Functional repair of the corticospinal tract by delayed transplantation of olfactory ensheathing cells in adult rats

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I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

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This Thesis is dedicated to my loving husband and parents
Abstract

Adult rats were trained to use their forepaws to retrieve a piece of food through a slit in the front of the cage. The dorsal corticospinal tract was then partially or totally destroyed on one side by a small focal stereotaxic radio-frequency lesion at the level of the first or second cervical segment. Complete destruction of one side of the corticospinal tract completely abolished the use of the ipsilateral forepaw for retrieval for at least 6 months after the operation. The group of rats with the smaller lesions (destroying up to 60% of the tract) the ipsilateral forepaw was used from the first time tested, but at a reduced level, which increased progressively and reached close to normal levels by 8 weeks. The rats with the larger lesions (destroying 60% or more of the tract) did not start retrieval until the second post-operative week, and were still severely impaired at 8 weeks. The corticospinal tract is capable of transferring functions to even very small numbers of surviving fibres (1% axons), which presumably did not carry this function before the lesion. It was indicated that the total number of retrievals by the ipsilateral forepaw showed a highly significant correlation (r=0.95) with the percentage of surviving axons.

The effect of directed forepaw retrieval recovery due to transplantation of olfactory ensheathing cells were examined. In lesioned rats, which had shown no retrieval for 8 weeks after operation, a suspension of olfactory ensheathing cells was stereotaxically injected into the lesion site. From between 1 to 3 weeks after transplantation, the rats started to use the forepaw of the transplanted side, initially, at a lower rate compared with the normal side, but progressively increasing with time. The transplanted rats showed 40% directed forepaw retrieval recovery. Anterograde tracing (Biotin dextran) showed regenerating corticospinal axons crossing the lesion area, travelling caudally in the distal part of the corticospinal tract, and forming terminal arborisations in the spinal grey matter.

A control group of rats with injection of olfactory ensheathing cell conditioned-medium and misplaced olfactory ensheathing cell transplants showed no functional recovery during the 8 weeks of training. Experimental results suggest that the transplanted cells must be placed in direct contact with the tract to form a bridge across the lesion.

These results indicate that the regenerated corticospinal tract axons induced by delayed transplantation of olfactory ensheathing cells provides a quantitatively limited (approximately around 1% regenerating fibres) but rapidly conducting new pathway across the lesioned site.
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Abbreviations used in text

α-MSH  melanotropin
ABC    avidin biotin dextran
ACTH   corticotropin
BDA    biotinylated dextran amine
BD     biotin dextran
BDNF   brain-derived neurotrophic factor
C      cervical
CaMIIk-α calcium/calmodulin–dependent protein kinase-α
CAMP   cyclic adenosine monophosphate pathway
CSPG   chondroitin sulphate proteoglycan
CNS    central nervous system
CST    corticospinal tract
DAB    diaminobenzidine
DAPI   4,6-diamidino-2-phenylindole, dihydrochloride
dFR    directed forepaw retrieval
DH     dorsal horn
DMEM   Dulbecco’s modification of Eagle’s medium
DMP-30 2,4,6 tri (Dimethylaminomethyl) Phenol
DNA    deoxyribose nucleic acid
DREZ   dorsal root entry zone
DSA    Dodecenyl Succinic Anhydride
E      embryonic day
EM     Electron microscopy
EM.PB  Electron microscopy phosphate buffer
f      Fibroblast-like cell
FN     fibronectin
GAP-43 growth-associated protein-43
GFAP   glial fibrillary acidic protein
GAG    glycosaminoglycan
GOD    Glucose Oxidase-Nickel
HBSS   Hank’s balanced salt solution
HRP    horseradish peroxidase
Les    Lesion
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<tr>
<td>mAB</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MAG</td>
<td>Myelin-associated glycoproteins</td>
</tr>
<tr>
<td>MNA</td>
<td>Methyl nadic Anhydride</td>
</tr>
<tr>
<td>MX</td>
<td>myelinated axon</td>
</tr>
<tr>
<td>NF</td>
<td>neurofilament</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NgR</td>
<td>Nogo receptor</td>
</tr>
<tr>
<td>NT-3</td>
<td>neurotrophin-3</td>
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<tr>
<td>NV</td>
<td>needle valves</td>
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<tr>
<td>OEC</td>
<td>olfactory ensheathing cell</td>
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<tr>
<td>OMgp</td>
<td>Oligodendrocyte-myelin glycoprotein</td>
</tr>
<tr>
<td>p75</td>
<td>low affinity neurotrophin receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PDL</td>
<td>poly-d-lysine</td>
</tr>
<tr>
<td>Pen-sterp</td>
<td>Penicillin and Streptomycin</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PLL</td>
<td>poly-l-lysine</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>r.m.p</td>
<td>revolution per minute</td>
</tr>
<tr>
<td>SC</td>
<td>Schwann cell</td>
</tr>
<tr>
<td>S</td>
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<tr>
<td>V</td>
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<tr>
<td>TESPA</td>
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# Chapter 1

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1.1: General introduction

In the adult mammalian central nervous system (CNS) cut axons are not able to grow back along their former pathways to reach their original targets (Cajal, 1928). Studies of successful regeneration that occurs naturally in the brain of lower vertebrates and in the peripheral nervous system (PNS) of all vertebrates raise the question of whether similar regeneration could occur in the adult mammalian CNS. Experimental strategies aimed at promoting axonal regeneration and resolving functional deficits suggest that it may be possible to stimulate repair after spinal cord injury.

An average of two new cases of spinal cord injuries are admitted to hospitals every day in the UK (BBC news, 10th April 2002). The incurable effects of human spinal cord injury are due to disconnection of nerve fibres from their targets and failure of repair. This leads to a loss of ascending sensory information and a loss of descending motor control. The major goal of repair is to restore the transmission of information across the lesion site. Sufficient regeneration of cut damaged nerve fibres to restore function is required.

In the last century one of the principle aims of research has been trying to restore a damaged pathway so that it will allow regeneration, possibly by transplantation of glial cells. Originally, Aguayo’s group, following Cajal’s original suggestion (Cajal, 1928), showed that peripheral glia such as Schwann cells (SCs) can attract the sprouting ends of cut central axons and allow them to grow for considerable distances (Richardson et al., 1980; Bray et al., 1987).

Since the observation of Graziadei and others, it has become known that olfactory neurons are continually formed throughout adult life (Graziadei and Montigraziadei, 1980). The olfactory nerves contain a unique type of ensheathing cell (the olfactory ensheathing cells, OECs), which have been shown to promote axonal growth and functional improvements in
different spinal cord injury models (Li et al., 1997, 1998; Ramón-Cueto et al., 1998, 2000). It was shown that these types of cells migrated in alignment with the glial cells of the host tract. OEC repressed axon branching, induced rapid growth of axons straight across the lesion and into the distal part of the CST (Li et al., 1998). It was shown that this regeneration could restore lost function such as directed forepaw retrieval (DFR) (Li et al., 1997).

While encouraging, the potential value of such transplants for human spinal cord injuries will have to take into account the considerable delay which will inevitably occur between the time of injury and the time when transplantation could be considered. A recent study by Lu et al. (2002) has reported an improvement of locomotor function after delayed transplantation of OECs into complete thoracic spinal lesion. These observations could be a breakthrough that ultimately leads to new treatments for people who have been paralysed by damage to their spinal cord.

The aim of this study was to investigate the potential of OECs to stimulate axon regeneration when transplanted 8 weeks after complete unilateral CST lesion in the spinal cord. The functional recovery of the animals was assessed by DFR.

In chapter 1, the reasons for the inability of the CNS to regenerate are discussed. Particular attention is given to the cellular events that follow spinal cord injury. The background to OECs and their potential therapeutic use in human spinal cord injuries is also discussed. A review of the literature concerning the transplantation of OECs as a potential future method to repair human spinal cord injuries is given.
Chapter 2 describes the techniques used in this study including microsurgery, immunohistochemistry, electron microscopy and behavioural testing. In chapter 3, the results of this study are presented. A detailed discussion is presented in chapter 4.

1.2: Overview of nervous system

The nervous system is divided into two main parts: the CNS, which consists of the brain and the spinal cord, and the PNS, which is composed of ganglia and peripheral nerves that lie outside the brain and spinal cord. These two systems are functionally connected.

1.2.1: The peripheral nervous system

The PNS consists of nerve endings, peripheral nerve trunks and ganglia, which link the CNS with other parts of the body. Many of the neurons in the PNS are either afferent or efferent with respect to the CNS. The PNS has two divisions, somatic and autonomic. Nerve endings may be classified as either sensory or effector. Sensory endings respond to mechanical, thermal or chemical stimulation, the nerve fibres to which they belong conduct action potentials to the CNS. Effector endings contact muscle or sensory cells and, under control from the CNS, produce movement or influence secretion. The somatic system provides sensory information to the CNS about muscle and limb position. The autonomic pathway is divided into the sympathetic and the parasympathetic nervous system. The autonomic divisions of the PNS consist of the motor system for the viscera, the smooth muscles of the body, and exocrine glands. In general, activation of the sympathetic system prepares the body for intense muscular activity; it is called 'the fight and flight' reaction. Whereas, parasympathetic activity is concerned primarily with mechanisms responsible for maintaining resting bodily functions, such as reducing heart rate and promotion of digestive activities.
1.2.2: The central nervous system

The CNS consists of six major divisions: the spinal cord, the medulla, the pons and cerebellum, the midbrain, the diencephalon and the cerebral hemispheres. The CNS contains three types of glia: astrocytes, oligodendrocytes and microglia.

The spinal cord is surrounded by three meninges: the dura mater, the arachnoid mater, and the pia mater. Further protection is provided by the cerebrospinal fluid. The spinal cord is composed of grey matter, which contains the cell bodies and dendrites of neurons and glial cells, and white matter, which consists of axons and glia grouped into tracts. The grey matter is seen on cross section as an H-shaped pillar with anterior and posterior grey horns, joined by a thin grey commissure containing the small central canal. The white matter is divided into anterior, lateral, and posterior white columns. The spinal cord is divided into four major regions; each of them contains numerous segments. These are cervical, thoracic, lumber and sacral segments.

The spinal cord receives afferent fibres from sensory receptors of the trunk and limbs, control movements of the trunk and limbs and provide autonomies innervations for most of the viscera. In addition, extensive connections with the brain, through ascending and descending nerve fibre tracts, both convey afferent information to higher centres and mediate their controlling influence over spinal mechanisms.

The two major pathways in the spinal cord of rats are the ascending and descending pathways. The ascending pathway conducts information from sensory receptors in the trunk and limbs to the brain. The descending pathway transmits motor commands from the brain to peripheral muscles.
The ascending pathway consists of three major pathways: A) posterior funiculus, B) lateral funiculus and C) anterior funiculus. The posterior funiculus pathway has been subdivided into fasciculus gracilis and fasciculus cuneatus. These pathways convey information on two point discrimination, vibration and conscious proprioception from nerves in the dorsal root ganglion to the ipsilateral nucleus gracilis and nucleus cuneatus. The lateral funiculus has three subdivisions including: 1) lateral spinothalamic, which carries sensations of pain and temperature from nerve ending receptors throughout body. 2) Posterior spinocerebellar and cuneocerebellar, these tracts carry muscle movement information from muscle spindles and tendon organs to the cerebellum. 3) The spinocerebellar tracts carry postural information from muscle spindle and Golgi tendon organs relating to an entire limb to the cerebellum. The spinothalamic pathways carry sensation of poorly localized crude touch, temperature and pain.

Descending pathways have six major tracts including: A) lateral corticospinal, B) anterior corticospinal, C) rubrospinal, D) tectospinal, E) reticulospinal tracts and F) vestibulospinal. The corticospinal tract (CST) is the major descending pathway extending from cerebral cortex affecting both motor neurons and sensory interneurons in the spinal cord.

In this study, the major focus is on the CST pathway (the main reasons for choosing CST are explained in section 4.2). The CST is a long descending central pathway involved in motor control (Stanfield, 1992). The CST is a massive bundle of fibres containing about one million axons. About one third these originate from primary motor cortex located in the precentral gyrus of the frontal lobe (Brodmann’s area). Another third originates from premotor area rostral to Brodmann’s area. The remaining third originates in the somatic sensory cortex. The corticospinal fibres then course through the internal capsule to reach the ventral portion of the midbrain. As they descend through the pons the fibres separate into small bundles of fibres. The fibres then regroup in the medulla to form medullary
pyramid. At this junction about three-quarters of the fibres cross the midline in the pyramidal decussation. The crossed fibres descend in the dorsal part of the lateral columns of the spinal cord, forming the lateral CST. The uncrossed fibres descend in the ventral column as the ventral CST (figure 1.1).
The lateral and ventral CST pathway in human. Motor fibres of corticospinal tract begin in cerebral cortex, cross over in medulla oblongata, and descend in spinal cord. There, they synapse with neurons whose fibres lead to spinal nerves that supply skeletal muscles. The location of CST in human is located laterally and ventrally (this image originally appeared in Hole's Essentials of Human Anatomy and Physiology, 7th Edition).
The most common causes of lesions in the CST are vascular occlusion of the middle cerebral artery in the internal capsule or tumour, trauma and demyelinating diseases. In primates with damage to the CST the deficit can be seen mainly in fine finger control.

In the rat, most of the CST neurons are located in the primary motor cortex and in the forelimb and hind-limb parts of the primary sensory cortex. In the primary motor cortex, CST neurons are located in layer 5, whereas in the primary sensory cortex the neurons are restricted to layer 5b (Miller, 1987). CST axons leave the cerebral hemispheres by passing through the midbrain and pons. Then CST fibres reach the medulla oblongata, where they form two prominent columns on the ventral surface. These are called the pyramids and for this reasons the term pyramidal tract may be used as an alternative name for CST. In the caudal medulla the fibres of the pyramids undergo subtotal decussation. 95% of CST axons decussate in the caudal medulla and run in the base of the dorsal columns (Brown, 1971), but 5% of axons run in an uncrossed ventral tract in the ventral funiculus (Joosten et al., 1993) (see figure 1.2).

CST axons terminate in all spinal laminae contralateral to the cells of origin. The terminal field of the CST are densest in laminae 3-7 of the dorsal horn and less dense in the ventral horn (Brown, 1971; Antal, 1984; Liang et al., 1991).
Figure 1.2

The dorsal and ventral CST pathway in rat. The descending pathway of CST axons from the sensorimotor cortex to the nucleus ruber, to the basilar pontine nuclei, and to the first or second cervical spinal cord. The black areas on the cross section of spinal cord represents the dorsal and ventral CST. (Modified from Schwab et al., 1998).
The CST is particularly concerned with the control of voluntary, discrete, skilled movements, sometimes referred to as fractionated movements. In the rat the CST plays a role in the control of skilled movements of the forelimb and therefore in locomotor function. The CST is also involved in the control of distal and proximal limb movements (Whishaw and Kolb, 1988). It has been shown that after pyramidotomy in the rat targeting problems occurred during reaching attempts (Castro, 1972). The CST lesions induced a stronger and more permanent deficit in reaching and grasping as demonstrated by a reduction of success rate in different skilled and spontaneous reaching tests (Whishaw et al., 1998).

Dysfunction in grasp and digit use also occurs following CST damage in other mammalian species including hamster (Kalil and Schneider, 1975) and cats (Gorska and Sybriska, 1980) (for review, see Joosten, 1997). Thus a digit deficit is common with a lesion of the CST (Schrimsher and Reier, 1993). Investigation to gain a better understanding of the functional role of the CST before and after spinal cord injury demonstrated that unilateral CST lesions led to deficits in directed forepaw retrieval tasks in rats (Li et al., 1997). The results from these experiments suggest that the rat CST provides an excellent model to study injury as well as regeneration of the CNS in mammals.

1.3: The peripheral nervous system following injury

Damage to the PNS is frequently reversible. Injured axons in the PNS successfully grow and establish synaptic contacts with denervated targets (Ramón y Cajal, 1928; Fawcett and Keynes, 1990). Neurons are able to regenerate and eventually restore functional connections to their peripheral axons. In contrast, regeneration of lesioned axons in the CNS of adult mammals, including human, does not occur.
Schwann cells (SCs) are unique cellular components of peripheral nerve. When a peripheral nerve is injured, the axons distal to the damage degenerate, but the SC and their basal lamina remain. It is also important to mention that SCs produce a number of trophic factors including nerve growth factor (NGF; Bandtlow et al., 1987; Heumann et al., 1987) and brain-derived neurotrophic factor (BDNF; Meyer et al., 1992) (see review Joosten, 1997). The immediate reaction of the peripheral nerve to damage is the recruitment of circulating macrophages to the site of lesion. The degenerated axons and myelin debris are removed over a period of 1-2 weeks. In addition to phagocytosing myelin debris, macrophages stimulate the proliferation of SCs. In vitro it has been shown that macrophages that have phagocytosed myelin produce a conditioned medium that is mitogenic for triggering change in SC behaviour, and in vivo macrophages begin to accumulate in the distal stump just before the period of SC proliferation (Perry et al., 1987). The SCs can secrete extracellular proteins such as laminin (Bunge et al., 1986; Bunge, 1993), which is known to modulate neurite outgrowth. All these components have been suggested to play a role in supporting neuronal survival and axonal regeneration.

1.4: The central nervous system following injury

Injuries to the CNS are particularly traumatic owing to the limited capacity of the CNS to repair itself. In the case of spinal cord, the distribution of nerve bundles that convey ascending sensory and descending motor information make the consequences of spinal cord injury especially devastating. Transection results in persistent sensory and motor dysfunctions for all body parts below the lesion site.

The highest rate of injury in Europe and North America occurs between the ages of 16 to 30. The causes are multiple in United States, about 40% are due to motor vehicle accidents, 25% are due to violence-related, 20% injuries to falls and 5-10% are due to sport-related accidents.
Spinal cord injuries can be classified as complete and incomplete. In majority of cases (approximately 55% in Europe and the United States), patients suffer from functionally incomplete spinal cord injuries. Immediately after injury most patients will have symptoms and signs of complete spinal cord transection. However in about one-third of patients, a proportion of the lost functions will return in the following weeks. The full extent of functional recovery can take between months to years to develop. This is due to the resolution of the oedema and inflammation affecting the surviving part of the partially transected cord. Complete injuries of the spinal cord can result in immediate loss of sensation and all voluntary movements below the lesion. If the lesion is at or above the C3 level, breathing can be affected. Lesions in the CST in humans can cause rapid and repeated contraction and relaxation in muscles that are important for precision movements, such as fine movements of the hands.

The diagram (figure 1.3) shows the effect and deficit of spinal cord injuries at different levels in human.

The incurable effects of human spinal cord injury are due to the disconnection of nerve fibres from their targets. In complete spinal cord transection, there is a total loss of ascending sensory information and a loss of descending motor control. In addition, abnormal behaviour patterns or sympathetic responses emerge as a result of the operation of spinal reflexes in an uncontrolled or exaggerated fashion. The major goal of repair (as opposed to palliative/rehabilitative measures) is to restore the transmission of information across the lesion site.
A schematic diagram shows the deficits and impairments might be caused due to spinal cord injury at different levels of human. (Adapted from www.spinalinjury.net)
1.5: Brief overview of axonal and glial responses after spinal cord injury

Damage to the CNS initiates a series of cellular and molecular events that evolve over several days and weeks. The main cell types involved in these changes are astrocytes, microglia, oligodendrocyte precursors and SCs (figure 1.4). The main cellular changes are briefly described in this section.
Figure 1.4

A schematic diagram summarizing the main events after long-term survival and unilateral spinal cord injury in adult rat. The zone of astrocytic scarring (ast) is coextensive with the zone of infiltration by Schwann cells (scw). Both cut and uncut axons passing from the areas of oligodendrocytes (oli), myelination (sheaths are shown grey) acquires peripheral type; Schwann cell myelin sheaths (shown in red). Both cut and uncut axons emit fine sprouts into a macrophage-filled central lesion area (mac). ML, midline and G, grey matter (Modified from Li and Raisman, 1995).
1.5.1: Preferential loss of oligodendrocytes

Due to injury the myelin sheaths are damaged and the oligodendrocytes die. Myelin debris is slowly removed by macrophages and microglia, but this may take several weeks (Li et al., 1999).

It has been shown that oligodendrocytes are selectively vulnerable to excitotoxic killing by glutamate (McDonald et al., 1998; Oka et al., 1993), which could be released from damaged axons.

1.5.2: Microglia and macrophages

Microglia in the undamaged brain have short branches. Following injury they exhibit various behaviours including activation, cell division, and migration to the site of injury. Immediately after the lesion there is an influx of microglial cells, which become more macrophage-like with time.

1.5.3: Astrocytes

In uninjured brain, astrocytes have a characteristic morphology. Their processes contact blood vessels and are interwoven around neuronal cell bodies. The initial event following injury is the death of the astrocytes. The astrocytes are identified by anti glial fibrillary acidic protein (GFAP) antibody. The astrocytes that are near to the site of injury are involved in making a glial scar. The scar consists of tightly packed, hyperfilamentous astrocytes, with many of their processes tightly apposed to one another with limited extracellular space, and many gap and tight junctions. It was shown that destroying one side of the CST in adult rat could cause obvious change in astrocytic arrangements (Li and Raisman, 1995). From 7 to 13 weeks following the injury in spinal cord, the astrocytes reach a point where the central area is completely walled off from the surrounding tissue.
by a solid mass of intensely GFAP-immunoreactive astrocytes and their processes (Li and Raisman, 1995).

1.5.4: Involvement of Schwann cells

SCs are the type of glia which myelinate the axons in the PNS. Under normal situations, SCs are not present in the CNS. However, after lesions in human spinal cord (Adelman & Aronson, 1972) and experimental animals (Brook et al., 1998; Gilmore & Sims, 1993; Li & Raisman, 1995; Olby & Blakemore, 1996), SCs invade the surrounding area of lesion in the spinal cord.

After spinal cord injuries in animal models, endogenous SCs respond after 10-14 days (Li & Raisman, 1999). The immigrant SCs are only found in the reactive area around the lesion. They do not enter the central lesion area of total tissue destruction, or the adjacent undamaged grey or white matter of the spinal cord.

It has been documented that the migration of endogenous SCs into the CNS is also induced by chemotoxic demyelinating lesions (Blakemore, 1976 and 1989), or by kainate lesions of the thalamus (Dusart et al., 1992).

The ingrowth of SCs after CNS injury might be due to two possible types of signals, A) the presence of myelin degeneration and B) the presence of exposed un-myelinated wide diameter axons (Li et al., 1999).

1.5.5: Persistence of cut axons and sprouts

Studies of electrolytic lesions in the CST of adult rats have shown that not only cut axons, but also uncut axons produce sprouts in the region of the lesion after the second postoperative week (Li et al., 1994). It was also reported that by increasing the survival
time after the lesion, all the sprouting axons remained in the lesioned area and the astrocytic scar was separated from the injury site by healthy tissue. A similar persistence was described by Fishman and Kelly (1984), who reported corticospinal axons had the ability to sprout even 7 months after a spinal cord lesion.

Although spontaneous regeneration of lesioned fibres is limited in the adult CNS, many people that suffer from incomplete spinal cord injuries show significant functional recovery (Burns et al., 1997). This recovery process can go on for several years after injury and depends on the reorganization of the circuits that have been spared by the lesion (Schwab and Raineteau, 2001). After incomplete lesions, the reorganization might occur at two levels: in pre-exciting circuits by modification of synaptic strength, or by the appearance of new circuits through sprouting (Schwab and Raineteau, 2001). Remyelination may also contribute to recovery of function.

1.6: Failure of axon regeneration in central nervous system after injury

Unlike injuries to the PNS, damage to the CNS is severe and irreversible. Early in neural development the extracellular matrix in both the CNS and PNS contains glycoproteins that are effective in promoting axon growth. However, with maturation important differences are observed. Two proteins, namely laminin (LN) and fibronectin (FN) are present in the PNS and are postulated to play a role in regeneration after injury. The CNS lacks these important proteins that are required for regeneration after injury.

Possible reasons for the abortive regenerative responses in adult mammalian CNS may be summarised as:

- Death of the injured neurons and oligodendrocytes (see section 1.6.1 below),
• Progressively dense astrocytic scar that acts as a barrier to axon growth (Reier et al., 1986) (see section 1.6.2 below), and

• Lack of supportive environment for such growth. Inability of the CNS environment to support growth and promote long-term survival appears to be due to both the presence of inhibitory molecules and a lack of growth-promoting signals (Schwab et al., 1990) (see section 1.6.3 below).

### 1.6.1: Death of Oligodendrocytes

The loss of oligodendrocytes following injury has already been mentioned in section 1.5.1. Previous studies have shown that after electrolytic lesions in the rat CST, the primary event in the peri-lesional reactive area is death of oligodendrocytes, with resorption of their myelin sheaths (Li et al., 1999). There is little known of the molecular mechanisms that trigger oligodendrocytes death.

### 1.6.2: Production of astrocytic scar around the lesion

According to this theory, the major cause for the failure of axon regeneration in the CNS is the inhibitory nature of the glial environment. One of the changes occurring in lesions of the spinal cord tract is a progressively denser and spreading astrocytic hypertrophy, resulting in a scar around the lesion (Reier et al., 1986).

