanned for undamaged axons, none of which were found. (C,F) Repair of crush after 5 days in culture. This preparation from a 5-day-old spinal cord, crushed and then cultured for 5 days shows disrupted structure as well as axonal profiles (star). In C, glial end feet once again contact the pia mater (arrow). Clear axonal profiles with normal structure are seen at high magnification in F. In general, growing axons were observed mainly close to the surface of the preparation. Scale bar in C is 1 μm (for A, B and C). Scale bar in F is 0.5 μm for D and F, 1 μm for E.

When preparations were maintained in culture for 5 days the site of the lesion appeared almost normal by light microscopy. The Toluidine Blue section in Fig. 1A contained abundant nuclei and stained material that had filled the space produced by the crush. In electron micrographs of lesions at 5 days, numerous degenerating neurones and extensive debris were still observed. At the marginal layer, however, in spite of the presence of debris, remarkably healthy looking neural and glial processes were observed (Fig. 2C,F). Neuronal processes were loosely packed and their size and structure closely resembled those of intact axons in the marginal layer of control preparations (Fig. 2A,D). Glial processes were observed in contact with the basement membrane at the pial surface. They
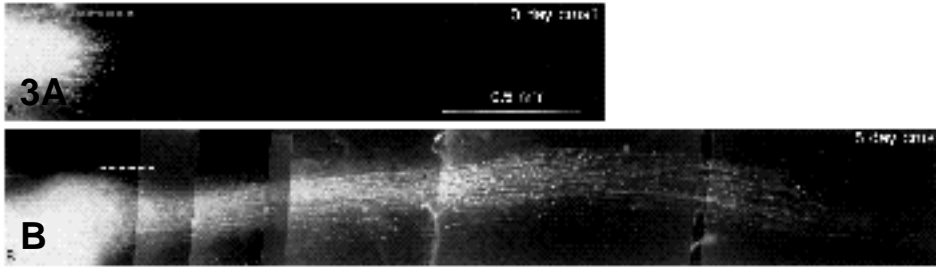
Fig. 3. Rate of growth of labelled fibres through crush. DiI was applied to crushed neonatal opossum spinal cord in culture by means of dye-coated microelectrodes. (A) 3 days after the crush had been made a few fibres entered the site of the lesion. (B) 5 days after the crush had been made in a different preparation abundant growth occurred through the crush and extended for 3mm beyond it. Similar observations were made at various stages in living preparations. The extent of the crush is marked by the dashed line.

probably corresponded to newly grown processes of radial glial cells. These electron microscopic studies confirmed observations made by light microscopy and indicate that axons grow through the lesion rapidly in culture.

Labelling of growing neurites in living preparations

The application of DiI on microelectrode tips was a highly effective way of staining axons in living preparations. Examples are shown in Fig. 3. During the first 3 days following the lesion, labelled axons had reached the edge of the crush in abundance, but only solitary processes entered and few or none were observed to traverse the lesion completely. In contrast, numerous undamaged axons on the other side of the DiI-coated microelectrode (i.e. away from the crush) had been labelled over long distances. Hence, the absence of labelled fibres within the crush at this time was due to a failure of growth rather than to defective labelling. Fig. 3B from another preparation shows the abundant growth through and beyond the lesion at 5 days. By this time fibres had grown 2–4mm beyond the site of the crush. These results are in accord with the physiological recordings (see Figs 5 and 6 and Treherne *et al.* 1992) in which volleys conducted across the crush were small or absent at 3 days and large at 5 days.

Precise fibre counts were not possible with DiI and light microscopy for several reasons. First, the individual axons, as shown in Fig. 2, are beyond the resolving power of conventional light microscopy; a dye-labelled profile could therefore represent a number of neurites. Second, since the entire spinal cord was examined as a whole mount, fibres or bundles of axons could be followed easily only at the surface in the absence of confocal microscopy. Nevertheless, what was plain was that abundant fibres such as those shown in Fig. 3 grew across the crush at 5 days compared to the solitary sparse outgrowth seen at 3 days. When rough comparisons were made between numbers of 'fibres' labelled in the crush at 5 days with those labelled away from the crush on the other side of the injection needle, a considerable discrepancy was apparent. Far fewer fibres were labelled at the crush, approximately one-fifth to a quarter of those undamaged fibres that had been labelled. Although such figures are quantitatively unreliable, they indicate both that

significant numbers of fibres grow through the crush and that fewer fibres had grown through by 5 days than were present before the lesion had been made.

