Investigations into long tract function following spinal cord injury and cell transplant therapy

A thesis submitted in fulfilment of the requirements of Degree of Doctor of Philosophy

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September 2013

Summary

Traumatic spinal cord injury (SCI) leads to severe functional deficits for which there are currently no effective treatments. About 50% of SCIs are incomplete leaving varying numbers of spared axons intact whilst damaging the cells which ensheathe them. These spared fibres provide targets for therapeutic interventions which aim to maximise their potential for supporting residual functions. In preclinical studies, functional outcomes are most commonly assessed using behavioural approaches. However they are unable to provide information on the mechanisms of recovery or differentiate between mechanisms occurring in the spinal cord and compensatory mechanisms occurring in the brain. This study had two main aims: Firstly to develop an electrophysiological protocol for assessing transmission in the ascending dorsal column pathway, to use this protocol to characterise the effects of contusion injuries of different severities and to investigate the time course of changes to long tract function following SCI. The second aim of this project was to use this protocol combined with behavioural testing to investigate the use of human lamina-propria mesenchymal stem cells (hLP-MSCs) as a potential therapy for spinal cord injury.

An electrophysiological approach was used to investigate function in rats subjected to T9 contusion injuries of the dorsal columns. Changes in the function of this pathway were assessed by recording sensory evoked potentials (SEPs) from the surface of the exposed somatosensory cortex, following stimulation of the contralateral sciatic nerve.

Functional effects of increasing injury severities were investigated in normal animals and animals 6 weeks after receiving contusion injuries of increasing severity. Maximum SEP amplitudes and isopotential plot areas were reduced with injury severity, and latency to sciatic SEP

onset was seen to increase in a graded fashion with increasing injury severity. SEP mapping revealed that the region of cortex from which SEPs could be recorded at or greater than certain amplitudes remained focused in the same location with increasing injury severity.

Animals were investigated at different time points from acute up to 6 months post injury. Acute investigation revealed that sciatic SEPs are ablated immediately following injury and after incomplete recovery stabilise within hours of injury. Maximum sciatic SEP amplitudes and cortical areas both show 2 phases of recovery: One at 2 weeks post injury and one at 6 months. Onset latencies are seen to increase initially before gradually returning nearer to normal levels by 6 months. SEP mapping revealed that the region of cortex from which SEPs could be recorded at or greater than certain amplitudes remained focused in the same location with increasing post injury survival time. Histological observations confirmed that the injury causes substantial damage to the dorsal columns.

To assess the effects of potential therapeutic hLP-MSC transplants, the functional effects of T9 150 Kdyn contusion injuries were investigated in medium injected controls and 3 week delayed hLP-MSC transplanted Sprague Dawley rats, at 10 weeks post injury. Behavioural testing was performed throughout, with terminal electrophysiological and immunohistological investigations performed at the end of the study.

Animals were behaviourally tested at pre- and post-operative time points for the duration of the experiment. Electrophysiological recordings suggest some recovery of function with time after injury. Two phases of recovery are seen, one at about 2 weeks after injury and the other at about 6 months after injury; however other measurements suggest hLP-MSC transplants had little or no effect on the functional integrity of the dorsal column pathway. Open field locomotor testing using the Basso, Beattie and Bresnahan (BBB) locomotor scale revealed

no differences between the recoveries of cell transplanted and control groups. Gait analysis was performed using the Digigait™ Imaging System revealing a trend for earlier recovery of co-ordination between forelimbs and hindlimbs in hLP-MSC transplanted animals compared to control animals. Moreover the step sequence data also suggested a better recovery of co-ordinated stepping in transplanted compared to medium injected animals. Dynamic weight bearing apparatus (BIOSEB) was used to measure the percentage of body weight borne on the forepaws and hindpaws, this demonstrated no effect of transplanted cells on postural changes. hLP-MSC transplants did not increase indicators of neuropathic pain in our model suggesting they are unlikely to exacerbate neuropathic pain following spinal cord injury. At present there are no immunohistochemical (IHC) markers that can be used to differentiate axons which have been remyelinated with central-type myelination from those which survived the injury. Thus, the degree of peripheral-type myelination was investigated as a simple way of assessing remyelination. This suggested that there was a greater degree of remyelination in transplanted animals, and that this was specifically in areas where transplanted cells were located.

Acknowledgements

First of all I would like to express the utmost gratitude to my supervisor Dr. John Riddell for his encouragement, guidance and support throughout my research and write up, as well as giving me the opportunity to advance my technical, analytical and surgical skills under his expert guidance.

I would like to say thanks to a number of other people whose help has been crucial in enabling me to complete this project. Extra-special thanks go to Andrew Toft who helped me at every single stage in more ways than I could begin to list, and without whom I would not be where I am today. Thank you to Dr. Dougald Scott for helping me with the electrophysiology experiments. Thank you to Dr. Susan Barnett and Dr. Susan Lindsey for providing me with purified stem cell cultures. Thank you also to everyone in the animal house for all their help and assistance in animal care. I would also like to express my gratitude to everyone in the University of Glasgow Spinal Cord group for all your encouragement and help during my research and write up.

I want to express my gratitude to the University of Glasgow and the MRC for providing the finances necessary to carry out this project as well as supporting me throughout my studies. I would also like to say an extra special thank you to all the fine people at Fyne Ales for supporting me throughout my write up, cheers guys!

I dedicate my thesis to my Mum, Dad, the rest of my family and to my dear friend Martina Hofner - thank you all so much for supporting me all these years - I would never have made it here without your encouragement, understanding and support.

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Abbreviations

^{o}C	Degrees Celsius
µ	Micro (10-6)
Ω	Ohm
A	Ampere
d	Day (s)
gm	Gram
h (r)	Hour (s)
i.m.	Intramuscular injection
i.v.	Intravenous injection
m	Milli (10^{-3})
min	Minute (s)
mm	Millimeter
n	Number
s	Second (s)
V	Volt
ANOVA	Analysis of variance
ASIA	American Spinal Cord Injury Association
BBB	Basso, Beattie, Bresnahan locomotor scale
BDNF	Brain-derived neurotrophic factor
CAP	Compound action potential
CDP	Cord dorsum potential
ChABC	Chondroitinase ABC
CNS	Central nervous system
CGRP	Calcitonin gene related peptide
CSPGs	Chondroitin sulphate proteoglycans
CST	Corticospinal tract
DCN	Dorsal column nuclei
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
GAGs	Glycosaminoglycans

GDNF	Glia cell line derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
hLP-MSC	Human lamina propria derived mesenchymal stem cell
MAG	Myelin-associated glycoprotein
NF	Neurofilament
NGF	Nerve growth factor
NgR	Nogo-66 receptor
NT-3	Neurotrophin 3
NT-4	Neurotrophin 4
OEC	Olfactory ensheathing cell
OMG	Oligodendrocyte-myelin glycoprotein
PBS	Phosphate buffered saline
PNS	Peripheral nervous system
SCI	Spinal cord injury
SEP	Sensory evoked potential

Author's Declaration

I declare that this thesis comprises my own original work, except where otherwise stated, and has not been accepted in any previous application for a degree. All sources of information have been referenced.

Jacob Peter Griffin

30th September 2013

Chapter 1

Introduction

1.1 Spinal cord injury

1.2 Epidemiology and aetiology

The spinal cord is a columnar projection of neuronal and support cells which extends caudally from the medulla oblongata in the brainstem and projects down the spinal canal of the vertebral column. It receives and processes sensory and autonomic information from the muscles, joints, skin and viscera, and carries motor signals to the musculature.

Injury to the spinal cord can disrupt the ascending and descending long white matter tracts which carry sensory and motor signals, respectively, to and from the brain. Injury can also damage the spinal grey matter in the dorsal, ventral and lateral horns which can result in the loss of local interneurones and motorneurones. SCI therefore has the capacity to produce deficits in motor, sensory and autonomic function, and due to the limited reparative capacity of the central nervous system these deficits are often permanent.

The number of people worldwide currently living with a SCI is unquantifiable due to inconsistencies in data reporting, however, reported annual incidences range from 10.4-83 per million population (Wyndaele and Wyndaele, 2006). Estimated annual incidence is reportedly 15 per million population in Western Europe, and 40 per million population in North America (Ackery et al., 2004; Cripps et al., 2011; National Spinal Cord Injuries Association Statistical Centre, 2013 - http://www.nscisc.uab.edu). In the UK an estimated 15-20 people per million population suffer a traumatic spinal cord injury (TSI) each year and a total of 40,000 people currently live with spinal cord injury (Bellack and Hersen, 1998).

The aetiologies of spinal cord injuries vary from country to country as well as between demographic groups. In the USA an estimated 36.5% of traumatic spinal cord injuries occur as a result of road traffic accidents, 28.5% as a result

of falls and 9.2% due to sports-related injuries (National Spinal Cord Injuries Association Statistical Centre, 2013 - http://www.nscisc.uab.edu). More developed countries tend to have lower rates of work-related injuries and a higher incidence of sports-related injuries when compared to developing countries; this is due to more developed nations having higher levels of worker safety as well as workers having more free time and greater disposable income (Sekhon and Fehlings, 2001).

1.3 General anatomy of the Spinal cord

The brain and spinal cord make up the central nervous system (CNS) and are protected from damage by physical and chemical barriers. The main defence against mechanical damage to the CNS is provided by the skull and vertebral column which encase the CNS and provide support and protection from blunt force and penetrating injuries. Physical protection is also provided by a tough three layered membranous barrier known as the meninges: the innermost pia mater, arachnoid mater and outermost dura mater surround the CNS and provide physical support (Kimmell, 2011 – see Figure 1.1). The subarachnoid space contains cerebrospinal fluid (CSF) which is involved in protecting the CNS by acting as a cushion against mechanical damage. The CNS is also protected by a selectively permeable membrane between the circulating blood and the extracellular fluid in the CNS known as the blood brain barrier (BBB). Protection is achieved through the selectivity of the tight junctions between endothelial cells that line the blood vessels of the CNS; this allows for chemical isolation of the CNS, safeguarding it from potentially damaging agents and microbes which circulate in the periphery, whilst allowing beneficial substances through via diffusion or active transport mechanisms.

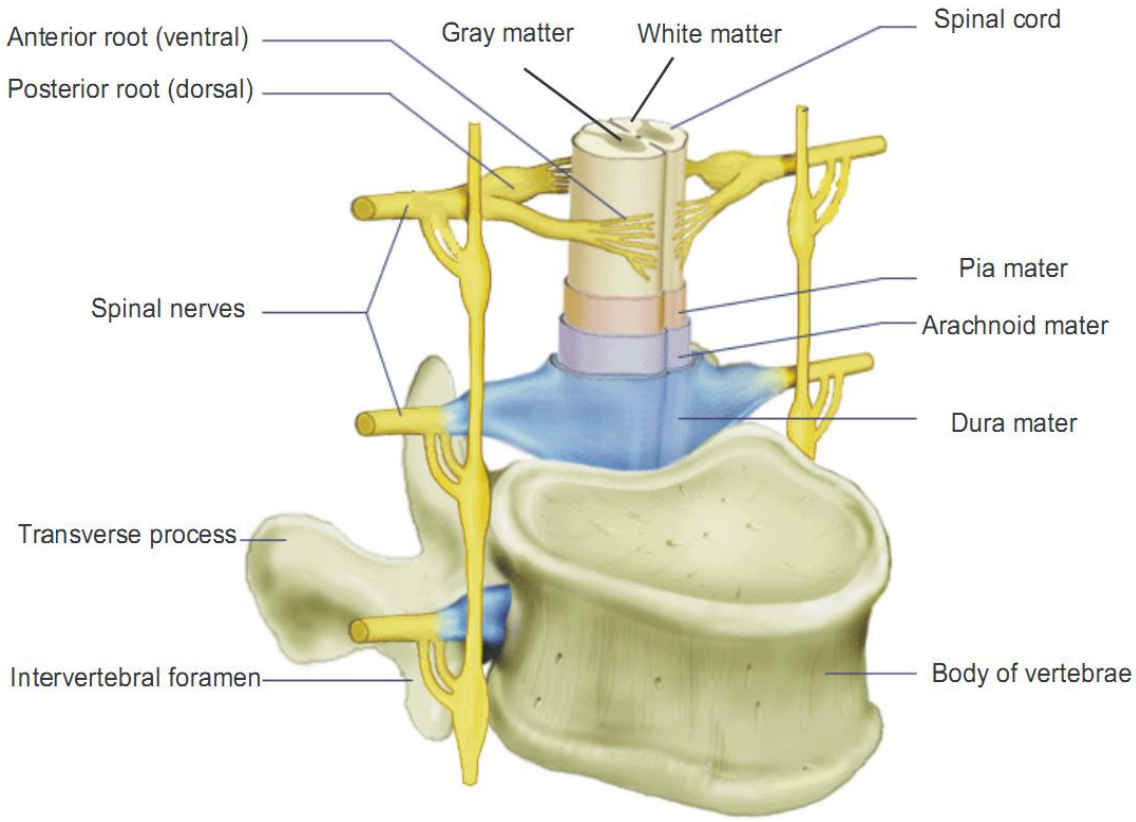


Figure 1.1 - Structure of the spinal cord *- In transverse section the spinal column can be seen to consist of a centrally located butterfly-shaped region of grey matter surrounded by white matter. The grey matter is largely composed of neuronal and glial cell bodies, whereas the white matter is composed largely of axonal fibre tracts. The spinal cord is protected from physical damage by the vertebral column and three tough meninges called the dura mater, the arachnoid mater and the pia mater. Each spinal segment contains two anterior/ventral roots which carry motor signals to the periphery and two posterior/dorsal roots which carry sensory signals to the CNS. The two roots on each side join to form paired spinal nerves. (Adapted from information available online at www.mmi.mcgill.ca, 2008).*

The human spinal cord contains millions of neuronal and glial cells which together act as the main pathways for the transmission of information to and from the peripheral nervous system. The spinal column consists of a central butterfly shaped column of grey matter surrounded by white matter fibre tracts (Figure 1.1). The spinal cord is divided into 31 segments: 8 cervical, 12 thoracic, 5 lumbar, 5 sacral and one coccygeal. Each spinal segment receives sensory input from the periphery via a pair of nerves known as the dorsal roots and transmits motor signals to the periphery via a pair of nerves known as the ventral roots. The dorsal roots enter the spinal column via the left and right dorsolateral sulci and ventral roots exit the spinal column via the left and right ventrolateral sulci. Ganglia known as the dorsal root ganglia are located on each dorsal root, and contain the cell bodies of afferent spinal nerves, whilst the cell bodies of efferent neurones are located in the ventral horn. The dorsal and ventral roots of each spinal segment meet to form paired spinal nerves before leaving the vertebral canal via the intervertebral foramina. The spinal cord contains two enlargements along its length: the cervical enlargement which extends from spinal segments C3-T2, and the lumbosacral enlargement which extends from spinal segments L1-S3. These enlargements exist due to the large numbers of nerves responsible for the innervation of the upper and lower limbs, respectively.

The adult spinal cord is shorter than the vertebral column and only occupies the upper part of the spinal canal, terminating at around the L1-2 vertebral region. Therefore the spinal cord segmental levels do not have a direct correlation with the spinal vertebral levels at all levels; this discrepancy increases as we travel down the cord from cervical to sacral levels (Figure 1.2).

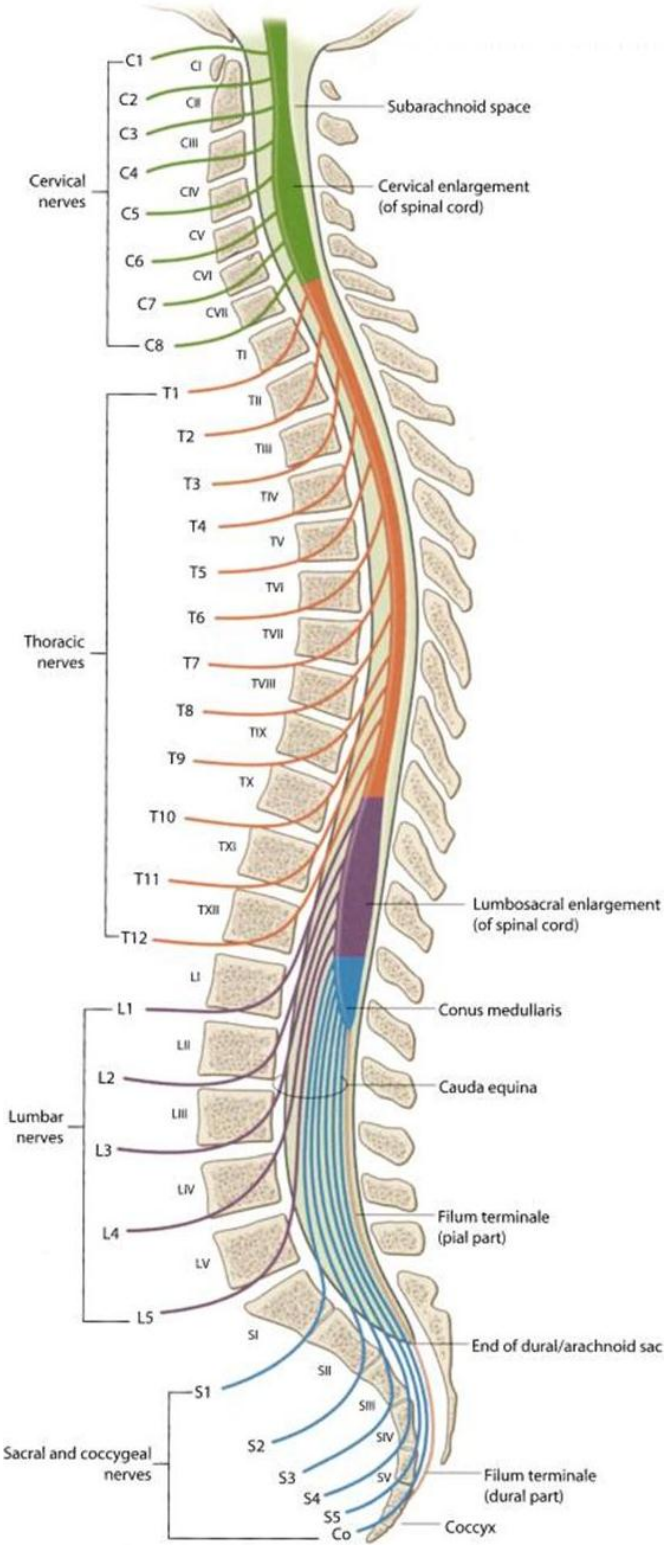


Figure 1.2 The relative locations of the spinal segmental levels, spinal nerve roots and vertebrae *- At birth the spinal cord segmental levels are located directly below the vertebral levels but the vertebral column grows faster than the spinal cord, thus as we grow the spinal cord ceases to occupy*

the lower third of the vertebral canal. This discrepancy increases until the time we stop growing, by which time the terminal portion of the cord, known as the conus medullaris, is located within the L1-2 vertebral region. The spinal roots of each segment continue to exit the intervertebral foramina of their correspondingly named vertebrae, thus at increasingly caudal spinal levels, the roots get longer such that the lumbar, sacral and coccygeal nerves extend below the level of the conus medullaris before exiting the spinal canal, and are collectively known as the cauda equina (From Drake et al., 2008, pg. 44).

1.4 Injury level and type

The specific effects of a SCI depend on the type of injury sustained and the level at which it occurs. SCIs can be classified as either complete or incomplete injuries, depending on the available neurological evidence for sparing of sensory and/or motor functions. Injuries are classified as incomplete when some degree of sensory and/or motor function remains below the injury level, which is defined as the lowest level that has normal neurological function. In complete injuries, no sensation or voluntary motor functions can be observed below the injury level.

Clinically, SCI severities are classified using the ASIA scale (American Spinal Cord Injury Association's International Standards for Neurological Functional Classification of Spinal Cord Injury - http://www.asia-spinalinjury.org), which allows clinicians to assign patients to one of 5 categories from A-E based on the observed level of injury, which is defined by touch and pinprick tests of sensory function and the strength of key muscles on both sides of the body following injury. A = complete: no motor or sensory function is preserved below the injury level. Patients that are assigned an ASIA A classification are least likely to recover any function. B = incomplete: sensory but not motor function is preserved below the neurological level and includes the sacral segments S4-S5. C = incomplete: motor function is preserved below the neurological level, and more than half the key muscles below the neurological level have a muscle grade less than 3. D = incomplete: motor function is preserved below the neurological level, and at least half the key muscles below the neurological level of injury have a muscle grade of 3 or more. E = normal: motor and sensory functions are normal and the patient may have had prior deficits or may have sustained an injury with no neurological damage.

Based on the ASIA scale, there has been a substantial reduction in the proportion of SCIs that are classified as complete in the last 40 years, from approximately two thirds to the current rate of less than a half. Many factors

have played a role in these statistics, including the increased awareness of SCI and the importance of immediate immobilisation and well planned post-injury care, better health and safety regulations as well as the use of drugs, such as anti-inflammatory drugs which appear to limit the effects of the secondary injury (Strauss et al., 2006), although this is still debated (Pinzon et al., 2008).

The level at which a SCI occurs determines the parts of the body in which function may be impaired or lost. Cervical level SCIs are the most commonly seen, accounting for approximately 45% of all SCIs, with thoracic level injuries accounting for approximately 29% and lumbar level injuries accounting for approximately 23% of all SCIs (Hasler et al., 2011). As shown in Figure 1.3, SCIs at the cervical level usually result in tetraplagia whereas SCIs at the thoracic (or lower) levels usually result in paraplegia. Tetraplegia, also known as quadriplegia, is characterised by paralysis in all limbs, although this does not have to be complete paralysis or loss of function. Injuries of this type will also affect the chest and abdominal muscles, often affecting respiratory and digestive functions. Paraplegia occurs when the injury level is at or below the level of the first thoracic spinal nerve and is characterised by complete or incomplete loss of function in the lower limbs and the abdomen up to the nipple line, with full function remaining in the upper limbs (Figure 1.3).

Lumbar and sacral SCIs (L1-S5) are characterised by decreased function in the legs, urinary tract and bowel. Lower thoracic SCIs (T9-T12) are capable of producing partial paralysis of the lower body and limbs; at higher thoracic levels (T1-T8) SCIs begin to also affect trunk control due to the decrease in abdominal muscle control, as well as beginning to adversely affect the sympathetic nervous system. Injuries at levels above the T1 spinal level start to affect arm and hand function, with C7-T1 injuries frequently causing deficits in hand and finger function whilst control of the arms remains. SCIs at the C7 spinal level are generally the highest level injuries with which patients can still live relatively independently. At the C6 level all function in the wrists and fingers is lost whilst wrist function remains, whereas at the C5 level

patients are only able to control the upper arms and shoulders with no control over their wrists. Individuals with injuries at the C4 level might retain some control over the upper arms and shoulders but this will be weaker than that seen with injuries at the C5 level. High cervical injuries (above the C3 level) typically result in deficits on head and neck movements as well as loss of diaphragm function, which if the patient survives the initial trauma, requires the use of an artificial ventilator to enable to patient to breath. Other common problems associated with SCI include neuropathic pain and/or spasticity (Burchiel & Hsu, 2001).

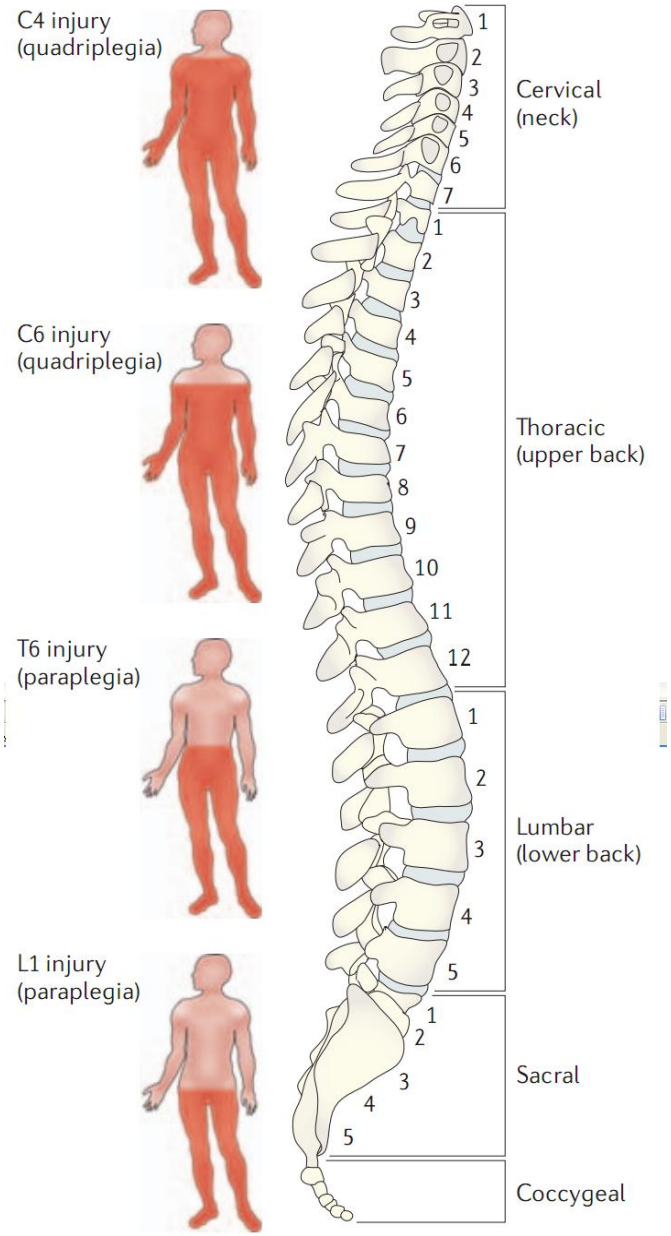


Figure 1.3 SCI level and associated functional deficits.

The severity of spinal cord injury and the level it occurs at both affect the type and degree of resultant functional deficits. SCIs at the cervical level usually result in tetraplegia, a symptom in which the individual experiences paralysis in all four limbs - though not necessarily complete paralysis or loss of function. SCIs at the thoracic level or below usually result in paraplegia, a symptom where the individual experiences paralysis of the lower limbs whilst retaining full function in the upper limbs. The area of paralysis is indicated in red (taken from Thuret et al., 2006).

1.5 Pathology following SCI

Due to the paucity of information from clinical injuries in the literature, much of our understanding of the events that follow the initial trauma of SCI come from studies that utilise animal models. Following SCI it is known that a complex sequelae of pathophysiological events occurs, which are summarised in Figure 1.4.

SCI is characterised by two stages of injury processes known as the primary and secondary injury response. During the primary injury response the mechanical force of the injury results in local axotomy as well as disruption of the spinal vasculature and cellular membranes. The secondary injury response rapidly follows this initial trauma and is characterised by a complex and time dependent series of pathophysiological events at and adjacent to the injury site; these ongoing sequelae include vascular disruptions, inflammation, oedema, excitotoxicity, demyelination, axonal degeneration, apoptosis and scar formation (Tator, 1995) (Figure 1.4). Whilst most of the cells that participate in the secondary injury response already reside in the spinal cord, others infiltrate the injured cord after being recruited from the circulatory system (Schwabb & Bartholdi, 1996).

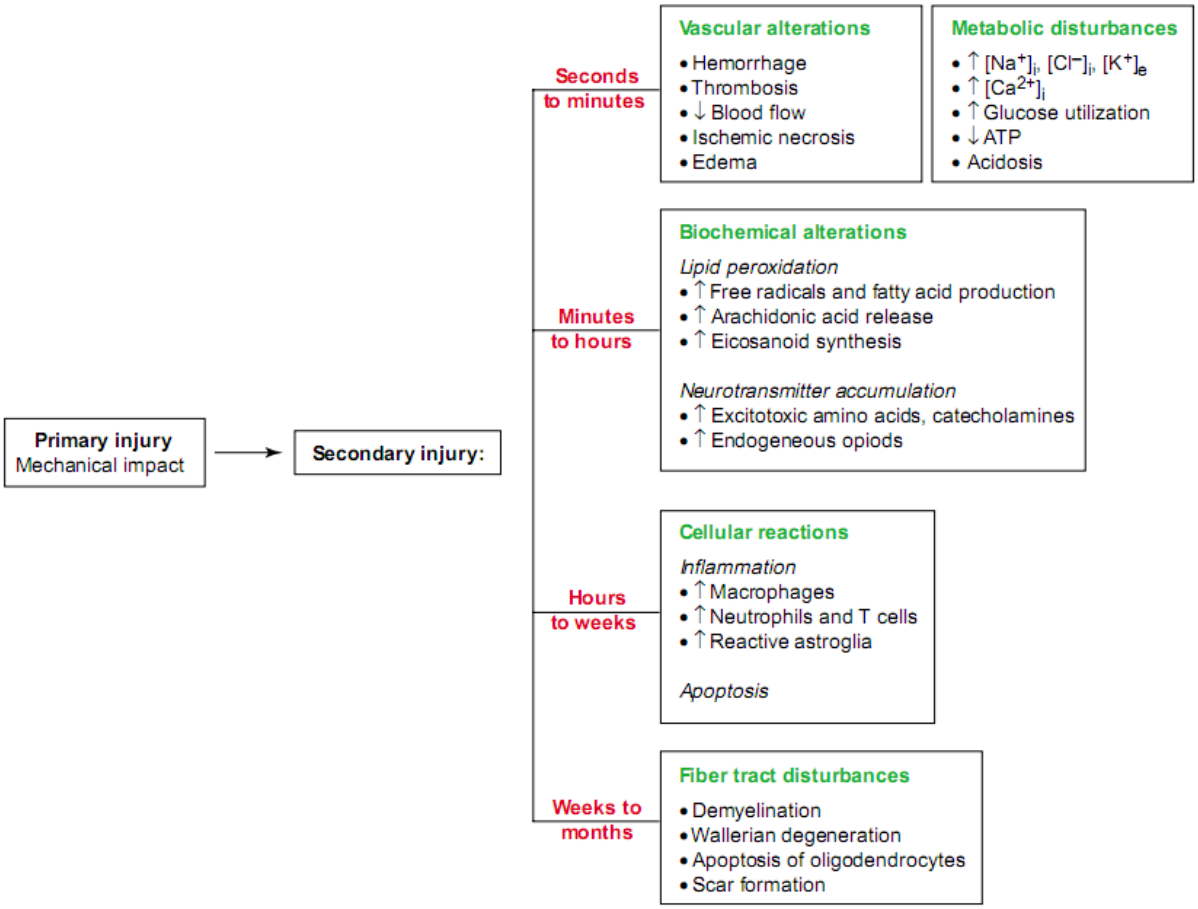


Figure 1.4 Pathogenesis of SCI *- Spinal cord injury occurs in two stages, referred to as the primary and secondary injury processes: The primary injury refers to the physical damage resulting from the initial mechanical impact on the cord whereas the secondary injury refers to a complex series of time-dependant pathophysiological processes that succeeds the primary injury, including disruption of the vasculature, excitotoxicity, inflammation, apoptosis, demyelination and axonal degeneration and dieback (taken from Bareyre and Schwab 2003).*

1.5.1 Necrosis, apoptosis and excitotoxicity

Mechanical injury to the spinal cord causes many cells to undergo immediate cell death, via a process called necrosis. This is often associated with inflammation and the severe depletion of cellular energy stores caused by disruption of the vasculature, and is characterised by cells swelling until their membranes rupture.

Later on, cells undergo another kind of cell death called apoptosis (programmed cell death), characterised by cells condensing before a highly controlled cascade of enzymatic reactions breaks the cell down into its constituent parts (reviewed by Beattie et al., 2000). Ionic disruption leading to a rapid influx of calcium has been shown to play a critical role in apoptosis (Mattson & Chan 2003). Calcium influx is induced by enzymatic modifications to Na+ channels in cellular membranes, leading to rapid influx of Na+ ions by causing reversal of the Na^{+}-Ca^{2+} exchangers, which in turn causes the rapid influx of Ca2+ through voltage-gated Ca2+ channels, leading to apoptosis (Nashmi and Fehlings et al., 2001; Mattson & Chan 2003; Iwata et al., 2004). Apoptosis has been shown to be ongoing for around three weeks to a month following injury in animal models (Crowe et al., 1997; Beattie et al., 2000) and around 2 months in humans (Emery et al., 1998).

Necrosis, apoptosis and excitotoxicity afflict many cell types following SCI, including neurones, oligodendrocytes, astrocytes and microglia (Beattie et al., 2000). Oligodendrocytes in particular are highly susceptible to degradation and have been shown to undergo apoptotic cell death in the proximity of the injury site as well as several segments rostral and caudal to the injury site in the degenerating white matter tracts, both in rats (Li et al., 1999) and in humans (Emery et al., 1998). As discussed earlier, microglia have been associated with oligodendrocyte apoptosis, though other factors have been implicated, including alterations to the level of trophic factor expression following injury (Krenz and Weaver, 2000).

Following injury, all types of glial and neural cells are at risk of excitotoxic cell death due to accumulation of the excitatory amino acids aspartate, glutamine, asparagine and glutamate. It has been shown that such molecules build up in the injured cord to levels capable of over activating AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) and NMDA (N-methyl-D-aspartate) receptors, leading to an influx of extracellular calcium which causes the release of cyctochrome c from mitochondria. Cytochrome c then binds to receptors on the endoplasmic reticulum and causes further calcium release, initiating a positive feedback loop whereby more cytochrome c is released from the mitochondria, leading to further release of calcium from the endoplasmic reticulum. This ultimately results in a massive increase in cytoplasmic calcium and cytochrome c concentrations, leading to the formation of the apoptosome which leads to the activation of a full caspase cascade and subsequent cell death (Mattson and Chan, 2003).

1.5.2 Disruption of spinal vasculature

Following SCI blood flow alterations are observable at and around the injury site. These alterations are required for the development of the destructive processes that take place in the secondary injury response (Mautes et al., 2000; Popa et al., 2010; Ng et al., 2011).

SCI invariably results in damage to the blood vessels at the site of injury, usually manifesting as intraparenchymal haemorrhage with a propensity for damaging highly vascularised regions and the central grey matter (Mautes et al., 2000). Bleeding is usually from the capillaries, arterioles and venules and very rarely from arteries such as the anterior spinal arteries (Tator et al., 1995). Over time haemorrhagic damage is seen to extend from the grey matter into the white matter (Mautes et al., 2000). More substantial bleeding rarely occurs within the cord; when it does occur, these larger bleeds, termed

hematomyelia, are occasionally accompanied by distension of the cord (Tator et al., 1995).

It has been shown that various proteinases including matrix metalloproteinases cysteine proteases, and serine proteases are upregulated following SCI in mice rats and human (Noble et al., 2002; Wells et al., 2003; Buss et al., 2007; Veeravalli et al., 2012). This upregulation causes excessive proteolytic activity in the injured cord, which causes further damage to the capillary endothelial cells that make up the blood brain barrier. As a result macrophages and neutrophils from the periphery are able to cross into the CNS, thus enhancing phagocytosis and protein breakdown (Noble et al., 2002; Wells et al., 2003; Buss et al., 2007).

Mechanical damage to the microvasculature has been shown to cause vasospasm, resulting in a reduction in arteriolar luminal area by up to 80% (Anthes et al., 1996). Thrombosis of small blood vessels has also been documented (Koyanagi et al., 1993). It has been shown that in brain injuries which result in subarachnoid haemorrhage, vasospasm can be induced by the breakdown products of red blood cells and endothelial cells, such as endothelin and oxyhemoglobin (Roux et al., 1995; Macdonald and Weir, 1991), similar mechanisms could feasibly act to induce or enhance the degree of vasospasm following damage to spinal vasculature caused by spinal cord trauma (Anthes et al., 1996). Combined, these processes are capable of causing ischemic injury in spinal cells by preventing spinal cells at and adjacent to the disruption from accessing the oxygen and nutritional support required to survive (Tanaka et al., 2005). In turn, inadequate nutrient and oxygen supply to the spinal tissue is capable of resulting in the spinal cord's failure to function properly in the first few weeks following injury (Krassioukov and Claydon, 2006). During the ischemic period, most cell types release numerous kinds of free radicals including reactive oxygen species and reactive nitrogen species which are known to play a significant role in progressive cell death during the secondary injury response (Liu et al., 2000; Jia et al., 2012).

Disrupting central control of the cardiovascular system occurs following complete cervical injuries and frequently results in hypotension and bradycardia, which can in turn lead to further damage to the cord (Weaver et al., 2012).

1.5.3 Oedema

It has been shown that following SCI extensive bleeding into the extracellular spaces of the spinal parenchyma plays a major contributing factor in the development of local oedema, which is maximal during the first hour after injury and then rapidly decreases (Griffiths and Miller, 1974). Oedema is known to start in the central part of the cord and to spread centrifugally into the white matter (Noble and Wrathall 1989), as well as extending considerable distances rostrally and caudally from the injury site (Wang et al., 1993). The degree and rate of spread is known to be highly dependant on injury severity (Griffiths and Miller, 1974).

Injury is known to cause blood vessels near the injury site to increase permeability, whilst the BBB remains intact. This process, known as vasogenic oedema, serves to exacerbate the oedema caused by bleeding from ruptured vessels, ultimately enhancing the deleterious effects that oedema exerts on the local tissue. These effects are believed to result mainly from localised tissue compression which further disrupts the vasculature and ultimately enhances necrosis (Griffiths and Miller, 1974).

1.5.4 Inflammatory response

SCI leads to a strong inflammatory response in an attempt to restore tissue homeostasis, with the recruitment of peripherally derived leukocytes (monocytes/macrophages, neutrophils and T-lymphocytes) and activation of

glial cells (astrocytes and microglia). This is accompanied by the up-regulation of soluble factors including cytokines and chemokines by activated glia. The release of cytokines and chemokines initiates intracellular signalling cascades which result in epithelial damage, alterations to vascular permeability and oedema (Schnell et al., 1999; Bareyre and Schwab 2003; Hagg and Oudega 2006). Using animal models of SCI, many such factors, including IL-1 and TNFα have been shown to be up-regulated as quickly as an hour following injury and are down-regulated again within 6-24 hours (Bartholdi and Schwab, 1997).

Inflammation is known to have strongly dichotomous effects on recovery following SCI; complex concomitantly occurring destructive and constructive processes occur during the post-injury inflammatory episode (Bethea, 2000). Reducing the inflammatory response has been shown to facilitate functional recovery (Sterling et al., 2004), despite this, the inflammatory response has also been shown to be advantageous to recovery through the elimination of cellular breakdown products from the injury site (Fleming et al., 2006; Rice et al., 2007).

Following SCI, the first group of cells to participate in the immune response are the local microglia. Using mouse models, it has been suggested that microglia may be activated within minutes following injury (Rice et al., 2007). Once activated, local microglia produce numerous pro-inflammatory molecules such as cytokines and chemokines as well as numerous mediators of cellular injury including various reactive oxygen species, reactive nitrogen species and proteases (Banati et al., 1993) all of which are capable of inducing apoptosis. Moreover, two of the reactive oxygen species up-regulated in activated microglia, nitric oxide and superoxide anion, have been shown to indirectly cause oligodendrocyte death (Li et al., 2005b). Despite this, several cytokines released by activated microglia are believed to have both direct and indirect neuroprotective and neuroregenerative actions (reviewed by David and Kroner, 2011).

Haematogenous macrophages arrive in the spinal cord within 2-3 days and remain there for weeks where they play one of the most active roles in the post-injury inflammatory period (Popovich et al., 1999). They are widely believed to have both beneficial and detrimental effects on recovery following SCI (Bethea 2000; David and Kroner 2011). Macrophages act to phagocytose cellular debris in and around the injury site as well as secreting factors that promote axonal growth and Schwann cell proliferation, which may lead to an increase in functional recovery. Concomitantly, it has been shown that a different subset of macrophages also synthesise and release numerous cytokines and reactive oxygen species which facilitate the inflammatory episode, cause severe membrane damage and contribute to further cell death (reviewed by David and Kroner, 2011 and Kigerl et al., 2012).

1.5.5 Demyelination

Like neurones, oligodendrocytes are extremely sensitive to the effects of SCI. When oligodendrocytes are injured or destroyed they cease to provide the insulating myelin sheath that axons require to function normally. This process, known as demyelination, impairs the axon's ability to conduct action potentials due to there being insufficient sodium channels distributed along the internodal axonal membrane regions (Black et al., 1991). Demyelination severely slows or blocks signal conduction through spared axons (Bostock and Sears, 1976; Bostock et al., 1978). As a result, a compensatory mechanism known as "channel spreading" occurs whereby the membrane channels which were tightly grouped around the nodes of Ranvier spread out along the axolemma to aid in signal transduction (Balentine, 1978; Griffiths and McCulloch, 1983; Nashmi et al., 2000; Karimi-Abdolrezaee et al., 2004).

Using animal models of SCI it has been possible to document the first phase of demyelination, called primary demyelination which is initiated by the primary injury response and involves the destruction or partial destruction of the

myelin sheath with axons remaining relatively structurally intact (Griffiths and Mcculloch, 1983). The initial loss of oligodendrocytes is complex and poorly understood. In animal models of SCI, primary demyelination has been attributed to the initial mechanical insult and resultant necrotic ischemia around the injury site (Salgado-Ceballos et al., 1998; Waxman 1989); whilst in humans it does not appear to be as common, with only sparse demyelination seen (Norenburg et al., 2004). However, in compression injuries in humans there has been shown to be a much more robust degree of demyelination (Bunge et al., 1993) observable at acute time points. As yet it remains unknown to what extent early demyelination might occur in humans following moderate SCI, as moderately injured human spinal tissue is normally only available at acute time points.

In animal models of SCI it has been reported that around 3 weeks after injury a second phase of demyelination begins, which results in further damage to myelin sheaths (Griffiths and Mcculloch, 1983). Totoiu and Keirstead reported chronic progressive demyelination following thoracic contusion injury in rats, which began between 2-4 weeks post injury and continued for up to 450 days (Totoiu and Keirstead, 2005). Where seen experimentally, later phases of oligodendrocyte loss have been linked with numerous factors involved in the secondary injury response, including reductions in the levels of trophic support molecules caused by axonal degeneration (Barres et al., 1993) as well as the delayed recruitment of macrophages to the injury site as part of the inflammatory response (Blight 1985). Despite these observations, several studies have been unable to corroborate the findings of Totoiu and Keirstead, 2005 (Lasiene et al., 2008; Powers et al., 2012). If however, as Totoiu and Keirstead have reported, chronic progressive demyelination occurs following contusive SCI, then this would present a potential therapeutic target for the treatment of SCI.

1.5.6 Axonal degeneration

Axons may be disrupted by the initial trauma or they may undergo anterograde neurodegeneration following apoptosis of the oligodendrocytes which ensheath their axons. This process is called Wallerian degeneration and may help explain the ongoing sensory-motor dysfunction following injury (Griffiths and Mcculloch, 1983). Wallerian degeneration is associated with disruption to the spinal vasculature (Sinescu et al., 2010), as well as ionic imbalances caused by the reversal of the Na^{+}-Ca^{2+} exchangers (Fehlings and Nashmi et al., 1996; Imaizumi et al., 1999; Iwata et al., 2004), resulting in an influx of calcium which induces cellular apoptosis (Mattson & Chan 2003), as discussed above. SCI also results in reduced expression of myelin-associated proteins, including myelin basic protein and myelin-associated glycoprotein, which results in abnormal myelination (Wrathall et al., 1998). Wallerian degeneration, also results in the loss of collateral projections, which accounts for the reduction in residual functions seen following the primary injury response. Axonal dieback also leads to a reduction in the degree of functional recovery that the later plastic changes are capable of inducing (see section 1.6.3.2).

1.5.7 The lesion scar

Various cytokines released by invading macrophages, leukocytes and lymphocytes in the inflammatory period lead to the recruitment of activated astrocytes, microglia, meningeal cells, oligodendrocytes and oligodendrocyte precursor cells around the injury site, resulting in the formation of a glial scar. Astrocytes are the most common and most important cell type associated with the glial scar, whilst fibroblasts of meningeal and perivascular origin also invade the injury site and form a fibrotic scar. The glial and fibrotic scars form a dense, highly organised, solid network, collectively known as the lesion scar, which acts as a barrier to axonal regeneration (Fawcett and Asher 1999; Faulkner et al., 2004; Fitch and Silver, 2008; Kawano et al., 2012).

Together the glial and fibrotic scars act as a physical and chemical barrier to regenerating axons. Many of the cell types involved, including oligodendrocytes, oligodendrocyte precursor cells, astrocytes, meningeal cells and activated microglia, are known to release factors which are inhibitory to axonal outgrowth (Fawcett and Asher 1999), as well as producing extracellular matrix molecules which have been shown to have potent growth inhibitory properties (Davies et al., 1999). Together these features combine to produce an environment which is highly non-permissive to axonal regeneration; however, some investigators have reported robust long distance axonal growth through lesion scars and into the sites of chronic SCI (Lu et al., 2007)

Despite the negative effects the lesion scar has on regenerating axons, it also plays a protective role in the recovery process by repairing the damaged blood brain barrier, preventing further degeneration and limiting the spread of damage to the surrounding, healthy tissue (Faulkner et al., 2004).

1.5.8 The lesion cavity

Following SCI the destruction of the grey matter at the injury epicentre sometimes leads to the formation of a cavity with a rim of preserved subpial myelinated axons (Beattie et al., 1997; Hayes and Kakulas, 1997). At the boundary between the cavity and the preserved axons, some studies have shown the presence of partially demyelinated axons (Blight and Decrescito, 1986; Salgado-Ceballos et al., 1998). It is believed that the myelinating cells are relatively more susceptible to injury than axons as unlike axons, their cell bodies are local to the injury site (Crowe et al., 1997; Emery et al., 1998; Frei et al., 2000; Yamaura et al., 2002).

The lesion cavity usually begins to form in the grey matter; this may be the case for numerous reasons, including simply that damage to the central canal causes leakage of CSF into the expanding extracellular space. Other

possibilities include the grey matter is inherently more rigid and thus fragile than the white matter, as revealed by studies in bovine cords (Ichihara et al., 2001), or that the comparatively denser vascular network of the grey matter makes it more susceptible to mechanical injury than the white matter (Tator, 1995). Irrespective of aeiteology, ongoing degeneration leads to the cavity extending outwards to include parts of the white matter whilst concomitantly spreading in the rostro-caudal plane, mostly in the grey matter, such that it can be seen extending several millimetres above and below the injury within a few weeks in animal models (Ek et al., 2010; James et al., 2011). Cavity size and the amount of uninjured or partially demyelinated tissue surrounding the cavity varies depending on the type, severity and location of the injury.

Single cavities are most commonly seen, though multiloculated cavities are also observed separated by fine trabeculae of connective tissue, blood vessels and a small number of axons. They are filled with extracellular fluid and frequently contain residual macrophages, connective tissue and blood vessels. Whilst fluid filled cavities provide a bad substrate for regenerating axons and therefore act as a physical barrier to regeneration, filled cavities provide a comparatively good substrate for regenerating axons (Norenberg et al, 2004). As the cavities expand over time, if multiloculated, individual cavities may coalesce. This condition, known as syringomyelia, only affects 4% of SCI patients but has been documented occurring from as little as 2 months up to 30 years following injury (Schurch et al., 1996).

1.6 Spontaneous recovery of function

As mentioned previously, spontaneous improvements in function are frequently reported following SCI, as reviewed by Fawcett et al., 2007. Spontaneous regeneration in the adult CNS is extremely limited, although some degree is observed in almost all cases in the days, weeks and years following injury; this may play a role in functional recovery. Synaptic plasticity in spared pathways

and the formation of new circuits by collateral sprouting may also contribute to the recovery of function (Raineteau and Schwab 2001).

1.6.1 Spontaneous recovery of function in humans

Following spinal cord injury in humans, highly predictable but limited degrees of functional recovery are known to occur (Brown et al., 1991; Fisher et al., 2005). However, functional recovery following incomplete SCIs is known to be much faster and extensive, yet highly variable. For example, following incomplete injuries some patients recover almost complete motor function, whereas in others it can be highly limited (Burns et al., 1997; Ditunno et al., 2000). It is known that spontaneous recovery of motor function in humans is rapid in the first few months following injury, but has usually reached a plateau between 12–18 months, at which point the injury is considered stable, although in some cases limited ongoing regeneration has been recorded up to several years following injury. Limited sensory recovery is also seen to follow the case time course as that seen for motor recovery (McDonald and Sadowsky, 2002; Fawcett et al., 2007).

The degree of spontaneous recovery of function observed is highly dependant on injury severity, with less severely injured patients more likely to experience greater degrees of functional recovery. Data from the US Model Systems, EMSCI and Sygen databases were reviewed by Fawcett et al., 2007 to reveal that approximately 10% of ASIA A patients are ultimately downgraded to ASIA B, and 10% to ASIA C due to the limited recovery of some sensory and motor function below the level of injury, respectively, in the first 12 months following injury. Between 15-40% of ASIA B patients were reassigned to ASIA C, and 40% to ASIA D. As discussed earlier, the majority of ASIA C patients are intimately reclassified as ASIA D; Fawcett et al., 2007 recorded this figure to be between 60-80%. Despite this, very few ASIA D patients ever recover fully

enough to be assigned an ASIA E score, indicating normal motor and sensory function (Fawcett et al., 2007).

1.6.2 Spontaneous recovery of function in animals

Several studies utilizing animal models of SCI have demonstrated spontaneous recovery of function (McKenna and Whishaw, 1999; Weidner et al., 2001; Bareyre et al,. 2004; Courtine et al., 2005; Ballermann and Fouad, 2006; Gulino et al., 2007), though the time course, extent and variability of functional recovery are seen to vary greatly between models utilising different injury types, severities, levels and species of animal (Noble and Wrathall, 1989).

Most animal models in which spontaneous recovery is seen show a much greater degree of functional recovery than we would expect to see in humans afflicted with comparable injuries. As discussed below, the most widely utilised functional assessments are behavioural tests, most of which have shown that varying levels of spontaneous recovery occur following injuries of different type, severity, level and in every species investigated. Behavioural assessments of locomotive abilities in animal models of SCI have shown that the prognosis for experimental animals is better than for humans. It is thought that this observation can be explained, in part, by the fact that quadrupeds have a wide range of compensatory modifications to gait which they can employ to compensate for the locomotor deficits caused by the injury (Rossignol et al., 2004; Ballermann et al., 2006).

1.6.3 Processes underlying spontaneous recovery of function

The physiological processes that underlie spontaneous functional recovery are complex and poorly understood. It is widely accepted that spontaneous

improvements in function cannot be attributed to axonal regeneration alone, and that other mechanisms such as remyelination and plasticity must also play critical roles, as discussed below.

1.6.3.1 Remyelination

Following injury in animal models, it is believed that a proportion of demyelinated and regenerating axons are remyelinated by resident oligodendrocyte precursor cells or by invading peripheral Schwann cells. Oligodendrocyte precursor cells (OPCs) have been shown to be recruited to the injury site where they can form myelin sheaths around denuded axons (Gensert and Goldman 1997; Keirstead and Blakemore, 1999). Schwann cells, which normally myelinate axons in the peripheral nervous system, have been shown to be capable of invading the injured spinal cord and remyelinating demyelinated and regenerating axons (Salgado-Ceballos et al., 1998), leading to improvements in function (Murray et al., 2001).

There has been debate as to the longevity of the spontaneous remyelination process, with some authors observing robust long term remyelination (Salgado-Ceballos et al., 1998; Powers et al., 2012). It has been reported that following thoracic contusion injury in the rat, the majority of spared axons were seen to be spontaneously remyelinated, as evidenced by at least one abnormally short internodal distance (<100 μm). These shortened internodes were significantly concentrated around the lesion epicentre where the axonal diameters were seen to be thinned by approximately 23% compared to their rostral and caudal zones. Using these observations it was predicted that the conduction velocity at the lesion epicentre would be reduced by 25% (Powers et al., 2012). Despite this, there still remains debate as to the longevity of the spontaneous remyelination process, with others reporting the process to be incomplete or abortive (Totoiu and Keirstead 2005). In recent years it has been reported that

OPCs can differentiate into Schwann cells as well as oligodendrocytes during the post-injury period (Zawadzka et al., 2010).

Endogenous remyelination has been demonstrated in primate models of SCI, where 7 months post-injury 15% of the newly divided cells were shown to display markers for mature oligodendrocytes, they were located at sites of injury induced demyelination where they ensheathed host axons (Yang et al., 2006).

Tissue from humans is only available from chronic time points, by which point most or all of the remyelinating processes might have been completed, after which point many of the remyelinated axons might have died back. Thus there is a paucity of evidence for remyelination in humans following SCI, especially at the acute stages of injury, though some studies looking at the effects of injury at chronic time points have reported Schwann cell remyelination of putatively demyelinated axons in the spinal cords of SCI patients (Guest et al., 2005). Methods of imaging the demylelination and remyelination that may occur in the acute stages of injury in primates (including man) are being developed by several laboratories using magnetic resonnance imaging (MRI), magnetization transfer imaging (MTI) and diffusion tensor imaging (DTI), as discussed by Dubois-Dalcq et al., 2008.

1.6.3.2 Plastic changes following injury

Changes in neural pathways and synapses due to changes in environment, behaviour, neural processes or changes resulting from injury are referred to as plastic changes, or plasticity. Once considered as static organs, the brain and spinal column are now known to undergo plastic changes throughout adult life. Neural contacts can be strengthened or weakened by the formation or pruning of synapses or simply through the strengthening or weakening of existing synaptic contacts. This process is termed ‘synaptic plasticity’ and was at one

point thought to be mainly limited to facilitating changes associated with learning and memory formation in the hippocampus (Gulino et al. 2007). It is now known that synaptic plasticity occurs throughout the CNS and plays an important role following injury, (Bareyre et al., 2004; Gulino et al., 2007). The term ‘anatomical plasticity’ refers to the formation of new circuits through sprouting and anatomical reorganisation (Bareyre et al. 2004). It is becoming increasingly apparent that anatomical plasticity plays an important role in the recovery of function following injury. As reviewed by Raineteau and Schwab, 2001, spontaneous sprouting of damaged axons has been documented following numerous kinds of experimental models of SCI, including contusion (Hill et al., 2001), partial transection (Fouad et al., 2001) and ischemic injury (von Euler et al., 2002). Cortical plasticity has also been shown to occur following SCI (Jain et al., 1997; Bazley et al., 2011; Aguilar et al., 2010). Importantly, plasticity has been linked with improvements in functional outcomes following injury in both experimental animals and humans (Raineteau and Schwab 2001; Edgerton and Roy 2002; Oudega and Perez, 2012).

Plasticity is known to occur at the spinal level in experimental models in response to SCI. Following peripheral nerve transection in the rat, Basbaum and Wall 1976 documented that the dorsal horn somatotopy undergoes reorganisation, whereby intact spared afferent fibres establish connections with deafferented cells, either through sprouting or unmasking of existing connections (Basbaum and Wall, 1976). It has been shown in mice and rats that sprouting in uninjured tracts in response to lesion injuries can result in the reestablishment of functional spinal circuitry leading to quantifiable improvements in functional outcomes (Weidner et al., 2001; Bareyre et al., 2005). Some studies have provided evidence for unlesioned tracts sprouting to form functional connections which ultimately take over the functions of lesioned tracts (Lawrence and Kuypers, 1968). In primate models, newly formed synapses have been observed in the spinal cord ipsilateral to hemisection injury (Aoki et al., 1986; Rosenweig et al., 2010). Other hemisection studies have shown that spontaneous plasticity so extensive that

the number of sprouting midline-crossing axons is enough to reconstitute an estimated 60% of pre-lesion axon density, leading to greatly improved behavioural outcomes (Rosenzweig et al., 2010).

