

INFLUENCES OF THE GLIAL ENVIRONMENT ON THE ELONGATION OF AXONS AFTER INJURY: TRANSPLANTATION STUDIES IN ADULT RODENTS

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SUMMARY

Tissue transplantation methods, previously used to study neural development, myelination and inherited disorders of myelin can be applied also to the investigation of repair and regeneration in the mammalian CNS. The elongation of axons from injured peripheral nerve or CNS has been studied in adult mice and rats by observing the growth of axons into PNS or CNS tissue grafts.

Following spinal cord injury and also after transplantation of optic nerves into the PNS there is axonal sprouting but these neuronal processes fail to elongate more than a few mm into the surrounding glia. On the other hand if segments of a peripheral nerve are grafted into the transected spinal cord, axons arising from spinal neurons and dorsal root ganglia become associated with the transplanted Schwann cells and elongate along the graft, approximately 1 cm. Recently the elongation of axons from spinal and medullary neurones was studied using a new experimental model which employed PNS grafts as 'bridges' to connect the spinal cord and the brain stem. In a series of adult C57BL/6J mice and Sprague Dawley rats, autologous segments of sciatic nerve were used to create 'bridges' between the lower cervical or upper thoracic spinal cord and the medulla oblongata. The spinal cord between these two levels was left intact.

Grafted segments examined by light and electron microscope 1–7 months after surgery were well innervated by Schwann cell ensheathed axons that had grown the entire length of the graft (2 cm in mice and 3.5 cm in rats). The origin and termination of these axons were determined by transecting the regenerated grafts and applying horseradish peroxidase to the cut ends. Retrogradely labelled neurones were found to be distributed widely in the gray matter of the spinal cord and medulla near the sites of insertion of the graft. Anterogradely labelled fibres coursing within the graft penetrated the CNS for short distances, approximately 2 mm.

These new results indicate that following CNS injury a conducive glial environment does allow spinal and brain stem neurones to elongate axons for distances that can be greater than those they usually extend for in the intact animal. This evidence that the regenerative response of similar axons differs in CNS and PNS neuroglia supports the hypothesis that influences arising from the environment play an important role in the success or failure of regeneration. The regenerative potentiality of central neurones may be expressed only when the CNS neuroglial environment is changed to resemble that in the PNS.

INTRODUCTION

Functional recovery from injury to the nervous system involves the restitution of damaged structures or their substitution by alternate neuronal pathways (Eidelberg & Stein, 1974; Schneider, 1979). Such reorganizations of neuronal activity may involve the regrowth of interrupted fibres and the extension of collateral branches from intact nerve cells (Liu & Chambers, 1958; Goldberger & Murray, 1978). To become effective, these responses often require substantial elongation of neuronal processes to establish appropriate terminal connections. Axons transected in the peripheral nervous system successfully re-grow over long distances. On the other hand, although initial regenerative responses are well documented for the injured central nervous systems of most animals (Björklund & Stenevi, 1979; Cotman & Nadler, 1978; Crutcher, Brothers & Davis, 1981; Raisman & Field, 1973), the subsequent axonal growth is generally limited to a few mm and regeneration is aborted (Cajal, 1928). Although other mechanisms may contribute to the failure of regeneration in the brain and spinal cord (Guth, 1974; Varon, 1977; Lund, 1978; Grafstein & McQuarrie, 1978; Veraa & Grafstein, 1981), the limited elongation of axons within the damaged CNS appears to be a fundamental element. In this review, we summarize experimental evidence suggesting that differences in the capacity of regenerating axons to elongate in the PNS and CNS may be more dependent on the environment in which these axons are located than upon the intrinsic properties of their neurones, a role for the neural environment that was postulated by Cajal (1928).

The neural environment and axonal growth

The hypothesis that the neural environment (Table 1) plays an important role in axonal regeneration implies that axons fail to elongate in the CNS because this environment either lacks the growth-promoting properties of the PNS or exerts inhibitory influences. The different cellular responses to nerve fibre interruption in the CNS and PNS support this concept. In peripheral nerves, axons regenerate along columns of Schwann cells surrounded by basal lamina, the so-called bands of Büngner. In the adult mammalian CNS, on the other hand, no such well-aligned structures as the bands of Büngner persist after injury; rather, intertwining cytoplasmic processes of glial cells form a dense mesh or scar at the site of injury.

