

TO MOTHER AND FATHER

献给亲爱的爸爸妈妈

AXON GROWTH IN THE ADULT RAT SPINAL CORD

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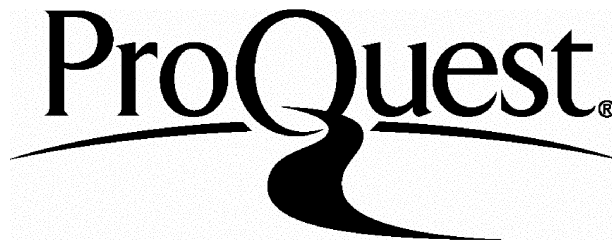
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ABSTRACT

The mouse species-specific marker, M6, showed that suspensions of embryonic mouse hippocampal neurons micro-injected into the cervical dorsal columns of immuno-suppressed adult rat spinal cord grew long, straight, and uniform axons rostrally and caudally for around 10 mm, with a clear preference for white matter. The donor axons dispersed among the host myelinated axons, parallel to them and to the aligned rows of interfascicular glial cell bodies.

After circumscribed lesions in the cervical corticospinal tract, the course of astroglial activation was followed by GFAP immunostaining at survivals up to about 3 months. After a dense astrocytic scar had formed, labelling of the descending axons by anterograde transport from unilateral stereotaxic medullary micro-injections of anterograde tracers showed that substantial numbers of cut axons persisted, without retraction, in the central, macrophage-filled lesion area. The ends of the cut axons were expanded into a variety of shapes, with profuse local branches, and myelinated as far as their expanded tips by endogenous Schwann cells.

A suspension of Schwann cells cultured from neonatal or adult rat sciatic nerve was micro-transplanted into the same positions as the lesions. In response to these exogenous Schwann cells, both ascending dorsal column axons and descending corticospinal axons sprouted much more rapidly and

profusely than after lesions. Both cut and uncut axons gave rise to parallel branches which extended for considerable distances, and fasciculated with each other and with other tract axons. The Schwann cell grafts were also invaded by masses of fine, tortuous, varicose branches, similar to the terminal arborisations in the adjacent grey matter. Electron microscopy and P₀ immunostaining showed that, from about 10 days after transplantation, the transplanted Schwann cells had myelinated a wide swathe of host axons.

Preliminary experiments with micro-injection of populations of Schwann cells transfected in culture with gene constructs directing the over-expression of NGF and NT-3 modified the axonal sprouting responses. Preliminary functional tests showed recovery in a fine paw-reaching test.

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江上小堂巢翡翠，苑边高冢卧麒麟。
细推物理须行乐，何用浮名绊此身。

杜 甫

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LIST OF ABBREVIATIONS

12D	a chemically defined culture medium (details in 2.1.1.)
ABC	avidin-biotin-HRP complex
ara-C	cytosine arabinoside
Asc	ascending tracts of the upper cervical spinal cord
BD	biotin-dextran
CNS	central nervous system
Co-GOD	glucose oxidase-cobalt DAB method
CST	corticospinal tract of the upper cervical spinal cord
DAB	3'3-diaminobenzidine dihydrochloride
DC	dorsal columns of the upper cervical spinal cord
DMEM	Dulbecco's modified Eagle's Medium
DMEMF	Dulbecco's Modified Eagle's Medium with 10% foetal calf serum
DPH	a mixture of dibutyl phthalate, polystyrene and Histoclear™
Dec	descending tracts of the upper spinal cord
EDTA	ethylenediaminetetraacetic acid
EM	electron microscopy
FCS	foetal calf serum
GFAP	glial fibrillary acidic protein
gm	grey matter of the upper cervical spinal cord
HRP	horseradish peroxidase
I.P.	intraperitoneally

MEM	Minimum Essential Medium
MEMF	Minimum Essential Medium with 10% foetal calf serum
milk-PBS	aqueous solution of dried powdered milk in 0.1 M PBS
NGF	nerve growth factor
Ni-GOD	glucose oxidase-nickel DAB method
NT-3	neurotrophin-3
p75	low affinity neurotrophin receptor
PB	phosphate buffer
PBS	phosphate buffered saline
PEI	polyethyleneimine
PLL	poly-L-lysine
TMB	tetramethylbenzidine
WGA-HRP	HRP conjugated with wheat germ agglutinin

CHAPTER I GENERAL INTRODUCTION

The purpose of this study is to investigate structural features of the long ascending and descending tracts of the dorsal columns of the adult rat spinal cord, and to explore experimental interventions that could provide information leading to a future method for repair of human spinal cord injuries.

The effects of spinal cord injury are due to severing axons. If those axons could be induced to regenerate back to their original targets, the injury could be repaired. However, when axons are severed in the adult mammalian brain and spinal cord they are unable to regenerate and grow back to their original targets (Cajal, 1928).

During development, axons are able to grow along their future pathways to reach their targets (e.g. Joosten *et al.*, 1989; Purves and Lichtman, 1985). What prevents the same axons from growing along those pathways in the adult?

If cut at an early stage during development axons are able to regenerate in the spinal cord (Bates and Stelzner, 1993; Xu and Martin, 1991). The crucial developmental time point at which immature axons fail to regenerate has recently been dramatically demonstrated in postnatal organotypic slice co-culture in this laboratory (Li *et al.*, 1995). By co-culturing different

combinations of entorhinal area and hippocampus at ages before and after the 12th postnatal day, it was shown that the failure of regeneration lies in the age of the axons, not the targets.

But even though cut adult axons are unable to regenerate along their correct pathways in adult brain or spinal cord, a number of studies show that, under appropriate circumstances, they can grow and form synapses:

(1) new synapses are formed by local sprouts in partially de-afferented adult neuropil (e.g. in the septal nuclei; Raisman, 1969; 1985), or the dentate gyrus (Cotman *et al.*, 1981; Steward *et al.*, 1976),

(2) when embryonic transplants are placed directly into adult host neuropil, damaged adult host brain axons are induced grow into the grafts, and make specific patterns of synaptic connections (Björklund, 1992; Field *et al.*, 1991; Zhou *et al.*, 1985),

(3) as has been described by Cajal (1928), cut central axons give rise to local sprouts in the white matter of central tracts, even though these sprouts are unable to grow beyond the damaged area or reach their original targets, and are described as 'abortive',

(4) cut central axons will regenerate into peripheral nerve grafts (Benfey and Aguayo, 1982; Berry *et al.*, 1988a; Richardson *et al.*, 1980),

(5) transplanted Schwann cells will induce sprouting and regeneration of cut central axons (Montero-Menei *et al.*, 1992; Neuberger *et al.*, 1992; Paño and Bunge, 1991),

(6) even when their cell bodies (such as motoneurons) lie in the CNS, axons can regenerate in the peripheral nervous system (Fawcett and Keynes, 1992; Guth, 1956), and

(7) cut central axons can regenerate in the CNS of cold blooded vertebrates (Fawcett, 1981; Fawcett and Gaze, 1981).

All these observations indicate that central axons retain the potential to regenerate even in adult life, and suggest that it is factors in the local tissue environment which determine whether or not growth is permitted.

There are many different theories as to why regeneration does not take place: blockage by astrocytic scar tissue (Reier, 1986), unavailability of aligned glial pathways, absence of suitable substrates bearing extracellular matrix-type molecules, absence of adequate growth factors, and the presence of inhibitory molecules (Dodd and Schuchardt, 1995; Guthrie and Pini, 1995; Luo and Raper, 1994; Schwab *et al.*, 1993).

There are several reasons why the spinal cord is an important area for study. Spinal lesions cause permanent disability in young people. Anatomically,

spinal lesions provide an example of a pure 'disconnection' * type lesion - i.e. the effects are due to severing axons, and local destruction of neurons has little part in the functional deficits. The long intraspinal tracts carrying motor and sensory functions in the rat dorsal columns are pure, compact, circumscribed bundles of axons which travel in one direction.

During development, the ascending sensory tracts are almost the earliest to form in the CNS, while the corticospinal tract develops postnatally, almost the latest in the CNS (De Kort *et al.*, 1985; Gribnau *et al.*, 1986; O'Leary and Terashima, 1988). Therefore a comparison of the regenerative potential of the two tracts provides examples of the behaviour of axons whose time of formation spans the entire range of development times.

The individual parts of this study were directed to a series of questions:

The demonstration that axons can extend for long distances and form new synapses in adult grey matter [(1) and (2) above], suggests that there is some special feature preventing regeneration in white matter. As a preliminary for future investigations of this feature, *CHAPTER III* examines the anatomical structure of the white matter tracts in the rat dorsal columns. What (glial) cells are present? How are they assembled into the tract structure?

* In the strict sense of the word, human spinal lesions cannot be called pure 'disconnections' of the long fibre tracts. There is additional damage to the dorsal roots, motoneurons, ventral roots, and spinal grey matter at the lesion site. However, while a lesion involving one or two segments at the cervical level might cause a band of impaired sensation in the neck, and a localised weakness of the neck muscles, these are probably transient (due to sprouting from the sensory and motor nerves from adjacent segments with intact innervation). From the patient's point of view, these effects cannot be compared with the destruction of the long fibre tracts, which causes permanent paralysis and anaesthesia of the entire body levels below the lesion.

Surprisingly, recent work has shown that the glial framework of adult central tracts readily allows the growth of axons from transplanted embryonic neurons (Davies *et al.*, 1993; 1994; Strömberg *et al.*, 1992; Wictorin *et al.*, 1990a; 1992; Wictorin and Björklund, 1992). Therefore, in *CHAPTER IV*, I have examined whether identified types of transplanted embryonic neurons can also grow through the spinal cord, and if so, whether that growth can be modified by the presence of target tissue.

Since the principal goal of studying regeneration in the spinal cord is the hope that a method may be found for the repair of human spinal cord injuries, it is important to know whether cut spinal axons can survive, and therefore could be stimulated to regenerate. If the axons were to retract, or the cells of origin die, then repair would be impossible.

In *CHAPTER V*, I have examined the persistence of axons in spinal lesions. Obviously the rat, with a life span of less than two years, cannot be a model for long term human spinal injury. Nonetheless, it is possible to observe relatively long term changes in the rat spinal cord.

This raises the question of how 'long-term' can be measured. Since the late effects of lesions are the formation of an astrocytic scar which 'walls off' the lesion site, I have progressively extended the survival time until sample animals showed that a dense scar had been formed, and then looked for the survival of cut axons and documented their sprouting pattern.

The most promising attempts at repair of CNS injuries come from the use of peripheral nerve grafts, or of transplanted cultured Schwann cells derived from peripheral nerve. In *CHAPTER VI*, I describe the effects of cultured Schwann cells transplanted into the ascending and descending tracts of the adult rat dorsal columns, where they have striking effects on the behaviour of the cut axon tips.

Compared with grafting pieces of peripheral nerve, the micro-injection of transplanted Schwann cells does much less damage to the host tissue. Moreover, the Schwann cells migrate into the host tissue, and become intimately integrated into it, by methods described in *CHAPTER VII*.

Transplantation, however, is not the only way of introducing Schwann cells into the spinal cord. It has been known for some time that lesions themselves induce endogenous Schwann cells from the surrounding peripheral nerves to migrate into the spinal cord. Obviously, this migration of endogenous Schwann cells does not result in repair of the spinal tract lesions.

In *CHAPTER VIII* I have described the cellular events following a lesion and leading to the ingrowth of endogenous Schwann cells. The histological evidence suggests that oligodendrocytic cell death, followed by invasion of microglia is important for subsequently inducing the ingrowth of the Schwann cells.

Compared with the immediate confrontation with transplanted exogenous Schwann cells, the ingrowth of endogenous Schwann cells into lesions is much more delayed, and this may account for their inability to induce axon growth.

The most optimistic observations with transplanted exogenous Schwann cells indicate that axon sprouts can elongate, but only within the extent of the graft. The Schwann cell grafts, therefore, are only a first step towards repair.

The ability of exogenous growth factors to stimulate axon growth in vivo (Higgins *et al.*, 1989) suggests a possible next step is to test the ability of exogenous growth factors to further extend the growth of cut axons under Schwann cell stimulation. It is possible to take advantage of the culture ex vivo phase (Tuszynski *et al.*, 1994a) of the Schwann cells to transfect them prior to transplantation with constructs directing the expression of specific growth factors (*CHAPTER IX*). Such a method of delivery takes advantage of the ability of the Schwann cells to integrate themselves intimately into the glial framework of the host tract.

The preliminary observations suggest that this genetic engineering approach is able to boost the already available axogenic effects of transplanted Schwann cells.

CHAPTER II EXPERIMENTAL PROCEDURES

2.1. Preparation of cells for transplantation

2.1.1. Hippocampal cells

Pregnant mice or rats (CBA and AS, both bred locally) were anaesthetized with 0.05-0.1 ml Sagatal (May and Baker, Dagenham, UK), and the embryos at 14-18 days post-conception (E14-18, where day of conception = E0) were removed aseptically, decapitated and the heads placed in a ice-cold chemically defined medium (12D) which contained 1:1 mixture of Ham's F12 medium and Dulbecco-Vogt Modification of Eagle's medium (Life Technologies Ltd, Paisley, UK) supplemented with 5 μ g/ml insulin, 100 μ g/ml transferrin, 20 μ M progesterone, 100 μ M putrescine and 30 μ M selenium (all components purchased from Sigma, Poole, UK; Bottenstein and Sato, 1979).

Under sterile conditions and using a dissecting microscope in a laminar flow hood the meninges were removed. The hippocampi were dissected out and cut into small pieces, which were washed in 10 ml of 0.1 M phosphate buffered saline (PBS), incubated in 2 ml of 0.1% trypsin (Sigma) in 0.1 M PBS in a 37°C water bath for 15 min, and the reaction was terminated by adding 200 μ l of foetal calf serum (FCS; Advanced Protein Products Ltd, UK). Excess trypsin was washed off by centrifuging the solution containing the hippocampal pieces at 1200 revolutions/min (rpm) for 5 min, removing the supernatant and re-suspending the hippocampal pieces in 10 ml of the medium. This was repeated three times. After the last centrifugation, the

hippocampal pieces were mechanically dissociated into a cell suspension by trituration with a fire-polished Pasteur pipette in 1 ml of the 12D medium. The cell density was checked by counting the cells in a haemocytometer. The final density was made up to approximately 1×10^7 cells/ml. DNase (25 ng/ml; Sigma) was added to the cell suspension to prevent the cells aggregating. The cell suspension was kept on ice throughout the operating period.

After the operations a sample of the remaining cell suspension was plated out in 35 mm culture dishes (Nunc, Life Technologies Ltd) coated with polyethylenimine (PEI; Sigma; coating was achieved by adding 1 ml of 0.05% PEI in 0.15 M borate buffer to the culture dish overnight) with 1.5 ml 12D medium and cultured for 3 days to check that the cells were still viable (Fig. 2.1).

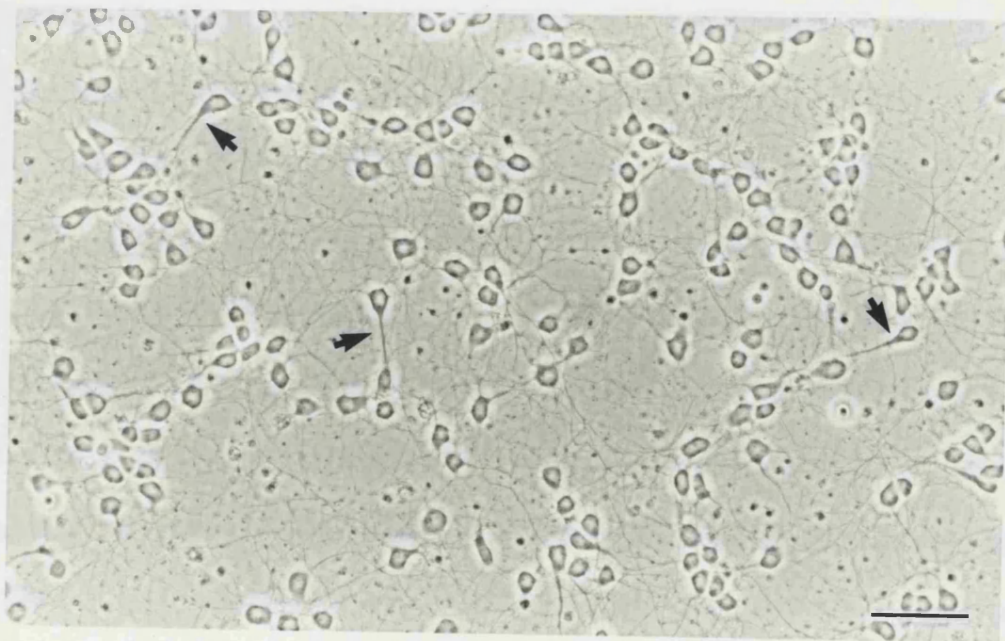
2.1.2. Schwann cells

Schwann cells were obtained from sciatic nerves of 2 day old postnatal rats of a locally bred AS strain. Under deep terminal anaesthesia (Sagatal) the animals were decapitated. In a sterile laminar flow hood the hind limbs were wiped with 70% alcohol, the sciatic nerves in the thigh were exposed and about 10 mm long segments dissected out, and placed into Dulbecco's modified Eagle's medium (DMEM; Life Technologies Ltd) supplemented with 10% FCS, 1 M glutamine, 1 M pyruvate, 20 μ g/ml penicillin and 20 μ g/ml streptomycin (DMEMF) in a 35 mm culture dish.

Figure 2.1

A sample of mouse hippocampal cell suspension remaining after transplantation was plated out in a 35 mm culture dish and cultured for 3 days. Arrows indicate three pyramid-like neurons.

Scale bar = 10 μm .



Under sterile conditions, the nerves (usually 10) were placed in parallel on a cutting disc, covered in a small amount of DMEMF, and 100 μ m segments were cut with a McIlwain tissue chopper. The nerve segments were enzymatically digested in 2 ml of DMEM with 0.15% type II collagenase (Sigma) and 0.1% trypsin (Lorne Laboratories Ltd, Reading, UK) in a 37°C water bath for 30 min. The reaction was terminated by adding 8 ml of DMEMF. After centrifuging the solution with the tissue at 1200 rpm for 5 min, removing the supernatant and re-suspending in DMEMF twice, the tissue pellet was suspended in 1 ml of DMEMF and triturated gently 5-10 times into a cell suspension with a fire-polished Pasteur pipette followed by a hypodermic needle (23 gauge). The cell suspension was then diluted with DMEMF (the volume added was approximately 1.5 ml for the nerves from 4 animals) and 1.5 ml of this cell suspension was plated into each poly-L-lysine (PLL; Sigma) coated 35 mm culture dish and incubated in a moist atmosphere enriched with 5% CO₂ at 37°C.

After 2 days when the cells became established in culture, 2×10^{-5} M of the mitotic inhibitor cytosine arabinoside (ara-c; Sigma) was added to the culture medium to suppress cell proliferation for 2 days and then washed with 3 changes of fresh DMEMF. After further culture for 1 day, the antibody-mediated complement cytotoxicity method (see below) was used to remove fibroblasts.

Antibody-mediated complement cytolysis method

1. The medium was removed from the culture dish.
2. The cells were washed gently with 0.1 M PBS (pH 7.2) twice.
3. 1 ml trypsinization solution (1 ml of 0.1 M PBS with 50 μ l 1% trypsin and 20 μ l of 100 Mm ethylenediaminetetraacetic acid (EDTA; molecular biology grade; Life Technologies Ltd) was added to each 35 mm culture dish.
4. The culture was viewed under an inverted microscope. As soon as the cells floated off the bottom of the culture dish, the reaction was terminated with DMEMF (1:1).
5. The cells were collected into a 12 ml conical tube (Bibby Sterilin Ltd, UK) and centrifuged for 5 min at 1200 rpm.
6. The supernatant was removed, a mixture of 100 μ l DMEM and 25 μ l of anti-Thy.1 IgM (Serotec, Oxford, UK) was added, and the cells were re-suspended.
7. After incubating for 40 min in a 37°C water bath, 2 ml DMEM was added to the tube which was centrifuged for 5 min. The supernatant was removed, the cells were re-suspended in a mixture of 100 μ l DMEM and 100 μ l complement (locally produced from guinea pig serum), and incubated for 40 min at 37°C.

8. The reaction was stopped with 8 ml DMEMF. The cell suspension was centrifuged for 5 min at 1200 rpm, the cells were re-suspended in DMEMF and plated on PLL coated 35 mm culture dishes.

After culture for an additional 2 days, the medium was removed, and the cells were rinsed gently twice with 0.1 M PBS and detached from the culture dish by trypsinization (see below).

Trypsinization Method

1. 1 ml enzyme solution (4 ml of 0.1 M PBS with 200 μ l of 1% trypsin and 20 μ l of 100 Mm EDTA) was added to each culture dish.
2. Under an inverted microscope, the culture dish was manually agitated for 20-30 seconds until the cells were detached from the bottom of the culture dish.
3. The reaction was stopped with 1 ml DMEMF. The contents of each culture dish were transferred to a 12 ml conical tube and made up to 10 ml with DMEMF.
4. The cell suspension was centrifuged for 5 min at 1200 rpm, the supernatant removed, 10 ml of DMEM added, centrifuged for 5 min, the supernatant removed, 1 ml of DMEM added, the cells were re-suspended and made up to 10 ml with DMEM. The cells were counted using a haemocytometer.

5. The final cell density was made into $1.5-2.0 \times 10^7$ cells/ml, DNase (25 ng/ml, Sigma) was added and the cell suspension kept on ice during transplantation.

After transplantation, a sample of the cell suspension was plated out in 35 mm culture dishes (Nunc) coated with PLL with 1.5 ml DMEM and cultured for 3 days to check that the Schwann cells were still viable (Fig. 2.2).

2.2. Surgical procedures

Adult female AS rats, body weight 180-220 g were used. Under tribromoethanol anaesthesia (20 mg/100 g body weight, I.P.), the hair over the area to be operated was removed and the skin wiped with 70% alcohol, the rat was held in a stereotaxic apparatus (Fig. 2.3) with the head stabilized using ear plugs and an incisor bar. The incisor bar was 5 mm below the ear plugs and the body was raised so that the spinal cord was level with the skull. The dorsal surface of the spinal cord was exposed by a dorsal laminectomy at the level of the first two to three cervical segments.

2.2.1. Hippocampal cell transplantation

2.2.1.1. Single transplant A glass micro-pipette with a bevelled tip of internal diameter 50-70 μm was mounted on a micro-manipulator (Prior Scientific Instruments Ltd, UK; Fig. 2.3) and about 2-3 μl cell suspension

Figure 2.2

A sample of Schwann cell suspension used for transplantation was plated out in a 35 mm culture dish and cultured for 3 days.

Scale bar = 10 μm .

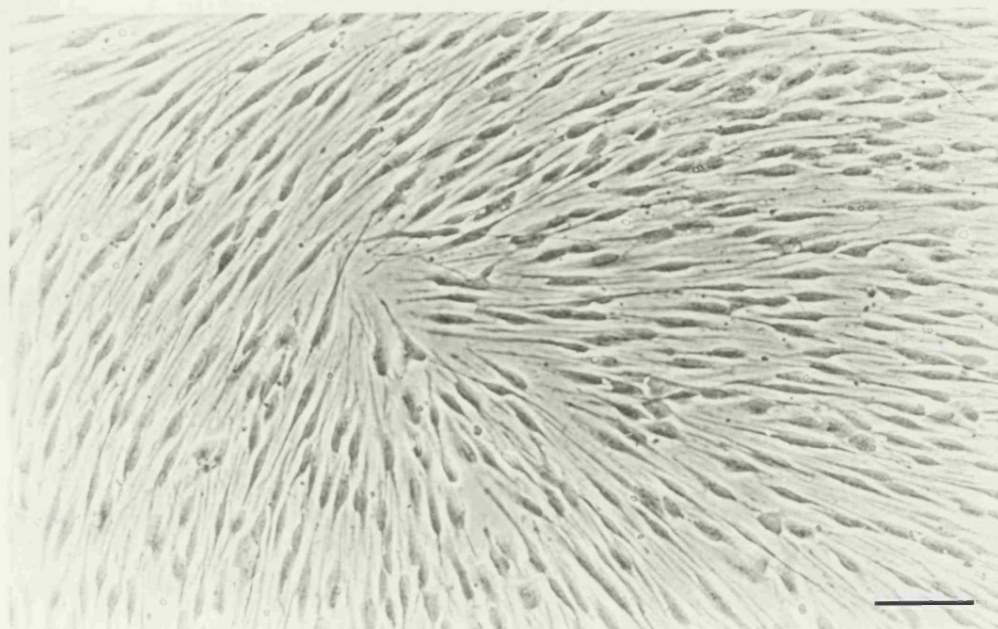
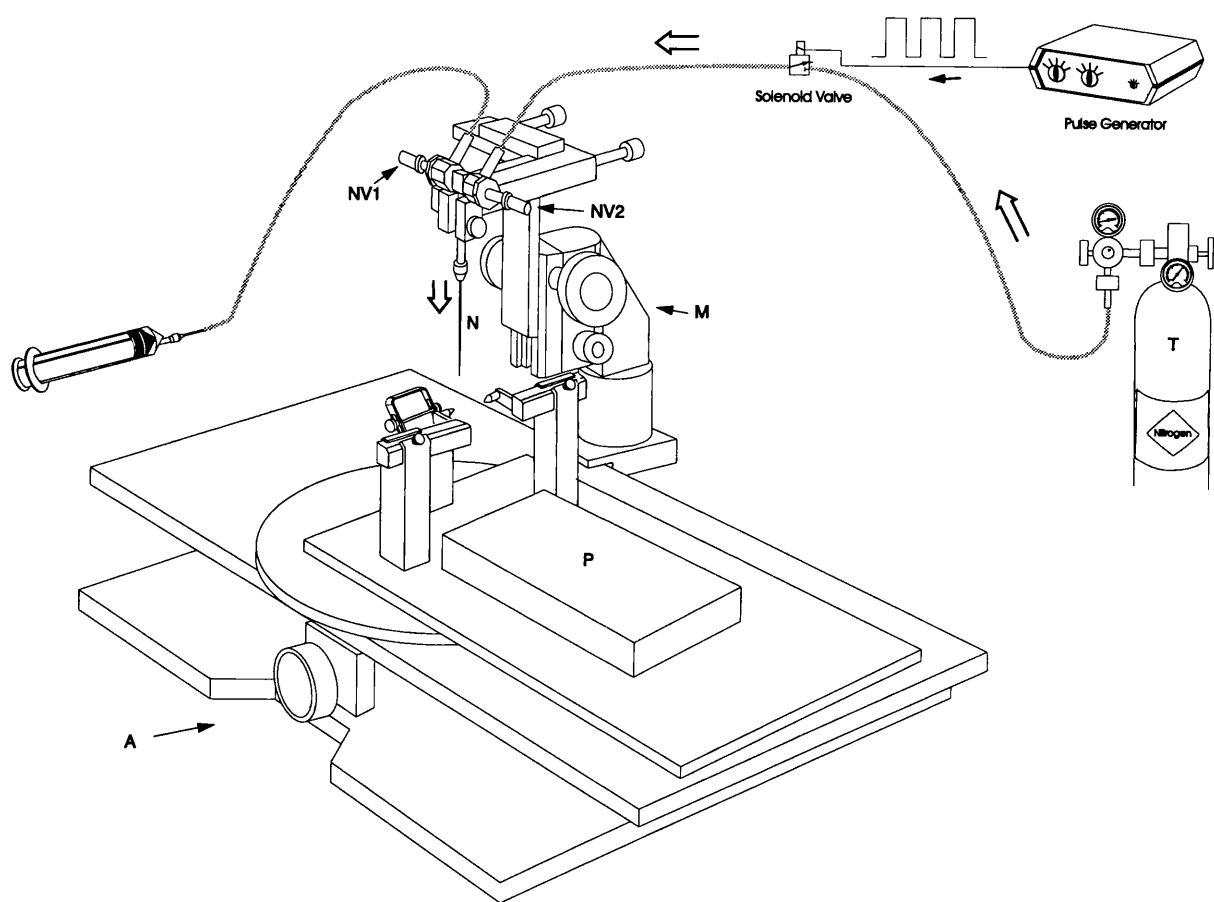


Figure 2.3

Schematic diagram of the system for micro-transplantation. A, stereotaxic apparatus; M, micro-manipulator; N, glass micro-pipette; P, platform for raising animal's body to level the spinal cord; T, air cylinder. NV1 and NV2 are needle-valves.

With NV1 open and NV2 closed the cell suspension or tracer solution can be loaded into the micro-pipette with a syringe. With NV2 open and NV1 closed, the contents of the micro-pipette can be expelled by pulsed air pressure. The injection is controlled by pulse rate and air pressure.



was drawn into the micro-pipette. With stereotaxic guidance the micro-pipette was inserted at a point about 0.2 mm lateral to the dorsal midline blood vessel to a depth of 0.4-0.5 mm from the pial surface for the ascending tracts or 0.7-0.8 mm for the descending corticospinal tract between level of the first and second cervical segments. 0.5-1.0 μ l of the cell suspension (i.e. about 5-10,000 hippocampal cells) was injected by a pulsed air pressure injection system (Fig. 2.3; Emmett *et al.*, 1990) into the ascending dorsal column tracts or the descending corticospinal tracts. Each injection took 1-2 min, and the micro-pipette was withdrawn slowly, the muscles and skin were sutured in layers under aseptic conditions.

2.2.1.2. Double transplants To investigate the possible effects of target tissue on the axons of transplanted cells, a further group of animals with E14 mouse hippocampal cell transplants received a simultaneous E16 rat hippocampal transplant, either 1-2 mm caudal to the mouse transplant on the same side of the spinal cord, or on the contralateral side. The surgery and the transplantation procedures were the same as for a single transplant.

2.2.2. Schwann cell transplantation

The operative procedure for transplanting Schwann cell was the same as that for transplanting hippocampal cells. 1.0 to 1.5 μ l of the cell suspension with a final density of $1.5-2 \times 10^7$ cells/ml (about 10-15,000 cells) was micro-injected into the ascending dorsal column or descending tracts.

2.2.3. Immuno-suppression

To prevent immune rejection the rats with the mouse transplants were maintained on cyclosporin A (Sandimmun, Sandoz; 10 mg/100 ml) in the drinking water from the time of operation throughout the survival period.

2.2.4. Lesions

Lesions were made electrolytically in the dorsal columns at the upper cervical level of the spinal cord. Adult female AS rats, body weight 180-220 g were used. The dorsal surface of the spinal cord was exposed by a dorsal laminectomy as described in surgery for transplantation. Under stereotaxic control a manipulator was used to insert an insulated stainless steel electrode (tip size: 0.10 μm , impedance: 5 M Ω , taper: 8°; A-M Systems Inc., Washington, USA) into the ascending dorsal column tracts or descending corticospinal tract (the same positions and depth as for transplantation). A locally made constant current device was used to pass a continuous constant anodal DC current of 10 μA for 4-7 min, and the electrode then withdrawn slowly. The muscles and skin were sutured in layers, under aseptic conditions.

The operated animals were left to survive for between 2 days and 4 months. Apart from the first few days after operation, none of the operated animals (with transplantation or lesion) had any detectable weight loss and/or abnormalities in their cage behaviour.

2.2.5. Extracellular axonal tracer injection

2.2.5.1. Retrograde labelling of transplanted hippocampal neurons

1-4 weeks after receiving an E14 mouse hippocampal transplant, the cervical part of the spinal cord was re-exposed by the same surgical procedure as for transplantation (see above), and 1 or 2 aliquots of 0.2 μ l of a 40% solution of horseradish peroxidase (HRP; Type VI, Sigma) in saline, or a 2.5% solution of HRP conjugated with wheat germ agglutinin (WGA-HRP; Sigma) were injected over 5-10 min through a micro-pipette (internal diameter 70-100 μ m) by the pulsed air pressure injection system at a position 0.5-0.7 mm below the dorsal pial surface, and 2-3 mm caudal to the transplant.

2.2.5.2. Anterograde labelling of axons of the dorsal columns

Ascending dorsal column axons The axons of the ascending dorsal columns were labelled by applying the anterograde tracer directly to the tracts. This is more practical than labelling the cells of origin by exposing large numbers of individual dorsal root ganglia.

40-48 hrs before sacrificing the operated animals, the dorsal surface of the spinal cord was re-exposed as for transplantation (see above), and the pulsed air pressure system used to inject a 40% solution of HRP in saline, or a 2.5% solution of WGA-HRP in saline into the dorsal columns through a micro-pipette (internal diameter 70-100 μ m) placed 0.4-0.6 mm below the dorsal pial surface of the spinal cord and 2-3 mm below the transplant or the lesion. Each animal received 1-2 injections, each injection took 5-10 min

and consisted of about 0.2 μ l of tracer solution. The micro-pipette was withdrawn slowly and the muscles and skin were sutured in layers, under aseptic conditions.

Descending corticospinal axons In a preliminary series of experiments, HRP was applied to the frontal cortex (1 and 2 mm posterior to the bregma, 2.5 mm lateral to the midline and 1.7-1.8 mm ventral to the dura, at the level of the cell bodies of origin of the corticospinal tract) and the anterogradely labelled axons visualized by the more sensitive TMB method (see 2.6). However, the large crystals of reaction product completely distorted the fine details of the axonal arborisations. Using the more gentle glucose oxidase method (see 2.6) the axonal arborisations were observed, but as the lower sensitivity of detection was unsuitable for such long axon transport, detectable terminal labelling could only be achieved by applying the tracer to the pyramidal tract at the medullary level, much closer to the site of the transplants or the lesions.

In the definitive series of experiments, the descending corticospinal axons were labelled by 2 adjacent stereotaxic injections into the pyramidal tract at the base of the medulla, rostral to the decussation (i.e. on the opposite side to the transplant or the lesion) 40-48 hrs (for biocytin) and 3 to 5 days (for biotin-dextran, BD) before sacrifice. The head was held in a stereotaxic apparatus with ear plugs and an incisor bar (5 mm below the ear plugs). The skull was exposed and a dental drill used to make a burr hole at a

position 11-11.5 mm posterior to the bregma, 0.3 and 0.6 mm lateral to the midline. A glass micro-pipette (internal diameter 100-150 μm) filled with 40% HRP or 5% biocytin (Sigma) in saline or 10% BD (10,000 MW, lysine fixable; Molecular Probes, Oregon, USA) in saline was lowered stereotactically into the base of the medulla (approximately 10 mm ventral to the dura). 0.2-0.4 μl of the tracer solution was injected with the pulsed air pressure injection system over 5 min, the micro-pipette withdrawn slowly and the skin sutured under aseptic conditions.

It cannot be excluded that a small amount of the HRP injection into the medullary pyramid might have also spread to label a small number of other descending axons passing through the ventro-medial part of the medulla. However, the investigations of the spinal cord referred only to the dorsal columns, and this would exclude most of the other descending tracts (e.g. rubrospinal, which runs in the lateral columns, Paxinos (1994)). A possible explanation of the axons selectively responding to NGF-secreting transfected Schwann cells (see *CHAPTER IX*) is that the anterograde tracer injection in the region of the medullary pyramids may label axons belonging to a p75-positive cholinergic projection descending in the dorsal columns.

2.3. Histological processing

2.3.1. Perfusion

2.3.1.1. For cryostat sectioning Animals were anaesthetized under a deep terminal dose of pentobarbitone (Sagatal). Through a wide thoracotomy, the

heart was exposed and the descending aorta clamped with haemostatic forceps. With a pair of fine scissors an incision was made to the right atrium and another to the left ventricle. A blunt hypodermic needle (0 gauge; cut flat and polished smooth) was inserted into the ascending aorta through the left ventricle. The blood was flushed out by perfusion of about 100 ml of 0.1 M PBS at room temperature for 5 min.

The cervical spinal cord was then exposed, very gently dissected out from the vertebrae and placed on a 25 mm diameter aluminium planchette (TAAB Laboratories Equipment Ltd, Reading, UK) with the dorsal part aligned with the flat bottom of the dish and rapidly frozen in crushed dry ice. The frozen tissue blocks were kept at -70°C until sectioning.

2.3.1.2. For fixation The blood was flushed out by the above procedure and 500 ml of various types of fixatives (according to the requirement of the following histological procedures) was perfused transcardially with about 50 cm of gravity at 4°C for 30-40 min.

2.3.2. Fixatives

2.3.2.1. Immunohistochemistry

Acetic alcohol A mixture of 5% glacial acetic acid and 95% of 96% alcohol (v/v).

Paraformaldehyde 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.2-7.4).

40 g of paraformaldehyde (EM grade; TAAB Laboratories Equipment Ltd) in 400 ml distilled water with 2 ml 1 N NaOH was heated to 60°C in a fume cupboard until the paraformaldehyde had completely dissolved. Distilled water was added to make up to 500 ml. After the solution had cooled 500 ml of 0.2 M PB was added. The fixative was filtered and the pH adjusted to 7.2-7.4 with HCl.

2.3.2.2. Palmgren silver stain

Carnoy's fixative A mixture of 60% absolute alcohol, 30% chloroform and 10% glacial acetic acid (v/v).

2.3.2.3. Detection of extracellular tracer

HRP 1% paraformaldehyde and 2% glutaraldehyde in 0.1 M PB (pH 7.3).

10 g paraformaldehyde in 400 ml distilled water was dissolved as for making 4% paraformaldehyde. After cooling 80 ml of 25% glutaraldehyde (EM grade; TAAB Laboratories Equipment Ltd) and distilled water were added to make up to 500 ml. 500 ml of 0.2 M PB was then added. The fixative was filtered and the pH adjusted.

WGA-HRP 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M PB (pH 7.4).

10 g paraformaldehyde in 400 ml distilled water was dissolved as above. 50 ml of 25% glutaraldehyde and distilled water were added to make up to 500 ml. 500 ml of 0.2 M PB was added. The fixative was filtered and the pH adjusted as above.

Biocytin 2% paraformaldehyde and 1% glutaraldehyde in 0.1 M PB (pH 7.4).

20 g paraformaldehyde in 400 ml distilled water was dissolved as above. 40 ml of 25% glutaraldehyde and distilled water were added to make up to 500 ml. 500 ml of 0.2 M PB was added. The fixative was filtered and the pH adjusted as above.

BD 4% paraformaldehyde, 0.15% glutaraldehyde and 0.4% picric acid in 0.1 M PB (pH 7.4).

40 g paraformaldehyde in 400 ml distilled water was dissolved as above. 6 ml of 25% glutaraldehyde, 4 ml saturated picric acid and distilled water were added to make up to 500 ml. 500 ml of 0.2 M PB was added. The fixative was filtered and the pH adjusted as above.

2.3.2.4. Electron microscopy (EM)

General EM 1% paraformaldehyde and 1% glutaraldehyde in 0.1 M PB (pH 7.2-7.4).

10 g paraformaldehyde in 400 ml distilled water was dissolved as above. 40 ml of 25% glutaraldehyde, 4 ml of 0.5% CaCl_2 , 5.4 g glucose and distilled water were added to make up to 500 ml. 500 ml of 0.2 M PB was added. The fixative was filtered and the pH adjusted as above.

Preservation of CNS myelin (method of Dr Gilmore) 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M PB (pH 7.3).

20 g paraformaldehyde in 400 ml distilled water was dissolved as above. 80 ml of 25% glutaraldehyde and distilled water were added to make up to 500 ml. 500 ml of 0.2 M PB was added. The fixative was filtered and the pH adjusted as above.

2.3.3. Tissue processing for electron microscopy

2.3.3.1. General electron microscopy After perfusing with the general EM fixative (see above), the cervical part of spinal cord was left in situ in the same fixative overnight at 4°C. The spinal cord was dissected out, washed in 0.1 M PBS and 200-300 μm horizontal Vibratome sections were cut (see below). The sections were trimmed to the desired area, collected in a washing solution composed of 0.002% CaCl_2 and 8% glucose in 0.1 M PB

for 1 hr, further fixed in 2% aqueous osmium tetroxide in 0.1 M PB for 1 hr, washed in the washing solution for 30 min, and dehydrated in an ascending series of alcohols (70%, 80%, 95% and 100%; three changes of 5 min for each step) and propan-2-ol (two 20 min changes; BDH Ltd, Poole, UK). The sections were immersed in 1:1 propan-2-ol and resin (TAAB Laboratories Equipment Ltd) for 1 hr, and 100% resin overnight at room temperature for resin infiltration. The sections were then flat embedded in resin on glass slides coated with mould-releasing compound (Formen-Trenmittal Hobby Fluid, Hobby Wholesale, UK). The resin was polymerized in a 60°C oven for 48 hrs.

2.3.3.2. Preservation of CNS myelin After perfusing with the fixative for preserving CNS myelin (see above) the cervical part of spinal cord was left in situ and kept in the same fixative overnight at 4°C. The spinal cord was dissected out and identical procedures as above (see 2.3.3.1) carried out until the dehydration stage. The sections were dehydrated in a more gentle and gradual manner in an ascending series of acetones (20%, 40% and 50% in two 5 min changes), alcohols (70%, 80%, 95% and 100% in three changes, each for 5 min) and 100% acetone (in three 5 min changes). The sections were immersed in 1:1 acetone and resin for 12 hrs, and 100% resin overnight at room temperature for resin infiltration. The sections were then flat embedded in resin on glass slides coated with mould-releasing compound (Formen-Trenmittal Hobby Fluid, Hobby Wholesale, UK). The resin was polymerized in a 60°C oven for 48 hrs.

2.3.3.3. Staining semithin and ultrathin sections The semithin sections were immersed in a mixture of equal amounts of 1% methylene blue in distilled water and 1% Azur II in 1% disodium tetraborate for 1 min, rinsed with distilled water and dried on a hot plate (70°C).

The grids with ultrathin sections were immersed in 0.3% uranyl acetate in methanol for 7 min, washed three times in methanol and air dried. They were then immersed in Reynolds lead citrate solution (see below) for 7 min, rinsed three times in distilled water and air dried.

Reynolds lead citrate

1. 100 ml of distilled water was boiled and then cooled in a sealed container (to decrease the amount of O₂).
2. 1.33 g of lead nitrate and 1.76 g sodium citrate 2H₂O were added consecutively to 30 ml of distilled water.
3. 8 ml of 1 N NaOH was added to the above solution to dissolve the lead nitrate and sodium citrate.
4. The distilled water (prepared as in step 1) was added to make the volume up to 50 ml.

2.3.4. Sectioning

2.3.4.1. Cryostat The frozen block of spinal cord was mounted horizontally on a specimen holder in OCT (Bright Cryo-M-Bed; Jencons Scientific Ltd,

Leighton Buzzard, UK), horizontal sections at thickness of 10 μm were cut from the flattened surface (dorsal spinal cord; in some cases sagittal and coronal sections were cut) on a cryostat microtome (Cryostat 2800 Frigocut-E, Leica, UK) and mounted on gelatin-coated slides (see below) and air dried for approximately 4 hrs at room temperature. For long term storage, the sections were kept at -70°C .

Slide cleaning and gelatin coating procedure

Racks of slides were immersed in distilled water with Decon 75 and sonicated for 20 min. After washing a few times in distilled water the slides were immersed in a solution containing 0.5% gelatin, 0.05% chromic potassium sulphate in distilled water for 1 min. The coated slides were dried at room temperature and stored in sealed plastic bags.

2.3.4.2. Vibratome After fixation by perfusion (see above), the cervical spinal cord was dissected out and left in the same fixative overnight. After rinsing in 0.1 M PBS, the spinal cord was trimmed with a razor blade and mounted on a metal block with Quick Set 404 adhesive (Loctite Corp., UK) with the dorsal spinal cord facing the blade. 60-100 μm (for axonal labelling) or 200-300 μm sections (for EM) were cut on a Vibratome (Series 1000; Technical Products International Inc, St. Louis, USA) and collected in 0.1 M PBS.

2.3.4.3. Freezing microtome Perfusing with the appropriate fixative was immediately followed with 500 ml of 10% sucrose in 0.1 M PB at 4°C for 30 min. The cervical part of spinal cord was removed and soaked in 20% sucrose in 0.1 M PB overnight. The spinal cord was mounted on an electrically cooled specimen block by freezing a small amount of PB around the bottom of the tissue. 50-100 μ m sections were cut on a sledge microtome (Reichert, Germany) and collected in 0.1 M PB.

2.3.4.4. Ultramicrotome The resin embedded section (see above) was cut out with a sharp razor blade and mounted on a block of resin. Semithin sections were cut with a glass knife at a thickness of 1.5-2 μ m on an ultramicrotome (Ultracut E, Leica, UK), mounted on gelatin coated slides, and dried on a hot plate (70°C). Ultrathin sections were cut with a diamond knife at an approximate thickness of 80 nm on the ultramicrotome and collected on copper grids.

2.4. Histological staining

2.4.1. Thionin

Air dried cryostat sections were fixed with acetic alcohol for 30 min and rehydrated through a descending series of alcohols (96%, 70% and 50%) to distilled water. The fixed and rehydrated cryostat sections, and sections which had undergone other histological or immunohistological procedures were immersed in 0.05% aqueous thionin solution for 2 min, dehydrated in

an ascending series of alcohols (70%, 96% and 100%), cleared in HistoClear™ (National Diagnostics, Aylesbury, UK) and mounted in a mixture of dibutyl phthalate, polystyrene and HistoClear™ (DPH).

2.4.2. Palmgren silver (Palmgren, 1960)

10 µm cryostat sections were fixed in Carnoy's fixative for 30 min, washed twice in absolute alcohol for 5 min, coated with celloidin (see below) for 1 min, hardened in 70% alcohol for 3-5 min, rehydrated in distilled water, placed in acid formalin (see below) for 10 min and washed thoroughly in distilled water. The sections were then incubated in a silver solution (see below) for 15 min, drained, left in the reducer at 40-45°C (see below) for 1 min, rinsed in 50% alcohol for 5-10 seconds, washed in distilled water, toned until the brown colour faded and washed in distilled water. After the reaction product was stabilized for 5 min in 5% sodium thiosulphate, the sections were counter-stained with thionin, dehydrated through an ascending series of alcohols (the celloidin was removed by the absolute alcohol), cleared in HistoClear™, and coverslipped in DPH.

Celloidin solution: 1% celloidin in a 50/50 mixture of absolute alcohol and ether.

Acidic formalin: A mixture of 25 ml formalin, 75 ml distilled water and 0.2 ml 1% nitric acid.

Silver solution: A mixture of 15% AgNO₃, 10% KNO₃, 0.05% glycine.

The solution was filtered before use.

Reducer: A mixture of 10 g pyrogallol, 450 ml distilled H₂O, 550 ml absolute alcohol and 2 ml 1% nitric acid. The mixture was allowed to stand for 24 hrs before use.

Toning solution: A mixture of 0.5% AuCl₂ and 0.1% glacial acetic acid.

Stabilizer: 5% sodium thiosulphate.

