M.D. Thesis

New Methodologies for the Treatment of Experimental Spinal Cord Injury

Sebastiano Bavetta

Department of Anatomy and Developmental Biology
University College London
and
Department of Neurosurgery
The Royal London Hospital
ABSTRACT

Spinal cord injury afflicts approximately 45,000 patients in the UK, many of whom are condemned to years of disability. However, new therapeutic approaches are being developed to reduce secondary injury and to stimulate regeneration. Immunophilin molecules are highly concentrated within the CNS, and their ligands FK506, Cyclosporin A and GPI 1046 have significant effects on neurons. There is also evidence that intestinal muscularis externa, containing myenteric plexus, can stimulate axonal regeneration following implantation into the CNS. This thesis has investigated these approaches to experimental spinal cord injury.

FK506 (n=66) and GPI 1046 (n=11) were administered to adult rats following partial transection of the thoracic dorsal columns. FK506 stimulated regeneration of severed sensory axons for short distances rostral to the lesion and also reduced the likelihood of axonal destruction compared to controls (n=45). This effect on axons was independent of protective effects on the parent cell bodies, which remain viable even without treatment. GPI 1046 did not influence axonal survival. Muscularis externa grafts were implanted into the spinal cords of Fischer rats and outcome was assessed functionally and histologically. These grafts had less effect on axonal regeneration in the spinal cord than in the striatum, and no clear effect on lesion size. The presence, within the intestine, of both BDNF and NT-3 mRNA was investigated using in situ hybridization, while the properties of dissociated intestinal muscularis externa smooth muscle cells were assessed with electron microscopy following implantation into the striatum.

This thesis has demonstrated that the effects of muscularis externa on injured axons are less in the spinal cord than in the striatum, but that its cells may be suitable for genetic modification prior to implantation. In addition the findings suggest that immunophilin ligands may have important therapeutic applications for spinal cord injury.
Chapter 1  Spinal cord injury: Introduction

Introduction
Clinical Aspects
Pathology
Therapy
Plan of Investigation
EXPERIMENTAL SECTIONS

Section I: Pharmacological Treatment of Spinal Cord Injury

Chapter 2  Immunophilins and spinal cord injury

Abstract
Introduction
Materials and Methods
Results
Discussion

Section II: The Investigation of Muscularis Externa as Graft Tissue in Spinal Cord Injury

Overview: Muscularis externa and CNS grafting

Chapter 3  Implantation of muscularis externa with mild spinal cord injury: Light and electron microscopy studies

Abstract
Introduction
Materials and Methods
Results
Discussion
Chapter 4  Implantation of muscularis externa using a new model of severe spinal cord injury: Functional and light microscopy studies
   Abstract
   Introduction
   Materials and Methods
   Results
   Discussion

Chapter 5  A study of the distribution of neurotrophin mRNA within the rat intestine
   Abstract
   Introduction
   Materials and Methods
   Results
   Discussion

Chapter 6  Cellular Properties of muscularis externa implanted into the CNS: an electron microscopy study
   Aims
   Introduction
   Materials and Methods
   Results
   Discussion

Chapter 7 General Discussion

BIBLIOGRAPHY
INVENTORY OF FIGURES

CHAPTER 2

Figure 2-1  Flow chart of possible mechanisms by which immunophilin ligands might produce axonal regeneration.

Figure 2-2  Camera lucida drawing used to assess maximum lesion length and width. The lesion area is shaded and the two arrows represent the maximum length and width. (SC = Spinal cord; L = Lesion)

Figure 2-3a  Regenerating axons (arrowheads) growing out of a lesion towards the surface of the spinal cord. Numerous macrophages (M) can be seen within the lesion (FK506-treated animal, killed 3 weeks after surgery; Scale Bar =100µm; dark-field microscopy).

Figure 2-3b  Regenerating fibres (arrowheads) growing along the axis of the cord within the lesion (L) when silastic and tissue glue were applied to the surface of the wound (FK506-treated animal, killed 3 weeks after surgery; Scale Bar =100µm; dark-field microscopy)

Figure 2-4  The swollen terminal of a regenerating axon within the degenerating dorsal columns approximately 10-15mm rostral to the lesion, growing in a cranial direction. (FK506-treated animal, killed 4 weeks after surgery; Scale Bar = 25µm; bright-field microscopy)

Figure 2-5a  Labelled axons (arrowheads) in ectopic positions in the thoracic cord rostral to the lesion. The axons can be seen on the surface of the spinal cord (SSC) probably within the meninges, approximately 2mm rostral to the lesion. (FK506-treated animal, killed 3 weeks after surgery; Scale Bar =100µm; dark-field microscopy).

Figure 2-5b  Regenerating axons (arrowheads) in an ectopic position within the severed stump of a dorsal root (R), which is next to the spinal cord (S), 2mm rostral to the lesion (FK506-treated animal, killed 2 weeks after surgery; Scale Bar = 200µm; dark-field microscopy)
Figure 2-6  The swollen terminal (arrow) of a regenerating axon can be seen approximately 10-15mm rostral to the lesion amongst several other longer, presumably spared, fibres (arrowheads). (FK506-treated animal, killed 4 weeks after surgery; Scale Bar = 25μm; bright-field microscopy)

Figure 2-7  Fibres in an abnormal position within the caudal spinal cord. Normal fibres (arrowheads) can be seen just to the left of the midline in the deepest part of the dorsal columns, with degenerating corticospinal tracts on either side (C), to the left of these fibres are co-lateral branches terminating in the grey matter (*). To the right of the midline is a fibre growing in an abnormal position (arrows) (FK506-treated animal, killed 2 weeks after surgery; Scale Bar = 200μm; dark-field microscopy)

Figure 2-8a  Axons (arrowheads) passing through an area of severe tissue injury within the lesion site. A prominent cluster of macrophages (M) and a cavity (C) are present within the lesion (FK506-treated animal, killed 3 weeks after surgery; Scale Bar = 200μm)

Figure 2-8b  A group of axons (arrowheads) passing through an area of severe tissue injury, with numerous pale macrophages (M) within the lesion site (FK506-treated animal, killed 3 weeks after surgery; Scale Bar = 100μm; dark-field microscopy)

Figure 2-9a  Transverse section of degenerating rostral dorsal columns (DC) showing labelled axons (arrows). On either side are the corticospinal tracts (CS), which comprising descending fibres are not degenerating. (FK506-treated animal, killed 3 weeks after surgery; Scale Bar = 50μm; dark-field microscopy).

Figure 2-9b  Line drawing showing fibres (arrows) at an intermediate depth within the dorsal columns (Scale Bar = 100μm) (CS = corticospinal tract; DC = dorsal columns)
**Figure 2-10a** LRS fibres (arrowheads) can be seen within the rostral degenerating dorsal columns, the lateral boundaries of which are indicated by arrows, approximately 8mm rostral to the lesion (FK506-treated animal, killed 8 weeks after surgery; Scale Bar = 50μm; dark-field microscopy).

**Figure 2-10b** LRS fibres (arrowheads) near the boundary with the corticospinal tract (CS) in the deepest part of the dorsal columns (FK506-treated animal, killed 2 weeks after surgery; Scale Bar = 100μm; dark-field microscopy).

**Figure 2-10c** No LRS fibres are visible within the degenerating dorsal columns (DC) of the rostral cord of a control subject killed 4 weeks after surgery (Scale Bar = 200μm; dark-field microscopy).

**Figure 2-11** Labelled axon terminals (arrowheads) in the ipsilateral nucleus gracilis (I). No terminals are seen in the contralateral nucleus (C). (FK506-treated animal killed 4 weeks after surgery; Scale Bar = 100μm; dark-field microscopy)

**Figure 2-12** Schematic diagram of a spinal cord lesion illustrating how FK506 could result in sparing of axons. On the right side, areas of primary and secondary destruction are proposed. On the left it can be seen that the deepest part of the dorsal columns (outlined in green) might be destroyed by the processes of secondary injury and might therefore be saved by protective therapy (DC: dorsal columns)

**CHAPTER 3**

**Figure 3-1** Transganglionic-labelled dorsal column axons at the boundaries of, and within, (arrows) a 2 week muscularis externa graft (Gr; boundaries indicated by dotted line) in the spinal cord. Labelled axons within the graft form bundles and appear to branch.

(Scale bar =60 μm)
Figure 3-2  Transganglionic-labelled dorsal column axons rostral (r) and caudal (c) to a 2 week muscularis externa graft (Gr). Some labelled axons are seen within the graft (arrows). (Scale bar = 100μm)

Figure 3-3  Transganglionic-labelled dorsal column axons (arrows) within a 2 week muscularis externa graft (Gr; boundaries indicated by dotted line) in the spinal cord (sc). (Scale bar =30μm)

Figure 3-4  Camera Lucida drawing showing the position of a muscularis externa graft 6 weeks after grafting in the spinal cord. The graft is entirely within the spinal cord, and impinges on different areas of the spinal cord. (dc-dorsal column, idc-injured dorsal column, lwc-lateral white column, gr-graft, p-peripheral nerve-like tissue, dh-dorsal horn, vh-ventral horn) (Scale bar = 1 mm)

Figure 3-5  Toludene blue stained section of a muscularis externa graft within the spinal cord, 6 weeks after grafting. (dc-dorsal column, gr-graft, p-peripheral nerve-like tissue, dh-dorsal horn) (Scale bar = 200 μm)

Figure 3-6  Electron micrograph of muscularis externa graft examined 6 weeks after implantation into the spinal cord. Myelinated (asterisk) and unmyelinated axons can be seen, loosely enveloped by fibroblast processes (fb). The size and extent of glial ensheathment of unmyelinated axons varies greatly. The other graft constituents, smooth muscle cells (m), fibroblasts (fb) and collagen fibrils can also be seen. (Scale bar = 500 nm)

Figure 3-7  Unmyelinated axons within a muscularis externa graft 6 weeks after implantation. Large diameter axons (arrows) and small diameter axons (arrowheads) are present in a compact bundle and are associated with what appears to be enteric glial cell cytoplasm (g). Smooth muscle cells (m) surround the nerve bundle. (Scale bar =1 μm)

Figure 3-8  Part of the interface between a muscularis externa graft and the lateral
white column of the spinal cord, showing a thin astrocytic glia limitans covered by basal lamina (arrowhead). Astrocyte processes protrude into the junctional zone, associated with a myelinated axon (a). Two Schwann cell myelinated axons (asterisks) are present at the interface, and a few small diameter axons (arrows) are present amongst the oligodendrocyte-myelinated CNS axons. (C-collagen fibrils in the expanded extracellular space around the graft) (Scale bar =1 μm)

**Figure 3-9** This region of the interface between the graft and the spinal cord is characterised by an expanded extracellular space containing collagen fibrils (C), abutting a thick astrocytic glia limitans (arrowheads). There is no sign of significant axonal sprouting in this region. (Scale bar =1 μm)

**Figure 3-10** Electron micrograph showing tissue resembling peripheral nerve found dorsal to the muscularis externa graft in the position indicated in Figure 3-4. Several Schwann cell myelinated axons are present within a compartment surrounded by fibroblast processes (arrowheads). Astrocyte processes are present amongst the axons and in some cases form mixed bundles with Schwann cells and/or axons (arrows). (Scale bar =2 μm)

**Figure 3-11** Smooth muscle cells (M) loosely associated with a capillary (C) extending into the lateral white column form a 6 week muscularis externa graft. An astrocytic basal lamina (arrowhead) separates the muscle cells from the axons in the spinal cord. (Scale bar =2 μm)
CHAPTER 4

Figure 4-1  Decker Microrongeur biopsy forceps, a 20cm ruler is included in the picture for comparison.

Figure 4-2  The modified Kopf small animal stereotactic frame used in these experiments, a 20cm ruler is included in the picture for comparison.

Figure 4-3  Schematic diagram showing the position of the tips of the biopsy forceps (DM) relative to the spinal cord (SC) seen in X-section. The open jaws of the forceps just touch the cord on either side of the dorsal vein (DV). The arrow indicates that the forceps are lowered vertically downwards into the cord.

Figure 4-4  Numerous labelled fibres (arrowheads) can be seen in the dorsal columns caudal to the lesion site (L). The more ventral spinal cord (SC) shows no labelling. No regenerating axons can be seen beyond the caudal margin of the lesion site (Animal studied at 8 weeks after surgery; Scale Bar = 400μm)

Figure 4-5  A few axons (arrowheads) are seen within residual spinal cord tissue (SC), caudal to the main lesion cavity (C). (Muscularis externa inserted 4 weeks previously; Scale Bar = 400μm)

Figure 4-6  Lesion site in a control animal (no graft) studied at 8 weeks. No regenerating axons are visible within the injured tissue (*). Some macrophages (M) are present and artefactual labelling is also present (Ar) (Scale Bar = 400μm)

Figure 4-7  Lesion site in an animal with muscularis externa grafted into the lesion site 4 weeks previously. Numerous macrophages (M) are seen within tissue that may be scar and / or graft (*). No regenerating fibres are seen. (Scale Bar = 200μm)

Figure 4-8  Lesion (L) within a thionine-stained section of spinal cord, in which a standard lesion was made post-mortem in a fixed tissue (Scale Bar = 500μm)
Spinal cord function following injury in control animals. Function was assessed within 3 hours of surgery (Day 1), 2 days later (Day 3), at 1 week and then weekly thereafter. Each animal is represented by a particular colour/symbol, and its progress can be followed through the post-operative period.

Spinal cord function following injury. A muscularis externa graft was inserted into the spinal cord lesion site. Function was assessed within 3 hours of surgery (Day 1), 2 days later (Day 3), at 1 week and then weekly thereafter. Each animal is represented by a particular colour/symbol, and its progress can be followed through the post-operative period.

CHAPTER 5

Intestine from 2 month animals hybridized for BDNF mRNA illustrating labelling within the myenteric plexus (arrow) and mucosa (arrowhead) (Scale bar = 100μm)

Intestine from 2 month animal hybridized for BDNF mRNA, the submucous plexus (arrow) and mucosa (arrowhead) can be seen deep to the smooth muscle layer (sm) (Scale bar = 50μm)

Neonatal intestine showing positive labelling for BDNF mRNA in the ENS (arrow) and mucosa (arrowhead) (Scale bar = 100μm)

1 week intestine showing positive labelling for BDNF mRNA in the ENS (arrow) and mucosa (arrowhead) (Scale bar = 100μm)

Ileal mucosa (arrowhead) is strongly positive for BDNF mRNA in this 1 month animal. ENS is also labelled (arrow) (Scale bar = 50μm)

Labelled (arrow) and unlabelled (arrowhead) ENS cells can be seen in a mosaic pattern within the myenteric ganglion of a colonic specimen from a 2 month animal hybridized for BDNF mRNA. (Scale bar = 25μm)
Figure 5-5a  Intestine from a 1 week animal showing positive labelling for NT-3 mRNA in the ENS (arrow) and mucosa (arrowhead) (Scale bar = 100μm)

Figure 5-5b  Colon from a 2 month animal showing positive labelling for NT-3 mRNA in the ENS (arrow) and mucosa (arrowhead) (Scale bar = 100μm)

Figure 5-6  (a) Section of colon from an adult animal processed for NT-3 mRNA. The arrow indicates a positively labelled ganglion cell while the arrowhead points to an unlabelled cell. (b) In situ hybridization for BDNF mRNA has been performed on the same tissue but the positive and negative labelling of the same ganglion cells has been reversed (Scale bar = 100μm)

CHAPTER 6

Figure 6-1  Cultured muscularis externa, 1 week after dissociation, has been immunohistochemically reacted for PGP 9.5. The strongly labelled cell body of a neuron (arrow) is shown, from which several neurites have emerged (arrowheads). Clumps of smooth muscle cells (M) are present in the background (Scale Bar = 50μm).

Figure 6-2  Toluidine blue-stained semi-thin section through the striatum, 1 year after implantation of dissociated muscularis externa cells. The graft (Gr) is clearly seen within the striatum (St), which contains bundles of myelinated fibres (*). A large blood vessel (BV) and several smaller vessels are present in and around the graft (Scale Bar = 50μm).

Figure 6-3  Electron micrograph showing a cluster of smooth muscle cells (M), 1 month after implantation. They appear to be partially enveloped by a fibroblast (F), which separates them from a nearby astrocyte (A), which has formed a glia limitans around the graft. A large group of
myelinated striatal axons (MY) is to one side, while on the other side is striatal neuropil (ST) (Scale Bar = 2μm).

**Figure 6-4** Loosely packed smooth muscle cells (M) separated by extracellular matrix and collagen fibrils (C), 1 year after implantation. Blood vessels (B) are also present within the graft. Myelinated axons (My) and an astrocyte (A) can be seen nearby (Scale Bar = 2μm).

**Figure 6-5** Smooth muscle cells (M) 1 year after implantation, showing a partial loss of the characteristic contractile phenotype. Although myofilaments are present, they appear to be poorly connected to the membrane (arrows) (A-astrocyte; Scale Bar = 500nm).

**Figure 6-6** Smooth muscle cells (M), of the secretory phenotype, which had migrated into the striatum (ST) can be seen loosely arranged around a blood vessel (B) and surrounded by extracellular matrix with large amounts of collagen (C) (1 month after surgery; Scale Bar = 1μm).

**Figure 6-7** Smooth muscle cells (M), of a more contractile phenotype, next to an endothelial cell (e), which forms part of the wall of a large venule (Lumen-L). There are enlarged extracellular spaces between the vessel and the striatal neuropil. A glia limitans, formed by astrocyte (A) processes covered by a basal lamina (arrowheads), can also be seen (1 month post-implantation; Scale Bar = 1μm).

**Figure 6-8** A well defined glia limitans (arrowheads) can be seen separating smooth muscle cells (M) from CNS tissue (C-collagen fibrils, A-astrocyte, My-myelinated axons; 1 year post-implantation; Scale Bar = 500nm).

**Figure 6-9** A bundle of unmyelinated axons (Ax), seen in x-section, run through the graft, surrounded by smooth muscle cells (M), which in some areas establish close contact with the bundle of axons (arrows) (1 year post-implantation; Scale Bar = 500nm)

**Figure 6-10** Unmyelinated axons (Ax) between smooth muscle cells (M) and astrocytes (A). Smooth muscle can be seen in close contact with glial
process (arrowheads), with no intervening basal lamina (1 year post-implantation; Scale Bar = 500nm)

**Figure 6-11** A bundle of myelinated axons (My) at the periphery of the graft. The axons are myelinated by Schwann cells (S) and are partially compartmentalized by fibroblast processes within a matrix of dense collagenous tissue (C) (Scale Bar = 2μm).

### TABLES

**CHAPTER 1**

Table 1-1 Causes of Hypoxia in Spinal Cord Injury

**CHAPTER 2**

Table 2-1 Review of the scientific literature on the effects of immunophilin ligands on neurons (SC = Spinal Cord, -ve = negative, Non-IS = Non-immunosuppressant)

Table 2-2 Series 1 treatment groups ( * figures in brackets represent animals that did NOT receive steroids).

Table 2-3 Series 2 treatment groups ( * figures in brackets represent animals that did NOT receive steroids)

Table 2-4 A comparison of the percentages of FK506-treated animals and controls with LRS fibres. The statistical significance of differences between treated and untreated groups is indicated. (Abbreviations: n/a - not applicable; n/s = not statistically significant)

Table 2-5 The effects of treatment and assessment variables on outcome following FK506 treatment (Figures refer to combined results of Series 1 and 2; the results of Cyclosporin A and GPI 1046 experiments are not shown) (Abbreviations: n/a - not applicable)

Table 2-6 Numbers and percentage of subjects with LRS fibres following MP treatment (Abbreviations: n/a - not applicable; n/s = not statistically
significant) (Figures refer to combined results of Series 1 and 2; the results of Cyclosporin A and GPI 1046 experiments are not shown)

Table 2-7  Lesion size, defined as the product of maximum length and width (mm$^2$), in Series 1 experiments. (Note: IQR= interquartile range, Mann-Whitney tests refer to pairs of data sets such as those receiving or not receiving FK506)

CHAPTER 4

Table 4-1  Scoring system for hindlimb motor control tests: each limb is assessed independently and the two scores are added to give an overall value of 0-12 which is then divided by 3 to give a score of 0-4.

Table 4-2  Lesion size (defined by length, depth or length x depth) in 13 control animals (2 of which had freeze-killed grafts inserted). The time at which each animal was killed is also indicated.

Table 4-3  Lesion size (defined by length, depth or ‘length x depth’) in 10 animals treated with muscularis externa grafts. The time at which the animal was killed ranged from 2 to 8 weeks.

Table 4-4  The lesions are grouped in fours (A-D) representing four separate lesions made in the post-mortem spinal cord of each animal. Lesion size is defined by length, depth or ‘length x depth’.

Table 4-5  Spinal cord function following injury in 10 control animals. A separate column represents each animal. The range, median and mean are indicated for each time interval at which an assessment was performed.

Table 4-6  Spinal cord function following injury in 6 animals, which received muscularis externa grafts. A separate column represents each animal. The range, median and mean are indicated for each time interval at which an assessment was performed.
PREFACE

The experimental chapters fall into two broad categories: Section I: Pharmacological Treatment of Spinal Cord Injury, dealing with immunophilin ligands (Chapter 2), and Section II: The Investigation of Muscularis Externa as Graft Tissue in Spinal Cord Injury. The latter includes work on the implantation of muscularis externa into the injured spinal cord (Chapters 3 and 4) and on properties of muscularis externa relevant to its potential use as a source of grafted cells for the CNS (Chapters 5 and 6). A wide variety of experimental techniques were used, and sometimes the same approach was modified in different sets of experiments. Therefore, although unconventional, separate ‘Materials and Methods’ sections were included with each experimental chapter, with the aim of increasing clarity, at the cost of some repetition. Chapter 1 reviews the clinical and experimental aspects of spinal cord injury. Chapter 7 is a general discussion, which does not attempt to reiterate what has already been said in Chapter 1 and in the discussion sections of the experimental chapters. Instead Chapter 7 is more speculative in nature, highlighting some general themes that arose from the experimental work, and suggesting questions for future investigation.

ACKNOWLEDGEMENTS

The author recognizes the particular debt owed to Professor G. Burnstock and Mr P.J. Hamlyn who acted as supervisors for the thesis and provided invaluable scientific advice. As well as his general encouragement and help throughout the author’s neurosurgical training, Mr Hamlyn played a vital role in encouraging the author to embark on this period of research. Professor Burnstock was able to provide a happy balance between guidance and independence to explore areas of interest to the author. In addition Dr P.N. Anderson acted as a mentor during the entire period of the thesis, providing crucial practical and intellectual guidance; without his help this
thesis would not have been possible. The author would also like to acknowledge the incisive comments and suggestions of Professor A.R. Lieberman during the writing of the section on immunophilin ligands. The author also wishes to thank Mark Turmaine, Doreen Bailey and Julia Winterbottom for their invaluable technical assistance.

The study was supported by The Royal Hospitals Neurosurgical Research Fund and the author would like to thank all those companies, charities and individuals, such as Mr and Mrs C.J. Ansell, which donated to the Fund, thus allowing the work to proceed. In particular large donations from The John and Lucille van Geest Foundation and the PF Charitable Trust were crucial in financing the work of this thesis.

Finally I would like to thank my wife Paula for tolerating the hardships involved in the past two years, my children Georgina, Francis and Isabelle for providing a constant source of distraction and entertainment and my parents for their encouragement and support.

**AUTHOR’S STATEMENT**

This study was conceived and carried out by the author over a three year period. The plan of investigation was developed by the author, in discussions with Professor G. Burnstock, Mr P.J. Hamlyn and Dr P.N. Anderson. All experimental work described, including surgery, tissue preparation, histological examination and statistical analysis was performed by the author.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA</td>
<td>$\gamma$-aminobutyric acid</td>
</tr>
<tr>
<td>GPI 1046</td>
<td>3-(3-pyridyl)-1-propyl (2S)-1-(3,3-dimethyl-1,2-dioxopentyl)-2-pyrrolidinedinecarboxylate</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-tetramethylbenzidine</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>Chi-squared</td>
</tr>
<tr>
<td>CT-HRP</td>
<td>Cholera toxin sub-unit B conjugated to horseradish peroxidase</td>
</tr>
<tr>
<td>CNTF</td>
<td>Ciliary neurotrophic factor</td>
</tr>
<tr>
<td>Cs A</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td>DIG</td>
<td>Digoxygenin</td>
</tr>
<tr>
<td>DCN</td>
<td>Dorsal column nuclei</td>
</tr>
<tr>
<td>ENS</td>
<td>Enteric nervous system</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factors</td>
</tr>
<tr>
<td>FET</td>
<td>Fisher’s exact test</td>
</tr>
<tr>
<td>FKBPs-12</td>
<td>FK506 binding protein-12</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial-derived neurotrophic factor</td>
</tr>
<tr>
<td>GAP-43</td>
<td>Growth Associated Protein of 43kda</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factors</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile range</td>
</tr>
<tr>
<td>LRS</td>
<td>Long rostral sensory</td>
</tr>
<tr>
<td>MP</td>
<td>Methylprednisolone sodium succinate</td>
</tr>
<tr>
<td>ME</td>
<td>Muscularis externa</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NT-4</td>
<td>Neurotrophin 4</td>
</tr>
</tbody>
</table>

19
<table>
<thead>
<tr>
<th>Neurotrophin 5</th>
<th>NT-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurotrophin-3</td>
<td>NT-3</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>NO</td>
</tr>
<tr>
<td>Nitric oxide synthase</td>
<td>NOS</td>
</tr>
<tr>
<td>N-methyl-D-aspartate</td>
<td>NMDA</td>
</tr>
<tr>
<td>N-nitro-L-arginine</td>
<td>L-NAME</td>
</tr>
<tr>
<td>Nuclear Factor of Activated T-cells</td>
<td>NFAT</td>
</tr>
<tr>
<td>Phosphate buffered saline</td>
<td>PBS</td>
</tr>
<tr>
<td>Protein gene product 9.5</td>
<td>PGP</td>
</tr>
<tr>
<td>Serotonin</td>
<td>5-HT</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>SOD</td>
</tr>
<tr>
<td>Thyrotropin releasing hormone</td>
<td>TRH</td>
</tr>
<tr>
<td>Transforming growth factor beta 1</td>
<td>TGF-β1</td>
</tr>
<tr>
<td>Transforming growth factor alpha</td>
<td>TGFα</td>
</tr>
</tbody>
</table>
CHAPTER 1

Spinal Cord Injury: Introduction

INTRODUCTION

CLINICAL ASPECTS OF SPINAL CORD INJURY
- Clinical features
- Investigation
- Treatment

PATHOLOGY
- Mechanisms of trauma
- Histological changes
- Pathogenesis and secondary tissue damage

THERAPY: EXPERIMENTAL STRATEGIES
- Pharmacological prevention of secondary injury
- The promotion of regeneration
- Regeneration- pharmacological treatment
- Regeneration- transplantation

PLAN OF INVESTIGATION
INTRODUCTION

Spinal cord injury was first described in the Edwin Smith papyrus as long ago as 2500 BC. An unknown Egyptian physician accurately described the features of traumatic tetraplegia and advised that the patient should be left to die (Swain et al. 1986) (Andres, 1989). The condition was later described by Hippocrates (470-370BC) and Galen (129-199AD). Paulus of Aegina advised removal of fractured laminae, while in the 16th century Ambrose Pare operated for depressed bone fragments impinging on the cord and nerve roots (Wolman, 1964). There were no further advances until the beginning of the 19th century and the rise of modern scientific medicine. The function and anatomy of the spinal cord became better understood through the writings of neurologists such as Marshall Hall (1790-1857), Charles Bell (1774-1842) and Brown-Sequard (1817-1894)(Andres, 1989). However, the clinical outlook remained appalling, and in the First World War 90% of patients died within one year of sustaining a spinal cord injury. It was not until the last 50 years that the prognosis improved through the work of pioneers such as Guttman at Stoke Mandeville (Swain et al. 1986).

Approximately 45,000 people are estimated to be affected by the consequences of spinal cord injury in the UK (Flint, 1988), with nearly a 1000 new cases each year (Flint, 1988) (Swain et al. 1986). In the USA the annual incidence is 3/100,000 with a prevalence of 50/100,000 (Kurtzke, 1984). Many of these injuries are in young people, with young men aged between 16-30 being particularly at risk (Marciano et al. 1995). Each year spinal cord injury in North America costs society $1.5 million for lifetime medical costs and lost earnings in addition to the personal suffering of the victims and their families (Tator and Fehlings, 1991).
CLINICAL ASPECTS

The causes of spinal cord injury have varied with time and geographical location, but recent figures for a spinal treatment centre in the UK (Swain et al. 1986) indicated that road traffic accidents accounted for approximately 55% of cases. Of these, motorcycle accidents were twice as common as car accidents, while injuries to pedestrians were the least common cause. Domestic and industrial accidents, such as falls down stairs or from ladders, accounted for just over 20% and sports injuries accounted for just under 20%, with diving, rugby and horse riding being particularly dangerous. The remaining 5% of cases were the result of criminal assault. The resulting injury occurred in the cervical spine in over 50% of cases, with the majority of the remainder in the thoracic region and at the thoraco-lumbar junction. The remaining 10% of cases occurred in the lumbosacral region (Swain et al. 1986) (Johnston, 1993). In about 10% of cases two separate sites within the cervical spine were injured (Johnston, 1993). Approximately half of all patients had complete injuries of the spinal cord with no residual neurological function below the level of the injury (Tator and Fehlings, 1991).

CLINICAL FEATURES

The clinical features of spinal cord injury vary in severity and location. There may also be associated injuries to the head, thorax, abdomen and long bones, which can obscure and exacerbate the effects of the spinal cord injury. For instance in an unconscious patient paralysis may be missed, while a tetraplegic patient with breathing problems is more likely to develop respiratory insufficiency in the presence of thoracic injury. The spinal injury itself causes pain due to bony and ligamentous injury and neurological dysfunction due to cord injury. There may be partial or complete loss of function below the level of the lesion, which results in paralysis, anaesthesia and autonomic problems. In thoracic and lumbar injuries the legs will be partially or completely paralysed, with brisk reflexes characteristic of upper motor neuron injuries. There may also be sensory loss with an upper level determined by the
location of the spinal injury. Cervical injuries produce weakness of all limbs, with a variable involvement of the arms depending on the precise level cervical cord injury. While weakness will be of the upper motor neuron type in the legs, in the arms there may also be features of lower motor neuron weakness which, together with the pattern of sensory loss, will help in determining the neurological level of injury. After some days there may be some improvement in neurological function in a few patients that is largely complete within a few months, but can sometimes continue for up to 2-3 years (Hughes, 1992).

Spinal cord injury will also result in autonomic dysfunction, which is more severe with cervical injuries. In the acute phase the main problems are with disruption of cardiovascular regulatory mechanisms and urinary retention due to sphincter disturbance. In cervical injuries the loss of intercostal nerve function will produce respiratory difficulties, and in cases of upper cervical lesions the phrenic nerves will also be lost with complete loss of respiratory control. All these problems require careful long-term management, and in addition as the acute phase is passed further problems manifest themselves. Contracture deformities of the limbs can arise and, in addition to urinary problems resulting from chronic retention and its treatment, there will be sexual dysfunction and in males reduced fertility. Sensory loss predisposes patients to developing pressure sores, which in turn can cause complications such as infection and amyloidosis.