After injury, cortical reorganisation is known to occur, with cortical areas receiving input from intact peripheral systems enlarging and invading the regions that have lost their peripheral inputs. Such cortical reorganisation involves both physiological and anatomical change, and has been observed in the somatosensory cortices of both experimental animals (Jain et al., 1997; Ghosh et al., 2009; Aguilar et al., 2010) and humans (Bruehlmeier et al., 1998). These changes have been detected immediately following injury in rats, accompanied by slower and more silent cortical spontaneous activity, representing a switch to a state of slow wave activity in the somatosensory network similar to that seen during slow-wave sleep; this is believed to play a crucial role in the early stages of cortical reorganisation following SCI (Aguilar et al., 2010). Substantial cortical reorganisation has also been shown to occur in the motor cortices of both experimental animals (Bareyre et al., 2004; Fouad et al., 2001) and humans (Levy et al., 1990; Raineteau and Schwab, 2001). Human studies have utilised fMRI to investigate cortical plasticity following SCI (Jurkiewicz et al. 2007). Individual sub-regions of the primary motor cortex are connected by a dense network of horizontal connections which are believed to facilitate such rapid changes (Raineteau and Schwab 2001). It has also been reported that cortical reorganisation occurs in the brain at the subcortical level following injury (Kaas et al., 1999; Bruehlmeier et al., 1998; McKenna and Whishaw 1999).

The spontaneous regenerative processes that occur following SCI, including remyelination, axonal sprouting and revascularisation make the differentiation and assessment of individual degenerative and regenerative processes all the harder (Blight and Young, 1989, Blight, 1993; Jeffery and Blakemore, 1997). Concomitantly, this also complicates the assessment of novel experimental therapies, especially as plasticity is known to exert negative and well as

positive effects. Plasticity in uninjured sensory tracts has been shown to be particularly prone to producing deleterious effects such as pain (Finnerup and Jensen, 2004; Kalous et al., 2009) and autonomic dysreflexia (Weaver et al., 2001 and 2006), although these findings were discredited in a more recent investigation (Kalous et al., 2007).

1.7 Injury type

In order to understand SCI mechanisms and to allow for the assessment of potential therapies, several animal models have been developed over the years, mostly focused on transection, compression and contusion injuries (reviewed by Rosenzweig and McDonald, 2004). As yet there is no one animal model that accurately replicates every aspect of traumatic SCI in humans; therefore several models are used to replicate different types and aspects of traumatic SCI using different species and injury methods (Anderson and Stokes, 1992). Caution should be exercised when interpreting the results as it has been shown that different strains of animal exhibit different behavioural outcomes following SCIs of different types (Mills et al., 2001).

Rodent models have been the most popular for the development of SCI models. This is largely due to their availability, cost, ease of care, relatively low susceptibility to infection and the existence of widely utilised functional assessment methodologies (Onifer et al., 2007; Toft et al., 2007; Sedy et al., 2008). Despite less being known about the recovery of function in mice, the transgenic potential of mouse models has led to an upsurge in their popularity in recent years (Pajoohesh-Ganji et al., 2010). Higher animal models, including cats, dogs and pigs have also been used on occasion (Blight and Young 1989; Jeffery et al., 2005; Navarro et al., 2012). It is known that there are significant neuroanatomical and functional differences between species; this complicates the development of models that can be used to assess human

therapies. Modelling SCI in non-human primates is therefore used in order to prove efficacy and safety before commencing clinical trials (Nout et al., 2012).

Transection

Transection injuries, whilst rarely seen in the clinical setting (eg. stab wounds), can be advantageous to model in animals. Partial or complete transections of the spinal cord are relatively simple to perform and allow investigators to examine regeneration across the lesion site (Schrimsher and Reier, 1993, Webb and Muir, 2002; Martinez et al., 2009) as such they provide excellent models for assessing neuroregeneration in long white matter tracts, and allow investigators to selectively injure designated fibre tracts in a highly reproducible manner. As such, transection injuries provide a useful and easy way of assessing the degree of axonal regeneration and functional recovery, spontaneous or induced (Takeoka et al., 2011; Ziegler et al., 2011). Many studies have utilised transection injuries, making them an attractive candidate for researchers wishing to make comparisons between studies.

Compression / Crush Injuries

Compression and crush injuries are capable of more accurately simulating the type of injury most frequently observed clinically. Compression SCI in rats results in severe ischemia and energy perturbations (Zhang et al., 1993), substantial neuronal loss at the injury epicentre and the development of a cystic cavity (Huang et al., 2007). They can be created by squeezing the cord using modified aneurysm clips (Rivlin and Tator 1978; Fehlings and Nashmi, 1995; Joshie and Fehlings 2002a and 2002b), subdural inflatable balloons (Martin et al., 1992; Vanicky et al., 2001) or placing a weight on the exposed cord (Black et al., 1986; Nystrom et al., 1988; Huang et al., 2007). By varying the degree and or duration of displacement, injury severity can be controlled (Joshi and Fehlings 2002b).

Contusion

Following contusion SCI *in vivo*, the primary injury response is known to closely reflect the sequelae observable in the majority of clinical cases and is characterised by axotomy and disruption of the vasculature and cell membranes. This initial mechanical injury is followed by a secondary response which continues for months to years following the initial injury and is characterised by continued disruption of the vasculature, excitotoxicity, oedema, inflammation, demyelination, Wallerian degeneration, glial and neuronal apoptosis, cystic cavity formation, micro-glial and (if the injury penetrated the meningese or damaged the BBB) macrophage infiltration (Blight, 1988; Bunge et al., 1993; Siegenthaler et al., 2007).

Histological examination of experimental contusion injury sites has shown that grey matter is more easily disrupted than white matter (Gensel et al., 2006; Ek et al., 2010; James et al., 2011). This is consistent with clinical observations which frequently show the creation of large cystic cavities in the grey matter, which expand further into the white matter with increasing injury severity and proximity to the injury epicentre (Edgar and Quail, 1994; Squier and Lehr, 1994).

Several observations could help explain why the injury affects the grey matter more severely than the white. It has long been known that the gray matter is more vascular than the white matter and is thus more vulnerable to the mechanical damage and vascular disturbances associated with the primary injury response (Craigie, 1920). Biomechanical analysis of bovine spinal cords *in vitro* has revealed that grey matter is more rigid, and thus more fragile than white matter (Ichihara et al., 2001). Also, experimental modelling of contusive SCI using gelatine filled tubes has shown that the longitudinal forces generated by compression are greatest in the centre of tube, in the region of the gray matter (Blight and Decrescito, 1986).

1.8 Assessments of Function

Functional assessments are required to enable correlation between the pathological and functional consequences of different injuries and intervention strategies. This allows inferences to be made about the integrity of damaged and spared tracts and grey matter circuitry and how these might change following injuries of differing severities, locations and types. Once a model is fully characterised, therapeutic interventions can be made and assessments of function compared to that seen without intervention.

Rodent behaviours have been assessed using numerous different types of tests, which can be broadly divided into behavioural tests in which an animal's movements are scored qualitatively and electrophysiological testing which quantitatively measures function in the neuronal circuitry, as discussed in detail below.

1.8.1 Behavioural testing

End point measures

End point measures are made by scoring the ability to achieve a predetermined goal. They are varied and specific to the test in question; examples of such tests include the time taken to successfully cross a horizontal ladder beam (Soblosky et al., 1997; Metz & Whishaw 2002), the grade of slope at which an animal can maintain a fixed position on a tilting surface (Rivlin and Tator, 1977) and number of foot-faults whilst walking on a grid (Metz et al., 2000). End point tests have also been devised to assess an individual limbs ability to achieve a set goal, such as the ability to reach for and grasp food (Ballermann et al., 2001; Montoya et al., 1991; Schrimsher and Reier, 1992) or to remove a sticker from their forehead or paw (Schrimsher and Reier, 1992, Onifer et al., 2005). Despite the advantages of using end point measures of behavioural function, they also have several caveats. For example,

compensatory behaviours are not differentiated from recovery behaviours, thus animals that learn to deal with deficits, which may lead to higher scores on end-point behavioural tests, are recorded the same as if recovering function (Whishaw et al., 1998; Mckenna & Wishaw 1999; Webb and Muir, 2002). Another caveat is the degree of variability observed in each animal's ability to achieve end point goals (Biesiadecki et al., 1999). It is thus a necessity to ensure that each animal's pre-injury score is recorded for use as an internal control when comparing with the scores it achieves post-injury; for this same reason it is important to exercise caution when comparing studies to ensure that the animals used in each study are of a similar age, sex and strain. It is also important to ensure that injury and control groups are capable of achieving similar scores prior to injury, which allows for more accurate comparisons to be made between the groups. Most behavioural tests that rely on end-point measures produce quantitative data; however in some instances the use of an ordinal scale has been applied (Basso et al., 1995). As discussed below in greater detail, caution should be exercised when interpreting or performing statistical analyses to the results of any behavioural test which utilises an ordinal scale.

Kinematic measures

"Kinematic measures" include anything from a qualitative description of an animal's movements to a continuous, quantitative record of an animal's movements; they are made by taking measurements of the whole body, limbs or body segments and how they move relative to each other and/or external reference points and are thus capable of producing either qualitative or quantitative data depending on the actual scoring system employed. Like all behavioural tests, different types of kinematic assessments have their advantages and disadvantages; the main advantages are that some types of kinematic test can be relatively easy and cheap to perform and are capable of detecting discrete differences in fine movements which would not be detectable using end-point measures alone (Sedy et al., 2008; Couto et al., 2008). The main disadvantages are that data acquisition in some types of test

can be highly labour and or time intensive, usually producing qualitative data (Whishaw & Pellis 1990; Whishaw et al., 1992; McKenna & Wishaw 1999; Alluin et al., 2011). Some kinematic tests make use of ordinal scales (Basso et al., 1995) which, as discussed in greater detail below, should be treated with caution when interpreting.

In some cases, through the use of sophisticated software and hardware, greater degrees of automation are possible, which can aid in the generation of quantitative data. Whilst the ability to analyse greater volumes of kinematic data and detect minor variances between conditions is highly advantageous, such systems are usually highly expensive (Alaverdashvili et al., 2008; Springer et al., 2010; Gravel et al., 2010).

- Locomotor assessment scales

Many kinematic tests have been developed to analyse functional abilities following spinal cord injury, mostly in the form of locomotor assessment scales which utilise ordinal scoring systems. The most widely used of these is the Basso, Beattie and Bresnahan locomotor rating scale (BBB scale), which scores an animal's hind-limb kinematic abilities on an ordinal scale from 0-21, with a score of 21 representative of uninjured animals and a score of 0 indicating no hind-limb motor function (Basso et al., 1995). This scoring system has been shown to be easily learned, highly reliable and capable of producing similar results from one lab to the next (Basso et al., 1995), and as a result has been widely apopted as the standard for locomotor assessment following SCI. Unfortunately, the BBB scoring system has been shown to have several limitations, mainly due to its reliance on an ordinal, non-linear scale in which the distinctions between each of the categories are defined arbitrarily, this has been shown to produce complications when interpreting the results, eg. an improvement of +1 at the lower end of the scale (<13) equates to the re-appearance of a gross motor ability, yet an improvement of +1 at the upper end of the scale (>13) equates to the re-appearance of a relatively discrete characteristic of normal locomotion (Metz et al., 2000); thus the

interpretation and analysis of data is complicated greatly. Whilst it could be argued that the only real option for statistical analyses is the use of non-parametric tests, there are currently no non-parametric tests available for use with repeated measures. The BBB scale is known to be less sensitive at the lower end of the scale, and had been shown to be more sensitive once animals start to achieve scores above 14 (Ferguson et al., 2004); this is likely due to the confounding effects of the injury, including inflammation and pain. Another limitation of the BBB score is that it relies heavily on the ability to accurately assess forelimb-hindlimb coordination, however, in injuries that affect the forelimbs the animal may exhibit severe functional deficits but remain coordinated.

In order to address the limitations of the BBB scale, several groups have made additions and modifications to the scoring system which improve its ability to differentiate between animals of different injury levels, severities and types (Lankhorst et al., 1999; Ferguson et al., 2004; Koopmans, 2005; Wong et al., 2009). Despite these scales still relying on ordinal scales of functional recovery, they do allow that recovery to occur in any order, which is their main advantage over the BBB scale. Despite this, the BBB is still a sensitive and reliable tool for assessing function following thoracic contusion injured rats and for serving as an effective method of making comparisons of results from different laboratories.

- Continuous Kinematic Tests

Continuous kinematic tests are a type of kinematic test capable of obtaining quantitative data from an animal's movements, usually through the use of cameras and sophisticated computer software. Such tests are capable of measuring a vast range of movement parameters, including the distances, speeds, and angles of each joints, limbs, and in some cases digits movements. Continuous kinematic tests lend themselves particularly to the detailed analysis of gait. Data were originally acquired by videoing the movement under

investigation and analysing the video frame by frame in a highly time- and labour-intensive process (Clarke, 1991; Cheng et al., 1997).

Since then, several systems have been designed which enable the semi-autonomous collection of numerous gait parameters, including systems which allow for the analysis of an animals gait, recorded from below, (Hamers et al., 2006) including several in which animals are made to walk at pre-determined speeds through the use of specially designed treadmills (Li et al., 2005b; Couto et al., 2008; Beare et al., 2009). Some authors have even elected to use systems that allow recording from several angles to enable analysis of movement in 3D (Couto et al., 2008). Several laboratories are currently experimenting with the use of Digital Motion X-ray (DMX) technology which allows for real-time x-ray scanning of animals performing behavioural tests, by using bones as points of reference (Alaverdashvili et al., 2008; Gravel et al., 2010).

Sensory testing

To measure and detect changes in the degree of cutaneous tactile sensitivity, testing known as aesthesiometry testing can be performed. By far the most commonly utilised aesthesiometry test is called the von Frey test; this involves using nylon filaments in a linear scale of physical force, applied to the skin one at a time with enough force to make the filament bend which may or may not elicit a response from the animal. If no response is observed then the filament is withdrawn after 2 seconds and the next strongest filament is applied; this is repeated until an avoidance response is elicited, as discussed in greater detail in Chapter 3.

The von Frey test is quick, easy and inexpensive to perform, and provides a highly sensitive, quantitative measure of cutaneous tactile sensitivity with limited inter-and intra-rater variability (Chaplan et al., 1994; Dixon et al., 1980). Such tests are usually performed on the plantar surfaces of the paws to

investigate changes in sensation and the development of tactile allodynia (Chaplan et al., 1994), though increasingly researchers are also using von Frey tests across the animals torso to investigate the response to mechanical stimulation nearer the injury level (Lindsey et al., 2000).

1.8.2 Electrophysiological testing

Electrophysiology is the study of the electrical properties of biological cells and tissues. Direct measurements of electric current or voltage change can be made from whole organs to single ion channels. Using animal models of SCI, electrophysiological recordings can provide a direct measure of the functional abilities of neurological systems and therefore avoids many of the complications that exist with behavioural testing, such as distinguishing functional recovery from adaptive mechanisms.

Cord dorsum potentials

A cord dorsum potential (CDP) is a series of waves that can be recorded from the skin overlying the vertebral column, or directly from the surface of the exposed spinal column. The characteristics of CDPs reflect the level of electrical activity in the ascending somatosensory white matter in the dorsal columns, as well as the activation in local circuits following electrical stimulation of peripheral nerves (Sedgwick et al., 1980).

CDPs can be recorded following stimulation of sensory tracts and are widely used to assess the functional abilities of pathways in the spinal column following injury as well as providing a way to monitor the health of the spinal column during spinal surgery (Sedgwick et al., 1980; Ahn and Fehlings, 2008). CDPs provide a sensitive and reliable method of monitoring the effects of experimental SCIs and the effects of potential therapeutic interventions, and are thus widely used in SCI research (Bradbury et al., 2002; Toft et al., 2007).

Somatosensory evoked potentials

A somatosensory evoked potential (SEP) is a series of waves that can be recorded from the somatosensory cortex. The characteristics of SEPs reflect the sequential activation of neural structures along the somatosensory pathways following electrical stimulation of peripheral nerves (Sedgwick et al., 1980).

When action potentials arrive in the somatosensory cortex they are capable of causing a population of cells, primarily located in cortical layer V to become depolarized, as ionic currents are leaked from the neurons. A collection of such depolarisation events generated by large groups of neurons firing simultaneously results in a field potential which can be recorded from a distance.

Recordings of SEPs are used to provide direct quantitative measures of the functional abilities of the ascending somatosensory pathways. SEPs are frequently used for the diagnosis and characterisation of neurological abnormalities in humans (Small et al., 1978; Dietz and Curt, 2006) and are capable of measuring dysfunction in the peripheral nerve, the spinal roots, the ascending somatosensory pathways of the spinal cord, the dorsal column nuclei in the medulla, the medial lemniscus pathways, the ventral posteriolateral nucleus of the thalamus, the posterior limbs of the internal capsules or the somatosensory cortices. SEPs are also widely used for intraoperatively monitoring the condition of the spinal cord in real time (Grundy, 1983). Such direct feedback on the condition of the neural networks and pathways allows surgeons to safely perform more aggressive surgeries, such as spinal tumor resection and the correction of deformities (Malhotra et al., 2010).

Using a clip compression model of SCI in rats it has been shown that a correlation exists between the amplitudes and latencies of SEPs and the severity of the injury; thus it was shown that SEPs can be used to assess the physiological integrity of the cord following injury (Fehlings et al., 1989).

These findings have been confirmed by other studies that also found correlations between injury severities, SEP amplitudes/latencies and the scores obtained in several behavioural tests, where lower amplitude, longer latency SEPs correlate with reduced behavioural scores (Nashmi et al., 1997). Thus it would appear that the electrical activity in the somatosensory pathways is linked to the functional abilities of the animal.

By comparing SEPs generated in normal animals with those generated in SCI animals with and without potential therapeutic interventions we can accurately and quantitatively measure the affects of both the injury and potential therapeutic interventions.

1.9 Repair strategies

As mentioned previously, the degree of spontaneous recovery following SCI is usually too limited to reinstate functional deficits completely. Consequently, many of the functional deficits caused by SCI are permanent. There are currently no therapeutic interventions which are capable of repairing the damage caused by SCI.

The complex sequelae that occur following SCI seriously hampers the recovery process, as discussed above. Several processes are required to occur in order to repair damaged tissue and restore functional deficits. Injured cells must first survive, only then might a damaged axon extend its cut processes to make connections with its original targets. In order for these connections to be functional, the axons must be remyelinated and the synaptic contacts must be functional. Repair strategies are thus designed to address one or more of these problems, and can be classified as one of four different approaches: the neuroprotective approach which attempts to reduce the damage caused by the secondary injury process and to preserve residual functions, the regenerative approach which attempts to create a environment that is permissive to axonal regeneration, the plasticity approach which attempts to create an

environment that promotes axonal sprouting and the remyelination approach which attempts to restore conduction deficits by attempting to create an environment which is conducive to remyelinating demyelinated and regenerating axons. Several different repair strategies have been employed to target each of these approaches in order to induce and support the reparative potential of each (Fawcett, 2002).

1.10 Therapeutic interventions

Numerous different therapeutic interventions are currently being used in phase I and phase II clinical trials, or are about to enter clinical trials (Kwon et al., 2010). No therapy with substantial beneficial effects has yet made it to phase III clinical trials or reached the clinic, although several treatment strategies which offer limited beneficial potential are currently available clinically. The only pharmacological therapy currently available involves the administration of high dose anti-inflammatory steroids such as methylprednisolone (reviewed by Breslin and Agrawal, 2012). If required, strategically timed surgical procedures designed to prevent further tissue damage have also been employed (reviewed by Fehlings and Perrin, 2006). The only other therapeutic interventions available following human SCI involve rehabilitative training designed to maintain and optimise residual functions and to reduce the effects of secondary problems such as muscle wastage, urinary tract infections and poor ventilation (McDonald et al., 2002; Behrman et al., 2006).

Whilst single therapies may prove sufficient to overcome individual injury-induced deficits associated with SCI, it is highly likely that multiple, simultaneously administered therapeutic interventions will be required to provide a solution for SCI treatment. Efficacious treatment strategies may involve treatments that provide a growth-permissive substrate in the lesion cavity, preserve residual neurons, reduce or prevent the secondary injury response, overcome the inhibitory effects of myelin debris, reduce or prevent

the inhibitory effects of the lesion scar, utilize neurotrophic factors to prevent axonal atrophy and promote axonal regeneration, promote spontaneous and/or exogenously enhanced remyelination and plasticity (Lu et al., 2004; Pearse et al., 2004; Houle et al., 2006). In the chronic stages, it is likely that rigorous rehabilitative training protocols will further aid functional recovery (McDonald et al., 2002).

1.10.1 Neuroprotection

Neuroprotection is the term used to describe all the different processes which act to reduce the effects of the secondary injury response and to reduce further tissue damage, thus preserving surviving spinal pathways and residual functions. Many neuroprotective therapies are currently being investigated to target the numerous different, concomitantly occurring, injury mechanisms that occur following traumatic SCI.

Methylprednisolone sodium succinate is a synthetic corticosteroid with potent anti-inflammatory properties. It is believed that methylprednisolone exerts its neuroprotective actions by scavenging free-radicals from the injury site (Hall, 1992) and down-regulating pro-inflammatory gene expression (Almon et al., 2002; Fu and Saporta, 2005). It is the most widely studied neuroprotective therapy for SCI and the standard clinical practice for acute SCI in the USA (reviewed by Bracken, 2012). When administered acutely, methylprednisolone has been shown to have beneficial effects on functional recovery in randomized, double-blind, placebo-controlled clinical trials (NASCIS II and III) (Bracken et al., 1990 and 1997). However, these studies have been criticized for problems with study design and statistic analysis (Coleman et al., 2000). Others have claimed that there is a lack of evidence for efficacy and a greater degree of clinical problems associated with methylprednisolone (Sayer et al., 2006; Suberviola et al., 2008).

Another promising neuroprotective agent is the cytokine erythropoietin, which has been shown to have potent anti-inflammatory and anti-apoptotic properties (Arishima et al., 2006). Erythropoietin has also been shown to restore vascular integrity, induce neural regeneration and act as a mediator of hypoxic preconditioning; erythropoietin thus has potential as a beneficial therapy for SCI (reviewed by Brines and Cerami, 2008). Despite this, erythropoietin has the disadvantage of being responsible for increasing the aggregation of thrombocytes and augmenting haematocrit (Brines and Cerami 2008). To combat these problems, mutant erythropoietin analogues, such as carbamylated erythropoietin (Kirkeby et al., 2008) and asialo-erythropoietin (Erbayraktar et al., 2003) have been created which do not evoke the same rheological complications.

Several other pharmacological interventions have been shown to be beneficial following SCI. Reducing the action of tumor necrosis factor (TNF) by inhibiting the CD95 receptor has been shown to decrease the degree of glial and neuronal apoptosis following SCI in mice (Demjen et al., 2004; Casha et al., 2005). Minocycline application has been shown to lessen the degree of apoptosis observed in oligodendrocytes as well as reducing the glial reaction. It is uncertain as to whether the surviving cells are functional, and it is not known how long they survive for; despite this, improvements in neurological function have been documented following minocycline application (Beattie, 2004; Stirling et al., 2004).

Despite the promising pre-clinical trials achieved with the application of neuroprotective pharmacological interventions, the vast majority of phase II/III clinical trials with such agents have failed to show significant efficacy (Dumont et al., 2001; Hawryluk et al., 2008).

Certain acutely implemented surgical interventions, designed to decompress the swollen cord as soon as possible following injury have also been shown to be beneficial in animal models (Mirza et al., 1999; Fehlings et al., 1999;

Papadopoulos et al., 2002). When performed within 24hrs of injury, decompression surgery has also been shown to result in improved outcomes in humans (Fehlings et al., 2012).

1.10.2 Neurotrophins

Neurotrophins are a class of small molecules capable of signaling to cells to differentiate or extend their neurites. In the injured spinal cord, the lack of neurotrophic molecules is a major contributing factor in the failure of neurons to regenerate.

There are numerous types of neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophins 3, 4 and 5 (NT-3/NT-4/NT-5), glia derived neurotrophic factor (GDNF) and fibroblast growth factor (FGF). Following injury, neurotrophins act by signaling to their target cells to upregulate the genes associated with regenerative processes, thereby enhancing cell survival (Bradbury et al., 1999; Ramer et al., 2000; Blesch and Tuszynski, 2003; Geremia et al., 2010). Neurotrophins also play an important role in targeting and regulating neuron-target interactions (Kobayashi et al., 1997; Kwon et al., 2002; Novikova et al., 2002).

NT-3 is perhaps one of the most promising neurotrophins studied, and as such it has received a lot of attention from different groups. It has previously been shown following SCI in the rat that a single injection of NT-3 at the lesion site was sufficient to promote sprouting of corticospinal axons (Schnell et al., 1994). It has also been shown that NT-3 is capable of partially ameliorating functional deficits, including when applied via acute cellular delivery into a hemisection injury (Grill et al., 1997) and also when injected alongside fetal spinal cord transplants 2-4 week following injury (Coumans et al., 2001). Whilst some studies have found other neurotrophins to be less able to promote axonal growth and sprouting (Bradbury et al., 1999) others have reported BDNF

to have neuroprotective and neuroregenerative effects (Namiki et al., 2000). The spinal cord contains different densities of receptors that correspond to each different neurotrophin, this may account for the different performance among those neurotrophins.

Despite their beneficial role following SCI, several complications exist with regards the application of neurotrophins for the treatment of SCI. For example, as well as their ability to ameliorate the effects of the secondary injury response, neurotrophins have been associated with the development of neuropathic pain (Pezet and McMahon 2006; Siniscalco et al., 2011). Other factors further complicate the use of neurotrophins for the treatment of SCI including the various degrees of permeation across the blood brain barrier seen with different neurotrophins (Pan et al., 1998), as well as the fact that different neurotrophins are known to play active roles at different time points following injury (Novikova et al., 2002). Thus the choice of neurotrophins, timing of applications and delivery methods require further investigation before viable clinical therapies can be developed.

1.10.3 Myelin inhibitors

Following injury several well characterized myelin-associated inhibitors of neurite growth are released in regions where oligodendrocyte death occurs. This plays a significant role in enhancing the growth-inhibitory characteristics of the injured cord and presents a substantial obstacle to regenerating axons.

The major CNS myelin-associated inhibitors of neurite growth include Nogo-A protein (GrandPré et al., 2000; Chen et al. 2000), myelin-associated glycoprotein (MAG) (McKerracher et al. 1994; Schachner and Bartsch 2000) and oligodendrocyte-myelin glycoprotein (OMgp) (Mikol and Stefansson, 1988; Wang et al., 2002); though several other molecules, including Semaphorin 4D and ephrin B3, are known to exert growth inhibitory effects on neurites

following SCI by acting as repulsive axon guidance cues as well as stimulating growth cone collapse (Moreau-Fauvarque et al., 2003; Benson et al., 2005).

1.10.3.1 Nogo

Nogo is the name given to a gene which gives rise to three different protein isoforms created by different splicing and promoter usage, these proteins are called Nogo-A, Nogo-B and Nogo-C and are found mainly in the endoplasmic reticulum. Nogo-A is a high molecular weight trans-membrane protein which is heavily expressed by oligodendrocytes (Schwab, 2004) and is the most intensively studied of all myelin inhibitors (Schweigreiter and Bandtlow, 2006).

Nogo-A contains 3 regions that are exposed to the extracellular space and which are inhibitors of CNS regeneration: A region of the C-terminus containing a 66 amino acid loop called Nogo-66, a central inhibitory domain known as NiG and a region on the N-terminus (reviewed by Wang et al., 2012). The Nogo-66 and NiG regions are both inhibitory to neurite outgrowth and are known to induce growth cone collapse (GrandPré et al., 2000; Oertle et al. 2003), whilst the N-terminus region is known to inhibit fibroblast spreading.

Several studies have utilized Nogo knockout mice to investigate the effects of the different Nogo isoforms on regeneration in the injured spinal cord (Simonen et al. 2003; Kim et al. 2003; Zheng et al. 2003; Lee et al., 2009). Whilst some saw modest improvements in axonal regeneration and function (Simonen et al. 2003; Kim et al. 2003), others did not (Zheng et al., 2003; Lee et al., 2009). This suggests that Nogo alone does not account for the lack of regeneration in the CNS, although other variables might account for these differences, such as the types of injuries used (Cafferty and Strittmatter, 2006), differences in the genetic backgrounds of the strains used (Dimou et al., 2006) and the compensatory up-regulation of alternate Nogo isoforms (Simonen et al., 2003).

The inactivation of Nogo-A has been achieved through the use of function-blocking antibodies as well as by interfering with its receptors and signalling pathways; this has been shown to result in substantial axonal regeneration below the injury level in both rats (Schnell and Schwab, 1990; Li et al., 2004; Liebscher et al., 2005) and non-human primates (Fouad et al., 2004; Freund et al., 2006; 2007). In rat and mouse models of SCI, blocking Nogo-A is known to result in substantial axonal regeneration and an impressive level of functional recovery (Schnell and Schwab, 1990; Schwab, 2004; Leibscher et al., 2005). Blockade of the Nogo-A receptor has also been shown to promote recovery of manual dexterity in non-human primate models of SCI (Freund et al., 2006).

1.10.3.2 Myelin-associated glycoprotein

MAG is a type I trans-membrane glycoprotein which is part of the immunoglobulin superfamily, which are known to important roles in cell adhesion, as such it contains five extracellular immunoglobulin Ig-like domains. In the rodent, alternate splicing of MAG mRNA yields two different isoforms, called simply, large (L) and small (S), which activate different signal transduction pathways in myelin forming cells (Lai et al., 1987; Salzer et al., 1987).

MAG is found in high quantities in both the oligodendrocytes of the CNS and to a lesser extent the Schwann cells of the PNS, localized in the periaxonal myelin sheaths, where it plays an important role in interactions between glial and neuronal cells (Sternberger et al. 1979; Poltorak et al., 1987; Schachner and Bartsch, 2000). Whilst MAG is not required for myelination, it plays crucial roles in enhancing long term axon-myelin stability as well as regulating the axonal cytoskeleton and helping to structure the nodes of Ranvier (reviewed by Schnaar and Lopez, 2009). MAG also appears to play an important role in axon-myelin stability through mediation of communications between neuronal

and glial cells, though these roles may differ between the CNS and the PNS (Schachner and Bartsch, 2000; Quarles, 2007). Combined, these properties contribute to MAG's role in preventing axonal degeneration (Nguyen et al., 2009). In addition to its axon-myelin stabilizing role, following injury MAG also acts as a potent inhibitor of axonal regeneration. Both the axon-stabilizing and growth-inhibiting roles played by MAG are mediated via 2 families of receptors in the axonal membrane, namely members of the sialoglycan family (gangliosides GD1a and GT1b) (Yang et al., 1996) and the aforementioned Nogo receptors (Liu et al., 2002).

In the injured spinal cord MAG is usually present in large quantities, bound to residual myelin debris. Its presence contributes to the potently growth inhibitory environment of the injured cord (McKerracher et al. 1994; Mukhopadhyay et al. 1994).

1.10.3.3 Oligodendrocyte-myelin glycoprotein

OMgp is a myelin associated inhibitory protein that is mainly expressed in long projection neurons and white matter in the spinal column (Mikol Stefansson 1988; Habib et al., 1998). Whilst OMgp is less well characterized than Nogo-A or MAG, it is known to also be a potently inhibitory molecule present in the injured spinal cord, and has been shown to be capable of causing growth cone collapse and inhibiting neurite growth (Wang et al. 2002a). When compared to Nogo-A or MAG, OMgp makes up a much smaller component of the myelin sheaths found in the CNS or PNS; however it may be present to a much greater degree in other non-myelinating cells (Huang et al, 2005).

All of the aforementioned myelin inhibitors are known to exert their effects, at least in part, through common receptors and co-receptors, including the NgR1-p75 receptor complex (Wang et al., 2002b) and PirB (Atwal et al., 2008).

Thus, antagonizing these receptors can impede the actions of all three myelin inhibitors and has been shown to induce a significant reduction in the degree of myelin inhibition (Atwal et al., 2008).

1.10.4 Lesion scar inhibitors

Following SCI, the formation of a lesion scar occurs as part of the endogenous local immune regulation and repair process. Whilst this leads to the creation of an environment which is inhibitory to regenerating axons, the lesion scar also has beneficial effects on recovery.

The injury site is characterized by features which are toxic to regenerating axons, such as the inflammatory response, an imbalance in the levels of ions and excitatory amino acids, free radicals, reactive oxygen. The formation of a lesion scar which encapsulates the injury site and isolates it from the rest of the spinal cord thus plays a beneficial role in the injury process (Rolls et al., 1999). Dichotomously, the lesion scar is also known to have potent growth inhibitory properties. The lesion scar consists mainly of reactive astrocytes and microglia/macrophages, all of which, though especially reactive astrocytes, release various chondroitin sulphate proteoglycans (CSPGs) with their glycosaminoglycan side chains (GAGs) into the extracellular space, where they act as potent growth-inhibitors (Morgenstern et al., 2002). CSPGs expression increases rapidly following injury, peaks within 2 weeks and persists for several months (Tang et al., 2003).

It is known that CSPGs are inhibitory to neurite growth across the injury site (Silver and Miller, 2004) and negatively affect axonal conduction through the injury site (Hunanyan et al., 2010). Injections of the bacterial enzyme chondroitinase-ABC (ChABC), which is know to digest the GAG sidechains of CSPGs, have been shown to reverse or prevent axonal conduction deficits

whilst simultaneously stimulating axonal growth and promoting locomotor recovery (Bradbury et al., 2002; Hunanyan et al., 2010). Functional recovery may be due to the stimulation of plastic changes around the injury site, as it has been observed as quickly as one week following injury (Bradbury et al., 2002); though axonal regeneration may also play a role, with some investigators not observing significant functional recovery until 42 days following injury (Hunanyan et al., 2010). Electrophysiological investigations have shown that ChABC induced reorganization of primary afferent terminals plays a role in improved functional outcomes (Cafferty et al., 2008). ChABC has also been shown to potently induce axonal sprouting in uninjured tracts contralateral to the injury, resulting in sprouting fibers crossing to the denervated side of the cord (Barritt et al., 2006). ChABC treatment has been shown to improve functional outcomes when applied up to 7 days following injury, which suggests it may have significant clinical potential (Garcia-Alias et al., 2008).

Several other growth inhibitory molecules have been shown to be upregulated in cells participating in the secondary injury response which are present in the lesion scar, including semaphorin 3, slit proteins and ephrin-B2, all of which further complicate our understanding of the causes of regenerative failure following SCI (reviewed by Silver and Miller, 2004), whilst also providing additional targets for potential therapeutic interventions.

1.10.5 Immunomodulatory treatments

As mentioned previously, the inflammatory response that occurs following SCI is known to have both beneficial and detrimental effects on outcome. Many studies have shown that modulating the immune response can affect functional outcomes following SCI.

Activated macrophages which participate in the inflammatory response are known to secrete a cytokine called tumor necrosis factor alpha (TNFα) as part of the immune response. Blocking TNFα has been shown to result in reductions in lesion size and significant functional improvements (Bethea et al., 1999). As discussed above, the immune response is also known to up-regulate the expression of numerous interleukins, such as IL-6 and IL-1. Blocking IL-6 receptors has been shown to reduce astrogliosis and the development of scar tissue, leading to improved functional outcomes (Okada et al., 2004), and administering IL-1 receptor antagonists has been shown to decrease the degree of injury-induced apoptosis (Nesic et al., 2001).

Macrophage recruitment and activation has been shown to have both detrimental (Blight, 1985; Blight, 1992) and beneficial (Rapalino et al., 1998; Popovich et al. 2002; Vallieres et al. 2006; Schwartz and Yoles 2005) effects on outcome following traumatic SCI; however, reduction of the macrophage response has also been shown to have beneficial effects on functional outcomes (Popovich et al., 1999).

1.10.6 Cell transplantation

Following most types of spinal cord injury, especially contusion injury, a large fluid filled cystic cavity develops centered around the injury site, as discussed in greater detail earlier. In order for regenerating axons to transverse the cystic cavity a growth permissive substrate is required; this could be achieved through the use of therapeutic cellular transplantations into the cavity. There are several advantages to using cellular transplants other than simply providing a supportive structure through which regenerating axons could grow. These include the possibility that transplanted cells might limit the amount of neuronal and glial cell death, reduce demyelination of surviving axons, promote the regeneration of surviving axons and supporting glia, induce remyelination, replace the damaged neural tissue and promote plasticity.

Many different cell types have already been investigated as potential therapies for treating the effects of SCI, including foetal tissue (Coumans et al., 2001), embryonic stem cells (McDonald et al., 1999), neural stem cells (Akiyama et al., 2001), olfactory ensheathing cells (OECs) (Franklin et al., 1996; Li et al., 1997; Ramón-Cueto et al., 1998; Riddell et al., 2004; Toft et al., 2007), oligodendrocyte precursor cells (Groves et al., 1993; Bambakidis and Miller 2004), Schwann cells (Duncan et al., 1981; Tuszynski et al., 1998) and mesenchymal stem cells extracted from bone marrow (Nandoe Tewarie et al., 2006; Vaquero and Zurita, 2009).

1.10.6.1 Stem cells

Stem cells are characterized by their capacity for self-renewal and differentiation into different cell types. Stem cells in the early embryonic phase are capable of differentiating into cells of any lineage and are therefore described as being totipotent. After a few divisions they becomes programmed to differentiate into cells from one of the three germ layers: the endodermal layer gives rise to the epithelium of the gastrointestinal, urinary and respiratory tracts, as well as the outer layers covering most of the internal organs; the mesodermal layer gives rise to bone, muscle, blood cells and mesenchyma (connective tissues); the ectoderm layer gives rise to nervous system and the epidermis (skin cells). Stem cells at this stage are still capable of further differentiation into the numerous cell types that each germ-layer gives rise to, and are therefore described as being multipotent.

It was believed that the process of differentiation from totipotent to multipotent stem cells was irreversible; however researchers have now proven the capability of multipotent stem cells to revert back and differentiate into cells from any germ-line in a process called transdifferentiation (reviewed by Tsonis, 2000). This capability, along with the capability of stem cells to be expanded in the quantities required for cellular transplantation therapies has

prompted a great deal of research into the use of stem cells for the treatment of several conditions that would benefit from cellular replacement and or repair, including treatments for SCI.

Stem cell therapies could be used to replace cells lost by SCI and prevent further loss by releasing or stimulating the release of neuroprotective factors as well as to stimulate axonal regeneration and remyelination. Numerous types of stem cells have been investigated as potential therapies for different types of SCI, with many appearing to show beneficial effects (reviewed by Coutts and Keirstead, 2008; Barnabé-Heider and Frisén 2008). Stem cells have been shown to play a role in several different processes which are believed to contribute to the restoration of functional deficits following SCI. These include the provision of trophic molecules which act to ameliorate damage and rescue neurons and oligodendrocytes, the creation of a substrate which is permissive to axonal growth and the remyelination of spared but demyelinated axons (Parr et al., 2007). Several groups have reported improved locomotor and/or sensory performances following stem cell transplantation into mouse (Cummings et al., 2005, 2006) and rat models of SCI (McDonald et al., 1999; Gu et al., 2012; He et al., 2013). Pre-differentiated OPCs have been shown to be a viable and promising candidate for cell transplantation therapies designed to promote remyelination and functional improvements (Keirstead et al., 2005)

It is difficult to accurately ascertain the mechanisms by which these functional improvements occur, be they through neuroprotective, neuroregenerative or other mechanisms, but it is likely that several mechanism are contributing to the improved functional outcomes which are seen. Despite this several human trials have been approved and are currently underway (Vogel et al., 2005, Illes et al., 2011), with some preliminary results suggesting modest functional improvements (Illes et al., 2011).

1.10.6.2 Schwann cells

Schwann cells are responsible for myelinating and stimulating the regeneration of axons in the PNS. They have become one of the most intensively studied types of cell for the treatment of SCI since the seminal work of Ramon Cajal, who in 1928 discovered their axonal growth promoting properties after grafting peripheral nerve grafts into the CNS (Ramon Cajal, 1928).

Schwann cells play an important role in spontaneous repair mechanisms following PNS injury, thanks largely to their innate ability to dedifferentiate, proliferate, migrate, express growth promoting factors, promote axonal regeneration and remyelinate demyelinated and regenerating axons (reviewed by Bunge and Wood, 2012); all of these properties are also highly advantageous for CNS repair. Schwann cells also offer the possibility of simple autologous cell transplantions; they can be quickly harvested from the peripheral nerves, easily purified and cultured in large enough numbers for transplantation.

Upon transplantation of cultured Schwann cells into spinal injury sites, researchers have documented their ability to promote regeneration of damaged axons and remyelinate demyelinated axons (Blight and Young, 1989; Li and Raisman 1994; Tuszynski et al. 1998; Kohama et al. 2001). However, the beneficial role that Schwann cells play is largely restricted to astrocytes-deficient areas due to the inhibitory interactions that have been shown to exist between the two cell types, thus limiting their potential as treatments for CNS repair (Baron-Van Evercooren et al. 1992; Shields et al. 2000; Lakatos et al. 2000). Despite this, several groups have reported remyelination leading to improved electrophysiological function following Schwann cell transplantation into animal models of SCI (Blight and Young, 1989; Kohama et al., 2001). Other studies have revealed that Schwann cell transplants can lead to improved locomotor function (Takami et al., 2002; Biernaskie et al., 2007; Ban et al., 2009).

It is clear that Schwann cells are promising candidates for repair of the injured spinal cord; however it has been shown that their reparative potential can be enhanced when applied in conjunction with other therapeutic interventions (Ban et al., 2011; Hu et al., 2013). Recently, clinical trials focusing on co-transplantation of Schwann and mesenchymal stem cells have been performed, revealing modest improvements in the ASIA sensory scale and proving the safety of the protocol (Yazdani et al., 2013).

1.10.6.3 Foetal cells

The ability of foetal cells to differentiate into different cell types has made them attractive candidates for treating a variety of neurodegenerative disorders. Whilst many studies have made use of foetal cell transplantations, the work done in Parkinson's disease is potentially their most successful application to date (Freed et al. 1992; Spencer et al. 1992).

Foetal cell transplants have been shown to reduce functional deficits following many types of SCI (reviewed by Reier et al., 1992). It has been shown that transplanted foetal cells release trophic factors which act to reduce cell death following injury, and may play a role in the observed axonal regeneration. Importantly, regenerating axons have been observed not only within, but also extending out of the transplanted region, which raises the possibility that functional connections might be reestablished (Reier et al., 1992). It has been shown that foetal stem cells are capable of significantly improving locomotor function when administered acutely following spinal cord contusion (Bottai et al., 2010).

Whilst the results from foetal transplant studies are highly promising, their potential for clinical application is restricted by their limited availability as well as the ethical issues the use of human foetal tissue raises, especially as the embryo is destroyed in order to obtain the required cells. However,

refining cell culturing methodologies may reduce the impact of these restrictions.

1.10.6.4 Olfactory ensheathing cells

The olfactory system occupies a unique position at the interface between the PNS and the CNS. Throughout adult life the axons of olfactory receptor cells are uniquely capable of regenerating, reentering the CNS and forming functional synaptic connections with their target neurons. The ensheathing cells of the olfactory neurons are called olfactory ensheathing cells (OECs) and have been shown to confer this ability by providing structural, functional and metabolic support to the regenerating axons (Doucette 1984 and 1991). OECs have been shown to support axonal growth and ensheathment in a similar fashion to Schwann cells, though are able to survive in the CNS (Farbman and Squinto, 1985; Chuah and Au, 1994). These properties make OECs highly promising candidates for transplantation therapies designed to treat neurodegenerative diseases of the CNS, with notable potential for the treatment of spinal injuries.

Over the last few decades, OECs have been used as experimental therapeutic interventions in numerous animal models of SCI, which have for the most part focused on axonal regeneration, remyelination and functional recovery. Of the injury models tested, most have utilized lesion, compression, contusion or chemical injuries, using mainly mice or rats. Numerous functional assessments and histological investigations have been used to evaluate the effects of OEC transplantation after SCI; these include a diverse range of behavioural tests, electrophysiological and immunohistological assessments.

A great deal of evidence has been collected that suggests OECs exert a positive influence on recovery after SCI, however findings have been mixed – for

example: Several studies have reported that OEC transplants promote long distance axonal regeneration (Ramón-Cueto et al., 2000; Li et al., 1998; Nash et al., 2002; Imaizumi et al., 2000; Ramer et al., 2004a) which may explain the observed improvements in function (see below), whilst others have failed to observe this (Ruitenberg et al., 2003, 2005; Lu et al. 2006; Ramer et al. 2004b; Andrews and Stelzner 2004; Deumens et al., 2013). It has recently been shown that differences in the source of OECs and the culture methods could account for differences in their ability to promote axonal survival and regeneration (Richter et al., 2005; Novikova et al., 2011). Differences have also been seen in the reported efficacy of OECs to improve behavioural assessment scores. Some researchers have observed that OEC transplants promote improvements in behavioural assessment scores (Ramón-Cueto et al., 2000; Plant et al., 2003; López-Vales et al., 2006), whilst others have failed to see any significant improvements in behavioural assessment scores (Takami et al., 2002; Riddell et al., 2004; Ruitenberg et al. 2005).

Electrophysiological testing around the injury site has revealed OEC-induced improvements in local circuitry with no observable long-distance axonal regeneration (Toft et al., 2007). This is indicative of OECs influencing mechanisms other than the promotion of axonal regeneration, such as neuroprotective and plastic processes, leading to improved local circuitry, which may account for the observed improvements in behavioural scores. This is backed up by previous studies which report enhanced levels of neuroprotection (Takami et al. 2002; Ramer et al. 2004a; López-Vales 2004; Sasaki et al., 2006) and local plasticity (Chuah et al., 2004) following OEC transplantation.

It has been proven that human OECs can be extracted and cultured from the human olfactory system (Barnett et al. 2000; Bianco et al. 2004; Féron et al., 2005; Mackay-Sim et al., 2008). Whilst 2-3 biopsies can be taken from the olfactory mucosa without affecting the patient sense of smell (Lanza et al.

1994; Féron et al. 1998), it is still hard to culture enough pure OECs to fill a human SCI cavity (Choi et al., 2008). Despite this and several other factors, such as cost, which may delay the development of OEC-based intervention strategies, several human trials are currently in progress worldwide (reviewed by Mackay-Sim and St. John 2011).

1.10.6.5 Mesenchymal stem cells

Mesenchymal stem cells are stromal cells that have the ability to self-renew and also exhibit multilineage differentiation; they can be derived from virtually all post-natal organs and tissues (da Silva Meirelles et al., 2006). As these cells types are derived from mature, differentiated tissues they have a more restricted potential for differentiation than embryonic stem cells. Whilst MSCs from several sources have been utilized for the treatment of numerous degenerative conditions, those derived from bone marrow are the most widely utilized and highly characterised subset of MSCs.

1.10.6.5.1 Bone marrow stromal cells

The stroma of the bone marrow contains a subset of heterogeneous, multipotent MSCs capable of differentiating into lineages of blood cell, skeletal tissue and stromal tissue. It has been claimed by some groups that these cells can then transdifferentiate into numerous cell types, including neuronal and glial cells (Woodbury et al., 2000; Qi et al., 2010), skeletal myocytes (Wakitani et al., 1995; Ferrari et al., 1998), cardiomyocytes (Martin-Rendon et al., 2008) and liver cells (Petersen et al., 1999), although some debate still remains (Castro et al., 2002; Mezey et al., 2003; Walczak et al., 2004), especially concerning their reported abilities for neuronal differentiation (Lu et al., 2004), as discussed in greater detail below. Extracting bone marrow, whilst comparatively invasive, avoids the ethical

concerns that exist about the use of cells of embryonic origin, thus making bone marrow derived cells attractive candidates for autologous stem cell transplantation strategies. As a result, there has been considerable interest in recent years in several cell types cultured from bone marrow for the treatment of numerous disorders (reviewed by Momin et al., 2010).

Much debate exists around the appropriate terminology for and the exact differentiative abilities of cells of bone marrow origin. They have been referred to as “bone marrow stem cells” as small proportions have stem cell like abilities, or as “mesenchymal stem cells” as they originate from the mesodermal germ layer, or “stromal cells / bone marrow stromal cells” (BMSCs) as they reside in the stroma of the bone marrow. This confusing terminology has made it hard to be certain of the true identity of the cells used by different research groups. Uncertainty also exists as to the true ability of these cells to differentiate and or transdifferentiate; this is largely due to the different purification and induction techniques used. For example, estimates of the percentage of BMSCs which are capable of being induced into the neural lineage vary considerably with different induction protocols; one group has estimated this to be around 1% for mice BMSCs (Sanchez-Ramos et al., 2000), whilst another group using a different induction protocol estimates that figure to be around 80% for human and rat BMSCs (Woodbury et al., 2000). Whilst we cannot ignore the species differences in the above observation, it remains a distinct possibility that the induction protocol plays a large part in the ability of BMSCs to differentiate into neural cells. It is therefore hard to gain a clear understanding of the abilities of BMSCs and their potential as therapeutic interventions for the treatment of SCI.

When transplanted into the injured spinal cord some researchers have reported that MSCs promote functional recovery (Hofstetter et al., 2002; Zurita and Vaquero, 2006; Quertainmont et al., 2012), although others have failed to confirm this observation (Lu et al., 2005; Parr et al., 2007). It has been shown that axonal regeneration is promoted within but not beyond the

transplant (Lu et al., 2005). Whilst some investigations have revealed that MCSs promote Schwann cell myelination of demyelinated axons (Lu et al., 2007), others have reported myelination by the MSCs themselves (Akiyama et al., 2002) although later studies have been unable to confirm this (Hunt et al., 2008). As reviewed by Uccelli et al., 2008, MSCs have been shown to play numerous other roles in SCI repair processes, including a role in modulating the release of trophic and anti-apoptotic molecules through interacting with cells involved in both the innate and adaptive immune system and by stimulating the release of anti-inflammatory cytokines (Uccelli et al., 2008).

A major complication associated with the use of MSCs is the lack of specific markers for their identification. In an attempt to address this problem, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) released guidelines to help identify human MSCs. The guidelines state that MSCs should maintain the ability to adhere to plastic when cultured under standard conditions; they should express CD73, CD90 and CD105, and lack expression of CD11b, CD14, CD19, CD34, CD45, CD79a and HLA-DR cell surface markers; lastly, they should have the ability to differentiate into adipocytes, osteoblasts and chondroblasts (Dominici et al., 2006). Whilst this has aided greatly in cell identification, there still remains a great deal of variation in the levels of marker expression seen between laboratories.

It remains a possibility that MSCs extracted from different tissues may have different properties that make them more or less able to combat the effects of spinal injury.

1.10.6.6 Mesenchymal stem cell co-transplantation studies

Recent studies have investigated the capacity for MSCs co-transplanted with OECs to improve functional outcomes following SCI. One group has reported improvements in behavioural scores and electrophysiological function with co-

transplantation (Deng et al., 2008), whilst others saw improvements in behavioural scores and improved electrophysiological function with transplants of either cell type alone, no synergistic benefits were seen with co-transplantation (Amemori et al., 2010).

Other investigators performing co-transplantation studies have found that allogenic MSCs and activated Schwann cells are capable of eliciting functional recovery, reducing the formation of the glial scar and promoting remyelination to a greater degree than either cell type alone (Ban et al., 2011).

1.11 Anatomy of the rat spinal cord

The rat spinal column contains 8 cervical, 13 thoracic and 6 lumbar segments. Like in humans, the rat spinal column contains a central region of grey matter which contains neuronal and glial cell bodies, surrounded by a region of white matter containing the axons of ascending sensory and descending motor neurons.

1.11.1 Dorsal column medial lemniscus pathway

The dorsal column medial lemniscus pathway is one of the main ascending pathways in the mammalian CNS and has a similar organization in rats and humans. It carries fine touch, vibration and conscious proprioceptive information from the body to the primary somatosensory cortices on the anterior parietal lobes. The dorsal columns contain two main groups of ascending fibres, the direct DC pathway and the postsynaptic DC pathway (Tracey and Waite, 1995). The DC pathways have been shown to be comprised of approximately 25% propriospinal fibres (Chung et al., 1987).

The cell bodies of primary sensory axons are located in the dorsal root ganglia of the dorsal root. They send projections into the PNS where they innervate

sensory receptors in skin, muscles and visceral tissues; as well as to the spinal cord, via the dorsal root, where they bifurcate into ascending and descending projections (Willis and Coggeshall, 2004) (Figure 1.5), these projections make up the direct DC pathway. Primary sensory axons, often termed ‘first order’ sensory neurons, are large myelinated fibers which transmit signals to the brain. Fibres in the descending branch have typically less well topographically organized distributions than the ascending projections, and only a small number of fibres project for two segments below their point of entry (Smith and Bennett, 1987). The centrally projecting branches ascend in the dorsal columns and form part of the dorsal column medial lemniscus pathway. The majority of ascending somatosensory projections originating in the lumbar region terminate in the spinal gray matter, with only an estimated 15% actually ascending all the way to the nucleus gracilis. Up to 63% of ascending somatosensory projections originating in the cervical region actually ascend all the way to the nucleus cuneatus (Willis and Coggeshall, 2004). Ascending somatosensory axons are known to be much more highly somatotopically organized than descending projections, for example, axonal projections from the lower limbs are known to ascend in the medial portions of the DCs in a tract known as the fasciculus gracilis, whilst sensory axons of the upper limbs ascend in the lateral portion of the DCs in a tract known as the fasciculus cuneatus (Figure 1.6) (Willis and Coggeshall, 2004). Fibres in both fasciculi are known to send collateral branches which project to numerous regions within the cord, including the dorsal and ventral horns as well as to the intermediate region (Willis and Coggeshall, 2004; Smith and Bennett 1987). Proportionally more of the first order cells from the cervical levels make synaptic connections with cells in the DCN, compared to those projecting from the lumbar levels (Smith and Bennett, 1987; Giuffrida and Rustioni, 1992).

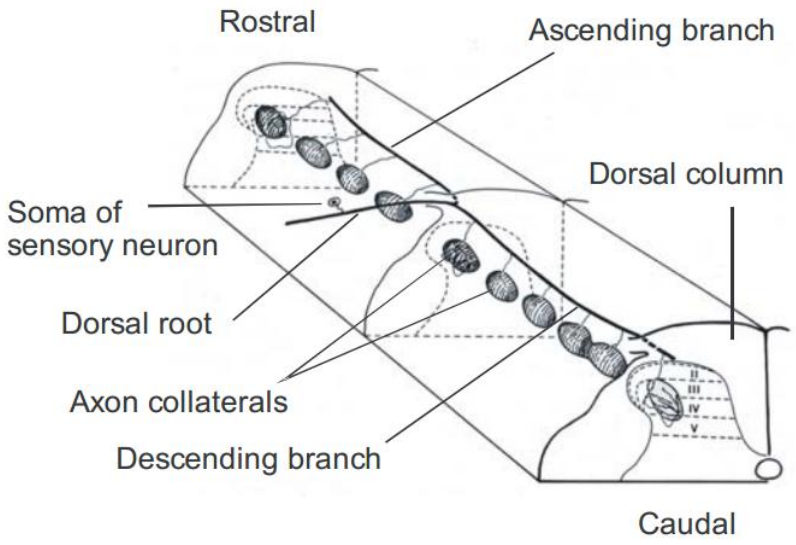


Figure 1.5 Schematic diagram showing the organization of ascending and descending sensory projections in the spinal cord - *The somas of primary sensory neurons are located in the dorsal root ganglia. It has two axonal projections, one to the spinal cord via the dorsal root, the other to the periphery where it innervates sensory receptors. The central projection bifurcates and sends ascending and descending axonal projections which have collateral terminals in the dorsal horn (Adapted from Brown et al., 1981).*

The first order neurons which reach the medulla in the brain stem make synaptic contacts with ‘second order’ neurons in the nucleus gracilus and nucleus cuneatus, respectively. On leaving the so called ‘dorsal column nuclei’, but whilst still in the medulla, each of these fasciculi decussates and continues to ascend to the thalamus in tracts known as the medial lemniscus fasciculi. The fibers in these tracts ascend and make synaptic contacts with third order neurones located in the ventral posteriolateral nucleus of the thalamus. The third order neurons then ascend in the posterior limbs of the internal capsules and synapse in layer IV of the primary somatosensory cortices, with the sensory information from the lower body positioned more medially and the sensory information from the upper body positioned more laterally (Figure 1.6).