2.5. Immunohistochemistry

2.5.1. M6

10 μ m cryostat sections were fixed in freshly prepared 4% paraformaldehyde for 20 min at room temperature, washed in several changes of 0.1 M PBS for 45 min, blocked in a 1% aqueous solution of dried powdered milk in 0.1 M PBS ('milk-PBS') for 30 min and incubated in 1:30 M6 antibody (Lund *et al.*, 1985) in 1% milk-PBS at 4°C in a humidified chamber overnight. The sections were then washed thoroughly in 0.1 M PBS, incubated in 1:100 anti-rat HRP-conjugated secondary F(ab')₂ antibody (Amersham, UK) in 1% milk-PBS for 2 hrs at room temperature and washed thoroughly in several changes of 0.1 M PBS for 45 min. The peroxidase

product was detected with the DAB method (see below) and intensified with the silver-gold enhancement method (see below). The sections were then washed in distilled water for 1 hr, counter-stained with thionin, dehydrated in an ascending series of alcohols, cleared with HistoClear™, mounted and coverslipped in DPH.

2.5.2. Thy-1.2

After fixing and washing (the same as for M6), the cryostat sections were pre-incubated in 1% milk-PBS, incubated in 1:1000 anti-Thy-1.2 antibody (Dupont, Wilmington, Delaware) in 1% milk-PBS in a humidified chamber at 4°C overnight, washed thoroughly in 0.1 M PBS, incubated in 1:100 anti-mouse HRP-conjugated secondary F(ab')₂ antibody (Amersham) in 1% milk-PBS for 2 hrs at room temperature. The DAB reaction (see below), silver-gold enhancement (see below), counter-staining and mounting were the same as for M6.

2.5.3. Py

After fixing and washing as for M6, the cryostat sections were pre-incubated in 1% milk-PBS for 30 min, then incubated with a 1:200 dilution of Py antibody (locally raised; Woodhams *et al.*, 1989) in 1% milk-PBS at 4°C in a humidified chamber overnight, washed thoroughly for 40 min in several changes of 0.1 M PBS, incubated for 2 hrs in 1:100 anti-mouse HRP-conjugated secondary F(ab')₂ antibody (Amersham) in 1% milk-PBS at room temperature. The DAB reaction (see below), silver-gold enhancement (see below), counter-staining and mounting were the same as for M6.

2.5.4. Low affinity neurotrophin receptor (p75)

10 μ m cryostat sections were fixed with ice-cold acetic alcohol for 30 min, washed with 0.1 M PBS for 40 min, blocked with 1% milk-PBS for 30 min, incubated in 1:10 anti-p75 antibody (Boehringer, Mannheim, UK) in an antibody diluent solution (1% bovine serum albumin and 0.05% azide in 0.1 M PBS) overnight in a humidified chamber at 4°C. The sections were then washed thoroughly in several changes of 0.1 M PBS for 40 min, incubated in 1:300 biotinylated anti-mouse secondary antibody (IgG; Vector Laboratories, Peterborough, UK) in antibody diluent for 30 min at room temperature, washed with 0.1 M PBS for 40 min, incubated in an avidin-biotin-HRP complex (ABC; Vector Laboratories) solution for 30 min and washed with 0.1 M PBS for 40 min. The HRP product was detected with the Ni-GOD method (see below) for approximately 5 min, counter-stained with thionin, dehydrated, cleared, mounted and coverslipped in DPH.

2.5.5. P₀

10 μ m cryostat sections were fixed with ice-cold acetic alcohol for 15 min at room temperature, washed in 0.1 M PBS for 10 min and 2 changes of Minimum Essential Medium (MEM; HEPES-buffered; Life Technologies) containing 10% FCS (MEMF) for 20 min, incubated in 1:200 anti-P₀ antibody (a gift from Dr J. Brockes) in MEMF overnight in a humidified chamber at 4°C, washed with 0.1 M PBS for 40 min, incubated in 1:200 biotinylated goat anti-rabbit secondary antibody (Vector Laboratories) in antibody diluent for 30 min at room temperature, washed with 0.1 M PBS for 40 min,

incubated in the ABC solution for 30 min and washed thoroughly with 0.1 M PBS. The sections were then reacted in the Ni-GOD solution for 3-5 min, washed with 0.1 M PBS, counter-stained with thionin, dehydrated, cleared and mounted.

2.5.6. Glial fibrillary acidic protein (GFAP)

10 μ m cryostat sections were fixed in ice-cold acetic alcohol for 30 min, washed with 0.1 M PBS for 40 min, blocked with 1% milk-PBS for 30 min and incubated in 1:1000 anti-GFAP antibody (Amersham) in antibody diluent overnight in a humidified chamber at 4°C. The sections were washed thoroughly with 0.1 M PBS, incubated in 1:300 biotinylated anti-mouse IgG secondary antibody (Vector Laboratories) in antibody diluent for 30 min at room temperature, washed with 0.1 M PBS for 40 min, incubated in the ABC solution for 30 min and washed with 0.1 M PBS for 40 min. The sections were then reacted in the Ni-GOD solution for about 5 min, counter-stained with thionin, dehydrated, cleared and mounted.

2.5.7. OX-42

10 μ m horizontal cryostat sections were fixed with ice-cold acetic alcohol for 5 min, washed for 40 min in 0.1 M PBS and blocked with 1% milk-PBS for 30 min. The sections were incubated in 1:2000 anti OX-42 (Sera Lab, UK) in 1% milk-PBS overnight in a humidified chamber at 4°C, washed thoroughly with 0.1 M PBS for 40 min, incubated in 1:300 biotinylated anti-mouse secondary antibody (IgG; Vector Laboratories) in antibody diluent for

30 min at room temperature, washed in 0.1 M PBS for 40 min and incubated in the ABC solution for 30 min. The sections were then washed with 0.1 M PBS for 40 min, reacted in the Ni-GOD solution for about 5 min, washed again with 0.1 M PBS for 40 min and counter-stained with thionin, dehydrated, cleared and mounted.

2.5.8. Control staining

In all immunostaining procedures control staining was carried out by incubating sections with the secondary antibody (but omitting the primary antibody) to exclude possibility of non-specific staining.

2.5.9. 3'3-diaminobenzidine (DAB) method

The 3'3-diaminobenzidine dihydrochloride (Sigma) was dissolved in a buffer (pH 5.8) which consisted of 1.6% sodium phosphate, 0.07% imidazole to a final concentration of 0.05%, filtered through filter paper (Whatman No. 1) and H₂O₂ was added to a final concentration of 0.003%. Sections were thoroughly washed with 0.1 M PBS and incubated in DAB solution until the required reaction intensity was achieved (the reaction intensity was monitored either with the naked eye or under a microscope, usually for about 3 min).

2.5.10. Glucose oxidase-nickel DAB method (Ni-GOD method; Shu *et al.*, 1988)

Sections were washed in 0.1 M PBS, rinsed in 0.1 M acetate buffer (pH 6.0) and incubated in a reaction solution as follows:

1. 0.1 M acetate buffer (pH 6.0)
2. 0.05% DAB
3. 0.04% ammonium chloride
4. 2.5% nickel ammonium sulphate
5. 0.25% β -D-glucose
6. 0.001% Glucose Oxidase

2.5.11. Silver-gold enhancement (Woodhams *et al.*, 1989)

To intensify the HRP-DAB reaction product, the sections were washed thoroughly in distilled water, immersed in 0.1% gold chloride for 5 min, and neutralised in 2.5% sodium sulphide for 5 min. They were then transferred to the developer (see below) and the reaction intensity was monitored either with the naked eye or under a microscope, usually for 2-4 min. Development was stopped by placing the sections in 1% acetic acid and the reaction product stabilised with 1% sodium thiosulphate. Between each step, the sections were washed in distilled water.

The developer consisted of 3 solutions which were mixed in a ratio of 2:1:1 in the order as shown below, immediately before use.

1. 4.17% Na_2CO_3
2. 0.2% NH_4NO_3 , 0.2% AgNO_3 and 1% $\text{SiO}_2 \cdot 12\text{WO}_3 \cdot 26\text{H}_2\text{O}$
3. the same solution as (2) with 0.73% formalin

2.6. Extracellular axonal tracer detection

2.6.1. HRP

2.6.1.1. Tetramethylbenzidine method (TMB method; Mesulam, 1978) The fixed floating sections which had been cut frozen were rinsed in distilled water for 3 min, incubated in the pre-reaction solution (see below) for 20 min at room temperature, developed in the enzymatic reaction solution (see below) for 20 min at room temperature and rinsed three times (1 min each) with the 'post-reaction solution' (see below). The sections were left in this solution for 1 hr, mounted on gelatin coated slide and left to air-dry for 5-24 hrs at room temperature. They were counter-stained with neutral red solution (see below) for 3 min and dehydrated and cleared according to the following schedule: 15 sec in distilled water; 15 sec in 70% alcohol; 15 sec in 95% alcohol; 2 x 15 sec in 100% alcohol; 1 min in Histoclear™ and then 5-10 min in a second bath of Histoclear™ and mounted with DPH.

Buffer at pH 3.3: 200 ml of 1.0 M sodium acetate, 190 ml of 1.0 M HCl and 200 ml distilled water were mixed. The mixture was then made up to 1000 ml with distilled water and the pH was adjusted to 3.3 with acetic acid or 0.2 M sodium hydroxide.

Pre-reaction solution

A: 5 ml of the pH 3.3 buffer and 1 ml of 10% sodium nitroferricyanide (Sigma) in distilled water were added to 92.5 ml of distilled water.

B: 5 mg of 3,3', 5,5' tetramethylbenzidine (Sigma) was dissolved in 2.5 ml of absolute alcohol at 37°C.

The two solutions were freshly made and were mixed just before use.

Enzymatic reaction solution: 4 ml of 0.3% H₂O₂ was added to 100 ml of the pre-reaction solution.

Post-reaction solution: 5 ml of the pH 3.3 buffer was added to 95 ml of distilled water.

Neutral red solution: 40 ml of 0.1 N acetate buffer (pH 4.8) was added to one litre of 1% aqueous neutral red solution. The solution was filtered before use.

2.6.1.2. Glucose oxidase-cobalt DAB method (Co-GOD method; Itoh *et al.*, 1979) The sections were briefly washed twice in 0.1 M Tris buffer (pH 7.6), placed in 0.5% CoCl₂ in Tris buffer for 10 min at room temperature, washed three times with Tris buffer and twice with 0.1 M PB (pH 7.3) for 20 min, incubated for 60 min in a 37°C oven in a freshly prepared solution containing 50 mg of DAB, 200 mg of β -D-glucose, 40 mg of NH₄Cl, 0.4 mg of glucose oxidase (Sigma) in 100 ml of 0.1 M PB (pH 7.3), washed in PB 3 times and mounted on gelatin coated slides. After air-drying for 5-24 hrs, the sections were counter-stained with neutral red as above, dehydrated in an ascending series of alcohols, cleared in HistoClear™ and mounted in DPH.

2.6.2. Biocytin and BD

The sections were washed in 0.1 M PBS and treated with 0.3% H₂O₂ in 0.1 M PBS for 15 min. After rinsing off H₂O₂ with 0.1 M PBS the sections were incubated in the ABC solution with 0.1% Triton in 0.1 M PBS overnight at 4°C and, the HRP product was detected with either the Co-GOD or the Ni-GOD method (see above). The reaction intensity was monitored under a microscope. Usually the Co-GOD solution needed about 60 min and the Ni-GOD solution about 20 min.

**CHAPTER III THE STRUCTURE OF THE LONG ASCENDING
AND DESCENDING TRACTS OF THE DORSAL COLUMNS OF
THE NORMAL ADULT RAT SPINAL CORD**

3.1. Introduction

The dorsal columns of the rat consist of (a) the sensory ascending fibres of the gracile tract and the cuneate tract, and (b) the descending, motor fibres of the corticospinal tract (Cajal, 1911; Matthews and Duncan, 1971). The ascending tracts are located in the dorsal part of the dorsal columns and contain axons predominantly from the Ia primary sensory afferents of the ipsilateral dorsal root ganglion cells. The descending corticospinal tracts are situated in the ventral part of the dorsal columns and their axons originate from the large pyramidal neurons of layer 5 in the primary somatosensory cortex and also from the agranular motor cortex (Terashima, 1995; Wise and Jones, 1977). The majority of corticospinal fibres decussate in the caudal medulla and descend in the ventral-most part of the dorsal columns (Porter and Lemon, 1993). Additional groups of fibres are uncrossed and descend in various portions (e.g. Vahlsing and Ferringa, 1980). The axons of the corticospinal tract terminate in all spinal laminae contralateral to the cells of origin, with dense terminations in laminae 3-7 of the dorsal horn and less dense terminations in the ventral horn (Brown, 1971; Casale *et al.*, 1988; Liang *et al.*, 1991; Porter and Lemon, 1993).

Matthew and Duncan (1971) recorded the differences in the fibre diameter and type between the ascending and descending tracts. The diameters of most corticospinal axons are between 0.3 and 3.0 μm . The diameters of the axons in the cuneate tracts, which are the largest fibre tracts in the dorsal columns, are between 3 and 8 μm and, the gracile tracts contain

axons with a range of diameters intermediate between the corticospinal and cuneate tracts, with most of the fibres being between 1 and 3 μm .

There have been few studies of the way in which the glial cells are integrated into the overall structural architecture of specific adult fibre tracts. Recently, however, Suzuki and Raisman (1992) described the regular arrangement of the glial framework of the adult rat fimbria. They showed that the adult rat fimbria consists of regularly spaced unicellular glial rows of considerable length, running in the longitudinal axis of the tract. The oligodendrocytes are contiguous, and the astrocytes are interspersed singly at a repeat distance between stretches of oligodendrocytes. They also showed that both astrocytes and oligodendrocytes have radial and longitudinal processes, which interweave with each other to form a rectilinear mesh work. Oligodendrocytes develop postnatally, and constitute over 85% of the total glial population in the dorsal columns in the mature adult (Matthews and Duncan, 1971). There is a dramatic postnatal developmental change in astrocytic GFAP immunoreactivity, the majority of astrocytes and radial glial cells being negative at birth and almost all becoming GFAP-positive by the end of the fourth postnatal week.

3.2. Materials and methods (For details see *CHAPTER II*)

3.2.1. Experimental animals

Adult AS female rats (body weight 190-220 g) were used for this study.

3.2.2. HRP labelling ascending axons

The ascending axons were labelled by injections of HRP into the ascending tracts of the dorsal columns at the level of cervical segment 4 or 5 (3 animals) and horizontal frozen sections were cut. Since the HRP was transported only for a short distance, the Co-GOD method, which offers better morphology, was used to detect the HRP reaction product.

3.2.3. HRP labelling descending corticospinal axons

The descending corticospinal axons were labelled by injections of HRP in the sensori-motor cortex (3 animals). 60 μm frozen sections of the cervical part of spinal cord were cut in the horizontal plane. The HRP reaction product was detected with the more sensitive TMB method, needed because the HRP was transported by the cortical pyramidal cells for a long distance from the cortex to the cervical level of the spinal cord.

3.2.4. Palmgren silver stain

Axons in the ascending and descending tracts were also studied with the Palmgren silver method (5 animals).

3.2.5. Staining of glial cells

To study the glial arrangement in the ascending and descending tracts, horizontal, coronal and sagittal cryostat sections were immunostained with GFAP and OX-42 (7 animals).

3.2.6. Semithin sections

Semithin sections were cut in a horizontal plane and stained with methylene blue to observe the tract structure (3 animals).

3.3. Results

3.3.1. Features of axons

3.3.1.1. Appearance of labelled axons The descending corticospinal axons labelled with HRP and detected with the TMB method had a dark black and particulate appearance. The HRP-labelled axons in the ascending tract and detected with the Co-GOD method also had dark black HRP reaction product but were more continuous and much smoother than the axons detected with the TMB method. All axons stained with the Palmgren silver method had a dark brown appearance.

Individual axons were of uniform diameter, with few branches, and parallel to each other in both the ascending and descending tracts (Fig. 3.1A,B). Sometimes an axon showed an isolated, single ovoid varicosity. Occasional fine axons had small, regularly spaced varicosities. Where the axons branched, the branches arose at right angles and turned laterally across the tract to reach terminal fields in the adjacent spinal grey matter. In some cases the entire axon turned laterally to leave the tract and enter the terminal field.

Figure 3.1

A: Axons in the normal adult rat corticospinal tract (CST) labelled with HRP and detected with the TMB method. Fine axon branches pass laterally (arrows) into the terminal fields in the adjacent spinal grey matter (gm). 60 μm frozen horizontal section.

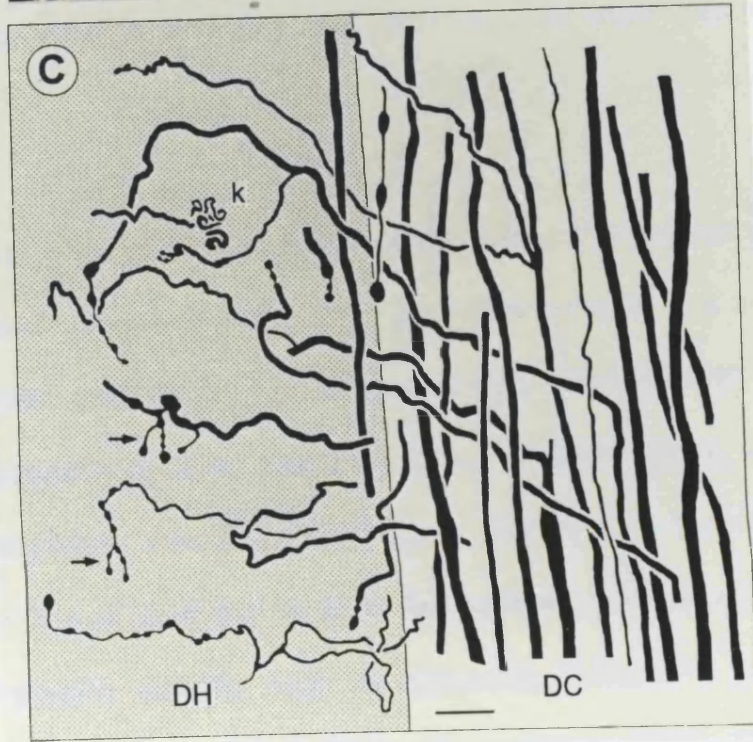
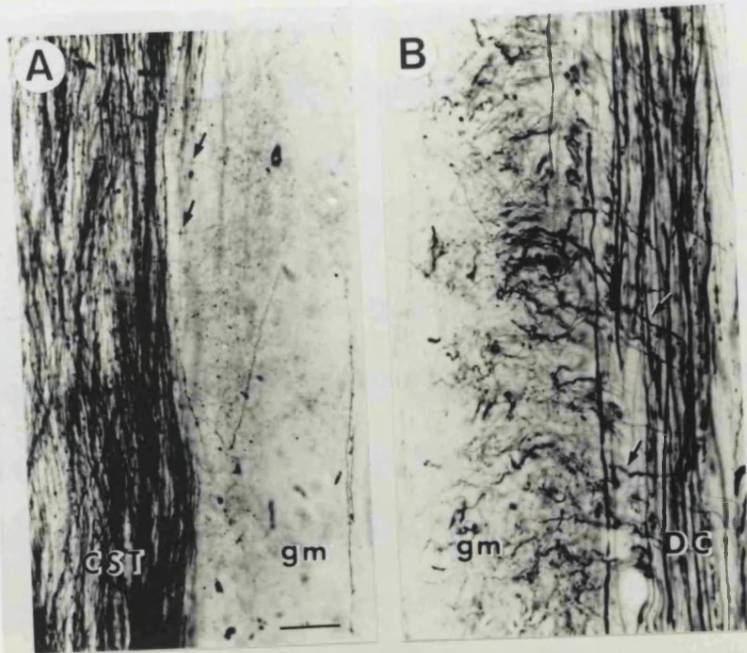
Scale bar = 100 μm .

B: Axons in the ascending sensory dorsal columns (DC) labelled with HRP and detected with the Co-GOD method. The branches of the axons (arrows) pass laterally and give rise to a dense terminal plexus in the adjacent grey matter of the dorsal horn. 60 μm frozen horizontal section.

Magnification as in A

C: Camera lucida drawing to illustrate the labelled axons of the ascending dorsal columns and their branches turning laterally into the grey matter of the dorsal horn (DH). In the terminal field, the axons have knot-like varicose segments (k), and fine terminal regions with bead-like varicosities, and caudally-directed dichotomous branches (arrows).

Scale bar = 25 μm .



3.3.1.2. Comparison of axons between the ascending and descending tracts In horizontal sections the diameters of the axons in the two tracts were obviously different, the ascending axons were generally thicker than the descending axons (Fig. 3.1A,B). There were a few large diameter axons in the descending corticospinal tract.

With the Palmgren silver stain the arrangement of the ascending and descending tracts could be distinguished on the coronal sections of the dorsal columns (Fig. 3.2). The differences in fibre diameter between the ascending and descending tracts were also clearly demonstrated in the coronal and sagittal sections (Fig. 3.3). The axons in the descending tracts were finer than those in the ascending tract and were weakly stained by the silver.

The preterminal parts of the corticospinal axons (Fig. 3.1A) generally left the tract in a caudo-lateral direction. Those terminating in the immediately adjacent medial part of the spinal grey matter bore a small cluster of rounded presynaptic boutons near their ends. In some cases, a subsidiary cluster of terminals was produced about 50-200 μm before the final termination. The preterminal parts of the ascending dorsal column axons also turned sharply laterally, with an ascending inclination, into the grey matter of the dorsal horn (Fig. 3.1B,C) where they could be very tortuous, with branches and varicosities. The finest branches frequently consisted of short dichotomous terminal segments directed caudally, and ending in a series of 2-3 small round presynaptic boutons.

Figure 3.2

Coronal cryostat section of the normal adult rat spinal cord stained with the Palmgren silver method to show the three tracts in the dorsal columns (separated by dashed lines): the ascending gracile tract (GT) and cuneate tract (CT), and the descending corticospinal tract (CST). Central canal (arrow).

Scale bar = 200 μm .

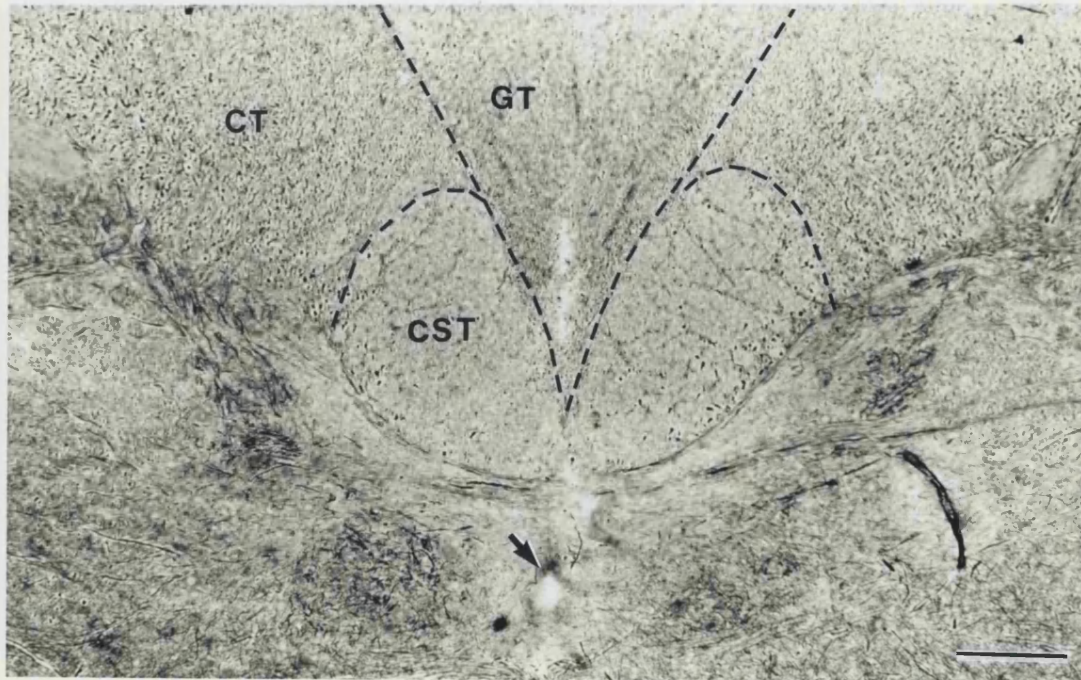
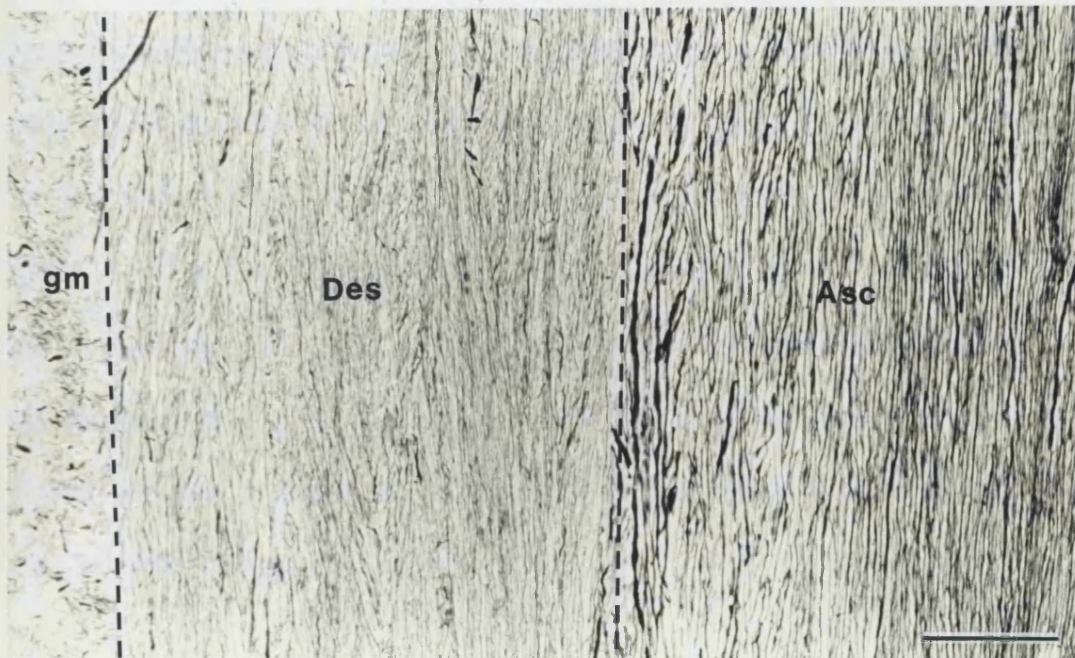


Figure 3.3

Sagittal cryostat section of the normal adult rat spinal cord stained with the Palmgren silver method to show the differences between the diameter of the axons of the ascending (Asc) and descending (Des) tracts (separated by dashed lines). gm, grey matter.

Scale bar = 100 μ m.



3.3.2. Glial framework

3.3.2.1. Interfascicular glial rows Since the tracts in the dorsal columns are almost completely neuron free, the cells stained by thionin on cryostat sections and by methylene blue on semithin sections were effectively all glial cells. In the horizontal and sagittal sections the cell bodies were assembled to form unicellular glial rows along the longitudinal axis of the tracts (Figs. 3.4B,5). The rows were unbranched, evenly placed and parallel to each other. Because it was not possible to cut sections which would include the full length of the glial rows the maximal length of the glial rows could not be determined. In the coronal cryostat sections the glial cell bodies formed an equidistant array (Fig. 3.4A).

Since the two tracts could be seen simultaneously in a single sagittal cryostat section, it was easy to compare the differences and arrangements of glial cells between the ascending and descending tracts. The glial rows of the descending corticospinal tract were closer together, longer and more compact with more glial cell bodies than in the ascending tracts, and the glial rows in the ascending tracts were more fragmented than the rows in the descending tract (Fig. 3.4B).

3.3.2.2. Astrocytes In the horizontal semithin sections, astrocytes could be identified by their large, pale nuclei, voluminous cytoplasm, and pale processes. The solitary astrocytes were interspersed singly at repeat stretches of about 4-8 oligodendrocytes in the glial rows of the descending

Figure 3.4

A: Spacing of the interfascicular glial rows shown on a thionin stained coronal cryostat section of the normal adult rat spinal cord. Spaces between cells are much smaller in the descending corticospinal tract (CST) than those in the ascending cuneate (CT) and gracile tracts (GT). Dashed lines separate the tracts; arrow points the central canal.

Scale bar = 200 μ m.

B: Thionin stained sagittal cryostat section of the normal adult rat spinal cord to show the interfascicular glial rows along the longitudinal axis of the spinal cord and the differences between of the glial rows of the ascending (Asc) tracts and descending (Des) (separated by dashed lines). The glial rows in the descending tract are longer and more compact with more glial cell bodies than in the ascending tracts, and the glial rows in the ascending tracts are more fragmented than the rows in the descending tract.

Scale bar = 50 μ m.

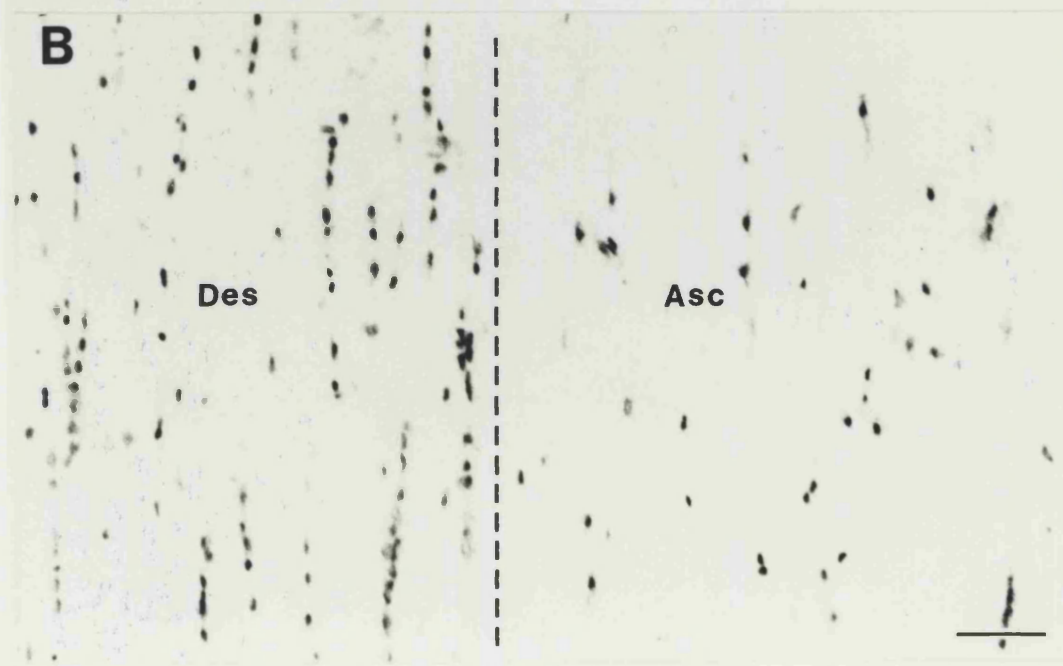
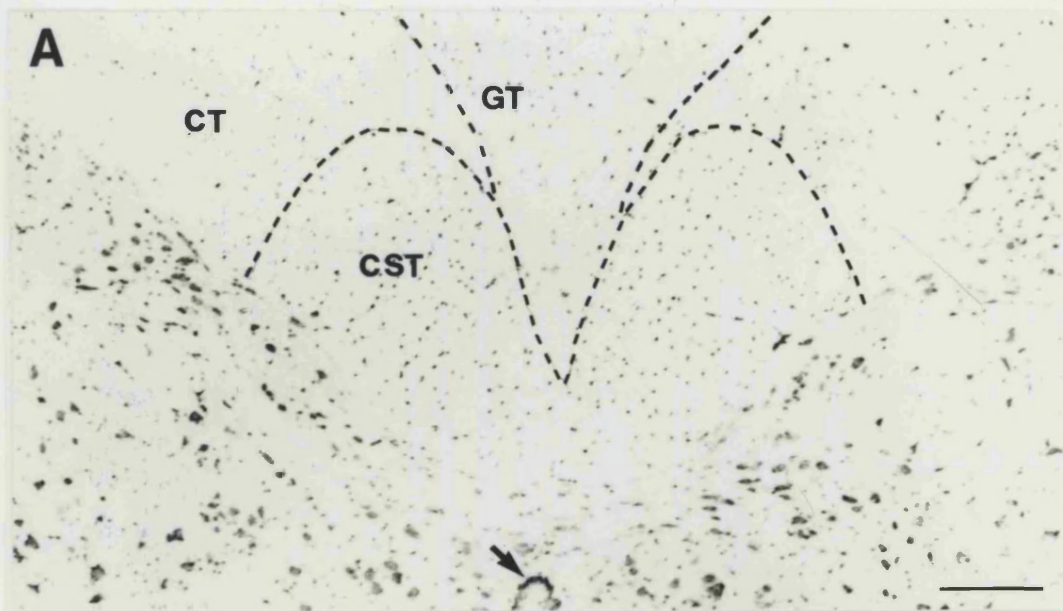
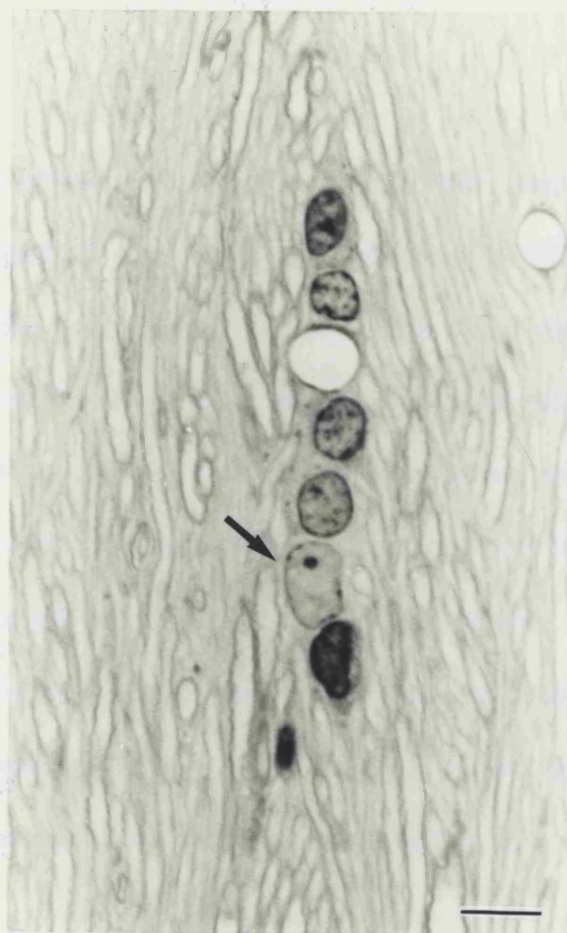


Figure 3.5

Semithin section to show an interfascicular glial row in the adult rat descending corticospinal tract. A solitary astrocyte with large, pale nucleus and voluminous cytoplasm (arrow) is located in a row of oligodendrocytes. Horizontal section stained with methylene blue.

Scale bar = 5 μm .



corticospinal tract (arrow in Fig. 3.5). This astrocytic arrangement was confirmed by GFAP staining in horizontal (Fig. 3.6) and sagittal cryostat sections.

GFAP staining also showed that astrocytes in both ascending tracts and descending corticospinal tract gave rise to two types of processes - longitudinal and radial processes (Fig. 3.6). The radial processes were the direct continuation of the cell body; they had a pyramidal shape tapering away from the cell body. The longitudinal processes arose from the cell body and from the radial processes, they exceeded the radial processes in number and had a different morphology from them. The longitudinal processes were straight, little branched, fine and untapering, and aligned along the longitudinal axis of the tracts. Thus the two types of astrocytic processes formed a rectilinear meshwork in the tracts.

The arrangement of astrocytes was similar in the ascending and descending tracts. The astrocytes in the descending corticospinal tracts were smaller with finer processes than those in the ascending tracts (Fig. 3.7).

3.3.2.3. Microglia The morphology and distribution of the microglia was shown with OX-42 immunostaining on the horizontal and sagittal cryostat sections. Some of the microglia were located in the interfascicular glial rows and some were dispersed singly among the tract axons at repeat

Figure 3.6

GFAP immunostained normal adult rat spinal cord to show the astrocytes (e.g. arrow) interspersed into the glial rows of the corticospinal tract. The astrocytes give rise to longitudinal and radial processes. Horizontal cryostat section, counter-stained with thionin.

Scale bar = 20 μm .

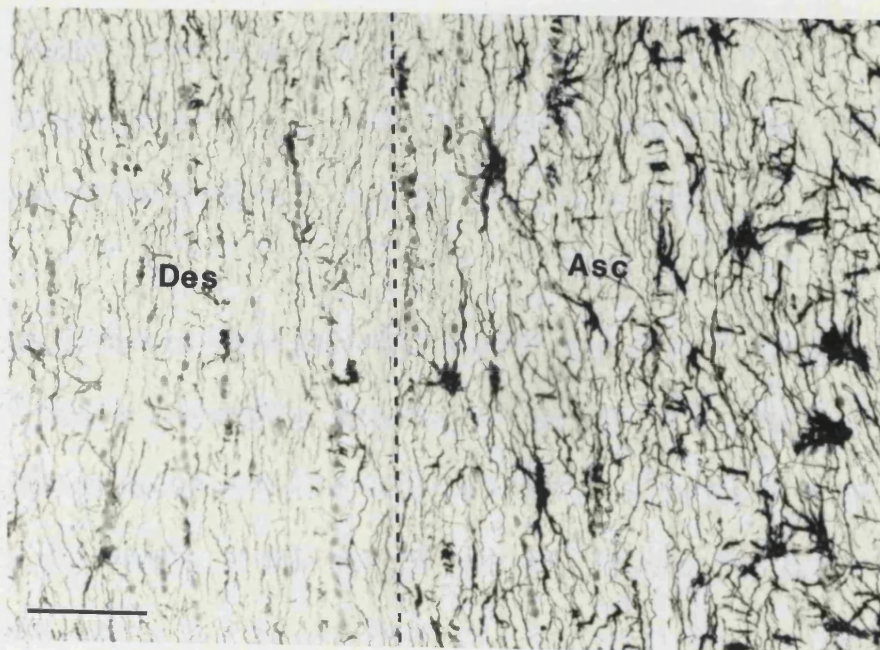


Figure 3.7

GFAP immunostained sagittal section of normal rat spinal cord to show the comparison of the astrocytes in the ascending and descending tracts (separated by dashed lines). The astrocytes in the descending corticospinal tracts (Des) are smaller with finer processes than those in the ascending tracts (Asc). Cryostat section, counter-stained with thionin.

Scale bar = 100 μm .

the same way as in the case of the other two, but here it is the *Des* cells that are the most numerous. The *Asc* cells are also present, but they are less numerous. The *Des* cells are the most numerous in the *Des* region, while the *Asc* cells are the most numerous in the *Asc* region. The *Des* cells are the most numerous in the *Des* region, while the *Asc* cells are the most numerous in the *Asc* region.



5.3.2.2. Comparison of the structure of the *Des* and *Asc* regions. The *Des* region is characterized by a more uniform, fibrous texture, while the *Asc* region is characterized by a more complex, cellular structure. The *Des* region is characterized by a more uniform, fibrous texture, while the *Asc* region is characterized by a more complex, cellular structure. The *Des* region is characterized by a more uniform, fibrous texture, while the *Asc* region is characterized by a more complex, cellular structure. The *Des* region is characterized by a more uniform, fibrous texture, while the *Asc* region is characterized by a more complex, cellular structure. The *Des* region is characterized by a more uniform, fibrous texture, while the *Asc* region is characterized by a more complex, cellular structure.

The results of the present investigation of the structure of the *Des* and *Asc* regions of the plant tissue are presented in the following figures. The results of the present investigation of the structure of the *Des* and *Asc* regions of the plant tissue are presented in the following figures. The results of the present investigation of the structure of the *Des* and *Asc* regions of the plant tissue are presented in the following figures. The results of the present investigation of the structure of the *Des* and *Asc* regions of the plant tissue are presented in the following figures.

stretches of about 3-5 oligodendrocytes in the glial rows of the descending corticospinal tracts. The cell bodies of the microglia were curved and their nuclei were smaller than those of the astrocytes. Their processes were thick and irregularly varicose and spiny, and radiated out from the cell body in different directions, but most were longitudinal (Fig. 3.8) and were shorter than those of the other types of glial cells in the tracts.

The arrangement of microglia in the ascending and descending tracts were easier to see in the sagittal than the horizontal plane. Unlike astrocytes, which differed in size in the ascending and descending tracts, there were no obvious differences in the size of the microglia in the ascending and descending tracts, but the processes of microglia in the corticospinal tract were longer and more uniform than in the ascending tracts.

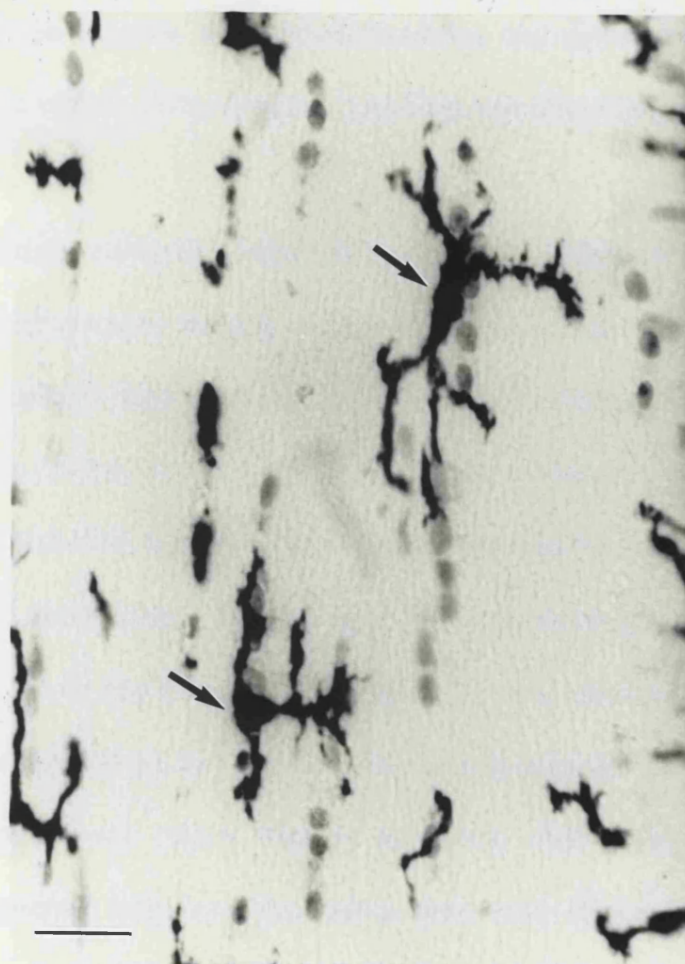
3.3.2.4. Comparison of interfascicular glial rows in the ascending and descending tracts The glial framework of the large diameter axon ascending tracts in the dorsal columns was clearly of the same pattern as that in the adjacent, smaller diameter axon descending corticospinal tracts. However, the spacing of the glial rows was much looser in the large diameter tracts (Fig. 3.4), and the sizes and packing density of the glial cells was different.

To make a full numerical assessment of these parameters is beyond the scope of the present investigation. It would require sampling methods (e.g. the dissector method of Coggeshall; Harding *et al.*, 1994; Pover *et al.*,

Figure 3.8

Horizontal cryostat section of adult rat spinal cord immunostained with OX-42 to show that the microglia (arrows) are located in the interfascicular glial rows. Their processes are thick and irregular radiating out from the cell body in different directions, but most are longitudinal and are shorter than those of other types of glial cells in the tracts.

Scale bar = 20 μm .



1993) to overcome the bias due to the differences in cell size, and cell orientation between the different cell types and tracts. However, the following sample counts and measurements are given in order to put numbers to the visible differences in packing density seen in the sections.

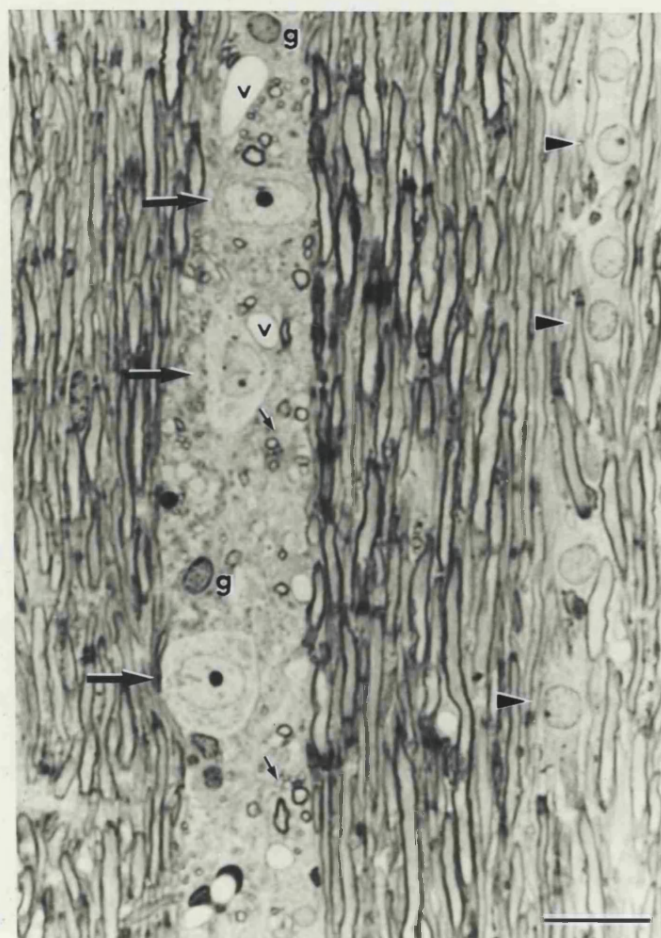
These numerical samples indicated (1) the distance between the interfascicular glial rows (sagittal thionin sections) in the sensory tracts was about $64\text{ }\mu\text{m}$, almost double that of $37\text{ }\mu\text{m}$ in the corticospinal tract, (2) the packing density of the OX-42-positive microglia in the ascending tracts was 87 cells/mm^2 , and 104 cells/mm^2 in the corticospinal tract. The intercellular spacing of $106\text{ }\mu\text{m}$ between the microglia in the ascending tracts was similar to that of $85\text{ }\mu\text{m}$ in the corticospinal tract, (3) the packing density of the GFAP-positive astrocytes in the ascending tracts was 30 cells/mm^2 , that in the corticospinal tract much higher, at about 153 cells/mm^2 , and the intercellular spacing between the astrocytes was about $266\text{ }\mu\text{m}$ in the ascending tracts and $64\text{ }\mu\text{m}$ (reflecting the much higher packing density) in the corticospinal tract, and (4) the overall glial cell density (thionin stain) in the sensory tracts was 521 cells/mm^2 , almost half that of 1123 cells/mm^2 in the corticospinal tract.