Physiological evidence for synapse formation by growing neurites

Maximal electrical stimulation of the spinal cord or mid brain in a freshly removed neonatal opossum CNS evokes a compound volley of action potentials, usually about 0.2mV in amplitude (Nicholls *et al.* 1989). It was shown in earlier experiments that such responses could be elicited by rostral or by caudal stimulation, that they were mediated in part directly and in part by way of synaptic connections and that they persisted virtually unchanged for up to 10 days in culture (Nicholls *et al.* 1989; Stewart *et al.* 1991). Treherne *et al.* (1992) found that these responses were abolished by crushing; 4 or 5 days later, however, marked recovery had occurred and the amplitude of the volley, though not fully restored, approached that seen in normal, undamaged CNS. These observations have been confirmed in the present experiments (see Figs 4, 5 and 6).

As in earlier experiments, the volley of conducted action potentials in repaired preparations showed two distinct peaks: a small early potential followed by a larger delayed potential with several peaks. Raised concentrations of Mg^{2+} block chemical synapses in opossum CNS as in other preparations. Thus, $15–25 \, \text{mmol} \, l^{-1} \, Mg^{2+}$ added to the bathing fluid rapidly blocked dorsal root–ventral root reflexes and abolished the second series of delayed peaks in volleys conducted through the spinal cord (Nicholls *et al.* 1989). It seemed likely that the second series of delayed peaks observed in repaired preparations also consisted of synaptically activated components. A further reason for suspecting this to be the case was the large degree of recovery of the electrical signal compared to the rather smaller numbers of fibres that grew across the crush (fewer than those present in undamaged preparations). Figs 4 and 5 show the results of experiments made in the presence of $25 \, \text{mmol} \, l^{-1} \, Mg^{2+}$ in normal and repaired preparations. In Fig. 4 most of the evoked volley was blocked by $25 \, \text{mmol} \, l^{-1}$ magnesium in both normal and 5 day crushed preparations (note the high gain in the lower series of records). Interestingly, the initial peak, which we assume corresponds to through conduction, was not blocked by raised $[Mg^{2+}]$. Additional evidence for the supposition that the initial component corresponded to through-fibre conduction is shown in Fig. 5. Here, in a 3 day preparation, the conducted volley showed only the initial component, with little evidence of synaptic activation. In this preparation, as in others at this stage, $25 \, \text{mmol} \, l^{-1} \, Mg^{2+}$ had virtually no effect on the amplitude of the potential.

The initial peak in repaired preparations was smaller, as expected, than that seen in normal controls. In opossum nervous system cultured for 5 days the amplitude of the initial peak was $22 \pm 3 \, \mu V$ (s.e. of mean, *N*=14) compared to $13 \pm 3 \, \mu V$ (*N*=9) in repaired preparations 5 days after crush. In contrast, the amplitudes of the second peaks, presumably corresponding to synaptic activation, were $160 \pm 26 \, \mu V$ (*N*=17) in normal preparations after 5 days in culture compared to $37 \pm 8 \, \mu V$ (*N*=9) in repaired preparations.

Experiments described so far provided physiological evidence that the delayed peaks represented activity mediated through synaptic connections. They did not, however, indicate whether the connections had been made by fibres that had grown through the

lesions. Fig. 6 shows an experiment in which $25\,\text{mmol}\,l^{-1}$ Mg^{2+} was applied locally to the spinal cord beyond the lesion. The arrangement is shown in the diagram. Staggered crushes were made as usual. The preparation was maintained in a bath with a partition that prevented fluid on one side from diffusing into the other. Both sides were separately perfused. The preparation showed early and delayed responses to electrical stimulation. The delayed responses, as before, were abolished within 30s by $25\,\text{mmol}\,l^{-1}$ Mg^{2+} with subsequent recovery 2min after replacing normal BME in the right-hand chamber. This experiment indicated that the blockage of synaptic transmission caused by magnesium
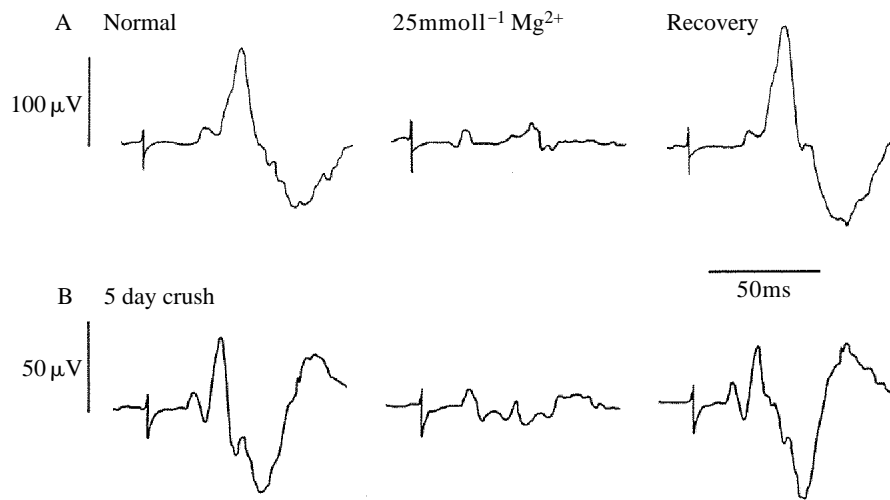