INVESTIGATION
On arrival in hospital any patient suspected of having a spinal injury should have X-rays of the spine, with special views if necessary to demonstrate the odontoid or the cervico-thoracic junction. If instability of the cervical spine is suspected, flexion / extension views may be needed. More precise delineation of vertebral fractures may require CT scanning, sometimes with myelography. In recent years MRI, which is particularly helpful in recognising damage to ligaments, discs and pre-vertebral tissues, has become more widely available (Johnston, 1993). Rarely, if diagnostic
difficulties arise, for instance in infants or unconscious patients, EMG may help to define neurological problems. Finally, tests of general well-being such as haemoglobin levels, renal function tests and blood gases will usually be required, particularly if there are associated injuries elsewhere.

TREATMENT
Spinal cord damage cannot be reversed and the aims of treatment are to prevent further neurological injury, to prevent and treat complications and to rehabilitate the patient.

If spinal cord injury is suspected, at the scene of the accident or in the early stages of hospital admission, then immediate immobilisation of the affected segment is imperative. Cervical injuries can be managed acutely by holding the head firmly between two hands until a firm Philadelphia style collar can be fitted or traction initiated (Johnston, 1993). Thoracic and lumbar injuries can be simply managed on a bed in the acute phase, but great care must be taken in moving the patient. When dealing with unconscious patients, for whom full assessment is not possible, the spine should initially be immobilised until injury has been excluded. Subluxations and dislocations can be reduced with traction or surgery. If investigation demonstrates that the spinal column is unstable the patient may be managed conservatively, for instance with a halo-brace or with prolonged traction, or surgically with internal fixation of the affected bony segments. Surgery has the advantage of allowing early mobilisation of the patient. Occasionally, surgical decompression of the spinal cord may be performed to prevent further damage. For patients in whom neurological deficits are present, methylprednisolone is often administered, as early as possible, with the aim of reducing secondary injury to the cord (Bracken et al. 1990) (Hall, 1992).

Any associated injuries must be vigorously treated since hypoxia or hypotension can exacerbate neurological damage. In addition, the acute complications of spinal cord
injury must be treated. In particular the patient will need catheterization, and ventilation
can be required in high cervical injuries.

Patients are ideally managed in centres with experience of spinal cord injury both in
the acute phase and particularly in the prolonged period of rehabilitation that follows.
The patient is taught methods, such as intermittent catheterization, of coping with the
long-term sequelae of spinal cord injury, and to maximise residual physical
capabilities and to adapt his lifestyle to the new disability.

PATHOLOGY

In the past, spinal injury was viewed as being caused largely by primary injury due to
direct, mechanical disruption to the nervous tissue. More recently, however, it has
become clear that the spinal cord usually remains physically intact and that neural
damage occurs in large part in the hours and days following injury. The pathological
processes involved in this secondary injury include metabolic, hypoxic, biochemical
and inflammatory derangements, which interact in a complex and unpredictable
fashion (Faden, 1993) (Tator and Fehlings, 1991). Another traditional view has been
that damaged axons do not regenerate. Recently this view has been challenged,
following the demonstration that regeneration does occur within the spinal cord
injury after axonal transection (Bregman et al. 1995a) (Cadelli and Schwab, 1991)
(Benfey and Aguayo, 1982) (Richardson et al. 1980b) (Wrathall et al. 1982)
1994b). The findings that secondary axonal damage can occur, and that axons can
regenerate have provided the conceptual foundation for an increase in interest in
spinal cord injury (Faden, 1993).
MECHANISMS OF TRAUMA

The spinal cord is protected from injury by the bones of the vertebral column, which permit a complex range of movements (flexion and extension, abduction and adduction and rotation). The lumbar vertebrae, which bear the weight of the upper body, and the thoracic vertebrae, which are buttressed by the ribs, can withstand considerable force. The slender and mobile cervical spine is much more vulnerable and is the site of most spinal injuries (Hughes, 1992).

Traumatic lesions of the spinal cord can be classified as direct, due to penetrating injuries, or indirect, due to fractures or fracture-dislocation. The former are rare in the UK, but may be caused by stab wounds or penetrating missiles, such as bullets, and can produce well defined neurological deficits such as Brown-Sequard Syndrome (Hughes, 1992) (Andres, 1989). Indirect injuries, which are much more common, result from forces applied to the spinal column. These forces can be resolved into flexion, extension, vertical compression and rotation (Johnston, 1993). The resulting vertebral injury will depend on the nature and site of these forces. Thus sudden hyperflexion of the cervical spine may tear the interspinous and posterior longitudinal ligaments as well as the annulus fibrosus of one or more intervertebral discs without causing a fracture. Forced extension or abduction may fracture the pedicles, laminae or interarticular processes, often with little radiological abnormality. There may be subluxation of adjacent vertebrae, which compromises the spinal canal. Forced flexion at any level in the spinal column can produce wedge fractures of the vertebral body, while vertical compression can result in burst fractures of the vertebral body, in such cases the cord can be damaged by bony fragments displaced posteriorly. Whatever the exact nature of the bony injury, if the spinal canal is compromised the result may be spinal cord injury which usually takes the form of acute compression or laceration. In addition there may be more prolonged stretching of the cord over a bony or cartilaginous protrusion, resulting in additional ischaemic injury (Hughes, 1992) (Tator and Fehlings, 1991) (Marciano et al. 1995).
HISTOLOGICAL CHANGES

The histological changes produced by spinal cord injury are a mixture of the consequences of reversible injury (such as tissue swelling and inflammation) and those related to cell death such as necrosis, apoptosis and eventually repair. A variety of histological changes have been described following spinal cord injury, the pattern of which will vary with the nature of the injury. Thus a crush injury would be expected to have different histological consequences than would a sharp transection. Histological changes will also vary with the time after injury and can be classified as early (1-3 weeks), intermediate (3 weeks to 2 years) and late (3 years onwards) (Hughes, 1992).

Early Pathological Changes: The immediate consequences of spinal cord trauma comprise axonal injury, damage to neuronal cell bodies and exudative phenomena (Hughes, 1992). Some features of exudative reaction can be demonstrated within five minutes of injury by electron microscopy, and within 15 minutes changes are apparent on light microscopy. The venules of the grey matter become congested with erythrocytes that also become extravasated into the perivascular spaces. Parenchymal haemorrhages appear in the grey matter associated with oedema of the white matter. Within two hours the haemorrhages in the grey matter increase. Polymorphs and lymphocytes will migrate to the vicinity of the lesion as part of an inflammatory cellular reaction. All these changes cause swelling of the spinal cord which becomes rounded and tense within the dura, obliterating the subarachnoid and subdural spaces. The damaged section of the cord thus becomes swollen and spindle-shaped, with a central region of softening punctuated at either end by an area of necrosis. Within four hours there is evidence of swelling and disintegration of axons and their myelin sheaths. As these changes progress during the first three weeks, axons that are mildly damaged show beading with silver stains, while axons that are more severely damaged will be represented by a row of silver droplets in the former position of the axon. The point of axonal transection may develop a terminal swelling commonly called an end bulb. The myelin sheaths will correspondingly show changes ranging
from a vesicular appearance to complete fragmentation of the myelin into fatty droplets. The cell bodies of neurons may be completely disrupted by direct injury or show the phenomenon of central chromatolysis, sometimes called axonal reaction, as a reaction to axonal injury (Hughes, 1992) (Marciano et al. 1995).

**Intermediate Pathological Changes:** After approximately 3 weeks the early changes subside and a reparative phase begins that lasts for 2 or more years. The oedema and parenchymal haemorrhages disappear and the lesion site may be replaced by a cyst cavity, which sometimes develops into post-traumatic syringomyelia. The polymorphs of the early period are replaced by lymphocytes and macrophages, including lipid phagocytes, which are present in areas where neurons or axons have been broken down. In areas of the spinal cord where damage is moderate, reactive astrocytic gliosis occurs, but in severely damaged areas, where glial cells are destroyed together with neurons, a connective tissue scar will be produced by fibroblasts. Surviving neurons demonstrate chromatolysis that may continue for up to 3 years and in some, but not all, populations of neurons this may progress to cell death (Schweitzer and Dohan, 1993). The long fibre tracts distal to the injury site undergo Wallerian degeneration. In the initial stages following injury the distal long fibre tracts are composed of relatively unperturbed tissue with an intact vascular supply, blood brain barrier and glia limitans. By 30 days post-injury there is depletion of abnormal axoplasm and an increase in the extracellular myelin dissociated from the axoplasm. Subsequently a cellular reaction develops with the appearance of macrophages and large astrocytes. By 60 days post-injury there are a few large astrocytes associated with unmyelinated and thinly myelinated axons (West and Collins, 1991).
Late Pathological Changes: By 3 years after injury the lesion site has been replaced by an acellular collagenous scar involving the cord and meninges. The regions above and below this show an intense astrocytic fibrous gliosis, consisting of a glial network with occasional astrocytes (Hughes, 1992).

PATHOGENESIS AND SECONDARY TISSUE DAMAGE
Damage to the spinal cord after acute traumatic injury can occur in two ways: primary mechanical damage, both at tissue and cellular level (Tator and Fehlings, 1991) and secondary injury from one or more of the additional pathological processes which are initiated by the primary injury. Primary mechanical damage can be:

- Compression
- Impact or missile
- Distraction
- Shear stress
- Laceration

Axonal injury is of critical importance in spinal cord injury and in recent years it has become apparent that axonal transection within the CNS often does not occur at the time of mechanical injury, but largely in the hours following trauma. Thus experimental mechanical injury may cause axonal stretching which does not result in immediate transection, but initiates changes, which include impairment of axonal transport, leading to axonal swelling and eventually axotomy between 24 and 72 hours later (Maxwell et al. 1991) (Povlishock, 1992).

In addition multiple biochemical, metabolic and vascular process are initiated within the CNS tissue by trauma. Some are useful responses to tissue damage, but others cause secondary injury. Damage caused by one process can initiate a cascade of further changes, which amplifies the damage. Thus hypoglycaemia or ischaemia can
directly damage cells, but in addition can cause release of the amino acid neurotransmitter glutamate, which is excitotoxic and stimulates an influx of calcium into the already vulnerable neuron and hence cell death.

Many mechanisms of secondary injury have been proposed including:

- Spinal cord haemorrhage
- Inflammatory changes
- Oxygen metabolism
  - Hypoxia
  - Free radical formation
- Biochemical changes
  - Excitotoxic amino acids
  - Reduced ATP production
  - Phospholipase loss and lipid breakdown
  - Endogenous opiates
- Electrolyte changes

It should be stressed that these processes are extensively inter-related and their separation into different categories is inevitably somewhat arbitrary and this is reflected in the large variety of classifications that have been created. Thus, for example, oxygen free radical damage could be classified as being a consequence of abnormal oxygen metabolism or as part of the inflammatory process, since free radicals are produced in response to re-perfusion following hypoxia but are made largely by leukocytes which are concentrated at the site of tissue damage as part of the inflammatory response.

**Spinal cord haemorrhage** may result from direct vascular damage or as a result of infarction. The presence of blood within the spinal cord tissue will have a number of harmful consequences through mechanisms described, in more detail, in the following paragraphs. The breakdown of extravasated erythrocytes into haemoglobin
and ferrous iron can lead to the formation of free radicals. In addition aggregation of red blood cells and platelets will occur with release of serotonin which will result in further coagulation and cellular aggregation, compromising spinal cord blood flow. Spinal cord haemorrhage will also cause arachidonic acid and eicosanoids release, polymorphonuclear leukocytes production of leukotrienes and activation of the kallikrein-kinin system exacerbating vasospasm and oedema (Marciano et al. 1995).

**Inflammation:** Traumatic injuries to the spinal cord cause an inflammatory response that begins within several hours and peaks after several days. This response includes the release of inflammatory mediators, induction of reactive microglia, infiltration of leukocytes, platelets and macrophages and increased vascular permeability with exudation and oedema. Inflammatory mediators including thromboxanes, peptidoleukotrienes, platelet activating factor, interleukin-1 and bradykinin can contribute to vasospasm and can also stimulate phospholipase activity (Faden, 1993). Increased vascular permeability and exudation caused by inflammation will in turn result in oedema which, within the confines of the spinal canal, leads to increased tissue pressure and hence reduced perfusion (Faden, 1993).

**Oxygen metabolism:** Hypoxia can produce either reversible or irreversible cell damage depending on the severity of the insult and on the vulnerability of the tissue. The CNS is particularly at risk from hypoxia because it cannot convert to anaerobic respiration in the absence of sufficient oxygen and neuronal death will occur after three to five minutes (Cotran et al. 1994).

The causes of hypoxia may be systemic, or localised to the vicinity of the injury. For instance hypoxia can result from reduced tissue perfusion due to systemic hypotension. This in turn could be the result of blood loss or generalised vasodilatation, which is a common sequel of the autonomic disturbances caused by spinal cord injury. Hypoxia can also result from a generalised reduction in the amount of oxygen transported by blood, which could be due to anaemia or
impairment of gaseous exchange by the lungs. Localised causes of hypoxia in spinal
cord injury include direct mechanical disruption of the vascular supply, thrombosis,
vasospasm and tissue oedema which can reduce perfusion pressure (Faden, 1993).
The causes of hypoxia are summarised in Table 1-1. The main effect of hypoxia is to
prevent normal aerobic respiration within the affected cells, producing a fall in the
ATP level and hence disruption of many energy dependent cellular processes (as
outlined below). If severe the changes become irreversible, particularly if significant
cell membrane damage is sustained as a result.

<table>
<thead>
<tr>
<th>SYSTEMIC CAUSES</th>
<th>LOCAL CAUSES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced Blood Flow i.e. Hypotension</td>
<td>Reduced Oxygen Content in Blood</td>
</tr>
<tr>
<td>Reduced Blood Volume e.g. haemorrhage</td>
<td>Reduced $pO_2$</td>
</tr>
<tr>
<td>Vasodilatation</td>
<td>Anaemia</td>
</tr>
<tr>
<td>Cardiogenic Shock</td>
<td>Vasospasm</td>
</tr>
<tr>
<td></td>
<td>Tissue Oedema</td>
</tr>
<tr>
<td></td>
<td>Loss of Local Autoregulation</td>
</tr>
</tbody>
</table>

Table 1-1 Causes of Hypoxia in Spinal Cord Injury

Free radicals are partially reduced oxygen molecules that have a single unpaired
electron in an outer orbital (Cotran et al. 1994). In such a state the free radical is
extremely unstable and enters into reactions with a wide variety of substances. Free
radicals are highly toxic to cell constituents with particularly deleterious effects if
this involves the cell membranes or nucleic acids. Free radicals can be produced either by normal endogenous oxidative reactions, by cellular absorption of radiant energy or as a result of metabolism of exogenous chemicals. The small amounts of free radicals produced during normal cellular metabolism are eliminated through a combination of spontaneous decay, endogenous anti-oxidants (such as Vitamin E and transferrin), and enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase. Free radical species can have beneficial effects and are normally harnessed to promote cellular digestion, particularly by leukocytes in the inflammatory reaction. If, however, the production and elimination of these free radicals becomes uncontrolled, then serious cellular and tissue damage can result. It is likely that free radicals have a role in secondary damage to the spinal cord following injury. In this context the free radicals are produced by polymorphonuclear leukocytes that infiltrate the injury site. Production is mediated by a wide variety of oxidative enzymes involved in oxidative metabolism in different parts of the cell. The most important species of free radical are superoxide ($\text{O}_2^-$), hydrogen peroxide ($\text{H}_2\text{O}_2$) and hydroxyl radicals (OH.). They are probably present in only very small amounts during the ischaemia that can occur after spinal cord injury, but following restoration of blood flow to the tissue they are produced in far greater quantities by incomplete reduction of oxygen in the mitochondria. This paradoxical effect of re-establishing adequate oxygenation is termed re-perfusion injury.

**Biochemical changes:** Adenosine triphosphate (ATP) depletion is a crucial result of many causes of deficient energy metabolism. ATP depletion can result from ischaemia due to a lack of oxygen for aerobic respiration, a lack of energy-rich substrates or from direct disruption of one or more of the steps involved in its production. Glucose is a major substrate for the production of ATP and its metabolism is reduced following spinal cord injury (Faden, 1993). ATP is required for many cellular synthetic and degradative processes, such as membrane transport, protein synthesis and function, lipogenesis and phospholipid synthesis. ATP depletion
is well recognised as contributing to the loss of integrity of the plasma membrane that leads to cell death (Cotran et al. 1994).

Phospholipid loss can result from reduced synthesis, due to a reduction in ATP, or from activation of endogenous phospholipases, for instance by an increase in intracellular calcium. The loss of phospholipid will itself tend to compromise the integrity of the plasma membrane but in addition degradation by phospholipases will lead to the accumulation of harmful catabolic products, including fatty acids such as arachidonic acid (Cotran et al. 1994) (Faden, 1993). These molecules can contribute to tissue oedema and inhibit Na⁺, K⁺-ATPase and can also be metabolised to form molecules, such as thromboxanes and leukotrienes, that can increase the inflammatory response and increase vasoconstriction. Phospholipid hydrolysis can also cause the release of toxic free radicals (Faden, 1993) (Marciano et al. 1995).

Serotonin (5-HT) rapidly accumulates at the site of spinal cord injury and its concentration remains elevated for some hours afterwards. 5-HT may contribute to secondary injury by promoting platelet activation and endothelial permeability, as well as causing vasoconstriction (Faden, 1993) (Marciano et al. 1995). 5-HT is also thought to cause secondary tissue damage by binding to amine receptors on axonal terminals that contain Thyrotropin Releasing Hormone (TRH), acting as a neurotransmitter (Marciano et al. 1995).

The amino acid neurotransmitters glutamate and aspartate are significantly increased at the site of spinal cord injury, with peak levels at 10-20 minutes after trauma. These amino acids cause excitotoxic over-activation of the N-methyl-D-aspartate (NMDA) receptor, and consequently a number of metabolic changes, of which intracellular calcium and sodium accumulation appear to be the most serious. These changes result in inappropriate cellular activation with damage to the affected neuron (Marciano et al. 1995) (Choi, 1992) (Faden, 1993) (Rothman and Olney, 1995).
Nitric oxide (NO) is an important neuronal messenger molecule which also mediates relaxation of blood vessels and cytotoxicity of activated macrophages. Nitric oxide synthase (NOS; L-arginine, NADPH: oxygen oxidoreductase) activity, which is responsible for the production of NO, is enhanced by NMDA receptor stimulation and there is evidence that NO mediates glutamate excitotoxicity in neurons. Within the CNS, NOS appears to be localized to specific populations of neurons, which may mediate NMDA killing of other nerve cells. The possible mechanisms by which NO kills cells is unknown. NO is itself a free radical and furthermore it can react with superoxide to yield peroxynitrate, which is extremely reactive. NO can also inactivate enzymes containing iron-sulphur complex, which are important in mitochondrial respiration (Dawson et al. 1993) (Lipton et al. 1993) (Dawson et al. 1991).

Endogenous opioids are released after spinal cord injury and may contribute to ischaemic secondary damage. The endogenous opioid dynorphin accumulates following trauma in proportion to the severity of injury, unlike other endogenous opioids, such as leucine enkephalin, methionine enkephalin and β-endorphin (Faden, 1993) (Marciano et al. 1995). Dynorphin activates the κ-receptor, which is the most common opiate receptor within the spinal cord. When administered centrally dynorphin causes tissue damage and exacerbates the consequences of spinal cord injury, while antibodies to dynorphin reduce tissue damage following spinal cord injury. Dynorphin may contribute to secondary injury by reducing spinal cord blood flow, by causing a release of excitatory amino acids or possibly by altering cellular metabolism (Faden, 1993).

**Electrolyte disturbances**: Intracellular Calcium is maintained at extremely low concentrations (less than 0.1μm compared to extracellular levels of 1.3μm), and most is sequestered in mitochondria and endoplasmic reticulum (Cotran et al. 1994). These gradients are maintained by energy dependent membrane-bound calcium carriers. Certain pathological stimuli can produce an influx of calcium ions, which
can in turn activate a number of enzymes with potentially deleterious cellular effects, such as damage to membranes, cytoskeletal elements and genetic material, as well as disruption of normal metabolism (Choi, 1988) (Choi, 1995).

A second divalent cation that has been implicated in delayed CNS injury is magnesium. This is intimately involved in the metabolism of ATP and may also play a role in cellular homeostasis by acting as a calcium antagonist. After spinal cord trauma there are marked decreases in the levels of intracellular free magnesium and in the total tissue magnesium at the injury site, which are related to the severity of the injury (Faden, 1993).

**Astroglial responses to injury:** Astrocytic swelling occurs as an early response to CNS trauma, and because astrocytes have a homeostatic role, these changes may be associated with tissue acidosis and excitotoxicity. The swelling of astrocytes may also contribute to more generalised tissue oedema. As described above astrocytic gliosis will develop in the vicinity of the lesion in the weeks following injury (Faden, 1993).

**THERAPY: EXPERIMENTAL STRATEGIES**

A greater understanding of the cellular and molecular processes involved in spinal cord injury, and the reasons why only limited neuroregeneration occurs, has led to new approaches to treatment. Treatment strategies can be aimed at the prevention of secondary damage or restoration of function in the damaged CNS. The latter might be achieved by promotion of axonal regeneration (either directly, by stimulation of axonal growth, or indirectly, by reduction of inhibitory influences on growth), or by replacement of cell loss by grafting. Even where neuronal loss is permanent,
treatment could be used to maximize function within the damaged tissue. Treatment techniques might act through more than one of these mechanisms. This new understanding of the pathological and cellular events involved in spinal cord injury has already produced the first clinical treatment. High dose steroids are now routinely given following injury to reduce secondary damage (Bracken et al. 1990) (Bracken et al. 1985) (Shepard and Bracken, 1994). A number of other techniques are being developed in animal models, many of which have produced encouraging, though as yet limited, results. This research has led to optimism that in the future some restoration of neurological function will be possible in a clinical setting.

PHARMACOLOGICAL PREVENTION OF SECONDARY INJURY

The attenuation of secondary injury represents the most promising avenue for the immediate development of new methods of treating spinal cord injury. Restoration of spinal cord function by regeneration is likely to involve more than one therapeutic intervention. In comparison, the reduction of secondary injury should be less complex and a single type of drug treatment could improve outcome.

Corticosteroids: Studies in animals have produced evidence of improved outcome following experimental injury and corticosteroid administration. Methylprednisolone has been claimed to influence secondary injury under experimental conditions through a number of mechanisms. It may reduce early inflammatory processes (including lipid peroxidation), re-establish sodium-potassium ATPase activity in the cell membrane, reduce neurofilament degeneration, improve acid-base balance, help maintain extracellular concentration of calcium and improve post-traumatic spinal cord blood flow (Bartholdi and Schwab, 1995) (Anderson et al. 1982) (Anderson and Means, 1985) (Anderson et al. 1985) (Braughler et al. 1987) (Means et al. 1981) (Young et al. 1988). These laboratory findings have led to multicentre clinical trials, which have demonstrated improved clinical outcome if methylprednisolone is administered within eight hours of injury (Bracken et al. 1990) (Bracken et al. 1985) (Shepard and Bracken, 1994) (Bracken et al. 1997).
**21-Aminosteroids (Lazaroids)** These compounds are 100 times more potent than conventional corticosteroids, but without the same glucocorticoid effects (Marciano et al. 1995). They are antioxidants, potent inhibitors of iron-dependent lipid peroxidation, membrane stabilisers and preserve post-traumatic blood flow (Bagenholm et al. 1996) (Boisvert and Hall, 1996) (Hall and Smith, 1991) (Hall et al. 1992). Clinical studies using the 21-aminosteroid Tirilazad in patients with spinal cord injury suggest that there may be some beneficial effects on outcome, though this is less than for treatment with methylprednisolone for 48 hours (Bracken et al. 1997).

**Free Radical Scavengers** Treatment with Vitamin E and Selenium can reduce experimental spinal cord injury by reducing free-radical damage. Vitamin E acts as a reducing agent and protects cell membranes from oxidation (Hall et al. 1992). Selenium is a cofactor for the enzyme glutathione peroxidase and thus helps scavenge intracellular hydrogen peroxide and lipid hydroperoxides (Marciano et al. 1995). Tirilazad also has free radical scavenging properties (Clark et al. 1995).

**Opiate Antagonists** such as naloxone have been found to produce enhanced neurological recovery in experimental spinal cord injury models. The mechanism for this unclear, though spinal blood flow is increased. Clinical trials, however, have revealed no benefits from the administration of naloxone (Bracken et al. 1990) (Bracken et al. 1992). It is possible that more selective κ-receptor antagonists may prove more effective in the future (Marciano et al. 1995).

**TRH** has been postulated to influence spinal cord injury through a number of mechanisms. It acts as a physiological opiate receptor antagonist, and can antagonise the effects of leukotrienes and platelet-activating factor. TRH may also enhance serotonin metabolism and thus reduce its potentially harmful effects. Recently TRH analogues with the advantages of greater potency and longer action have been introduced (Marciano et al. 1995).
Inhibitors of Excitotoxicity  Glutamate can act via the NMDA receptor to produce excitotoxic damage by calcium influx (Choi, 1995). Nimodipine, diltiazem and nifedipine are calcium channel blocking agents and appear to have beneficial effects on the outcome of experimental spinal cord injury. NMAD receptor antagonists such as MK-801 and dextrorphan may also improve outcome (Liu et al. 1997) (Marciano et al. 1995).

NOS inhibitors such as N-nitro-L-arginine (L-NAME) and L-NG-nitroarginine that reduce NO concentrations could in theory be protective for the CNS, however they are also potent vasoconstrictors and have been found to damage the spinal cord and cause strokes in hypertensive rats (Yezierski et al. 1996) (Cohen et al. 1996) (Hitchon et al. 1996).

THE PROMOTION OF REGENERATION

Regeneration does not normally occur within the injured mammalian CNS and several reasons for this, alone or in combination, have been suggested. Adult mammalian neurons may have entered an irreversible level of differentiation in which they are simply unable to react to injury with neuronal sprouting. Recently, however, clear evidence has emerged that, given appropriate experimental stimulation, damaged axons can be induced to regenerate (Bregman et al. 1995a) (Cadelli and Schwab, 1991) (Benfey and Aguayo, 1982) (Richardson et al. 1980b) (Wrathall et al. 1982) (Wrathall et al. 1984) (Guenard et al. 1993) (Paino and Bunge, 1991) (Paino et al. 1994b) (Berry et al. 1996). Neurons damaged by spinal cord injury must therefore either lack some stimulus for axonal regeneration or are inhibited from regenerating by some external influence. For instance axonal regeneration may be dependent on soluble factors, such as neurotrophins or some form of direct contact with supporting cells such as glia. There is also evidence that regeneration might be blocked, either by inhibition due to myelin-associated neurite growth inhibitory proteins produced by oligodendrocytes (Bregman et al. 1995b) or...
by the mechanical effects of scar tissue, including astrogliosis (Compston, 1994) (Logan and Berry, 1993) (Logan et al. 1994). This consists of a dense scar of astrocyte fibrillary tangle and collagen, which obstructs the path of growing axons (Compston, 1994) (Faden, 1993).

In spite of the emergence of evidence that regeneration does occur within the spinal cord injury after axonal transection (Bregman et al. 1995a) (Cadelli and Schwab, 1991) (Benfey and Aguayo, 1982) (Richardson et al. 1980b) (Wrathall et al. 1982) (Wrathall et al. 1984) (Guenard et al. 1993) (Paino and Bunge, 1991) (Paino et al. 1994b), substantial problems remain to be resolved if these experimental successes are to be applied in a clinical context. Long-tract axons that pass through the spinal cord can approach 1 metre in length, yet proven regeneration has usually amounted to a few cm, at most. Although some improvement in function could possibly occur through local regeneration and the formation of novel synaptic connections, more substantial recovery would require the restoration of the original pathways. Even if axons could be made to grow over long distances, this would not be in itself sufficient to produce useful re-constitution of damaged neuronal circuitry. For restoration of the pre-morbid pathways it would be necessary that growing axons reach the appropriate region of the CNS and, having arrived, establish functional connections with appropriate neurons, while not developing any abnormal connections. Furthermore, it is possible that even if each of these processes required for successful regeneration could be induced to occur, functional restoration would fail because the temporal sequence of events was not correct. For instance, the Wallerian zone is initially inhibitory to axonal growth but with time may become permissive. By this time, however, the potential for axonal outgrowth may have diminished and, additionally, scarring at the injury site may have produced an impenetrable physical barrier. It may be that the therapeutic interventions most likely to prove successful in ameliorating functional loss after injury are those that allow the injured CNS to re-iterate the sequence of events associated with development. Thus
eventual treatment may require several interventions, each at an appropriate time and perhaps in different regions of the CNS.

**REGENERATION- PHARMACOLOGICAL TREATMENT**

**Gangliosides** are complex acidic glycolipids that are present in high concentrations in CNS cells. They form a major component of the cell membrane, in which they are situated mainly in the outer leaflet of the membrane bilayer. Gangliosides play important roles in the physiological operations of the nervous system and changes in ganglioside composition occur in the mammalian CNS not only during development, but also in ageing and in several neuro-pathological situations (Skaper and Leon, 1992). Gangliosides, like GM1, may protect neurons against amino acid-related neurotoxicity, improve neurite outgrowth in vitro, induce regeneration and sprouting of neurons and restore neuronal function after injury in vivo (Constantini and Young, 1994) (Skaper and Leon, 1992) (Marciano et al. 1995). Clinical evaluation of the therapeutic potential of gangliosides is underway (Marciano et al. 1995) (Geisler et al. 1991).

**Inhibitory Factors:** Axonal growth in development and regeneration is subject to control by a number of different molecules. These may influence different aspects of development and perhaps regeneration, including growth cone guidance (eg netrins and semaphorins), axonal outgrowth and sorting (eg neural cadherin, polysialylated neural cell adhesion molecules and L1) and neural connectivity (cell adhesion molecules, dystroglycan and agrin) (Aubert et al. 1995). The interpretation of the possible roles of such molecules is complicated by the fact that their effects may vary in different situations within the CNS (Zhang et al. 1997). A greater understanding of these mechanisms has led to the development of an experimental treatment of spinal cord injury directed at oligodendroglia-myelin associated membrane protein, which appears to act as a potent inhibitor of neurite outgrowth. Administration of IN1 antibodies to neutralise this protein appears to facilitate experimental axonal regeneration within the corticospinal tract of the spinal cord and elsewhere in the
CNS in adult rats. The regenerated axons appear to survive for prolonged periods and are associated with functional recovery (Bregman et al. 1995b) (Cadelli and Schwab, 1991) (Kapfhammer and Schwab, 1994) (Schnell and Schwab, 1990) (Schnell and Schwab, 1993). However it should be noted that antibodies cannot easily reach neurons within the CNS following systemic administration. To overcome this problem an antibody-producing cell line was implanted, and in order to prevent immune rejection Cyclosporin A was administered. Immunosuppressants may be associated with effects on axonal regeneration and survival (see Chapter 2) and might therefore have influenced the outcome. The regeneration of axons may also be physically blocked by the formation of scar tissue at the lesion site, which is increased by transforming growth factor β1 (TGF-β1); antibodies to TGF-β1 may reduce scar formation and therefore facilitate axonal regeneration (Logan and Berry, 1993) (Logan et al. 1994).