3.3.2.5. Midline tissue The midline of the dorsal columns was occupied by a very characteristic densely packed tissue, about $25\text{ }\mu\text{m}$ wide (Fig. 3.9). This tissue contains a cell type resembling undifferentiated glia (with rather dense nuclei), not stained by GFAP or OX-42. In addition there are numbers

Figure 3.9

Semithin section of the midline tissue of the adult rat dorsal columns. Small neurons (large arrows) have pale vesicular nuclei, with prominent nucleoli and substantial cytoplasm with Nissl bodies. The glial cells (g) are small and undifferentiated. Compared with the adjacent myelinated fibre tracts, there are many more micro-vessels (v). Small arrows indicate small clusters of very fine, myelinated axons. An adjacent interfascicular glial row (arrowheads) is included for comparison.

Scale bar = 20 μm .



of small, solitary neurons, identified by typical neuronal characteristics in semithin sections (pale, vesicular nuclei, with prominent nucleoli, and substantial cytoplasm with Nissl bodies) with synapses in electron micrographs. The tissue also contains numbers of very small, myelinated axons and is clearly more densely vascularised by micro-vessels than the adjacent white matter tracts.

3.4. Discussion

3.4.1. Axons

The present study confirmed that axons in the descending corticospinal tracts are more densely packed than those in the ascending fibres and much finer although there are a small number of large diameter fibres. The ascending tracts have much larger diameter fibres.

3.4.2. Interfascicular glial rows

Many studies (e.g. Bayer and Altman, 1975; Bunge, 1968; Lin and Connor, 1989; Matthews and Duncan, 1971) have illustrated the longitudinal rows of interfascicular oligodendrocytes in central white matter tracts. The study on the fimbria (Suzuki and Raisman, 1992) further illustrated that the interfascicular rows contain all three types of glial cells, in a series of regular, repeating units consisting of stretches of closely packed interfascicular oligodendrocytes separated by solitary astrocytes, and microglia. The present study shows that, as in the fimbria, the glial cells in

the dorsal columns form interfascicular rows along the longitudinal axis of the spinal cord similar to those in the fimbria (Fig. 3.10). The glial rows in the ascending tracts are less uniform and appeared more fragmented than in the descending tracts. This could be due to the ingrowth of fascicles of axons from the dorsal roots breaking up the longitudinal bundles. The spacing between the interfascicular glial rows seems to be related to the diameter of the axons, since in the small axon diameter tract the rows are spaced much closer than in the larger axon diameter tract.

3.4.3. Astrocytes

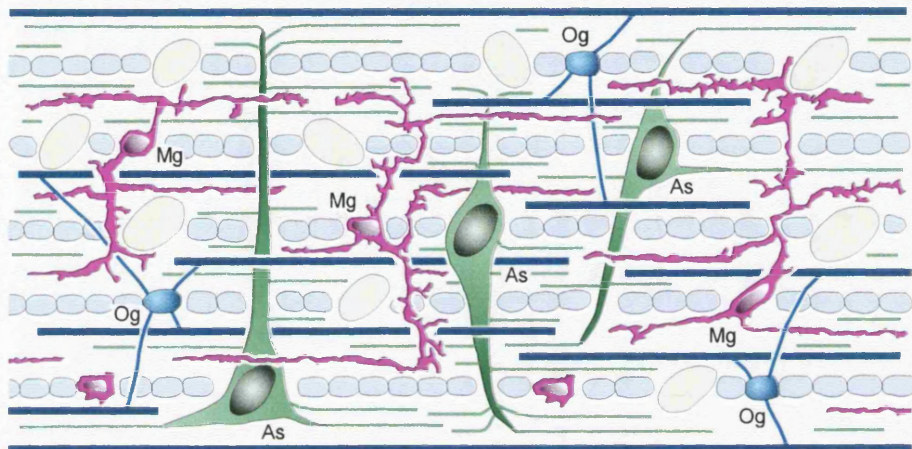
The overall astrocytic arrangements of the ascending and descending tracts are similar. The diameter of the processes and size of the cell bodies in the ascending tracts are larger than in the descending tract. The astrocytes seem to be highly responsive to the axonal size: in the smaller diameter axon corticospinal tract the astrocytes were both more numerous and also more densely packed. Although the present study does not include a strict quantitative calculation of the total numbers of axons, it seems possible that the astrocyte/axon ratios might be held more constant between the two tracts, i.e. compared with the sensory tracts, the increased numbers and density of astrocytes in the corticospinal tract could reflect the increased numbers and density of axons.

3.4.4. Microglia

OX-42 immunostaining shows that, in contrast to the astrocytes there is no obvious differences between the size of the microglia in the two tracts. The

Figure 3.10

A schematic diagram showing the arrangement of astrocytes (green, As), oligodendrocytes (blue, Og), and microglia (magenta, Mg) as found in the normal ascending and descending white matter tracts of the spinal dorsal columns. The glial cell bodies are arranged in unicellular rows. The astrocytes lie singly, evenly spaced from each other, the microglia are similarly spaced, but the oligodendrocytes lie in contiguous rows of around 10 cells. All three glial cell types generate processes which enter into the longitudinal axis of the tract.



processes in the corticospinal tract are more regular and longer than in the ascending tracts which could be due to the fact that the glial rows in the ascending tracts are more fragmented. In general, the microglia possess several long processes running along the longitudinal axis of the spinal cord as described by Lawson *et al* (1990) in the mouse. The uniformity of the cell density between the two tracts suggests that the microglia are insensitive to the differences in fibre size.

3.4.5. Midline tissue

This is the first description of a complex, organised tissue in the adult spinal midline. The midline tissue of the dorsal columns has an undifferentiated type of glial cells, not stained with GFAP or OX-42. They are probably derived from the embryonic roof plate (Snow *et al.*, 1990). They may be comparable to the midline glia of the optic chiasma described by Marcus *et al* (1995), and thought to be involved in the selective repulsion of the ipsilateral (non-crossing) retinal ganglion cell axons.

A transient population of midline neurons has been described in the optic chiasma, where they are among the earliest forming neurons in the diencephalon (Guillery *et al.*, 1995; Sretavan *et al.*, 1995). These neurons seem to play a decisive role in the subsequent patterning of axons, since their destruction prevents crossing of the retinal axons. While the dorsal midline neurons are permanent in the adult spinal cord, they may also have an important role for developmental patterning of axon pathways.

3.5. Summary

The dorsal columns in the rat can be divided into the ascending cuneate and gracile tracts and descending corticospinal tract. The structure of the normal rat dorsal columns was studied by extracellular HRP labelling, immunostaining of GFAP, OX-42 and the Palmgren silver method.

The axons in the ascending and descending tracts were straight, unbranched and parallel to each other, but are different in diameter.

As in the fimbria, the glial cells in the two tracts form interfascicular rows which run along the longitudinal axis of the spinal cord. There were more cells in the glial rows of the descending corticospinal tract than in the ascending tracts and the glial rows in the ascending tracts were more fragmented than the rows in the descending tract. The processes and cell bodies of the astrocytes in the descending corticospinal tract were finer and smaller than in the ascending cuneate and gracile tracts.

In contrast to the astrocytes, the arrangement and size of the microglia in all tracts were similar but the processes in the corticospinal tract were more regular and longer than those in the ascending tracts.

**CHAPTER IV AXON GROWTH FROM EMBRYONIC
HIPPOCAMPAL NEURONS TRANSPLANTED INTO THE DORSAL
COLUMN TRACTS OF THE ADULT RAT SPINAL CORD**

4.1. Introduction

It has been suggested that adult tracts in the central nervous system are non-permissive for the regrowth of cut adult axons. Schwab and colleagues (Schnell and Schwab, 1990; Schwab *et al.*, 1993) have proposed that this is due to an inhibitory molecule which is associated with mature oligodendrocytes present in myelinated fibre tracts of the adult mammalian central nervous system. However, there are several recent studies showing that transplanted embryonic donor neurons are able to grow long axons in adult myelinated fibre tracts in a number of different sites in the brain and spinal cord (Fujii, 1989; 1991; Strömberg *et al.*, 1992; Wictorin *et al.*, 1990a; 1992; Wictorin and Björklund, 1992).

The micro-transplantation technique (Emmett *et al.*, 1990) which has been developed in our laboratory causes minimal damage to the host tracts and enables small quantities of donor cell suspension to be injected into precisely defined sites. By transplanting mouse embryonic neurons into adult rat brain, the donor neurons and their axonal projections can be identified by species-specific murine allelic markers such as Thy-1.1 (Davies *et al.*, 1993) or M6 (Lund *et al.*, 1985; Wictorin *et al.*, 1991). Embryonic mouse hippocampal neurons micro-transplanted into the rat fimbria (Davies *et al.*, 1993) produced a beam of rapidly elongating axons which followed a course determined by the longitudinal axis of the host tract glia and axons (Davies *et al.*, 1993; Suzuki and Raisman, 1992). Recently, Davies and colleagues

showed that axons from the same type of hippocampal donor cells grew equally effectively when they were micro-injected into host tracts such as the corpus callosum, through which hippocampal axons never normally travel (Davies *et al.*, 1994). Observations from a number of other types of intentionally mismatched transplants (e.g. superior colliculus into corpus callosum) have also confirmed that - unlike denervated terminals fields, which are only permissive for reinnervation by correctly matched specific inputs (Zhou *et al.*, 1985, 1989) - central fibre tracts are permissive for the growth of axons from transplanted embryonic neurons obtained from regions which do not project through those tracts in the normal adult brain.

I have used the mouse-to-rat intra-tract micro-transplantation approach to investigate whether the long motor and sensory myelinated fibre tracts of the adult rat spinal cord can also provide a permissive environment for interfascicular axon growth, and whether axons from mismatched embryonic hippocampal donor tissue can grow in the spinal cord.

Can the donor axons still recognize their correct targets? Would they be stopped or deflected by introducing appropriate targets? To investigate this, a second transplant, consisting of rat embryonic hippocampal tissue was introduced as a target into the spinal cord.¹ This experimental design is able to test whether the proximity of 'correct' (i.e. hippocampal) targets can

¹ In a previous study, I have shown that, in organotypic slice co-culture (Li *et al.*, 1993), correct axon-target specificity is preserved in cross species mouse-rat confrontations.

(a) prevent the growing intrafascicular hippocampal axons from elongating further in the host tract, or (b) deflect them or make them produce collaterals (O'Leary and Terashima, 1988).

4.2. Materials and methods

4.2.1. Cells for transplantation

Donor cells were obtained from E14 or E18 mouse hippocampi, or E16 rat hippocampi. The cell suspension was made at a final concentration 1×10^7 cells/ml. 0.5-1.0 μ l of cell suspension (approximately 5,000-10,000 mouse or rat hippocampal cells, which would include neurons and also glia and precursors) was micro-transplanted into the ascending or descending tracts of the dorsal columns of adult AS rat spinal cords by the pulsed air pressure injection system.

4.2.2. Transplantation

4.2.2.1. Single transplants 10 rats received E14 mouse donor cells in the ascending tracts with survival times of 7 days ($n=3$), 10 days ($n=4$) and 14 days ($n=3$). 26 animals received E14 donor cells in the descending corticospinal tracts with survival times of 2 days ($n=3$), 3 days ($n=3$), 6 days ($n=4$), 10 days ($n=4$), 14 days ($n=4$), 21 days ($n=3$), 43 days ($n=2$) and 48 days ($n=3$). 5 animals received E18 mouse donor cells (2 for the ascending and 3 for the descending tracts) and with a survival time of 8 days. 5 animals received E16 rat donor cells (as a control for M6

immunostaining, 2 for the ascending and 3 for the descending tracts) and with survival times of 6-8 days.

4.2.2.2. Double transplants 10 rats received an E14 embryonic mouse and also an E16 embryonic rat transplant in the descending corticospinal tract on the same side of the spinal cord (the rat neurons and their axons would not stain with the mouse specific marker M6, and therefore would not interfere with the identification of the M6-positive mouse donor axons). The rat transplant was placed 1-2 mm caudal to the mouse transplant. The operated animals were sacrificed at 6 days (n=4), 8 days (n=3) and 22 days (n=3). 4 animals received a mouse transplant on one side and a rat transplant on the other side and were allowed to survive for 8 days.

4.2.3. Staining

10 μ m horizontal cryostat sections were used for thionin staining and Py immunostaining to identify transplanted hippocampal CA3 pyramidal cells and M6 immunostaining was used to identify the axonal growth from the mouse transplants.

4.2.4. HRP retrograde labelling

HRP was injected into the dorsal columns 0.5-0.7 mm below the dorsal surface and 2-3 mm caudal to the E14 mouse hippocampal transplant 2-4 weeks after transplantation (5 animals, n=3 at 2 weeks, n=2 at 4 weeks)

to confirm the axonal projections from the transplant. 60 μm horizontal sections were cut and the retrograde perikaryal HRP was detected with either the TMB or the Co-GOD method. The TMB detection method was more sensitive, but because of its smaller crystals the Co-GOD method gave better morphology.

4.2.5. Semithin sections

4 animals with E14 mouse transplants were processed for semithin sections to show more detail in the transplants. The sections were cut in the horizontal plane and stained with a mixture of methylene blue and Azur II.

4.3. Results

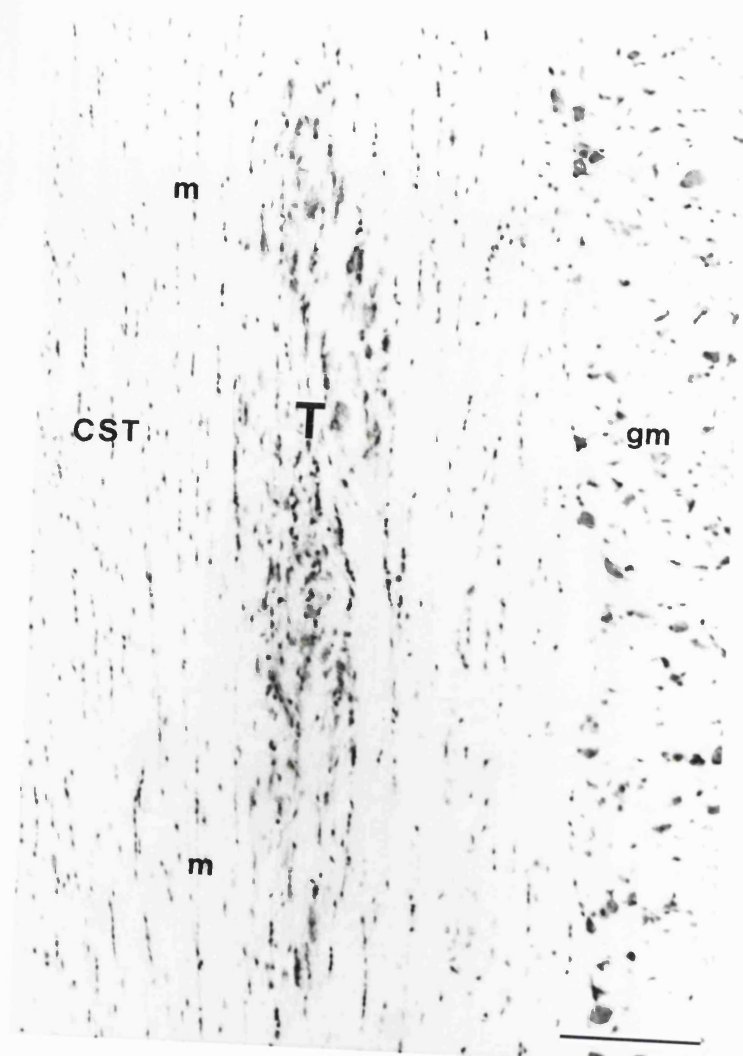
4.3.1. Survival and morphology of the transplants

Thionin staining Sections stained with thionin showed that the transplants formed spindle-shaped compact masses, about 1 mm in length and 0.3-0.5 mm wide, and elongated along the long axis of the host ascending and descending spinal tracts (Fig. 4.1). The transplants contained different sizes of cells from large hippocampal neurons to small glia within the transplant. There was little sign of host tissue damage surrounding the transplants. The appearance of the transplants changed little over survival times from 6 to 48 days.

Figure 4.1

An intraspinal spindle-shaped transplant (T) of E14 mouse hippocampal cells in the corticospinal tract (CST). m, midline; gm, grey matter. Horizontal cryostat section, thionin stain. Survival time, 6 days.

Scale bar = 200 μ m.



Semithin sections Semithin sections (Figs. 4.2,3) showed that transplanted embryonic cells contained well differentiated neurons with dendrites ramifying into a compact neuropil. The transplants were densely vascularised and flanked by an interconnecting system of prominent, wide-diameter blood vessels. The host myelinated fibre bundles were minimally disorganised at the transplant site. The transplanted hippocampal cells made direct contact with the axons of the host tract. The host myelinated axons ran through the transplant readily penetrating between the neurons of the transplant mass.

Py immunostaining Py is a monoclonal antibody that selectively stains a cytoplasmic neurofibrillary component in the hippocampal CA3 pyramids (Woodhams *et al.*, 1989). 5 animals at survivals of 43 days and 48 days were used for Py immunostaining. The horizontal cryostat sections showed that many CA3 pyramidal-like neurons survived well in the transplants in the myelinated adult spinal tracts. Scattered through the transplant, the majority of the large donor neurons had abundant, filamentous Py-positive material in the cytoplasm of the somata and main dendrites (Fig. 4.4), thus resembling normal adult hippocampal CA3 pyramids in situ.

4.3.2. Donor axonal projections in the tracts

4.3.2.1. M6 immunohistochemistry

Immunostaining The embryonic mouse transplants were intensely stained with M6 antibody and at low power had an overall appearance of a dark

Figure 4.2

Semithin section stained with methylene blue and Azur II to show an E18 mouse hippocampal transplant (T) in the corticospinal tract (CST). The transplant has good contact with the host tract and is densely vascularised. Arrows, close contact with undisturbed myelinated fibres of the host corticospinal tract. Horizontal section. Survival time, 38 days.

Scale bar = 200 μm .

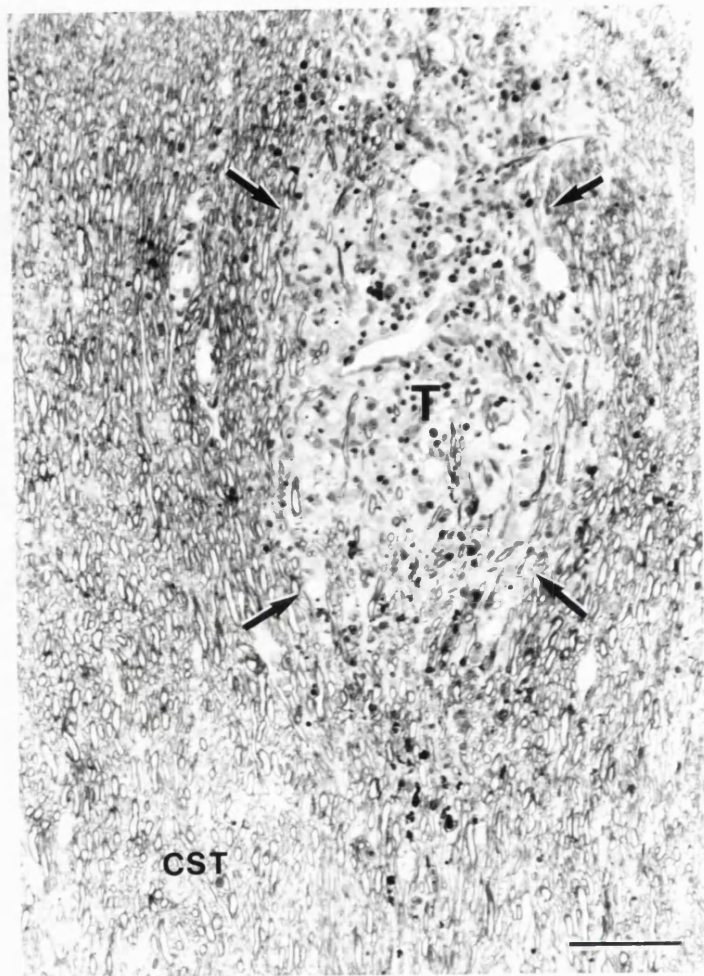


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Scale bar = 200 μm .

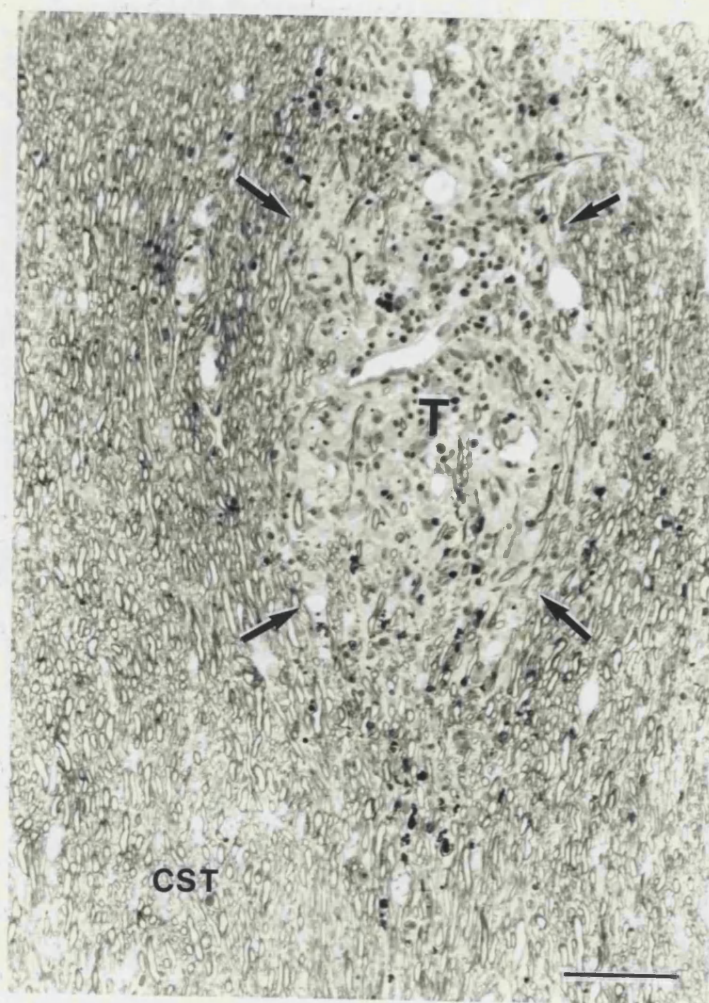


Figure 4.3

Higher magnification of a horizontal semithin section to show that the transplanted embryonic hippocampal cells contain well differentiated neurons and host myelinated axons (arrows) running through the transplant. Survival time, 38 days.

Scale bar = 10 μm .

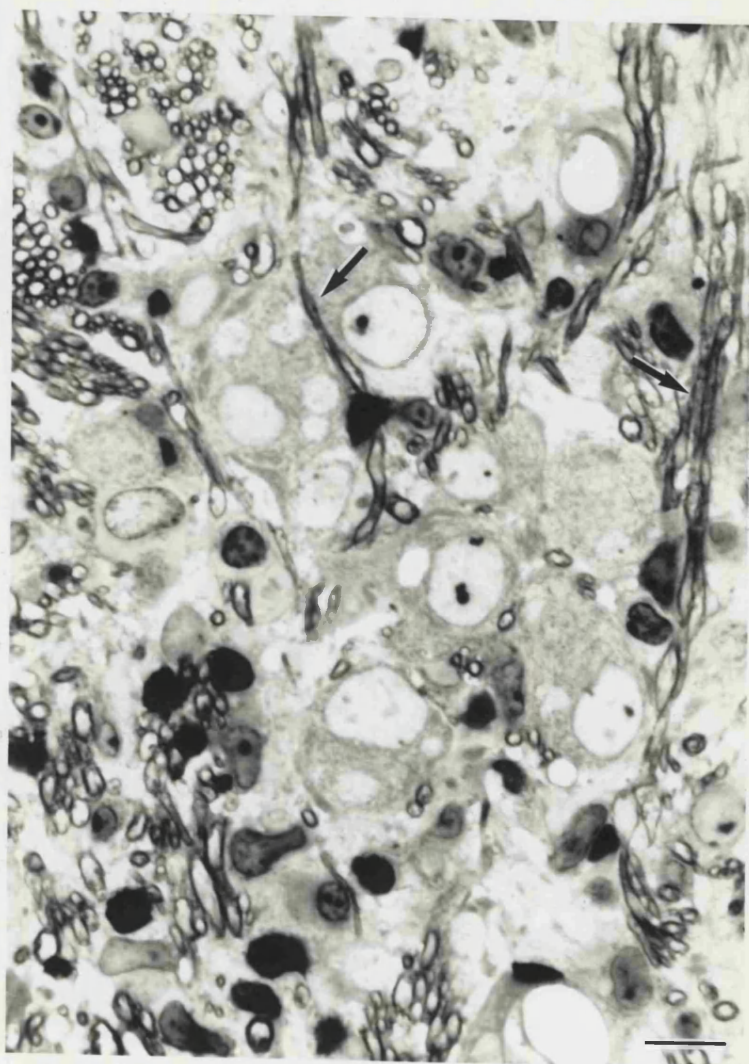


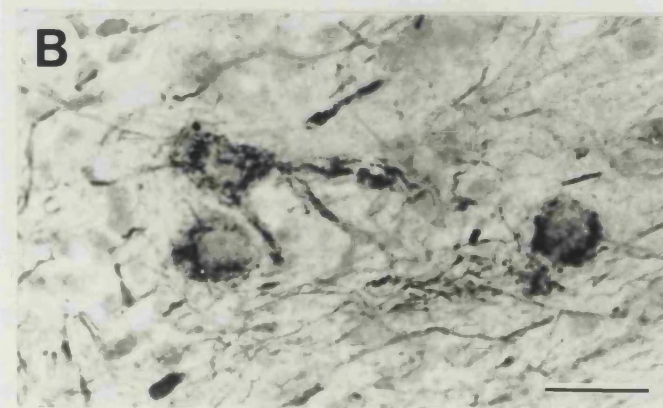
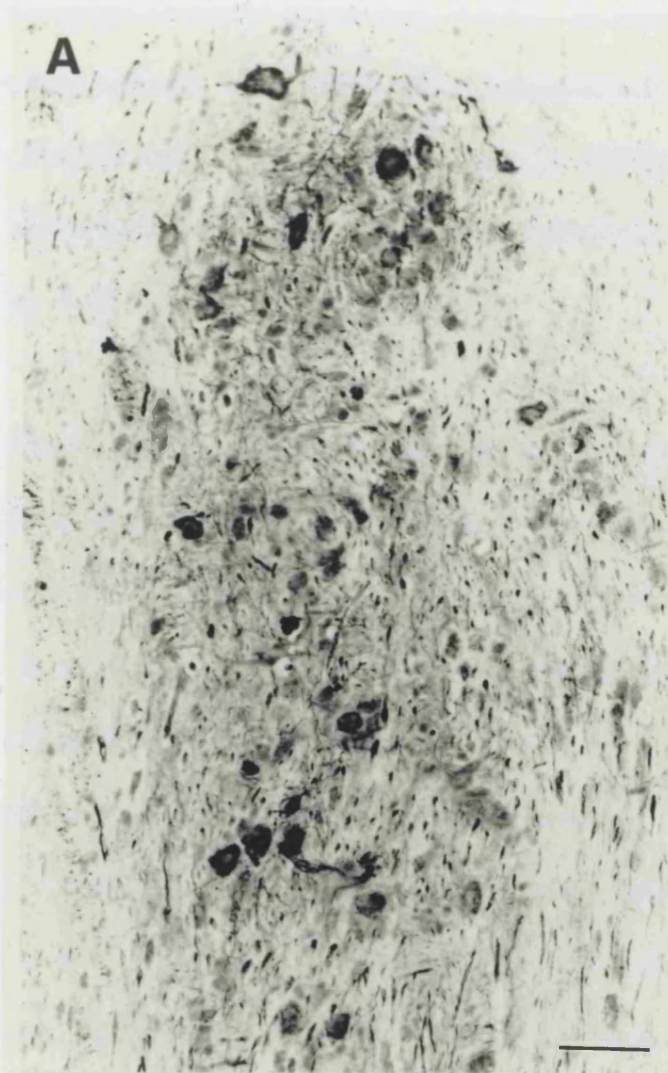
Figure 4.4

A: An intraspinal transplant of E14 mouse hippocampal cells with large numbers of large, CA3-like neurons stained by the Py monoclonal marker for CA3 pyramids.

Scale bar = 50 μ m.

B: High power view of three large Py-positive cells. Horizontal cryostat section. Survival time, 43 days.

Scale bar = 25 μ m.



brown spindle-shaped compact mass (Fig. 4.5A). In control experiments when E16 rat hippocampal cells were micro-transplanted into host spinal tracts no M6-positive cells or axons were present, although with thionin staining the transplants showed a similar morphology to the mouse transplants (Fig. 4.6).

Axon growth The embryonic mouse transplants gave rise to abundant M6-positive axonal projections into both the host ascending and descending spinal cord tracts. There were no qualitative nor quantitative differences between the axon growth from E14 transplants and that from E18 transplants in either the ascending or descending tracts. M6-positive axonal projections could be seen as they left the transplants. The projections separated into individual axons (Figs. 4.5,7) caudal and rostral to the transplants in the tracts. As they extended, the axons showed no tendency to fasciculate with each other. They were straight, uniform and untapering, about 1 μm in diameter. The axons were parallel to each other, intermingled with and in direct contact with the host axons, and parallel to the rows of host interfascicular glial cell bodies (Fig. 4.7B). Occasional collaterals were emitted at right angles to the main axonal stems. The collaterals could be traced for about 10-20 μm , but were too few to assess whether they were selectively directed towards specific host target structures.

Figure 4.5

A: M6-positive mouse E14 hippocampal transplant (T) with axonal projections in the corticospinal tract (CST). gm, grey matter; m, midline.

Scale bar = 250 μm .

B: M6-positive axons leaving the caudal pole of the transplant (open arrows indicate the same position in A and B). Horizontal cryostat section, counter-stained with thionin. Survival time, 20 days.

Scale bar = 100 μm .

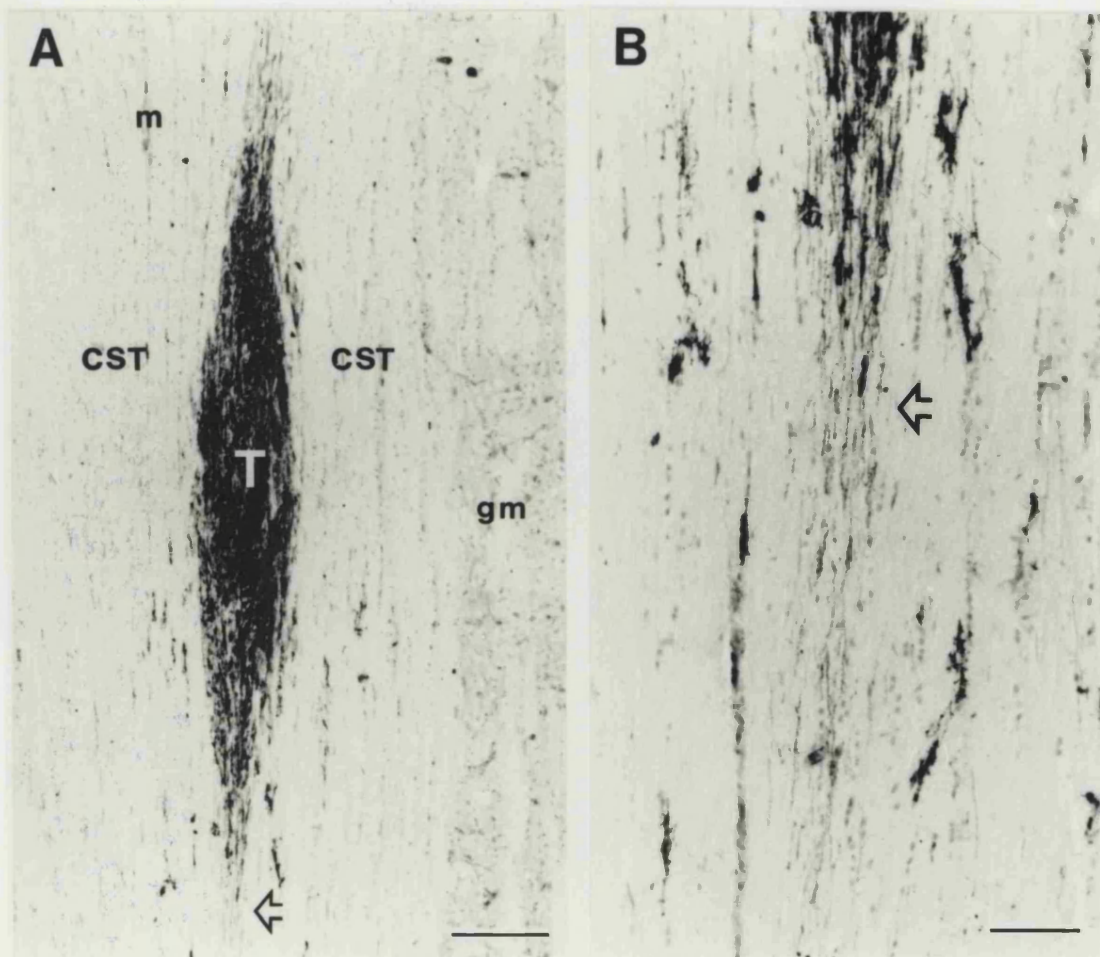


Figure 4.6

Control, M6-negative E16 rat hippocampal transplant (R) in the corticospinal tract (CST). gm, grey matter; m, midline. Horizontal cryostat section. Survival time, 6 days.

Scale bar = 200 μ m.

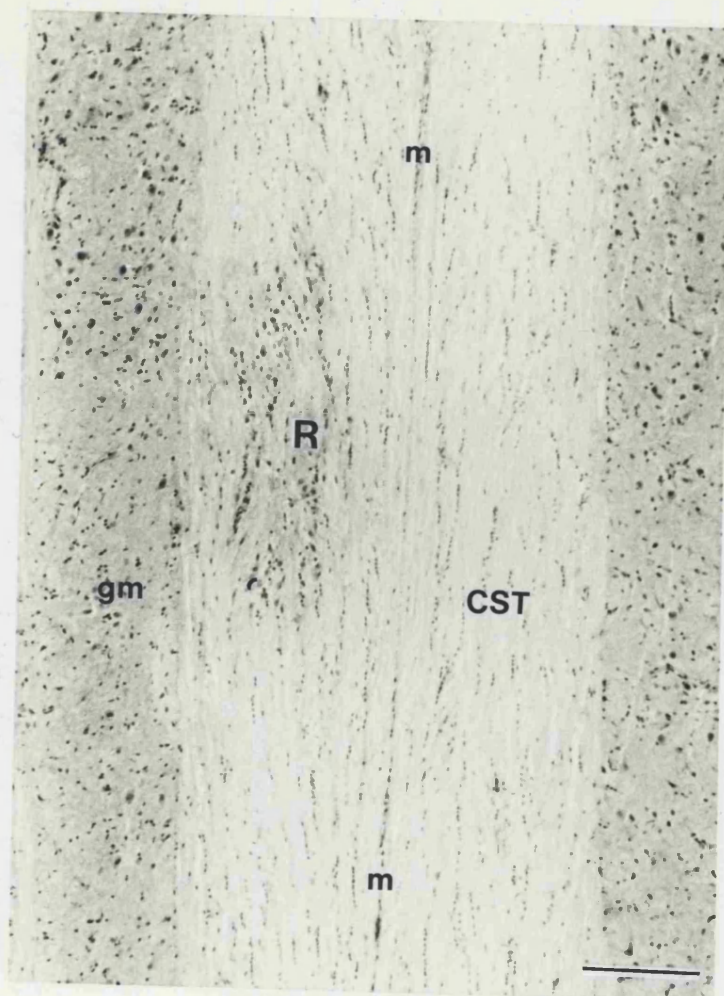


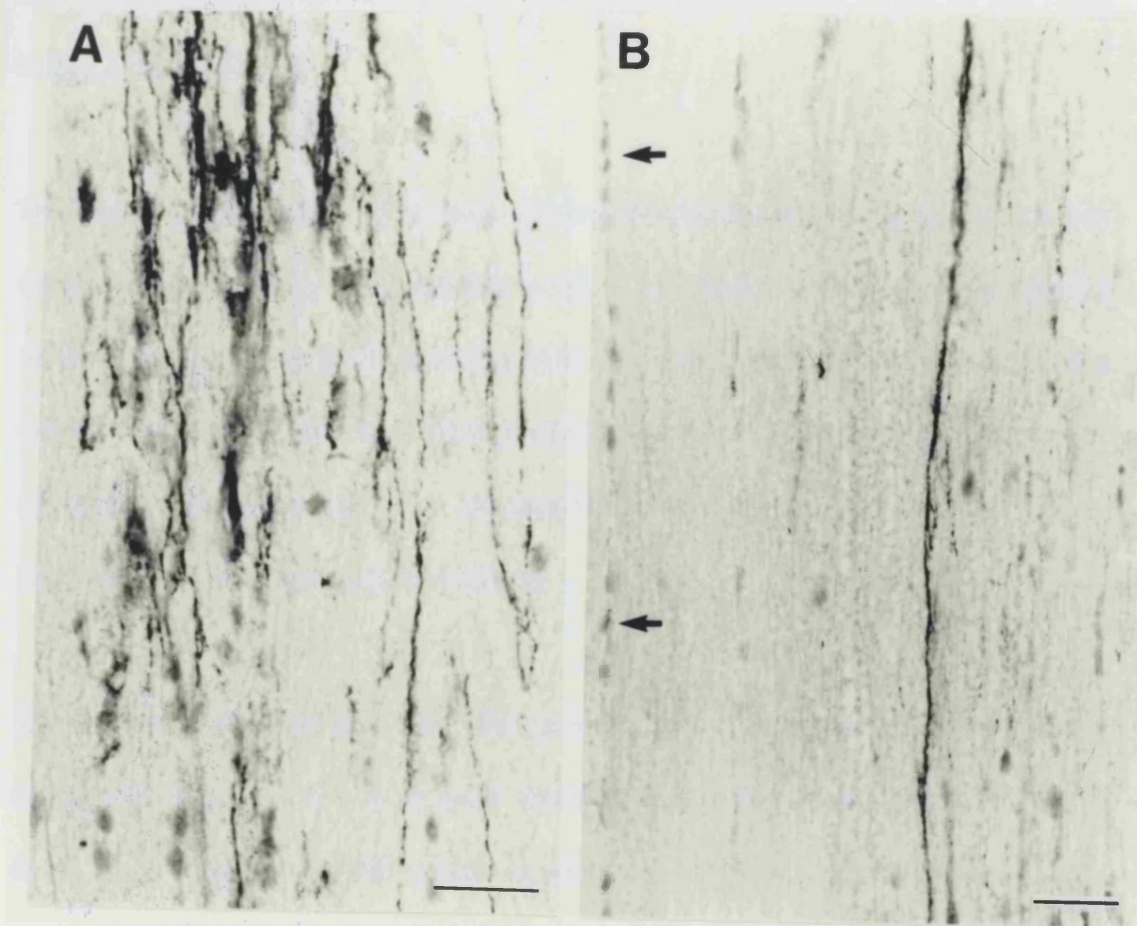
Figure 4.7

A: M6 immunostained loose fascicle of axons caudal to an E14 hippocampal transplant. The axons do not tend to fasciculate with each other, they are parallel to each other and intermingled with host tract axons. Survival time, 20 days.

Scale bar = 25 μ m.

B: Single M6-positive donor axon running parallel to the host axons (seen as faint background striation) and an aligned interfascicular glial row (arrows) in the corticospinal tract. Horizontal cryostat section, counterstained with thionin. Survival time, 20 days.

Scale bar = 25 μ m.



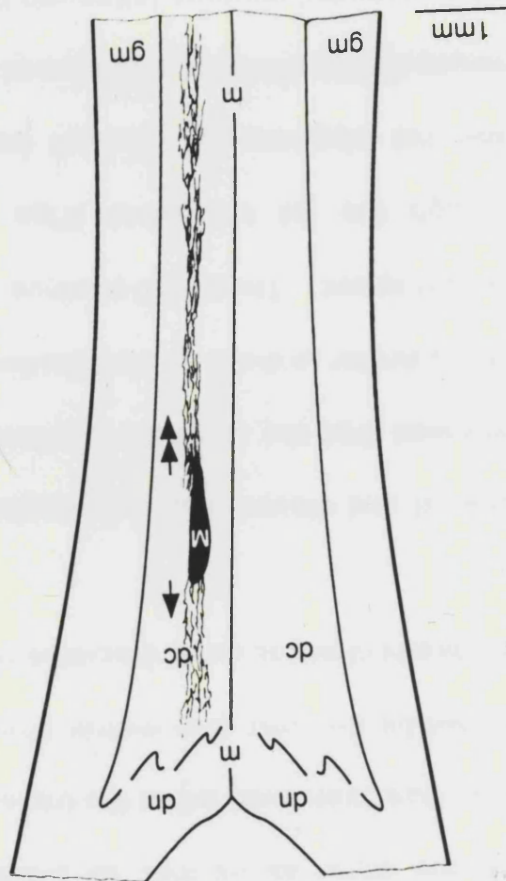
The donor axons projected in approximately equal numbers in both rostral and caudal directions (Figs. 4.5,8). The caudal projections could be traced further, since at the high cervical position of the transplants the rostrally directed projection fibres had already reached the rostral limits of the host tracts (Fig. 4.8).

The transplanted donor cells were either in the host ascending dorsal column tracts or descending corticospinal tract. The transplants projected parallel 'beams' of axons in both directions in the same tract. The extent of the rostral and caudal donor axonal projections were the same regardless of whether they followed the ascending host dorsal column axons or descending host corticospinal axons.

Time course of axon growth The axon growth from transplanted mouse embryonic neurons could be seen from 2 days after transplantation. By 6 days large numbers of M6-positive axons had grown into the host spinal tracts. The axons appeared to all grow at the same speed and the leading edge of the wave of growing axons advanced at a rate of up to 0.5 mm per day for the first 6-10 days, after which time they slowed down. At longer survival times, the numbers of M6-positive axons became less (as the M6 immunostaining was down-regulated). They reached a maximum distance of around 8 mm from the transplants by 6-7 weeks (the longest time examined).

Figure 4.8

Camera lucida drawing of a mouse E14 hippocampal transplant (M) which gives rise to M6-positive axonal projections passing rostrally (single headed arrow), and caudally (double headed arrow). dc, dorsal columns; dn, dorsal column nuclei; gm, grey matter; m, midline. Survival time, 20 days.



Termination The present M6 material does not give a definitive impression of the formation of terminal fields. Where the transplants lay in the grey matter, or at the junction of white and grey matter, the grey matter surrounding the transplant was also diffusely M6-positive (asterisks in Fig. 4.9A), suggesting that the donor axons may be breaking up into a fine preterminal arborisation. However, even when the transplanted neurons lay almost entirely surrounded by the host grey matter (Fig. 4.9), major donor long axonal projections clearly chose to run in fascicles of host white matter.

In the material with the rat and mouse double transplants (Fig. 4.10), the M6 immunostaining showed that the mouse transplants gave rise to M6-positive axonal projections similar to the axonal projections from the material with the single mouse transplant. These M6-positive mouse axons grew directly into and through the rat transplant (Figs. 4.11,12A). The transplanted embryonic rat hippocampal neuropil became M6-positive, suggesting that the mouse donor axons have produced a very fine terminal plexus around the rat hippocampal neurons (although this was beyond the limits of resolution of the present material). In every case, however, considerable numbers of large, unbranched M6-positive mouse donor axons passed directly through the embryonic rat hippocampal transplant, with no sign of branching. These axons continued to run caudally in the host spinal tract for long distances beyond the rat hippocampal transplant (Fig. 4.11). There was no indication that these caudal projections were less dense or less extensive after having passed through the rat transplant than those which travelled unhindered down the host spinal tract in the rats with a

Figure 4.9

A: An E14 mouse hippocampal transplant (T), which is surrounded by a halo of diffuse, terminal field-like staining (asterisks) in the host spinal grey matter (gm), generates a major rostrally directed projection of long axons which follow, and are intermingled with a curving bundle of host axons (open arrow) passing through the grey matter. (The small arrows indicate examples of comparable, but smaller host axon bundles running through the grey matter). wm, white matter of the host dorsal columns.

Scale bar = 250 μm .

B: Enlargement of the area (indicated by the open arrow) in A. Horizontal cryostat section, counter-stained with thionin. Survival time, 6 days.

Scale bar = 100 μm .

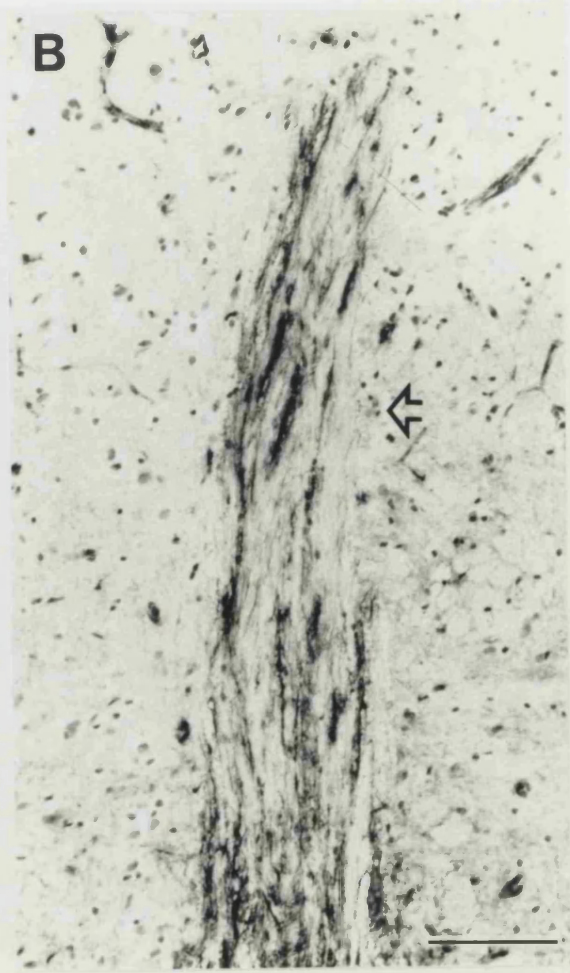


Figure 4.10

Low power view to show the position of an E14 mouse hippocampal transplant (M) aligned rostrally to an E16 rat hippocampal transplant (R) in the corticospinal tract (CST). gm, grey matter; m, midline. Horizontal cryostat section, with thionin counter-staining. Survival time, 8 days.

