The use of olfactory ensheathing cells to promote regeneration of axons of central nervous system neurons

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DECLARATION

I, Ahmed Ibrahim, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated as such in the thesis.

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ABSTRACT

The mammalian central nervous system (CNS), including that of humans, has a poor capacity to repair and regenerate after injury. The outcome of brain and spinal cord injuries is a devastating loss of function and profound disability to the patient and tremendous socio-economic burden to society.

In this thesis, I first developed a behavioural model of brachial plexus dorsal root avulsion injury quantifying the subsequent behavioural deficit. Secondly, I transplanted olfactory bulb ensheathing cells in the dorsal root lesions and carried out functional, anatomical, electrophysiological assessments.

The data showed that while dorsal roots avulsion injury of C6 to T1 created a permanent climbing deficit, the lesioning of 3 or fewer roots produced a less severe form of the deficit and rats were still able to climb by masking the effects of the lesion with time.

After transplanting OECs into the dorsal root lesions, 70% of rats had restoration of paw grasping function, starting from 2-3 weeks post surgery while none of rats without OEC transplant recovered climbing function. The transplanted cells induced a mass of reactive tissue which served as a bridge for regenerating axons to cross over into the spinal cord. Individual axonal fibres were detected (labelled with anterograde axonal tracer) crossing the dorsal root entry zone, entering the spinal cord, arborising within the laminae of the spinal cord grey matter.

6-8 weeks after receiving OEC transplants, 7 out of 8 rats had cord dorsum field potentials detected at the cord after stimulating the median nerve. In the control group of 4 rats with chronic lesions but without OECs transplant, none showed cord dorsum potential nor cuneate responses up to 8 weeks after surgical intervention.

I concluded that OEC transplants promote recovery of paw grasping functions and electrophysiological transmission in a dorsal root injury model.

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Ahmed Ibrahim

Dedicated to my late father and my mother

LIST OF ABBREVIATION

BDA	Biotinylated Dextran Amine
BP	Brachial plexus
BPAI	Brachial plexus avulsion injury
BPI	Brachial plexus injury
CD	Cord dorsum
CDP	Cord dorsum potential
CNS	Central nervous system
CSPGs	Chondroitin Sulphate proteoglycans
DR	Dorsal root
DREZ	Dorsal root entry zone
DRG	Dorsal root ganglion
F	Fault (paw placement error)
GFAP	Glial fibrillary acid protein
GFP	Green fluorescent protein
HG	Horizontal grasp
LN	Laminin
MAG	Myelin associated glycoprotein
NGF	Neurotrophic growth

OEC	Olfactory ensheathing cells
Omgp	Oligodendrocytes myelin associated glycoprotein
ONF	Olfactory nerve fibroblast
PBS	Phosphate Buffer Saline
PFA	Paraformaldehyde
PNS	Peripheral nervous system
RTA	Road traffic accident
SCI	Spinal cord injury
SEM	Standard error of the mean
T	Touch (paw touches the grid but does not grasp the bars)
TBI	Traumatic brain injury
VG	Vertical grasp (Paw grasps the vertical bar)

PUBLICATIONS

- Restoration of hand function in a rat model of repair of brachial plexus injury

  Ahmed G. Ibrahim, Peter A. Kirkwood, Geoffrey Raisman and Ying Li Brain: 2009; 132; 1268–1276

- Permanent loss of fore-paw grasping requires complete deprivation of afferent input from a minimum of four dorsal roots of the rat brachial plexus

  Ahmed G. Ibrahim, Geoffrey Raisman, Ying Li Experimental Neurology: 2009; 215; 142–145

- Olfactory ensheathing cells: ripples of an incoming tide?

  Ahmed Ibrahim, Ying Li, Daqing Li, Geoffrey Raisman, Wagih S El Masry Lancet Neurol: 2006: 5; 453–57

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CHAPTER ONE

1 CHAPTER 1: INTRODUCTION

Few injuries to the body lead to a more profound and permanent loss of physical, cognitive and emotional function than brain or spinal cord injuries, and currently there are few or no effective treatment options available. The devastating disabilities of neurotrauma disproportionally affect the young in their prime. Both the acute and the chronic healthcare related needs of these patients as well as the associated loss in productivity places a tremendous socio-economic burden on the affected families and even the society at large. Each individual case of traumatic brain injury (TBI) in the United States is said to cost 436,000 USD in immediate healthcare needs at the time of injury [Hoshizaki *et al.* 2012]. The life time aggregate healthcare cost of those surviving severe head injury alone amounts to billions of dollars a year to a high income country in the western hemisphere [Berkowitz 1993].

The recent advances in providing systematic approach to trauma care and resuscitation using Advanced Trauma Life Support have resulted in improved survival of trauma patients, even in cases of severe TBI or spinal cord injuries (SCI) [van Olden *et al.* 2004]. This will increase the relative prevalence of neurotrauma patients surviving the initial insult and subsequently living with neurological disabilities, which makes the search for an effective repair of central nervous system (CNS) damage more relevant.

The incidence of SCI varies from 25 to 59 per million of population: common causes include road traffic accidents (RTA), falls or penetration injuries [Price *et al.* 1994;Devivo

2012]. In SCIs, aside from the loss of motor, sensory and autonomic function below the spinal injury level, the loss of independence has immeasurable psychological impact.

The nature and mechanisms which prevent CNS axons from repairing or regenerating after injury in a similar manner to the peripheral nervous system (PNS) neurons that has a robust capacity to regenerate is not yet fully understood. Studies have been ongoing for decades to identify and remove these CNS inhibitory factors. The successful discovery of an effective therapeutic agent or agents will not only benefit neurotrauma cases but also several neurological degenerative conditions such Parkinson's, dementia and more commonly cerebrovascular attacks.

1.1 The degeneration and regeneration of the nervous system

The mammalian neuronal cells form an astoundingly complex network of fibres capable of consistently and reliably communicating information along its circuit of axons, dendrites and synapses. Injury to the CNS can cause damage both at the anatomical and/or functional levels disrupting this continuity. The highly organised CNS system however, lacks the ability to repair itself in the adult; and in an attempt to protect the integrity of the rest of the circuit, the injury site becomes sealed off with activated astrocytic calls creating scar tissue.

Both the CNS and the PNS undergo sequence of events called Wallerian degeneration after injury, named after Augustus Waller in 1850. However, there some important differences in the processes in the two tissues [Stoll *et al.* 1989]. In the PNS the Schwann cells play a

central role in initiating Wallerian degeneration and the subsequent re-growth of axons [Stoll, Griffin, Li, and Trapp 1989;Perry *et al.* 1995;Fernandez-Valle *et al.* 1995]. Within hours to days of injury Schwann cells trigger the degeneration process when they cease to manufacture myelin [Trapp *et al.* 1988]. The axonal cytoskeleton then disintegrates with the Schwann cells and macrophages efficiently clearing up the myelin break down products by phagocytosis to set the stage up for the process of regeneration [George *et al.* 1995;Waller 1851]. Schwann cells then proliferate and together with fibroblasts release neurotrophic and growth factors such as nerve growth factor (NGF), fibroblast growth factor (FGF) and brain-derived growth factors (BDNF) that promote axonal outgrowth [Piirsoo *et al.* 2010;Watabe *et al.* 1995;Santos-Silva *et al.* 2007]. Within days of injury a robust re-growth of axons at rate of 1mm a day along their original trajectory capable of successfully re-innervating the peripheral targets to restore function takes place [Lunn *et al.* 1990;Ramer *et al.* 2000]. The response of CNS to injury however, is a process of degeneration that is soon followed by a failure of any meaningful regeneration process. The question then arises as to why the CNS neuronal tissue loses the capacity for spontaneous regeneration?

1.2 Failure of regeneration in the CNS and at the dorsal root transition zone

There are many hypotheses postulated to explain lack of CNS regenerative capacity including a) loss of innate re-growth ability of CNS neurons, b) the presence of abundant hostile molecular inhibitors to regeneration in the CNS environment and c) the presence of astrocytic or glial scar.

1.2.1 The loss of innate ability to regenerate in the adult CNS

Although degeneration occurs in the adult CNS following injury to a more or less similar manner, there is a distinct lack of an increase in the trophic factor expression to the degree that occurs in the PNS [Berry *et al.* 1996;Anderson *et al.* 1998;Bradbury *et al.* 1999]. The integrity of the blood brain barrier in the CNS also precludes the recruitment of circulating macrophages to the site of injury while the PNS induces fibroblasts and chemotactically attract macrophages to secrete a vast array of neurotrophic factors including FGF, BDNF, Glial Derived Neurotrophic Factor (GDNF), ciliary neurotrophic factor, and insulin like growth factors [Wang *et al.* 2008;Steinmetz *et al.* 2005;Ramer *et al.* 2002]. For instance, there is a 7 fold increase of NGF in the PNS compared with the CNS [Heumann *et al.* 1987].

In the immature mammalian CNS however, the necessary neurotrophic factors are secreted and are effective where restoration of function is possible [Varga *et al.* 1995;Kalil and Reh 1979;Bregman and Goldberger 1983]. Fujimoto et al reported that injury of the foetal spinal cord does not induce the same level of intensive scarring or inflammation and shows a good degree of regeneration [Fujimoto *et al.* 2006]. Studies have also shown that after transection of corticospinal tract in the immature neonatal spinal cord, severed fibres regenerated across the injured spinal segment and successfully found their distal targets [Bregman *et al.* 1989]. Even in the immediate post natal period considerably less degree of gliosis was observed and regeneration was still possible [Blackmore and Letourneau 2006; Fry *et al.* 2003; Barrett *et al.* 1984; Hase *et al.* 2002]. Thus the antenatal and immediate post natal periods can be viewed as regeneration permissive suggesting that the CNS tissue does have an innate capability to regenerate but that this is lost soon after birth for reasons that are yet to be fully understood.

1.2.2 The presence of hostile regeneration inhibiting factors in the CNS molecular environment

Aguayo and colleagues in a ground breaking work explored the idea of transplanting PNS tissue, a regeneration permitting system, into the adult CNS and reported that CNS axons were able to grow into the PNS grafts [Aguayo *et al.* 1981; Richardson *et al.* 1980; Richardson *et al.* 1982]. This important observation supported the hypothesis that CNS neurons do retain the capacity to regenerate in the right cellular or molecular surroundings and the hypothesis shifted to the idea that the astrocytic CNS environment is itself inhibitory to regenerating axons. This led many to search for the identity of these inhibitory factors and to devise strategies to neutralise them. The astro-glial scar tissue and myelin or myelin degradation products are reported to be potent inhibitors of regeneration as detailed below.

1.2.3 The inhibitory effects of the astro-glial scar

In order to maintain the physiological integrity of the CNS, glial cells such as oligodendrocytes, astrocytes and microglia respond to injury by forming a gliotic barrier [Fitch *et al.* 1999; Fitch and Silver 2008; Myer *et al.* 2006]. Similar injury induced intense gliosis occurs on the CNS side of the dorsal root (DR) transitional zone [Fraher 1999]. After injury there is significant upregulation of components of the glial tissue such as, extracellular matrix molecules including Chondroitin Sulphate proteoglycans (CSPGs) and semaphorin III [Horner and Gage 2000]. *In vitro* these molecules including CSPGs were found to be potent inhibitors of neurite growth and of axonal regrowth [Galtrey and Fawcett 2007; Busch and Silver 2007; Davies *et al.* 1999; Fitch and Silver 1997; Horner and Gage 2000]. There is no such up regulation and over-expression of these molecules in the injured immature CNS [Pasterkamp *et al.* 1999; Fidler *et al.* 1999].

1.2.4 Regeneration- inhibiting myelin degradation products

After axonal degradation the clearance of myelin break-down products in the CNS is inefficient with phagocyte cells recruited two to three days later than in the PNS [Popovich *et al.* 2001; Bell *et al.* 1994; Kim and de 2005]. The blood-brain barrier limits intravascular macrophages from entering CNS injury site further hindering the clearance of myelin degradation products [Bartholdi *et al.* 1997; Hickey 2001; Schnell *et al.* 1999]. Therefore, CNS myelin proteins persist at the injured site for longer than in the PNS.

A number of adult myelin proteins have now been identified to be inhibitory to neuronal regeneration [Varga, Bandtlow, Erulkar, Schwab, and Nicholls 1995; Savio *et al.* 1989]. Berry reported that non-myelinated axons were able to regenerate after chemical axotomy but no regeneration occurred in any fully myelinated axons [Berry 1982]. Myelin proteins such as myelin-associated glycoproteins (MAG) and oligodendrocytes myelin glycoprotein (OMgp) are thought to be potent inhibitors of regeneration [McKerracher *et al.* 1994; Mukhopadhyay *et al.* 1994; Wang *et al.* 2002; Schwab and Caroni 1988]. OMgp is a CNS glycolipid phosphomembrane found in not only oligodendrocytes but also in neurons [Wang, Kim, Sivasankaran, Segal, and He 2002; Cafferty *et al.* 2010]. In the PNS Schwann cells down regulate MAG after injury when they become transformed to the non-myelinating phenotype after injury. Such down regulation in oligodendrocytes does not occur in the CNS.

1.2.5 NOGO-A

Schwab and colleagues isolated proteins from central oligodendritic myelin which is reported to be an inhibitor of neurite growth in vitro [Schwab and Caroni 1988]. The

inhibitory molecule was later cloned and is termed NOGO-A. NOGO-A is expressed by neurons and oligodendrocytes and not by Schwann cells thus, it does not play a role in inhibiting regeneration in the PNS. Schwab et al developed monoclonal antibody against NOGO-A called IN-1 to block its inhibitory effects [Schwab and Thoenen 1985; Caroni and Schwab 1989; Chen *et al.* 2000]. The inhibitory effects of NOGO-A are mediated by a receptor called NgR which is expressed in neurons, glial cells.

There was considerable optimism around the discovery of the anti NOGO-A monoclonal antibodies [Bandtlow and Löschinger 1997]. In non-human primates administration of intrathecal anti NOGO-A antibodies restored hand dexterity after cervical cord hemisection by inducing axonal re-growth and compensatory sprouting [Freund *et al.* 2006; Schwab 2004]. Anatomical observation from these studies showed that in addition to axonal growth beyond the lesion sites there was considerable local growth of axons at the lesion site i.e. local sprouting [Cafferty *et al.* 2008]. Treatment with anti-NOGO-A antibodies has been reported to stimulate re-growth of severed axons of thoracic spinal cord and the sprouting of intact axons [Gonzenbach and Schwab 2008]. Further studies have however shown that the role of anti NOGO-A has been found to be less effective than was first suggested after experiments in NOGO knockout mice [Simonen *et al.* 2003; Zheng *et al.* 2003; Kim *et al.* 2003]. Additionally, work carried out to block NOGO-A receptor, (NgR), has disappointingly shown only limited restoration of function or regeneration. Recently, a receptor called PirB has been described by Atwal et al, which like NgR, also binds Omgp, Mag and NOGO that may explain the limited benefit gained by blocking NgR alone [Atwal *et al.* 2008].

Strategies of applying cellular transplant to induce axonal repair have the advantage of circumventing the need to individually characterise mechanism of action of each molecule involved.

1.3 Brachial plexus root avulsion injury as a subtype of SCI

The spinal nerve roots that constitute the brachial plexus are made up of two segments, a peripheral one that outgrows from the dorsal root ganglion (DRG) to the periphery and a shorter central preganglionic (CNS) segment that connects the DRG with the spinal cord. The preganglionic root closer to the spinal cord has astrocytic finger like processes that project outwards into the peripheral segment. Injury in this central segment disrupts sensory and motor fibres causing anatomical disconnection and loss of motor and sensory function which is essentially a CNS sensory deafferentation injury. Restoration of function can only be achieved if axons are able to elongate and cross the transition zone from dorsal root to spinal cord and form the correct connections that to enable communication of information through an exchange of axonal impulses between the CNS and the PNS.

In clinical setting such injuries are encountered after RTAs when motorcycle riders violently fall on an outstretched sustaining severe traction forces capable of severing the spinal nerve roots of the brachial plexus from the spinal cord [Midha 1997]. There is considerable variation in the exact nature and extent of these lesions which can occur either in the post-ganglionic peripheral segment or in the preganglionic central segment including complete avulsion of the roots from the cord with an associated rupture of the theca. Avulsion injury at least one nerve root after brachial plexus (BP) injures is fairly common affecting the majority (70%) of patients [Narakas 1993]. In the UK there are an

estimated 450 to 500 brachial plexus injuries (BPI) each year [Goldie and Coates 1992;Webb *et al.* 2002]. Untreated the clinical outcomes of BP roots avulsion injury are permanent paralysis, sensory loss in the upper limb and in the majority severe neuropathic pain [Htut *et al.* 2006;Koliatsos *et al.* 1994]. This lack of recovery after root injury is similar to that observed after cross section injury of the spinal cord. Thus the severance of the central segment of the nerve root from the spinal cord leads to CNS deafferentation comparable to transverse injury of the dorsal column [Carlstedt 1997;Kachramanoglou *et al.* 2011]. Successful repair of avulsed roots requires axonal elongation and growth to reconnect the peripheral nervous system with that of the dorsal horn in the CNS.

1.4 Current treatment strategies of adult brachial plexus injury

1.4.1 Management of ventral root lesions

In current clinical practice the management of ventral root brachial plexus avulsion injuries is aimed at restoring motor function particularly in the shoulder and the elbow. The surgical techniques employed to achieve this are microsurgical nerve reconstructions including nerve transfer, nerve repair with or without a graft and in some cases functioning free muscle flap transfer [Yang *et al.* 2012;Bertelli and Ghizoni 2003]. The microsurgical reconstructions of injured nerves are frequently reserved for brachial plexus injures that are less than a year old. Nerve repair maintains the native neuronal pathways to re-innervate the affected muscles. A nerve graft may be required in order to achieve tension free nerve repair obtained from a functionally less important nerve. In a meta-analysis Yang et al reported patients undergoing direct nerve repair surgery recovered muscle power of up to 3-4/5 [(medical research council (MRC)] grade in shoulder abduction and a grade 3 or better power in elbow flexion [Yang, Chang, and Chung 2012].

This technique is ideal for focal nerve laceration or transection injuries particularly in the postganglionic nerve segments.

When there is extensive damage to the brachial plexus the use of nerve graft or nerve transfer strategies are preferred [Songcharoen 2008;Terzis and Papakonstantinou 2000;Kandenwein *et al.* 2005]. This technique involves harvesting a donor nerve of less important function e.g. spinal accessory, intercostal or regional collateral nerves for transfer to re-innervate a more valued recipient nerve and muscle e.g. musculocutaneous for elbow flexion or suprascapular for shoulder abduction [Bertelli and Ghizoni 2003;Yang, Chang, and Chung 2012]. The authors report restoration in elbow flexion power of MRC grade 3 following treatment. Commonly used nerves for transfer are thoracic intercostals from T3 to T5 which can be accessed at the time of exploration of brachial plexus injury. The co-morbidities associated with nerve transfer surgery to the donor nerve site also need to be considered carefully. A disadvantage of using intercostal nerve transfer is the involuntary arm movement with coughing or sneezing. Reports show no outcome advantage of nerve transfer over nerve repair in shoulder abduction, however, for elbow flexion combined nerve repair and nerve transfer had better outcome [Yang, Chang, and Chung 2012;Garg *et al.* 2011].

The use of microsurgical techniques to repair severed ventral root axons is likely to fail in chronic ventral brachial plexus injury of more than a year old due to the loss of motorneurons [Bertelli *et al.* 2011]. An alternative is transplanting free functioning muscle harvested either locally or from a distal site, for instance, free gracillis myocutaneous,

trapezius or latissimus dorsi [Chuang 2010]. This is purely palliative therapy and is also useful when microsurgical nerve reconstruction therapies have failed.

Other newer surgical techniques include intra-plexus nerve transfer of ipsilateral or contralateral C7 nerve with limited success. Songcharoen et al reported median nerve motor recovery following C7 neurotisation to a biceps grade of 3 or 4 in about 20% of patients [Songcharoen *et al.* 2001]. Lin et al reported bicep muscle motor power of 3 or 4/5 after C7 nerve transfer to two recipient nerves in the hand and sensory improvement [Lin *et al.* 2011].

More recently, strategies of re-implanting the avulsed ventral roots directly into the spinal cord have shown encouraging results with grade 3-4 in proximal muscle power [Carlstedt *et al.* 1995]. In one exceptional case there was restoration of shoulder abduction to an MRC muscle power grade of 5 and a grade of 4 in the elbow and forearm muscles respectively, but with no restoration of functionally useful distal hand muscles function [Carlstedt *et al.* 2004].

Despite the above advances in microsurgical techniques to treat ventral BP root injuries, the outcome still remains poor and patients carry tremendous disabilities. Nerve transfer techniques target particular muscle groups and not the repair of the all the paralysed limb and as such are considered palliative treatment options only. Moreover, the re-innervations of distal forearm and hand functions using these techniques have been singularly disappointing [Berger *et al.* 1990].

1.4.2 Dorsal root BP injuries

When the dorsal roots of the brachial plexus are avulsed from the spinal cord the subsequent loss of sensation including touch, pain, temperature and propioception are irreversible and can be debilitating. The majority (60 to 80%) of patients sustaining these injuries develop severe and often intractable central neuropathic pain [Carlstedt 2009;Berman *et al.* 1998]. The severed axons do not have the capacity to regenerate or repair spontaneously or even when reimplanted back into the spinal cord [Siegal *et al.* 1990]. At present there are limited viable treatment options available to reverse the loss of function or to manage the pain.

The causes for the failure of spontaneous recovery or regeneration after avulsion injury of the dorsal roots are multi-factorial and hypotheses are abound. Ramon y Cajal in a seminal work first drew attention to the fact that the dorsal root entry zone itself was a major barrier to regenerating axons of the dorsal root ganglion (DRG). Cajal as well as later investigators showed that axons of the central segment of the DRG were indeed able to regenerate but only as far as the interface of the spinal cord entry zone where the regeneration abruptly stops and the growing axons change direction and/or grow backwards eventually forming stable growth endings [Aguayo, David, and Bray 1981;Kliot *et al.* 1990;Carlstedt 1985;Liuzzi and Lasek 1987;Li *et al.* 2004;Carlstedt 1997].

The consistently poor outcome of untreated root avulsion injury due to failure of regeneration makes the dorsal root injury model suitable for testing the efficacy of putative therapeutic agents that promote axonal regeneration. Various therapeutic agents have been applied to the lesioned dorsal root so as to induce regeneration. The

regenerative capacity of dorsal root has been reported to increase with conditioning peripheral nerve injury [Lu and Richardson 1991;Chong *et al.* 1996]. Chong et al lesioned the sciatic nerve at the time of transecting lumbar dorsal root and reported that more vigorous outgrowth of regenerating axons from the DRG were seen [Chong, Woolf, Turmaine, Emson, and Anderson 1996]. The authors noted that there was vigorous regenerating axons at the interface of the dorsal roots and the spinal cord when the sciatic nerve was simultaneously lesioned than not. However, only a minority of these regenerating axons entered the spinal cord.

While Ramer et al, in attempting to focus on intrinsic factors to support regenerating axons applied neurotrophic factor (NT3, BDNF, NGF and GDNF) after crush injury to thoracic dorsal roots and, reported recovery of pain sensation and crossing of axons into the spinal cord 10-15 days after treatment with NT3 and BDNF [Ramer, Bishop, Dockery, Mobarak, O'Leary, Fraher, Priestley, and McMahon 2002]. The authors concluded that neurotrophic support promotes axonal regeneration across the DREZ. However, there are a number of shortcomings with this study. Firstly, the crush injury was applied in the segment of the root midway between the DRG and the spinal cord leaving a generous stump. Secondly, the crush injury model is liable to leave spared axons and the reported re-entry of axons into the spinal cord after the short observation period of 10-15 days can be accounted for by these uncrushed axons. Thirdly, there was no control for cross contamination of the tracer labelling adjacent collateral nerve roots which may be responsible for transporting the tracer to the dorsal columns. It is likely that the observed functional and histological outcome resulted from recovery of partially lesioned or spared axons or sprouting.

Other investigators also used the dorsal root model to assess the effect of blocking myelin associated inhibition on regeneration. Harvey et al administered NOGO receptor blockers into the ventricle of rats and reported significant regrowth of myelinated sensory axons but not unmyelinated axons after a crush injury [Harvey *et al.* 2009]. Recent evidence however, suggests that blockage of NOGO receptors promotes sprouting rather than regeneration. In addition, the fact that only fully myelinated axons rather than any unmyelinated fibres were observed raises a strong possibility that these mature axons were in fact spared fibres.