Neurotrophic factors: Since the discovery of nerve growth factor (NGF) (Levi-Montalcini, 1987), a family of related proteins has been identified. These proteins, known as neurotrophins, include Brain Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3), Neurotrophin 4 (NT-4), and Neurotrophin 5 (NT-5). The neurotrophins are homodimeric molecules produced by enzymatic cleavage of precursor polypeptides (Levi-Montalcini, 1987). Their actions are mediated by the trk family of high-affinity tyrosine kinase receptors, and the p75 low affinity receptor (Hefti et al. 1993). In addition a number of other factors may have neurotrophic effects including ciliary neurotrophic factor (CNTF), fibroblast growth factors (FGFs), epidermal growth factor (EGF), transforming growth factor alpha (TGFα), transforming growth factor beta (TGF-β), glial-derived neurotrophic factor (GDNF) and insulin-like growth factors (IGF) (Loughlin and Fallon, 1993).

Neurotrophic factors can be defined as 'endogenous soluble proteins regulating survival, growth, morphological plasticity or synthesis of proteins for differentiated functions of neurons' (Hefti et al. 1993). As implied by this definition, neurotrophins
are likely to have several actions depending on their anatomical or developmental context. A key concept in the understanding of neurotrophins is that they are target-derived; thus a neurotrophic factor with actions on a particular type of neuron will be produced by the target tissue with which axons from those neurons make contact (Hefti et al. 1993). It is thought that neurotrophins may be taken up at the ends of the axons and either transported back to the cell body itself or induce some signal which is then transported to the perikaryon. In the context of development it is likely that only those neurons that establish contact with appropriate target cells, and hence a supply of neurotrophic factors, will survive. Thus, since one action of neurotrophins may be to promote survival of neurons during development, it is possible that severing of axons in the context of CNS injury might interrupt this target-derived signal and lead to neuronal death (Hefti et al. 1993). It appears that after brain injury there is an increase in the concentration of neurotrophins around the site of damage (Lindvall et al. 1994). However this may not always be rapid enough or sufficient to prevent secondary cell death in all neurotrophin dependent neurons (Cotman and Nieto-Sampedro, 1985). In other experimental systems, however, administration of neurotrophic factors has been shown to reduce the effects of injury induced by axotomy (Diener and Bregman, 1994) and excitotoxins (Compston, 1994) (Faden, 1993) (Mocchetti and Wrathall, 1995).

Neurotrophic factors, including NGF, BDNF and NT-3, are also capable of stimulating neurite outgrowth and the expression of key enzymes for neurotransmitter synthesis that may be up-regulated to compensate for reduced innervation (Mocchetti and Wrathall, 1995). Specific neurotrophins appear to have effects on particular populations of neurons, thus NT-3 in particular appears to have very powerful effects on regeneration of severed corticospinal tract axons in the injured spinal cord (Grill et al. 1997) (Schnell et al. 1994). In addition, other growth factors such as CNTF and FGFs not only sustain survival of injured neurons, but also stimulate re-vascularization and glial responses to injury (Compston, 1994).
There are several problems associated with the potential pharmacological use of neurotrophic factors in the CNS. Neurotrophic factors are proteins and will not cross the blood brain barrier, they therefore cannot be administered systemically. To overcome this problem alternative delivery techniques have been tested, including intrathecal administration using infusion pumps, slow-release devices inserted into the CNS and implantation of tissue grafts that synthesise active molecules, so-called biological minipumps (Hefti et al. 1993) (Grill et al. 1997). Neurotrophic factors may also produce unwanted side effects through their actions on neurons. For instance, NGF has been implicated in the potentiation of pain perception (Andreev et al. 1995) (McMahon et al. 1995), and because neurotrophic factors act not only on function, but also on the structure of the CNS, unwanted effects could be more prolonged.

**REGENERATION- TRANSPLANTATION**

Although the limitation of axonal damage and the stimulation of endogenous repair of spinal cord axons are of great importance, the loss of neurons themselves may also contribute to the neurological deficits associated with spinal cord injury. Cell implantation may be required in these situations (Compston, 1994).

Cell grafting could improve neurological function through several different mechanisms. For instance, implanted neurons could restore damaged neuronal circuitry to a pre-morbid pattern, or they might lead to the creation of new and different neuronal pathways, which nevertheless produce a functional benefit. Grafts could also release biologically active molecules, such as neurotrophic factors, that enhance the survival or regeneration of host neurons. Alternatively the transplants could improve the function of host tissue by making more transmitter available in the vicinity of the implant, either by stimulating release from the host neurons or by release from the graft itself. The function of surviving CNS tissue could also be enhanced if grafting increased an inadequate blood supply by improving vascularisation, or if grafted cells were able to replace the supporting glial cells,
which have vital roles in neuronal function, survival and regeneration. Finally it has
been suggested that the trauma to the CNS involved in implanting cells may, in some
circumstances, have beneficial effects (Rosenfeld, 1994) (Compston, 1994)
(Blakemore and Franklin, 1991). One or more of these mechanisms may be relevant
to a single experimental model of cell implantation into the CNS and, for example,
the effects of cell transplantation in models of Parkinson’s disease have been
variously attributed to most of these factors (Bjorklund, 1991) (Rosenfeld, 1994).

A crucial, although often neglected, factor in the potential usefulness of candidate
cells for implantation is the degree to which they are regulated by their host
environment. Thus it might be envisaged that implanted neurons, similar to the host
cells that they are replacing, would be closely regulated by cues from the host CNS.
In contrast, other cell types may be poorly regulated within the host. Thus cell lines
might proliferate in an uncontrolled fashion. Similarly cells, such as fibroblasts that
have been genetically modified to create biological-minipumps, might release an
excess of biologically active molecules (Bjorklund, 1991). A further issue in
interpreting claims of therapeutic benefit from grafted cell is that of
immunosuppression. In order to prevent graft rejections, many experiments have
involved the administration of immunosuppressant drugs that have protective and
regenerative effects on neurons (see Chapter 2), and might therefore influence
functional outcome.

Cell transplantation has been applied to several pathologies affecting the CNS.
Perhaps the most extensively studied condition is Parkinson’s disease, in which a
variety of cell types have been implanted to compensate for the loss of dopaminergic
(Bakay and Sladek, Jr., 1993) (Cunningham et al. 1994) (Date et al. 1996) (Jaeger et
al. 1990). Although the precise effects of the implanted cells is uncertain,
improvements in functional outcome have been reported. These findings have led,
somewhat controversially, to on-going clinical trials (Rosenfeld, 1994) (Borlongan et
al. 1996b) (Hitchcock, 1994) (Madrazo et al. 1991), in spite of the fact that the optimum experimental treatment protocol is far from established for many issues. Questions concerning surgical technique, candidate selection, the optimal brain regions for implantation and the optimal tissue for implantation all remain to be resolved.

In addition to Parkinson's disease, experimental transplantation techniques have been applied to experimental models of other degenerative diseases. These include Huntington's disease, in which embryonic striatal cells and NGF-secreting fibroblasts have been used as tissue sources (Rosenfeld, 1994; Lindvall, 1995). The degeneration of the brain's cholinergic systems is one of the main factors in the pathogenesis of Alzheimer's disease and Purkinje cell degeneration results in cerebellar ataxia. Cell implantation techniques have been used in experimental models of both of these degenerative conditions (Rosenfeld, 1994). In addition, transplantation has been used in the experimental treatment of other non-degenerative diseases, including demyelinating diseases, such as disseminated sclerosis, cerebral infarction and spinal cord injury (Rosenfeld, 1994). There is great interest in neural transplantation in spinal cord injury, and in this context the grafts may have several roles. They may (1) act as a local relay between neurons, (2) supply appropriate neurotransmitters to compensate for the loss of supraspinal input, (3) act as a bridge through which axons will grow and re-connect with an appropriate target by providing chemical and / or mechanical guidance for host neurons to grow across the lesion, (4) rescue axotomized neurons or replace damaged neurons in the grey matter (Zompa et al. 1997).

A wide variety of tissues have been implanted into the CNS, each with potential advantages. In experimental studies the choice of tissue is largely determined by considerations of technical feasibility and efficacy. However, if clinical applications are envisaged then a further set of criteria must be considered. Of primary importance is that of safety, for instance the implantation of unmodified cell lines could lead to
tumour formation. Allografts have the disadvantage that immune rejection will occur, while ethical considerations have held back the use of human foetal tissues. The following list describes a few of the tissues that have been implanted into models of spinal cord injury.

**Foetal tissue**, in general comprising largely CNS neurons, has proven more useful than adult CNS tissue in experimental implantation models of spinal cord injury where the aim has been to replace lost cells (Bernstein-Goral and Bregman, 1993) (Howland et al. 1995) (Iwashita et al. 1994) (Jakeman and Reier, 1991) (Reier et al. 1992) (Reier et al. 1986) (Anderson et al. 1995) (Bregman et al. 1993) (Gimenez et al. 1996) (Theele et al. 1996) (Vaquero et al. 1992). It is able to survive for prolonged periods and interact with host tissues (Clowry et al. 1991) (Reier et al. 1986). Foetal cells are not fully committed developmentally and can therefore exhibit greater plasticity and potential for regeneration following transplantation than adult cells. Embryonic spinal grafts have neuroprotective effects, and when placed in the injured spinal cord of neonatal rats will prevent cell death of axotomized neurons, an effect that appears to be dependent on the use of spinal cord donor tissue (Bregman and Reier, 1986) (Kunkel-Bagden and Bregman, 1990). Embryonic spinal cord tissue grafted into lesioned spinal cord integrates into the host tissue and sends fibres into the surroundings spinal grey matter while, at the same time, host fibres project into the graft, and at least in some cases, implanted neurons may establish appropriate synaptic connections (Reier et al. 1986). Furthermore these new connections may be functional, since post-operative functional assessment suggests that grafting improves outcome (Kunkel-Bagden and Bregman, 1990) (Liu et al. 1995) (Miya et al. 1997) (Stokes and Reier, 1992) (Tessler et al. 1997) (Zompa et al. 1997).

**Peripheral nerve implantation** Following peripheral axotomy damaged axons will regenerate, even without therapeutic intervention. This observation has led to the use of peripheral nerves as a source of tissue for implantation into the injured CNS (Benfey and Aguayo, 1982) (Campbell et al. 1992) (Cheng et al. 1996) (David and
Aguayo, 1981) (Matsuyama et al. 1995) (Moissonnier et al. 1996) (Richardson et al. 1980b) (Richardson and Ebendal, 1982) (Richardson et al. 1982) (Richardson and Issa, 1984) (Woolhead et al. 1998) (Wrathall et al. 1982). CNS axons, which would normally be expected to show little regenerative response, will grow into the graft. Regenerating axons within the peripheral nerve grafts are often ensheathed by Schwann cells (Wrathall et al. 1982), which are thought to play an important role in axonal regeneration (Berry et al. 1988) (Matsuyama et al. 1995), possibly by secreting biologically active soluble substances, such as neurotrophins, that stimulate outgrowth of neurites (Richardson and Ebendal, 1982). The regenerating axons may grow along the grafts for considerable distances, but do not re-enter the CNS (Campbell et al. 1992) (Moissonnier et al. 1996). Different populations of CNS neurons may demonstrate different regenerative responses to peripheral nerve grafts (Woolhead et al. 1998). Recently it has been claimed that multiple peripheral nerve grafts, in combination with fibrin tissue glue and possibly growth factors, will permit axons to grow into the distal stump of the transected spinal cord with some restoration of function (Cheng et al. 1996). This finding has yet to be confirmed.

**Glial cells:** Schwann cells are believed to have a crucial role in promoting axonal regeneration in peripheral nerves and have therefore been used for implantation into the CNS. In addition to the implantation of peripheral nerve segments (see above), Schwann cells have been implanted into the injured spinal cord in purified dissociated form, either directly (Guenard et al. 1993) (Kuhlengel et al. 1990) (Paino et al. 1994a), or within semi-permeable polymeric channels that provide a bridge for axons to grow across the injury site (Chen et al. 1996) (Guenard et al. 1993). These experiments confirmed that Schwann cells tend to migrate towards demyelinated axons within the CNS (Blakemore and Franklin, 1991) and ensheath and myelinate these axons. Schwann cells also stimulated rapid and abundant growth of both ascending and descending axons into grafts. However, regenerated axons did not grow from the graft into the distal cord tissue (Xu et al. 1997). Oligodendrocytes have been transplanted into the CNS, usually with the aim of re-establishing axonal
myelination in models of demyelinating diseases (Blakemore and Franklin, 1991). Astrocytes are considered to be inhibitory to axonal regeneration but have been investigated as implants in the CNS. They may be important for the efficient remyelination of axons by oligodendrocytes (Bradbury et al. 1995) (Wang et al. 1995) (Hatton and Garcia, 1992a) (Emmett et al. 1991) (Emmett et al. 1990) (Franklin et al. 1991). Recently ensheathing cells from the olfactory bulb have been transplanted into the injured spinal cord. These cells share some characteristics with both Schwann cells and astrocytes, but are confined to the CNS. Implantation of these cells induced elongative growth of severed corticospinal axons and limb function was improved. These cells therefore represent a promising source for transplantation into the injured spinal cord, particularly since autotransplantation may be possible in a clinical setting (Li et al. 1997).

**Immortalised cell lines** have been grafted into the CNS, often with the aim of delivering biologically active macromolecules. Such cells have the advantage over primary cells, such as neurons or glia, that they can be grown in large numbers in culture without the need for a living donor. Moreover, once implanted they tend to survive in greater numbers than primary cells (Anton et al. 1995), thus making it more likely that any significant biological effect they may possess will be expressed (Zompa et al. 1993) (Pedersen et al. 1995) (Cattaneo et al. 1994). The clear problem with such cells is, however, the tendency to neoplasia (Hatton et al. 1992b), which would preclude their use in most clinical situations. In order to overcome this problem some workers have enclosed grafted cells within a semi-permeable polymer capsule, with an appropriate molecular weight cut-off, that allows inward diffusion of nutrients and outward diffusion of neurotransmitters and some macromolecules, but prevents the passage of immunoglobulins or cells. This technique therefore prevents the formation of tumours by physically sequestering the transplanted tissue and, in addition, prevents the immune rejection of the grafts (Jaeger et al. 1990) (Aebischer et al. 1991). Others have created temperature-sensitive cells that will replicate in
cultures but not at body-temperature, and will therefore not form tumours after transplantation (Barnett et al. 1993).

**Genetically modified tissue** The development of new techniques in molecular biology has created the possibility of genetically-modifying tissue prior to implantation so that their functional properties are enhanced. While it may be possible to modify neurons in such a way that they are more likely to form new circuitry with host cells, the majority of research into the genetic-modification of implanted tissue has centred on the creation of ‘biological mini-pumps’. In such a system, genes are inserted into the graft cells by genetic manipulation with the aim of enhancing the synthesis and secretion of biologically active molecules, and hence altering the function or structure of the host CNS.

CNS function could be enhanced, without altering the structure, for instance by modifying cells to synthesise enzymes that produce neurotransmitters. Investigators pursuing this approach have genetically-modified cells, to produce dopaminergic transmitters, which have been implanted in animal models of Parkinson’s disease with some success (Cunningham et al. 1994) (Rohrer et al. 1996). In spinal cord injury, however, a local deficiency of neurotransmitters is likely to be less important than damage to long fibre tracts. Gene-products that either reduce secondary tissue damage or promote regeneration are therefore more promising. The neurotrophins, which are known to influence neuronal survival, differentiation and neurite outgrowth, may be particularly effective if delivered to the injured CNS (Senut et al. 1995). Other potentially useful gene products might include a variety of other neurotrophic factors such as, for example, CNTF and GDNF, as well as angiogenic factors that could improve the blood supply to the vicinity of the area of injury (Doering, 1994).

A number of possible methods for introducing the gene-products into cells exist, including the direct introduction of DNA, or the use of viral vectors designed
specifically for this purpose (Doering, 1994) (See Chapter 7). In the context of the ex vivo insertion of the gene into cells, which are later implanted, the retroviruses have been the most widely used group. They have the advantage that disabled forms can be used which are therefore safe. In addition, because they will only infect populations of cells that are actively dividing, neurons within the mature CNS will not be directly affected and are therefore not exposed to the neuropathic effects of other viral vectors such as herpes simplex and adenovirus, which infect neurons (Doering, 1994) (Howard et al. 1997).

The other main variable is the type of cell to be genetically modified and then implanted into the CNS. These may be either immortalised cell lines or primary cells. The former have the advantage of being readily available and of growing well in culture, but have the disadvantage that they may continue to grow in an uncontrolled fashion following transplantation (Chen et al. 1996) (see above). Primary cell implants, such as fibroblasts, astrocytes or muscle cells are much less likely to be neoplastic (Cunningham et al. 1994), although slow growing tumors can also occur with primary fibroblast implants (Tuszynski et al. 1994). In addition primary cells obtained from the eventual recipient of the graft are autografts and would therefore avoid the problem of immune rejection. In selecting a cell type to act as source for primary cell implants the following factors should be considered:

- Availability, particularly if clinical applications were being considered. Thus neurons or glia would not be freely available for autografting, while foetal tissues are difficult to obtain and in any case raise ethical issues.
- Suitability for genetic modification. For instance neurons, which do not divide, cannot be infected by retroviruses.
- Other properties of the graft tissue might, in some way, increase the beneficial effects of the gene product or alternatively might have effects that are detrimental to survival or regeneration.

Primary skin fibroblasts have been used for the creation of genetically-modified implants. The results have been encouraging, however this type of cell may not
represent the best tissue source, since in some sites the grafts have grown to form tumours within the CNS (Tuszynski et al. 1994). Moreover, fibroblasts secrete a number of molecules such as collagen and fibronectin (Tuszynski et al. 1994). Although it is possible that some of these may be beneficial to the repair of spinal cord injury, this may not necessarily be the case, and therefore other cell types may be preferable. The complexity of the processes that follow injury to the nervous system means that the relative merits of different cell types can only be assessed experimentally.

Significant progress has been made with this type of approach to cell implantation, and experimental treatments have been developed for models of Parkinson's disease and Alzheimer's disease (Rosenberg et al. 1988) (Gage et al. 1991) (Cunningham et al. 1994). In models of spinal cord injury genetically modified fibroblasts have been used to deliver NGF, BDNF and NT-3 (Tuszynski et al. 1996) (Senut et al. 1995). The latter, in particular, has produced very promising results, with evidence that corticospinal tract axons can regenerate over long distances as well as partial functional recovery (Grill et al. 1997).

Omentum has been used to create vascularized autografts in the injured spinal cord (Neil-Dwyer et al. 1996). This approach differs significantly from all other types of treatment by implantation in that it consists of whole tissue, still attached to its vascular supply in the mesentery, rather than dissociated cells, or small fragments of tissue (Neil-Dwyer et al. 1996). Furthermore, the omentum is applied to the surface of the damaged cord rather than being placed within it. It is believed that omentum may increase the blood supply available to the injured spinal cord, by encouraging the formation of new blood vessels (MacMillan and Stauffer, 1991), possibly by producing lipid angiogenic factor (Neil-Dwyer et al. 1996) (Goldsmith et al. 1984). Omentum has also been found to contain a variety of active molecules including neurotransmitters, trophic factors and vasoactive substances (Neil-Dwyer et al. 1996) (Goldsmith et al. 1987). The procedure has been used in human studies, usually in
the chronic phase, with little apparent benefit and significant morbidity (Clifton et al. 1996) (Sgouros and Williams, 1996).

**Myenteric Plexus**, together with other components of the intestinal muscularis externa, including glial cells, interstitial cells of Cajal, fibroblasts and smooth muscle, have been implanted into the CNS. The results have been variable with significant axonal sprouting seen in the striatum, but more limited regeneration elsewhere (Lawrence et al. 1991) (Tew et al. 1992). Nevertheless the results have been promising and, in view of practical and ethical advantages of this tissue source, the implantation of muscularis externa into the injured spinal cord has been a major theme of this thesis (see ‘Section II – Overview’ for further discussion of this tissue source).

**PLAN OF INVESTIGATION**

A variety of new techniques with the potential for treating spinal cord injury have emerged recently. Two of these, treatment with immunophilin ligands and implantation of intestinal muscularis externa, containing myenteric plexus, were chosen for investigation, looking at the effects of each, on axonal regeneration and neuroprotection.

Regeneration was assessed by studying a model of dorsal column injury, usually in association with peripheral nerve crush. The central processes of dorsal root ganglion sensory neurons within the dorsal columns were visualised, either by transganglionic labelling following injection of CT-HRP into the sciatic nerve or by electron microscopy. In addition, the potential protective effects of treatment were investigated using either a simple transection model with retrograde labelling of
axons or a new model, which allowed for easy insertion of graft tissue in standardised lesions. The latter was assessed both histologically, measuring lesion size and the presence of labelled axons, and in terms of hindlimb function.

Treatment strategies involved the systemic administration of a variety of immunophilin ligands, including the immunosuppressant drugs FK506 and Cyclosporin A and the non-immunosuppressant GPI-1046, either alone or in combination. Further treatment variables included whether the sciatic nerve was crushed at the time of spinal cord injury and whether steroids were administered. Dorsal column axons and lesion size were investigated at time periods ranging from 1 to 12 weeks. In one series of experiments treatment was assigned at random to eliminate the possibility of operator bias.

Experiments were also carried out to assess the effects of intestinal muscularis externa implanted into the spinal cord in different injury models to delineate the effects of implantation on both regeneration and protection. In addition the properties of muscularis externa which might make it an appropriate tissue for implantation into the CNS were investigated using in situ hybridization to determine the distribution of mRNA for NT-3 and BDNF within the rat intestine. Finally electron microscopy was used to define the behaviour of muscularis externa cells implanted into the CNS. Following stereotactically-guided injection of cultured dissociated muscularis externa into the striatum, the grafts were assessed for cell survival, migration and neoplasia.
Experimental Findings

-Section I-

Pharmacological Treatment of Spinal Cord Injury
CHAPTER 2

Immunophilin Ligands and Spinal Cord Injury

ABSTRACT

INTRODUCTION

MATERIALS AND METHODS

RESULTS

DISCUSSION
ABSTRACT

The aim of this study was to investigate the effects of the immunophilin ligands FK506, Cyclosporin A and GPI 1046 on ascending axons following a lesion of the thoracic spinal cord.

The dorsal columns of adult rats were partially transected at T8/9 and in some cases the sciatic nerve was crushed to stimulate regeneration. FK506 (0.5 or 2.0 mg/kg) and GPI 1046 (10 or 40 mg/kg) were administered subcutaneously 5 times per week, while Cyclosporin A (30mg/kg) was injected intraperitoneally 3 times a week. Some animals received two 30mg/kg doses of methylprednisolone. After survival times of 1-12 weeks dorsal column axons were labelled transganglionically with cholera toxin B-HRP.

FK506 stimulated regeneration of some severed sensory axons for short distances rostral to the lesion. FK506 also reduced the likelihood of axonal destruction due to secondary injury in treated animals (n=66) compared to controls (n=45) (p<0.05). This effect on axons was independent of protective effects on neuronal cell bodies, which remain viable even without treatment. A combination of FK506 and methylprednisolone afforded greater axonal protection than methylprednisolone alone (p<0.05), but axonal survival was not affected by: sciatic nerve crush, dose of FK506 or survival time after injury. GPI 1046 (n=11) did not affect axonal survival, while the actions of Cyclosporin A treatment (n=6) did not reach statistical significance. FK506 treatment did not significantly influence lesion size.

A protective effect of drug treatment on axonal survival, independent of effects on the survival of the neuronal cell body, has not been previously demonstrated. These findings may have important therapeutic implications for spinal cord injury as well as cerebral diffuse axonal injury and internal capsule strokes.
INTRODUCTION

The immunophilins FK506 Binding Protein-12 (FKBP-12) and cyclophilin are intracellular proteins with important roles in immunosuppression (Schreiber, 1991). More recently evidence has emerged that immunophilins may also be involved in promoting axonal regeneration (Gold et al. 1994) (Steiner et al. 1997) (Lyons et al. 1994) (Howland et al. 1995) (Steiner et al. 1997) (Gold et al. 1997a) (Gold et al. 1995) (Wang et al. 1997b) (Wang and Gold, 1997a) (Palladini et al. 1996) (Teichner et al. 1993) (Gold et al. 1997b) and neuroprotection (Sharkey and Butcher, 1994) (Butcher et al. 1997) (Dawson et al. 1993) (Matsuura et al. 1996) (Hugon et al. 1997) (Ide et al. 1996) (Tokime et al. 1996) (Drake et al. 1996) (Kitamura et al. 1994) (Yagita et al. 1996) (Bochelen et al. 1997) (Matsuura et al. 1996) (Folbergrova et al. 1997) (Li et al. 1997) (Matsuura et al. 1997b) (Matsuura et al. 1997a) (Uchino et al. 1995) (Steiner et al. 1997). Although no physiological ligands for these molecules have been identified, FKBP-12 and cyclophilin will bind respectively to the immunosuppressant drugs, FK506 and Cyclosporin A (Cs A), which are routinely used to prevent allograft rejection following transplant surgery (Briffa and Morris, 1998; Starzl et al. 1989) (Shapiro et al. 1990) (Showstack et al. 1989). These drugs form complexes with immunophilins present in lymphocytes. This results in immunosuppression through effects on calcineurin (Liu et al. 1991), another widespread intracellular protein (Bierer et al. 1990). A third immunophilin ligand, GPI 1046 (3-(3-pyridyl)-1-propyl (2S)-1-(3,3-dimethyl-1,2-dioxopentyl)-2-pyrrolidinedinecarboxylate), will specifically bind to FKBP-12 but is not an immunosuppressant (Steiner et al. 1997). The potential importance of immunophilins in the nervous system is indicated by the fact that FKBP-12 is highly concentrated in peripheral nerves (Lyons et al. 1995) and in the brain (Steiner et al. 1992), where its concentration is far greater than in the immune system.
MECHANISMS OF ACTION

FK506 has 2 distinct binding domains that are responsible for its biological actions (Schreiber, 1991). One region interacts with a specific part of the FKBP-12 molecule to form a calcineurin-binding 'effector domain' (Liu et al. 1991), which inhibits calcineurin phosphatase activity and thus increases levels of phosphorylated calcineurin substrates (Liu et al. 1991). Within the immune system the production of Interleukin-2 is only stimulated by non-phosphorylated NFAT (Nuclear Factor of Activated T-cells), since it is only in this form that it can be translocated from the cytoplasm to the nucleus. FK506 administration leads to an increase in the phosphorylated form of NFAT, and hence immunosuppression (Liu et al. 1991). Inhibition of neuronal calcineurin activity leads to increased phosphorylation of substrate molecules such as GAP-43 (Growth Associated Protein of 43kDa) which is well established as playing an important role in regeneration in the peripheral and central nervous systems (Skene, 1990) (Skene, 1989). The inhibition of calcineurin phosphatase activity will also diminish nitric oxide synthase activity and this may have neuroprotective consequences (Dawson et al. 1993). A second region of FK506 binds to a site on the immunophilin molecule with rotamase (peptidyl prolyl cis-trans isomerase) activity, as a result of which events dependent on rotamase activity are inhibited (Liu et al. 1991). The binding of GPI 1046 to immunophilins also inhibits this rotamase activity, but does not have an immunosuppressant action because the GPI 1046 / FKBP-12 complex lacks an effector domain and therefore does not bind calcineurin (Steiner et al. 1997) (Figure 2-1).

EFFECTS OF IMMUNOPHILIN LIGANDS IN THE NERVOUS SYSTEM

There is increasing evidence that immunophilin ligands play an important role in promoting neuroregeneration, both in vitro (Lyons et al. 1994) (Steiner et al. 1997) and in vivo (Table 2-1). Thus in the PNS the rate of axonal regeneration and functional recovery following sciatic nerve injury is enhanced by immunophilin ligands (Gold et al. 1994) (Gold et al. 1995) (Steiner et al. 1997). Similarly these drugs promote regeneration in response to brain injury (Steiner et al. 1997), but do
not appear to cause abnormal neuronal sprouting in the undamaged nervous system (Steiner et al. 1997). In addition immunophilin ligands may have protective effects on cultured neurons (Dawson et al. 1993) and in the CNS (Sharkey and Butcher, 1994) (Butcher et al. 1997) (Matsuura et al. 1996). Thus cortical damage induced by experimental middle cerebral artery occlusion is reduced by FK506 (Sharkey and Butcher, 1994) (Butcher et al. 1997) and GPI 1046 may protect serotonin-containing neurons in the somatosensory cortex (Steiner et al. 1997). FK506 improves the functional outcome of spinal cord injury produced by photothrombosis (Madsen et al. 1996). Although lesion volumes are unaffected in this model, treatment increases the density of GAP-43 immunoreactive neurons in the vicinity of the injury site (Madsen et al. 1996). However the specific effects of FK506 on neurons following this type of photothrombotic lesion have not yet been determined.
<table>
<thead>
<tr>
<th>Drug</th>
<th>In Vitro Regeneration</th>
<th>In Vitro Protection</th>
<th>In Vivo Regeneration</th>
<th>In Vivo Protection</th>
<th>Functional</th>
</tr>
</thead>
<tbody>
<tr>
<td>FK 506</td>
<td>(Lyons et al. 1994)</td>
<td>(Dawson et al. 1993)</td>
<td>(Gold et al. 1994)</td>
<td>(Sharkey and Butcher, 1994)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Chang et al. 1995) -ve</td>
<td>(Moriwaki et al. 1996) -ve</td>
<td>(Kitamura et al. 1994)</td>
<td>(Kitamura et al. 1994)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Ferreira et al. 1993) -ve</td>
<td>(McDonald et al. 1996) -ve</td>
<td>(Matsuura et al. 1997b)</td>
<td>(Matsuura et al. 1997a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Steiner et al. 1996) -ve</td>
<td>(Wang et al. 1997b) No effect</td>
<td>(Steiner et al. 1997)</td>
<td>(Steiner et al. 1997b)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Steiner et al. 1997)</td>
<td>(Gold et al. 19977b)</td>
<td>(Gold et al. 19977a)</td>
<td>(Steiner et al. 1997)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2-1**

Review of the scientific literature on the effects of immunophilin ligands on neurons (SC = spinal cord, -ve = negative [suggesting an adverse effect on regeneration or survival], non-I.S. = non-immunosuppressant)
**PLAN OF INVESTIGATION**

In this investigation a model of dorsal column transection was employed to clarify the effects of immunophilin ligands on regeneration and survival of spinal cord axons, with specific reference to the central axonal processes of dorsal root ganglion sensory neurons. Experiments were performed with the following aims:

To assess the effects of immunophilin ligands on primary sensory axons in the dorsal columns following a transection injury of the spinal cord, with specific reference to:

- Possible effects on axonal regeneration
- The course and termination of any regenerating axons
- Possible effects on axonal survival of secondary injury
- Effects of FK506 on lesion size

To determine factors which might influence the outcome of treatment with immunophilin ligands:

- To assess the dose dependency of FK506 and GPI 1046
- To assess whether the effects of a combination of immunophilin ligands are additive
- To assess whether any effects of these drugs is dependent on sciatic nerve crush
- To assess whether any drug effect is enhanced or inhibited by the additional administration of steroids at the time of injury
- To determine whether isolating the wound site with silastic affects outcome

To clarify the molecular pathways by which regeneration is promoted, by studying the effects on neuroregeneration of the immunophilins ligands FK506, GPI 1046 and Cyclosporin, which differ in their molecular actions.
MATERIALS AND METHODS

SURGICAL METHODS
Female Sprague Dawley rats (weight 150-200g) were deeply anaesthetized with an inhaled mixture of 1.5% halothane (Mallinckrodt Veterinary Ltd, UK), 3% nitrous oxide and 1.5% oxygen. The surgical site was appropriately shaved and cleaned with antiseptic solution.