Conditions in the neural environment may also contribute to the more successful regrowth of axons observed in amphibians, fish and newborn mammals. In *amphibians and fish*, CNS regeneration is usually more effective than in mammals (Murray, 1976; Michel & Reier, 1979; Forehand & Farel, 1979; Wood & Cohen, 1979). In these submammalian species, the glial cells may guide and facilitate axon growth and regeneration (Singer, Nordlander & Egar, 1979; Katz & Lasek, 1979; Wood & Cohen, 1979). In *newborn mammals*, the transection of CNS fibre tracts can be followed by a growth of axons over distances greater than those observed after injury in older animals (Kalil & Reh, 1979), although some of the axons observed in these experiments may have been due to the arrival of developing fibres rather than regeneration. The enhanced elongation in these animals may reflect the paucity of glial cells in young animals (Brizzee & Jacobs, 1959; Fulcrand & Privat, 1977) and/or developmental changes in the properties of these cells, including their tendency to form

Table 1. *Components of the neural environment*

	PNS	CNS
Sheath cells	Schwann cells	Oligodendrocytes, astrocytes
Other cells	Fibroblasts, mast cells	Microglia
Extracellular matrix	Basal lamina, collagen	—
Diffusable factors*	?	?

* Although diffusable factors that influence neurite growth have been recognized *in vitro*, the role of such substances in regeneration has not been established *in vivo*.

less dense glial scars (Sumi & Hager, 1968). Furthermore, regeneration in newborn animals may also be more successful because of the relatively short distances that are required for axons to reach their targets.

The growth facilitating effects of various components of the neural microenvironment have been investigated in tissue culture. Surface characteristics of the substrate such as its adhesiveness, can influence branch formation and the course of axonal growth *in vitro* (Letourneau, 1975; Sidman & Wessells, 1975). Furthermore, Schwann cells may have trophic effects on neurones (Varon & Bunge, 1978). In dissociated cultures, Schwann cells and fibroblasts can substitute for nerve growth factor (NGF) to support the survival and neuritic growth of sympathetic or dorsal root ganglion cells (Burnham, Raiborn & Varon, 1972). *In vitro* studies have also demonstrated factors other than NGF have trophic influences on specific cell populations (Edgar, Barde & Thoenen, 1981). Such factors can be derived from glial tumour cells (Monard *et al.* 1975), Schwann cells (Varon, Skaper & Manthorpe, 1981) or cardiac muscle cells (Adler & Varon, 1979; Edgar *et al.* 1981). The requirements of cultured neurones for some of these factors changes during development (Barde, Edgar & Thoenen, 1980).

The use of experimental transplants to study regeneration and neural repair

Tissue transplantation techniques used to study neural development (Harrison 1934; Harris, 1979), myelination (Aguayo, Charron & Bray, 1976) and inherited disorders of myelin formation (Aguayo, Bray & Perkins, 1979), have also been applied to the investigation of repair and regeneration in the mammalian nervous system. Two main strategies are available:

(a) *Neural tissue transplants* in which neurones from foetal or newborn animals survive and may establish connections with the brains of adult hosts (Stenevi & Björklund, 1978; Kromer, Björklund & Stenevi, 1979; Perlow *et al.* 1979; Rosenstein & Brightman, 1979);

(b) *Grafts of central or peripheral glia* into the peripheral nerves or central nervous system of adult animals (Aguayo *et al.* 1979). Such grafts were employed to assess interactions of axons from host neurones in the PNS or CNS with transplanted glia or Schwann cells to compare the effects of different environments on axonal growth (Aguayo *et al.* 1979).

In the present report we review the results of experiments in which we have used this approach to assess influences that favour or impair axon elongation after injury.