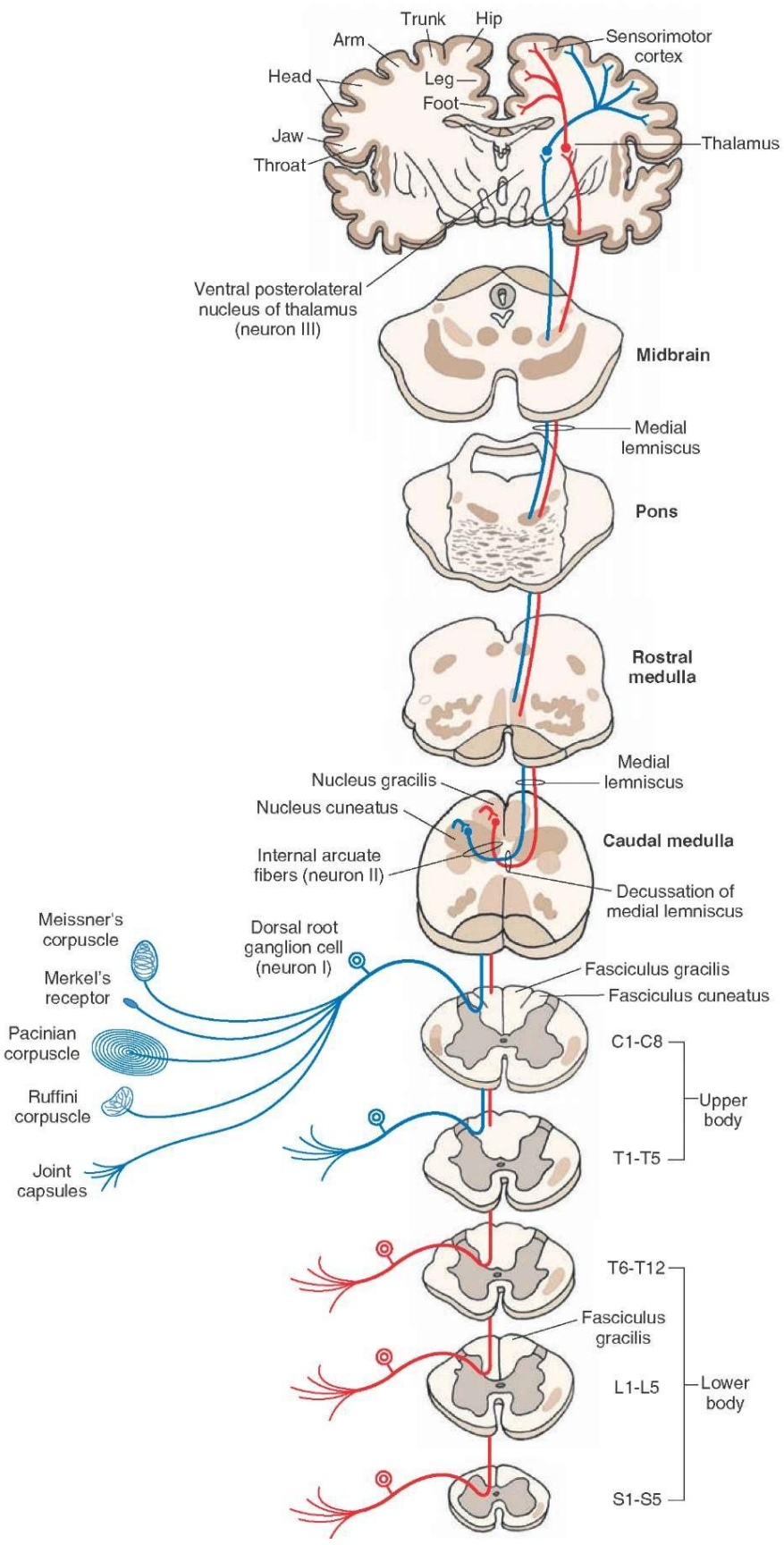

Trunk
Hip
Arm
Sensorimotor cortex
Head
Leg
Foot
Jaw
Thalamus
Throat
Ventral posterolateral nucleus of thalamus (neuron III)
Midbrain
Medial lemniscus
Pons
Rostral medulla
Medial lemniscus
Nucleus gracilis
Nucleus cuneatus
Caudal medulla
Internal arcuate fibers (neuron II)
Decussation of medial lemniscus
Meissner's corpuscle
Dorsal root ganglion cell (neuron I)
Fasciculus gracilis
Fasciculus cuneatus
Merkel's receptor
Pacinian corpuscle
C1-C8
Upper body
Ruffini corpuscle
T1-T5
Joint capsules
T6-T12
Fasciculus gracilis
L1-L5
Lower body
S1-S5

Figure 1.6 Dorsal column medial lemniscus pathway - *Schematic diagram of the dorsal column medial lemniscus pathway showing first order large myelinated ascending sensorimotor fibres from the forelimb (blue) and hindlimb (red) ascending in the fasciculus cuneatus and fasciculus gracilus, respectively. Fibres in these tracts make synaptic contacts with second order neurons in the nucelus gracilis and nucleus cuneatus, collectively known as the dorsal column nuclei (DCN), which in turn project to make synaptic contacts with third order neurons in the ventral posteriolateral nucleus of the thalamus. The tracts then decussate in the thalamus and ascend in the medial lemniscus pathways and make synaptic contacts with neurons in layer V of the ipsilateral sensorimotor cortex (from http://what-when-how.com/wp-content/uploads/2012/04/tmp1499.jpg, 2013).*

Studies in the rat have shown that approximately 25% of axons in the DC pathways are unmyelinated and contain calcitonin gene related peptide (CGRP), suggesting that they might be involved in transmitting signals from either the viscera or peripheral nociceptors to the DCN (McNeill et al., 1988; Tamatani et al., 1989; Patterson et al., 1990).

The other DC pathway, called the postsynaptic DC pathway consists of neurons located in the nucleus proprius, just ventral to the substantia gelatinosa in laminae III, IV and V, which receive input from primary afferent collaterals and in turn send projections that make synaptic contacts with second order cells in the DCNs, which are positioned in a highly somatotopically organised fashion. Cells in the nucleus proprius also send projections to cells in the external cuneate nucleus which lies laterally to the cuneate nucleus and relays proprioceptive signals from the upper extremities to the cerebellum, as well as to lemniscal neurons which send projections to the ventrobasal thalamus and then outputs to the primary somatosensory cortex (Rinvik and Walberg, 1975; de Pommery et al., 1984; Giesler et al., 1984; Cliffer and Giesler, 1989).

1.12 Summary of study aims

The aims of this thesis were as follows:

1. The aim of chapter 2 is to use direct electrophysiological, behavioral and anatomical techniques to investigate and characterise the effects of contusive SCIs of different severity on the functional changes occurring in ascending long white matter pathways. This information will be used to select an injury severity that produces moderate functional deficits which are detectable up to 6 months post injury, and this model will be used to characterise the time course of secondary damage and any spontaneous recovery of functions.
2. The aim of chapter 3 is to combine the injury model designed in chapter 2 with electrophysiological, behavioral and anatomical investigations to determine whether the delayed transplantation of a recently characterised subset of mesenchymal stem cells can promote the recovery of function through remyelination or other mechanisms of repair.

Chapter 2

Responses of the somatosensory pathways to contusion injury

2.1 Introduction

SCI disrupts both ascending and descending axonal pathways, resulting in the loss of motor, sensory and autonomic function. Substantial spontaneous recovery has been observed in both experimental animals and in humans in the days, weeks and months following injury (Burns et al., 1997; Weidner et al., 2001; Ballermann and Fouad 2006; Fawcett et al., 2007). Spontaneous recovery has been investigated using mainly behavioural and anatomical approaches but the mechanisms responsible remain largely undefined.

It is known that adult CNS neurones have an intrinsic capacity for regrowth over long distances although anatomical evidence has shown this regrowth to be inhibited by the inhospitable environment that results following SCI (David and Aguayo 1981). Thus it is unlikely that axonal regeneration contributes significantly to spontaneous recovery; consequently, research has largely focused on mechanisms such as remyelination and plasticity.

Following SCI it is known that a population of fibres in the long white matter pathways are partially demyelinated but remain intact (Griffiths and McCulloch, 1983). It has been demonstrated that some segmentally demyelinated axons retain an ability to conduct action potentials, even where the demyelinated region exceeds several internodes. Predictably, conduction velocities are much slower and the refractory periods of transmission are much longer in demyelinated axons; this is partly due to the redistribution of ion channels which spread out into the exposed internodal regions (Black et al., 1991; Felts et al., 1997).

It has been shown that infiltrating Schwann cells and oligodendrocyte precursor cells act to remyelinate the exposed axons, leading to functional improvements which can be measured electrophysiologically (James et al., 2011). Experimental contusion injuries have been shown to be particularly good at recreating the types of demyelination seen clinically, and thus provide an excellent model for

studying the effect of spontaneous and induced remyelination. The time course of the loss and potential recovery of functional abilities has not yet been fully characterised following moderate thoracic contusion injury.

Molecular and anatomic techniques have been employed to study several types of so-called 'injury-induced plasticity' which may play a role in spontaneous functional recovery following SCI. Studies looking at animals in which spontaneous recovery is observed have revealed that both injured and the remaining uninjured axons undergo conformational changes including synaptic plasticity and axonal sprouting. Shortly after injury, inactive synapses and pathways may be transformed into a functional form (Merzenich et al., 1984). At later time points the targets of partially disrupted projections have been shown to upregulate the number of receptors to bind a reduced number of neurotransmitter molecules (Gulino et al., 2007) and spared axons have been shown to have the potential to sprout to form new functional synapses, thus both responses have the potential to partially ameliorate the effects of the injury (Brus-Ramer et al. 2007; Bareyre et al., 2004; Weidner et al., 2001). Whilst limited plasticity has been observed in the spinal cord following injury, extensive cortical plasticity has also been shown to rapidly occur following SCIs of different types (Jain et al., 1997; Bazley et al., 2011; Aguilar et al., 2010). Injury-induced plasticity in supra-spinal systems has been linked with improvements in functional outcomes following injury in both experimental animals and humans (Raineteau and Schwab 2001; Edgerton and Roy 2002; Oudega and Perez, 2012).

The above mechanisms have been investigated mainly using molecular, cell and anatomical approaches, and in some studies behavioural tests have been employed in order to determine their influence on functional outcome. However, whilst behavioural tests are capable of providing measures of an animal's ability to perform certain tasks which allow us to make comparisons between experimental groups, they are unable to ascertain the processes which underlie any observed changes. Behavioural tests are unable to differentiate functional changes in the spinal cord from functional changes occurring supra-spinally and

can therefore reveal nothing about the functional changes in spinal pathways. To make inferences about the function of spinal tracts they must be investigated directly using an electrophysiological approach.

To determine the functional effects of injuries on long white matter tracts and therapeutic interventions, direct electrophysiological investigations of injured and uninjured pathways are required. This allows for changes in function to be attributed to changes seen in the investigated spinal pathways or to other, potentially supraspinal mechanisms. Ideally such investigations should be designed so that direct functional measures can be related to anatomical and behavioural measures. There is a paucity of such studies in the literature, with researchers thus far choosing to largely focus only on anatomical and behavioural testing alone.

The assessment of potential therapies for SCI would be aided greatly by the accurate characterisation of direct functional measures in both normally functioning pathways and pathways subjected to contusion injury. The aims of the experiments described in this chapter are therefore: 1) to ascertain whether an electrophysiological approach can be used to detect functional changes in the ascending long white matter tracts in the dorsal columns following a contusion injury; 2) to design an injury protocol which will allow for direct functional (electrophysiological) measures to be made; 3) to determine and characterise the time course of changes to long tract function that might occur following contusion injury.

To assess functional changes in the ascending dorsal column pathways, sensory evoked potentials (SEPs) evoked by stimulating ipsilateral radial and sciatic nerves were recorded from the exposed contralateral somatosensory cortex. In the first part of the study SEPs were compared between groups of normal animals and animals 6 weeks following T9 contusion injuries of different severity. After selecting an appropriate injury severity, SEPs were recorded at different time points from the acute time point up to 6 months post-injury.

2.2 Materials and methods

2.2.1 Animals

All the experiments were approved by the Ethical Review Process Applications Panel of the University of Glasgow and performed in accordance with the UK animals (Scientific Procedures) Act 1986.

Observations reported in this chapter are based on the investigation of 98 adult male Sprague Dawley rats (Harlan, Loughborough, UK). Of these, 30 animals were used to investigate the effects of injury severity on cortical SEPs and 45 animals were used to investigate the time course of changes that might occur to SEPs. A further 23 normal animals were investigated electrophysiologically and were used as controls for both studies. The remaining 2 animals were seen to have received contusion injuries in which the impactor tip glanced the bone at the edge of the laminectomy window and were thus excluded from the study on the grounds that they had not received the correct severity contusion injury based on the exclusion criteria described below.

Of the 30 animals used to investigate the effects of injury severity, 11 received a 150 Kdyn injury, 12 received a 175 Kdyn injury and 7 received a 200 Kdyn injury. All animals were allowed to recover for 6 weeks before being investigated electrophysiologically.

In total 45 animals were used to investigate the time course of spontaneous recovery following contusive spinal cord injuries of 150 Kdyn force. Of these, electrophysiological investigations were performed on 10 animals 3-4 days following injury, 8 after 2 weeks, 15 after 12 weeks and 12 after 6 months recovery. Additionally, animals that received 150 Kdyne injuries in the survival study were used as a 6 week survival group here (n=11).

For comparison, peripheral nerve evoked SEPs were also recorded from 23 normal animals which were used as a control group in each study. Some on these animals were subsequently used to investigate the effects of injury at the acute time point and made a further acute group in the injury severity study (n=12).

2.2.2 Contusion Injury

All the contusion injuries in this study were carried out by Dr. John Riddell.

2.2.2.1 Drugs

Contused animals routinely received the following peri-operative medication as required:

Buprenorphine (Vetergesic) - analgesic: 0.05 mg/kg, administered subcutaneously, once at the time of induction and once again the following morning.

Carprofen (Rimadyl) - non-steroidal anti-inflammatory: 5 mg/kg, administered subcutaneously, once at the time of induction and subsequently if direceted by the vetenarian if recovery seemed slower than usual.

Amfipen - antibiotic: 22.5 mg/kg, administered subcutaneously, once preoperatively.

Enrofloxacin (Baytril) - antibiotic: 5 mg/kg, administered subcutaneously, twice daily from day of surgery for 7 days.

Saline: 0.2-0.4 ml/100 gm body weight, administered subcutaneously, two or three times daily from day of surgery for up to three days, then as required.

An ophthalmic ointment was applied as required to prevent drying of the eyes during surgery.

2.2.2.2 Contusion injury procedure

The dorsal surface of the spinal cord was subjected to a midline contusion injury at the T9 level. This location was selected as it is easily surgically accessible and allows electrophysiological assessment of sensory input originating in the hindlimb. Contusion at this level interrupts the ascending dorsal column pathways which carry somatosensory signals from the hindlimbs to the brain in long white matter tracts. Surgical instruments and materials were sterilised and precautions were taken to minimise the risk of infection.

Animals were anaesthetised with 5% isoflurane and oxygen at the time of induction, and 1-3% isoflurane and oxygen during surgery. The vertebral column was exposed from T8-T10 and the spinal cord was exposed by laminectomy of the T9 vertebra. The animal was placed in a stereotaxic frame and the vertebral bodies of T8 and T10 were clamped with lockable Adson forceps to stabilise the spine. A contusion injury was induced using the commercially available Infinite Horizon Impactor™ (Precision Systems and Instrumentation, LLC, Lexington, KY, USA; Figure 2.1).

Briefly, after centring the impactor tip to the middle of the exposed spinal cord, a computer controlled stepper motor was activated, lowering the impactor tip at approximately 120 m/s to contuse the exposed spinal cord until a user-defined force was reached, after which the impactor tip was immediately retracted (Figure 2.2). Computer generated graphs of force and displacement vs. time were then created by the Infinite Horizons Impactor (IHI) software before being saved and stored offline for further analysis (Figure 2.3).

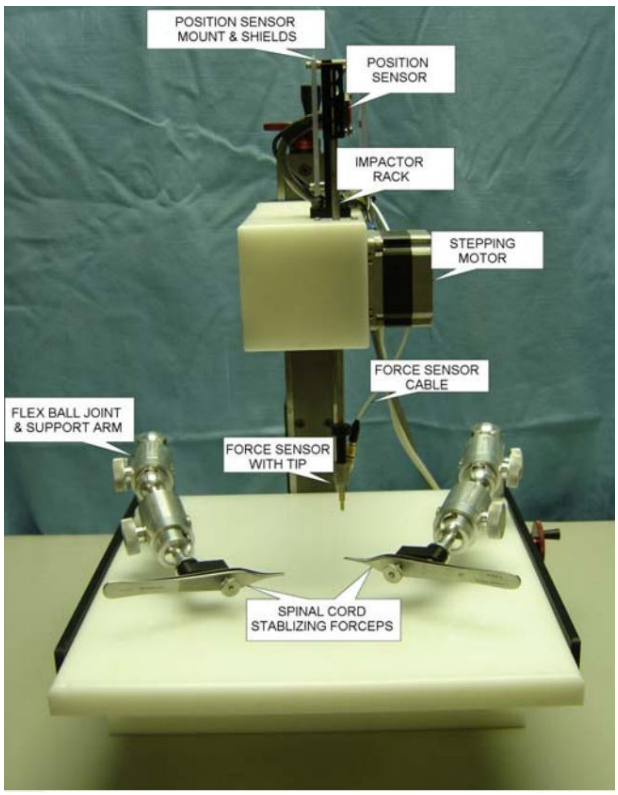


Figure 2.1 The Infinite Horizons Impactor*. The adjustable support arms were positioned and locked into place so that the lockable stabilising forceps could be used to clamp the animal into position. The impactor tip was then moved and lowered into position just above the exposed spinal cord using adjustable control wheels. Once in position the computer-controlled stepping motor is activated, driving the impactor rack downwards. The force sensor and impactor tip are located at the lower end of the impactor rack, which is in turn connected to the position sensor. The position and force sensors both connect to a control unit which interfaces with a control computer via a serial port (Image taken from the PSI-IH Impactor user manual 2007).*

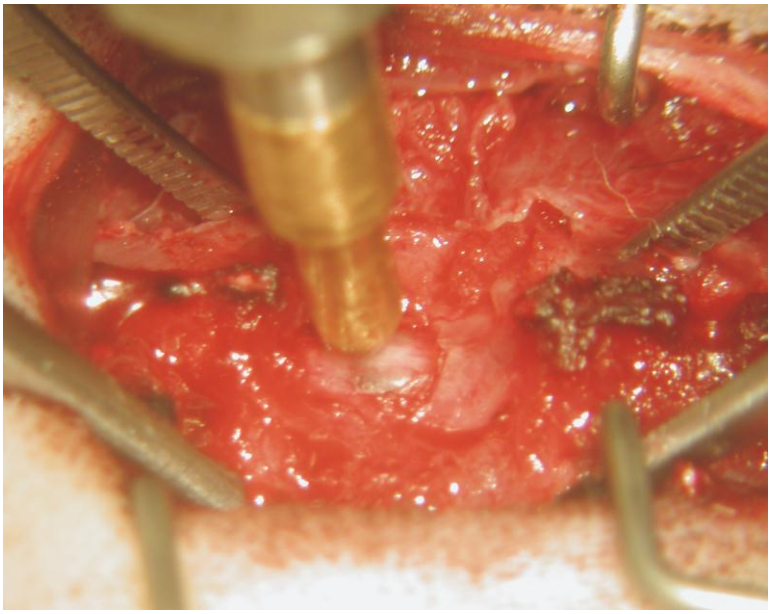


Figure 2.2 Contusion injury. *Photograph of the injury procedure immediately following contusion injury. The contusion injury procedure involved performing a laminectomy to remove the T9 vertebrae, and rigidly fixing the T8 and T10 vertebrae using lockable Adson forceps, the impactor tip of the Infinite Horizons Impactor was then positioned over the exposed T9 spinal cord and a computer-controlled contusion injury of user-defined force was performed.*

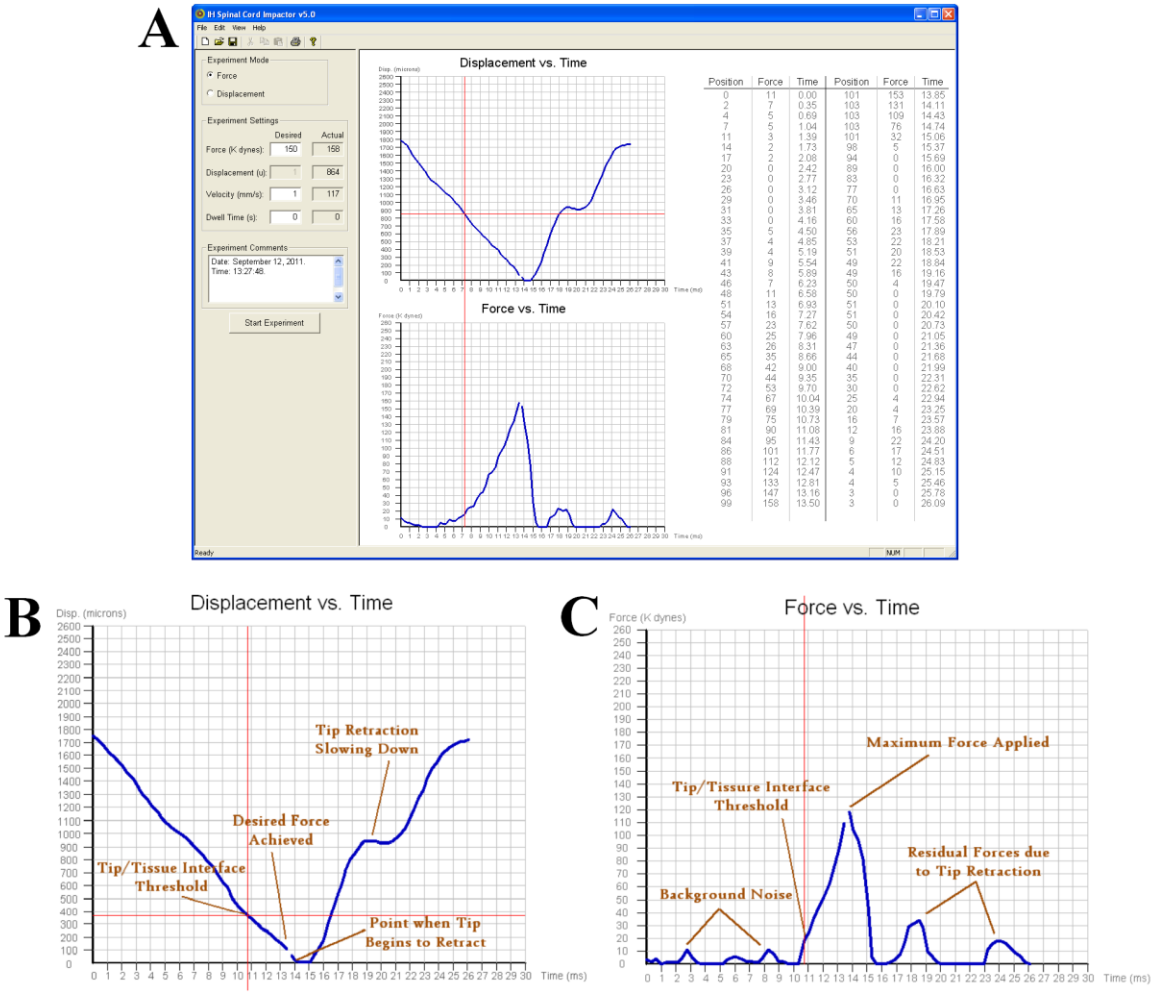


Figure 2.3 The Infinite Horizons Impactor software. *(A) the Infinite Horizons Spinal Cord Impactor software (Precision Systems and Instrumentation, LLC, Lexington, KY, USA) showing the data generated by a contusion injury of 150 Kdyn. (B) An example of a displacement vs. time graph with key points labelled. (C) An example of a force vs. time graph with key points labelled. The impactor-tip/tissue interface is recorded at the point where a force of 20 Kdyn is first measured, at which point a vertical red line is drawn through both graphs to indicate the time at which the initial impact occurred. A horizontal red line is then drawn on the displacement vs. time graph through the tip/tissue interface, indicating the displacement of the tip into the tissue. The tip travels slightly beyond the point at which desired force is achieved before the motor is able to*

overcome its inertia and begin retracting the tip (adapted from the PSI-IH Impactor user manual 2007).

Any anomalous graphs were indicative that we had hit or grazed the bone surrounding the laminectomy, causing either a blunted peak, or a bump/second peak on the ascending part of the force vs. time graph, usually resulting in a severely reduced displacement distance. Such graphs indicated that the animal had not received the correct injury severity and therefore the animal was excluded from our investigations. This procedure allowed for highly accurate injuries, with a high degree of consistency within and across groups of animals, to be included in the study.

In the animals which were allowed to recover post-injury, a 10-0 nylon suture was placed in the dura mata at the injury level to allow the location to be accurately identified during electrophysiological experiments and to aid in confirmation of the segmental level at the end of the experiment. It has been seen from previous studies in this laboratory that this is the most dependable and accurate way of locating the injury site post-mortem. The wound was sutured with 3-0 vicryl and closed using wound clips. Post-operative drugs were routinely administered as described previously. In total, 100 animals received a contusion injury, of which 85 animals were allowed to recover post-injury, and were recuperated for 24 hours in a warm environment with free access to food and water before being allowed to recover for up to 6 months in normal animal housing conditions. A further 15 animals that had received a contusion injury underwent electrophysiological investigation immediately, whilst the remaining two animals were excluded on the grounds that they had not received the correct severity contusion injury based on the exclusion criteria described above.

All injury severities that were investigated immediately produced severe deficits in hind limb function which severely impeded the animal’s ability to locomote normally for 3-4 days for some animals in the least serve and permanently in some of the animals in the most severe injury group. Despite

this, all animals were able to move around their cages and were able to eat and drink with the aid of their fore limbs, which remained unaffected by the injury procedure. To encourage eating in their recovery period the animals were given Heinz chocolate flavoured baby food, which was placed at ground level in their recovery cages.

As injuries at this level are known to prevent normal bladder function, the animals bladders were manually expressed twice daily by gently pressing the abdomen until normal function resumed as assessed by three consecutive failed attempts at expressing the bladder, usually this was seen within 7 days of injury in all groups, with function in the least severely injured seen to recover first.

2.2.3 Electrophysiological experiments

In the injury severity study, 30 animals were allowed to survive for 6 weeks post-injury before being investigated electrophysiologically to ascertain the effects of different injury severities on cortical SEPs. In the time course study a further 45 animals were investigated electrophysiologically in order to characterise the interplay between injury progression and spontaneous recovery from the acute to the chronic stages; 23 normal animals were investigated similarly and made up a control group for each study. The anaesthetic regime, preparatory surgery, post injury recovery and electrophysiological protocol were the same for animals in each experimental group.

2.2.3.1 Anaesthesia

It was found that gaseous isoflurane provided the most stable anaesthesia for the preparatory surgery and i.v. pentobarbital the most stable for electrophysiological recordings. Anaesthesia was thus induced with 5% isoflurane in oxygen and maintained with 1-3% isoflurane in oxygen during the initial surgical procedures, after which frequent doses of sodium pentobarbital (10mg/kg i.v.) were administered as required. During dissection the depth of anaesthesia was assessed by monitoring pedal withdrawal reflexes, corneal reflexes, arterial blood pressure and electrocardiogram activity. Additional doses of anaesthesia were administered if pedal or ocular (blink) reflexes were evoked or if blood pressure was abnormally high or increased on pinching. During the recording phase of the experiment, the animal was paralysed and artificially ventilated (see below). Once paralysed we were unable to use pedal withdrawal or ocular reflexes to monitor anaesthesia levels, so anaesthetics were routinely administered at a rate commensurate with that required before paralysis. During the recording period, the arterial blood pressure's response to noxious stimuli was monitored and if any response could be elicited then additional anaesthetic top ups could be administered, although this was rarely required.

2.2.3.2 Preparatory Surgery

The animal was induced as detailed above and placed in a supine position on an operating table. Core body temperature was continuously monitored and maintained close to 37^{o}C using a rectal temperature probe, a heating blanket and a radiant heat source. Adhesive tape was used to secure each of the limbs in position and needle electrodes placed in the skin of both forelimbs and the right hindlimb to allow for the continuous monitoring of the animals

electrocardiogram. Once surgical levels of anaesthesia had been achieved, heart rate was typically between 360-400 beats/minute. Prior to the commencement of surgery, atropine sulphate (0.05 mg/kg i.p.; Martindale Pharmaceutical, UK) and dexamethasone sodium phosphate (2-3 mg/kg i.m.; Faulding Pharmaceuticals Plc, UK) were injected to reduce bronchial constriction and salivary secretions, and to prevent swelling of the spinal cord and brain (as evidenced by bulging of the brain or cord at the site of laminectmy and/or craniotomy), respectively. Before making any skin incisions an animal fur clipper was used to remove fur from the relevant areas. All fine surgery was performed with the aid of a dissecting microscope with foot controller (Zeiss S5, Germany). A soldering iron or electrocautery (Eschmann Equipment, TDB 50, UK) was used to cauterise any areas of bleeding.

2.2.3.3 Tracheal, venous and arterial cannulations

Tracheal, jugular vein and carotid artery cannulations were performed to allow for artificial respiration, intravenous drug administration and monitoring blood pressure, respectively. Whilst under the gaseous anaesthetic regime described above, the skin overlying the trachea was incised and retracted, and the superficial muscle overlying the trachea was separated. The right carotid artery was then exposed by blunt dissection and an approximately 2cm length was carefully separated from the vagus nerve and ligated distally. The proximal end was clamped and a cannula inserted proximally (in the direction of the heart) and tied into place before the clamps removal. The cannula was filled with sterile heparinised saline (1% heparin in saline) to maintain patency by preventing blood clot formation. This allowed for the continual monitoring of arterial blood pressure which was maintained above 80mm Hg (typically 100-120mm Hg) throughout the experiment. The right jugular vein was exposed in a similar fashion and

cannulated to allow for the administration of intravenous drugs. The muscle overlying the trachea was retracted, a small length of trachea was separated from the surrounding tissue and a small opening was created such that a cannula could be inserted proximally and tied into position. Aspiration of lung secretions from the treachea was performed from time to time to ensure that the trachea remained patent. All cannulae were secured to the skin on the animals chin with threads to prevent them from being accidentally dislodged and the skin incision was closed with Michel clips.

Cannulation of the carotid did not appear to have negative effects on SEPs through reduced cortical blood flow, and has been a standard procedure for some time in similar studies.

2.2.3.4 Sciatic nerve dissection

To dissect the sciatic nerve the animal was placed in a prone position and the skin overlying the dorsal aspect of the left hind limb was incised from the hip joint proximally, to the knee joint distally. The biceps femoris was retracted by blunt dissection and the sciatic nerve exposed. A length of sciatic nerve was dissected free (approximately 2 cm) and sectioned distally. A suture loop was tied to the distal end of the sciatic nerve and loosely tied in order to lift it without damage. Cotton soaked in sterile saline was used to cover the exposed nerve and the skin incision was held closed with a tissue clip to prevent the nerve from drying out.

2.2.3.5 Radial nerve dissection

In order to dissect the superficial branches of the left radial nerve, the animal was kept in a prone position and the skin overlying the right dorsal

aspect of the fore limb was incised from the shoulder joint proximally, to the elbow joint distally. The tensor of the triceps brachii muscle was cut using an electrocautery (Eschmann Equipment, TDB 50, UK) and the superficial branches of the radial nerve were exposed and dissected free (approximately 2cm) from the surrounding tissues using blunt dissection. The branches were sectioned distally and a suture loop tied around the end of the radial nerve so it could be lifted without damage. Cotton soaked in sterile saline was used to cover the exposed nerve and the skin incision was held closed with a tissue clip to prevent the nerve from drying out.

2.2.3.6 Laminectomy

A laminectomy was performed to expose the lumbar (L3-4) segments of the spinal cord to allow for the monitoring of CDP traces which were used to ensure that the sciatic nerve was both functioning properly and maximally activated. Briefly, a midline incision was made from the mid thoracic to mid lumbar regions. The skin was retracted and the thoracolumbar fasciae of the latissimus dorsi muscles separated (partially removed if required) from the vertebral column. The posterior arches of the T13-L2 vertebrae were then carefully removed using small bone rongeurs to expose the L3-4 spinal cord. If required, bone wax was used to prevent any bleeding from the cut edges of the vertebrae with the addition of gel foam to prevent any further bleeding. Throughout the laminectomy procedure, great care was taken to avoid damaging the spinal cord. When not recording CDPs, cotton soaked in sterile saline was used to cover the exposed spinal cord and the skin incision was held closed with tissue clips to prevent the cord from drying out.

2.2.3.7 Transfer to recording frame

After the preparatory surgery was completed the animal was transferred from the surgical table to the recording frame and the animals head was secured using ear bars and an incisor bar.

2.2.3.8 Artificial ventilation

To prevent movements whilst stimulating the peripheral nerves it was necessary to paralyse the animal using a neuromuscular blocker, after which it was necessary to apply artificial ventilation. After aspirating the trachea, the animal was paralysed with pancuronium bromide (Sigma, induced with 0.3 mg/kg i.v. with top up doses of 0.04mg every 30 minutes or as required). The animal was then immediately ventilated with air by connecting a respiratory pump (6025 Ventilator, UGO Basile, Italy) to the tracheal cannula. Carbon dioxide levels were monitored (Micro-Capnometer, Columbus Instruments, USA) and maintained at around 4% throughout the experiment by adjusting the ventilation rate and or stroke volume (typically 75-100 strokes per minute and 10-20 ml/stroke).

2.2.3.9 Craniotomy

A unilateral craniotomy in the right hemisphere was performed using a fine dental drill, small bone rongeurs and watch makers forceps, extending from 4mm rostral to 4mm caudal of Bregma and 5mm laterally, exposing the somatosensory cortex. Bone wax was used to prevent any bleeding from the cut edges of the cranium with the addition of gel foam to prevent any further bleeding, if required. The dura was carefully opened and removed from the exposed cortical surface using fine tweezers and iris scissors. Great care was taken so as to avoid damaging the cortex. An example of an exposed sensorimotor cortex is shown in Figure 2.4.

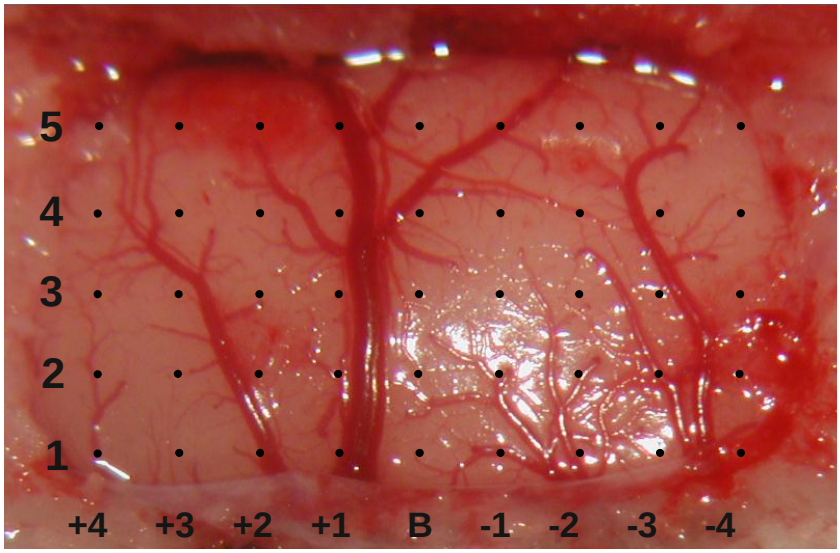


Figure 2.4 The cortical SEP recording grid. *A craniotomy was performed to expose the somatosensory cortex of the hemisphere contralateral to the stimulated peripheral nerves. The dura was removed and SEPs were recorded using a silver ball electrode in a grid pattern over the cortex, with 1 mm spacing between the locations, all relative to Bregma (B), + indicates rostral, - indicates caudal (Image provided by Toft and Riddell).*

2.2.3.10 Paraffin pools

Paraffin pools were created around the posterior sides of the left forelimb, hindlimb and craniotomy by tying threads passing through the edge of the skin incisions to the frame. The pools were filled with paraffin at approximately 37^{o}C so that all exposed tissues were covered. This provided insulation to prevent current spread to other tissues on electrical stimulation of the peripheral nerves, as well as to reduce heat loss and prevent tissue drying.

2.2.3.11 Stimulation of the sciatic nerve

The sciatic nerve was carefully mounted on bipolar silver wire electrodes immersed in the paraffin pool created from the skin flaps of the hindlimb surgical site. Care was taken to prevent the nerve coming into contact with any of the surrounding tissues and the electrode was secured firmly into place once positioned. The nerve was electrically stimulated using single square wave stimulation pulses of 0.2 ms duration and up to 500 μA.

2.2.3.12 Stimulation of the radial nerve

The radial nerve was carefully mounted on bipolar silver wire electrodes immersed in the paraffin pool created from the skin flaps of the hindlimb surgical site. Care was taken to prevent the nerve coming into contact with any of the surrounding tissues and the electrode was secured firmly into place once positioned. The nerve was electrically stimulated using single square wave stimulation pulses of 0.2 ms duration and up to 500 μA, the stimulation parameters were set using the protocol described in section 2.2.3.16.

2.2.3.13 Monitoring the condition of the sciatic nerve

To check that the sciatic nerve was both functioning normally and maximally activated it was stimulated and a CDP waveform was recorded from the exposed surface of the cord just below the level of the contusion injury at the L3-4 border. A normal sciatic evoked CDP is composed of a stimulus artifact, an afferent volley and a cord dorsum potential representing the summed activity of circuits local to the recording electrode. For the purpose of these experiments, where we were only interested in activity in long tracts rather than that of local circuits, therefore we were mainly concerned with any changes observed in the afferent volley. An example of a normal L4 sciatic evoked CDP trace depicting the separate components of the waveform is shown in Figure 2.5.

2.2.3.14 Peripheral nerve stimulation and SEP recording setup

The electrophysiological setup involved stimulating the sciatic and radial nerves and recording the evoked potentials from the cortical surface, as shown in Figure 2.6. Briefly, when stimuli were applied to the radial and sciatic nerves with bipolar hook electrodes, potentials were recorded across the surface of the contralateral somatosensory cortex. SEPs generated by alternately stimulating the radial and sciatic nerves were recorded at different positions across the cortical surface using a silver ball electrode (Figure 2.4).

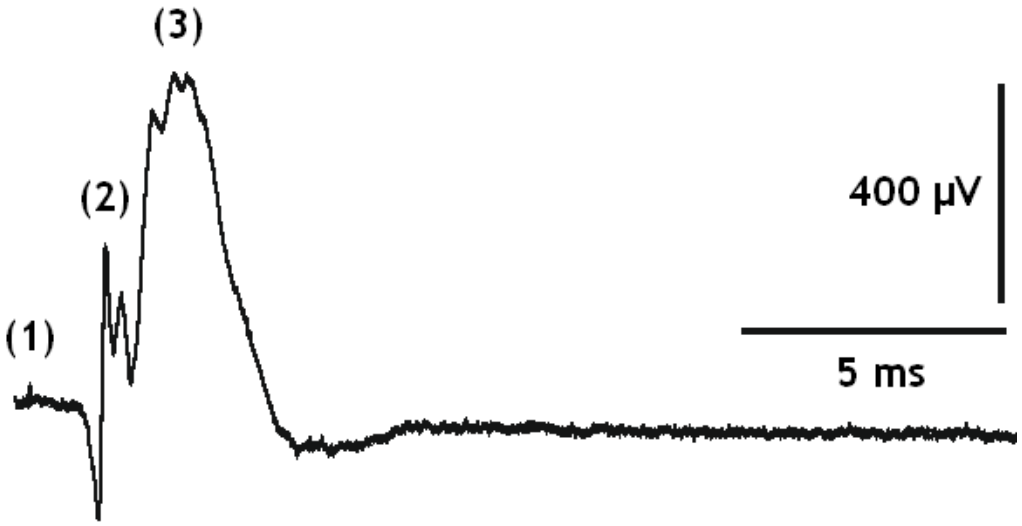


Figure 2.5 Sciatic evoked CDP recorded from L4 *– An example of a CDP recorded from the dorsal surface of the spinal cord at the L4 level, showing the different components of the waveform: 1 = Stimulus artifact, 2 = Afferent Volley, 3 = Cord dorsum potential.*

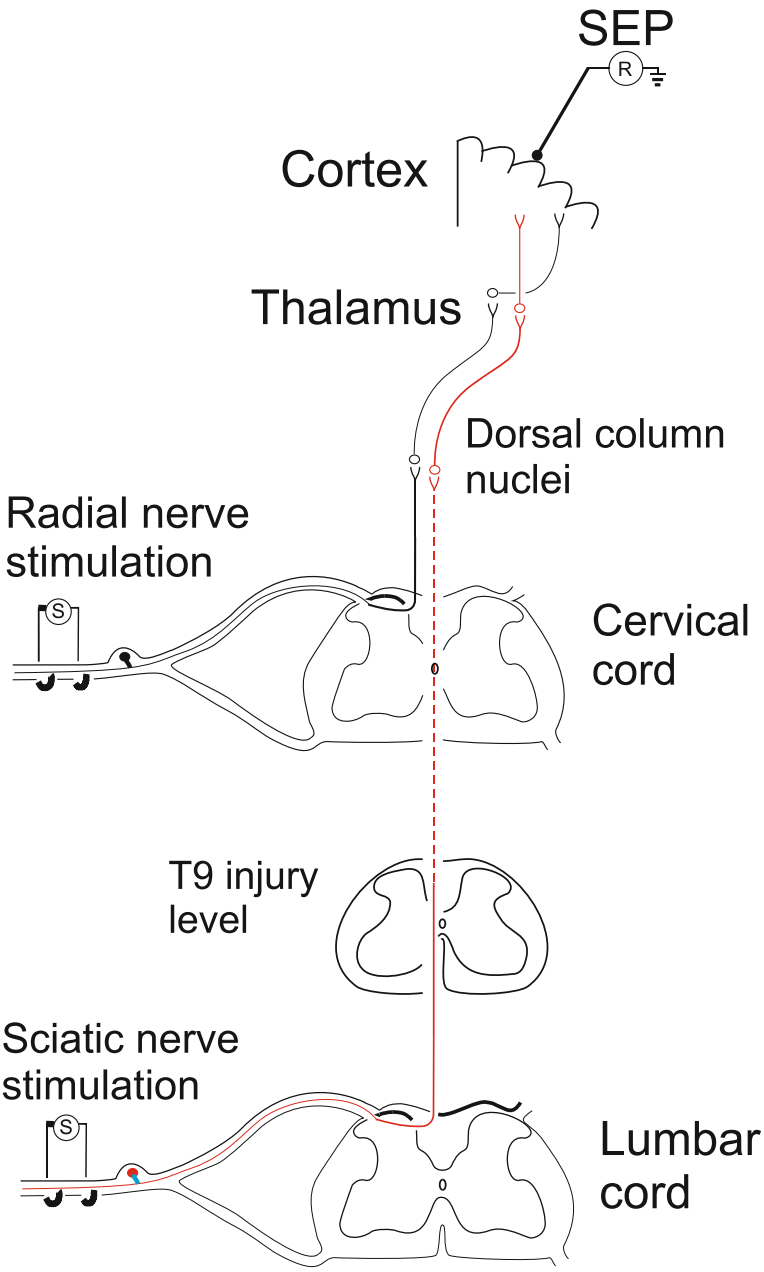


Figure 2.6 Schematic diagram of electrophysiological setup

Schematic diagram showing the injury level and the stimulating (S) and recording (R) electrodes used to assess radial and sciatic SEPs. The ascending dorsal column pathways were bilaterally injured at the T9 level, interrupting the ascending signals from the sciatic nerve whilst leaving the ascending radial pathway in tact (Image provided by Toft and Riddell).

2.2.3.15 Components and measurements of SEPs

SEPs consist of a stimulus artifact which indicates the onset of stimulation, followed after a brief interval by a strong depolarisation known as the SEP waveform which represents a summation of the post-synaptic activity evoked in the cortex in response to peripheral nerve stimulation, as shown in Figure 2.7 A; the SEP waveform reflects the number and strength of these cortical connections and are largest at locations which receive more inputs from the pathways activated by the stimulated peripheral nerve. Various measurements can be made from SEPs in order to provide quantitative measurements of the ability of the stimulated pathways to transmit the stimulus from the peripheral nerve to the cortex, as shown in Figure 2.7 B. The latency of the SEPs arrival in the cortex provides useful information about the maximum conduction speeds in the activated pathways, whilst the amplitude of the negative component is capable of providing useful information on the strength of the transmitted signals, and the latencies of the negative and positive peaks are capable of providing useful information about the synchronicity of the action potentials arrival in the cortex, which in turn is a reflection of the range of conduction speeds in the activated pathway.

2.2.3.16 Method of recording SEPs

To assess function in the ascending sensory pathways, SEPs from the surface of the contralateral somatosensory cortex were recorded. Recordings were made using a silver ball electrode placed on the surface of the cortex, with the indifferent electrodes placed on nearby scalp muscle. Recordings were made in a grid pattern with 1 mm spacing between recording locations. The electrode positioning was guided with reference to a 1 mm graduated paper strip which was placed alongside the exposed somatosensory cortex. The grid

was centred at 1 mm lateral to Bregma and extended from 4 mm rostral to 4 mm caudal and 4 mm lateral to bregma, giving a total of 36 recording locations (Figure 2.4). The first recording electrode was used to locate the position at which the SEP elicited by electrical stimulation of the radial nerve was maximal; this position was fixed and the electrode remained static throughout the experiment. The second recording electrode was used to locate the position at which the SEP elicited by electrical stimulation of the sciatic nerve was maximal. Once found, the locations where SEPs of the greatest amplitude could be recorded were used to ascertain the stimulus intensity which evoked the maximal response. The stimulus intensity was then set at a value that was supramaximal for both the radial and sciatic-evoked SEPs (typically 100-200 μA) to ensure that the peripheral nerves were maximally activated in a consistent manner. Stimulating with supramaximal intensities has been standard practice in similar studies for some time now, and in this preparation this was seen to consistently and maximally activate both radial and sciatic nerves such that recordings of cortical SEPs could reliably be made.

The roving electrode was then moved around the exposed somatosensory cortex and recordings were taken from each of the recording locations in the grid in order to map the varying responses at different locations. Alternate stimulation of the sciatic and radial nerves evoked SEPs which were recorded from the surface of the somatosensory cortex. Amplitude attenuation is known to occur with rapidly repeated stimuli, to avoid this, the stimulus repetition rate was fixed at 2 Hz, and to reduce the background noise averaged recordings were made of 30 sweeps recorded from each cortical location (Abbruzzese et al., 1990; Fujii et al., 1994). Examples of averaged sciatic and radial–evoked SEPs recorded at each cortical location in representative normal and 6 weeks survival animals are shown in Figures 2.8 and 2.9, respectively.

It was noted that the accumulation of cerebrospinal fluid tended to short circuit the recording electrodes, resulting in smaller potentials. Therefore, throughout the recording of SEPs, great care was taken to remove fluid from the surface of the cortex. Due to this potential source of inaccuracy, the fluid was removed after moving the roving electrode to each new recording location and also during recording if the SEPs were seen to be reducing in amplitude whist collecting recordings from the same location.

All recordings of electrical potentials were digitised using a CED 1401+ interface (Cambridge Electronic Design, Cambridge, UK) before being stored on a computer at a sampling rate of 20 kHz, without filtering. Averaging and taking measurements of latencies and amplitudes of electrical potentials were performed using Signal software (Cambridge Electronic Design, Cambridge, UK).

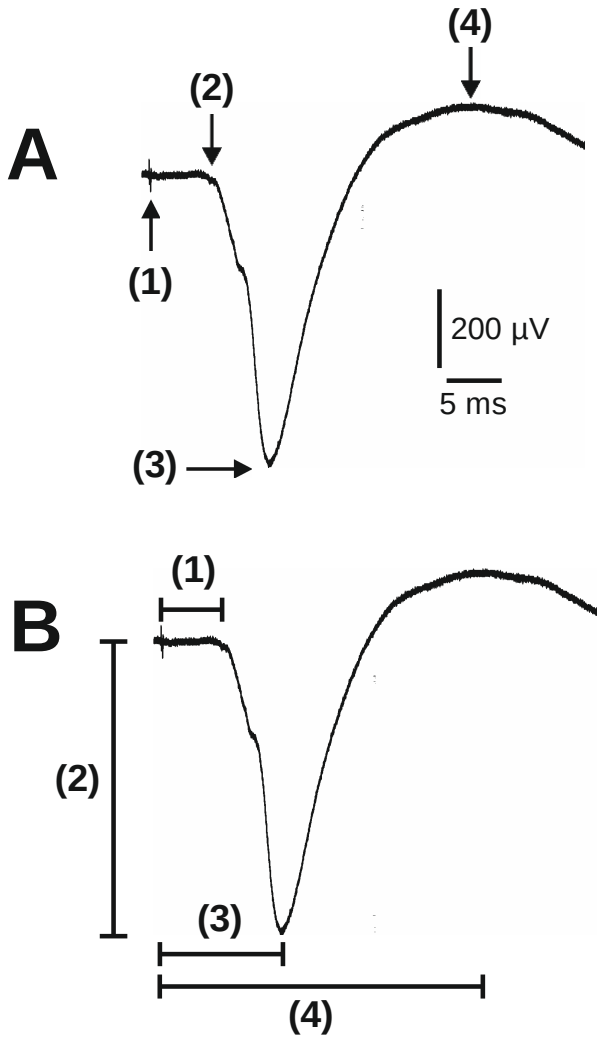


Figure 2.7 Examples of radial SEP waveforms. *Examples of SEPs recorded from the exposed somatosensory cortex of a normal (non-lesioned) animal following stimulation of the radial nerve. All recordings were averages of 15 sweeps and displayed using the standard electrophysiological convention of having positivity downwards. (A) numbered arrows point to different components of the surface recordings: (1) stimulus artifact, which indicates the timing of the stimulation; (2) onset of the positive component; (3) peak positive component; and (4) peak negative component of the SEP. (B) numbered bars indicate the different waveform measurements that were made: (1) latency to onset of the positive component; (2) amplitude of the positive component; (3) latency to peak positive component; (4) latency to peak negative component (Image provided by Toft and Riddell).*

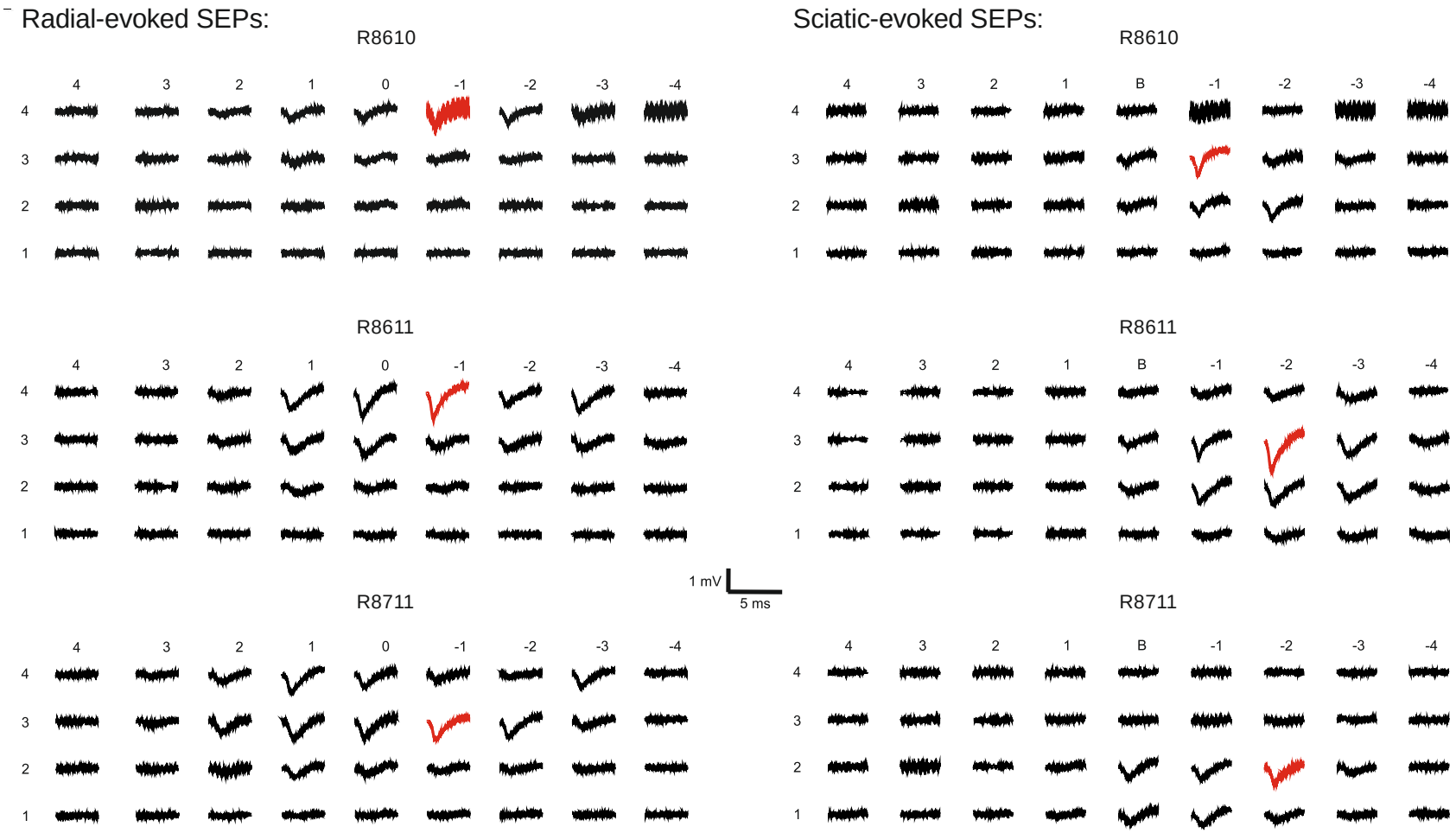


Figure 2.8 Examples of averaged normal radial and sciatic-evoked SEP traces *- recorded in three representative uninjured animals, showing the overlapping cortical locations from which radial and sciatic SEPs*

were recorded, and the relatively consistent locations and amplitudes at which radial and sciatic-evoked SEPs of maximal amplitude were recorded (shown in red). All locations are shown in mm relative to Bregma (B).