Astrocytes have two roles in response to CNS injury which are as follows: a) astrocytic processes invade large regions of extra-cellular space produced by progressive degeneration of the neuronal and glial elements (Vaughan et al., 1970; Vaughan and Pease, 1970; Raisman, 1977), and b) astrocytes act as a barrier to the CNS cellular environment (Berry, 1979; Reier et al., 1983a; Mathewson and Berry, 1985) (see review Fawcett and Asher, 1999).
Over the years, there have been at least two fundamental ways that astrocytes have been viewed in relation to the problem of CNS regeneration: a) they form obstructive physical barriers to elongating axons; b) they lack the appropriate trophic, or growth-promoting properties conducive to axonal outgrowth (Reier et al., 1984). Different types of experiments *in vivo* and *in vitro* have shown that astrocytes can have an inhibitory effect on axon regeneration (Anders & Hurlock, 1996; Davies et al., 1997; Fawcett et al., 1989; Reier et al., 1988; Fitch and Sliver, 1999) (see review Fawcett and Asher, 1999).

At the site of CNS injury, the glial scar contains extracellular matrix molecules including chondroitin sulphate proteoglycans (CSPGs). There is up regulation of the amount of CSPG around the lesion starting after 4 or 5 days and it remains high for many weeks (Bradbury et al.; 2002; Fawcett et al., 1999; Fitch & Silver, 1999).

1.6.3: The presence of inhibitory molecules

It is now well established that axons of the adult CNS are not capable of regeneration after injury. Changes in the cellular environment and in the neuronal response to the environment are both responsible for the absence of regeneration by mature axons (Li and Raisman, 1993; Schwab and Bartholdi, 1996). Much of the axon growth inhibitory activity in the CNS is associated with myelin, and a number of individual proteins that inhibit axon growth, have been identified.

The myelin-associated molecules that might prevent axon regeneration in damaged CNS are as follows:

- NI250/nogo (see section 1.6.3.1 below)
- Myelin-associated glycoprotein (MAG) (see section 1.6.3.2)
- Oligodendrocyte-myelin glycoprotein (OMgp) (see section 1.6.3.3)
1.6.3.1: NI250/nogo

There are three different isoforms of nogos that exist. Nogo-A is localized to CNS myelin and is highly expressed by oligodendrocytes but not SCs. Nogo-B and -C are found in certain neurons and several non-neural tissues, although their functions are unknown (Schnell & Schwab, 1993; Schwab et al., 1993). Nogo-A is present immediately after injury where the damage has actually disrupted myelinated axons. The 66 amino acid surface loop of Nogo is inhibitory for axonal growth in culture and acts via a Nogo-66 receptor (NgR) expressed by neurons and localized to their axons in vitro (GrandPre et al., 2000; Fournier et al., 2001). The growth cone collapsing activity is mediated via a calcium-dependent mechanism (Bandtlow et al., 1993).

The expression of Nogo in oligodendrocytes but not in SCs may contribute to the failure of axonal regeneration in the adult mammalian CNS as opposed to the adult PNS (Wang et al., 2002).

1.6.3.2: Myelin-associated glycoprotein (MAG)

When oligodendrocytes differentiate and begin to myelinate CNS axons they synthesize glycoproteins that actively repress axon outgrowth.

MAG switches from promotion to inhibition during development (Mukhopadhay et al., 1994). It was shown that elevating endogenous levels of cyclic adenosine monophosphate pathway (cAMP) effectively blocked the inhibition of axonal regeneration by MAG (Cai et al., 1999). It was also shown that MAG inhibition could be converted to attraction by elevating neuronal cAMP, and attraction can be converted to inhibition by blocking a downstream effector of cAMP, protein kinase A (PKA; Mukhopadhay et al., 1994). Importantly, developmental plasticity of spinal tract axons in neonatal rat pups in vivo is dramatically reduced by inhibition of PKA (Cai et al., 2001). Thus, the switch from
promotion to inhibition by MAG, which marks the developmental loss of regenerative capacity, is associated with a developmentally regulated decrease in endogenous neuronal cAMP levels.

1.6.3.3: Oligodendrocyte-myelin glycoprotein (OMgp)

OMgp is the most recently identified of the inhibitory components of myelin (Wang et al., 2002). The inhibitory activity of OMgp in in vitro experiments appears to be as potent as that of MAG (Mukhopadhay et al., 1994) and Nogo-A (Chen et al., 2000). All three proteins have a similar distribution in the myelin sheaths, suggesting that all are likely to contribute to growth inhibition in the adult CNS. It has been suggested that OMgp acts through a Nogo receptor (Fournier et. al., 2001) and its associated receptor complex. Interfering with the OMgp and Nogo-receptor pathway, may allow lesioned axons to regenerate after injury following in vivo situations (Wang et al., 2002).

1.7: Different possible ways of repairing the central nervous system after injury

Successful regeneration depends upon the ability of injured axons to survive, re-grow, and reconnect with their original targets. As described above, the capacity for intrinsic repair after mammalian CNS injury is limited because the mature CNS can neither generate new neurons nor initiate substantive functional axonal regenerative responses to damage. One of the principle aims of regenerative research for the last century or more has been to attempt to restore the damaged neuronal pathway to a state, which will allow regeneration.

A summary of the in vivo and in vitro studies (table 1.1) reveals that the associated studies published so far contain a number of variable experimental parameters such as the...
composition of the transplant, its species origin, the lesion model system used and the test for the determination of functional recovery.
Table 1.1

Summary of different approaches used for regeneration in adult spinal cord. BDNF, brain derived neurotrophic factor; SC, Schwann cells; mAB, monoclonal antibody; CST, corticospinal tract; CSPG, chondroitin sulphate proteoglycan; ChABC, chondroitinase.

<table>
<thead>
<tr>
<th>Reference</th>
<th>System</th>
<th>Approach</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ramón y Cajal, 1928; David &amp; Aguayo, 1981</td>
<td>In vivo adult rat Spinal cord transection</td>
<td>Transplantation of peripheral nerve into injured spinal cord</td>
<td>Induce sprouting of cut CNS axons</td>
</tr>
<tr>
<td>Li and Raisman, 1994</td>
<td>In vivo adult rat CST lesion</td>
<td>Transplantation of cultured SCs into CST lesion</td>
<td>Induce sprouting of cut CNS axons and myelinated axons are formed</td>
</tr>
<tr>
<td>Li et al., 1997</td>
<td>In vivo adult rat CST lesion</td>
<td>Transplantation of OEC into CST lesion</td>
<td>Induce sprouting and can leave the transplant area and myelinated axons are formed</td>
</tr>
<tr>
<td>Reier et al., 1986; Jakeman &amp; Reier, 1991</td>
<td>In vivo adult rat Spinal cord transection</td>
<td>Embryonic graft into CST</td>
<td>Can establish a short-range intersegmental circuitry in the spinal cord</td>
</tr>
<tr>
<td>Schwab, 1988</td>
<td>In vitro adult rat</td>
<td>CNS myelin contains defined neurite growth inhibitory proteins (NI-35/250). Antibody (mAB IN-1) was raised against NI250</td>
<td>Myelin becomes a more permissive environment</td>
</tr>
<tr>
<td>Schnell et al., 1994</td>
<td>In vivo adult rat Spinal cord transection</td>
<td>Introducing NT-3 and BDNF neurotrophic factors into CST</td>
<td>The neurotrophic factors can enhance CST sprouting</td>
</tr>
<tr>
<td>Cai et al., 2001</td>
<td>In vivo neonatal rat pups with hemi-section spinal cord</td>
<td>Inhibiting a down-stream effector of cAMP</td>
<td>cAMP controls the developmental loss in ability of axons to regenerate</td>
</tr>
<tr>
<td>Reference</td>
<td>System</td>
<td>Approach</td>
<td>Results</td>
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<tr>
<td>Guest et al., 1997</td>
<td><em>In vivo</em> adult rat</td>
<td>Pieces of peripheral nerve plus acidic fibroblast growth factor into lesioned CST</td>
<td>An improvement in hind limb function.</td>
</tr>
<tr>
<td>Wang et al., 2002</td>
<td><em>In vivo</em> adult rat</td>
<td>Interfering with the OMgp and Nogo-receptor</td>
<td>Lesioned axons to regenerate after injury</td>
</tr>
<tr>
<td>Schnell &amp; Schwab, 1993</td>
<td><em>In vivo</em> adult rat</td>
<td>Application of IN-1 antibody to lesioned spinal cord</td>
<td>Neutralization of the neurite outgrowth inhibitory proteins and led to long distance re-growth of CST axons</td>
</tr>
<tr>
<td>Boyd et al., 2004</td>
<td><em>In vivo</em> adult rat</td>
<td>Application embryonic OECs infected by LacZ retrovirus</td>
<td>Labelled OECs made no direct contact with axons and there were no myelination in the transplant</td>
</tr>
<tr>
<td>Lu et al., 2001 &amp; 2002</td>
<td><em>In vivo</em> adult rat</td>
<td>Transplanting cells cultured from olfactory mucosa</td>
<td>Olfactory mucosa cells are able to induce functionally useful regeneration of adult axons</td>
</tr>
<tr>
<td>Bradbury et al., 2002</td>
<td><em>In vivo</em> adult rat</td>
<td>Blocking the inhibitory activity of CSPG by ABC</td>
<td>Promoted regeneration of both ascending and descending CST axons</td>
</tr>
<tr>
<td>Priestly et al., 2002</td>
<td><em>In vivo</em> dorsal roots injury</td>
<td>Adding NT-3 into the lesion and synthetic fibronectin conduits implanted into the lesion</td>
<td>The damaged axons become stimulated by NT-3 and the fibronectin mats supported the axons become myelinated by Schwann cells</td>
</tr>
</tbody>
</table>
1.7.1: Transplantation of peripheral nerve

The PNS has the ability to regenerate after injury (Ramón y Cajal, 1928; Fawcett and Keynes, 1990; Son and Thompson, 1996). It has been reported that peripheral nerve grafts are able to induce growth of cut central axons and can be used to direct the growing sprouts to the terminal field (Berry et al., 1988; Campbell et al., 1992).

In 1996, Cheng and his colleagues developed a multiple peripheral nerve approach in combination with other strategies to enhance regeneration in the completely transected spinal cord of the adult rat. They transplanted 18 pieces of peripheral nerve into a 3-5 mm gap in rat spinal cord (Cheng et al., 1996). The peripheral nerves were collected from intercostal nerves from the same animal. The pieces were stabilized into the lesion with fibrin glue and a growth factor called acidic fibroblast growth factor, which has been reported to prevent lesioned CST axons from dying, was fed into the graft (Guest et al., 1997). According to their report on a single rat (which has never been repeated) there was an improvement in hind limb function.

To extend the principle of peripheral nerve grafting, SCs have been isolated from peripheral nerves (Bunge, 1975).

1.7.2: Transplantation of cultured Schwann cells

Following spinal cord injury, a gap is created that cannot be crossed by regenerating axons unless a bridge between both cord stumps is provided. Successful repair would require neurons to use the bridge containing a supportive substrate for regeneration. These bridges can be tubes containing cells such as SCs, OEC or olfactory mucosa. SCs play a major role in the regenerative capacity of peripheral nerves (Fawcett and Keynes, 1990).
The preparation of SCs using tissue culture techniques may favourably influence repair of CNS injuries (Kromer and Cornbrooks, 1985). Such SC population are generally prepared from prenatal or neonatal peripheral nerves (Wood, 1976; Brockes et al., 1979). Several researchers have investigated the transplantation of cultured SCs (Wrathall et al., 1984; Kromer and Cornbrooks, 1985; Kuhlengel et al., 1990; Martin et al., 1991, 1993; Painó and Bunge, 1991 Montero-Menei et al., 1992; Neuberger et al., 1992; Li and Raisman, 1994).

Li and Raisman, (1994) injected SCs into a circumscribed lesion in the CST (Li and Raisman, 1994). From day four after transplantation, a central mass of p75 (low affinity neurotrophin receptor) immuno-positive SCs and cuffs of SCs along the perivascular space of blood vessels was shown. Six weeks after transplantation, p75 immunoreactivity had been down regulated, but P0 immunostaining revealed that the SCs were still present and they had been myelinating the host CST in the transplant region. These results indicated that SCs were intimately integrated into the myelinated adult host CST where they induced local sprouting of the cut and uncut CST fibres. However, it was clearly observed that cut sprouting axons were unable to extend sufficiently along the SC graft.

In addition to transplantation of rat SCs from adult nerves, SCs dissociated, expanded and purified from adult human donors have been transplanted into the injured spinal cord of an immunodeficient (‘nude’) rat (Guest et al., 1997a, b). It was shown that there are a number of axons found in the graft and that they were also myelinated (Guest et al., 1997a). Behavioural tests (Basso et al., 1995) were carried out and it was observed that there was a modest but statistically significant improvement in functional recovery.

In a study by Bunge, (1995) it was shown that the ability of SCs seeded into semi permeable guidance channels promoted both motor and sensory axonal regeneration in the adult rat thoracic spinal cord (Bunge et al., 1995).
In 2002 Takami et al., transplanted cultured rat purified SCs, or purified OEC or both, into spinal cord of an adult rat with T9 contusion injury. It was reported that SC grafts contained more myelinated axons than SC/OEC or OEC alone. The results indicated that a SC graft is more effective in preserving spinal tissue sparing than OECs alone. It was also shown there was a significant improvement in hindlimb locomotor performance in animals with SC grafts. In these studies the evidence that the improved function is due to fibre regeneration is not convincing.

1.7.3: Transplantation of embryonic neurons

The strategy of grafting into injured adult spinal cord has two possible benefits: (a) acting as a bridge, allowing regenerative axons to cross the lesion and grow directly into intact spinal cord segment and b) rescuing axotomized neurons in the spinal cord and brain (Bregman et al., 1993).

Akesson and Kjaldgaard (1998) reported that solid pieces of human embryonic spinal cord can minimize the cystic deformations following surgically induced cavity lesions in the rat adult spinal cord.

Bregman and McAtee (1993) designed an experiment to determine if the differences in the magnitude of the anatomical plasticity of host pathways in the presence of transplants is reflected in differences in recovery of function between neonatal and adult. Newborn and adult rats received a midthoracic "overhemisection." Immediately following the hemisection embryonic (E14) spinal cord transplants were placed into the lesion site. The growth of descending pathways into the transplants was substantially greater in density and spatial extent after lesions at birth than at maturity. Fetal spinal cord transplants promoted recovery of motor function in both newborn and adult operates.
Coumans et al. (2001) investigated the axonal regeneration and functional recovery after complete spinal cord transection in rats by delayed treatment with transplants and neurotrophins. Fetal spinal cord transplants and neurotrophins were used to influence axonal regeneration in the adult rat after complete spinal cord transection at a midthoracic level. Transplants were placed into the lesion cavity either immediately after transection (acute injury) or after a 2-4 week delay, and either vehicle or neurotrophic factors were administered exogenously via an implanted minipump. Regeneration from supraspinal pathways and recovery of motor function were increased when transplants and neurotrophins were delayed for 2-4 weeks after transection rather than applied acutely.

1.7.4: Transplantation of neural stem cells

The term neural stem cell is used to describe cells that 1) can generate neural tissue or are derived from the nervous system, 2) have some capacity for self-renewal and 3) can give rise through asymmetric cell division into different cell types including; neurons, astrocytes and oligodendrocytes (Reynolds and Weiss, 1996).

Neural stem cells exist not only in the developing mammalian nervous system but also in the adult nervous system of all mammalian organisms, including humans. The vast majority of cells in the nervous system are generated during the embryonic and early postnatal stages, but new neurons are continuously added in certain regions of the adult brain (Altman and Das, 1965).

Neural stem cells can be isolated from the walls of the ventricular system of the adult CNS as well as from the dentate gyrus of the hippocampus (Lois and Alvarez, 1993; Morshead et al., 1994; Weiss et al., 1996; Palmer et al., 1997). Stem cells in the subventricular layer give rise to immature neurons that migrate along the rostral migratory stream to the
olfactory bulb, where they differentiate and integrate as interneurons (Luskin, 1993; Lois and Alvarez-Buylla, 1994).

Potential uses of stem cells in repair include transplantation to repair missing cells and activation of endogenous cells to provide self-repair. Over the last few years, there has been progress with the more established paradigms, in which neural stem cells are transplanted into experimentally lesioned animals, in order to assay their capacity for repair.

McDonald and his colleges in 1999 have studied the effect of delayed transplantation of neural differentiated mouse embryonic stem cells into a rat with traumatic spinal cord injury. Histological analysis 2-5 weeks later showed that transplant-derived cells survived and differentiated into astrocytes, oligodendrocytes and neurons and migrated as far as 8 mm away from the lesion edge. Furthermore, analysis of gait (beam walking) demonstrated that the transplanted rats showed hind-limb weight support and partial hind-limb coordination compared to the control rats.

1.7.5: Blocking inhibitory molecules

As it was mentioned in section 1.6.3, there are different types of myelin-associated molecules, which might prevent axon regeneration in the damaged CNS. One of these molecules is called Nogo-A, which is expressed by oligodendrocytes (CNS myelin) and it is believed that these molecules have contributed to the failure of axonal regeneration in the adult CNS (Schnell & Schwab, 1993; Schwab et al., 1993).

In vivo studies showed that preventing myelin formation or using a monoclonal antibody (mAb IN-1) that neutralized the activity of myelin protein Nogo-A (Schwab et al., 1988,
Chen et al., 2000) promoted regeneration of corticospinal fibres in the lesioned spinal cords of adult rats (Schwab and Bartholdi, 1996; Schnell & Schwab, 1990).

Current efforts are aimed at blocking Nogo-A as well as other growth-inhibitory molecules by: a) *in vivo* application of antibodies against these molecules; b) blocking the receptors to which they bind; or c) pharmacologically manipulating the downstream signalling pathways induced by these inhibitory signals in growing neurites (Moore, 2001; Pantev et al., 1998). One way to deliver these agents is using a subcutaneous pump that injects antibodies and other blocking agents directly into the cerebrospinal fluid that bathes the spinal cord.

Several experiments using the antibody IN-1 showed successful long distance regeneration of lesioned axons in adult rat CNS. This suggests that Nogo-A plays an important role in restricting spontaneously occurring regeneration.

In a study by Hunt et al. (2002) it was shown that NgR expression was weakly expressed in spinal cord neurons, but in other parts of CNS i.e. cerebral deep nuclei it showed higher expression. In this study it was suggested that Nogo-66 and NgR are not the only reason behind the absence of regeneration in CNS.

As it was mentioned in section 1.6.2 CSPGs are inhibitory to axon growth *in vitro* (Silver et al., 1991), and regenerating axons stop at the CSPG-rich region *in vivo* (Davies et al., 1999). The inhibitory activity of CSPG is through the actions of its protein core, the glycosaminoglycan (GAG) chain. The inhibitory CSPG can be removed both by digesting away the GAG chains with chondroitinase, and by removing the strong negative charge of GAG chains (Bradbury et al., 2002; McKeon et al., 1995).
Bradbury et al., (2002) have showed the functional effect of the chondroitinase (ChABC) after spinal cord injury. To test the functional effects of degrading chondroitin sulphate (CS)-GAG after spinal cord injury, chondroitinase ABC (ChABC) was delivered to the lesioned dorsal columns of adult rats. It was showed that intrathecal treatment with ChABC degraded CS-GAG at the injury site, unregulated a regeneration-associated protein in injured neurons, and promoted regeneration of both ascending sensory projections and descending corticospinal tract axons. Their results demonstrated that CSPGs are important inhibitory molecules in vivo and suggested that their manipulation will be useful for treatment of human spinal injuries.

1.7.6: Application of neurotrophic factors

It is well known that neurotrophic factors play important roles in neurite outgrowth and guidance as well as cell survival (for reviews see Tessier-Lavigne and Goodman, 1996). There is evidence that neurotrophic factor NT-3 can enhance corticospinal axons sprouting in the spinal cord (Schnell et al., 1994) and may be involved in mediating target innervation during development (Yee and O’Leary, 1996).

Schnell et al. (1994) investigated the effect of injecting either NT-3 or its neurotrophin family member BDNF or NGF on regenerative sprouting of transected CST fibres in adult rat spinal cord. Injection of NT-3 or NGF into lesioned spinal cord just rostral to the lesion gap increased the regenerative sprouting of the transected CST fibres. From these results it was concluded that exogenously applied NGF shifted the re-growth of CST axons to a more dorsal location in the spinal cord. It was shown that there was no re-growth of labelled CST fibres in areas caudal to the lesion (Femandaz et al., 1993). It was suggested that the balance between permissive and inhibitory factors is crucial for the regeneration of injured CST fibres in the adult spinal cord.
It was found that NT-3 supports the functional regeneration of sensory axons into the adult dorsal root after injury (Ramer et al., 2000 & Priestley et al., 2002). In 2001, it was shown by the same group that NT-3 promotes up regulation of the growth-associated protein (GAP-43) in large diameter sensory axons when it is delivered at the time of injury in the dorsal root entry zone (DREZ). It was also shown that when the NT-3 treatment was delayed for one week after injury in the DREZ, axons regenerate only a short distance across the DREZ compared to immediate treatment (Ramer et al., 2001). The explanation for short in-growth of axons after delayed NT-3 treatment was that it was mainly due to axons encountering degenerated myelin.

In addition to various members of the neurotrophin family, there are different neuro­peptides such as melanotropin (α-MSH) and corticotropin (ACTH), which causes stimulation or re-growth of lesioned axons in adult rat spinal cord (Joosten et al., 1995).

It remains to be seen whether sprouting of axons after administration of neurotrophic factors can lead to functional recovery after spinal cord injury. However, it has been shown that in traumatized adult rat spinal cord, the systemic application of α-MSH during the first weeks after compression injury led to a small but significant neurological and electrophysiological improvement in spinal cord function (Van de Meent et al., 1997).

1.7.7: Preventing the astrocytic scar around the lesion

As mentioned above (section 1.6.2) the astrocytic scar acts as a dense barrier which cut axons cannot cross (Li et al., 1995). It has been reported that in animals with olfactory glia transplanted into the lesion, CST fibres could cross the astrocytic scar (Ramon-Cueto et al., 2000; Li and Raisman, 1997).
1.7.8: Transplantation of olfactory ensheathing cells

Through work by Graziadei and others, it has become known that olfactory neurons are continually formed throughout life (Moulton, 1974; Barber, Raisman, 1978; Wilson, Raisman, 1981; Graziadei & Montigraziadei, 1974). This has led to the view that olfactory neurons are in a state of continual turnover (Hinds and McNelly, 1981), and as a result, olfactory nerves are in a state of high turnover, and able to regenerate into the olfactory bulb (i.e. the CNS) throughout adult life. Thus the glial cells of the adult olfactory nerves are permissive to continuous axon growth.

This observation is central to the design of the research presented here. In section 1.8, the anatomy of an olfactory bulb is given.

1.7.9: Transplantation of rat mucosal OEC

The potential therapeutic role of OECs extracted directly from the olfactory bulb in the repair of the injured spinal cord has been highlighted already. OECs appear to provide a highly favourable substrate for axonal regeneration, partly because they secrete extracellular matrix molecules and neurotrophic factors. The ability of OECs to migrate and overcome the astrocytic scar and integrate into the normal host spinal cord tissue is important. But surgery to remove an olfactory bulb in humans for autografting is not attractive. Another possible source of OECs, which has come under recent scrutiny, is the olfactory mucosa in the nasal lining. Here OECs ensheaths the olfactory sensory axons of the first cranial nerve along its entire length from its origin in the olfactory mucosa through the lamina propria and into the cranium via the cribriform plate. Lu and colleagues have identified that lamina propria resides immediately beneath the olfactory epithelial layer of the mucosa (Lu et al., 2002).
Biopsy of human olfactory mucosa is relatively straightforward and has been carried out under local anaesthesia (Féron et al., 1998, 1999). In the rat, the lamina propria has been separated from the overlying epithelium, and OECs were grown in vitro (Féron et al., 1999). Lu demonstrated in 2002 that transplantation of OECs from nasal olfactory mucosa promote axonal regeneration after complete transection of the spinal cord in adult rat. In that study it was shown that olfactory lamina propria grafts could promote functional recovery even when the initial transection was made 4 weeks previously (Lu et al., 2002). These investigators demonstrated that OEC-containing tissue from the mucosa has regenerative properties similar to those of cultured OECs obtained from the bulb.

1.8: The olfactory system

Newly formed olfactory nerve fibres leave the olfactory receptors situated in the olfactory mucosa in the region of nasal cavity and pass through the cribriform plate and form terminals in the olfactory bulb in the CNS. There are five major layers in the olfactory bulb and each layer has a special cell type. I shall discuss each layer separately (see figure 1.5).

1.8.1: Olfactory nerve layer

The most superficial layer of the olfactory bulb is the olfactory nerve layer, which contains axons from the primary olfactory neurons and glial cells. The olfactory nerves contain not only nerve fibres, but also a special type of glial cell, which later came to be called OEC (Ramón-Cueto et al., 1993; Raisman, 1985; Doucette, Devon, 1993).

1.8.2: Glomerular layer

Immediately under the olfactory nerve layer is the glomerular layer. The specialized glia (OECs) at the surface of the olfactory bulb have rough superficial processes permeating the bundles of olfactory nerves and accompanying them into the glomerular layer. Olfactory axons enter the glomerulus and synapse upon the dendrites of mitral cells. The OEC
processes enclose huge numbers of unmyelinated olfactory axons and form the inner and outer layers of the capsule (see figure 1.3). The diagram shows that the OECs accompany the incoming olfactory axons all the way to their synaptic terminations in the CNS.