Transplantation of Schwann cells into peripheral nerves. Allogenic and xenogenic nerve segments or *in vitro* preparations of Schwann cells were transplanted into the transected sciatic nerves of immune-suppressed mice where they ensheathed and

myelinated axons regenerating from the host nerve (Aguayo *et al.* 1977a, b). When after regeneration of the grafted nerves, the immune suppression was discontinued the allo- or xenogenic sheath cells were rejected and the segments of nerve at the site of the original graft became ensheathed by Schwann cells migrating from the host (Aguayo *et al.* 1977b, 1979). This Schwann cell migration may have been guided and facilitated by the axons and basal lamina that survived the rejection of the grafted (donor) Schwann cells. Thus, the transplanted Schwann cells had acted as temporary 'bridges' to facilitate axonal regrowth across the nerve gaps. The recent finding that Schwann cells grown *in vitro* can be transplanted into peripheral nerve, as well as spinal cord (Duncan *et al.* 1981), provides a new experimental technique for the study of cell interactions in the PNS and CNS and also suggests that it may be possible to use cells cultured *in vitro* for sheath cell replacement and experimental neural repair.

Transplantation of peripheral nerve segments into the CNS. Although Cajal (1928) and several other investigators (Tello, 1911; Sugar & Gerard, 1940; Clark, 1943; Kao, Chang & Bloodworth, 1977) demonstrated the reinnervation of peripheral nerve segments grafted into the spinal cord, it remained to be determined whether any axons within these grafts were derived from intrinsic spinal neurones or if all were the result of regrowth from neighbouring spinal roots (Sugar & Gerard, 1940; Clark, 1943). The experiments described below were designed to answer this question using contemporary morphologic techniques.

In adult female rats, a segment of the thoracic spinal cord was resected and replaced by an autologous sciatic nerve graft 1 cm long. Ten days to four months later, the animals were killed and the graft and its junctions with the spinal cord were examined by light and electron microscope. All grafts of more than 3 weeks duration were richly innervated with myelinated and unmyelinated axons, even if the dorsal spinal roots entering the graft site and their ganglia had been avulsed. At the junction between the graft and the central nervous tissues, myelinated and unmyelinated axons were observed within dome-shaped structures containing astrocytic processes and surrounded by basal lamina. Occasional nodes of Ranvier were seen in which one heminode had peripheral myelin and the other central myelin.

Retrograde cell labelling with horseradish peroxidase (HRP) (Mesulam, 1978) indicated that some of the axons within the graft originated from intrinsic spinal cord neurones and others were derived from the dorsal root ganglia below the graft (Richardson, McGuinness & Aguayo, 1980). In another group of animals, a tritiated leucine-proline mixture was injected into the lumbar dorsal root ganglia three months after grafting a peripheral nerve segment into the transected thoracic spinal cord. Radioautography showed that anterogradely labelled axons originating in the dorsal root ganglia several segments below the level of the graft had ascended in the dorsal columns and entered the graft. Some of these labelled axons crossed the grafts from the caudal end of the cord but did not appear to re-enter the rostral spinal stump (Richardson, Aguayo & McGuinness, 1981). These experiments indicate that axons from dorsal root ganglia as well as from spinal neurones in the proximity of the spinal cord transection are capable of growth along the PNS grafts for distances of approximately 1 cm. These axons may have originated by regrowth of damaged fibres or from the extension of collaterals from uninjured neurones.

Transplantation of CNS glia into peripheral nerves. Interactions between regenerating

Peripheral axons and transplanted CNS glia were studied in mice and rats by grafting segments of optic nerve 5 mm in length into the main trunk of the sciatic nerve or one of its branches (Aguayo *et al.* 1978; Weinberg & Spencer, 1979). Such optic nerve grafts contain astrocytes and oligodendrocytes but not neurones. Following transplantation the axons within the optic nerve segments degenerated but (in contrast to the rapidity with which myelin and axon remnants were cleared from degenerating peripheral nerves) such debris was observed in the grafts for several months. The majority of axons arising from the proximal stump of the recipient peripheral nerve bypassed the transplant and re-entered the distal stump. However, the glial transplants were penetrated by some peripheral axons that became ensheathed by astrocytic processes and were occasionally myelinated by oligodendrocytes. The longitudinal growth of most of these axons was limited to less than 1 mm with only a few axons reaching the distal end of the graft.