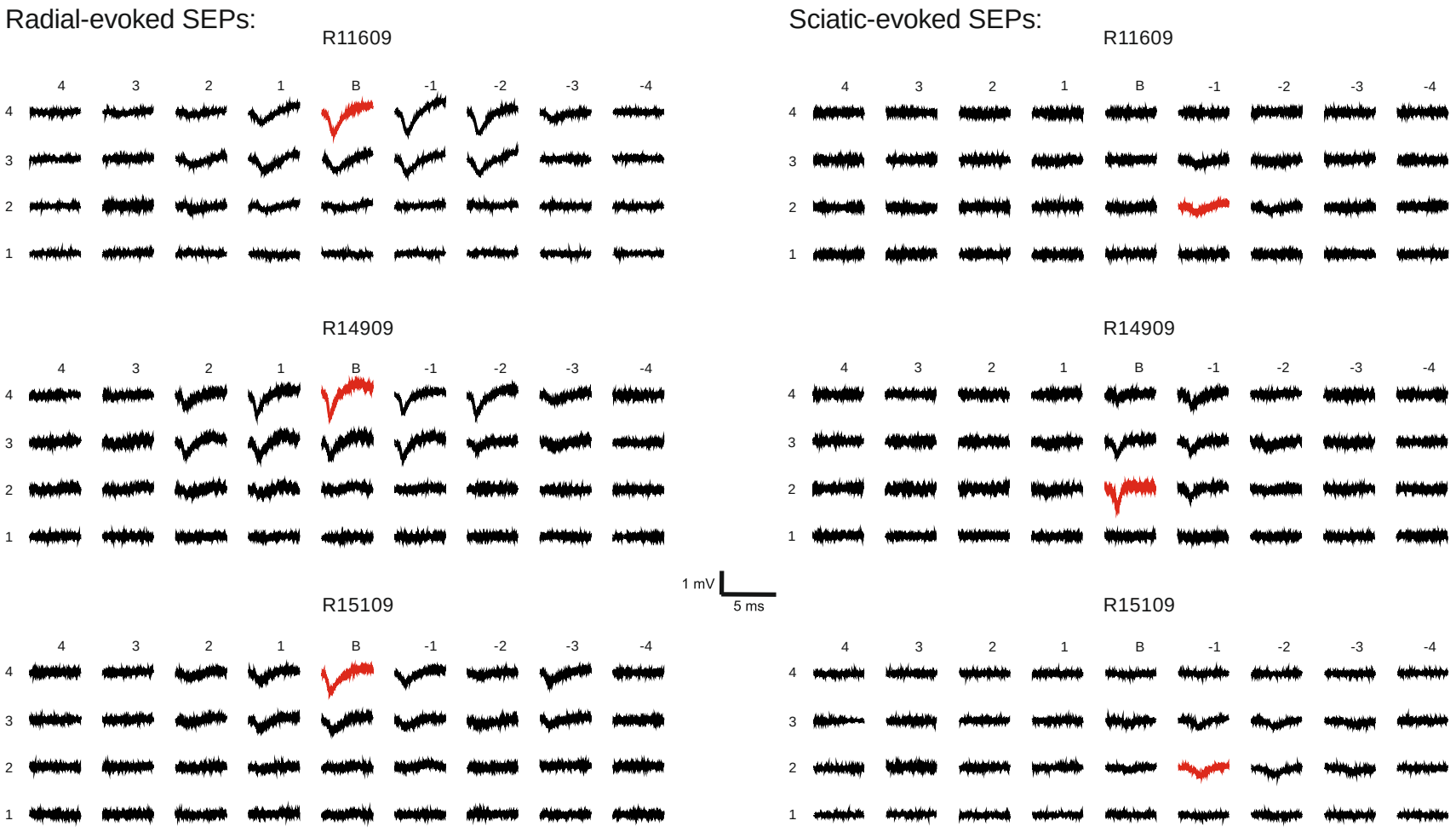


Figure 2.9 Examples of averaged injured radial and sciatic-evoked SEP traces *- recorded in three representative animals 6 weeks following 150 Kdyn contusion injuries at T9, showing the overlapping cortical locations from which*

radial and sciatic SEPs were recorded, and the relatively consistent locations and amplitudes at which radial and sciatic-evoked SEPs of maximal amplitude were recorded (shown in red). All locations are shown in mm relative to Bregma (B).

2.2.3.17 Recording the acute recovery of sensory-evoked potentials

Once the normal map had been completed, twelve of the animals in the uninjured control group were injured and used to make up the acute group. To allow monitoring of the acute recovery the radial recording electrode was left in place at the end of the normal map and the roving electrode was placed back where the largest sciatic SEPs had been recorded. SEP recordings were made starting immediately following injury and continued at 5 minute intervals until amplitudes had stabilised. Only once SEP amplitudes had stabilised were acute SEP maps made.

2.2.4 Technical considerations with peripheral nerve stimulation

To obtain a quantitative measure of the summed strength of action potentials in the dorsal column-medial lemniscus pathway which can be compared between animals, it is necessary to be able to consistently activate the same pathway to the same extent from animal to animal. This is achieved relatively simply when using peripheral nerve stimulation, where care can be taken to ensure that the same branches of the nerve are stimulated in each animal and by applying a supra-maximal stimulus intensity.

Occasionally a transient drift in SEP amplitude was observed. This could be controlled for by monitoring the sciatic SEP amplitude throughout the experiment. If a drop in radial SEP amplitude was observed, stimulation of both sciatic and radial nerves was stopped to allow time for the radial SEP amplitude to return to the level observed prior to the drifting. Thus, any change in the radial SEP amplitude was interpreted as the system drifting, and once it had returned to normal levels the amplitudes of sciatic SEPs could once again be trusted to be genuinely maximal.

As described earlier, the stability of the radial SEP allows for the normalisation of sciatic SEP amplitudes such that they can be made directly comparable between animals. This is required as SEP amplitudes vary between normal uninjured animals, and as such must be normalised by expressing the

sciatic SEP amplitude as a percentage of the maximal radial SEP amplitude recorded in the same animal.

2.2.5 Method for supramaximal activation of peripheral nerves

In each animal, supramaximal activation of the radial and sciatic nerves was achieved by finding the threshold for activation and then slowly increasing the stimulus intensity until the SEP amplitude was seen to be maximal. Supramaximal stimulus intensities were then used throughout the rest of the experiment.

2.2.6 Perfusion fixation

At the end of electrophysiological experiments, animals were transcardically perfused with mammalian Ringer's solution followed by paraformaldehyde fixative at the end of the electrophysiological experiment for histology. Animals were perfused through the right ventricle with mammalian Ringer's solution, containing 0.1% lidocaine to help dilate the blood vessels and remove blood from the circulatory system. This was immediately followed by 1L 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS), pH 7.4. The injury site was then located and carefully removed for post fixation. Post fixation was achieved by placing the cord in the same fixative solution overnight in which 30% sucrose was added for the purpose of cryoprotection.

2.2.7 Histological processing

After washing in 0.1M PBS, the injury site was identified by locating the marking suture in the dura and blocks containing the whole injury site were prepared, it was seen that the whole injury site could usually be contained within a 6 mm block. A notch was made in the ventral, caudal aspect of the block in approximately half the blocks, the remaining blocks were left intact. 60µm sections were cut (parasagitally in notched blocks and transversely in

intact blocks) at -20°C using a Leica CM1850 cryostat (Leica Microsystems GmbH, Wetzlar, Germany). Sometimes, where the injury was extensive, it was difficult to cut whole sections or the sections fell apart after cutting, this was particularly problematic, especially in transverse sections, and precluded the quantification of lesion cavity volume. Sections were washed in 0.3M PBS double salt (PBSD), and then incubated for 30 min in 50% ethanol. After washing three times for 10 minutes each in 0.1M PBS, sections were treated with fluorescence histochemistry techniques.

Sections were incubated in anti-NF200 (1:1000, Sigma) and anti-GFAP (1:1000, Dako) primary antibodies at 4°C for 72hrs, which label the axons and the astrocytes (contusion lesion boundaries) respectively. Sections were then washed in PBSD and incubated in secondary antibodies, raised in the appropriate species, conjugated to fluorophores (Alexa 488 1:500, Rhodamine 1:100) for 3-4 hours at room temperature or overnight at 4°C. After which, all sections were washed in PBS three times for 10 minutes each, mounted on plane glass slides in anti-fade mounting medium (Vectashield: Vector Laboratories, UK), before being coverslipped and the edges of the coverslip sealed with nail varnish and stored at -20°C in darkness.

2.2.8 Microscopy

2.2.8.1 Verification of contusion injury

Phase contrast light microscopy was used to view sections containing the lesion cavity in order to determine the perimeter of the cavity and the relationship to the boundary between grey and white matter. Epifluorescence microscopy was also used to observe neurofilament and GFAP immunolabelling in the sections.

2.2.9 Off-line analysis of electrophysiological data

Averaged records were inspected offline with Signal software (Cambridge Electronic Design, Cambridge, UK) to measure the amplitudes and latencies of the SEPs recorded at each location. Maximum amplitude and latency data were collected in Microsoft Excel spreadsheets before being imported into Prism 4 software (Graphpad Software Inc, USA) for graphing. Data from the SEP maps were collected in Microsoft Excel spreadsheets and imported to 3D Field software (Version 3.0.9.0, Copyright 1998–2007, Vladimir Galouchko, Russia) to create isopotential contour plots of the cortical areas in which SEPs were recorded. These data were then imported to Prism 4 software for graphing.

2.2.10 Statistical analysis

All electrophysiological data were statistically analysed using Prism 4 software (Graphpad Software Inc, USA). Analysis of variance (ANOVA) and post-hoc Tukey's multiple comparison tests were employed to reveal the difference among different groups.

2.3 Results

2.3.1 Electrophysiological assessment of long tract functions following contusion injury.

Sensory evoked potentials (SEPs) evoked by stimulating ipsilateral radial and sciatic nerves and recorded from the contralateral somatosensory cortex were used to assess function in the ascending dorsal column pathways following contusion injury at T9. Two main studies were performed:

Injury severity study

To assess the effects of injury severity, recordings were made of peripheral nerve-evoked SEPs from 30 animals receiving injuries of 150 kdyne (n=11), 175 kdyne (n=12) and 200 kdyne (n=7). All of the animals were investigated at 6 weeks post injury.

Time course study

To assess changes in long tract function with time after injury, peripheral nerve-evoked SEP recordings were made from animals 45 receiving injuries of 150 kdyne at 3-4 days (n=10), 2 weeks (n=8), 12 weeks (n=15) and 6 months (n=12) following injury. Animals that received 150 Kdyne injuries in the injury severity study were used as a 6 week survival group here (n=11).

For comparison, peripheral nerve evoked SEPs were also recorded from 23 control animals, 12 of which were made from animals subsequently used to investigate the effects of injury at the acute time point.

In either study, if whilst mapping SEPs the experiment had to be terminated early due to the animal losing condition then only the data obtained from full maps were included.

Comparison of body weights and injury severities

It was necessary to limit the variable parameters in each experiment to ensure that any differences we observed in the electrophysiological recordings were

solely due to differences in injury severity or survival time. One method that was employed was to ensure that the animals from different experimental groups received injuries of as similar a severity as possible. Contusion injuries of similar force will produce varied injury severities when applied to spinal cords of different sizes due to the impactor tips ability to damage a greater or lesser proportion of the cord depending on its size. It was therefore necessary to select animals for injury that were of a similar body weight and would therefore have similar sized spinal columns. To ensure this was strictly adhered to, each animal's weight at the time of injury and the injury parameters provided by the Infinite Horizons software were recorded and graphed for comparison.

Injury severity study

Animals from each of the different injury severity groups had similar body weights at the time of injury, as shown in Figure 2.10 A. At the time of electrophysiological assessment, individuals from each experimental group had maintained similar body weights, as shown in Figure 2.10 B. Data produced by the Infinite Horizons Impactor show that the actual injury force that each animal was subjected to was similar between animals within each experimental group (Figure 2.10 C). When comparing actual injury force between experimental groups it is evident that each experimental group received distinctly different force contusions and distinctly different cord displacements, both of which increased in a graded fashion with increasing injury severity (Figure 2.10 C-D). It should be noted that the spread of data points in the displacement graph shown in Figure 2.10 D is thought to result from the non-uniform nature of the vasculature on the dorsal surface of the cord.

Time course study

Animals from the different time course groups had similar body weights at the time of injury, with the exception of animals from the acute injured group which were selected to be of a weight which was more comparable to that seen in other groups at the time of electrophysiology, as shown in Figure 2.11 A-B. This was a deliberate decision to make the electrophysiological recordings more comparable between groups, which we decided was of more

importance than ensuring that animals were of a similar weight at the time of injury. Data produced by the Infinite Horizons Impactor show that the actual injury force that each animal was subjected to was similar between animals within each experimental group. Animals from different experimental groups were shown to have received similar force contusions and similar cord displacements, which suggests that they received similar severity injuries and are thus directly comparable (Figure 2.11 C-D).

2.3.2 Injury severity and its effects on SEPs

Typical SEP traces showed clearly that injury severity has a graded effect on SEPs, with more severe injuries linked to smaller amplitude SEPs which are slower to arrive in the cortex and are more temporally diffuse, as shown in Figure 2.12. To ascertain if these observations were significant, quantitative measures were made; the maximum radial and normalised sciatic SEP amplitudes, the cortical locations within which radial and sciatic SEP amplitudes were 80% and 15%, respectfully, of the averaged maximal radial SEP amplitude, and normalised latency to radial and sciatic SEP onset were determined in each animal as discussed below.

Maximum SEP amplitudes

Radial and sciatic SEPs were recorded at each location on the grid shown in Figure 2.4. Whilst radial SEP amplitudes were kept as absolute values, sciatic SEP amplitudes were normalised by expressing each as 15% of the maximal radial SEP amplitude recorded in the same animal. The maximum radial and sciatic SEP amplitudes recorded from animals within each experimental group were then statistically analysed using ANOVA with Tukey's multiple comparisons and plotted as shown in Figures 2.13 A & B. Whilst Figure 2.13 A shows no change in maximum radial amplitudes, Figure 2.13 B shows that maximum sciatic SEP amplitudes were reduced in a graded fashion with increasing injury severity and that the maximum SEP amplitudes recorded in each of the injured experimental groups were significantly different from that recorded in normal animals. A breakdown of the statistical analysis is shown in Table 2.1.

SEP isopotential areas

The amplitudes of the radial and sciatic SEPs evoked at each cortical recording site were used to construct radial and sciatic isopotential contour; these represent the areas of cortex from which radial and sciatic SEPs are 80% or 15%, respectively of the largest radial SEP amplitude recorded in each animal. The radial and sciatic isopotential contour areas from animals within each experimental group were then averaged, statistically analysed using ANOVA with Tukey's multiple comparisons, and plotted as shown in Figures 2.14 A-B. This showed that there is no observable trend between injury severity and radial isopotential contour size, and statistic analysis revealed that no significant difference exists between the radial SEP isopotential areas recorded in each experimental group (Figure 2.14 A). However, as shown in Figure 2.14 B, the sciatic SEP isopotential areas were seen to be reduced in a graded fashion with increasing injury severity, and the SEP isopotential areas recorded in each of the injured experimental groups were significantly different from that recorded in normal animals. A breakdown of the statistical analysis is shown in Table 2.1.

Latency to SEP onset

The latencies to the onset of both radial and sciatic SEPs were measured in each animal and normalised to account for the differences in conduction distance seen from animal to animal by expressing each as the % difference between the actual conduction distances measured in each animal and the maximum radial and sciatic conduction distances found across the groups, respectively, as shown in Figures 2.15 A-B. It can be seen from Figure 2.15 A that radial latencies to SEP onset remain unaffected by injury severity, with ANOVA and Tukey's multiple comparisons revealing no significant difference between the groups. However, as shown in Figure 2.15 B, the latency to sciatic SEP onset was increased in a graded fashion with increasing injury severity, and the latencies to the onset of sciatic SEPs in each of the injured groups were significantly different from that recorded in normal animals. A breakdown of the statistical analysis is shown in Table 2.1.

2.3.3.1 Injury severity and its effects on the cortical locations from which SEPs could be recorded

To investigate the distribution of SEPs in normal and injured animals, two approaches were used: Firstly the locations from which maximum SEPs could be recorded were identified, and then complete cortical SEP maps of the cortex were used to construct isopotential contour plots of where SEPs could be recorded.

SEPs evoked by peripheral nerve stimulation were investigated in normal animals (n=23) as well as in 150 Kdyn (n=11), 175 Kdyn (n=11) and 200 Kdyn (n=7) injured groups. Whilst maximum radial and sciatic SEP amplitudes were recorded in every animal investigated, one animal from the normal group and two from the 150 Kdyn and 5 of the animals in the 200 Kdyn group lost condition before completion of the sciatic SEP map.

Locations of maximum SEP amplitudes

After performing full radial and sciatic SEP maps of the cortex, the cortical locations at which SEP amplitudes were maximal could be identified. This was achieved by recording the cortical locations at which the maximal amplitude radial and sciatic SEPs were recorded in each animal in the normal and injured groups. Figures 2.16 A-B show grids representing the cortex relative to Bregma (B), with 1 mm spaced grid points representing the locations from which SEP recordings were made. The location at which maximum SEP amplitudes were recorded in each animal is shown by an asterisk coloured to represent the normal or different injury groups.

Figure 2.16 A shows the locations at which maximum radial SEP amplitudes were recorded in each individual animal in each experimental group. It can be seen that the majority of maximum radial SEP amplitudes were recorded within 1 mm of Bregma -1 mm, lateral 4 mm, with normal and injured groups appearing to display roughly the same distribution and a focus on Bregma -1 mm, lateral 4 mm. This shows that the location of maximum radial SEP amplitudes does not appear to change following T9 contusion injuries of up to 200 Kdyn force.

Figure 2.16 B shows the locations at which maximum sciatic SEP amplitudes were recorded in each individual animal in each experimental group. It can be seen that the majority of maximum sciatic SEP amplitudes were recorded within 1 mm of Bregma -1 mm, lateral 2 and 3 mms, with normal and injured groups appearing to display roughly the same distribution and a focus on Bregma -1 mm, lateral 2 and 3 mm. This shows that the location of maximum sciatic SEP amplitudes does not appear to change following T9 contusion injuries of up to 200 Kdyn force.

Locations of normalised SEP isopotential contour plots

To investigate possible changes in the cortical regions from which SEPs can be recorded following different injury severities the data was normalised as described earlier so that the data obtained from the SEP maps was less affected by variations in the maximal SEP amplitudes. These normalised data were used to create isopotential plots: contour lines delineating the area of cortex from which SEP amplitudes were at or greater than specified %s of the maximum radial SEP amplitude recorded in each animal.

Using the electrophysiological approach previously described, the radial and sciatic SEP amplitudes were recorded at each location and normalised by expressing them, respectively, as 80% and 15% of the maximum radial SEP amplitude recorded in the same animal. Isopotential plots were then created, delineating the areas of cortex from which radial SEPs were ≥80% and sciatic SEPs were ≥15% of the maximum radial SEP amplitude recorded in each animal. Figure 2.17 shows the individual 80% radial and 15% sciatic isopotential contour plots recorded in each animal in each of the experimental groups. As shown in Figures 2.17 A and B, in normal animals, despite a degree of variation, the radial SEP isopotential plots are clearly centred on Bregma -1 mm, lateral 4 mm and the sciatic on Bregma -1 mm, lateral 2 and 3 mms. Despite some variation the overall location and apparent size of radial SEP isopotential contours appears to remain relatively constant following 150 Kdyn, 175 Kdyn and 200 Kdyn injuries (Figures 2.17 C, E and G). As shown in Figures 2.17 D, F and H, whilst the location of sciatic SEP isopotential contours does not appear to change following contusion injuries of any of the severities

investigated, the area of cortex that each isopotential plot delineates appears to be reduced in a graded manner with increasing injury severity.

To make it easier to compare the isopotential plots from different groups the radial and sciatic SEP amplitudes recorded at each cortical location in each animal within a group were averaged so that average isopotential contour plots could be created. Due to the slight variation in the individual isopotential contours centres of focus, once averaged, the maximum SEP amplitude as a % of maximum radial SEP amplitude was reduced. Therefore the normalization %s had to be reduced in order to display the isopotential plots from each experimental group on the same cortical grid. Figures 2.18 A and B show averaged radial and sciatic isopotential contour plots from groups of normal 150 Kdyn, 175 Kdyn and 200 Kdyn injured animals with radial SEPs normalised to 80%, and the sciatic to 10% of the maximum radial SEP amplitude recorded in each animal. These plots show that the size and location of normalised, averaged radial SEP isopotential plots is not substantially different when comparing normal animals or any of the injury severities investigated (Figure 2.18 A), however, whilst the cortical locations of averaged sciatic SEP isopotentials appear to remain relatively constant, the size is reduced in a graded fashion with injury severity (Figure 2.18 B).

2.3.3.2 Choice of injury severity for further investigation

In the selection of an injury model on which to assess the effects of spontaneous functional changes or functional changes brought about through intervention strategies, it is advantageous to select an injury severity that causes persistent functional deficits. As functional deficits spontaneously change with time following injury or with the application of therapeutic interventions, long lasting and fairly moderate functional deficits are required in order to assess the potential for improvement or worsening over time.

Figure 2.13 A shows that 6 weeks following contusion injuries of 150 Kdyn the maximum SEP amplitudes that can be detected are reduced to approximately 50% of that seen in normal animals. This shows that up to 6 weeks following a

150 Kdyn injury at T9, deficits are seen to be moderate and persistent such that potential future changes to radial SEP amplitude that improved or worsened functional ability should be detected using this model. Both 175 Kdyn and 200 Kdyn contusion injuries were seen to result in sciatic SEP amplitudes of less than 50% of those seen in normal animals at 6 weeks post-injury (Figure 2.13 B); these more severe injuries would therefore be less useful in assessing any potential worsening of function.

As discussed in chapter one, it is also desirable to select an injury model which causes deficits which are severe enough to be persistent without being so severe that the animals cannot perform behavioural tests. After observing the 150 Kdyn injured animals recover in their home environments it was clear that whilst functional deficits were present up to six weeks post injury, the animals appeared capable of being behaviourally tested soon after injury. The deficits observed in animals from both the 175 Kdyn and 200 Kdyn injured groups appeared severe enough that the animals would be unable to perform behavioural testing.

2.3.4 Post-injury survival time and its effects on SEPs

The time course of changes to SEPs evoked by peripheral nerve stimulation following contusion injuries of 150 Kdyn severity was investigated using 45 additional animals. SEP recordings were made from animals receiving injuries of 150 Kdyn force at different time points post injury; several time points were investigated, including 3-4 days (n=10), 2 weeks (n=8), 12 weeks (n=15) and 6 months (n=12) following injury. Data obtained from the 150 Kdyn injured animals in the previously described severity study were also used to as a 6 week survival group (n=11), and data from the normal animals used in the previously described injury severity study were also used as a control group in this study (n=23); 12 of the animals included in the normal control group received 150 Kdyn contusion injuries immediately after the normal electrophysiological investigations were complete. The animals were then investigated electophysiologically and the data obtained were used to investigate the acute injury time point (n=12).

The effects of acute injury on SEPs

The data on the acute functional effects of contusion injury were obtained from 12 animals that first underwent normal electrophysiological investigation and formed part of the control group, immediately after which each animal received a 150 Kdyn contusion injury at T9 as described earlier. The effects this had on radial and sciatic evoked SEPs were investigated acutely using an electrophysiological approach. During the acute recovery recordings, if sciatic SEP amplitudes were seen to stabilize, normal SEP maps were performed and the data were included as an acute group in the time course study below.

Initial electrophysiological examination of acutely injured animals showed that contusion injury completely ablates sciatic SEPs, as shown in the typical SEP traces in Figure 2.19 which have been coloured to indicate whether normal or to indicate the time post injury. It can be seen that immediately following injury the SEPs begin to gradually recover, with more acute time points associated with smaller amplitude SEPs which are characteristically slower to arrive in the cortex and more temporally diffuse. The recovery of SEPs was investigated over the acute period using the electrophysiological approach described below.

During the pre-injury normal SEP map, the cortical location at which maximal amplitude sciatic SEPs could be recorded was noted. After completion of the map the recording electrode was returned to this location for the duration of the acute experiment. For comparison, radial and sciatic SEP recordings were made immediately prior to and immediately following the injury, and then again at five minute intervals until either the SEP amplitudes stabilized or the animal began to lose condition and the experiment had to be terminated.

Figure 2.20 shows a series of graphs showing the radial and sciatic SEP amplitudes that were recorded before injury and then at five minute intervals thereafter in every acutely injured animal. It can be seen that in every animal investigated sciatic SEP amplitudes were completely ablated as a result of the injury, and radial SEP amplitudes are seen to be considerably attenuated. Radial SEP amplitudes are usually seen to have recovered by 5 minutes post

injury, and appear to stay roughly stable for the duration of the experiment. Despite variability in time course and completeness of each animal's recovery, in most animals, sciatic SEP amplitudes took around 10-15 minutes to start recovering and around an hour to stabilize. A representative example can be seen in the R13610 recovery graph in Figure 2.20 which shows the initial ablation of the sciatic SEP amplitudes and a considerable reduction in radial SEP amplitudes with injury; despite some variation in recorded SEP amplitudes, it can be seen that in this animal the general trend is for radial SEP amplitudes to recover to around pre-injury levels within 5 minutes following injury and for sciatic SEP amplitudes to have stabilised by around one hour following injury. It can be seen in Figure 2.21 that averaged data confirmed that by 5 minutes post injury, radial SEP amplitudes had returned to normal and that roughly one hour after injury both the absolute and normalised sciatic SEP amplitudes had stabilised.

As a consequence of the lengthy and invasive surgical procedures, some animals began to lose condition; consequently only 5 animals were sufficiently stable enough for post-injury SEP maps to be made (see below), and one of these animals lost condition before completion of the sciatic map.

The time course of changes to SEPs from acute to chronic injury

The time course of changes in long tract function from normal up to 6 months post injury were investigated in animals subjected to a 150 Kdyn injury at T9. Radial and sciatic SEPs were investigated after different survival times using the previously described electrophysiological approach.

Figure 2.22 shows typical sciatic SEP traces which demonstrate the time course of changes after injury. Initially, contusion injury completely ablates sciatic SEPs, but over time they begin to recover. More acute time points are associated with SEPs which are smaller in amplitude, longer in latency to onset and greater in temporal dispersion. Over time maximal SEP amplitudes can be seen to gradually return towards normal, with longer recovery times linked to larger amplitude SEPs which are quicker to arrive in the cortex and less temporally diffuse (Figure 2.22).

To ascertain if data obtained from the different groups were significantly different, the maximum normalised radial and sciatic SEP amplitudes, the cortical locations within which radial and sciatic SEP amplitudes were 80% and 15%, respectively, of the averaged maximal radial SEP amplitude, and normalised latency to sciatic SEP onset were determined in each animal as discussed below.

Maximum SEP amplitudes.

Maximum radial and sciatic SEP amplitudes were measured in each animal and whilst maximum radial SEP amplitudes were kept as absolute values, sciatic SEP amplitudes were normalised to 15% of the maximum radial SEP amplitude recorded in the same animal. Data from all the animals in each group were then graphed and analysed (Figure 2.23). Figure 2.23 A reveals that no significant difference was observed between the maximum radial SEP isopotential areas recorded in each experimental group. Surprisingly however, a fairly substantial increase in the radial SEP amplitude was seen at the acute time point, after which amplitudes return closer to normal and remain fairly constant up to 6 months post injury (Figure 2.23 A). Figure 2.23 B shows that immediately following injury sciatic SEPs were significantly reduced, and remained that way with some recovery by two weeks then little change up to 12 weeks post injury; despite this the 6 months time point shows evidence for a possible secondary phase of recovery with sciatic SEP amplitudes no longer significantly different from normal. The statistical tests employed were one-way ANOVA with post-hoc Tukey's multiple comparisons (Table 2.2).

SEP isopotential areas

Isopotential plots were constructed to represent the areas of cortex from which we recorded radial and sciatic SEPs ≥80% and ≥15%, respectively, of the maximal radial SEP amplitudes recorded. Data from all the animals in each group were then averaged, graphed and analysed (Figure 2.24). Figure 2.24 A reveals that no significant difference was observed between the maximum radial SEP isopotential areas recorded in each experimental group. Though a trend was observed for the radial isopotential area to increase slightly with time following injury, a quite substantial dip was noticed in the 6 weeks

survival animals. Figure 2.24 B shows that the sciatic SEP isopotential areas were significantly reduced following injury, again with some recovery seen by 2 weeks and little further recovery up to 12 weeks post injury. Again, the 6 month group showed evidence of a possible secondary phase of recovery. The statistical tests employed were one-way ANOVA with post-hoc Tukey's multiple comparisons (Table 2.2).

Latency to SEP onset

The latencies to the onset of both radial and sciatic SEPs were measured in each animal and normalised by expressing each as the % difference between the actual conduction distances measured in each animal and the maximum radial and sciatic conduction distances found across the groups, respectively. Data from all the animals in each group were then averaged, graphed and analysed (Figure 2.25). Figure 2.25 A reveals a small and non-significant increase in radial SEP onset latency up to the 2/4 day time point, followed by a gradual decline at each time point up to 6 months post injury, by which point onset latency was shorter than in normal animals, though not significantly so. Figure 2.25 B shows that following injury, SEP onset latencies were significantly increased up to the 3-4 days time point, after which they were reduced at each time point investigated up to 6 months post injury. At every time point investigated, sciatic onset latencies were significantly different from normal, except from the 6 months time point. This observation might be indicative of an ongoing recovery process. The statistical tests employed were one-way ANOVA with post-hoc Tukey's multiple comparisons (Table 2.2).

2.3.4.2 Survival time and its effects on the cortical areas from which SEPs could be recorded

To investigate the distribution of SEPs in normal and injured animals, two approaches were used: Firstly the locations from which maximum SEPs could be recorded were identified, and then complete cortical SEP maps were used to construct isopotential contour plots of where SEPs of predetermined sizes could be recorded.

Locations of maximum SEP amplitudes

After performing full radial and sciatic SEP maps of the cortex, the cortical locations at which SEP amplitudes were maximal could be identified. Figures 2.26 A-B show grids representing the cortex relative to Bregma (B), with 1 mm spaced grid points representing the locations from which SEP recordings were made. The location at which maximum SEP amplitudes were recorded in each animal is shown by an asterisk coloured to represent the normal or different injury groups.

The locations at which maximum radial SEP amplitudes were recorded in each individual animal in each experimental group were recorded in Figure 2.26 A. It can be seen that the majority of maximum radial SEP amplitudes were recorded within 1 mm of Bregma -1 mm, lateral 4 mm, with normal and injured groups appearing to display roughly the same distribution and a focus on Bregma -1 mm, lateral 4 mm. This shows that the location of maximum radial SEP amplitudes does not appear to change from the normal to the 6 month post injury time points.

Figure 2.26 B shows the locations at which maximum sciatic SEP amplitudes were recorded in each individual animal in each experimental group. It can be seen that the majority of maximum sciatic SEP amplitudes were recorded within 1 mm of Bregma -1 mm, lateral 2 and 3 mms, with normal and injured groups appearing to display roughly the same distribution and a focus on Bregma -1 mm, lateral 2 and 3 mm. This shows that the location of maximum sciatic SEP amplitudes does not appear to change from the normal to the 6 month post injury time points.

Locations of normalised SEP isopotential contour plots

To investigate potential changes in the cortical regions from which SEPs can be recorded following different injury severities, SEP isopotential contour plots were created from which the cortical areas from which SEPs of certain chosen %s of the largest radial SEP could be investigated.

By normalizing the data as described below, the data obtained from the SEP maps was less affected by variations in the maximal SEP amplitudes. These normalised data were used to create isopotential plots: contour lines delineating the area of cortex from which SEP amplitudes were at or greater than specified %s of the maximum radial SEP amplitude recorded in each animal.

Using the electrophysiological approach previously described, the radial and sciatic SEP amplitudes were recorded at each location and normalised by expressing them, respectively, as 80% and 15% of the maximum radial SEP amplitude recorded in the same animal. Isopotential plots were then created, delineating the areas of cortex from which radial SEPs were ≥80% and sciatic SEPs were ≥15% of the maximum radial SEP amplitude recorded in each animal. Figure 2.27 shows the individual 80% radial and 15% sciatic isopotential contour plots recorded in each animal in each of the experimental groups. As shown in Figures 2.27 A and B, in normal animals, despite a degree of variation, the radial SEP isopotential plots are clearly centred on Bregma -1 mm, lateral 4 mm and the sciatic on Bregma -1 mm, lateral 2 and 3 mms. Despite some variation the overall location and apparent size of radial SEP isopotential contours appears to remain relatively constant following regardless of post-injury survival time (Figures 2.27 C, E, G, I, K and M). As shown in Figures 2.27 D, F, H, J, L and M, the location of sciatic SEP isopotential contours does not appear to change following different post-injury survival times, though some change in isopotential area is clear, as quantified earlier.

To make it easier to compare the isopotential plots from different groups, the radial and sciatic SEP amplitudes recorded at each cortical location in each animal within a group were averaged so that average isopotential contour plots could be created for each group. As with the injury severity group, due to the slight variation in the individual isopotential contours centres of focus, once averaged, the maximum sciatic SEP amplitude as a % of maximum radial SEP amplitude was reduced. Therefore the normalization % had to be reduced in order to display the isopotential plots from each experimental group on the same cortical grid. Figures 2.28 A and B show averaged radial and sciatic

isopotential contour plots from groups following different post-injury survival times, with radial SEPs normalised to 80%, and the sciatic SEPs normalised to 15% of the maximum radial SEP amplitude recorded in each animal. These plots show that the location of normalised, averaged radial SEP isopotential plots does not change following injury (Figure 2.28 A). No change is seen in the cortical locations of averaged sciatic SEP isopotentials at any time point following injury, however as quantified earlier, isopotential contour size is seen to be reduced immediately following injury then appears to increase with increasing post-injury survival time.

2.3.5 Histology

The injury model employed here is designed to investigate changes in function in white matter tracts, specifically the ascending dorsal column system. Using a cryostat, attempts were made to cut the injury site in to serial sections which would allow us to calculate the volume of the lesion cavity, however despite our best attempts the injury epicenters proved too fragile to be sectioned in tact. Despite this, we were able to section and process tissue from immediately either side of the injury epicenter, which allowed us to perform dark field microscopy on tissue sections from within the injury site (Figures 2.29). This revealed that our contusion injuries cause substantial damage to the dorsal columns. Whilst the dorsal columns are seen to be severely disrupted with a cavity at the tissue core, a rim of spared white matter tissue can be seen surrounding the cavity.

In future studies the use of paraffin embedding should be investigated as a method of providing greater stability when cutting sections; due to time constraints this was not possible in this study.

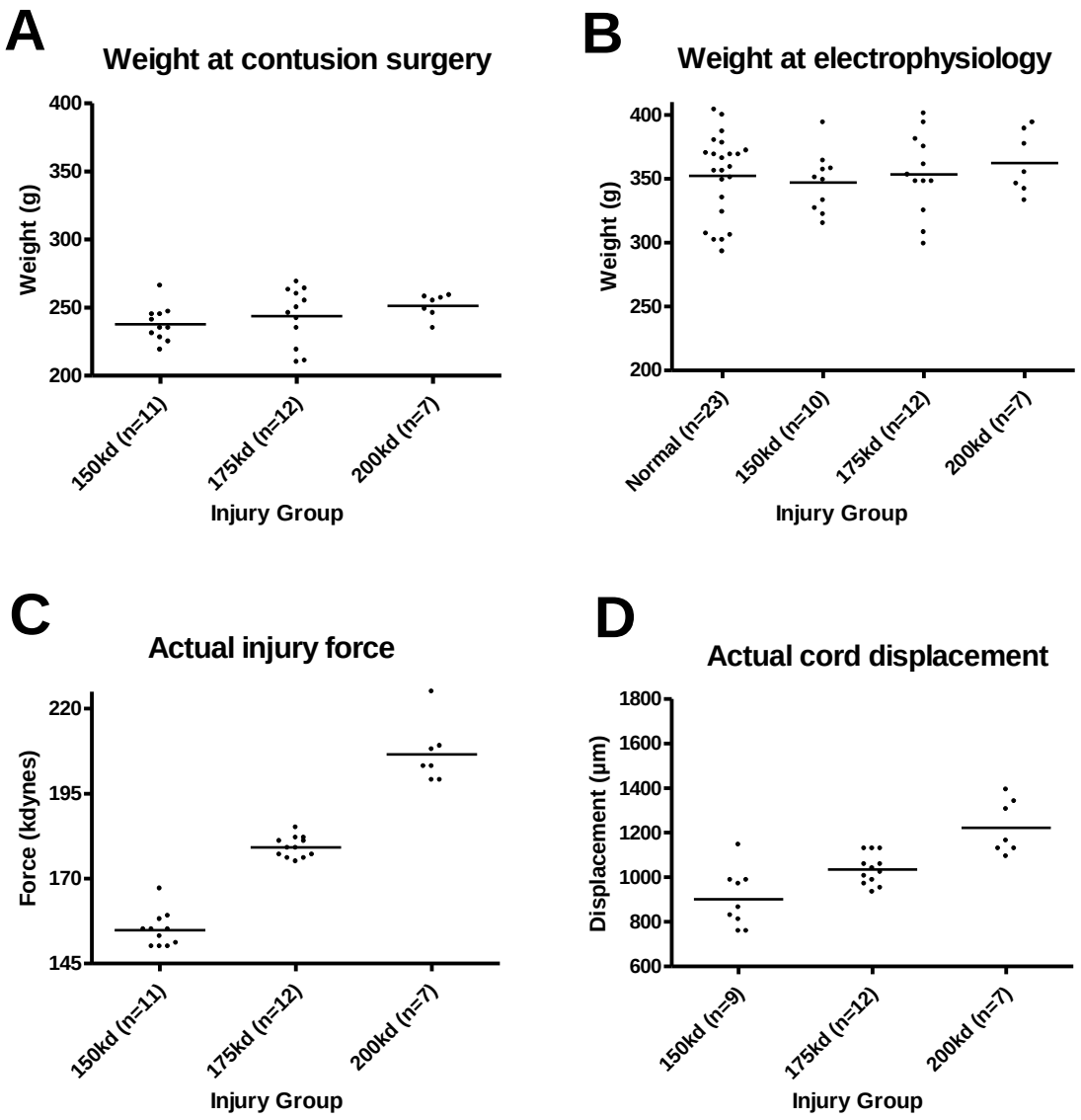


Figure 2.10 Weights, injury forces and cord displacements of individual animals in the injury severity study*. A) Body weight at the time of contusion surgery in each injury group. B) Body weight at the time of electrophysiology in normal animals and in each injury group. C) Actual contusion injury forces recorded in animals in each injury group. D) Actual cord displacement during contusion injury in each injury group. Bars = means.*

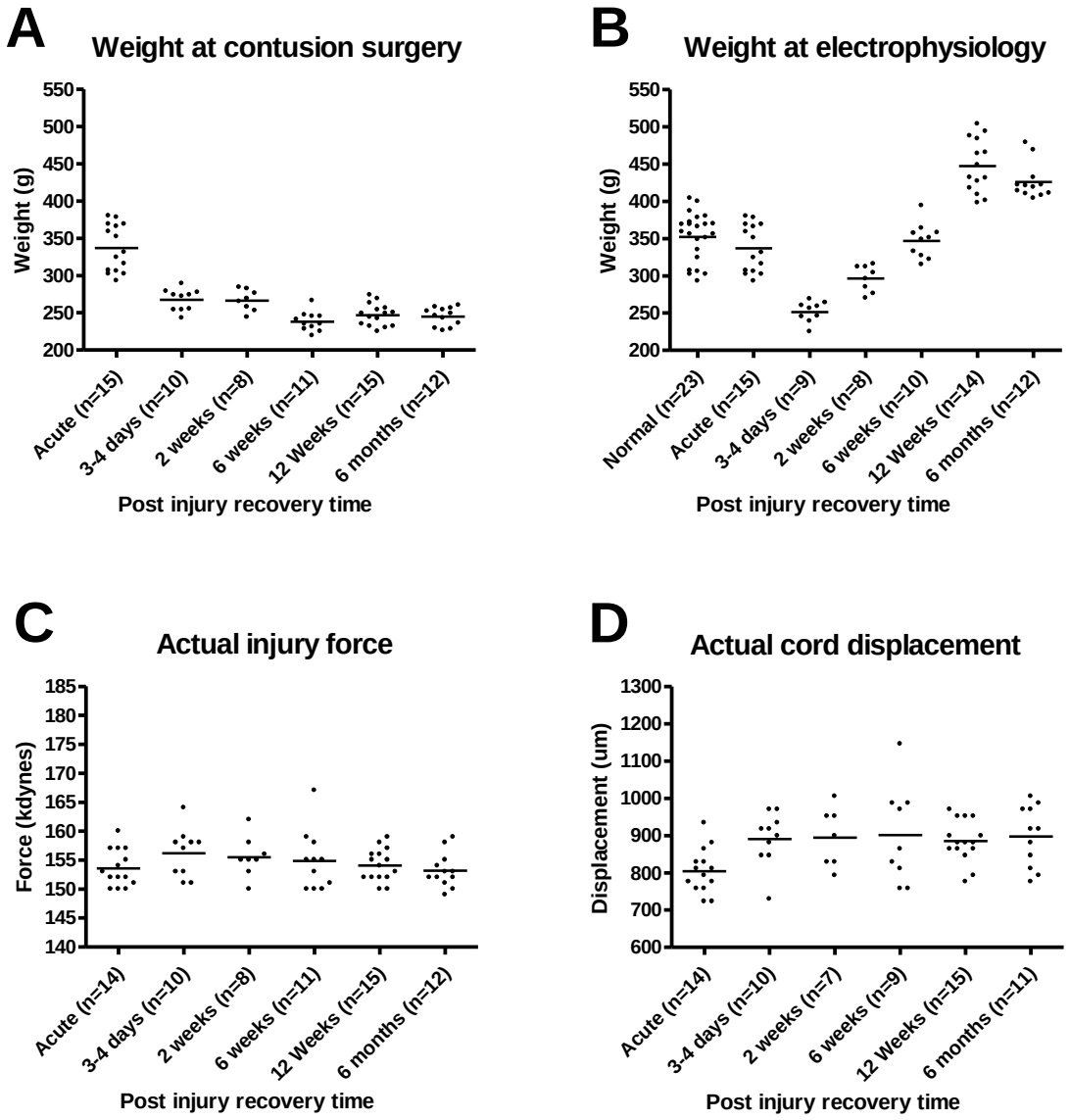


Figure 2.11 Weights, injury forces and cord displacements of individual animals in the time course study*. A) Body weight at the time of contusion surgery in each of the different post injury recovery groups. B) Body weight at the time of electrophysiology in the normal group and in each of the different post injury recovery groups. C) Actual force of contusion injury in each of the different post injury recovery groups. D) Actual cord displacement as a result of contusion injury in each of the different post injury recovery groups. Bars = means.*

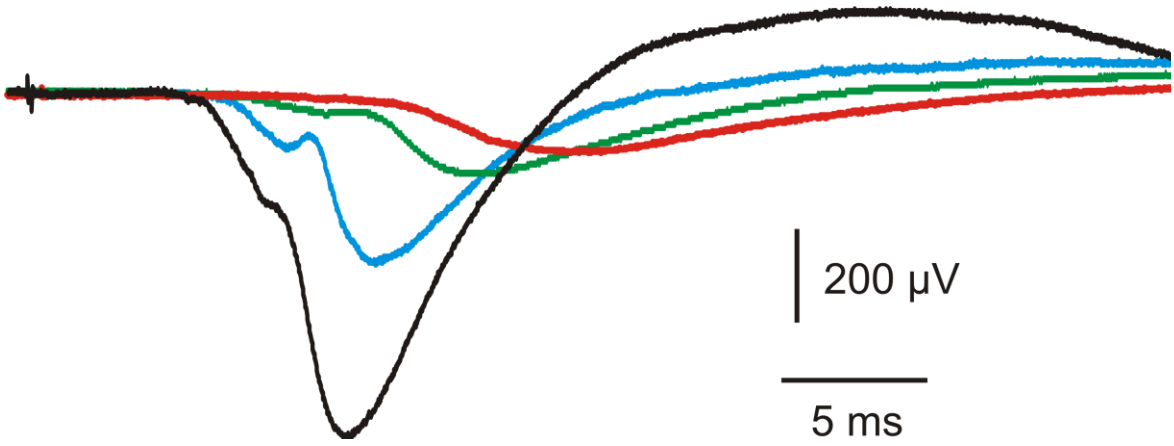


Figure 2.12 Sciatic SEPs demonstrating the effects of injury severities. *Examples of sciatic SEPs averaged from 15 sweeps, recorded from a normal animal and animals 6 weeks after receiving contusion injuries of increasing severity (Black = Normal animal, Blue = 150 Kdyn injured animal, Green = 175 Kdyn injured animal and Red = 200 Kdyn injured animal).*

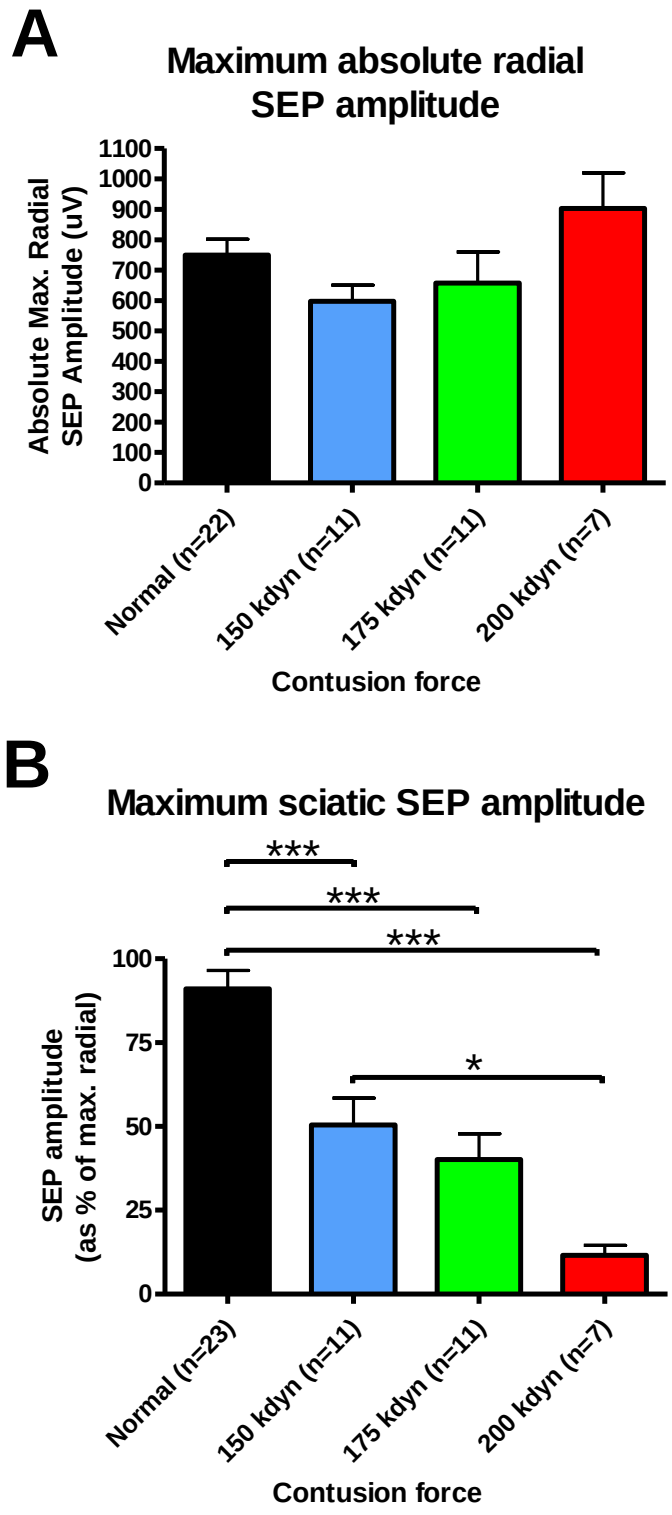


Figure 2.13 Effects of injury severity on maximum SEP amplitude. *(A) Maximum amplitudes of radial SEPs in normal animals and animals 6 weeks following T9 contusion injuries of increasing severity. (B) Maximum sciatic SEPs in normal animals and animals 6 weeks following injuries of increasing severity, expressed as a % of the maximum radial SEP amplitude recorded in each animal. (*p<0.05; **p<0.01; ***p<0.001; one-way ANOVA Tukey's test. Error bars = SEM).*

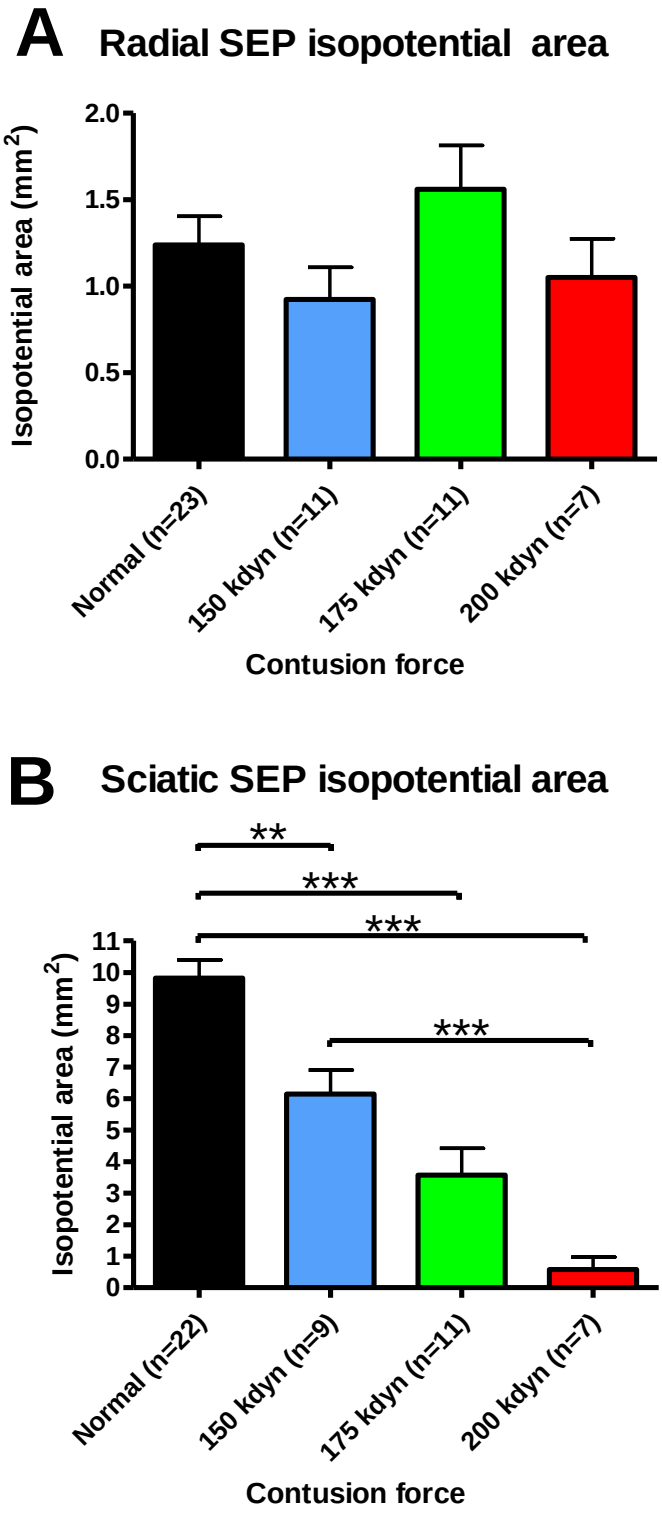


Figure 2.14 Effects of injury severity on isopotential contour areas. *(A) Cortical areas from which radial SEPs at or exceeding 80% of the maximum radial SEP recorded in normal animals and animals 6 weeks following injuries of increasing severity. (B) Cortical areas from which sciatic SEPs at or exceeding 15% of the maximum radial SEP recorded in normal animals and animals 6 weeks following injuries of increasing severity. (*p<0.05; **p<0.01; ***p<0.001; one-way ANOVA Tukey's test. Error bars = SEM).*

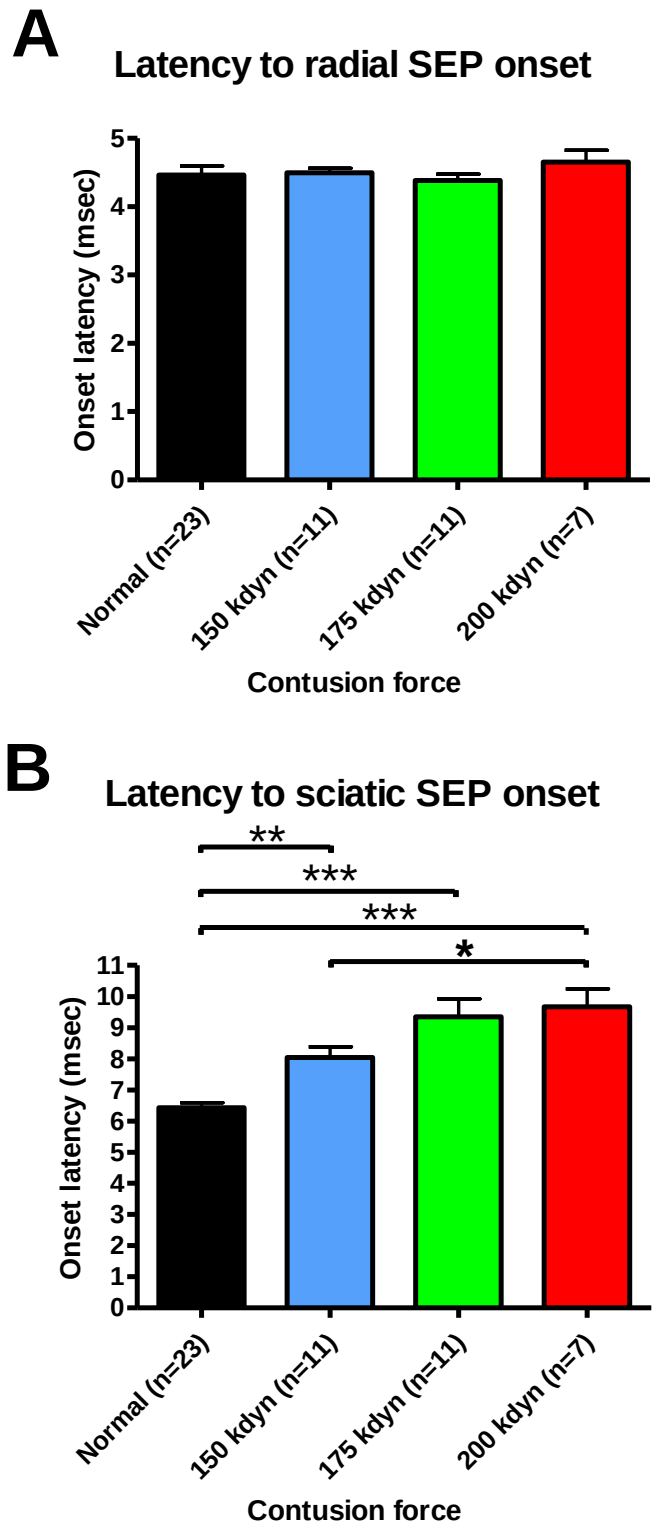


Figure 2.15 Effects of injury severity on the latency to SEP onset. *(A) Latency to onset of radial SEPs in normal animals and animals 6 weeks following injuries of increasing severity. (B) Latency to onset of sciatic SEPs in normal animals and animals 6 weeks following injuries of increasing severity. (*p<0.05; **p<0.01; ***p<0.001; one-way ANOVA Tukey's test. Error bars = SEM).*