Other therapeutic strategies include cell-mediated therapies with various cell types such as glial cells, Schwann cells and neuronal stem cells [Kliot, Smith, Siegal, and Silver 1990;Pearse *et al.* 2007]. Kliot et al transplanted embryonic astrocytes into crushed dorsal root lesions and reported limited axonal regeneration and arborisation in the grey matter of the spinal cord in only 23% of treated animals [Kliot, Smith, Siegal, and Silver 1990]. The lack of experimental success coupled with the difficulty of obtaining embryonic cells in the clinical setting made this a less favourable option.

Amongst the leading candidates of the cellular based therapies are olfactory ensheathing cells (OECs). The efficacy of these cells in promoting regeneration was first tested on the DR injury model [Ramón-Cueto and Nieto-Sampedro 1994]. The authors transplanted purified OECs into a severed single thoracic nerve and reported axons were traversing the lesion to innervate the laminae in the grey matter of the spinal cord. Another group [Pascual *et al.* 2002] injected purified OECs into L6 to S2 dorsal roots lesions and reported

a return of bladder function 6 weeks after surgery. In our own laboratory, we observed transplanting OECs into a single lumbar DR lesion promoted formation of a bridge like tissue over which the severed axons were able to cross into the spinal cord and continued to travel up to 10mm cranially [Li, Carlstedt, Berthold, and Raisman 2004].

1.5 OECs and the olfactory sensory system

The olfactory system uniquely in the body has the capacity, even in the adult, to continuosly re-grow axons that extend from nasal mucosa to reach the olfactory bulb within the brain [Graziadei and Montigraziadei 1978;Mackay-Sim and Kittel 1991;Carlstedt 1997]. The progenitor cells responsible for peripheral OECs are found in the neuroepithelial basal cell layer in the PNS where they divide and differentiate into mature neuronal cells that re-grow axonal processes that reach the CNS [Doucette 1984;Raisman 1985]. Doucet and Raisman first suggested the presence of these astrocyte like cells, later named OECs [Doucette 1995], in the nasal mucosa that ensheath and escort the olfactory axons through the hostile PNS-CNS interface [Chuah and Au 1991;Norgren, Jr. *et al.* 1992].

OECs were originally thought to arise from the olfactory placode [Barraud *et al.* 2010]. Recent evidence shows that they orginate from the neural crest: they share morphological and molecular characteristics of Schwann cells. Interestingly, OECs also share some features with astrocytes [Gong *et al.* 1994;Pixley 1992] expressing glial fibrillary acid protien but they can also be identified by the low affinity nerve growth factor receptor, p75 [Chuah and Au 1991], S100β [Barber 1982;Richter and Roskams 2007;Jani and Raisman 2004] and fibronectin [Valverde and Lopez-Mascaraque 1991]. To date there is no single marker that has been reported to be uniquely expressed by OECs. OECs have

also been reported to be able to secrete a large number of growth factors that could potentially support and maintain axonal growth such as neurotrophins, epithelial growth factor, platelet derived growth factors, insulin like growth factor [Lipson *et al.* 2003;Au *et al.* 2007].

OECs have been shown to also lay down channels through which regenerating axons could pass through to cross the PNS-CNS interface and reach the olfactory bulb [Graziadei *et al.* 1979]. Even when the olfactory bulb was removed in the neonate olfactory axons were still able to enter and synapse with frontal cortex [Graziadei *et al.* 1978]. This suggests that the OECs have the ability 'open the door' for axons to access the surface of the brain crossing the PNS/CNS interface. The concept of transferring the OECs with these properties into an induced lesion including the dorsal root injury model has obvious merit.

The reparative properties of OECs have been tested in various animal models giving mixed results of the capacity for OECs to induce axonal regeneration. The majority of studies [Ruitenberg *et al.* 2005;Li *et al.* 1997;Ramón-Cueto and Nieto-Sampedro 1994;Navarro *et al.* 1999;Ramón-Cueto *et al.* 1998;Ramón-Cueto *et al.* 2000;Imaizumi *et al.* 2000b;Imaizumi *et al.* 2000a;Boruch *et al.* 2001;Lu *et al.* 2001;Lu *et al.* 2002;Nash *et al.* 2002;Ruitenberg *et al.* 2002;Ruitenberg *et al.* 2003;Graziadei *et al.* 1980;Huard *et al.* 1998;Mackay-Sim and Kittel 1991;Pascual, Gudino-Cabrera, Insausti, and Nieto-Sampedro 2002] report OECs can promote regeneration of injured CNS axons while others however, [Gomez *et al.* 2003;Steward *et al.* 2006;Takami *et al.* 2002;Riddell *et al.* 2004], have failed to find any evidence to support the efficacy of OECs to promote regeneration. Gomez et al also used purified OECs transplanted in rhizotomised rats but failed to show axons

regenerating through the dorsal root entry zone. Similarly Riddell et al observed only limited in-growth of axons after lesioning and transplantation of purified OECs in a suspension[Riddell, Enriquez-Denton, Toft, Fairless, and Barnett 2004]. There are important distinctions in the experimental methods between ours and that of these groups that failed to find evidence that OECs induce axonal regeneration. These groups injected the OECs in a purified form in a suspension which would be retained with difficulty at transplanted location. Our own culturing method allows for production of a gel-like matrix within which the cells become embedded, which in our view allows for better retention of the OECs at the transplanted site of the injury enhancing the interaction between lesions and OECs facilitating the consequent repair. In this study I used the above method of culturing to obtain olfactory bulb OECs for transplant into dorsal root lesions.

1.6 Aims of this study

The aim of this study was to test the efficacy of OECs to induce regeneration of severed axons restoring function in an animal model that has clinical relevance. To this end I first developed an animal model of brachial plexus injury and once I established a stable and reproducible behavioural model, I transplanted cultured adult OECs into the lesions created and assessed the recovery in behavioural function. However, this behavioural outcome measure was insufficient to demonstrate axonal regeneration. Therefore, in addition to the functional outcome, I carried out detailed anatomical and sophisticated immunohistochemisry staining to observe the effects and interactions of transplanted OECs with host tissues. The DRGs of the transplanted roots were injected with an anterograde axonal tracer and histological processing of tissue carried out identified regenerating axons as they crossed the spinal cord and arborised within the spinal cord. In addition, I carried out electrophysiological experiments, in collaboration with Dr Peter Kirkwood (Sobell Department, Institute of Neurology), to assess restoration of functional and successful synaptic transmission after OEC transplantation.

1.7 Developing the dorsal root injury model

1.7.1 Anatomy of brachial plexus in the rat and the lesioning method used

The dermatomal innervations in the rat are remarkably similar to those in humans. The rat has BP sensory roots of C5 to T1 which form a brachial plexus that supplies the upper limb from the shoulder to the axilla [Lee *et al.* 2008;Takahashi and Nakajima 1996;Bertelli *et al.* 1992;Bertelli, Mira, Gilbert, Michot, and Legagneux 1992].

The dorsal roots have a pre-ganglionic/central and post-ganglionic/peripheral segments [Takahashi and Nakajima 1996]. Lesioning of dorsal root could either be induced by crushing the fibres using forceps or by transecting the dorsal roots with scissors. Crushing injury represents a mechanical type of compression injury for a sufficiently long duration so as to induce a disconnection injury. The disadvantages of this type of lesion are that it does not closely replicate the clinical condition of brachial plexus avulsion injury but rather a mechanical compression type similar to that seen in degenerative radicular conditions. Moreover, some of the dorsal roots fibres may inadvertently escape uncrushed or become only partially crushed capable of recovering spontaneously that can erroneously interpreted as induced by our intervention. Reproducibility of the crushing force applied by different experimenters or even by even at different times is also another concern. This variability makes interpretation of the behavioural outcome difficult with a degree of certainty.

The transection of the central segment of the dorsal roots used here on the other hand allows the completeness of lesioning to be visually verified under the operating microscope at the time of surgery which reduces the likelihood of fibres escaping uncut. Sharp transection injury inherently has a high degree of reproducibility both within laboratories and between different laboratories without a great deal of pre-training. However, one of the disadvantages is in that such a precise laceration lesion of the roots does not accurately reflect the violent and traumatic shearing forces that cause root avulsion seen clinically in BP injury. Attempts to faithfully reproduce such an avulsion injury in animal models would create unpredictable injury pattern making interpretation of outcomes more unreliable. In this thesis I transected the dorsal roots flush with the spinal cord without leaving a stump.

1.7.2 Behavioural or clinical assessments after dorsal root transections

The behavioural assessment method needed to be sensitive enough to discriminate between unlesioned and lesioned rats, allow detection of the magnitude of lesioned number of roots and any subsequent improvements that may occur after interventions.

Deafferentation injuries result in the loss of touch, vibration, proprioception as well as pain and temperature [Pitcher *et al.* 1999;Wu *et al.* 2009;Cook and Moore 2006;Montagne-Clavel and Oliveras 1996]. Behavioural assessment can be conducted to evaluate the integrity of the above modalities of the sensory system. For instance, assessing pain sensation by placing the paw on the lesioned side onto a hot plate and recording the time taken for animals to withdraw the said paws relies on co-operation of the rat that could potentially introduce unpredictable bias. Similarly, using Von Frey hair filaments to assess pressure sensation requires manipulations or even restraining of the animals which can raise anxiety levels of the rats influencing pain threshold levels which in turn can adversely interfere with the results [Sainburg *et al.* 1993;Ghez *et al.* 1990;Pitcher, Ritchie, and Henry 1999;Hargreaves *et al.* 1988].

Of all the sensory modalities, the loss of proprioceptive sensation appears to be difficult to quantify or to assess directly. However, behavioural effects of loss of proprioception (in-coordination) can be observed clinically in coordinated behavioural movements such as, grooming, manipulation of pellets or the drinking nozzle etc. These movements require continuous proprioceptive input for successfully coordinated goal orientated movement. Mott and Sherrington first reported that monkeys failed to use a limb for mobilising, grasping fruit and climbing robes after deafferentation injury from C4 to T4. Restraining

the contralateral unlesioned limb led the monkeys to reach for food with the deafferented limb but their aim was inaccurate. They concluded that sensory function was imperative to execute all purposeful motor function and the limb was essentially paralysed [F.W.Mott and C.S.Sherrington 1895]. Taub et al however, reported that monkeys were able to perform accurate pointing at visual targets even with a fully deafferented forelimb [Taub *et al.* 1975]. They suggested that the loss of motor coordination in the absence of somatosensory input can be overcome by visually assisted purposeful movement, although the deafferented limb remained clumsy and had impaired movement [Vaughan, Jr. *et al.* 1970;Rothwell *et al.* 1982;Bossom 1974]. The level of impairment was accentuated when vision was obscured. The impairment of coordination is likely to be exaggerated after deafferentation if the task assessed requires a high level of paw movement accuracy.

Both the wild and laboratory rats have a natural ability to climb up poles, drains, and cages in search of food, water etc. Successful climbing is such a demanding task that requires continuous fine motor adjustments of many muscle groups across multiple joints to be executed [Field *et al.* 2003]. Efficient climbing, unlike walking, could potentially expose a relatively minor deficit that would otherwise be undetectable. Additionally, the rats have dorsally located eyes which will limit visual cues of the immediate climbing area to guide final limb positioning particularly when their heads are held up. The attractiveness of the climbing test lies in the fact it requires minimum investigator intrusion or interference with the performance of the rats. In this thesis, I assessed climbing deficit after transection of various number of dorsal roots or combinations of roots to identify a clear behavioural model of deafferentation injury.

CHAPTER TWO

2 CHAPTER 2: MATERIALS AND METHODS

2.1 Animal subjects

All experiments in this study were conducted in accordance with the UK's Animals (Scientific Procedures) Act 1986. In all experiments locally bred female rats Albino Swiss strains were used. These rats were isogenic, phenotypically identical and weighed between 180g and 200g at the onset of the experiments. Throughout the experiments the rats had unrestricted free access to food and water at all times. Adequate measures were taken to minimise pain and discomfort the experiments were terminated early if the rats developed any signs of self harm or autotomy.

2.2 Anaesthesia

In all experiments I either used an inhalational anaesthetic agent or three different parenteral anaesthetic agents injected into the peritoneal cavity depending on the type of experiments undertaken or when the animals are perfused.

2.2.1 Parenteral Anaesthesia

2.2.1.1 Tribromoethanol (Avertin)

Tribromoethanol has been used in our own laboratory as an effective rat anaesthetic agent. A volume of 0.5 mls was administered via an intra-peritoneal injection at a dose of 20mg per 100g of body weight in the lower abdominal quadrant to avoid injury of abdominal organs. Five to 10 minutes after injection the rat did not respond to painful pinch stimuli applied to the hindpaws. When re-operating a lower dose of tribromoethanol at a dose concentration of 10mg per 100g of weight was used. The rats

recovered from the effects of anaesthesia in a warm cage heated by an electric blanket till fully mobile.

2.2.1.2 Urethane

Urethane is a long acting anaesthetic agent that was used for all electrophysiological experiments. It was also administered via intraperitoneal route at dose of 1.4gm per Kg. Urethane had a disadvantage that it can become deactivated by strong light and thus was kept in a dark glass bottle. It was also thought to potentially be a carcinogenic agent and needed to be handled with extra care.

2.2.1.3 Pentobarbitone

This anaesthetic agent provided deep and irreversible anaesthesia that was used for rats undergoing perfusion for histological processing. 1ml at a dose of 40mg per Kg was administered intraperitoneally.

2.2.1.4 Inhalational Anaesthesia

Two inhalational anaesthetic agents, halothane or isoflurane, were used that were delivered though a face mask from a pre-calibrated anaesthetic machine. A dose of 0.5 to 2L per minute was required to reach and maintain adequate anaesthesia. The delivery of inhalational agents could be more easily titrated to reach the desired levels of anaesthesia. Recovery of the rats from the anaesthesia was also much faster taking only 5-10 min for the rats to walk, drink or even start eating compared to the minimum of 15-

20 min required to reach the same level of alertness when using the intra-peritoneal injection of tribromoethanol.

2.3 Surgery for transection of dorsal roots

The transection of the dorsal roots was carried out under an operating microscope. Once the rat was fully anaesthetised an incision was made in the back of the neck starting at the cranio-cervical junction to the upper thoracic spine. The subcutaneous tissue was dissected and the left paraspinal neck muscles retracted laterally from the spinous processes exposing the laminae. The spinal levels were confirmed by counting down from C1. In the rat the C2 and T2 spinous processes were consistently the most prominent and could easily be palpated. Hence they were useful to serve as anatomical landmarks to aid identification of the vertebral levels of the spine being operated on. Left sided cervico-thoracic hemi-laminectomies were carried out at the required level. The thin and transparent dura revealed the dorsal roots as they enter the dorso-lateral spinal cord. The dura was then incised and the dorsal roots were selectively transected flush against the spinal cord.

To exclude anastomoses between rootlets, extra care was taken to ensure that transection of dorsal roots was made as close to the spinal cord as possible without leaving a stump behind. In all cases the completeness of transaction of the roots was always verified by reflecting the cut ends of the roots away from the spinal cord and by sweeping an instrument to detect any uncut rootlets. The cut ends of the roots were then replaced back against the spinal cord to their pre-lesion anatomical position. The number of roots

lesioned at one time was determined by the design of the experiment and varied from between single to six adjacent dorsal roots from C5 to T1.

In the majority of rats the dorsal roots were accompanied by radicular arteries which varied markedly in size between individual rats. Dividing the radicular arteries caused minimal bleeding and there were no incidence of cord infarction encountered. The cervical roots exited the cord in a more horizontal trajectory compared to the thoracic roots which had a more acute angle of exit from the spinal column. It was noteworthy, that only a minority of the rats (30%) had accessory rootlets running in parallel with the main root.

At the end of the surgery the wounds were closed in two layers and the rats were placed in a warm cage to recover from anaesthesia before placing back into their regular housing cages.

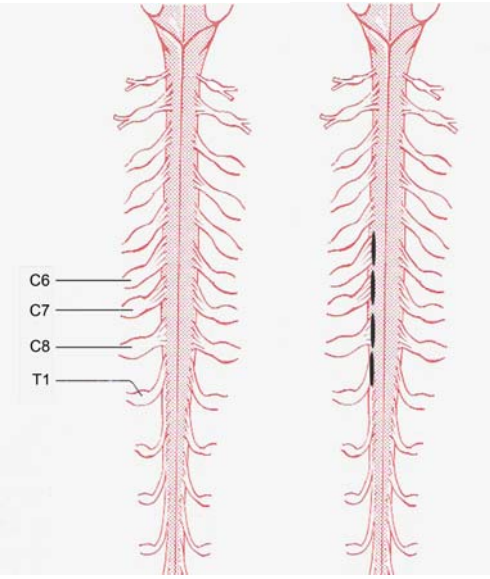

Figure 2-1. Schematic diagram of a rat spinal cord showing with the four cervical roots labelled and the location of the lesioning indicated in black.

2.4 OEC cell culture methods

Throughout this thesis the OECs used for transplantation were harvested from olfactory bulbs of adult isogenic rats. The olfactory bulbs were dissected free from the brain tissue after terminally anaesthetising the rats. The outer layers of olfactory nerve and the glomerular of the olfactory bulb were dissected and dissociated in 0.1% trypsin at 37°C for 15 minutes. Once fully trypsinized the tissue was plated on 35mm dishes coated with poly-*L*-lysine. The cells were then cultured in DMEM-F12 medium containing 10% foetal calf serum (Gibco 3133-028) for just over 2 weeks. After this time each dish yielded a mixture of about 1.5 million cells consisting of 50% OECs and 50% fibronectin-positive olfactory nerve fibroblasts (ONF) (Fig 2-2). This composition quickly changes in favour of ONFs if the culturing period was prolonged beyond the 17 days. The time frame for transplantation of optimum cell mixture was found to be between 14 to 17 days.

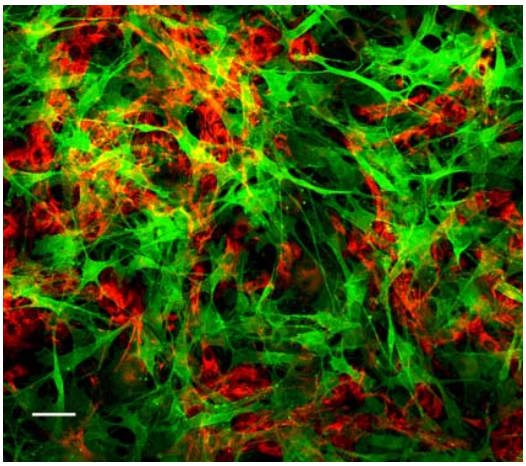

Figure 2-2. Showing OECs and ONFs in culture at 14 days. OECs are P75 positive labeled in red and fibronectin positive ONFs labeled in green.

2.4.1 Transfection of OECs with lentivirus

A lentivirus was used to transfect OECs in culture prior to transplantation with green fluorescent protein (GFP) in order to identify the cells in histological specimens. Transfection was carried out 2 to 5 days before transplantation; the OEC culture dishes were washed with fresh DMEM-F12 medium and incubated for 12 hours in the same medium containing the recombinant, replication-incompetent lentivirus vectors expressing enhanced green fluorescent protein (GFP-LV, a gift from Professor Luigi Naldini, San Raffaele Telethon Institute for Gene Therapy, Milan, Italy; packaged in our lab). The virus was then added to the culture medium and incubated for around 24 hours after which time 95% of the cells expressed green-fluorescence confirmed in confocal preparations.

2.4.2 Preparation of OECs for transplantation

The gel-like matrix of cell culture containing the OECs and the ONFs was scraped off the dish with a commercially available polythene spatula (Costar, Corning, NY) and cut into 4-5 pieces measuring 5mm in diameter transplanted at a density of 2.0-2.5 x 10^7 cells/ml (Fig 2-3). The cut pieces were then carefully transplanted into the lesion placing them between the cut ends of the dorsal roots and the spinal cord. Transplanting the cells embedded within the matrix not only avoids loss of cells during transfer, but also prevents diffusion of the cells away after transplant from the site of the lesion. Once the OEC transplants were in place a few drops of fibrin glue were applied to maintain the transplanted cells-dorsal root arrangement in place (Tisseel Kit, Baxter, Thetford, UK).

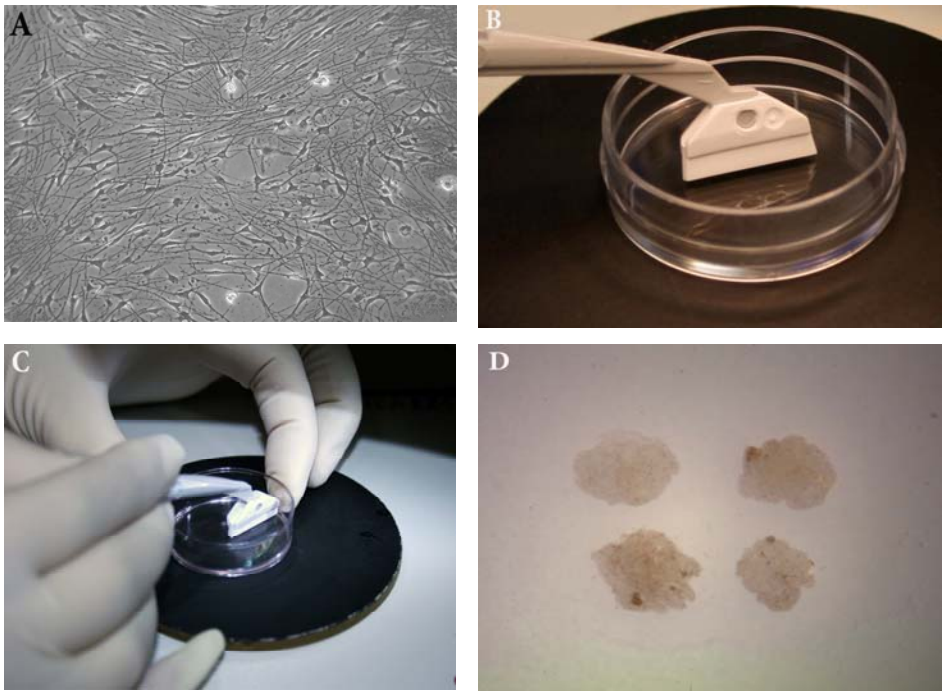

Figure 2-3. Showing preparation of OECs for transplantation. A) Shows cells in culture dish, B) scraping spatula and C) scrapping technique with the cut up portions of cells with matrix ready for transplanting in (D)

2.4.3 Regenerating axonal tracing method with biotinylated dextran amine (BDA)

A tracer or molecular marker that is transported from the cell body (anterograde transport) to the distal synapse along the axons was used to identify individual fibres. The marker is introduced distal to the lesion and detected more proximally. Of the number of tracers available commercially, we opted to use BDA to trace dorsal root axons from their origin at the DRGs (SIGMA UK). As BDA is not a transganglionic tracer it needed to be injected directly into the DRGs. The anterograde transport of BDA takes 10 to 14 days to reach the tracts in the spinal cord [Li, Carlstedt, Berthold, and Raisman 2004].

The DRGs were technically difficult to access surgically. I initially attempted the standard midline surgical approach with wide dissection, however, this was soon abandoned due to the excessive and often fatal bleeding was encountered when dissecting lateral and adjacent to the vertebral bodies presumably from epidural venous plexus. I thereafter, developed a paramidline plane which was a more direct route but required extensive dissection of the underlying muscle. The muscle fibres were obliquely arranged and following the direction of the fibres led to their insertion point at the lateral edge of the vertebral bodies. Immediately below the muscle insertion points, located in a groove covered by paraspinal muscles were found the DRGs. Once exposed the DRGs were injected through an oblique trajectory of the needle. There was minimal bleeding through this approach. The prominent C2 and T2 spinous processes were used as anatomical landmarks to help identify the correct levels.

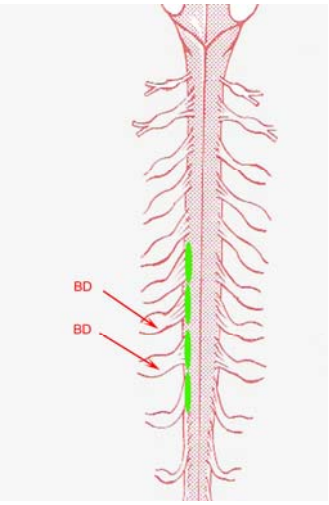

Figure 2-4. Schematic diagram of spinal cord and dorsal root indicating the injection site for BDA (indicated as BD on the diagram) at C7 and C8 root

A volume of 1-2µl of BDA, at a concentration of 10% in saline, was injected directly into the DRGs using a glass micropipette with an internal diameter of around 50µm. Two or three separate injections were carried out. To minimise the risk of BDA contaminating and reaching the spinal cord through adjacent intact roots, at least 2 roots above the injection levels and 2 below the level were transected. All rats that underwent tracing studies had extended transections from C4 to T2 and only C7 and C8 DRGs injected with BDA.