The results of these experiments suggest four conclusions: (i) CNS transplants are less receptive to the regeneration of peripheral axons than are peripheral nerve grafts or the distal stumps of transected peripheral nerves; (ii) the connective tissue that forms at the margins of the grafts is not an impenetrable barrier to axon growth because some axons enter the glial grafts although they fail to continue to elongate within them; (iii) glial cells survive in the graft and are able to ensheath and myelinate penetrating axons; (iv) early synaptogenesis with neurones, a phenomenon shown in experiments involving transected spinal cords (Bernstein & Bernstein, 1971), could not be the cause of the limited axonal growth observed in these grafts because the transplanted optic nerve segments contained no nerve cells.

The observations in the optic nerve grafts are also in concordance with the demonstration that injured dorsal root axons regenerate as far as the PNS-CNS boundary but only a few enter the spinal cord (Stensaas, Burgess & Horch, 1979; Perkins *et al.* 1980).

Peripheral nerve grafts as 'bridges' for regenerating CNS axons

The sciatic and optic nerve graft experiments described above provided the basis for more recent studies designed to determine the potential of axons injured in the CNS to elongate for distances equivalent to some of the long projecting neurone systems in the intact CNS (David & Aguayo, 1981).

Autologous segments of the sciatic nerve (2 cm in mice and 3.5 cm in rats) were used to prepare 'bridges' between the medulla oblongata and the lower cervical or upper thoracic spinal cord in a series of adult C57BL/6J mice and Sprague-Dawley rats. The bridging nerve grafts were placed extraspinally within the subcutaneous tissues in the back of the animals (Fig. 1). Through a small laminectomy an incision was made with a 150 μ m glass rod into the dorsal spinal cord and medulla to insert the ends of the nerve 'bridges'. The spinal cord between these two levels was left intact. Animals survived without apparent neurologic deficits and were sacrificed between 1 and 7½ months after grafting.

Examination of the grafts by light and electron microscope showed that they were well innervated by axons ensheathed by Schwann cells (Fig. 2). The origin and termination of these axons was determined by transecting the regenerated grafts and applying horseradish peroxidase to the cut ends (Mesulam, 1978; Richardson *et al.*

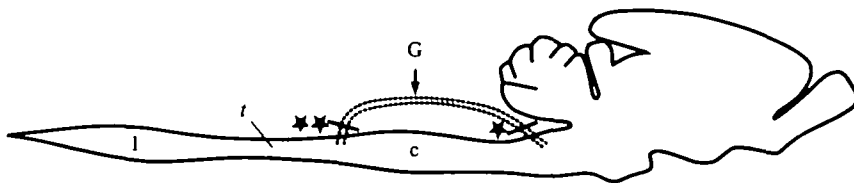


Fig. 1. Diagram of sagittal section of rat CNS with a peripheral nerve graft (G) linking the medulla oblongata and the upper thoracic spinal cord (t). The elongation of axons was measured as the distance between the site of HRP application and the labelled cells in the CNS. For this purpose, when neurones were sought in the medulla the tracer was applied to the nerve 30 mm from the brain stem (**). Conversely, when the growth of spinal neurones was investigated, the graft was cut close to the brain stem (*). Anterograde labelling was obtained by applying HRP to the short stumps.

1980). Retrogradely-labelled neurones were found in several nuclei of the medulla and in the gray matter of the spinal cord in areas that were close to the insertion of the graft (Figs. 3, 4). Anterogradely-labelled fibres penetrated the CNS for distances that did not exceed 2 mm (Fig. 5). The results of these experiments indicate that the PNS 'bridges' contain axons from spinal cord and brain stem neurones. Under such experimental conditions, such axons are capable of a growth that approximates 1.5 cm in mice and 3 cm in rats, distances that are equal to or greater than the lengths of axons from many of these neurones in intact animals.