1.8.3: External plexiform layer
The next layer deep to the glomeruli layer is a layer with a relatively low cell density but very dense neuropil. It is called the external plexiform layer. In this layer the dendrites of the mitral cells are mingled with the dendrites of tufted cells and granule cells within the layer.

1.8.4: Mitral cell layer
This is a thin layer containing the somata of mitral cells. They are arranged in almost a monolayer. The primary dendrites make connections with the periglomerular cells. The secondary dendrites of mitral cells may extend up to the external plexiform layer.

1.8.5: Granule cell layer
The granule cell layer is the deepest neuronal layer in the bulb. This layer contains many small granule cell neurons. Frequently three to five granule cells are arranged in row-like aggregates of tightly packed somata. The dendrites of granule cells are located in the external plexiform layer.
Figure 1.5

Schematic diagram of the primary olfactory pathway to show the peripheral position of the primary sensory neurons (above the cribriform plate) and their entry into the CNS (below the cribriform plate) at the surface of the olfactory bulb where they synapse with different type of cells. (Adapted from website: flavor.monell.org/~loweg/OlfactoryBulb.htm).
Mucus Layer
- Supporting Cells
- Receptor Cells
- Olfactory axons

Cribriform Plate

Glomerular Layer
- Periglomerular Cells
- Tufted Cells
- Mitral Cells
- Granule Cells
1.9: What are olfactory ensheathing cells?

Morphological and immunohistochemical studies have demonstrated that OECs are derived from precursor cells residing in olfactory epithelium (Chuah and Au, 1991). During development, they migrate from the epithelium and extend processes to ensheathe the olfactory axons. Olfactory neurons undergo high turnover and are replaced continually throughout adult life from a stock of stem cells that lie in the olfactory epithelium of the nasal mucosa (Barber, 1982; Graziadei, 1979; Graziadei, 1980). OECs ensheathe the olfactory axon bundles in the peripheral part (i.e. mucosa) and extend into the CNS at the olfactory bulb (Doucette, 1993). The OEC have a unique arrangement in which they give rise to very fine cytoplasmic processes that enclose huge numbers of unmyelinated olfactory axons (Doucette et al., 1984). A study by Raisman has shown that OECs have a special relationship with olfactory nerve fibres, accompanying them from the olfactory nerve fibre layer all the way to their synaptic terminal in the glomeruli of the olfactory bulb (Raisman et al., 1985).

Olfactory ensheathing glia displays a very different morphology in vitro, depending on the age of tissue donor and culture conditions. Ensheathing glias have been cultured from embryos (Chuah, 1991, Doucette, 1993), neonates (Barber et al., 1982, Barnett, 1993) and adult (Barber et al., 1982, Ramón-Cueto et al., 1992 and 1993) rats and mice.

When cultures are set up from embryonic olfactory bulb, they contain neurons, astroglia and ensheathing glia (Fracek et al., 1994). It should be noted that embryonic cultures give different morphology in serum-free or serum-containing medium. Cells grown in medium with serum appeared flat and bipolar, but in chemically defined medium their morphology changes in to spindly or stellated bipolar (Doucette, 1993) cells.
Two major different cell types are present in primary cultures of adult rat olfactory nerve and glomerular layer of the olfactory bulb: Fibroblast-like cells (F cells) (Barnett et al., 2000; Li et al., 1998) and Schwann-like cells (S cells) (Franklin et al., 1996; Imaizumi et al., 1998). Ensheathing glia can be purified, using an antibody against low affinity p75-nerve growth factor receptor (L-NGFR) (Ramón-Cueto et al., 1994).

It is believed that a third type of cell exists in OEC cultures, which is a stem cell. Stem cells in the olfactory bulb have two different origins. The deepest layers of the bulb contain adult stem cells. These are derived from a rostral migratory stream of neural stem cells arising from the ependymal layers of the anterior horns of the lateral ventricles (Weiss et al., 1996). The second region of stem cells is the superficial layer of the olfactory bulb. The stem cells are derived from the olfactory lamina propria mucosa.

To establish whether neonatal or adult OECs have different properties after transplantation the different cell types would need to be identified. This has been complicated due to lack of specific and definitive markers to distinguish the transplanted cells from host SCs and fibroblasts growing into the operated area. More recently OECs have been labelled by adenoviral-mediated (Ruitenber et al., 2003) and retroviral-mediated (Lakatos et al., 2002) transfer of LacZ (Ruitenber et al., 2002) or adenoviral transfer of GFP (Li et al., 2003).

It has been reported that OECs are capable of myelinating axons when they are co-cultured with dorsal root ganglion neurons (Devon et al., 1992 and 1995). OECs appear to myelinate axons after transplantation into demyelinated areas of the CNS (Franklin et al., 1996). The first transplantation study was performed in 1994 by Ramón-Cueto and Nieto-Sampedro who demonstrated that transected dorsal root axons are able to enter into the spinal cord after OEC transplantation. The use of OECs from adult olfactory bulb was also
reported to promote the regeneration of injured adult rat spinal cord. Li et al., 1997 performed a unilateral CST lesion in adult rat spinal cord and OEC transplantation into the lesion. When both types of cells are transplanted into CST lesions, each of them has distinct behaviours. The Schwann-like cells make intimate contact on a one-to-one basis with the CST axons and later myelinate them. The fibroblast-like cells adhere to each other in clusters, making multiple intercellular junctions, lie in a collagen-containing matrix and form an outer, perineurial-like sheath around the inner cores of 5-10 myelinated regenerating axons. Ramón-Cueto et al., 1998 performed a complete transection of the adult rat spinal cord by removing a whole spinal cord segment. Schwann cell-seeded guidance channels were grafted to bridge the gap and a suspension of purified adult OECs was injected into cord stumps. In a control group the guidance channel had no OECs and the axons were unable to grow into spinal cord, but animals with OEC injections into the channels showed long distance axonal regeneration.

Garcia-Alias et al. (2004) showed that after an acute transplantation of purified adult OECs into a photochemical injury at the T8 spinal cord segment, functional recovery and electrophysiological responses were improved.

All studies reviewed earlier indicate that OEC are unique cells with unique properties and can be strong candidate for promotion of axonal regeneration.

Boyd et al., 2004 gave a different type of conclusion. In this study the adult rats were induced a clip compression injury at T10 level of the spinal cord and then after one week delay embryonic OECs which were infected with LacZ retrovirus were implanted into the lesion. The labelled OECs showed no direct contact with axons and there were no myelinated axons in the middle of transplant. This study suggested that the role of OECs as
a beneficial mediator on axon growth and functional recovery after spinal cord injury requires that the cells form a continuous bridge across the lesion.
Figure 1.6
A schematic representation of the glial capsule ensheathed by OECs in the glomerular layer. Olfactory axons (pink) enter the glomerular layer and make connection with the dendrites of the mitral cells. The OECs (pale green) ensheathe the olfactory axons and make a capsule. Astrocytic processes (green) ensheathe the dendrites from the middle capsule layer. (Adapted from Raisman, 2001).
Olfactory ensheathing cell processes

Olfactory axons

Expansion of astrocytic process

Astrocytic processes ensheathing dendrites

Dendrites of mitral and periglomerular cells
1.10: Aim of study

Injury to the adult mammalian spinal cord results in progressive tissue damage, which causes permanent functional deficits. Experimental strategies aimed at promoting axonal regeneration and restoring functional deficits suggest that there may be a window of opportunity for repair after spinal cord injuries (Bunge, 2001). Several cellular grafting strategies result in some behavioural improvements of experimentally induced paralysis (Li et al., 1997, Ramón-Cueto et al., 2000). To analyse the effects of alteration of the cellular environment of cut axon tips in vivo, experimental models have been chosen in order to maximize the degree of control and the precision of monitoring the tissue rearrangements induced by lesions and transplantation.

The focus of this study is spinal cord, and the reasons are as follows: First, it provides a relatively pure example of axotomy, since the long, white matter fibre tracts can be severed during their course without damage to the cells of origin or to the postsynaptic cells in the terminal fields and the incidental damage to spinal grey matter in small lesions has only minor local functional consequences. Second, the study responds to the challenge of curing human injuries that largely affects young and healthy people and leads to permanent disabilities.

In this project, the major focal point is on the CST pathway. The CST is along the descending central pathway, restricted to mammals, and involved in motor control (Stanfield, 1992). Lesions in the CST of humans affect precision movements, such as fine movements in the hands.
1.10.1: Objectives

The objectives of this study are as follows:

1. To produce a constant and localized heated lesion in only one side of the CST of adult rats at the level of the first and second cervical segments. Li et al. (1994) used electrolytic lesions to destroy the CST. Therefore, it was essential to find out the precise combination of voltage and time required to produce constant lesions.

2. To use a range of different lesion sizes to investigate the relationship between the proportions of spared corticospinal axons and the degree and rate of recovery of DFR.

3. To provide the new information that with incomplete lesions, both the rate and the degree of return of ipsilateral DFR are a function of the numbers of surviving CST fibres. Previous studies of CST lesions have largely relied on injections of anterograde tracers (such as biotin dextran) to identify corticospinal axons. However, even with very extensive injections of tracer there is always the possibility that small numbers of surviving corticospinal axons may not have been labelled. The demonstration (Terashima et al., 1994; plus ours) of selective immunostaining of corticospinal axons by calcium calmodulin kinase IIα (CaMIIk-α) antibody gives an independent assessment, which avoids the problem of uptake of an injected anterograde label.

4. To confirm whether delayed transplantation of OECs could restore lost DFR. In a previous study (Li and Raisman, 1997) it was found that OECs
transplanted at the time of operation into unilateral lesions of the CST induced a recovery of DFR by the ipsilateral forepaw starting from about 10 days after transplantation. While encouraging, the potential value of such transplants for human spinal cord injuries, it must be taken into account the considerable delay, which will occur between the time of injury and the time when transplantation could be considered. In a previous study (Li and Raisman, 1997), return of DFR was reported after 10 days in one out of a preliminary series of three rats in which transplantation of OECs was delayed for two months after CST lesions.

5. To examine by immunohistochemistry of semi-thin sections and electron microscopy the detailed pattern of myelination of the regenerated axons due to OEC transplantation.

6. To measure the growth of regenerated axons from the OEC transplant to the terminal field by using an anterograde tracer, biotinylated dextran amine (BD) (Brandt, 1992). The regenerated axons from OEC transplantation were viewed by confocal microscopy.
Chapter 2

Materials and Methods

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2.0: General materials and methods

In this project five primary techniques were used: microsurgery, immunohistochemistry, electron microscopy, cell culture and behavioural testing. The microsurgery technique involved making consistent and reproducible heat lesions of the corticospinal tract (CST) on one side, transplantation of OECs into the lesioned CST and injection of a tracer into the brain to measure the axon growth after cell transplantation. Different types of immunohistochemistry and electron microscopy were used to visualize regenerating cortical spinal axons within the transplant. The method used for quantitative analysis of CST function after injury and following cell transplantation was directed forepaw retrieval (DFR). In this chapter, all aspects of each technique will be described in detail.

A summary of all animals and the histological procedures were taken on them in this study is presented in table 2.1.
Table 2.1

The lists to show all the rats were used, the different histological procedures were processed, survival time and the number of weeks the rats were tested for DFR recovery. MB, methylene blue; NF, neurofilament; FN, fibronectin and GFAP, glial fibrillary acidic protein.
<table>
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<tr>
<td>2 complete CST lesion</td>
<td>(Control Group)</td>
<td>OECs culture Medium transplantation</td>
<td>BD tracer staining on the cryostat sections</td>
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<tr>
<td>2 complete CST lesion</td>
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<td>Long-term complete lesion</td>
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<td>2 complete CST lesion</td>
<td>(Control Group)</td>
<td>Long-term complete lesion</td>
<td>MB, NF, GFAP, FN and P0 on the semi-thin sections</td>
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2.1: Developing a complete unilateral lesions

The first stage of my project was, to develop a constant and localized heated lesion on one side of CST by using a radio-frequency lesion generator (lesion generator model, RFG-3C RF, Burlington, Mass, USA). The lesion was produced by combination of three parameters including voltage, resistance and time. The lesion size and position was monitored histologically after 1 week survival.

2.1.1: Surgical procedures

To produce a complete lesion and complete loss of DFR function on one side of CST in adult rats, a lesion generator was used. The lesion had to be big enough to destroy all the fibres on only one side of the CST. The procedures used in this study are a modification of those used by Li and Raisman (1994). Adult (200-230 g body weight) female rats of a locally inbred Albino Swiss Strain (AS) were used and all procedures conformed to Home office regulation and the Animals (Scientific Procedures) Act 1986. The rats were anaesthetised by intraperitoneal injections of 2% 2,2,2-tribromoethanol (Aldrich Chem. Co. USA) (Avertin) solution, 200 mg/ Kg body weight. Avertin is made up in 2% 2,2,2-tribromoethanol, 0.08% absolute ethanol, and 0.02% of 2-methylbutan-2-ol (BDH, UK) and 100% distilled water (see Appendix). The solution was stored in the dark in a refrigerator. Fur over the area to be operated was removed and the skin wiped with 70% alcohol. The rat was mounted in a stereotaxic frame with the neck flexed and the head stabilized using ear plugs and an incisor bar. The dorsal surface of the spinal cord at the level of the first to second cervical segments was exposed through a midline incision of the skin, parting the supraspinal muscles in midline, and incising the atlanto-occipital membrane. The identification of the midline depends mainly on the presence of the median sagittal venous pattern. However, this is not absolutely consistent, leading to some variation in electrode placement. Since the size of the lesions (0.5 – 1.0 mm) was little greater than that of the target CST (0.5 mm) slight errors in electrode placement lead to
sparing of varying amounts of the target. A stainless steel electrode of 250 μm diameter with 8° tapered tip and impedance of 5 MΩ, insulated to within 1mm of the tip (A-M System, WA, USA), was inserted to a depth of 0.8-1.0 mm at a point 0.5 mm lateral to the midline. Roughly equal numbers of rats were operated on right and left sides. The CST was destroyed by heat lesion. After making lesions the muscles were repositioned, and the skin sutured. Silk non-absorbable sutures (Ethicon, Belgium) were used to close each wound.

In order to produce a precise and complete lesion, a variety of different combinations of voltage and lesion time were tried. The resistance of the electrode was kept constant with 2.0-2.5 KΩ.

The operation was performed on a total of 52 rats in order to relate the balance of voltage and heating time and to observe histological changes. In these 52 rats after making the lesion, one week later histological analysis were done on them to find out the size of the lesion.

The lesion size was dependent on the two parameters, voltage and time. In some animals I kept the time constant and the voltage was the variable. For example, one rat received 14 volts and 2 minutes time lesion. After histological analysis, it was decided that the next rat should receive 2 minutes duration and voltage was changed to 10.

In some other group of rats the voltage was kept constant (10 volts) and the time was the variable, for example, one rat received a lesion with 10 volts and 2 minutes duration. After histological staining, another rat was decided to have 10 volts and 30 seconds time. Finally I decided to increase the time from 30 seconds to 1 minute.
Therefore this work revealed that 10 volts for a period of 1 minute was required to destroy one side of the CST fibres (figure 2.1).

The surgical procedures throughout the project are the same as it is explained in this section.
Figure 2.1

Adult rats received unilateral lesion in CST between first or second cervical segments of spinal cord. The constant lesion was produced by a combination of 10 volts and 1-minute time via the radio-frequency lesion device.
Lesion/Transplante region

Cervical

Thoracic

Lumbar

Sacral

V = 10 V
T = 1 min

Radio frequency lesion device
2.1.2: Perfusion for cryostat sectioning

Animals were anaesthetized with a deep terminal dose of pentobarbitone (Rhone Merieux, Sagatal). Through a wide thoracotomy, the heart was exposed and the descending aorta clamped with haemostatic forceps. With a pair of fine scissors an incision was made to the right atrium and another to the left ventricle. A blunt hypodermic needle (0 gauge; cut flat and polished smooth) was inserted into the ascending aorta through the left ventricle. The blood was flushed out by perfusion of about 100 ml of 0.1 M phosphate-buffered saline (PBS) at room temperature for 5 min.

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

2.1.3: Histology for accuracy of corticospinal tract lesion

The accuracy of the lesion was assessed one week after operation. The immunohistochemical analysis was performed on sections to check if all the axons were destroyed on the lesion side. Neurofilament (Serotec, UK) and calcium/calmodulin-dependent protein kinase II (CaMIIk-α Zymed Lab Inc, San Francisco, CA, USA; Terashima et al., 1994) staining were performed.

The expression of CaMIIk-α is weak during early postnatal periods, and strong in the adult stage (Terashima, unpublished data). CaMIIk-α is a prominent enzyme in the mammalian brain that phosphorylates a variety of substrate proteins (Yamauchi and Fujisawa 1980; Kennedy and Greengard, 1981; Bennett et al., 1983). This enzyme has two different isoforms α and β. In 1994 Terashima studied the distribution of these isoforms within rat
spinal cord by using monoclonal CaMIIk-α antibody. In rat, both dorsal and ventral CST fibres were strongly immunopositive for CaMIIk-α antibody (Yamauchi et al., 1994). Therefore CaMIIk-α staining of CST provided a suitable tool to assess the accuracy of lesion.

The rats were perfused with PBS and the spinal cords were taken out and immediately frozen on dry ice. Serial horizontal and coronal sections (20 μm thickness) were cut in a Reichart-Jung cryostat (model CM3050, Leica, Germany) at -15°C and thaw mounted onto TESPA (3-Aminopropyltriethoxy Silane; Sigma, UK) coated Super Frost microscope slides (BDH; Poole, UK) (see Appendix). In each case 20 μm cryostat sections were fixed in acid/alcohol. Sections were blocked in 2% milk and then incubated in the 1/1000 primary antibody dilution in a humid chamber overnight at 4°C. All primary antibodies were made in 2% dry milk powder (Marvel) to the required dilution. Following overnight incubation in primary antiserum, sections were washed in PBS and immunoreactivity detected using a 1/500 dilution of biotinylated anti mouse and anti rabbit secondary antibody (Vector Laboratories, UK) for CaMIIk-α and neurofilament respectively. The slides were incubated for 30 minutes in avidin biotin dextran (ABC) (Vector, UK). Sections were washed for 1 hour. The reaction product was then developed by using glucose oxidase-nickel DAB (GOD) method (see Appendix; Shu et al., 1988). The sections were then counterstained with 1% neutral red (see Appendix; which stains cell nuclei; Sigma, UK). Following counterstaining the slides were rinsed in distilled water and dehydrated through a series of graded ethanol solutions (up to 100%). The staining was completed by a 15-minute incubation in Histoclear, and mounted in DPX (LAMB, East Sussex, UK).

Immunohistochemistry analysis confirmed that 10 volts for a period of 1 minute was required to destroy one side of CST fibres.
2.2: Behavioural Investigation

The CST is necessary for skilled forelimb use. A test exists to look for the absence and presence of a DFR, which the rats reach through an aperture to obtain food reward (Whishaw et al., 1986). The use of forepaw for retrieving pellets involves different muscles, which these measurements were based on the description of (Whishaw et al., 1993). Food pellet retrieval requires coordinated activation of both proximal and distal forelimb muscles, including advancing the forepaw to the pellet (proximal muscles), grasping the pellet (distal muscles), and then retracting the limb into the testing cage (proximal muscles) while the paw maintains a grip on the pellet (distal muscles). Above all the test requires the paw to be correctly orientated in space to reach the pellet. This is important to note because after CST lesions this function is lost without any other obvious defects in forepaw strength or use (e.g. in handling food or walking).

2.2.1: Behavioural study before making lesion

The cage used for DFR test was made of clear Plexiglas Perspex, so that the rats could be filmed from any perspective (Whishaw and Pellis, 1990). A box was 25 x 35 x 30 cm high. Five centimetres form the inside of each front wall was a 2 cm wide slit that extended from the floor to a height of 15 cm. On the outside of the wall, in front of the slit, mounted 3 cm above the floor, was a 2 cm wide by 3 cm long shelf. Food pellets (dried spaghetti) were presented by forceps on the floor of the shelf (see figure 2.2). The indentation were placed 2 cm away from the inside wall of the box and were cantered on the edges of the slit through which the rats reached. Rats were free to reach with either forelimb through the slit. The normal rats were tested for one session a day for 3 days.

Training was administrated in such a way when a rat made a successful reach, a short pause preceded presentation of the next food pellet. Thus, prior to each reach, the rat had to
reposition itself for the next food pellet. During each session food pellets were presented until the rats had made 50 retrievals through the aperture. Numbers of retrievals were recorded, whether the right or left paw was used. It was very important to use the rats that used both paws regularly for making the lesions. The majority of rats use both paws more or less equally. The small number of rats showing an asymmetry of use greater than 15/35 were not used. By this behavioural task, 27 rats were chosen to have the CST lesions.
Figure 2.2

Skilled directed forepaw retrieval task. Illustrated is a rat reaching through an aperture to retrieve a food pellet. In the cage there is a 2 cm wide and in front of the slit a shelf is mounted 3 cm above the floor. Foods were held by forceps on the shelf for rats to retrieve with either forepaw.
2.2.2: Behavioural investigation after making the lesion and transplantation

27 rats had CST lesions exactly the same as explained in surgical procedures (section 2.1.1). It is crucial to insert the electrode 0.5 mm away from midline, otherwise the 10 volts and 1-minute time could not destroy whole CST on one side. Due to this essential accuracy, 27 rats were operated only 7 of them had complete lesions. The rest of 20 animals had incomplete lesions. The functional recoveries of the incomplete lesions were assessed against the extent of lesions. The estimation of extent of lesion will be explained in section 2.3.

The animals recovered from anaesthesia and showed no abnormalities in their normal cage behaviour. Three days after lesions were made, the behavioural tests were repeated. To determine the accuracy of reaching, a category of scoring was made. If the rats made reaching movement, where either paw was inserted through the slit of the box, even though the food pellet was not grasped, the movement was scored as retrieval.

The complete lesion resulted in a functional loss on the ipsilateral forepaw. Next, I restrained the contralateral forepaw by wrapping the forepaw with a light sleeve (Whishaw, 1986), which prevented the rat from reaching through the slit with contralateral forepaw. This restrained test was only done on the complete lesioned rat for once a week for period of three weeks.

In the incomplete lesions, although both paws were used, the rats showed preferential use of the paw on the non-lesioned side. When the number of reaches on both sides totalled 50 counts, the test was stopped. The success of the complete and incomplete lesions were tested post-operatively, three times a week, for a period of 8 weeks by observing the ability to reach with both paws.
The rats, which received any type of cell transplantation, were tested 3 times a week for a further 8 weeks. The control group with complete lesions and not showing DFR at 8 weeks post-lesion were maintained for a further 16 weeks, during which time they were tested once a week.

2.2.3. Video recording

Video records were made with a Sony (Tokyo, Japan) Video 8 CCD VII portable camera with a shutter speed of 1/2000 second. Illumination for high-shutter speed filming was provided by a two arm-Nikon (Tokyo, Japan) MII cold light source. Frame by frame analysis at 20-25 frames per second was provided by a Sony Video 8 recorded or through a computer-based frame grabber (Whishaw and Pellis, 1990).

2.3: Estimation of the extent of incomplete lesions

The 20 rats with incomplete lesion and 7 rats with complete lesion were chosen for studying the abolition of DFR. The ideal way of assessing the extent of lesions was to choose the centre of lesion for each animal. The depth of the lesion is about 1mm as a result the centre of lesion is about 500 μm.

Serial coronal spinal cord sections (20 μm thickness) were cut in a Leica CM 3050 cryostat at -15°C and thaw mounted onto TESPA coated slides and were stained with CaMIIk-α antibody, dehydrated, and counterstained with 1% neutral red.

In order to determine the extent of incomplete lesions, it was important to choose the sections, which showed largest area of lesion i.e. the centre of lesion. As I explained in section 2.1.1, the sizes of lesions were around 0.5mm (width) and 1mm (depth). For each animal 50 cryostat sections (20 μm thickness) were collected. These 50 sections were
stained with CaMIIk-α. Around section 25th was about the centre of the lesion. In every case I selected three sections 24th, 25th and 26th of cross-sections of each rat for estimation of the lesion size.

Photomicrographs of the normal and lesioned CSTs were taken (×10 magnification). A 5×5 mm² grid was superimposed onto the photomicrograph and the number of squares that were within the lesioned area and normal side of CST were counted. This method was repeated three times on the three chosen sections and the average was taken. In my counts, the numbers of 5×5 mm² squares in normal side of CST were between 156 and 160. The volumes of spared spinal tissues of incomplete lesions were represented as a percentage of uninjured CST site.

2.4: Delayed repair of complete corticospinal tract lesions by transplantation of OECs, adult SCs and pure peripheral nerve fibroblasts

22 rats with complete lesions, which had shown no functional recovery during 8 weeks training, were chosen for this part of study. The 12 rats were received OECs transplant, 2 received pure peripheral nerve fibroblasts and 2 received Schwann cell transplants.

For control group of this part of study, 2 rats received OECs culture medium and were used for BD tracer. Another 2 rats were used for only BD tracer without any transplantation and 2 rats were used for semi-thin sections staining.