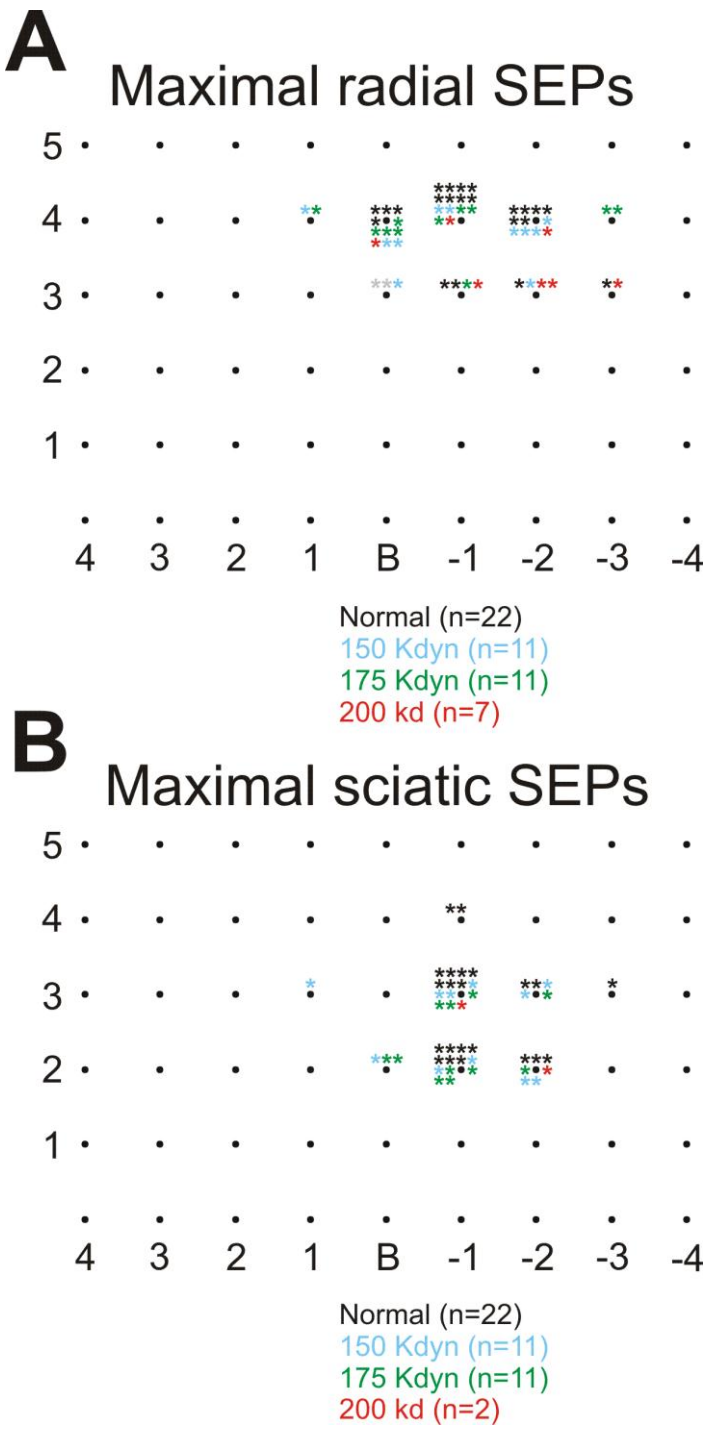


Figure 2.16 Cortical locations of maximum radial and sciatic SEPs recorded 6 weeks following contusion injuries of different severity. *(A) Cortical locations of maximum radial SEPs recorded in individual animals. (B) Cortical locations of maximum sciatic SEPs recorded in individual animals. Asterisks represent individual animals and colours represent different experimental groups. (1 mm spacing between recording locations relative to Bregma (B); caudal = negative).*

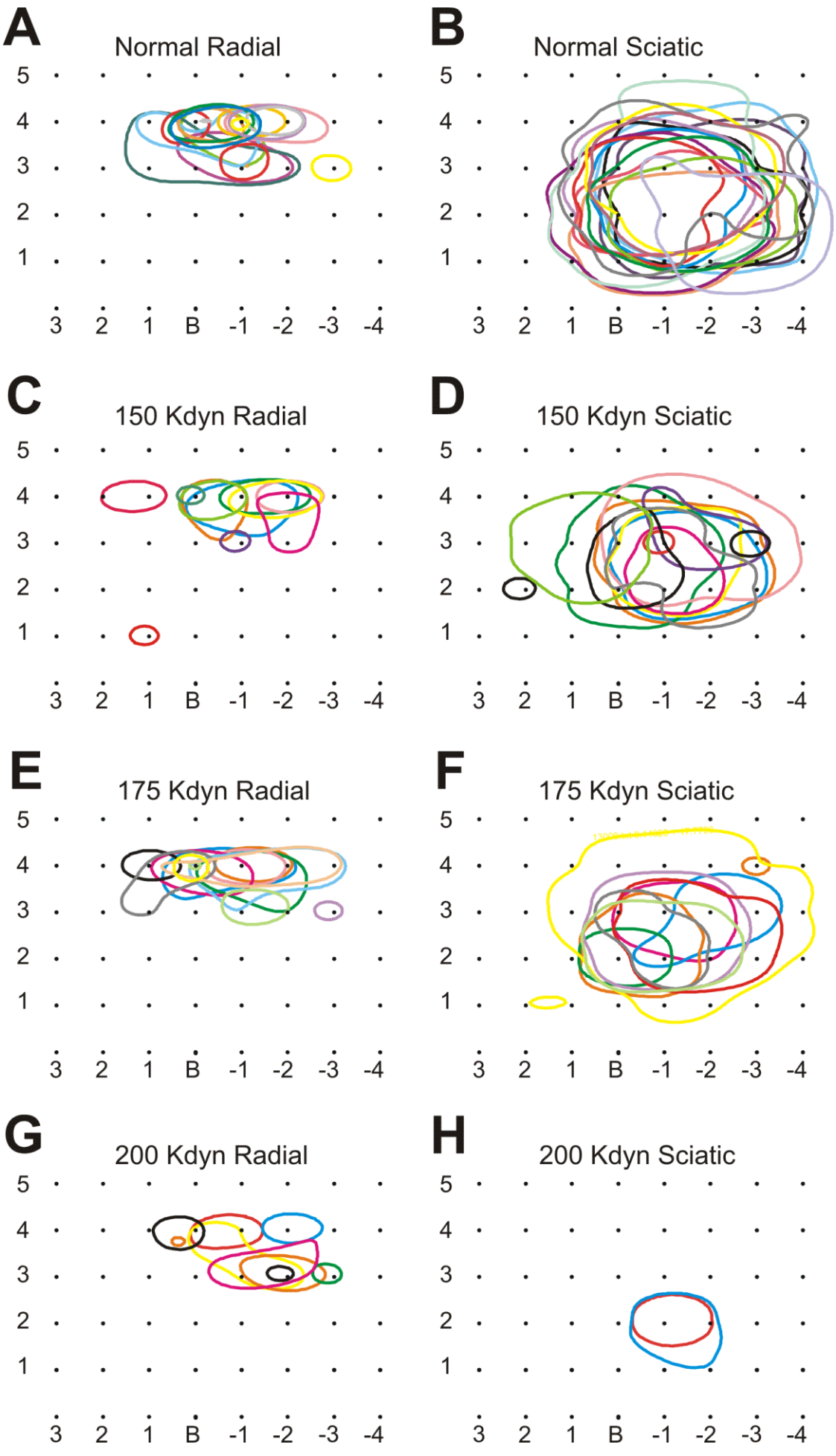

A
Normal Radial
B
Normal Sciatic
C
150 Kdyn Radial
D
150 Kdyn Sciatic
E
175 Kdyn Radial
F
175 Kdyn Sciatic
G
200 Kdyn Radial
H
200 Kdyn Sciatic
5
4
3
2
1
3 2 1 B -1 -2 -3 -4

Figure 2.17 Individual isopotential contour plots of normal animals and animals receiving 150 Kdyn, 175 Kdyn and 200 Kdyn injuries*. Individual radial and sciatic isopotential contour plots normalised to 80% and 15% of the maximum radial SEP amplitude, respectively. Different colours were used to represent isopotential plots from different animals. A) Individual normal radial isopotential plots (n=22), B) Individual normal sciatic isopotential plots (n=22), C) Individual 150 Kdyn group radial isopotential plots (n=11), D) Individual 150 Kdyn group sciatic isopotential plots (n=11), E) Individual 175 Kdyn group radial isopotential plots (n=11), F) Individual 175 Kdyn group sciatic isopotential plots (n=11), G) Individual 200 Kdyn group radial isopotential plots (n=7), H) Individual 200 Kdyn group sciatic isopotential plots (n=2). (1 mm spacing between recording locations relative to Bregma (B); caudal = negative).*

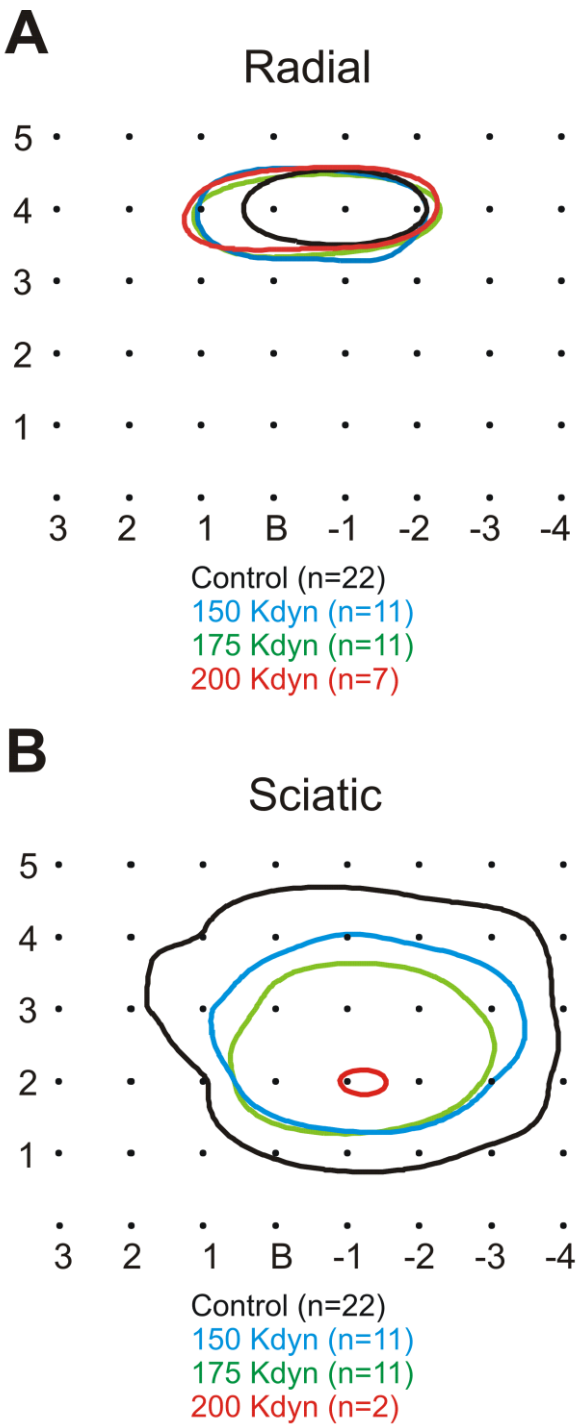


Figure 2.18 Averaged and normalised radial and sciatic SEP isopotential contour plots recorded in normal animals and groups receiving different injury severities. *A) Isopotential contour plots encompassing the area of cortex within which radial SEP amplitudes were at least 80% of the averaged maximal radial SEP amplitude. B) Isopotential contour plots encompassing the region of cortex within which sciatic SEPs could be recorded with amplitudes at least 10% of the averaged maximal radial SEP amplitude. (1 mm spacing between recording locations relative to Bregma (B); caudal = negative).*

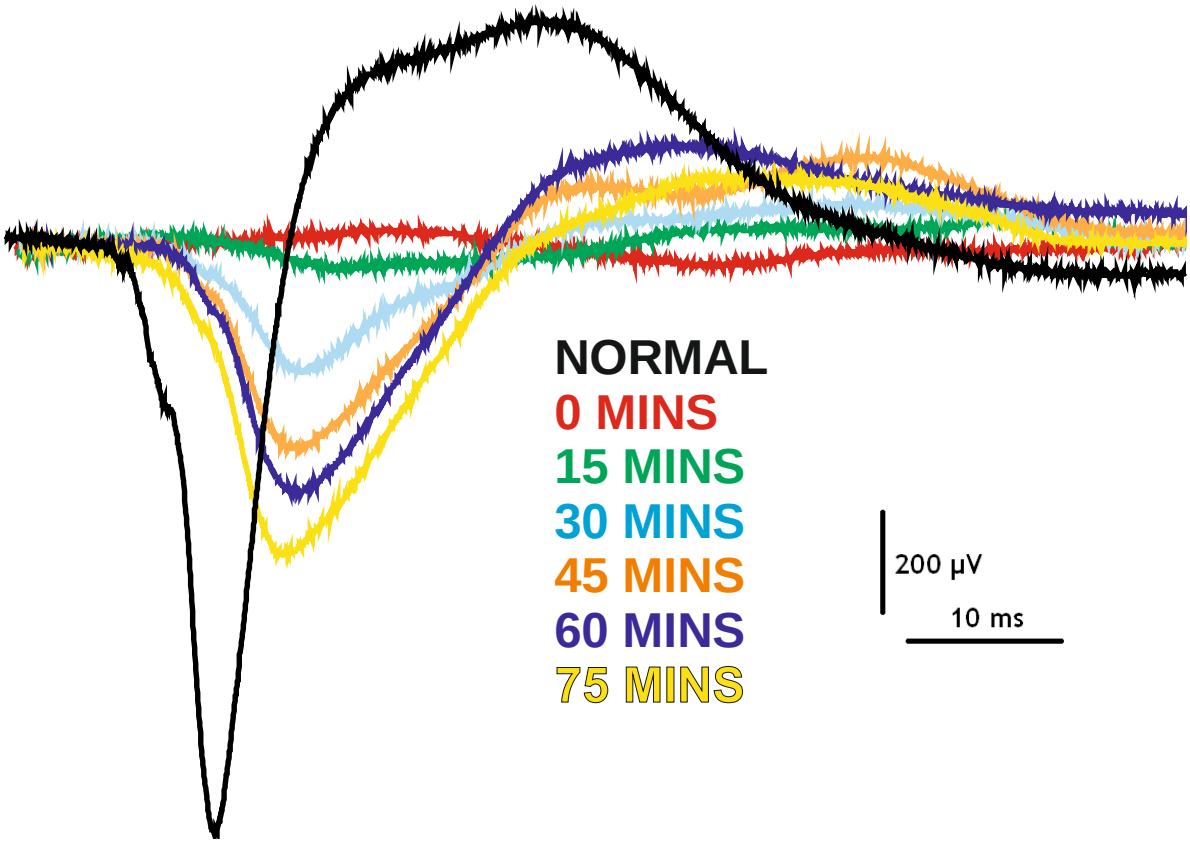
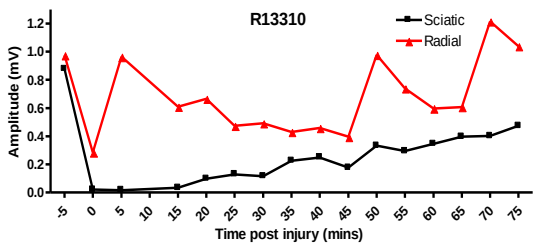
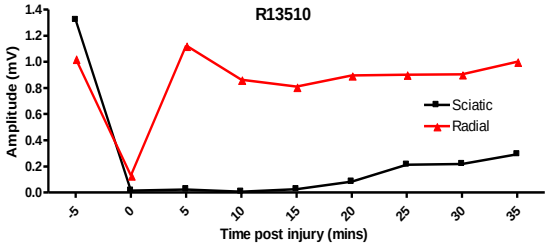
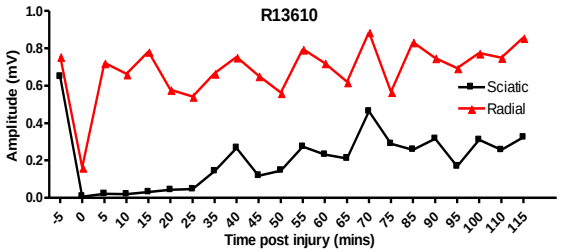
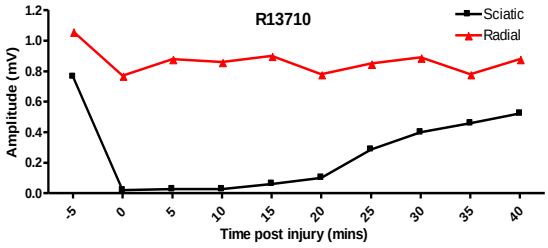


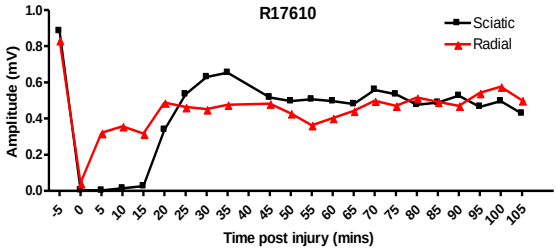
Figure 2.19 Example sciatic SEPs at different time points following acute 150 Kdyn contusion injury. *Examples of averaged SEP waveforms recorded from the cortical surface following stimulation of the sciatic nerve in a representative animal. Recordings were made immediately prior to injury (normal) and at 5 minute intervals starting immediately following a 150 Kdyn contusion injury.*

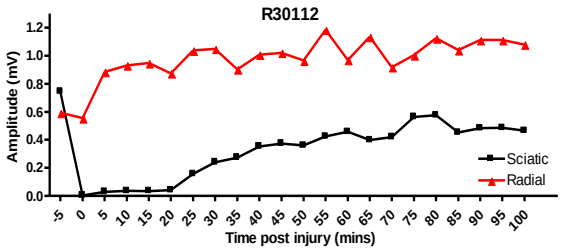

R13310
Sciatic
Radial
Amplitude (mV)
Time post injury (mins)

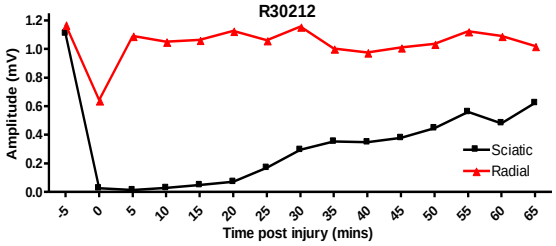

R13510
Sciatic
Radial
Amplitude (mV)
Time post injury (mins)

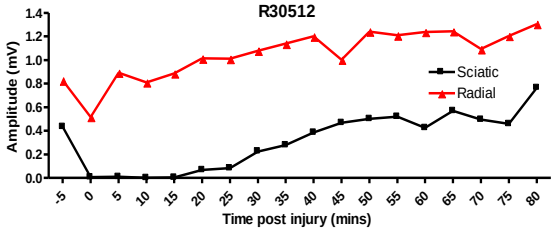

R13610
Sciatic
Radial
Amplitude (mV)
Time post injury (mins)

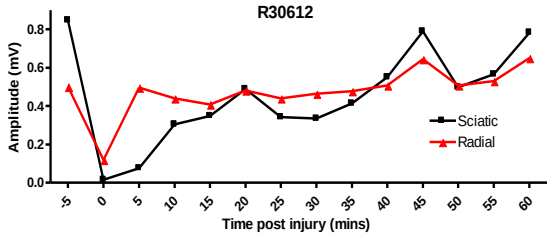

R13710
Sciatic
Radial
Amplitude (mV)
Time post injury (mins)

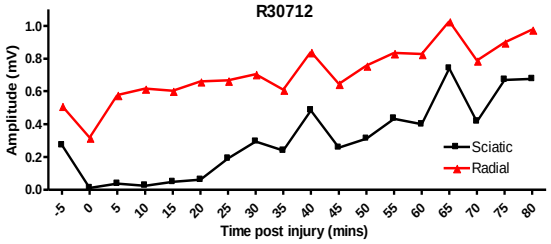

R17610
Sciatic
Radial
Amplitude (mV)
Time post injury (mins)

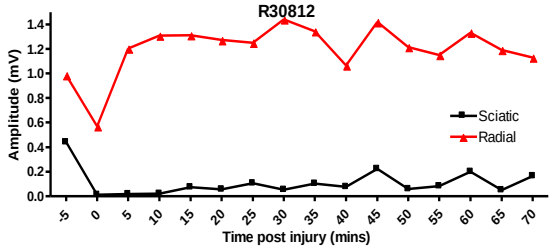

R30112
Sciatic
Radial
Amplitude (mV)
Time post injury (mins)


R30212
Sciatic
Radial
Amplitude (mV)
Time post injury (mins)


R30512
Sciatic
Radial
Amplitude (mV)
Time post injury (mins)


R30612
Sciatic
Radial
Amplitude (mV)
Time post injury (mins)


R30712
Sciatic
Radial
Amplitude (mV)
Time post injury (mins)


R30812
Sciatic
Radial
Amplitude (mV)
Time post injury (mins)

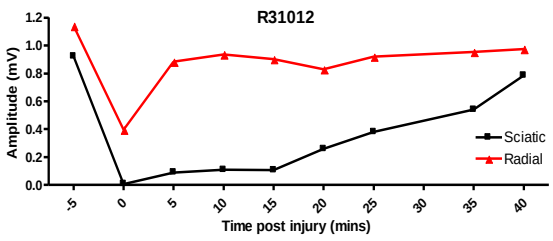

R31012
Sciatic
Radial
Amplitude (mV)
Time post injury (mins)

Figure 2.20 Time course of SEP recovery in individual animals following acute T9 150 Kdyn contusion injury. *Sciatic and radial SEPs were recorded immediately before and immediately after injury, and then again at 5 minute intervals until sciatic SEP amplitudes began to stabilize or the animal began to lose condition and the experiment had to be terminated. Red = Radial SEP amplitude / Black = Sciatic SEP amplitude.*

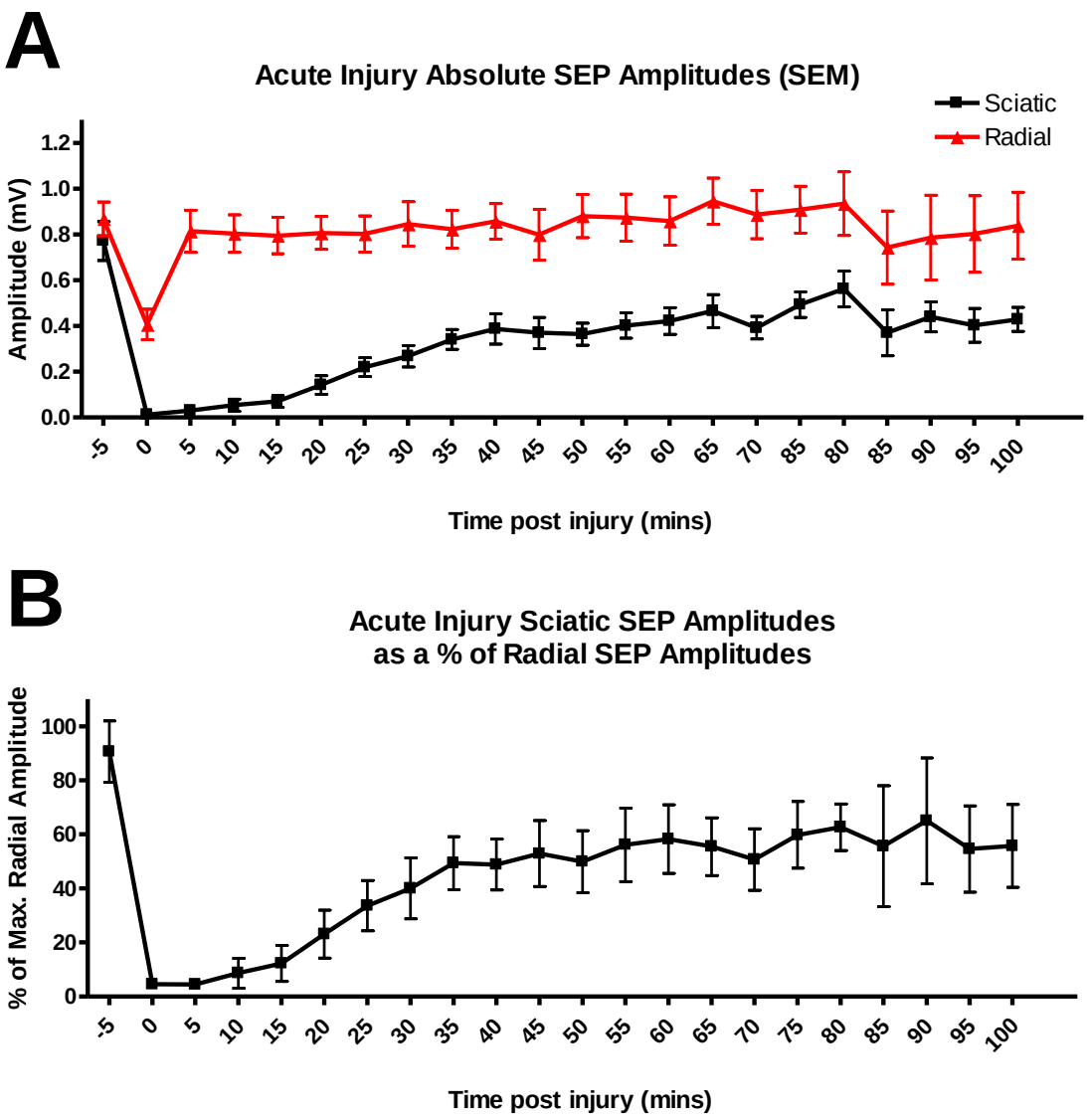


Figure 2.21 Averaged time course of SEP recovery following acute T9 150 Kdyn contusion injury. *Sciatic and radial SEPs were recorded immediately before and immediately after injury, and then again at 5 minute intervals until sciatic SEP amplitudes began to stabilize or the animal began to lose condition and the experiment had to be terminated. A) Sciatic and radial-evoked SEP amplitude recovery, expressed as an averaged amplitude (error bars = SEM, Red = Radial SEP amplitude / Black = Sciatic SEP amplitude; N=12), B) Averaged sciatic SEP amplitude recovery, expressed as a percentage of radial the SEP amplitude recorded at each time point (error bars = SEM; N=12).*

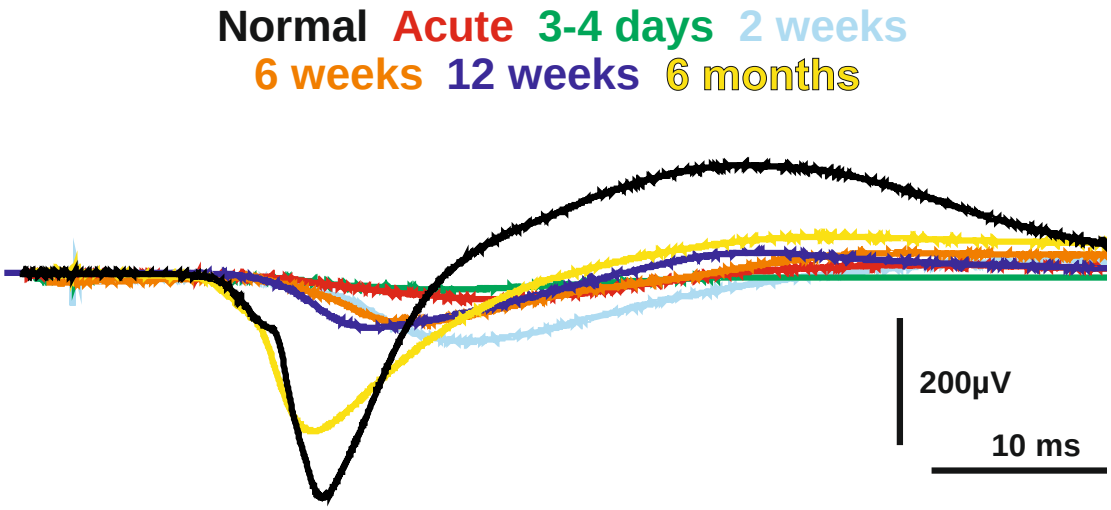


Figure 2.22 Example sciatic SEPs at different time points following 150 Kdyn contusion injury. *Sciatic SEPs recorded from a normal animal and at different time points following a 150 Kdyn contusion injury. SEP amplitudes are reduced and latencies increased following injury, but slowly return towards normal in the 6 months following injury.*

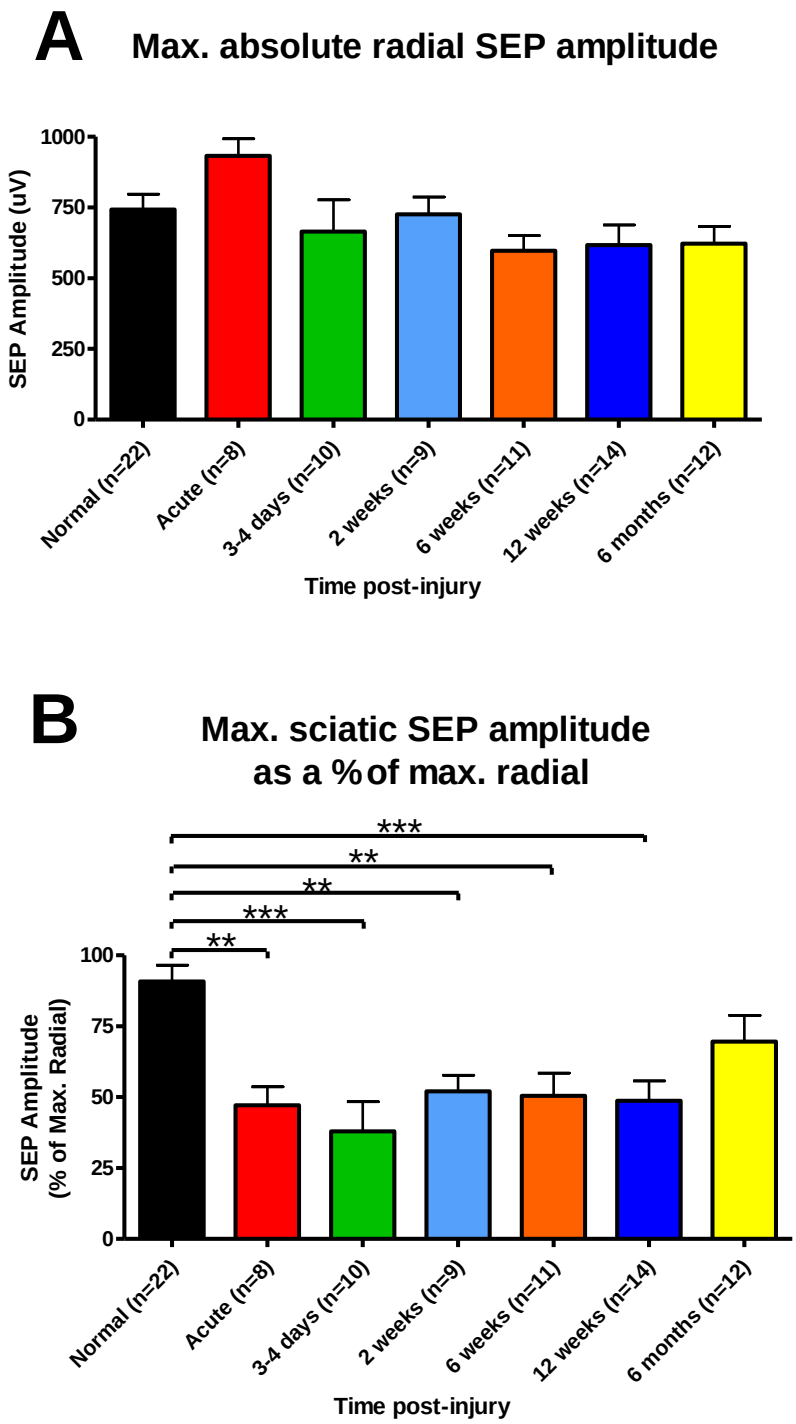


Figure 2.23 Maximum SEP amplitudes recorded in 150 Kdyn injured animals. *Maximum radial (A) and sciatic (B) SEP amplitudes, expressed as 80% and 15% of the maximum radial SEP amplitudes recorded in animals up to 6 months following 150 Kdyn contusion injury, respectively. Groups of normal animals (n=22) were investigated, as well as animals following acute injury (n=8) and following 3-4 days (n=10), 2 weeks (n=9), 6 weeks (n=11), 12 weeks (n=14) and 6 months (n=12) survival (**p<0.01; ***p<0.001; one-way ANOVA Tukey's test. Error bars = SEM).*

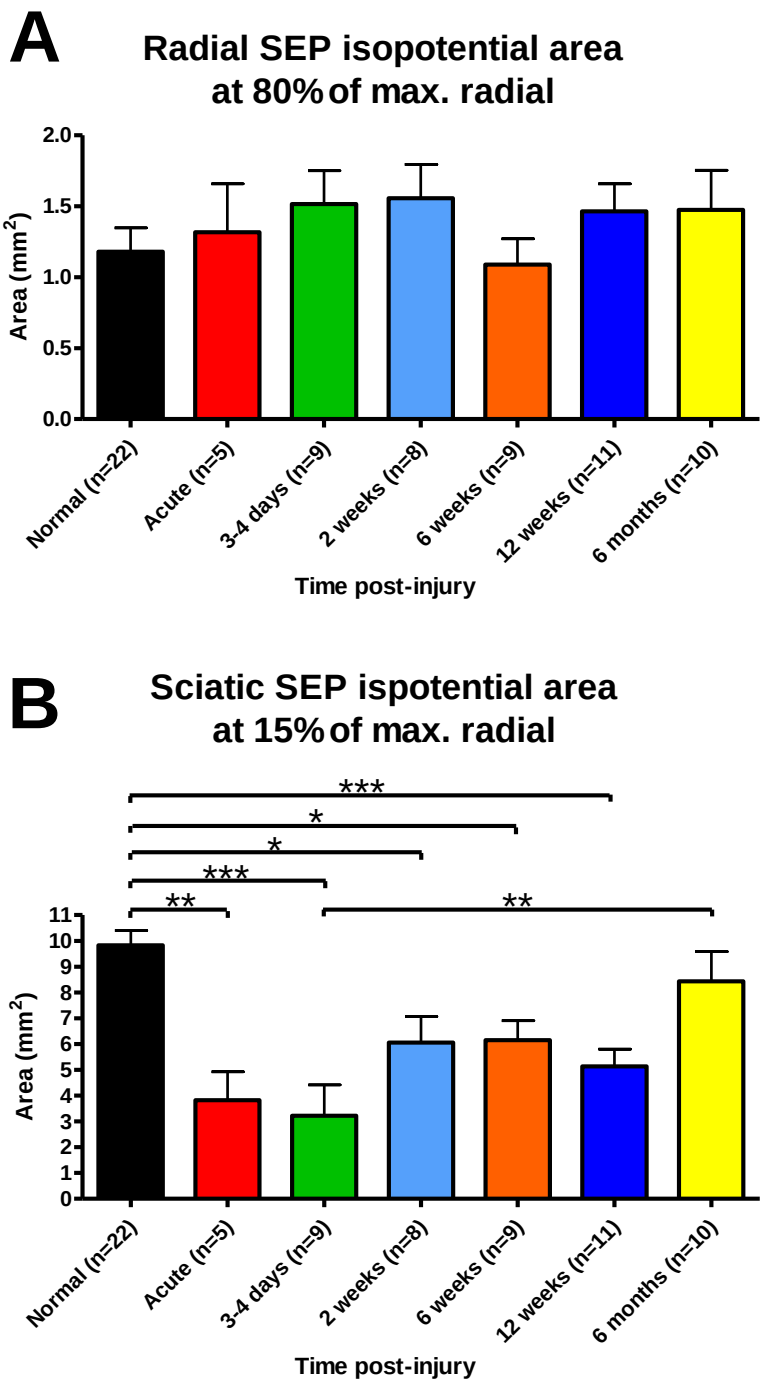


Figure 2.24 SEP isopotential contour areas recorded in 150 Kdyn injured animals. *Radial (A) and sciatic (B) SEP isopotential areas, expressed as 80% and 15% of the maximum radial SEP amplitudes recorded in animals up to 6 months following 150 Kdyn contusion injury, respectively. Groups of normal animals (n=22) were investigated, as well as animals following acute injury (n=5) and following 3-4 days (n=9), 2 weeks (n=8), 6 weeks (n=9), 12 weeks (n=11) and 6 months (n=10) survival (*p<0.05; **p<0.01; ***p<0.001; one-way ANOVA Tukey's test. Error bars = SEM).*

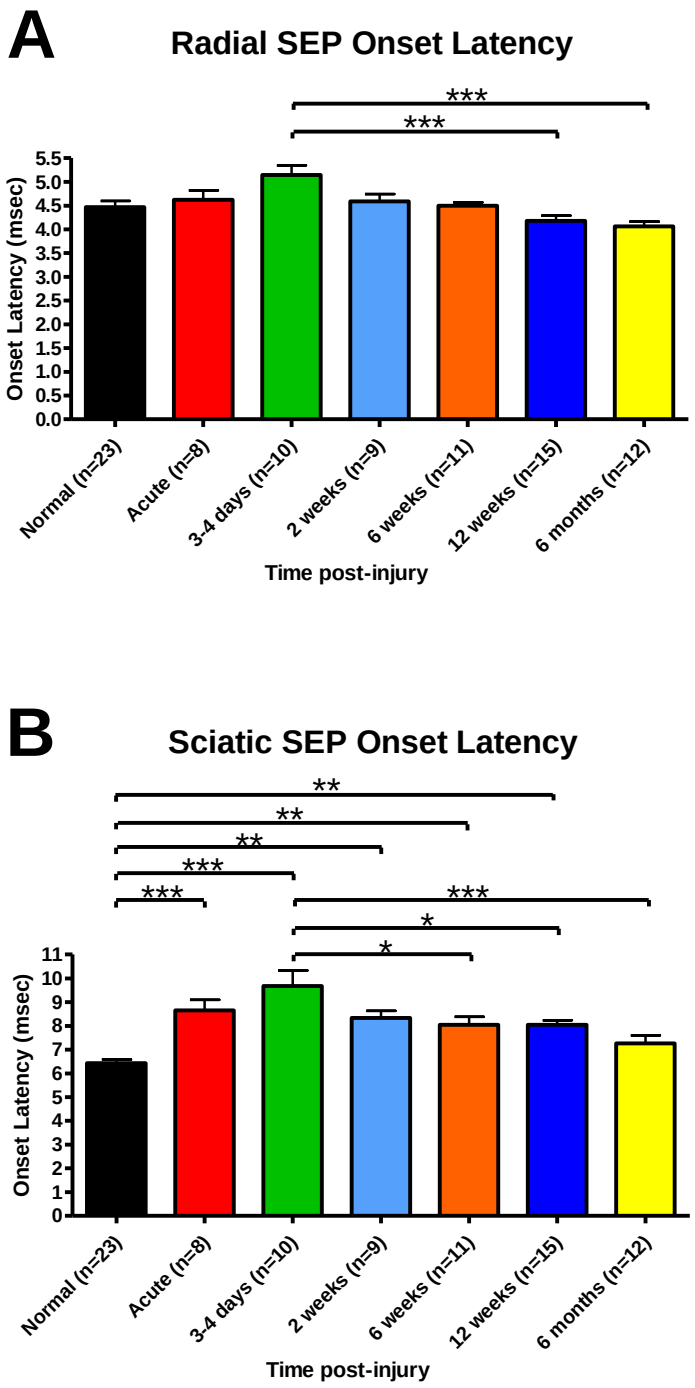


Figure 2.25 Latency to SEP onset recorded in 150 Kdyn injured animals. *Radial (A) and sciatic (B) SEP onset latencies, recorded in animals up to 6 months following 150 Kdyn contusion injury. Groups of normal animals (n=23) were investigated, as well as animals following acute injury (n=8) and following 3-4 days (n=10), 2 weeks (n=9), 6 weeks (n=11), 12 weeks (n=15) and 6 months (n=12) survival (*p<0.05; **p<0.01; ***p<0.001; one-way ANOVA Tukey's test. Error bars = SEM).*

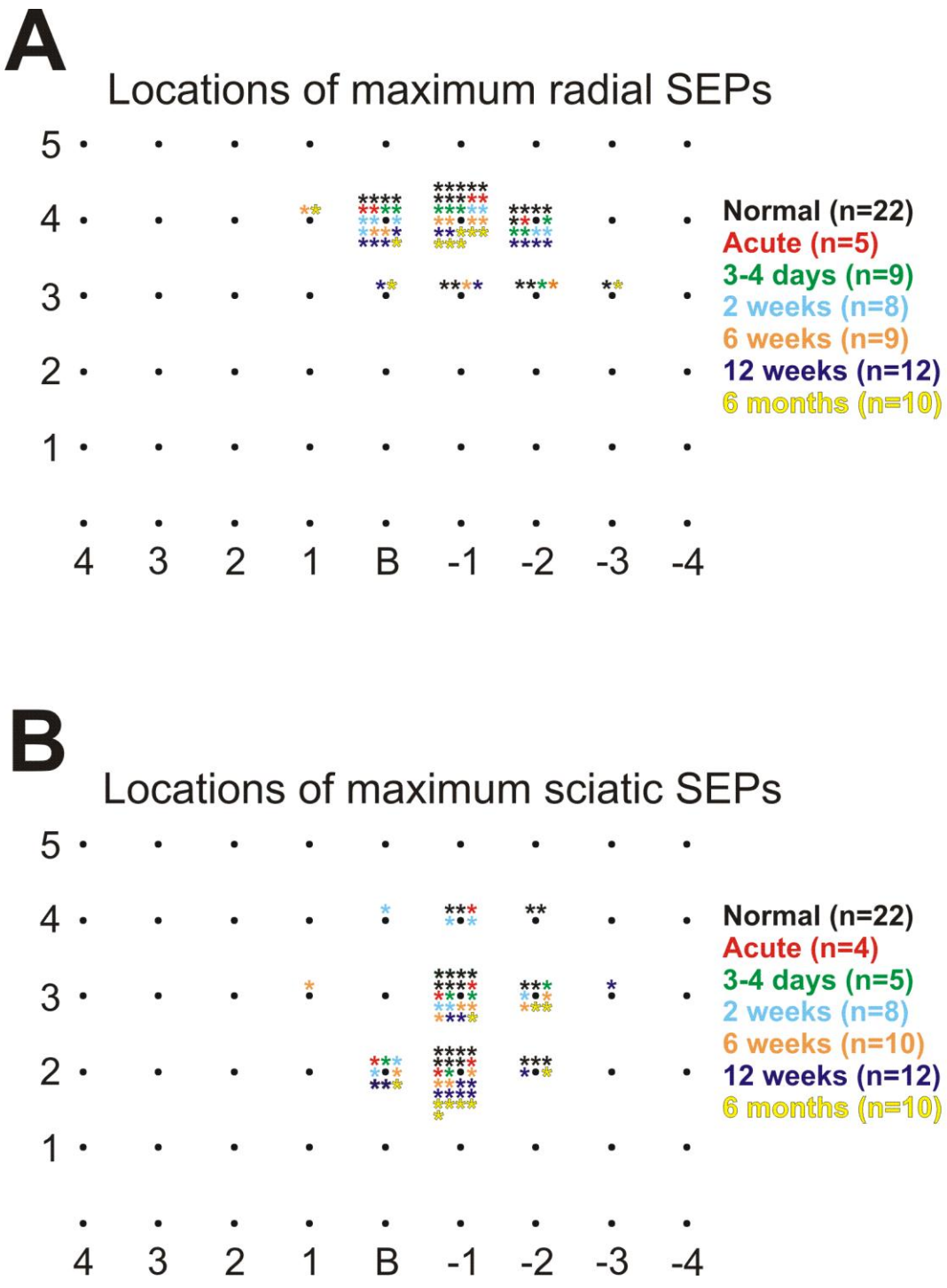
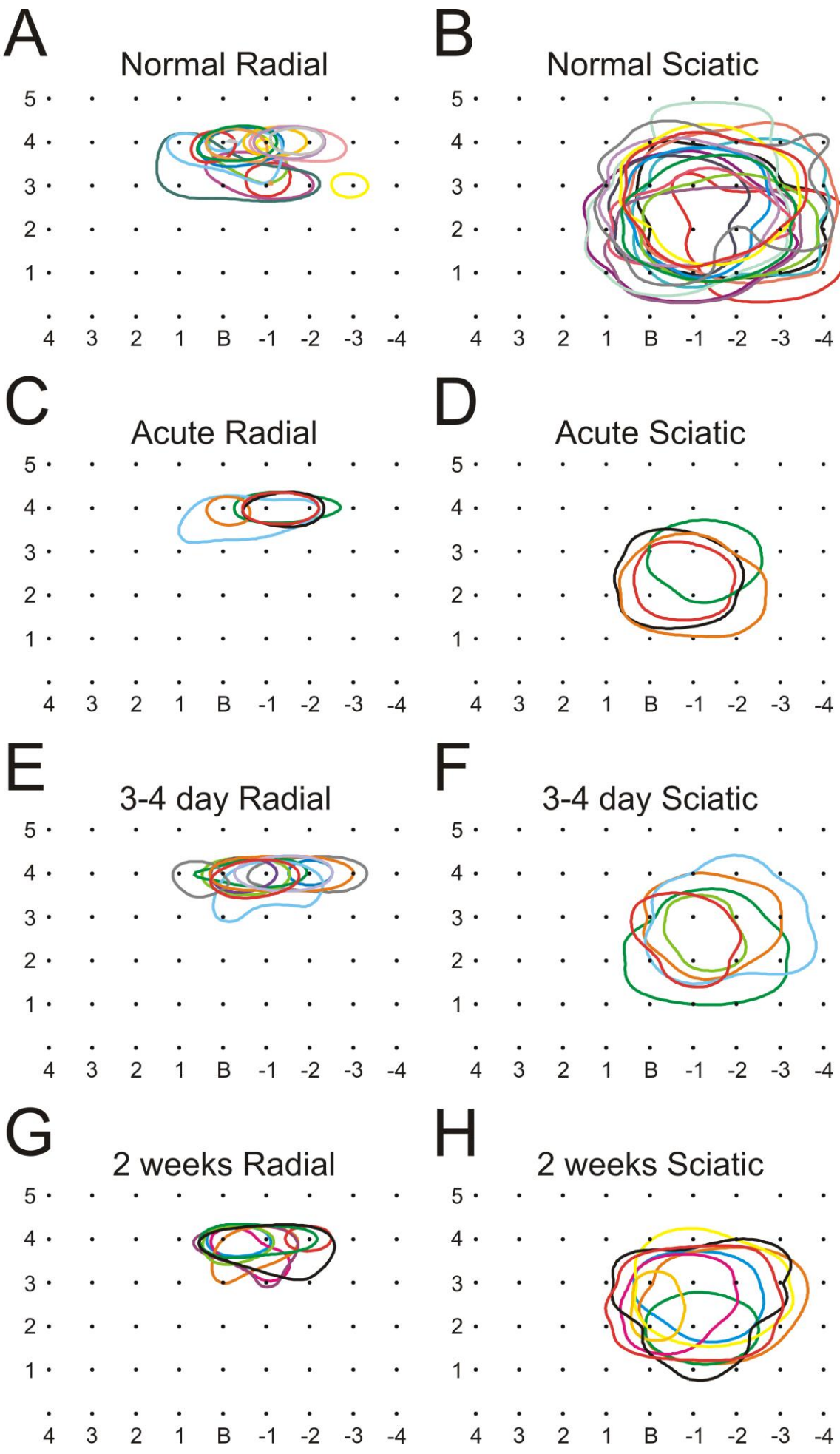


Figure 2.26 Cortical locations of maximum radial and sciatic SEPs recorded at different post-injury time points *(A) Cortical locations of maximum radial SEPs recorded in individual animals, (B) Cortical locations of maximum sciatic SEPs recorded in individual animals. Asterisks represent individual animals and colours represent different experimental groups.*


A
Normal Radial
B
Normal Sciatic
C
Acute Radial
D
Acute Sciatic
E
3-4 day Radial
F
3-4 day Sciatic
G
2 weeks Radial
H
2 weeks Sciatic
5
4
3
2
1
4 3 2 1 B -1 -2 -3 -4

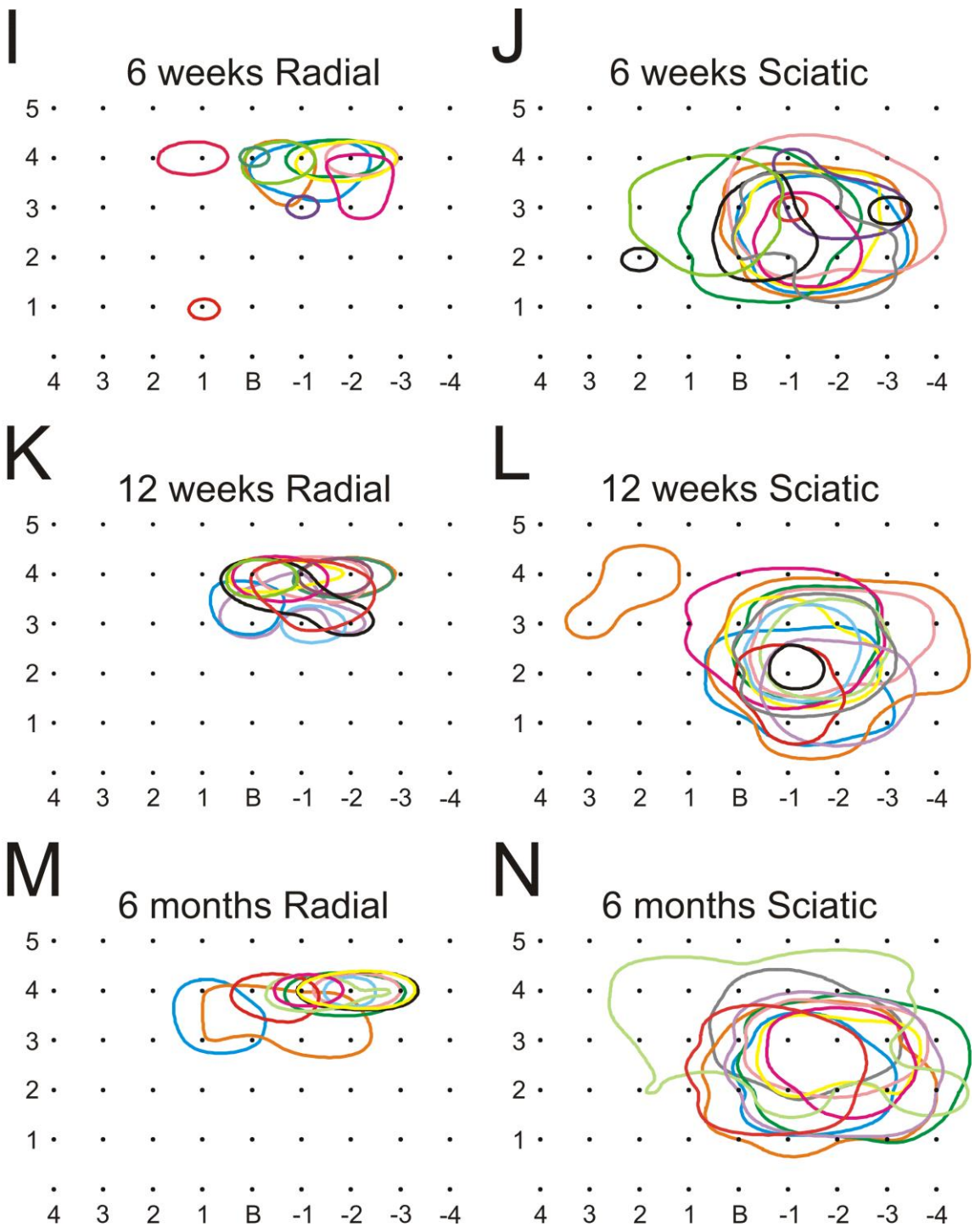


Figure 2.27 Individual isopotential contour plots of normal animals and animals receiving 150 Kdyn injuries at T9*. Radial and sciatic contours were normalised to 80% and 15% of the maximum radial SEP amplitude measured in each animal, respectively. Different colours have been used to represent data from different individual animals. Individual normalised radial isopotential contours from A) normal animals (n=22), and animals C) acutely (n=5), E) 3-4 days (n=9), G) 2 weeks (n=8), I) 6 weeks (n=9), K) 12 weeks (n=12) and M) 6 months (n=10) following 150 Kdyn contusion injury. Individual normalised*

sciatic isopotential contours from B) normal animals (n=22), and animals D) acutely (n=4), F) 3-4 days (n=5), H) 2 weeks (n=8), J) 6 weeks (n=10), L) 12 weeks (n=12) and N) 6 months (n=10) following 150 Kdyn contusion injury.