This new experimental approach has several advantages that are important for the study of the origin, course, length and termination of regenerating axons:

(i) because most of each peripheral nerve 'bridge' is located extraspinally, there is little risk that the HRP applied to the transected nerve to identify cells in the spinal cord and brain stem could reach the CNS by interstitial spread and label neurones spuriously;

(ii) by selectively positioning the nerve 'bridges', it is possible to direct the course of axons from and into specific regions of the CNS including the cerebral hemispheres (Benfey & Aguayo, 1981);

(iii) the length of axonal growth can be determined by measuring the distance between the level of HRP application and the labelled cell bodies;

(iv) the terminations of regenerated axons can be visualized by anterograde labelling;

(v) because experimental animals are not paralysed and retain bowel and bladder function after this procedure, in contrast to the animals grafted after a complete spinal cord transection, their care and survival is greatly facilitated.

DISCUSSION

The failure of axons to regenerate as effectively in the mammalian CNS as in peripheral nerves has been attributed to several mechanisms including a primary inability of CNS neurones to support the regrowth of their processes, early synapse formation with other neurones (Bernstein & Bernstein, 1971), a blocking effect of the astrocytic and connective tissue scar (Windle, 1956), autoimmunity (Berry & Riches, 1974) and differences between the blood-brain and blood-nerve barriers (Kiernan, 1979). Without excluding the possibility that these mechanisms are operative, our approach has been to investigate the general conditions that influence the axons of CNS neurones

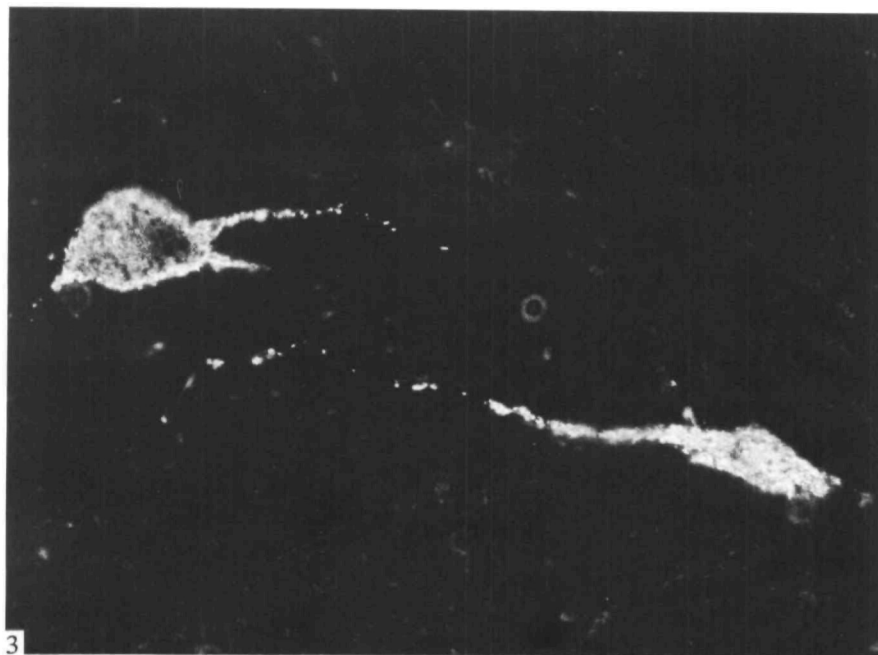
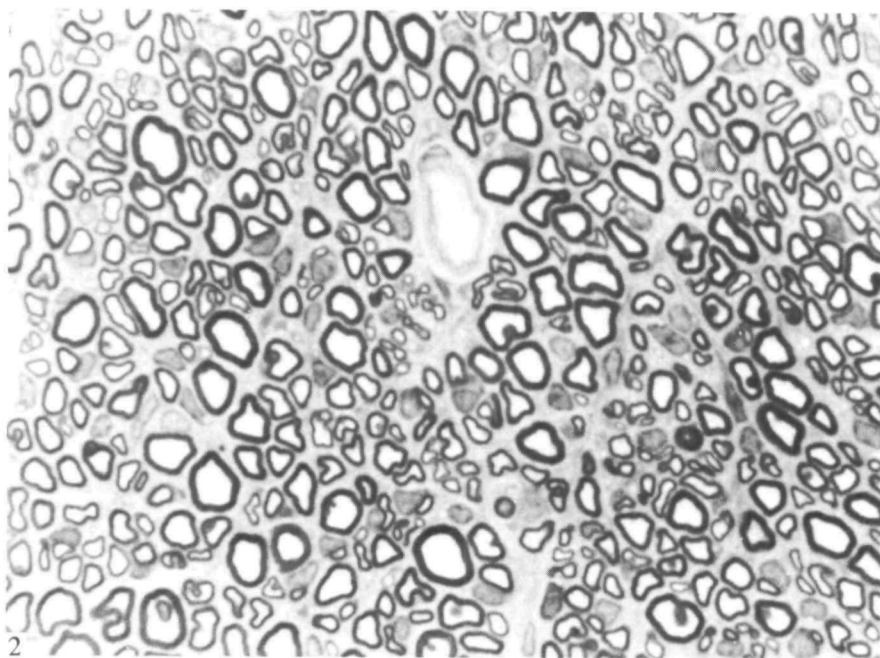