Scale bar = 200 μ m.

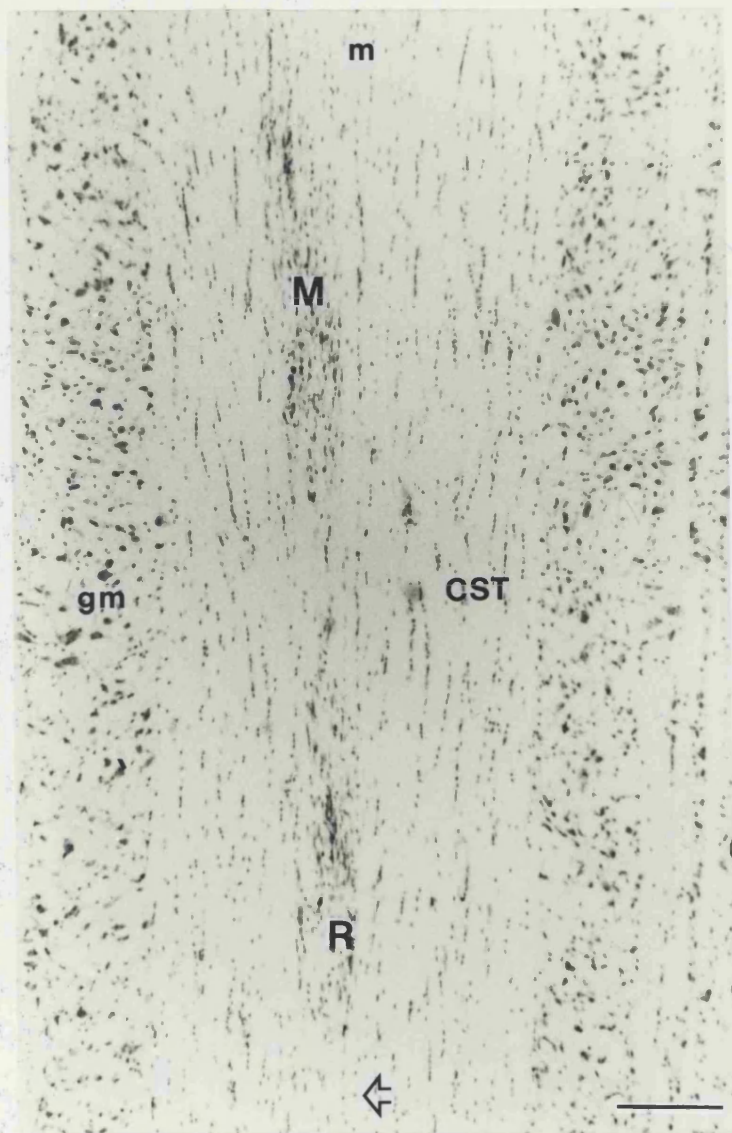


Figure 4.11

M6-positive axonal projections (arrows) which have arisen from the rostrally placed E14 mouse transplant (not shown in this photograph), passing caudally from the caudal pole of the rat hippocampal transplant (R). Open arrow indicate the same position in figure 4.10. Compare with the axons leaving the caudal end of a single mouse graft in Fig. 4.5B. Horizontal cryostat section, counter-stained with thionin. Survival time, 8 days.

Scale bar = 50 μ m.

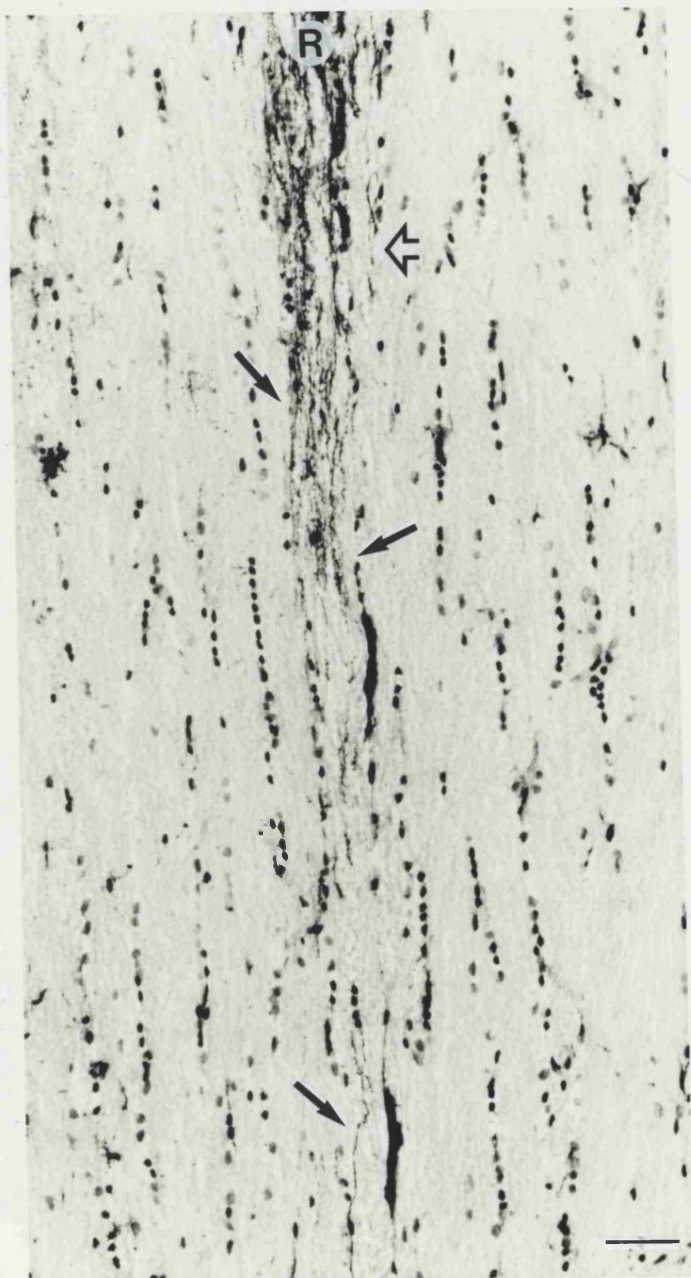


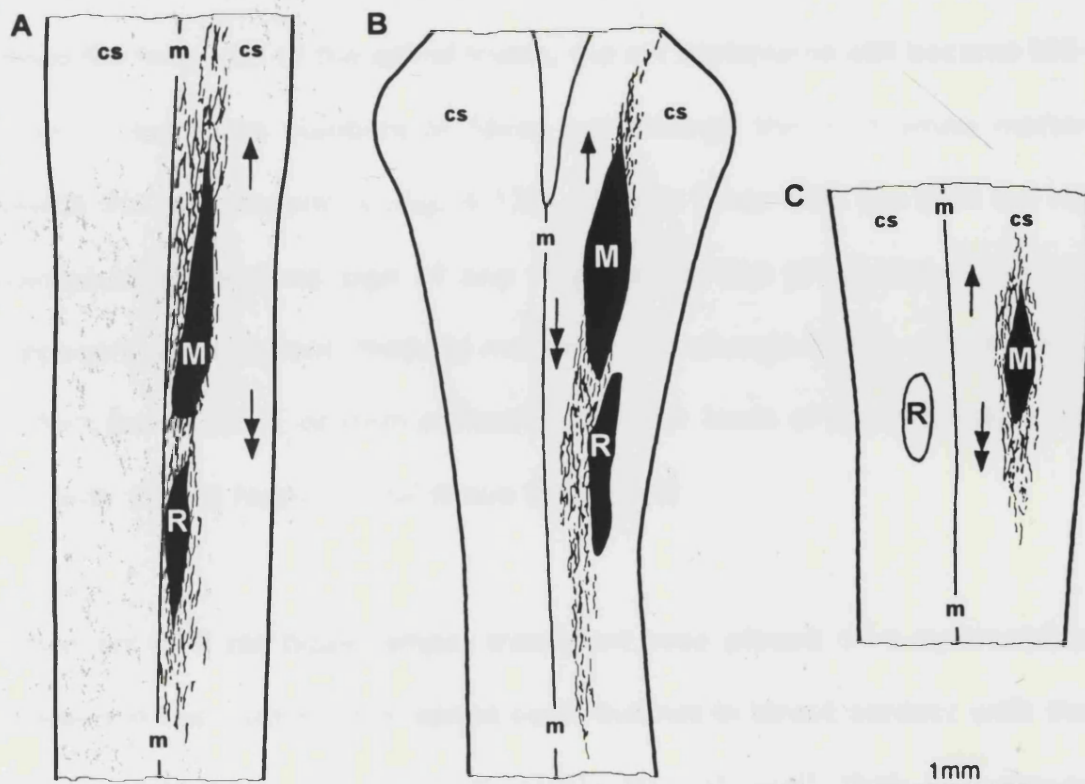
Figure 4.12

Camera lucida drawings of the M6-positive axonal projections rostrally (single headed arrows) and caudally (double headed arrows) from E14 mouse hippocampal cell transplant (M) in the corticospinal tracts (cs) with a second transplant of E16 rat hippocampal cells (R).

A: In a case where the mouse and rat transplants are aligned in the longitudinal rostro-caudal axis of the host corticospinal tract (as in Fig. 4.10), the caudally directed mouse axonal projections pass into and through the rat transplant.

B: In a case where the mouse and rat transplants are partially misaligned, the caudally directed mouse axonal projections pass the rat transplant without deflection (micrograph in Fig. 4.13).

C: In a case where the mouse and rat transplants are on opposite sides of the midline (m), the mouse axonal projection is completely uninfluenced by the rat transplant, which receives no projection and is M6-negative. Survival times, 6-8 days. which travelled unhindered down the host spinal tract in the rats with a single mouse transplant. (cf. Fig. 4.11 with the axons leaving the caudal end of a single mouse transplant in Fig. 4.5B).



single mouse transplant. (cf. Fig. 4.11 with the axons leaving the caudal end of a single mouse transplant in Fig. 4.5B).

When the embryonic mouse and rat transplants were only partially aligned along the long axis of the spinal tracts, the rat transplants still became M6-positive, but large numbers of fibres ran through the host white matter beside the rat transplants (Fig. 4.12B). These fibres that ran past the rat transplant showed no sign of any response to the proximity of the rat hippocampal transplant: they did not show any change in size, nor did they deflect their course, or form collaterals or other kinds of branches directed towards the rat hippocampal tissue (Fig. 4.13).

When an E16 rat hippocampal transplant was placed in a symmetrical position in the contralateral spinal cord, but not in direct contact with the mouse transplant, the M6 immunostaining showed that the mouse transplants formed M6-positive projections with the same distribution as in the experiments with mouse transplants alone. The M6-positive axons did not deviate towards the rat transplants, and did not form collaterals or branches in that direction. There were no axons crossing the midline, and the rat transplants were not stained with M6 (Fig. 4.12C).

Figure 4.13

Caudally directed M6-positive mouse axons (e.g. arrows) by-passing the rat transplant (R) from the area medial to the rat transplant as shown in drawing shown in Fig. 4.12B. Horizontal cryostat section, counter-stained with thionin. Survival time, 14 days.

Scale bar = 50 μ m.

4.3.2.2. Axon growth by HRP retrograde labelling

The presence of donor axonal projections in the host spinal tracts was confirmed by intraspinal injections of HRP. In 5 out of 7 animals the position of the HRP injection was directly below the transplants and there were numerous labelled neurons in the transplant. The neurons labelled with intra-cytoplasmic granular HRP reaction product were of various sizes and some of them were large pyramids with apical and basal dendrites (Fig. 4.14). In 2 cases where the HRP injections were lateral to the axis of the transplant, no labelled neurons could be seen in the transplants.

4.4. Discussion

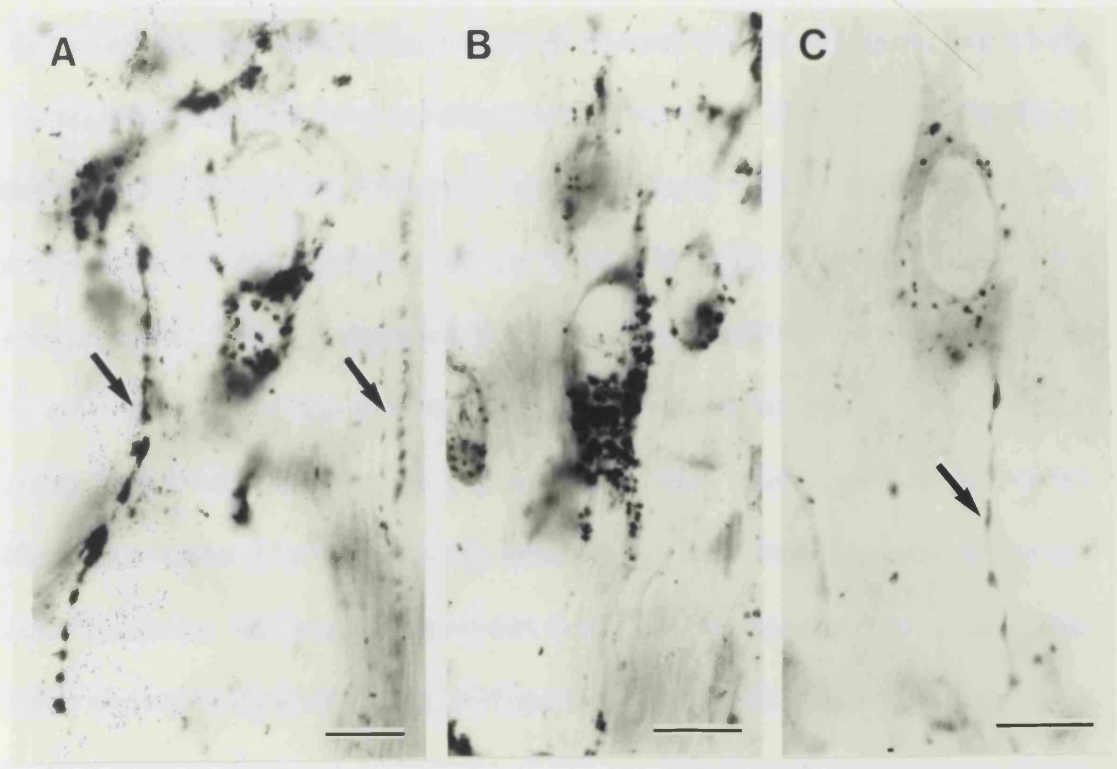
Long intrafascicular axon growth is highly reproducible and can be seen from embryonic mouse hippocampal neurons injected into the adult rat spinal cord. The effect is equally robust in the ascending and descending tracts of the adult rat dorsal columns. In both tracts, the axons grow in both rostral and caudal directions from the transplants at up to 0.5 mm per day for the first 6-10 days, after which time they slow down, reaching a final maximum distance of around 8 mm from the transplants by 6-7 weeks. The presence of donor axonal projections at 2-4 weeks was confirmed by retrograde transport of HRP. There is a progressive decrease in numbers of M6-positive axons after about one month. This may be due to down-regulation, or to failure of access of the antibody (e.g. due to myelination; Victorin *et al.*, 1991) or there may be a loss of axons.

Figure 4.14

Transplanted E14 hippocampal neurons retrogradely labelled by an injection of HRP.

A,B: HRP labelling detected with the TMB method. **C:** HRP labelling detected by the Co-GOD method. Arrows, the particulate HRP reaction product in retrogradely labelled axons. 60 μm horizontal frozen sections. Survival times, 28 days after transplantation, followed by a further 48 hours after HRP injection.

Scale bar = 10 μm .



Wictorin et al (1990a) used a human neurofilament antibody to show that suspensions of human embryonic striatal neuroblasts transplanted into the striatum could grow axons down the host myelinated corticofugal pathways as far as the upper spinal cord in adult rats, and they subsequently reported long axon growth from suspensions of human embryonic spinal cord cells transplanted into the long myelinated fibre tracts of the lateral columns of the adult rat spinal cord (Wictorin and Björklund, 1992), and showed that axons travelled for distances up to 10 mm in both directions in the host spinal tracts. They suggested that the remarkably long axon growth of human neuroblasts transplanted into rat brain could be a reflection of a greater intrinsic growth capacity of the human axons, which in normal circumstances of the large human brain must cover much greater distances than rat axons. Although the present study has not used human donor cells, it still shows comparably long, bi-directional intra-fascicular axon growth (at least 8 mm) in the dorsal columns of the rat spinal cord from mouse donor tissue - a situation where the donor neurons came from a species smaller than the host.

It is not clear why this interfascicular axon growth is not prevented by inhibitory molecules, such as those postulated to be expressed by mature myelinating oligodendrocytes (Savio and Schwab, 1990; Schnell and Schwab, 1990; Schwab *et al.*, 1993). Wictorin et al (1990a) suggested that axons formed by embryonic neurons may not have receptors to make them susceptible to the inhibitory molecules that prevent the regeneration of cut adult axons.

The present study shows that the overall pattern of M6 axonal projections which are formed by intraspinal micro-transplants of suspensions of embryonic mouse hippocampal neurons is very similar to that described for the same type of cells injected into the fimbria or corpus callosum (Davies *et al.*, 1994), and has four characteristics: 1. the adult myelinated tracts are permissive to growth of axons from inappropriate ('mismatched') types of donor tissue, 2. there is no clear indication that the growth is affected by denervation, 3. the growing axons show a strong preference for white matter, and 4. the pattern of growth strictly follows the underlying glial and axonal tract architecture.

4.4.1. Growth of inappropriate axons

Wictorin and colleagues reported that axon growth in systems where the donor material was taken from regions appropriate to the host transplantation site (Wictorin *et al.*, 1989, 1990b, 1991), and inappropriate tissue (such as cerebellum) did not grow so well. In contrast, Davies *et al.* (1994) reported that axons from several types of completely inappropriate donor regions grew both as fast, and as far as those from the correct regions, and with similar patterns of intra-tract distribution. Thus, transplants of cortical, hippocampal and superior collicular donor tissue each produced the same patterns of growth of long interfascicular axons in the adult corpus callosum. Since hippocampal axons are not present at any time in the normal developing or adult spinal cord (Stanfield *et al.*, 1987), the intraspinal projections observed in the present study must also be regarded as mismatched. It should be remembered, however, that the donor cell

suspensions contain many different kinds of cells, at different stages of maturity (including neuroblasts). Whilst it is possible that the intrafascicular projections arise from types of cells which do not normally project to the spinal cord, it is also possible that the environment of the host tract transforms the properties of some of the donor cells (Renfranz *et al.*, 1991).

4.4.2. Damage

Present experiments used a micro-transplantation technique which minimised (but cannot totally avoid) damage to the host tracts. However, the fact that the axons travelled both rostrally and caudally in both tracts strongly suggests that damage to host axons did not have a major influence on the pattern or extent of donor axon growth; otherwise, one might expect that the transplant projections would follow the 'beam' of degenerating distal fragments of host axons and run preferentially rostrally in the ascending tracts, and caudally in the descending tracts.

4.4.3. Preference for white matter

As in the experiments of Wictorin and Björklund (1992), the present transplant situation shows that there is a major preference for growth along white matter as opposed to grey. Even when transplants lie in the grey matter, or at its junction with the white, the axons project selectively along white matter bundles. In the double transplant situation, transplanted rat hippocampal neuropil, containing postsynaptic sites appropriate to the hippocampal axons, becomes M6-positive only if the transplant is in direct contact with the beam of donor axons. However, considerable numbers of

elongating mouse axons continue to grow, undeflected along the host white matter, beyond the rat transplant. The inability of the axons to cross the midline is possibly associated with the specialized midline tissue described in *CHAPTER III*.

4.4.4. Tract structure

There have been few studies of the organisation of the different non-neuronal cell types making up the skeleton of central fibre tracts (Bayer and Altman, 1975; Lin and Connor, 1989; Matthews and Duncan, 1971). In previous studies from this laboratory it was shown that adult central fibre tracts have a regular rectilinear glial skeleton, with an overlapping continuum of longitudinal astrocytic processes aligned along the axonal axis (Suzuki and Raisman, 1992), and a similar glial framework also can be seen in the spinal tracts (*CHAPTER III*). Long interfascicular axon growth from embryonic neurons micro-transplanted into minimally disturbed tracts is strictly parallel to the longitudinal (axonal and glial) processes of the host tract (Davies *et al.*, 1994). The present experiments show that the same pattern of axon elongation occurs in the spinal cord, and add that even the interposition of neuropil is unable to abolish the predominant facilitatory effect of the longitudinal host tract elements on the elongation and orientation of the growing embryonic axons.

4.4.5. Interpretation

Over the survival times studied, it is clear that the environment of the myelinated motor and sensory tracts of the dorsal columns of the adult rat

spinal cord is permissive for the rapid and long growth of axons from immature donor neurons.

Because the present experiments have examined time courses of less than 2 months (beyond which the M6 marker ceases to be useful (Wictorin *et al.*, 1991), it is not known whether the long intraspinal axon projections are permanent, nor whether at longer times they do leave the tracts and enter host postsynaptic fields and terminate by forming synapses. The survival times used in this study could be the equivalent of an early, exuberant phase of non-specific axon developmental growth along pathways, which is later re-modelled to match specific connectional requirements (O'Leary *et al.*, 1990; Simon and O'Leary, 1992).

4.5. Summary

Using the micro-transplantation technique, a suspension of embryonic mouse hippocampal cells was injected into the cervical level of the ascending dorsal column tracts or descending corticospinal of an immuno-suppressed adult rat spinal cord. The mouse specific marker M6, showed that the transplanted embryonic neurons grew long, straight, uniform axons in both the ascending and descending tracts that passed both rostrally and caudally. The donor axons did not fasciculate with each other, but were intermingled with the host myelinated spinal axons, parallel to them and to the host interfascicular glial rows. The donor axons extended at about 0.5 mm per day for the first 6-10 days, after which time they slowed down, gradually

reaching a maximum distance of around 8 mm from the transplants by 6-7 weeks. The presence of the donor axonal projections was confirmed by retrograde cellular labelling of the donor cells from injections of HRP about 2 mm caudal to the transplants.

Immunostaining showed that the bundles of M6-positive axons in the host myelinated fibre tracts were surrounded by areas of finely granular, diffuse M6-positive material in the surrounding host grey matter. This was especially noticeable in the situation where a smaller host fibre was separated from the main fibre tract and passed through neuropil areas. It was clear that M6-positive donor axons travelled further in the white matter than in the grey.

To examine whether the embryonic mouse hippocampal axons would be attracted to neuropil containing appropriate postsynaptic sites, a second transplant of embryonic rat hippocampal cells was micro-injected caudally to the mouse transplant. M6 immunostaining showed that when the rat transplants were placed where they could make direct contact with the beam of mouse axons, the rat transplants became M6-positive; this suggested that they had been innervated by the mouse axons. The rat transplants did not prevent a large number of mouse axons continuing caudally in the host spinal tracts. When the rat transplants were placed to one side of the beam of donor projection, the long projecting mouse axons did not deviate from their original course.

These results indicated that the long ascending and descending myelinated fibre tracts of adult rat spinal cord could provide a permissive environment for the rapid growth of axons from embryonic donor neurons originating from a region not normally projecting to the spinal cord. The route taken by these axons is determined by the internal structure of the host spinal tracts. Donor interfascicular axons are able to pass, without deviation, through or beside tissue containing appropriately matched postsynaptic targets and are not attracted by them.

CHAPTER V LESIONS IN ADULT RAT SPINAL TRACTS

5.1. Introduction

It has generally been considered that the sprouts emitted by cut central axons are transient, and are later retracted (e.g. Cajal, 1928), and that the proximal parts of the cut axons die back (Pallini *et al.*, 1988). Like other central axons, the corticospinal axons do not regenerate after lesions. Some experimental observations have suggested that cut axons undergo retrograde axonal degeneration leading to their retraction from the lesion site (Firkins *et al.*, 1993; Kalil and Schneider, 1975; Lahr and Stelzner, 1990).

There is great interest in the possibility that transplants of cultured Schwann cells might be able to induce and guide regeneration of cut axons in the central nervous system (Chen *et al.*, 1991; Martin *et al.*, 1993; Montero-Menei *et al.*, 1992; Montgomery and Robson, 1993; Neuberger *et al.*, 1992; Paíno *et al.*, 1994). A number of laboratories have described strategies to induce regeneration of damaged corticospinal axons by local application of blocking antibodies, growth factors, or Schwann cells to the area of the spinal lesion (Reier *et al.*, 1992; Schnell and Schwab, 1990; Tuszynski *et al.*, 1994a,b; Xu *et al.*, 1995). However, if there was a progressive retraction of cut axons from the lesion site with increasing time, then it might be expected that these repair strategies would become progressively less effective in chronic lesions.

Lesions in the central tract not only cut axons, but also destroy the glial

framework (Suzuki and Raisman, 1992). One of the marked changes occurring in lesions of adult central tracts is a progressively dense and spreading astrocytic hypertrophy, resulting in a scar, which is usually thought to be a barrier to axon growth (Obouhova *et al.*, 1994; Reier, 1986). It is important therefore to know when the astrocytic scar starts to form and whether the cut axons persist long enough at the lesion site for them to be stimulated to regenerate by therapeutic interventions.

The present study used an electrode micro-lesion approach which caused minimal disturbance to the adjacent host tract architecture, and a precise anterograde axon label was used to re-evaluate whether cut axons of the ascending and descending tracts of the adult rat spinal cord can survive in the lesion area. Do all cut axons die back after the lesion? If not, are they able to persist despite formation of the glial scar?

For this purpose, electrolytic lesions were made in the ascending and descending tracts of the dorsal columns at the level of the first two cervical segments of the adult rat spinal cord. The early responses (within 24 hrs) of the host ascending and descending axons were studied by the Palmgren silver staining method, and the later changes with anterograde axonal labelling. At increasing times after the lesion the progressive development of the astrocytic scar was monitored by GFAP immunohistochemistry. Once the scar was fully developed, the descending corticospinal fibres were labelled by intrapylaridal injections of BD, or examined in the electron microscope.

5.2. Materials and methods (For details see *CHAPTER II*)

5.2.1. Lesions

Lesions were made in the ascending or descending tracts of the dorsal columns in adult AS rats by passing a DC current through an electrode. Recovery was rapid after the operation, with the animals showing no obvious functional deficits or abnormalities in their cage behaviour.

5.2.2. Palmgren silver stain

5 animals with the descending corticospinal lesions at survivals of 4 hrs ($n = 2$) and 18 hrs ($n = 3$) were studied with the Palmgren silver method. 4 animals with lesions in the ascending tracts were also allowed to survive for 4 hrs ($n = 2$) and 18 hrs ($n = 2$), and stained with the Palmgren silver method.

5.2.3. GFAP immunohistochemistry

GFAP immunostaining was used to monitor the formation of the astrocytic scar in the descending corticospinal tract. 28 animals with the descending corticospinal lesions were allowed to survive for 1 day ($n = 2$), 4 days ($n = 3$), 1 week ($n = 3$), 3 weeks ($n = 3$), 5 weeks ($n = 3$), 6 weeks ($n = 2$), 7 weeks ($n = 3$), 8 weeks ($n = 3$), 9 weeks ($n = 3$) and 13 weeks ($n = 3$).

5.2.4. Extracellular labelling

29 animals with lesions in the descending corticospinal tract received injections of HRP, biocytin or BD in the pyramidal tract at the base of the

medulla. The animals were allowed to survive for 4 days (n = 2), 1 week (n = 3), 2 weeks (n = 3), 3 weeks (n = 4), 4 weeks (n = 3), 5 weeks (n = 4), 6 weeks (n = 4), 9.5 weeks (n = 3) and 13 weeks (n = 3).

9 animals with lesions in the ascending tracts received injections of HRP in the dorsal columns at a distance of 1-2 vertebral segments below the lesions. They were allowed to survive for 2 weeks (n = 3), 3 weeks (n = 3), 4 weeks (n = 3). 100 μ m serial horizontal sections were cut through the lesion area. Sections of the spinal cord with injections of HRP were detected by the Co-GOD method and the sections of the spinal cord with injections of biocytin or BD were incubated overnight in the ABC solution with 0.1% Triton and detected by the Ni-GOD method.

5.2.5. Semithin sections and EM

14 animals with corticospinal lesions were prepared for the EM study at survivals of 1 week (n = 3), 2 weeks (n = 3), 3 weeks (n = 3), 4 weeks (n = 3) and 4 months (n = 2). 200-300 μ m Vibratome sections were processed by the method modified for the preservation of CNS myelin, and flat embedded in resin. Semithin and ultrathin sections were cut in a horizontal plane.

5.3. Results

5.3.1. General morphology of the lesion

Horizontal sections stained with the Palmgren silver method showed that the

DC current produced small, compact, ovoid lesions of around 0.5-1.0 mm diameter and around 1 mm in length (Fig. 5.1) which were localised to either the ascending dorsal column tracts or the descending corticospinal tract. The centre of the lesion consisted of an area of total destruction, which later became filled by debris and exudate, and was rapidly populated by macrophages (e.g. Figs. 5.2,3). Adjacent to the lesion centre, there was an area of partial tissue damage in which semithin and ultrathin sections showed that many of the tract components were destroyed (oligodendrocytes, myelin, some axons) but where many axons and the longitudinal astrocytic tract framework remained intact.

Because of the 100 μ m thick sections used for the study, it was possible to be certain, in many cases, that axons and their swollen cut ends were contained completely within the thickness of the section, thus confirming that these axons had been cut. In contrast to cut axons, the uncut axons could be traced along their whole length into the un-lesioned area of the tract, in the same section.

5.3.2. Responses of the host astrocytes

The reaction of the host astrocytes to the lesion was examined in GFAP immunostained horizontal sections, at different survival times in the lesioned

Figure 5.1

Palmgren silver staining to show the typical size and location of a lesion in the corticospinal tract (CST). Swellings (arrows) at the ends of cut axons at 18 hrs after lesion (Les). Horizontal cryostat section.

Scale bar = 100 μ m.



Figure 5.2

A: HRP labelling to show that a long term lesion (Les) stimulates (probably nodal) coarse, irregular, short sprouts (arrows) from an adjacent uncut corticospinal axon. CST, corticospinal tract. Survival time, 45 days.

Scale bar = 100 μm .

B: High power photomicrograph of A (arrow area). HRP labelling with the Co-GOD detection method, counter-stained with neutral red. 100 μm horizontal freezing microtome section. Survival time, 45 days.

Scale bar = 25 μm .

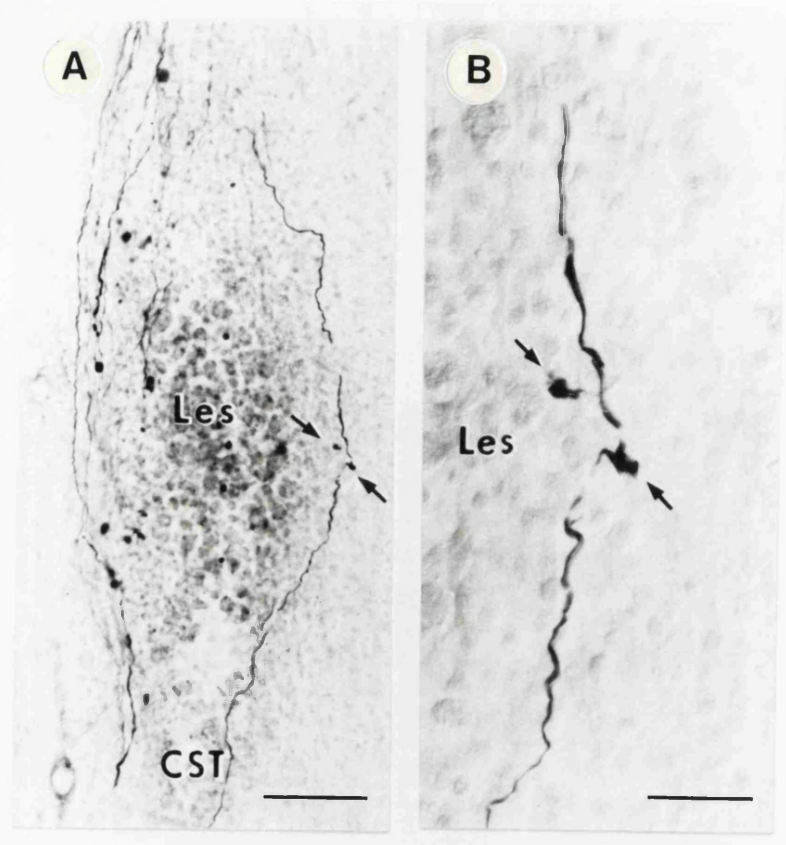


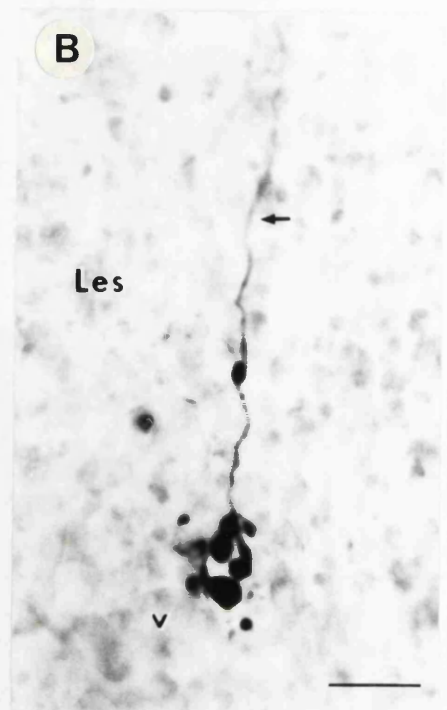
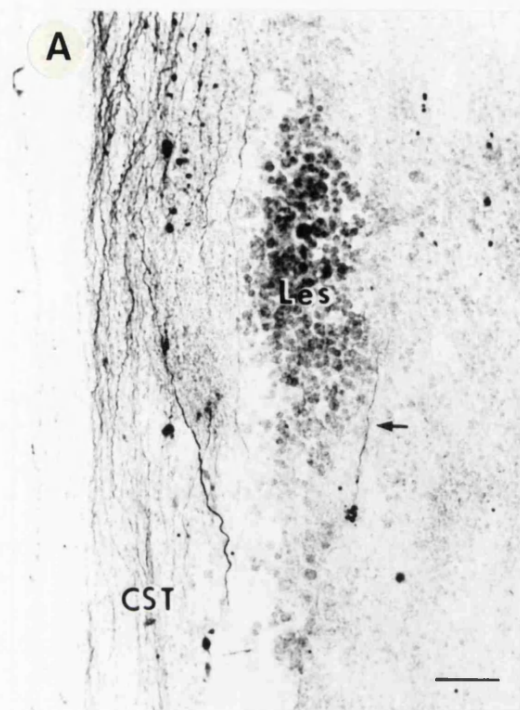
Figure 5.3

A: Low power photomicrograph of a lesion in the corticospinal tract (CST) to show the multiple varicosities (v) and recurved segments at the expanded cut end of a corticospinal axon (arrow) which persists in the entirely destroyed, macrophage invaded area of a long term lesion. Les, lesion.

Scale bar = 100 μ m.

B: High power view of A (arrow area). 100 μ m horizontal freezing microtome section, HRP labelling and detected with the Co-GOD method, counter-stained with neutral red. Survival time, 38 days.

Scale bar = 25 μ m.



corticospinal tract. There was a progressive intensification of GFAP immunoreactivity in the astrocytic cell bodies and their processes from 1 week to 13 weeks after the lesion.

For the first few days after the lesion, there was no obvious change in the arrangement of the astrocytes around the lesion and the astrocytic processes maintained their predominant longitudinal orientation. From 1 week onwards (Figs. 5.4,5) there was a noticeable increase in the size of individual astrocytic cell bodies and their processes, especially those bordering the edges of the central lesion area (Fig. 5.5A). The astrocytes in the lesioned corticospinal tract underwent a progressive hypertrophy which spread out from the lesion. By 7 weeks this progressive hypertrophy had reached the point where the central area appeared to be completely walled off from the surrounding tissue by a solid mass of intensely GFAP immunoreactive astrocytes and their processes. From 7 weeks onwards the astrocytic scar area continued to increase in width and density, and by 13 weeks the lesion area was completely surrounded by a dense astrocytic scar.

5.3.3. Responses of the host axons

5.3.3.1. Responses of the ascending and descending axons to the lesions

within 24 hours Because of the friability of the tissue, it was difficult to get intact sections with short term lesioned tissue (4-24 hrs) to demonstrate the cut ends of the axons with the anterograde labelling. This was a mechanical

Figure 5.4

Development of the astrocytic scar in the corticospinal tract at 1 (**A**), 3 (**B**), 7 (**C**), and 13 (**D**) weeks after lesions. Asterisk, central lesion area; m, midline. Horizontal cryostat section, GFAP immunostain with thionin counter-staining.

Scale bars = 100 μm .

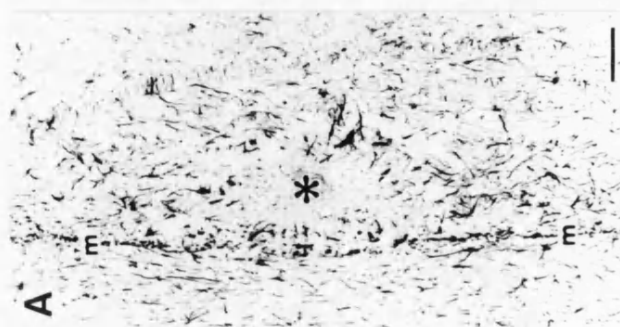
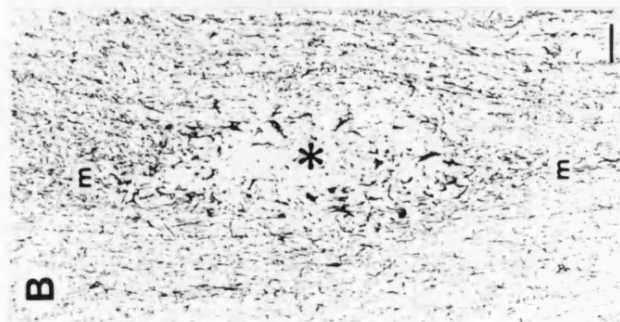
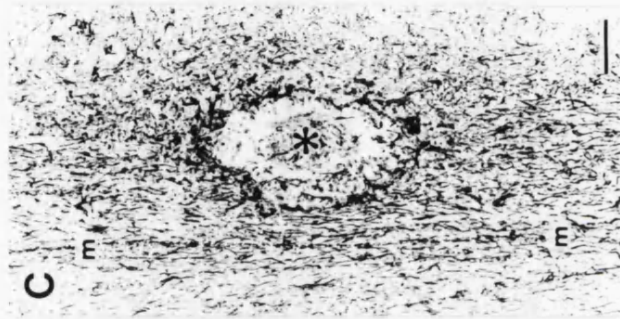
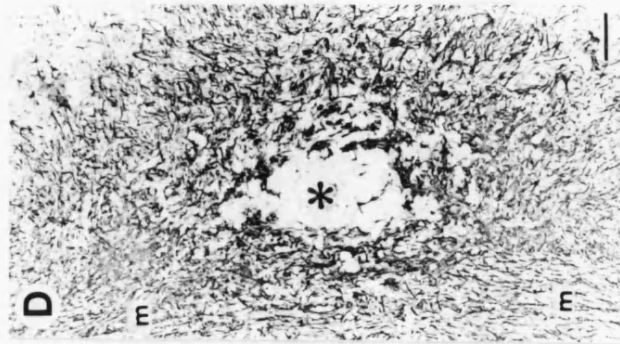
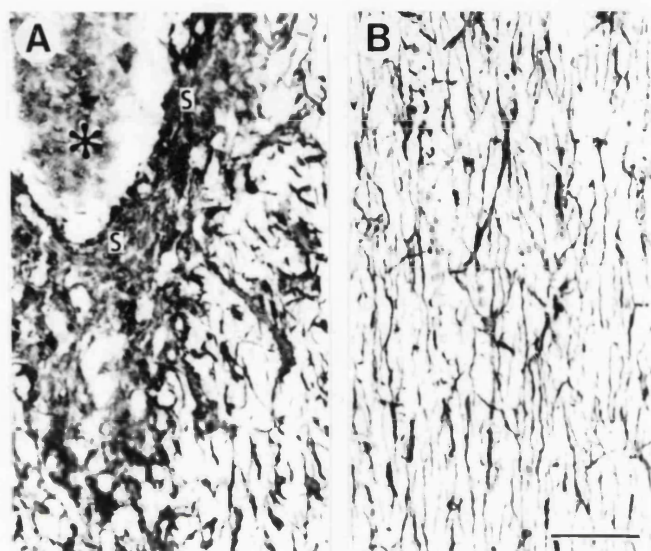


Figure 5.5

A: GFAP immunostaining to show the edge of a dense astrocytic scar (S), surrounding the central lesion area (asterisk) at 9.5 weeks after corticospinal tract lesion.

B: Array of slender, uniform longitudinal astrocytic processes in the normal corticospinal tract at the same magnification as A (as a control to demonstrate the degree of hypertrophy). Horizontal cryostat section, GFAP immunostain with thionin counter-staining.

Scale bar = 50 μm .



problem associated with the free floating frozen sections cut on the sledge microtome. The fluid-engorged lesion area was so fragile that the large, dense axon swellings attached to their parent stems could not be preserved. The responses of the axons to the short term lesion were therefore studied using cryostat sections and Palmgren silver stain.

Cut and uncut axons In the case of short term lesions, the responses of cut axons in both the ascending and descending tracts could be seen clearly by Palmgren silver stain. The ends of the cut axons at the border of the lesions were swollen (Fig. 5.1). This effect was most marked in the proximal stumps, attached to the cell bodies - i.e. at the rostral edge of the lesion for the descending fibres, and the caudal edge for the ascending fibres. The lesion centre contained many degenerating fibres which were isolated from the main tract axons. The lesions also induced en passant varicosities in the adjacent uncut axons.

5.3.3.2. Responses of the ascending and descending axons to the lesions at 4 days to 6 weeks

Cut axons The cut axons could be seen both in the central macrophage-filled area of complete tissue destruction, and also in the immediately surrounding areas, which had been partially damaged. The tract still retained a framework of astrocytic processes.

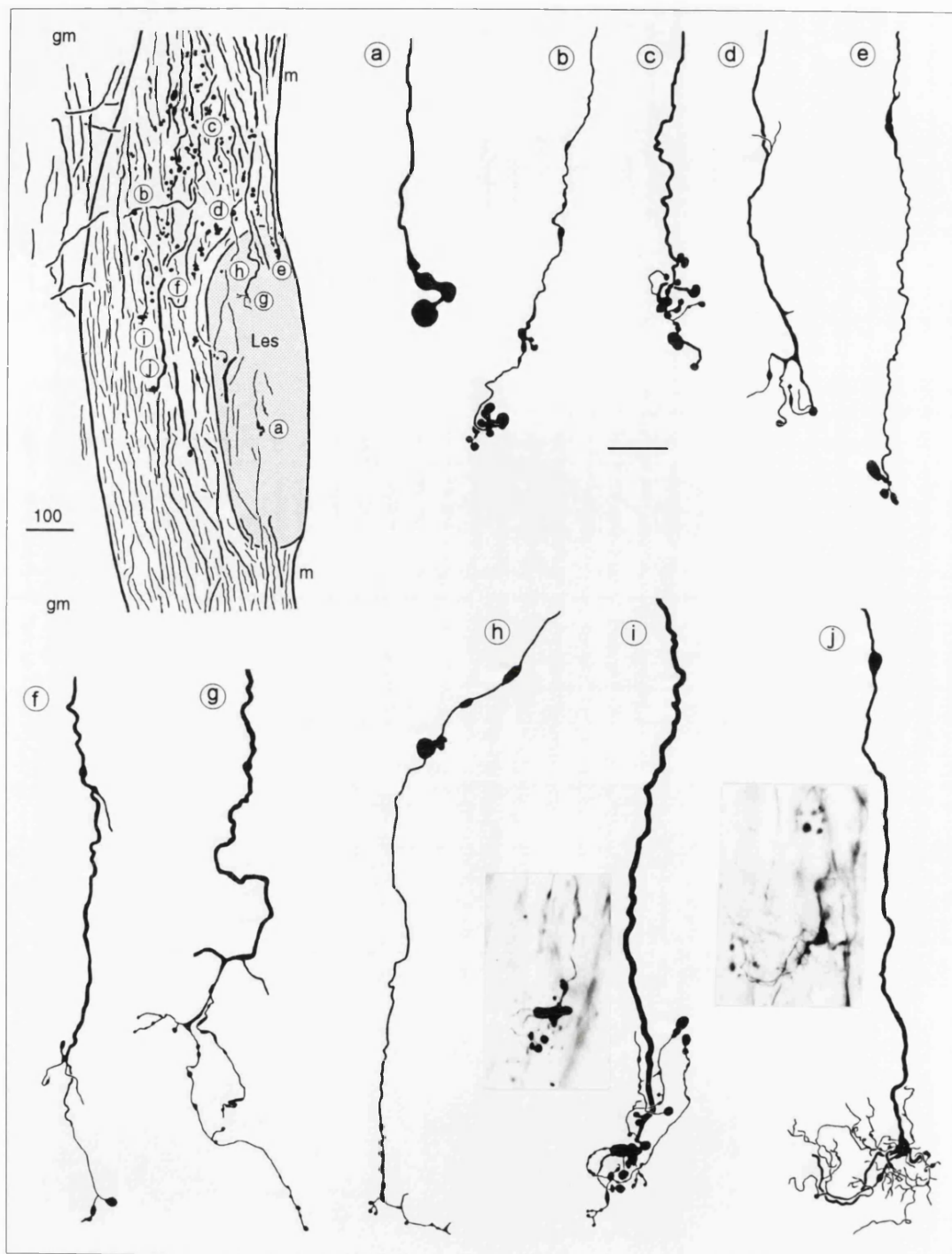
At 4 days, HRP labelled material confirmed that both cut and uncut axons expanded into varicosities (as had been shown by the Palmgren silver stain). By 6 weeks there were considerable numbers of completely severed axons still remaining in the lesion area, with local sprouting in both the ascending dorsal column tracts and descending corticospinal tract. These severed axons showed a progressive increase in swelling or sprouting at their cut ends (Fig. 5.3). These effects were seen prominently in the descending corticospinal tracts, but also to a lesser extent in the ascending tracts. The cut axons had long segments, uniform in diameter, which could be followed for a distance of 0.5 to 1 mm towards the lesion. During this part of their course they were straight, and showed almost no indication of damage (except for an occasional single varicosity) until they terminated abruptly, either within the macrophage filled area of the lesion centre or in the surrounding tract (Figs. 5.3,6). The cut ends did not appear to have retracted; some may have advanced a short distance into the macrophage area. The cut ends showed a variety of morphologies, but there was no obvious relationship between the location of the cut endings and the shape of the ending.