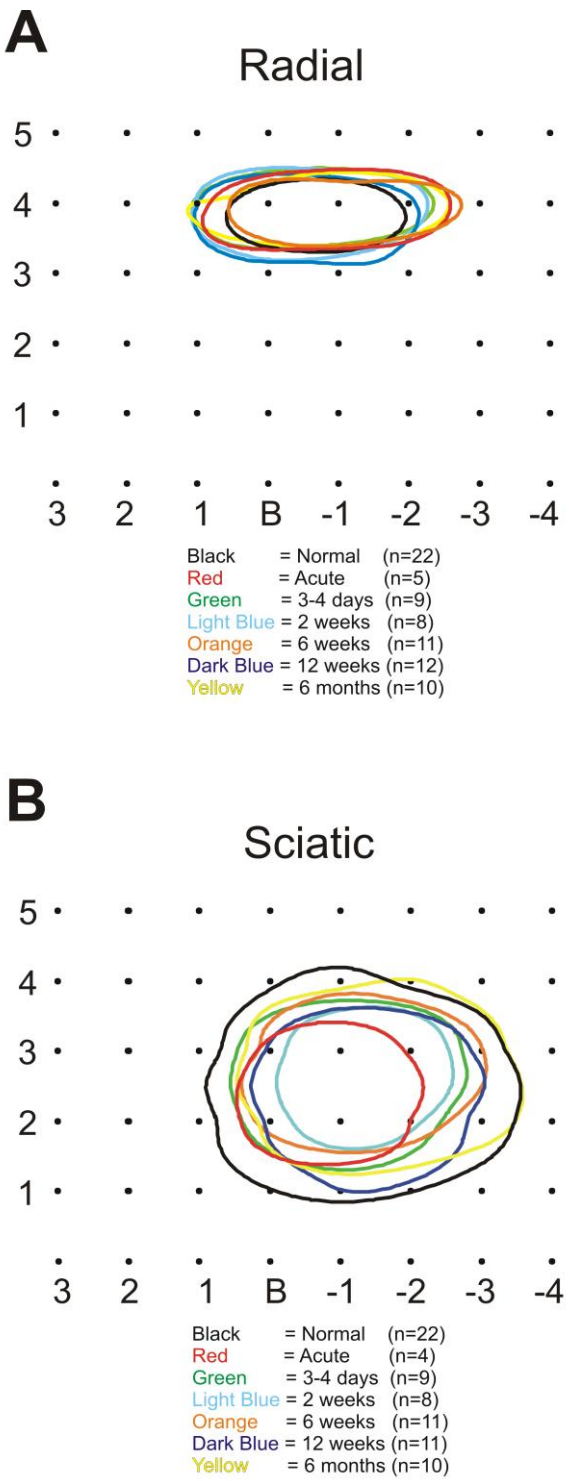


Figure 2.28 Averaged radial and sciatic isopotential contour plots of groups of animals recorded at different post-injury time points *A) Isopotential contour plots encompassing the area of cortex within which radial SEP amplitudes were at least 80% of the averaged maximal radial SEP amplitude. B) Isopotential contour plots encompassing the region of cortex within which sciatic SEPs could be recorded with amplitudes at least 15% of the averaged maximal radial SEP amplitude. All distances are in mm relative to Bregma (B).*

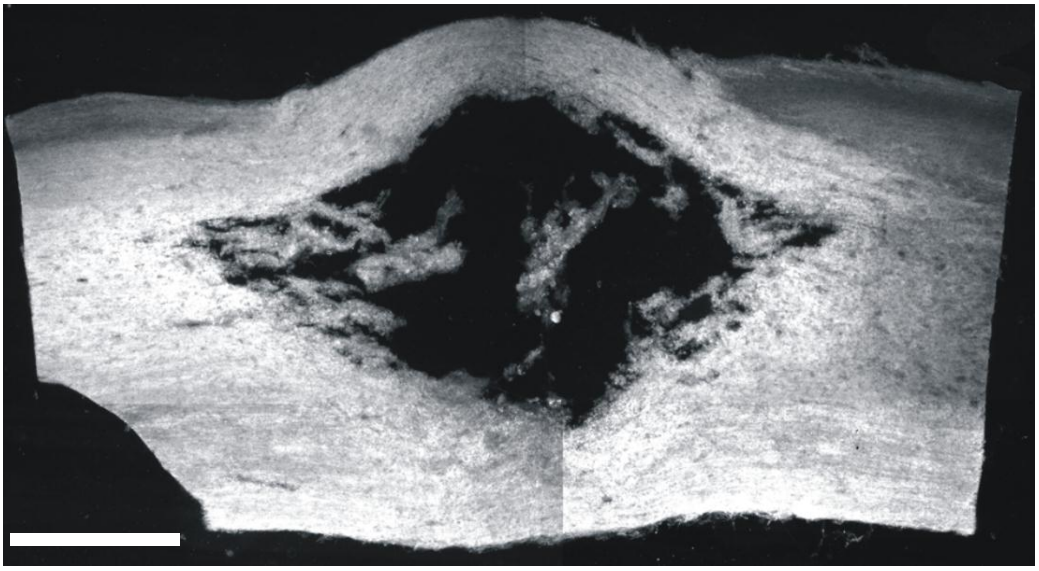


Figure 2.29 Dark field histology of contusion injury site*. Transverse section from an animal that received a 150 Kdyn force injury. T9 contusion injuries cause substantial damage to dorsal white matter tracts. Whilst the dorsal columns (DC) are severely disrupted, a cavity forms at the tissue core but a rim of spared white matter tissue encloses the cavity. Scale bar = 1 mm. Notch = Ventral and caudal.*

Table 2.1 Statistic analysis of electrophysiological data obtained from injury severity groups. *Results of ANOVA and Tukey's post-hoc multiple comparison tests performed on each experimental group included in the injury severity study.*

	Sciatic Isopotential Area 15% Max Radial	**Radial Isopotential Area 80% Max Radial**	**Sciatic SEP Max. Amplitude as % Max. Radial**	**Radial Max. SEP Amplitude**	**Sciatic Latency to SEP Onset**	**Radial Latency to SEP Onset**
One-way ANOVA	P<0.0001****	P > 0.05 (ns)	P<0.0001****	P > 0.05 (ns)	P<0.0001****	P > 0.05 (ns)
Tukey's Multiple Comparison Test	-	-	-	-	-	-
Normal vs 150 Kdyn	P < 0.01**	P > 0.05	P < 0.001***	P > 0.05	P < 0.01***	P > 0.05
Normal vs 175 kdyn	P < 0.001***	P > 0.05	P < 0.001***	P > 0.05	P < 0.001***	P > 0.05
Normal vs 200 kdyn	P < 0.001***	P > 0.05	P < 0.001***	P > 0.05	P < 0.001***	P > 0.05
150 Kdyn vs 175 kdyn	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
150 Kdyn vs 200 Kdyn	P < 0.001***	P > 0.05	P < 0.05*	P > 0.05	P < 0.05*	P > 0.05
175 Kdyn vs 200 Kdyn	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05

Table 2.2 Statistic analysis of electrophysiological data obtained from time course groups. *Results of ANOVA and Tukey's post-hoc multiple comparison tests performed on each experimental group included in the time course study.*

	Sciatic Isopotential Area 15% Max Radial	**Radial Isopotential Area 80% Max Radial**	**Sciatic SEP Max. Amplitude as % Max. Radial**	**Radial Max. SEP Amplitude**	**Sciatic Latency to SEP Onset**	**Radial Latency to SEP Onset**
One-way ANOVA	P<0.0001****	P > 0.5 (ns)	P<0.0001****	P > 0.5 (ns)	P<0.0001****	P<0.0001****
Tukey's Multiple Comparison Test	-	-	-	-	-	-
Normal vs Acute	P < 0.01**	P > 0.5	P < 0.01**	P > 0.5	P < 0.001***	P > 0.05
Normal vs 3-4 days	P < 0.001***	P > 0.5	P < 0.001***	P > 0.5	P < 0.001***	P < 0.05
Normal vs 2 weeks	P < 0.05*	P > 0.5	P < 0.01**	P > 0.5	P < 0.01**	P > 0.05
Normal vs 6 weeks	P < 0.05*	P > 0.5	P < 0.01**	P > 0.5	P < 0.01**	P > 0.05
Normal vs 12 weeks	P < 0.001***	P > 0.5	P < 0.001***	P > 0.5	P < 0.01**	P > 0.05
Normal vs 6 months	P > 0.05	P > 0.5	P > 0.05	P > 0.5	P > 0.05	P > 0.05
Acute vs 3-4 days	P > 0.05	P > 0.5	P > 0.05	P > 0.5	P > 0.05	P > 0.05
Acute vs 2 weeks	P > 0.05	P > 0.5	P > 0.05	P > 0.5	P > 0.05	P > 0.05
Acute vs 6 weeks	P > 0.05	P > 0.5	P > 0.05	P > 0.5	P > 0.05	P > 0.05
Acute vs 12 weeks	P > 0.05	P > 0.5	P > 0.05	P > 0.5	P > 0.05	P > 0.05
Acute vs 6 months	P > 0.05	P > 0.5	P > 0.05	P > 0.5	P > 0.05	P > 0.05
3-4 days vs 2 weeks	P > 0.05	P > 0.5	P > 0.05	P > 0.5	P > 0.05	P > 0.05
3-4 days vs 6 weeks	P > 0.05	P > 0.5	P > 0.05	P > 0.5	P < 0.05*	P > 0.05
3-4 days vs 12 weeks	P > 0.05	P > 0.5	P > 0.05	P > 0.5	P < 0.05*	P < 0.001
3-4 days vs 6 months	P < 0.01**	P > 0.5	P > 0.05	P > 0.5	P < 0.001***	P < 0.001
2 weeks vs 6 weeks	P > 0.05	P > 0.5	P > 0.05	P > 0.5	P > 0.05	P > 0.05
2 weeks vs 12 weeks	P > 0.05	P > 0.5	P > 0.05	P > 0.5	P > 0.05	P > 0.05
2 weeks vs 6 months	P > 0.05	P > 0.5	P > 0.05	P > 0.5	P > 0.05	P > 0.05
6 weeks vs 12 weeks	P > 0.05	P > 0.5	P > 0.05	P > 0.5	P > 0.05	P > 0.05
6 weeks vs 6 months	P > 0.05	P > 0.5	P > 0.05	P > 0.5	P > 0.05	P > 0.05
12 weeks vs 6 months	P > 0.05	P > 0.5	P > 0.05	P > 0.5	P > 0.05	P > 0.05

2.4 Discussion

In this chapter it has been shown that SEPs recorded across the surface of the sensorimotor cortex which are due to impulses conveyed from hindlimb sensory afferents along the dorsal column system of the spinal cord, provide a sensitive measure of the severity of contusion injury of the thoracic spinal cord. It was also shown that such recordings can be used to investigate the time course of functional changes in the dorsal column system following injury.

Rationale for the use of SEPs as an indicator of long white matter tract function

One of the major mechanisms underlying loss of function following spinal cord injury is damage to white matter tracts, which compromises their ability to transmit impulses properly. One of the major ascending long white matter pathways responsible for transmitting sensory information from the skin to the sensory cortex is the dorsal column medial lemniscal pathway. The first order neurones in this pathway are sensory neurones with somas in the dorsal root ganglia, each of which has a central axon projecting into the dorsal columns. A proportion of these axons ascend in the dorsal column pathways and project to the dorsal column nuclei in the brainstem. Here signals are relayed to the thalamus via the medial lemniscus pathway and subsequently the sensory cortex. SEPs represent the activity of synapses and neurons in the sensorimotor cortex and, although other pathways converge on the cortex, the early negative component of the SEP is mediated entirely by impulses transmitted along nerve fibres ascending in the dorsal columns of the spinal cord (Angel and Berridge, 1974).

The axons that make up the dorsal columns are topographically organised so that those entering the cord more caudally occupy a more medial position than those that enter more rostrally – for example, in the rat the sciatic nerve afferents enter the cord primarily through the 4^{th}, 5^{th} and 6^{th} lumbar dorsal roots.

Although in rats the sciatic nerve is a mixed nerve, containing muscular and cutaneous afferents and fibres of proprioceptive origin, it is the large diameter cutaneous afferents that can be found in this medial location within the fasciculus gracilis in the dorsal white matter (Angel and Berridge 1974, Fern et al., 1988), whereas the radial nerve afferents enters the cord almost exclusively through the C6 dorsal roots and ascend in the fasciculus cuneatus, which is located lateral to the fasciculus gracilis. Thus by centring our impactor tip in the middle of the dorsal columns we are able to focus our injury on the tracts being investigated.

Following electrical stimulation of afferents in hindlimb nerves, the dorsal column pathway conveys this activity along the spinal cord to the dorsal column nuclei in the brain stem and then via the thalamus to layer iV of the sensory cortex. Since the primary afferent fibres forming this pathway enter the spinal cord below the level at which the contusion injury was made their function is affected by damage to the dorsal column fibres. This is the rationale for using the SEPs evoked by electrical stimulation of the sciatic nerve in the hindlimb as a measure of how the transmission of impulses along this pathway are affected by injury.

Three measures of the effectiveness of transmission along the dorsal column pathway were recorded. These were the maximal amplitude of the SEP, the area within which SEPs of a given amplitude were evoked and the shortest latency of the SEPs.

SEPs provide a measure of the severity of an experimental contusion injury

By measuring SEPs from groups of animals subjected to contusion injuries of graded severities we were able to show that each of the parameters of the SEPs we chose to measure were correlated with injury severity. Results show that there is a clear correlation between injury severity groups and SEPs and that each of the SEP parameters measured shows a change that follows this relationship.

The observed reduction in sciatic maximum amplitude and reduced isopotential area suggests that fewer dorsal column fibres are contributing to the SEPs

evoked after injury and that more of these fibres are damaged the greater the force used to produce the injury. The increased latency to onset of sciatic SEPs after injury also correlates with injury severity, and could be due to two main mechanisms: increased latency at supraspinal relays or reduced conduction velocities in the dorsal columns:

Increased latency at supraspinal relays

The loss of dorsal column fibres contributing to the evoked potentials could increase the time taken for the relay of impulses in the dorsal column nuclei, thalamus and layer iV of the cortex. Transmission of impulses through these relays depends on convergence of inputs and temporal summation which leads to action potentials being generated in the postsynaptic neurons. Increased latencies can lead to reduced synchronicity which means actions arriving at target neurons at different times so reduced temporal summation. This happens more quickly the more synchronous and powerful the input, however if the input is reduced due to loss of fibres or the input is less synchronous because of a reduced conduction velocity in spared fibres then both of these effects would lead to longer delays at each of the relays and a longer overall latency.

Reduced conduction velocities in the dorsal columns

Increased latencies to the onset of SEPs could be due to reduced conduction velocities in the spared fibres as a result of damage to the fibres produced by the injury. It could also potentially occur if the contusion preferentially damaged larger diameter fibres, if for example they were more susceptible to injury than medium diameter fibres.

As the increased latency to SEP onset could be due to an effect directly on conduction along the dorsal column fibre or on the delay in the relays of the dorsal column pathway it is not possible to directly deduce the change in conduction velocity of the dorsal column fibres as a result of the injury and damage such as demyelination.

Time course data

SEPs are abolished immediately on injury

This thesis represents the first time that SEP recovery has been investigated at such an acute time point using an electrophysiological approach. Here we show for the first time the immediate abolition of SEPs with spinal contusion injury, as well as their re-emergence and partial recovery over the minutes to hours post-injury.

Most SCI research has thus far focussed on what happens in secondary injury i.e. how injury to axon progresses to eventual axonal death over longer time periods. Much less research has focused on acute changes in function after axonal injury, especially transient changes. However, the early effects of CNS injury have been investigated in the guinea pig optic nerve, where soon after injury damage to the nodes of ranvier, leading to 'nodal blebs' leading to loss of conduction that recovers over time (Maxwell et al., 1991). Others have reported that dysregulation of ionic concentration gradients caused by leaking from damaged cell membranes prevents signal transduction, this is seen to recover to an extent over time in axons that have not been axotomised (Borgens, 1988)

Another possible explanation for this observation is that the acute loss of function results from the initial mechanical damage caused by the contusion. McCulloch and Griffiths reported disruption of myelin sheaths and partial demyelination within 1½ hrs of impact injuries (Griffiths and McCulloch, 1983).

Use of radial SEPs for normalising sciatic SEP data

Although the transmission of sensory input entering the spinal cord below the injury will be affected by the injury, the radial nerve enters the spinal cord through cervical dorsal roots, well above the injury level, and is not therefore directly affected by it. As a result the maximum radial SEP amplitudes, latencies to SEP onset and isopotential areas remain fairly constant in animals regardless of different batches, injury severities or survival times. Despite others reporting the expansion of the forelimb cortical representations following thoracic transection injury (Aguilar et al., 2010; Ghosh et al., 2009 and 2010), here we found no such expansion or movement in the centre of focus, though this may result from the different injury models used.

In these experiments the only time we observed any substantial change in radial SEPs was immediately following acute injury, with amplitudes dropping substantially before rapidly recovering, usually within 5 minutes. Whilst the poorly defined phenomena of 'spinal shock' may play a role, it is more likely a result of the more general shock e.g. neurogenic shock that leads to changes in autonomic function which are transient. i.e. the injury could have resulted in a short term change in general physiology that was manifest in a change in the radial SEP acutely because SEPs are so sensitive in general (Dumont et al., 2001)

Despite this, the maximum radial SEP appears to be constant and stable in both amplitude and location of cortical focus, which makes it ideally suited to normalising data obtained from sciatic SEPs and thus validates the normalisation protocol used here.

How do the electrophysiologically detected changes in function correlate with behavioural changes in function.

A major difficulty when attempting to correlate electrophysiological observations with behavioural measures is the absence of behavioural tests that specifically depend on the integrity of the dorsal column pathway. In preliminary experiments we found that complete dorsal column lesions made with a wire knife device did not abolish withdrawal reflexes from the hindlimbs.

Implications of observations for understanding spinal cord injury and developing therapies

These findings suggest that most of the damage to long white matter tracts results directly from the injury and occurs at the time of the injury, and that secondary mechanisms do not appear to lead to much additional damage if recovery seen in acute experiments is compared to 3-4day survival animals. This implies that there may be limited opportunities for a neuroprotective strategy since there may not be much to protect against. However, it is possible that neuroprotective strategies could speed the recovery of function.

Despite seeing considerable repair and recovery with time, others have reported that the repair and optimisation of function in fibres that are spared may not be complete (James et al., 2011); this was demonstrated experimentally by cooling

exposed rat cords following injury and recording from single fibres which were more responsive with cooling than without, thus offering a therapeutic target which could be effectively utilized even in the chronic stages of injury. Combined these findings suggest that strategies for enhancing repair or restoring/optimising function could be beneficial. Other potential therapeutic strategies include optimising myelination, improving the quality of endogenous remyelination and compensating for the lack of myelin or suboptimal remyelination.

Technical issues

Injury severity

The infinite horizons impactor with its force feedback controller optimises the consistency with which injuries with regards to mechanical properties. However, some variation is inevitable because of differences in the degree of stretch of the cord, the amount of bone removed by the laminectomy, the precise alignment of the impactor tip with spinal cord midline and the effect this will have on the injury properties; there will also be biological variability, for example in the pattern of blood supply.

Variation was minimised via several strategies, including having the same operator carry out all of the injuries, by removing any animals in which bone strikes were indicated by the graphical output of the impactor and using animals with as similar weight as possible, though acute animals were a compromise between weights at injury and weights at electrophysiology.

Another potential source of variation is the anaesthetic used during the electrophysiological recordings; unpublished observations from this laboratory show changes in SEPs amplitude between sodium pentobarbatal, ketamine or urethane anaestheasia. We therefore selected the anaesthetic known to have least effect on SEPs, sodium pentobarbital, for use in all SEP recordings

Electrophysiology

Efforts were made to reduce the degree of variability in the collection of electrophysiological data, including the monitoring of and removal of fluids to avoid shorting the electrodes and ensuring that the level of anaestheasia was maintained as level as possible. Fluctuations in cortical excitability are well known and may be due to changes in anaesthetic level or changes in physiological conditions. These changes in excitability affect transmission through each of the relays of the dorsal column system – the dorsal column nuclei, the thalamus and the cortex- and can therefore result in significant changes in SEP amplitude. Physiology was maintained within close limits by monitoring and controlling core temperature, blood pressure and end tidal CO2. Supramaximal stimulation was used to avoid any drifting in stimulation parameters; this was determined as described earlier, by raising the stimulus intensity incrementally until maximum maplitude SEPs were observed, and then stimulating with a supramaximal intensity.

As discussed previously, recordings of radial evoked SEPs were used to normalize the recordings of sciatic evoked potentials by expressing the maximal sciatic SEP amplitudes as a percentage of the maximal radial SEP amplitudes recorded in each animal. The sciatic evoked SEPs could be normalised in each animal on the grounds that the forelimb SEPs will not be affected by the injury, and by using this as a reference, the effects of drifts in the conditions of recording of the sciatic SEPs could be reduced.
This approach assumes no change in radial SEP as a consequence of injury or with time following injury, and was thus validated by the observations made here. Additionally, several different SEP parameters were quantified in order to increase the possibility of detecting changes.

Advantaged of using SEPs to measure white matter function

SEPs recorded from the cortical surface have greater sensitivity than other methods such as single fibres recordings or recording of SEPs by less invasive methods. This is because recording SEPs does not rely on sampling enough fibres to find those that are still conducting, but instead activates all fibres and detects the summed actions of all spared fibres; it is thus a more sensitive method of detecting and measuring residual function after injury. Consequently, SEP recordings have therefore detected recovery of function at much earlier

time points (within minutes of the injury) than has previously been reported. For the same reasoning the absence of any detectable activity immediately following the contusion in the acute injury experiments can be considered a reliable observation and is not attributable to insufficient sensitivity of the assessment method. The failure of James et al, 2011 to observe early recovery is probably explained by the sampling issue referred to above.

The invasive approach adopted here provides maximal control over the stimulation and recording conditions. This is important in obtaining consistent and reliable results. The use of skull screws for recording or the use of needle or cuff electrodes to activate primary afferents does not provide the same level of control due to the slight variability of the cortical location from which maximal SEPs can be recorded and the possibility of not maximally activating peripheral nerves due to movement of cuff electrodes. This may explain the failure of these approaches, despite involving the use of SEP recording, to detect functional recovery at early time points. Invasive approaches such as that used here which are capable of accurately characterising the effects of injuries on the cortical locations from which maximal amplitude SEPs can be recorded will assist in more accurately locating skull screws and thus aid the refinement of such non-invasive approaches.

Disadvantages of using SEPs to monitor long white matter tract function

That there are two disadvantages of using the full dorsal column lemniscal pathway. Firstly, we cannot rule out compensatory changes in synaptic efficacy within the relays – but this does not appear to interfere with the utility of the SEPs as an indicator of injury severity because the different SEP parameters were correlated with injury severity even at 6 weeks, by which time plastic changes can be expected to be established; secondly, the conduction velocity of afferents cannot be directly determined only an indirect indication of changes obtained. This is because the summation of post-synaptic potentials at synapses onto neurons in the relay nuclei of the pathway also contributes to determining the latency between stimulation and onset of the SEPs generated. Thus it is not possible to directly determine changes in the conduction velocity of dorsal column fibres using SEPs because changes in latency do not directly relate to changes in conduction velocity but may also reflect longer delays for

transmission in the relays of the pathway. The approach used here measures function in the population of damaged fibres as a whole rather than individual fibres so that the latency information applies only to the fastest conducting fibres contributing to the SEP, conduction delays in the fibre population as a whole cannot be determined. Stimulation of the ascending dorsal column pathways results in the generation of both anterograde and retrograde action potentials. Retrograde compound action potentials (CAPs) have been used to provide an accurate measure of the conduction properties in long white matter tracts as they allow for recordings to be made directly from the stimulated nerve without the added complication of synaptic relays distorting the properties of the recorded CAP. In this respect, stimulating the spinal cord and recording retrograde action potentials from the dorsal roots provides a method of providing much more accurate information on the conduction properties of tracts or individual single fibres that make up the ascending dorsal column system. Such recordings are of great utility when assessing the effects of therapies on the conduction properties of fibres in the dorsal column pathways, which can provide accurate information on the integrity of nerve fibres, allowing for inferences to be made about the integrity of their myelination (James et al., 2011; Bei and Smith, 2012). Despite this, here we elected to use anterogradely conducting action potentials to assess the summed functional capabilities in the tracts under investigation, taking in to account the effects of signal amplification at each of the cortical relays; this provides a method of quantitatively assessing the direct inputs to the cortex and hence allows for a more accurate measure of an injury's effects at the cortical level.

Chapter 3

Investigation of human lamina propria mesenchymal stem cells as a potential therapy for spinal cord injury

3.1 Introduction

Mesenchymal stem cells (MSCs) are multipotent stromal cells with the ability to self replicate, proliferate and differentiate into numerous mesenchymal tissues. It has been shown that MSCs can be isolated from virtually all post-natal organs and tissues (da Silver Meirelles et al., 2006).

In studies looking at neuronal regeneration, MSCs derived from bone marrow (BMSCs) have been studied the most intensively (Dezawa et al., 2001; Hofstetter et al., 2002; Keilhoff et al., 2006; Krampera et al., 2007), though several other sources have also been investigated, including the umbilical cord, (Cao et al., 2009) and adipose tissue (Gimble et al., 2007). Whilst it has been shown that BMSCs from humans and rats can be induced to differentiate in to neural cells (Woodbury et al., 2000), when compared with neural tissue derived stem cells BMSCs have been shown to have a lower neurogenic potential (Song et al., 2007). Some investigators have even observed improved neurological function with BMSC transplantation following compression injury despite no cells being observable as early as 7 days post transplantation (Quertainmont et al., 2012). It is thus likely that the reparative capabilities of BMSCs come from their ability to modulate the environment in to which they are transplanted, rather then their differentiative capabilities (van Poll et al., 2008). Numerous studies have reported that BMSCs release anti-inflammatory and anti-apoptotic molecules as well as several trophic factors known to play active roles in supporting axonal growth, immunomodulation, angiogenesis, remyelination and protecting against apoptotic cell death (Caplan and Dennis, 2006; de Silva Meirelles et al., 2006; Uccelli et al., 2011).

When transplanted into the injured spinal cord, BMSCs have been shown to migrate to the site of injury where they reduce the degree of neuro-degeneration (Quertainmont et al., 2012) whilst concomitantly enhancing neuro-regeneration (Lu et al., 2004), although in many instances it has been shown that the transplanted cells and their effects appear to be limited to the transplant site (Hofstetter et al., 2002; Lu et al., 2005, 2006; Boido et al., 2012). Whilst several investigators have reported improved function after SCI with BMSC transplants (Chopp et al., 2000; Hofstetter et al., 2002; Wu et al.,

2003; Zurita et al., 2004; Ohta et al., 2004; Quertainmont et al., 2012), others have not seen such improvements (Ankeny et al., 2004; Lu et al., 2005).

A recent study utilizing *in vitro* MSC cultures suggested that human MSCs isolated from the lamina propria of the nasal mucosa (hLP-MSCs) might have greater therapeutic potential for the treatment of SCI than those isolated from bone marrow (Lindsay et al., 2013). It was reported that when compared to BMSCs, hLP-MSCs have significantly greater clonogenic capabilities, have a higher level of mitotic activity and are able to induce significantly greater degrees of myelination *in vitro* (Lindsay et al., 2013). Isolating MSCs from the nasal lamina propria also provides a more easily accessible and less invasive source of cells for autologous transplantation.

It has been shown that hLP-MSCs secrete a factor or factors which enhance myelination *in vitro*. Adding conditioned medium from cultures of hLP-MSCs increases the myelination of neurites in dissociated embryonic spinal cord cultures grown on an astrocyte monolayer (Figure 3.1). Interestingly, human bone marrow derived MSCs tend to inhibit myelination in these same cultures. The increased myelination promoted by the hLP-MSCs involved central type myelination by oligodendrocytes in the culture system. These observations suggested that hLP-MSCs might provide a means of enhancing the remyelination of spared fibres if transplanted into the site of a spinal cord injury. The aim of this chapter was therefore to combine electrophysiological and behavioral approaches to determine whether hLP-MSCs, when transplanted into an animal model of contusive SCI, would by promoting remyelination and possibly other mechanisms in addition, improve functional outcome following injury.

3.2 Methods

3.2.1 Experimental Design

Figure 3.2 summarises the experimental design. As the *in vitro* investigation of hLP-MSCs suggested that remyelination was a potential mechanism, we elected to use a contusion injury model which is known to result in demyelination of spared fibres. The focus was on the assessment of long white matter tracts and

the injuries were therefore made at a T9 level. Transplants of cells (or injections of medium in control animals) were made 3 weeks after injury – a time point at which we have shown that conduction along the dorsal column pathway has stabilized following injury (see chapter 2). Immunosuppression (cyclosporine, 20 mg/kg s.c.) was begun two days before transplantation and continued daily throughout the study. Functional outcomes were assessed by behavioural testing performed throughout the study period and extending for 7 weeks after the transplantation of cells or medium injections. At the end of the testing period, long tract function (ascending dorsal column somatosensory pathway) was assessed in electrophysiological experiments under terminal anaesthesia. At the end of this each animal was perfusion fixed and histological processing of the injury sites performed to assess the survival of transplanted cells as well as to investigate aspects of repair using immunohistochemistry and confocal microscopy.

3.2.2 Animals

Observations reported in this chapter are based on the investigation of adult male Sprague Dawley rats (Harlan, Loughborough, UK). Sprague Dawley rats were chosen as they are known to perform behavioural tests more readily than other strains. Before beginning the investigation, animals were screened for the ability to run on the Digigait™ treadmill. In total 60 animals were screened and 29 animals were excluded from the study due to not being able to run on the Digigait™ treadmill, with 31 capable of running sufficiently well to be included in the study. Each of these animals was subjected to a 150 Kdyn contusion injury at the T9 level, of which 16 later received hLP-MSC transplants into the lesion cavity at three weeks post injury, and 15 later received injections of medium and acted as controls. Two of the hLP-MSC transplanted animals were used to assess cell survival and distribution at different time points post-injury (10 days and 6 weeks post transplantation); the remaining 14 transplanted animals, and the 15 control animals were allowed to survive for 7 weeks post transplantation, throughout which time their functional abilities were investigated using various behavioural tests; at

the end of the study, all animals were investigated using electrophysiological techniques as described in chapter 2.

A summary of the testing protocol timeline is given in the schematic diagram shown in Figure 3.2, and the number of animals used at each point are shown in Figure 3.3, and described in detail below.

At the start of the experiment, animals were randomly assigned to one of two groups and each group was assigned to receive either cellular transplants or medium injections, to avoid bias this was performed by a member of the animal house staff who was blind to the experimental procedure. All subsequent surgeries and behavioural tests were performed with the researchers blind to the experimental groups, unless where practically impossible, for example the surgeon was able to see if cells or medium were being injected into the injury site.

3.2.3 Extraction, purification and culture of hLP-MSCs

3.2.3.1 Preparation of human lamina propria mesenchymal stem cells

All cell culture was performed by Dr. Susan Lindsey in Professor Sue Barnett's laboratory in the Beatson Institute, University of Glasgow.

The cells used for transplantation came from three middle aged donors undergoing nasal septoplasty operations from which nasal biopsies were obtained with informed consent. The cells were grown in culture and conditioned media obtained from each of the cultures was confirmed to enhance myelination when assessed using the myelinating cultures (Figure. 3.1). The cells were transduced to express green fluorescent protein (GFP) prior to transplantation. Similar numbers of animals were transplanted with cells from each of the three donors and these were matched by control animals which were injured in the same way but were injected with medium instead of cells (Figure 3.5). Here we elected to use the same media as the hLP-MSCs were cultured in as a control so we could attribute any differences

to the cells or the media provides the only way of differentiating the effects of the cells from that of the media they were cultured in. Whilst is may be advantageous to fill the injury cavity with any cell type, which could act as a beneficial substrate, no cell line has been sufficiently characterised as to fully understand its effect in the injured cord and thus make for an accurate control. Also, due to potential differences in cell size it would make accurate comparisons difficult to make as transplanted cell counts would either differ or fill the cavity to a greater or lesser degree.

Briefly, biopsies were kept on ice in Hanks balanced salt solution (HBSS) containing penicillin (100 units/ml), streptomycin (100 µg/ml) and fungizone (Amphotericin B, 1.25 µg/ml) (all, Invitrogen, UK). After mincing with a scalpel the tissue was digested using 1.33 % collagenase (Sigma-Aldrich, UK) for 20 min followed by incubation with soya bean trypsin inhibitor and DNAse to reduce clumping (0.04 mg/ml bovine pancreas DNAse, 3.0 mg/ml bovine serum albumin-fraction A in L15; Sigma-Aldrich). Cells were mechanically dissociated by pipetting, then triturating through a 23G needle. The digested tissue was then grown in minimum essential medium alpha (αMEM, Invitrogen) containing 10 % foetal bovine HyClone© serum (αMEM: 10%) (Thermo Scientific, UK) and incubated at 37 °C and 7 % CO_2, changing half of the medium every 2-3 days. After 5 days a heterogeneous monolayer of spindle shaped cells would develop. Purification of human MSCs was performed using an EasySep® Human CD271 positive selection kit (StemCell Technologies Inc, Grenoble, France). After purification, the cells, now termed hLP-MSCs, were only used up to passage 6.

3.2.4 Contusion Injury

All the contusion injuries and cell transplants in this study were carried out by Dr. John Riddell.

The T9 spinal cord was subjected to a 150 Kdyn contusion injury using the same procedure as described in chapter 2. A suture was placed in the dura

over the injury site at a known location so that it could be identified in the later cell transplantation procedure, and a piece of latex was used to cover the exposed cord and held in place with 6-0 Vicryl (Ethicon Inc, Johnson and Johnson, USA), which reduced the adhesion of scar tissue to the dura, and aided in re-exposure of the injury site at the time of transplantation. Animals routinely received the same peri-operative medication as described in chapter 2.

3.2.5 Immunosuppression

To reduce the chances of cellular transplants being immunologically rejected, animals received daily subcutaneous injections of cyclosporine (20 mg/kg subcutaneously) starting 48 hours prior to transplantation and continuing for the duration of the experiment. In order to ensure that any observed changes were solely down to the presence of transplanted hLP-MSCs, the control animals were also immunosupressed throughout in a similar fashion. All the animals used in this study were immunosupressed with daily injections of cyclosporine.

3.2.6 Delayed cell transplantation

Transplantation surgery was performed 3 weeks after injury to ensure that the protocol could be replicated using autologous transplants in humans following SCI. This delay also helped to minimize the chances of transplant rejection due to the elevated immune response that is known to occur immediately following injury (Dumont et al., 2001a).

3.2.6.1 Transplantation of hLP-MSCs into the injury site

In order to ascertain if hLP-MSCs transplants are able to improve function in the ascending dorsal column pathway we aimed to transplant cells directly into the lesion cavity that forms following the contusion injury. Briefly, a glass

micropipette with an internal tip caliber of between 40-70µm was loaded with cells (concentrated into a slurry containing ~100,000-200,000 cells per microlitre), mounted on a stereotaxtic manipulator and inserted into the lesion cavity through a slit in the dura to a depth of 1200µm. Between 10-50µl of the cell suspension was then injected, by applying brief (40ms) pressure pulses (Picoinjector, WPI, Srasota FL, USA), as the pipette was slowly raised to a depth of around 200µm or until the cells overflowed. Animals were then sutured and clipped closed before being allowed to recover in a warm environment with free access to food and water.

3.2.6.2 Media injections into the injury site

As a control for the animals which received cell transplants, a group of animals received injections of the same media as was used to suspend the cells: minimum essential medium alpha (aMEM, Invitrogen, UK) containing 10 % foetal bovine HyClone© serum (Thermo Scientific, UK), supplemented with 2 mM EDTA (Sigma-Aldrich, UK). Injections were made using the same procedure and volumes as were used for cellular transplants.

The wound was closed and analgesia, antibiotics and subcutaneous saline routinely administered, as described earlier. Animals were placed in a warm environment with free access to food and water and allowed to recuperate. Animals were allowed to survive for 7 weeks before electrophysiological assessment.

3.2.7 Cell survival

Two animals received 150 Kdyn injuries and hLP-MSC transplants at 3 weeks post injury and were allowed to survive for 10 days and 6 weeks each, after before being perfused and the injury sites removed to assess cell survival. The remaining 14 transplanted animals were allowed to recover for 7 weeks post-transplantation. At the end of the study all animals were sacrificed via

perfusion fixation, as described in chapter 2, and the injury sites removed and processed with the immunohistological techniques described in detail below.

3.2.8 Behavioural testing

All 15 control animals and 14 cell transplanted animals that were due to undergo electrophysiological assessment were assessed using numerous behavioural tests, as described in detail in the next section.

3.2.8.1 The DigiGait™ Imaging System

Initially animals were selected for this study via a rigorous acclimatisation process in which animals were slowly introduced to the Digigait™ apparatus for 2-5 minutes each day for a week. In order to test compliance, once acclimatised each animal was tested on the Digigait™ system pre-operatively and its ability or inability to run on the treadmill was noted. It was seen that around half of the animals tested were able to run on the Digigait™. Batches were then made up entirely from animals that had shown a pre-operative ability to run on the treadmill.

All 14 animals that received hLP-MSCs transplants and all 15 animals that received medium injections were tested using the Digigait™ Imaging System, in total 8 cell-transplanted and 10 medium injected animals were able to run on the treadmill.

Animals that could run without problem were tested with the DigiGait™ Imaging System (Mouse Specifics, Boston, MA, USA) on a weekly based, starting 1 week before injury and continued weekly for the duration of the experiment (Figure 3.2). The Digigait™ System utilises treadmill running and ventral plane videography to provide a non-invasive method for quantitatively and semi-autonomously comparing numerous spatial and temporal indices of gait dynamics and posture, including stride length, stance width, stance duration, braking duration, swing duration, propulsion duration, paw angle and stride frequency as previously described (Li et al., 2005).

Animals were trained to run at 22 cm/s on a transparent treadmill and recorded from below with an 80 Hz high speed video camera. All 14 animals that received hLP-MSCs transplants and all 15 animals that received medium injections were tested using the Digigait™ Imaging System, of these 8 cell-transplanted and 10 medium injected animals were able to run at the desired speed for the duration of the study, the 10 animals which were unable to run at the desired speed were excluded from this part of the study.

Using the DigiGait™ imaging software, sections of video in which the animal was moving at a relatively constant speed and in a straight line were selected and saved for offline analysis. The aim was to record a minimum of four 2-3 second long clips for each animal at each time point. Animals were initially acclimatized on the treadmill with the motor speed set to zero. The treadmill was started at 12 cm/s at the start of the first testing session, and then raised to 22 cm/s; thereafter the treadmill was started at 22 cm/s. Previous work in this laboratory has shown that this method produces higher levels of compliance than if treadmill speed is increased incrementally. To reduce stress and fatigue, animals were only ever kept inside the Digigait™ machine for a maximum of 10 minutes in any one testing session, and if they fatigued before sufficient runs had been collected they were given several hours to recover before trying again.

It was observed early on that most animals were unable to run sufficiently well on the treadmill in the first week post-injury, so it was decided to miss this time point so as not to stress the animals, and instead we started testing at two weeks post-injury.

Analysis of DigiGait™ videos

Videos were backed up and processed using the Digigait™ analysis software. The snout was blocked to prevent it falsely being identified as a paw. This was achieved by telling the software which direction the animal was running in and the snout blocker manually adjusted to be as small as possible whilst still blocking the snout to ensure that fore paw placements were not blocked. Colour filters were adjusted such that paw placements were detected but tail,

genitals and the area around the snout were not. Stance and swings were manually corrected if required by comparing the plotted temporal history of paw placements with the actual video. The software then allowed for the deletion of any false stances and the merging of stances on either side of any false swings. Once videos were manually corrected the DigiGait™ analysis software analysed them frame by frame and automatically calculated numerous different spatial and temporal indices of gait dynamics and posture which was output to a Microsoft Excel spreadsheet.

3.2.8.2 BBB locomotor assessment scale

In order to assess voluntary locomotor abilities, each animal was assessed using the standardized Basso, Beattie and Bresnahan (BBB) locomotor assessment scale (Basso et al., 1995), which as discussed in greater detail in chapter 1, provides a simple, quick, cheap and non-invasive method of assessing open field locomotion. The BBB test is a commonly used behavioral test used by researchers investigating the effects of spinal cord injury, and thus it provides an invaluable way for researches to understand the true severity of injuries performed by other researchers.

Animals were placed one at a time into a shallow circular pen of ~1.5m diameter and allowed to locomote freely for a minimum of 3 minutes whilst 2 assessors scored the animal's locomotor abilities on a 21 point ordinal scale (Basso et al., 1995). All animals intended for electrophysiological investigation were assessed using the BBB scale at 5 days post-injury and then once weekly for the duration of the study. Animals which scored above 11 on the BBB scale by day 5 post-injury were considered to have not been injured properly and were removed from the study; fortunately, only one of the animals that received transplants and none of the animals in the control group fell into this category. If the assessor's scores differed then extra time was allowed until a consensus could be reached. If an animal was scored on the border between two points, or if the assessors failed to reach consensus, then the lower of the two scores was assigned.

Animals were also scored using the BBB subscore (Lankhorst et al., 1999) by allowing them to locomote freely in the open field whilst two trained assessors scored them on a 7 point scale, in a similar fashion to BBB scoring. Again, if the animal was scored on the border between two points, or if the assessors failed to reach consensus, then the lower of the two scores was assigned.

Analysis of open field locomotion

3.2.8.3 BBB subscore

To increase the accuracy of open field locomotor assessment, all animals were also assessed using the BBB subscore on the same days as they were tested using the BBB. As discussed in greater detail in chapter 1, the BBB subscore utilises a non-ordinal 7 point scale which requires 2 assessors to observe the animal freely locomoting in the same circular pen as is used for the BBB scale and score each animal's locomotor abilities. The BBB subscore was designed to allow for a more accurate assessment of functional deficits than the BBB scale, especially once higher motor functions return; this greater degree of accuracy was achieved due to the BBB subscore scale being non-ordinal and as such points could be awarded for behavioural measures occurring in any order (Lankhorst et al., 1999).

3.2.8.4 Sensory testing

Animals were assessed for changes in sensation or the development of mechanical allodynia using von Frey hairs, a series of 20 monofilaments in a linear scale of physical force which can be used to assess changes in touch sensitivity in response to mechanical stimulation which have been shown to occur following spinal contusion injury (Hulsebosch et al., 2000).

Von Frey filaments are made from hygroscopic materials and their bending forces are significantly affected by humidity and temperature (Andrews, 1993). Consequently, care was taken to ensure that the temperature and humidity levels in the testing room were maintained at a constant level, typically between 50-80% humidity and 20-25°C.

Animals were assessed for increased pain sensitivity using a von Frey hair applied to the plantar surface of the forepaws. The testing protocol relied on the up and down method (Dixon, 1980) to determine the 50% withdrawal threshold. According to Dixon, this method requires 6 responses in the immediate vicinity of the 50% threshold. Response counting was retrospectively started from the response elicited immediately prior to crossing the threshold for eliciting a response.

3.2.8.4.1 Forepaw plantar von Frey hair testing

Each animal was tested using von Frey hairs applied to the plantar surface of each forepaw. Hindpaw testing was not performed as the relationship of this to pain is uncertain (Baastrup, et al., 2010). Animals were tested using the up-down technique described by Dixon et al., 1980, by placing them one at a time into an enclosure with a grated floor which permitted access to the plantar surface of the paws and given 5-10 minutes to acclimatize to the new environment before testing began. In order of increasing force, different von Frey hairs were then applied to the plantar surface of the forepaws with enough force for the filament to bend slightly. Each filament was held in this position for 2 seconds or until the animal initiated an avoidance response. If no response was observed after 2 seconds the filament was removed and the animal was given 3 minutes recovery time before the filament with next higher force was applied similarly, this process was repeated until a response was initiated. Avoidance responses were usually restricted to the animal lifting and repositioning the paw being tested, however on occasion animals were observed vocalizing, trying to bite or jumping away from the filament.

Animals were tested once pre-operatively and once again at 10 weeks post-injury. The levels of mechanical sensitivity were quantified using the up and

down method of Dixon et al., 1980 to determine the 50% withdrawal threshold. All tests were performed by an experimenter who was blind to each animal's condition, in order to avoid conscious or unconscious bias which may have invalidated the results.

3.2.8.4.2 Girdle von Frey hair testing

To assess the development of at level pain in response to mechanical stimulation, each animal was tested using von Frey hairs applied to the girdle at 2cm caudal and 2cm lateral to the injury site, bilaterally. To aid in the location of these sites the animals were marked using permanent marker at the time of injury, the marks were checked weekly throughout the experiment and reapplied if they began to fade. Animals were placed one at a time into a normal cage without any bedding or lid and given 5-10 minutes to acclimatize to the new environment before testing began. A single von Frey filament of (2.44g force) was applied to each location and held against the skin so that the filament bent. This was held in place for 2-3 seconds or until it evoked an avoidance response. This was repeated 10 times in each location, giving the animal at least 30 seconds to recover in between, and the number of avoidance responses recorded in each location was averaged. Avoidance responses were varied and included head turning, biting, running away or jumping. Care was taken to not confuse normal movement, such as relocating within the pen, with an avoidance response, if such movements were observed then the animal was given 3 minutes to recover before testing was resumed.

Analysis of girdle von Frey test

Animals were tested by applying a 2.44 g forced von Frey filament at 2 locations on each animal's girdle, set at 2 cm below and 2 cm lateral to midline, bilaterally. The filament was applied with enough force to slightly bend it, this was performed ten times on each side and one point was awarded for each avoidance response observed, as discussed in detail earlier. The scores from each side were then averaged and graphed, as shown below.

3.2.8.4.3 Dynamic weight bearing test

Animals were assessed for the development of allodynia using a newly developed dynamic weight bearing (DWB) device (Bioseb, Boulogne, France), capable of assessing postural equilibrium in animals in the open field.

Animals were placed one at a time into a Plexiglass enclosure measuring 22 x 22 x 30 cm with a floor sensor consisting of an array of 1936 captors arranged in a 44 x 44 grid (10.89 mm^2 per captor), and allowed to locomote freely for 4 minutes whilst the weights borne by each limb in each paw placement were recorded and stored for offline analysis (Figure 3.5A). Testing sessions were also videoed from the lateral plane to allow the raw data from each paw placement to be synchronised with the video using Bioseb's DWB v1.3 software to aid in offline analysis (Figure 3.5B). Recorded paw placements were considered valid when $\geq$4 g was detected on a single captor, with a minimum of 2 adjacent captors detecting $\geq$1 g. When these parameters were detected and weight distribution was stable for more than 0.5 s, the validated paw placements were assigned as either left or right hind paw or fore paw by a trained assessor after reviewing the video and a scaled map of the activated captors. Mean values for the weights borne by each limb were calculated by dividing the average weight borne by each limb by the average length of time of each limbs validated paw placements.

In preliminary tests performed in our laboratory by Dr. Ahmed Emraja, it was discovered that in normal animals, during periods when all four paws are in contact with the floor, animals consistently bear significantly more weight on their hind paws than their forepaws (Figure 3.5C). Sham surgery at the T9 level had no effect on this (Figure 3.5D), but following a contusion injury at the T9 level there was a redistribution of weight such that some of the weight normally borne by the hindpaws is shifted to the forepaws (Figure 3.5E). There was no spontaneous recovery of this for at least 6 weeks after the injury (Figures 3.5F-G), making this an ideal assessment of posture on which to test therapies for SCI.

The DWB test was performed on all the animals tested prior to injury in order to create a set of baseline data for comparison. Each of the 15 medium injected control animals and the 13 hLP-MSC transplanted animals (that achieved normal BBB scores at 5 days post injury) were tested again at 10 weeks post-injury.

Analysis of dynamic weight bearing test

As discussed earlier, each animal was placed in the DWB enclosure and allowed to locomote freely for 4 minutes as the software automatically recorded the timing and weight borne by each paw placement. Each video was reviewed alongside the recordings made by the pressure transducers and each paw placement was manually validated. The mean values for the weights borne by each limb were calculated by dividing the average weight borne by each limb by the average length of time of each limbs validated paw placements and expressed as a percentage of each animal's body weight.

3.2.9 Electrophysiology

3.2.9.1 Anaesthesia

All animals were anaesthetised as described in Chapter 2 during the preparatory surgery and electrophysiological assessment.

3.2.9.2 Preparatory surgery

All preparatory surgery and the protocols for electrophysiological assessment were exactly the same as described in Chapter 2, (including the use of CDPs to monitor function in the sciatic nerve and dorsal columns below the injury level).

3.2.9.3 Recordings of sensory evoked potentials

To assess function in the ascending dorsal column pathways of the spinal cord and the effect of hLP-MSC transplants, SEPs evoked by electrical stimulation of the radial and sciatic nerve were recorded from the contralateral sensorimotor cortex. Recordings of SEPs were made whilst stimulating the radial and sciatic nerve with supramaximal stimulus intensity. Stimulating and recording protocols were the same as described in Chapter 2. Whilst recording SEPs, great care was taken to remove fluid from the surface of the cortex to avoid inaccuracy of recordings.

Recordings of electrical potentials were digitised (Cambridge Electronic Design 1401+ interface, Cambridge, UK) and stored on a computer at a sampling rate of 20 kHz, without filtering. Averaging and measurements of the amplitudes and latencies of electrical potentials were performed using Signal software (Cambridge Electronic Design, Cambridge, UK).

3.2.9.4 Perfusion fixation

All animals were transcardically perfused at the end of their electrophysiological assessment so that the injury site could be preserved and removed for histological processing, as described in Chapter 2.

3.2.10 Histological processing

3.2.10.1 Histological processing of spinal cord for contusion site and cell distribution

Approximately 3 cm of spinal cord centered on the middle of the injury site was dissected out of each animal and cut in to three 8mm blocks, one centered on the middle of the injury site and one each from immediately rostral and immediately caudal to the injury block. Most animals had their cords cut in the parasaggital plane to preserve information about the

distribution of hLP-MSCs in the rostro-caudal axis. When cut parasaggitally, rostro-ventral and caudo-ventral notches were made on the rostral and caudal blocks, respectively, and either a rostro-ventral or caudo-ventral notch was made on each of the injury blocks, favoring the end that seemed firmest. Some animals were cut in the transverse plane, for which no notch was made so that the injury site could be optimally preserved. Blocks were cut into 60 µm sections at -20^{o}C using a Leica CM1850 cryostat (Leica Microsystems GmbH, Wetzlar, Germany). Sometimes, where the injury was extensive, it was difficult to cut whole sections or the sections fell apart after cutting, this was particularly problematic, especially in transverse sections, and precluded the quantification of lesion cavity volume. Sections were washed in 0.3M phosphate buffered saline (PBS double salt), and then incubated for 30 min in 50% ethanol. After washing three times for 10 minutes each in PBS, sections were treated with fluorescence histochemistry techniques.

Sections were incubated in combinations of anti-NF200 (1:1000, Sigma), anti-GFAP (1:1000, Dako), anti-P0 (1:100, Santa Cruz), anti-laminin (1:100, Sigma) and anti-GFP (1:1000, Serotec) primary antibodies at 4^{o}C for 72hrs, which were used to label the axons, reactive astrocytes, peripheral type myelin, the basement membrane of the basal lamina and green fluorescent protein, respectively. Sections were then washed in PBSD and incubated in secondary antibodies, raised in the appropriate species, conjugated to appropriate fluorophores (Alexa 488 1:500, Rhodamine 1:100 and DL649 1:500; Jackson Immunoresearch, USA) for 3-4 hours at room temperature or overnight at 4^{o}C. All sections were then washed in PBS three times for 10 minutes each, mounted on plane glass slides in anti-fade mounting medium (Vectashield: Vector Laboratories, UK), before being coverslipped and the edges of the coverslip sealed with nailvarnish and stored at -20^{o}C in darkness.

3.2.11 Microscopy

3.2.11.1 Verification of contusion injury

Phase contrast light microscopy was used to view sections containing the lesion cavity in order to determine the perimeter of the cavity and the relationship to the boundary between grey and white matter. Epifluorescence

microscopy was also used to observe NF200, GFAP, P0, laminin and GFP immunolabelling in the sections.

3.2.11.2 Distribution of hLP-MSCs

Sections taken from animals that had received hLPs-MSC transplants were examined with epifluorescence microscopy (Nikon Eclipse E600) in order to confirm transplanted cell survival and to document their distribution. Two animals were used to assess cell survival in injured animals at 10 days and 6 weeks following injury. Confocal microscopy was used to illustrate the distribution of the transplanted hLP-MSCs, see below.

3.2.12 Off-line analysis of SEPs

As described in Chapter 2, averaged SEP records were created from which measurements were taken offline using Signal software (Cambridge Electronic Design, Cambridge, UK). Data were collated in Microsoft Excel spreadsheets and imported to Prism 4 software (GraphPad Software Inc, USA) for graphing. Data from the SEP maps were collected in Microsoft Excel spreadsheets and imported to 3D Field software (Version 3.0.9.0, Copyright 1998–2007, Vladimir Galouchko, Russia) to create isopotential contour plots of the cortical areas in which SEPs were recorded. Contour measurements were made in the 3D field software and the data were averaged and imported to Prism 4 software for graphing.

3.2.12.1 Conduction latency of SEPs in the dorsal column-medial lemniscus pathway

SEP traces were analysed in order to calculate the conduction latencies in the dorsal column-medial lemniscus pathway. This was performed in 13 hLP-MSC transplanted and 15 medium injected control animals by measuring the time delay between stimulus application and the arrival of potentials in the cortex

using Signal software (Cambridge Electronic Design, Cambridge, UK). Latencies were normalised with respect to the different conduction distances by measuring each animal's conduction distance and correcting conduction latencies by the percentage difference between the conduction distance recorded in each animal and the maximum conduction distance recorded in any animal.

3.3 Results

3.3.1 Assessment of long tract function after contusion injury with delayed hLP-MSC transplants

In order to assess the effects of delayed hLP-MSC transplants on long tract function, 14 animals received 150 kdyn contusion injuries at T9; three weeks later they received injections of GFP labeled hLP-MSCs directly into the injury epicenter. Each animal's functional capabilities were monitored by behavioural testing and long tract function was assessed directly using an electrophysiological approach, as described below.

For comparison 15 animals received similar injections of medium and were assessed thereafter by researchers blind to their experimental group.

GFP labeling allowed the transplanted cells to be easily identified in the histological analysis detailed below.

3.3.2 Comparison of body weights and injury severities

To ensure that any differences we observed in the electrophysiological recordings were solely due to the presence or absence of hLP-MSCs, it was necessary to limit the variable parameters in each experiment. Firstly, animals were randomly assigned to experimental groups by a member of the animal house staff blind to the experimental protocol. For this same reason it was necessary to ensure that the animals from different experimental groups received injuries of as similar a severity as possible. Contusion injuries of similar force will produce varied injury severities when applied to spinal cords of different sizes due to the impactor tips ability to damage a greater or lesser proportion of the cord depending on its size. It was therefore necessary to select animals for injury that were of a similar body weight and would therefore have similar sized spinal columns. To ensure this was strictly adhered to, each animal's weight at the time of injury and the injury

parameters provided by the Infinite Horizons software were recorded and graphed for comparison, so that any anomalous animals could be considered for exclusion.

As shown in Figure 3.6, individuals included in the both experimental groups had similar body weights throughout the study. Whilst body weight did increase through the 10 weeks of testing, animals from both the medium injected and hLP-MSC transplanted groups were of a similar weight at the time of contusion injury surgery, medium injection/cell transplantation surgery and terminal electrophysiology. We can therefore be sure that the animal's cords were damaged in as equally a proportional manner as possible, that each transplant or injection was made into as similar an injury site as possible and that the electrophysiological data was obtained in as similarly sized animals as possible.

The Infinite Horizons Impactor is able to record the actual force pushing back against the impactor tip and the displacement of the cord; these data can be used to ensure that all the animals received as similar an injury as possible. Figures 3.7A-B show that the animals from each group received injuries of similar force and cord displacements.

By monitoring and where possible reducing variables such as these as much as is feasibly possible, we can increase confidence that any changes seen between the groups result from the hLP-MSCs vs. control differences.

3.3.3 Behavioural testing

3.3.3.1 Assessment of function using Digigait™

Animals were tested using the Digigait™ system at weekly intervals from pre-injury to 9 weeks post-injury. In total 9 of the 14 animals that received hLP-MSCs transplants and 10 of the 15 animals that received medium injections were able to run on the Digigait™ treadmill throughout the testing period, and so were included in this part of the study – the animals that were either

unable or stopped running were kept for BBB testing, BBB subscore testing, DWB testing, sensory testing, electrophysiological testing and histological investigation.

Whilst many of the gait indices recorded by the Digigait™ Imaging System showed no differences between the groups, there were three which did appear to display differences between hLP-MSC transplanted and medium injected groups:

Gait symmetry

Figure 3.8A shows the observed changes in gait symmetry; that is, a ratio of the frequency of forelimb steps divided by the frequency of hindlimb steps. In normal animals, because forelimb and hindlimb stepping is fully coordinated i.e. the frequency of forelimb and hindlimb stepping is the same, this ratio is one. After injury there is a loss of this coordination reflected in an increase in this ratio. This loss of forelimb-hindlimb co-ordination is seen in both the transplanted and medium injected animals since both received the same injury. Both groups of animals also show a recovery of this co-ordination with time after injury. That is to say, the ratio of forelimb to hindlimb steps returns towards one. It can be seen in Figure 3.8A that the recovery of gait symmetry in the hLP-MSC transplanted animals occurs faster and at the end of the experiment is slightly more complete than that seen in the medium injected animals. This suggests that hLP-MSCs helped enhance the recovery of gait symmetry. Figure 3.8B shows the absolute gait symmetry values obtained in normal animals and animals from both experimental groups at the time points which appeared to have the greatest difference, namely 7, 8 and 9 weeks post injury. A two way ANOVA test was performed on these data, revealing that there is no statistically significant difference between the groups at any of the time points investigated ($P>0.05$). The size of the standard error bars in Figure 3.8B indicates that the variation seen within each group was high enough to prevent the observed trend for hLP-MSC transplanted animals to recover gait symmetry faster, from being significantly different.