After injecting BDA rats were observed for a further two weeks to allow sufficient time for the tracer to be transported into the spinal cord and beyond. The experiments were terminated by perfusing the rats with 4% paraformaldehyde ready for histological processing.

2.5 Electrophysiological experimental methods

I collaborated with an electrophysiologist to carry out experiments to test whether electrical stimulus of a peripheral nerve can generate evoked field potentials in the cord dorsum and/or the cuneate nucleus before and after transplanting OECs in dorsal root lesions. Stimulating and recording wire electrodes were used in the electrophysiological experiments.

The rats undergoing electrophysiological experiments were terminally anaesthetised using urethane (1.4gmkg^{-1} I.P.) and the trachea, jugular vein and carotid artery cannulated. Throughout the experiments, physiological monitoring included recording of heart rate, blood pressure and body temperature. Optimal conditions of the rat were maintained by keeping the body temperature at 37-38ºC and the mean arterial blood pressure above 80 mm Hg by administering intravenous Hartmann's solution, typically at a rate of 1ml/hr, with occasional boluses of fluid required to boost systolic blood pressure. The median nerve was electrically stimulated and recordings were made via recording electrodes from the cord dorsum as well as the cuneate nucleus.

2.5.1 Median nerve dissection for electrophysiological experiment

The left forelimb was secured on to a frame in the supinated and externally rotated position with the ventral aspect of the limb uppermost. The median nerve was dissected and identified as the midline nerve structure and dissecting it further to the level just above the elbow. Part of the aconeus and the biceps muscles were excised and the skin flaps were raised to create a paraffin oil pool to protect the nerve from dehydrating.

2.5.2 Spinal cord and cuneate exposure for cord dorsum potential measurement

For the wider surgical exposure required for electrophysiological experiments the laminectomies were extended to C4 cranially and to T2 caudally. The cord dorsum recording electrodes were placed at the dorsal entry zone referenced on the local paraspinal muscles.

In rats which had previously underwent hemilaminectomies, surgical dissection was extended to cover the full laminae to include C4 to T2. Extra care was taken in these rats so as not to disrupt the dense connective tissue and glial scar that develops over the lesion site which needed to be trimmed down to as close to the cord as was judged to be safe in order to maintain the integrity of the dorsal root-spinal cord and scar complex. Despite this the remaining scar still forced the placement of the recording electrodes to a more medial location to the dorsal horn by about 0.5 mm (Fig 2-5).

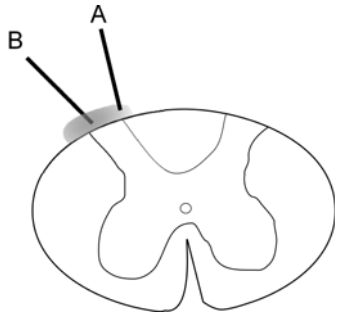

Figure 2-5. Schematic diagram of a cross section of spinal cord showing the scar (grey shaded area) that develops after surgery and the resulting medial displacement of recording electrodes in previously operated on rats (A) and the optimal placement of the electrodes in unlesioned rats with no scars (B).

In animals undergoing cuneate recordings, occipital craniectomy and C1 laminectomy were performed to expose the caudal medulla. Skin flaps were again raised around the spinal cord to form pools of warm paraffin oil to protect the cord from dehydration throughout the recording process. To minimise movement artefact the head and the thoracic spinous processes were secured on to a frame before placing the recording electrodes on to the cord dorsum. However, paralysing the rats in order to eliminate breathing movement was not found to be necessary.

2.5.3 Electrical stimulation of the median nerve and recordings potentials at the cord dorsum

The stimulating platinum wire electrodes were placed on the median nerve above the wrist and a recording electrodes were placed on the surface of the dorso-lateral spinal cord as close as to the dorsal root entry zone as possible with another control recording electrode placed on a more proximal location on the median nerve that monitored the

nerve volley conduction of the impulses. The cuneate recording electrodes were placed on the left dorsum of the medulla, 1.3 mm caudal and 0.7 mm lateral to obex, on the cuneate nucleus.

The electrodes placed on the median nerve were stimulated by a constant current stimulus with a duration of 0.1 ms pulses at a rate of once per second. The evoked potential signals were conventionally amplified and band-pass filtered (10Hz–10kHz) and recordings of around 50-300 responses were averaged using a computer software (Spike II). Negative deflections were shown as downward curves in the graphs.

On account of the more medial placement of the cord dorsum recording electrodes signals were probably modestly attenuated relative to the normal animals.

At the end of each experiment rats were terminated with anaesthetic overdose and perfused with 4% paraformaldehyde fixative for histological processing.

2.6 Behavioural analysis

The brachial plexus lesion disrupts the function of the dorsal roots to transmit sensory function including tactile, pain and temperature as well as vibration and proprioception back to the central nervous system. Loss of any or all of these sensory modalities impairs the ability of the individuals to interact with their surrounding environment appropriately. The behavioural disability that develops as a result of disrupted integration of sensory input with fine motor output was observed in a climbing apparatus and the climbing deficits was quantified using an objective, robust, reliable and reproducible method.

2.6.1 Climbing frame

The climbing frame was a metre long secured at 15° inclination from the vertical made up of a grid of horizontal bars welded together 5cm apart and vertical bars at 1cm apart (Fig 2-6). On reaching the flat platform at the top of the frame the climbing run was completed. No incentive was required or provided for the rats to climb and reach the top of the platform.

Figure 2-6. Showing the climbing frame (left) and an intact rat climbing the grid (right)

2.6.2 Climbing test

In a pilot group of animals, the left brachial plexus dorsal roots were transected in various combinations in order to evaluate the suitability of the climbing behavioural test and to establish an assessment method.

On a weekly basis, the rats were placed at bottom of onto the frame with the snout facing upwards and allowed to run twice up the rungs towards the platform at the top. The same experimenters carried out the tests at roughly the same time of day so as not to unsettle

the rats. Prior to commencing any of the experiments the rat underwent a test run to identify hesitant climbers and around 5% of the rats were found to be persistently poor climbers and were excluded from entering the pool of animals available for the experiments.

No more than two successive climbs were performed to minimise the effects of fatigue on their performance. All the climbs were videotaped and the clips were later played back in slow motion on a frame by frame basis to analyse the pattern of climbing paying particular attention to the left side noting the placement and the quality of the paw grasps. Occasionally the rats stopped half way through the climb or turned back to the bottom of the frame and for the purpose of analysis only data of completed climbs was included.

2.7 Histology

2.7.1 Animal perfusion and fixation

Under deep terminal anaesthesia induced by pentobarbitone (Sagatal) the rats were perfused. A wide thoracotomy was performed and the descending aorta clamped with haemostatic forceps. The right atrium was then incised to drain venous blood and a ventriculostomy was made on the left ventricle through which a hypodermic needle (0 gauge; cut flat and polished smooth) was inserted to cannulate the ascending aorta. The blood was first flushed out by 50-100mls of 0.1M PBS at room temperature for 5 min followed by slow perfusion with a further 500mls of PBS or 4% paraformaldehyde for 30minutes. The cervico-thoracic vertebral column was then dissected out and placed in

either PBS or fixative for a further 24-48 hours at 4°C before carrying out histological processing.

2.7.2 Tissue preparation for sectioning using a cryostat microtome

Under dissecting microscope the spinal cord and spinal roots from C1 to T3 were carefully dissected free from the vertebral skeleton. Extreme delicacy and care were needed to avoid disrupting the continuity of the roots or the scar tissue in lesioned area. The dissected tissue was placed in 10% then 20% sucrose solutions until fully saturated and the tissue sank to the bottom of the container.

The spinal cord and spinal roots were deep frozen using crushed dry ice. The frozen block of spinal cord was mounted on a specimen holder in embedding compound (Bright Cryo-M-Bed; Jencons Scientific Ltd, Leighton Buzzard, UK), cutting the flattened surface either in horizontal or coronal axial plane at thickness of 16µm for histology or immunostaining processing or 50µm sections were used for detecting of BDA. The sections were mounted on gelatine-coated slides and dried for at least 4 hours at room temperature with a fan. For long term storage, the sections were kept at -70°C. Mounting the sections required experience and perseverance to avoid air bubbles or other debris from being mounted which cause artefacts and adversely affect the quality of the slides.

2.7.3 Histology and Immunohistochemistry

2.7.3.1 Thionin staining

The sections were fixed with acetic alcohol (95% ethanol, 5% glacial acetic acid) for 30 min and rehydrated through immersion in a series of descending concentration of alcohols (96%, 70% and 50%) and finally in distilled water. Sections were then immersed in 0.05% aqueous thionin solution for 2 min, dehydrated by submerging in ascending concentration alcohols (50%, 70%, 96% and 100%). Sections were cleared in Histoclear™ (National Diagnostics, Aylesbury, UK) and mounted in a mixture of dibutyl phthalate, polystyrene and Histoclear™ (DPH) and air dried.

2.7.3.2 Immunohistochemistry

The rats were perfused with paraformaldehyde fixative for all immunohistochemistry staining. After a minimum of 24 hour of storing the tissue in a fixative at 4°C the relevant spinal cord segment was dissected and dehydrated by immersing it in 10-20% sucrose. The spinal cord segment was then deep frozen using dry ice and sectioned at 16 µm thickness using a cryostat.

2.7.3.3 Neurofilament staining

Neurofilaments are major supporting cytoskeleton components of peripheral and central axons in mammals. For neurofilament staining cryostat cut sections were incubated overnight at 4°C in 2% milk solution containing 1:500 heavy chain polyclonal anti-rabbit antibodies (Serotec, AHP245). After a minimum of 12 hour incubation with primary antibodies the sections were washed with PBS and again incubated in 2% milk containing

1:500 anti-rabbit secondary antibodies for 2 hours at room temperature (Alexaflour red) before washing and mounting.

2.7.3.4 Double immunostaining of GFAP and LN

To differentiate CNS from PNS tissue a double staining method using antibodies against astrocytic antigen glial fibrillary acid protein (GFAP) and antigens against peripheral nerve marker laminin were used. Sections 16µm thick were incubated in 2% milk containing 1:1000 GFAP antibodies (mouse monoclonal, Sigma UK) and 1:500 anti-laminin antibodies (rabbit polyclonal Sigma, UK) overnight at 4°C. The sections were then incubated in secondary antibodies (1:400 anti rabbit and anti mouse (Alexafluor Red or Green) for 1-2 hours in the dark at room temperature before washing and mounting.

2.7.3.5 BDA detection

14 days after BDA was air-injected into the DRGs at 10% concentration the rats were perfused using 4% paraformaldehyde as described above. Thicker cryostat sections at 25-30µm were cut and incubated in 1:400 of Streptavidine (Alexafluor 546. Invitrogen, molecular probes, inc. Eugene OR) overnight at 4°C and washed thoroughly before mounting.

2.8 Protocols for preparations of solutions

2.8.1 Phosphate Buffer

To prepare a phosphate buffer solutions A and B were added together. Solution A was made by adding 31.2gm of 0.2M NaH2PO42H2O to make up to 1 litre with distilled water.

Solution B was made using 28.4gm of 0.2M Na2HPO4 (Analar) which was added to distilled water to make up 1 litre solution. To make the buffer at pH 7.4 23ml solution A was added to 77ml of solution B. 8.76g of NaCl and 0.2g of KCL were added to 50mls of PBS.

2.8.2 Paraformaldehyde

Paraformaldehyde fixative solution was prepared at a concentration of 4% in 0.1M phosphate buffer (pH 7.2-7.4) by adding 40g of paraformaldehyde (EM grade; TAAB Laboratories Equipment Ltd) in 400ml distilled water with 2ml (40 drops) 1M NaOH. This solution was heated to 60°C in a fume cupboard until the paraformaldehyde had completely dissolved. Distilled water was then added to make up to 500ml. After the solution had cooled 500ml of 0.2M PB was added. The fixative was filtered and the pH adjusted to 7.2-7.4 with HCl.

2.8.3 Phosphate Buffer Saline (PBS)

To make PBS buffer solution 0.359 of NaH2PO4.2H2O, 3.19g of NaH2PO4.12H2O and 9g of NaCl were all added to distilled water to make up a litre of the buffer solution.

2.8.4 Tribromoethanol Anaesthetic agent (Avertin)

Tribromoethanol was used as the parenteral anaesthetic agent was prepared by adding 2g of 2,2,2-tribromoethanol (Aldrich Chem. Co, USA Cat No T4,840-2) to a mix of 2ml of 2-methylebutan-2-01 with 8ml of absolute ethanol. This was stirred vigorously for up to 30 min or until completely dissolved. The solution was then stored in 4°C refrigerator but was

warmed to room temperature before use. The dose used for rats was 1ml per 100g of body weight.

2.8.5 Fluorescence staining of BDA with Alexaflour

After perfusing rat with 4% paraformaldehyde (PFA) and fixing tissue in 4% PFA at 4°C for overnight I transferred tissue to 10% sucrose till fully saturated (tissue sank to bottom of container when fully saturated), then in 20% sucrose and finally left overnight. Cryostat sections were cut at thickness of 25 to 35 um and air dry slides for at least 2 hours. The sections were fixed again in 4%PFA for 30 min then washed with PBS three times at 30 minutes each. The slides were then incubated in 1:400 of Alexaflour (546) Streptavidin conjugate in antibody diluent for a minimum of 2 hours at room temperature or overnight at 4c. The sections were then thoroughly washed with PBS three times before mounting the sections and left to dry.

2.8.6 Neurofilament staining protocol

For the purpose of neurofilament staining the rats were perfused with PBS or PBS followed by the 4% paraformaldehyde, and post-fixed tissue overnight with 4% paraformaldehyde at 4°C. The tissue was then transferred into a 10% sucrose solution followed by immersion into 20% sucrose until sinking and sections were then cut on the cryostat at appropriate thickness. The sections were air dried for at least one hour before staining. The dry slides were fixed for 30 minutes with 4% PFA then washed in PBS 3 times at 30 minutes each. The sections were blocked in 2% milk-PBS-triton for 30 min and incubated in milk-PBS with 0.1% triton containing neurofilament antibody (heavy chain polyclonal anti-rabbit 1:500 from Serotec) overnight at 4°C. The sections were then washed in PBS five times at 30 min

each and incubated in milk-PBS-triton with secondary antibody (anti-rabbit 1:500) for 2 hours at room temperature. The sections were finally washed in PBS and mounted.

CHAPTER THREE

3 CHAPTER 3: THE DESIGN, VALIDATION AND USE OF A BEHAVIOURAL ASSESSMENT METHOD FOR BRACHIAL PLEXUS INJURY MODEL

(Ibrahim et al, Permanent loss of fore-paw grasping requires complete deprivation of afferent input from a minimum of four dorsal roots of the rat brachial plexus, Experimental Neurology 215 (2009) 142–145)

3.1 Summary

A climbing behavioural assessment method was designed with the aim of finding an accurate, robust and reproducible scoring method so as to determine the nature and extent of the neurological deficit that arises from an induced deafferentation brachial plexus injury.

Seventy six female rats (76) were divided into unlesioned (n=17) and lesioned groups (n=59) where single or multiple brachial plexus dorsal roots were transected. Transection of a single brachial plexus dorsal root, between C5 and T1, was carried out in 8 of the 59 rats. A further 8 rats had two adjacent dorsal roots of C6 and C7 transected, 16 rats had three adjacent dorsal roots (C6 to C8). Transections of four dorsal roots were carried out in two sets of rats; 9 had lesioning of C5 to C8, the upper four roots and 18 rats had transection of C6 to T1, the lower brachial plexus roots. The climbing performance of lesioned and unlesioned rats was then compared in the behavioural climbing apparatus as described in Chapter 2.

The results showed that the intact rats grasped the bars a mean of 7.0 ± 0.1 times per metre; grasping was completely abolished after transection of C6 to T1 dorsal roots with a mean score of 0.1 ± 0.06 grasps per metre. This deficit in grasping remained unchanged for the entire test period of 8 weeks. Sectioning of C5-8 or C6-8 resulted in milder pattern of deficit whereas transection of 2 adjacent roots caused only minor deficits. There was no detectable climbing deficit after sectioning only a single root.

3.2 Introduction

There is usually an unpredictable but spontaneous recovery of function in the first few months after most spinal cord injuries. In order to distinguish the spontaneous recovery from that induced by intervention a very large number of patients is needed makes conducting clinical trials somewhat prohibitive. Good experimental animal models of SCI aim to control for as many variables as possible which in turn restricts the relevance of the findings in the unpredictable nature of clinical injuries.

In this thesis, I avoided the challenges of distinguishing the effects of therapy from spontaneous recovery of function observed after SCIs by focusing on a specific and focal CNS deafferentation injury that has a stable natural history with no known meaningful recovery: the brachial plexus avulsion injury model. This lesion disrupts afferent sensory and efferent motor connections leading permanent and irreversible loss of these functions in the affected upper limb [Havton and Carlstedt 2009;Goldie and Coates 1992]. In many, the injury to the dorsal roots causes persistent central pain that is severe and difficult to treat [Htut, Misra, Anand, Birch, and Carlstedt 2006]. The predictable clinical outcome of root avulsion injury makes it an attractive model to develop and use as a bench mark to test the efficacy of putative regeneration promoting therapies. The added experimental benefit of using this injury is in its focal and discrete size requiring fewer cells and has shorter distance needed for regenerating axons to traverse. Conversely, this fact may limit extrapolation of findings from small lesions into larger sizes injuries in humans

A number of lesioning methods such as crush or transection injuries have been described [Wang, King, Ossipov, Rossomando, Vanderah, Harvey, Cariani, Frank, Sah, and Porreca

2008;Ramer, Bishop, Dockery, Mobarak, O'Leary, Fraher, Priestley, and McMahon 2002;Gomez, Averill, King, Yang, Perez, Chacon, Ward, Nieto-Sampedro, Priestley, and Taylor 2003;Ramón-Cueto and Nieto-Sampedro 1994;Riddell, Enriquez-Denton, Toft, Fairless, and Barnett 2004;Blits *et al.* 2004]. While, root crushing injury replicates a mechanical compression of the nerves, akin to incomplete spinal cord injury where there can be spontaneous recovery, I have opted to induce a sharp transection lesion of the dorsal roots to guard against incomplete division of the nerve fibres and thus make the model more closely representative to the clinical complete BPI roots avulsion injury.

The behavioural deficit that develops after the induced DR injury needs to be quantified in an accurate, robust and reproducible assessment method. DR lesions are deafferentation injuries which will lead to loss of sensory function including touch, pain, temperature, vibration and proprioception. My aim was to evaluate one of these sensory modalities in a minimally invasive behavioural test that could accurately reflect the neurological deficit. The test needed to be demanding and robust enough not to allow the rats to mask the deficits by developing compensatory strategies.

Rodents often climb naturally to explore their surrounding environment e.g. cage tops etc. In a previous experimental paradigm our group tested climbing behaviour to evaluate motor function after long tract injury before and after OECs transplantation [Li *et al.* 2003]. I adopted the climbing test to in sequential brachial plexus DR transection injury model to assess if it's applicable. While it is not difficult to accept the rational for testing climbing to quantify motor function, the suitability of this test to assess the integrity of the sensory dorsal root functions has not been previously demonstrated.

3.3 Materials and methods

Female adult AS rats weighing 180-200g were used for these experiments. The surgery to transect the roots and the climbing tests were carried out as described above (Chapter 2 Material and methods). Briefly, hemilaminectomies were carried out to access the dorsal spinal cord where the dorsal root rats were completely transected flush against the spinal cord without leaving a stump. After one week recovery period the rats were videotaped while climbing a 1m grid 2 separate and consecutive runs once a week for a minimum duration of 8 weeks.

The climbing cage (Fig 2-6) was one metre long grid of horizontal and vertical bars leaning at a slope of 15% with a platform at the top. There was no reward offered or punishment administered at the end of the climbs. The rats were simply placed on the lowest rung of the frame and freely allowed to climb while being videotaped. To prevent de-motivating the rats the test was only repeated twice at any one time. The video clips of the climbs were later analysed in slow motion on a frame by frame basis.

A total of 17 unlesioned intact rats had their climbing assessed. In the surgical arm there were a total of 59 rats which underwent transection of a single or multiple DRs. Eight (8) of the 59 rats had transection of a single root with 51 rats undergoing multiple root lesions. Of these, 8 rats had two adjacent roots (C6 and C7) transected and 16 other rats had three roots transected (C6 to C8). There were two groups of rats with four root transections; 9 rats had lesioning of C5 to C8 and another 18 rats had C6 to T1 roots lesioned (table 3-1).

At the end of the experiment animals were perfused and the spinal cord and attached roots tissue complex were processed for analysis.

Table 3-1. Summary of transected roots and climbing videos

Roots sectioned (number of rats)	No of climbs assessed
Unlesioned (n=17)	**102**
Single root lesion (n=8)	**128**
C6 and C7 (n=8)	**128**
C6,7and C8 n (16)	**256**
C5,6,7 and C8 (n=8)	**66**
C6,7,8 and T1 (n=18)	**666**

3.3.1 Statistics

The data was analysed using STATA or SPSS which were commercially available statistical softwares with guidance from a qualified statistician. The means are quoted with standard errors of the mean or the 95% confidence intervals. The differences between means of the different groups were analysed using a t-test with the result considered significant if the P was <0.05. In order to be able to use the t-test the data must be normally distributed. However, given the large sample size, an assumption that the real mean of the population tended towards a normal distribution was made.

3.4 Results I: preliminary observations of climbing performance after dorsal root lesioning

The terminal event of ach paw reach was analysed to determine the final placement of the paw and the movement of individual digits in grasping the frame. The general efficiency of the climb and characteristics of the errors were noted.

3.4.1 Climbing of Intact rats

A total of 192 metre climbing runs clips of unlesioned rats (n=17) were assessed to define normal climbing patterns. The intact rats did not require prior training or familiarity with the grid and were found to be excellent natural climbers. The observations showed that the rats lead with each of the forepaws alternately and the hindpaws quickly follow behind.

3.4.2 Climbing after transection of a single root lesion

Similar to the intact group, rats with single root transection did not show detectable levels of climbing deficits. The single root deafferentation injury was insufficient to cause a deficit in either paw placement or grasping. Hence, I decided to proceed to test the cumulative effects of multiple adjacent dorsal root transections.

3.4.3 Climbing after transection of two adjacent roots

After lesioning of C6 and C7 dorsal roots, a mild deficit developed when occasional errors were made in paw placement onto the climbing frame compared to the near perfect climbing

performance of the intact rats. The subtle deficit was insufficient to cause disability or impair the climbing of the rats to any degree.

3.4.4 Climbing after transection of three adjacent roots

The lesioning of 3 adjacent roots from C6 to C8 was then carried out. This now caused noticeable climbing errors causing loss of the smooth paw climbing action replacing it by jerky and hesitant movements that led to inaccurate paw placements and inefficient paw grasping to propel them upwards.

On closer analysis of the clips of the lesioned rats, a repeating pattern of errors were occurring which appeared to correlate with the number of dorsal roots transected; being greatest after three root lesions. These errors or mistakes in paw placement broadly fell into one of the following four main categories (table 3-2).

Table 3-2. Climbing categories of the end of paw-reaching events in the preliminary scoring method

Event Category	Description
Fault (F)	The left forepaw reach ended in completely missing the bars and going through the grid
Touch (T)	The left forepaw reached and touched the bars but did not grasp
Horizontal grasp (HG)	The left forepaw grasped the horizontal bar
Vertical grasp (VG)	The left forepaw grasped the vertical bar

3.4.5 Use of preliminary scoring method for comparison of performance of unlesioned and lesioned rats

The video clips of the unlesioned and the lesioned rats were re-run and each rat had a score for the above four categories. Attention was paid to the performance of the paw on the lesioned side which was the left forepaw.

3.4.5.1 Fault (F) score

While the median F score in intact animals was 0 per metre of climb, the median F in the two root lesioned rats was 2. The median F score significantly increased to 6.5 after transecting three adjacent roots (Fig 3-1).