An incision was made along the back and a laminectomy was performed at the T8-T9 level to expose the dura, which was then widely opened (by first making a hole with a needle, then enlarging it with microsurgical scissors). A small hole was made in the pia on the dorsal surface of the cord using a fine needle and this was enlarged across the midline using microsurgical scissors, at the same time cutting the dorsal vein. After haemostasis had been achieved, a fine needle (25G) was swept across the dorsal aspect of the cord 2 or 3 times, to a depth of 1.5mm, with the aim of transecting the dorsal columns. In 4 animals a much larger spinal cord lesion was made using a Decker microrongeur (1 x 5mm straight cup)(Johnson and Johnson, MA, USA) attached to a stereotactic frame, which removed the dorsal aspect of the cord to a depth of 2mm.

In some animals with standard lesions the wound in the dorsal columns was covered with a thin layer of silastic, fixed in place with Histoacryl tissue glue (B. Braun Melsungen AG, Germany). The muscle around the wound was closed with 3.0 silk sutures (Ethilon, UK), and the skin along the back was clipped together (Mitchel clips). In a subset of these animals the left sciatic nerve was exposed in the thigh and crushed with fine forceps at the same operating session, to stimulate the regeneration of the central processes of primary sensory neurons passing through the dorsal columns. All rats received 1 mg of Finadyne (Schering-Plough Animal Health, UK) and Amoxycillin (SmithKline Beecham, UK) administered subcutaneously.
PREPARATION AND ADMINISTRATION OF DRUGS

**FK 506** was obtained from Fujisawa Ireland Ltd (Ireland). 1mg was dissolved in 200 μl of absolute ethanol, and this solution was mixed with Intralipid (Pharmacia Laboratories Ltd, UK) in the ratio of 2 volumes to 3 volumes. Individual animals were given either low or high dose regimes (0.5 or 2.0 mg/kg), by subcutaneous injection 5 times per week, starting within 30 minutes of surgery. The treatment regime and dosage was continued until the animal was killed. Control animals were treated with an identical regime of vehicle alone.

**GPI 1046** was obtained from Guilford Pharmaceuticals (Baltimore, USA). 12 mg was dissolved in 20 μl of absolute ethanol and, on the day of administration, was mixed with Intralipid in the ratio 1 volume to 14 volumes. Individual animals were given either low or high dose regimes (10 or 40 mg/kg) as described above for FK506. Control animals were treated as for the FK506 group.

**Cyclosporin A** was obtained from Sandoz Pharmaceuticals (UK), in the form of ‘Concentrate for Infusion’ (50 mg/ml). This was mixed with Intralipid in the ratio 1 volume to 2 volumes and administered by intraperitoneal injection 3 times per week at a dose of 30 mg/kg. The initial dose was given within 30 minutes of surgery and the treatment was maintained until the animal was killed. Control animals received injections of vehicle.

**Methylprednisolone sodium succinate (MP)** was obtained from Upjohn (USA). It was dissolved in sterile distilled water at a concentration of 30 mg/ml. Each animal received 2 doses of 30 mg/kg by subcutaneous injection. The first dose was administered within 30 minutes of surgery and the second and final dose 24 hours later.
TRANSGANGLIONIC LABELLING, FIXATION AND HISTOCHEMICAL PROCESSING

After survival periods of 1 to 12 weeks each animal in the study was re-anaesthetized with halothane. The left sciatic nerve was exposed in the thigh and injected with 0.02mg of cholera toxin sub-unit B conjugated to horseradish peroxidase (CT-HRP)(List Biological Laboratories, USA), dissolved in 1µl distilled water.

Three to four days later the animal was over-dosed with a combination of halothane and 0.5-1 ml of intraperitoneal pentobarbitone sodium (Sagatal, Rhone-Merieux, Ireland). The heart was exposed and the right atrium incised. A needle was inserted into the left ventricle and the rat was perfused with approximately 400ml of 0.1M phosphate buffer at room temperature, followed by 500-750ml of 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1M phosphate buffer at room temperature. The spinal cord was exposed and the segmental level was determined with reference to the entry of nerve roots from the sciatic nerve. The entire spinal cord and brainstem was removed and stored overnight at 4°C in 30% sucrose. Blocks were cut from different levels of the spinal cord and brainstem and then frozen in OCT (Miles Inc, USA).

Horizontal longitudinal sections were cut at a nominal thickness of 50µm on a cryostat and placed in 0.1M phosphate buffer filled reaction wells in serial order. The free-floating sections were then reacted with 3,3'-5,5'-tetramethylbenzidine (TMB)(Sigma, UK) to visualize transganglionically transported label. Briefly, 0.3g sodium nitroferricyanide was dissolved in 280 ml of distilled water and 15 mls of sodium acetate buffer (pH 3.3). A solution of 0.015g TMB in 7.5 ml of absolute alcohol was prepared. The two solutions were then mixed in the ratio 1:39 (TMB: sodium nitroferricyanide). Sections were immersed in this mixture at 4°C for a period of 20 minutes, following which 0.01% hydrogen peroxide was added in a ratio of 1 part in 25. After a further 5 minutes this mixture was replaced by a new solution.
containing all three reagents in the same proportions as defined above and this process was repeated at 5 minute intervals over the course of a 30 minute reaction period. At the end of this time the sections were rinsed several times in sodium acetate buffer, and then air-dried, dehydrated and cover-slipped.

**TREATMENT GROUPS**

In all, 151 animals were used in this study, of which 134 yielded useful results; 9 were discarded because of inadequate transganglionic labelling and 8 animals died post-operatively. Of the latter, only 1 of the deaths, due to a urinary infection in an animal receiving FK506, was likely to have been due to treatment, all others were due to surgical complications or were unexplained. Death rates in treatment groups did not differ significantly. Two main sets of experiments were carried out (Series 1-76 animals and Series 2-54 animals). In addition 4 further animals treated with low dose FK506 and sciatic nerve crush had larger lesions made using biopsy forceps, as described above. The results of these 4 experiments were analyzed separately from those of Series 1 and 2.

**Series 1 (Initial experiments):** In the first series 76 animals were divided into 5 groups: Group 1 received FK506 treatment; Group 2 received GPI 1046; Group 3 received Cyclosporin A; Group 4 received a combination of Cyclosporin A and GPI 1046, each according to the standard regimes, and Group 5 (controls) received vehicle only. The FK506 and GPI 1046 groups were further divided into subgroups receiving high and low dose regimes and in each group some animals also received methylprednisolone (see Table 2-2). Survival periods before analysis varied between 1 and 12 weeks. Initial analysis of the material in the first series was not carried out ‘blind’.
<table>
<thead>
<tr>
<th>Group</th>
<th>Main Treatment</th>
<th>Total Number in Subgroups</th>
<th>Additional treatment with MP *</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FK506: Low Dose</td>
<td>31</td>
<td>14 (17)</td>
</tr>
<tr>
<td></td>
<td>FK506: High Dose</td>
<td>7</td>
<td>3 (4)</td>
</tr>
<tr>
<td>2</td>
<td>GPI 1046: Low Dose</td>
<td>6</td>
<td>0 (6)</td>
</tr>
<tr>
<td></td>
<td>GPI 1046: High Dose</td>
<td>5</td>
<td>3 (2)</td>
</tr>
<tr>
<td>3</td>
<td>Cyclosporin A</td>
<td>6</td>
<td>6 (0)</td>
</tr>
<tr>
<td>4</td>
<td>Cyclosporin A + GPI 1046</td>
<td>2</td>
<td>2 (0)</td>
</tr>
<tr>
<td>5</td>
<td>Controls</td>
<td>19</td>
<td>6 (13)</td>
</tr>
<tr>
<td></td>
<td>Total Number</td>
<td>76</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2-2**

Series 1 treatment groups (* figures in brackets represent animals that did NOT receive steroids).
Series 2 (Randomized experiments): Because of the possibility of observer bias, a second series of experiments was performed in which 54 rats underwent the same surgical procedures as those in the first series, but were randomly assigned to one of two treatment groups post-operatively. Group 1 animals were treated with FK506 (low dose only) in precisely the same regime as used in the first series; Group 2 animals were controls and received injections of vehicle only. In each group some animals were also treated with MP (see Table 2-3). The survival period for all animals in the second series was 7-15 days after injury. Interpretation of the histological results was conducted without any knowledge of the treatment category of individual animals.

<table>
<thead>
<tr>
<th>Group</th>
<th>Main Treatment</th>
<th>Total Number in Subgroups</th>
<th>Additional treatment with MP *</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FK506: Low Dose</td>
<td>28</td>
<td>16 (12)</td>
</tr>
<tr>
<td>2</td>
<td>Controls</td>
<td>26</td>
<td>13 (13)</td>
</tr>
<tr>
<td></td>
<td>Total Number</td>
<td>54</td>
<td></td>
</tr>
</tbody>
</table>

Table 2-3

Series 2 treatment groups ( * figures in brackets represent animals that did NOT receive steroids)
ANALYSIS OF MATERIAL
Serial longitudinal (horizontal) sections through the lesion site and dorsal columns rostral to the lesion and transverse sections through the dorsal column nuclei were systematically examined in bright and dark field and labelled axons recorded. Particular attention was given to trying to identify tips of labelled axons. In addition, lesion size was assessed by making camera lucida drawings of dark-field images of the lesion at its maximum extent, allowing a calculation of the maximum length and width of the lesion (Figure 2-2).

Differences between the frequencies of labelled axons in different treatment subgroups were compared using contingency tables and a chi-squared ($\chi^2$) test, or if numbers were too small, a one-tailed Fisher's Exact Test (FET). Non-parametric statistics were used to calculate the median and interquartile range (IQR) of lesion size in different treatment groups, and differences were analyzed using the Mann-Whitney test.

RESULTS

REGENERATING FIBRES
Series 1 included results from 74 animals (38 received FK506, 11 GPI 1046, 6 Cyclosporin A and 19 were controls). Severed primary sensory axons could be traced to the caudal margin of the lesion and, in many cases, large numbers of axonal sprout terminals could be seen, apparently terminating within the lesion. Often these neurites appeared to be orientated towards the surface, and occasionally appeared to be growing out of the cord (Figure 2-3a). However this was not observed in animals in which silastic had been glued over the surface of the injured dorsal columns to
isolate the wound from the surrounding tissues (Figure 2-3b). The sprouting reaction was usually less pronounced in animals that did not receive immunophilin ligand treatment or a sciatic nerve crush. There were examples of axons apparently terminating in the spinal cord rostral to the lesion in all three treatment groups although not controls, with the clearest examples among the FK506-treated subjects. The swollen terminals of rostral regenerating fibres were located within the degenerating dorsal columns (Figure 2-4) or, sometimes, in abnormal rostral positions such as the grey matter, meninges or dorsal root stumps (Figure 2-5). Occasionally the terminal swellings of regenerating axons were present among other, much longer, labelled fibres (Figure 2-6) more than 1 cm rostral to the lesion, suggesting that the latter might provide some form of guidance to the growing axons. Sprouting fibres were also present in abnormal positions caudal to the lesion, such as the contralateral dorsal columns (Figure 2-7), where they sometimes appeared to be growing in a caudal direction. Caudal sprouting fibres of this type were even seen occasionally in control animals.

**LONG ROSTRAL SENSORY FIBRES**

In 29% of FK506-treated animals in Series 1 (Table 2-4), axons were found extending through the lesion cavity (Figure 2-8) and well beyond it. These are referred to as long rostral sensory (LRS) fibres. LRS axons were confined within the boundaries of the rostral dorsal columns (Figure 2-9). Furthermore, when more rostral levels of the spinal cord and brain stem were examined, labelled fibres could be seen along the entire length of the ipsilateral dorsal columns (Figure 2-10), terminating unilaterally at their normal destination in the nucleus gracilis of the medulla oblongata (Figure 2-11). In the remaining 71% of FK506-treated subjects there were no fibres present within the degenerating dorsal columns. In contrast, only 11% of control animals were found to have LRS fibres (Table 2-4). Among the 54 animals entered into the randomized protocol, LRS fibres were found in 29% of those treated with FK506, in contrast to 8% of controls (Table 2-4). This difference was significant (p<0.05, FET). Moreover the results of FK506 treatment, and controls, in series 1 and
2 were essentially the same, and the figures for both groups were therefore combined to give an overall result for all 66 animals treated with FK506 and all 45 controls (Table 2-4). This produced a clearly significant difference between FK506 treatment and controls ($p = 0.01$, $\chi^2$ Test).

<table>
<thead>
<tr>
<th>Category</th>
<th>Treatment Groups</th>
<th>Numbers</th>
<th>% with LRS fibres</th>
<th>$\chi^2$ (statistical significance)</th>
<th>Fishers Exact Test (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Series 1</td>
<td>FK506</td>
<td>38</td>
<td>29%</td>
<td>n/a</td>
<td>0.105 (n/s)</td>
</tr>
<tr>
<td></td>
<td>No FK506</td>
<td>19</td>
<td>11%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Series 2</td>
<td>FK506</td>
<td>28</td>
<td>29%</td>
<td>n/a</td>
<td>$&lt;0.05$</td>
</tr>
<tr>
<td></td>
<td>No FK506</td>
<td>26</td>
<td>8%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All Experiments</td>
<td>FK506</td>
<td>66</td>
<td>29%</td>
<td>0.01</td>
<td>$&lt;0.01$</td>
</tr>
<tr>
<td></td>
<td>No FK506</td>
<td>45</td>
<td>9%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2-4**

A comparison of the percentages of FK506-treated animals and controls with LRS fibres. The statistical significance of differences between treated and untreated groups is indicated. (Abbreviations: n/a - not applicable; n/s = not statistically significant)
TREATMENT VARIABLES AND LRS FIBRES

Although LRS fibres were more frequent with a lower dose of FK506 (31% compared to 14% with the higher dose; see Table II- VI) the difference did not reach statistical significance. None of the 4 animals in which larger spinal cord lesions were made using biopsy forceps had evidence of LRS fibres. The significance of this could not be evaluated because of the small group size. Animals assessed at different survival periods after dorsal column injury were classified into three groups: those examined at 15 days or less, those examined at 16-40 days and those examined at more than 40 days after operation (Table 2-5). The percentage of animals with LRS fibres did not change significantly in groups with progressively longer survival times, whether or not treated with FK506. Even in the earliest survival period, LRS fibres could be traced for the entire length of the rostral dorsal columns and there was no evidence of a ‘front’ of axons terminating in the rostral cord; indeed regenerating axons were extremely rare more than 5mm beyond the lesion. None of the animals treated with GPI 1046 had LRS fibres. The difference between FK506 and GPI 1046 treated animals was significant (p<0.05, FET). In the group of 6 animals treated with Cyclosporin A only one had LRS fibres. The significance of this could not be evaluated because of the small group size. Neither of the 2 animals treated with a combination of Cyclosporin A and GPI 1046 was found to have LRS fibres, though both showed evidence of some local regeneration. The two drugs act on different immunophilins and it is therefore possible that their effects could be additive, however the small numbers involved obviously precluded any statistical analysis. There was no evidence that sciatic nerve crush influenced the percentage of animals with LRS fibres (Table 2-5), in contrast to local regeneration rostral to the lesion which was contingent on peripheral nerve injury. The presence of silastic did not affect the likelihood of LRS fibres (Table 2-5). This is in contrast to the finding that when silastic and tissue glue were used to cover the spinal cord injury site, the growth of regenerating fibres towards the surface of the cord was largely prevented. This suggests that the regenerating fibres grow towards tissues, such as muscle, found around the spinal column, which may perhaps secrete neurotropic factors.
## Table 2-5

The effects of treatment and assessment variables on outcome following FK506 treatment (figures refer to combined results of Series 1 and 2; the results of Cyclosporin A and GPI 1046 experiments are not shown) (Abbreviations: n/a - not applicable)

<table>
<thead>
<tr>
<th>Variable</th>
<th>FK 506</th>
<th>Control Experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>% with LRS fibres</td>
</tr>
<tr>
<td>High FK506</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>Low FK506</td>
<td>59</td>
<td>31</td>
</tr>
<tr>
<td>Time (days)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;16</td>
<td>35</td>
<td>26</td>
</tr>
<tr>
<td>16-40</td>
<td>20</td>
<td>35</td>
</tr>
<tr>
<td>&gt;40</td>
<td>11</td>
<td>27</td>
</tr>
<tr>
<td>Crush</td>
<td>54</td>
<td>30</td>
</tr>
<tr>
<td>No Crush</td>
<td>12</td>
<td>25</td>
</tr>
<tr>
<td>Silastic</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>No Silastic</td>
<td>36</td>
<td>28</td>
</tr>
</tbody>
</table>
**METHYLPREDNISOLONE**

In comparison with FK506, the administration of MP had no significant effect on the frequency of LRS fibres (23% with MP treatment vs 19% in controls; Table 2-6). Moreover, when treatment groups were further subdivided, the percentage of animals with LRS fibres were as follows: FK506 and MP- 33%; FK506 only-24%; MP only-5%; No treatment-12%. The combination of FK506 and MP differed significantly from treatment with MP alone (Table 2-6).

**LESION SIZE**

Whereas FK506 treatment was clearly associated with the presence of LRS fibres, it made no clear difference to lesion size expressed as the product of maximum length and maximum width (Table 2-7), or to the maximum length of the lesion (ie its caudo-rostral extent) (Median values: FK506 treatment-3.19mm; Controls-3.38mm). Although the median value of lesion size with FK506 was slightly smaller the difference was not statistically different when analyzed using the Mann-Whitney test. There was similarly no evidence that MP significantly influenced lesion size (Table 2-7).
<table>
<thead>
<tr>
<th>Category</th>
<th>Numbers</th>
<th>Treatment Groups</th>
<th>% with LRS fibres</th>
<th>$\chi^2$ (statistical significance) (p)</th>
<th>FET (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Experiments</td>
<td>52</td>
<td>MP</td>
<td>23%</td>
<td>0.57 (n/s)</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>59</td>
<td>No MP</td>
<td>19%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sub-categories</td>
<td>33</td>
<td>FK506 + MP</td>
<td>33%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>MP only</td>
<td>5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>FK506 only</td>
<td>24%</td>
<td></td>
<td>n/s</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>No Treatment</td>
<td>12%</td>
<td></td>
<td>n/s</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>MP + FK506</td>
<td>33%</td>
<td></td>
<td>n/s</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>FK506 only</td>
<td>24%</td>
<td></td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>MP only</td>
<td>5%</td>
<td></td>
<td>n/s</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>No Treatment</td>
<td>12%</td>
<td></td>
<td>n/s</td>
</tr>
</tbody>
</table>

**Table 2-6**

Numbers and percentage of subjects with LRS fibres following MP treatment

(Abbreviations: n/a - not applicable; n/s = not statistically significant) (Figures refer to combined results of Series 1 and 2; the results of Cyclosporin A and GPI 1046 experiments are not shown)
<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Total number of animals</th>
<th>Median lesion size (mm²)</th>
<th>IQR (mm²)</th>
<th>Mann-Whitney(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All FK506</td>
<td>28</td>
<td>3.63</td>
<td>1.8 to 5.7</td>
<td>0.396</td>
</tr>
<tr>
<td>No FK506</td>
<td>26</td>
<td>4.65</td>
<td>3.4 to 5.6</td>
<td></td>
</tr>
<tr>
<td>All MP</td>
<td>29</td>
<td>3.95</td>
<td>1.9 to 5.6</td>
<td>0.69</td>
</tr>
<tr>
<td>No MP</td>
<td>25</td>
<td>3.95</td>
<td>3.3 to 6.0</td>
<td></td>
</tr>
<tr>
<td>FK506 + MP</td>
<td>16</td>
<td>3.99</td>
<td>2.2 to 5.4</td>
<td>0.7</td>
</tr>
<tr>
<td>MP only</td>
<td>13</td>
<td>4.22</td>
<td>2.7 to 5.7</td>
<td></td>
</tr>
<tr>
<td>FK506 only</td>
<td>12</td>
<td>3.61</td>
<td>2.0 to 6.0</td>
<td>0.46</td>
</tr>
<tr>
<td>No treatment</td>
<td>13</td>
<td>4.65</td>
<td>3.4 to 5.5</td>
<td></td>
</tr>
<tr>
<td>MP + FK506</td>
<td>16</td>
<td>3.99</td>
<td>2.2 to 5.4</td>
<td>0.98</td>
</tr>
<tr>
<td>FK506 only</td>
<td>12</td>
<td>3.61</td>
<td>2.0 to 6.0</td>
<td></td>
</tr>
<tr>
<td>No treatment</td>
<td>13</td>
<td>4.65</td>
<td>3.4 to 5.5</td>
<td>0.78</td>
</tr>
<tr>
<td>MP only</td>
<td>13</td>
<td>4.22</td>
<td>2.7 to 5.7</td>
<td></td>
</tr>
<tr>
<td>No treatment</td>
<td>13</td>
<td>4.65</td>
<td>3.4 to 5.5</td>
<td></td>
</tr>
</tbody>
</table>

Table 2-7

Lesion size, defined as the product of maximum length and width (mm²), in Series 1 experiments. (Note: IQR= interquartile range, Mann-Whitney tests refer to pairs of data sets such as those receiving or not receiving FK506)
DISCUSSION

FK506 promotes the survival of ascending dorsal column axons following spinal cord injury by reducing axonal destruction due to the secondary pathological processes that result from the initial surgical injury. This effect on axonal survival is independent of any effects on the viability of the parent cell body. In addition FK506 treatment, in combination with a sciatic nerve crush, promotes limited regeneration of severed primary sensory axons rostral to the lesion within the spinal cord, an effect which was entirely absent in control animals.

LRS fibres were most commonly found in animals treated with FK506. Their presence might be due to either regeneration of severed axons along the length of the dorsal columns or by sparing of some axons by the initial lesion. However it is unlikely that the LRS fibres had regenerated since, even at 7-14 days after injury, they extended approximately 6cm from the lesion to the dorsal column nuclei (DCN) in the medulla. This would imply rates of axonal regeneration of up to 8mm day, even discounting a latent period for growth through the lesion. The maximum recorded rate of regenerative axonal growth past an experimental lesion is less than 5mm /day (Lundborg, 1988) (Verdu and Navarro, 1997). FK506 treatment following sciatic nerve crush increases axonal growth rates (Wang et al. 1997b), nevertheless the maximum rate of axonal regeneration between the 12th and 15th days after injury is still only 5.1mm/day, even without any delay associated with growth through the lesion site, which would reduce the overall axonal growth rate still further. It is therefore extremely unlikely that regenerating axons could have reached the DCN within 7 days. Furthermore, when animals were classified according to the length of their post-operative survival, there was no progressive increase in the percentage with LRS fibres, or indeed the numbers of LRS fibres within individual animals, as might be expected if they were regenerative in nature. In addition, a ‘front’ of regenerating axon terminals within the rostral dorsal columns was not seen in any experiment. Indeed axons terminating more than 5mm rostral to the lesion were extremely rare.
Further evidence that LRS fibres had been spared arises from the observation that they were never present in the right dorsal column and that their terminals were found exclusively in the left DCN. Since ascending axons were labelled by CT-HRP injected into the left sciatic nerve, a unilateral distribution of LRS fibres would be expected if they had been spared. It is less likely that regenerating axons would have produced this asymmetrical pattern since this would imply that, as they emerged from the disorganized lesion site, they recognized and grew along the ipsilateral degenerating dorsal column while avoiding the contralateral one. Finally, LRS fibres were not present in any of the 4 animals in which larger lesions had been made and, while the numbers were too small for statistical analysis, such a finding might be surprising if LRS axons resulted from regeneration. Thus, the evidence indicates that LRS fibres were spared by the initial transection injury, although in a very few cases axons were found terminating more than 5mm rostral to the lesion. These longer regenerating axons were seen exclusively in the presence of other fibres that extended all the way to the DCN and may thus have provided some form of guidance to assist the limited regenerative processes. Although it is possible that in a minority of cases some axons might have regenerated up to the DCN, such long distance growth was never demonstrated in the absence of spared axons and could therefore not be identified.

Spared fibres were significantly more common among the FK506 treated animals than controls. It is therefore probable that in some animals a few axons were not transected at the time of surgery, but without FK506 treatment the processes of secondary injury were sufficient to cause irreversible axonal damage so that no axons were seen beyond the lesion in over 90% of cases (Figure 2-12). The possibility that significant secondary injury, which could cause delayed axonal destruction, did indeed occur with this transection technique is suggested by the finding that the median axial length of lesions produced by a transverse linear incision was over 3mm. Furthermore spared axons could be traced through the lesion, surrounded on all sides by clear evidence of pathology, suggesting that the secondary injury extended.
the boundary of the lesion well beyond the original incision. Although in some cases spared fibres were present in the deepest parts of the dorsal columns, in others the fibres were relatively superficial with evidence of injury deeper within the cord. This pattern could have arisen if, as the needle was swept laterally, some fibres were not cut, but were pushed away and simply stretched over the blade. FK506 treatment increased the probability that axons, spared by the initial lesion, were able to survive the effects of secondary injury. Significant operator bias towards the creation of larger surgical injuries among controls in the non-randomized experiments was highly unlikely given the near identical results in the randomized series. Although spared fibres were demonstrated in 29% of animals treated with FK506, this may in fact under-represent the effects of the drug on axonal survival, since in an unknown proportion of animals all the dorsal column axons may have been completely transected at the time of surgery thus precluding the possibility that treatment could reduce secondary axonal destruction.

It is possible that the effect of FK506 on axonal sparing could result from its immunosuppressant actions reducing the severity of secondary injury. It has been suggested that spinal cord injury may be associated with an autoimmune reaction that is reduced by immunosuppression (Teichner et al. 1993) (Palladini et al. 1996). However, a dose of 1mg/kg of FK506 is sufficient to produce increased peripheral nerve regeneration, but not enough to prevent immune rejection of neuronal transplants in rats (Gold et al. 1994) (Sakai et al. 1991), and in this study 0.5 mg/kg elicited a sparing effect. Alternatively the ability of axons to survive secondary injury might be enhanced by FK506 binding to FKBP-12 within neurons, or in associated glia where FKBP-12 may also be present (Lyons et al. 1995). A direct protective action on axons within the CNS is consistent with our finding that the effects of FK506 on lesion size are less marked than on axonal sparing, or indeed on functional outcome (Madsen et al. 1996).
Our results did not give a clear indication as to whether Cyclosporin A also protects axons, primarily because the number of animals treated with Cyclosporin A was too small, but previous studies suggest that it has protective effects on neurons, both in vitro (Dawson et al. 1993) and in vivo (Butcher et al. 1997), provided it is given in adequate doses to ensure sufficient concentrations within the CNS. However GPI 1046 does not protect axons from secondary injury in this model, at doses which have been shown to stimulate axonal sprouting within the brain (Steiner et al. 1997). This would suggest that, unless a localized reduction in the bioavailability of GPI 1046 occurs within the injured spinal cord, reduced calcineurin phosphatase activity is important in mediating the protective effects of FK506 on dorsal column axons, since the GPI 1046/FKBP-12 complex lacks a calcineurin-binding effector domain. Such a mechanism would be compatible with the finding that calcineurin is co-localized with FKBP-12 within the CNS (Steiner et al. 1992). Moreover rapamycin, which competes with FK506 for a common binding site on FKBP-12, but does not inhibit calcineurin activity, will block the neuroprotective actions of FK506 on the cerebral cortex following ischaemic injury (Sharkey and Butcher, 1994).

The mechanism by which FK 506 might protect axons has yet to be clarified, however it is possible that it is the same as that which mediates protection of neurons. A number of molecular mechanisms have been proposed to result in cellular neuroprotective effects of FK506. Calcineurin inhibition might protect neurons by preventing the dephosphorylation of nitric oxide synthase (NOS), thereby diminishing its catalytic activity (Dawson et al. 1993) and reducing the formation of nitric oxide (NO), an important component of glutamate excitotoxicity (Dawson et al. 1991). NO itself can also increase the formation of other free radical species (Lipton et al. 1993) leading to cellular damage and, moreover, FK506 is known to reduce superoxide formation in neutrophils (Nishinaka et al. 1993). However, there is evidence that FK506 does not protect neurons from excitotoxic cerebral injuries, implying that neuroprotective mechanisms in experimental models of stroke might not be antiexcitotoxic (Butcher et al. 1997). Recently evidence has emerged that in vitro
protective effects of FK 506 on neurons might be mediated by a reduction in the active form of the nuclear protein c-Jun (Hugon et al. 1997) (Buschmann et al. 1997).

The clinical effects of spinal cord injury are caused, in large part, by the transection of the long-tract axons of neurons, many of the cell bodies of which survive the injury. The concept that drug therapy might reduce such axonal loss is fundamental to most pharmacological approaches to spinal cord injury that attempt to reduce secondary damage and hence ameliorate the functional outcome. However, many studies have only examined the numbers of surviving neurons or lesion size, sometimes predominantly involving the grey matter (Das, 1989a). Others have demonstrated that treatment can increase the numbers of spared myelinated axons (Blight, 1994), but have not distinguished between protective effects on the entire neuron and the axon alone. Since transected dorsal column axons originate from dorsal root ganglion cells, which survive axonal injury even without treatment (Groves et al. 1996) (Dent et al. 1996), the effects of FK506 on axonal sparing must be independent of viability of the cell body. There has been no previous explicit experimental demonstration of this basic distinction between the possible effects of neuroprotective drugs. The concept of treatment-induced axonal sparing is also pertinent to the study of neuroregeneration since the histological and functional outcomes of these two neurobiological processes are potentially similar and must therefore be distinguished.

FK506 stimulates axonal regeneration, probably by a direct effect on axons rather than by an immunosuppressant action. Evidence for this comes from the finding that FK506 produced neurite extension only if a peripheral axotomy, of the sciatic nerve, was performed. The effect of the peripheral nerve injury is to augment intracellular molecules which are important in neuroregeneration, including GAP-43 and FKBP-12 mRNA (Lyons et al. 1995). This would suggest that, following axotomy, FK506 is able to exert a greater effect by increased binding to the greater number of FKBP-12
molecules available within neurons or perhaps associated glial cells. Binding to FKBP-12 could increase regeneration by inhibiting calcineurin activity and hence GAP-43 de-phosphorylation. The increased levels of phosphorylated GAP-43 causes its affinity for calmodulin to be reduced (Skene, 1990) and hence a rise in free calmodulin. This results in activation of calcium-calmodulin dependent enzymes leading to increased growth cone activity (Skene, 1990). Cyclosporin A, which like FK-506 inhibits calcineurin, may also stimulate axonal regeneration. It has been claimed that following complete spinal cord transection Cyclosporin A promotes regeneration of many long-tract axons beyond the lesion and improves functional outcome (Teichner et al. 1993) (Palladini et al. 1996). However, in the injured sciatic nerve Cyclosporin A is much less effective than FK506 in promoting regeneration (Wang et al. 1997b) and the regenerative responses to any drugs observed in our study appeared to be much more limited than those reported previously (Teichner et al. 1993) (Palladini et al. 1996). The role of calcineurin in axonal regeneration therefore remains uncertain, particularly since non-immunosuppressant analogues of FK506, including GPI 1046, promote neurite outgrowth in spite of lacking calcineurin inhibitory activity (Steiner et al. 1997) (Steiner et al. 1997). Alternative explanations of the effects of FK506 that do not depend on calcineurin inhibition are also possible. Thus FKBP-12 is associated with molecules such as IP₃ and TGFβ1 and FK 506 could act by disrupting these complexes, though the concentrations required for in vitro stimulation of neurite outgrowth are much lower than are required to cause dissociation (Wang et al. 1997b). Other molecules which bind to FKBP-12 are currently being investigated (Lai et al. 1997) and could mediate alternative mechanisms of action.