In this section all procedure required for preparation of OECs, method of transplantation into the CST injury model and histology of OECs in the culture dish are presented.
2.4.1: Procedure of culturing Olfactory ensheathing cell

The method used for cell culture was that of Ramón-Cueto and Nieto-Sampedro (1992), but without purification. Adult (3-6 months old) female AS rats were killed by decapitation and olfactory bulb were collected in 10 ml of PBS solution. After removal of the pia, the outer including nerve fibre and glomerular layers were dissected away from the rest of bulb. The glomerular tissues were washed few times in PBS. Then the tissues were transferred into another dish with 2ml of PBS solution and 200 µl of 1% trypsin (Worthington, USA supplied by Lorne Lab, Reading UK) and left in an incubator at 37°C for 15 minutes. Trypsinization was stopped by adding the growth medium with a mixture of Dulbecco’s modification of Eagle’s medium (DMEM) and Ham’s F-12 medium (D/F-12; 1:1 mixture, Sigma Chem. Co) supplemented with 10% fetal calf serum (GlobePharm, Surrey, England). The tissues were allowed to settle and then 20 µl DNAse solution (Sigma, UK) was added to the tissues and triturated with fire-polished Pasteur pipette for few times. The tissue suspension was centrifuged at 1200 revolution per minute (r.p.m). for 5 min. The medium was removed, and re-suspended in 2 ml of medium. The cells were then plated out on poly-d-lysine (Pdl) (Sigma, UK; wt, 30,000-70,000; 10 µl/ml) coated dishes. The cells were incubated at 37°C, 5% CO₂ and the medium was changed after 4-5 days and then twice a week the cells were feed for period of 17 days.

2.4.2: Preparation of OECs for transplantation

After 14-17 days in culture, the OECs were ready to be transplanted into the CST lesions. To prepare cell suspension for the transplantation, the growth medium was aspirated off from the culture dishes and was washed gently with 0.1 M PBS (pH 7.2). The cells were de-attached from the dishes by adding HBSS, Ca²⁺, Mg²⁺ -free for 2 minutes. Then the cells were lifted from the dish by using 0.1% trypsin (Worthington, USA supplied by Lorne Lab, Reading UK) and left in an incubator at 37°C for 2 minutes. As soon as the cells floated off the bottom of the culture dish, the reaction was terminated by adding 500
μl growth-medium to each dish. The cells were collected into a 12 ml conical tube (Bibby Sterilin Ltd, UK) and centrifuged for 5 minutes at 1200 r.p.m. The supernatant was removed from the tube. It was essential, for effective transplantation, that the cells were resuspended in DMEM/F12 (GIBCO BRL, UK) solution without the fetal calf serum. This is because fetal calf serum may cause rejection in the animals. The cell suspension was kept in ice for one hour before the transplantation. The cells were counted using a haemocytometer. The final density was made at 2.0-2.5 \times 10^7 \text{ cells/ml}. The cell suspension was kept in ice during transplantation.

2.4.3. Transplantation of OECs into the lesioned corticospinal tract

In this stage of experiments the rats were going under second time of anaesthetic injection, therefore it was important to reduce the anaesthetic by 0.5 ml. For example, a rat with 250g weight, the volume of anaesthetic had to be injected was 2 ml.

To ensure the cells were injected into the centre of the lesion, a detailed drawing and exact co-ordinates of the lesion position made for each rat before making the lesion. Due to the second operation it was very difficult to judge the midline in the spinal cord therefore in some animals two injections were used.

The micro-transplantation technique by air pressure injection system and a glass micropipette (Brook et al., 1993) was used to reduce trauma at the site of injury. 5 μl of a suspension (the suspension of cells procedures explained below) containing around 100,000 cultured cells was injected using a solenoid-driven air pressure system (Emmett et al., 1990) into the lesion site using a glass micropipette.

The system for micro-transplantation was done by air pressure with using two needle valves (NV). With NV1 open and the NV2 closed the cell suspension or tracer solution can
be loaded into the micropipette with a syringe. With NV2 open and NV1 closed, the contents of the micropipette can be expelled by pulsed air-pressure. The injection was controlled by pulse and air pressure (see figure 2.3).
Figure 2.3
Illustrates the micro-transplantation air pressure system. Needle valves (NV1) open and the NV2 closed then the cell suspension or tracer solution can be loaded into the micropipette with a syringe. For injection the cell or tracer into the spinal cord NV2 open and NV1 closed. The injection was controlled by air pressure through a tank and pulse generator connected to the NV1.
2.4.4: Histology of cultured OECs

After 14-17 days, the cultured cells had segregated into clusters of the two major cell types (Li et al., 1998): Schwann-like cells and fibroblast-like cells.

In order to show what type of cells had been transplanted into lesioned rats two different antibodies were used. After transplantation, a sample of the cell suspension was plated out in 35 mm culture dishes coated with Pdl with 1.5 ml DMEMF and cultured for one day to check that the OECs were still viable. A suitable cell surface antigen to select SCs is the p75 (low affinity nerve growth factor-receptor), which is widely expressed on SCs in vitro, but not on fibroblasts. The cells were stained for p75 and FN (fibronectin) antibodies.

Cells were washed with 0.1 M PBS (pH 7.4), fixed for 30 min in acid/alcohol (see Appendix), blocked with 0.5% milk at room temperature. Then the cells were incubated with p75 antibody (1/100 dilution) and fibronectin (1/1000 dilution) with 0.1% Triton at 4°C overnight. The following day, after washes in PBS, cells were incubated with Alexa anti-mouse (p75, green) (Molecular probes, Eugene, Oregon, USA, 488nm wave length) and Alexa anti-rabbit secondary antibody (FN, red) (1/400, Molecular probes, Eugene, Oregon, USA, 568 nm wave length) for one hour at room temperature. After final washes in PBS, the cell culture was then counterstained with 1/500 of 1 µg/ml stock solution DAPI (4,6-diamidino-2 phenylindole, dihydrochloride) (Molecular probes, Eugene, Oregon, USA, 461 nm wave length). Then culture dish was washed with PBS, cover slipped with aqueous mounting medium and viewed by confocal microscope.

2.4.5: Procedure of culturing adult Schwann cells

Adult Schwann cells cultures were obtained from sciatic nerves of an adult (2.5 months old) female AS rats as described by Morrissey et al., 1991.
The rats were killed by decapitation and both sciatic nerves were dissected out from midthigh and placed into sterile PBS. After the epineurium, connective tissue, and blood vessels were stripped off with fine forceps. Each nerve was 3-4 cm long. The nerves were placed in fresh Petri dish in 0.1 M PBS containing penicillin (50 U/ml) and streptomycin (0.05 mg/ml; supplied internally from Media services at the National Institute for Medical Research, London, UK). The tissue was washed twice with PBS. The tissues were chopped into 1mm explants with the tissue chopper (Mickle laboratory, Co. Ltd). Then the chopped nerves were rinsed with fresh PBS/pen-strep. 8-10 pieces of nerve were plated out on 35mm coated dishes containing 750μl of a growth medium consisting of a mixture of DMEM and 10% fetal calf serum (GlobePharm, Surry, England). The tissues were incubated at 37°C, 5% CO₂. The medium level was kept low during the initial plating to prevent the explants from floating off the surface of the culture dish. The explants were examined several times a week for the emergence of migratory cells. As there were no neurons in the explants, and thus no neurite extension, the outgrowth was entirely cellular. When the outgrowth around the explants reached a near-confluent monolayer (7-10 days), the explants were lifted off the surface of the culture dish with fine forceps and placed in new dishes with fresh medium. The numbers of passages were dependant on the purity of SC needed for transplantation. From the first passages of the explants the majority of cells migrating out from the explants were fibroblasts. These dishes with pure fibroblast were used for control study. All the passaged dishes with fibroblast were kept and fed twice a week with growth medium for two weeks.

Nerve pieces were transferred to a fresh 35 mm Petri dish every week for 6 weeks and were feed twice a week. After six transfers, explants were devoid of fibroblasts and contained only SCs. Explants were replated on to 35 mm Petri dishes with 1.25 U/ml dispase (Boehringer Mannheim, Mannheim, Germany), 5% collagenase (Sigma) and were left in an incubator at 37°C in 5% CO₂ overnight. After incubation, the tissues were
collected into sterile tube with growth medium and then were centrifuged at 1200 r.p.m. for 5 min and were washed 2-3 times in DMEM/10% FCS. The tissue was triturated with a 0.5mm diameter needle and syringe for few times. Once fully dissociated the cell suspension was diluted and plated on poly-L-lysine (PLL Sigma, UK; wt, 30,000-70,000; 10 μl/ml) dishes. After two days the medium was removed and replaced with growth medium supplemented with 2 mM (1.5 μl) forskolin (Sigma, UK) and 10 μg/ml (1.5 μl) PDGF (platelet derived growth factor; Gibco, BRL, UK). These SCs were ready to use after 3-4 days.

SCs were identified in living cultures on the basis of cell soma and nuclear morphology using phase contrast microscopy. Cells with long bi- or tri-polar processes (that were approximately 5-7 times the width of the cell body) and oval nuclei were counted as SCs while fibroblast (the only other abundant cell type) were identified by a much more flattened polymorphic shape with larger round nuclei.

2.4.6: Preparation of adult Schwann cells suspension for transplantation

The procedure of making cell suspension was the same as OECs transplantation.

For pure fibroblasts suspension after adding HBSS, Ca^{2+}, Mg^{2+} -free for 2 minutes, the cells were removed from the dish by a cell scraper (Costar). The fibroblasts were strongly attached to the dish. The rest of the procedure was exactly the same as section 2.4.2.

2.4.7: Histology of cultured adult Schwann cells

After transplantation, pure fibroblasts and adult SCs, a remaining sample of cell suspension were plated out in 35 mm culture dishes coated with PLL with 1.5 ml DMEM and cultured for one day. The double staining of p75 and fibronectin was done the dishes as in section 2.4.4.
2.5: Perfusion of rats with transplantation for electron microscopy and immunostaining

The transplanted rats and the control group rats were perfused (explained in section 2.1.2) survival period of 16 weeks with 100ml of PBS followed by a mixture of 1% paraformaldehyde and 1% glutaraldehyde (see Appendix) in 0.1 M PBS at PH 7.4 at 4°C for 30 min and left in fixative at 4°C overnight. The cervical spinal cord was removed and 700 μm cross sections were cut using a tissue chopper. The sections were washed with washing solution followed by a mixture of distilled water, glucose and 0.5% CaCl₂ in 0.1 M PBS (see Appendix) for 15 minutes. The sections were post-fixed for 2h in 2% (0.2M) aqueous osmium tetroxide Johnson Matthey Chemical Limited, England). Then the tissues were dehydrated in graded alcohols and incubated in 50:50 acetone and resin (TAAB Lab, UK) overnight at room temperature. TAAB resin contains embedding resin (51%), methyl nadic anhydride (5.2%) (MNA), dodecenyl succinic anhydride (42%) (DDSA) and 2,4,6 tri(dimethylaminomethyl) phenol (1.8%) (DMP-30). Then the sections were infiltrated in pure TAAB resin for at least 6 hours. All the steps from fixing in osmium up to incubation in pure TAAB resin were done by tissue processor (Leica, Germany). Then the sections were embedded in fresh pure TAAB resin and left at 60°C for 2 days to be hardened.

2.6. Histological procedures of semi-thin section

Two-micrometer semi-thin sections were cut using an ultra-microtome (Reichert-Jung, Ultracut, Germany) in a horizontal plane, mounted onto a slide and stained with 1% methylene blue and Azur II (see Appendix) in distilled water and dried on a hot plate (60°C). Neurofilament and FN (Life Technologies, USA) immunostaining were used for identifying the axons and the transplanted cells. Glial fibrillary acidic protein (GFAP)
(Sigma, USA) was used to stain the astrocytic scar around the lesion. P0 (gift from J. J. Archelos, Max Planck Institute, Munich) was used to identify peripheral type myelin around the lesion as well as the regenerated axons in the transplant.

With each type of staining, the semi-thin sections were incubated overnight at 60°C. Then sections were left for 10 minutes in matured sodium ethoxide diluted 1:1 with ethanol in the dark for clearing the resin around the sections. Sections were quenched of endogenous peroxide activity in 3% H₂O₂ (Sigma, UK)/methanol for 20 minutes and 8% formic acid for 10 minute (W. F. Blakemore, personal communication). The slides were washed with 0.1% Triton in PBS for 10 minutes. The sections were blocked in 5% milk. The slides were incubated with primary antibody in a humid chamber overnight at 4°C. The dilution for P0 antibody was 1/3000, NF; 1/500, fibronectin; 1/2000 and GFAP; 1/500. All the primary antibodies were made in 2.5% normal horse serum/PBS + 0.2% Triton to the required dilutions. Following overnight incubation in primary antiserum, sections were washed in PBS with 0.1% Triton and immunoreactivity detected using a 1/300 dilution of biotinylated anti mouse and 1/500 anti rabbit secondary antibody (Vector Laboratories, UK) for P0 and neurofilament respectively. This was followed by ABC and subsequently visualized using DAB buffer (see Appendix). The sections were washed with distilled water and dried on a hotplate.

2.7: Histological procedures for ultra-thin section

Semi-thin sections stained with methylene blue and Azur II was used to assess overall morphology of the graft. Areas of particular interest in the blocks were trimmed. Ultra-thin sections were cut with a diamond knife at an approximate thickness of 80 nm on the ultramicrotome and collected on copper grids. The grids with ultra-thin sections were immersed in 0.3% uranyl acetate in methanol for 7 min, washed three times in methanol and air-
dried. They were then immersed in Reynolds lead citrate solution (see Appendix) for 7 minutes, rinsed three times in distilled water and air-dried.

2.8: Surgical procedures for anterograde tracer

Biotinylated dextran amine (BD) (Brandt, 1992) was chosen in this study as an anterograde tracer. This method was used for rats with cell transplantation and functional recovery, to measure the regenerated axon growth from the transplant to the terminal field. In addition BD tracer was also used for control group rats with complete lesions and no function recovery.

8 weeks after receiving cell transplantation rats were anaesthetised for a third time. The volume of 2% Avertin had to be reduced by 1ml. For example the rat with 250g weights received 1.5ml anaesthetic.

The rat skull was secured in the horizontal position. After incision of the skin, the skull was exposed, bregma and lambda were determined and location of forelimb sensory and motor cortices were determined by measuring positions on the skull relative to bregma (Paxinos et al., 1998). The skull was exposed and a dental drill used to make a burr hole at a position 1-2 mm posterior to the bregma, 2.0-2.5 mm lateral to the midline and on the side opposite to the spinal lesion. 0.3-0.5 μl of 10% solution of BD (10,000 MW, Molecular Probes, Eugene, Oregon, USA) in saline, pH 7.4 was injected over 5-10 minutes by the pulsed air pressure system through a 70 μm glass capillary tube (Baxter, McGaw Park, USA; see figure 2.4). The injection needle was kept in place for an additional 3 minutes to minimize leakage on withdrawal. The injection points had to be repeated a few times in different places in the motor cortex for labelling more axons in CST. After injection the skin was closed. The animals were left for a period of two weeks.
Figure 2.4
The pathway of CST in rat. Scheme of the descending pathway of CST axons from the sensorimotor cortex (A) to the nucleus ruber (B), to the basilar pontine nuclei (C), and to the first or second cervical spinal cord (D) with the sensory-motor cortical BD tracer injection site in motor sensory cortex and the lesion site in the CST (arrow). The insert in the top left corner indicates the levels of the cross sections. Modified from Schwab et al., 1998.
Injection site
Motor sensory cortex

Nucleus ruber

Cerebral peduncle
Basilar pontine nuclei
Decussation
CST
Lesion
2.8.1: **Histology of anterograde tracer**

After two weeks the animals were perfused under deep anaesthesia, by thoracotomy and aortic cannulation, using 100 ml of 0.1 M PBS followed by 500 ml fixative solution containing 4% para-formaldehyde, 0.15% glutaraldehyde and 0.4% picric acid fixative solution, pH 7.4 for 40 minutes (see Appendix). The spinal cord was collected and left in 10% sucrose (BDH, UK) for 4 hours and then left in 20% sucrose over night in 4°C. The following day the spinal cord was sectioned at 60 μm using a cryostat. The sections were mounted onto 1% gelatine coated slides (see appendix). Afterwards the sections were left to dry at room temperature for a few hours.

Two different ways of developing BD were used on the sections. One-way was to incubate the sections with 1/200 green fluorescence Streptavidin, Alexa (Molecular probes, Eugene, Oregon, USA, 488 nm wave length) and 0.1% Triton over night in the dark at 4°C. The following day the sections were washed with PBS for one hour and then counter stained with 1/300 dilution of red propidium iodide. Then the sections were washed with PBS and cover slipped with aqueous mounting medium.

The other way was to incubate the sections with ABC and 0.1% Triton over night at 4°C. The next day the BD was subsequently visualized using glucose oxidase-nickel DAB method (GOD). In some sections BD tracer and GFAP double staining were used to show the sprouting of axons and the heavy astrocytic scar around the lesion. After BD tracer was developed, in dark brown colour with GOD method, primary 1/500 monoclonal GFAP antibody was incubated on the slides overnight at 4°C. Then for detection of GFAP, peroxides substrate kit (VIP, Vector Laboratories, UK) was used and the final colour was pink.
2.9: Fluorescence and Confocal microscopy

Confocal laser scanning microscopy was carried out using a Leica TCS SP1 mounted on a DM RBE upright fluorescent microscope. The system had four-laser lines 351 nm UV laser, 488 nm blue laser, 543 nm green laser and a 633 red laser. Images were captured using the Leica confocal software and adjusted for brightness and contrast using Adobe Photoshop.
Chapter 3

Results

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3.1. Intact corticospinal tract

This section had focuses on the use of various types of staining to show the structure of the normal CST. In addition the skilled directed forepaw retrievals (DFR) test will be analysed in more detail.

3.1.1. Histology of intact corticospinal tract

The corticospinal axons were identified by selective immunostaining for CaMIIk-α (Terashima, 1995) on cryostat sections (figure 3.1) and BD tracer labelling (figure 3.2). CaMIIk-α staining shows compact, well delineated tract of about 0.5 mm diameter at the level of the first two cervical segments, and located in the ventromedial part of the dorsal columns (figure 3.1, A & B). The CST consists of approximately 50,000 axons on each side (Li et al., 1997). The majority of axons are myelinated and around 1 μm diameter. In semi-thin sections stained with methylene blue and Azur II (figure 3.3, A), and neurofilament (NF) (figure 3.3, B) the CST axons are distinguishable from the larger myelinated ascending sensory axons in the dorsal column. The NF staining shows that the axons in CST are much finer and smaller.
Figure 3.1

The intact rat CST in 20 μm horizontal section (A) and cross section (B) at the level of the cervical 1 stained with CaMIIk-α. Arrowhead, central canal; x, fibres leaving the pyramidal decussation. Counterstained with neutral red. Scale bars, 500 μm (in A & B).
Figure 3.2

Confocal image of horizontal 60 μm section showing BD anterograde labelled CST axons (green) in both sides at the level of the cervical 1. Dashed line, midline. Counterstained with propidium iodide (red). Scale bar, 100 μm.
Figure 3.3

Histology of intact CST. The 2 μm coronal semi-thin section stained with methylene blue (A) and NF-antibody (B). The CSTs are outlined in B. Arrowhead, central canal; DH, dorsal horn. Scale bars, 500 μm (in A & B).
3.1.2. Skilled directed forepaw retrievals

Analysis of specific features of the forelimb used for retrieving the food pellets was performed. Briefly, pellet retrievals require coordination and detailed movements, which are as follows: 1) Using primarily the upper arm, the reaching limb was lifted from the floor so that the paw was aligned with the midline of the body. 2) As the limb was lifted, the paw was supinated, and the wrist was partially flexed. 3) The limb was advanced directly through the slot towards the food target. 4) When the paw was over the target, the wrist was slightly extended and then the paws flexed to grasp the food. 5) Then retracting the limb into the box while maintaining a grip on the pellet.

Some representative frames of a reach drawn from film of a lateral view are shown in figure 3.4. Movements of sniffing the food are shown in frame 1-2 and reaching movements to grasp the food are shown in frame 3-5 (figure 3.4). Figure 3.5 clearly shows that the normal rats were able to reach for and retrieve food from the shelf with either paw. The retrieval performance was scored by counting the successful retrievals for each limb. If the rat made a reaching movement in which a paw was inserted through the slit of the cage, the movement was scored as DFR even if the food pellet was not grasped.

Each movement was based on activation of both distal and proximal muscles including, advancing the forepaw to the pellet (proximal muscles), grasping the pellet (distal muscles), and then retracing the limb into the cage (proximal muscles) while the paw held the pellet (distal muscles).
Figure 3.4

Lateral view of retrieval in normal rat. Movement of the nose to locate the food in frame 1-2, extension of limb to grasp the food through the slit in the cage in frame 3-5. The foods were hold by forceps on the shelf for rats to retrieve with either forepaw.

Adapted and modified from Whishaw and Pellis, 1990).
Figure 3.5

Frontal view of retrievals in normal rat. In frame A rat retrieving a piece of food with left forepaw, B) right forepaw and C) both paws. Film, 25 frame per second.
3.2. Corticospinal tract lesion and functional recovery

To develop a complete unilateral lesion in the CST, 52 rats were used to relate the balance of voltage and heating time. The lesion size was dependent on the two parameters voltage and time. Therefore different voltages and times were tested for each rat and then a one-week later histological analysis was done on the spinal cord to assess the size of the lesion. This histology revealed that 10 volts for a period of 1 minute was required to destroy all fibres of the CST tract on one side.

It is important to mention that, the electrode had to be inserted 0.5 mm away from midline, otherwise the 10 volts and 1-minute time could not destroy whole the CST on one side. This meant that of the 27 rats operated only 7 had complete lesions. The remaining 20 animals had incomplete lesions. All lesions were largely confined to the CST, and they destroyed an ovoid area of tissue around 0.5 mm in width and 1.0 mm in rostrocaudal length.

The completeness of the lesions was verified in semi-thin sections, and by the total absence of anterograde BD labelling caudal to the lesion in a complete series of contiguous horizontal sections through the entire width of the tract, or by total absence of CaMIIk-α staining throughout the entire cross sectional area of the tract 1mm caudal to the lesion. In the case of incomplete lesions the degree of sparing of the CST was expressed as a percentage of the area of remaining CaMIIk-α staining.

3.2.1. Complete corticospinal tract lesion

As mentioned above 52 rats were used to estimate lesion size. By changing the two parameters voltage and time, the size of the CST lesion change. Figure 3.6 shows semi-thin cross-sections, stained with methylene blue to show three different lesions sizes, which were produced by changing the voltage and time. Figure 3.6 A, is an example to show that
with 15 volts and 2 minutes the lesion produce is a very large and destroyed both sides of
the CST plus central canal (figure 3.6 A). 10 volts for 2 minutes (figure 3.6 B) caused a
lesion, which was not as big as the one in figure 3.6 A, but the lesion still damaged both
sites of the CST. The lesion in figure 3.6 C had 10 volts for 30 seconds. This lesion was
not even big enough to destroy one site of the CST. After trying different voltages and
times, 10 volts for 1-minute gave a constant and localized lesion destroying the CST on
only one side making minimal damage to the contralateral site and central canal (figure
3.10 A & B).
Lesion size varies by changing voltage and time parameters of the radio-frequency lesion maker. Semi-thin coronal sections of rat’s spinal cord stained with methylene blue. A) 15 volts for 2 minutes lesion gave very large lesion, which destroyed both sides of CSTs plus central canal (dashed line for showing the midline). B) With 10 volts for 2 minutes the lesion still affected both sites of CST. C) 10 volts for 30 seconds lesion was not big enough to destroy one site of CST. Arrowhead, central canal. Survival time, one week. Scale bar, 500µm.
3.2.1.1: Histology of cryostat sections of complete corticospinal tract lesion

Light microscopic immunohistochemistry of CaMIIk-α and NF for axons and GFAP for astrocytes showed, the area of total destruction of the host spinal tissue was clearly demarcated from the myelinated fibre bundles of the adjacent intact spinal tracts with their associated cellular framework of astrocytes, oligodendrocytes, and microglia. Due to the lesion, all the components were completely changed. Within the lesioned area, all CNS components were completely eliminated.

Seven rats from the complete lesion group were used for different staining on the cryostat sections. NF immunohistochemistry on horizontal sections showed that the ends of the axons at the edge of the lesions had sprouted and the centre of lesion contained macrophages and tissue debris but neither axons nor glia (figure 3.7, A). The completeness of the lesions was shown in horizontal sections through the entire width of the tract by labelling CST axons rostral to the lesion (Les) on the left and total absence of CaMIIk-α staining in the left CST caudal to the lesion (figure 3.7, B). Horizontal sections immunostained with GFAP after 8 weeks post-operation showed a progressive astrocytic scar around the lesion. In the CST lesioned section, the astrocytic scar contained larger and more densely packed astrocytes with more abundant and hypertrophic processes. By two months the central area appeared to be completely 'walled' off from the surrounding tissue by a solid mass of intensely GFAP-immunoreactive astrocytes and their processes (figure 3.7, C & D), in agreement with previous study (Li et al., 1995).