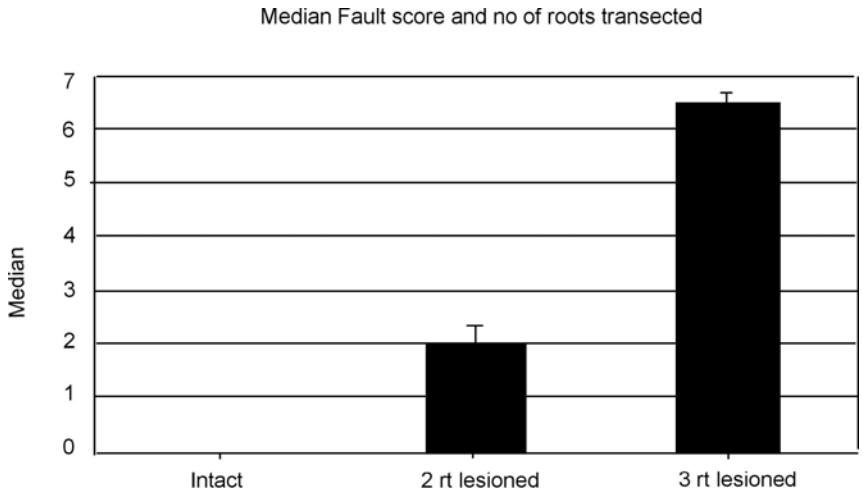

Figure 3-1. Graph showing progressive increase in median fault score with increasing number of roots lesions

3.4.5.2 Vertical Grasp (VG) score

The median VG score of the intact rats was 6 per metre climbed. Following two roots transections there was an increase in median of 9 VGs per metre whereas after three roots transections there was sharp drop to a median of 2 VG per metre (Fig 3-2).

The rats which underwent transection of 2 roots appeared to favour grasping the vertical bars to the horizontal bars for reasons that were unclear. The rise in VG in two root lesioned rats however, was offset by a corresponding drop in HG score compared to normal level (Fig 3-3).

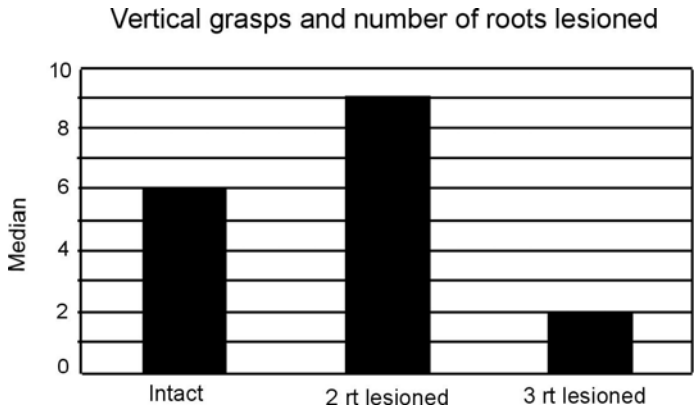

Figure 3-2. Median VG score and number of roots lesioned (x-axis). The initial increase in VG after two roots are transected compared to score of intact rats and the sharp drop after three roots were transected

3.4.5.3 Horizontal Grasp (HG) and Touch (T) scoring events

The paw reaches that ended in HG and T were infrequent in both lesioned and unlesioned rats, although there was a tendency for frequency of T to increase the more roots were transected. However, these events were found to be unhelpful in discriminating between intact and lesioned rats and also in detecting the severity of the injury. The median for these parameters was 0 as they were found to be rare occurrences and hence the mean was calculated.

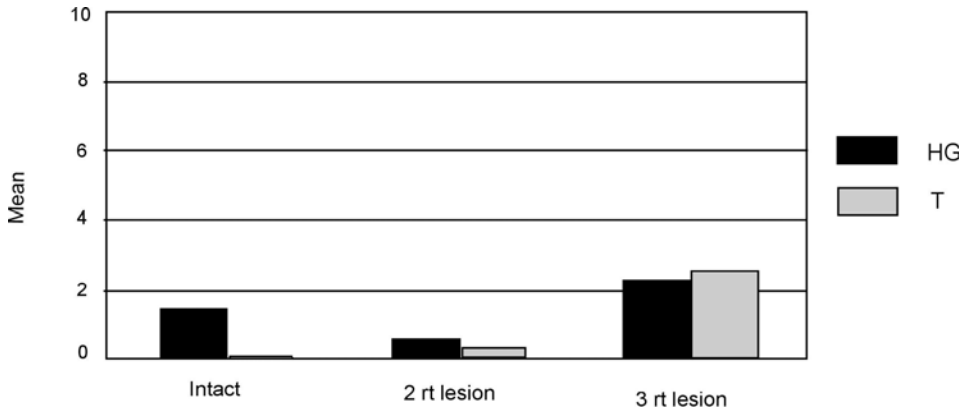

Figure 3-3. The mean scores of HG and T in the unlesioned and lesioned rats showing the infrequent occurrences of these score categories

3.4.5.4 Rationale for re-defining the preliminary scoring method

The above score method projected the different types of paw reaching events. However, not all the different events scored above were sensitive enough to the number of roots lesioned and thus subsequently would be unlikely parameters to detect any beneficial effects that may develop after a given intervention. In particular, the horizontal grasp and the touch Category were poor at discriminating between the climbing performances of the rats with various numbers of roots lesioned. For instance, the mean and median of the HG score of unlesioned and 3 roots lesioned rats were the same while there were none were recorded for the two root lesioned rats.

Of all the different scores, the fault (F) score was found to best correlated with the sequential increase of the number of roots transected (Figs 3-1 and 3-4). The grasp scores, though increased after two roots were transected initially, there was a noticeable drop after

transection of 3 roots. These two parameters also occurred with more frequencies that the HG and T.

It was thus decided that the categories needed to be redefined to better reflect the effect of increasing number of root lesions.

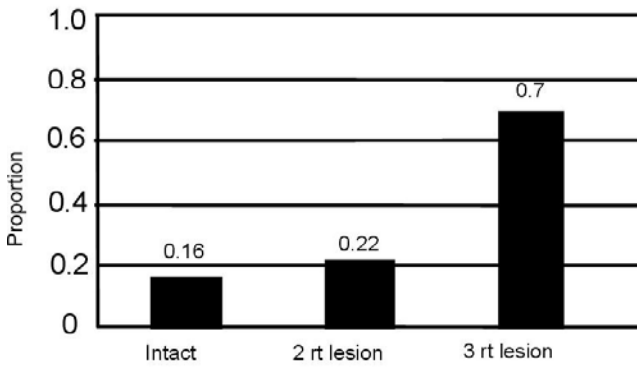

Figure 3-4. The proportion of paw reaches that ended in faults and the number of roots lesioned

3.4.5.5 Redefining the scoring categories

It became clear that each reach of the paws ended in a terminal event of either a successful grasp or an error of varying degree of severity. Furthermore, climbing events that ended with a grasp of the horizontal bars functionally aided the rats in propelling themselves upwards. Thus, all VG and VH grasps were considered useful and combined into a single score of grasp G. Similarly, paw reaches that ended with the digits physically contacting but failing to grasp the bars were considered as errors of climbing. Thus the Touch category was abolished and the scores were combined with fault scores.

3.4.5.6 Grading of severity of faults

Paw reaches that ended in minor Faults had little or no adverse effect on climbing to one that curtailed the capacity of the rats to climb efficiently. When the faults were severe the paw ended up protruding through the grid. The protruded paws hindered climbing when it could not be corrected in time for the next forepaw movement forward. Thus, the extent to which the paws protruded beyond the grid were graded (table 3-3). Thus, at the end of each paw reach, the rats had two scores per the metre of climbing: a "Grasp" and "Fault" scores.

Table 3-3. Grading of the faults according to severity of paw protrusion

Level of paw protrusion	Fault score
Paw reaches the grid but does not grasp the bar	**1**
Paw misses the bars and protrudes through grid to the wrist level	**2**
Paw protrudes through to the elbow level	**3**
Paw protrudes through the grid to the shoulder level	**4**

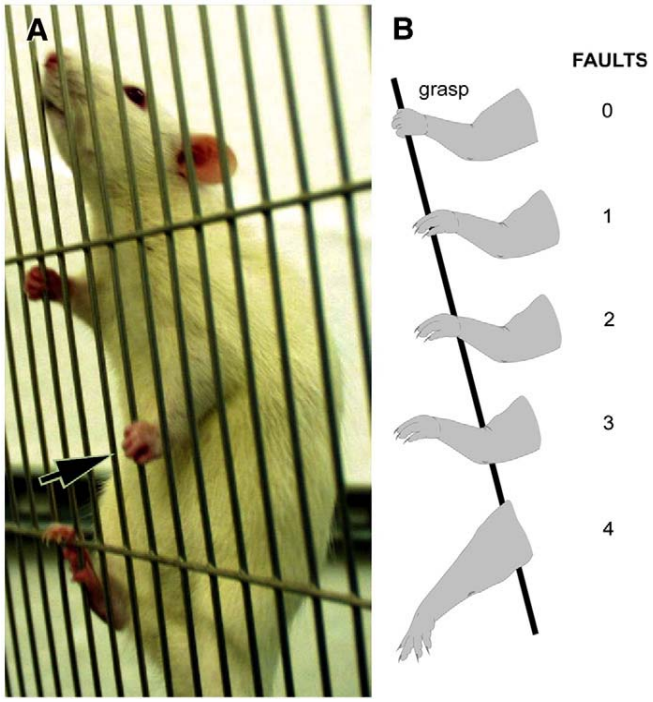

Figure 3-5A and B. Rat climbing the grid with the normal grasp (arrowed) of the left fore-paw. Fig 3-5B. Scoring of paw grasps or faults in reaching, G = successful grasp, 1 = reach the level of the bars but not grasp, 2 = paw protrudes through the bars as far as the wrist, 3 = protrusion as far as the elbow, to 4 = paw protrusion as far as the axilla.

3.4.5.7 Validation of climbing scoring system

In order to validate the scoring method two additional investigators were blinded to the status of the number of roots transected and were asked to independently score 60 runs for 10 randomly selected lesioned and unlesioned rats. The independent of grasp or fault scores of all the investigators were collected and analysed. The inter-observer correlation was remarkably high with correlation with inter-observer ratio R=0.93 for scores of observers 1 and 3 and 0.68 for scores observer 1 and 3. The analysis of the rest of the data was carried out by observer 3 who was blinded to the status of rats scored (Fig 3-6).

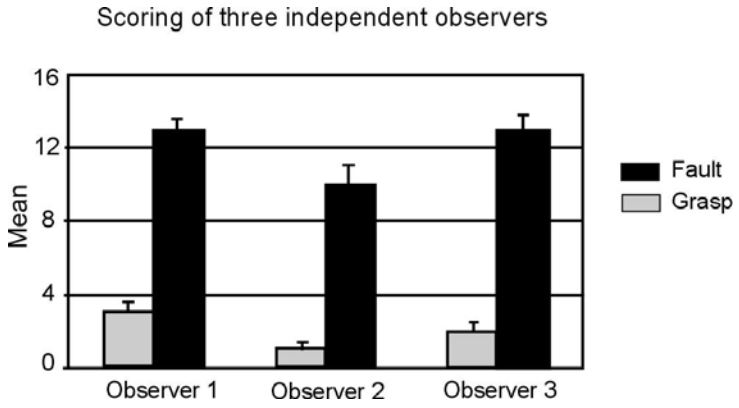

Figure 3-6. Mean fault and grasps of 10 randomly selected rats scores by three independent observers showing the good level of inter observer agreement, particularly between observers 1 and 3 more than agreement of observer 2 with the other two observers

3.4.5.8 Two consecutive runs as independent variables

There were no clinically obvious differences between runs 1 and 2. Comparison in the scores of mean fault and grasp between the first and the second climbs runs revealed that there was no statistically significant between the scores either ($p<0.89$).

3.5 Results II: scoring using redefined categories

The two behavioural categories of Touch and the Fault scores were inadequate to detect increased number of roots transected. The difference in the climbing scores between a 2 root lesioned rats and a 4 root lesioned rat were similar. Therefore, I decided that these 2 categories could not be used as tools to detect any potential benefits of future interventions. The Touch scores were thus added to the fault score to be called Fault score. The Grasp score was calculated by adding the two grasping scores of VG and HG.

3.5.1 Climbing of unlesioned rats

In 102 climbs recorded, the 17 intact rats rarely made mistakes grasping the bars completed the one metre climb in a mean of 7.1 ± 0.4 (SEM) (95% CI 6.8, 7.6) grasps. These rats also rarely misplaced the paws on the grid resulting in a small mean fault score of only 0.98 ± 0.1 (95% CI 0.9, 1.6) per metre climbed.

3.5.2 Single root lesioned rats

The level of climbing paw function accuracy of rats after single dorsal root lesioning was high and comparable to unlesioned rats again. Using the combined scores of faults and grasps, the single dorsal root lesioned rats had a mean fault score of 1 ± 0.1, and a mean grasp score of 8 ± 0.2 per climb.

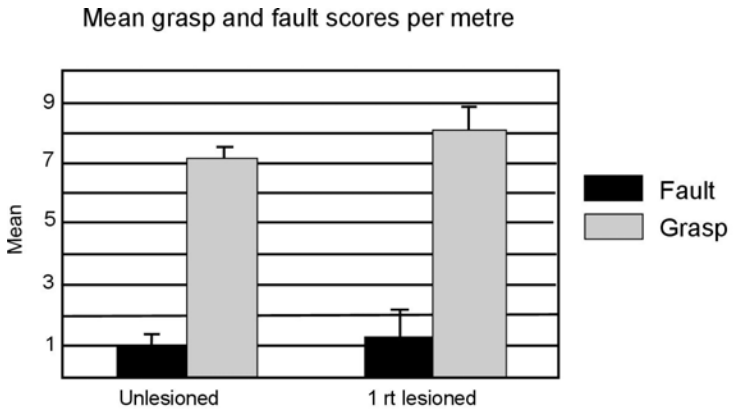

Figure 3-7. Similar scores of mean fault and grasp were scored by the unlesioned and the single root lesioned rats. Error bars 97% CI.

3.5.3 Two root lesioned rats

The rats with 2 root lesions had a mean fault score of 3 ± 0.4 (SEM) and an average of 9 ± 0.3 grasps per climb over 6-8 weeks. There was an increase in the fault score after 2 roots were lesioned while the number of grasps conversely also increased after 2 roots were transected but sharply fell after 3 roots were cut. After lesioning of 2 roots only, the rats were able to grasp and still climb efficiently with compensatory increase in the number of grasps by the intact contralateral paw.

3.5.4 Three root lesioned rats

In 256 climbs (n= 16) of rats with 3 root transected, the mean fault score was 7.1 ± 0.4 and the mean grasp score was 3.9 ± 0.2. The fault score was much higher than the 1 ± 0.1 of

unlesioned rats ($p<0.001$). Similarly the mean number of grasps for rats with three root lesions was much reduced to the normal score of 8 per run ($p<0.001$).

3.5.5 Climbing scores of three root lesioned rats over time

In the three root lesioned rats there was however a trend towards a reduction in the mean fault scores over the 8 weeks period of observation (Fig 3-8 B). Using a multi-level mixed effect linear regression model (run in Stata for Windows software, Stata Corp LP, TX, USA) there was no statistically significant difference in the first three weeks ($p=0.13$), however, when compared to the first week the scores from week four to week 8 the reduction in fault score was highly significant (t-test $p<0.001$).

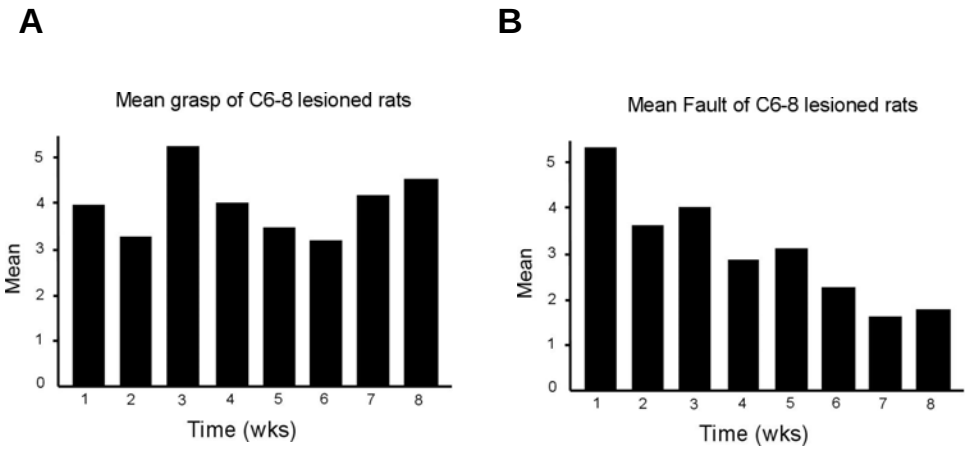

Figure 3-8 A & B. Means of grasps and fault of C6-8 root lesioned rats over the observation time. The graph shows that while the mean grasp remains relatively stable over time (A), there was trend towards a gradual reduction in the fault score over time in (B)

The observation that there was a trend towards improvement in fault score after 3 root lesions, however small, I carried out transection of four roots in order to obtain a more stable lesion with time.

3.5.6 Rats with 4 dorsal root transection

I carried out transection of 4 adjacent roots out of the 5 dorsal roots that make up the brachial plexus. Two sets of rats had transection of four roots either from C5-8 (n=9) or from C6-T1 (n=18). While inclusion of T1 dorsal root within the lesion segment impairs distal paw function, the C5-8 lesion impairs more proximal sensory function from the shoulder area.

3.5.6.1 C5 to C8 root transection

Following this combination of dorsal root lesions the rats completed the climbs with a mean grasp score of 3.9 (95% CI: 3.2 - 4.6) and a mean fault score of 8.9 (95% CI: 5.35 - 10.3). Thus the climbing scores after lesioning C6 to C8 and C5 to C8 were similar. The mean grasp score for this group was 3.9 compared to the 3.1 for the C6-8 lesioned rats. And the mean fault score was 8.9 per climb for the C5-8 lesioned rats and 7.1 for C6-8 roots lesioned rats. Thus additional lesioning of the C5 nerve root contributed little to increased distal paw function disability, which was surprising but not entirely unexpected.

3.5.6.2 C6 to T1 root transections

The analysis of 288 climbs completed runs after rats had C6-T1 lesions revealed that the mean fault score was now a massive 21.9 ± 1 per metre, additionally, the rats now had completely lost the grasping function by the ipsilateral paw managing to score 0.1 ± 0.06 Grasps per metre (95%CI: 0.05 - 0.3) Fig 3-6. The inclusion of T1 DR in the lesioning with at least adjacent 3 DR roots created a remarkable climbing deficit. There was almost complete inability of the rats to grasp the bars after lesioning which was a very promising finding.

It was now important to examine the severity of the injury with time to assess if it remains stable or there was a spontaneous recovery of function.

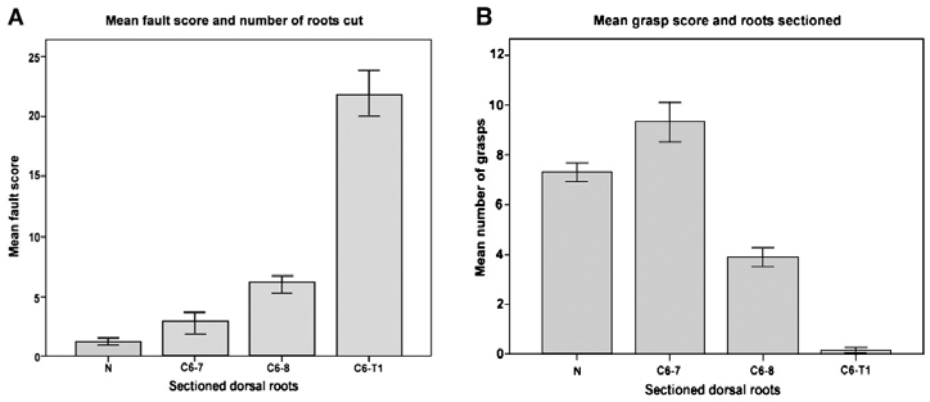

Figure 3-9. A and B, Summary graph of mean fault and grasp scores for normal controls (N), and after 2 (C6 & C7), 3 (C6-8) and 4 (C6-T1) roots were lesioned. The graph shows the marked increase in fault score as more roots are transected. 3-9B, shows the grasp score for the same group of rats showing the almost complete abolition of grasping transection of C6 to T1

3.5.7 Weekly climbing scores for C6 to T1 lesioned rats

Unlike for the three roots where there was a decline in the mean fault score per run over time (half as many faults in wk 8 compared to wk 1), after lesioning of C6 to T1 lesioning there was no such decline and there was no difference in the mean fault score between the first and the last observation week (Fig 3-10) in comparison to the falling fault score with time after C6-8 root lesions (Fig 3-8B).

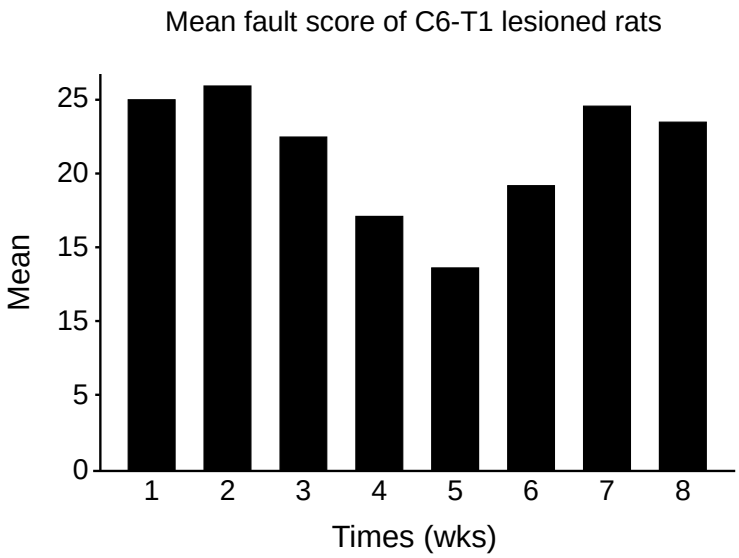

Figure 3-10. Graphs showing the mean fault scores after C6 to T1 lesions over time in rats with C6 to C8 roots lesions over 8 weeks observation

Table 3-4. Summary of fault and grasp climbing scores for all groups of rats

Roots Sectioned	Climbs Scored	Mean fault (95% CI)	Mean grasp (95% CI)
Unlesioned rats	102	1.0 *(0.9, 1.2)*	7.2 *(6.8, 7.6)*
C6, C7, C8 or T1 (single root lesion)	128	1.3 *(0.9, 1.6)*	8.1 *(7.6, 8.7)*
C6-7 or C7/8 or C8-T1 (2 roots)	128	2.7 *(1.8, 3.6)*	9.3 *(8.5, 10.1)*
C6-8 (3 roots)	256	7.1 *(6.3, 7.7)*	3.9 *(3.5, 4.3)*
C5-C8 (4 roots)	144	8.9 (5.35, 10.3)	3.9 (3.2, 4.6)
C6-T1 (4 roots)	288	21.9 *(20.0, 23.8)*	0.1 *(0.05, 0.3)*

3.5.8 Correlation of types of faults and number of roots transected

At attempt was made to examine the distribution of the type of faults according to number of roots lesioned expecting the severe faults to be more common in increased number of roots lesioned. In the C6-T1 root lesioned rats type 3 fault score was the commonest fault type followed by the minor fault score of type 1 (Fig 3-11). Although I expected to observe more minor fault score after two roots transection the mean fault scores of types 1, 2 and 3 roots were almost evenly distributed. Because the distribution of fault however did not appear to

correlate with the number of roots transected, further sub analysis of the types of faults was not carried out for all rats.

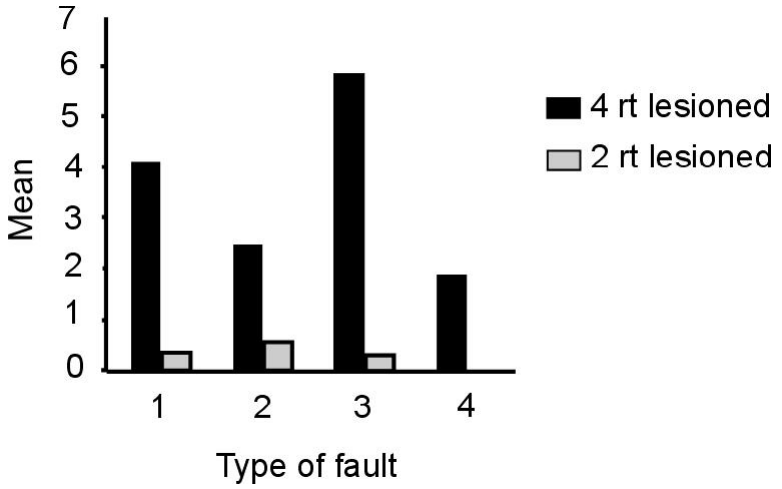

Figure 3-11. Mean score of types of fault per metre of climbing in 2 and 4 root transected rats

3.6 Histology

The histological preparation of the intact dorsal root entry zone showed dome like CNS astrocytic tissue projection that extends into the peripheral nerve segment in the PNS (Figs 3-12A and B). The transection of the dorsal roots was carried out as close to the spinal cord junction as possible to ensure that the CNS tissue at the root entry zone is lesioned. The lesion line is at the CNS/PNS transition zone as demonstrated by the white line in Fig 3-12A.