Many of the large and small diameter axons terminated in a mass of large varicosities, connected by very short, narrow segments (Fig. 5.6b,e), and often recurved configurations (Fig. 5.3). In other cases, the cut ends were only slightly expanded, but gave rise to a profusion of fine sprouts which formed arborisations, exceeding 50 μm across, in the region of the cut axonal end (Fig. 5.6c,i,j). Some the axonal arborisations were thread-like

Figure 5.6

Top left: low power camera lucida drawing (scale bar = 100 μm) of a lesion of the corticospinal tract to show the location of the various cut axon endings illustrated in a-j. The endings a,e,g,h are in the entirely destroyed, macrophage invaded area (Les); the remainder, although also belonging to cut axons, lie within an area of diffuse axon damage in the adjacent corticospinal tract which still contains an array of parallel uncut axons and presumably intact astrocytic organisation. Photomicrographs included for i,j were labelled with HRP and detected with the Co-GOD method. 100 μm horizontal frozen section. Survival time, 45 days.

Scale bar = 25 μm .



and non-varicose, ending freely (Fig. 5.6j), others bore small, spherical varicosities en passant or terminal, and resembled the presynaptic boutons in terminal fields (Fig. 5.6c,d,i). These terminal branches did not extend for more than 100-150 μm (Fig. 5.6f,g,h) in the lesion area.

Uncut axons The morphologies of the uncut axons and their responses to the lesion in both ascending and descending tracts were demonstrated clearly using anterograde labelling. The uncut axons could be induced to sprout, by stimulation from the lesions over the period from 2 to 6 weeks after operation, for a distance of up to 200 μm on either side of the lesion centre. Axons en passant developed characteristic strings of contiguous spherical varicosities (Fig. 5.7). Uncut axons passing in direct contact with the lesion centre were strongly stimulated, and gave rise to short, club-like sprouts (Fig. 5.2).

5.3.3.3. Responses of the descending corticospinal axons to the lesions at 9.5 and 13 weeks

Cut axons After a sufficient time for the formation of a dense astrocytic scar (as demonstrated by GFAP immunostaining) at 9.5 and 13 weeks, the axons of the corticospinal tract were anterogradely labelled by injecting BD into the pyramidal tract. BD labelling showed that the sprouts of cut corticospinal axons had persisted for 13 weeks in the centre of the macrophage-filled lesion and also in the area of the astrocytic scar. There were no obvious morphological differences between of the

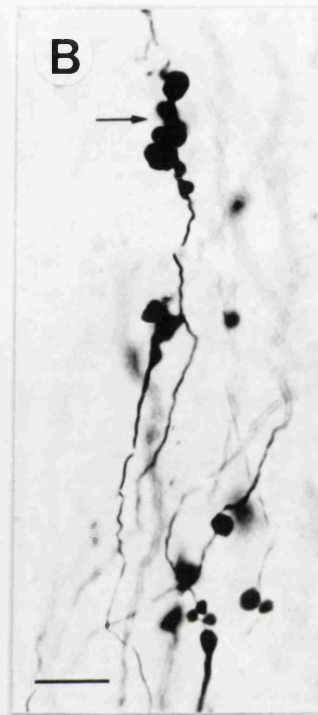
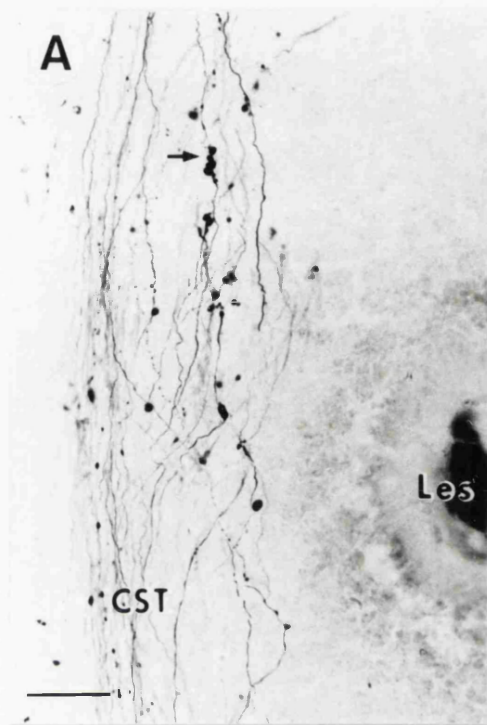
Figure 5.7

A: HRP labelled corticospinal axons to show contiguous clusters of axon varicosities (e.g. arrow) in the uncut corticospinal tract (CST). Les, lesion.

Scale bar = 100 μm .

B: High power view of A. 100 μm horizontal frozen section, HRP labelling and detected with the Co-GOD method, counter-stained with neutral red. Survival time, 16 days.

Scale bar = 25 μm .



sprouts seen before and after the scar formation and there were no systematic differences in the distribution or extent of sprouting between the material at 9.5 weeks and 13 weeks after the lesion. The cut ends of the BD labelled axons were greatly expanded, and gave rise to multiple, BD-filled sprouts (Figs. 5.8,9), which showed variation in length and branching, some were profusely radiating while others were varicose. The sprouts arborised freely among the macrophages of the central lesion area, but were not able to cross the lesion to reach the distal part of the host tract. As the exact size and position of the BD injection varied in different experiments, it was only possible to make a rough, semi-quantitative assessment of the numbers of sprouting cut axons surviving in the lesion: in a complete series of adjacent sections from two animals at 9.5 weeks, 35 BD-labelled cut axons sprouting in the lesion area could be identified (12 and 23) as compared with 32 (15 and 17) in two animals at 13 weeks.

Uncut axons At 9.5 and 13 weeks there were no further obvious morphological changes in the uncut axons compared with those at 6 weeks. The main stems of the uncut corticospinal axons which pass beside the region of the lesion were also labelled by the BD, Some of these uncut axons gave rise to collateral sprouts directed into the macrophage-filled central lesion area (Fig. 5.10). In addition, large varicosities formed on the main stems of some uncut axons as they passed through the zone of partially damaged tract tissue on either side of the central macrophage containing lesion area. In some cases these varicosities were contiguous,

Figure 5.8

Photomicrograph (**A**) and camera lucida drawing (**B**) to show a sprout from a cut corticospinal axon in the macrophage-filled central lesion area. 100 μm horizontal Vibratome section, BD labelling and detected with the Ni-GOD method, counter-stained with neutral red. Survival time, 13 week.

Scale bar = 25 μm .

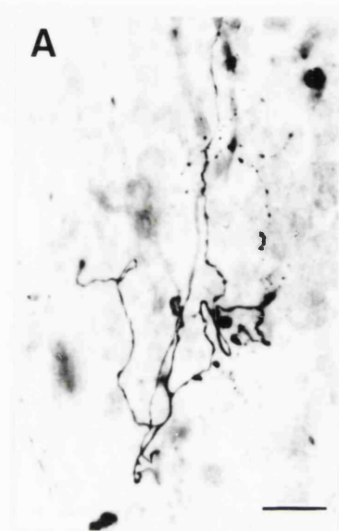


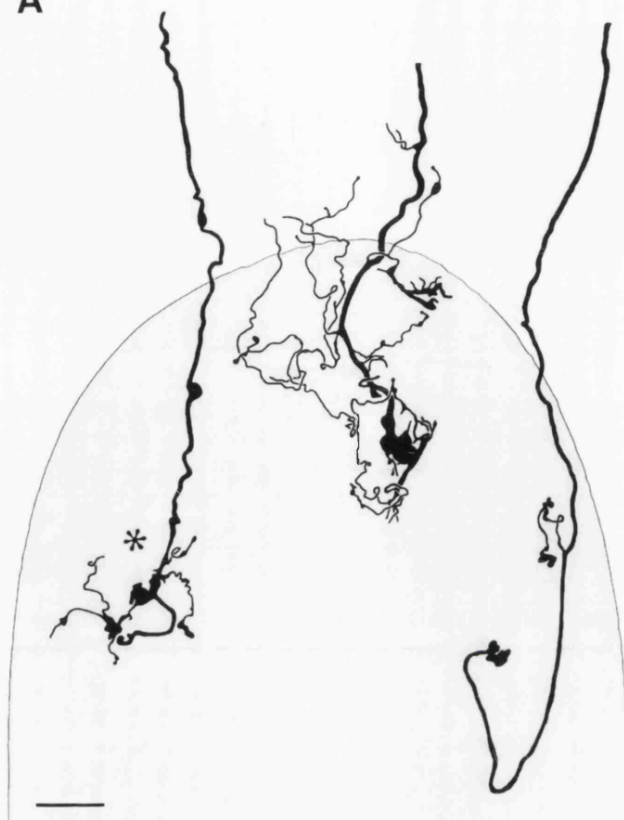
Figure 5.9

A: Camera lucida drawing of 3 cut corticospinal axons in the macrophage-filled central lesion area.

B: Photomicrograph of the axon indicated by an asterisk in A. 100 μm horizontal Vibratome section, BD labelling and detected with the Ni-GOD method, counter-stained with neutral red. Survival time, 9.5 weeks.

Scale bars = 25 μm .

A



B

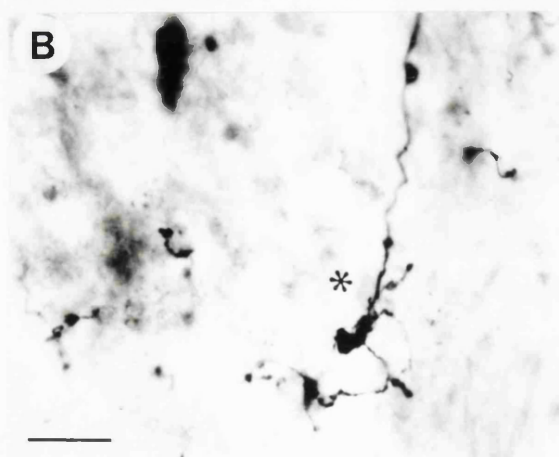
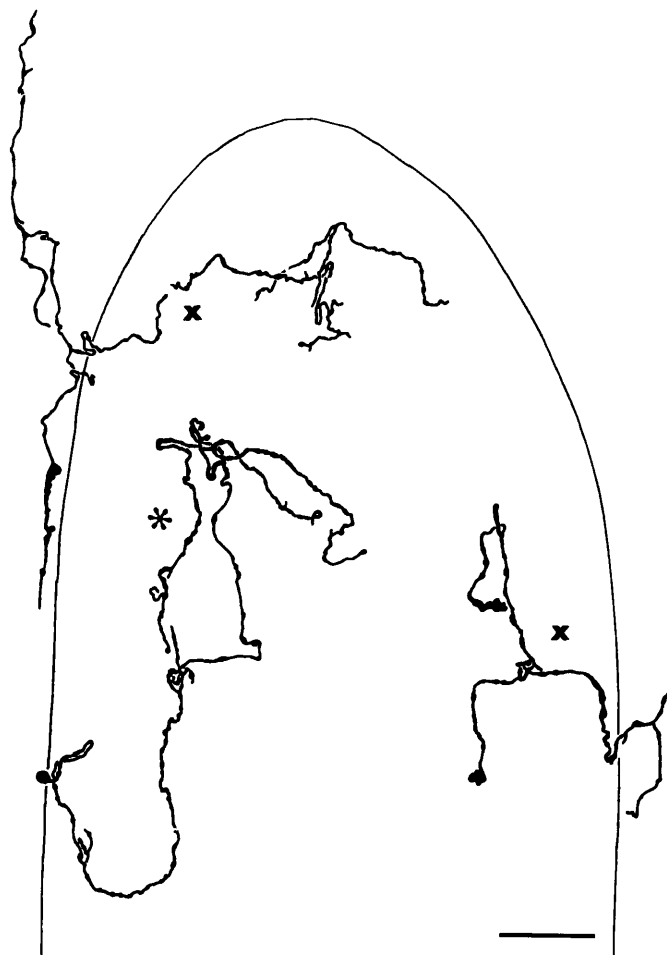


Figure 5.10

Camera lucida drawing of fine branches of corticospinal axons entering the macrophage-filled central lesion area at 13 weeks. The branches (x) are collaterals arising from axons passing by the lesion. Asterisk indicates sprouts whose position is shown by arrow in Fig. 5.11D. 100 μm horizontal Vibratome section, BD labelling and detected with the Ni-GOD method, counter-stained with neutral red. Survival time, 13 weeks.

Scale bar = 50 μm .



or had developed into complex structures, with large, irregular swellings, sometimes separated by narrow axonal segments folded backwards and forwards on each other (Fig. 5.11).

5.3.4. Semithin sections and EM

The observations from semithin sections showed that the distal parts of the corticospinal axons underwent Wallerian degeneration passing caudally from the lesion (Fig. 5.12), resulting in a compact 'beam' of degenerating fragments. These degenerating fragments were phagocytosed by macrophages in the tract. Rostrally from the lesion, there was a smaller 'beam' of partial axonal fragmentation, which affected only a proportion of the axons, but was also associated with ingrowth of phagocytic macrophages.

The EM results confirmed that the centre of the lesion consisted of an amorphous mass of debris which was rapidly permeated by macrophages, and later also developed a loose connective tissue stroma, containing blood vessels and some astrocytic processes. Surrounding the region of the lesion was an area of densely packed, hypertrophic astrocytic processes with massed cytoplasmic filaments, which corresponded to the scar region of the GFAP immunostained material.

In the region of partial tissue damage, there was an area with loss of oligodendrocytes and myelin and wide diameter demyelinated axons, from

Figure 5.11

Uncut, anterograde labelled corticospinal axons adjacent to the macrophage-filled central lesion area. x at 2 weeks, y at 13 weeks after lesion.

A: Photomicrograph of 100 μm horizontal frozen sections; HRP labelling with the Co-GOD detection method. **C:** Photomicrograph of 100 μm horizontal Vibratome sections; BD labelling with the Ni-GOD detection method. **B:** Camera lucida drawings of A and C.

Scale bars = 50 μm .

D: low power photomicrograph to show position of the axon y in the corticospinal tract adjacent to the macrophage-filled central lesion area (CL); arrow indicates the position of the sprouts marked by asterisk in Fig. 5.10.

Scale bar = 100 μm .

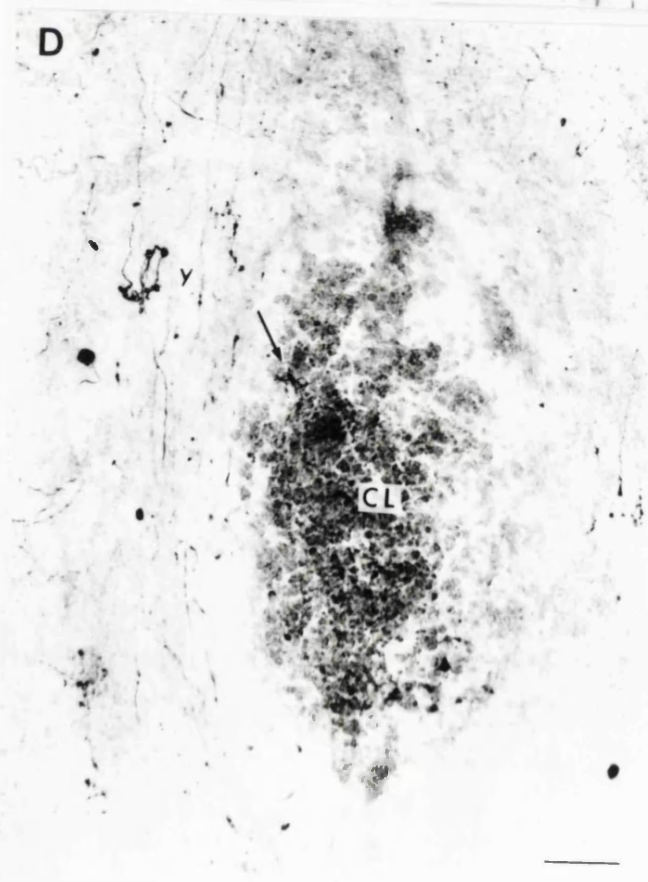
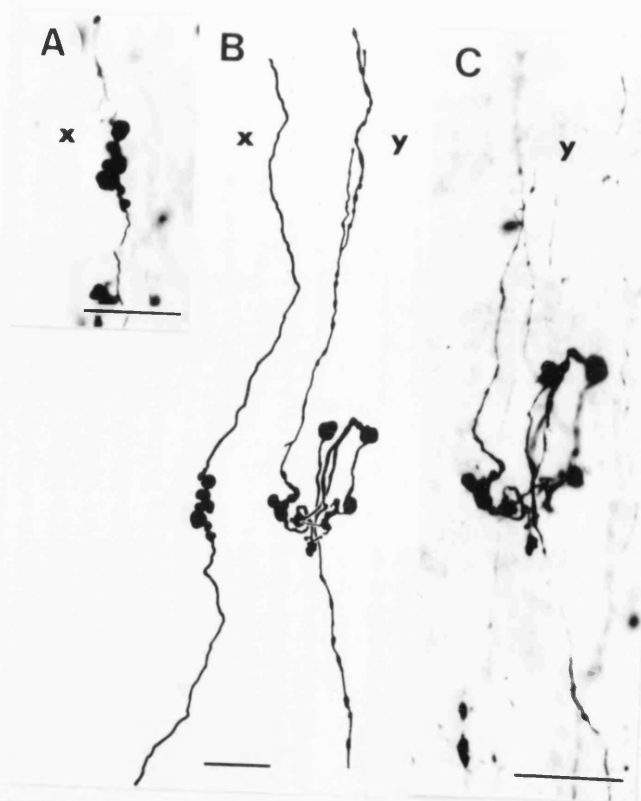
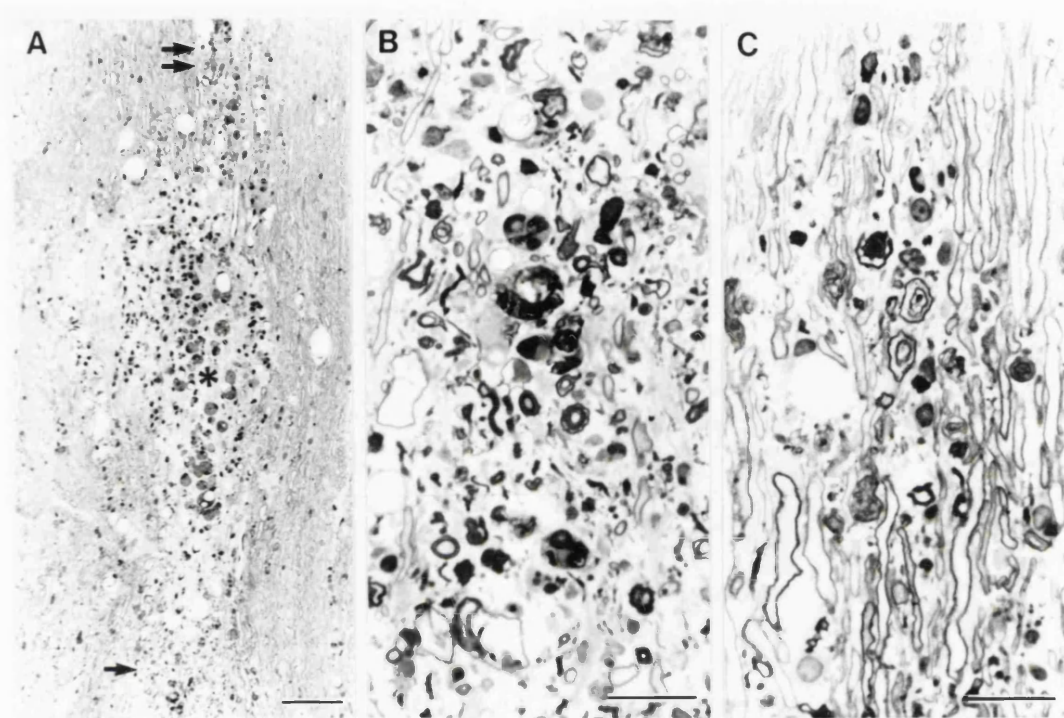


Figure 5.12

A: Low power view through a corticospinal tract lesion (*) containing macrophages (small round cells); single arrow, tract caudal to the lesion showing anterograde ('Wallerian') degeneration of a wide beam of axons; double arrow, tract rostral to the lesion, showing a smaller beam of axonal fragments surrounded by intact axons. **B,C:** High power views of the tract caudal (B), and rostral (C) to the lesion. Horizontal semithin sections, methylene blue stain. Survival time, 4 weeks.

Scale bars = 20 μm (in A); 50 μm (in B,C).



1 week after the lesion. From 3 weeks after lesion, a few of these axons had become re-myelinated by host endogenous Schwann cells which had migrated into the lesion area. At 4 months, most demyelinated axons were re-myelinated by the host Schwann cells. The astrocytic scarred region flanking the central lesion area contained a wide layer in which the axons were re-myelinated by Schwann cells (Fig. 5.13A).

Further away from the centre of the lesion, where the zone of Schwann cells ceased, the tract fibres remained myelinated by their original oligodendrocytes (Fig. 5.13). In many cases it was possible to see a node in which one segment of an axon (as it entered or left the adjacent area of undamaged tract) was myelinated by an oligodendrocytic process and the adjacent segment of the axon (passing through the damaged area) had been re-myelinated by a Schwann cell (shown diagrammatically in Fig. 5.14).

Endogenous Schwann cells could be seen in the lesion area in semithin and EM sections 3 weeks after the lesion. These Schwann cells had dark cytoplasm and a characteristic pattern of boldly marked nuclear heterochromatin (Fig. 5.13B). In contrast to the oligodendrocytes, the Schwann cells established a one-to-one relationship with the axons, and the Schwann cell bodies lay in direct contact with the axonal segments that they myelinated. The Schwann cells were wrapped by a basal lamina on the surfaces which faced the extracellular connective tissue space. The large terminal expansions of the cut axons (Fig. 5.15) were wrapped in a single

Figure 5.13

A: The different zones (separated by dashed lines) around a lesion. M, macrophages invading the central area of complete tissue destruction; S, adjacent area with swathe of uncut host corticospinal axons myelinated by ingrowing endogenous Schwann cells; O, oligodendrocyte-myelinated area of normal corticospinal axons. Horizontal semithin section stained with methylene blue.

Scale bar = 500 μm .

B: Horizontal electron micrograph showing a region of axons re-myelinated by endogenous Schwann cells (S). Survival time, 4 months.

Scale bar = 5 μm .

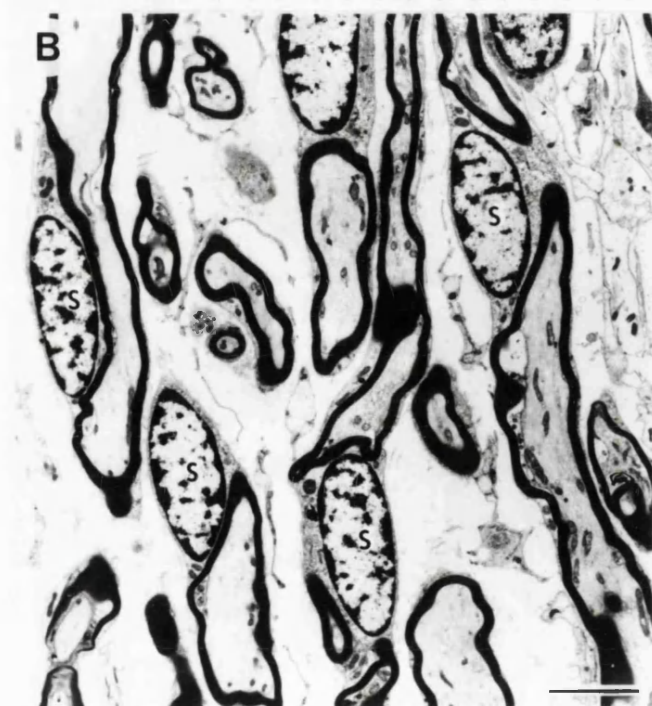
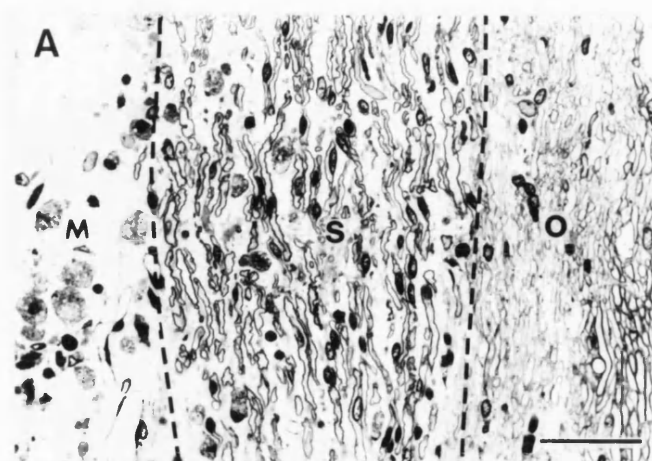


Figure 5.14

A schematic diagram summarising the main conclusions at long survivals. The zone of astrocytic scarring (ast) is coextensive with the zone of infiltration by Schwann cells (scw). Both cut and uncut axons passing from the areas of oligodendrocyte (oli) myelination (sheaths shown pale) acquire peripheral type, Schwann cell myelin sheaths (shown in black) as they enter the Schwann cell/astrocytic scar area. The massively expanded end of the cut axon (asterisk) is ensheathed, but not myelinated, by Schwann cells. Both cut and uncut axons emit fine sprouts into the macrophage-filled central lesion area (mac).

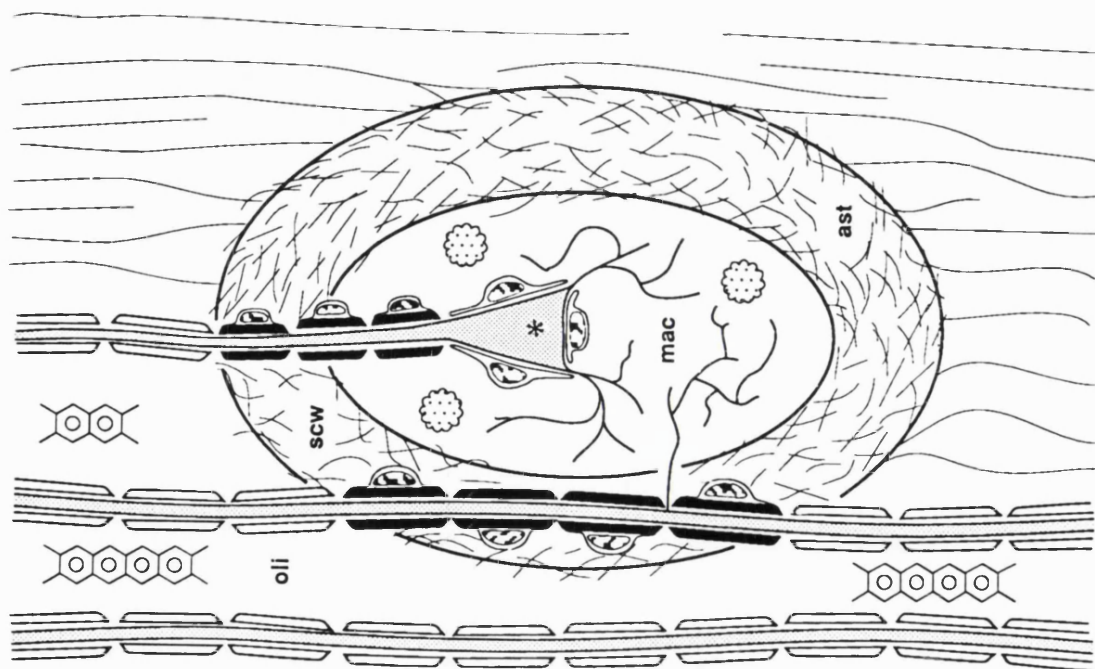
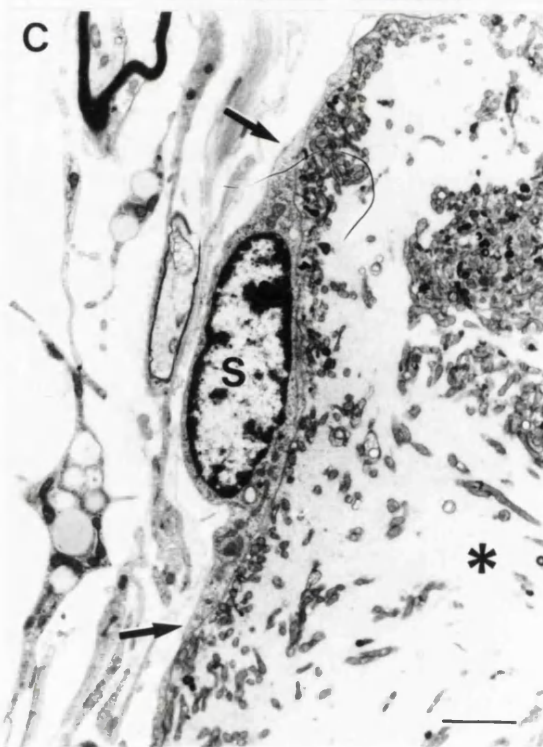
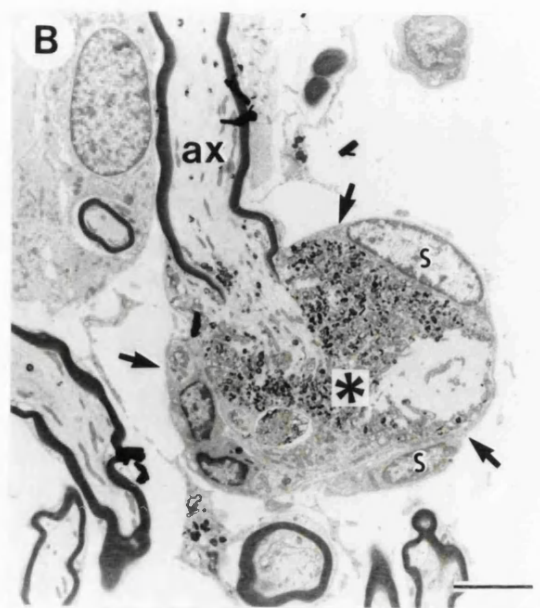


Figure 5.15

A,B: Horizontal electron micrographs showing cut ends of 2 corticospinal axons (ax) in the edge of the central lesion area, re-myelinated by endogenous Schwann cells (S) as far as the greatly expanded cut tips (asterisks) which are ensheathed by a fine rim (arrows) of dark cytoplasm belonging to satellitic Schwann cells stretched out around their circumference. **C:** Enlargement of the lower Schwann cell shown in A (arrow). Survival time, 4 months.

Scale bars = 5 μm (in A,B); 2.5 μm (in C).



layer of electron dense cytoplasm derived from Schwann cells applied directly to the axonal plasma membranes. The Schwann cell myelin had the typical dense appearance of peripheral myelin, with a wider periodicity than central myelin, and the sheaths were much thicker and in the present material better fixed than the oligodendrocytic myelin in the adjacent tract (Fig. 5.13).

5.4. Discussion

The present study differs from other studies where lesions were made in the spinal cord, in that (1) the electrode micro-lesion technique caused considerable less disturbance to the host tract architecture; (2) an anterograde tracing technique used was able to detect the fine morphological details of the responding cut and uncut host spinal axons. This resulted in being able to demonstrate that (a) the ascending axons as well as the descending corticospinal axons respond to the lesion, (b) the lesion is stimulatory for axon sprouting, and (c) considerable numbers of cut axons persist, without retraction at the level of the lesion even after the formation of an astrocytic scar.

5.4.1. Lesions stimulate axon sprouting

Initially the lesions caused the ends of the cut axon to swell and also produced en passant swellings in cut and uncut axons. After about 2 weeks, the uncut axons adjacent to the lesion had produced clusters of large

contiguous varicosities, and the ends of the cut axons had given rise to exuberant local sprouting, which was confined to the region of the cut ends and showed no sign of gaining access to the distal, undamaged regions of the host tracts. On the other hand, the greatly increased area of surface membrane provided by the luxuriant local terminal sprouting at the lesion site could provide an important expansion of surface receptors for growth factors. This suggests that the cut axons could go on to further regeneration if they could be provided with the appropriate stimuli and guidance.

Although it is well established that the cells of origin of the corticospinal tract and the ascending dorsal columns are not killed by axotomy at the spinal level (Kalil and Schneider, 1975; Lahr and Stelzner, 1990; Lieberman, 1971; Merline and Kalil, 1990). it has been reported that cut axons undergo retrograde degeneration (Kalil and Schneider, 1975), and retract from knife-cut lesions (Firkins *et al.*, 1993; Lahr and Stelzner, 1990). However, the axon tracing methods used in such studies were not optimal for detecting the responses at the cut ends of the axons. The present observations based on a less atraumatic lesion approach, combined with a morphologically more sensitive anterograde axonal label, show that cut axons do persist at the lesion site in considerable numbers.

5.4.2. Mechanism

The stimulatory effects of lesions, as also noted by Cajal (1928), may be

due to a number of factors, such as damage to astrocytes and oligodendrocytes, demyelination, the presence of degeneration products or the invasion of macrophages. Damage to the axon itself may be a stimulatory factor for cut axons, but the lesions also stimulated sprouting by uncut axons.

5.4.3. Persistence of cut axons and sprouts

With increasing survival times, and well after the formation of a dense astrocytic scar, both the cut and the uncut corticospinal axon sprouts remained in the lesion area, branching freely among the macrophages (Fig. 5.14). A similar persistence was described by Fishman and Kelly (1984), who reported corticospinal axons sprouting into the grey matter for at least 7 months, at the level of a spinal lesion. Van Den Pol and Collins (1994) used neurophysin immunoreactivity to give a superb demonstration of survival and sprouting of cut paraventriculo-spinal axons in the adult rat.

5.4.4. Retrograde degeneration

The fact that some axons survive axotomy does not exclude the possibility that others degenerate. The corticospinal tract lesions in the present experiments produced extensive 'Wallerian' degeneration of the distal segments of the cut axons which passed down the tract caudal to the lesion. However, the presence of a smaller, but definite beam of retrograde axonal degeneration extending back into the region containing the proximal segments of the cut axons rostral to the lesion suggests that there is also

some 'die-back' (Pallini *et al.*, 1988; Schnell and Schwab, 1993) affecting at least a proportion of the cut corticospinal axons.

Most authors consider that (in contrast to the neonate), the cortical neuronal cell bodies giving rise to the spinally projecting axons do not die after axotomy at the spinal level in the adult (Firkins *et al.*, 1993; McBride *et al.*, 1989; Merline and Kalil, 1990). It has generally been assumed that this ability of the corticospinal neurons to survive loss of the spinal parts of their axons is related to the large number of 'sustaining' collaterals (Cajal, 1928) which are emitted by these axons at supraspinal levels (O'Leary *et al.*, 1990), and which remain intact after lesions at the spinal level. In a recent study, however, Dusart and Sotelo (1994) reported that cerebellar Purkinje cells survived for long periods after their axons had been cut so close to the cell body that no collaterals remained.

5.4.5. Ingrowth of Schwann cells

A characteristic of the present long term lesions was the ingrowth of Schwann cells. It has been extensively documented that the migration of endogenous Schwann cells into the parenchyma of the central nervous system (and their subsequent myelination of central axons) is induced by chemotoxic demyelinating lesions (Blakemore, 1976; Blakemore and Crang, 1989; Harrison, 1987), by prevention of developmental myelination by destruction of oligodendrocytic precursors by neonatal X-irradiation (Gilmore and Duncan, 1968; Gilmore and Sims, 1993; Sims and Gilmore, 1983), by

kainate lesions of the thalamus (Dusart *et al.*, 1992), and by transplantation of genetically modified fibroblasts into the spinal cord (Tuszynski *et al.*, 1994b). The present study confirms these reports, and demonstrates that the ingrowing Schwann cells re-myelinated demyelinated segments of spinal axons. In the present material both cut and uncut corticospinal tract axons were re-myelinated by Schwann cells, and the large expanded ends of the cut axons were ensheathed by Schwann cells (Figs. 5.14,15).

The observation (Sims and Gilmore, 1994) that the endogenous Schwann cells which invade neonatally X-irradiated spinal cord provide pathways for the ingrowth of regenerating dorsal root axons is evidence that ingrowing Schwann cells have axogenic properties. Schwann cells are known to synthesize many neurotrophic molecules, as well as surface and extracellular matrix adhesion molecules which promote neurite growth (Paño *et al.*, 1994), and it has recently been reported that the Schwann cell myelin protein P₀ promotes neurite elongation in the partially transected spinal cord (Yazaki *et al.*, 1994).

Transient sprouting of cut axons also occurs after lesions in other tracts in the brain (Cajal, 1928), where ingrowth of endogenous Schwann cells has not generally been reported, but possibly the presence of endogenous Schwann cells which had migrated into the lesion area in the present corticospinal tract lesions may help to maintain the long term persistence of cut axon sprouts.

5.4.6. Importance of the long term persistence of cut axon sprouts in a spinal lesion site

The present observations that cut axons can persist and maintain massive localised sprouting even after formation of a dense astrocytic scar is highly encouraging for the future development of localised interventions designed to induce regenerative growth of the cut axons. In terms of the possibility of future repair, it has been suggested that cut axons can be induced to elongate by local application of those specific growth factors to which they have receptors (e.g. Magal *et al.*, 1993; Tuszynski *et al.*, 1994a,b).

5.5. Summary

Lesions were made with a stainless steel electrode by passing DC current in either the ascending dorsal column tracts or descending corticospinal tract of adult rat spinal cord at the level of the first two spinal segments. The responses of the host axons to the lesions were studied by the Palmgren silver stain and anterograde transport of HRP, biocytin or BD. The Palmgren silver stain was used for short term lesions (within 2 days after operation), and anterograde labelling was used for the lesions from 4 days to 13 weeks. The tracer was micro-injected into the dorsal columns at a distance of 1-2 spinal segments below the lesions to label the ascending axons, or injected into the pyramidal tract at the base of the medulla to label the descending corticospinal axons.

The Palmgren silver stain showed that within 2 days after the lesion, the ends of the cut axons were swollen, the central lesion area contained many degenerating fibres which separated from the tract axons. The lesions also induced en passant varicosities in the adjacent uncut axons.

2 to 6 weeks after the lesion, anterograde labelling showed that both cut and also adjacent uncut axons in both ascending and descending tracts responded to the lesions. The cut ends were expanded into a variety of shapes - large, complex bulbous and recurved and many had profuse local branches with or without small, terminal-type varicosities. The swollen ends of the cut and uncut axons, emitted extensive arborisations of sprouts which could be seen either in the macrophage-filled lesion centre or in the area surrounding the lesion centre.

At increasing times after the lesions, immunostaining of GFAP showed that compared with the original framework of longitudinal astrocytic processes characteristic of the normal corticospinal tract, the astrocytes in the lesioned corticospinal tract underwent a progressive hypertrophy which spread from the lesion, increasing in intensity from 1 week after the lesion. By 7 weeks, the astrocytic scar started to form around the lesion area and the lesion area was completely surrounded by a dense astrocytic scar at 13 weeks.

When the astrocytic scar was heavily formed at 9.5 and 13 weeks, anterograde labelling BD was used to examine whether sprouts from the cut

corticospinal axons were still remaining in the lesion area. BD labelling showed that the sprouts of cut corticospinal axon persisted in the macrophage-filled lesion centre and also in the area of the astrocytic scar at 13 weeks after the lesion. There was no obvious morphological differences between the sprouts seen before and after the scar formation.

The EM results showed that there were only a few corticospinal axons that were re-myelinated at 3 weeks (with peripheral myelin formed by endogenous Schwann cells which had migrated into the lesion area). By 4 months the region of the astrocytic scar was densely colonised by host Schwann cells, which now had re-myelinated a wide swathe of both cut and uncut axons. The cut axons were re-myelinated by Schwann cells as far as their large terminal expansions, which were sheathed, but not myelinated, by satellitic Schwann cells. Thus, at survivals long enough for the formation of a dense, astrocytic scar, cut corticospinal axons retain extensive terminal and collateral arborisations even in the macrophage-filled central lesion area, and are re-myelinated or ensheathed by endogenous Schwann cells.

**CHAPTER VI SPROUTING INDUCED BY TRANSPLANTATION
OF SCHWANN CELLS INTO THE ASCENDING AND
DESCENDING TRACTS OF THE ADULT RAT SPINAL CORD**

6.1. Introduction

Peripheral nerve transplants are able to induce growth of cut adult central axons and to direct the growing sprouts to distant terminal fields where they can make structural and functional synaptic connections with appropriate targets (Berry *et al.*, 1988a; Campbell *et al.*, 1992; Carter *et al.*, 1989; Keirstead *et al.*, 1989; Knoop *et al.*, 1993; Thanos, 1992). There are a number of limitations to this approach. Apart from the inevitable mechanical damage caused by the physical insertion of the nerve, the most favourable transplants only pick up a proportion of the host fibres, the axons leaving the transplants show only restricted penetration of the central target tissues (Vidal-Sanz *et al.*, 1987, 1991), and abnormal connections may be formed (Zwimpfer *et al.*, 1992). The transplants are selective for certain categories of axons (Benfey *et al.*, 1985; Morrow *et al.*, 1993), and it has been reported that other types of axons, such as the corticospinal tract, (Richardson *et al.*, 1982, 1984) may not respond.

Even if the above limitations could be overcome, peripheral nerve 'bridges' would not offer a complete solution to spinal tract lesions: the long ascending and descending spinal tracts recruit axons from many different sites, at all levels of the brain and spinal cord, and deliver them to targets which are no less dispersed. Therefore, it is not physically possible to devise peripheral nerve bridges which would selectively and correctly reconnect all the different components of such a dispersed system of

connections. Repair would occur if after bridging the lesion, the regenerating axons were able to regrow correctly along their original trajectories. One aim for a bridge would be to pick up axons from different sources, convey them across the lesion, and then allow the different groups of axons to re-enter the host tracts and complete the remainder of their courses through those tracts.

It has been shown that the 'axogenic' effects of peripheral nerve transplants depend on the presence of viable Schwann cells in the nerve (Berry *et al.*, 1988a,b; Hagg *et al.*, 1991; Paíno and Bunge, 1991; Smith and Stevenson, 1988). Consequently, a number of workers have investigated the transplantation of cultured Schwann cells (Kromer and Cornbrooks, 1985; Kuhlengel *et al.*, 1990a; Martin *et al.*, 1991, 1993; Montero-Menei *et al.*, 1992; Neuberger *et al.*, 1992; Paíno and Bunge, 1991; Wrathall *et al.*, 1984). These transplantation techniques cause less physical damage to the host tract than transplanting a piece of peripheral nerve. The present study has attempted to decrease the operative damage further by using a pulsed air pressure delivery technique (Emmett *et al.*, 1990) to micro-transplant small volumes of a suspension of cultured peripheral nerve Schwann cells through a stereotaxically guided glass micro-pipette (Brook *et al.*, 1993) into circumscribed locations in the ascending tracts of the dorsal columns or descending corticospinal tracts in the adult rat spinal cord.

Most previous studies of spinal axon regeneration have used retrograde cell labelling (e.g. Richardson *et al.*, 1982, 1984), which is only suitable if the axons have travelled for some distance from the lesion in a peripheral nerve or other transplant, or axon identification by immunohistochemistry (Martin *et al.*, 1991, 1993) or by anterograde labelling (Schnell and Schwab, 1993) using the TMB reaction for the HRP. In the present study the anterograde transport of HRP or biocytin was used to identify the ascending and descending axons. Compared with the coarse crystals formed by the TMB method, the Co-GOD detection method made it possible to obtain much finer details of the responses of the cut and uncut spinal axons to the transplanted Schwann cells, and therefore to re-examine the apparent failure of corticospinal axons to respond to Schwann cells in peripheral nerve transplants (Richardson *et al.*, 1982, 1984), and whether the corticospinal axons can sprout in response to contact with Schwann cells.

6.2. Materials and methods (For details see *CHAPTER II*)

6.2.1. Donor cells

Donor Schwann cells were obtained from sciatic nerves of 2 day postnatal rats of the AS strain. The cell suspension was made at a final concentration $1.0-1.5 \times 10^7$ cells/ml.

6.2.2. Transplantation

$1.0-1.5 \mu\text{l}$ of cell suspension (approximately 10-15,000 cells) was injected

into the ascending dorsal column tracts or descending corticospinal tract of the adult AS rat spinal cord at the level of the first two cervical segments by the pulsed air pressure injection system.

6.2.3. Anterograde labelling

The responses of the ascending and descending axons to the transplanted Schwann cells were studied by labelling axons using anterograde transport of HRP or biocytin. To label the descending corticospinal axons, tracer was injected into the pyramidal tract at the level of the medulla. 31 animals with transplanted Schwann cells were used to study descending axons with survival times of 2 days (n = 3), 4 days (n = 3), 6 days (n = 3), 8 days (n = 3), 10 days (n = 3), 12 days (n = 3), 16 days (n = 3), 20 days (n = 3), 35 days (n = 4) and 45 days (n = 3).

To label the ascending axons, tracer was injected into the dorsal columns at a distance of 1-2 vertebral segments below the Schwann cells transplants. 11 animals were used to study the ascending axons with survival times of 6 days (n = 3), 12 days (n = 4) and 21 days (n = 4).

Serial 60-100 μm horizontal sections through the entire transplant were cut on a freezing microtome or Vibratome. The sections of the spinal cord with the HRP injections were detected with the Co-GOD method and the sections of the spinal cord with injections of biocytin were incubated overnight in the ABC solution, followed by detection with the Co-GOD method.

6.2.4. p75 Immunohistochemistry

The transplanted Schwann cells were identified with p75 immunostaining. 12 animals with transplanted Schwann cells in the descending corticospinal tract were allowed to survive for 1 week (n = 3), 2 weeks (n = 3), 4 weeks (n = 3) and 6 weeks (n = 3). 5 animals with transplanted Schwann cells in the ascending tracts were allowed to survive for 1 week (n = 3) and 2 weeks (n = 2). 10 μ m horizontal cryostat sections were used for the immunostaining.

6.3. Results

6.3.1. Morphology of the transplants

p75 immunostaining showed that transplanted donor Schwann cells survived well in both ascending and descending tracts of the dorsal columns. The transplants formed ovoid masses (e.g. Figs. 6.1,2,3) of around 0.5 mm in diameter and around 1 mm in length in both ascending and descending tracts. Most of the transplanted donor cells were p75-positive. The Schwann cells formed a central cluster from which cells migrated out as cuffs along the numerous new blood vessels formed in the transplant region.

6.3.2. Responses of the host axons to the transplanted Schwann cells

The micro-injection technique not only introduced donor Schwann cells into the host tracts, but also damaged host axons at the injection site. The

Figure 6.1

Low power view of mass of Schwann cells transplanted into the upper cervical level of the right corticospinal tract (CST), immunostained for P75. Arrow, typical perivascular flare of Schwann cells leaving the graft to ensheath a blood vessel. gm, grey matter; m, midline. Horizontal cryostat section, p75 immunostain with thionin counter-staining. Survival time, 7 days.