Axonal transection is a critical factor in many neurological conditions including head injury and strokes of the internal capsule, as well as spinal cord injury. Any drug treatment that reduced axonal loss would clearly have important implications for these conditions; however several issues remain to be addressed. This study has investigated only one axonal population within the spinal cord, and it is possible that
FK506 is less protective of other axons. Moreover the state of myelination of the spared fibres is unknown, and is critical for normal axonal conduction (Blight, 1994). There is indeed some clinical evidence that immunosuppressants affect myelination, cases having been reported of both FK506 and Cyclosporin A related leukoencephalopathy, which involves demyelination of the parieto-occipital region and resolves rapidly when the immunosuppressant is stopped. It may be that calcineurin is involved in the regulation of myelination in the normal CNS (Dawson, 1996). Further unresolved issues include the timing of the first dose, the duration of therapy and the optimum dosage. Our results suggest that increasing the dose of FK506 from 0.5 to 2.0 mg/kg reduces the likelihood of spared fibres though the difference was not significant. Other investigators, however, have used a wider range of doses with neurological effects arising between 0.1 to 10 mg/kg (Madsen et al. 1996) (Sharkey and Butcher, 1994) (Wang et al. 1997b), and the equivalent dose might be different in humans. Finally, the effects of FK506 on functional recovery in a crush model of spinal cord injury, which most closely replicates clinical injuries, has yet to be investigated.

In spite of these reservations, evidence from this, and other studies, gives reason for optimism. Immunosuppressants are already commonly prescribed to prevent allograft rejection and their pharmacology is therefore well established. Administration can be intravenous or oral and unlike peptide neurotrophins, for instance, they readily reach their target tissues. Although in this study FK506 was administered throughout the post-operative period of up to 12 weeks, in order to stimulate neuroregeneration, there is already experimental evidence that spinal cord function is improved by only three doses of FK506 given within the first 48 hours after injury (Madsen et al. 1996), and it is therefore likely that neurological treatment would not need to be prolonged, as it is after transplant surgery, thus avoiding the complications of long-term administration. Furthermore a temporal therapeutic window appears to exist, since intravenous FK506 will reduce cortical damage in a model of cerebrovascular accident when administered at 120 minutes, though not at 180 minutes, after injury.
(Butcher et al. 1997), although this time period may differ for other pathologies and locations. In addition, it appears that FK506 does improve spinal cord function after photothrombosis injury (Madsen et al. 1996), which accords with our finding that spared axons must be at least partly functional, since CT-HRP was transported to the thoracic dorsal columns following sciatic nerve injection. At present MP is the only drug routinely administered after acute spinal cord injury (Bracken et al. 1990) (Hall, 1992) (Bracken et al. 1992) and, although direct comparisons should be treated with caution, these results provide no evidence that FK506 reduces the beneficial effects MP, as can occur with other drug combinations (Constantini and Young, 1994). In fact, the results suggest that the combination of FK506 and MP is significantly more effective than MP alone in protecting axons.

**CONCLUSIONS**
FK506 enhances the regeneration of severed primary sensory axons for short distances in the rostral spinal cord, but in addition it promotes axonal survival independent of any effects on the survival of the parent cell body. This might occur due to a reduction in secondary injury through effects on lymphocytes or due to a more direct protective action on neurons or glia. These effects may be mediated by calcineurin inhibition since GPI 1046 does not protect axons. The findings of this study have potentially important clinical implications in spinal cord injury, head injury, cerebrovascular accidents and several other neurological conditions.
Figure 2-1  Flow chart of possible mechanisms by which immunophilin ligands might produce axonal regeneration.
Camera lucida drawing used to assess maximum lesion length and width. The lesion area is shaded and the two arrows represent the maximum length and width.

(SC = Spinal cord; L = Lesion)
Figure 2-3a

Regenerating axons (arrowheads) growing out of a lesion towards the surface of the spinal cord. Numerous macrophages (M) can be seen within the lesion (FK506-treated animal, killed 3 weeks after surgery; Scale Bar =100μm; dark-field microscopy).
Figure 2-3b

Regenerating fibres (arrowheads) growing along the axis of the cord within the lesion (L) when silastic and tissue glue were applied to the surface of the wound (FK506-treated animal, killed 3 weeks after surgery; Scale Bar =100μm; dark-field microscopy)
Figure 2-4

The swollen terminal of a regenerating axon within the degenerating dorsal columns approximately 10-15mm rostral to the lesion, growing in a cranial direction. (FK506-treated animal, killed 4 weeks after surgery; Scale Bar = 25μm; bright-field microscopy)
Labelled axons (arrowheads) in ectopic positions in the thoracic cord rostral to the lesion. The axons can be seen on the surface of the spinal cord (SSC) probably within the meninges, approximately 2mm rostral to the lesion. (FK506-treated animal, killed 3 weeks after surgery; Scale Bar =100μm; dark-field microscopy).
Figure 2-5b

Regenerating axons (arrowheads) in an ectopic position within the severed stump of a dorsal root (R), which is next to the spinal cord (S), 2mm rostral to the lesion (FK506-treated animal, killed 2 weeks after surgery; Scale Bar = 200μm; dark-field microscopy)
Figure 2-6

The swollen terminal (arrow) of a regenerating axon can be seen approximately 10-15mm rostral to the lesion amongst several other longer, presumably spared, fibres (arrowheads). (FK506-treated animal, killed 4 weeks after surgery; Scale Bar = 25μm; bright-field microscopy)
Figure 2-7

Fibres in an abnormal position within the caudal spinal cord. Normal fibres (arrowheads) can be seen just to the left of the midline in the deepest part of the dorsal columns, with degenerating corticospinal tracts on either side (C), to the left of these fibres are co-lateral branches terminating in the grey matter (*). To the right of the midline is a fibre growing in an abnormal position (arrows) (FK506-treated animal, killed 2 weeks after surgery; Scale Bar = 200μm; dark-field microscopy)
**Figure 2-8a**

Axons (arrowheads) passing through an area of severe tissue injury within the lesion site. A prominent cluster of macrophages (M) and a cavity (C) are present within the lesion (FK506-treated animal, killed 3 weeks after surgery; Scale Bar = 200μm)
Figure 2-8b

A group of axons (arrowheads) passing through an area of severe tissue injury, with numerous pale macrophages (M) within the lesion site (FK506-treated animal, killed 3 weeks after surgery; Scale Bar = 100 μm; dark-field microscopy)
Figure 2-9a

Transverse section of degenerating rostral dorsal columns (DC) showing labelled axons (arrows). On either side are the corticospinal tracts (CS), which are not degenerating since they comprise descending fibres. (FK506-treated animal, killed 3 weeks after surgery; Scale Bar = 50μm; dark-field microscopy).
Figure 2-9b

Line drawing showing fibres (arrows) at an intermediate depth within the dorsal columns (Scale Bar = 100μm) (CS = corticospinal tract; DC = dorsal columns)
**Figure 2-10a**

LRS fibres (arrowheads) can be seen within the rostral degenerating dorsal columns (the lateral boundaries of which are indicated by arrows) approximately 8mm rostral to the lesion (FK506-treated animal, killed 8 weeks after surgery; Scale Bar = 50μm; dark-field microscopy).
Figure 2-10b

LRS fibres (arrowheads) near the boundary with the corticospinal tract (CS) in the deepest part of the dorsal columns (FK506-treated animal, killed 2 weeks after surgery; Scale Bar = 100μm; dark-field microscopy).
Figure 2-10c

No LRS fibres are visible within the degenerating dorsal columns (DC) of the rostral cord of a control subject killed 4 weeks after surgery (Scale Bar = 200μm; dark-field microscopy).
Figure 2- 11

Labelled axon terminals (arrowheads) in the ipsilateral nucleus gracilis (I). No terminals are seen in the contralateral nucleus (C). (FK506-treated animal killed 4 weeks after surgery; Scale Bar = 100μm; dark-field microscopy)
Figure 2-12
Schematic diagram of a spinal cord lesion illustrating how FK506 could result in sparing of axons. On the right side, areas of primary and secondary destruction are proposed. On the left it can be seen that the deepest part of the dorsal columns (outlined in green) might be destroyed by the processes of secondary injury and might therefore be saved by protective therapy (DC: dorsal columns)
Experimental Findings

-Section II-

The Investigation of Muscularis Externa as a Graft Tissue in Spinal Cord Injury
OVERVIEW: MUSCULARIS EXTERNA AND CNS GRAFTING

The intestine consists of an inner mucosal layer, surrounded by the submucosa, muscularis externa and an outer serosal layer. The muscularis externa comprises an outer longitudinal and inner circular layer of smooth muscle, with the enteric nervous system (ENS) lying between them. Other cell types such as interstitial cells of Cajal, glial cells and fibroblasts are closely associated with the ENS.

The gastrointestinal tract has a unique intrinsic innervation that is highly complex and controls many of its motor, secretory and absorptive functions (Furness and Bornstien, 1995). The ENS runs from the oesophagus to the anus and is composed largely of the ganglionated submucous and myenteric plexuses, located respectively within the submucosa and between the inner circular and outer longitudinal smooth muscle layers of the muscularis externa. Individual ganglia are extensively interconnected via nerve fibres contained in internodal strands (Furness and Bornstien, 1995). ENS neurons receive inputs from the CNS via parasympathetic and sympathetic pathways, but also from sensory neurons and interneurons within the ENS itself (Jessen and Burnstock, 1982) (Costa and Brookes, 1994). Effector neurons, which may be excitatory or inhibitory, supply the muscularis externa, mucosa and vasculature. Motoneurons innervating smooth muscle are located mainly in the ganglia of the myenteric plexus, while most secretomotor neurons supplying the mucosa are found within the submucous plexus. It contains a heterogeneous population of several million neurons, a number that is comparable with that in the spinal cord (Costa and Brookes, 1994) (Furness and Bornstien, 1995) (Furness and Costa, 1980) (Karaosmanoglu et al. 1996).
The ENS exhibits a high degree of functional autonomy and differs from the rest of the peripheral nervous system in a number of ways (Jessen and Burnstock, 1982) (Costa and Brookes, 1994) (Furness and Bornstien, 1995). Neurons are arranged in complex circuitry utilizing a wide variety of neurotransmitters including acetylcholine, \( \gamma \)-aminobutyric acid (GABA), adenosine triphosphate (ATP), serotonin, nitric oxide and many peptides. Glial cells with features similar to astrocytes are also present within the ENS (Gershon and Rothman, 1991). However, unlike the CNS it has a notable regenerative capacity and demonstrates significant plasticity following changes in its structure. Its cells also demonstrate plasticity and regeneration following experimental manipulation. Thus ENS neurons exhibit strong regenerative responses when cultured in vitro (Saffrey et al. 1991) (Saffrey et al. 1992). Similarly, experimentally-induced stenosis will induce enlargement of enteric neurons and glia (Gabella, 1984), while chemical ablation of the myenteric plexus results in hypertrophy of submucosal neurons (See et al. 1990). Moreover several different types of intrinsic nerve fibres will regenerate across a surgical anastamosis of guinea pig intestine (Galligan et al. 1989).

A major problem affecting the application of neural transplantation techniques to human disease is the availability of a suitable source of donor tissue. Selection of such a source must take account of ethical considerations, such as those arising from the use of foetal tissue (Hoffer and Olson, 1991) and technical problems of immune rejection. The use of autografted muscularis externa would avoid both these issues and has the additional advantage that it can be obtained in large amounts from the patient’s own intestine without harmful consequences (Jaeger et al. 1993). Moreover, muscularis externa might provide a valuable source of cells for grafting into the nervous system for a number of reasons. The ENS contains a wide variety of neurons and glia, which show structural and functional similarities with the CNS, but which exhibit a notable degree of plasticity and a capacity for axonal regeneration after injury.
A muscularis externa graft implanted into the injured CNS could improve outcome in several ways. In the acute phase following injury the graft could reduce secondary injury, for instance by the release of neuroprotective factors or by modulation of the biochemical, metabolic or inflammatory processes which produce secondary neuronal damage. The grafts could also act by stimulating neuroregeneration, perhaps by the release of neurotrophic factors or the provision of a matrix to support and guide regenerating axons. When ENS neurons are included in the graft tissue the possibility exists that damaged neuronal circuitry could be replaced by the transplanted cells. This could be either in a similar, though not necessarily identical, pattern to the pre-morbid network or in a novel pattern that nevertheless is of some functional benefit to the patient. Even when no significant structural alteration is produced by the transplant there could be benefits to the neurological status by improvements in the function of damaged spinal cord, mediated by increased neurotransmitter availability or release, or by increasing blood flow.

Recently, muscularis externa and its derivatives have been transplanted into the CNS (Lawrence et al. 1991) (Tew et al. 1992) (Tew et al. 1993) (Tew et al. 1994) (Tew et al. 1995a) (Tew et al. 1996) (Jaeger et al. 1993) (Tew et al. 1995b). There is evidence from both in vitro and in vivo studies that muscularis externa and its derivatives have significant effects on axonal growth in the CNS. Thus co-culture of striatal neurons with whole myenteric plexus increases neurite outgrowth of CNS neurons; this effect is less marked with enriched myenteric glial cells or neurons. Non-ganglionic cells have no effect. Furthermore, the results suggest that this increased neurite elongation is independent of any effect on striatal cell numbers and is likely to be mediated by one or more soluble factors produced by myenteric glial cells, possibly acting together with signals from the myenteric neurons (Hopker et al. 1994) (Hopker et al. 1995) (Hopker et al. 1996).

Muscularis externa transplanted into the rat striatum (caudatoputamen) will survive and elicit a vigorous sprouting response from axons in the surrounding brain
parenchyma with in-growth of both myelinated and unmyelinated axons into the graft (Tew et al. 1992) (Tew et al. 1995b). The presence of myelination is of interest since the enteric ganglia of rodents do not normally contain myelinated fibres. A similar sprouting response is elicited by implants of either isolated myenteric plexus (Tew et al. 1994) (Tew et al. 1993) or smooth muscle (Tew et al. 1995a). Thus, grafts consisting of colonic smooth muscle and associated interstitial cells of Cajal, from which myenteric plexus tissue has been removed, are well integrated into the brain, with in-growth of blood vessels, 3-6 weeks after implantation. A vigorous axonal reaction is induced by the smooth muscle cells, with many bundles of small unmyelinated axons visible around the graft. Similar bundles are also seen within the grafts, probably representing in-growth from the surrounding brain. In addition some larger diameter myelinated axons penetrate the graft, suggesting in-growth of supporting cells from the surroundings. It would thus appear that intestinal smooth muscle grafts in the absence of myenteric plexus tissue can induce axonal sprouting from CNS neurons. Although smooth muscle cells make up the bulk of the tissue other cell types such as the interstitial cells of Cajal, which have features common to smooth muscle cells and fibroblasts and exhibit pacemaker activity, are also present in small numbers and could have a role in inducing axonal sprouting (Tew et al. 1995a). The regenerative response to muscularis externa grafts is abolished by freeze-killing the grafts prior to implantation, implying that some property of the living cells of the grafts, rather than the surgery, is responsible for the axonal sprouting (Tew et al. 1992). There is thus an interesting difference between the results of in vitro studies, which indicate that smooth muscle cells have little effect on neurite outgrowth (Hopker et al. 1994), and in vivo studies that show a considerable promotion of axonal sprouting by muscle grafts (Tew et al. 1992). This apparent difference may occur because many of the sprouting neurites seen around grafts implanted into the striatum arise from neurons whose cell bodies are located outside the striatum. Intrinsic striatal neurons, such as those used in the in vitro studies, may not respond as vigorously to the presence of smooth muscle cells.
Although muscularis externa implants in the spinal cord survive and become histologically integrated (Jaeger et al. 1993), their effect on nearby axons may be less marked than in the striatum. Myenteric plexus cells have also been implanted into the denervated hippocampus survive, but are not reported to induce axonal sprouting (Lawrence et al. 1991). This suggests that the strength of the regenerative response to muscularis externa and its derivatives is stronger in the striatum than in other regions of the CNS (Lawrence et al. 1991) (Tew et al. 1992).

The work presented here was carried out to further investigate the possible effects of muscularis externa grafted into the spinal cord, following either major or minor injury to the spinal cord. The properties of cells of the muscularis externa have also been investigated, with specific reference to their production of the neurotrophins BDNF and NT-3 and their survival and proliferation when implanted into the CNS.
CHAPTER 3

Implantation of muscularis externa with mild spinal cord injury: Light and electron microscopy studies

ABSTRACT

INTRODUCTION

MATERIALS AND METHODS

RESULTS

DISCUSSION
ABSTRACT

Small pieces of muscularis externa (intestinal smooth muscle and myenteric ganglia) were implanted in the dorsal columns of the left side of the thoracic spinal cord. The animals were allowed to survive for 2-6 weeks after implantation. Electron microscopy revealed that the grafts had been invaded by a variety of axons, many of which were myelinated by Schwann cells but not by oligodendrocytes. Transganglionic labelling with cholera toxin B-HRP conjugate showed that dorsal column axons were present in the grafts, providing unequivocal evidence that axons from primary sensory neurons regenerated into the grafts from the CNS tissue. There was little evidence of extensive axonal sprouting in the CNS tissue surrounding the grafts, in marked contrast to the production of large numbers of axonal sprouts surrounding similar grafts in the corpus striatum.

INTRODUCTION

Muscularis externa and its derivatives stimulate axonal sprouting when implanted into the striatum (Tew et al. 1992; Tew et al. 1993; Tew et al. 1994; Tew et al. 1995a; Tew et al. 1995b; Tew et al. 1996; Tew et al. 1995b) (see ‘Overview’ above). Although muscularis externa implants in the spinal cord survive and become histologically integrated, there is some evidence their effect on regeneration of axons, from either the graft itself or its surroundings, may be less marked than in the striatum. Thus allografts consisting of cultured and re-aggregated rat muscularis externa implanted into the lower thoracic spinal cord following the production of a penetrating lesion
reduce cavitation in the injured spinal cord (Jaeger et al. 1993). At 3 weeks such
grafts are well integrated into the surrounding spinal cord with vascular in-growth.
However, only half survive to 8 weeks, due to immune rejection. There is no
evidence of projection of axonal fibres from the graft to the spinal cord but some
ramifying fibres, growth cones and synapses are present within the implants which
might be projections from spinal cord neurons. Myelinated fibres associated with
Schwann cells can be seen within the grafts. Since myelination is not a feature of the
myenteric plexus, this suggests that enteric cells may be differentiating to produce
myelin or that Schwann cells from peripheral nerves may migrate into the spinal cord
in response to some unidentified factor produced by the graft (Jaeger et al. 1993). As
noted above the grafts implanted by Jaeger et al (1993) consisted of cultured and re-
aggregated rat muscularis externa. It therefore remains to be clarified whether
undissociated tissue grafts, such as those used by Tew et al (1992), have effects on
axonal regeneration in the spinal cord similar to those on the striatum.

Experiments were therefore performed in order to clarify whether implantation of
muscularis externa grafts into the spinal cord of adult rats stimulates the production
of regenerative axonal sprouts and, if so, whether there is any indication that
regenerating axons enter the grafts from the spinal cord. In the initial experiments
described in this section a surgical technique was used which caused only mild
damage to the dorsal columns, in order to minimize the degree of secondary injury.
This limited injury technique was chosen to study axonal regeneration since it is
possible that the hostile cellular environment associated with severe secondary
pathological process might inhibit any effects of muscularis externa on regeneration.
The grafts and the surrounding spinal cord were investigated either using electron
microscopy or using tracers injected into the sciatic nerve to visualize the ascending
sensory axons within the dorsal columns and allow their identification within the
grafted tissue.
MATERIALS AND METHODS

SURGICAL METHODS
Inbred adult male Fischer rats (200 to 400g) (supplied by Harlan-Olac, UK) were used in these experiments. Donor rats were killed using an overdose of halothane and small portions of distal ileum were removed. The intestinal contents were removed and the muscularis externa peeled away from the underlying layers. This was then divided into 0.5 mm² pieces which were washed four times in Hanks Balanced Salt Solution (GIBCOBRL, UK) containing antibiotics and anti-mycotics. Recipient rats were deeply anaesthetized with an inhaled mixture of 1.5% halothane (ICI, UK), 3% nitrous oxide and 1.5% oxygen. The animals were appropriately shaved and cleaned with antiseptic solution.

An incision was made along the back of the anaesthetized recipient rats. A laminectomy was performed at the T8-T9 segment to expose the dura, which was then widely opened (by first making a hole with a needle, then enlarging it with microsurgical scissors). A small piece of muscularis externa (0.2mm²) was inserted into the left side of the spinal cord to a depth of 2mm at the T8 segment and the site of implantation marked with a fine suture (10/O Ethilon) in the dura. The muscle around the wound was closed with 3/O sutures, and the skin along the back was clipped together using Mitchel clips. The left sciatic nerve was exposed in the thigh and crushed with fine forceps in all animals to stimulate the regeneration of the central processes of primary sensory neurons passing through the dorsal columns. The animals were allowed to survive for 2-6 weeks. The dorsal roots were severed from the spinal cord at the site of implantation in two animals destined for electron microscopical analysis, in order to facilitate the determination of the origin of axons within the grafts. The muscle layer was sutured and the skin clipped. All rats had 1 mg of Finadyne (supplied by Schering-Plough Animal Health, UK) and Amoxycillin (Smith Kline Beecham, UK) administered subcutaneously.
HISTOLOGICAL ASSESSMENT

Following terminal anaesthesia with halothane and intraperitoneal pentobarbitone sodium 0.5-1 ml (Sagital, Rhone-Merieux, UK), the heart was exposed, the right atrium was opened and a perfusion cannula was inserted into the left ventricle. Animals destined for transganglionic labelling studies were perfused with 0.1M phosphate buffer (pH 7.4), followed by 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1M phosphate buffer (pH 7.4). The spinal cord was dissected out, with the level of the graft being determined with reference to the entry of nerve roots from the sciatic nerve. Tissues were stored overnight at 4°C in phosphate buffer containing 30% sucrose. In the case of animals selected for electron microscopy the fixative was 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1M Millonig's buffered phosphate. The spinal cord was dissected out as described above.

The spinal cord, at the level of the implant, was removed and stored in fixative overnight at 4°C. The spinal cord was then placed in a mould and covered with a quick embedding gelatin-albumen solution, for cutting. Transverse sections were cut on a vibrotome (200 μm), osmicated and block stained in 2% uranyl acetate, before being processed into Araldite resin. The whole spinal cord section was mounted on a block and semi-thin (0.5 μm) sections were cut on an Ultracut E microtome. Semi-thin sections were stained with Toluidine blue and the area containing the graft was identified. The block was then trimmed down and ultra-thin sections were cut and collected on copper grids, stained with lead citrate and then examined in a Jeol 1010 electron microscope.

Three days prior to perfusion, anesthetized animals were injected in the sciatic nerve with 0.02mg of cholera toxin sub-unit B conjugated to horseradish peroxidase (List Biological Laboratories, USA) dissolved in 1μl of water. Following perfusion specimens of spinal cord were slowly frozen in OCT (Miles Inc, USA) and 40μm thick parasagittal longitudinal sections of the cord were cut directly onto gelatin-subbed slides using a cryostat. After allowing the sections to dry in air they were
reacted to visualize the transganglionic label. 0.3g sodium nitroferricyanide was dissolved in 280ml of distilled water and 15mls of sodium acetate buffer (pH 3.3). 0.015g of 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma, UK) was dissolved in 7.5mls of absolute alcohol. The two solutions were then mixed in the ratio 1:39 (TMB: sodium nitroferricyanide). Sections were immersed in this mixture at 4°C for a period of 20 minutes following which 0.01% hydrogen peroxide was added in a ratio of 1 part in 25. After a further five minutes this mixture was replaced by new solution containing all three reagents in the same relative quantities and this process was repeated at five minute intervals over the course of a 30 minute reaction period. At the end of this time the sections were rinsed several times in sodium acetate buffer, and then air dried, dehydrated and coverslipped. Labelled fibres were visualized using dark field microscopy.

RESULTS

TRANSGANGLIONIC LABELLING
All grafts had survived at 2 weeks after implantation (n=4). When examined using dark field microscopy, large numbers of labelled fibres could be identified within the dorsal columns. These fibres were relatively fine with punctate labelling but many became thickened where they abutted the edge of the graft. Labelled dorsal column axons could be traced into the grafts: some of these resembled the fine diameter dorsal column axons but other labelled structures were much thicker and may have included bundles of axons (Figures 3-1 to 3-3). The number of transganglionically labelled axons in the grafts was always much smaller than that present in the dorsal columns.
ELECTRON MICROSCOPY

Living grafts were found in all animals examined. All grafts were found entirely within the spinal cord, always in contact with grey matter and abutting the dorsal and sometimes the lateral white columns (Figures 3-4 and 3-5). Five grafts were examined in detail (3 weeks survival, n=2; 6 weeks survival, n=3). There were no obvious differences between the three and six week grafts. The grafts contained much smooth muscle, enteric ganglia, fibroblasts and collagen fibrils. There was little evidence of necrosis within the grafts. The ganglia in the grafted tissue were morphologically similar to those observed in the gut, although there were some holes within the ganglionic neuropil. Clearly damaged CNS tissue, containing enlarged extracellular spaces, abnormal axons and some debris-laden microglia was present at the interface with the graft, but deeper into the spinal cord the CNS tissue was relatively normal.

Axons (both myelinated and unmyelinated) were observed within all the grafts (Figure 3-6). Myelinated axons were found throughout the grafts, not just in the periphery. Many axons were myelinated by cells with the morphological features of Schwann cells (including the presence of distinct basal lamina and mesaxon, the glial cell bodies always being closely associated with the ensheathed axon). Oligodendrocyte-myelinated axons were not found in the grafts. The axons usually appeared to be grouped together, between large "bundles" of closely packed smooth muscle. They were often partially or completely surrounded by flattened fibroblast processes, which appeared to be forming perineural compartments separating the axons from the rest of the graft (Figure 3-6). Myelinated axons were identified close to (within 2μm of) myenteric neurons in the grafts. There were also examples of large-diameter unmyelinated axons within the grafts (Figure 3-7), and many small axons (Figures 3-6 and 3-7), some of which contained transmitter vesicles. In the two animals in which the dorsal roots of the segments immediately adjacent to the level of the graft were surgically removed, myelinated axons were observed throughout the
grafts and there was no apparent reduction in the number of axons apparently myelinated by Schwann cells compared to grafts in animals with intact dorsal roots.

The interface observed between the grafts and the spinal cord varied in appearance. In some areas, there was a thin discrete glia limitans, covered by basal lamina, delineating the graft (Figure 3-8). This was particularly the case where the grafts abutted the grey matter. Other areas were gliotic (Figure 3-9), being dominated by hypertrophic astrocyte processes, and in the injured dorsal columns it was difficult to define a glia limitans (not shown). Axons of different sizes were found near the interface, but small unmyelinated axons were only observed in small groups (Figure 3-8) and were most common where the grafts abutted the grey matter.

In all grafted animals, including those in which the adjacent dorsal roots had been extirpated, Schwann cell myelinated axons were observed between the graft and the spinal cord tissue (in large numbers in some cases). The composition of these regions resembled peripheral nerve tissue (Figure 3-10). In suitable sections this tissue could be seen to extend from the dorsal surface of the cord to the graft, apparently occupying the lesion tract made during implantation. In other areas smooth muscle cells could be seen apparently migrating away from the compacted graft usually loosely associated with blood vessels (Figure 3-11).
DISCUSSION

The findings presented here demonstrate that grafts of myenteric plexus and smooth muscle of the muscularis externa of the intestine can survive within the spinal cord and are invaded by axons which are morphologically distinct from those normally found in the enteric plexuses. Some of the invading axons originated from the surrounding CNS tissue. In contrast to previous studies with similar muscularis externa grafts implanted into the corpus striatum (Tew et al. 1992), large bundles of small unmyelinated axonal sprouts were not observed surrounding grafts in the spinal cord. This indicates that graft implantation produces less sprouting in the spinal cord than it does in the striatum, and/or that regenerating CNS axons in the spinal cord have a different morphology compared with those in the striatum and were therefore not recognized. Although the grafts remained mainly as a compacted mass, some smooth muscle cells appeared to migrate into the spinal cord tissue in association with blood vessels.

The results of the tracing study demonstrate that the central processes of primary sensory neurons (found within the dorsal columns) do grow into the graft from the CNS tissue, although the extent of this innervation, detected by transganglionic labelling, was variable, difficult to quantify and appears to be less than has been reported for other types of grafts (Tuszynski et al. 1996) (Tuszynski et al. 1994). A number of different types of axon were found within these grafts. There were examples of small unmyelinated axons, larger unmyelinated and myelinated axons. Which of these individual axons, detected by electron microscopy, represented axons which regenerated into the grafts from the dorsal columns was not determined, but it is likely that they included some of the larger axons, since dorsal column axons are predominantly myelinated. Since the processes of enteric neurons are not myelinated, the myelinated axons within the grafts probably originated from neurons outside the grafted tissue. Some of these axons may have come directly from dorsal roots passing over or entering the cord at the level of the grafts. Although there was no obvious
reduction in the number of myelinated axons observed in grafts where the nearby
dorsal roots had been removed, Schwann cell myelinated axons were also seen at the
graft/spinal cord interface in these animals. It is possible that some axons from the
severed dorsal roots may have reached the grafted region even in these animals.

In all the grafts examined, axons were myelinated by cells that had the morphological
features of Schwann cells; myelinating cells with the morphological characteristics of
oligodendrocytes were not observed. Schwann cells have been reported to perform
the myelinating role in other grafts in spinal cord (Tuszynski et al. 1996) (Tuszynski
et al. 1994). The origin of the Schwann cells in the present study is unclear. They
could have invaded from the periphery via the dorsal roots after disruption of the glial
limitans (Franklin and Blakemore, 1993). It is also possible that Schwann cells are
resident within the muscularis externa (they may be associated with the extrinsic
innervation of the enteric nervous system). The observation that no oligodendrocyte-
myelinated axons were observed within the grafts is in contrast to observations on
similar grafts in the brain (Tew et al. 1992) and in contrast to observations on
peripheral nerve grafts in the brain (Campbell et al. 1992), both of which are
penetrated by oligodendrocyte processes. We have no explanation for this difference
except that it may reflect differences between spinal and cranial oligodendrocytes.

The observation that no large bundles of axonal sprouts were present around the
muscularis externa grafts in the spinal cord is important because of the contrast with
the effects of similar grafts implanted into the corpus striatum (Tew et al. 1992). In
the latter case large fascicles of small-diameter axonal profiles were found at the
graft/brain interface, and some of these were shown to extend into the grafts. Similar
bundles of axonal sprouts have been observed around peripheral nerve grafts in the
thalamus (Campbell et al. 1992) and corpus striatum (unpublished observations) of
adult rats. In the present study, small clusters of small-diameter putative axonal
sprouts were seen around the grafts, particularly at the interface with the grey matter
of the dorsal horn, but they were not sufficiently different from structures normally
present in the spinal cord to be identified confidently as regenerating axons. The
difference in the effects of muscularis extema grafts in the striatum compared with
the spinal cord raises the possibility that axons in the spinal cord are less able to
initiate a regenerative response to the combination of axotomy and muscularis
externa-derived factors. This could be the result of a lack of intrinsic determinants of
axonal regeneration responsive to axotomy and muscularis extema, or the presence of
inhibitory influences. There is increasing evidence that different populations of CNS
neurons vary enormously in their capacity to regenerate their axons (Morrow et al.
1993) (Vaudano et al. 1993) (Woolhead et al. 1998) (Benfey et al. 1985) and indeed
the spinal cord and cerebrum exhibit a differential response following grafting of
genetically modified fibroblasts secreting NT-3, which induce sprouting of host
neurites only within the spinal cord (Senut et al. 1995). It therefore seems likely that
in the present study the injured axons in the spinal cord respond to different
influences, perhaps NT-3 (Senut et al. 1995) (McMahon et al. 1994).