Stride Frequency

Using the Digigait™ system it was possible to record changes in stride frequency, a measure of the average number of forelimb or hindlimb steps taken per second, in medium injected and hLP-MSC transplanted animals from pre-injury to 9 weeks post-injury (Figure 3.9A); this provides an interesting insight into the mechanisms underlying recovery of co-ordinated stepping. It might be expected that the loss of co-ordination following a T9 contusion injury would be because the hindlimbs slow down after injury; however, the data in Figure 3.9A show that this is not the case. Surprisingly, the loss of gait symmetry shown in Figure 3.8A-B is actually explained by the forelimbs increasing their stepping frequency after injury, while that of the hindlimbs initially changes very little (Figure 3.9A). This is confirmed in Figure 3.9B which shows the difference in forelimb-hindlimb stride frequency recorded in normal animals and animals from both experimental groups at the time points which appeared to have the greatest difference, namely 7, 8 and 9 weeks post injury. It can be seen that the injury initially caused the difference between forelimb and hindlimb stepping frequency to increase, though by 7-9 weeks the difference in stride frequency can be seen to be recovering in hLP-MSC transplanted animals faster than in medium injected animals. Figure 3.9A shows that from around 2 weeks following injury, the forelimbs begin to slow a little and the hindlimbs speed up a little. Thus, a more similar stride frequency i.e. closer co-ordination of forelimb and hindlimb stride frequency is achieved by adaptations of both the forelimbs and hindlimbs to a new common stride frequency rather than by normalisation of the stride frequency to that before the injury; thus it can be seen that animals employ compensatory mechanisms which, combined with limited functional recovery act to return stride frequency towards normal levels. Two way ANOVA tests were performed on these data, revealing that although there is a trend, no statistically significant difference exists between the forelimb and hindlimb data from each of the groups at any of the time points investigated ($P>0.05$).

Alternate stepping

The coordination of all four limbs is important in quadrupeds and can be used to measure function in the spinal cord. In normal animals the predominant pattern of stepping is an alternate pattern which follows the sequence illustrated in Figure 3.10Bi of left HL followed by left FL followed by right HL

followed by right FL and so on. Before injury this sequence occurs for about 90% of the steps. However, after injury this sequence is known to break down and becomes uncoordinated, instead following one of several non-alternate step sequence patterns such as the example shown in Figure 3.10Bii. Animals stepping sequence patterns were recorded by Digigait™; Figure 3.10Biii shows a representative example of a partially coordinated animal as shown in the Digigait™ software.

Figure 3.10A shows the step sequence data recorded in transplanted vs. control animals as a % of normal alternative stepping sequence. It can be seen that a greater recovery of co-ordinated stepping occurs in transplanted compared to medium injected animals. Whilst the % alternate steps do not recover fully in either group, the transplanted animals tend to show some improvement which is not matched by the control animals (Figure 3.10A). Despite this trend, a two way ANOVA test performed on these data revealed no statistically significant difference between the groups at any of the time points investigated ($P>0.05$).

3.3.3.2 Assessment of locomotor function using the BBB locomotor assessment scale and the BBB subscore

As discussed earlier, locomotion in the open field can be assessed by two trained testers observing each animal as it was allowed to freely locomote in a shallow pen for 4 minutes. Each animal is then scored out of 21 on the BBB locomotor assessment scale and out of 7 for the BBB subscore using the respective scoring scales.

In total, 13 animals which received hLP-MSC transplants and 15 medium injected animals were assessed using the BBB locomotor assessment scale and the BBB subscore. The time course of recovery of locomotion in hLP-MSC transplanted and medium injected groups, as measured by the BBB locomotor assessment scale and BBB subscores, is shown in the graphs shown in Figures 3.10 A-B. It can be seen that the contusion procedure resulted in substantial reductions in the BBB and BBB sub-scores assigned to both cell-transplanted

and medium injected animals by 5 days post-operatively. However, both experimental groups were then seen to begin to recover their locomotor abilities at similar rates, though whilst substantial recovery was observed, the locomotor abilities of both the hLP-MSC transplanted and the medium injected groups were seen to plateau without returning to pre-operative levels. Two-way ANOVA statistical tests were performed to ascertain if there were any significant differences between the data obtained from each group at each time point; it was seen that there were no significant differences observed between groups at any of the experimental time points for the duration of the study, as measured via both tests (Figure 3.11A-B).

3.3.4 Sensory testing

3.3.4.1 Forepaw plantar von Frey testing

The 50% withdrawal thresholds of the forepaws were determined using the up and down method described by Dixon, 1980, as described earlier. Results obtained in normal uninjured animals were compared with those obtained in injured animals that had received either medium injections or hLP-MSC transplants. As shown in Figure 3.12, after performing one-way ANOVA with Tukey's post-hoc analysis, it was seen that the force required to cross the 50% withdrawal thresholds of uninjured animals was significantly higher than either the medium injected or cell transplanted groups. Although no significant difference was observed, a trend can be seen in which medium injected animals tend to have a lower 50% withdrawal threshold than cell transplanted animals, suggesting that hLP-MSC transplants might play a role in reducing forepaw sensitivity up to 7 weeks post-transplantation (Figure 3.12).

3.3.4.2 Girdle von Frey testing

To assess the development of tactile allodynia in response to mechanical stimulation, a single calibrated von Frey filament (2.44 hair / 0.04 g) was applied to two regions located on each animal's back, at 2 cm lateral and 2

cm caudal to the injury level, bilaterally, as described earlier and shown in Figure 3.13A.

Previous work performed in this laboratory by Dr. Ahmed Emraja using T9 contusion injuries of 200 Kdyn force showed that injury can lead to an increased number of avoidance responses to girdle von Frey stimuli for up to 6 weeks following injury, whilst sham operations produced no such increases to pain sensitivity (Figure 3.13B). It is therefore not unreasonable to assume that contusion injuries of 150 Kdyn force should evoke increases in tactile allodynia recorded across the girdle.

In order to assess if the application of hLP-MSCs had any effect on tactile allodynia, the number of avoidance responses that girdle von Frey testing produced in uninjured animals was compared with that produced in the same animals after they had received 150 Kdyn contusion injuries at T9, followed by either medium injections (n=15) or hLP-MSC transplants (n=14) at 3, 4, 8 and 10 weeks post-injury. As shown in Figure 3.13C, when compared to the number of responses made by animals preoperatively, the number of avoidance responses produced in uninjured animals was significantly increased in both medium injected and cell transplanted groups by 3 weeks post operatively (one way ANOVA – Tukey's post-hoc test). At every post-operative time point tested, no significant difference in the number of avoidance responses was observed between medium injected and cell transplanted groups (2 way ANOVA, $P>0.05$), though a trend was seen for reduced pain sensitivity in the cell transplanted group (Figure 3.13C).

3.3.5 Dynamic weight bearing testing

Each animal was therefore assessed using the DWB apparatus as described previously. As mentioned previously, normal baseline data was obtained from all the animals prior to injury (n=29), which showed that in normal animals approximately 35% and 65% of their total body weight is placed on the forepaws and hindpaws, respectively, as shown by the dashed red line on the graphs in Figure 3.14.

By 7 weeks following either hLP-MSC transplantation or medium injection, animals from both medium control (n=15) and cell transplanted (n=14) groups were tested again, revealing a clear trend whereby the percentage of total body weight placed on the forepaws had increased to around 40% and the total body weight placed on the hindpaws had decreased to around 60% (Figure 3.14). A student's T-test was performed on the data, which revealed that there was no significant difference between the % body weights placed on either the fore paws or hind paws of the medium injected vs. hLP-MSC transplanted groups. This could result from a number of mechanisms, including the development of allodynia in the hind paws which causes animals to transfer a greater proportion of their weight to the fore paws; alternatively, this may result from a compensatory mechanism adopted by the animals to compensate for the deficits caused by the injury.

3.3.6 Electrophysiological assessment of long tract function in hLP-MSC transplanted SCI animals

In order to assess the effects of hLP-MSC transplants on function in the ascending dorsal column white matter pathways following SCI, groups of hLP-MSC transplanted (n=14) and control animals (n=15) were investigated using the electrophysiological approach described earlier.

As described in greater detail below, SEPs evoked by stimulating ipsilateral radial and sciatic nerves were recorded from the contralateral somatosensory cortex at ten weeks post-injury in animals receiving either 3 weeks delayed hLP-MSC transplants or for comparison, similar injections of medium.

hLP-MSC transplants and their effects on SEPs

As before, SEPs were recorded in order to quantify any differences that may exist between the groups. Maximum SEP amplitudes, the cortical areas from SEPs could be recorded and the latency to SEP onset, were recorded in each animal as discussed below.

Maximum SEP amplitudes

Maximum radial and sciatic SEPs were measured in each animal, as described earlier. Whilst radial SEP amplitudes were kept as absolute values, sciatic SEP amplitudes were normalised by expressing each as 15% of the maximal radial SEP amplitude recorded in the same animal; the results are shown in Figures 3.14 A-B. A student's T-test was performed on the data, revealing no significant difference between the maximum amplitude radial or sciatic SEPs recorded in each of the groups (Figures 3.14 A-B).

SEP isopotential areas

Maximal radial and sciatic SEP amplitudes were measured in each animal and normalised to 80% and 15% respectively, of the maximal radial SEP amplitude recorded in the same animal. These data were used to construct isopotential contour plots which delineate the area of cortex from which radial and sciatic SEPs could be recorded which were ≥80% or ≥15%, respectively of the largest radial SEP amplitude recorded in each animal; the results are shown in Figures 3.15 A-B. A student's T-test was performed on the data, again revealing no significant difference between the groups (Figures 3.16A-B).

Latency to SEP onset

Latencies to radial and sciatic SEP onset were recorded from individual animals in each group and normalised to account for the different conduction distances as described earlier; the results are shown in Figures 3.17A-B. A student's T-test was performed on the data, which again revealed no significant difference between the groups (Figure 3.17B).

Together these data reveal that hLP-MSCs appear to have no significant effect on long tract function, following 150 Kdyn contusion injury at T9.

3.3.6.1 The effects of hLP-MSCs transplants on the cortical locations from which SEPs could be recorded

Medium injected control group

Peripheral nerve-evoked SEP recordings were made in 15 medium injected control animals at 10 weeks post-injury. In each animal, clear surface potentials were evoked by stimulation of both the radial and sciatic nerves. SEP amplitudes were normalised by expressing the radial and sciatic SEP amplitudes recorded at each location as 80% and 15% respectively, of the maximum radial amplitude recorded in each individual. Normalised radial SEPs from each animal were relatively consistent and could be recorded from Bregma 0 mm to -2 mm at 4 mm lateral to Bregma (Figure 3.18A), and normalised sciatic SEPs could be recorded from Bregma 0 mm to -3 mm from 2-4 mm lateral to Bregma (Figure 3.18B). Normalised radial and sciatic SEP amplitudes from all animals were then averaged and plotted as isopotential contour lines which encompass the area of cortex within which SEP amplitudes were 80% and 15%, respectively, of the averaged maximal radial SEP amplitude. As shown in Figure 3.19A, this revealed that the centre of focus for averaged radial SEPs was located from Bregma -1 mm to -2 mm at 4 mm lateral to Bregma, and the centre of focus for sciatic SEPs was located around Bregma -1 to -2 mm rostrocaudally at 2-4 mm lateral to Bregma (Figure 3.19B).

The centres of focus seen here are the same as were recorded in normal animals in chapter 2 (eg. Figures 2.17 A-B and Figures 2.18 A-B), which indicates that medium injections have no effect on the cortical locations from which SEPs can be recorded.

hLP-MSC injected group

Peripheral nerve-evoked SEP recordings were made in 14 hLP-MSC transplanted animals at 10 weeks post-injury. In each animal, clear surface potentials were evoked by stimulation of both the radial and sciatic nerves; amplitudes were normalised as before. Normalised radial SEPs from each animal were relatively consistent and could be recorded from Bregma 0 mm to -2 mm at 4 mm lateral to Bregma (Figure 3.18C), and normalised sciatic SEPs could be recorded from Bregma 0 mm to -3 mm from 2-4 mm lateral to Bregma (Figure 3.18D). Normalised radial and sciatic SEP amplitudes from all animals were then averaged and plotted as isopotential contour lines, as described earlier. As shown in Figure 3.19A, this revealed that the centre of

focus for averaged radial SEPs was the same as was seen in the control group, located from Bregma -1 mm to -2 mm at 4 mm lateral to Bregma. The centre of focus for sciatic SEPs was also the same as was seen in the control group, located around Bregma -1 to -2 mm rostrocaudally at 2-4 mm lateral to Bregma (Figure 3.19B).

Together these results indicate that hLP-MSCs have no significant effect on the cortical locations from which SEPs can be recorded.

3.3.7 Histology

Survival of transplanted cells

Sections of spinal cord from the injury sites of all animals were processed for anatomical observations. Sections from all transplanted animals were inspected for the presence of GFP labeled transplanted hLP-MSCs. Figure 3.20A shows an example of a transplant site from an animal used in a pilot study which shows many surviving cells 10 days after transplantation into a T9 150 Kdyn contusion injury. Figure 3.20B shows an equivalent section from one of the animals from the main study perfused 7 weeks after transplantation which has fewer labeled cells. Both of these animals received daily injections of Cyclosporin which was essential for survival of the cells. Evidence of labeled cells was observed in all 13 of the transplanted animals used in the main study but in some animals the numbers of surviving cells were low and in all animals the numbers of cells were less than are typically seen in our experience when performing syngeneic transplants of primary cells such as OECs or Schwann cells. For comparison, Figure 3.20C shows GFP labeled OECs 4 weeks after being transplanted into a cervical contusion injury. This syngeneic transplant (F344 donor and recipient animals) did not require Cyclosporin treatment.

Injury cavities

Sections from the injury site were investigated to determine the degree to which transplanted cells and/or extracellular matrix had filled the cavities that typically form in the weeks following injury. The injury sites were classified as completely filled, partially filled or cavitated (Figures 3.21A-C).

The frequency with which injury sites of these types were observed in the transplanted and medium control groups is shown in Table 3.1. In more than half the transplanted animals (8 of 14) the injury site was completely filled with cells or extracellular matrix rich in laminin, whereas only 3 of the 15 medium injected animals had injury sites completely filled with matrix. Concomitantly, the non-transplanted group showed a higher proportion of partially matrix filled or cavitated injury sites.

Assessment of myelination

The standard method of assessing demyelination and remyelination is to use electron microscopy to assess the axonal diameter to myelin thickness ratio, known as the G-ratio. Remyelinated axons have smaller G-ratios than normally myelinated cells, which in the absence of a remyelination specific antibody is the only way of differentiating remyelinated cells from cells that survived the injury with their myelin sheath intact.

As we prioritised investigating the fate of transplanted cells, the opportunity for investigating myelination using an anatomical approach was limited due to the limited tissue and fact that we could not use the same tissue for both immunohistochemistry and electron microscopy due to the need for different perfusion and tissue processing protocols.

Sections from both medium injected and transplanted animals were processed using antibodies for axons (neurofilament) and a constituent of peripheral type myelin (P0). P0 immunolabelling was seen in both medium injected and transplanted animals (see Figures 3.22A-B), but preliminary analysis suggested that there was a wider distribution of P0 in transplanted animals and that this was specifically in areas where transplanted cells were located. P0 immunolabelling frequently showed a close association with neurofilament labelled nerve fibres and was present both within and out with the transplanted injury site (Figure 3.22C). The labelling within the injury site most likely represents myelination of regenerating nerve fibres while that out with might reflect remyelination of spared fibres.

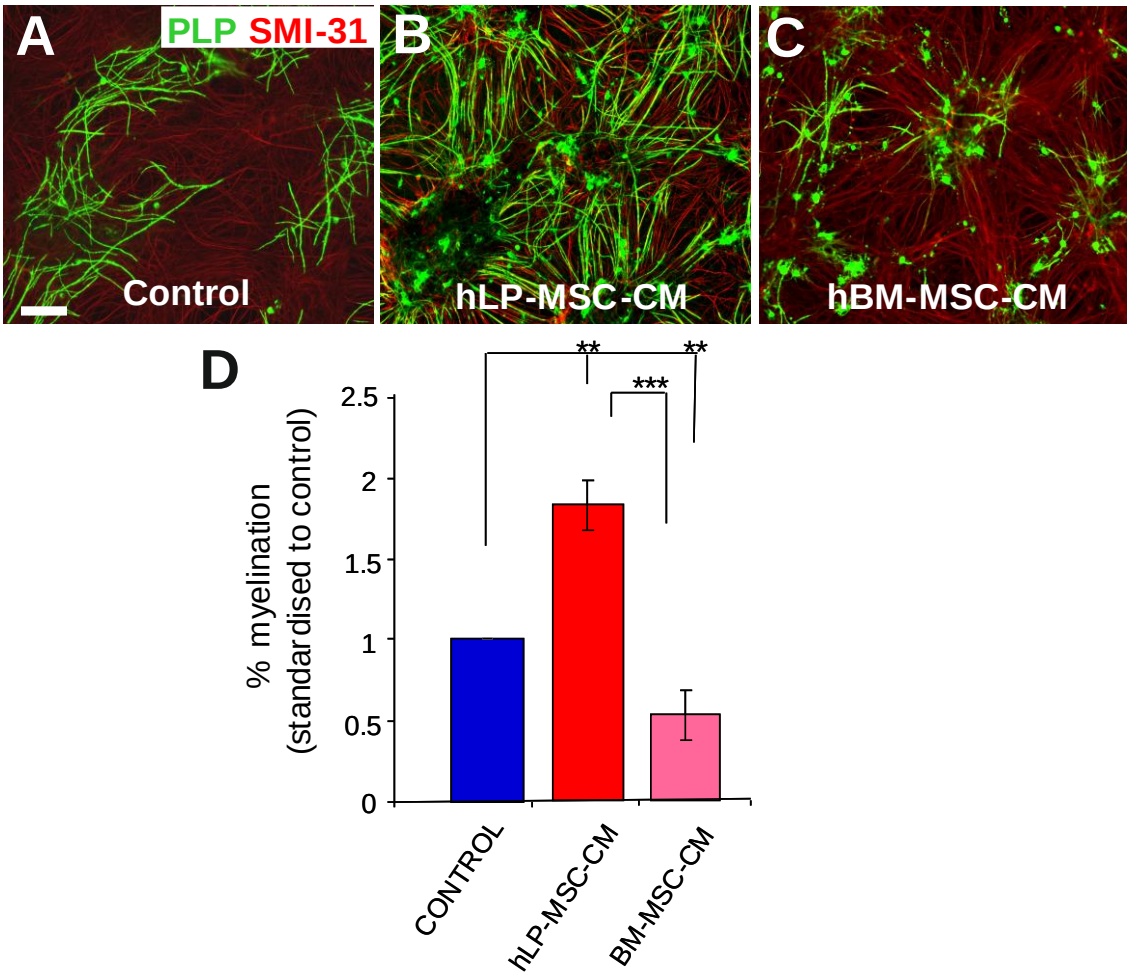


Figure 3.1 hLP-MSCs enhance oligodendrocyte myelination in dissociated spinal cord cultures*. The rational for using hLP-MSCs came from preliminary in vitro myelination studies. A) Spontaneous oligodendrocyte myelination was observed in dissociated spinal cord culture. B) The presence of hLP-MSC conditioned media enhances the degree of oligodendrocyte myelination. C) The presence of hBM-MSC conditioned media reduces the degree of oligodendrocyte myelination. D) Quantitative analysis reveals that when compared to control cultures (n=9), hLP-MSC conditioned media (n=5) promotes a highly significant increase in oligodendrocyte myelination, whereas hBM-MSC conditioned media (n=9) produces a highly significant reduction in the amount of oligodendrocyte myelination (Error bars = SEM). PLP = Myelin proteolipid protein - marker for oligodendrocyte myelin, SMI-31 = Neurofilament protein - marker for axons and dendrites; **p<0.01; ***p<0.001; one-way ANOVA Tukey's test. Scale bar = 100 µm. (Adapted from Lindsay et al., 2013).*

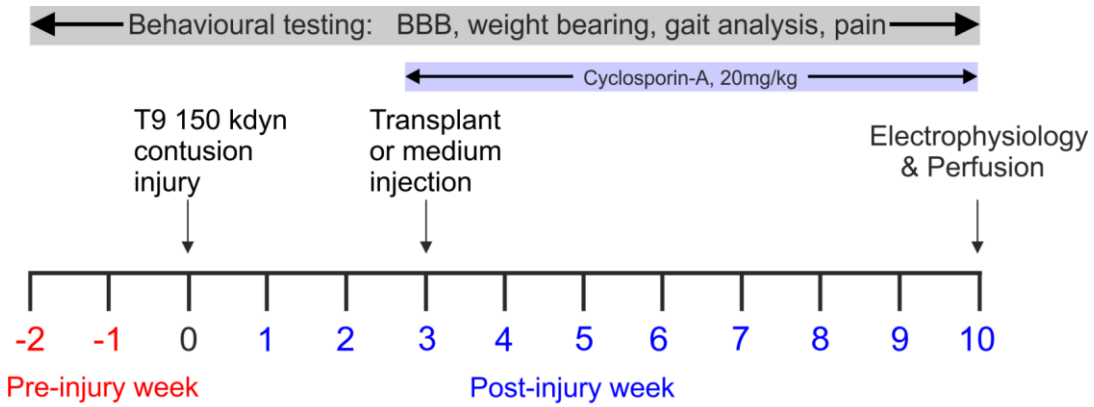


Figure 3.2 Experimental design *- Schematic diagram summarizing the experimental design.*

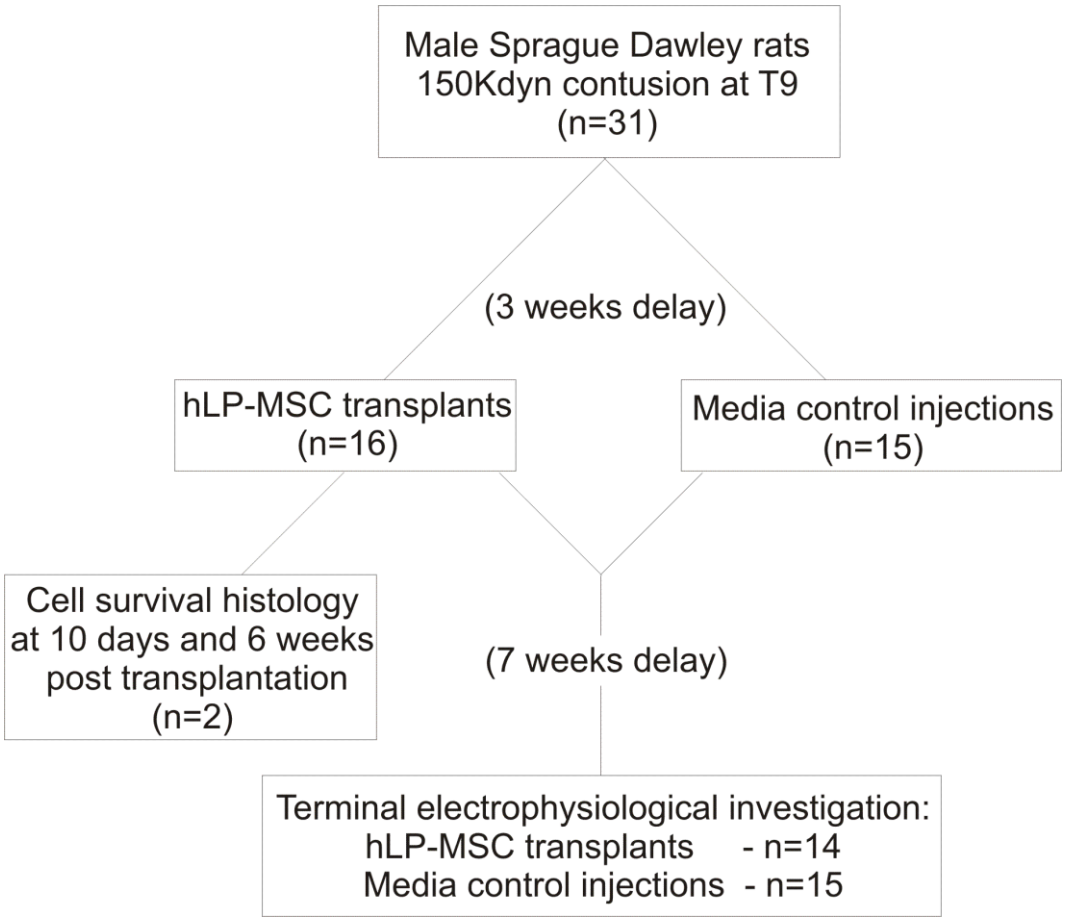


Figure 3.3 Flow diagram of animal use- *Flow diagram detailing the n numbers used at each point in the experimental design.*

	hLP-MSCs	Medium
Donor 1	4	3
Donor 2	5	5
Donor 3	5	7
Total	**14**	**15**

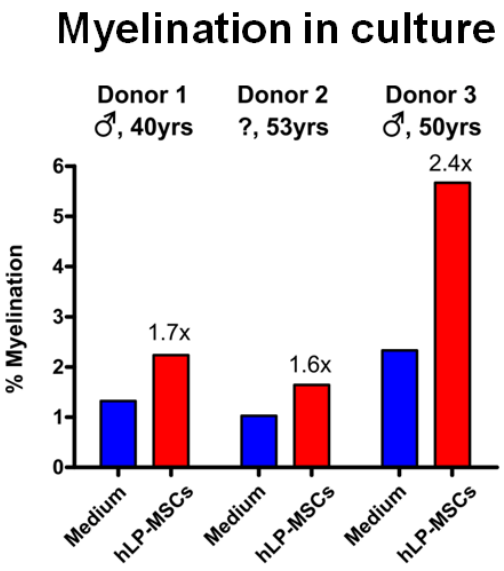


Figure 3.4 Source of transplanted hLP-MSCs and myelination in culture promoted by conditioned medium. *Right: hLP-MSCs were obtained from three individuals; after culturing, cells from each individual were transplanted into several injured rats with similar numbers of rats concommittantly receiving injections of unconditioned media. Left: Conditioned medium taken from the cells from each donor promoted myelination in culture. (Unpublished work performed by Dr. Susan Lindsay).*

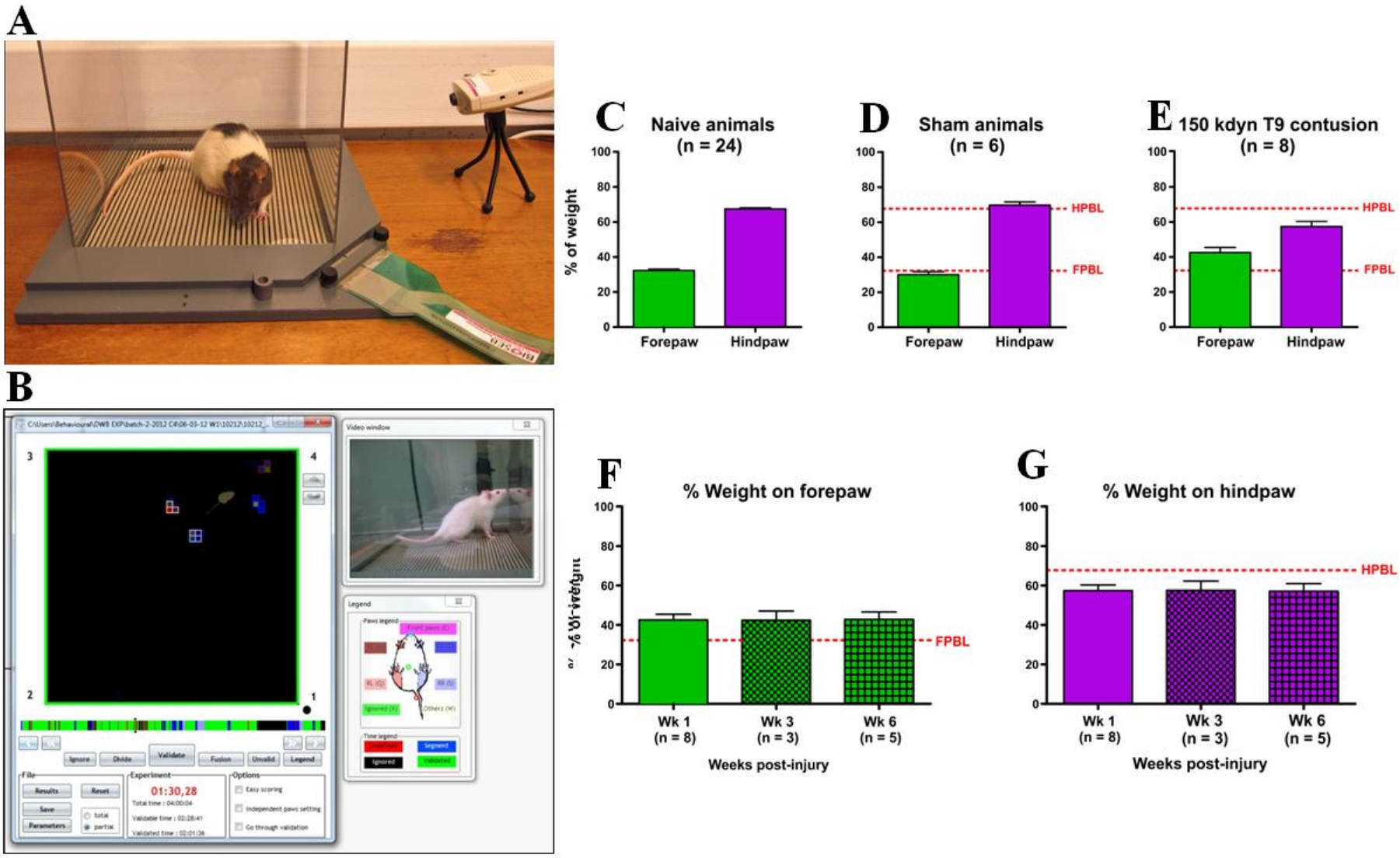

A
B
C
Naive animals
(n = 24)
% of weight
Forepaw
Hindpaw
D
Sham animals
(n = 6)
HPBL
FPBL
E
150 kdyn T9 contusion
(n = 8)
F
% Weight on forepaw
Wk 1
(n = 8)
Wk 3
(n = 3)
Wk 6
(n = 5)
Weeks post-injury
G
% Weight on hindpaw

Figure 3.5 - Dynamic Weight Bearing (DWB) test*. A) DWB apparatus and B) DWB software analysis of paw pressures. C) In Naïve animals more weight is distributed to the hindpaws than the forepaws. D) After sham surgery there is no change in the distribution of body weight. E) T9 150 Kdyn contusions lead to a redistribution of body weight from the hindpaws to the forepaws. F) There is no change in this redistribution at up to 6 weeks post-operatively (All = SEM; FPBL = Forepaw Baseline; HPBL = Hindpaw Baseline; unpublished observations by Dr. Ahmed Enraja, 2013).*

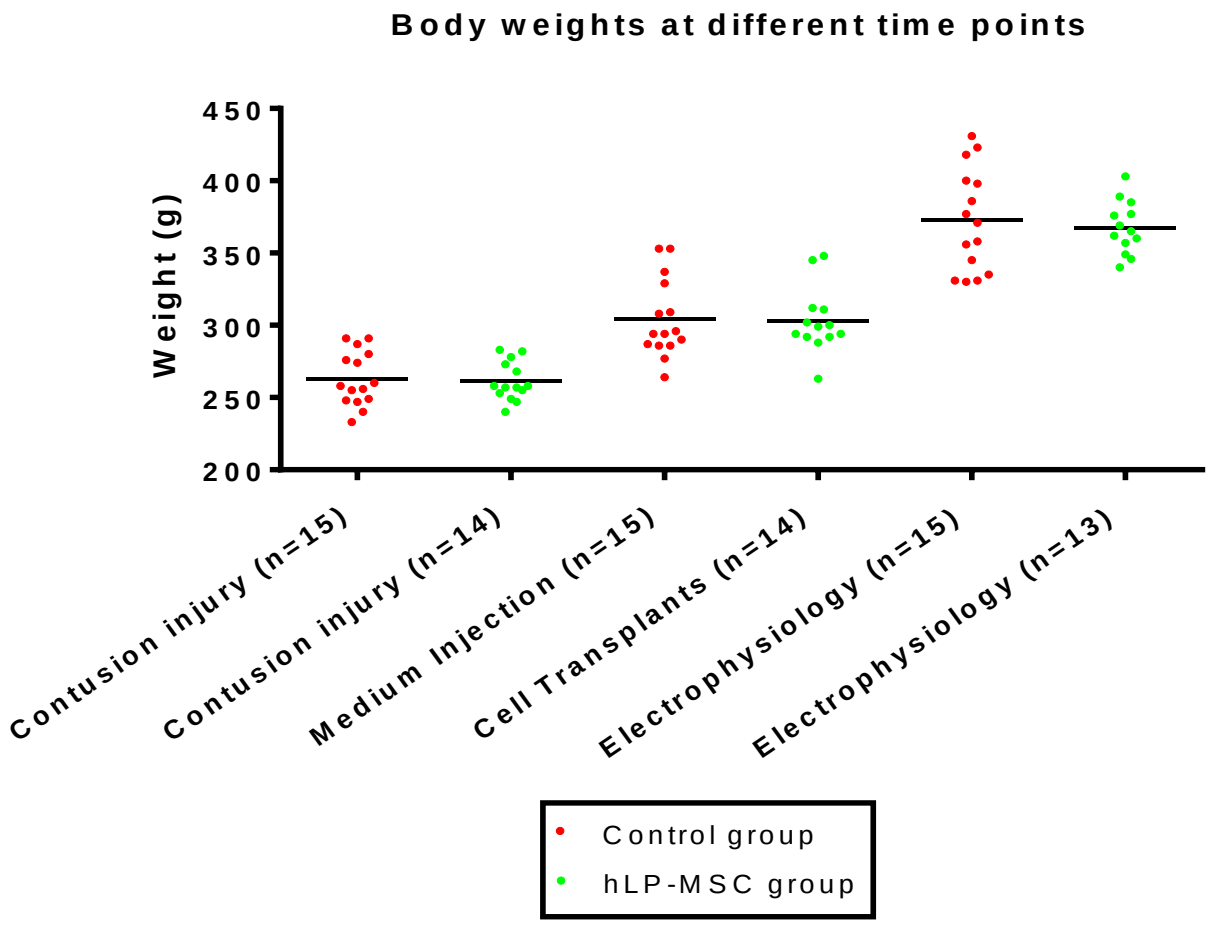


Figure 3.6 Individual body weights at different points throughout the study. *The body weights of individual animals in both the medium injected control group and the hLP-MSC transplanted group were recorded at the time of contusion surgery, media injection/cell transplantation surgery and terminal electrophysiological surgery. Student's T-test's were performed on data sets from each time point, revealing no significant differences between the groups at any of the time points (all p>0.05; Bars = means).*

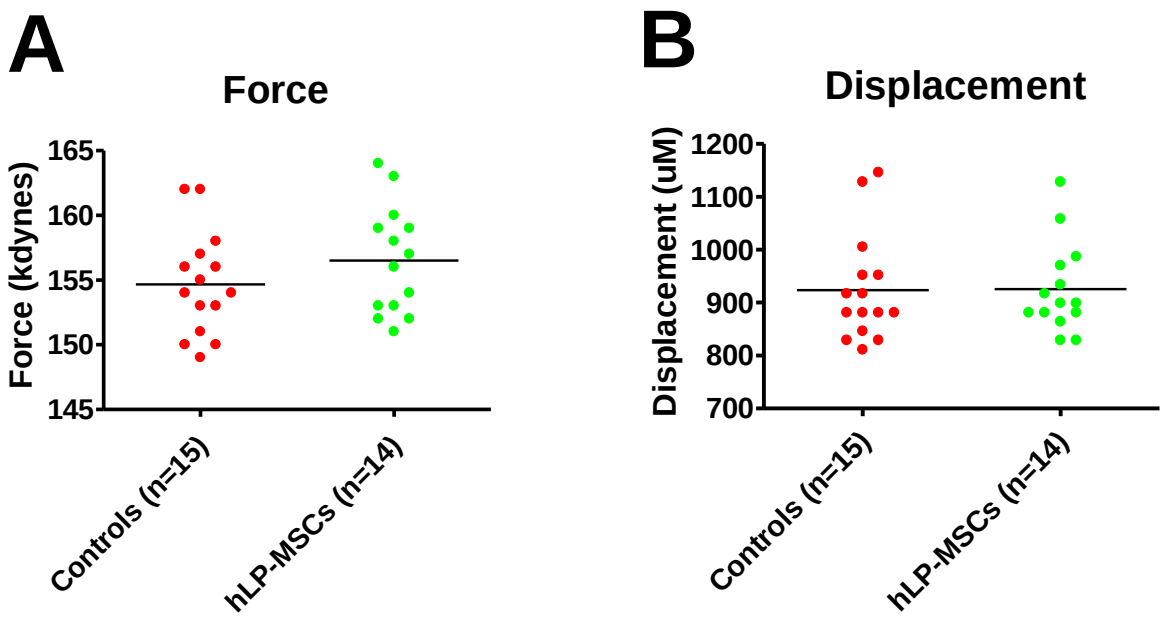


Figure 3.7 Contusion injury impact force and tissue displacement. *Both experimental groups received similar contusion injuries. All animals received similar force injuries resulting in similar degrees of tissue displacement. Student's T-test's revealed no significant differences between injury force or displacement between the groups (all p>0.05; Bars = means).*

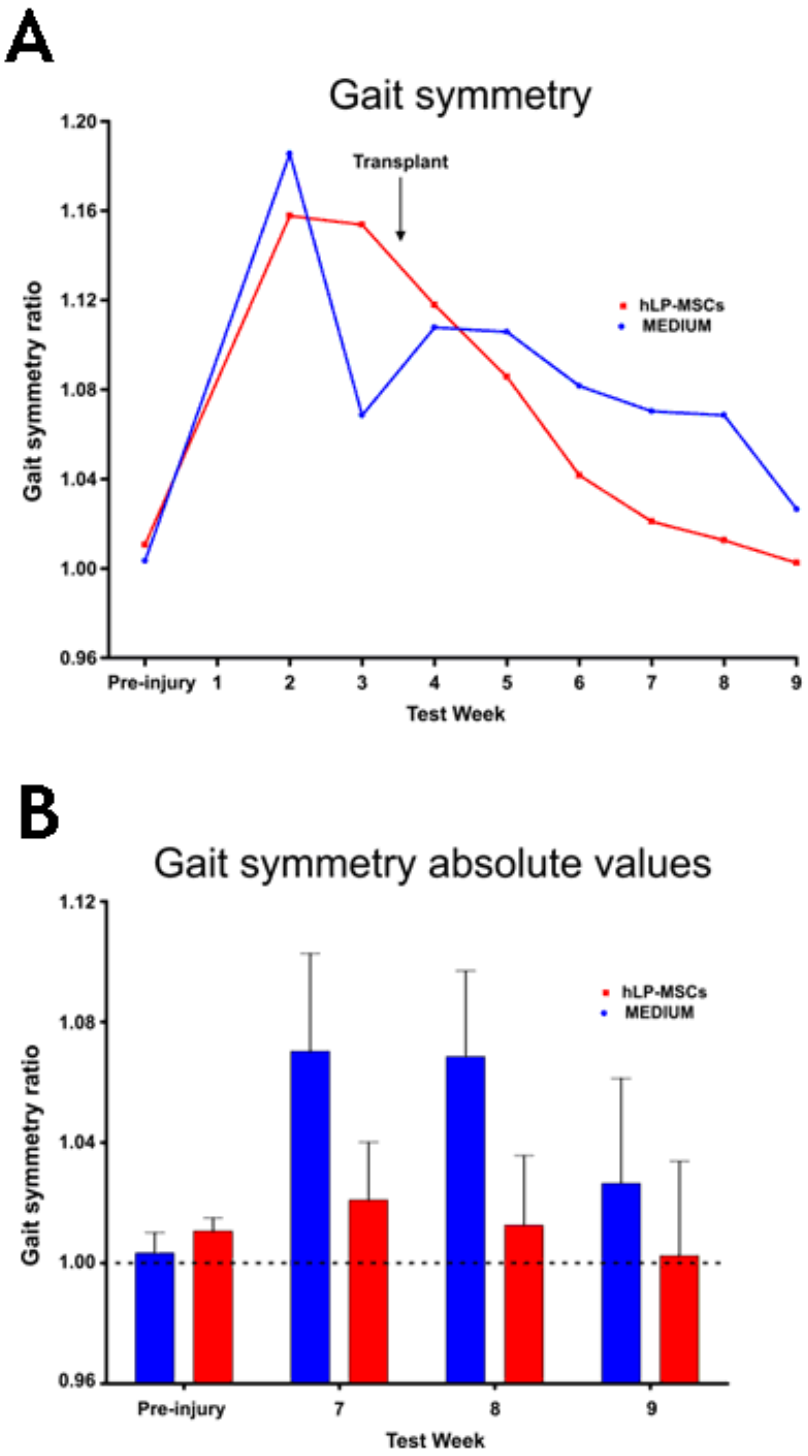


***Figure 3.8 Gait symmetry**. Digigait™ data revealed a trend for earlier recovery of gait symmetry in hLP-MSC transplanted animals compared to control animals. A) Gait symmetry ratio of FL-HL steps in hLP-MSC transplanted and medium injected groups over time. B) Gait symmetry absolute values at pre-injury and three of the most chronic post-injury time points studied (Error bars = SEM; hLP-MSC group - N=9 / Medium group - N=10. All = 2 way ANOVA, P>0.05).*

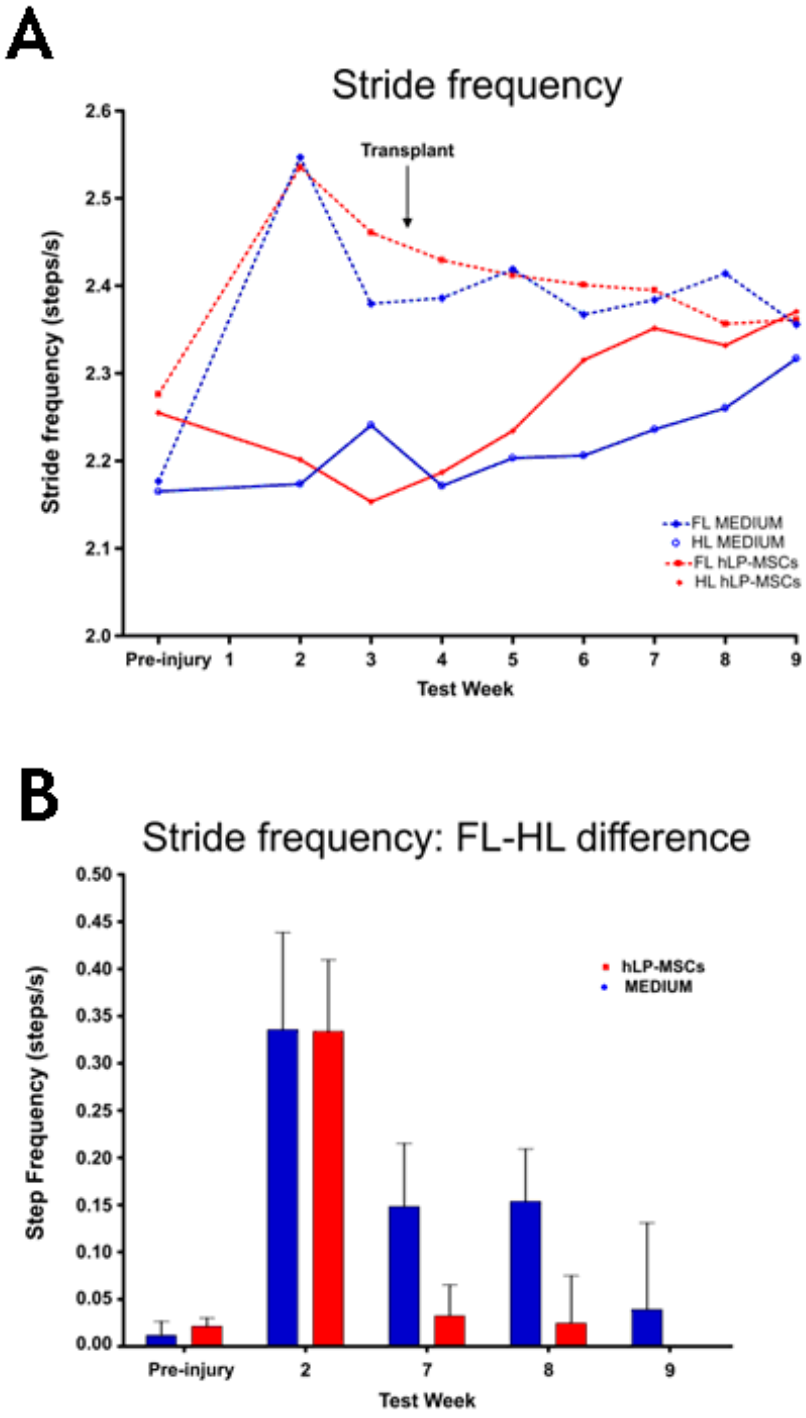


Figure 3.9 Stride Frequency. *Digigait™ data revealed a trend for earlier recovery of stride frequency in hLP-MSC transplanted animals compared to control animals. A) Stride frequency in hLP-MSC transplanted and medium injected groups over time. B) Differences in stride frequency absolute values at pre-injury and three of the most chronic post-injury time points studied (Error bars = SEM; hLP-MSC group - N=9 / Medium group - N=10. All = 2 way ANOVA, P>0.05).*

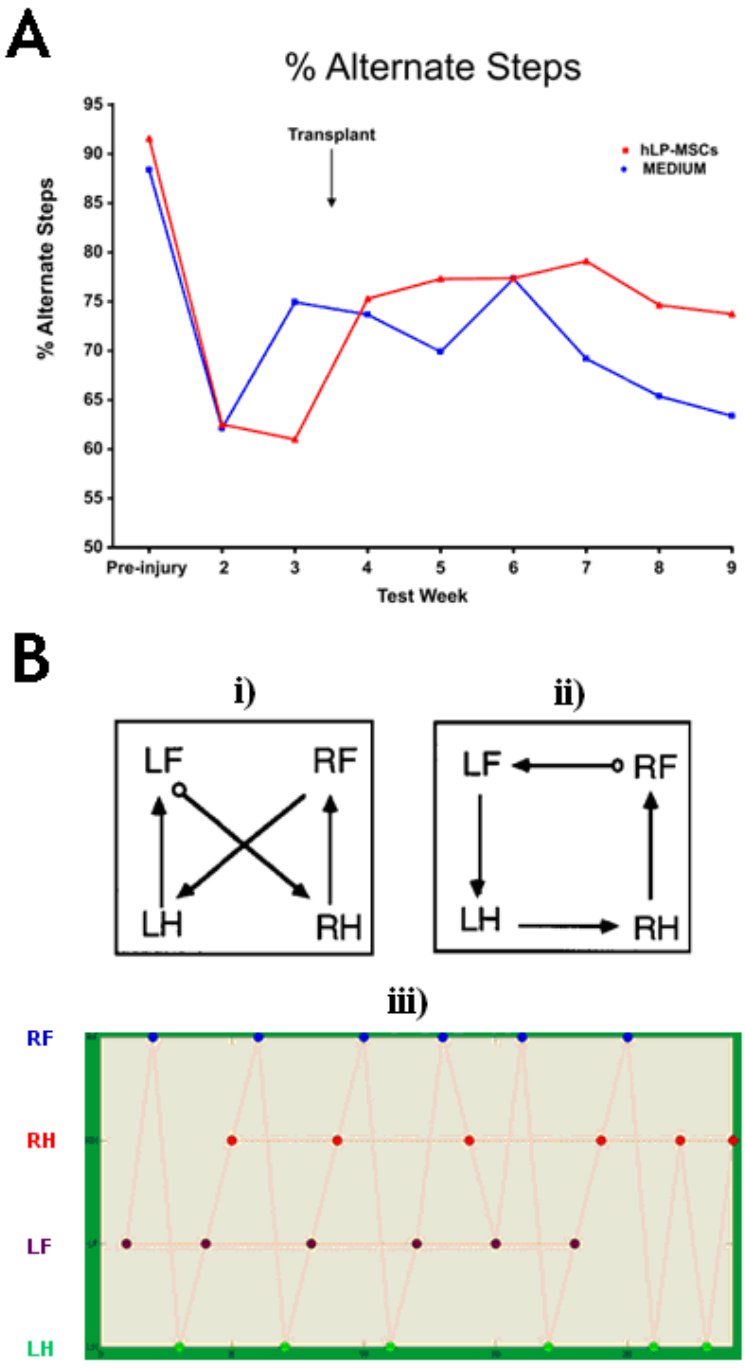


Figure 3.10 Alternate stepping. *Digigait™ revealed a trend for earlier recovery in hLP-MSC transplanted animals compared to control animals. A) % alternate stepping over time B) Digigait™ method of recording alternate steps, with i) Showing normal alternate stepping sequence, ii) showing dyscoordination and iii) Schematic diagram showing Digigait™ method of recording step sequence. (L = left, R = right, F = forelimb, H = Hindlimb). hLP-MSC group - N=9 / Medium group - N=10. All = 2 way ANOVA, P>0.05.*

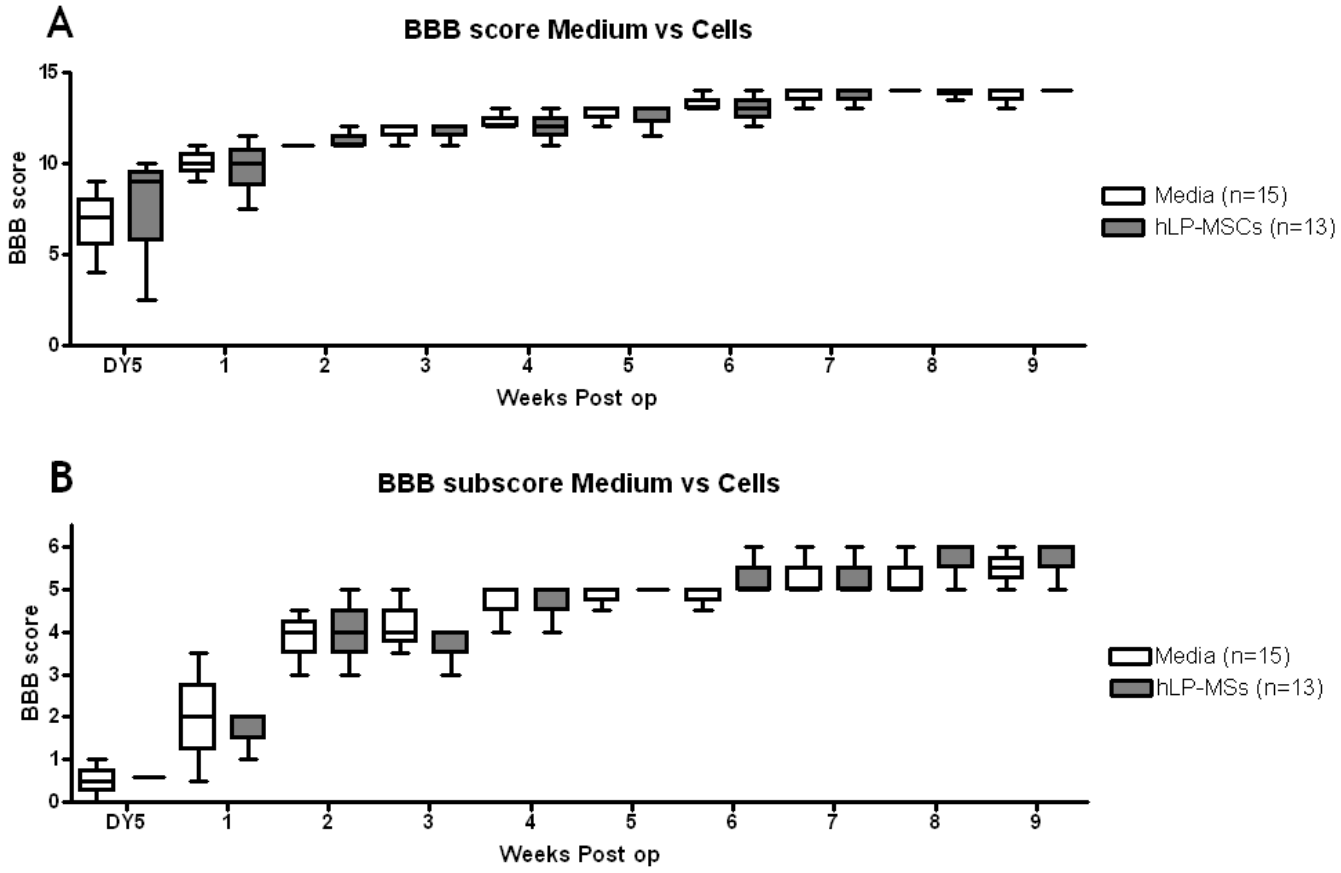


Figure 3.11 Assessment of locomotor abilities in the open field. *A) The BBB locomotor assessment scale and B) The BBB subscore were used to assess locomotor abilities in hLP-MSC transplanted and medium injected animals, at 5 days post-operatively and then once weekly for the duration of the study (both = 2 way ANOVA, P>0.05. Plotted as the median and interquartile range).*

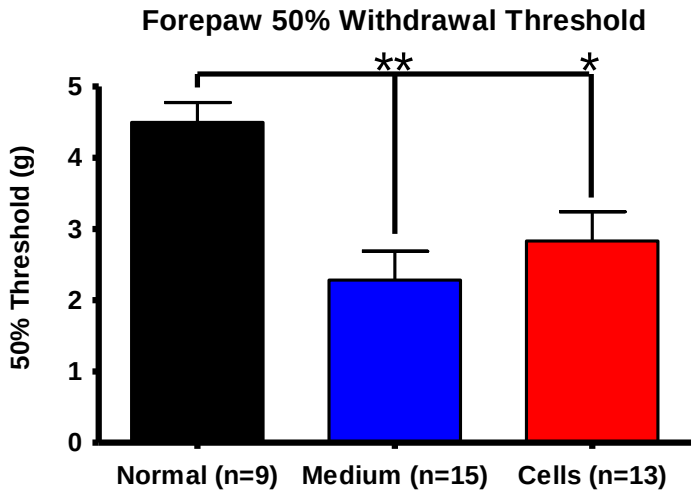


Figure 3.12 Forepaw 50% withdrawal thresholds. *Von Frey filaments were applied to the forepaws of normal, uninjured animals (n=9) and in groups of animals that received contusion injuries of 150 Kdyn force followed by 3 week delayed medium injections (n=15) or hLP-MSC transplants (n=13). In injured groups, testing was performed once, at 10 weeks following the injury (One way ANOVA, Tukey's post-hoc test. Error bars = SEM; *=p<0.05 / **=p<0.01).*