Scale bar = 200 μ m.

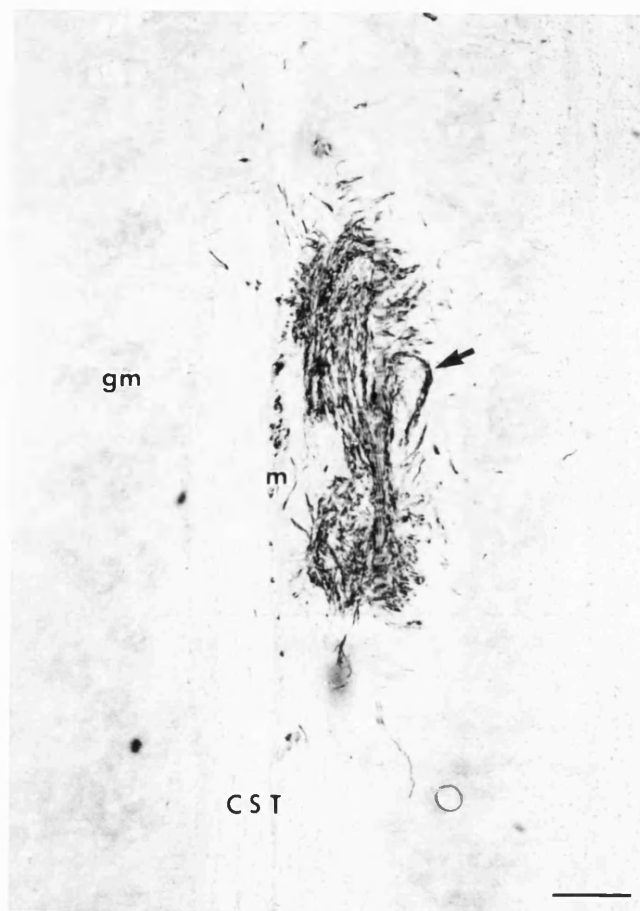


Figure 6.2

Biocytin labelled corticospinal axons (CST) to show the Schwann cell transplant (SC) with a hot spot (arrow) containing a knot-like mass of tortuous segments (camera lucida drawing in Fig. 6.3A). 100 μm horizontal Vibratome section, detected with the Co-GOD method and counter-stained with neutral red. Survival time, 5 days.

Scale bar = 50 μm .

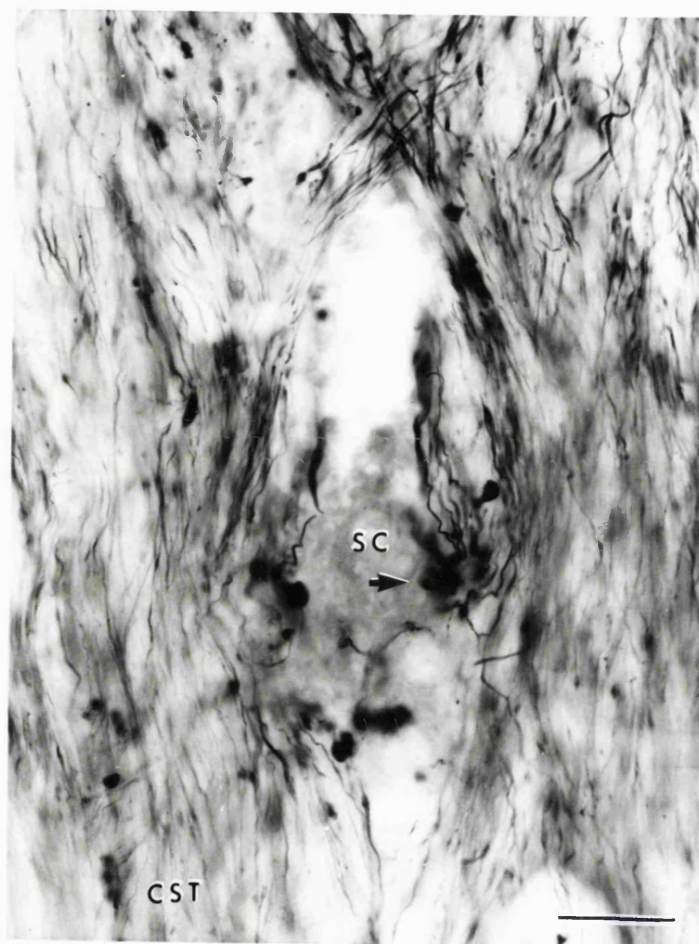
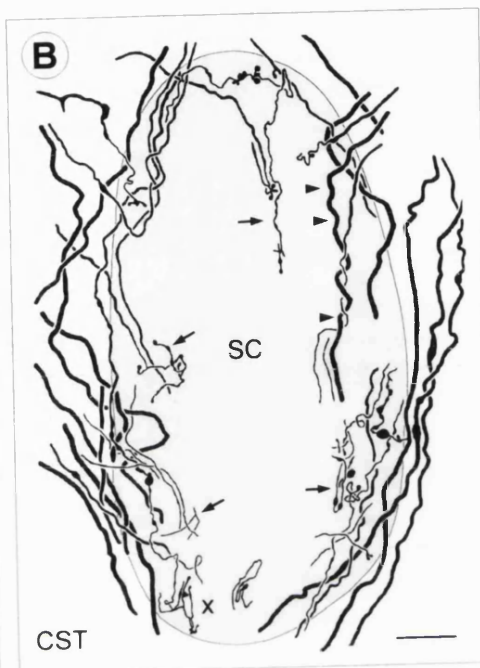
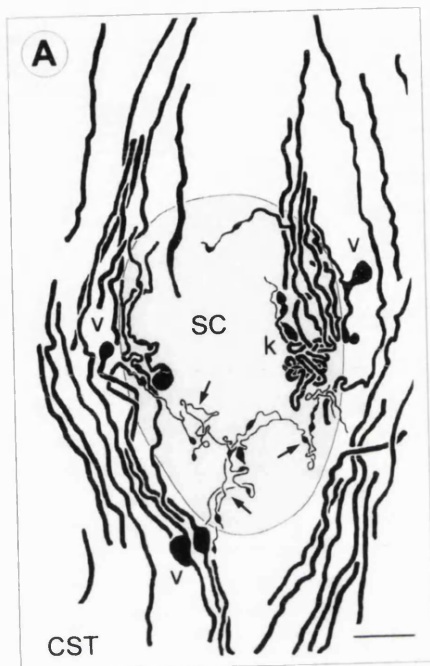


Figure 6.3

A: Camera lucida drawing of biocytin labelled corticospinal axons in of Fig 6.2 to show varicosities (v), a knot-like mass of tortuous segments (k), and fine terminal plexus-like extensions (arrows) into the Schwann cell transplant (SC). Survival time, 5 days.

B: Camera lucida drawing of HRP labelled corticospinal axons to show the invasion of the superficial area of a Schwann cell graft by fine, terminal plexus-like axons (arrows; x is shown enlarged in Fig. 8B,D). Arrowheads, a bundle of uncut corticospinal axons crossing the surface of the graft. Survival time, 8 days.

Scale bars = 25 μ m.



Schwann cells also had effects on uncut axons, which could be traced in continuity through the host tract beside and beyond the transplant region (e.g. Fig. 6.4B). Anterograde labelling showed that both ascending and descending axons responded to the transplanted Schwann cells. In both cut and uncut host axons, the transplanted Schwann cells induced branching (Fig. 6.5A,C); re-orientation (loops and tortuosity); and changes in diameter (narrowing and varicosities; Fig. 6.5A).

6.3.3. Branching

The host corticospinal axons (for discussion of the identification of the descending axons see *CHAPTER II*) started responding to the transplanted Schwann cells from 2 days after transplantation. Branching occurred very rapidly and was well established by 2 days after transplantation (the earliest time point studied, since 48 hrs was needed for the anterograde transport of the HRP tracer). Labelled host axons gave rise to one or a number of branches (Fig. 6.5A,C). When the branch points were multiple, they were usually separated by 50-100 μm segments of unbranched parent axon, suggesting that the sprouts might be arising at the nodes of Ranvier of the myelinated axons (e.g. Fig. 6.6A; Tomatsuri *et al.*, 1993). The branches were usually associated with varicosities or tortuosity of the parent axon, and could consist of small, short spurs (Figs. 6.5C,6B,C) or could be at least 100 μm long (e.g. Fig. 6.5). In many cases the axons narrowed, or the branches were very fine (e.g. Figs. 6.6,7), and second and third order branching occurred (e.g. Figs. 6.5C,7).

Figure 6.4

A: Camera lucida drawing of a bundle of corticospinal axons gives rise to three fine, tortuous varicose axons (arrow) which loop into the surface of a Schwann cell transplant (SC). CST, Corticospinal tract; Survival time, 5 days.

B: Camera lucida drawing of a single, uncut corticospinal axon gives rise to a multiple varicose segment (v) which loops into the surface of an adjacent Schwann cell transplant. Survival time, 8 days.

C: Camera lucida drawing of a terminal plexus-like array (x) of fine, tortuous, varicose axons (one of which - arrow - is a branch of an apparently uncut corticospinal axon) invading the surface of a Schwann cell transplant. Survival time, 5 days.

100 μm horizontal frozen sections, labelled with HRP and detected with the Co-GOD method, counter-stained with neutral red.

Scale bars = 25 μm .

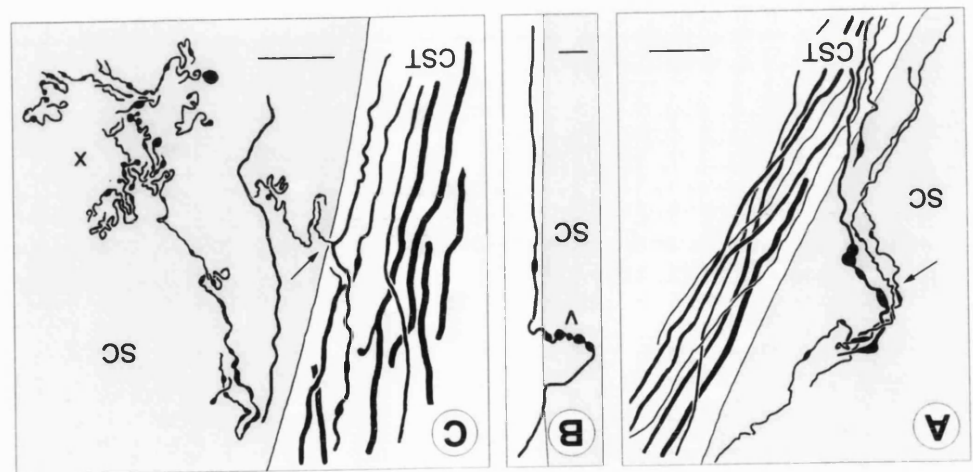


Figure 6.5

Camera lucida drawings of HRP-labelled axons in Schwann cell transplants in the corticospinal tract.

A: A corticospinal axon (x) whose main stem (s) gives rise to a fine, parallel varicose branch (arrows). Survival time, 5 days.

B: A fine corticospinal axon (presumed newly formed) with small varicosities gives rise to a knot-like segment (k) from which arises a small, stalked, preterminal bouton-like structure (x) before continuing as a fine straight segment (p) parallel to a large (presumed uncut) corticospinal axon with large varicosities. Survival time, 8 days.

C: A varicose corticospinal axon gives rise (arrow) to several branches with a strong tendency to fasciculate (f). Survival time, 8 days.

Scale bars = 25 μm .

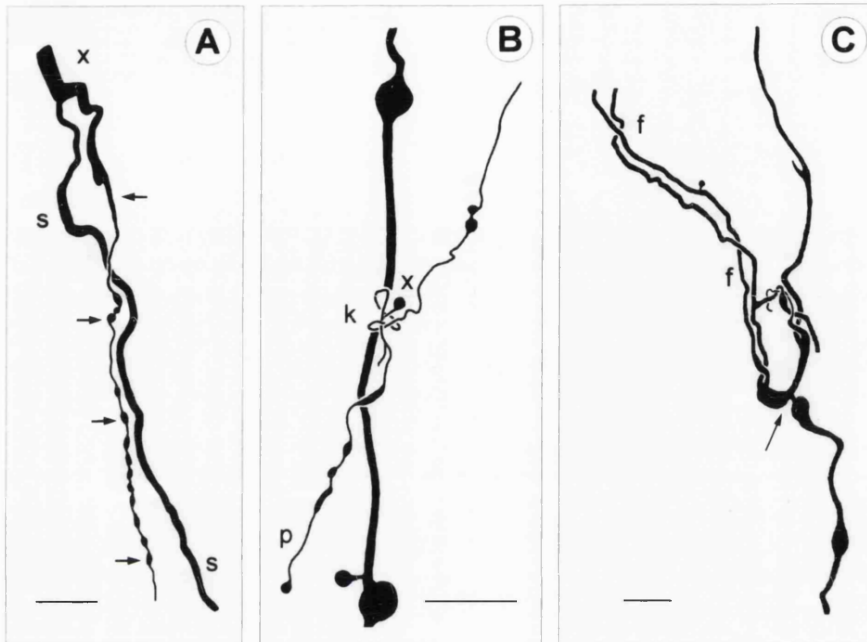


Figure 6.6

Camera lucida drawing of HRP labelled axons to show the morphological stages of axon growth of ascending dorsal column axons in Schwann cell transplants.

A: Cut end showing multiple varicose branching (arrows). **B:** Probable uncut axon (u) with a short, spur-like branch (s), and a long parallel (presumed newly formed) branch ending in a narrow segment (arrow) bearing a small terminal varicosity. **C:** A cut ascending axon with 2 short, spur-like branches (s) and a short-stalked varicosity (v) progressively narrows into a fine, increasingly tortuous and varicose terminal segment (arrow) ending in a small varicosity. **D:** A HRP labelled axon in the ascending dorsal column tract. 100 μm horizontal frozen sections, labelled with HRP and detected with the Co-GOD method, counter-stained with neutral red. Survival times, A,C 6 days, B,D 13 days.

Scale bars = 25 μm .

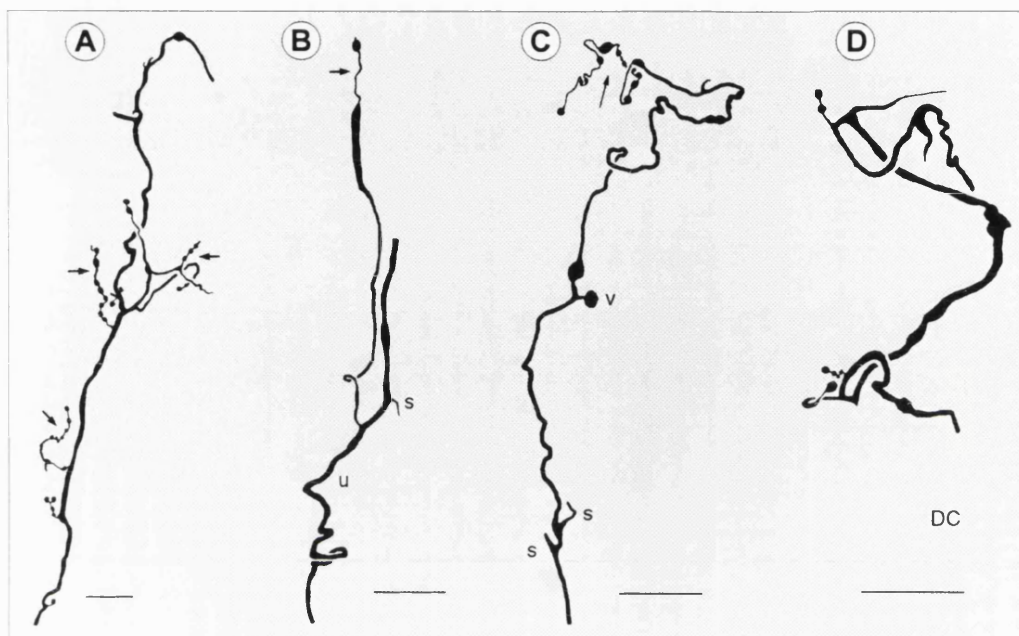


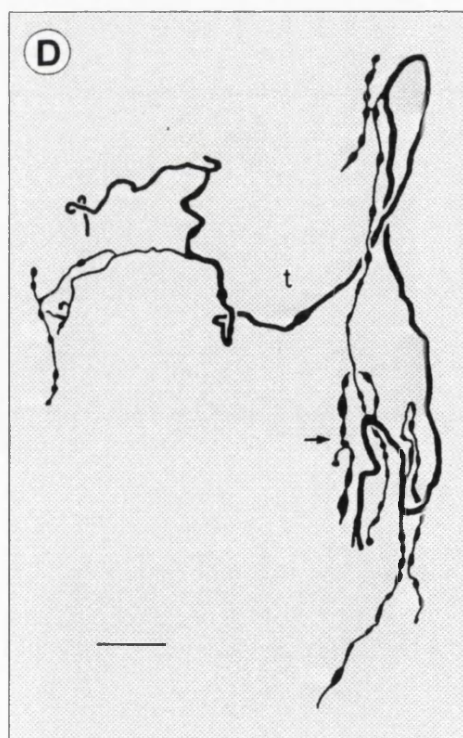
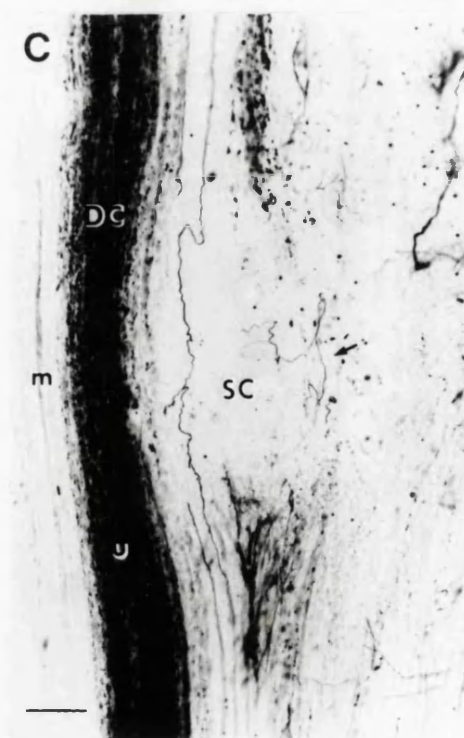
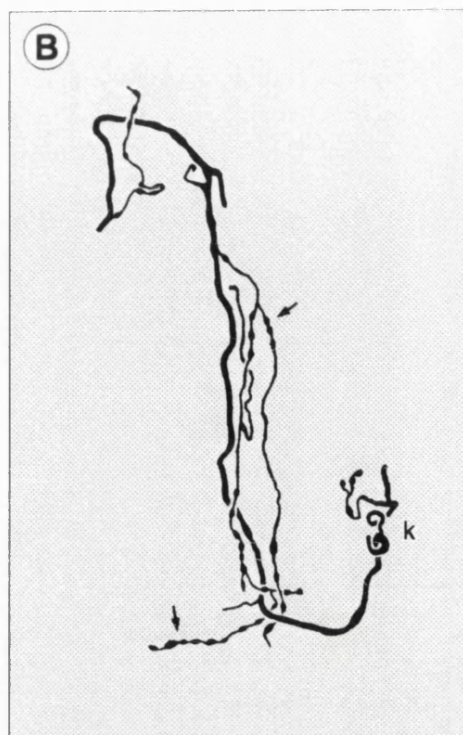
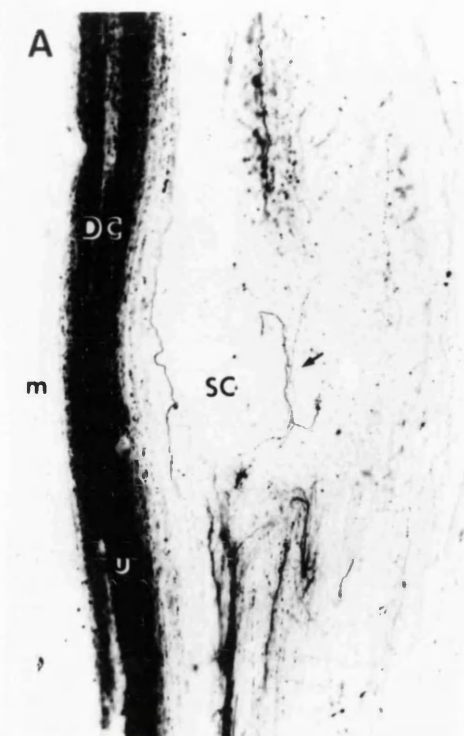
Figure 6.7

A, C: HRP labelled cut and uncut ascending dorsal column axons. Medially is a dense band of HRP-labelled, uncut axons (u); laterally, some of axons cut by the Schwann cell transplant (SC) gives rise to a multiply branched axonal sprout (arrow). m, midline; DC, dorsal column.

Scale bar = 100 μm .

B, D: Camera lucida drawing of the sprouting axon from A and C to show a knot-like mass of tortuous segments (k), transverse turning (t) and varicose dichotomous branching (e.g. arrow). 100 μm horizontal frozen sections, labelled with HRP and detected with the Co-GOD method, counter-stained with neutral red. Survival time, 6 days.

Scale bar = 25 μm .



The initial orientation of the branches tended to follow the direction of the parent axon - i.e. rostrally for the ascending axons (Fig. 6.6A,B) and caudally for the descending axons (Fig. 6.5A). After some initial searching, all axon segments tended to take up a longitudinal rostro-caudal arrangement, and branches from a single parent stem often fasciculated closely with each other (e.g. Fig. 6.5C), with the parent axon, and with other adjacent axons. They were presumably also in parallel with the longitudinal astrocytic processes of the host tract glial skeleton (Davies *et al.*, 1994). The diameter of the branches seemed to depend on the host tract, the wider diameter ascending dorsal column axons produced larger arborisations (Fig. 6.7) than the finer, descending corticospinal axons (e.g. Fig. 6.3).

6.3.4. Re-orientation (loops and knots)

Many labelled horizontal sections showed that segments from uncut axons in the host tract looped into the Schwann cell transplants, passed through them, and returned again to continue their caudal course in the host tract (Fig. 6.4A,B). These segments often showed many varicosities, although the varicosities were separate from each other. Very fine axons often made multiple local changes of direction, so that they formed knot-like segments (Fig. 6.5B) before taking up a final, straight course, parallel to the rostro-caudal longitudinal axis of the tract.

6.3.5. Changes in diameter (narrowing and varicosity)

Axons narrowed down to very fine segments which formed a terminal-like plexus in the Schwann cell transplants (Figs. 6.3B,4C). The branched terminal segments were very tortuous, with many tiny varicosities, similar to the presynaptic boutons found in their normal terminal fields (see below). Where the structure could be unambiguously identified to form the free end of a sprout (i.e. it was located deep within a section, and not cut on its way to adjacent sections) it consisted either of a varicosity, or a fine, un-expanded free sprout. Terminal-like plexuses did not occur uniformly over all the interface, but tended to cluster in 'hot spots' (arrows in Figs. 6.2,3).

Morphological differences between the terminations of the ascending and descending axons were reflected in their responses to Schwann cell transplants. Thus, the ascending dorsal column axons tended to have more branches, at wider angles, and larger arborisations, and varicose dichotomous terminal branches, as seen in the terminal field in the dorsal horn (cf. Fig. 3.1B,C; Fig. 6.7). In contrast, the descending corticospinal axons were much finer, and formed masses of tight curls (e.g. Fig. 3.1A; Figs. 6.3,4C) with small presynaptic bouton-like varicosities which resembled the normal corticospinal arborisations in the medial spinal grey matter (Fig. 6.8).

In the material with longer survival times after transplantation (Fig. 6.9), a reduced number of fine tortuous axonal segments were still present, and the

Figure 6.8

Comparison of the mode of branching of a normal corticospinal axon terminating in the medial part of the spinal grey matter (gm) adjacent to the corticospinal tract (CST; box in A, enlarged in C) with the terminal-like branching of a corticospinal axon (box in B, enlarged in D) entering a Schwann cell transplant (SC; low power in Fig. 3B). Both axons narrow into branched terminal segments bearing en passant or terminal varicosities. Note the disorganisation of the corticospinal axons in the transplanted material (B) compared with the normal parallel tract (A). Survival time, 8 days.

Scale bar = 25 μm .

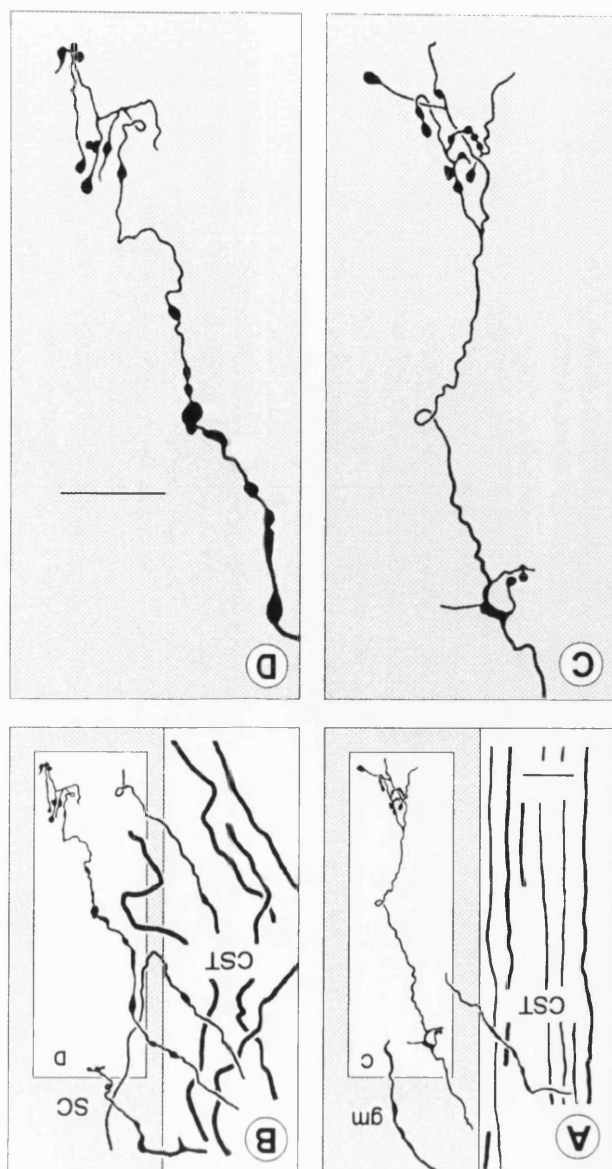
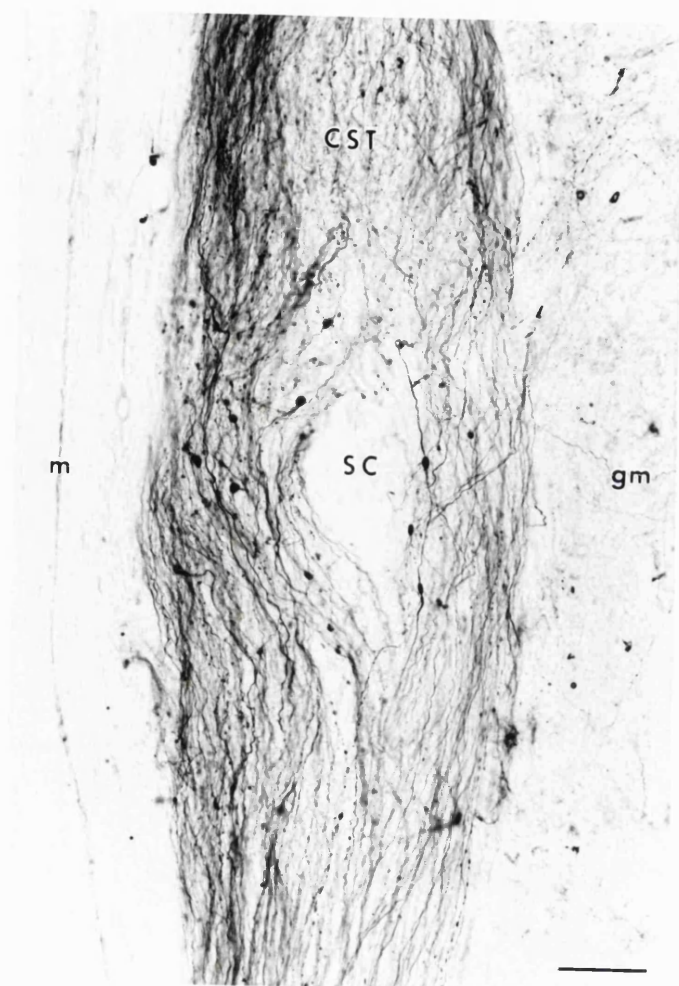


Figure 6.9

Photomicrograph of long term Schwann cell transplant (SC) to show a reduced number of fine tortuous axonal segments are still present and surrounded by axon varicosities in the corticospinal tract (CST). m, midline; gm, grey matter. 100 μ m horizontal frozen sections, labelled with HRP and detected with the Co-GOD method, counter-stained with neutral red. Survival time, 21 days.

Scale bar = 100 μ m.



axons retained their varicosities. Unlike the long term lesioned material, the varicosities remained spaced singly on the axons. In some cases the islands of surviving Schwann cells were surrounded by looped varicose axons (Fig. 6.10). The branches, however, were rarely seen after about 2 weeks. This may be due to retraction; alternatively, the axons may have elongated, and the branches extended into and through the adjacent normal segments of the tract, so that the branch points would have become dispersed and difficult to find among the normal parallel host fibres.

6.4. Discussion

In comparison with previous reports of peripheral nerve or cultured Schwann cell transplants into the spinal cord, the main differences in the present study were (1) introduction of a micro-transplantation technique which caused minimal disturbance to the host tract, and (2) use of an anterograde tracing technique which was able to detect the fine morphological details of the responses of both cut and uncut host axons. It was, therefore, possible to show that both the ascending axons of the dorsal columns and also descending corticospinal axons respond to the presence of transplanted Schwann cells.

Compared with previous experiments (see also Paíno and Bunge, 1991), the present study showed that there was branching and the formation of terminal field-like plexuses. These were characteristic responses to the

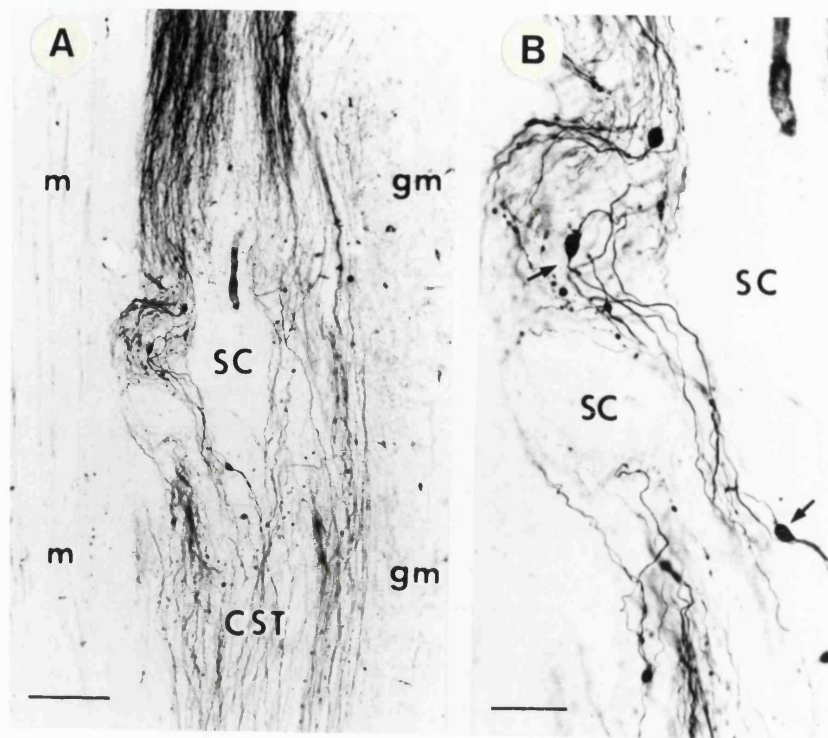
Figure 6.10

A: Photomicrograph of a long term Schwann cell transplant (SC) to show that a reduced number of fine tortuous axonal segments are still present, and the axons have retained their varicosities. The transplant is surrounded by looping varicose corticospinal axons (arrow in B). CST, corticospinal tract; gm, grey matter; m, midline.

Scale bar = 100 μm .

B: High power view of A. 100 μm horizontal frozen sections, labelled with HRP and detected with the Co-GOD method, counter-stained with neutral red. Survival time, 21 days.

Scale bar = 50 μm .



Schwann cell transplants. The Schwann cell effects were rapid in both the ascending and descending tracts, and the sprouts were much longer than those in the lesion experiments (*CHAPTER V*). The Schwann cell transplants induced far greater, and more persistent tortuosity of the uncut host axons than lesions alone, and the surface of the Schwann cell transplants was invaginated by an abundance of very thin axon segments which were either straight or gave rise to masses of densely curled fibres with small en passant or terminal spherical expansions. These resembled presynaptic boutons (although there was no indication of any tissue element providing a postsynaptic structure). In many cases the branches passed through knot-like convolutions, but ultimately extended in parallel with existing tract axons and fasciculated with them. The reduced incidence of these structures in the longer term Schwann cell transplants may have been due to maturation and elongation of the branches, although we cannot exclude that they may have retracted.

Several authors, using both peripheral nerve transplants (Richardson *et al.*, 1982, 1984) and transplantation of cultured Schwann cells (Kuhlengel *et al.*, 1990b; Martin *et al.*, 1991, 1993) have detected a sprouting response in the ascending dorsal column branches of the centrally projecting axons of the dorsal root ganglion cells by either retrograde cell labelling (Kuhlengel *et al.*, 1990b; Richardson *et al.*, 1982, 1984) or immunohistochemistry (Martin *et al.*, 1991, 1993). Using the morphologically more sensitive anterograde HRP labelling technique, the present study clearly showed that corticospinal

axons can also respond to intraspinal Schwann cell transplants, although they are perhaps unable to extend sufficiently along peripheral nerve transplants to be detected by retrograde tracers applied at some distance from the transplant site.

Mechanism The stimulatory effects of transplanted peripheral nerve or cultured Schwann cells on cut central axons are well established (references above). The present study suggests that transplanted Schwann cells also provide powerful stimuli for the sprouting of uncut axons.

Compared with the descending axons, the more exuberant sprouting which Schwann cell transplants induced in the ascending axons may be partly due to their larger diameter (and/or the proximity to the cell bodies). On the other hand, it should be remembered that, whereas corticospinal axons never normally come into contact with Schwann cells, the ascending dorsal column sensory fibres are the central branches of dorsal root ganglion cells which are normally apposed by Schwann cells along their entire peripheral branches, their cell bodies, and the first parts of their centrally directed branches in the dorsal roots. It would not therefore be unexpected if their cut central branches were particularly responsive to contact with Schwann cells.

It has been reported that Schwann cells can secrete a number of growth factors which act on neurons (e.g. Bandtlow *et al.*, 1987; Leibrock *et al.*,

1989; Meyer *et al.*, 1992; Neuberger and De Vries, 1993), and these would presumably be part of the mechanism of the sprouting has been observed in the present experiments. Despite the overlapping specificity in growth factors and their receptors (for a review see Korsching, 1993), different types of nerve cells do show cell-type specific differences. Although it has been reported that peripheral nerve transplants may be selective for particular types of axons (Benfey *et al.*, 1985; Morrow *et al.*, 1993; Richardson *et al.*, 1982, 1984), the present observations show that both ascending dorsal column axons and descending corticospinal respond to injections of Schwann cells.

6.5. Summary

A suspension of Schwann cells, cultured from neonatal sciatic nerve, was used for transplantation. 1-1.5 μ l of cell suspension at final concentration $1.0-1.5 \times 10^7$ cells/ml was injected into the ascending or descending tracts of the adult rat spinal cord at the level of the first two cervical segments through a glass micro-pipette by the pulsed air pressure injection system. The transplanted donor Schwann cells were identified with immunostaining of p75. The responses of the ascending and descending axons to the transplanted Schwann cells were studied by anterograde labelling with HRP or biocytin into the pyramidal tract or the dorsal columns.

p75 immunostaining showed that transplanted donor Schwann cells survived well in both the ascending and descending tracts. The transplants formed ovoid masses of around 0.5 mm diameter and around 1 mm length in either the ascending or descending tracts. Most transplanted donor cells were p75-positive, and these p75-positive Schwann cells migrated out from the central cell cluster of the transplant as cuffs along the blood vessels of the transplant region.

The anterograde labelling showed that both ascending and descending axons sprouted in response to the transplanted Schwann cells. The responses of the host axons to the Schwann cells were much more rapid than to lesions alone (*CHAPTER V*). The descending corticospinal axons started to sprout from 2 days after transplantation. The axons which had been cut at the time of the transplantation and adjacent uncut axons gave rise to the branches in both the ascending and descending tracts. Unlike the localised sprouting seen after the long term lesions (*CHAPTER V*), these branches extended for considerable distances parallel to and fasciculating with each other and with the host tract axons. In addition, a mass of fine, tortuous, varicose branches invaded the superficial parts of the Schwann cell transplants, where they formed arborisations with small bead-like expansions resembling presynaptic boutons. As in their normal terminal fields, the arborisations formed by the corticospinal axons were smaller and finer than those formed by the ascending axons.

The physical implantation of the Schwann cells would in itself cause some degree of damage. Since (as the previous chapter showed) sprouting occurred after electrolytic lesions with no Schwann cell injections, it was necessary to control for the lesion effect in the Schwann cell transplants. Because the electrolytic lesion was of a different character to the physically expansive effect of Schwann cell implantation, a further series of 2 controls was prepared by injection into the same site of 1.5 - 2.0 μ l of DMEM without Schwann cells.

10 days later these 2 cases had very few varicosities (5-10 - i.e. less than 1% of what is seen after Schwann cell transplants), and occasional short, simple sprouts (less than 100 μ m), which were totally different from the long, branched, varicose arborisations seen after Schwann cell transplantation.

In contrast to previous studies claiming that corticospinal axons do not elongate into peripheral nerve transplants (Richardson *et al.*, 1982), the present results showed that the axons in both ascending tracts of the dorsal columns and descending corticospinal tract sprouted in response to contact with the transplanted Schwann cells.

**CHAPTER VII INTEGRATION OF TRANSPLANTED SCHWANN
CELLS INTO THE CORTICOSPINAL TRACTS OF THE ADULT
RAT SPINAL CORD**

7.1. Introduction

The study in *CHAPTER III* described the basic normal glial structure of the ascending and descending tracts of the dorsal columns of the adult rat spinal cord. The unicellular glial rows in the spinal tracts contained the cell bodies of oligodendrocytes, astrocytes and microglia. Astrocytes were interspersed singly between 4-8 contiguous oligodendrocytes, and microglia were similarly interspersed between 4-6 oligodendrocytes. There are also other earlier studies which have investigated the structure of central fibre pathways (Bayer and Altman, 1975; Lin and Connor, 1989; Matthews and Duncan, 1971). Suzuki and Raisman (1992) have shown a similar glial framework in the fimbria. The fimbria has a skeleton made up of rows of glial cell bodies in which groups of 5-10 contiguous oligodendrocytes are interspersed by solitary astrocytes. These rows of cell bodies are surrounded by regions consisting of densely packed myelinated and unmyelinated axons into which both astrocytes and oligodendrocytes project a regular array of radial and longitudinal processes.

This adult glial structure is permissive to the rapid growth of axons from embryonic neurons transplanted into the tracts of the spinal cord by the micro-injection technique that causes minimal disturbance to the glial framework of the host tract (see *CHAPTER IV*). Under these circumstances the newly growing axons follow the longitudinal axis of the host tract, intermingled with the host axons and glial processes.

It has been demonstrated that transplanted cultured Schwann cells are able to induce regeneration of cut central axons (Kromer and Cornbrooks, 1985; Kuhlengel *et al.*, 1990a; Martin *et al.*, 1991, 1993; Montero-Menei *et al.*, 1992; Neuberger *et al.*, 1992; Paíno and Bunge, 1991; Wrathall *et al.*, 1984), although these observations were carried out in situations where a major lesion had already been caused (Montero-Menei *et al.*, 1992). The study in *CHAPTER VI* showed that Schwann cells induced local sprouting and elongation of cut corticospinal axons when a minimally traumatic micro-transplantation technique was used to introduce them into the spinal cord.

Since the micro-transplantation technique leaves the host tract structure relatively undisturbed, it is possible to study the pattern of interaction of the donor Schwann cells into the organised structure of the host tract. In an earlier study (Brook *et al.*, 1993) it was found that micro-transplantation of Schwann cells into the fimbria rapidly induced the formation of blood vessels, and the donor cells migrated initially along them, and then were integrated as elongated bipolar cells interspersed with the axons of the host tract. This study of the fimbria did not show myelination of the host axons by the transplanted Schwann cells. However, in the spinal cord, a number of studies have shown that either endogenous or exogenous Schwann cells transplanted into demyelinating lesions myelinated spinal axons (Blakemore, 1976; Blakemore and Crang, 1985; Duncan *et al.*, 1988; Gilmore and Duncan, 1968).

To understand how the transplanted Schwann cells induce the responses of host adult axons, it is necessary to understand exactly how these micro-transplanted Schwann cells are integrated into the host tract structure. The present experiments were designed to reveal the mode of incorporation and the pattern of migration of exogenous Schwann cells micro-transplanted into the adult rat corticospinal tract under conditions which induce sprouting of corticospinal axons.

7.2. Materials and methods (For details see *CHAPTER II*)

7.2.1. Donor cells

Schwann cells were obtained from the postnatal sciatic nerve and maintained in culture for 1 week, fibroblasts were reduced by the antibody-mediated complement cytotoxicity method. The cell suspension was made up at a final concentration $1.0-1.5 \times 10^7$ cells/ml in defined medium.

7.2.2. Transplantation

$1.0-1.5 \mu\text{l}$ of the cell suspension (approximately 10-15,000 cells) was injected into the corticospinal tract of the AS adult rat spinal cord at the level of the first two cervical spinal segments by the pulsed air pressure injection system.

7.2.3. Immunohistochemistry

7.2.3.1. p75 23 animals with transplanted Schwann cells were allowed to

survive for 1 day (n = 2), 4 days (n = 2), 7 days (n = 3), 10 days (n = 5), 42 days (n = 4), 110 days (n = 3) and 143 days (n = 4). 10 μ m horizontal cryostat sections were prepared for p75 immunostaining.

7.2.3.2. P₀ 12 animals with transplanted Schwann cells were allowed to survive for 7 days (n = 3), 42 days (n = 3), 110 days (n = 2) and 143 days (n = 4). 10 μ m horizontal cryostat sections were used for P₀ immunostaining.

7.2.4. Semithin sections and EM

A total of 14 animals with transplants of Schwann cells in the descending corticospinal tract were used for the EM study with survival times of 3 days (n = 3), 5 days (n = 3), 10 days (n = 3), 14 days (n = 3) and 25 days (n = 2). The animals were perfused and prepared for the EM study by the normal EM procedure. Semithin and ultrathin sections were cut in a horizontal plane.

7.3. Results

7.3.1. Morphology of the transplants

The horizontal sections immunostained with p75 showed that transplanted donor Schwann cells within the spinal cord were densely p75-positive (Figs. 7.1,2). The transplants formed ovoid masses of around 0.5 mm in diameter and about 1 mm in length in the descending corticospinal tracts. Outside

the spinal cord, the Schwann cells of the peripheral dorsal root bundles were strongly stained with the p75 antibody. Apart from this, the only p75-positive structures in the spinal cord were a small number of scattered, very fine, lightly p75-positive host axons (Fig. 7.1).

Semithin and ultrathin sections showed that the host myelinated fibre tracts were minimally disorganised by the micro-transplantation technique. The transplants made good contact with the host myelinated tract, with host axons running through the Schwann cell transplants. The transplants were densely vascularised, with many wide-diameter blood vessels formed in the transplants (Fig. 7.3).

7.3.2. Migration pattern of the transplanted Schwann cells

From 4 to 7 days after transplantation, the transplants consisted of a central mass of heavily p75-positive Schwann cells with prominent, curving blood vessels arising from them. These blood vessels were surrounded by elongated p75-positive cells (Figs. 7.1,2). Schwann cells migrated out from the central mass as 'ribbon-like' cells (as shown by Brook et al (1993) in the fimbria) and the perivascular cuffs were in strict rostro-caudal alignment with the longitudinal axis of the host tract fibres. These migrating Schwann cells took two distinct forms:

Figure 7.1

Horizontal section to show the central mass of transplanted Schwann cells (SC) in the corticospinal tract with perivascular cuff (asterisk). Arrowheads, Schwann cells in rows of host glial cell bodies; large arrows, elongated bipolar interfascicular Schwann cells; small arrows, host p75-positive corticospinal axons. Cryostat section, p75 immunostain with thionin counter-staining. Survival time, 7 days.

Scale bar = 100 μm .



Figure 7.2

High power view of p75 immunostained Schwann cell transplant in the corticospinal tract to show the perivascular cuffs of p75-positive Schwann cells around transplant blood vessels (b). Horizontal cryostat section with thionin counter-staining. Survival time, 7 days.

Scale bar = 50 μ m.