Fig. 2. Cross-section of a peripheral nerve graft at the mid point between the brain stem and spinal cord. The axons are normally ensheathed by Schwann cells and myelin (light micrograph, $\times 1060$).

Fig. 3. Two labelled neurones in the nucleus reticularis lateralis are seen in the medulla after HRP was applied to the caudal end of the graft, 30 mm from the brain stem (dark field micrograph, $\times 530$).

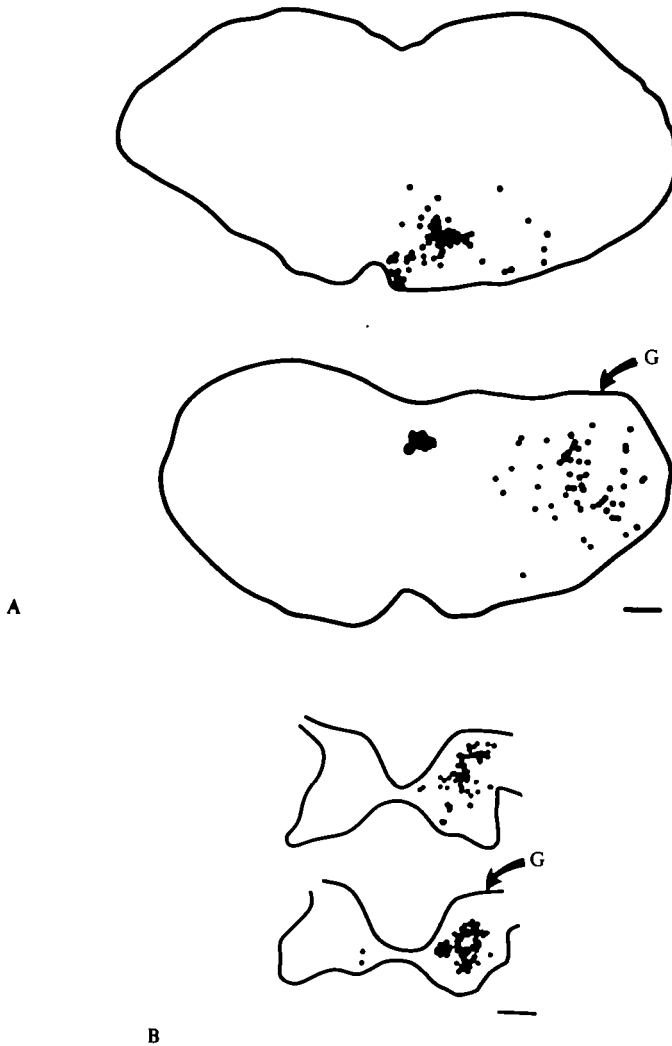


Fig. 4. Diagrams illustrating the position of HRP labelled neurones in (A) medulla and (B) spinal cord after HRP application to the graft.

elongate *in vivo*. Our experiments have demonstrated that while PNS axons with a known capacity to regenerate fail to elongate in a CNS glial environment (Aguayo *et al.* 1978), the axons from intrinsic CNS neurones will grow into segments of peripheral nerves (Richardson *et al.* 1980; David & Aguayo, 1981; Benfey & Aguayo, 1981). The studies of peripheral nerve 'bridges' (David & Aguayo, 1981) also indicate that axons in the injured CNS are able to elongate for unprecedented distances within such peripheral nerve grafts. Although regenerating axons only penetrate damaged CNS tissues for short distances, it would appear that glial cells within the denervated CNS remain responsive to the influences of regenerating axons and are capable of producing myelin (Aguayo *et al.* 1978; Weinberg & Spencer, 1979).