In the case of ascending dorsal column axons, the left sciatic nerve crush should have
initiated a strong regenerative response in the injured neurons (Chong et al. 1996)
(Richardson and Verge, 1987). It is also interesting to speculate that axonal growth
inhibitory molecules may be more concentrated in the spinal cord than in the brain.
For example, tenascin-C, an extracellular matrix molecule with powerful inhibitory
effects on neurite outgrowth under some circumstances, is expressed at injury sites in
the spinal cord (Zhang et al. 1997), but there are no published studies of its
expression around injury sites in the striatum. Alternatively, axonal sprouts produced
in response to graft implantation in the spinal cord may have been difficult to
recognize. It is possible, for example, that some of the large axonal profiles around
the grafts were axonal sprouts, and it seems reasonable to suggest that the
regenerating dorsal column axons, being the central processes of primary afferent
neurons, might resemble regenerating axons in peripheral nerves. None the less, the
number of such axons around the grafts could not have been great and must have
been much lower than that found around grafts in the brain.
Figure 3-1

Transganglionic-labelled dorsal column axons at the boundaries of, and within, (arrows) a 2 week muscularis externa graft (Gr; boundaries indicated by dotted line) in the spinal cord. Labelled axons within the graft form bundles and appear to branch. (Scale bar = 60 μm)
Figure 3-2

Transganglionic-labelled dorsal column axons rostral (r) and caudal (c) to a 2 week muscularis externa graft (Gr). Some labelled axons are seen within the graft (arrows). (Scale bar = 100μm)
Figure 3-3

Transganglionic-labelled dorsal column axons (arrows) within a 2 week muscularis externa graft (Gr; boundaries indicated by dotted line) in the spinal cord (sc). (Scale bar =30μm)
Figure 3-4
Camera Lucida drawing showing the position of a muscularis externa graft 6 weeks after grafting in the spinal cord. The graft is entirely within the spinal cord, and impinges on different areas of the spinal cord. (dc-dorsal column, idc-injured dorsal column, lwc- lateral white column, gr-graft, p-peripheral nerve-like tissue, dh-dorsal horn, vh-ventral horn) (Scale bar = 1 mm)
Figure 3-5

Toluidene blue stained section of a muscularis externa graft within the spinal cord, 6 weeks after grafting. (dc-dorsal column, gr-graft, p-peripheral nerve-like tissue, dh-dorsal horn) (Scale bar = 200 μm)
Figure 3-6

Electron micrograph of muscularis externa graft examined 6 weeks after implantation into the spinal cord. Myelinated (asterisk) and unmyelinated axons can be seen, loosely enveloped by fibroblast processes (fb). The size and extent of glial ensheathment of unmyelinated axons varies greatly. The other graft constituents, smooth muscle cells (m), fibroblasts (fb) and collagen fibrils can also be seen. (Scale bar = 500 nm)
Unmyelinated axons within a muscularis externa graft 6 weeks after implantation. Large diameter axons (arrows) and small diameter axons (arrowheads) are present in a compact bundle and are associated with what appears to be enteric glial cell cytoplasm (g). Smooth muscle cells (m) surround the nerve bundle.

(Scale bar =1 μm)
Figure 3-8

Part of the interface between a muscularis externa graft and the lateral white column of the spinal cord, showing a thin astrocytic glia limitans covered by basal lamina (arrowhead). Astrocyte processes protrude into the junctional zone, associated with a myelinated axon (a). Two Schwann cell myelinated axons (asterisks) are present at the interface, and a few small diameter axons (arrows) are present amongst the oligodendrocyte-myelinated CNS axons. (C-collagen fibrils in the expanded extracellular space around the graft) (Scale bar =1 μm)
This region of the interface between the graft and the spinal cord is characterised by an expanded extracellular space containing collagen fibrils (C), abutting a thick astrocytic glia limitans (arrowheads). There is no sign of significant axonal sprouting in this region. (Scale bar = 1 μm)
Figure 3-10

Electron micrograph showing tissue resembling peripheral nerve found dorsal to the muscularis externa graft in the position indicated in Figure 3-4. Several Schwann cell myelinated axons are present within a compartment surrounded by fibroblast processes (arrowheads). Astrocyte processes are present amongst the axons and in some cases form mixed bundles with Schwann cells and/or axons (arrows). (Scale bar =2 μm)
Smooth muscle cells (M) loosely associated with a capillary (C) extending into the lateral white column form a 6 week muscularis externa graft. An astrocytic basal lamina (arrowhead) separates the muscle cells from the axons in the spinal cord. (Scale bar = 2 μm)
CHAPTER 4

Implantation of muscularis externa using a new model of severe spinal cord injury: Functional and light microscopy studies

ABSTRACT

INTRODUCTION

MATERIALS AND METHODS

RESULTS

DISCUSSION
ABSTRACT

The effects of muscularis externa grafts on secondary spinal cord injury were investigated. A large lesion was made in the spinal cord by removing the dorsal columns using biopsy forceps guided by a stereotactic frame. This allowed the easy insertion of a muscularis externa graft into the resulting defect. An assessment of hindlimb function was made in the post-operative period and the spinal cord was examined histologically after 2-8 weeks.

Large lesions were present in all spinal cords examined. There was no significant difference in the median lesion size (length x depth) between grafted animals (5.9 mm²) and controls (6.2mm²). However, there was considerable variation in lesion size between individual animals, even when they received the same treatment (interquartile range: grafted animals = 4.4 to 9.9mm²; controls = 5.3 to 8.2mm²). A separate series of experiments in which the same injury technique was applied to fixed post-mortem spinal cords, was associated with a lesser degree of variation and significantly smaller lesions (median- 2.1mm²), suggesting that the injured spinal cord in living animals suffers considerable secondary injury with this technique. Few regenerating fibres were seen within the lesion site in grafted animals and none in controls. Hindlimb function showed considerable variation but by 3 weeks all animals were essentially fully recovered, making it difficult to compare treated and control animals.

There was no clear evidence that muscularis externa reduced secondary injury. However, the limitations of this new model of spinal cord injury did not allow a definitive assessment of the effects of muscularis externa on the severely injured spinal cord to be made.
INTRODUCTION

METHODS OF SPINAL CORD INJURY
A wide variety of animal models have been developed to study spinal cord injury, each with different characteristics. Thus, depending on the question being investigated or the species being used, different techniques may be more or less appropriate. Each model involves injury techniques that may result in neuronal cell death or the transection of axons within the long tracts of the white matter, with functional disconnection across the lesion. Damage is produced by a combination of primary and secondary injury (See Chapter I) and different injury techniques produce primary and secondary injury in different proportions. Techniques that produce mild injuries may not result in any measurable functional deficits, while more severe injury may produce paraplegia, which may be complete or incomplete, permanent or transient. Transient paraplegia results from sub-threshold injury to some neurons, which causes a loss of function, but not cell death or axonal transection. As these injury processes subside there is a recovery of neuronal function (Das, 1989a). In addition, the CNS may exhibit plasticity, with undamaged pathways compensating for those that have been lost. In models of spinal cord injury using rats there is often spontaneous recovery within 1-2 weeks, which is usually complete, or close to complete. Any residual deficits are due in part, to injuries to the musculoskeletal system, rather than neurological damage.

The large variety of methods for experimental spinal cord injury bears witness to the fact that there is no ideal technique. The following list represents a simple classification of spinal cord injury techniques:

- Dynamic eg weight drop
- Static eg placement of weights on the cord, forceps crush, aneurysm clip, inflatable balloon
- Incision eg knife, needle
- Laceration eg scissors
- Others eg thermal or photo-coagulation injuries

Weight drop and static crush injuries have the advantage of producing significant secondary injury, thus reproducing some aspects of clinical injury. Furthermore, since the meningeal membranes are not cut, the injury site is not penetrated by external tissues, such as muscle, with the result that the analysis of pathological processes is simpler (Das, 1989a). These techniques do not, however, produce reliable axonal transection, unless very severe, and therefore may make the study of axonal regeneration difficult.

Many variations on the weight-drop theme have been described (Blight and DeCrescito, 1986). In this type of model a defined weight is dropped from a specific distance, established for a particular set of experiments. A consistent amount of energy is thus imparted to the spinal cord tissues allowing quantitative analysis (Das, 1989a). There are, however, problems with this approach (Blight, 1988). The exact position and angle at which the weights impact on the cord may vary, and the spinal cord has to be stabilized so that movement of the flexible underlying bony spine and thorax does not dissipate the energy of impact (Dohrmann et al. 1978). Although solutions to these problems involving stabilization of these structures have been proposed (Blight and DeCrescito, 1986) (Ford, 1983), some variation in the degree of neurological injury between animals still occurs due to differences in size and biomechanics of individual spinal cords. These problems have led to the development of more elaborate devices that allow tissue impact with independent control of compression and contact velocity (Anderson, 1982) (Somerson and Stokes, 1987). Although relatively reliable, there is still some variation in injury with cord size, and the complexity of the systems makes them difficult to use in a general laboratory.
Because of the problems of dynamic techniques, an alternative approach has been developed employing static compression of the spinal cord. The degree of compression can be defined by the force applied or by the thickness to which the cord is reduced, and this, together with the duration of the insult, determines the severity of the injury. Reproducibility is limited by the same types of problems that affect weight-drop techniques, although inter-operative problems are more easily detected since the compression lasts longer and can be carried out under more direct vision. A variety of methods have been developed to compress the cord, but there is limited experimental evidence to allow direct comparison of the reliability of the different methods, which include:

- Inflation of a cuff surrounding the cord (Tator and Deecke, 1973)
- Compression with a balloon positioned over the dorsal surface of the cord (Martin and Bloedel, 1973) (Hansebout et al. 1981)
- Compression between the blades of an aneurysm clip, with a force determined by the choice of clip (Tator et al. 1984).
- Compression to a set thickness, between the tips of specially designed forceps (Blight, 1991)

These static crush methods involve prolonged spinal cord compression that may superimpose ischaemia on the initial contusion injury (Black et al. 1988). In comparison the sudden impact of a weight-drop injury is more like the instantaneous trauma associated with most human spinal cord injuries. Furthermore there is some evidence that weight-drop techniques may produce a more uniform pattern of injury through the cord than some static techniques that produce pathological changes mainly in the dorsal part of the cord (Black et al. 1988).
Researchers studying regeneration have often used a simple stab wound (incision) in which the spinal cord is simply cut with a sharp instrument such as a needle. This type of injury is relatively simple to perform and the lesion can be checked under direct vision (Das, 1989a). It is associated with clear primary axonal injury, and relatively less secondary injury in comparison with crush models. Stab wounds produce lesions that are anatomically discrete, while crush models are associated with diffuse injury throughout the thickness of the cord, with intermingling of intact and severed axons. Stab wounds are therefore a useful model for the study of axonal injury since, in theory, regenerating axons can be readily distinguished from spared ones although, as discussed in Chapter 2, this distinction is not always easy to make. The relative paucity of secondary injury associated with stab wounds makes it a poorer model for studying the effects of possible neuroprotective therapies. Laceration injuries involve more severe cutting injuries of the spinal cord, usually using microscissors, and result in some tissue loss. This type of lesion produces both discrete primary injury, as the long tract fibres are transected and secondary injury and thus combines some of the advantages of both crush and stab wound techniques (Das, 1989a).

A NEW MODEL OF SPINAL CORD INJURY

In this study an attempt was made to devise an animal model which involved both primary and secondary injury, produced lesions of consistent severity and also allowed graft material to be inserted directly, and atraumatically, into the lesion site. The outcome was assessed histologically to determine any effects of graft insertion on lesion size and axonal morphology of dorsal root ganglion sensory neurons at the spinal cord injury site. A final aim was to produce a lesion that was not severe enough to result in dangerous complications of spinal cord trauma, such as urinary retention, hypothermia and autophagia (Das et al. 1989b), while at the same time producing sufficient injury to result in measurable neurological deficits.
The rat has been extensively investigated in previous spinal cord injury research. Fischer rats were therefore chosen for these experiments to facilitate comparison with other studies, and to allow the application of species-specific molecular and histological techniques. Furthermore the rat is hardy and inexpensive. Crucially, unlike guinea pigs, inbred strains, such as Fischers, are readily available, permitting the transplantation of tissues between animals without causing immune rejection.

Simple insertion of a graft into the spinal cord will usually produce a relatively small, though variable, degree of damage (Das, 1989a), and while this type of model is useful in studying axonal regeneration, it differs significantly from clinical spinal cord injuries. Unfortunately, animal models with injuries, such as crush or weight-drop, which produce significant secondary injury, and therefore more closely reproduce clinical damage to the spinal cord, do not allow for the insertion of a graft at the injury site. This would require a second injury during the act of implantation, thus increasing the degree of inter-operative variation. A laceration model was therefore felt to be the most appropriate model. It was considered that the use of microscissors to remove tissue would be unlikely to produce sufficient consistency. A small sharp hook was tried but this too produced highly variable injury due to tearing of spinal cord tissue, even when attached to a stereotactic frame so that it could be positioned to a standard depth.

Biopsy forceps (Decker microrongeur) were therefore chosen with the aim of removing a consistent ‘bite’ from the dorsal cord, thus producing a combination of primary injury, as axons are transected and secondary injury resulting from the laceration. It was expected that secondary injury would also be produced by compression of the cord as the blunt tips of the biopsy forceps were lowered, and that a cavity would therefore result into which the graft could be atraumatically inserted. In order to standardize the lesion, the forceps were attached to a stereotactic frame and each bite was made to a standard width and depth (as determined respectively by the aperture of the jaws of the forceps and the depth to which they were inserted into
the spinal cord). The lesion depth and duration of the injury were chosen to produce significant tissue damage and functional deficit without producing cord transection.

Tests of spinal cord function were carried out in the post-operative period, with the aim of following the course of functional deficits resulting from spinal cord injury. It was expected that the tests would demonstrate an immediate partial loss of function followed by a further deterioration in the first few days and finally a prolonged recovery, corresponding respectively to the underlying pathological processes of primary and secondary injury and recovery. Specific tests of hindlimb function were chosen to be reproducible and simple, without the need for special equipment as is required for the inclined plane test (Rivlin and Tator, 1977) or cutaneous trunci muscle reflex test (Blight, 1991). Functional tests based on a modified Tarlov scale have been described that fulfill these criteria following a crush model of spinal cord injury (Blight, 1991), and in this study two sets of these tests were used. The ‘toe-spread’ response involves a vertical movement of the whole animal, which will elicit a characteristic hindlimb toe-spreading response related to the free-fall reflex of cats. This behaviour depends on signals from the brain, which pass via the lumbar spinal cord and indicate the integrity of descending spinal pathways. The results can be scored on a scale of 0 (no reflex) to 4 (normal) as described below. Evaluation of hindlimb motor control was performed using a simple set of tests and observations (see below) and could again be scored on a scale of 0-4. These tests of hindlimb function evaluated both descending motor control (weight support and locomotion) and segmental function (proprioceptive placing and withdrawal) (Blight, 1991).

In order to assess the consistency of the surgical aspects of this injury technique a further series of experiments was performed. The same injury technique was applied to postmortem spinal cords that had already been perfused with fixative, thus eliminating a number of variables associated with living tissue. These experiments allowed evaluation of the extent of the initial injury.
MUSCULARIS EXTERNA AND LARGE SPINAL CORD INJURY

Muscularis externa grafts in the spinal cord appear to have a weaker effect on axonal regeneration in the spinal cord than elsewhere in the CNS (see Chapter 3). However, there remains the possibility that muscularis externa could reduce the degree of secondary injury, which is critical in clinical spinal cord injury. Thus components of muscularis externa may produce biologically active molecules (Hopker et al. 1994). It is possible that such molecules, which may include neurotrophins, could reduce neuronal cell death. Moreover, there is some evidence that the presence of muscularis externa grafts reduces cavitation at the site of spinal cord injury (Jaeger et al. 1993).

In order to test the hypothesis that muscularis externa might reduce secondary injury a series of controlled experiments were performed using the model of severe spinal cord injury described above. Graft survival, neuronal sprouting in the vicinity of the graft, secondary injury using a histological measure of lesion size and functional outcome were assessed.

MATERIALS AND METHODS

SURGICAL METHODS

Inbred adult male Fischer rats (200 to 400g) (supplied by Harlan-Olac, UK) were used. Donor rats were killed using halothane and small portions of distal ileum were removed. Intestinal contents were removed and the muscularis externa peeled away from the underlying layers. This was then divided into pieces of approximately 2mm² which were washed four times in Hanks Balanced Salt Solution (GIBCOBRL,UK) containing antibiotics and anti-mycotics. Freeze-killed grafts were prepared by placing the tissue on aluminum foil that was then plunged into liquid nitrogen. Freezing and thawing were repeated four times.
Recipient rats were deeply anaesthetized with an inhaled mixture of 1.5% halothane (ICI, UK), 3% nitrous oxide and 1.5% oxygen. The animals were appropriately shaved and cleaned with antiseptic solution. An incision was made along the back of the anaesthetized recipient rats. A laminectomy was performed at the T8-T9 segment with a wide clearance of bone. The dura was exposed and then widely opened (by first making a hole with a needle, then enlarging it with microsurgical scissors). To reduce respiratory movements of the spinal column the dorsal spines of vertebrae immediately rostral and caudal to the lesion were clamped and fixed to a rigid frame.

A Decker microrongeur (2 x 5mm straight cup) (Johnson and Johnson, MA, USA) (Figure 4-1) was attached to a modified Kopf small animal stereotactic frame (Figure 4-2). The jaws were opened to a separation 1mm and aligned longitudinally in relation to the spinal cord. The microrongeur was then lowered towards the exposed spinal cord so that the separated tips just touched the surface on either side of the midline (Figure 4-3). The device was then lowered 2mm and the jaws closed firmly. After 1 minute the microrongeur was withdrawn. This produced a concave cavity on the dorsal aspect of the cord. After haemostasis had been obtained the wound was either simply closed or had a muscularis externa graft positioned in the cavity prior to closure. The muscle layer was sutured and the skin clipped. All rats had 1 mg of Finadyne (Schering-Plough Animal Health, UK) and Amoxycillin (Smith Kline Beecham, UK) administered subcutaneously.

A total of 30 animals were assigned to the following groups:

- Fresh ME grafts (n=12), of which 10 were assessed histologically at 2 to 8 weeks after injury, and 6 had functional tests performed.
- Controls (n=18), comprising 2 animals with freeze-killed grafts and the remaining 16 animals with no graft material implanted. 13 were assessed histologically at 2 to 8 weeks after injury, and 10 were assessed for functional outcome.
HISTOLOGICAL ASSESSMENT

Three days prior to perfusion, animals were re-anaesthetized and 0.02mg of cholera toxin sub-unit B conjugated to horseradish peroxidase (List Biological Laboratories, USA) dissolved in 1μl of water was injected into the sciatic nerve exposed in the thigh. Following terminal anaesthesia with halothane and intraperitoneal pentobarbitone sodium 0.5-1ml (Sagital, Rhone-Merieux, UK), the heart was exposed. The right atrium was incised and a needle inserted into the left ventricle. Animals were perfused with 500ml of 0.1M phosphate buffer at room temperature, followed by 500ml of 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1M phosphate buffer also at room temperature. The spinal cord was dissected out and the level was determined with reference to the entry of nerve roots from the sciatic nerve. Tissues were stored overnight at 4°C in 30% sucrose in 0.1M phosphate buffer.

Following perfusion, specimens of spinal cord were slowly frozen in OCT (Miles Inc, USA) and 40μm thick parasagittal longitudinal sections of the cord were cut directly onto gelatin-subbed slides using a cryostat. After allowing the sections to dry in air they were reacted to visualize the transganglionic label. Briefly, 0.3g sodium nitroferricyanide was dissolved in 280ml of distilled water and 15mls of sodium acetate buffer (pH 3.3). 0.015g of 3,3',5,5'-tetramethylbenzidine (TMB)(Sigma, UK) was dissolved in 7.5mls of absolute alcohol. The two solutions were then mixed in the ratio 1:39 (TMB: sodium nitroferricyanide). Sections were immersed in this mixture at 4°C for a period of 20 minutes, following which 0.01% hydrogen peroxide was added in a ratio of 1 part in 25. After a further five minutes this mixture was replaced by a new solution containing all three reagents in the same relative quantities and this process was repeated at five minute intervals over the course of a 30 minute reaction period. At the end of this time the sections were rinsed several times in sodium acetate buffer, and then air dried, dehydrated and coverslipped. Labelled fibres were visualized using dark field microscopy.
Camera lucida drawings were made of the lesion site at the level of its maximum overall size. A drawing of a gratacule scale at the same magnification was also made. This allowed the calculation of the maximum longitudinal (rostro-caudal) extent and the maximum depth of the lesion. The product of these two figures was calculated to give an indicator of overall lesion size.

**MEASUREMENT OF LESION SIZE IN POSTMORTEM SPINAL CORD**

Four Fischer rats (Harlan Olac, UK) were overdosed with halothane and intraperitoneal pentobarbitone and perfused with fixative as described above. The lower thoracic spinal cord was widely exposed by dissection including opening of the dura. Four adjacent lesions were then made in the spinal cord with a Decker microrongeur using the methodology described above. This portion of spinal cord was removed and immersed overnight in 30% buffered-sucrose. The specimen was frozen in OCT (Miles Inc, USA) and 50μm horizontal sections through the lesion, were cut directly onto gelatin-subbed slides. The sections were allowed to dry overnight in air and then immersed for 30 seconds in thionin solution. They were then dehydrated through alcohols and coverslipped. The depth of each lesion was estimated by counting the number of consecutive sections in which each lesion was visible, while the maximum longitudinal extent was calculated using the camera lucida technique described above.

**FUNCTIONAL ASSESSMENT**

Animals were periodically assessed for neurological function in the weeks following surgery. Motor tests (i.e. ‘toe spread response’ and assessment of hindlimb motor control) were performed prior to surgery, approximately 3 hours after surgery (day 1), 2 days later (day 3), at 1 week after surgery and weekly thereafter. The results of the tests were given numerical values according to the level of neurological function
(Blight, 1991). The addition of the scores for toe-spread response and hindlimb motor control gave an overall score of 0-8.

**Toe-Spread Response:** The rat was held suspended by hand from the back and moved briskly downwards for a few inches, while observing movements of the hindlimb. The response for each leg was graded independently on a scale of 0 (no detectable response) to 2 (relatively normal with the hindlimb digits being spread wide apart for several seconds). Mild injuries led to the reflex being present but reduced in strength and this was graded as 1. The scores of both legs were combined on a scale of 0-4.

**Hindlimb Motor Control:** The animals were observed on an open table top to evaluate their ability to support weight and use their hind limbs in walking. With the animals lightly restrained the reflex response to gently extending each of the hindlimbs was recorded. A normal animal briskly replaced the extended limb below the body. The response of the animals to pinching the toes of an extended hindlimb with the fingers was also examined; with a normal animal briskly withdrawing the limb in a typical flexion reflex. The results of these tests were translated into a four point cumulative scale, see Table 4-1. (Note: the initial score is 0-12, but by dividing by 3, a value on the scale 0-4 is obtained for comparison with the other assessment scales).
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Right leg</th>
<th>Left Leg</th>
<th>Total Possible Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal Placing</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Weak Placing</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>No Response</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pinch Test</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal Withdrawal</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Weak Withdrawal</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>No Response</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Observation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well co-ordinated walking</td>
<td>--</td>
<td>--</td>
<td>4</td>
</tr>
<tr>
<td>Weight support and limited walking</td>
<td>--</td>
<td>--</td>
<td>2</td>
</tr>
<tr>
<td>No weight bearing</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Overall score</td>
<td></td>
<td></td>
<td>0-12</td>
</tr>
</tbody>
</table>

**Table 4-1**

Scoring system for hindlimb motor control tests: each limb is assessed independently and the two scores are added to give an overall value of 0-12 which is then divided by 3 to give a score of 0-4.

**STATISTICAL ANALYSIS**

Non-parametric statistics were used (though the mean and standard deviation are also quoted simply to aid comparison with other studies). Thus for each data set the median and interquartile ranges were calculated and in comparing different groups of values the Mann-Whitney test was used.
RESULTS

HISTOLOGICAL ASSESSMENT

Large numbers of labelled axons could be seen within the caudal dorsal columns (Figure 4-4) and traced to the margins of the lesion zone, often becoming thicker as they abutted the lesion. Although a small number of axons could be seen entering the lesion zone (Figure 4-5) they were even sparser than those in animals in which grafts had been inserted into areas of mild injury (Chapter 3). No regenerating axons were seen within the lesion site, or freeze-killed grafts, among control animals (Figure 4-6), with axons terminating well caudal to the lesion (Figure 4-4).

In both grafted and control animals examined with dark-field microscopy, major tissue damage and cavitation could be seen around the lesion site. The residual cord in this area was often very thin with the lesion extending down to the ventral white matter. There was usually a very large cavity at the lesion site itself, with numerous macrophages within the lesion and in the surrounding tissues (Figure 4-7). No clear histological difference between the animals examined at different survival times was seen. Where muscularis externa grafts had been implanted they were often difficult to recognize among the scar tissue at the injury site (Figure 4-5), in contrast with grafts implanted into sites of mild injury that were usually readily identified (Chapter 3). This may have resulted from tissue damage by the pathological processes associated with secondary injury and / or processing artefact.

Among the control animals the maximum depth of lesions varied from 1.0 to 2.1mm, while the length varied from 2.7 to 8.5mm. The overall indicator of lesion size (length x depth) varied from 3.2 to 12.6 mm² (median: 6.2; interquartile range: 5.3 to 8.2 mm²) (Table 4-2). Among the grafted animals the maximum depth of lesions varied from 0.9 to 1.6mm, while the length varied from 3.5 to 8.7mm. The overall indicator of lesion size (length x depth) varied from 4.0 to 11.3 mm² (median: 5.9;
interquartile range: 4.4 to 9.9mm$^2$) (Table 4-3). The results indicate that lesion size in experimental and control groups is very similar, with a relatively wide range of values. The small differences between the two groups does not reach statistical significance (Mann-Whitney test, p=0.69). There was no clear trend in the lesion size for either grafted animals or controls at different survival times.

**SIZE OF MULTIPLE LESIONS IN POST-MORTEM SPINAL CORD**
Lesions could be clearly seen in thionin -stained tissue and followed down through consecutive sections (Figure 4-8). The maximum depth of lesions varied from 0.9 to 1.6mm, while the length varied from 1.2 to 2.8mm. The overall indicator of lesion size (length x depth) varied from 1.3 to 3.1 mm$^2$ (median: 2.1; interquartile range: 1.8 to 2.4mm$^2$) (Table 4-4). The values for overall lesion size differed significantly from the living control animals (Mann-Whitney Test p<0.05). The rostro-caudal length of lesions in the postmortem spinal cords also differed significantly from the living control animals (Mann-Whitney Test p<0.05), but the lesion depth did not (Mann-Whitney Test p=0.07).
<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Features</th>
<th>Length (mm)</th>
<th>Depth (mm)</th>
<th>Length x Depth (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>Freeze-killed</td>
<td>2.7</td>
<td>1.2</td>
<td>3.2</td>
</tr>
<tr>
<td>8</td>
<td>Freeze-killed</td>
<td>4.7</td>
<td>1.2</td>
<td>5.6</td>
</tr>
<tr>
<td>2</td>
<td>No graft</td>
<td>8.5</td>
<td>1.3</td>
<td>11.1</td>
</tr>
<tr>
<td>2</td>
<td>No graft</td>
<td>4.8</td>
<td>1.3</td>
<td>6.2</td>
</tr>
<tr>
<td>4</td>
<td>No graft</td>
<td>4.1</td>
<td>1.4</td>
<td>5.7</td>
</tr>
<tr>
<td>4</td>
<td>No graft</td>
<td>4.3</td>
<td>1.1</td>
<td>4.7</td>
</tr>
<tr>
<td>8</td>
<td>No graft</td>
<td>4.7</td>
<td>1.3</td>
<td>5.9</td>
</tr>
<tr>
<td>8</td>
<td>No graft</td>
<td>5.2</td>
<td>1.5</td>
<td>7.8</td>
</tr>
<tr>
<td>8</td>
<td>No graft</td>
<td>6.0</td>
<td>2.1</td>
<td>12.6</td>
</tr>
<tr>
<td>8</td>
<td>No graft</td>
<td>7.5</td>
<td>1.0</td>
<td>7.5</td>
</tr>
<tr>
<td>8</td>
<td>No graft</td>
<td>5.0</td>
<td>1.3</td>
<td>6.5</td>
</tr>
<tr>
<td>8</td>
<td>No graft</td>
<td>8.5</td>
<td>1.0</td>
<td>8.5</td>
</tr>
<tr>
<td>8</td>
<td>No graft</td>
<td>4.9</td>
<td>1.1</td>
<td>5.4</td>
</tr>
<tr>
<td><strong>Total Number</strong></td>
<td></td>
<td><strong>13</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Median</strong></td>
<td></td>
<td>4.9</td>
<td>1.3</td>
<td>6.2</td>
</tr>
<tr>
<td><strong>IQR</strong></td>
<td></td>
<td></td>
<td></td>
<td>5.3 : 8.2</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td></td>
<td>5.5</td>
<td>1.3</td>
<td>7.0</td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td></td>
<td>1.7</td>
<td>0.3</td>
<td>2.6</td>
</tr>
</tbody>
</table>

**Table 4-2**

Lesion size (defined by length, depth or length x depth) in 13 control animals (2 of which had freeze-killed grafts inserted). The time at which each animal was killed is also indicated. (Abbreviations: IQR-interquartile range; SD-standard deviation)
<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Features</th>
<th>Length (mm)</th>
<th>Depth (mm)</th>
<th>Length x Depth (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Fresh Graft</td>
<td>4.4</td>
<td>1.1</td>
<td>4.8</td>
</tr>
<tr>
<td>2</td>
<td>Fresh Graft</td>
<td>4.5</td>
<td>1.0</td>
<td>4.5</td>
</tr>
<tr>
<td>4</td>
<td>Fresh Graft</td>
<td>3.3</td>
<td>1.3</td>
<td>4.3</td>
</tr>
<tr>
<td>4</td>
<td>Fresh Graft</td>
<td>4.5</td>
<td>1.3</td>
<td>5.9</td>
</tr>
<tr>
<td>8</td>
<td>Fresh Graft</td>
<td>3.7</td>
<td>1.6</td>
<td>5.9</td>
</tr>
<tr>
<td>8</td>
<td>Fresh Graft</td>
<td>4.4</td>
<td>0.9</td>
<td>4.0</td>
</tr>
<tr>
<td>8</td>
<td>Fresh Graft</td>
<td>5.6</td>
<td>1.3</td>
<td>7.3</td>
</tr>
<tr>
<td>8</td>
<td>Fresh Graft</td>
<td>8.7</td>
<td>1.3</td>
<td>11.3</td>
</tr>
<tr>
<td>8</td>
<td>Fresh Graft</td>
<td>7.5</td>
<td>1.5</td>
<td>11.3</td>
</tr>
<tr>
<td>8</td>
<td>Fresh Graft</td>
<td>6.5</td>
<td>1.3</td>
<td>8.5</td>
</tr>
<tr>
<td><strong>Total Number</strong></td>
<td><strong>10</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Median</strong></td>
<td></td>
<td>4.5</td>
<td>1.3</td>
<td>5.9</td>
</tr>
<tr>
<td><strong>IQR</strong></td>
<td></td>
<td></td>
<td></td>
<td>4.4 : 9.9</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td></td>
<td>5.3</td>
<td>1.3</td>
<td>6.8</td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td></td>
<td>1.7</td>
<td>0.2</td>
<td>2.8</td>
</tr>
</tbody>
</table>

**Table 4-3**

Lesion size (defined by length, depth or length x depth) in 10 animals treated with muscularis externa grafts. The time at which the animal was killed ranged from 2 to 8 weeks. (Abbreviations: IQR-interquartile range; SD-standard deviation)
<table>
<thead>
<tr>
<th>Lesion</th>
<th>Depth (mm)</th>
<th>Length (mm)</th>
<th>Lesion Size (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.3</td>
<td>2.1</td>
<td>2.7</td>
</tr>
<tr>
<td>B</td>
<td>1.6</td>
<td>1.8</td>
<td>2.8</td>
</tr>
<tr>
<td>C</td>
<td>1.2</td>
<td>1.8</td>
<td>2.1</td>
</tr>
<tr>
<td>D</td>
<td>1.0</td>
<td>2.1</td>
<td>2.1</td>
</tr>
<tr>
<td>A</td>
<td>1.0</td>
<td>2.8</td>
<td>2.8</td>
</tr>
<tr>
<td>B</td>
<td>0.9</td>
<td>1.9</td>
<td>1.6</td>
</tr>
<tr>
<td>C</td>
<td>1.1</td>
<td>1.9</td>
<td>2.0</td>
</tr>
<tr>
<td>D</td>
<td>0.9</td>
<td>2.0</td>
<td>1.7</td>
</tr>
<tr>
<td>A</td>
<td>1.1</td>
<td>2.2</td>
<td>2.3</td>
</tr>
<tr>
<td>B</td>
<td>1.5</td>
<td>1.6</td>
<td>2.4</td>
</tr>
<tr>
<td>C</td>
<td>1.0</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>D</td>
<td>1.3</td>
<td>1.8</td>
<td>2.3</td>
</tr>
<tr>
<td>A</td>
<td>0.9</td>
<td>2.2</td>
<td>1.9</td>
</tr>
<tr>
<td>B</td>
<td>1.4</td>
<td>2.3</td>
<td>3.1</td>
</tr>
<tr>
<td>C</td>
<td>1.0</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>D</td>
<td>1.1</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td><strong>Total Number</strong></td>
<td><strong>12</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Median</strong></td>
<td><strong>1.1</strong></td>
<td><strong>1.9</strong></td>
<td><strong>2.1</strong></td>
</tr>
<tr>
<td><strong>IQR</strong></td>
<td></td>
<td></td>
<td><strong>1.8 to 2.4</strong></td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td><strong>1.1</strong></td>
<td><strong>1.9</strong></td>
<td><strong>2.2</strong></td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td><strong>0.2</strong></td>
<td><strong>0.4</strong></td>
<td><strong>0.5</strong></td>
</tr>
</tbody>
</table>

**Table 4-4**

The lesions are grouped in fours (A-D) representing four separate lesions made in the post-mortem spinal cord of each animal. Lesion size is defined by length, depth or length x depth. (Abbreviations: IQR-interquartile range; SD-standard deviation)
FUNCTIONAL ASSESSMENT

Ten control animals were assessed functionally over a period of up to four weeks. (8 had a standardized lesion alone while a further 2 had a lesion and additional insertion of a freeze-killed muscularis externa graft, which was presumed to be biologically inactive). The functional score was measured, as described above, on the day of injury (day 1), 2 days later, at 1 week and weekly thereafter. The results are shown in Table 4-5 and Figure 4-9. Following an initial deterioration immediately after injury (day 1: median 3.0) there was a subsequent fall in the median score by day 3 (median 1.75) followed by a recovery in function which was largely complete by 21 days. It was not possible to distinguish between the results of control animals with freeze-killed grafts and those with no graft. There was a relatively wide range of scores at any given time in the 10 animals studied (Table 4-5). Thus within a few hours of injury the range was 1 to 7.33, while at 1 week it was 0.66 to 7.