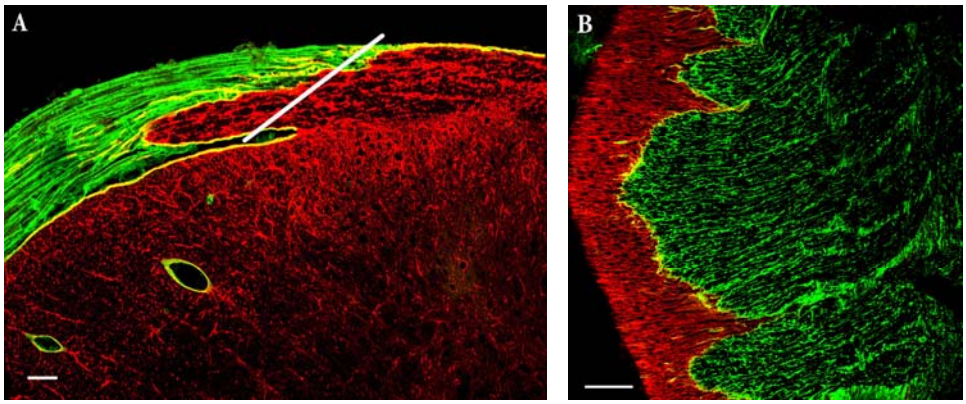

Figure 3-12. Double stained sections of intact cervical dorsal roots; cross-section (A) and horizontal (B) slides with double staining for CNS GFAP (stained red in A and green in B) and PNS NF (stained green in A and red in B) showing the dome like projections of the astrocytic processes into the laminin dominated PNS environment. The white line on A indicates the line of transection of the dorsal roots. Scale bar 50µm

The spinal cords of randomly selected lesioned rats were processed for immunohistochemistry to examine the extent of the transection injury of the roots. Staining of continuous series of adjacent histological sections showed the peripheral stumps of the roots to be completely separated from the spinal cord by an area of around 50-100µm which became infiltrated by macrophages and other inflammatory cellular elements. The slides

showed that after transection injury the astrocytic surface of the spinal cord loses the dome like structures and becomes sealed off (Fig 3-13).

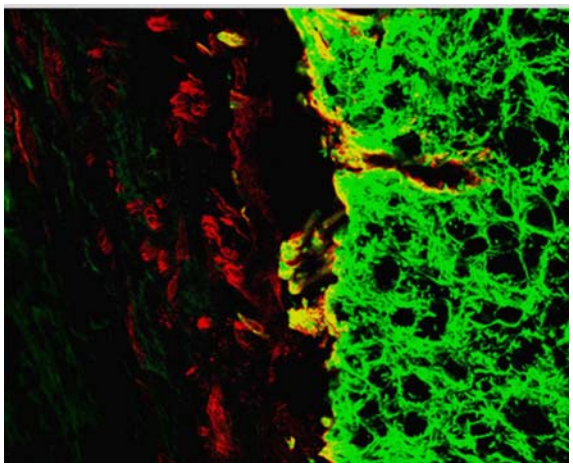

Figure 3-13. Confocal imagine showing the lesion site between the spinal cord (GFAP labelled green) and the cut end of the dorsal root (laminin stained red).

3.6.1 Effect of unintentional sparing of nerve fibres after in C6 to T1 lesioning

Three rats after 4-root lesions were unexpectedly able to maintain a high level of distal forepaw grasping function achieving a mean grasp score of 8 and a mean fault score of 6 per climb. The mean fault score of these rats was at least three folds lower than was expected and the grasp score was on par with that of unlesioned rats. Careful histological processing showed that these rats had a degree of sparing of up to around 15% of the normal dorsal root fibres in the T1 dorsal roots.

The importance of T1 dorsal root function in climbing was underscored by this result. T1 supplies the distal digits and thus sparing of even a small proportion of the fibres can be responsible for significant functional retention. This means even restoration of small proportion of fibres may restore function. This observation was in line with the functional

outcome of rats with C5 to C8 lesioning sparing T1 nerve roots. These C5-8 root lesioned rats also had a good degree of distal paw function.

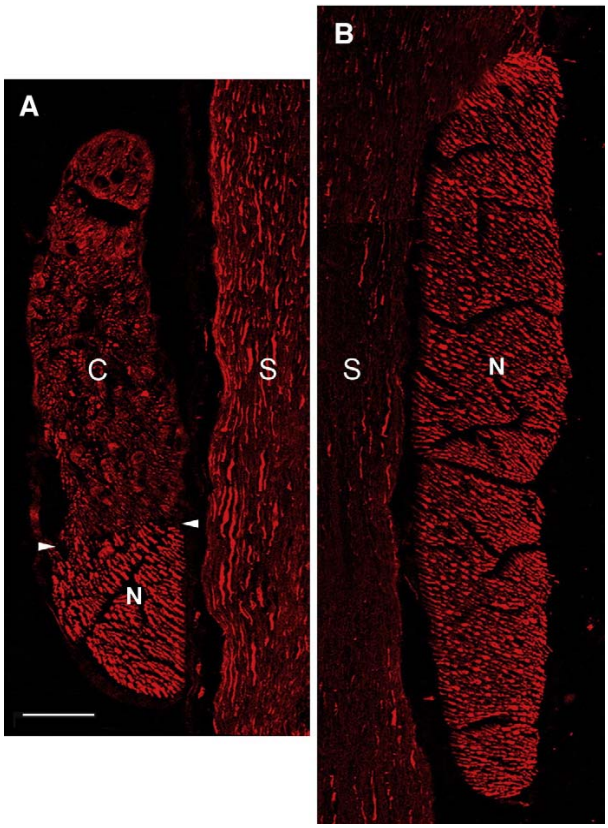

Figure 3-14 A and B. A) A horizontal section through the partially cut T1 dorsal root ventral to its entry point into the spinal cord (S). The caudal part of the nerve root (N) has around 15% of normally arranged, spared fibres comparable to the intact side (B). This compact region of normal fibres is demarcated by a sharp line (arrowheads; representing the plane of section of the edge of the knife blade) from the rostral part of the root, which contains fine, disorganised neurofilament positive material (C); this probably includes fine, abortively regenerating axons peripheral to the cut, and/or residual degenerating debris from die back of the cut axons. B) Contralateral intact T1 dorsal root (N) at a comparable level to A. Neurofilament immunostaining, survival time, 8 weeks. Scale bar for both A and B, 50μm.

3.6.2 Self harm or autotomy

In 12% of all rats used in the experiment, signs of self harm or autotomy developed affecting the ipsilateral forepaw. Autotomy developed at any time from 2 weeks after surgery onwards without prior warning. The onset of this self induced injury was encountered as late as 8 weeks post surgery or as early as the first week post surgery. As soon as any signs of injury appeared in the paws, which ranged from superficial skin abrasion to loss of the entire digits, the experiment were immediately terminated. While only 10% of rats with C6-T1 roots interventions showed evidence of self harm, 15% of 3 root lesioned rats showed signs of autotomy. The overall autotomy rate occurred more in the OEC transplanted rats than those who did not receive the cells at a ratio of 3:1. However, it was noteworthy the highest rate of autotomy occurred in the C5 to C8 lesioned rats, which is the group that did not receive OEC transplants. The reason why sparing T1 nerve root triples the rate of autotomy even in the absence of OEC transplants remains unclear.

Partial denervation in fact may be more a predisposing factor than presence of pain. Autotomy in the rats may imply that OEC transplantation restores propioceptive sensory function restoring coordinated muscle movement but may not necessarily restore cutaneous sensory function.

The possibility of transplanting OECs somehow induced autotomy, which is of course a major cause for concern clinically. The idea that neurological improvement after transplanting OECs would be accompanied by severe pain is not very attractive proposition for patients. There is still controversy about the cause of autotomy and some reports suggest that in fact complete denervation alone is sufficient to trigger it. Recent case-report of a cognitively normal man

with deafferentation injury self-inflicted injury to the exclusively denervated areas of his thumb to the extent he lost an entire thumb without having any pre-existing pain. [Frost *et al.* 2008;Kachramanoglou, Li, Andrews, East, Carlstedt, Raisman, and Choi 2011].

There are very little predictive factors identified as trigger factors for autotomy. The self inflicted injury occurred when 2 or more roots were lesioned. The number of roots transected did not correlate with onset of autotomy, in fact more autotomy was observed after 3 roots were transected than when 4 roots were cut. It is difficult to conclude from the above results that OECs induce autotomy; lesioning of C5-8 alone without transplanting OECs caused the highest ratio of autotomy.

3.7 Discussion

The scoring system developed for quantifying a primary sensory induced injury in a climbing apparatus has been presented and validated. The complete unilateral deaffrentation of 4 adjacent dorsal roots from C6 to T1 leads to a marked disability abolishing almost completely the grasping function of the forepaw on the operated side during climbing [Ibrahim *et al.* 2009a]. The disability that develops after C6 to T1 lesioning was immediate and more importantly the effect was permanent with no improvement in paw function observed over the test period, indicating that sprouting of adjacent intact roots [Liu and Chambers 1958] was not able to rescue this function. Lesions of 3 or 2 dorsal roots also resulted in climbing deficit but to a much lesser degree while rats with lesions of only single dorsal root hardly showed any deficit in locating or grasping the grid bars.

Upper brachial plexus lesions of C5-8 lesions failed to induce the same level of the disability. This observation underscored the importance of the function of the distal T1 dermatome in the skilled fine hand function. This lack of recovery in climbing function after induced C6 to T1 four root lesions makes this a suitable behavioural animal model in which to assess the efficacy of olfactory ensheathing cells which have been reported to have properties that promote axonal regeneration. This model has an advantage of the relatively small size compared to a transverse lesion of the spinal cord.

Why does the selective lesioning of dorsal roots only, leaving the ventral motor roots intact to innervate the muscles fully, create a measurable climbing behavioural deficit? This climbing function impairment is in the face of preservation of the functionally important motor movements such as walking, manipulating food, grooming, presumably because of central

pattern generators [Barriere *et al.* 2008]. Firstly, the relative size of the of the rats' head and dorsal position of the eyes leaves the rat unable to directly see the grid while climbing on the frame. Secondly, the grasping action during climbing was observed to be initiated by physical contact of the paws with the bars which triggers a reflex flexion of the digits as has been recognised in humans [Lemon *et al.* 1995;Johansson 1998]. This is similar to the primitive human grasp reflex seen in infants and after neurological degenerative condition in adults which is triggered by sensory input [Seyffarth and Denny-Brown 1948].

A successful grasping action requires an intact sensorimotor system that incorporates both ascending sensory impulses of propioceptive joint position and tactile sensations; and descending motor pathways to enable co-ordination and manipulation of upper extremity [Johansson 1998;Sainburg, Poizner, and Ghez 1993;Sainburg *et al.* 1995;Seyffarth and Denny-Brown 1948]. The climbing faults occur when the paw either cannot detect the physical contact with the bar is made (scored as fault type 1) or when there is more significant impairment of all joint position sense that results in entirely missing the grid (scored as grade 4). In this respect lesioning of four adjacent dorsal roots of C5-8 while leaving the T1 root intact impaired the grasping function of the rats to a much less degree than when the C6 to T1 roots were transected.

Conversely, individual lesioning of single dorsal roots of between C5 to T1 does not cause any significant behavioural impairment. In the rat, as in humans, there is overlap of the roots with a dermatomes being supplied by more than one root segment which compensated for the loss of a single root.

In the rats with unintended incomplete lesions, which showed good climbing performance, histological analysis showed there was sparing of around 15% of the T1 root. The fact that a relatively small number of fibres can mediate useful function is an encouraging indication that even partial anatomical repair may bring significant functional benefit.

In conclusion, lesioning four dorsal brachial plexus roots must include T1 to result in loss of grasping ability of the ipsilateral paw. The loss of grasping after lesioning is a clear indicator of complete 4 root lesions and provides a reliable benchmark which can be used to assess the efficacy of transplanting OECs in restoring climbing function.

CHAPTER FOUR

4 CHAPTER 4: RESTORATION OF GRASP FUNCTION BY TRANSPLANTATION OF OLFACTORY ENSHEATHING CELLS INTO SEVERED BRACHIAL PLEXUS DORSAL ROOTS

(Restoration of hand function in a rat model of repair of brachial plexus injury [Ibrahim *et al.* 2009b]

4.1 Summary

The transection of four dorsal roots (C6-T1) permanently abolished a complex, goal-directed fore-paw grasping in the rat (Chapter 3). In this chapter, I transplanted OECs into the C6-T1 transection behavioural model and assessed the paw function in the climbing frame.

A total of 85 rats underwent the following experiments randomly divided into three groups: (1) unlesioned control group (n=19), (2) lesioned control group (n=20) and (3) lesioned and OEC transplanted rats (n=35). In a separate group of rats, (n=11) the dorsal root ganglia were injected with an anterograde axonal tracer biotinylated dextran amine (BDA) to label the DR axons. Of these 11, 7 had received OEC transplantation to identify regenerating axons. The BDA injected group of rats did not have behavioural studies carried out.

The rats were allowed to climb a one metre grid while the total number of successful grasps and the total faults were scored. Rats in group 1 (unlesioned control group) had a mean grasp score of 7.1 (± 0.2) per climb. Of the 20 lesioned control rats in group 2 (lesioned control), 16 completely lost the ability to grasp the bars and four had a mean score of 0.2 (± 0.08). Of 35 rats that received transplanted OECs in group 3 (lesioned and transplanted with OEC), grasping in 25 rats (71%; the 'responders') returned 2 to 3 weeks after transplantation

achieving a mean grasp score of 2.9 (± 0.22), the remaining ten (29%; the 'non-responders') failed to grasp.

In immunohistochemistry study, I observed that the green fluorescent protein (GFP) labelled transplanted OECs were retained at the lesion sites throughout the 8-12 weeks after transplantation. The transplanted cells created an intense reaction between the cut end of the dorsal root and the spinal cord forming long interdigitation of astrocytic processes with the PNS tissue. Anterograde BDA axonal tracing confirmed that regenerating fibres originating from the DRGs were able to enter the spinal cord traversing the CNS/PNS bridge like tissue arborising within the spinal cord.

4.2 Introduction

Skilled hand function in man is crucial to independence and thus when lost, for instance as a result of injury to the brachial plexus roots, has a devastating personal outcome. Contrary to the commonly held belief, a survey of people with cervical spine injury revealed that restoration of hand and arm function was prioritised highest, even above the ability to walk again [Simpson *et al.* 2012;French *et al.* 2010]. Significant loss of upper limb function can result not only from a transverse or axial cervical spine cord injury but also from a focal avulsion injury of the intra-dural central segment of the brachial plexus nerve roots.

In the last chapter I described a model by inducing lesions of the rat brachial plexus dorsal roots of C6 to T1 which was found to cause a permanent loss of paw grasping function. The irreversible loss of function after avulsion injury both in the clinical and experimental paradigms is due to the lack of regeneration of the severed dorsal roots across the PNS/CNS interface [Perkins *et al.* 1980;Carlstedt 1997].

There are many factors cited in the literature for the failure of axonal regeneration in the CNS and in the dorsal root injury model. One of the main barriers to axonal repair reported is the glial scar that develops at the injury site of CNS tissue [Anders and Hurlock 1996;Asher *et al.* 2001] [Bradbury and Carter 2010]. Intense gliosis also develops after dorsal root entry zone injury at the interface between the CNS and PNS in line with other SCI injuries [Fraher 1999;Stensaas *et al.* 1979;McPhail *et al.* 2005;Raisman 1985;Doucette 1984]. There are however cells in the olfactory mucosa and bulb that even in the adult, can traverse the PNS/CNS overcoming the inhibitory forces by extending axons that arise in the mucosa (PNS) to reach the olfactory bulb (CNS) [Ramón-Cueto and Nieto-Sampedro 1994;Li, Carlstedt,

Berthold, and Raisman 2004;Muneton-Gomez and et al 2003;Navarro, Valero, Gudino, Fores, Rodriguez, Verdu, Pascual, Cuadras, and Nieto-Sampedro 1999]. Thus there is obvious merit in the idea of transplanting OECs along with their unique properties into the dorsal root injury paradigm to support regenerating axons of the DRGs in re-entering the spinal cord.

Various groups have tested the reparative properties of OECs in the dorsal root model and while more have reported the cells promoted regeneration [Li, Carlstedt, Berthold, and Raisman 2004;Navarro, Valero, Gudino, Fores, Rodriguez, Verdu, Pascual, Cuadras, and Nieto-Sampedro 1999;Ramón-Cueto and Nieto-Sampedro 1994;Lu, Féron, Ho, Mackay-Sim, and Waite 2001], a few have failed to find evidence regeneration was induced by these cells [Gomez, Averill, King, Yang, Perez, Chacon, Ward, Nieto-Sampedro, Priestley, and Taylor 2003;Riddell, Enriquez-Denton, Toft, Fairless, and Barnett 2004;Steward, Sharp, Selvan, Hadden, Hofstadter, Au, and Roskams 2006;Ramer *et al.* 2004a]. In our own laboratory [Li, Carlstedt, Berthold, and Raisman 2004] we observed that transplantation of OECs, embedded within their own matrix, in a single lumbar dorsal root lesion induced regenerating axons to re-enter the spinal cord crossing the PNS/CNS interface at the dorsal root entry zone.

Similar beneficial reparative effects of these cells have also been reported in various spinal cord injury models [Li, Field, and Raisman 1997;Li *et al.* 1998;Ruitenberg, Levison, Lee, Verhaagen, Harvey, and Plant 2005;Cao *et al.* 2004;Guest *et al.* 2008;Kubasak *et al.* 2008;Andrews and Stelzner 2004;Polentes *et al.* 2004;Ma *et al.* 2010;Wu *et al.* 2008;Plant *et al.* 2003;Munoz-Quiles *et al.* 2009;Ramón-Cueto, Cordero, Santos-Benito, and Avila 2000].

The rat model developed (chapter 3) and previously described [Ibrahim, Raisman, and Li 2009a] is well suited to be used as a benchmark in which to test the efficacy of OECs in restoring sensory functional recovery.

4.3 Materials and methods

A total of 85 adult female Albino Swiss (AS) rats were used for this experiment. Nineteen rats were observed as normal controls with another 20 rats undergoing surgery to transect C6-T1 roots without OECs and a group of 35 rats that had transection as well as immediate transplantation of OECs.

The olfactory ensheathing cells were harvested from adult olfactory bulbs of inbred female rats. The cultures were monitored until confluent reaching around 1.5 x 10^6 cells / 35mm dish at around 14-16 days, at which time the cellular mixture was composed of 50% OECs accompanied by 50% olfactory nerve fibroblasts (ONFs). The ONFs contributed to the formation of the endogenous matrix within which the OECs were embedded ready for transplantation. Prior to transplanting, the OECs were transfected with a lentiviral vector that expresses green fluorescent protein (GFP) that allows histological identification of the transplanted cells in preparations (chapter 2).

4.3.1 Behavioural assessment

All behavioural climbing assessment and scoring was carried out by an assessor who was unaware of the treatment status of individual rats. The 19 intact rats were assessed while climbing the frame as above. For the surgical groups (both OEC transplanted and lesioned

controls) climbing behaviour assessment started one week after surgery. Weekly for 8 weeks all rats were placed snout upwards on the lower bars of a 1 metre grid and allowed to climb freely to reach the horizontal platform at the top of the grid. All climbs were video recorded and analysed in slow motion. For each climb, the rats were awarded two scores- a grasp score when the rats were able to fully grasp the bar and a fault score graded according to severity.

4.3.2 Biotinylated Dextran Amine (BDA) tracing of regenerating axons

The injection of BDA tracer in the preganglionic segment of the dorsal roots was technically difficult because the preganglionic root segments in the cervico-thoracic roots are short and relatively inaccessible located within the spinal canal. For this thesis I developed a surgical method to expose the DRGs safely where I could inject the BDA directly.

A total of 11 rats had transection of dorsal roots with OEC transplants as treatment group and without transplants as controls (n=4 control rats and n=7 lesioned and transplanted rats). A volume of 1-2µl of BDA was air injected at a dose of 10% into the C7 and C8 DRGs of each rat.

To minimise the possibility of BDA contamination through adjacent roots, the two adjacent most roots (C5 and C6 above) and (T1 and T2 below) were transected. 14 days after injection the rats were perfused and fixed with 4% paraformaldehyde for histological processing and immunohistochemistry staining of the BDA.

4.3.3 Histology and immunochemistry

Full details of tissue processing for histology are described in Chapter 2. Briefly, the rats were perfused with PBS followed by 4% paraformaldehyde at the end of the experiments. The spinal cord with the attached dorsal roots were dissected out and after fixing the tissue for a minimum of 24 hours cryostat slides of continuous serial sections through C6 to T1 in both the longitudinal and cross-sectional planes were prepared.

4.4 Results 1: Behavioural outcome

4.4.1 Unlesioned control group (n=19)

A total of 19 intact control rats were assessed while climbing before undergoing transection surgery. In the 304 completed climbs the mean grasp score was 7.1 ± 0.2 (SEM) per metre with the mean fault score of 1.3 ± 0.2 per metre of climbing.

4.4.2 Lesioned untransplanted control rat group (n=20)

Out of 20 rats with C6 to T1 transection without transplants, 16 completely lost the ability to grasp the bars. The remaining four showed only occasional grasps, with a mean score of 0.2 ± 0.08. Overall, these 20 rats had a high incidence of faults, with a mean score of 20.8 ± 0.5.

4.4.3 Lesioned and OEC transplanted group of rats (n=35)

The behavioural assessment of these rats was also carried out by an investigator blinded to whether the rats were transplanted or not and to the climbing status. After transplanting OECs, 25 of the 35 rats (71%; the "responders") demonstrated a return of paw grasping which started from 2 to 3 weeks after transplant surgery and continued throughout the observation period of 8 weeks (Fig 4-1). The mean grasp score for the 'responders' was 2.9 ± 0.22 (SEM) and a significantly lower fault score of 13.8 ± 0.06 (t-test $p<0.001$). This represented around 40% of the pre-lesion grasping function level. In general the rats missed the grid less and were able to grasp the bars more frequently while climbing. The difference of mean grasp scores of the group with OEC transplanted and the lesioned but not OEC treated rat groups was also statistically significant (t-test $p< 0.01$).

The remaining 10 rats (29%; the "non-responders"), however failed to perform more than two grasps (for the whole group) over the entire 8-week observation period. The non-responders had a mean fault score of 19.3 ± 0.4 (comparable score of the lesion alone rat group of 20.8 ± 0.5). The overall mean fault score for both responders and non responders of the rats with OEC transplants was 17.6 ± 0.2. This score was significantly different from the score of the lesioned rats without OEC transplants (mean fault 20.8 ± 0.5, $p<0.01$). The mean grasp score of 1 ± 0.09 per metre which was significantly different from the lesioned and untreated group (mean grasp 0.2 ± 0.08, $p<0.003$).

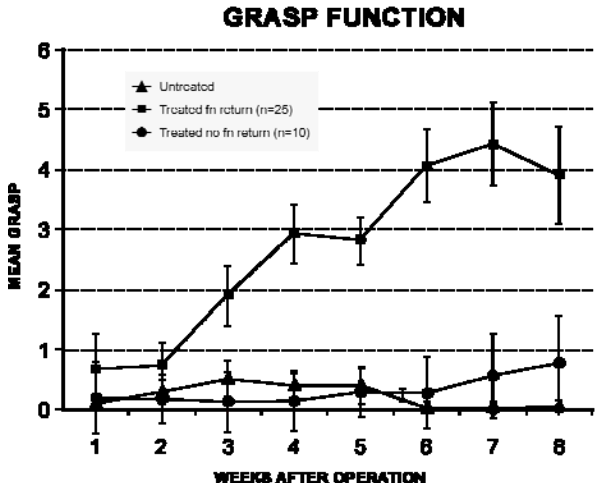

Figure 4-2. Recovery of grasping. Squares, transected rats with transplants ('responders' n=25) over the observation period. Triangles, non-responders (n=10); circles, rhizotomy without transplants (n=20). Group means (±SEM). Mean grasping for intact rats is 7.1.