Fig. 4. Comparison of conduction through normal and lesioned spinal cords. (A) In normal preparations, stimulation of the caudal or the rostral end of the cord elicited a compound action potential with an initial small peak and a delayed larger peak. $25\,\text{mmol}\,l^{-1}$ Mg^{2+} blocked the large delayed peak without markedly influencing the early potential. Block occurred within 30s and recovered 1–2min after replacing normal BME in the chamber. (B) A similar experiment in a preparation that had been crushed 5 days previously. Crushes were applied at C7 and T1 and extended beyond the mid-line. Although the conducted volleys were smaller (note voltage scale), the results were very similar to those observed in normal preparations. $25\,\text{mmol}\,l^{-1}$ Mg^{2+} rapidly and reversibly blocked delayed peaks.
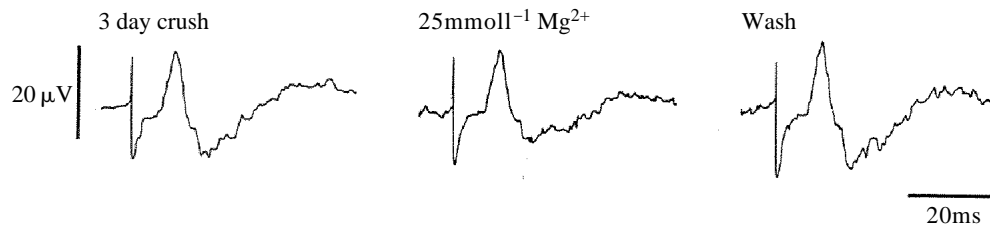

Fig. 5. At early stages of repair, 3 days after crushes had been made, only the initial component of the volley was conducted through the crush. This early small component was, as in Fig. 4, not blocked by $25\,\text{mmol}\,l^{-1}$ Mg^{2+}. This experiment suggested that the initial small volley was due to conduction in through fibres and was not mediated by way of synaptic connections.
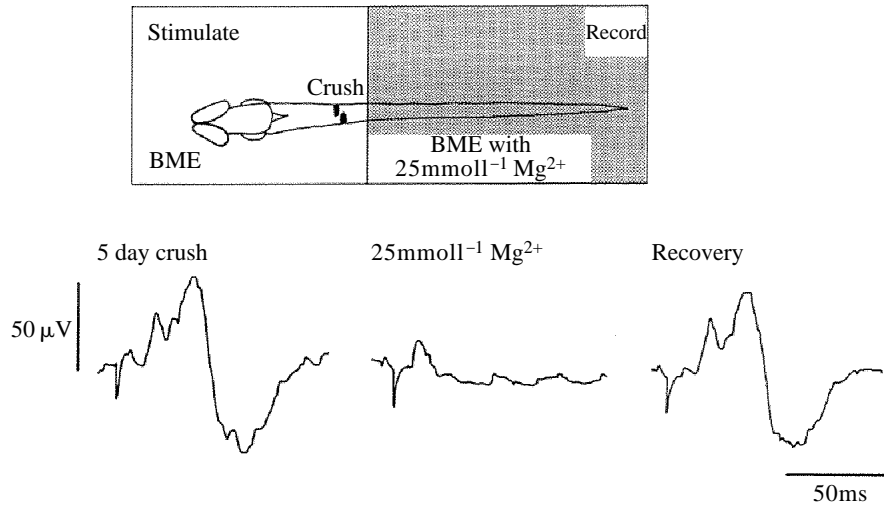
Fig. 6. Effects of applying $25\,\text{mmol}\,l^{-1}$ Mg^{2+} to opossum spinal cord beyond a crush lesion. The upper diagram shows the arrangement for stimulating and recording with suction electrodes. The preparation had been crushed 5 days earlier at the site indicated in the diagram. The partition separating the two chambers was distal to the crush. When $25\,\text{mmol}\,l^{-1}$ Mg^{2+} was applied to the distal part of the cord, from which recordings were made, the late components of the volleys were blocked. The block occurred rapidly (within 30s) and was reversible. Dye applied to the recording chamber did not spread to the site of the crush since the partition fitted well and both chambers were separately perfused. This experiment suggested that fibres that had grown through the crush had formed synaptic connections.