6 animals were injured in the same way but had living muscularis externa grafts implanted at the lesion site. The pattern of deterioration between days 1 and 3, followed by practically complete recovery by week 3 was essentially the same as in the control animals (Table 4-6 and Figure 4-10). Moreover, the functional scores at equivalent times after injury were similar. Thus at 1 week the median score was 2.78 (controls: 3.26), with a range of 0.66 to 3.33 (controls: 0.66 to 7). The 2 sets of data did not differ significantly (Mann-Whitney Test).

In summary there was evidence of continuing deterioration in neurological function in the first 2 days after injury followed by near complete recovery at 21 days in both groups, with nothing to suggest that the muscularis externa grafts influenced functional outcome.

There were no premature deaths among the lesioned animals. In a few animals there was some degree of urinary retention for a few days, that was treated by gentle manual pressure until intrinsic bladder activity led to spontaneous micturition.
<table>
<thead>
<tr>
<th>Time</th>
<th>Animal:</th>
<th>Range</th>
<th>Median</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4</td>
<td>5 6 7</td>
<td>8 9 10</td>
<td></td>
</tr>
<tr>
<td>Pre-op</td>
<td>8 8</td>
<td>8 8</td>
<td>8 8</td>
<td>8 8</td>
</tr>
<tr>
<td>Day 1</td>
<td>3.3 7.3</td>
<td>1.3 3.6</td>
<td>1 4</td>
<td>2.6 1</td>
</tr>
<tr>
<td>Day 3</td>
<td>2.6 4</td>
<td>3 2 0.33</td>
<td>0.66 0.66</td>
<td>0.66 0.33 to 4</td>
</tr>
<tr>
<td>1 week</td>
<td>3 7 0.66</td>
<td>5.33 1.66</td>
<td>2.33 1 4.33 3.33 4</td>
<td>0.66 to 7</td>
</tr>
<tr>
<td>2 week</td>
<td>7.6 8 7</td>
<td>7.33 8 7.66</td>
<td>3 7.66 7 8</td>
<td>3 to 8</td>
</tr>
<tr>
<td>3 week</td>
<td>8 8 7.66</td>
<td>8 8 8 7.66</td>
<td>7.66 7.66 7.66 7.66</td>
<td>7.66 to 8</td>
</tr>
<tr>
<td>4 week</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 4-5**

Spinal cord function following injury in 10 control animals. A separate column represents each animal. The range, median and mean are indicated for each time interval at which an assessment was performed.
<table>
<thead>
<tr>
<th>Time</th>
<th>Animal:</th>
<th>Range</th>
<th>Median</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4 5 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-op</td>
<td>8 8 8 8 8 8</td>
<td>n/a</td>
<td>8.00</td>
<td>8.00</td>
</tr>
<tr>
<td>Day 1</td>
<td>6.66 7 1.33 1.33 1.33 to 7</td>
<td>4.00</td>
<td>4.08</td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>2.66 3.33 0 1 0 to 3.33</td>
<td>1.83</td>
<td>1.75</td>
<td></td>
</tr>
<tr>
<td>1 week</td>
<td>2.33 3.33 1.33 6 0.66 3 0.66 to 3.33</td>
<td>2.67</td>
<td>2.78</td>
<td></td>
</tr>
<tr>
<td>2 week</td>
<td>8 7.66 6.66 7.66 5 7.66 5 to 8</td>
<td>7.66</td>
<td>7.11</td>
<td></td>
</tr>
<tr>
<td>3 week</td>
<td>8 8 7.66 8 8 8 7.66 to 8</td>
<td>8.00</td>
<td>7.94</td>
<td></td>
</tr>
<tr>
<td>4 week</td>
<td>8 8 n/a 8</td>
<td>8.00</td>
<td>8.00</td>
<td></td>
</tr>
</tbody>
</table>

**Table 4-6**

Spinal cord function following injury in 6 animals, which received muscularis externa grafts. A separate column represents each animal. The range, median and mean are indicated for each time interval at which an assessment was performed.
DISCUSSION

These experiments indicate that this model of spinal cord injury succeeded in producing significant secondary injury. Thus the size of the lesion was significantly smaller in post-mortem spinal cord (median: 2.1; IQR: 1.8 to 2.4 mm²), compared to controls (median: 6.2; IQR: 5.3 to 8.2mm²) (Mann Whitney, p<0.05). Secondary injury is further suggested by the finding that the length of lesions in controls ranged up to 8.5mm, far longer than the width of the instrument jaws.

This model of spinal cord injury was, however, significantly flawed since the outcome, assessed both by lesion size and hindlimb function at 1 week, showed considerable variation between animals. This variation occurred, in spite of efforts to standardize the lesion. A consistent injury technique was used, involving the same size of the biopsy forceps and the same depth of lesion, determined using a stereotactic frame. Variation in the severity of experimental spinal cord injury has been a common problem during the development of many different injury techniques (Blight, 1991). There are several possible reasons for the inter-operative variation seen in these experiments. The dimensions of the spinal cord at the injury site is known to affect lesion size (Blight and DeCrescito, 1986), and such variation may have occurred in this model, either because of differences in the size of individual animals (+/- 10%), or because of differences in the spinal level of the lesion (+/- 1 spinal segment). Although every effort was made to achieve consistency in operative technique, this was not always achieved, due to one or more of the following factors:

- Cord injury during laminectomy
- Errors in assessing contact of forceps with cord, prior to inserting them into the cord tissue
- As the jaws of the forceps were closed they may not have cut cleanly through the spinal cord and hence, as the instrument was withdrawn, there may have been a degree of tearing of spinal cord tissue
• Variation in vascular supply and hence vascular damage caused by a given lesion
• Movement of the spinal cord with respiration (in spite of fixation of adjacent vertebrae)
• Systemic complications such as variable blood loss and hypotension

It is clear that several of these factors are exclusive to living subjects and this is confirmed by the fact that the variation in lesions made in post-mortem cords was less pronounced than in the control animals.

This model of spinal cord injury produced large lesions and functional deficits in the week after surgery, which were often very marked. However, by 3 weeks hindlimb function scores had returned to normal, thus limiting its usefulness as an experimental method. The recovery in spinal cord function was presumably due to residual ventral cord tissue compensating for the loss of the dorsal columns and dorsal corticospinal tract. This model would therefore have failed to demonstrate any beneficial effects of a graft.

This apparently complete recovery in spinal cord function in the weeks following injury may have been because the tests of function were not sufficiently discriminating to detect minor neurological deficits, which may be masked or compensated for by intact functions (Kunkel-Bagden et al. 1993). It is possible that a more complete set of hindlimb function tests (Kunkel-Bagden et al. 1993) or more sensitive tests, such as the cutaneous trunci muscle reflex (Blight, 1991) and the inclined plane test (Rivlin and Tator, 1977), might have demonstrated some residual deficit in these experiments. It is also possible that if the same experimental methods had been applied to other species, such as the guinea pig, clearly detectable permanent hindlimb deficits might have resulted, since functional recovery may be less complete than in the rat. Alternatively the creation of larger lesions in the spinal cord of rats might have allowed the production of readily detected permanent deficits. It should be noted, however, that in this series even the animals with the
largest lesions, in which the residual spinal cord was often very thin, demonstrated complete recovery of the functional score. This finding contrasts with previous evidence from studies using compression injuries, which have produced permanent functional deficits (Black et al. 1988) (Black et al. 1986). Thus laceration injuries of the spinal cord may be less suitable than compression injuries for the creation of lesions associated with prolonged impairment of hindlimb function. However, compression injury would require a second insult to the cord if graft material were to be inserted, increasing the possible inter-operative variation.

Muscularis externa implanted into large spinal cord lesions had only a limited effect on axonal regeneration in the spinal cord. However, it should be noted that in these experiments the sciatic nerve was not crushed (since it was considered that this would interfere with functional assessment). The regenerative capacity of the axons arising from DRG sensory neurons in these experiments would therefore have been less than was the case with minimal spinal cord injury (Chapter 3). Furthermore, grafts had no significant effect on lesion size. It therefore appears that, at least as measured by this model, muscularis externa does not reduce the processes of secondary injury. However, an effect on lesion size alone is an incomplete indicator of therapeutic efficacy following spinal cord injury. It is possible that muscularis externa could act directly on axons or neurons to promote their survival, for instance by the production of neurotrophins, independently of any effects on the pathological processes associated with secondary injury which influence lesion size. Such an effect would not have been detected by the techniques employed in these experiments (See also Section II: Overview).
Figure 4-1

Decker Microrongeur biopsy forceps, a 20cm ruler is included in the picture for comparison.
Figure 4-2

The modified Kopf small animal stereotactic frame used in these experiments, a 20cm ruler is included in the picture for comparison.
Figure 4-4

Numerous labelled fibres (arrowheads) can be seen in the dorsal columns caudal to the lesion site (L). The more ventral spinal cord (SC) shows no labelling. No regenerating axons can be seen beyond the caudal margin of the lesion (Animal studied at 8 weeks after surgery; Scale Bar = 400µm)
Figure 4-5

A few axons (arrowheads) are seen within residual spinal cord tissue (SC), caudal to the main lesion cavity (C). (Muscularis externa inserted 4 weeks previously; Scale Bar = 400μm)
Figure 4-6

Lesion site in a control animal (no graft) studied at 8 weeks. No regenerating axons are visible within the injured tissue (*). Some macrophages (M) are present and artefactual labelling is also present (Ar) (Scale Bar = 400μm)
Lesion site in an animal with muscularis externa grafted into the lesion site 4 weeks previously. Numerous macrophages (M) are seen within tissue that may be scar and / or graft (*). No regenerating fibres are seen. (C-cavity) (Scale Bar = 200μm)
Figure 4-8

Lesion (L) within a thionin-stained section of spinal cord, in which a standard lesion was made post-mortem in a fixed tissue (Scale Bar = 500μm)
Spinal cord function following injury in control animals. Function was assessed within 3 hours of surgery (Day 1), 2 days later (Day 3), at 1 week and then weekly thereafter. Each animal is represented by a particular colour/symbol, and its progress can be followed through the post-operative period.
Figure 4-10

Spinal cord function following injury. A muscularis externa graft was inserted into the spinal cord lesion site. Function was assessed within 3 hours of surgery (Day 1), 2 days later (Day 3), at 1 week and then weekly thereafter. Each animal is represented by a particular colour/symbol, and its progress can be followed through the post-operative period.
A study of the distribution of neurotrophin mRNA within the rat intestine
ABSTRACT

The cellular localization of BDNF and NT-3 mRNA within the ileum and colon of post-natal rats was examined by in situ hybridization. Both BDNF and NT-3 mRNA were found in the myenteric and submucous plexuses of the enteric nervous system (ENS), as well as in the intestinal mucosa. The pattern of labelling seen in the enteric ganglia indicated that mRNA for both neurotrophins was present in neurons. The two species of mRNA molecule were not always co-localized within the ENS. The overall pattern of expression of each type of mRNA was the same in both ileum and colon, and was similar in neonatal and adult intestine.

INTRODUCTION

Neurotrophins may have important roles in the development, normal function and pathology of the ENS and possibly other intestinal tissues (see Overview: Muscularis externa and CNS grafting). Although there is evidence that neurotrophins are present within the developing and adult gastrointestinal tracts (Berkemeier et al. 1991) (Hohn et al. 1990) (Maisonnier et al. 1990a) (Kuroda et al. 1994) (Scarisbrick et al. 1993), a greater knowledge of their cellular distribution is crucial to a fuller understanding of their function within the intestine. Data from humans using immunohistochemical techniques (Hoehner et al. 1996) suggests that BDNF and NT-3, as well as Trk A, B and C, are present within the ENS; however, the site of synthesis of neurotrophins within the intestine has not previously been investigated. In this study the technique of in situ hybridization was used to localize mRNA for the
neurotrophins BDNF and NT-3 within the ileum and colon of the rat in order to determine the likely site of synthesis of these neurotrophins. This methodology allows accurate cellular localization of mRNA both within the ENS and in other intestinal tissues. The possibility that changes in mRNA occur as the rat matures was examined by studying animals at ages ranging from 1 day to 3 months.

**MATERIALS AND METHODS**

Male Fischer rats supplied by Harlan-Olac (UK) were studied in either the neonatal period, at one week, one month or over 2 months. Each rat was overdosed with halothane to produce terminal anesthesia. 2mm lengths of intestine were quickly removed and immediately fresh frozen in OCT, immersed in isopentane pre-cooled with liquid nitrogen. Separate specimens of distal ileum and colon were obtained from animals in the 2 older age groups. 10μm transverse sections of each specimen were cut using a cryostat and thaw-mounted onto slides coated with 3-aminopropyl-triethoxy-silane.

In situ hybridization was carried out as previously described (Zhang et al. 1995). Briefly, slide mounted specimens were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for at least two hours. After washing in PBS, sections were treated with 0.1M hydrochloric acid and washed in PBS, incubated in 0.1M triethanolamine containing 0.25% acetic anhydride, and then washed with PBS, dehydrated in an ascending ethanol series and air dried. Pre-hybridization was carried out at 37 °C for at least three hours and hybridization overnight at 55 °C with 3 μl/ml concentrations of digoxygenin (DIG)-labelled antisense probes in hybridization buffer.
NT-3 and BDNF plasmids were provided by Regeneron (Maisonpierre et al. 1990c)
(Maisonpierre et al. 1991) and prepared using standard methods (Sambrook et al.
1989), then linearized using the appropriate enzyme (NT-3 plasmid consisted of 800
base pair Xhol fragment encoding the precise rat NT-3 coding region inserted into the
Stratagene plasmid, pKS version (Maisonpierre et al. 1990c) (Maisonpierre et al.
1990b); BDNF plasmid consisted of 1127 base pair rat BDNF cDNA cloned into
Bluescript SK (Maisonpierre et al. 1991)). DIG-labelled RNA probes were made
according to the methods in the DIG RNA Labelling Mix (Boehringer Mannheim,
Germany) and checked using a northern blot (Sambrook et al. 1989). The amount of
DIG-labelled probe was determined using a dot blot (Boehringer Mannheim, 1996).

After hybridization, sections were washed in standard saline citrate containing 50%
formamide at 55 °C. Sections were equilibrated with buffer 1 (100mM Tris-HCl,
150mM NaCl, pH7.5), then incubated in modified buffer 2 (1% Boehringer blocking
reagent, 0.5% bovine serum albumin fraction V; Sigma in buffer 1) and incubated
with alkaline phosphatase-coupled antibodies to digoxigenin (Boehringer Mannheim,
Germany) at a dilution of 1:350 in modified buffer 2 overnight at 4 °C. Sections were
washed in buffer 1, equilibrated in buffer 3 containing 0.34 mg/ml 4-nitroblue
tetrazolium chloride (Sigma) and 0.175 mg/ml 5-bromo-4-chloro-3-indolylphosphate
(Sigma). Development was stopped by washing with buffer 4 (100mM Tris-HCl,
1mM EDTA, pH 8.0), following which the slides were mounted with a coverslip.
The specificity of the hybridization signal was verified by comparing adjacent
sections processed under identical conditions in which either sense probes were used
or all probes were omitted.
RESULTS

The expression of BDNF and NT-3 mRNA was studied in the intestines of 15 animals, using in situ hybridization. Of these, 3 were neonatal (less than 24 hours post-partum), 3 were one week old, 3 were one month old and 6 were aged more than 2 months when studied. Both BDNF and NT-3 mRNA were detected, by in situ hybridization, in the intestines of animals at all ages studied. Control sections, either with no probe added or sense probe for either BDNF or NT-3 mRNA, were consistently negative.

BDNF mRNA was found to be present within both the myenteric and submucous plexuses of the ENS in animals aged 1 month or more (Figure 5-1), as well as in neonatal and 1 week animals (Figure 5-2). In addition there was clear evidence of BDNF mRNA expression within the mucosa at all ages studied. The strongest labelling in the mucosa was seen within the crypts (Figure 5-3). No differences in the pattern of expression of BDNF mRNA were detected in the distal ileum and colon of animals aged 1 month or more. The pattern of labelling in the enteric ganglia had a mosaic-like appearance, with some cells positively labelled but others showing no staining, as if BDNF mRNA was present within some, but not all, neurons (Figure 5-4).

The general pattern of NT-3 mRNA expression in the intestine was found to be similar to that of BDNF mRNA. Positive labelling was detected within both the ENS and mucosa at all ages examined (Figure 5-5). Again, a mosaic pattern of labelling was seen in the enteric ganglia, and mucosal cells within the crypts were strongly labelled. As with BDNF mRNA, the distribution of NT-3 mRNA expression was similar in the distal ileum and colon in animals of 1 month or older.

The pattern of labelling of both BDNF and NT-3 mRNA seen within the enteric ganglia suggested that both species of neurotrophin mRNA are present in neurons,
but that neither probe hybridized to all neurons (Figure 5-4). In order to determine whether BDNF and NT-3 mRNA were present in the same sub-population of neurons, consecutive sections from the same intestinal specimen were hybridized with antisense probes to NT-3 and BDNF mRNA. Study of these sections indicated that NT-3 (Figure 5-6a) and BDNF (Figure 5-6b) mRNA were not always present in the same cells, but were, at least in some cases, present in separate populations of neurons.

**DISCUSSION**

These results indicate that mRNA for the neurotrophins BDNF and NT-3 are present in the intestinal mucosa and the myenteric and submucous ganglia of rat ileum and colon. The presence of BDNF and NT-3 mRNA in these locations suggests that BDNF and NT-3 proteins are themselves likely to be present in the intestine. Previous studies have demonstrated that whole gut from adult animals contain transcripts of NGF, NT-3 and NT-4/5 (Berkemeier et al. 1991) (Hohn et al. 1990) (Maisonpierre et al. 1990a) (Kuroda et al. 1994) and that mRNA transcripts for NGF, BDNF and NT-3 are present within the submucosa of the developing stomach of rats (Scarisbrick et al. 1993), but this is the first in situ hybridization study to define the distribution of BDNF and NT-3 mRNA within the intestinal wall. The results are similar to those obtained from human specimens using immunohistological techniques, which have shown that BDNF and NT-3 are present in the ENS, with BDNF localized to enteric ganglion cells and NT-3 is present in enteric neurons and perhaps glia. Immunoreactivity is also described in the intermuscular basal lamina and in contact with smooth muscle (Hoehner et al. 1996). However, such immunohistological studies do not unequivocally indicate the site of neurotrophin synthesis since the presence neurotrophic factors in the ENS could be as a result of either local synthesis or of accumulation of molecules produced elsewhere. The
finding of neurotrophin mRNA in the ENS and mucosa suggests that they are in fact made locally. The presence of neurotrophin mRNA suggests that BDNF and NT-3 might have functional significance within the intestine, particularly since neurotrophin receptors have also been demonstrated in the gastrointestinal tract (Baetge et al. 1990) (Sternini et al. 1993) (Hoehner et al. 1996) (Lamballe et al. 1994) (Tessarollo et al. 1993) (Sternini et al. 1996).

The ENS demonstrates several unusual properties that may be dependent on neurotrophic support (Saffrey and Burnstock, 1994). For instance, the ENS is unique in its capacity to alter its morphology during normal intestinal peristalsis and is also remarkable for the regenerative capability and plasticity that its cells can demonstrate following experimental manipulation. Thus ENS neurons exhibit strong regenerative responses when cultured in vitro (Saffrey et al. 1991) (Saffrey et al. 1992). Similarly, experimentally-induced stenosis will induce enlargement of enteric neurons and glia (Gabella, 1984), while chemical ablation of the myenteric plexus results in hypertrophy of submucosal neurons (See et al. 1990). Moreover several different types of intrinsic nerve fibres will regenerate across a surgical anastomosis of guinea pig intestine (Galligan et al. 1989). The finding of mRNA for both BDNF and NT-3 in neurons of the mature sub-mucous and myenteric plexuses in this study, suggests that these neurotrophins have a role in maintaining functional or morphological differentiation and possibly cellular survival in the ENS. Furthermore, the finding that BDNF and NT-3 mRNA are produced by some, but not all, cells within the ENS and that the cells involved may, sometimes, differ for the two neurotrophins would suggest that each neurotrophin may have specific and differing target cells and effects. It is known that both sensory and secretomotor neurons of the ENS project to the ileal and colonic mucosa via the non-ganglionated mucosal plexus (Costa and Brookes, 1994) (Furness and Bornstien, 1995) and the strong expression of both neurotrophins within this layer suggests that they might have a role in maintaining the innervation of the mucosa, which undergoes a rapid turnover and may therefore require constant regeneration of its nerve supply. Alternatively the
presence of BDNF and NT-3 in the mucosa could be unrelated to the ENS since neurotrophic factors are able to influence non-neuronal tissues (Scarisbrick et al. 1993) (Trupp et al. 1995) (Levi-Montalcini, 1987). Their presence in the mucosa might thus reflect actions on cells unrelated to the nervous system.

The significance of neurotrophins in the development of the ENS remains uncertain. NT-3 promotes the development of enteric neurons and glia from precursors immunoselected from the embryonic rat or chick gut in vitro (Gershon et al. 1993). However BDNF, NT-3, and the receptors Trk A, B and C are absent from the intestine until after ENS precursor cells have completed their main period of migration and early phenotypic differentiation (Hoehner et al. 1996). Furthermore, the ENS of neonatal homozygous mice lacking NT-3 appears to develop normally (Farinas et al. 1994). There is, therefore, no clear evidence that enteric neurons are dependent on neurotrophic support for their differentiation or survival. Changes in neurotrophin expression at different postnatal ages might indicate that the neurotrophins are exerting specific effects at different stages of maturation and might thus be guiding later development of the ENS. The finding that there were no major differences in neurotrophin mRNA distributions between neonatal animals and those of 2 or more months makes it less likely that, in postnatal animals, there is a clear role for BDNF and NT-3 in the maturation of the ENS. However, NT-3 has also been found to exert trophic effects on postnatal rat enteric neurons in culture and the continued expression of neurotrophins and their receptors during postnatal life may indicate a role for these factors in the maintenance of the ENS.

These results show, for the first time, that BDNF and NT-3 are synthesized in the intestine, both in the ENS and mucosa, and therefore extend previous reports of the presence of neurotrophins and their receptors within the gastrointestinal tract. These findings suggest that neurotrophins are likely to play a significant functional role within the developing and mature intestinal ENS and mucosa.
Figure 5-1a

Intestine from 2 month animals hybridized for BDNF mRNA illustrating labelling within the myenteric plexus (arrow) and mucosa (arrowhead) (Scale bar = 100\,\mu m)
Figure 5-1b

Intestine from 2 month animal hybridized for BDNF mRNA, the submucous plexus (arrow) and mucosa (arrowhead) can be seen deep to the smooth muscle layer (sm) (Scale bar = 50μm)
Figure 5-2a

Neonatal intestine showing positive labelling for BDNF mRNA in the ENS (arrow) and mucosa (arrowhead) (Scale bar = 100μm)
Figure 5-2b

1 week intestine showing positive labelling for BDNF mRNA in the ENS (arrow) and mucosa (arrowhead) (Scale bar = 100μm)
Figure 5-3

Ileal mucosa (arrowhead) is strongly positive for BDNF mRNA in this 1 month animal. ENS is also labelled (arrow) (Scale bar = 50µm)
Figure 5-4

Labelled (arrow) and unlabelled (arrowhead) ENS cells can be seen in a mosaic pattern within the myenteric ganglion of a colonic specimen from a 2 month animal hybridized for BDNF mRNA. (Scale bar = 25µm)
Intestine from a 1 week animal showing positive labelling for NT-3 mRNA in the ENS (arrow) and mucosa (arrowhead) (Scale bar = 100μm)
Figure 5-5b

Colon from a 2 month animal showing positive labelling for NT-3 mRNA in the ENS (arrow) and mucosa (arrowhead) (Scale bar = 100μm)
Figure 5-6

(a) Section of colon from an adult animal processed for NT-3 mRNA. The arrow indicates a positively labeled ganglion cell while the arrowhead points to an unlabeled cell. (b) In situ hybridization for BDNF mRNA has been performed on the same tissue but the positive and negative labeling of the same ganglion cells has been reversed (Scale bar = 100μm)
CHAPTER 6

Cultured muscularis externa implanted into the striatum of adult rats: Ultrastructural studies

ABSTRACT

INTRODUCTION

MATERIALS AND METHODS

RESULTS

DISCUSSION
ABSTRACT

The muscularis externa of the intestine comprises smooth muscle enteric neurons, glial cells, fibroblasts and interstitial cells of Cajal. In this study muscularis externa from rats aged 1 week was dissociated and cultured for 7 days. When examined using immunohistochemistry, the cultures were found to contain a variety of cells including many smooth muscle cells and some neurons. Dissociated muscularis externa cells were grafted into the striatum of adult rats and studied using electron microscopy after periods of 1 week to 1 year. It was found that smooth muscle cells survived in the CNS for up to 1 year, and showed no tendency to form tumors. They maintained their morphological differentiation and remained mainly aggregated at the implantation site. However, some cells migrated for a short distance into the surrounding brain where they were often loosely associated with blood vessels. Extracellular matrix, in which collagen fibrils were present, was often deposited around smooth muscle cells. Small numbers of myelinated and unmyelinated axons, but no neuronal perikarya were identified within the grafts. The regenerative response of axons around the graft was not as prominent as that found around undissociated muscularis externa implanted into the striatum in previous studies.

INTRODUCTION

Neurotrophins, and other protein molecules, have considerable therapeutic potential in the CNS. However, the administration of such molecules is difficult because they do not cross the blood brain barrier and therefore the implantation of cells into the CNS has been used to deliver potentially beneficial molecules. A number of different types of cells have been used as grafts, including peripheral nerve (David and

The cells of the muscularis externa have a number of properties that suggest that they might provide a valuable source of tissue for grafting into the nervous system. Furthermore, smooth muscle cells from muscularis externa might be genetically-modified prior to implantation (Tuszynski et al. 1994) (Howard et al. 1997) to secrete molecules within the CNS. For instance non-enteric fibroblasts, have been transfected with retroviruses to produce neurotrophin molecules, but the potential usefulness of such cells is limited by the risk of tumor formation (Tuszynski et al. 1994), and it is possible that the use of enteric smooth muscle cells might avoid this problem. The muscularis externa has the further advantage that it is readily available in large amounts from the recipient’s own intestine, thus avoiding the danger of immune rejection, while being free from the ethical problems arising from the use of foetal cells (Hoffer and Olson, 1991).

In assessing the potential usefulness of dissociated muscularis externa cells as grafts, their survival and proliferation (which, if uncontrolled, could lead to tumor formation) are clearly important. The effects of living muscularis externa cells on the surrounding CNS will depend on several factors including the maintenance of differentiation and the degree of cell dispersal that, if rapid and widespread, will
reduce the local effects of the implant. In this study electron microscopy has been used to investigate these aspects of the behaviour of implanted muscularis externa cells within the striatum of adult rats over periods ranging from 1 week to 1 year.