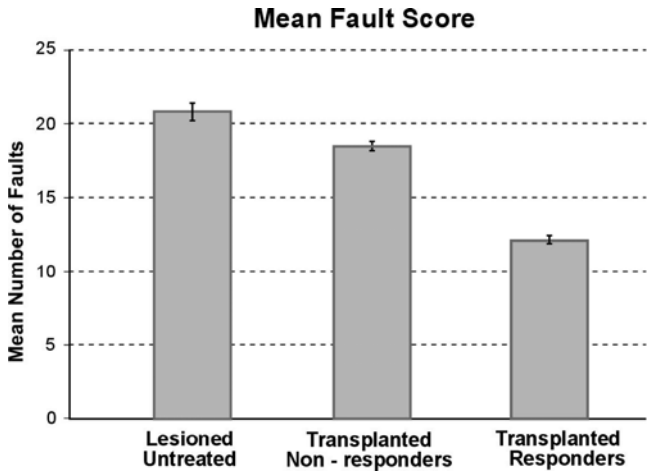

Figure 4-3. Mean Fault scores. Transected group of rats without transplants (n = 20), Non-responding transplanted rats (n = 10) and responding transplanted rats (n = 25).

Table 4-1. Summary of fault and grasps climbing scores of intact, lesioned but untreated and OEC treated rats

Lesion	Climbs scored	Mean grasp ±SE	Mean fault ±SE
Intact (n=19)	304	7.1 ± 0.2	1.3 ± 0.2
C6-T1 transection only (n=20)	262	0.2 ± 0.08	20.8 ± 0.5
C6-T1 transected and OEC transplanted (n=35)	354	1 ± 0.09	17.6 ± 0.2

4.5 Results 2: histology and immunohistochemistry

All rats were perfused using either BPS or PFA for storage and later processing for histology when necessary.

4.5.1 Immunohistochemistry

I have presented below selected immunohistochemistry stained histological slides. We found that the GFP labelled transplanted OECs were retained at the transplanted site between the dorsal roots and the spinal cord for the duration of the observation period in a viable state (Fig 4-3).

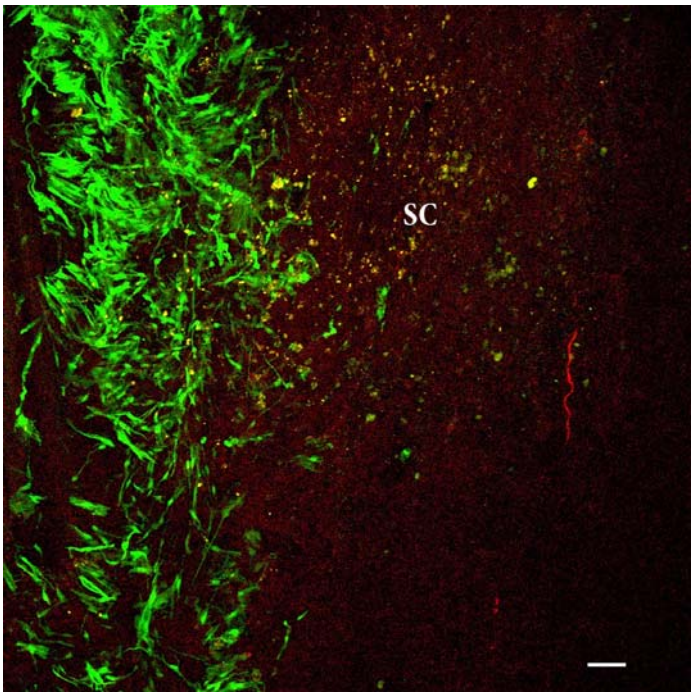

Figure 4-4. Green fluorescent protein (GFP) labelled OECs 8 weeks post transplant retained at the transplanted site. Dorsal roots (Left), spinal cord (SC);_scale bar = 100 µm

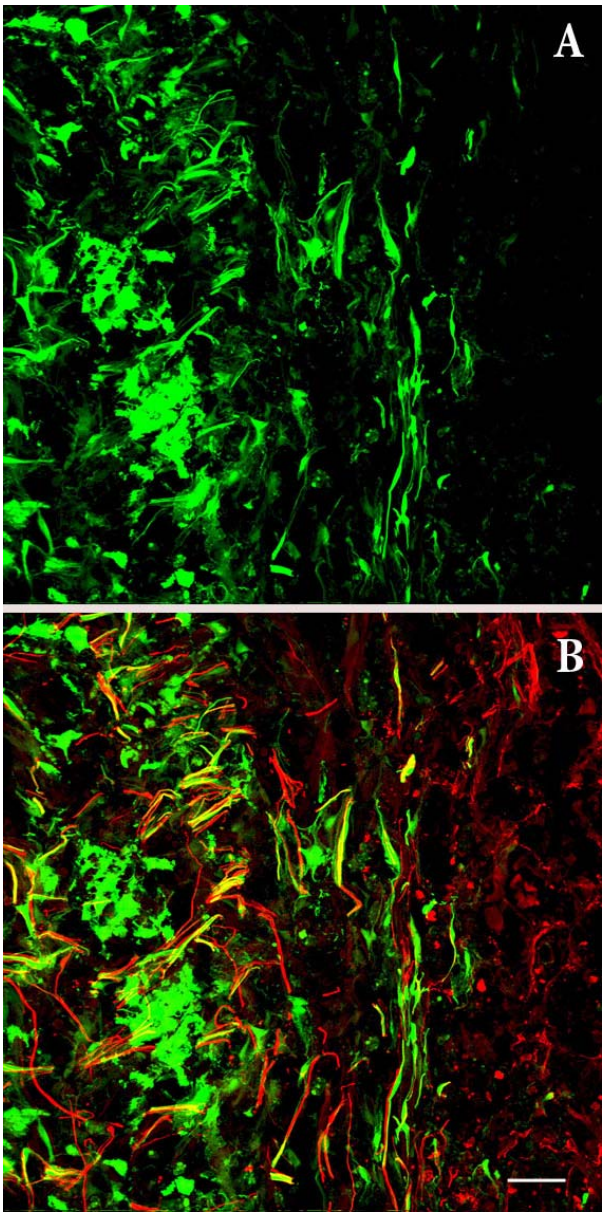

Figure 4-5 A and B. Transplanted OECs (GFAP, green) at the lesion site (A), and the interaction of the transplanted OECs and the presumed regenerating dorsal root axons stained with neurofilament antibody (red) can be seen (B). The horizontal and the vertical alignments of both OECs and neurofilament stained axons follow one another Scale bar 300 micron

In most cases, the majority of transplanted OECs remained at the lesion site. In some cases, a small amount of OECs migrated into the spinal cord and elongated with host axons that were labelled either by injection of BDA or immunostaining of NF.

The transplanted OECs induced powerful interactions between the cut end of the dorsal root and the spinal cord end forming long interdigitation of astrocytic processes with PNS axons (Fig 4-5). This was in stark contrast to the little or no interaction evident when the cut ends of the dorsal root ends are apposed to the spinal cords without the intervening layer of OECs (Fig 4-6).

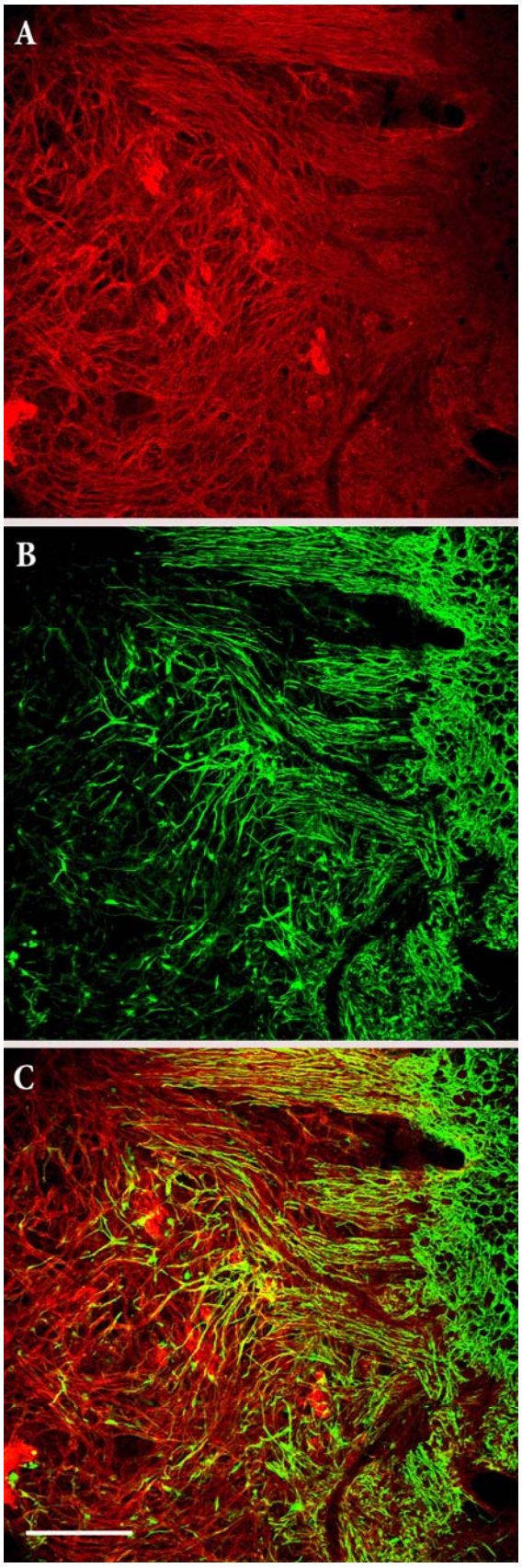
A
B
C

Figure 4-6. Double immunostaining of Laminin (red) and GFAP (green), to show the peripheral tissue stained red by Laminin (A) and the CNS tissue stained green with GFAP (B); C shows the extensive outgrowth of astrocytic processes (green stained by GFAP) that were induced by transplanted OECs and were seen to extend from the spinal cord peripherally for some distance intimately intertwining with the PNS forming a bridge to allow regenerating axons to cross the DREZ and re-enter the spinal cord. Scale bar = 200μm.

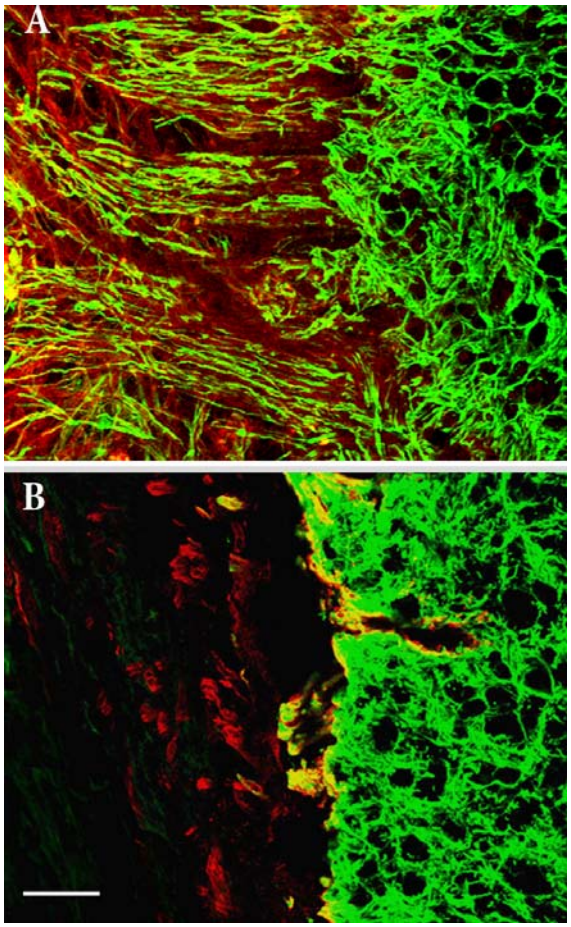

Figure 4-7 A and B. Showing the comparison of astrocytic reaction of the transplanted OEC and untransplanted rats. A) shows the transplanted OEC induced interdigitation of the GFAP positive astrocytic processes (GFAP green) and its absence in transected but not transplanted rats with the laminin positive peripheral nerve tissue stained red (B). Confocal imagine, double immunostaining of GFAP (green) and Laminin (red). Scale bar = 100μm.

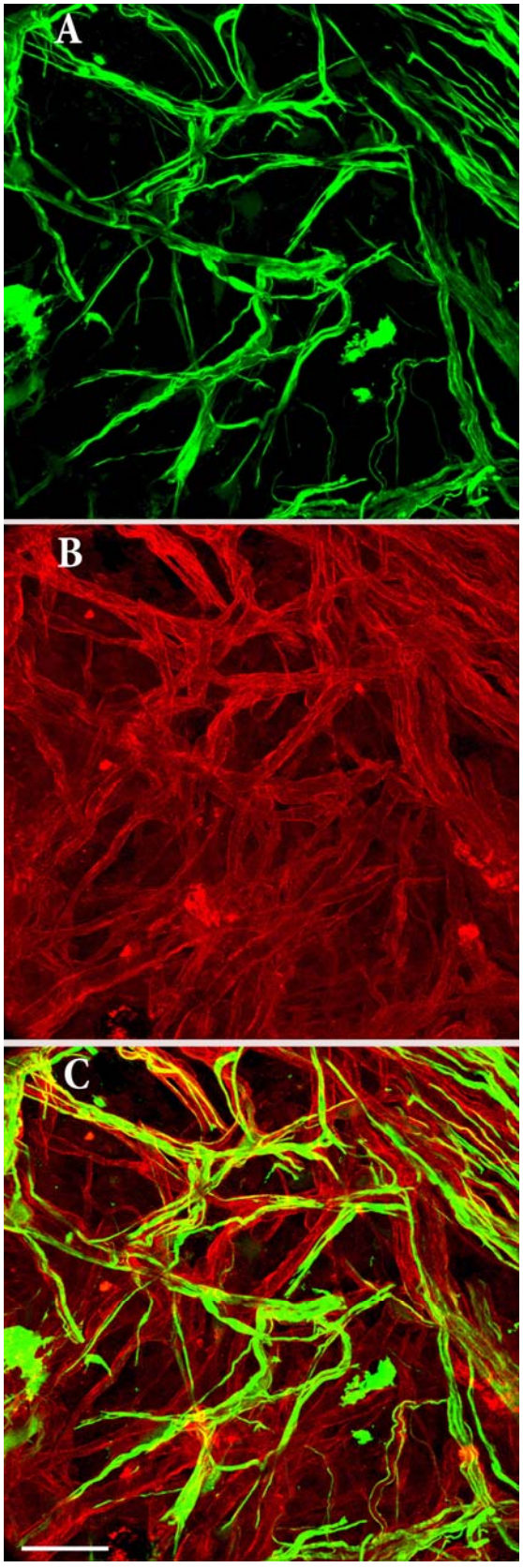
A
B
C

Figure 4-8. High power view to show the interaction of the CNS (GFAP positive, green) and the PNS (laminin positive, red) induced by transplanted OECs (8 week survival), Confocal imagine, double immunostaining of GFAP (green) and Laminin (red) Scale bar = 30 μm.

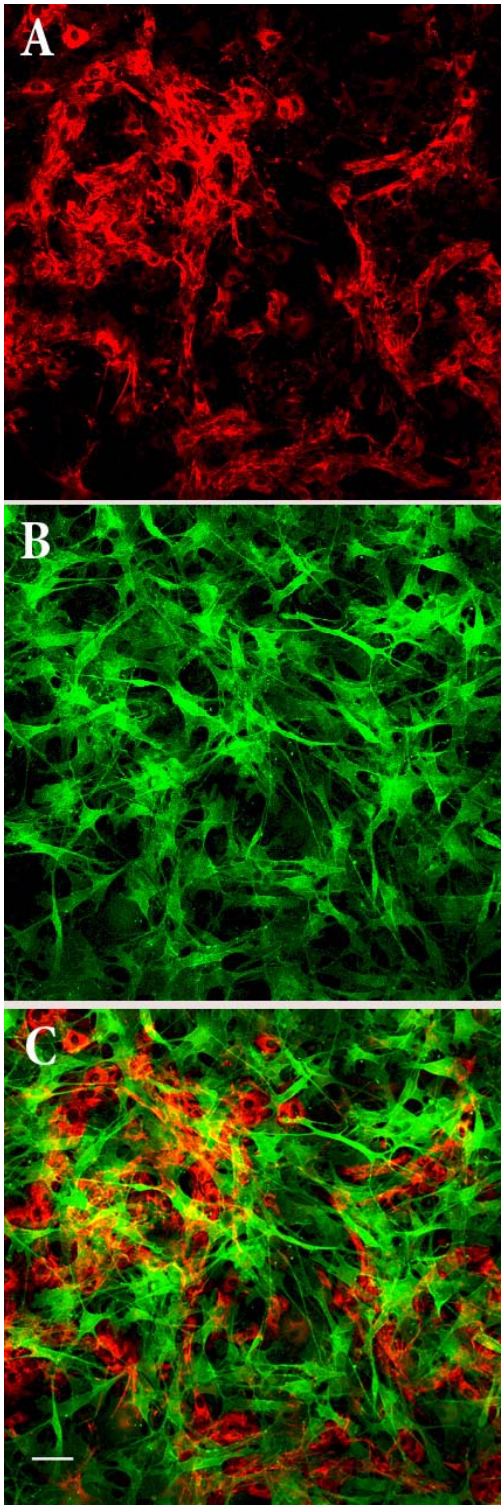
A
B
C

Figure 4-9. Confocal imagines showing a typical olfactory bulb cells after 15 days in culture. The double immunostaining shows fibronectin positive OECs (A, red) and the P75 positive ONFs meshwork (B, green) and the overlay imagine (C) depicting the matrix within which the OECs contained. The optimum culture composition for transplantation is usually reached around 50% OECs and 50% ONF at around 14-16 days in culture. Scale bar = 600μm.

4.5.2 Axonal tracing result

Seven rats underwent anterograde axonal tracing experiments where the DRGs were injected with BDA and 14 days later the rats were perfused. The BDA experiments showed important findings in this thesis. Firstly, I discovered that the labelled axons were indeed ensheathed by the OECs on a one to one basis (Fig 4-10). Secondly, the experiments served to demonstrate ensheathed axons crossing the normally hostile transition zone from dorsal root into the spinal cord. Thirdly, the axons were seen to be arborising within the dorsal column of the spinal cord. It is not clear whether these axons were projecting up or downwards. The overall aim of these experiments was to serve as a proof of principle of axons re-entering the spinal cord and to compliment the behavioural as well as the electrophysiological data. Thus attempts were not made to quantify the proportion of axons labelled by BDA.

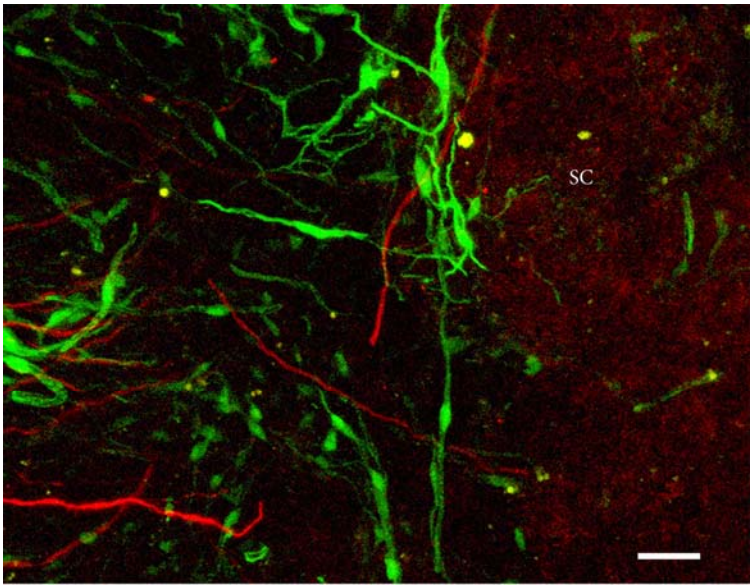

Figure 4-10. BDA labelled wide diameter of regenerating dorsal root axons (red) crossing into the spinal cord (SC) induced by transplanted OECs (green, left). Confocal imagine. Horizontal section, survival time 12 weeks, scale bar = 50µm.

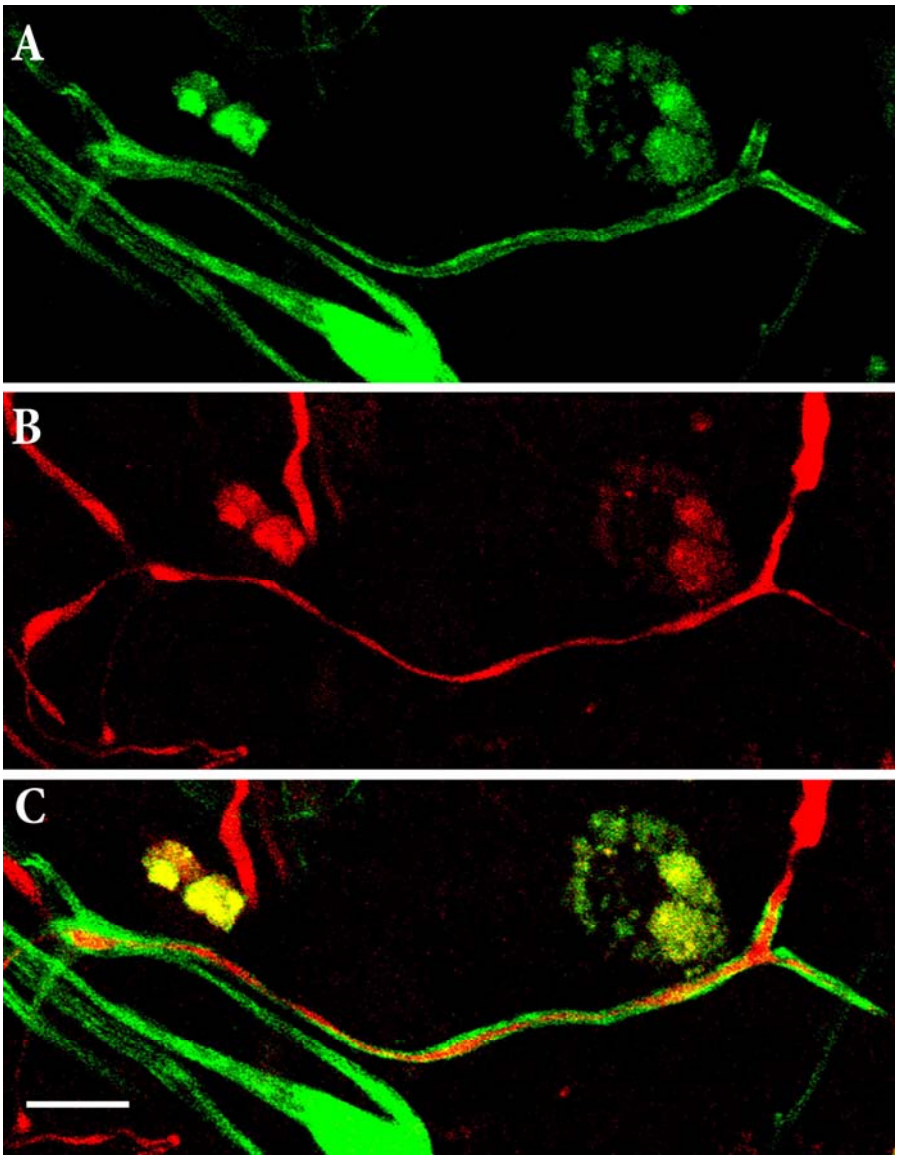

Figure 4-11. High power confocal imagines to show the GFP labelled elongated process of a transplanted OEC (green) with a central 'hollow' (clear) lumen (A). A single BDA labelled DR axon (red, B) and the combination of BDA labelled axon the hollow lumen now containing the re-growing ensheathed new fibre (C). Horizontal section, survival time 10 weeks. Scale bar = 20µm.