had occurred distal to the crush and that newly grown fibres had made synaptic connections.

Discussion

These experiments confirm our initial finding (Treherne *et al.* 1992) that a neonatal mammalian CNS is capable of repair *in vitro*, without grafts or special manipulations (Aguayo *et al.* 1991; David and Aguayo, 1981; Savio and Schwab, 1990; Schnell and Schwab, 1990; Schwab, 1991; Vidal-Sanz *et al.* 1987).

Here we present evidence that fibres growing through the site of a crush extend far beyond it and make synaptic connections. Our experiments have not revealed whether the outgrowth is from axons that had been cut or from newly grown fibres reaching the lesion for the first time. The direct observation of outgrowth in living preparations offers a promising approach to this problem. Similarly, one can hope to confirm by electron microscopy that new synapses are present.

At what age is the ability of the opossum CNS to regenerate lost? In 18-day-old North American opossums, neurones grew around rather than across a spinal cord lesion *in vivo* (Martin and Xu, 1988: Xu and Martin, 1989, 1991): so, it would appear that some capacity for repair is lost by this stage. Similar observations have been made in eutherian (or placental) mammals, where lesions of the corticospinal tract lead to growth of axons

around the lesion to innervate more caudal areas in neonatal rats (Bernstein and Stelzner, 1981, 1983), cats (Bregman and Goldberger, 1982, 1983) and hamsters (Kalil and Reh, 1979, 1982). Thus, the exceptional nature of opossums under a week old (compared with eutherian mammals) resides in their late postnatal development, rather than in the structure or properties of their CNS. Furthermore, it has recently been shown that *embryonic* (E16) rat spinal cord can repair itself *in vitro* by growth of axons through a lesion to restore electrical conduction (Saunders *et al.* 1992).

That some regeneration can occur in adult mammalian CNS has been demonstrated by Aguayo and his colleagues (David and Aguayo, 1981; Vidal-Sanz *et al.* 1987; Aguayo *et al.* 1991), who applied grafts of peripheral nerves as conduits for growth and guidance. Similarly, Schwab and others (Savio and Schwab, 1990; Schnell and Schwab, 1990; Schwab, 1991) have shown that regeneration of CNS axons can occur in adult rats that have been pre-treated with X-rays or with antibodies directed against inhibitory factors in myelin. Together, these experiments suggest that adult mammalian CNS lacks substrates suitable for promoting neurite outgrowth and contains molecules that inhibit regeneration. Opossum pups at 3 days contain only a few radial glial cells and no myelin. Hence, the environment in this developing CNS is favourable for regrowth.

Certain advantages of the intact opossum CNS in culture have emerged. First, regeneration occurs rapidly: after waiting for only 4 days or so (not weeks or months) regrowing neurites can be followed in the living CNS. As a result, numerous experiments under various conditions can be effectively carried out. Second, there is good evidence for synapse formation. A third obvious advantage is that the recovery occurs in tissue culture, so that growth factors and antibodies can be applied at known concentrations and their effects assayed.

The results presented here set the stage for an in-depth study of regeneration. Experiments are now in progress to analyse how neurites grow across the area of the lesion and to assess the role of pia mater, radial glia and pioneer fibres for guidance (S. Blackshaw, K. J. Muller, J. G. Nicholls and Z. Varga, in preparation). Other problems of interest include the specificity of the new connections that are formed by regenerating axons. In a culture dish it becomes possible also to analyze in real time how molecules that promote or inhibit outgrowth influence the efficacy of regeneration and restoration of synaptic connections after injury.

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