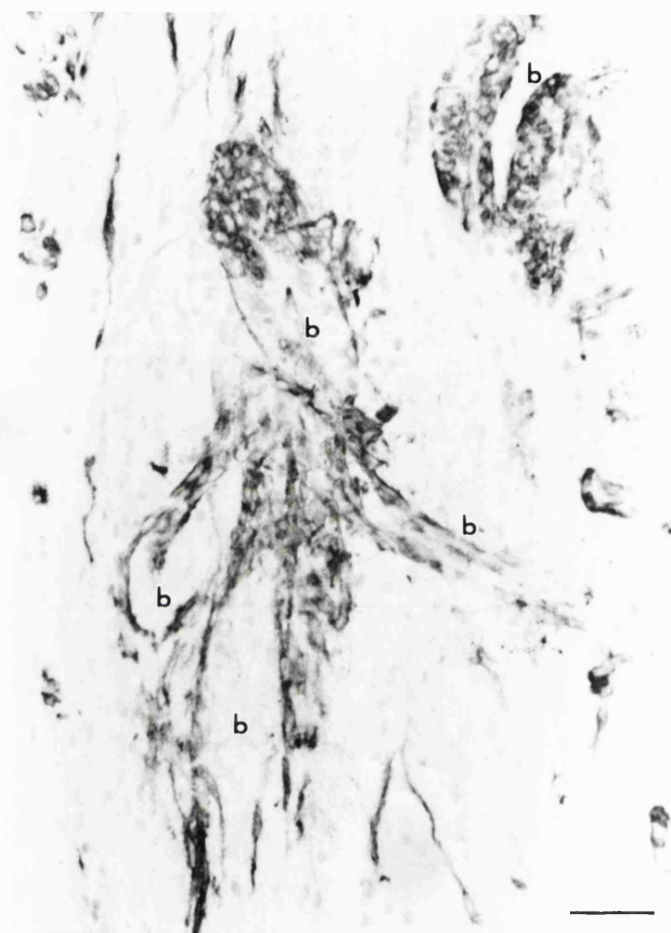
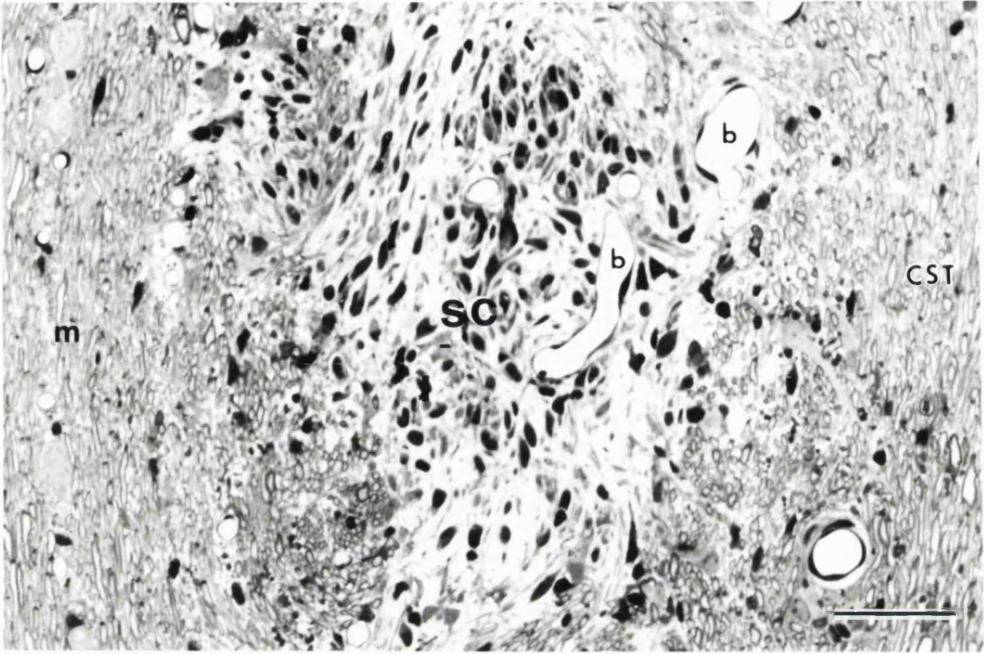


Figure 7.3

Horizontal semithin section to show that the Schwann cell transplant (SC) is densely vascularised, there are some wide-diameter blood vessels (b) formed inside the transplant. The host myelinated fibre tracts are minimally disorganised by the micro-transplantation technique. CST, corticospinal tract; m, midline; Survival time, 8 days.

Scale bar = 50 μ m.



(1) There were some heavily p75-positive cells, with rather square outlines, which were completely devoid of processes around the transplants. These cells were inserted either singly, or as contiguous rows of cells into the linear rows of oligodendrocytic and astrocytic cell bodies making up the glial skeleton (Suzuki and Raisman, 1992) of the host corticospinal tract (Figs 7.4,5,6A). In some cases the p75-positive material had a peripheral distribution, leaving a clear rim of perinuclear cytoplasm. Where the transplant was close to the midline, similar non-process bearing Schwann cells became embedded in the midline glial structure (m in Fig. 7.7).

(2) Most p75-positive Schwann cells did not enter the rows of glial cell bodies, but migrated parallel to each other along the tract regions occupied by the host corticospinal tract nerve fibres. This group of Schwann cells were elongated into slender, thread-like, symmetrical bipolar structures, about 100-120 μm long, with a bulge caused by the centrally located nucleus (Figs. 7.7,8). The bipolar interfascicular Schwann cells were either insinuated singly among the host nerve fibres, or else intertwined into small, rope-like clusters (asterisks in Figs 7.6B,8B).

In the material from 6 weeks after transplantation and onwards, there were very few p75-positive cells seen in the transplant region, and after 3 months there were no p75-positive cells, although now Schwann cells could be identified by P₀ immunohistochemistry, semithin sections and electron microscopy.

Figure 7.4

Low power photomicrograph of p75 immunostaining to show several parallel rows of host glial cell bodies containing numerous p75-positive, non-process bearing transplanted Schwann cells (arrows) in the corticospinal tract. Arrowhead, an elongated interfascicular bipolar Schwann cell migrating rostrally out of the main mass of transplanted Schwann cells (SC). The area of the asterisk is enlarged in figure 7.5. Horizontal cryostat section, p75 immunostain with thionin counter-staining. Survival time, 7 days.

Scale bar = 100 μ m.



Figure 7.5

Enlargement of area marked with asterisk from figure 7.4. Arrows, to show separate and contiguous square-shaped non-process bearing p75-positive Schwann cell bodies interspersed in the rows of glial cell bodies of the host corticospinal tract. Horizontal cryostat section, p75 immunostain with thionin counter-staining. Survival time, 7 days.

Scale bar = 25 μ m.

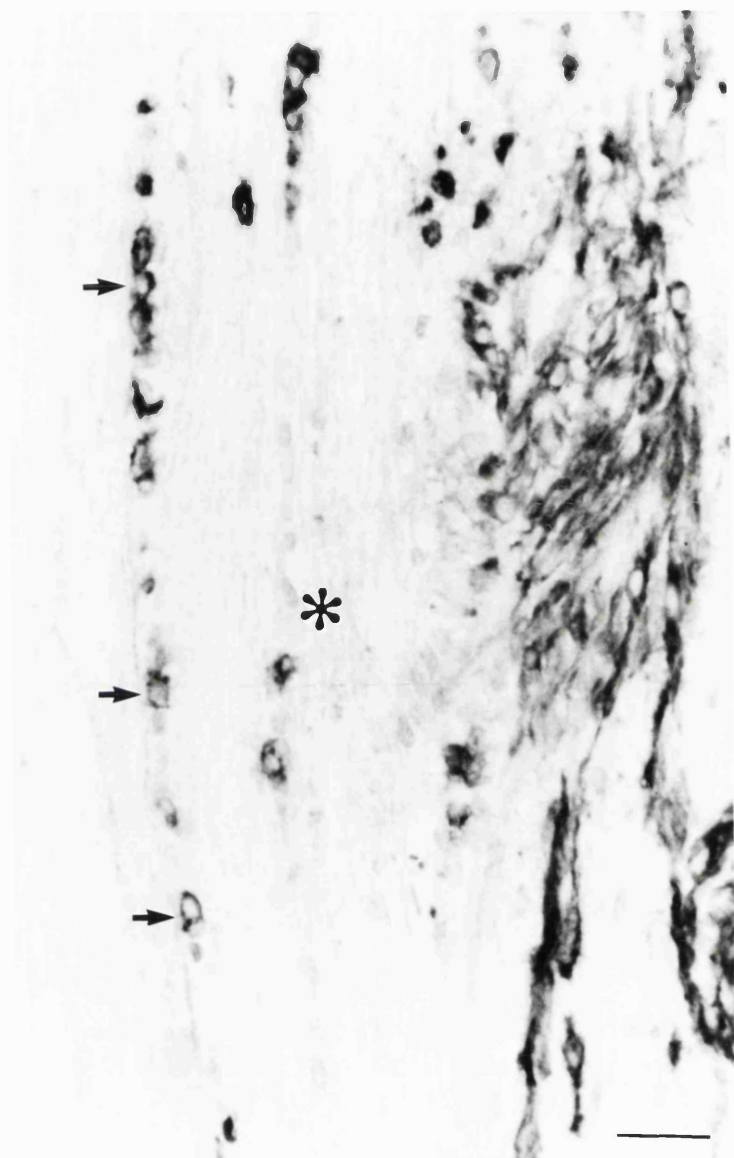


Figure 7.6

Camera lucida drawings of the two types of migrating p75-positive donor Schwann cells in the corticospinal tract:

A: Incorporated into the rows of host glial cell bodies.

B: Elongated, bipolar interfascicular Schwann cells. Asterisks, cells intertwined into rope-like groups (cf. Fig. 8B). Survival 7 days.

Scale bar = 20 μ m.

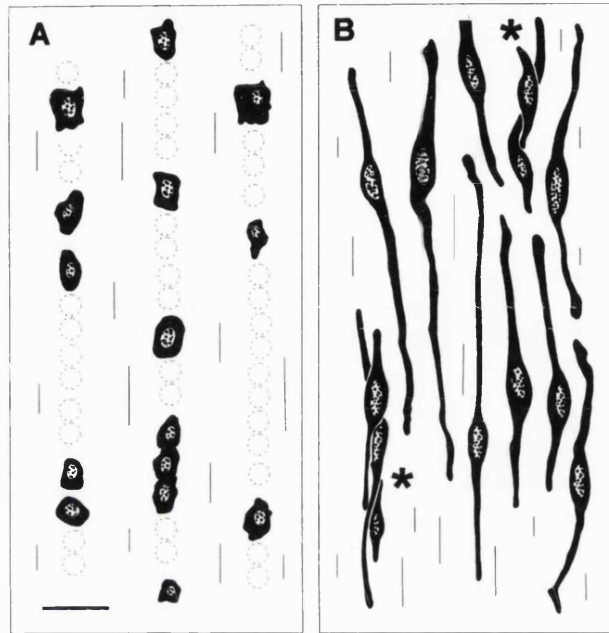


Figure 7.7

p75 immunostained transplanted Schwann cells in the corticospinal tract to show a group of elongated interfascicular bipolar Schwann cells (e.g. arrows) lying just rostral to the tip of a transplant (not shown). m, midline. Horizontal cryostat section, p75 immunostain with thionin counter-staining. Survival time, 7 days.

Scale bar = 100 μ m.

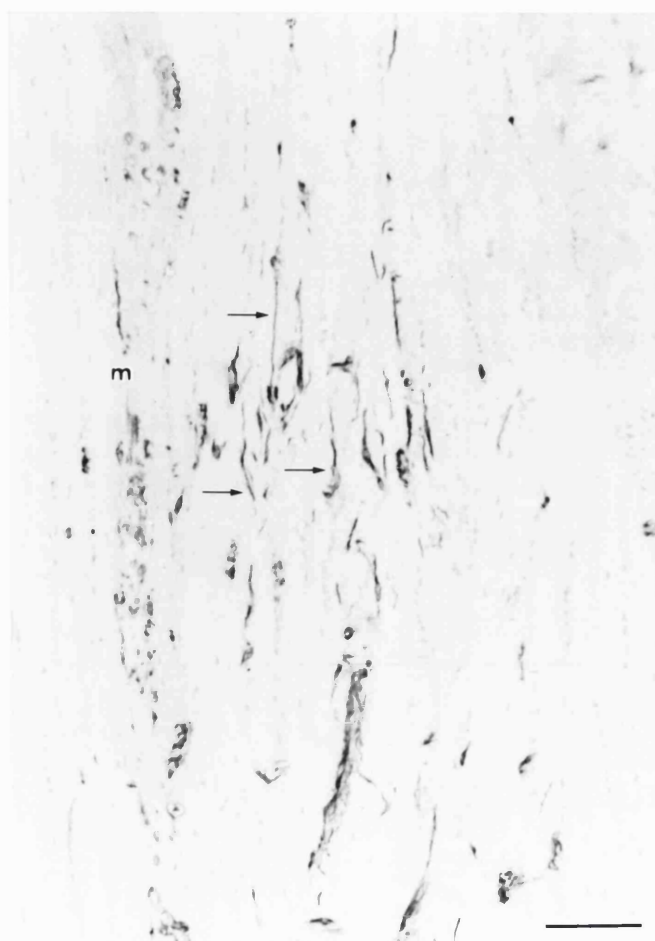


Figure 7.8

p75-positive transplanted Schwann cells in the corticospinal tract.

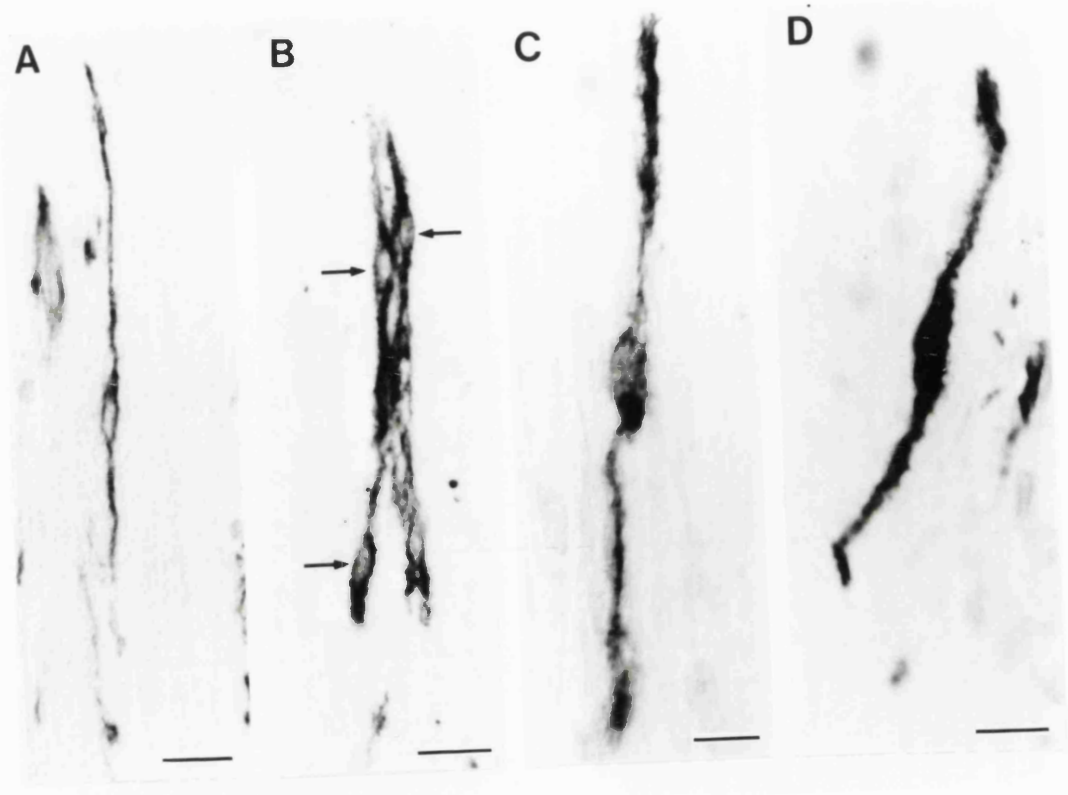
A, C, D: To show the single elongated interfascicular bipolar Schwann cells.

B: To show a group of elongated interfascicular bipolar Schwann cells (nuclei indicated by small arrows) intertwined into a rope-like structure.

Horizontal cryostat section, p75 immunostain with thionin counter-staining.

Survival time, 7 days.

Scale bars = 20 μm (in A,B); 10 μm (in C,D).



7.3.3. Myelination by the transplanted Schwann cells

In the normal and operated animals, the peripheral Schwann cell sheaths of the host dorsal roots outside the spinal cord were intensely P₀-positive (Fig. 7.9). P₀ immunostaining was not found in any structure within the spinal cord parenchyma at 7 days after Schwann cells transplantation, but became prominent from 6 weeks onwards. The central mass of the transplant was completely permeated by elongated P₀-positive structures (Fig. 7.10), and small P₀-positive bundles, corresponding to bundles of myelinated fibres were found in the adjacent corticospinal tract, running both rostro-caudally and also in some cases orientated towards the grey matter (Fig. 7.11). At high magnifications, the cytoplasmic P₀ reaction product had a highly characteristic, 'bubbly' appearance (Fig. 7.12).

Semithin and ultrathin sections showed the presence of Schwann cells forming thick, densely stained peripheral type myelin with a one-to-one cell/axon relationship in the transplant region, from 10 days after transplantation (Fig. 7.13A). Most host axons in the transplant were myelinated by the transplanted donor Schwann cells. The transplanted Schwann cells were elongated and parallel to the host fibre tracts. Electron microscopy confirmed that this represented thick, dense peripheral myelin formed by electron dense Schwann cells with characteristic clumped nuclear heterochromatin and basal lamina (Fig. 7.13B).

Figure 7.9

A: Intensely P₀-immunoreactive myelin sheaths surrounding small fascicle of dorsal root axons applied to the pial surface (arrows) of the adult rat spinal cord.

Scale bar = 100 μ m.

B: Control section of a dorsal root of the adult rat spinal cord without primary P₀ antiserum. Horizontal cryostat section, P₀ immunostain with thionin counter-staining.

Scale bar = 100 μ m.

A



B



Figure 7.10

Horizontal section to show intensely P₀-immunoreactive myelin sheaths in a Schwann cell transplant (T) in the corticospinal tract. The central mass of the transplant is completely permeated by elongated P₀-positive structures. Cryostat section, P₀ immunostain with thionin counter-staining. Survival time, 42 days.

Scale bar = 25 μ m.



Figure 7.11

High power view of a group of transversely orientated P₀ immuno-positive Schwann cell myelinated axons running laterally towards the host grey matter. Arrows, two longitudinally, rostro-caudally orientated Schwann cell myelinated sheaths. Horizontal cryostat section, P₀ immunostain with thionin counter-staining. Survival time, 42 days.

Scale bar = 25 μ m.

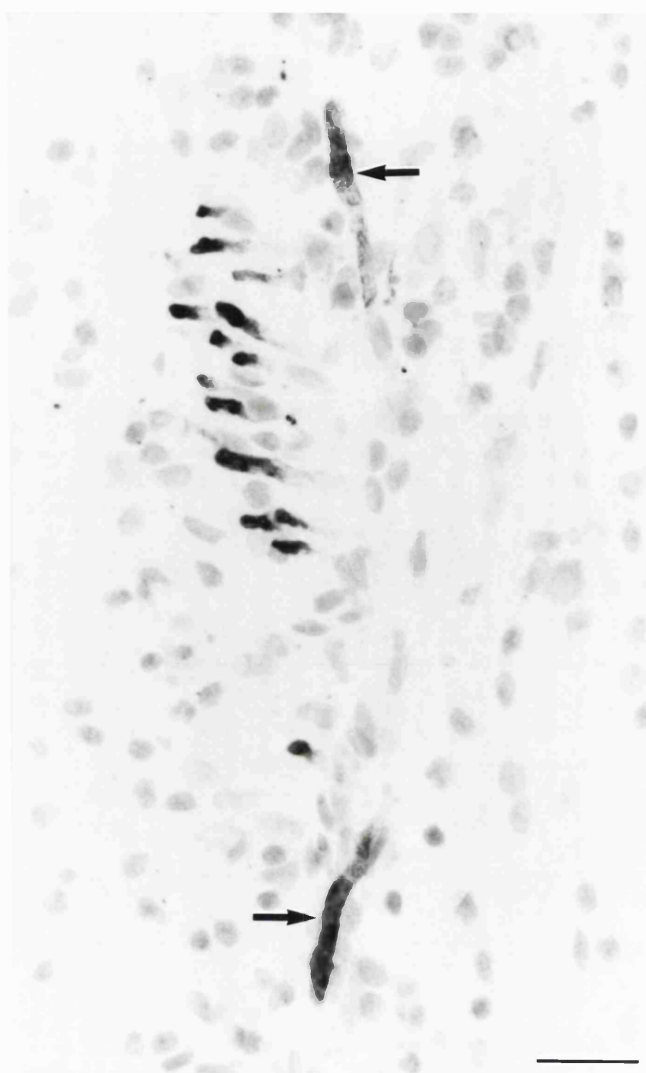
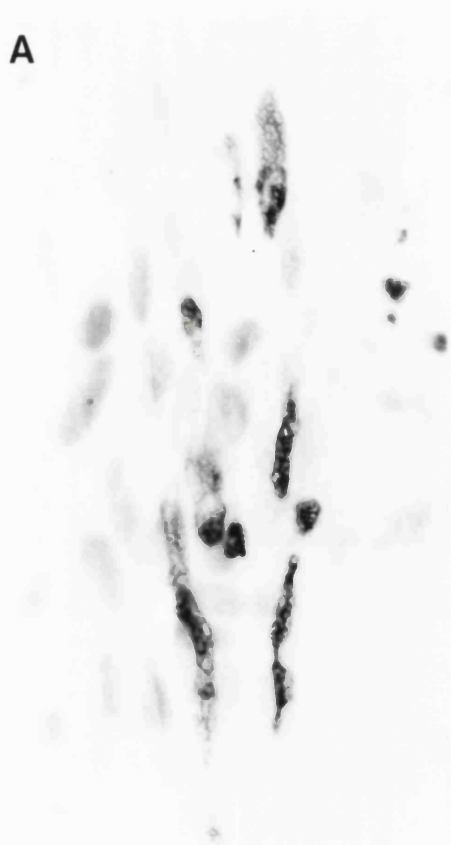


Figure 7.12

A, B: High power photomicrographs to show the 'bubbly' appearance of P₀ immunostaining in longitudinally orientated donor Schwann cell myelinated host corticospinal tract axons. Horizontal cryostat section, P₀ immunostain with thionin counter-staining. Survival time, 42 days.

Scale bars = 20 μ m.

A



B

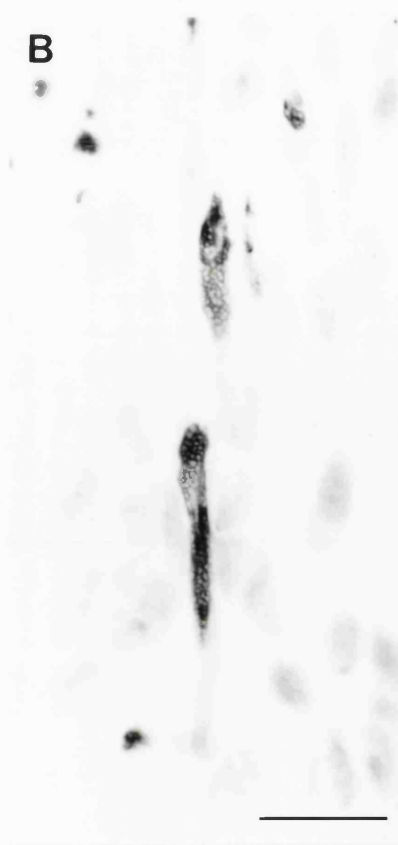


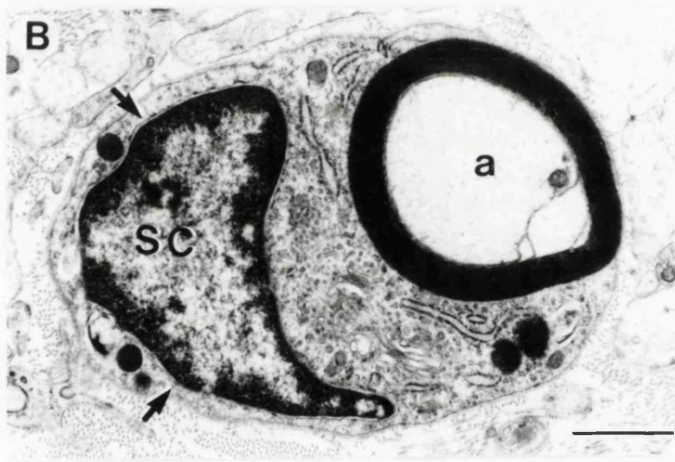
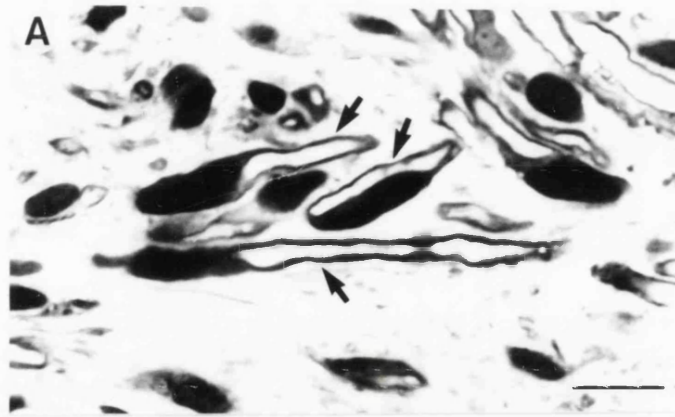
Figure 7.13

A: Semithin section showing the thick, dense peripheral myelin sheaths formed by transplanted Schwann cells (e.g. arrows) in the corticospinal tract. Horizontal section stained with methylene blue. Survival time, 25 days.

Scale bar = 10 μm .

B: Electron micrograph of a transverse section across a transplanted Schwann cell (SC), surrounded by a basal lamina (arrows) and forming a thick myelin sheath around a corticospinal axon (a). Survival time, 25 days.

Scale bar = 2.5 μm .



7.4. Discussion

The migration of transplanted cultured Schwann cells along the perivascular space of blood vessels (many of which have formed in response to the transplantation), and also as single interfascicular elongated bipolar cells along the host corticospinal tract are similar to the observations made in the fimbria (Brook *et al.*, 1993; Raisman *et al.*, 1993). Two further observations from present study were made in the corticospinal tract - (1) the incorporation of the perikarya of non-process-bearing Schwann cells into the rows of host oligodendrocytic and astrocytic cells bodies, and (2) the myelination of host axons.

7.4.1. Schwann cells in the rows of glial cell bodies

The entry of the donor Schwann cells into the rows of host glial cell bodies, and their interspersal among those cell bodies indicates that the rows of glial cell bodies present are a favourable tissue micro-environment for the integration of the foreign Schwann cell bodies into the host tract structure. The regular rows of oligodendrocyte and astrocytic cell bodies (Suzuki and Raisman, 1992) are a characteristic feature of adult central tracts. Studies on the fimbria (Suzuki and Raisman, 1995) show that during development these orderly adult rows are the end point of a complex process which includes radial migration of astrocytic precursors from the ependymal surface, cell division in the body of the tract, and immigration of oligodendrocyte precursors (Timsit *et al.*, 1992; Yu *et al.*, 1994).

The first cells accumulating in the body of the fimbria are not arranged in rows. The adult arrangement is not achieved until about the second week of postnatal life, indicating that there is some continuous influence which progressively directs the cell bodies into the long unicellular rows. The present observations on the incorporation of transplanted Schwann cells suggest that this influence continues to direct cells into the rows even in adult life.

7.4.2. Interfascicular Schwann cells

The donor Schwann cells are also able to enter the regions of densely packed axons surrounding the rows of cell bodies. But whereas the Schwann cell bodies in the glial cell body rows do not form processes, the interfascicular Schwann cells form 100-120 μm long processes elongated along the long axis of the tract. This consistent difference in morphology suggests that the local tissue micro-environmental influences in the two compartments of the tract are quite different. The larger size of the interfascicular Schwann cells, and their formation of major processes is probably due to the influence of axonal contact, which is known to have major effects of Schwann cell division (Jessen and Mirsky, 1991; Mirsky and Jessen, 1990) and phenotype in culture (e.g. formation of basal lamina; Obremski *et al.*, 1993a,b).

7.4.3. Schwann cell phenotype and myelination

Apart from its value as a marker of the behaviour of the transplanted

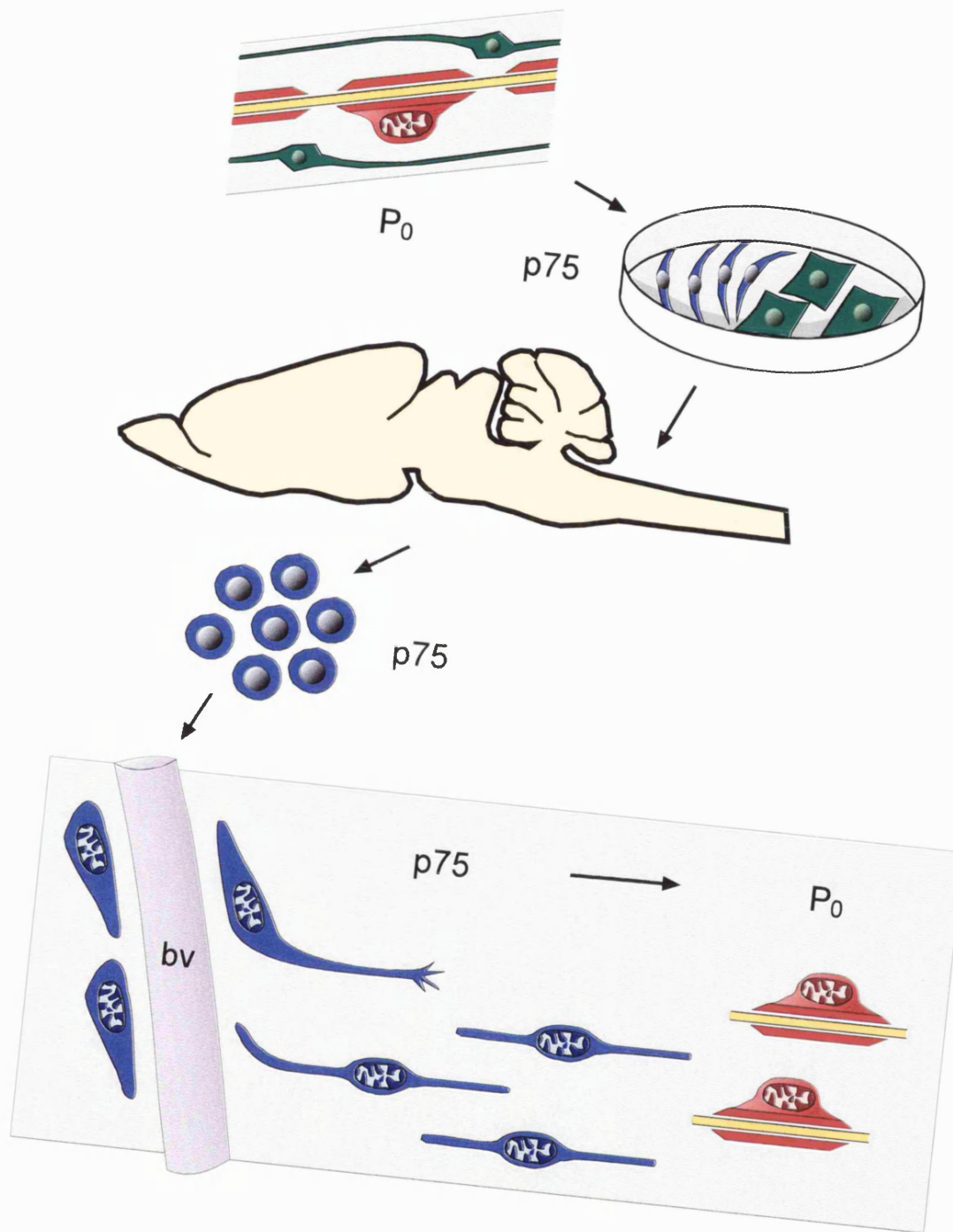
Schwann cells, Anton et al (1994) have recently provided fascinating evidence that p75 may be involved in the migratory activity of Schwann cells in culture. This raises the possibility that p75 may also have a similar functional significance for the marked migration in the present in vivo spinal cord material. Although the donor Schwann cells later lose p75 immunoreactivity, the appearance of P₀-immunoreactivity, and the semithin sections and electron micrographs show that a number of them survive and develop into a myelinated form, which is incorporated as a long-term, stable component of the host tract structure. The conversion of the donor Schwann cells from an early p75-positive, P₀-negative, non-myelinating form to a later p75-negative, P₀-positive, myelinating form (Fig. 7.14) is similar to the sequence of events in normal peripheral nerve development (Jessen and Mirsky, 1991; Mirsky and Jessen, 1990) and regeneration (Taniuchi *et al.*, 1988). The re-myelination of spinal axons by exogenous and endogenous Schwann cells has been previously reported in demyelinating lesions (Blakemore, 1976; Blakemore and Crang, 1985; Duncan *et al.*, 1988; Gilmore and Duncan, 1968), and the material in *CHAPTER V* shows that in focal electrolytic lesions of the corticospinal tract the endogenous host Schwann cells grow into the lesion and re-myelinate the axons.

7.4.4. Significance of Schwann cell incorporation into central tracts

Micro-transplantation of cultured Schwann cells results in the formation of a 'mixed' tissue in which the highly orderly and complex glial structure of the central tract is retained, but which incorporates the specific cellular

Figure 7.14

A summary of the phenotypic changes in transplanted Schwann cells. Schwann cells, either from neonatal, unmyelinated sciatic nerve, or from adult, myelinated sciatic nerve, where they express myelin markers such as P₀ protein (red), are purified from fibroblasts (green) in culture, where they cease expressing myelin proteins, and become p75-positive (blue). After transplantation into the adult spinal cord, they form initial p75-positive clusters, induce formation of blood vessels (bv), migrate as bipolar cells along the tract interfascicular glial rows, and finally settle down, down-regulate p75, myelinate host axons (yellow), and re-express the myelin markers such as P₀ (red).



element from peripheral nerves which is needed for axon regeneration (Fig. 7.15). The Schwann cell component may have several functions - inducing axon growth, providing pathways for sprouts, suppressing astrocytic scarring, and the P₀ component of the Schwann cell myelin could be a stimulant for axon growth (Yazaki *et al.*, 1994). Such 'mixed' tracts could form the basis of a future strategy for repair of cut central axons.

7.5. Summary

Donor Schwann cells were obtained from neonatal sciatic nerve. After maintaining in culture for 1 week and purifying with the antibody-mediated complement cytolysis method, a suspension made at a final concentration $1.0\text{-}1.5 \times 10^7$ cells/ml in defined medium. $1.0\text{-}1.5 \mu\text{l}$ of cell suspension was micro-injected into the descending corticospinal tract of the adult AS rat spinal cord by the pulsed air pressure injection system at the upper cervical level. Immunostaining for p75 showed that most transplanted donor cells were p75-positive with a central mass of transplanted Schwann cells, and cuffs of elongated Schwann cells along the perivascular space of blood vessels. Schwann cells leaving the central mass and perivascular cuffs migrated in strict orientation along the rostro-caudal axis of the host corticospinal tract. In the comparable study by Brook *et al* (1993) it was shown that at 1 hour after operation the Schwann cells are tightly concentrated in a solid mass at the injection site. Even by 1 day only a few cells have started to migrate out of this site for no more than $100 \mu\text{m}$. This indicates that the later spread of Schwann cells is due to a true migration and not physical dispersal caused by the force of the injection.

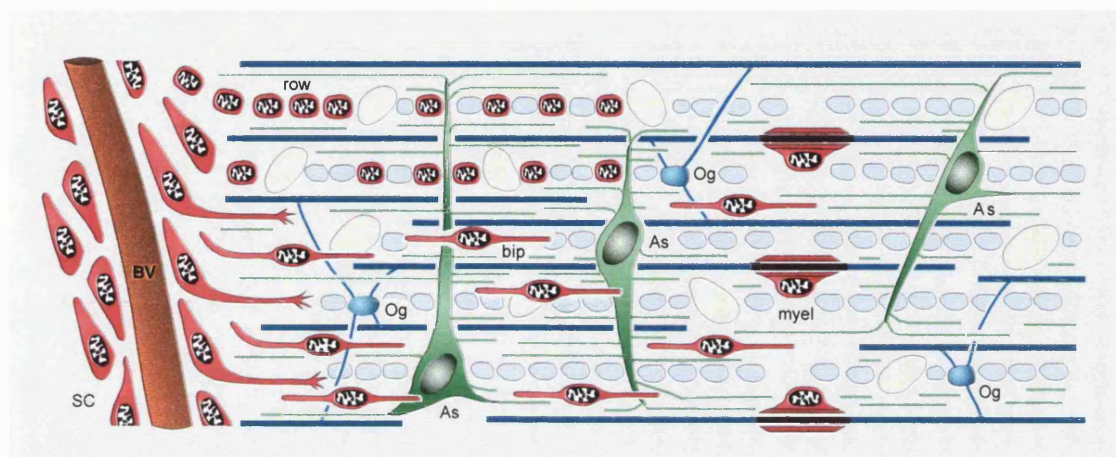
element from peripheral nerves which is needed for axon regeneration (Fig. 7.15). The Schwann cell component may have several functions - inducing axon growth, providing pathways for sprouts, suppressing astrocytic scarring, and the P₀ component of the Schwann cell myelin could be a stimulant for axon growth (Yazaki *et al.*, 1994). Such 'mixed' tracts could form the basis of a future strategy for repair of cut central axons.

7.5. Summary

Donor Schwann cells were obtained from neonatal sciatic nerve. After maintaining in culture for 1 week and purifying with the antibody-mediated complement cytotoxicity method, a suspension made at a final concentration 1.0-1.5 x 10⁷ cells/ml in defined medium. 1.0-1.5 μ l of cell suspension was micro-injected into the descending corticospinal tract of the adult AS rat spinal cord by the pulsed air pressure injection system at the upper cervical level. Immunostaining for p75 showed that most transplanted donor cells were p75-positive with a central mass of transplanted Schwann cells, and cuffs of elongated Schwann cells along the perivascular space of blood vessels. Schwann cells leaving the central mass and perivascular cuffs migrated in strict orientation along the rostro-caudal axis of the host corticospinal tract.

Figure 7.15

A schematic representation of the normal astrocyte (As, green) and oligodendrocytic (Og, blue) meshwork of a myelinated CNS tract into which Schwann cells (SC, red) migrate from the perivascular position around a blood vessel (BV, brown) either as non-process bearing cells in the rows of glial cell bodies (row), or as elongated interfascicular bipolar cells (bip), and re-myelinate the corticospinal axons (myel, red). (Note that, compared with the previous figures, the rostro-caudal axis of the corticospinal tract is represented horizontally).



p75-positive transplanted Schwann cells formed two distinct patterns of migration: (1) row Schwann cells: non-processbearing, rather square-shaped Schwann cells inserted singly or in groups among the rows of host oligodendrocytic and astrocytic cell bodies, and (2) interfascicular: individual, bipolar Schwann cells, about 100-120 μm long, with central nuclei, migrating singly or intertwined in rope-like groups interspersed among the axons of the host corticospinal tract.

Immunostaining for P₀ protein showed that transplanted Schwann cells readily myelinated the host axons. Semithin section and electron microscopy study confirmed that transplanted Schwann cells myelinated the host axons and myelination started about 10 days after transplantation.

These observations show that Schwann cells can be intimately integrated into the cytoarchitecture of the myelinated adult host corticospinal tract. Their integration involves direct interaction with all the cell elements present in the tracts and respects the complex and regular organisation of the host tract glial cells.

**CHAPTER VIII MICROGLIAL INVASION OF SPINAL LESIONS
PRECEDES THE INGROWTH OF SCHWANN CELLS**

8.1. Introduction

Apart from transplantation of exogenous cultured cells, endogenous Schwann cells can be induced to enter the spinal cord and brain by a number of different lesion procedures (Blakemore, 1976; Blakemore and Crang, 1989; Dusart *et al.*, 1992; Gilmore and Duncan, 1968; Gilmore and Sims, 1993; Harrison, 1987; Sims and Gilmore, 1983), of which a crucial element seems to be the destruction of oligodendrocytes in the adult, or the prevention of their formation during development. These observations arose incidentally, when, as a control for the studies of transplantation of Schwann cells (see *CHAPTER VI*), small lesions were made in the rat corticospinal tract. These lesions alone induce the ingrowth of endogenous Schwann cells which re-myelinate a wide swathe of uncut demyelinated corticospinal axons passing beside the lesion, as well as cut axons terminating in the lesion (see *CHAPTER V*).

As described in *CHAPTER V*, the endogenous Schwann cells did not appear in the lesion until 3 weeks after operation. Preceding this, the demyelinated regions showed a rapid accumulation of microglia (Gehrmann *et al.*, 1995; Lawson *et al.*, 1990). The microglial accumulation was prominent by 1 week after operation. The present Chapter describes the temporal and spatial relationships between the microglial and Schwann cell accumulations.

8.2 Materials and methods (For details see *CHAPTER II*)

8.2.1. Lesion

Lesions were made in the descending corticospinal tract of the dorsal columns in adult AS rats by passing a DC current of 10 μ A for 4-7 min through an electrode.

8.2.2. Immunohistochemistry

8.2.2.1. OX-42 15 animals with the lesions at survivals of 7 days (n = 3), 14 days (n = 3), 21 days (n = 3), 28 days (n = 3) and 49 days (n = 3) were used for OX-42 immunostaining.

8.2.2.2. p75 14 animals with the lesions at survivals of 7 days (n = 3), 14 days (n = 4), 21 days (n = 4) and 30 days (n = 3) were used for p75 immunostaining.

8.2.3. Semithin sections and EM 14 animals with corticospinal lesions at survivals of 1 week (n = 3), 2 weeks (n = 3), 3 weeks (n = 3), 4 weeks (n = 3) and 4 months (n = 2) were used for EM study. 200-300 μ m Vibratome sections were processed using the method for preservation of CNS myelin and flat embedded in resin. Semithin and ultrathin sections were cut in a horizontal plane. Semithin sections were stained with a mixture of methylene blue and Azur II and ultrathin with uranyl acetate and lead citrate.

8.3. Results

8.3.1. General morphology of the lesions

The general morphology of the lesion has been described in *CHAPTER V*. The majority of the lesions were confined to the corticospinal tract on one side; in some cases crossing the midline to involve a small medial part of the opposite tract. The central part of the lesions was an area of complete tissue destruction, which became rapidly filled with amoeboid macrophages, and later vascularised. Caudal and aligned with this, was a dense band of anterograde axonal degeneration, which consisted of the distal fragments of the cut axons, and was invaded by macrophages. Aligned in a similar position rostrally was a lesser band of similar axonal fragmentation, probably representing retrograde axonal fragmentation of the proximal segments of the corticospinal axons.

On either side of the central area were cushion-like regions of tract where the tissue was partially destroyed, and partially surviving (Fig. 8.1). In this area there seemed to be an almost complete loss of oligodendrocytes, but many axons remained intact but demyelinated. Anterograde axonal tracing (see *CHAPTER V*) showed that some axons were severed at this level. To a large extent, the tract astrocytes remained, and progressively formed a hypertrophic scar (see *CHAPTER V*). On either side of this cushion area of partial damage the corticospinal tract was largely normal.

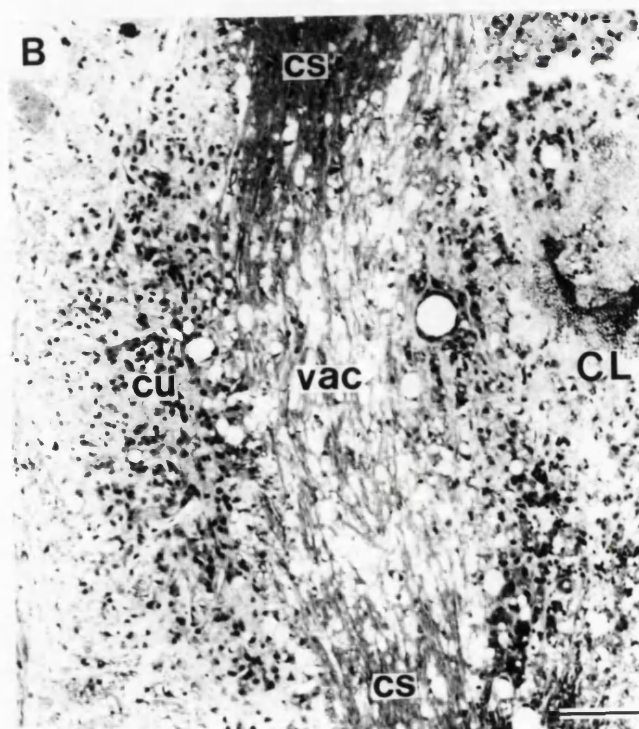
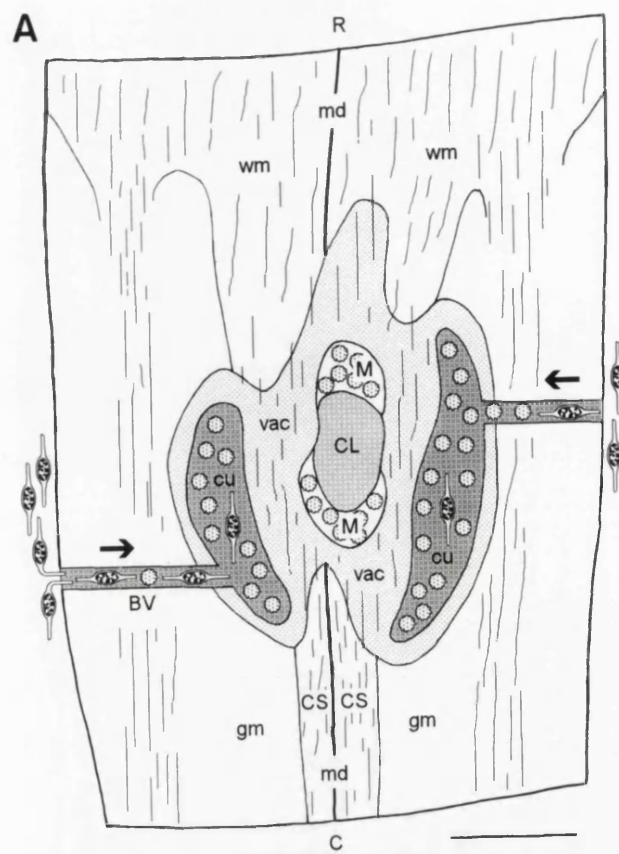
Figure 8.1

A: Semi-schematic drawing from a semithin section showing the hypercellular cushions on both sides of a lesion crossing the midline (md). The central lesion area (CL) is capped rostrally (R) and caudally (C) by macrophages (M), and flanked by a vacuolated region (vac) which is invaded by a hypercellular cushion (cu) of mainly macrophages, but also occasional Schwann cells (spindle shaped with marked nuclear heterochromatin pattern) which migrate (arrow) along radial blood vessels (BV). gm, spinal grey matter. Survival, 1 week.

Scale bar = 500 μ m.

B: Photomicrograph of a semithin section showing the left cushion area (cu) from A. vac, vacuolated area with axons continuous from the adjacent regions of the corticospinal tract (cs); CL, central lesion area. Methylene blue and azur II stain. Survival, 1 week.

Scale bar = 100 μ m.



8.3.2. Responses of host microglia

At 1 week after the lesion, the 'cushion' areas of partial tissue damage were densely infiltrated with small dark cells with dark nuclei. These cells had the OX-42-positive phenotype (Fig. 8.2) and the morphology typical of microglia (Gehrmann *et al.*, 1995; Lawson *et al.*, 1990).

8.3.3. p75 Immunohistochemistry

At 1 week survival p75 immunohistochemistry showed occasional thread-like cells (presumed Schwann cells) around the lesion. Blood vessels running radially from the pial surface to the lateral aspect of the lesion were surrounded by thread-like, p75-positive cells, elongated along the long axis of the vessels (Fig. 8.3). Outside the pial surface, the dorsal root fascicles were outlined by p75-positive Schwann cells, and from these areas masses of thread-like p75-positive cells also extended radially into the subjacent spinal cord (Fig. 8.3).