Several questions raised by these studies of CNS regeneration in the adult mammalian CNS require further exploration:

(i) Do the axons that elongate within the PNS grafts originate by regrowth of damaged CNS fibres, by sprouting from uninjured nerve cells or by both mechanisms?

(ii) Are different neuronal populations capable of the same regenerative responses? It is not known if the neurones that have been identified in these experiments represent a special group of cells or if they are examples of a general rule that applies to all neurones.

(iii) Are long pathways, such as the corticospinal tract, capable of axonal regrowth?

(iv) Are regenerating axons able to establish functional synaptic connections with neurones in the limited areas of the CNS they penetrate.

(v) Are specific chemical substances and/or the surface properties of axons and glia responsible for the differences in axonal regeneration in the peripheral and central neural environment?

If the conclusion that interactions between neurones and their glial environment play a determinant role in the failure of CNS regeneration is corroborated, the study of the molecular basis of these interdependencies may lead to experimental approaches aimed at the modification of neuronal and glial responses to CNS injury. If axons from CNS neurones are able to establish functional connections with cells in the target regions to which they have been directed, it may also be possible to devise experimental strategies that will permit selected populations of axons to bypass damaged CNS tissues and connect with specific groups of neurones at a distance.

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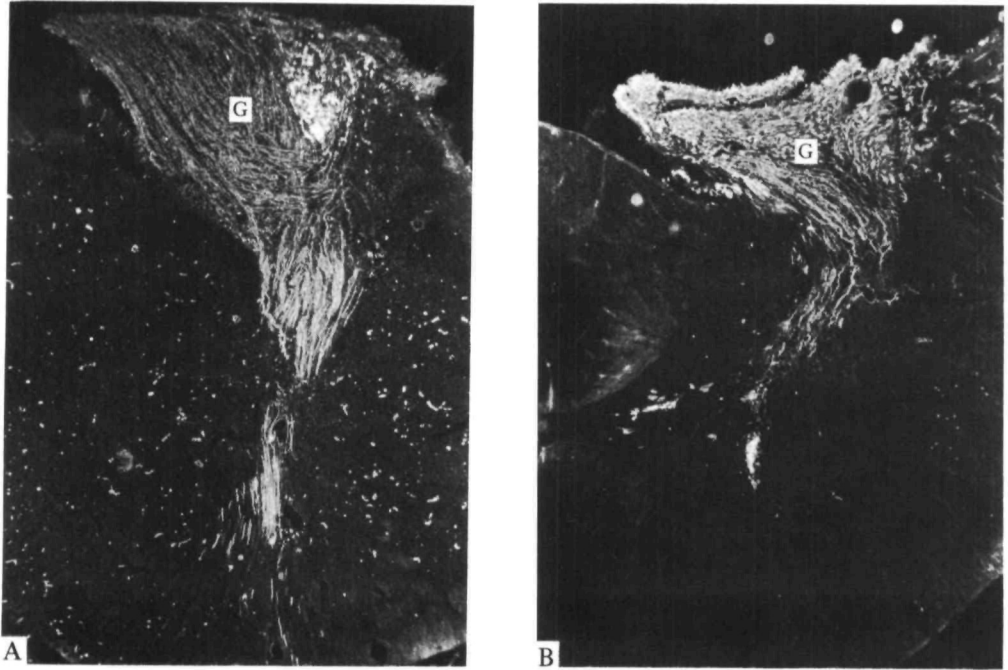


Fig. 5. Cross-section of the medulla oblongata (A) and spinal cord (B) illustrating the course of axons at the junction between the PNS graft (G) and the CNS tissues. The anterograde tracing of these axons was obtained by the application of HRP to the transected graft 5 mm away from the surface of the neuraxis.

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