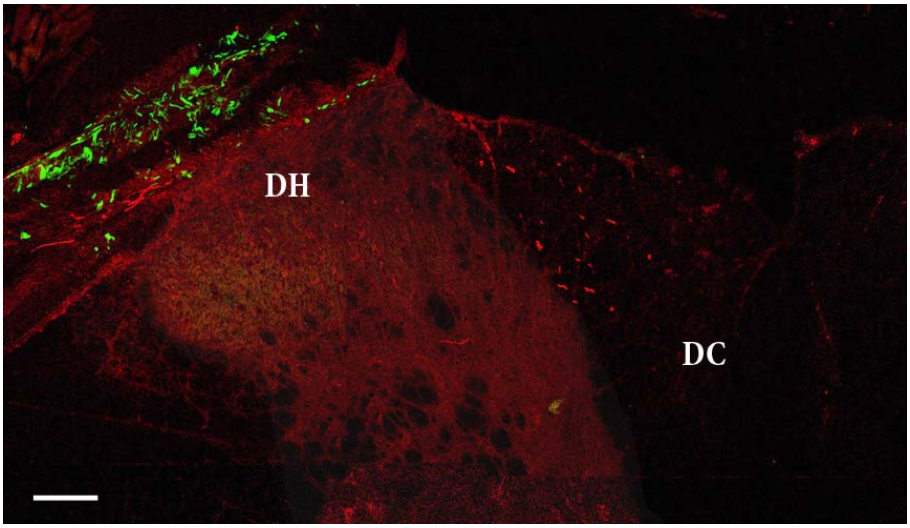

Figure 4-12. Montage of cross sections of a repaired dorsal root and the spinal cord. The GFP positive transplanted OECs (green) are apposed to the surface of the dorsal horn (DH). BDA labelled dorsal root axons and their fine arborisations could be seen in the root and the dorsal column (DC). Cross section slide, confocal imagine, survival time 12 wks, scale bar = 100μm.

4.6 Discussion

In this chapter I transplanted OECs into C6-T1 dorsal root lesions and subsequently carried out behavioural observation over 8 weeks period.

The data showed that OEC transplantation induced a return of grasping function in about two thirds of the rats. The recovery started 2-3 weeks after treatment and progressively continued over the observation period of 8 weeks. The extent to which recovery may continue beyond the observation period remains unknown. The recovery observed was not a full restoration of climbing function back to normal levels. However, the rats regained approximately 40% of the mean grasping action per metre of climb. This level of grasping was functionally significant in aiding the rat perform the task. In clinical terms, this may mean the difference between having the independence to feed one's self and from being fed by others.

The fact that rats that had lesions alone did not regain grasping supported the idea that the recovery was induced OECs. In addition, the lack of recovery in the lesioned alone group occurred even when performing the weekly climbing exercise as part of the behavioural study, showing that exercise alone was insufficient to restore function.

It is difficult to give a definitive explanation for the failure of 30% of the transplants to restore function. The failure for restoration of function could occur if 1) the transplanted graft of cells did not survive, 2) the cells survived but the axons failed to cross in sufficient numbers or 3) the axons made erroneous and non functional connections. The number of fibres needed for partial functional recovery may be relatively few. In rats where 15% of dorsal root fibres

inadvertently escaped lesioning there was preserved function of a mean of almost 4 grasps per metre. Thus, few axons that manage to cross the PNS/CNS boundary could have profound impact on function.

The immunohistochemistry data shown above makes it clear that OECs create an environment where astrocytic processes project into the peripheral segments of the dorsal root whereby the provide the bridging mechanism for axons to cross over (Figs 4-5 & 4-6). The unique ensheathing property of an elongated OEC can be seen in Fig 4-9B where single axons is shown wrapped around by OEC process. The OEC appear to be protecting and escorting the axons through the dorsal root into the cord. The BDA labelled regenerating axons were also demonstrated crossing into the spinal cord as well as arborising in the laminae of the dorsal horn (Fig 4-9).

Riddell et al who reported limited or no reparative properties of OECs that were transplantation in a suspension injected into the spinal cord commented that the lack of high concentration of cells at the transplant site if the cells were introduced in a suspension [Riddell, Enriquez-Denton, Toft, Fairless, and Barnett 2004]. In my study transplanted OECs were cultured for 14-16 days. By that time the culture has reached an optimum composition of OECs and ONFs mixture. The OECs are embedded in the extracellular matrix laid down by the ONFs which in our view is essential in reducing the risk of losing the cells during both the transplantation process and thereafter retaining in the lesion sites (Fig 4-3).

In the next chapter I conducted electrophysiological experiments to test the regenerating axons that crossing the PNS/CNS interface are not only anatomical but also functional and able to transmit electrical impulses to the spinal cord.

Currently, the clinical practice of surgical repair of brachial plexus avulsion by re-implantation of avulsed roots via a peripheral nerve graft provides a degree of recovery of synkinetic movement by the large proximal arm muscles, but there is persistent central pain, there is no sensory recovery, and useful hand function is not restored. The above finding suggests that modifying the above surgical practice to transplant OECs into the severed roots could restore beneficial sensory function in patients.

CHAPTER FIVE

5 CHAPTER 5: RESTORATION OF CORD DORSUM AND CUNEATE REPOSNSES AFTER TRANSPLANTING OECs INTO DORSAL BRACHIAL PLEXUS ROOT LESIONS

5.1 Summary

In 16 rats of lesioned and unlesioned rats electrophysiological experiments were carried out to test if regenerated axons could transmit action potentials from the periphery to the spinal cord. The median nerve was located and stimulated while recordings were made of incoming potentials at the cord dorsum (CD) and the cuneate nucleus in the medulla.

In unlesioned rats the cord dorsum potentials (CDPs) were found to be maximal at C6 or C7 DRs (amplitudes 440 – 1020μV, mean 761μV). In acutely transected rats the CDP was abolished and in two rats a mean CDP of 6μV persisted. The cuneate recordings in intact animals showed complex waveforms (amplitudes 125, 133μV, mean 129μV). After DR transections the mean cuneate potential was 3.5μV. In 4 rats with chronic lesions, all CDPs and cuneate potentials were completely abolished.

After transplanting OECs, 7 out of 8 rats showed negative going CDP responses, 6-8 weeks after surgery but the waves had a notably slower time course. All the 4 rats where cuneate recording was carried out showed synaptic responses, but again of longer latency and slower time course.

Of the 8 rats transplanted with OECs and tested elctrophysiologically, 6 recovered grasping function. 5 of the recovered 6 rats had clear evidence of negative CDP wave (amplitudes 23 – 174μV), with one showing no detectable CDP responses. The 2 rats that showed no functional recovery showed negative CDP responses with amplitudes of 6 and 37μV. Three of four transplanted rats that had functional recovery showed relatively large the cuneate evoked potential responses of 15, 27 and 54μV negative amplitudes. The fourth, which had not recovered grasping, showed a response of lower amplitude, 7μV.

5.2 Introduction

I have shown so far data to support that transplanting OECs into DR transection model restores grasping function as evaluated on the climbing test. In addition, anatomical evidence showed how OECs create enhanced interlocking of CNS and PNS tissue. Furthermore, regenerating axons were shown as they crossed into the spinal cord using an anterograde axonal tracer, BDA. However, though the above evidence of recovery of function following transplantation of OECs implies functional axonal reconnections have been re-established it does not provide direct evidence of synaptic and postsynaptic communications.

Hence, I have designed a collaborative experiment with Dr Peter Kirkwood, a senior and experienced spinal cord electrophysiologist, to investigate the synaptic and post synaptic function of OEC induced regenerating axons.

5.3 Materials and methods

Sixteen rats were divided into unlesioned and lesioned groups. Eight rats were underwent dorsal roots transection only; 4 acute and 4 chronic (6-8 weeks) lesions; and the other 8 rats had lesioning following by immediate transplantation of OECs. Climbing function was evaluated both before and after intervention. After 6 to 8 weeks post transplant surgery and weekly behavioural testing the rats underwent electrophysiological experiments by an experimenter who was unaware of the status of the rats with regards to both transplantation and climbing behavioural performance.

5.3.1 Electrophysiological experiment

The rats were terminally anaesthetised and physiological parameters including heart rate, blood pressure and body temperature (kept 37-38ºC) were continuous monitored. Extended laminectomies from C4 to T2 were performed in both the acute and chronic lesioned rats.

Extra care was taken in previously lesioned rats not to disturb the relationship between the surgical scar tissue and the underlying roots and spinal cord. The extradural components of the scar were trimmed as close to the spinal cord as possible to minimise the medial displacement of the recording electrodes. In animals where cuneate responses were recorded an occipital craniectomy and C1 laminectomy were carried out to expose the caudal medulla. The spinal cord was covered with saline soaked cotton wool balls to keep it moist.

The median nerve was exposed from the wrist to above the elbow with the forelimb positioned in supination and external rotation. The anconeus and biceps muscles were partially resected raising skin flaps to create a paraffin oil pool to protect the nerve for the duration of the experiments.

5.3.2 Recording of cord dorsum and cuneate evoked potentials

Stimulating platinum wire electrodes were placed on the median nerve to deliver a stimulus of 0.1ms duration repeated every second at 10x the threshold (10T) necessary to evoke a spinal cord potential. The integrity of conduction of median nerve action potentials was monitored via a further electrode placed more proximally a few millimetres above the elbow (reference wire on nearby muscle). The CDPs were recorded with a platinum wire electrodes placed on the surface of spinal cord at or close to the dorsal root entry zone, referenced on surrounding exposed neck muscle (Fig 2.5). Recording from the cuneate was obtained via electrodes placed on the left medulla 1.3 mm caudal and 0.7 mm lateral to obex, referenced on the paraspinal muscles.

Evoked potential signals were conventionally amplified (at 10000 gain) and band-pass filtered (10 Hz – 10 kHz). The recorded activity was averaged over 50-300 sweeps using a computer programme (Spike 2, CED, UK). Throughout this study negative polarity is recorded by a downward deflection.

5.3.3 Stimulus strength

A very similar relationship between stimulus strength and the synaptic responses is seen for both the cord dorsum and the cuneate responses in unlesioned and lesioned and treated group of animals, showing that a similar range of afferent fibres generated the responses in both groups (Fig 5-1).

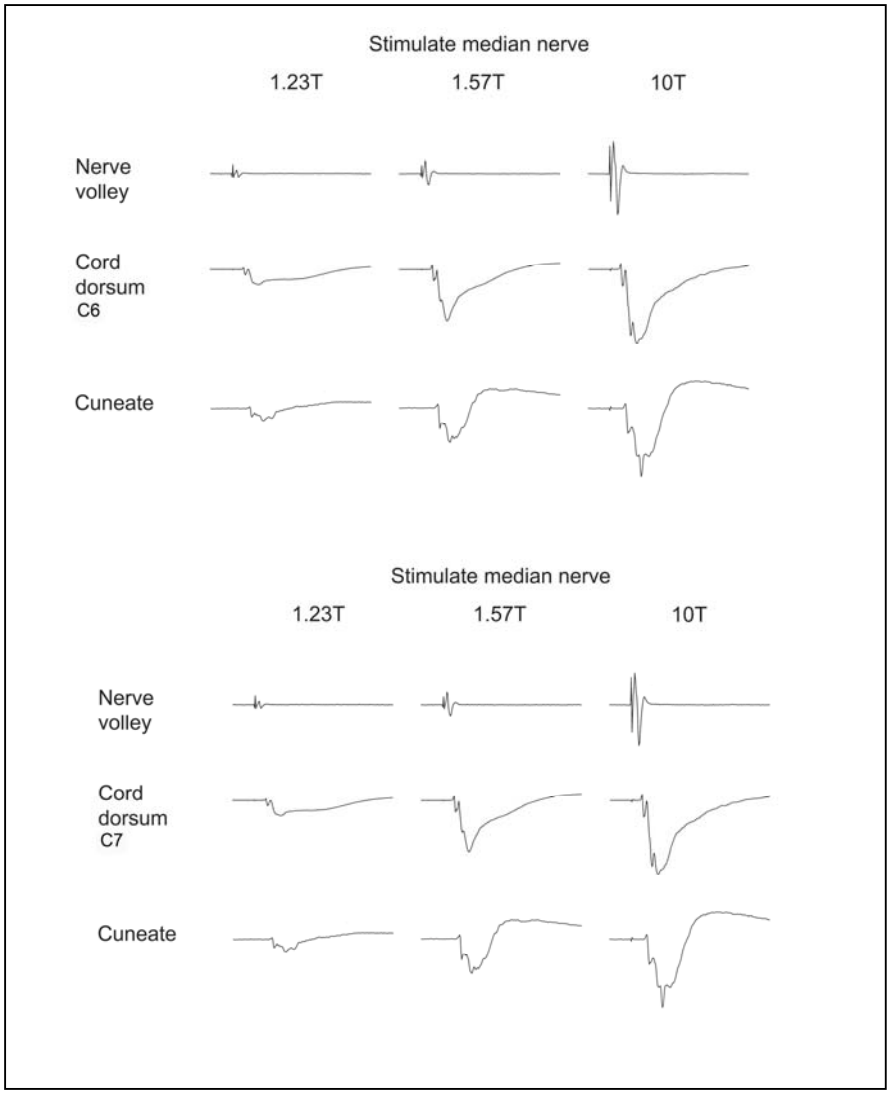

Figure 5- 1. Showing the relationship between stimulus strength and cord dorsum and cuneate responses at C6 (top) and C7 roots (bottom) in intact animals. The responses to increasing thresholds were similar in both CD and the cuneate of these intact animals.

5.4 Results

5.4.1 Normal Unlesioned Control Rats

Median nerve stimulation tests in 4 unlesioned animals with intact roots yielded cord dorsum potentials (Fig.5-2**A**) comprising a small initial positivity, followed by negativity representing a conducted volley plus synaptic activity in the dorsal horn [Willis 1980]. CDPs tests were carried out from C5 to T1, and were found to be maximal at C6 or C7 (amplitudes 440 – 1020 μV, mean 761 μV). Cuneate recordings made in 2 of the normal rats showed evoked potentials in both (Fig.5-2**B**) with complex waveforms (amplitudes 125 and 133 μV) that represent a combination of ascending volleys plus synaptic activity [Andersen *et al.* 1964;Sen and Møller 1991].

5.4.2 Rats with Acute lesions

After lesioning dorsal roots from C5 to T1 in 4 previously intact rats resulted in completely elimination of CDP negativity in two of these rats (Fig 5-2**C**), but showed a small persistent negativity in the other two (2μV and 22μV recorded at C6 level at the cuneate (Fig. 5-2**D**). In 2 rats where cuneate recording was carried out, revealed complete abolition of cuneate evoked potential in one, and a persistent small residual potential with amplitude of 7μV remained in the other. In the rats with the persistent CDP after transection the dorsal roots were reflected back and away from the spinal cord to detect any spared or undivided rootlets. Despite ensuring all roots and any visible rootlets from C5 to T1 were divided fully the small negative CDP remained.

5.4.3 Rats with Chronic lesions

In 4 rats with chronic C6 to T1 dorsal root transection without OEC transplants (at survival times of 6-8 weeks post surgery), none showed any CDP negativity (Fig 5-2E). In addition, in 3 rats where cuneate recordings were also carried out none showed detectable evoked potentials (Fig 5-2F). In all of the 8 rats with dorsal root transections, both acute and chronic, the initial positivity in the CDP waves remained without being followed by a subsequent negativity, representing remote recordings from the cut ends of the dorsal roots [LORENTE de N.O.R. 1947].

5.4.4 Rats with lesions and OEC Transplants

Recordings were made in 8 rats 6-8 weeks after lesioning of C6 to T1 roots followed by OEC transplants. Climbing test were performed weekly prior to electrophysiological experiments. The spared C5 dorsal root was transected acutely at the time of electrophysiological preparations. Recordings in all lesioned and transplanted animals showed initial positive CDP wave, followed, in 7 out of 8 rats, by a negative wave which had a notably slower time course compared to normal wave (Fig. 5-2 **I**). The negativity in CDP recordings were present from C5 - T1 and were maximal at C6 or C7. Recordings were made from the cuneate in 4 of the 8 lesioned and transplanted rats. All 4 rats had synaptic responses, but of longer latency and slower time course than normal (Fig. 5-2 **J**)

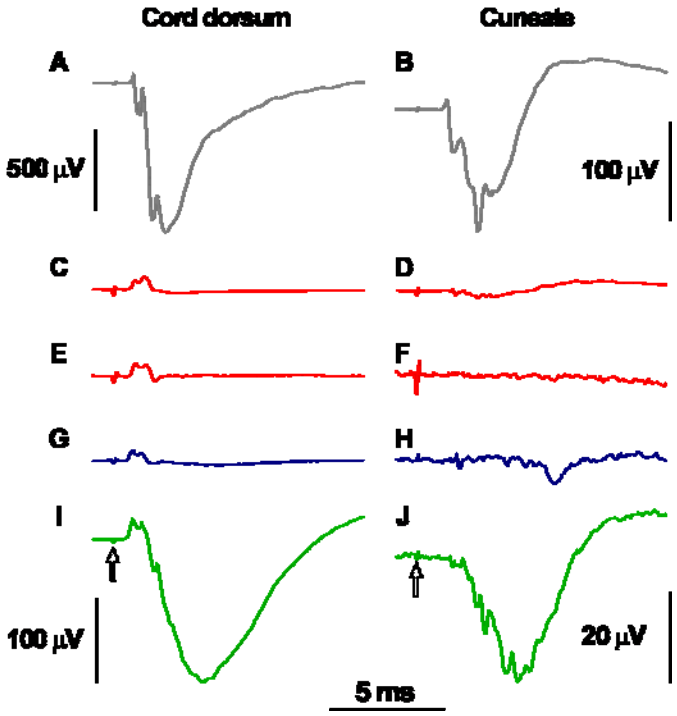

Figure 5- 2 A-J. Electrophysiological responses. Stimulation of the median nerve at 10 times nerve threshold (stimulation time indicated by vertical arrows). Simultaneously recorded responses from the cord dorsum of C6 or 7 (left column) and the cuneate nucleus (right column). (A and B) normal animal; (C and D) the same animal after acute section of DRs C4-T2; (E and F) a rat with chronic root transection without transplant; (G and H) a transplanted rat showing no recovery of grasping ('non-responder'); (I and J) a transplanted rat showing good restoration of grasping ability ('responders'). The upper calibration apply to A-D, the lower calibrations apply to E-J. Responses are displayed with negative down. Responses averaged (110-272 per trace).

5.4.5 Control for spared fibres

The possibility of leaving some fibres unlesioned is a concern when interpreting all various outcome measures. In order to study the effect of spared roots on CDP characteristics in one animal all C5 to T1 dorsal roots were transected except a small part (around 20%) of the cranial end of C7 was left intact. CDP was then recorded to quantify the response. The spared rootlet transmitted a negativity recorded at C6 of 78 µV in amplitude, which represented 8%

of the 950 µV recorded before the root was transected (Fig. 5-3). In contrast the 2 observed residual CDPs after rhizotomy above (2 µV and 22 µV) represented 0.4% and 2.3% respectively of the CDPs before root transection, and thus were much smaller than the 8% represented by the response in Fig. 5-2.

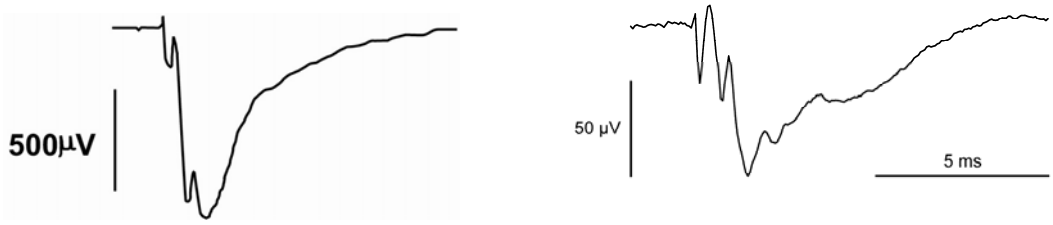

Figure 5- 3. CDP recorded at C6 from median nerve stimulation in a normal rat (left) and after sectioning dorsal roots C5 - T1 in previously intact rat except for C7 which was partially transected sparing about 20% of the rostral end of the root. The amplitude in the intact rat was 950 (left) compared to the markedly reduced amplitude of 78 µV in rat with partial sparing of C7 nerve root (right) which represented 8% of the normal before the roots were transected (Note the vertical amplitude calibration bar on the left is 10x that of the right).

5.4.6 Correlation of electrophysiological findings with behavioural outcome

The functional data indicated that none of the animals with chronic dorsal root lesions without OEC transplants showed recovery in left paw function. Likewise electrophysiological test found congruent outcome as no CDP responses were detected in these rats.

In 5 of 6 rats with OEC transplants there was recovery of paw grasping function which was in complete agreement with the electrophysiological tests in these rats which showed clear evidence of a negative CDP waves (amplitudes 23 – 174µV). One of the OEC transplanted rats

had no detectable CDP responses despite showing behavioural evidence of recovery. In 2 rats with OEC transplants in which grasping had not been restored showed negative CDP responses with amplitudes of 6 and 37µV.

Cuneate recordings in all the four lesioned and transplanted rats showed negative going responses. Three of four of these rats had recovery of grasping and relatively large the cuneate evoked potential responses of 15, 27 and 54µV negative amplitudes. The fourth, which had not recovered grasping, showed a response of lower amplitude, 7µV (Fig. 5-2H).

5.4.7 Possible underestimate of CDP amplitudes in chronically lesioned animals

The possible underestimate of CDP potentials was investigated in 2 of the lesioned rats with OEC transplants and showing substantial CDPs, by recording the CDPs at several points spaced by approximately 0.5 mm across the dorsal columns. The figure also shows that the amplitudes of the responses in the two animals varied linearly with distance, allowing an estimate of the amplitude at the normal site by extrapolation. This amplitude was about 25% greater than was recorded, i.e. the responses were probably underestimated by around 20%.

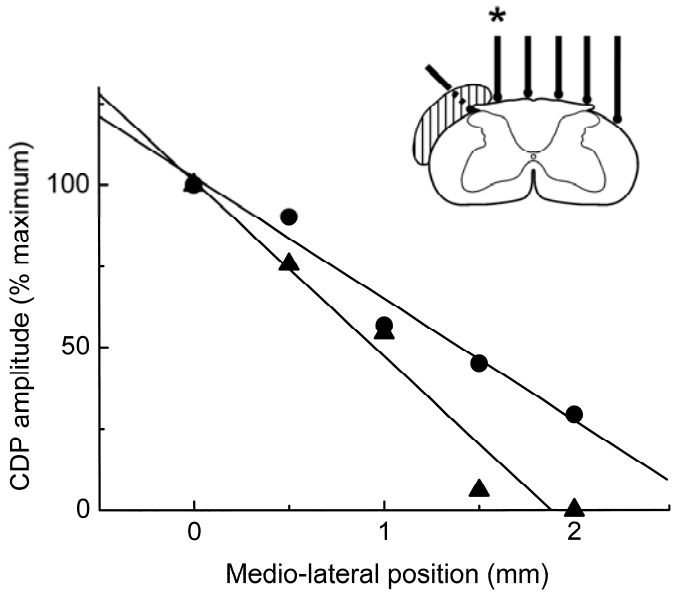

Figure 5- 4. Amplitudes of CDPs at C7 recorded at different points across the cord dorsum for two rats with OEC transplants. The insert sketch indicates the approximate positions of the electrodes (vertical lines) in one of these, with the non-resected, adherent connective tissue shown hatched. The maximum amplitude in each rat was recorded at the most lateral exposed part of the dorsal columns (asterisk in insert). The recording position used in the normal animals was assumed to be at -0.5 mm (angled electrode in the insert). The two lines are regression lines for each rat (different symbols for each). The intercepts on the abscissa give estimated CDP amplitudes at -0.5 mm, an average for the two animals of about 125%.

5.5 Discussion

I described here findings from sensitive electrophysiological experiments showing axonal regeneration promoted by OEC transplants make successful synaptic connections within the dorsal Colum and the cuneate nucleus in the spinal cord. These regenerated axons can successfully transmit nerve volleys and evoke synaptic field potentials in the cord dorsum and the cuneate nucleus. However, the tests show that the restored potential responses, vary from the normal.

The restored responses for both the cord dorsum and the cuneate showed long-duration positive waves following the initial negative waves, just as in the controls. These positive waves are believed to arise from primary afferent depolarization, responsible for presynaptic inhibition. The restored responses therefore represent connections that access complex, normal circuitry in both the spinal cord and the cuneate. It must also be taken into account that these long-duration responses also represent an underestimate: they are somewhat attenuated by the filter settings.

The CDP waveform in unlesioned rats consisted of initial stimulus artefact followed by a compound wave comprising of a sharp negative deflection with mean amplitude of 761µV, as well as short lived small double positive deflections of differing latency approximately 100µV lasting roughly 1ms. The negative polarity represents synaptic activation while the positive deflection is likely to represent incoming nerve volley from mixed calibre of fibres. The faster of the two positive deflections occurs immediately after onset of the negative wave.