At 13 weeks survival, the picture was similar, except that there was a moderate increase in the number of thread-like p75-positive cells around the lesion area itself.

8.3.4. Semithin sections and electron microscopy

8.3.4.1. 1-2 weeks The tissue of the cushion areas had a sponge-like appearance due to massive vacuolation. The cushion areas were densely packed with cells, the most prominent being small, angular microglia, of

Figure 8.2

A corticospinal tract lesion (Les), stained with OX-42 to show the dense infiltration with macrophages. The arrow indicates the typical cushion-like area in the adjacent grey matter which is densely infiltrated with macrophages and microglia. Survival time, 2 weeks days.

Scale bar = 200 μm .

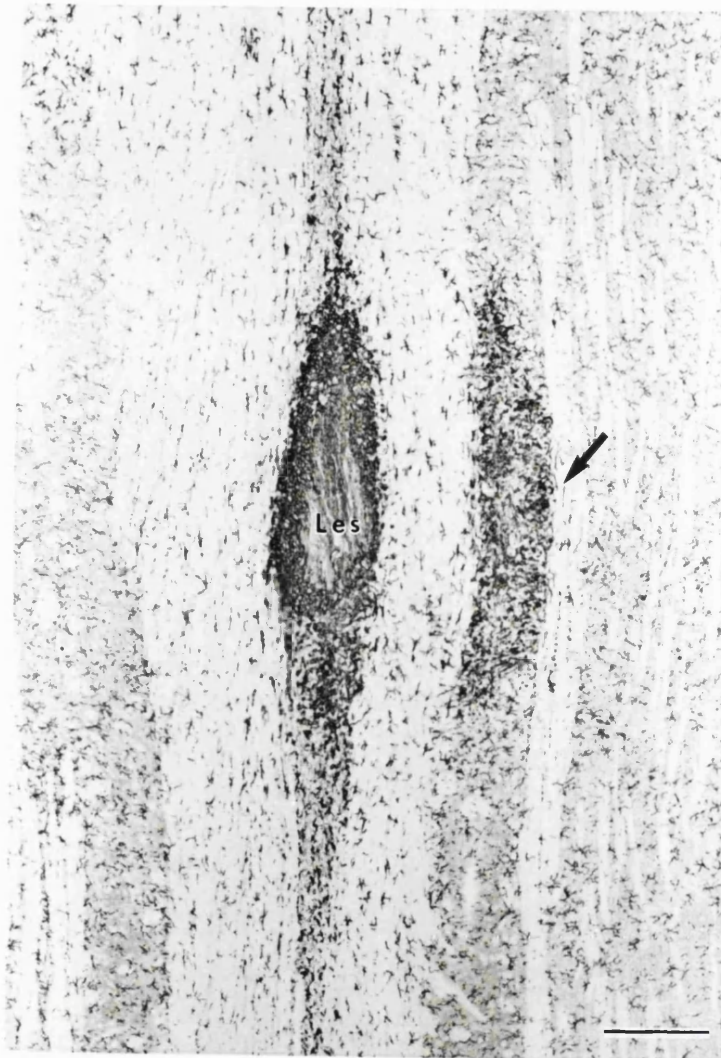
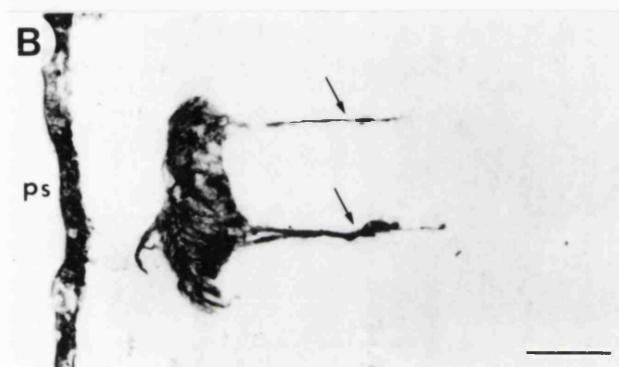


Figure 8.3

Trails (arrows) of elongated, p75 immunoreactive Schwann cells (individual cells are not visible at this magnification) running radially through the spinal cord from the lateral, pial surface (ps) towards the lesion along a blood vessel (in A), or fine fascicles of dorsal root fibres (in B). Survivals, 1 week (A), 3 weeks (B).

Scale bar = 50 μ m.



irregular shape. The microglial nuclei were electron dense, with even denser chromatin condensations. Their surfaces bore short, irregular micro-villi. Their cytoplasm contained the organelles typical of activated microglia - dense, whorled bodies (usually called 'myelin figures', although probably not derived from myelin) and lipid droplets. The most characteristic organelles, which were confined to the microglia of the cushion areas, were angularly folded linear, electron-dense inclusions with finger-print-like striations (which will be referred to as 'myelin prints') which showed their derivation from engulfed myelin (Fig. 8.4). In general these microglia were adjacent to completely demyelinated, wide diameter axons (axons of such diameter are all myelinated in the normal tract; Fig. 8.5).

These myelin-engulfing microglia of the cushion areas differed markedly from the huge, rounded amoeboid macrophages present in the central area of complete tissue destruction. (see also Dusart and Schwab, 1994).

The cushion areas contain quite significant numbers of cells in mitosis (Fig. 8.6), suggesting that local cell proliferation was also contributing to the hypercellularity of the cushion areas. Some of these are identifiable (by their cytoplasmic content) as microglia. There are also occasional, highly electron dense pyknotic cells.

The cushion areas contained large numbers of wide diameter unmyelinated axons, as well as axonal varicosities, some very large, which were filled by

Figure 8.4

A: Electron micrograph of a macrophage from the region of demyelination, showing the characteristic folded linear cytoplasmic phagosomes (e.g. arrows) consisting of ingested myelin sheaths, and lipid droplets (e.g. arrowheads).

Scale bar = 2 μm .

B: Enlarged view of a phagosome to show the finger-print-like striations (arrow) derived from the myelin sheath.

Scale bar = 0.5 μm .

C: Macrophage from the central lesion area showing the different type of cytoplasmic phagosomes resulting from ingestion of tissue debris. Survival, 2 weeks.

Scale bar = 2 μm .

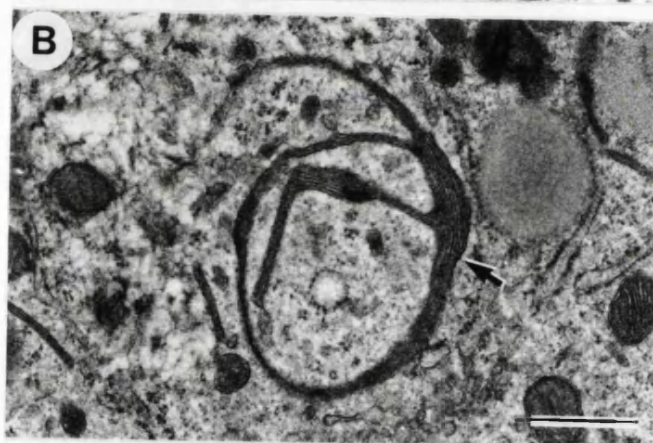
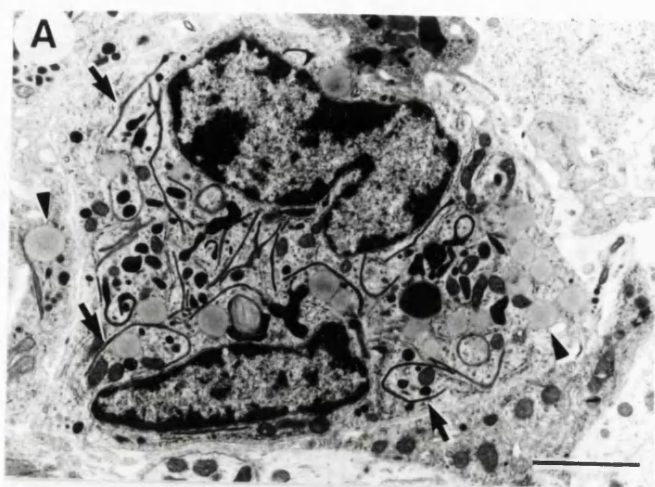


Figure 8.5

Electron micrograph of a macrophage (M) containing myelin phagosomes (e.g. arrow) lying in contact with a number of wide diameter demyelinated axons (x). Survival, 2 weeks.

Scale bar = 2 μm .

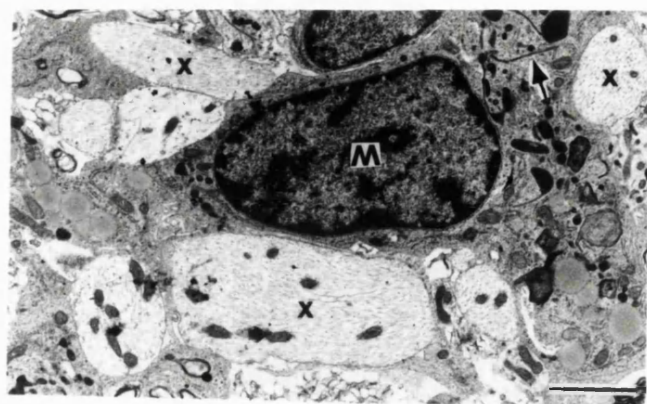
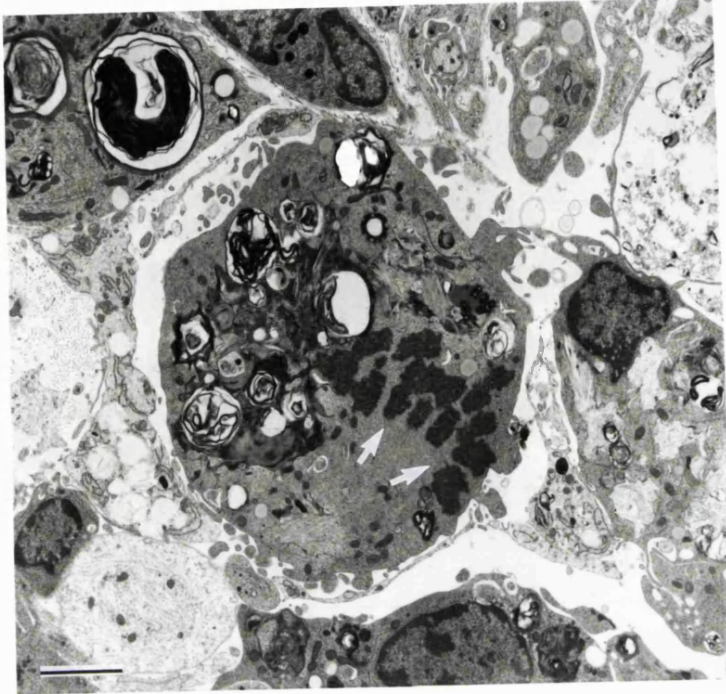


Figure 8.6

Electron micrograph of a spinal lesion showing a macrophage, identified by moderately electron dense cytoplasm and laminar dense inclusion bodies, in the process of mitosis. Arrows, metaphase chromosomes. Survival time, 3 weeks.

Scale bar = 2.5 μm .



various types of organelles, such as clusters of dense core vesicles, mitochondria and thick fascicles of neurofilaments. Astrocytes were present, but both oligodendrocytes and oligodendrocytic myelin were absent from this hypercellular cushion area.

8.3.4.2. 3-4 weeks In the cushion areas, the vacuolation was much reduced. Although still hypercellular, with densely clustered microglia, by 3 weeks there were areas with small groups of Schwann cells (identified by axon ensheathment and peripheral type myelination) aligned with the axons, and many areas with bundles of unmyelinated axons. Many mitoses were present. By 4 weeks Schwann cells were markedly more numerous, and the unmyelinated axons were fewer and solitary.

The Schwann cells had medium dense nuclei, with some chromatin condensations, but were less dense and boldly marked than those of the microglia. Compared with microglia, the Schwann cells had a more rounded form, usually with an elongated, ovoid shape. The smooth surfaces lacked micro-villi but were covered by a single basal lamina where they faced an extracellular space. The cytoplasm did not contain the dense bodies and lipid droplets of microglia or macrophages, and did not contain myelin 'prints.'

The Schwann-cell-myelinated axons formed straight, parallel fascicles, although individual axons often had a tight, corkscrew-like tortuosity. Some

of the longer myelin segments had multiple, quite closely spaced Schmidt-Lantermann incisures (Fig. 8.7).

Virtually all the Schwann cells were involved in ensheathing or myelinating axons. In some cases where the axons were still unmyelinated, they were of wide diameter, and had one-to-one relationships to the ensheathing Schwann cells (Fig. 8.8), suggesting that these axons had been demyelinated and were now in the preliminary stages of re-myelination. Fig. 8.9 shows a Schwann cell in which a single wide diameter axon is ensheathed by a few turns of loose membrane with some indication of one or two turns of myelin compaction, suggesting that this is an early stage of myelination. Since the wide diameter, unmyelinated axons embedded in single Schwann cells were not seen at the longer survival times, this suggests that all Schwann cell axon ensheathments go on to myelinate. We did not find Schwann cells ensheathing multiple unmyelinated axons (as in peripheral nerve development; Bunge, 1968).

The very large terminal expansions of the cut axons were wrapped in a single layer of electron dense cytoplasm derived from Schwann cells applied directly to the axonal plasma membranes.

Further away from the centre of the lesion, the zone of Schwann cells ceased, and the tract fibres were myelinated by oligodendrocytes, which were not in direct contact with the axons they myelinated, but lay in typical

Figure 8.7

Electron micrograph showing a Schmidt-Lantermann cleft (arrow) in a host axon thickly myelinated by an endogenous Schwann cell in a corticospinal tract lesion. Survival time, 4 months.

Scale bar = 2 μ m.

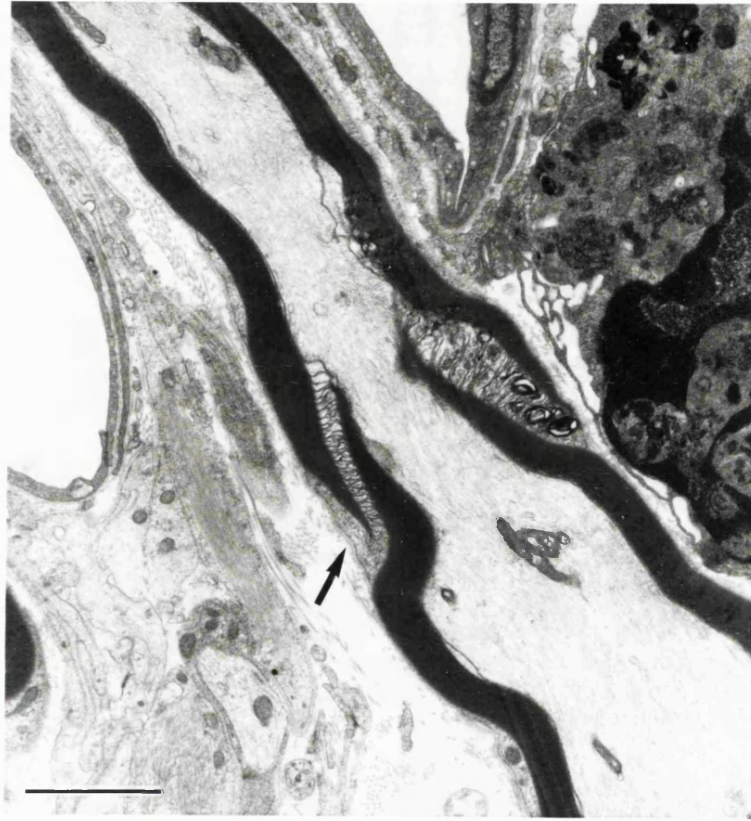


Figure 8.8

Electron micrographs showing re-myelination of wide diameter (demyelinated) corticospinal axons by endogenous Schwann cells.

A: A heavily Schwann-cell-myelinated axonal segment (s) lies adjacent to a lightly, oligodendrocyte-myelinated segment (o) embedded in a hypertrophic astrocytic process (as); arrows, node of Ranvier. Survival, 4 months.

B: One-to-one relationship showing a single wide diameter axon (x) embedded in (but not yet re-myelinated by) a Schwann cell (S). Survival, 4 months.

C: Unmyelinated axon segments (un) surrounded by a single layer of dark Schwann cell cytoplasm (arrows), adjacent to Schwann-cell-myelinated segments (my); as, multiple astrocytic lamellae. Survival, 4 months.

Scale bars = 5 μ m.

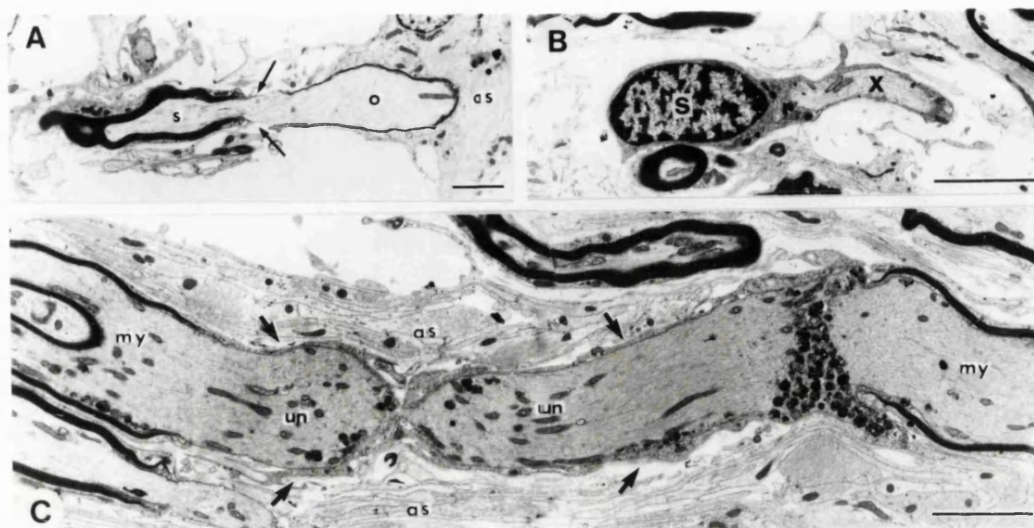


Figure 8.9

Electron micrograph of an endogenous Schwann cell in the presumed early stages of myelination, making initial, still incompletely compacted loops around an axon in a corticospinal tract lesion. Survival time, 3 weeks.

Scale bar = 2 μ m.



rows of contiguous cells, as in undamaged tracts (Suzuki and Raisman, 1992). The oligodendrocyte myelin was thinner, less well-preserved by our present fixation method, and had a narrower periodicity than the Schwann cell myelin. In many cases it was possible to see a node in which one segment of an axon (as it entered or left the adjacent area of undamaged tract) was myelinated by an oligodendrocytic process and the adjacent segment of the axon (passing through the damaged area) was myelinated by a Schwann cell (Fig. 8.8). Because the Schwann cells myelinated nodes in a one-to-one fashion, while each oligodendrocyte can myelinate 30-40 nodes (Suzuki and Raisman, 1992), the Schwann cell density needed to achieve full myelination in the cushion areas was much greater than that of the oligodendrocytes in the adjacent, regions of undamaged tract.

8.3.4.3. Long term (4 months) By 4 months survival, the flanking zones of partial tissue destruction had lost their vacuolated appearance. There were wide swathes of axons myelinated by Schwann cells.

8.4. Discussion

8.4.1. Ingrowth of Schwann cells

It is well established that the ingrowth of endogenous Schwann cells into the parenchyma of the central nervous system (and their subsequent myelination of central axons) is induced by chemotoxic demyelinating lesions (Blakemore, 1976; Blakemore and Crang, 1989; Harrison, 1987), by

prevention of developmental myelination by destruction of oligodendrocytic precursors by neonatal X-irradiation (Gilmore and Duncan, 1968; Gilmore and Sims, 1993; Sims and Gilmore, 1983), by kainate lesions of the thalamus (Dusart *et al.*, 1992), and by transplantation of genetically modified fibroblasts into the spinal cord, but not into the brain (Tuszynski *et al.*, 1994b).

It is possible that the ingrowth of Schwann cells into the present corticospinal tract lesions may have been enhanced by the method of injury (an electrolytic lesion, with deposition of iron in the tissues from the anodal current passed through the stainless steel electrode), which appears to be selectively toxic to oligodendrocytes in the tissue surrounding the central lesion area (see below).

Sims and Gilmore (1983) found that endogenous Schwann cells invaded the spinal cord by 2 weeks after local X-irradiation in neonatal rats, Felts and Smith (1992) found Schwann cell re-myelination at 3 weeks after an adult demyelinating lesion, although Dusart *et al* (1992) did not observe endogenous Schwann cells in the thalamus until 1 month after a kainate lesion. In our material at 2 weeks after the lesion the hypercellular 'cushion' areas were still made up almost entirely of macrophages, with only the occasional Schwann cell. At this time, the sections immunostained for p75 showed aligned thread-like cells along the course of radially oriented blood vessels and along incoming dorsal root fibres approaching the lateral aspect

of the lesion from the pial surface of the spinal cord. The identification of these p75-positive cells as Schwann cells is based on their elongated shape, and the fact that after 4 months Schwann cells (characterised ultrastructurally by axon ensheathment and by the formation of peripheral myelin) have accumulated in the lesions. The routes of Schwann cell ingrowth are in agreement with previous reports (Gilmore and Duncan, 1968; Gilmore and Sims, 1993; Raine *et al.*, 1978).

The present material confirms previous reports that both endogenous Schwann cells and transplanted exogenous Schwann cells can re-myelinate demyelinated spinal axons (Blakemore and Crang, 1989; Gilmore and Sims, 1993), and as in adult corticospinal tract lesions (described in *CHAPTER V*), both cut and uncut axons are re-myelinated. As noted also by Sims and Gilmore (1983), physical constraints dictate that the re-myelinating Schwann cells must enter at once into a one-to-one relationship with the demyelinated axon segments rather than - as in normal peripheral nerve development - passing through a preliminary stage of multiple unmyelinated ensheathment (Bunge, 1968; Webster *et al.*, 1973).

8.4.2. What induces the ingrowth of Schwann cells?

The present lesion procedure results in a major loss of oligodendrocytes, possibly because of complement components gaining access through opening of the blood brain barrier (Scolding *et al.*, 1989; Wren and Noble, 1989). The tissue responds to the presence of the dead oligodendrocytes

by a rapid and massive accumulation of macrophages, which phagocytose the dead cells together with their myelin. The ingestion of myelin is indicated by the characteristic striated type of folded, linear phagosomes present in the macrophages in the 'cushion' area. The removal of the dead oligodendrocytes and their myelin exposes long segments of axons of a diameter so wide that they would never normally remain unmyelinated, thus confirming that demyelination has occurred.

Powerful mitogenic stimuli for Schwann cells in vitro are emitted by macrophages which have ingested myelin (Baichwal *et al.*, 1988), an effect specific to processing of myelin basic protein, (Baichwal and Devries, 1989), by macrophage cytokines (Khan and Wigley, 1994), and growth factors (Davis and Stroobant, 1990; Ridley *et al.*, 1989), and also by axonal membranes (Ratner *et al.*, 1988); in addition, axons regulate Schwann cell phenotype by diffusible molecules (Bolin and Shooter, 1993). The present findings that the Schwann cells appear selectively in the region adjacent to the central lesion area, and that they myelinate demyelinated host axons in this area, from which the host oligodendrocytes have been depleted, are consistent with the view that myelin-containing macrophages and demyelinated wide diameter axonal segments provide major stimuli for Schwann cell ingrowth in vivo. It has also been reported (Baron-Van Evercooren *et al.*, 1992, 1993) that transplanted exogenous Schwann cells are attracted to spinal lesions. The present observation that there was no appreciable excess of Schwann cells over the numbers needed to form

myelin, suggests that Schwann cell ingrowth ceased as soon as all axons had been re-myelinated. This is supported by the finding that only small numbers of the Schwann cells at any one time expressed p75 protein, which is probably involved in promoting Schwann cell migration (Anton *et al.*, 1994), but is down-regulated on contact with axons (Taniuchi *et al.*, 1988).

Taken together, the present observations suggest the following series of events: destruction of oligodendrocytes in the corticospinal tract triggers removal of myelin sheaths by activated microglia which in turn induce the ingrowth and proliferation of Schwann cells which are thus brought into contact with the demyelinated axon segments which they then ensheathe and myelinate.

8.5. Summary

Small, circumscribed electrolytic lesions were made in the corticospinal tract at the upper cervical level of the adult rat spinal cord. On either side of this area a considerable swathe of uncut host corticospinal axons became demyelinated as a result of the death of oligodendrocytes. By 1 week after the operation, this demyelinated region had become densely infiltrated by OX-42 immunoreactive macrophages and microglia which had removed the myelin sheaths. The microglia were identified in electron micrographs by characteristic angular, laminated dense cytoplasmic bodies which are as the remnants ('prints') of phagocytosed myelin. A few thread-like p75

immunoreactive Schwann cells could be seen migrating into the lesion along blood vessels and dorsal root fibres.

From 3 weeks semithin sections and electron microscopy showed the presence of Schwann cells giving rise to one-to-one unmyelinated type of ensheathment of axons as well as typical peripheral type myelination. By 4 months all Schwann cells were involved in myelination of axons, and there were no further signs of unmyelinated type ensheathments.

These observations suggest that the initial response to demyelination is the accumulation of microglia whose phagocytosis of the myelin is the stimulus for the ingrowth and proliferation of Schwann cells which are thus brought into contact with the demyelinated axons which they then myelinate.

**CHAPTER IX PRELIMINARY OBSERVATIONS ON THE
AXOGENIC EFFECT OF SCHWANN CELLS TRANSFECTED
WITH GENE CONSTRUCTS DIRECTING THE OVER-SECRETION
OF NGF AND NT-3**

9.1. Introduction

While the Schwann cell transplants were able to induce sprouting of long ascending and descending spinal tract axons in the dorsal columns, the axon sprouts are largely confined to the transplants. This could be due to the fact that the Schwann cells provide a more attractive environment than the host tract, so that the axons have no inducement to leave the graft. One possibility for overcoming this failure would be to boost the growth of the nerve fibres by increasing the level of available growth factors.

The preparation of an in vitro dividing population of Schwann cells prior to transplantation offers an excellent opportunity for modification by transfection with growth factor and other genes before transplantation, a procedure referred to as ex vivo (Choi and Gage, 1994) and reported for fibroblasts (Tuszynski *et al.*, 1994b) and more recently Schwann cells (Tuszynski *et al.*, 1994a). According to Gage's observations fibroblasts and Schwann cells modified to over-secrete NGF induce dense sprouting of adult host cholinergic axons (Higgins *et al.*, 1989).

Dr Nick Mazarakis has prepared populations of Schwann cells transfected with gene constructs directing the expression of NGF and NT-3 in vitro. The rate of proliferation of the transfected cells was insufficient to be convenient for cloning, but the whole, mixed population was tested for levels of secretion and then aliquots used for micro-injection into the upper cervical

levels of the adult rat corticospinal tract. After appropriate survival times, the descending corticospinal axons were labelled by an intra-medullary injection of BD.

9.2. Materials and methods (Details see *CHAPTER II*)

9.2.1. Cells for transplantation

The primary Schwann cell culture was prepared as described in *CHAPTER II* and the cells were then genetically modified to secrete NGF. Cells used for transplantation were generated by infection with retroviral containing media from Ψ -2 ecotropic producer cell lines containing recombinant retroviral constructs. The NGF producing cells were provided by Dr Xandra O. Breakefield and were used as described in Frim et al (1993). NT-3 retrovirus producer cells PA317-LNT3RNL were kindly provided by Dr Senut and Professor Gage, and transfected into Schwann cells by Dr Mazarakis.

9.2.2. Transplantation

The Schwann cell suspension was made at a final concentration of $1.0\text{-}1.5 \times 10^7$ cell/ml. $1.0\text{-}1.5 \mu\text{l}$ of cell suspension (approximately 10-15,000 cells) was micro-injected into the descending corticospinal tract of AS adult rat spinal cord by the pulsed air pressure injection system. 3 animals received transplants of the NGF secreting Schwann cells and 3 animals NT-3 secreting Schwann cells.

9.2.3. Anterograde labelling of corticospinal axons

After 10 days survival, BD was injected into the pyramidal tract at the base of the medulla, rostral to the decussation on the opposite side to the transplant. Tissue preparation and BD detection were the same as described in *CHAPTER II*.

9.3. Results

9.3.1. Nerve growth factor

The transplants over-secreting NGF appeared to be attractive to BD-labelled descending axons in the dorsal columns. This was particularly seen when the labelled beam of axons was to one side of the transplant, and the axons became deflected so as to enter the Schwann cell mass (Fig 9.1). The NGF-induced axons tended to be long, rather thin, and poorly varicose, with few branches. However, with the present small sample size and the variation in the size of the BD injections (and hence the numbers of labelled axons) it will be necessary to have further repeats to confirm that this is a consistent finding.

9.3.2. NT-3

The transplants over-secreting NT-3 had a striking effect on the host tract axons. Thick axons entered the transplant, were highly tortuous, and had large varicosities (Figs. 9.2,3).

Figure 9.1

A transplant of a population of Schwann cells (SC) transfected with a construct directing the over-expression of NGF. Fine descending axons are labelled by anterograde transport of BD from an injection into the contralateral medullary pyramid. The descending axons are neither conspicuously varicose, nor branched, but appear to be deflected so as to enter the transplant. m, midline; Survival time, 10 days.

Scale bar = 50 μ m.



Figure 9.2

A transplant of a population of Schwann cells (SC) transfected with a construct directing the over-expression of NT-3. The transplanted cells are surrounded by a mass of wide diameter, highly varicose corticospinal axons. The axons are labelled by anterograde transport of BD and detected with the Ni-GOD method. Both the density of these axons, and their size considerably exceed those found after any transplants of untransfected Schwann cells. 100 μm horizontal Vibratome section. Survival time, 10 days.

Scale bar = 50 μm .

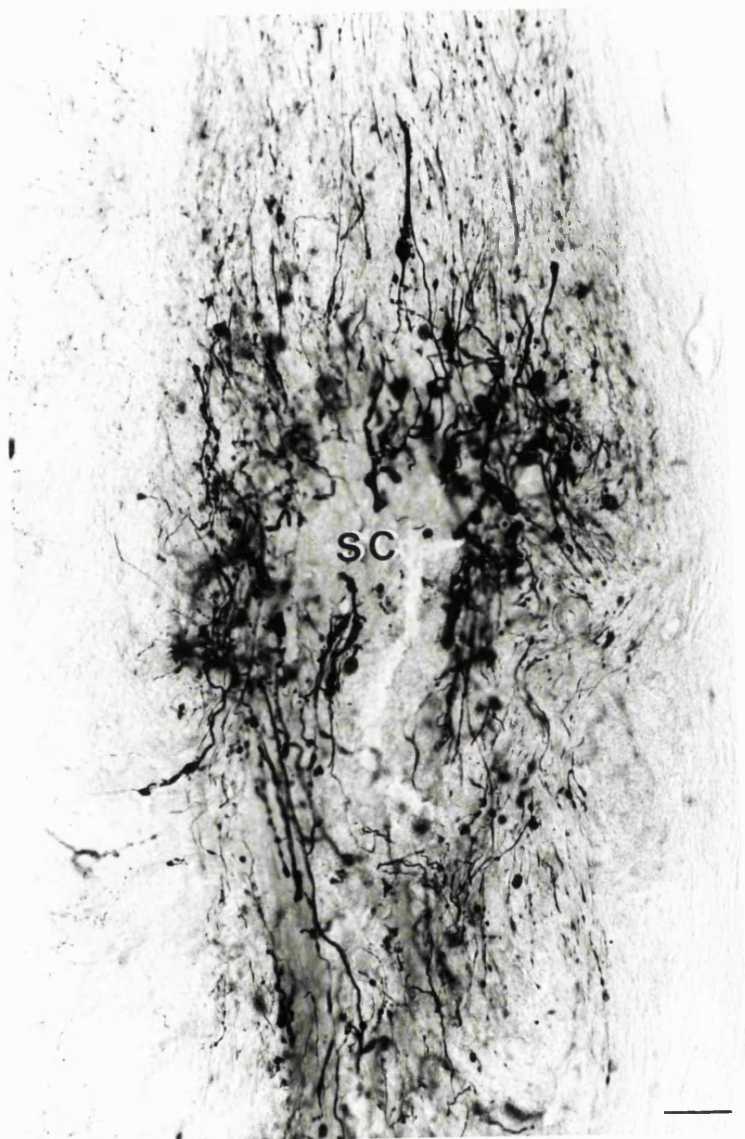
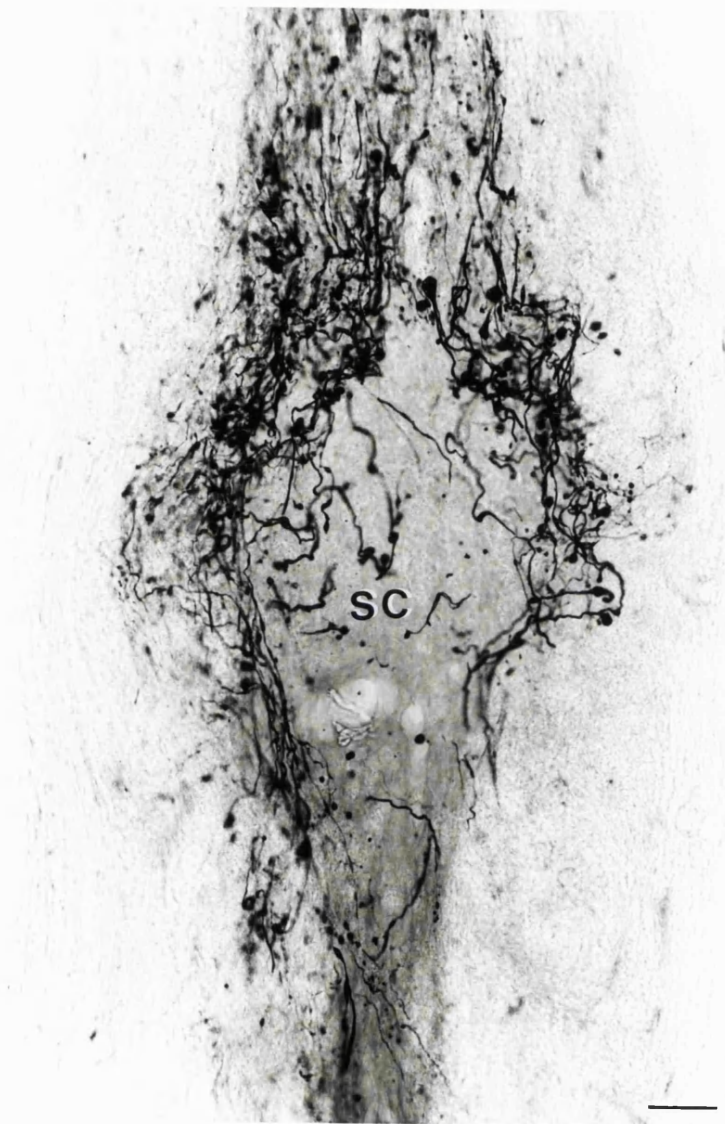


Figure 9.3

Another example to show effect of NT-3 transfected Schwann cells (SC) on the corticospinal axons. 100 μm horizontal Vibratome section. Survival time, 10 days.

Scale bar = 50 μm .



In the case of the NT-3 transplants the encrustation of axons in every section of all the transplants was always very much denser than in any section with untransfected Schwann cell transplants. This strongly suggests that the NT-3 is increasing the power of the Schwann cells to attract host corticospinal axons.

9.4. Discussion

These small samples will require further confirmation and quantification. However, even with the small numbers, there was a marked and consistent qualitative difference between the straight, uniform, virtually unbranched ingrowth into the NGF transplants, and the highly tortuous, thickened, varicose axons which accumulated heavily around the NT-3 transplants. In a parallel study of the same population of NT-3 over-secreting Schwann cells transplanted into the thalamus or hypothalamus, a strikingly similar axonal morphological change was also consistently observed (Daqing Li, personal communication).

It is possible that the NGF transplants select different types of axons from the NT-3 transplants. In particular, the fine diameter, non-varicose fibres responding to the NGF-secreting transplants could belong to a descending cholinergic system (see Fig. 7.1). Tuszynski et al (1994b) showed the response of spinal p75-positive axons to grafts of fibroblasts or Schwann cells (Tuszynski *et al.*, 1994a). In their case they assumed the axons were

sensory. It is unlikely, however, that the axons in the present situation are sensory, since they were labelled by a BD anterograde tracer injection at the medullary level. The nature of the descending axons responding to the NGF secreting Schwann cells in the present material could perhaps be clarified by a double labelling study using p75 immunoreactivity to characterise cholinergic axons.

The striking effect of NT-3 on corticospinal axons is in agreement with a study of Schnell et al (Schnell *et al.*, 1994) who introduced NT-3 protein into a lesion of the corticospinal tract. There is also evidence for an effect of NT-3 on ascending sensory axons (Zhang *et al.*, 1994).

At this preliminary stage, the observations do not allow any firm conclusions beyond the fact that transfection of Schwann cells with gene constructs directing the over-expression of NT-3 (and possibly NGF) in vitro prior to transplantation greatly potentiate the axogenic ability of Schwann cells subsequently transplanted into the adult corticospinal tract.

CHAPTER X GENERAL DISCUSSION

10.1. Lesions

The majority of published accounts of spinal lesions describe the formation of a cavity, consisting of necrotic tissue and debris, and usually opening widely to the pial surface. It has generally been thought that this is an inescapable effect of injury to spinal tissue, related perhaps to some 'aggressive' feature of macrophages or microglia (Giulian and Robertson, 1990; Giulian *et al.*, 1993).

The present micro-lesions and micro-transplants placed deep in the dorsal columns do not cause cavitation, and the operated areas become sealed off from the surface. This suggests that in situations where cavitation occurs, it is probably ischaemic (see also Dusart and Schwab, 1994), due to interference with the pial blood supply.

10.2. Glial structure

Examination of the cellular structure of the dorsal columns showed that both ascending and descending tracts have a glial framework similar to that described for the fimbria and other central tracts (Bayer and Altman, 1975; Bunge, 1968; Lin and Connor, 1989), and that there is no qualitative difference between the glial framework of the very early forming ascending sensory tracts, and the very late forming motor corticospinal tracts.

There were, however, some striking quantitative differences. The size of the astrocytes, and the spacing of the interfascicular glial rows correlated with the diameter spectrum of the axons. Possibly the astrocytes are related to a relatively fixed number of axons; to accomplish this in the wider diameter axon tract, the astrocytes would have to be larger. The same applies to the glial row spacing.

The microglia, however, seemed less affected by axon diameters. They were of similar size and spacing in the two tracts, despite the major differences in axon diameter. Therefore the morphological differentiation of the astrocytes and the microglia are regulated differently.

The specialised midline glial cells (*CHAPTER III*) are probably derived from the embryonic roof plate, which is a tissue which prevents axons from crossing the midline (Snow *et al.*, 1990). What may be a comparable group of midline cells has been described in the optic chiasma (Marcus *et al.*, 1995), where it has been proposed that they provide a mechanism for selectively deflecting the growth cones of the axons of the future ipsilateral projection, and thus preventing them crossing the midline.

10.3. Growth of axons

As has been demonstrated in other adult central white matter tracts (Davies *et al.*, 1993; 1994), the interfascicular glia of the main body of the adult

spinal dorsal columns permit the rapid, long distance elongation of axons from transplanted embryonic neurons. These axons showed no preference for ascending or descending tracts, and travelled equally in rostral or caudal directions in either tract. Since they were derived from a completely foreign type of axon (hippocampal) it is clear that the ability to grow in long adult tracts does not require specific matching of axon type to pathway.

After lesions of the long spinal tracts the cut adult axons can sprout, but, in contrast to the embryonic axons, these sprouts do not elongate along the glial pathway. Nonetheless, at least some adult sprouting axons are maintained for long periods, and this offers at least some hope of future repair.

10.4. Endogenous Schwann cells

In view of the use of transplanted exogenous Schwann cells to attempt to induce growth of cut adult axons, it is interesting that adult spinal lesions induce the ingrowth of endogenous Schwann cells. This ingrowth occurs in a region of oligodendrocytic cell death, and is preceded by a highly localised, regulated microgliosis, which may itself be necessary to generate the stimuli for the Schwann cell ingrowth. The observation that endogenous Schwann cells grow into adult spinal lesions raises the question of why they do not induce axon regeneration.

Study from *CHAPTER VI* shows that when exogenous Schwann cells were introduced at the same time as the axons were damaged (by the injection procedure), the axonal growth response was immediate. *CHAPTER V* study shows that the axonal response after lesions is much delayed, endogenous Schwann cells do not begin to appear the lesion area until some time between 2 and 3 weeks. Thus the differences in the timing of the axon sprouting response is correlated with the differences in the time when Schwann cells are present.

Possibly the failure of the later sprouts induced by endogenous Schwann cells to elongate was because they found the astrocytic framework of the tract to have been distorted and closed up by the astrocytic scarring reaction. This is summarised in Fig. 10.1.

10.5. Exogenous Schwann cells

The rapid induction of axonal sprouts by transplanted exogenous Schwann cells may provide a more favourable situation for advance of the axon sprouts, since the astrocytic scar has not yet formed, and the axon growth may therefore be unimpeded. The early sprouts induced by transplantation of exogenous Schwann cells showed evidence of elongation and fasciculation as if they were finding and growing along the longitudinal astrocytic framework of the host tract (Davies *et al.*, 1994; Suzuki and Raisman, 1992) in a similar manner to axons of embryonic neurons transplanted into adult corticospinal tracts (see *CHAPTER VI*).

Figure 10.1

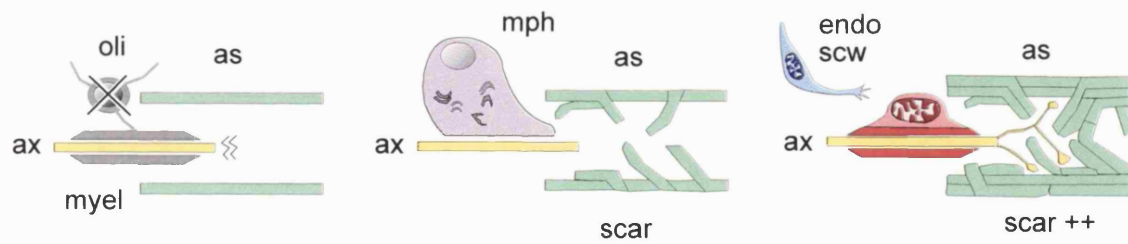
Top line: The immediate introduction of cultured exogenous Schwann cells (exo scw, blue) by an atraumatic method results in the transplanted cells migrating as bipolar, p75-positive cells (bip, blue) into the host tract aligned with the astrocytes (as, green), inducing sprouting of axons (ax, yellow), inducing P₀ and other myelin proteins (myel, red) and myelinating the axons (*CHAPTER VI*).

Bottom line: When a lesion is made, oligodendrocytes (oli, grey) are killed, macrophages (mph, lilac) are activated to invade and proliferate, ingest the cellular and myelin fragments, and induce the ingrowth of endogenous Schwann cells (endo scw, blue), but by this time, the inflammatory tissue has had time to induce astrocytic scarring sufficient to block axon elongation (*CHAPTER V*).

Exogenous Schwann cells



Endogenous Schwann cells



Experiments from this laboratory (Brook *et al.*, 1994), in which Schwann cells were transplanted by extrusion across the thalamus and choroid fissure showed that Schwann cells can indeed both induce the formation of new axon tracts (in locations where long axons are not normally present), and also provide a pathway able to carry the axons out of the diencephalon, across the choroid fissure, and back again into the hippocampus.

The exogenous Schwann cells are able to induce sprouting in both descending and ascending tracts, and the sprouts take 3 main forms: (1) elongation, (2) fasciculation, and (3) formation of pseudo-terminal field like arborisations.

There is little indication of the extent to which the axon sprouts are able to re-enter the adult tract at the other side of the transplants. The present material, however, cannot totally exclude the possibility that some of the cut axons are in fact re-entering the host tracts.

10.6. Genetic engineering

I have attempted to boost the axogenic effects of transplanted Schwann cells by using transfection of genes directing the secretion of NGF and NT-3. The latter (at least) shows definite signs of increasing and modifying the axon response.

10.7. Functional recovery

There is some problem detecting the formation of new connections in a system such as this, where the lesion and/or transplant is very small, and a large number of axons remain uncut.

Functional work on the motor deficits after lesions of the corticospinal tract in a number of different species, and in man, show that the commonest deficits are in fine finger control, and especially in thumb movements in primates and man (Porter and Lemon, 1993). There does not seem to be a localisation of different muscles or movements in the tract. Rather, the failure with partial lesions (even quite large partial lesions) is in movements which require a great deal of coordination.

I have carried out preliminary experiments with a finger reaching test requiring fine coordination for retrieval of food pellets (Montoya *et al.*, 1991). The animals were given training for 10 - 14 days before operation. After circumscribed upper cervical corticospinal tract lesions of the type used in *CHAPTER V*, all animals were impaired on the operated side from the 5th day after operation (the first day tested). However, they recovered rapidly, and by the 12th day, their performance was not different from pre-operative.

This return of function may be due to transfer of the function to surviving pathways. In further experiments I plan to construct a time course of the recovery process to see whether this will show differences according to the size and/or position of the lesion. Only when such a baseline has been constructed will it be possible to use the test to decide whether transplanted Schwann cells can make any improvement.

It is not known how many steps will have to be taken to achieve repair of adult spinal cord injuries, but the present study has described a cellular transplantation approach which has a major axogenic effect on cut spinal axons, and which provides a baseline for testing for beneficial effects of newly discovered genes by transfection of the Schwann cells prior to transplantation.

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APPENDIX NUMERICAL DATA

Numbers of labelled corticospinal axons				
	1 mm rostral to graft	At the level of the graft/lesion	Cut axons in the graft/lesion	Uncut CST axons sprouting into the graft/lesion
Expt number	Schwann cell grafts			
TS1	300	50	20	30
TS2	260	45	18	27
Expt number	Lesions			
SL1	190	20	9	11
SL2	80	11	5	6

The numbers of labelled descending corticospinal axons associated with 2 animals (TS1 and TS2) with grafts and 2 animals (SL1 and SL2) with lesions of the corticospinal tract at the level of cervical segment. The axons were counted 1 mm rostral to the graft/lesion, or at the level of the graft/lesion, where they are further sub-divided into cut axons, and uncut axons sprouting into the graft/lesion.

TS1,2: label HRP in the medullary pyramid, survival 1 week.

SL1,2: label BD in the medullary pyramid, survival 9 weeks.

Cut and uncut axons were defined as in 5.3.1 (last para) on page 104.