In normal and lesioned rats, the peripheral SC sheaths of the host dorsal root outside the spinal cord were intensely immunoreactive for P0. 8 weeks after lesion, the endogenous SCs are seen to remyelinate demyelinated axons especially at the edges of the lesion and along the needle tract from the dorsal surface of the spinal cord. A layer of SC myelination is seen to surround the lesion (figure 3.7, E & F).
Figure 3.7

Horizontal sections of rat spinal cord in the presence of NF, CaMIIk-α, GFAP and P0 immunohistochemistry of complete CST lesion. A) NF staining showed, sprouting of axons around the lesion area (Les) and no staining in the middle of lesion. B) CaMIIk-α showed complete destruction of axons in only one side and normal side (CST), C) GFAP showed astrocytic scar formation around the lesion area, E) P0 (black), demonstrated the peripherally myelinated type around the lesion and small arrow indicate P0 immunostaining of host SCs in the dorsal root, D and F) enlargement of the area outlined by the rectangle in C and E) enlargement of the area outlined by the rectangle in D. Sections were between C1 and C2 level. Counterstained with natural red. Survival time, 8 weeks. Scale bars, 500 μm (in A, B, C & E); 100 μm (in D & F).
As explained in section 2.3, in order to estimate the extent of completeness of lesions, serial cryostat cross-sections (20 μm) across the lesion were collected for each rat. The cross-sections were from the surface of the lesion all the way to the bottom of the lesion (1mm depth). All sections for each rat were stained with CaMIIk-α. It is clear that from 3.8 A, the lesion was centred on only one side of CST and which had approximately 60% lesion. In figure 3.8 C, shows 90% lesion on the left CST and the right site had been slightly lesioned as well. Sections E, F and G were taken from the centre of the lesion. It is absolutely clear that there is no CaMIIk-α staining on the left hand site. Section 3.8 H, is a section below the lesion.
Figure 3.8
Serial cryostat cross-sections of the complete CST lesion stained with CaMIIm-α. The sections were collected from the surface to the bottom of the lesion of one rat. Section A, B and C are from the surface of the lesion; D, E and F are from the centre of the lesion. Sections G and H are from the bottom of the lesion. Counterstained with neutral red. Survival time, 8 weeks. Scale bar, 500 μm.
3.2.1.2. Histology of semi-thin sections after complete corticospinal tract lesions

Six rats with complete lesions were used for semi-thin immunohistochemistry. The lesions were identified by total loss of NF staining, and the centre of lesion was occupied by a few macrophages. The components of the lesion in the CST were identified by light microscopic immunohistochemistry of methylene blue, GFAP, NF and P0. In addition to semi-thin staining, electron microscopy was also used.

The histology of semi-thin sections confirmed exactly the same results as the histology on the cryostat sections. Semi-thin sections and electron microscopy confirmed that the centre of the lesion consisted of an amorphous mass of debris and degenerated axons (figure 3.9), which was rapidly permeated by macrophages and later also developed a loose connective tissue stroma with blood vessels. There was an absence of central type myelin (3.10 A, C). The surrounding region contained densely packed, hypertrophic astrocytic processes with massed cytoplasmic filaments, which corresponded to the scar region of the GFAP immunostained section (figure 3.10, E & F). P0 antibody showed that the injured axons in the CST were surrounded by typical peripheral-type myelin and were continuous with a thick a cuff extending along the electrode track all the way to the surface of the spinal cord (figure 3.10, G & H). Immunostaining for FN clearly shows the position, size and shape of the lesion surrounded with blood vessels (figure 3.10, I, J).
Figure 3.9

(A) A low power micrograph of ultra-thin cross section of complete CST lesion. It shows the central type myelin has degenerated (white arrows). (B) Camera lucida drawing shows the overall location and size of lesion in CST, (outlined in gray). This area is indicated by the rectangular box (heavy solid line) in B indicates that the micrograph is taken form the centre of lesion. Arrowhead, central canal; DH, dorsal horn; Les, lesion. Survival time, 8 weeks. Scale bar, 5 μm (in A).
Figure 3.10

Semi-thin coronal sections of rat spinal cord following a complete CST lesion in the presence of (A & B) methylene blue, (C & D) NF, (E & F) GFAP, (G & H) FN and (I & J) P0, immunohistochemistry of complete CST lesion. Sections were between C1 and C2 level. For each staining the sections are adjacent to each other. Right column (B, D, F, H & J), enlargement of the area outlined by the rectangle in left column. Arrowhead, central canal and Les, lesion. Survival time, 16 weeks. Scale bars, 500 µm (in A, C, E, G & I); 100 µm (in B, D, F, H & J).
3.2.1.3. Histology of biotinylated dextran amine tracer in complete lesions

The six rats in control group were used for BD tracer study. Six rats with complete lesion were divided into two groups. Four rats were kept for a period of six months and the other two rats were injected with OEC conditioned medium into CST and kept for further 8 weeks.

To be certain which axons were cut, the sections were taken in a horizontal plane as close as possible to the longitudinal axis of the fibres and included a segment of at least 12 mm of the CST. This consisted of 1-3 mm rostral to the lesion and at least 5 mm caudal to it.

The BD labelled axons were developed by two different methods. The sections from a rat with OEC conditioned medium were developed with A) green fluorescence Streptavidin, Alexa (figure 3.11). B) with DAB (dark brown colour) and GFAP for astrocytic scar (pink colour) (figure 3.12).

The dense astrocytic scar surrounds the central lesioned area. In the control group, the cut ends of the BD-labelled axons were greatly expanded and gave rise to multiple, BD-filled sprouts. In the middle of the lesion a few macrophages can be seen. DAB and green fluorescence staining showed that the ends of the cut axons at the edge of the lesions were swollen and they do not seem to cross the lesion site (figure 3.11 & 3.12). The completeness of the lesions was verified by the total absence of anterograde BD labelling through the entire width of the tract.
Figure 3.11

Confocal image of horizontal 60 μm section showing BD anterograde labelled CST axons (green) rostral to the lesion (Les) on the left and complete absence of axons in the left CST caudal to the lesion (d). The contralateral CST is unlabelled as the BD was only applied to the ipsilateral cortex. Dashed line, midline. Counterstained with propidium iodide (red).

Survival time, 6 months. Scale bar, 100 μm.
Figure 3.12

60 μm horizontal cryostat section of CST, which has been labelled with BD (DAB, black colour). Edge of dense astrocytic scar (GFAP, immunohistochemistry) surrounding the lesion is shown in pink. This result confirms that the cut CST axons still persist in the proximal tract rostrally as far as the cut surface, but none progresses beyond this level. B, Enlargement of the area outlined by the rectangle in A. Survival time, 6 months. Les, lesion. Scale bars, 100 μm (in A), 50 μm (in B).
3.2.1.4. Directed forepaw retrieval after complete corticospinal tract lesions

From 3 days after operation the rats were introduced to the testing cage and the use of the right and left paws was scored for 50 retrievals three times a week for 8 consecutive weeks after operation.

19 rats never used the ipsilateral forepaw for retrieval at any time during the postoperative period of 8 weeks (n= 13, figure 3.16, filled triangles) or 24 weeks (n= 6). The rats in which the histology showed complete destruction of the CST corresponded exactly to the rats, which did not show any use of the ipsilateral forepaw for DFR during the entire 8 weeks post-operative period.

In response to food presentation, the detailed movement of complete CST lesioned rats was as follows: 1) It was clear that the limb was lifted from the floor of the cage 2) the upper arm was not aligned with the body midline. 3) The animals did not use the proximal muscles to extend their arms through the slit 4) The rats tried very hard to use the ipsilateral forepaw, since repeated short range distal movements (wrist flexions) were readily performed in response to presentation of the food pellets.

This meant that the reaching performance of ipsilateral forepaw was scored as none. Even when the contralateral forepaw was restrained, the rats were unable to carry out retrieval with the ipsilateral forepaw, and instead attempted to retrieve the food with the tongue or snout. The test was terminated when the rats made 50 successful retrievals with the contralateral paw, (the contralateral forepaw was inserted through the slot in the cage). Figure 3.13 shows that the rat could not use the ipsilateral forepaw for retrieval and all of retrievals were done with the other forepaw. Frame B illustrates that the ipsilateral forepaw was positioned on the front wall of the cage. This is part of a video clip of the rat trying
very hard to push the ipsilateral forepaw through the slit but failing and hitting the front wall of the cage (figure 3.13, B).

Apart from the defect in retrieval, no impairment of the ipsilateral forepaw in other functions, such as grasping the pellet, walking along a cage edge, climbing, grooming, picking up and handling food was found.
Figure 3.13
Illustrates the frontal view of forepaw retrieval in the rat with a CST lesion on the right side. A) Rat preferred to use the unlesioned side (left forepaw) for retrieving the food. B) Ipsilateral forepaw (right forepaw, arrow) was misplaced and could not push through the slit. C) Finally after failure of using the lesioned side, the unlesioned site was used again to retrieve food. Survival time, 8 weeks. Film, 25 frame per second.
3.2.2. Incomplete corticospinal tract lesions and functional recovery

As it was explained in chapter 2.1.1, to make complete and constant lesions the electrode had to be inserted 0.5 mm away from midline and 0.8-1.0 mm depth, otherwise the 10 volts and 1-minute time could not destroy one side of the CST. The unavoidable minor variations in electrode positioning resulted in varying degrees of destruction of the CST. Of the 40 rats with CST lesions, 19 rats had complete CST lesions. The remaining operated rats had incomplete lesions and they were chosen to make a comparative study between the functional recovery and the extent of lesions. In the case of incomplete lesions the degree of destruction of the CST was expressed as a percentage of the area of remaining CaMIIk-α staining.

3.2.2.1. Histology of incomplete lesions

To estimate the extent of lesion size, a photomicrograph of normal and lesioned CSTs stained with CaMIIk-α antibody was taken (×10 magnification). A 5×5 mm² grid was superimposed on the photomicrograph and the number of squares that were within the lesioned area were counted. This lesion area was expressed as a percentage of the area of the normal contralateral CST (figure 3.14). Subsequent histology showed that each had a degree of sparing of the CST fibres as assessed by the presence of CaMIIk-α immunostaining in the distal CST 1 mm caudal to the lesion.

The variation in the degree of destruction of the CST as seen in CaMIIk-α immunohistochemistry, was categorized into six bins: 2-19% (n=4 rats), 20-39% (n=4), 40-59% (n=4), 60-79% (n=5), 80-99% (n=4) and 100% (n=13).

In figure 3.15 shows that the difference between no spared in 100% lesion and small percentage (1-2%) of sparing axons in the CST, which can support some return of DFR.
Figure 3.14

The extent of axon loss from the CST caudal to the lesions is assessed by placing a 5 mm squared grid over photomicrographs of CaMIIk-α immunostained, 10 μm thick cross sections through the distal CST 1 mm caudal to the lesion (to aid comparison all lesions are shown on the right side of the figure). The extent of the loss of CaMIIk-α staining varies from none (A, Intact), in 5 increasing steps of 20% to 100% (B-F). Counterstained with neutral red. Survival time, 8 weeks. Scale bars, 500 μm (in A-F).
A. Intact

B. 20%

C. 40%

D. 60%

E. 80%

F. 100%
Figure 3.15

10 µm thick cross sections through the distal CST 1 mm caudal to the 100% lesion (A) and sparing about 2% (C). B and D of the CaMIIk-α positive axons (boxed area enlarged in A and C). Dashed line, midline. Counterstained with neutral red. Survival time, 8 weeks. Scale bars, 500 µm (in A & C); 200 µm (in B & D).
3.2.2.2. Recovery of directed forepaw retrieval after incomplete corticospinal tract lesions

There are a number of changes after CST lesions. The contralateral forepaw was used more often than the ipsilateral paw during the test. It was clear that the ipsilateral limb was lifted from the floor and after a number of short range distal movements (wrist flexions), finally the rat used the proximal muscles to extend their arms through the slit.

The reaching performance was scored by counting the successful retrievals. Due to the lesion the animals prefer to use the contralateral forepaw. When the total number of retrievals by both forepaws achieved 50, the test was stopped. It is important to mention that the number of short-range distal movements depended on the size of the lesion. With smaller lesions (2%-50%), the rats made a small number of short-range movements (1 or 2, wrist movement), but with larger lesions (60%-95%) the animals tried very hard and after (5 or 7, wrist movement) ultimately achieved successful retrieval.

In the 21 rats with dorsal CST lesions, the damage extended form 2% to 100% of the cross section of the spinal cord. The animals with 1-19% lesion had an initial deficit on the first postoperative week of testing. The number of paw retrievals on the lesioned side was an average of 15 out 25 (25 is the maximum number of retrievals that rat can achieve with one forepaw). However, this deficit rapidly resolved by the second week of testing compared with intact animals. By the end of 8 weeks the animals had full functional recovery.

Rats with 20-39% lesions showed very similar functional recovery compared to 1-19% lesions. The total numbers of paw reaching after 8 weeks training was 45.
The rats with 40-59% (n=8) lesions showed 8-10 retrievals in first week of training. The functional recovery was up to 38 retrievals after 8 weeks. Functional deficits were more severe after 40-59% lesion compared to 1-19% and 20-39% lesions.

Interestingly, animals with lesions of 60-79% (n=5) could not reach on the first week after operation and these deficits were very severe on the second and third week of training. By the end of the 8-week behavioural test, these rats recovered a maximum of 18 retrievals.

21 rats showed a degree of return of DFR by the ipsilateral forepaw starting at 1 or 3 weeks after operation, with a consistent progressive increase in DFR by the ipsilateral forepaw over the 8 post-operative weeks (figure 3.16). Histologically, these 21 rats all had a degree of sparing of the CST. The extent of DFR in the 5 groups of the lesion sizes was directly related to the degree of destruction of the CST. The three groups of rats with the smaller lesions started retrieval at the first post-operative week, and reached close to normal by 8 weeks. The two groups of rats with the largest lesions did not start retrieval until the second post-operative week, and were still severely impaired at 8 weeks, although on a rising curve, which suggested that greater recovery would have been acquired at longer survivals. Lesions sparing only around 1% of the CST axons (figure 3.15 C, D shows a case with around 400 axons) were still compatible with spontaneous return of DFR, although after a delay of 3 weeks.

The average extent of individual dorsal CST lesions was compared with the locomotor deficits assessed by the DFR (figure 3.17). In rats with even a very small percentage (<20%) of remaining CST, there was a return of forepaw movement on the operated side (corresponding to 4 retrievals out of 25). With about 90% of CST fibres remaining the rats reached the maximum score of 25 points. The correlation between histological evaluation
and the directed forepaw retrievals score was highly significant in rats with dorsal CST lesions ($R^2 = 0.94$).
Figure 3.16

Changes with time (X-axis in weeks) in the number of retrievals carried out by the forepaw ipsilateral to the lesion (Y-axis) in each testing period (out of a total of 50 retrievals by both forepaws). The rate of recovery of DFR over the 8 postoperative weeks is inversely proportional to the amount of the CST destroyed by the lesion. Percentage destroyed: open circles, 1-19%, open squares, 20-39%, open triangles, 40-59%, filled circles, 60-79%, filled squares, 80-99%, filled triangles, 100%. Survival time, 8 weeks.
The correlation between spared CST and the DFR score. It shows the highly significant correlation ($R^2=0.96$) between the number of retrievals by the ipsilateral forepaw (Y-axis) and the proportion of spared CST axons (X-axis). Survival time, 8 weeks.
3.3. Delayed transplantation of olfactory ensheathing cells into complete lesioned corticospinal tract

12 rats with absence of DFR after 8 weeks testing received OEC transplants. All rats in this group were tested for DFR 3 times weekly for 8 weeks after transplantation.

In this section, the immunohistochemistry data from the OEC culture dish and the immunoreactivity of semi-thin and ultra-thin sections for electron microscopy will be analysed. In addition, the data from labelling the regenerated axons by anterograde tracer and behavioural study will be presented.

3.3.1. Characterization of olfactory ensheathing cells in culture

It is still not clear what the similarity is between the cells that have been defined in vivo as ensheathing the olfactory nerves as they enter the olfactory bulb (Doucette, 1984) and the cells that have reparative effects after culture and transplantation. It was necessary to culture the cells for 15-17 days before transplantation, this allows for a considerable amount of cell death, cell division and differentiation to take place.

Primary cultures of adult olfactory bulb were immunostained while in the culture dish, as described in the experimental procedures. After 15 days in the culture, two different antibodies (p75 and FN) were used to identify the various types of cells. Immunostaining (figure 3.18) showed that after 15 days, the cultured cells had segregated into clusters of the two major cell types described by Doucette and Devon (1994), Barnett et al., (1993), and Ramón-Cueto and Nieto-Sampedro (1992): (a) p75, S100 positive or Schwann-like cells (S cells), and (b) FN positive or fibroblasts-like cells (F cells). It has been shown that each dish of 35 mm yields ~1.0 million cells, of which ~ 50% are p75 positive and ~50% are FN positive.
The S cells showed the distinctive spindle-shaped morphology (Brockes et al., 1979), usually bipolar with an oval cell body (figure 3.18, green). The F cells (figure 3.16, red), which tended to grow under the S cells, were flatter with nuclei, which were larger, rounder and strongly positive for FN. By the end of two weeks in culture the F cells were in roughly equal in numbers to the S cells, and remained so after transplantation.
Figure 3.18

Characterization of olfactory ensheathing cells in culture. Confocal image of p75/FN fluorescence double immunohistochemistry of OECs. S cells were stained with p75 (green), F cells were stained with FN (red). The cells were counterstained with DAPI (blue). Survival time, 15 days. Scale bar, 100 μm.
3.3.2. Histology of semi-thin and cryostat sections after delayed transplantation of olfactory ensheathing cells

Subsequent histology (see below) showed in 10 rats that the CST had been completely destroyed and the lesion site completely filled with transplanted OECs. The OEC transplants were clearly demarcated from the surrounding host tissue as can seen by FN staining. Figure 3.19 A, shows a longitudinal section of the transplant stained with fibronectin. At the ventral end of the transplant, where the injection started, the donor cells form a large rounded ball with diameter up to 500 μm. From the dorsal edge of the rounded ball, the transplanted cells continue as an elongated column, which ascends vertically along the withdrawal track of the extrusion pipette to form the central column. The OEC transplant extends caudally within the boundaries of the host CST for distances of 7.0 mm at 8 weeks after transplantation. The transplants were clearly identified as solid, highly vascularised tissue masses with an intense, patterned FN staining. The transplants were around 0.5 mm in transverse diameter, elongated to around 1.0 mm in the dorso-ventral direction (figure 3.20, A & 3.21, B). The regenerating axons become ensheathed by P0-immunoreactive myelin throughout the transplant (figure 3.19, B & C).

p75 immunostaining, while useful for identifying the S cells in culture, and at short term survivals after transplantation, was not helpful in the present material as it was down-regulated after about 3 weeks, although the presence of the S cells were later identifies by P0 immunostaining for peripheral myelin (figure 3.20, B). In 10 rats studied in cross section histology, P0 immunohistochemistry for peripheral myelin clearly showed that regenerating axons were myelinated by OECs during their course through the transplants. The clarity of the P0 staining in cross section enabled me to count the total number of fibres crossing the transplants (figure 3.20 B, C). For 10 rats, the mean and standard error was 532±58.
The results show that under the influence of OECs, regenerating axons can grow through an environment despite the presence of activated astrocytes. The transplants did not completely reverse the astrocytic hypertrophy, but they seemed to reduce it and the astrocytic processes showed more of a tendency to rearrange themselves as a rather more 'open' surface (figure 3.21, C & D). The complete lesion with GFAP staining illustrates that there is a closed surface of astrocytic scar around the lesion (figure 3.10, E, F).

In 2 rats the transplants (stained with FN) had been mis-placed dorsally, in the ascending dorsal columns, and did not occupy the lesioned CST in the ascending dorsal columns. PO immunohistochemistry illustrates the presence of peripheral type myelin (figure 3.22, E & F). As it is clear from figure 3.22, the lesions consisted of circumscribed cystic areas bigger than 0.5-1.0 mm in transverse diameter, which can destroy about 1% of the contralateral side of the lesion. This case clearly shows that inside the lesion there is no NF staining (figure 3.22, C & D).
Figure 3.19

Migration of OEC in lesioned CST. A, Montage of 3 horizontal 10 μm thick cryostat sections showing the selective migration (arrows) of the intensely fibronectin-positive transplanted cells from the injection site (IS) caudally for 7 mm along the distal CST. B & C, Horizontal sections adjacent section to A, showing migrating OEC column stained with P0 in the middle lesion/transplant region. Sections were counter stained with neutral red. Survival time, 16 weeks. Scale bars: 500 μm (in A); 100 μm (in B & C).
Figure 3.20

Histology of rats with delayed OEC transplantation into complete lesioned CST. A, Cross section showing a transplant stained with anti-FN antibody in the CST. 2 μm semithin sections counterstained with methylene blue and Azur II. Arrowhead, central canal. B, Adjacent section stained with anti-P0 antibody to show the regenerating axons (about 200-300) myelinated by OEC (peripheral-type) myelin. C, Enlargement of the area outlined by the rectangle in B. Survival time, 16 weeks. Scale bars, 100 μm (in A & B); 50 μm (in C).
Figure 3.21

The reaction of the astrocytic scar due to OEC transplantation. A, 2 μm semi-thin coronal section stained with FN. C, adjacent section to A, stained for GFAP, to show the considerable reduction in astrocytic hypertrophy in comparison to figure 3.8 E & F. B & D are the enlargement of the area outlined by the rectangle in A & C. Section A counterstained with methylene blue and Azur II. Arrowhead, central canal and black arrow, OEC transplant. Survival time, 16 weeks. Scale bars, 500 μm (in A & C); 100 μm (in B & D).
Figure 3.22

Misplaced OEC transplant (white arrow) in the dorsal columns of a rat with a lesion (Les) of the CST. A) Stained with methylene blue and Azur II, B) stained with FN to show the location of the transplant, C) NF, and E) P0. 2 μm semi-thin adjacent section. D & F are enlargement of the area outlined by the rectangle in C & E. Arrowhead, central canal. Black arrow, P0-positive staining in the dorsal root fibres. Survival time, 16 weeks. Scale bars, 500 μm (in A, C & E); 100 μm (in B, D & F).
3.3.3. Electron microscopy of ultra-thin sections after delayed transplantation of olfactory ensheathing cells

Ultra-thin cross sections (figure 3.23 & 3.24) confirm that the axons are myelinated by peripheral type myelin and remain devoid of any central glial cell types (astrocytes, oligodendrocytes).

S cells myelinated a single axon in a collagen-containing extracellular space (figure 3.23). The S cells are surrounded by basal lamina (figure 3.23, arrowhead) and are often separated from each other by abundant extracellular deposits of collagen fibrils (Berthold, 1978). The F cells (see figure 3.23, indicated by F) are elongated, with paler cytoplasm and paler nuclei and a more irregular shape. They are located in the collagen-containing extracellular space and they do not contact the host axons. In the cross sections, the sheet-like curving F cell processes can be seen to form layers like shells, enclosing groups of axons and their associated S cell wrapping. Some of the F cells aggregate in close contact with each other, at these points the membrane thickens over much of the contact areas.

Figure 3.24 shows the one-to-one relationship between the axon and peripheral type myelin (see figure 3.24, indicated by M). The S cell has a big overall rounded dark nucleus (figure 3.24, indicated by N).

Figure 3.25 shows number of axons (figure 3.25, indicated as X), which are myelinated by an S cells (figure 3.25). Bundles of 9 axons are wrapped by outer sheaths made up of F cells (figure 3.25, indicated as F).
Figure 3.23

A, Electron micrograph of a cross section of a delayed OEC transplant into a complete lesion. There is a bundle of fine non-myelinated axons (nx) enwrapped by S cell processes (S), and myelinated axon (x) in a collagen-containing extracellular space (C), with an outer sheath of curving sheets of loosely apposed F cell processes (F). Part of basal lamina showed by arrowhead. B, Camera lucida drawing shows the location of OEC transplant (outlined in black, T) in the CST. The area indicated by rectangular white lined box is that from which ‘A’ is taken. Survival time, 16 weeks. Scale bars, 1 μm (in A), and 1 mm (in B).
Figure 3.24

Electron micrograph of a cross section of a delayed OEC transplant into a complete lesion. The S cell making the myelin sheath (M) around the axon (X) was cut at the level of the nucleus (N). The peripheral-type myelin is formed on a one-to-one basis by S cells. E, extracellular space. Basal lamina is marked by arrowheads. C, collagen fibres in the endoneurium. Survival time, 16 weeks. Scale bar, 1 μm.
Figure 3.25
Electron micrograph of a cross section through a column of axons e.g. (x) taken from the centre of the transplant/lesion and myelinated by S cells. A bundle of 9 axons and their myelinated cells are wrapped by outer sheath of F cells (F). In the middle of myelinated axons, there is a growth cone (GC). Survival time, 16 weeks. Scale bar, 5 μm.
3.3.4. Functional recovery after delayed transplantation of olfactory ensheathing cells

It was shown clearly by histological and functional studies that the complete absence of DFR over an 8 weeks period after CST lesions only occurs when subsequent histology shows that the CST on one side has been completely destroyed. It therefore enables us to identify animals with complete CST lesions since they showed no DFR during 8 weeks after the CST lesions and it was these rats that were chosen for OEC transplantation. After OEC transplantation rats were tested three times a week for another 8 weeks.