**MATERIALS AND METHODS**

**CULTURE OF MUSCULARIS EXTERNA**

Inbred Fischer rats (Harlan Olac, UK) were used. Muscularis externa for tissue culture was obtained from the distal ileum of animals aged 5-10 days, which had been killed by an overdose of pentobarbitone. After rinsing in Ca-Mg-free Dulbecco’s phosphate buffer solution (Life Technologies, UK), the muscularis externa was cut into 1-2mm lengths and left overnight at 4°C in a solution of 0.25% trypsin (Life Technologies, UK) and Dnase (Sigma, UK) at 10μg/ml. The tissue was then triturated at 15 minute intervals for 90 minutes at 37 °C. The resulting cell suspension was removed and the remaining tissue was further dissociated with collagenase (Boehringer Mannhiem, Germany) for a further 30 minutes. The second cell suspension was added to the earlier one in foetal calf serum (Flow, UK). Following centrifugation the cell dissociates destined for implantation were grown on plastic at an initial density of 4x10⁶/flask at 37°C with 5% CO₂ in medium 199 supplemented by 10% foetal calf serum.

**IMMUNOHISTOCHEMISTRY**

Dissociated muscularis externa cells to be used for immunohistochemistry were grown under similar conditions but on collagen-coated cover-slips within 4-well multi-dishes (Marathon, UK). After 1 week the cultured muscularis externa cells were fixed as follows: debris was rinsed off with Ca-Mg-free medium, which was
then replaced with 4% paraformaldehyde for 1 hour at 4°C. The cells were then rinsed six times with 80% ethanol over 1 hour, followed by 0.1M phosphate buffer solution (PBS) for 10 minutes, then PBS-triton 0.1% for 10 minutes and finally twice with PBS for 10 minutes, all at room temperature. At the end of the rinsing process PBS-Azide was added and the cells were stored at 4°C.

For the purposes of immunohistochemistry muscularis externa cell cultures were incubated in blocking serum (10% normal goat serum in 1% bovine serum albumin) for 1 hour at room temperature. This was then replaced for a further hour at room temperature with a solution of PGP (protein gene product 9.5) rabbit-polyclonal antibody (Ultraclone, UK) in blocking serum at a concentration of 1 in 200. This was incubated overnight at 4°C and biotinylated anti-rabbit IgG secondary antibody (Vector, UK) was then added at a concentration of 1 in 200 for 90 minutes at room temperature. The cells were then washed again three times in 0.1M PBS and a solution of Avidin-Biotin complex (Vector, UK) was added for 1.5 hours (relative volumes of: Solution A to Solution B to 0.1M PBS were 2 to 2 to 100). Following 3 further washes the cells were incubated with Diaminobenzidine solution (Sigma, UK) for 5 minutes and then rinsed again with 0.1M PBS. They were then left to dry overnight at room temperature. Following dehydration through alcohols, the cells were cleared and mounted onto slides.

**PREPARATION OF CELLS FOR IMPLANTATION**

After a culture period of approximately 7 days the cells were detached from the flask with 0.125% trypsin in Ca-Mg-free medium at 37°C. An equal volume to 10% foetal calf serum (Flow, UK) was added to the cell suspension, which was centrifuged at 900 r.p.m. for 5 minutes. The pellet was re-suspended in medium 199 supplemented by 10% foetal calf serum and a cell count was performed to determine the total number of cells present. The cells were again centrifuged at 900 r.p.m. for 3 minutes and the resulting pellet was stored on ice until implantation shortly afterwards.
SURGERY

All surgical procedures were performed while the recipients (Fischer rats- 250-400g) were deeply anaesthetized with a mixture of halothane, oxygen and nitrous oxide and all animals were treated post-operatively with analgesic (Flunixin, Schering Plough, UK) and antibiotic (amoxyccillin, Beecham, UK). Implantation of dissociated muscularis externa cells was carried out with the recipient held in a modified Kopf small animal stereotactic frame. A midline sagittal incision was made to expose the skull. A hole was drilled in the right parietal bone 0.3mm anterior to bregma and 3mm lateral to the midline and the dura was opened. The suspension of dissociated muscularis externa cells was taken up into a 10μl Hamilton syringe, which was then fixed to the stereotactic frame and positioned so that its tip was in contact with the surface of the brain. It was then lowered 5mm into the brain and 2 μl of the cell suspension was injected into the striatum over the course of 2 minutes. After the syringe had been withdrawn the skin was closed with 3/0 silk sutures (Ethilon, UK).

ELECTRON MICROSCOPY

At intervals of 1 week, 1 month and 9-12 months after implantation, recipient animals were killed by an overdose of pentobarbitone and were perfused transcardially with fixative (4% paraformaldehyde, 0.5% glutaraldehyde in 0.1M Millonig's buffered phosphate). The brain was removed and stored in fixative overnight at 4°C. Transverse sections were then cut on a vibrotome (200μm), osmicated and block stained in 2% uranyl acetate, before being processed into Araldite resin. The whole spinal cord section was mounted on a block and semi-thin (0.5μm) sections were cut on an Ultracut E microtome. Semi-thin sections were stained with Toluidine blue and the area containing the graft was identified. The block was then trimmed down and ultra-thin sections were cut and collected on copper grids, stained with lead citrate and then examined in a Jeol 1010 electron microscope.
RESULTS

Immunohistochemical studies of cultured dissociated muscularis externa using antibodies to PGP 9.5 demonstrated a variety of cells, many of which appeared to be smooth muscle. The smooth muscle cells exhibited only weak, background, staining in contrast to strongly immunoreactive cells, which had the morphological features characteristic of neurons (Figure 6-1). The number of neurons varied between cultures, but was generally far lower than the number of smooth muscle cells. Some cells with features suggestive of glia and fibroblasts were seen but could not be confidently identified with PGP immunohistochemistry.

All the operated animals remained in good health throughout the study, with no discernible neurological deficits. The striatum was examined at intervals of 1 week (3 animals), 1 month (3 animals) or 1 year (3 animals) after the implantation of dissociated muscularis externa cells. When semi-thin sections of striatum, stained with toluidine blue, were examined the implantation site was readily identified (Figure 6-2). The grafts were more darkly stained than the striatum and were often associated with prominent blood vessels. There was no evidence of mitosis in the surviving smooth muscle cells and there was no indication that they had formed any expanding tumor mass or that they were causing distortion or compression of the adjacent CNS (Figure 6-2).

When specimens were examined using electron microscopy, smooth muscle cells could be easily identified in the vicinity of the implantation site in all three age groups (Figure 6-3), although the degree of differentiation and separation of cells was variable (Figure 6-4). These cells had the same cellular morphology as smooth muscle in the intestine, with actin filaments, dense bodies within the cytoplasm and caveolae at the cell surface. However, myofilaments were less obviously attached to the cell membrane than in muscle cells in the gut wall and generally appeared to have a more secretory, and less contractile, phenotype (Figure 6-5).
Smooth muscle cells were usually loosely clustered together around the area of implantation (Figure 6-3). Some cells, however, appeared to have migrated up to 100-200μm into the surrounding brain, and in particular were often found around blood vessels (Figures 6-6). They could be distinguished from intrinsic vascular smooth muscle by the fact that they were not integrated into the vessel wall and by their presence around capillaries, which would not normally possess a smooth muscle layer. When striatum was examined at a slightly greater distance from the implantation site there was no evidence that any muscularis externa cells had migrated there, even after 1 year. Smooth muscle cells were often associated with large venules in the brain near the site of implantation (Figure 6-7).

CNS and smooth muscle were sometimes separated by fibroblasts, probably derived from the graft (Figure 6-3). In some areas a well-defined glia limitans had formed which abutted smooth muscle (Figure 6-8). Extracellular matrix had been extensively deposited around the smooth muscle cells and collagen fibrils were present in large amounts within the extracellular matrix (Figure 6-8).

Although no neuronal cell bodies were identified within the grafts, some unmyelinated axons could be found growing among the smooth muscle cells, either singly or in bundles (Figures 6-9). Smooth muscle cells often made close contact with glial processes within such bundles, with no intervening basal lamina (Figure 6-10). Schwann cell myelinated axons were present within the graft in the 1 year age group (Figure 6-11).
DISCUSSION

Cells with the characteristic morphology of smooth muscle were clearly identifiable within the striatum using electron microscopy, even 1 year after implantation. Since smooth muscle cells are not normally found in the CNS, their presence clearly indicates an intestinal origin. Fibroblasts were also seen around the implantation site and, in view of their frequency, these too were likely to have originated from the graft, although a non-intestinal derivation cannot be excluded. Axons in the graft could have originated from neuronal perikarya in the brain or from neurons present in the muscularis externa cultures. However, the number of enteric neurons that survived implantation by this protocol must have been low, since no perikarya were found within the graft. Collagen was frequently found around the smooth muscle cells as part of the extracellular matrix.

The finding that dissociated smooth muscle cells survived for up to 1 year within the striatum accords with the results of previous studies which have shown survival of muscularis externa after grafting into the CNS. Thus whole muscularis externa (Tew et al. 1992), and its smooth muscle (Tew et al. 1995a) and myenteric plexus (Tew et al. 1993) (Tew et al. 1994) (Jaeger et al. 1993) derivatives, can survive within the CNS for at least 6 weeks. Moreover, the NADPH-diaphorase containing sub-population of enteric neurons can survive for a year after implantation of undissociated myenteric plexus into the striatum (Tew et al. 1996).

There was no evidence of uncontrolled proliferation, or tumor formation arising from the muscularis externa cells. This is in contrast with studies in which immortalized cell lines have been used as CNS grafts with subsequent tumor formation (Rohrer et al. 1996) (Chen et al. 1996). Even primary cell cultures have sometimes led to problems with tumor formation; for instance fibroblasts, genetically modified to secrete neurotrophins, have been associated with slowly growing masses within the spinal cord and hence the risk of CNS compression (Tuszynski et al. 1994). It should
be noted, however, that although our results indicate that muscularis externa does not form tumors within the striatum, this does not necessarily exclude this outcome elsewhere in the CNS, since there is evidence both from implantation of cell lines (Zompa et al. 1993) and primary fibroblasts (Tuszynski et al. 1994) that the site of implantation can profoundly affect the tendency to tumor formation.

Smooth muscle cells did not spread far into the striatum, but were found mainly in clusters at the graft site. However, some cells appeared to have migrated short distances away from the implantation site. This migration of intestinal smooth muscle cells has not previously been demonstrated in whole tissue grafts (Tew et al. 1992) (Tew et al. 1995a) and dissociated cells would therefore appear to have a greater tendency to spread away from the implantation site. A variety of other types of dissociated cells, including tumor cells (Pedersen et al. 1995) (Yamada et al. 1994), immortalized cell lines (Cattaneo et al. 1994), Schwann cells (Raisman et al. 1993) (Brook et al. 1993), CNS glia (Emmett et al. 1990) (Hatton and Garcia, 1992a) (Emmett et al. 1991) (Bradbury et al. 1995) (Wang et al. 1995) (Lachapelle et al. 1994) and neurons (Macklis, 1993) (Demierre et al. 1990), will also exhibit migration within the CNS following transplantation. Smooth muscle cells were frequently seen in loose association with blood vessels. This suggests that the spread of cells away from the graft site is not simply due to the physical act of injection but that active cell migration is an important part of the mechanism of dispersal. Such migration could be a response to a metabolic gradient between the blood and CNS or an unidentified tropic factor produced by the capillaries. Alternatively, the perivascular (Virchow-Robin) spaces might provide a physically favourable environment for the migrating cells to accumulate and move in. Similar attraction towards, and along, blood vessels occurs with other cell implants such as glia (Gout and Dubois-Dalcq, 1993) (Hatton and Garcia, 1992a) (Emmett et al. 1991) and Schwann cells (Brook et al. 1993) (Raisman et al. 1993).
Axons with typical morphological features of regenerating CNS axons were seen in and around the grafts, either singly or in bundles. In the year long experiments some had become myelinated, apparently by Schwann cells. Schwann cell myelination has previously been reported to occur in grafts of enteric smooth muscle, but not of complete muscularis externa. Therefore, in this respect, the cultured muscularis externa grafts resemble grafts of muscle dissected free from plexus, perhaps indicating that enteric neurons did not survive implantation in this protocol. The regenerating fibres could have arisen from either striatal neurons, afferents to the striatum, or fibres passing through the striatum. However electron microscopy cannot distinguish which of these populations of cells the neurites had grown from. The signal for the regenerative response is unknown, but since it is abolished by freeze-killing of grafts derived from muscularis externa (Tew et al. 1992) (Tew et al. 1995a), it is likely to be a soluble substance derived from living cells. Although the cells of the rat ENS are able to produce BDNF and NT-3 mRNA, this does not appear to be the case with intestinal smooth muscle (see Chapter 5), which are, however, still able to induce a sprouting response (Tew et al. 1995a). Therefore some other unidentified factor is probably involved, possibly produced by the intestinal cells of Cajal, which are present throughout the muscularis externa (Tew et al. 1995a). The regenerative response was significantly less pronounced than that following implantation of un-dissociated muscularis externa (Tew et al. 1992) or smooth muscle (Tew et al. 1995a) into the striatum. This could be because in this study cells were implanted using a stereotactic frame which would be likely to cause less damage to the CNS than a free-hand technique which had previously been used (Tew et al. 1992) (Tew et al. 1995a). The CNS damage itself might act as a stimulus to regeneration and hence explain the lesser response seen in this study. An alternative explanation might be that in this experiment fewer muscularis externa cells were implanted into the striatum than in previous studies. A third possibility might be that the process of culturing the muscularis externa leads to the relative loss of a key extracellular component or cell type, such as enteric neurons or interstitial cells of Cajal.
This study has demonstrated that cultured, dissociated smooth muscle cells derived from muscularis externa show several properties that might be useful for experimental and therapeutic grafting into the CNS. The grafts can survive for periods of at least 1 year, but do not form tumors within the brain. The cells themselves largely maintain their morphological differentiation and are loosely aggregated in the vicinity of the implant, thus allowing interactions with the adjacent CNS. It is thus possible that smooth muscle cells from the muscularis externa could provide suitable cell for the genetic modification with retrovirus to allow localized synthesis of biologically active molecules in a graft implanted into the CNS.
Figure 6-1

Cultured muscularis externa, 1 week after dissociation, has been immunohistochemically reacted for PGP 9.5. The strongly labelled cell body of a neuron (arrow) is shown, from which several neurites have emerged (arrowheads). Clumps of smooth muscle cells (M) are present in the background (Scale Bar = 50μm).
Figure 6-2

Toluidine blue-stained semi-thin section through the striatum, 1 year after implantation of dissociated muscularis externa cells. The graft (Gr) is clearly seen within the striatum (St), which contains bundles of myelinated fibres (*). A large blood vessel (BV) and several smaller vessels are present in and around the graft (Scale Bar = 50µm).
Figure 6-3

Electron micrograph showing a cluster of smooth muscle cells (M), 1 month after implantation. They appear to be partially enveloped by a fibroblast (F), which separates them from a nearby astrocyte (A), which has formed a glia limitans around the graft. A large group of myelinated striatal axons (MY) is to one side, while on the other side is striatal neuropil (ST) (Scale Bar = 2μm).
Loosely packed smooth muscle cells (M) separated by extracellular matrix and collagen fibrils (C), 1 year after implantation. Blood vessels (B) are also present within the graft. Myelinated axons (My) and an astrocyte (A) can be seen nearby (Scale Bar = 2µm).
Figure 6-5

Smooth muscle cells (M) 1 year after implantation, showing a partial loss of the characteristic contractile phenotype. Although myofilaments are present, they appear to be poorly connected to the membrane (arrows) (A-astrocyte; Scale Bar = 500nm).
Figure 6-6

Smooth muscle cells (M), of the secretory phenotype, which had migrated into the striatum (ST) can be seen loosely arranged around a blood vessel (B) and surrounded by extracellular matrix with large amounts of collagen (C) (1 month after surgery; Scale Bar = 1 μm).
Figure 6-7

Smooth muscle cells (M), of a more contractile phenotype, next to an endothelial cell (e), which forms part of the wall of a large venule (Lumen-L). There are enlarged extracellular spaces between the vessel and the striatal neuropil. A glia limitans, formed by astrocyte (A) processes covered by a basal lamina (arrowheads), can also be seen (1 month post-implantation; Scale Bar = 1μm).
Figure 6-8

A well defined glia limitans (arrowheads) can be seen separating smooth muscle cells (M) from CNS tissue (C-collagen fibrils, A-astrocyte, My-myelinated axons; 1 year post-implantation; Scale Bar = 500nm).
Figure 6-9

A bundle of unmyelinated axons (Ax), seen in x-section, run through the graft, surrounded by smooth muscle cells (M), which in some areas establish close contact with the bundle of axons (arrows) (1 year post-implantation; Scale Bar = 500nm)
Figure 6-10

Unmyelinated axons (Ax) between smooth muscle cells (M) and astrocytes (A). Smooth muscle can be seen in close contact with glial process (arrowheads), with no intervening basal lamina (1 year post-implantation; Scale Bar = 500nm)
Figure 6-11

A bundle of myelinated axons (My) at the periphery of the graft. The axons are myelinated by Schwann cells (S) and are partially compartmentalized by fibroblast processes within a matrix of dense collagenous tissue (C) (Scale Bar = 2μm).
CHAPTER 7

General Discussion
A variety of questions have been addressed in the course of this thesis, using several different experimental techniques. However, some general themes and topics of a more speculative nature have emerged that warrant consideration separately from the individual experimental chapters. In addition this section provides an opportunity to discuss some general issues concerning the experimental work, and how the research might be extended in the future.

The results that have been presented confirm recent evidence from several sources that, given appropriate treatment, regeneration of axons within the spinal cord is possible. Indeed both treatment with immunophilin ligands and muscularis externa can stimulate limited axonal regeneration and, in addition there is clear evidence that FK506 can protect axons. The finding that a single treatment can influence both axonal regeneration and protection indicates that great care must be taken in defining these two actions, because their histological and functional effects may be very difficult to distinguish and confusion could therefore arise in the interpretation of experimental results. The finding that FK506 promotes both axonal regeneration and survival illustrates that the relationship between the two is complicated, since some other molecules, such as c-Jun, can increase both regeneration and neuronal death (Herdegen et al. 1997).

This problem with the distinction between regeneration and protection is often complicated by the fact that research within a particular laboratory may be focused on only one of these processes. This problem arose in the course of the experiments on FK506 described here. The main interest in the initial stages of the investigation was on increased regeneration, with the consequence that the experimental protocol was not one that would normally be used for the demonstration of protective effects. The use of this methodology, in which individual dorsal root ganglion sensory axons were labelled, had the positive, though unforeseen, consequence of demonstrating that FK506 protects axons even when the parent cell bodies were spared. This would
not have been shown by more conventional methodologies for the study of neuroprotection. As might be expected, however, there were problems associated with the study of protective effects using tools designed for studying regeneration. Once it was clear that regeneration was unlikely to have produced long rostral sensory fibres, the design was modified. Thus all subsequent experiments were randomized and assessed in a ‘blind’ fashion, at a short time interval after injury. Also as many variables as possible were eliminated since they would complicate the more statistical analysis required for the demonstration of a protective effect. In the randomized experiments a single dosage of FK506 was used, animals were all treated for a period of 1-2 weeks and no other immunophilin ligands were administered. Furthermore sciatic nerve crush, performed in order to stimulate regeneration, was omitted in a far greater proportion of animals.

The analysis of neuroprotective treatment for CNS injury is complicated. Lesion size was one of the parameters measured as part of the assessment of the effects of immunophilin ligands and muscularis externa. However, lesion size alone is at best only a crude indicator of damage to the CNS and is an even worse indicator of protective effects on spinal cord function following injury, because much of the functional loss results from the transection of the long-fibre tracts of the white matter. The area of visible damage used to calculate lesion size may correlate poorly with the amount of axonal transection. Indeed in some studies of spinal cord injury, the lesion predominantly involves the grey matter, and the calculated lesion size will be only indirectly related to long-tract function (Bartholdi and Schwab, 1995). A similar problem is that many studies of potential protective therapies assess survival of neuronal cell bodies, rather than axons. In fact the parent cell bodies of transected axons often survive the injury (Groves et al. 1996), though the neuron’s principle function, the transmission of data along axons, is of course lost. The experiments on immunophilin ligands suggested a drug effect on axonal viability, independent of the parent cell body. This has not previously been demonstrated and such an effect has
clear implications not only for spinal cord injury but also strokes of the internal capsule and diffuse axonal injury associated with cerebral trauma.

Although conventional models used for the assessment of protective effects of treatment may be flawed, the method used here for the assessment of the effects of immunophilin ligands on spinal cord injury also had limitations. Principal among these was the fact that the frequency of complete primary transection was not known. At best, with FK506 treatment, 29% of animals showed evidence of spared fibres and it is possible that all the remaining 71% had undergone complete primary transection and therefore the figure of 29% with spared fibres indicated complete protection of axons that survived the primary injury. Alternatively it is possible that the primary lesion was never complete and all remaining axons were destroyed by secondary injury in over two thirds of treated animals. While it is probable that the real effect is somewhere in between these two extremes, it is clear that the possible range of effectiveness of FK506 demonstrated by these experiments is very great. It should also be noted that the scoring system was qualitative, in the form of positive (i.e. spared fibres) or negative (no spared fibres) and the effects of treatment are therefore rather crudely demonstrated in comparison to a quantitative scoring system. Finally the study did not provide direct evidence (hindlimb function tests) or indirect evidence (myelination of axons) as to whether the spared fibres were functional.

The finding that FK506 has significant protective effects on axons opens up many possibilities for future scientific and clinical research. The major component of clinical spinal cord injury is crushing of the spinal cord, and a clear priority is to determine the effects of FK506 using either a static or dynamic compression model, preferably in conjunction with tests of functional recovery (see Chapter 4). Such a model might further allow the assessment of whether FK506 has protective effects on other populations of axons such as corticospinal axons. The presence of axons within the long-tracts of the spinal cord is necessary but not sufficient for normal function, which depends also on the state of myelination, which could usefully be investigated following FK506 treatment, using specific markers for myelin or perhaps electron
microscopy. In addition, from a clinical perspective a number of laboratory experiments would be of value to optimize the administration of FK506 following spinal cord injury. Unresolved issues include the optimum dose, the duration of treatment and most crucially the timing of the first dose. If the therapeutic window is small, for instance the drug had to be administered in less than 1 hour, then the chances of many patients benefiting would be severely curtailed. A crucial advantage of FK506 as a potential treatment for spinal cord injury is the fact that it is already in widespread use for transplantation patients and is relatively safe, thus avoiding the necessity of Phase I trials. Preliminary work has, therefore, already commenced to design a clinical study, with the aim of establishing the safety of the drug, given in a relatively high dose. It is envisaged that approximately 50 patients, including controls, might be randomized into the study. FK506 would be given as an initial intravenous bolus within 24 hours of injury, probably at the highest dose currently recommended for transplant surgery, which is 0.1mg/kg/24 hours. This would be at the lower end of the dose range that has been used successfully in animal models. However, in the context of human spinal cord injury, this would be a reasonably large dose when account is taken of species differences and the fact that administration would be intravenous and therefore more effective than the subcutaneous injections used in most animal experiments. Treatment would be for 2 weeks, although a shorter course might be effective. A full neurological assessment would need to be performed within 24 hours of admission, at 6 weeks, 6 months and 1 year, using a standard protocol such as the Frankel scale or the American Spinal Injury Association motor score. In addition, a range of simple clinical and metabolic measures of systemic health would be taken.

More basic research is also envisaged. Experiments could be undertaken to probe the possible mechanisms by which FK506 prevents secondary injury to nerve fibres. Thus NO levels at the site of spinal cord injury could be measured, while in situ hybridization could be used to determine the distribution of immunophilins in the spinal cord after injury. FK506 treatment could also be combined with other forms of
therapy that may promote regeneration, such as the administration of neurotrophins, or IN-1 antibodies directed at the inhibitory oligodendroglia-myelin associated membrane protein. It would also be important to determine whether FK506 has protective effects elsewhere in the CNS. Finally opportunities exist to develop analogues of FK506 with potent calcineurin activity.

There is clear evidence that muscularis externa can exert a powerful effect on axonal regeneration within the striatum, and this provided the rationale for investigating its effects within the spinal cord. However, the results presented here show that regeneration following implantation into the spinal cord is less than in the striatum. This finding is consistent with previous evidence that different populations of neurons differ in their regenerative response to treatment (Woolhead et al. 1998), and in particular that there is regional variation in the regenerative effects of muscularis externa grafts (Lawrence et al. 1991) (Tew et al. 1992). It is interesting to speculate how this situation may have arisen. The differential effect of muscularis externa grafts within the CNS could be the result of differences in the injured axons in the different sites. Axons arising within, or passing through, the striatum, may possess receptors for molecules derived from muscularis externa that stimulate axonal sprouting, whereas spinal axons may lack those receptors. If this were the case a different form of treatment might preferentially cause regeneration of spinal cord axons. It is possible, however, that axons within the spinal cord may in general be intrinsically less able to regenerate, or that the molecular environment is inhibitory to axonal regeneration. Such a situation could have arisen for evolutionary reasons. Regeneration and sprouting within the mature CNS brings the potential for repair of damage, but also for the formation of aberrant sprouts and inappropriate synaptic connections, with the possibility of harmful consequences. In regions, such as the spinal cord, where even a small proportion of wrong connections could significantly impair normal motor and sensory function, a reduced regenerative response would
confer a survival advantage. In other regions of the CNS where small abnormalities would cause fewer functional problems, there would be less evolutionary pressure to suppress axonal sprouting. A similar, though distinct, explanation arises from the concept that the CNS exhibits plasticity and that this is bound to the process of learning. The function of the spinal cord pathways is relatively fixed and the formation of new connections would not be of benefit. In other regions, such as the cortex, which may be modified by experience, there is a greater degree of plasticity. If axonal sprouting following injury and the structural changes associated with plasticity were related, then it would not be entirely surprising that muscularis externa implanted into the spinal cord had less effect than in the striatum, with its rich cortical innervation.

The research findings on the use of muscularis externa as a graft tissue suggest several avenues for future investigation. The work with in situ hybridization could be extended to study distribution of mRNA for other neurotrophic factors within the intestine. In addition, it would be of interest to study the presence of neurotrophin mRNA in cultured muscularis externa. Some preliminary work was performed for BDNF and NT-3 mRNA, as part of this thesis. The results suggested that both species may have been present within a minority of the cultured cells, but background labelling was also present in control cultures, making it difficult to be certain of the validity of the findings. In some further experiments cultured muscularis externa cells were implanted into the striatum. After 1-2 weeks sections were taken through the graft within the striatum and in situ hybridization was performed for BDNF and NT-3 mRNA. Although there was slightly increased labelling in the vicinity of the graft this was not sufficiently clear to draw definitive conclusions.

NT-3 promotes the regeneration of corticospinal axons in the injured spinal cord (Schnell et al. 1994) (Grill et al. 1997). Although it seems unlikely that a great deal of NT-3 is produced by cultured muscularis externa smooth muscle cells, other properties of these cells suggest that they may be good candidates for genetic
modification to create biological minipumps in order to deliver NT-3 to the site of spinal cord injury. Since the discovery, by Crick and Watson in 1953, that DNA forms a double helix there has been a rapid expansion of knowledge in the field of molecular biology (Watson et al. 1992; Glover et al. 1984). This has led to profound new insights into the molecular basis of genetics, protein synthesis and structure, development, disease, the immune system and evolution. These technical advances include the transfer of novel genes to create genetically engineered cells. Although it is possible to introduce the relevant DNA sequence directly into cells by either physical or chemical means, it is generally more effective to insert the DNA sequence into the genome of a viral vector which has been specifically designed to enter the cell. For safety reasons such viral vectors are usually designed to be deficient in certain essential genes, which render them unable to replicate except in specific situations. For instance retroviral vectors are usually designed so that they can replicate only in specific packaging cells, which are grown in culture to produce the viral particles. In addition to the desired DNA sequence, all viral vectors include regulatory sequences, such as promoters and enhancers and sometimes markers that allow their presence within cells to be identified (Howard et al. 1997) (Doering, 1994).

In the context of research into the CNS, DNA has been introduced either in vivo or ex vivo. In the former the DNA is delivered directly to CNS cells, by injection of recombinant viral vectors into the brain or spinal cord. Ex vivo techniques involve the insertion of the DNA into cells in culture which are then implanted within the CNS to create biological minipumps (see also Chapter 1) (Doering, 1994). Members of the following viral groups have been used for the genetic modification of cells: herpes simplex viruses, adenoviruses, adeno-associated viruses and retroviruses, each with specific advantages and disadvantages (Howard et al. 1997; Doering, 1994). Retroviruses contain viral RNA that is converted to DNA by the viral enzyme reverse transcriptase following infection of a cell. The viral DNA is integrated into the host genome where it stays, replicating alongside host DNA at each cell division. This
integrated provirus steadily produces viral RNA, which serves both as mRNA templates for viral protein synthesis and as genomic RNA for the creation of new viruses that are assembled in the cytoplasm and shed from the cell surface with little effect on the cell’s health. The viral genome thus becomes a permanent part of the host cell genome and the cell should express any new gene inserted into the viral RNA. This makes retroviruses efficient vectors for the transfection of mammalian cells with new genes. Retroviruses designed to serve as vectors usually include a marker gene, like the β-galactosidase gene, as well as the foreign gene to be expressed. Most of the viral structural genes will be missing so these vectors cannot replicate under normal conditions. To prepare a virus stock cloned proviral DNA is transfected into a packaging cell, which will contain an integrated provirus with all its genes intact but lacking the DNA sequence recognized by the packaging apparatus. Thus the packaging provirus cannot package its own RNA but will allow the vector RNA to be packaged into infectious viruses. These vector viruses cannot replicate but can be introduced into appropriate target cells, which will therefore carry the integrated provirus but will never produce any new retrovirus particles.

An initial aim of the thesis was to extend the work on implantation of muscularis externa using retrovirus to genetically modify the smooth muscle cells of cultured muscularis externa to produce neurotrophins such as NT-3. A substantial amount of preliminary work was in fact undertaken using the retrovirus pLXSG (supplied by Professor Kageyama, Institute for Immunology Kyoto University, Japan), which was grown in the packaging cell line E86, following transfection with the calcium phosphate method. The use of selection medium permitted the identification of virally infected cells, and the supernatant from these cultures was harvested for virus particles. Titration of the virus was performed using rodent NIH-3T3 cells to assess the concentrations of the virus within the supernatant. When satisfactory viral titres had been obtained, an attempt was made to transfect smooth muscle cells from cultured muscularis externa. Unfortunately, in spite of repeated efforts, successful transfection could not be conclusively demonstrated and the work was suspended to
pursue more fruitful lines of investigation. However, the problems are likely to have been technical in nature, either related to the specific retroviral vector or the conditions of attempted transfection. Indeed had time been available it was planned to try again with a pLRNL retroviruses (supplied by Dr Gage, La Jolla, California, USA). The basic concept of using smooth muscle from the intestine and a retroviral vector to create biological minipumps, remains valid and should be explored in the future.

In conclusion, this thesis has confirmed that the recent advances by many researchers in basic neuroscience are presenting exciting new opportunities for the development of new approaches to the clinical management of spinal cord injury.


Folbergrova, J., Li, P.A., Uchino, H., Smith, M.L. and Siesjo, B.K. (1997) Changes in the bioenergetic state of rat hippocampus during 2.5 min of ischemia, and prevention


Vaudano, E., Woolhead, C.L., Anderson, P.N., Lieberman, A.R. and Hunt, S.P. (1993) Molecular changes in Purkinje cells (PC) and deep cerebellar nuclei (DCN) neurons after lesion or insertion of a peripheral nerve graft into the adult cerebellum. Society of Neuroscience Abstracts 622, 7