Recordings from cuneate nucleus in intact animals showed even more complex waveform with mixed positivities superimposed on an overall negative polarity waveform of mean amplitude of 129μV. This represents the incoming nerve volley followed by the negativity triggered by the depolarisation of the cell membrane and synaptic activity.

Six of 8 lesioned rats that received OEC transplants showed substantial spinal cord and/or cuneate responses to stimulation of the median nerve. The latency to evoked potential peak in animals with OECs transplants increased by 2.5 times greater than normal (mean durations at half amplitude 4.71 ± 0.37 *vs* 1.88 ± 0.17 ms, SEM). This delay in time course could be due to the slower conduction velocity in regenerated small diameter fibres that are yet to fully re-myelinate. Another factor may be the disconnection of primary afferent receptor fields leads to vacant synapse permitting neighbouring intact dorsal root axons to sprout and occupy vacant terminals thus altering synaptic threshold potentials for regenerated axons that re-establish connections with their original terminals [Houle *et al.* 1996;Shortland and Fitzgerald 1991].

Alternatively, apoptosis of glial and neuronal cells in the dorsal horn after dorsal root lesions may reduce responses due to loss of target synapses [Chew *et al.* 2008;Polgar *et al.* 2005]. In addition, presynaptic inhibition may also play a role in attenuating synaptic response at the dorsal horn. Presynaptic inhibition regulates the distribution and size of receptive fields of spinal neurons by inhibiting the first central synapses of primary afferents located at the dorsal column [Rudomin and Schmidt 1999;Lidierth 2006]

Even if small number of regenerated axons find their target neurons and make functional synaptic connections then functional return could be possible. This was suggested by the observation that when there was a partial transection of the T1 dorsal root, the rats maintained a functionally useful level of climbing function. Keyvan-Fouladi et al had also previously observed a return of paw reaching function after repair of an estimated 1% of fibres in the corticospinal lesion [Keyvan-Fouladi *et al.* 2003].

5.5.1 Effect of surgical scar on amplitude of evoked potentials

The above recorded measurements of response in amplitude in transplanted rats probably underestimated the strength of synaptic input that they represent in comparison with normal animals. The OEC treated rats had a thick scar at the previous lesion and transplant site which displaced the placement of the recording electrodes away from the dorsal horn. To measure the effect of recording from a less than the optimal position two rats with OEC transplants had CDPs recorded at several points across the cord dorsum starting from the lateral edge of the scar continuing medially to the midline at 0.5mm intervals. The restricted CDP recording at the edge of the scar medial to the optimal position at the root entry zone in the transplanted rats underestimates the evoked potential amplitude by about 20% (Fig 5-4).

In this study the transection rather than crush dorsal root injury paradigm was used in order to cause maximum damage to the dorsal roots so there was little or no chance of axons escaping uninjured. Crush injuries are widely used in the dorsal root injury model however, there are reports suggesting some axons do survive such injuries [Wang, King, Ossipov, Rossomando, Vanderah, Harvey, Cariani, Frank, Sah, and Porreca 2008;Harvey, Lee, Qian,

Weinreb, and Frank 2009]. Previously, in Chapter 3, I showed that sparing of axons resulted in rats maintaining grasping function in our behavioural assessment.

In this study all rats with lesion alone that underwent behavioural assessment had profound loss of grasping function. Electrophysiological tests, 6 to 8 weeks after root transection and behavioural assessments of rats with lesion alone showed complete abolition of evoked field potentials both in the cord dorsum and the cuneate to median nerve stimulus in all four rats tested. This is a sensitive test that indicates no sprouting or regenerated axons were able to cross into the spinal cord in the absence of OEC transplants. The delayed testing is significant in that sufficient time had passed for any regenerating axons to cross into the spinal cord and form synaptic re-connections. The rats in this cohort had been undergoing weekly climbing tests indicating that exercise alone was inadequate at promoting regeneration or inducing sufficient sprouting to effect behavioural performance.

During acute transection of C5 to T1 dorsal roots in 4 rats, two had similar complete abolition of any evoked potentials in the spinal cord. Of the remaining two animals however, one had a persistent but small negative deflection in CDP but not in the cuneate where all responses were abolished. In the other animal there were small persistent negativities in both CDP and the cuneate raising the potential for presence of spared fibres.

5.5.2 Possibility of spared fibres

The possibility of having incomplete lesioning being responsible for the return of function needs to be considered. I opted for transecting rather than crushing the roots in order to minimise the possibility of fibres escaping the induced injury. In addition, extreme care was taken during the original lesioning surgery when the transected trunk of the dorsal root was reflected away from the DREZ to actively look for any small accessory rootlets that may still be intact.

The presence even of the small residual evoked potential response as suggested in the 2 acutely lesioned animals was a matter of concern for the interpretation of recovery of function. Hence we performed a control experiment in one animal where C5 to T1 dorsal root were fully sectioned except for C7 root, which was 80% transected. Prior to lesioning a CDP negativity of 950 μV in amplitude was recorded at C6 level. After partial transection, with around 20% of fibres left intact, the CDP response was found to be 78 μV in amplitude, which corresponds to a survival of 8% in the CDP response (Fig 5-3).

The observed small residual CDPs in the 2 animals were 2 μV and 22 μV which represented 0.4% and 2.3% respectively of the CDPs before roots transection, and thus were even smaller. Furthermore, the amplitudes of the CDPs in the lesioned rats with transplants were up to 8 times larger than even the largest of these residual CDPs, markedly augmented by OECs.

Crucially, none of the 4 chronically lesioned rats without OECs transplants showed any evoked responses. These were the more matched controls for the OEC transplanted rats and thus the

fact that the majority of those rats that received OECs grafts clearly showed electrophysiological responses is that much more reassuring. This result was obtained by an electrophysiologist unaware of the transplantation status of the above rats.

These results together with the functionally observed restoration of function strongly indicate that OECs guide, support and promote to re-enter the spinal cord and re-establish correct neuronal connections with appropriate synaptic communications. This was in concert with the anatomical finding of tracing BDA regenerated axons crossing the DREZ and showing them arborising in the dorsal horn and the dorsal column (Fig 4-11).

CHAPTER SIX

6 CHAPTER 6: GENERAL DISCUSSION

The profound paw handicap developed after four DR transections causes loss of sensory input of touch, pain, temperature, and propioception (joint and muscle position sense). The observed paw deficit was not due to motor paralysis, as the ventral roots remain intact, but rather due deafferentation injury.

Of the sensory modalities adversely affected by the deafferentation injury, the loss of cutaneous sensation that provides tactile feedback of physical contact with an object and the proprioceptive input that guides skilled hand muscle adjustments balancing the action of the agonist and antagonist muscles to exert the required force in order to complete a given task, such as climbing, are most relevant [Sainburg, Ghilardi, Poizner, and Ghez 1995]. Performing demanding physical activities such as, stepping on ladders or climbing up a grid relies on accurate input of proprioceptive information and assisted with visual clues when necessary.

When there is loss of proprioception in humans, patients are able to correct significant ataxia using vision to estimate depth and distance of the ground. In the above climbing apparatus our albino rats were disadvantaged by their poor vision and hampered even more as they would be unable to see the grid to compensate for inaccurate paw placement as the grid lies outside their line of sight of the dorsolaterally located eyes. This may account for why there was relatively minor disability when the rats were walking in the cage, grooming etc even after four root transection, but the deficit was augmented by a climbing task.

The recovery or regeneration of the avulsed axonal fibres is hampered by the same factors that hinder axonal regeneration in CNS injuries such as stroke or head injury. The permanence of the disability that develops after dorsal root lesioning permits this animal brachial plexus injury model to be used as bench mark to test putative regeneration promoting interventions such as OECs.

Glial scar develops at the site of CNS after dorsal roots injury, and is thought to create barriers to axonal regeneration [Caroni and Schwab 1989;Bradbury and Carter 2010;Fraher 1999;Asher, Morgenstern, Moon, and Fawcett 2001;Bradbury and Carter 2010;Fawcett and Asher 1999;Silver and Miller 2004;Anders and Hurlock 1996;McPhail, Plunet, Das, and Ramer 2005;Filbin 2003;Fabes *et al.* 2006;Yiu and He 2006;Low *et al.* 2008;Bradbury *et al.* 2002;Schwab 2004]

In an effort to induce regeneration, many have described different types of intervention such as, molecular agents [Wang, King, Ossipov, Rossomando, Vanderah, Harvey, Cariani, Frank, Sah, and Porreca 2008;Schwab 2004;Bradbury, Moon, Popat, King, Bennett, Patel, Fawcett, and McMahon 2002;Udina *et al.* 2008;Steinmetz, Horn, Tom, Miller, Busch, Nair, Silver, and Silver 2005;Ramer, Bishop, Dockery, Mobarak, O'Leary, Fraher, Priestley, and McMahon 2002] or applying cellular transplants [Kliot, Smith, Siegal, and Silver 1990;Ramón-Cueto and Nieto-Sampedro 1994;Montero-Menei *et al.* 1992;Li, Carlstedt, Berthold, and Raisman 2004;Li and Raisman 1997].

Of the different cellular transplants, OECs are amongst the leading candidates. OECs in their native environment function to support axons from the nasal mucosa to cross the important CNS/PNS barrier to reach the olfactory bulb in the CNS. Transplantation of OECs could induce repair of intraspinal tracts [Li, Field, and Raisman 1997;Bunge 2002] and DRs [Li, Field, and Raisman 1997;Ramón-Cueto and Nieto-Sampedro 1994;Toft *et al.* 2007;Pascual, Gudino-Cabrera, Insausti, and Nieto-Sampedro 2002] with fewer studies reporting contradicting results [Riddell, Enriquez-Denton, Toft, Fairless, and Barnett 2004;Gomez, Averill, King, Yang, Perez, Chacon, Ward, Nieto-Sampedro, Priestley, and Taylor 2003].

The source of OECs in most transplant studies, including in my thesis, is the olfactory bulb. A number of groups, including our own, are currently studying the properties of human OECs [Barnett *et al.* 2000;Choi *et al.* 2008;Bianco *et al.* 2004;Feron *et al.* 1998].

OECs can be obtained from human nasal mucosa biopsy which has obvious advantages of ease of surgical access and carries relatively minor side effects as compared to obtaining the cells from human olfactory bulb. Mackay-Sim and colleagues reported on an autologous OEC transplantation in patients with spinal cord injuries to be safe with no adverse symptoms or signs reported [Mackay-Sim *et al.* 2008].

In the brachial plexus dorsal roots lesioning model I transplanted cultured OECs and carried out behavioural, anatomical, and electrophysiological experiments showing OECs restore function and induce axonal regeneration of the severed central segment of dorsal roots. The transplanted GFP labelled OECs were found to survive up to the 12 days post transplant. Anatomical data showed that the transplanted OECs caused intense interaction between the

CNS and PNS cellular surfaces and created bridge like structures that connected the two surfaces.

It is likely that the endogenous matrix used in our laboratory allows for better retention of the graft at the site of the lesion. The OECs in culture remain confined within a matrix produced by the ONFs, a picture reminiscent of the way platelets become trapped by activated fibrinogen to form a blood clot (Fig 4-8). It is our conviction that the cellular composition transplanted and the method of cell transfer determines the concentration of cells available to interact with injured surfaces.

After transplanting OECs in the same four root lesion, the behavioural data showed that 70% of rats recovered grasping function which started 2-3 weeks after transplantation and soon became functionally and clinically significant, reaching half the level of normal grasping function per metre. Failure in recovery in 30% of the treated rats could be due to loss of the transplanted OECs, formations of non functional connections or inability to bridge the gap between the roots and the spinal cord. However the transplanted OECs consistently generated prominent astrocytic processes that migrated from the CNS into the PNS intimately interlocked with fibres of the DRs. Such outgrowth was absent in rats that had transections of the DR without transplanted OECs.

BDA labelled DR axons were traced from the DRG as they crossed the spinal cord through the mass of bridging tissue in the treated rats and were seen to project through the dorsal columns. The calibre of the axons detected was smaller than normal but this was not formally

quantified. Although contamination of BDA through adjacent segments of the injection site was controlled for by transecting 2 roots above and 2 below the injected level, I did not carry out histological processing of the adjacent spinal segments to show there was no detectable BDA. This and the fact that the number of BDA labelled axons crossing into the spinal cord were also not related to the behavioural function to demonstrate the axons were functional. Instead electrophysiological experiments were carried out to investigate the functional connection established by the regrowing axons.

The failure of untreated rats to recover and the fact that most of the treated rats regained function suggested that regenerated axons are delivering useful information that can be integrated in the afferent system to finely adjust final paw positioning. Behavioural evidence alone is insufficient to differentiate sprouting or regeneration induced functional recovery. Loss of paw function after lesioning of 3 dorsal roots or less was followed by spontaneous return of function [Ibrahim, Raisman, and Li 2009a]. Such spontaneous recovery is likely to be mediated through sprouting of adjacent intact axons. Extending the lesioning to four adjacent dorsal roots resulted in a stable behavioural disability which could not be overcome by sprouting alone. Transplanting OECs restored function after four DR lesions which I propose to be due to regeneration. However, it is conceivable that the observed functional recovery after OEC transplant is mediated by both axonal regeneration and by augmenting sprouting of uninjured axons. In this study I demonstrated axonal elongation of BDA labelled axons across the dorsal root entry zone which arborise in the dorsal columns. These axons originated at the BDA injected DRGs of the fully transected roots and elongated to reach the spinal cord. This elongation of the axons can be termed regeneration however further work is needed to provide evidence regenerating axons extending beyond the dorsal column to reach their target nucleus.

To confirm that the regenerated axons create functional connections, I carried out collaborative electrophysiological experiments where recording with made in the spinal cord and in the cuneate, more centrally. The median nerve was stimulated peripherally (it has primary sensory fibres to the four lesioned DRs) and the conducted potential volleys were recorded from the cord dorsum. Six of 8 lesioned rats that received OEC transplants showed substantial spinal cord and/or cuneate responses to stimulation of the median nerve but the latency increased by 2.5 times greater than normal (mean durations at half amplitude 4.71 ± 0.37 *vs* 1.88 ± 0.17 ms, SEM). In the chronic DR lesioned animals CDP was abolished by the lesioning. The amplitudes of the CDPs in the OEC treated rats were up to eight times larger than even the biggest residual CDP seen in untreated rats. This electrophysiological evidence of recovery of transmission showed that the synaptic connections were restored but the responses had a longer latency than expected. Together these observations indicate that sensory inputs received via the regenerated axons can be integrated to perform a complex, goal-directed behaviour.

However, correlation of the electrophysiological and behavioural observations raised interesting points. In the OEC treated group, 1 rat showed no detectable CDP responses despite showing behavioural recovery and 2 rats showed CDP responses without behavioural improvements, though the amplitudes were relatively small (6 and 37μV). There are significant individual anatomical variations in dermatomal innervations and potentially this may explain the unexpected results. It was however, extremely important that in the matched control group for treated rats, which were the chronically lesioned animals, and none showed any cord dorsum or cuneate electrical responses when tested up to 8 weeks following transections.

A common complicating factor for both these lesions is the possible microscopic exchange of function that could occur without having anatomical connections or anastomoses to account for it. Sprouting of adjacent intact roots into denervated area can also complicate matters further in the quest to exclude input from surviving fibres.

Once regenerating DR fibres are able to cross the spinal cord they first arborise in the dorsal laminae of the spinal cord grey matter before sending ascending and descending fibres in the dorsal columns. These axons could follow quite complex pathways including long distance elongation through myelinated tracts, to form functioning contacts with distant synaptic destinations. This was observed in the electrophysiological experiments where there were postsynaptic responses from the cuneate in which these fibres terminate.

In order to provide further supporting evidence that regenerated axons were indeed responsible for the restoration of the behavioural function as well as the electrophysiological responses re-lesioning of the dorsal roots could be attempted. However, during re-do surgery and histological processing it became apparent that at the lesion site a thick astroglial scar that was adherent to the cord and the DR had developed. Re-lesioning would involve transection of the dorsal root along with the adherent scar increasing the risk of damaging the causing cord or causing bleeding. The cause of any loss of function subsequent to re-lesioning could then be attributed to the associated injuries rather than the re-severing of regenerating axons. Furthermore, re-lesioning would also not address the argument of whether the return of function was due to regenerating axons or sprouting even if the re-lesioning was performed more distally in the DR leaving the astroglial scar intact.

6.1 Potential effects of abnormal connections

The observations on repair of sensory roots in the rat model, while functionally useful in the restoration of forepaw grasping, are still too preliminary to indicate to what extent the connections they re-establish deviate from the normal pattern. After root avulsion there are changes in the pattern of connections occur at all levels of the CNS in humans [Lundborg and Rosén 2007] and a significant loss of neurons in the target areas of the dorsal horn has also been reported [Chew, Leinster, Sakthithasan, Robson, Carlstedt, and Shortland 2008]. It is not clear to what extent the denervated central target sites may be reinnervated by adjacent surviving fibres. After clinical repair of peripheral nerve injuries touch stimuli are often wrongly localised [Lundborg and Rosén 2007] probably because regenerated axons fail to find their original target. In patients with root avulsion, where deafferentation pain is a prominent feature, it is reported that reconnection between the spinal cord and the affected arm has been of benefit in alleviating the pain [Berman *et al.* 1996]. This suggests that repair of avulsed root axons are more likely to alleviate than to provoke or exacerbate a pain state, although this remains a risk which must be balanced against the considerable benefits which would accrue from motor and sensory repair.

6.2 Clinical Prospects of transplanting OECs

The potential prospect of transplantation of OECs to restore function after BPI is enormously appealing for the following reasons:

1) Autologous mucosal OECs can be harvested, cultured and transplanted into the patient with injury confers massive advantages in avoiding lifelong immunosuppressive treatments that would be needed for allograft. 2) In comparison to SCI, BPI root lesions represent discreet injuries that have no known documented spontaneous recovery and are potentially bridgeable

with cell-embedded matrix. 3) The surgery to reimplant spinal roots is already established and carried out in specialised centres. However, a number of obstacles remain to be overcome before considering translating the above techniques in clinical brachial plexus injury.

6.3 Further basic science research

A marked improvement in the current OEC culture yields needs to be established in order to obtain sufficient OEC numbers for transplantation. The rat dorsal roots are no more than a few millimetres wide and the lesion induced by transection created a gap of 0.5 mm easily traversed with our OEC embedded matrix transplants. The human dorsal root, spinal cord and the lesion created by avulsion injury however are many folds that of the rat spinal cord.

Although various authors have already carried out transplantation of OECs in clinical settings, further experimental data of beneficial effects after transplanting nasal mucosal OECs is required before extrapolating the results of olfactory bulb cells. Although harvesting human olfactory bulb cells is technically feasible, it carries serious risks of causing further disability or even death and is not a practical option. At present there are conflicting reports regarding the efficacy of peripherally derived OECs in promoting regeneration [Ramer, Au, Richter, Liu, Tetzlaff, and Roskams 2004a;Ramer *et al.* 2004b;Yamamoto *et al.* 2009]. Feron et al first carried out auto-transplantation of cultured mucosal OECs in patients with SCIs but have not demonstrated beneficial outcome [Feron *et al.* 2005].

Controversially, Lima et el carried out transplantation of autografts of unpurified uncultured whole nasal mucosa in 7 patients with chronic cervico-thoracic SCI injuries after debriding the

site of the injury. This group interestingly did not have any safety issues; in particular there was no incidence of infection reported. Furthermore, they reported significant beneficial effects in 2 patients, pain in one patient and increased numbness in another 18 months after surgery [Lima *et al.* 2006]. At present there is limited basic science data to support the use of whole nasal mucosa which contains more respiratory cells than olfactory ensheathing cells and the process of culturing is considered essential.

Another group (Huang et al) obtained OECs from olfactory bulbs of 3-4 months aborted foetuses, and have claimed improvements in light touch, pin prick and motor function [Huang *et al.* 2006a;Huang *et al.* 2006b]. However, the assessment of the reported improvement was not carried out by independent examiners and due to ethical and regulatory issues it would be difficult to corroborate the results.

Therefore, it is my view that further basic research is required to rigorously assess outcome of transplanting mucosal sells in transection injury model of BPI before progressing further. Success in mucosal OEC transplant in BPI can then be potentially applied to the wider field of spinal cord injury, subcortical strokes, degenerative neurological conditions and cranial nerve lesions.

6.4 Summary

In a series of rats, I developed a dorsal injury model for the human brachial plexus injury that often arises in road traffic accidents. I described the effects of unilateral transection lesions of the DRs in a behavioural test by allowing the rats to climb a one metre grid made up of horizontal and vertical bars. A minimum of four adjacent dorsal root transections were required to develop a permanent climbing deficit of the ipsilateral forepaw. Unlesioned rats grasped the bars with a mean of 7.0 times per metre. The grasping function was completed abolished in the rats that had transection of C6 to T1 DRs with a mean score of 0.1 ± 0.06 grasp per metre. The climbing deficit remained unchanged over the 8 week test period. The transection of less than 4 roots provided a lesser deficit and the rats were able to mask the effects with time.

Using this model I assessed efficacy of OEC transplantation on lesioned and a matching control group on behaviour and anatomy. While the lesioned rats score a mean grasp of 0.2 (± 0.08), 71% of the OEC treated rats scored a mean of 2.9 (± 0.22) per metre with the rest failing to recover the loss of paw function. The immunohistochemistry study showed that the transplanted OECs induced an intense reactive projections of astrocytic projection into the PNS that was completely absent in lesion untreated rats. The axonal tracer BDA injected into the DRGs of treated animals showed that the regeneration axons crossed the PNS/CNS interface and also within the dorsal column of the spinal cord.

To investigate if the regeneration axons in the treated rats re-establish synaptic transmission to the spinal grey matter and cuneate nucleus, in another series of lesioned and unlesioned rats I performed electrophysiological experiments in collaboration. The median nerve was

stimulation while recording potentials record at the CD and the cuneate. The CDPs were maximal at C6 or C7 (amplitudes 440 – 1020 μV, mean 761 μV) and the cuneate recordings showed complex waveforms (amplitudes 125 and 133 μV).

In 4 acutely transected rats the mean CDP detected was 6μV and the mean cuneate potential was 3.5μV down from a mean of 133μV pre-lesion. In 4 with transections of DRs 6-8 weeks after lesioning electrophysiological tests revealed that all CDPs and cuneate potentials were completely abolished. In 7 out of 8 rats where recordings were made 6-8 weeks after transplanting, negative CDP responses were observed which had a notably slower time course compared to normal. All 4 of the 8 lesioned and treated rats, where cuneate recording was carried out, showed synaptic responses but again of longer latency and slower time course.

On correlating the behavioural and electrophysiological tests, 5 of 6 rats with OEC transplants that had recovered paw grasping function also had clear evidence of negative CDP wave responses (amplitudes 23 – 174μV); 1 of these 6 rats had no detectable CDP responses despite showing behavioural recovery. In 2 rats with OEC transplants in which grasping had not been restored showed negative CDP responses with amplitudes of 6 and 37μV. Cuneate recordings in all the four lesioned and transplanted rats tested showed negative responses. Three of four of these rats had observed recovery of grasping and relatively large the cuneate evoked potential responses of 15, 27 and 54μV negative amplitudes. The fourth, which had not recovered grasping, showed a response of lower amplitude, 7μV.

The outcome could then determine further applications of OECs in CNS injuries or guide experimental work to further evaluate and tackle issues that may arise.

CHAPTER SEVEN

7 CHAPTER 7: CONCLUSION

In summary, the data from this thesis suggests that the dorsal root injury paradigm can be a useful surrogate for the study of effects of putative CNS axonal regeneration promoting interventions. Of the many therapies reported, I have tested the efficacy of transplanted OECs in this model but equally other candidates could be used in the model.

The majority of OEC treated rats recovered lost climbing paw function as tested in our apparatus. Transplanted OECs induced bridge like tissue between the spinal cord PNS environment and the spinal cord CNS astro-gliotic environment. The regenerating dorsal root axons were shown to be crossing into the spinal cord and were also detected in the dorsal horn and the dorsal column. The findings also suggest that the regenerated axons are able to form functioning connection and thus restoring function. Rats that had not received OEC did not regain grasping function. Furthermore, the function of regenerated axons was tested by electrophysiological experiments and the results strongly suggest that synaptic transmission had been re-established.

This result shed some light on potential efficacy of OECs which would be expected to be repeated in other similar studies and eventually the findings translated to treat human brachial plexus avulsion injuries.

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