In the present series of experiments, 12 animals received OEC transplants and 10 showed a return of DFR beginning between 1-3 weeks after transplantation, which continually increased over the next 8 weeks to a maximum of 4 – 11 reaches (normal rats being 25; figure 3.26). It is clear that the 10 rats with correctly positioned OECs into CST showed 40% functional recovery after 8 weeks training (figure 3.27). In 2 rats, the OEC transplants were in the ascending dorsal columns, dorsal to the lesioned CST (figure 3.22). In these cases there was no return of DFR over the 8 weeks after transplantation.

It was obvious that there were changes in the lesioned rats in the forepaw movement compared to the normal rats without any lesions. The major change was that the ipsilateral forepaw was never used for retrieval during the 8 weeks after the lesion, but subsequently the rats were able to start to reach for the pellet. The sequence of movements of retrieval were as follows: 1) The forepaw was lifted from the floor. 2) As the limb was lifted, the wrist was partially fixed. 3) The limb was advanced indirectly through the slot. The forepaw hit the front wall of the cage. 4) After few attempts the limb was extended through the slit. It is also noteworthy that lesioned rats misplaced the forepaw through the slit, and this specific movement was quite slow and weaker than to the other movements such as lifting, wrist and grasping movements.
Frame by frame analysis from video clips demonstrated that OEC transplanted rats were able to advance the ipsilateral forepaw through the slit (figure 3.28, A). It was observed that during the training, the rat preferred to use the unlesioned forepaw for retrieving the food (figure 3.28, B).
Figure 3.26

The onset and progressive increase in numbers of paw retrievals (Y-axis) achieved in each of the thrice weekly tests over the 8 weeks (X-axis) after transplantation in the series of 10 rats which had showed no DFR in the 8 week period after lesioning and preceding transplantation of OECs. The approximate size and position of the maximal extent of each of the transplants is indicated in solid black on the thumbnail outlines of the spinal cord. Survival time, 16 weeks. Scale bar, 1 mm.
Figure 3.27

Forepaw retrievals counts from non-operated (n=25, normal), lesion alone (n=8) and lesion plus OEC transplant (n=10). Rats with OEC transplantation showed 40% DFR recovery compare to the rats with normal rats. Survival time, 16 weeks.
Number of retrievals

- Normal
- Lesion
- Lesion plus OEC
Figure 3.28

Illustration of extending the ipsilateral forepaw (right side, arrow) through the slit after OEC transplantation into the CST. A) Ipsilateral paw (right side, arrow) was able to retrieve the food pellet. B) Rats used the unlesioned site (left forepaw) for retrieving food. Survival time, 16 weeks.
3.3.5. Histology of biotinylated dextran amine tracer

BD anterograde tracing of the regenerating CST axons was carried out in 4 rats with transplanted OECs and post-transplantation return of DFR. As described in a previous study by Li et al., (1997), the cut CST axons enter the transplants and traverse them as a rostro-caudal tract of parallel fibres, crossing the lesion/transplant site, and re-enter the caudal part of the CST, where they continue as straight, largely unbranched fibres (figure 3.29). In these samples, the regenerated CST axons were seen at different distances caudal to the transplant site. In each rat, the maximum distance the axons travelled was measured. In one case the regenerated CST axons were seen to extend for up to 11 mm caudally. Throughout their course they emit branches, which leave at right angles and travel for up to 400 µm in a lateral direction into the adjacent grey matter, where they brake up into arborisations (figure 3.29, B & C). In the other three cases, the regenerated axons are visible caudal to the transplant for a distance of 9.5 ± 0.65 (mean ± SEM; n=4).
Figure 3.29

A) Camera lucida drawing of the position of BD labelled CST axons passing from the proximal tract (pt) through a transplant (tr) and caudally into the distal CST (dt) and forming arborisations in the adjacent grey matter (gm) lateral to the tract. Dashed line, midline; solid line is border between CST and adjacent grey matter. Green shading shows areas photographed in B & C. B & C) Confocal images showing BD anterogradely labelled CST axons (green) passing caudally through the distal CST with collaterals travelling medially at right angles, and generating a terminal arborisation in the grey matter. C) The regenerated CST axons were seen to extend for up to 11 mm from the injection site, the long distance is represented by zig zag in A. Horizontal 60 µm thick cryostat sections caudal to an OEC transplant placed in a complete CST lesion. Counterstained with propidium iodide (red). Survival time, 16 weeks after lesion, 8 weeks after transplant. Scale bars, 250 µm (in A top part, B), 100 µm (in A bottom part & C).
3.4. Delayed transplantation of adult Schwann cells into complete lesioned corticospinal tract

8 weeks after complete CST lesions and no return of DFR, 2 rats were transplanted with adult SCs prepared from sciatic nerve. The recovery of DFR and histology on semi-thin sections will be presented.

3.4.1. Characterization of adult Schwann cells in culture

Primary adult SCs were isolated from the sciatic nerve of the adult rat. In this study adult SCs were that were 95% pure transplanted into the lesioned CST (figure 3.30). The purity of the SCs was estimated by counting the number of SCs relative to the total number of cells. The cultures contained S and F cells. The S cells were stained with p75 and F cells, were stained with FN (figure 3.30). The SC nuclei were small, oval and brightly labelled and the F cells nuclei were larger, round and pale.
Figure 3.30

Characterization of adult Schwann cells in culture. Confocal images of p75/FN fluorescence double staining, showing 95% purity of adult SCs. SCs were stained with p75 (green), fibroblasts were stained with FN (red). The cells were counterstained with DAPI (blue). The purity of SCs is determined by counting the number of SCs relative to the total number of cells. A sample of adult SCs suspension used for transplantation was plated out in a 35 mm dish and cultured for one day. Survival time, 4 weeks. Scale bar, 100 μm.
3.4.2. Histology of semi-thin sections of delayed adult Schwann cell transplanted rats

Transplantation of adult SCs into complete CST lesions were done after 8 weeks training. The behavioural test was continued for a further 8 weeks after transplantation. The animals were then ready for immunohistochemistry analysis. FN immunohistochemistry on the semi-thin sections showed that the transplants were located in the CST. The transplants were clearly identified as vascularised tissue masses, with an intense patterned FN staining. In addition, figure 3.31 C and D clearly shows that there are axons stained with NF antibody in the transplant.
Figure 3.31

Histology of rats with delayed adult SCs transplantation into complete lesioned CST. A) 2 μm semi-thin coronal section stained with methylene blue. B) Adjacent section stained with FN antibody to show the transplant location. C) Adjacent section stained with NF antibody to show the axons in the middle of the transplant. D) Is the enlargement of the area outlined by the rectangle in C. Arrowhead, central canal; dashed line, midline; solid line to outline CST position and DH, dorsal horn. Survival time, 16 weeks. Scale bars, 500 μm (in A, B & C); 100 μm (in D).
3.4.3. Recovery of directed forepaw retrieval after adult Schwann cell transplantation

Rats with adult SC transplantation showed a return of DFR beginning between 3-4 weeks after transplantation, which continually increasing over the next 8 weeks to a maximum of 2-4 retrievals (figure 3.32). In comparison, OEC transplanted rats, showed return of DFR within 1-2 weeks after transplantation and the maximum numbers of retrievals were 11.
The onset and progressive increase in numbers of paw retrievals (Y-axis) achieved in each of the thrice weekly tests over the 8 weeks (X-axis) after transplantation in the series of 2 rats which had showed no DFR in the 8 week period after lesioning and preceding transplantation of adult SCs. The approximate size and position of the maximal extent of each of the transplants is indicated in solid black on the thumbnail outlines of the spinal cord. Survival, 16 weeks. Scale bar, 1mm.
3.5. Delayed transplantation of pure fibroblasts into complete lesioned corticospinal tract

Two rats were transplanted with 99% pure fibroblasts. Three different stains were done on the semi-thin sections. The DFR study will be presented below.

3.5.1. Characterization of pure fibroblasts in culture

Pure fibroblasts were cultured from adult sciatic nerve. From the first passages of the explants the majority of cells migrating out from the explants were fibroblasts. In the double staining of p75/FN no S cells (green) seen, more than 99% of the cells were stained with FN (red) (see figure 3.33).
Confocal image of p75/FN fluorescence double immunostained pure fibroblasts stained with FN (red). There are no SCs (green). The cells were counterstained with DAPI (blue). The purity of fibroblasts is determined by counting the number of fibroblasts relative to the total number of cells. Survival time, 14 days. Scale bar, 100 μm.
3.5.2. Histology of semi-thin sections of delayed pure fibroblasts transplants

Two different antibodies were used on the semi-thin sections; these were FN and NF. FN staining illustrated the position of the graft in the CST lesion. The staining was much fainter and not as compact as OEC transplants and there were many areas not filled with transplanted cells (figure 3.34, B). Large numbers of host macrophages had invaded the centre of lesion/transplant region. No NF-positive staining was observed in the transplants (figure 3.34, C & D).
Figure 3.34

Histology of rats with delayed pure fibroblast transplantation into complete lesioned CST
A) 2 μm semi-thin coronal section stained with methylene blue. B) Adjacent section stained with FN antibody to show the transplant location. C) Adjacent section stained with NF antibody to show there are no axons in the transplant. D) Is the enlargement of the area outlined by the rectangle in C. Arrowhead, central canal. Survival time, 16 weeks. Scale bars, 500 μm (in A, B & C); 100 μm (in D).
3.5.3. Directed forepaw retrieval function after pure fibroblast transplantation

Two rats with complete CST lesion, which were transplanted with a suspension of 99% fibroblasts showed no DFR recovery after 8 weeks postoperative training. The absence of functional recovery was not due to misplaced transplants. This was confirmed with FN staining. NF staining further strengthen the results to show that there was no regenerated axons in the transplant.
Chapter 4

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4.1. General discussion

The failure of axon regeneration and the failure of remyelination in the adult CNS are two important areas of interest that occupy the minds of neuroscientists.

The first part of this study indicated that DFR was completely abolished for at least 8 weeks after complete unilateral destruction of the CST. In rats where part of the CST was spared, there was an initial loss of retrieval on the lesioned side, followed by a progressive return over 8 postoperative weeks of testing. The rate and degree of recovery were proportional to the percentage of surviving CST axons.

In the second part of the study, lesioned rats with a suspension of OECs injected into the lesion site showed DFR recovery in one to three weeks after transplantation. The rats started to use the forepaw of the transplanted side, initially at a low rate compared with the normal side, but this progressively increased with time.

In the third section of the study, the 8 weeks delayed transplantation of 95% pure adult peripheral nerve SCs into the complete CST lesions, showed DFR recovery after four to five weeks following transplantation, but at a much lower level than the rats had OECs transplants.

In the control group, transplantation of pure fibroblasts and OEC-conditioned medium into complete CST lesions showed no functional recovery at all during 8 weeks post-training. There was also no recovery even when the survival time was extended up to 6 months.
In this chapter, the logic and literature review behind this study design will be explained. This includes the method of creating lesions and the reason for choosing the CST as a model. The argument for use of delayed OEC and SC transplantation for regeneration will also be presented. In the third section of this chapter the correlation of functional recovery related to the degree of CST damage will be discussed.

4.2. Study design

The potential value of cell transplantation for human spinal cord injuries will have to take into account the inevitable delay seen in clinical practice between the time of injury and the time when the condition of the patient is sufficiently stable to be certain that no further spontaneous recovery can be expected without transplantation. The experiments presented attempt to provide a model to correlate the extent of damage, the level of spontaneous functional recovery and the effects of OECs in regeneration of complete CST lesions.

The following sections present the justifications for A) developing a precise method for producing a constant lesion, B) choosing the CST as a model, C) using delayed transplantation.

4.2.1. Creating spinal cord lesions in an animal model

Careful examination of the various methods used to injure the mammalian spinal cord is of the utmost importance in understanding the impact of experimental lesions. Several different techniques have been used to make lesions in the spinal cord.

These are compression (e.g. inflated balloons or weight drop) (Fehlings and Tator, 1988; Das, 1989), ischaemic (e.g. clips) (Wallace et al., 1990), heat (e.g. radio-frequency) (Li and
Raisman, 1995), transection (e.g. cutting or removing a segment) (Ramón-Cueto et al., 2000) and photochemical (e.g. organic dye, erythrosine B) (Watson, 1987; Bunge et al., 1994) of spinal cord.

In this study, it was important to produce a precise and complete lesion of only one motor tract pathway so that we could be sure that the regenerative responses were due to OEC transplantation and not due to sparing of axons.

Each method of compression lesioning (inflated balloons or weight drop) has its own typical characteristics. The weight drop technique (Allen, 1911; modified by Beattie et al., 1988) allows a significant correlation between the anatomical extent of the lesion and the motor deficiency (Bresnahan et al., 1991; Basso et al., 1995). Ischaemic injury has been created using aneurysm clips (Wallace et al., 1990). In 1987, a photosensitising organic dye (Rose Bengal, erythrosine B) was injected into the spinal cord and irradiated with a light beam of appropriate wavelength. The light was absorbed by the dye resulting in free radical production (Watson, 1987). Vascular thrombosis, resulting from an intravascular photochemical reaction induced by a Rose Bengal/laser beam interaction led within a few days to an extensive area of tissue deterioration (Bunge et al., 1994). Verdu et al., 2001, also used a photochemically damaged spinal cord model.

All the techniques mentioned above require a pre-injury laminectomy to expose the spinal cord. This is in contrast to the human clinical situation where in most cases of spinal cord trauma is due to a closed injury. Martin et al., 1992 described an elegant model to produce closed traumatic injuries to spinal cord of adult rats, using an extradurally positioned,
inflatable balloon. Close correlation could be noted between histopathological damage, the physical parameters of compression and the severity of the behavioural deficits.

In addition rats take a longer time to recover when lesions produced by methods such as removing a segment of spinal cord (Ramón-Cueto et al., 2000) or dropping a weight on to the spinal cord (Schrimsher et al., 1992) are used. Both can cause uncontrolled levels of extensive damage to adjacent motor and sensory fibres outside the CST. Lesions caused by dropping weights are not constant and cannot be created as precisely as surgical lesions. For any type of experimental lesion in the spinal cord, the most common complication is the large variation in the amount of anatomical and physiological damage of the injured axons.

In general, in compression lesions, there is a central area of total destruction in the fibre tracts, surrounded by partially damaged tracts. The regenerative response of the more compressed tracts may differ from the relatively undamaged ones. This causes an additional difficulty in interpreting the regenerative response of CNS after transplantation. The variation in anatomical damage could make a difference in regenerative responses. An important aspect of the methodological issue relates to the completeness of the lesions. In complete lesions it is difficult to record accurately the extent of behavioural recovery.

Most laboratories have worked with contusion lesions or surgical transections of the spinal cord. While these compound lesions mimic the situations found in clinical injuries, they also cause variable degrees of damage to the surviving tissue. The tips of the severed axons find themselves confronted with a number of different situations and considerable misalignment with the distal parts of their original pathways.
Li and Raisman (1995) devised a micro-lesion or keyhole approach using much smaller and precisely controlled electrolytic lesions, placed at a fixed level within the CST on one side. These lesions provide analysable results, which can be compared easily with the intact fibres of the adjacent spinal tracts, but it was not easy to produce the lesions bigger than 0.5 mm diameter. In my study, a radio-frequency device was used, which can totally destroy one side of the CST by heat and repeatedly produce lesions of the same size and shape. The underlying aim of a radio-frequency lesion is to create a small, ovoid area of damage deep in the spinal cord with minimal damage to adjacent tracts. It was intended, as far as possible to limit damage to any other part of the spinal cord, or other tracts whose damage might influence the functional data.

The stereotaxic approach to the CST requires precision. Positioning depends on the exact angle of flexion of the neck. The identification of the dorsal midline of the spinal cord is made difficult by variations in the pattern of veins associated with the dorsal median sulcus. Nonetheless, a stereotaxic approach in rat spinal cord, does allow accuracy sufficient for a lesion of 0.5-1.0 mm diameter to be placed in such a way as to get consistent total destruction of a tract of around 0.5 mm diameter. The inevitable sparing of parts of the CST by ‘mis-placed’ lesions in some cases was integrated into the overall study design, this allowed a quantitative correlation of the amount of spared tract with the degree of recovery of DFR (see section 4.3.2).

The CST was selected as the best tract for making the lesions, for the following reasons: the CST has been investigated due to its well-defined anatomic and functional characteristics. Traditionally, in higher mammals the CST is considered to be the prime source of motor commands to the spinal cord and skilled movements. Although, neither
CST injury resulted in only minor deficits in locomotor capacity (Metz et al., 1998; Muir and Whishaw, 1999). Therefore, injured animals recover well and can be kept for longer periods of time under regular animal husbandry conditions. The very specific deficits occurring after CST lesions are restricted to fine motor control, particularly of hand and finger movements (Whishaw, 1993).

As it was explained in section 2.2.2, localised radio-frequency lesions can produce a functional deficit that can be clearly observed and recorded. In this project a pilot experiment was set up to look for the absence of DFR in complete lesions and return of DFR in transplanted rats. The failure of DFR following complete CST lesions is a failure of orientation, due to the inability to extend the forelimb through the slit and place it in a position where the wrist flexors would be able to bring the paw into contact with the food pellet. There was no obvious asymmetry in the use of the forelimbs in walking, grooming and in handling the food once it has been grasped.

4.2.2. Transplantation of olfactory ensheathing cells

Several strategies aimed at providing future treatments for spinal cord injuries are under investigation. These include (1) encouraging the survival and growth of damaged axons using neurotrophins (Blesch et al. 1999; Bradbury et al. 2000; Ramer et al. 2000; Bamber et al. 2001), (2) targeting the downstream signalling molecules which promote axonal outgrowth, for example, cAMP (Stichel & Muller, 1998; Qiu et al. 2002) and lastly (3)
neutralizing inhibitory molecules associated with the failure of axonal regeneration, e.g. enzymatic treatments for chondroitin sulphate proteoglycans (Bradbury et al. 2002), antibodies to Nogo receptors (Brittis & Flanagan, 2001; Huber & Schwab, 2000; McKerracher & Winton, 2002).

One of the most promising candidates for cellular transplant-mediated repair of the CNS lesions is the OEC. The primary olfactory system is an unusual tissue in that it can support neurogenesis throughout life (Graziadei, 1973; Graziadei et al. 1979; Farbman, 1990). In addition, newly generated olfactory receptor neurons are able to grow into the CNS environment of the olfactory bulb tissue and reform synapses. It is thought that this unique regenerative property depends in part on the presence of OECs. For this reason it was though by many scientists that OECs are strong candidate for repair of CNS injuries.

It has been reported that OECs can remyelinate experimentally created demyelinated axons (Franklin et al. 1996; Imaizumi et al. 1998; Barnett et al. 2000; Kato et al. 2000) and can promote regeneration (Li et al. 1998; Ramon-Cueto et al. 1998, 2000; Nash et al. 2002). Ramon-Cueto et al., (1998) showed that by transplantation of purified OECs from adult rats into complete transection spinal cord, OECs were capable of promoting long-distance axonal regeneration. Other scientists have used different injury model such as dorsal root entry zone and transplanted cells from adult donor animals and purified the cells using immunopanning (Ramon & Nieto-Sampedro, 1994; Navarro et al. 1999). In some studies in which remyelination of experimentally denuded axons were demonstrated (Franklin et al. 1996), cell grafts were prepared from neonatal animals. Although it is unlikely that these differences in cell preparation techniques can explain the contrasting results, the influence of the cell preparation method and the effect that resulting mixture of cells has on
the effectiveness of OEC grafts is emerging as an important issue. Axonal regeneration and/or improved functional recovery after spinal cord injury have been reported using purified OEC grafts (Ramon-Cueto & Nieto-Sampedro, 1994; Ramon-Cueto et al. 1998, 2000; Navarro et al. 1999; Nash et al. 2002; Pascual et al. 2002), non-purified cell preparations obtained from the olfactory bulb (Li et al. 1997, 1998, Keyvan-Fouladi et al., 2003) and even nasal olfactory tissue implanted directly after removal (Lu et al. 2001, 2002). There are, however, suggestions that the cells with which they are transplanted may influence the properties of OECs. For example, meningeal cells are reported to enhance the remyelinating properties of OECs (Lakatos et al. 2003), and some consider that the fibroblasts contained in non-purified cell cultures prepared from the olfactory bulb are crucial to their regenerative properties (Raisman, 2001).

This raises the question of which types of cells are important for effective OEC grafts? There are several reports, based mainly on evidence from behavioural testing, that transplants of OECs can provide a remarkable improvement in recovery of function after spinal cord injury (Li et al. 1997, 2003; Ramon-Cueto et al. 2000; Lu et al. 2001, 2002; Nash et al. 2002). The axonal regeneration obtained using transplants of OECs, although apparently modest (e.g. Li et al. 1998; Ramon-Cueto et al. 1998, 2000), might provide a sufficient repair for the functional benefits observed. Li et al (1998) reported that unilateral lesions of the dorsal CST abolish DFR for the ipsilateral forepaw. In their study cultured OECs were transplanted into the lesion site at the time of injury resulting in functional recovery. The effect of non-purified OECs obtained from olfactory bulb was studied in my delayed CST injury model.
4.2.3. Transplantation of Schwann cells and pure fibroblasts

One of the most promising initiatives for repair of CNS lesions was Cajal’s proposal that transfer of glial cells from an area where axons can regenerate may transfer the property of regeneration to an area where regeneration does not occur. The original idea was to transfer SCs, either as nerve grafts or as cultured cells into CNS lesions. These transplants indeed induce substantial growth of cut central axons. However, the axons show only a slight aptitude to leave the grafts and re-enter the host CNS. In experiments where detached dorsal roots were re-apposed to the spinal cord, axons are similarly reluctant to cross the boundary from the Schwann cell environment into the astrocytic environment of the spinal cord. The discovery that the replacement of adult olfactory neurons leads to structural and functional regeneration of the olfactory nerves into the CNS led to the suggestion that transplantation of the glial cells of this system, the OECs, might be more effective in repairing CNS lesions, and there a now a number of studies verifying this.

Schwann cells have many of the properties desirable for use in transplant-mediated repair. They can be readily harvested from the peripheral nerve and easily purified and grown in culture in large numbers from both rat and human tissue. The reparative abilities of SCs and OECs in the adult spinal cord have been studied in partial and complete transection models (Ramón-Cueto et al., 2000; Plant et al., 2001; Takami et al., 2002). The simple and seemingly obvious conclusion that the improved repair is mediated by the transplanted OECs leaves a number of unanswered issues, which may be appreciated by reference to lesions of the CST. As has been described in other lesions of the spinal cord, lesions of the CST induce the ingrowth of endogenous SCs. These SCs, however, are not sufficient to produce detectable functional repair. The possibility remains, however, that OEC
transplants have in some way potentiated the ingrowth of endogenous SCs or made them more effective at repair, and therefore that it is the SCs that are mediating the recovery.

This question is difficult to answer since there is no phenotypic character enabling transplanted OECs to be distinguished unambiguously from endogenous SCs. In addition obtaining OECs from the olfactory bulb is more complicated than harvesting Schwann cells from the sciatic nerve. I therefore undertook series of experiments using a reparative paradigm defined in a study using OEC transplants. This injury model system was consistent and quantitatively robust, it provided an excellent baseline to compare the effects of SC transplants. In this study I have used this paradigm to compare the effects of OEC transplants with transplants of a mixture of 95% pure adult peripheral nerve cultured SCs and 100% pure fibroblasts.

4.2.4. Survival and location of the transplants

So far the majority of repair studies have been conducted with transplantation taking place at the same time as the lesions were made (Ramón-Cueto et al., 2000; Ramón-Cueto et al. 1998; Imaizumi et al., 1998). This will certainly not be the case in human clinical trials, both for practical reasons, but also because the unpredictability of ‘spontaneous’ repair means that the prognosis for any individual spinal injured patient may not be clear until anything up to a year after injury.

Plant et al., (2003) demonstrated that when the delivery of transplants was delayed for a period of time after spinal cord transection, the amount of axonal growth and the amount of recovery of function were dramatically increased in comparison to immediate transplantation. Weidner et al., (1999) suggested that downside of delayed transplantation
is the possibility that the regenerative potential of the PNS and CNS may decrease over
time due to events such as cellular degeneration, scar formation and down-regulation of
growth promoting molecules.

In our study, the reasons for the 8 weeks time delay were:
1) To make sure the CST lesions were complete and that there was no spontaneous
functional recovery after lesion. Following incomplete lesions even small numbers of
surviving fibres (only around 1%) can mediate useful return of function. This is crucial in
my study that to make sure that the lesions were complete and that no spared axons were
observed by staining.

2) The life span of a rat is around two year and as the rat is ageing the functional recovery
performances are not as clear as when the rats were younger.