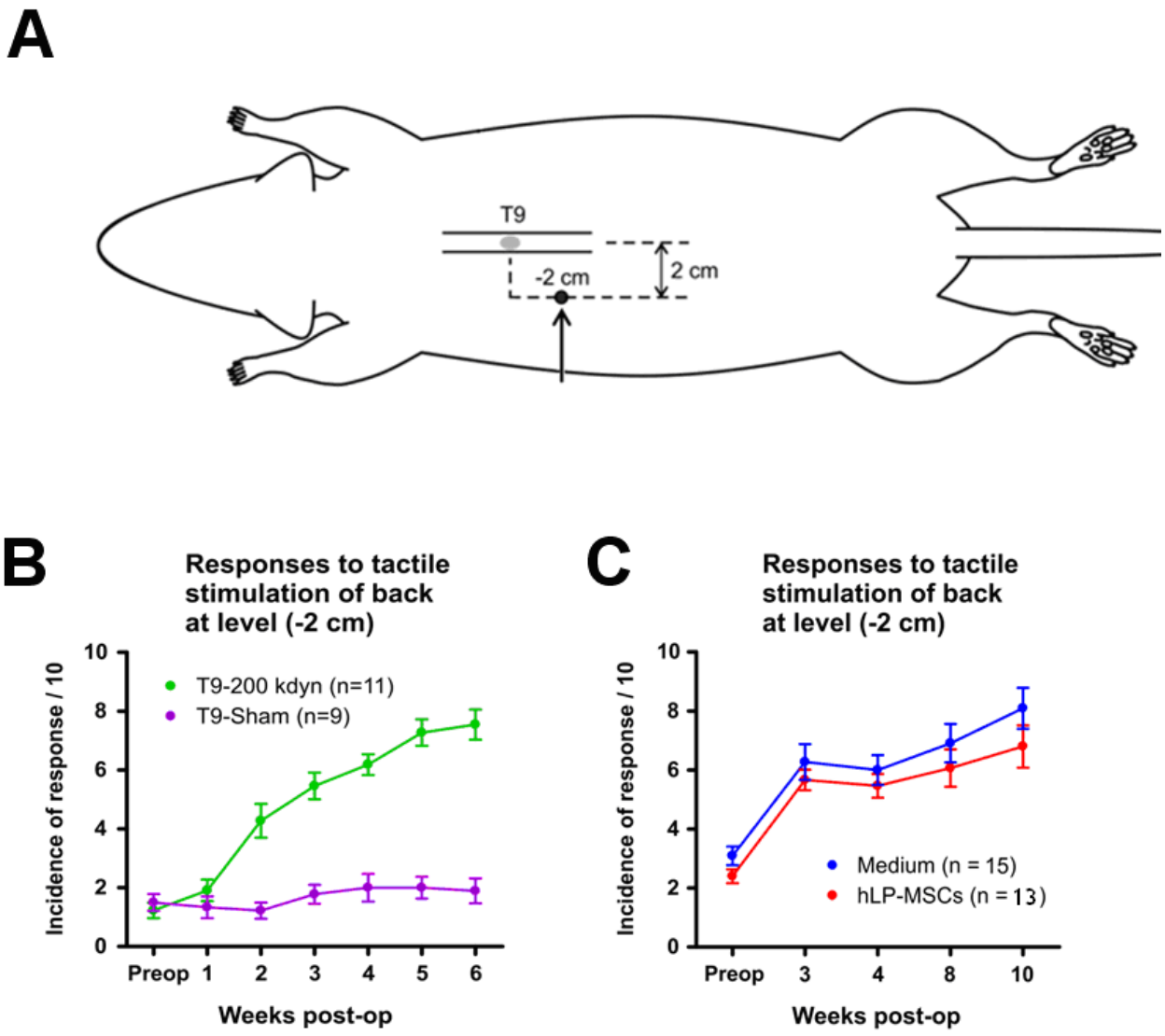


Figure 3.13 Pain sensitivity testing. *A) A von Frey hair (2.44 hair / 0.04g) was applied to skin 2cm caudal and 2cm lateral to the injury location to assess the development of tactile allodynia. B) Previous studies have shown that allodynia develops at this level by 2 weeks following a 200 Kdyn or 150 Kdyn contusion injury, C) Transplanted and control groups developed allodynia similarly by 3 weeks following 150 Kdyn contusion injury and sensitivity remained elevated for up to 10 weeks post injury (2 way ANOVA - P>0.05. Error bars = SEM. Figures A and B provided by Dr. Ahmed Emraja).*

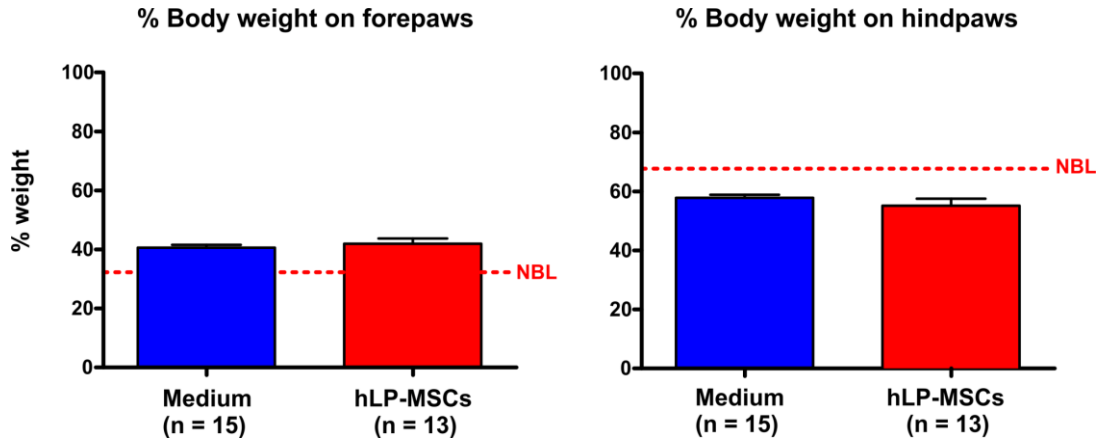


Figure 3.14 Dynamic weight bearing (DWB) tests. *The distribution of weight applied to forelimbs vs. hindlimbs was recorded using the DWB test in hLP-MSC transplanted animals and medium injected controls (NBL = Normal base line levels for uninjured animals; both = Student's T-test - P>0.05. Error bars = SEM).*

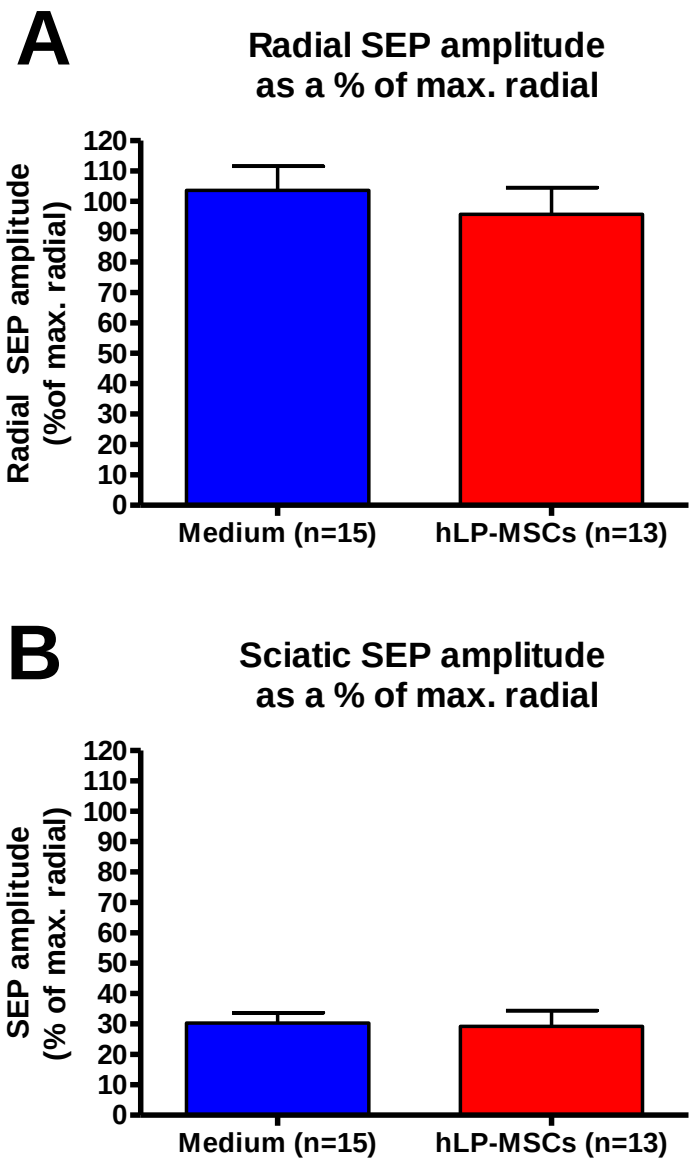


Figure 3.15 Maximum SEP amplitudes. *Maximum SEP amplitudes recorded in hLP-MSC transplanted and medium injected controls A) Maximum radial SEP amplitude expressed as a % of the maximum radial SEP amplitude recorded in each animal, B) Maximum sciatic SEP amplitude expressed as a % of the maximum radial SEP amplitude recorded in each animal, (Student's T-tests - all = P>0.05. Error bars = SEM).*

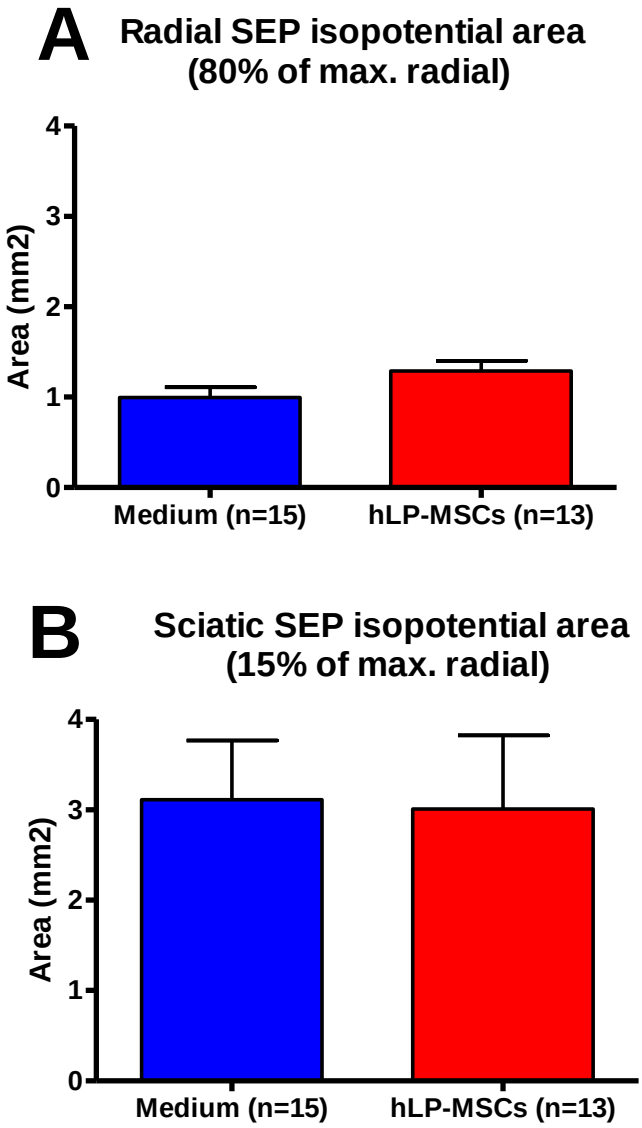


Figure 3.16 SEP isopotential areas*. SEP isopotential contour plots recorded in hLP-MSC transplanted and medium injected controls. A) Maximum 80% radial SEP isopotential area, B) Maximum 15% sciatic isopotential areacorded in each animal, (Student's T-tests – all = P>0.05. Error bars = SEM).*

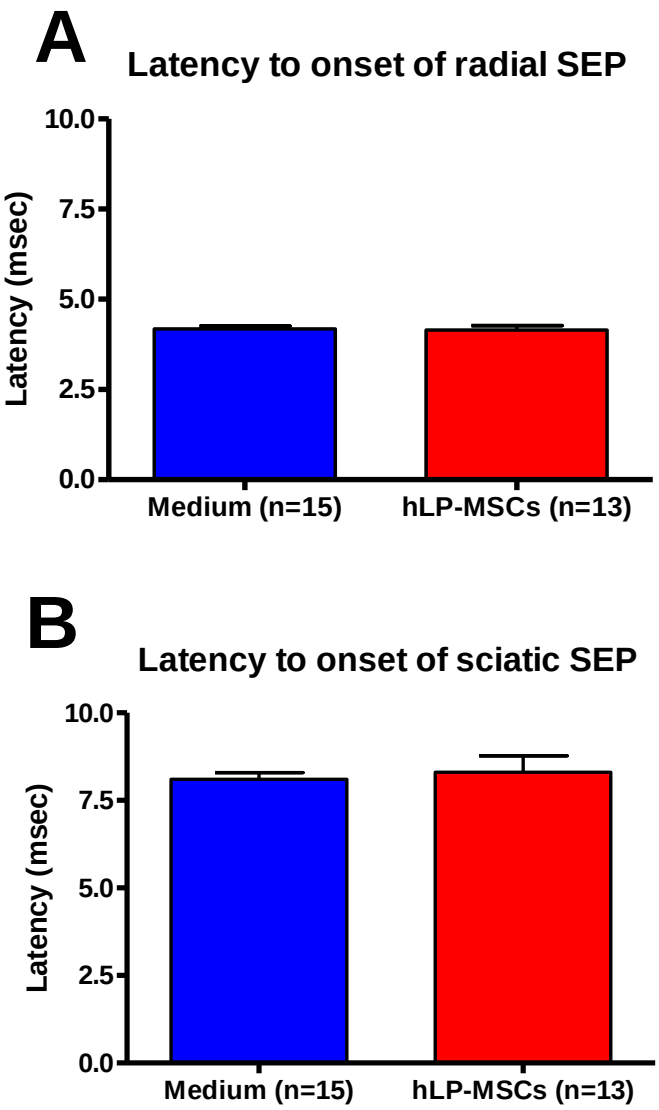


Figure 3.17 Latency to SEP onset*. Latency to SEP onset recorded in hLP-MSC transplanted and medium injected controls. A) Latency to radial SEP onset, B) Latency to sciatic SEP onset (Student's T-tests - all = P>0.05. Error bars = SEM).*

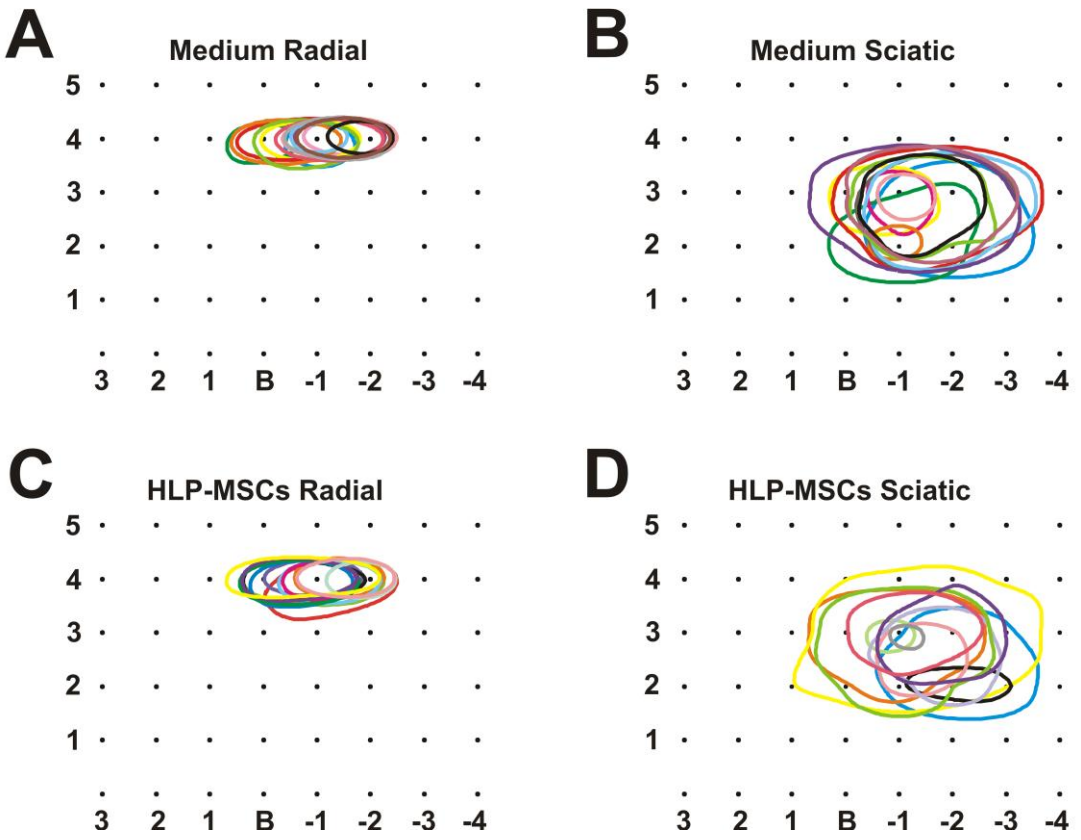


Figure 3.18 Individual SEP isopotential contour plots of medium injected and hLP-MSC transplanted animals. *Individual isopotential contour plots encompassing the areas of cortex within which radial and sciatic SEP amplitudes were at least 80% and 15% of the maximal radial SEP amplitudes recorded in each animal, respectively. A) Individual radial isopotential contour plots from medium injected animals (n=15), B) Individual sciatic isopotential contour plots from medium injected animals (n=15), C) Individual radial isopotential contour plots from hLP-MSC transplanted animals (n=14), D) Individual sciatic isopotential contour plots from hLP-MSC transplanted animals (n=14). All distances are in mm relative to Bregma (B).*

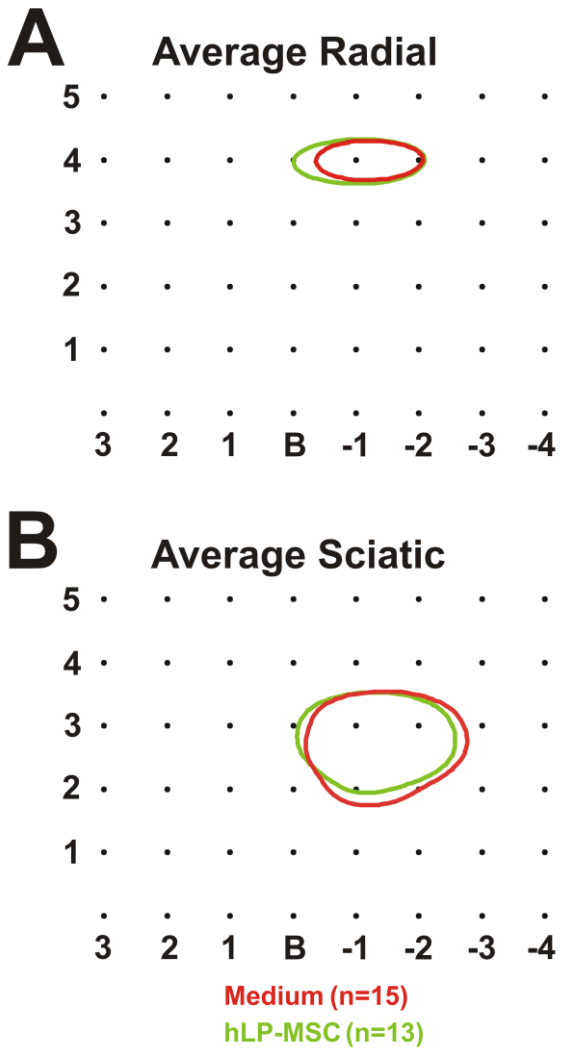


Figure 3.19 Averaged radial and sciatic SEP isopotential contour plots recorded in medium injected and hLP-MSC transplanted groups. *A) Isopotential contour plots encompassing the area of cortex within which radial SEP amplitudes were at least 80% of the averaged maximal radial SEP amplitude. B) Isopotential contour plots encompassing the region of cortex within which sciatic SEPs could be recorded with amplitudes at least 15% of the averaged maximal radial SEP amplitude. All distances are in mm relative to Bregma (B) Red = Medium injected group (n=15); Green = hLP-MSC transplanted group (n=14).*

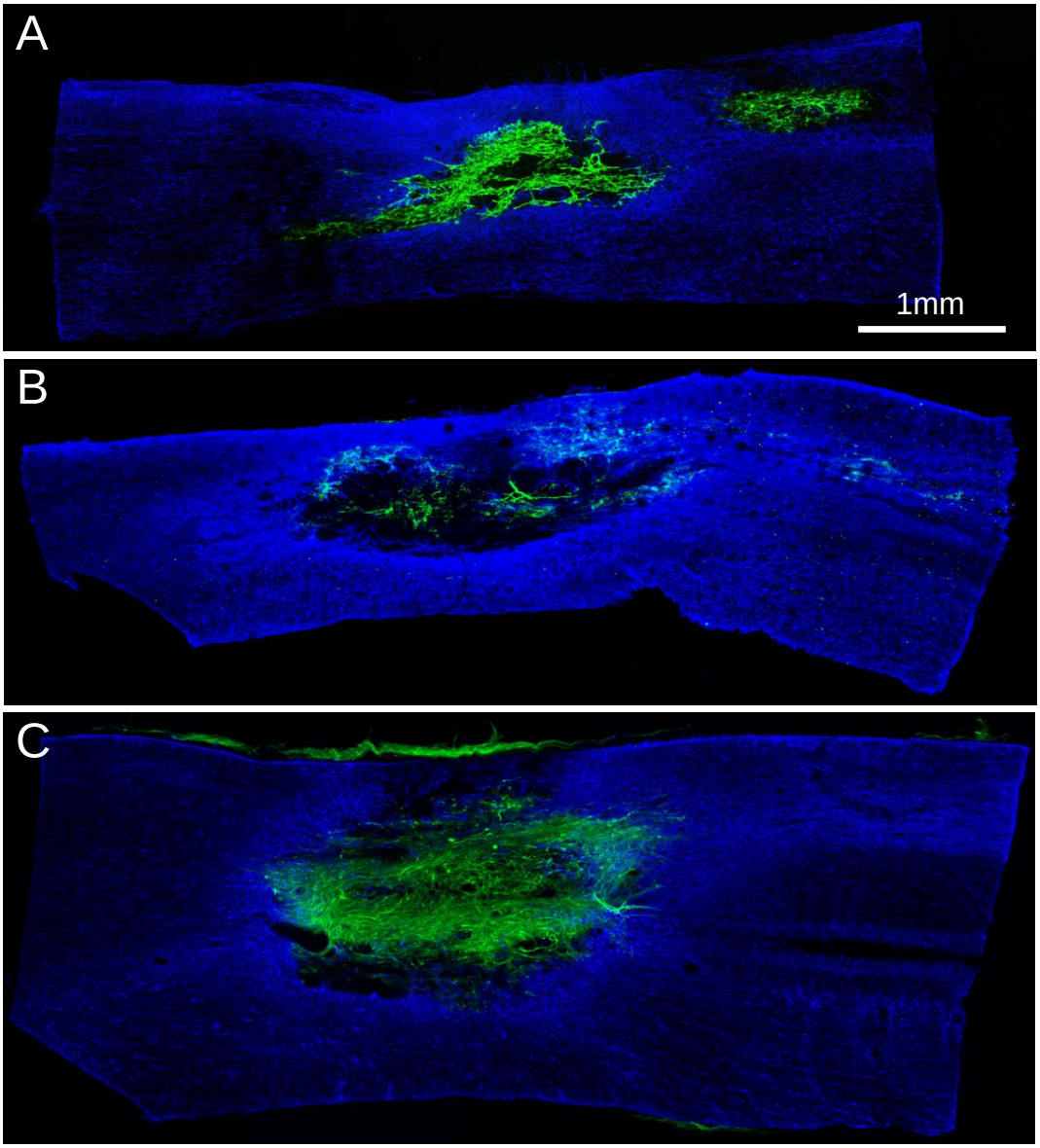


Figure 3.20 Cell survival following delayed transplantation into the contused spinal cord*. Confocal microscope images showing the survival of transplanted cells at and around the injury epicenter of spinal cords subjected to contusion injuries of 150 Kdyne force at T9. A) hLP-MSC transplants at 10 days post transplantation. B) hLP-MSC transplants at 6 weeks post transplantation. C) Syngeneic F344 rat OEC transplants at 4 weeks post transplantation into a cervical level contusion injury, for comparison. (GFAP = blue, GFP-transplanted cells = green (in A&B), olfactory ensheathing cells = green (in C)).*

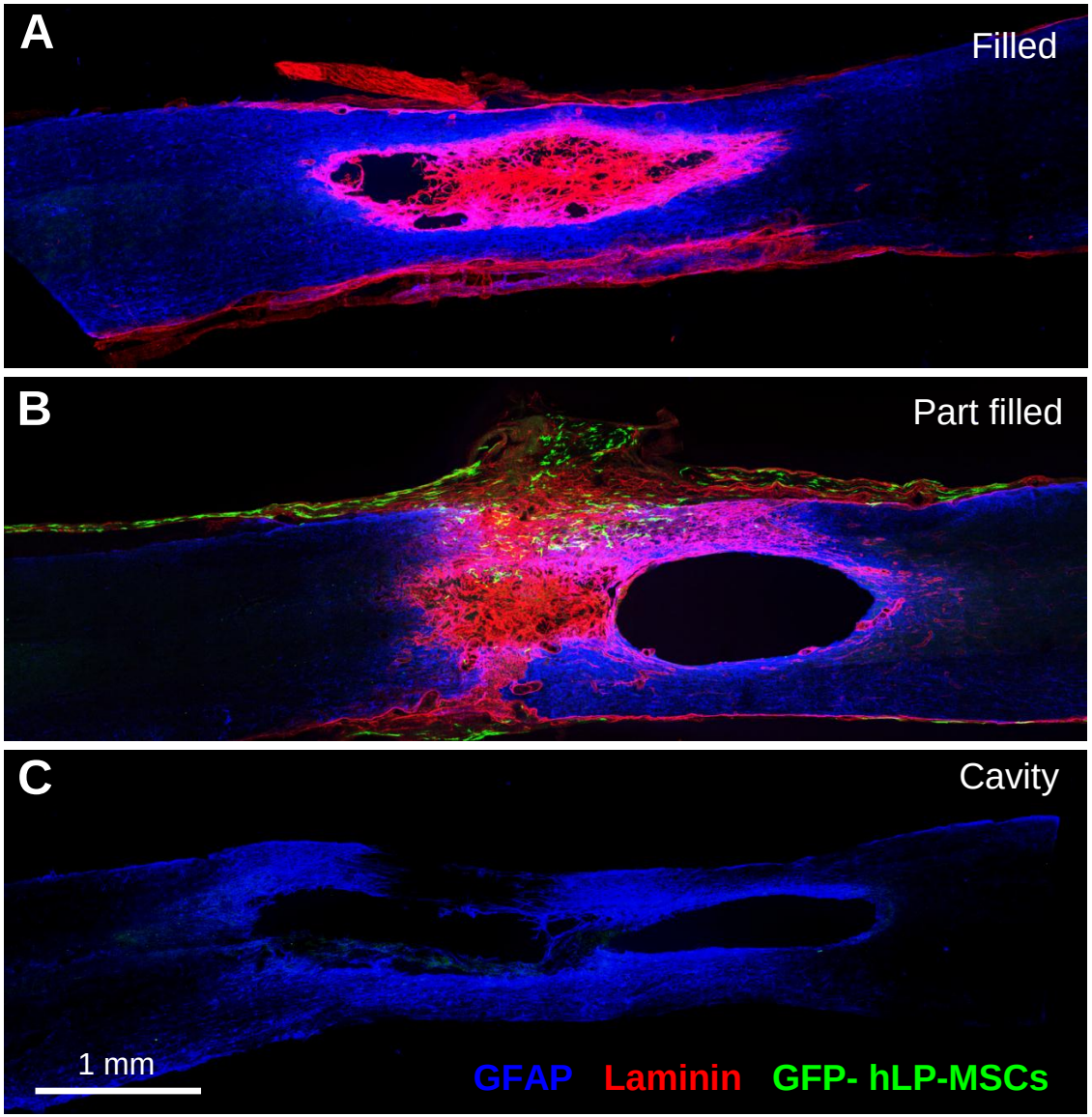


Figure 3.21 Assessment of injury cavity filling*. Confocal microscope images showing the different degrees of cavitation infilling at the injury site 10 weeks following injury and 7 weeks following hLP-MSC transplants or medium injections. A) An example of filled cavities in a control animal. B) An example of a partially filled cavity in a transplanted animal. C) An example of an empty cavities in a medium injected animal (GFAP = blue, Laminin = red, GFP-hLP-MSCs = green).*

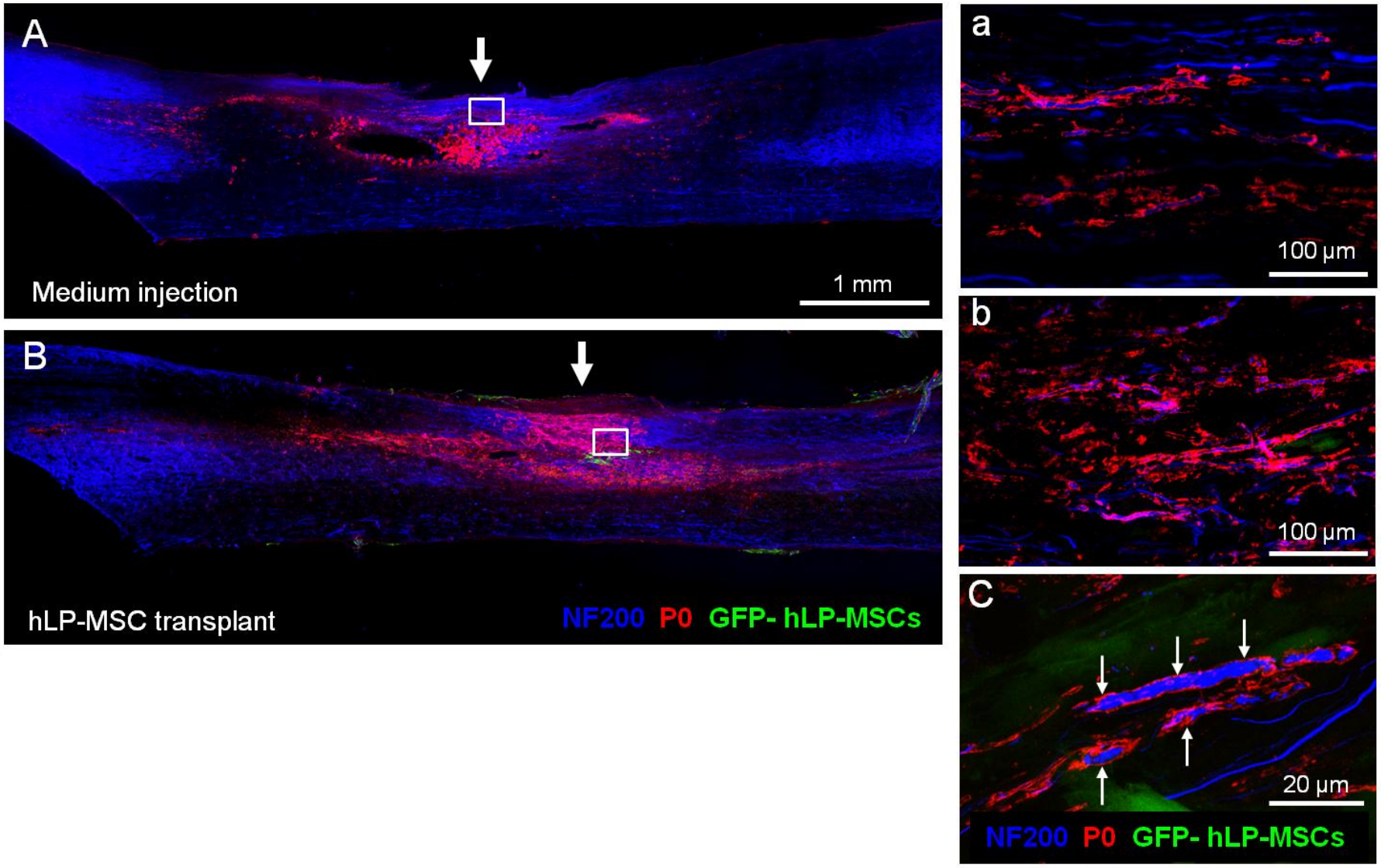

A
Medium injection
1 mm
B
hLP-MSC transplant
NF200 P0 GFP- hLP-MSCs
a
100 µm
b
100 µm
C
20 µm
NF200 P0 GFP- hLP-MSCs

Figure 3.22 Assessment of myelination and remyelination with peripheral type myelin. *P0 labelled cells in T9 150 Kdyn injury sites 10 weeks following injury and 7 weeks following media injection or hLP-MSC transplantation. A) Parasagittal sections from A) medium injected and B) hLP-MSC transplanted animals showing immunolabelling for P0, a component of peripheral type myelin. a and b show boxed areas in A and B at higher power. C) High power confocal image of P0 immunolabelling associated with neurofilament labelled axons.*

Table 3.1 Cavity Infilling in hLP-MSC transplanted and medium injected animals. *Incidence of matrix infilling of cavities at the injury sites in transplanted and medium injected animals, 7 weeks post transplantation/injection. hLP-MSC group - n=14 / Medium group - n=15.*

	hLP-MSCs	Medium
Filled	57% (8)	20% (3)
Part filled	36% (5)	66% (10)
Cavity	7% (1)	14% (2)

3.3.8 Discussion

The electrophysiological recordings reported here suggest some recovery of function with time after injury. hLP-MSC transplants appeared to have little or no effect on the functional integrity of the dorsal column pathway. BBB open field locomotor testing did not reveal any difference between the recoveries of cell transplanted and control groups. Gait analysis, performed using the Digigait™ imaging system revealing a trend for earlier recovery of co-ordination between forelimbs and hindlimbs in hLP-MSC transplanted animals compared to control animals. Moreover the step sequence data also suggested a better recovery of co-ordinated stepping in transplanted compared to medium injected animals. DWB testing was used to measure the percentage of body weights borne by each of the forepaws and hindpaws, demonstrating no effect of transplanted cells on postural changes. hLP-MSC transplants did not increase indicators of neuropathic pain suggesting they are unlikely to exacerbate neuropathic pain following spinal cord injury. Immunohistological analysis suggested that there was a wider distribution of P0 (mature peripheral-type myelin immunoreactivity) in transplanted animals and that this was specifically in areas where transplanted cells were located.

Potential reasons for absence of electrophysiologically detectable improvements in dorsal column transmission

Researchers using similar injuries have shown persistent demyelination (James et al., 2011; Totoiu and Keirstead, 2005) so it is reasonable to assume that the model used here should produce similar demyelination which offers a therapeutic target for improving function.

Since there should be a therapeutic target for the cells to act on i.e. to improve the completeness of myelination, several explanations exist which could explain the current findings. One possibility is that the cells do not survive in sufficient numbers to result in a robust enhancement of remyelination – although this argument is confounded by the fact that we have demonstrated the cells surviving

for up to 6 weeks post transplantation, and arguably this is when they should be having an effect on the ongoing process of endogenous myelination. When interpreting this observation we must remain cognisant of the fact that cell transplants designed to promote processes such as repair of myelination need not be an intrinsic part of the structure of repair, and need not necessarily form the myelin sheath themselves; it should be possible and might be desirable for the transplanted cells to die once their job is done. Other explanations include the possibility that the cells are not distributed optimally for secreted factors to reach OPCs close to demyelinated dorsal column fibres, or that the cells simply do not do *in vivo* what they do in an *in vitro* system.

It also remains a possibility that the electrophysiological assessment is not sensitive enough to detect improved remyelination and/or functionally superior myelination, for example, with better internodal distance and nodal structure and distribution of ion channels. Also, there remains a possibility that endogenous remyelination is as complete as possible, and that there remains other reasons which could explain why demyelination is persistent and the factors produced by the transplanted cells cannot overcome these.

Enhanced recovery speeds shown by the longitudinal behavioural tests may reflect an increased speed of remyelination encouraged by the cells but that this benefit does not lead to a long term advantage because in the absence of cells, endogenous mechanisms eventually lead to the same degree of myelination, this raises the possibility that in injuries where there is more persistent demyelination that the factors produced by the hLP-MSCs may provide a more significant advantage.

As discussed earlier, without the use of electron microscopy to assess the G-ratios of myelinated axons, we are unable to assess the degree of remyelination and as such are reliant on IHC techniques. With no specific marker for central remyelination, we are only able to assess the degree of peripheral type myelination. Whilst this technique cannot measure remyelination by oligodendrocytes, it does provide some indication of the injured cords ability to support remyelination of demyelinated axons.

Mechanisms underlying promotion of improved gait

It has been shown that locomotion and gait are controlled primarily by pathways in deeper or more ventral parts of the spinal cord (Raineteau et al., 2002).
As a result, studies in which the effect of lesions in different areas of the spinal cord have been investigated suggest that a large proportion of the dorsal white matter, including the dorsal columns, can be damaged before any obvious changes in locomotion can be detected. Thus, the electrophysiology of the dorsal columns and the gait analysis are therefore complementary measures of functional outcome which assess different pathways in the spinal cord. Gait analysis showed that whereas transplants have no obvious effect on dorsal column transmission, the recovery of co-ordinated stepping showed a trend for more rapid and more complete in transplanted animals compared to non-transplanted controls. Compensatory mechanisms may account for the discrepancy between functional improvements recorded by electrophysiological testing and behavioural testing. For example, here we report that reinstating co-ordination of forelimb and hindlimb stride frequency is achieved by adaptations of both the forelimbs and hindlimbs to a new common stride frequency rather than by normalisation of the stride frequency to that before the injury; thus it can be seen that animals employ compensatory mechanisms which, combined with limited functional recovery act to return stride frequency towards normal levels. It remains to be determined whether the mechanisms by which gait has been shown to be improved in this study during treadmill based locomotion would be the same in overland locomotion where animals have the possibility of reducing the speed of locomotion. A recent investigation into the effects of intraspinal OEC transplantation on gait and long tract function in spinal-cord-injured companion dogs showed similar improvements in forelimb-hindlimb coordination with no improvement in long tract function measured via a non-invasive transcranial magnetic SEP and MEP stimulation and recording protocols (Granger et al., 2012). However in this study the scores for individual forelimb and hindlimb scores were not given, so it is unknown whether or not similar compensatory adaptions to gait were made.

Another potential mechanism by which cell transplants might improve functional outcome is via a neuroprotective effect. However, this is unlikely to be involved in the functional recovery in gait seen in this study as the transplants were made three weeks after the injury, by which time point we consider it to be a chronic injury as previous studies have shown that the function of the dorsal column system has stabilized by this time point. Plus, since it is unlikely that an autologous transplant strategy would allow for transplants earlier than a few weeks after injury, a neuroprotective mechanism is unlikely to be relevant in this scenario.

Whilst there is no direct evidence that cell transplants promote plasticity, it remains a possibility. Because the study design was optimised to assess long tract function it provides less information about mechanisms such as plasticity but plasticity at the low thoracic level (the level of the transplant) is unlikely to significantly affect gait co-ordination and promotion of plasticity remote from the transplant site.

P0 immunolabelling

Since P0 is a marker for peripheral type myelin, its presence in the injury cavity shows that there is Schwann cell myelination occurring. There were some indications that P0 immunolabelling might be greater in transplanted animals. For one of the transplanted animals this was seen to be concentrated in areas where transplanted cells were located, and this appeared to be located deeper within the cord than was typical for non-transplanted animals. James et al., 2011 did not report such ventral P0 labeling, but rather reported P0 labeled cells present more dorsally in the dorsal columns after 4 wks post-injury; thus suggesting that the more ventrally located labeling reported here could be related to the transplanted cells.

One explanation for the peripheral type myelin seen here is that Schwann cells are attracted to the injury site and are encouraged to migrate more ventrally. Another viable explanation is that the factors produced by transplanted hLP-MSCs are capable of inducing OPCs to differentiate into Schwann cells (Zawadzka et al., 2010) which then form peripheral type myelin in the injury site.

Further work is required to validate these findings but if they can be confirmed then more efficient remyelination of more ventral locomotor tracts may explain the trend for more rapid recovery of coordinated locomotion seen in the transplanted animals in this study.

Axonal ingrowth

Ingrowth of neurofilament immunolabelled axons was seen within the transplanted injury sites and also within the matrix filling the injury site of non-transplanted animals. Although the extent of this axonal ingrowth was not quantified, the density of axons appeared greater in the transplanted animals. Axons could be seen at the centre of what was clearly an infilled injury cavity which was devoid of any spared tissue. Because of this and the tortuous morphology of these axons there is no doubt that these represent regenerating rather than spared fibres (Steward et al., 2003). As we had no reason to suspect these axons to show bridging axonal regeneration no tract tracing was carried out in these experiments. Since these axons form blind endings within the transplant or matrix infill, the regeneration is likely to be of no functional importance. It is possible that the regeneration that occurs in the axon within the injury site stimulates sprouting of additional axon collaterals from more distal portions of the fibres. This might conceivably lead to improved function locally but there is no evidence for this at present and it would require a different electrophysiological approach in which local circuit activity could be recorded from around the injury area. Investigating cord dorsum potentials provides an excellent way of achieving this (Toft et al., 2007).

Reasons for limited cell survival

Although transplanted cells could be detected in all of the transplanted animals, their numbers were relatively low and less than in previous cell transplantation studies we have carried out in our laboratory using, for example, syngeneic animal cells.

The immune response immediately following injury is one of the largest barriers to transplant survival, though by simply delaying transplantation until the immune response begins to subside, as in this study, researchers are able to increase the likelihood that cells will survive after transplantation. Xenotransplants invariably require hosts to be immunosupressed in order to ensure cell survival.

As well as delaying transplants until the immunological response had started to subside, we also provided immunosuppression with daily injections of cyclosporin beginning 24hrs prior to transplantation and maintained daily throughout the experimental period. Despite this, it remains possible that the immunosuppressive regime may be inadequate to ensure long term transplant survival and that the immunological response which occurred prior to immunosuppression has effects which last beyond the commencement of immunosuppression. Whilst these factors are likely involved in contributing to the loss of transplanted cells, numerous other factors will also play a significant role in the loss of cells, including a lack of trophic support and their introduction to an environment with extreme electrochemical imbalances as a result of the secondary injury sequale.

The gross structure of the injured cord might also play a role in limiting cell survival. For example, this injury model produces large cavities which transplanted cells would find it hard to survive in the centre of, due to the lack of metabolic support and the ongoing effects of the secondary injury. Additionally, our histology showed that in some animals the cavities were septated and that while one cavity showed evidence of transplanted cells the adjacent cavity did not. This is presumably because the injection, which was made at the injury epicentre filled one cavity but that the septae prevented cells spreading throughout the injury site. This could be improved in future by making multiple injections.

Immunosuppression – problem of testing autologous transplant strategy with human cells in animals

A problem that occurs when studying human cells in animal models is that it is necessary to suppress the immune system using immunosuppressive drugs or to conduct experiments in animals in which the immune system is compromised by a

genetic mutation (nude animals). In studies where it is envisaged that grafts would in any case be allogeneic, this is less of a problem because it can be argued that immunosuppression would have to be a part of the therapy. In this case, however, it is envisaged that the grafts would come from the patient themselves i.e. the patient would act as their own donor, and that this would avoid the need for immunosuppression. This is seen as the big advantage of using cells from this source. However, this means that immunosuppression in the animal model is not a realistic feature but is a necessary condition. Although nude rats might be an alternative to an immunosuppressive treatment and could offer an advantage in terms of promoting better graft survival, they have the disadvantage that the injury occurs in an animal lacking an immune response. Giving immunosuppression a few days before the transplant has the advantage over nude rats that the injury at least develops with the usual immunoreactive response. In nude rats it is possible that the injury environment is not a good model of the clinical situation since the immune response to traumatic injury is considered an important component of the response to injury and the secondary sequallae, without which the nude rats will respond to the injury in a significantly different manner to that seen with functional immunological systems, ie. clinically.

Preclinical and clinical trials

This investigation was designed to assess the beneficial potential of hLP-MSCs with a view to one day developing an autologous transplantation strategy. Whilst this is the first time that hLP-MSC have been assessed *in vivo* for the treatment of SCI, many other pre-clinical investigations have used MSCs from different sources and used different purification protocols, routes and timings of transplantation; whilst these studies have resulted in vastly different outcomes, all have demonstrated that MSCs are safe to transplant into the CNS, and the majority have demonstrated that transplanting MSCs into the injured cord has a positive effect on recovery (reviewed by Forostyak et al., 2013). Despite the variability in protocols and efficacies seen in pre-clinical studies, many labs have already entered into clinical trials, with to date several hindered people having been

made the recipients of MSCs for the treatment of SCI (reviewed by Kwon et al., 2013). Whilst it is clear that the different protocols and cell sources investigated are capable of eliciting different effects on the sequalea that follows SCI – this, combined with the findings reported in this study raise questions as to why clinical trials are already underway before any firm consensus has been reached on the optimal protocols for extraction, purification, culturing and transplantation of MSCs.

General discussion and future directions

Electrophysiological approaches for investigating function after spinal cord injury

How it has been used and what it has shown in this project

In this project we have demonstrated that SEP recording can provide an indicator of the severity of injury to the dorsal columns. We have then used this approach to provide the first accurate and complete information on the time course of changes in function of white matter pathways of the spinal cord after a traumatic injury.

Electrophysiological testing is the only way of providing direct information on what is happening to local circuits and pathways in the spinal cord after injury and treatments. This project has demonstrated that this approach is possible for recordings of long white matter pathways.

Advantages over other electrophysiological approaches/previous studies

This *in vivo* and invasive approach under terminal anaesthesia is the first time this protocol has been used to provide an accurate picture of changes occurring directly within the spinal cord at the level of long tract function. Other approaches have been used in an attempt to obtain this important information. However, single fibre sampling has a more limited resolution as only a subset of the injured fibres can be assessed and as such may not allow investigation of severe injuries or at time points soon after injury. Whilst investigation of retrograde compound action potentials is capable of providing detailed information about the integrity of the tracts and individual fibres investigated, this approach remains limited in resolution as it is not capable of selectively measuring the function in the whole tract. Unfortunately, non-invasive methods of recording SEPs appear to have not yet reached a sufficient point of refinement to be able to provide an alternative means of obtaining this information; again there appears to be a lack sensitivity in the early stages after injury and they are also subject to a great deal of variability.

General disadvantages / limitations of an electrophysiological approach

Although there are some clear and valuable advantages in using an electrophysiological approach to obtain information on the function of pathways and circuits directly at the spinal cord level, there are a number of difficulties and limitations to this approach.

Anatomical restrictions mean that only certain pathways can be studied because only some are conveniently arranged such that they can be maximally activated in a selective way to allow for quantitative comparisons between animals and treatment groups. Similarly, only certain pathways are accessible and convenient for recording from in a manner that allows for recording and assessing total or massed activity in the pathway i.e. summed activity – this is usually in the form of surface potentials that can be recorded in a consistent manner. In practice this means the dorsal column pathway and corticospinal pathway.

Electrophysiological tests are unable to differentiate positive and negative changes in an animal's condition. Changes in function can be good or bad, for example, an increase in the strength of a pathway might be due to regeneration or plasticity and the new connections might be functionally useful and provide an improved functional outcome or they may be inappropriate and of no functional benefit. It is even possible that they may be maladaptive and lead to problems with pain or spasticity, or autonomic problems. As a result it is desirable to perform behavioural testing along-side electrophysiology, though as behaviour depends on integrated functions and is rarely critically affected by a single pathway, there is no behaviour that directly depends on the function of one single pathway. Correlating electrophysiological changes in function with behaviour is less of an issue if the underlying mechanisms for change are neuroprotective mechanisms or mechanisms such as remyelination which should not alter connectivity but preserve more of the previous fibres and their existing connections.

Stem cell therapies

Stem cell therapies for the treatment of SCI have received a great deal of focus due to the numerous reports that they can positively influence recovery following SCI; including the reported involvement of various cell lines in the regulation of trophic factors and cytokines *in vivo*, the ability to positively influence neighbouring cells via paracrine secretions, extracellular matrix molecules and the secretion of neurotrophic factors as well as their ability to transdifferentiate into cell types which are known to play important roles in recovery following SCI.

Recently a great deal of focus has been placed in MSCs of various origins for the treatment of SCI. Most focus has been placed on MSCs of bone marrow origin, whilst the reported effects of MSCs have been varied many authors have reported improvements in functional recovery recorded both electrophysiologically and behaviourally. Recent *in vitro* studies have shown that MSCs isolated from the human nasal lamina propria have greater myelinating abilities than BMSCs, though as reported here, hLP-MSCs did not produce the same level of functional recovery *in vivo* as has been reported with BMSCs. Thus whilst here we report modest trends suggesting improved functional outcomes with hLP-MSCs, the differences between the *in vitro* and *in vivo* preparations appear to be enough to curb the hLP-MSCs beneficial effects on functional recovery to such an extent that are exceeded *in vivo* by those reported following MSC of alternate origin, including BMSCs.

Despite the variability in the reported pre-clinical efficacies of stem cells at ameliorating the effects of SCI, several clinical trials are currently underway. As there is currently no consensus on the optimal source, isolation, purification or culturing or transplantation protocols used in pre-clinical trials, it would seem that clinical trials may have been started prematurely. In order to maximise the efficacy of clinical studies, it would seem logical to wait until consensus is reached in the aforementioned protocols in pre-clinical studies. These same problems also exist in the development of stem cell therapies for other CNS diseases; this is perhaps a

product of the extremely high potential seen for such therapies combined with the massive incentive to prevent further damage and ameliorate functional deficits.

Future directions

Extending the approach to obtain information on local circuit (grey matter) function

The current work demonstrates the usefulness of an invasive *in vivo* electrophysiological approach for obtaining information on the function of white mater tracts after traumatic injury. A similar approach would be valuable in providing information about local circuit function. Local circuit functioning can be investigated most simply and straight forwardly using cord dorsum potential recording. This approach has been used to investigate the effect of OEC transplants on CDPs evoked by sensory afferents in the dorsal roots in a wire knife injury model (Toft et al., 2007). It provided evidence that OEC transplants have a neuroprotective effect if transplanted acutely. However, so far there has been no investigation of how contusion injuries affect local circuits in the grey matter around the injury or how function in the grey matter changes with time after injury. This information would be potentially valuable in indicating how much damage occurs at the initial injury and how much additional damage occurs as a result of secondary injury mechanisms. By investigating the time course of changes in function after the injury, the time over which the secondary mechanisms continue to cause deterioration in local circuit function could be determined. This approach could also indicate whether there is any recovery in this function and over what period this occurs and when a stable situation indicative of the chronic stage of the injury is reached. Such information would be valuable in indicating how much potential there is for reducing the impact of traumatic spinal cord injury by using neuroprotective strategies. It would also indicate the time window within which such treatments would need to be administered to be effective. This information does not exist either for animal models or in humans despite its obvious importance in the development of neuroprotective strategies for treating spinal cord injury. Investigation of other treatments based on other strategies would also benefit from knowledge of what represents a chronic time point in an animal model since it is necessary to know before considering transplantation whether a treatment is likely

to be of benefit to newly injured (acute injury) patients or could benefit existing patients with long term (chronic) injuries.

Potential for developing a chronic recording approach in the future

The present study obtained valuable information on the time course of changes in function of long white matter pathways over time. However, to obtain this information, large numbers of animals had to be used in acute experiments conducted at different time points. This has a cost implication in terms of the expense of the experiments and is very time consuming to perform. It would also be desirable to minimise the numbers of animals used in future studies. Although, chronic recording approaches adopted so far do not seem to have the appropriate sensitivity of accuracy it is possible that they could be improved and used to obtain longitudinal information from the same animal over a period of time after the injury. For example it may be possible to use the information generated in this study on the location of maximal SEPs to guide the best placement of electrodes for chronic implantation. These locations were fairly consistent and injury did not significantly shift the distribution of these maximal recording locations. Similarly, it may be possible to implant cuff electrodes around peripheral nerve such as the sciatic nerve or forelimbs nerves in order to provide better control over stimulation parameters.

Thus chronic recording of SEPs, if carefully approached – for example using appropriate types of electrodes appropriately sited so as to have good control over stimulation of the nerve, might offer an adequate way of assessing dorsal column function and might provide a means of following changes due to treatments in a longitudinal fashion.

As discussed above, one of the disadvantages of using SEPs as a way of monitoring function in long white matter tracts is that it is a pathway which involves relays. Although these relays are probably an advantage in terms of increasing the

sensitivity of the approach, they have the disadvantage that they complicate interpretation of latency changes in the pathway. Recordings directly from the dorsal column fibres represent an advantage in this respect. However, this may be difficult to achieve as a recovery procedure as stimulation of the dorsal columns strong enough to activate the majority of primary afferents usually causes muscle contractions and temporary paralysis and artificial ventilation would therefore probably be required. Preliminary experiments in our lab have suggested that the alternative possibility of recording directly at the site of termination of the ascending dorsal column fibres in the dorsal column nuclei is not very promising because the resulting potentials that can be recorded from the surface of the brain stem are relatively small but also rather spread out and complex in form (i.e. consisting of several peaks). This would make quantification difficult although the earliest latency response could probably be determined. Also the cerebellum has to be retracted slightly to provide adequate access for these recordings.

Cell transplantation and the future of therapies for spinal cord injury

The most definitive answer to whether myelination is enhanced in transplanted animals would be to investigate this by using electron microscopy and quantifying the proportions of axons that are demyelinated, normally myelinated and remyelinated with peripheral versus central type myelin. Given that the survival of cells shown in this study was poor in comparison to when transplants have been made into syngeneic animals, experiments using different immunosuppressive regime would have to be considered. Another alternative would be to use animal cells, which have yet to be investigated. This would remove the need for immunosuppression since the study could be carried out in an inbred rat strain using syngeneic cells. The survival of the cells in this model should be good and the immune response would not be compromised in any way. The down side of this approach is that there may be differences between animal cells and human cells. Another approach could be to use nude or athymic rats system. The *in vitro* evidence for enhanced myelination was obtained using conditioned media obtained from cultures of human cells but tested on neural cells from animals grown in

culture. Whilst there are some immune cells present in the culture but this clearly does not represent a normal immune system. It is therefore not clear whether immune responses are necessary or contribute to the mechanism of enhanced myelination.

A further possible approach would be to investigate the factor or factors that is produced by the LP-MSCs and is within the conditioned media secreted by them and added to the myelinating cultures. If the important factor(s) could be identified then it might be possible to deliver these in some way to the injury site. This might be possible by engineering cells to over-secrete the factors and therefore produce them in a higher concentration with the potential for greater effectiveness. It might also be possible having identified the factor(s) that are important to isolate these and to deliver them to the injury site using an intrathecal cannula with its tip placed close to the injury and an implanted osmotic pump. This could be used to deliver the factors over a prolonged period. However, the factors would need to be stable at body temperature and they would need to penetrate the spinal cord tissue readily. Intravenous delivery might be an alternative but would be less targeted and may not reach the spinal cord as readily as direct delivery, although disruption of the blood brain barrier as a result of the injury may facilitate this.

This thesis provides valuable insights into long tract function in the dorsal columns following moderate contusion injury at T9, as well as providing a well characterised model on which to test therapeutic interventions. Using this model we have investigated the effects of hLP-MSC transplants on function in long tracts and have provided promising directions for future investigations.

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