3) The cellular events after spinal cord injury are very important and may be beneficial for
regeneration. These include sprouting of cut and uncut fibres, formation of new, abnormal
terminal fields, die-back of axons and/or neuronal atrophy or death. In the lesion site there
is immediate death of oligodendrocytes, activation of microglia, invasion of macrophages,
and progressive astrocytic hypertrophy (scarring), together with cavitation, changes in
vascular density and distribution, fibrosis and invasion of SCs (for refs see Li et al., 1999).
It was reported that around 4 weeks after CST lesions, P0 (peripheral type myelin)
immunoreactivity start to show up around the lesion but no staining was observed in the
middle of lesion. Therefore the situation facing a delayed transplant is quite different from
that of an immediate transplant. Some of these changes may be beneficial (e.g. sprouting,
vascularisation), others detrimental (e.g. die back, scarring and cavitation).
Coumans et al., 2001, transplanted fetal spinal cord and neurotrophins into a complete spinal cord transection at a midthoracic level. Transplants were placed into the lesion cavity either immediately after transection (acute injury) or after a 2-4 week delay, and neurotrophic factors were administered exogenously via an implanted minipump. Host axons grew into the transplant in all groups. It was reported that regeneration from supraspinal pathways and recovery of motor function were dramatically increased when transplants and neurotrophins were delayed until 2-4 weeks after transection rather than when they were applied acutely. In a previous study by Li et al., (1997), it was found that in rats where OECs transplanted at the time of operation into unilateral lesions of the CST there was a recovery of DFR by the ipsilateral forepaw by about 10 days after transplantation. It is likely that the differences between acute and delayed transplant injury conditions are not restricted only to the local tissue environment, rather there may be differences in the ability of neurons themselves to mount a regenerative response. The process of re-exposing the lesion site 2 weeks or more after injury and clearing away the glial scar at the injury site before placement of the transplant may actually elicit a 'conditioning lesion' (Coumans et al., 2001; Neumann and Woolf, 1999). Although in my study the glial scar was not cleaned away, the cells were injected using a solenoid-driven air pressure system (Emmett et al., 1990) into the lesion site using a glass micropipette. In my opinion, the air pressure may have caused the astrocytic scar to be broken up making some gaps within the heavy astrocytic scar that could enable some axons to grow through the lesion/transplant area.

One of the closest publications to the present work is that of Lu et al (2002) who showed that transplantation of olfactory mucosa derived cells into complete spinal transections caused an increased rate of recovery in the BBB scale. Two very significant aspects of this
work are (1) the use of cells from the mucosa rather than (as in my studies) from the olfactory bulb, and (2) the recovery after delay. The value of (1) is that the mucosa will be more accessible than the bulb for obtaining adult human cells from the patient. It is important in the future to test whether mucosal cells are equally good if not better in my system. The value of (2) is that it implies that the maturation of the glial scar is not an irrevocable obstacle, and that repair does not absolutely require instant transplantation, which is obviously impossible in human injuries. A problem with the study Lu et al is that the BBB scale is a multiple function aggregate, with a subjective element, and the return of function is not, as in my case, the return of something absolutely lost, but simply an acceleration of a recovery which does occur even in the absence of transplants. Because of this difference, it is in this case more likely that the transplants may be acting by stimulating existing systems (remyelination, sprouting, secretion of growth factors, recovery of blood supply etc) rather than by inducing regeneration of cut fibres which are essential for that function. One reason to think that regeneration of cut fibres is less likely in the Lu et al study is the size of the lesion. My data indicate that the transplants must form a complete anatomical bridge. This is possible in small, circumscribed stereotaxic radiofrequency CST lesions, it is a much more difficult proposition in spinal transection followed by resection of a 3-4 mm gap, especially with the limited numbers of cells available, and the difficulty of retaining them at the operated site.

In my study a key methodological challenge was to ensure the cells were injected exactly into the centre of the lesion. A detailed drawing of the pattern of surface blood vessels and the exact co-ordinates of the lesion site was made for each rat, these records were used to mark the exact area for cell transplantation. Even so in two cases, the OEC transplants were mis-placed, 1-2 mm away from the lesion site in the ascending dorsal columns, and
did not occupy the lesioned CST (figure. 3.22), this resulted in no return of DFR over the 8 weeks after transplantation.

Microtransplantation of the cell suspension was minimally traumatic to both donor cells and the host structures, and provided rapid and intimate contact between grafted cells and host tissue. This allowed the behaviour of the transplanted cells and the host response to be studied under the same conditions, which minimised the damage such as haemorrhage, which is inevitably caused by the transplantation of solid pieces of peripheral nerve or intact tissue fragments (David and Aguayo, 1981).

4.2.5. Labelling of the regenerated axons by the anterograde tracer

My study showed that regenerating axons, which crossed the lesion/OEC transplant site and re-entered the caudal part of the CST were few. Therefore to label these few axons by using an anterograde tracer was difficult. The reasons behind the problem of uptake of an injected anterograde label were as follows: 1) after lesion there is retrograde cell death, which is completed within about 1-month post-axotomy (Houlé and Ye, 1999) and thus a reduced population of neurons will be available after this period. 2) During microtransplantation of cells into an injury site, the glial scar that has formed around the initial lesion could be broken up by an air pressure system (explained in section 2.4). Even microtransplantation of cells can cause further damage to the axons.

Previous studies of CST lesions have largely relied on injections of anterograde tracers (such as biotin dextran) to identify corticospinal axons. However, for a quantitative study, even with extensive injections of tracer it is difficult to be certain that the entire population of cells has been labelled in every case. Failure to label even a small number of surviving
axons can give a false impression as to the completeness of a lesion. The demonstration (Terashima et al., 1994; Li et al., 1998) of selective immunostaining of corticospinal axons by CaMIIk-α antibody gives an independent assessment which avoids the problems associated with uptake of an injected anterograde label. CaMIIk-α antibody staining provided an additional information and showed that with incomplete lesions, both the rate and the degree of return of ipsilateral DFR were a function of the percentage of surviving CST fibres.

4.3. Correlation of functional recovery with the extent of CST lesion

Axons of CNS in contrast with PNS do not regenerate after injury. There are several reasons for failure of the regenerative process, which were explained in section 1.6. To provide a brief recapitulation, the essential features of this failure are as follows.

When CNS axons are damaged the synaptic boutons that connect with the cell body are stripped away in a process that involves microglia. The lesion site itself changes considerably, with immediate death of oligodendrocytes, activation of microglia, invasion of macrophages, and progressive astrocytic hypertrophy (scarring), together with cavitations, changes in vasculature, fibrosis and invasion of SCs (Li et al. 1999). In addition, demyelinated axons in the spinal cord may be remyelinated by ingrowth of endogenous SCs around the injury site.

In this section I will discuss few points such as; the complexity of the CST in rat and the relation to DFR, the importance of complete and incomplete lesions in human and the
relationship between the proportions of spared corticospinal axons and the degree and rate of DFR recovery.

4.3.1. DFR study after complete CST lesion

Traditionally, in mammals the CST is considered to be the prime source of voluntary movements (Maendly, 1981). My results indicate that after complete unilateral destruction of the dorsal CST, DFR function is abolished absolutely for a period of 6 months. CST injury resulted only in deficits of hand and finger movements (Whishaw et al., 1993). In my study it was found that even increasing the motivational drive by preventing the use of the contralateral forepaw for DFR did not induce the rats to use the ipsilateral forepaw. It was shown in a control group that there was no recovery even when the survival was extended to 6 months. Whether DFR would return at even longer survival periods, or under more intensive training seems unlikely, since even after lesions sparing only the barest detectable minimum of fibres, recovery had started by 3 weeks. The defect in DFR is not associated with either paralysis or any obvious weakness of the affected forepaw. Moreover, motivation is clearly intact, since DFR movements of the affected forepaw are readily initiated, but they are abortive.

A complex coordinated movement such as DFR (together with all the other necessary adjustments of body position, balance and posture needed to make the arm and paw retrieval effective) involves activity of many supra- and intra-spinal pathways. Although a number of different ascending and descending spinal tracts certainly contribute components to the overall performance of DFR (Weidner et al., 2001; McKenna et al., 1999), the use of small circumscribed lesions confirms my earlier finding that severance of the dorsal CST, which does not affect other tracts, such as the rubrospinal tract (in the
lateral columns), or the ventral CST (in the ventral columns), and which causes only minor and variable damage to the ascending (sensory) dorsal columns nevertheless completely abolishes ipsilateral DFR, and there is no return of this function for up to 6 months. In other words, these other pathways cannot replace the loss of DFR function after a complete lesion in dorsal CST.

Küchler et al. (2002) devised an experiment to find out which forepaw muscles are involved in forelimb movements such as reaching and grasping. In this study, an anterograde tracer (BD), was combined with injections of a cholera toxin α-subunit, into selected groups of forelimb muscle to analyse in detail the rubrospinal tract projection to the forelimb areas. It was concluded that three populations of forelimb muscles were distinguished: distal (paw), intermediate (forearm), and proximal muscles (upper arm).

Further evidence for the complexity of relating the various movement used in reaching to proximal/distal muscles comes from studies of associated muscle electromyography, which suggest that most limb muscles are active during most of the components of reaching. The CST is involved in both proximal and distal limb movements (Whishaw et al., 1993), for example, digit movements and supination of the paw are produced by distal musculature whereas most of the other movements such as grasp, reaching and lifting are produced by proximal musculature of the upper arm. It was reported that latissimus dorsi, triceps and biceps are the major muscles involved in forelimb reaching movements in rats (Hyland and Jordan, 1997). It is possible to speculate that according to Kücher et al., (2002) and Hyland et al., (1997), most of these muscles involved in reaching activity, are located in the proximal region of forelimb.
A study by Weidner et al., 2001 reported that DFR recovery was completely abolished if dorsal CST lesions were followed 5 weeks later by ventral CST lesions. Rats with ventral CST lesions exhibited short-term functional deficits, even this tract comprises less than 5% of all axons in the CST. It was also suggested that increases in ventral CST axon contacts correlate with functional recovery in dorsal CST lesioned rats, implying that the ventral CST may exert a more important contribution to skilled forepaw function than previously appreciated. In contrast, my results indicate that DFR is completely abolished for at least 8 weeks after complete unilateral destruction of the dorsal CST. As the histology of the series of Weidner et al. was not fully reported it is difficult to resolve these differences. In my experiments, the use of small circumscribed lesions confirm that complete severance of the dorsal CST which did not affect other tracts (such as the rubrospinal tract in the lateral columns), the ventral CST (in the ventral columns), and which causes only minor and variable damage to the ascending (sensory) dorsal columns nevertheless completely abolishes ipsilateral DFR, and there is no return of function. I feel it unlikely that these other pathways can maintain DFR after complete loss of the ipsilateral dorsal CST.

The major differences between the above study and my study are: A) In my study small-circumscribed dorsal CST lesions did not damage the ventral CST, and caused only minor and variable damage to the ascending (sensory) dorsal columns but the ipsilateral DFR was completely abolished, and there was no return of this function for up to 6 months. B) In the Weidner study, tungsten wire knife was used for making dorsal CST lesions, which might not have caused complete lesions. Therefore functional recovery might be due to spared dorsal CST. In my experience even the survival of 1% of dorsal CST fibres would mediate such recovery. Unless there was absolute certainty that the first lesion totally destroyed the dorsal CST, there is no validity to the claim of functional recovery.
A behavioural study by Whishaw et al., (1998), showed that rats with CST lesions were also impaired in success of paw reaching compared to rats with red nucleus lesions. This suggested that the CST was more involved in forelimb guidance than was the rubrospinal tract (Whishaw et al., 1998). An anatomical and electrophysiological study (Küchler et al. 2000) showed the rubrospinal tract was in close apposition to the distal and intermediate muscle in the forelimb, but was consistently absent in the proximal muscles. It was observed that a lesion in the CST could cause more severe and permanent deficits in reaching and grasping (Whishaw et al., 1998). In contrast, a lesion in the red nucleus could induce permanent locomotor gait deficits (Muir and Whishaw, 2000). These behavioural results suggested that the red nucleus was involved in a more general control of groups of forelimb muscles, whereas the precise control of individual proximal limb muscles relates to the CST.

On the basis of this evidence, it was decided that DFR was an appropriate behavioural test for this study. Due to the small and localized lesion in the CST, other motor behavioural tests were not required.

4.3.2. DFR after incomplete CST lesions

Part of this study was to establish the correlation between rate and degree of post-lesional recovery of DFR with the number of surviving CST fibres. Histological estimation of CST lesion demonstrated that sparing axons from CST correlated well with the functional outcome. Together these results strongly indicated that after spinal cord lesion, a small residual population of CST fibres could provide sufficient input to initiate locomotor movements.
It was found that a small proportion of spared CST fibres can mediate large effects of functional recovery after spinal cord injury, but the location of these axons within the CST is not crucial. This suggests that the CST does not have specific subgroups of fibres dedicated to specific functions. The delayed return of DFR after incomplete CST lesions showed that when partially destroyed, the surviving part of the CST was capable of re-establishing functions with even very small numbers of surviving fibres (figure 3.12).

The return of DFR (at around 3 weeks) in a group of rats with 98% lesions showed that the CST is capable of transferring functions to even very small numbers of surviving fibres, which presumably did not carry this function before the lesion. In large lesions in human, there is always an element of function which is permanently lost, and which can leave an injured person with a severe and intractable disability. Presumably the tracts, which have been severed, mediate a unique type of information, which is unable to get round the lesion by any sprouting or re-learning mechanism. If so, only reconnection of the cut fibres will suffice for repair. This model seems to fit the obligatory relationship between the CST as a whole and DFR.

Although spontaneous regeneration of lesioned fibres is limited in the adult CNS, many people with incomplete spinal cord injuries show significant functional recovery. This spontaneous functional recovery depends on two different mechanisms: A) the reorganization of circuits that have been spared by the lesion and B) the formation of new circuits through collateral sprouting of lesioned and unlesioned fibres (Raineteau et al., 2001).
Structural rearrangements of axons, dendrites, or synapses can lead to behavioural compensation (Klintsova et al., 1999). It was shown clearly in a study by Kleim et al., (1998) that three groups of adult female rats were allocated to an acrobatic condition (AC), a voluntary exercise condition (VX), or an inactive condition (IC). Acrobatic condition animals were trained to traverse an elevated obstacle course requiring substantial motor coordination to complete. VX animals were housed with unlimited access to running wheels and IC animals received no motor training but were handled briefly each day. Results showed the AC animals to have significantly more parallel fibres to Purkinje cell synapses than both the VX and IC animals. Complex motor skill learning, leads to an increase in synapse number within the cerebellar cortex. A study by Klintsova et al., (1999) indicated that synapse addition and/or loss are associated with different types of learning.

A number of authors have previously found that persistence of a relatively small number of axons at the injury site can sustain neurological function. For example, Windle et al., 1958 studied cats with incomplete transections of the lower thoracic cord and reported that retention of some motor function occurred if 1-10% of pathways were intact. These results are consistent with our finding that persistence of a relatively small population of myelinated axons can result in significant motor recovery. The differences in the proportion of axons necessary for maintenance of forelimb function in the cat (Blight, 1983) and rat (Fehlings and Tator, 1995) may be accounted for by differences in the injury model, species, and method of behavioural assessment.

In a subsequent series of studies, cats with incomplete spinal cord injury showed spontaneous partial functional recovery that was further improved stability and weight
bearing by using a treadmill (Lovely et al., 1986). In human patients treadmill training is increasingly used in rehabilitation after incomplete spinal cord injury (Wernig et al., 1998). Interestingly it was shown that systematic treadmill training did not provide additional improvement in several functional tests (i.e. narrow beam crossing, grid walking and open field activity) in adult rats with incomplete dorsal CST and rubrospinal tract lesions. It is important to mention that while lesions of the dorsal tracts (i.e. the CST and rubrospinal tract) severally impair precision movements (i.e. DFR) (Wishaw et al., 1997; Li and Raisman, 1997), they allow good functional recovery of locomotion including weight bearing (Muir and Wishaw, 1999; Metz and Schwab, 1998).

In another study it was shown that after selective dorsal column lesions at the C2 level in rat, DFR completely recovered within a few days (McKenna and Whishaw, 1999). It is worth mentioning that these dorsal column lesions caused partial CST lesions. Although a number of different ascending and descending spinal tracts contribute to DFR, our results and McKenna et al., (1999) data confirmed that CST has a vital role in DFR.

4.4. Do OECs have advantages over Schwann cells for regenerating of corticospinal axons?

One of the principle aims of neural regeneration research has been to attempt to restore the connections of any damaged pathway to a state that allows regeneration. Many of these have attempts involved transplantation of glial cells. OECs and SCs have so far been the best candidates for repair of spinal cord in different studies. Both OECs and SCs have been shown to promote axonal re-growth and remyelination in the CNS under experimental conditions (Aguayo et al., 1987; Li et al., 1997; Imaizumi et al., 1998).
OECs posses several characteristics that are very similar to SCs. One of these similarities was seen in the morphology of cells in the culture dish. To date there appear to be only two properties that may be used to distinguish the fibroblast-like cell (F cells) and Schwann-like cell types (S cells). It is clear from this study and other data, after culturing the OECs and adult SCs, the cultured cells segregate into clusters of the two major cell types, S cells and F cells (Devon and Doucette, 1992; Li et al., 1997).

Putting the \textit{in vivo} results such as the DFR functional recovery together with focal CST lesions in this study I conclude that:

1. Complete unilateral CST lesions cause a stable and complete loss of DFR despite the ingrowth of endogenous host SCs,
2. Injection of cultured OECs induce a substantial and consistent recovery of DFR (and a regeneration of axons across the graft and back into the host),
3. Injection of cultured adult peripheral nerve SCs induce a much smaller, but still measurable and consistent recovery of DFR
4. Pure fibroblast transplants do not produce any functional repair.

These observations are consistent with the view that SCs do have a limited value in repair of the CST.

The following section is divided into two main parts: 1) The cellular aspects (e.g. migration of cells, interaction with astrocytes) of OECs and adult SCs in spinal cord injury, and 2) the effect of these two cell types on functional recovery.
4.4.1. Myelination and migration by OECs and Schwann cells

OECs under experimental conditions can be induced to generate myelin sheaths both in vitro (Doucette, 1990) and in vivo (Franklin et al., 1996; Li et al., 1998), although this has been questioned because of contamination with SCs (Cui et al., 2003). The finding that myelin sheaths formed are of peripheral composition and morphology (e.g. expression of P0, Franklin et al., 1996; Kato et al., 2000) is indicative of their common 'peripheral' developmental origin. OECs in our study showed P0 staining in the middle of the transplant. The electron microscopic sections confirmed that all the regenerated axons were myelinated by peripheral type myelin and were never seen in the CNS without any injury.

One of the major observations of OECs in the delayed transplantation model is the migration of cells and the growth of the regenerated corticospinal axons. The transplanted OECs migrated for considerable distances (up to 7 mm, figure, 3.19) from their point of insertion. However, it appeared from our results that the axons regenerated further than the front of the OEC migration, suggesting that the axons had not simply been 'towed' behind migrating cells, but were able to leave the OEC environment and re-enter the CNS glial environment (Li et al., 1997). The longest regenerating axons that could be traced to the terminal field by anterograde BD labelling and re-enter the caudal part of the CST were 11 mm (figure, 3.29, in this case). A study by Ramón-Cueto et al. showed that OECs migrated widely into distal stump of the host cord, unlike SCs, which completely fail to migrate (Ramón-Cueto et al., 1998).

A study by Takami et al., 2002 reported that cultured SCs are more effective in promoting axonal sparing/regeneration than OEC or a combination of both cells (SC/OEC) in a one-week-old moderately contused adult rat thoracic spinal cord. It was demonstrated that all
grafts (SC, OEC and SC/OEC) promoted regenerated axons, but SC grafts exhibited the greatest number of myelinated axons. In addition, SC grafted rats showed significant hindlimb performance compared to OEC transplantation (Takami et al., 2002). It is difficult to interpret their data. The lesions are highly variable, and the effects on hindlimb function are more so. It is not clear how much hindlimb function would return without grafting. In general hindlimb function is not highly dependent on long descending tracts in the spinal cord. The authors make little attempt to decide whether the SC grafts were as extensive or more extensive than the OEC grafts. This missing information would have a major bearing on deciding to what extent the grafted cells actually make contact with damaged axons and act as a bridge to their target tracts. If the cells do not act as a bridge, then they cannot be expected to have a functional effect. But whether they form structural bridges depends not so much on the cells themselves as on the mode of transplantation, the size of the lesions etc, on which the paper gives no details on these things. The histological read-outs - viz. the area of tissue spared, and the degree of proximity of cut CST axons to the graft - have no known functional significance, and the authors present no back up data to say that changes in these histological parameters are in any way related to hindlimb function.

Recovery of ipsilateral DFR after complete unilateral CST lesions in my study occurs only when OECs are transplanted into the lesion site. Transplants misplaced in the dorsal columns, although only 1-2 mm away from the lesion site, did not cause recovery of function (figure 3.22). Although the transplanted OECs migrating for up to 11 mm within the distal CST, their migratory ability is not sufficient to cross tissue boundaries such as those between adjacent white matter tracts. It was concluded from this study, in order to make an effective repair, the cells must be placed in direct contact with the tract.
4.4.2. Interaction between OECs and SCs with host astrocytes

The success of axon regeneration and remyelination that distinguishes the PNS from the CNS is correlated with the difference in their respective glial environments. In injured CNS astrocytes appear as a key component of a major impediment to axonal regeneration (Ramón y Cajal, 1913; Reier et al., 1989). Many scientists have been attracted to the idea that SCs from the PNS might hold the key to overcoming regeneration in the CNS. More recently attention has focused on the olfactory glia, which have a particular ability to support axonal regeneration in adult mammal.

There are several lines of evidence suggest that OECs will achieve myelination in the face of heavy astrocytic scar around the injury. First, OECs mingle intimately with astrocytes in the olfactory nerve fibre layer of the olfactory bulb in a naturally occurring cohabitation that occurs between SCs and astrocytes (Doucette, 1990). Second, OECs and SCs interact completely differently with astrocytes when they are in culture: OECs and astrocytes tend to intermingle while SCs and astrocytes segregate from one other (Lakatos et al., 2000). Third, transplanted OECs appear to migrate more readily within the CNS than transplanted SCs (Ramón-Cueto et al., 1998). SC migration is strongly inhibited by astrocytes (Wilby et al., 1999). On the other hand, regenerated axons achieved by OEC transplantation can grow through regions of extensive astrocytes scarring (Li et al., 1997).

Astrocytes become reactive in response to lesions of the CNS. The reactivity includes hypertrophy of the cell body, increased arborisation of processes and up-regulation of intermediate filaments such as GFAP. Reactive astrocytes produce CSPG, which can inhibit neurite out growth (Bovolenta, 1993). It was reported that CSPG was upregulated when astrocytes were cultured with SCs but not when astrocytes were grown with OECs.
(Lakatos et al., 2000). Verdu et al (2001) made large photochemical lesions destroying most of the dorsal half of the spinal cord and claimed that transplantation of cultured OECs caused a reduction in the hypertrophic astrocytic reaction to injury. It is not clear whether these animals received any functional benefit, as the hindlimb and sensory responses improve spontaneously after these lesions. The huge cavitation lesions (up to 16.74 mm3) could not possibility have been filled or bridged by the amounts of OECs injected (60,000 cells in 3 μl), and the authors make no attempt to describe any regeneration of axonal connections.

The mechanism of the beneficial effect of OECs on astrocytic scars is not clear. The astrocytic scar is, after all, made up of processes of living cells, and these cells, which have hypertrophied and tangled their processes into the scar in response to the initial injury, may well be capable of reversing the process when confronted with OECs. A similar 'opening up' of the astrocytic interface is seen at the normal point of entry of the olfactory nerves into the glomeruli of the olfactory bulb. It seems likely that OECs can have a similar effect on astrocytes in other areas, such as in the lesioned CST. Such an effect is described in a recent paper by Li et al (2004) on regeneration of dorsal root axons across an OEC bridge into the spinal cord.

It has been suggested that the expression of N-cadherin on SCs may mediate the adhesion of SCs to astrocytes (Wilbey et al., 1999). However, OECs also express N-cadherin, which does not appear to play a role in their interaction with astrocytes (Lakatos et al., 2000). In my histological study GFAP staining was intensified in the astrocytes surrounding the lesion, with a dense wall of astrocytic processes closing off the lesion cavity (figure 3.10; E & F). Although OEC transplants did not completely reverse the astrocytic hypertrophy,
they seemed to reduce it and the astrocytic processes showed more of a tendency to rearrange themselves as a rather more 'open' surface (figure 3.21 C & D). It was concluded from this project and other experiments that, transplanted OECs were able to reduce the neurite inhibitory factors associated with astrogliosis (Verdu et al., 2001; Li et al., 1997 & 1998) compared with the non-transplanted controls.

It has been well documented that although transplanted SCs can promote CNS axon regeneration and remyelination, their ability to do so is impeded by the presence of astrocytes. Thus, transplanted SCs have limited remyelination capacity in the presence of astrocytes (Franklin and Blakemore, 1993). On the other hand transplanted OECs behave differently from the SCs in the presence of astrocytes. There is evidence suggesting that within the nerve layer of the olfactory bulb the OECs co-exist alongside astrocytes (Doucette, 1984, 1991). It was hypothesized that OECs have advantages over SCs in repairing CNS, and this is due to an increased ability of transplanted OECs to integrate and migrate within the astrocytic environment (Barnett et al., 2000).

4.4.3. Functional recovery after OECs transplantation

The function of DFR is a complex one involving sensory systems, motivation, a degree of learning and memory, and the defect is also a complex one since the paw can be used for all other functions that have been observed. It is unlikely that such a defect involves higher level systems than the spinal cord. The reason that the lesion of the CST affects them is that it cuts off access to these higher-level systems.

The recovery of such a complex function after transplantation can occur in many ways, such as use of other undamaged pathways, or new abnormal patterns of connection formed
by sprouting, or by local effects such as improving the milieu for existing axons, e.g. by remyelination or by reducing the local damage, such as the size of the lesion (Plant et al., 2003). The value of sprouting for functional recovery has been supported by several recent publications from the Schwab group (Raineteau et al., 2002) who show that there is increased sprouting of corticospinal and rubrospinal axons after treatment with the anti-myelin antibody IN-1 and return of functions such as food pellet retrieval (Z'Graggen et al., 1998). However, I feel that in my experiments, the most likely cause of the return of function is the regeneration of those fibres, which originally carried that function. This is borne out by the facts that (1) the function is not lost if even a small number of CST axons survive, and (2) the function is only returns in those animals where the CST has regenerated.

In this study, it is demonstrated that transplanted OECs are capable of inducing regeneration of injured CST axons and recovery of DFR (figure, 3.27).

Two lines of evidence indicate that the remyelination was indeed from the transplanted OECs and not from endogenous SCs. First, there is no recovery of ipsilateral DFR after complete unilateral dorsal CST lesions. We must assume that these tracts carry specific information which cannot be satisfactorily accessed via alternative or sprouted pathways, and that the functional recovery seen after transplantation is due to the transplanted OECs inducing regeneration of the cut CST fibres across the lesion. Second, the endogenous SCs have no effect on the DFR functional recovery. This was confirmed by long-term survival (6 months) in a control group with complete unilateral CST lesions.

The study with partial CST lesions shows that very small numbers of spared fibres are sufficient to allow return of DFR. In the OEC transplant study considerably fewer BD
labelled axons cross the transplants than in the previous study with immediate transplantation (Li et al. 1997). Even so, the small numbers of axons regenerating in the delayed transplants – around 1% - are sufficient to mediate a return of function. The present findings show that valuable amounts of functional benefit can occur from the reconnection of relatively small numbers of the right type of fibres, and this is an encouraging sign for future human therapy.

Figure 3.27 show that most of the local terminal field formed by the regenerating CST axons are at different distances from the grafted lesions, which contributes to the functional recovery. In this study there is no indication of the extent to which the cortical somatotopic map is restored, nor whether fibres can regenerate much further, for example to lumbar levels. These connections might have an abnormal distribution, therefore a great deal of functional readjustment would be required to enable the rat to incorporate this misdirected information into a functional retrieval performance.

The limited amount of data available on the pattern of termination of the cut CST axons after regenerating through the OEC grafts indicates that the arborisations are in the normal terminal zone of CST axons in lamina V. The data is insufficient to say whether these new terminations are of normal size and extent, nor do I have any retrograde labelling data to say from which cells in the cortex they arise. It would be of great interest in a future study to see to what extent the cortical pattern is re-established after CST regeneration: do the fibres from the arm area of cortex distribute to the cervical levels and the leg areas to the lumbosacral levels.
The CST axons terminate largely in the cervical region, and in general, over a large number of species, including human, their function is predominantly forelimb and small hand muscles, with little effect on hindlimb locomotion. Therefore although the high cervical lesion is ideal for studying forepaw use, there might be other descending systems that would be better for studying hindlimb use.

The overview of possible alternative explanations for the recovery of DFR are: a) remyelination of damaged axons by OECs (P0 positive staining in the centre of transplant), b) OECs might act as isolators, ensheathing regenerating axons and preventing their exposure to chemo-repellent molecules of myelin and reactive glia, as they do in the olfactory bulb (Pasterkamp et al., 1998). OECs produce variety of adhesion molecules and growth factors (Ramón-Cueto, 1998), which can accompany growing axons through the inhibitory environment. It can be suggested that successful axon growth after adult injury is the result of a balance of positive and negative signals located in highly localized sites. If a preponderance of positive signals is present, then growth will proceed even in the presence of inhibitory molecules (Silver, 1994; Schwab et al., 1993). A study by Jones et al., 2003 reported that by transplanting fibroblasts (genetically modified to secrete NGF) into the lesioned dorsal column spinal cord, the regenerated axons cross a region of CNS injury expressing inhibitory molecules such as CSPG.

It is worthy of note that environmental enrichment, behavioural experience and cell transplantation can each influence neuronal plasticity and recovery of function after brain damage (Dobrossy and Dunnett, 2001). The studies of Rosenzweig, Diamond and colleagues in the 1960's, showed that enriched environments (i.e. greater opportunity for activity and play) produce significant changes in the structural anatomy of the brain and branching of dendrites. A specific aspect of this study is that not only the transplanted
tissue has to survive but also that the axons have to establish the right anatomical circuits, or in other words ‘learning to use the transplant’. It was concluded that the efficiency of any cell-based treatment in damaged circuits in the CNS depends on the behavioural experience and training of the host animal (patient) (Dobrossy and Dunnett, 2001).

In considering the practical use of transplanted cells in clinical lesions, the more effective OECs have the disadvantage that present day techniques for obtaining them, from the nasal mucosa for autografting, provide only limited numbers of cells. In most cases, autotransplantation would require preparation of SCs from adult nerve obtained at biopsy. It is substantially more difficult to extract SCs from adult nerve than from embryonic or perinatal nerve because the adult tissue contains a more fully developed epineurium and perineurium composed of fibroblasts (Bunge et al., 1989), and substantial amounts of connective tissue within these layers as well as in the endoneurium (Bunge, 1986). If SCs are to be useful for grafting into patients with spinal cord injuries, it will be necessary to have a reliable method for procuring large populations of SCs from relatively small samples of peripheral nerve. Overall, it is important to be able to freeze and store SC for future clinical use in matched donors, however, it has been shown that storage of peripheral nerve by freezing and thawing can diminish their ability to support regeneration (Levi et al., 1994).

4.4.4. How do OEC and Schwann cell promote CNS regeneration?

Histological and behavioural examinations of the lesioned CST in this study support the conclusion that transplantation of either OECs or SCs separately are capable of facilitating regeneration of axons within the spinal cord. I have shown that cultured adult OECs injected into the CST 8 weeks after lesion, induced restoration of DFR and regeneration of
axons across the lesion. As has been described in other lesions of the spinal cord, lesions of
the CST induce the ingrowth of endogenous SCs. These SCs, however, are not sufficient to
produce detectable functional repair. The possibility remains, however, that the OEC
transplants have in some way potentiated the ingrowth of endogenous SCs or made them
more effective at repair, and therefore that it is the SCs that are mediating the recovery.
Since it is difficult to answer this question, I showed that transplanted SCs (n=2) did
indeed restore function, but at a much lower level than OECs. In a control group rats
received pure fibroblasts transplantation had no reparative effect. Taken together, these
results indicate that regenerating axons induced by OEC or SC transplants allow limited
number of rapidly and securely conducting axons to extend a considerable distance across
the transplantation site.

SCs and OECs are closely related cell types. Phenotypically they cannot be distinguished
after transplantation. Both ensheathe axons and showed peripheral-type of myelination
pattern with large cytoplasmic and nuclear components outside of the myelin. It was also
reported by Imaizumi et al., 2000 that by transplanting OECs or SCs into the transected
dorsal columns of the rat spinal cord similar peripheral type myelin around regenerated
axons were observed.

Li et al., 1997 reported a similar pattern of myelinated axons following transplantation of
OECs into a CST lesion site, but as the regenerating axons re-entered the host CNS they
were remyelinated by oligodendrocytes.

Numbers of studies have shown that CNS axons can regenerate through peripheral nerve
grafts (David and Aguayo, 1981) or in an environment where glial inhibitory proteins have
been neutralized by antibodies (Schwab et al., 1993). It is well established that OECs (Ramón-Cueto, 1995) and SCs (Taniuchi et al., 1988) can express low affinity NGF p75 receptors and SCs also express NGF after axotomy (Windebank and Poduslo, 1986). OECs have been shown to express adhesion molecules, such as neural CAMs, laminin and L1 (Kafitz and Geer, 1997; Kleitman et al., 1988). Whether the presence of NGF and their receptors can contribute to regeneration of axons after transplantation of OECs and SCs is yet not known.

Anderson et al. (1998) have looked directly at the molecules up-regulated on SCs and regenerating axons due to the SC transplantation into the CNS. Certainly, in injured peripheral nerve, there is secretion of different trophic factors from SCs such as NGF and BDNF. Therefore after transplanting the SCs into the CNS they may still have the property of attracting axons. Many of the adhesion molecules have been reported to have important roles in promoting axon growth. SCs express low affinity NGF p75 receptors and NGF after axotomy (Windebank et al., 1986). Anderson et al (1998) suggest that repair of injuries in brain and spinal cord may also benefit from some form of gene therapy for axotomised neurons.

Some studies have demonstrated that by adding neurotrophic factors to SC grafts they can attract more axons. Bunge and colleagues have developed a model in which a semi-permeable tube containing SCs was implanted into the dorsal column of spinal cord. The number of axons was greatly increased by adding NT-3 or BDNF into one end of the graft (Xu et al., 1995).
The presence of neurotrophic factors might create a supportive environment for axon growth. One exciting possibility is that a combination of growth factors and adhesion molecules could provide a permissive substrate for endogenous SC invasion. There is a possibility that there is a putative chemotactic effect of OECs on endogenous SCs. Transplanted OECs into the injury site express a variety of other growth factors that could promote growth and survival of surrounding cells after transplantation including acidic and basic fibroblast growth factors, transforming growth factor-α, platelet-derived growth factors-α and -β and insulin-like growth factor-I and -II (Newman et al., 2000).

The stimulatory effects of OECs on CST axon growth demonstrate that cut spinal axons, which do not normally regenerate, can be made to do so. In view of the idea that the failure of regeneration is due to local inhibitory influences associated among others with astrocytic molecules upregulated by lesions (Fitch and Silver, 1997) the stimulatory effects of the transplanted OECs are obviously able to override them. A similar view, based on a different type of cell transplant is expressed by Jones et al (2003) who have provided evidence that spinal cord axons of various types extend further into areas where lesions have induced upregulation of Chondroitin sulphate proteoglycan (NG2) expression in the presence of transplants of fibroblasts genetically engineered to express NGF. From this they conclude that the inhibitory effect of molecules such as NG2 is not absolute but can be overridden if the lesioned axons are provided with a sufficient growth stimulus.

An important concern for effectiveness of cell transplantation to induce axonal regeneration is to determine if regenerated axons can go through the lesion site. In this study and in other studies (Imaizumi et al., 2000 and Li et al., 1997) donor cells were
found within the lesion centre and were able to bridge the cut spinal cord and provide a scaffold for the regenerating axons.

It was also shown in my study that after a lesion in the CST, no axons could cross the injury site, but after OEC transplantation, a few axons made the right connections. It can be suggested that OEC transplants with permissive molecules have balanced out the inhibitory environment of lesion. Possible explanations for the functional improvement in the OEC transplanted rats include both enhanced plasticity and regeneration of axons across the lesion site.

OECs from adult or embryonic animals surgically implanted into various spinal cord injury models showed axonal regeneration (Ramón-Cueto, 1998; Li et al., 2003), remyelination of axons (Franklin et al., 1996; Lakatos et al., 2003) and improved functional recovery (Ramón-Cueto et al., 1998; Li et al., 2003; Keyvan-Fouladi et al., 2003). Recent attempts to demonstrate similar myelination pattern with adult rat OECs have been unsuccessful (Plant et al., 2002; Boyd et al., 2004). Boyd et al., 2004 showed adult rats with a clip compression injury in the spinal cord. Embryonic OECs, which were infected with LacZ retrovirus were implanted into the lesion, these labelled OECs showed no direct contact with axons and there were no myelinated axons in the middle of transplant and they did not form a basal lamina. The OECs had remained localized to the implantation site, and it seems unlikely that they had been transplanted in such a way as to form a continuous tissue bridge. In my study adult OECs migrated up to 7 mm after transplantation into the CST lesion.
The direct comparison between this study and my results are difficult because of the age
difference of the animals from which the OECs were harvested, as well as differences in
the purification procedures and type of injury model which have been used in this study.
The other differences between Boyd et al experiments and other studies is that other
scientists have implanted unlabeled neonatal and adult rats OECs into the damaged spinal
cord, but Boyd used LacZ-expressing OECs isolated from embryonic day 18 rats. Another
explanation for the inability of fetal OECs to myelinated damaged spinal cord is that the
expression of LacZ (or its protein product β-galactosidase) interferes with myelin
synthesis. The Boyd injury model was done by clip compression in spinal cord, which
caus ed a large lesion. In my opinion injecting 4 μl of a suspension of OECs could not fill
the gap in the lesion. In my study, with a much smaller localized CST lesion 5 μl OECs
solution was needed to fill up the cavity. The most likely explanation of the failure in these
experiments is that the injection of a suspension of cells into a large lesion is unable to
place enough cells, and retain them in a position where they can actually contact the
damaged axons. It seems quite unrealistic to assume that OECs can act at a distance from
the damaged axons.

4.5. Failure of functional recovery after pure fibroblast transplantation

Operated control rats included those that received pure peripheral nerve fibroblasts or OEC
conditioned medium showed no DFR recovery after 8 weeks post-operation. The BD
labelling demonstrated that there were no axons crossing the lesion/transplant site. This
suggests either that fibronectin extracellular matrix of the graft was nonconductive to
corticospinal axon growth or that host glial responses at the graft/host interface inhibited
axon penetration into the graft.
In agreement with our results, a study by Jin et al., 2002 suggested that control rats in which the lesion was filled with unmodified fibroblasts showed no functional recovery after 8 weeks post-operation. On the other hand, all rats with lesion and genetically modified fibroblasts to produce BDNF showed fewer deficits in their functional recovery compared to the control group. Other studies by Tuszynski et al., 1996 and 1994 confirmed that only genetically modified fibroblasts could provide an environment suitable for axon growth in injured spinal cord. It is important to mention that most of studies were chosen to concentrate on the rubrospinal system, not the CST.

An experiment designed by Grill et al. (1997) demonstrated that in the dorsal hemisection lesions which received fibroblasts genetically modified to produce NT-3 there was a degree of functional recovery. The major difference between this study and my study is that in my injury model pure fibroblasts without any modification were transplanted into the lesion.

**4.6. Future plans**

Obviously these findings are encouraging for potential future clinical applications, although it must be accepted that the small CST injuries we study do not compare with the much larger cavities and scarring seen in severe human contusion injuries. However, with rat lifetimes of less than two years it is impossible in this species to model the effects of human injuries, which may have occurred two or more decades previously. Small human cortical lesions have major devastating effects.

There are several important and clinically relevant points, which should be taken into consideration in relation to repairing CNS (i.e. spinal cord). Delayed repair will be a
necessity in any future clinical trials, if only because the unpredictability of 'spontaneous' repair means the prognosis for any individual spinal injured patient may not be clear for up to a year after injury. In the present study I have attempted to provide a model of a delayed transplantation paradigm. The most important conclusion of the present observations is that the previously reported reparative effect of immediate transplantation OECs on DFR (Li et al., 1997) can still be obtained when the transplantation is delayed for 8 weeks after the lesion.

The future goal is to test other cells including OECs derived from human olfactory bulb or rat mucosa into the same experimental model (i.e. complete CST lesion), in order to determine which of these will provide meaningful functional restoration. The stable CST injury model is consistent and quantitatively robust, it will provide an excellent baseline to compare the effects of human OEC transplants.

4.6.1. Transplantation of human OEC into complete corticospinal tract lesions

This study and other recent studies have shown that rodent OECs are able to support axonal re-growth when transplanted into the experimental models of spinal cord injury (Li et al., 1997; Ramón-Cueto et al., 1998) and are able to form myelin sheaths around regenerated axons (Franklin et al., 1996; Li et al., 1997; Imaizumi et al., 1998; Ramón-Cueto et al., 1998). All these data indicate that transplanted OECs have advantages over SCs. The advantages include the ability to migrate and integrate within areas of astrocytosis that are characteristic of damaged CNS (Franklin and Barnett, 1997).
The major issue regarding their therapeutic potential is whether cells similar to rat OEC exist in human olfactory tissue and whether they could be transplanted into experimental models like the CST lesion model.

In a few studies, OECs isolated from human olfactory bulb during neurosurgery have been used to show that human OECs share many properties with their rat counterparts, including expression of low affinity nerve growth factor receptor p75 and similar growth factor requirements. Cultured OECs have been transplanted into the demyelinated dorsal funiculus in the spinal cord of adult rat (Barnett, 2000). This study showed that human OECs differentiated into a myelinating phenotype that remyelinated the demyelinated CNS axons. Kato et al (2000) reported that transplants of adult human OECs derived from olfactory nerves were transplanted into the demyelinated spinal cord of immunosuppressed adult rats. The results provide evidence that adult human OECs are able to produce Schwann cell-like myelin sheaths around demyelinated axons in the adult rat spinal cord.

4.6.3. Transplantation of rat mucosal OECs into complete corticospinal tract lesions

The potential therapeutic role of OECs from the olfactory bulb in the repair of lesioned spinal cord is well illustrated in this study. However there are a number of difficulties involved in obtaining these cells. These include: 1) the surgical procedure of removing the inaccessible human olfactory bulb, 2) culturing the cells from the olfactory bulb of patients would be unlikely to produce enough material to bridge the large lesions seen in human spinal cord injuries. These difficulties could be avoided if the OECs used were from another source. OECs can also be obtained from the olfactory lamina propria in the nose. This tissue is accessible via simple biopsy through the external nares (Féron et al., 1998).
Other advantages of using mucosal OECs are that they would be from the patients themselves, which would reduce the likelihood of immune rejection, and large quantities of cells could be prepared for transplantation.

Recently, Lu et al. (2001) suggested mucosal OECs promoted partial restoration of function in animals with spinal cord transection. Recoveries of function were observed in hind limb movements and ability to bare weight on the hind limb.

One final point reflects the fact that these studies were done on the immediate post injury in spinal cord, it would be very interesting to look at the potential role of rat mucosal OEC in my delayed transplantation model from histological and behavioural point of view.

4.7. Conclusion

It was only a few years ago that the view was that spinal cord axons could not be made to regenerate. Now, as explained above, there are several treatments that could induce regeneration in complete spinal cord injury models. The two treatments (OECs and SCs transplantation) used in this study had effect on functional recovery. It was demonstrated that transplanted OECs derived from adult rat olfactory bulb are capable of regenerating axons in the adult rat spinal cord injury.

The important points of this study can be summarized:

Incomplete lesions

- Rats with partial lesions of the CST have a deficit in DFR. The rate and degree of recovery is proportionate to the number of spared CST fibres.
• The CST is capable of transferring functions in even a very small number of surviving fibres (1% axons).

• Possible factors, which were contributing to functional recovery were: vascularisation, sprouting of new connections and reorganization of existing connections.

**Complete lesions**

• Complete destruction of the dorsal CST on one side at the level of the first cervical segment completely abolished the use of ipsilateral forepaw for retrieval for at least 6 months after operation.

**Complete lesion with delayed OEC transplantation**

• When OECs transplanted into the delayed lesions showed recovery of DFR in rats.

• The small numbers of axons regenerating in the delayed transplants – around 1% (526±58 myelinated fibres) are sufficient to mediate a return of the function.

• Transplanted OECs migrated for up to 7 mm *within* the distal CST, their migratory ability was not sufficient to cross tissue boundaries such as those *between* adjacent white matter tracts
Rats with misplaced transplants in the dorsal columns, although only 1-2mm away from the lesion site, did not recover function. To be effective, the transplanted cells must be placed in direct contact with the tract to be repaired.

It remains possible that transplanted cells may induce recovery of function at least in part by stimulation of uncut fibres or by humoral mechanisms, possibly associated with the intense vascularisation of the transplants. But whatever the mechanism, the functional outcome is clear: only transplants bridging the lesion are able to restore function. In terms of future human therapy the encouraging aspects of the present findings are (a) that recovery of function is induced by transplants in situations where no spontaneous recovery otherwise occurs, and (b) that the beneficial reparative effects of donor transplants can still be obtained when transplantation is delayed for 8 weeks after the lesions.
Chapter 5

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Appendix

Glucose Oxidase-Nickel DAB method (Ni-GOD; Shu et al., 1988)

Stock Reaction Solution contained:

- 0.1 M sodium acetate buffer
- 0.04% ammonium chloride
- 2.5% nickel ammonium sulphate

100 ml of above stock solution was taken and items below were added to it:

- 0.001% glucose oxidase
- 0.05% 3,3 diaminobenzidine (DAB, Sigma chemical Co, UK)
- 0.2% β-D- (+) glucose (Sigma, USA)

Sections that have been incubating overnight in ABC are washed in 0.1 M PBS for 1 hour.

A rinse in 0.1M acetate buffers is given before applying GOD.

Fixatives used for immunohistochemical staining

Acid Alcohol

- 5 ml of Glacial Acetic Acid
- 95 ml of 96% alcohol

4% Paraformaldehyde in 0.1M PB (pH 7.4)

- 40 gm paraformaldehyde dissolved in 500 ml of distilled water
- Heat to 60°C then add 40 drops of NaOH
- Cool. Add 500 ml 0.2 M phosphate buffer
- Filter
Fixative for BD labelling:

4% Paraformaldehyde, 0.15% Gluteraldehyde and 0.4% picric acid in 0.1M PB (pH 7.4-7.6)

- 40 gm paraformaldehyde in 400 ml distilled water dissolved as above
- 6 ml of 25% Gluteraldehyde, 4ml saturated picric acid and distilled water were added to make up to 500 ml
- 500ml of 0.2 M of PB was added
- Fixative was filtered and pH adjusted

4% paraformaldehyde fixative (1 litter)

- 40g of paraformaldehyde were dissolved in 500ml of distilled water.
- Heat to 60°C then 40 drops of NaOH was added
- 500ml of electron microscopy (EM) phosphate buffer (PB) was added to the solution
- The solution was filtered and the pH was adjusted to 7.4.

1% gelatine coated slides

- Wash the slides in the running water for 30 minutes
- Rinse in distilled water
- Dip into subbing solution
- Leave to dry overnight
- Keep it in the 4°C
Subbing solution

- 100 ml distilled water
- 0.5% Gelatine (Prolabo, France)

Heat to 80°C to dissolve and add 0.1% potassium sulphate. Then cool it down and filter it.

1% neutral red counterstaining

- A) 0.2 M Acetic acid glacial in distilled water
- B) 27g Sodium Acetate in 1000ml distilled water
- Take 200ml of A solution and add 300ml of B solution and make the 4.8 pH
- Then add 1% of neutral red powder into the solution

Preparation of TESPA coated slides

- The slides were laced into racks
- Immerse slides in acetone for 5 minutes
- The slides were incubated into 2% TESPA for 5 minutes
- Slides were washed twice in acetone for 5 minutes
- Followed by a rinse in distilled water and left to dry in room temperature.

Preparation of antibody dilute:

Adding 1% Bovine serum albumin (BSA) into PBS and 0.5% sodium Azide in to the solution.

Tribromoethanol Anaesthesia (Avertin)

- 2 grams of 2,2,2-tribromoethanal (Aldrich Chem Co. USA)
- 2 ml 2-methylbutan-2-ol
• 100 ml distilled water
• 8 ml absolute ethanol

The mixture is kept in the dark and in a refrigerator.

**Methylene Blue and Azur II (staining for semi-thin sections)**

• 1% Methylene Blue (BDH, Analar, England) in distilled water
• 1% Borax (di-sodium tetraborate) (BDH, Analar, England) in distilled water
• 5 gm of Azur II (Raymond A. LAMB, Middlesex, England) was added into the 1% Borax
• Then the two solution 1% Methylene Blue and 1% Borax plus Azur II were mixed together, filtered and was left to mature for one month in the dark.

**DAB buffer for staining of semi-thin sections**

• 142 g sodium di-hydrogen orthophosphate 1-hydrate (NaH$_2$PO$_4$.2H$_2$O, BDH, England)
• 27 g Imidazole (BDH, England)
• Mix both in 1 litre distilled water

**Electron microscopy (EM) fixative (1% glutaraldehyde, 1% paraformaldehyde; pH 7.4)**

To make up 1 litter of EM fixative:

• 10 g of Paraformaldehyde
• 400 ml of distilled water
• The solution was heated to 60 °C then 10 drops of Sodium hydroxide (NaOH) was added to the solution
• After the mixture was cooled down 25% glutaraldehyde (40 ml) was added
- Then the solution was made up to 500 ml with adding more distilled water
- 500 ml of electron microscopy phosphate buffer (EM.PB) was added
- 5.4 g glucose and 0.5% CaCl₂ (4 ml) were added to the solution and then the mixture was filtered and the pH was adjusted to 7.4.

**Washing solution (0.1M)**

To make 100 ml of washing solution:

- 50 ml of EM.PB
- 50 ml of distilled water
- 8 g of glucose
- 0.5% CaCl₂ (0.4 ml)

**Buffer for Osmium (0.2 M)**

To make 100 ml of Osmium buffer:

- 100 ml of EM.PB
- 14 g Glucose

**Osmium**

One capsule of Osmium was broken into 25 ml of distilled water and the bottle was wrapped up in foil and the mixture should be made one night before use.

For 2% Osmium tetraoxide, dilute 1:1 with 4% osmium and add 1 drop of 0.5% CaCl₂ for every 10 ml.

Reynolds lead citrate for electron microscopy:

- 100 ml of distilled water was boiled and then cooled in a sealed contained (to decrease the amount of O₂)
• 1.33 g of lead nitrate and 1.76 g sodium citrate-2H₂O were added consecutively to 30 ml of distilled water.

• 8 ml of 1 N NaOH was added to the above solution to dissolve the lead nitrate and sodium citrate.

• The distilled water (prepared as in step 1) was added to make the volume up to 50 ml.