Investigating local circuit function following spinal cord injury and cell transplant therapy

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Institute of Neuroscience and Psychology

College of Medical, Veterinary and Life Sciences

University of Glasgow

By

Dr Syed Hamid Habib

MBBS, PGD (EBM & E)

March 2015

Summary

Spinal cord injury (SCI) leads to severe functional deficits and currently there are no effective treatments. In addition to the initial mechanical damage to axons, neurons and glia, traumatic injury also triggers a complex array of cellular and molecular events. Some of these processes are thought to contribute to mechanisms of progressive damage (secondary mechanisms) whilst others concurrently promote limited repair. Functional outcome depends on the interaction of these two processes. However, the interacting dynamics and time course with which they impact the functional outcome is not fully understood. Secondary processes are studied with molecular and anatomical approaches and loss of axons or neurons are used to infer reduced function. Similarly, recovery of functional outcome is mostly assessed using behavioural approaches. However, these are unable to differentiate recovery due to compensatory plastic changes in the brain which might contribute to recovery. Therefore, the first aim of the thesis was to develop an electrophysiological approach to directly assess functional changes in spinal cord circuits following a contusion SCI. The second aim was to use this protocol combined with behavioural testing in order to investigate the use of human embryonic stem cell derived mesenchymal stem cells (hESC-MSCs) as a potential therapy for spinal cord injury.

An electrophysiological approach was used to assess spinal cord function after contusion injuries at the cervical (C6) level of the rat spinal cord. Contusion injuries due to high velocity impact were made using an Infinite Horizon Impactor device. Cord dorsum potentials (CDPs) evoked by supramaximal electrical stimulation of a radial nerve (to activate sensory fibres) or within the pyramids (to activate corticospinal fibres) were used to measure changes in the function of these fibre systems in the vicinity of the injury. Animals were investigated at different time points from acute up to 6 months post injury to characterise the temporal progression of changes in spinal cord function.

Contusion injury produced an immediate substantial reduction in sensory circuit function which was highly localized to the site of the impact. However, CDPs continued to be evoked both above and below the injury site. This suggests that the main sensory axons in the dorsal column which carry impulses past the level of the injury are not substantially affected and deterioration of function at the

injury centre is a result of damage to the neurons and axon collaterals located at the site of impact. Further worsening occurred over the following 3 days but was more marked above the level of the injury possibly because it is due to demyelination of the main sensory axons. However, CDP mapping at 2 weeks showed almost complete recovery of the potentials above this compared to 3 days reflecting a repair mechanism which is possibly remyelination. The fact that CDP amplitudes at 2 weeks are very close to those recorded immediately after injury suggests that most of the loss of function produced by the contusion is due to primary mechanical trauma and that secondary mechanisms contribute relatively little to the loss of function. CDP amplitudes recorded 4 weeks, 7 weeks and 3 months after injury remained closely similar to 2 weeks suggesting a prolonged period of stability in the sensory system. However, a later phase of deterioration is observed at 6 months below the injury possibly due to extension of the injury cavity causing further neuronal loss. Histology also revealed significantly larger cavities in 6 month survival animals.

CDPs evoked by stimulation of the corticospinal tract below the injury level were profoundly depressed immediately following a contusion reflecting extensive damage to the main component of the CST in the dorsal columns while potentials above the injury were not acutely affected. There was a further reduction in CDP amplitudes rostral to the injury at 3 days due to demyelination or die back of CST fibres in the dorsal columns. Substantially this improved at 2 weeks and then remained stable up to 6 months. Below the injury there was a gradual increase in the strength of corticospinal connections evident by an increase in CDP amplitudes at 7 weeks which was maximal by 3 months. This reflects plasticity in the spared corticospinal projection probably those in the lateral component.

Mesenchymal stem cells (MSCs) from bone marrow (BM) have been reported to promote repair and some functional improvement in animal models. However, autologous transplantation of BM-MSCs has several disadvantages such as the need for invasive harvesting, variable cell quality and slow proliferation. We have successfully derived MSC-like cells from human embryonic stem cells (hESCs) and have found that these cells show anatomical evidence of repair after transplantation in an animal model of SCI (Mohamad, 2014). Delayed transplants of hESC derived MSCs (hESC-MSCs) were performed in a cervical (C6) contusion

(175 kdyn) injury model. These cells were transplanted 3 weeks after injury and immunosuppression was begun two days prior to this and continued for the remainder of the study. Functional outcomes were evaluated behaviourally using grip strength and horizontal ladder walking to assess improvement in forelimb function. In electrophysiology experiments cord dorsum potentials (CDPs) evoked by supra-maximal electrical stimulation of a radial nerve or within the pyramids were used to measure changes in the function of these fibre systems in the vicinity of a spinal cord injury at 7 weeks post-transplant.

There was a substantial reduction in the gripping ability immediately after the contusion injury followed by a modest spontaneous recovery. Weekly assessment of the grip strength score before and after transplantation revealed no differences in hESC-MSC transplanted and control groups of animals. Similarly, ladder walk analysis revealed loss of co-ordinated forelimb-hindlimb stepping and increased errors of forepaw placement on the ladder rungs after injury. There was no difference in the recovery of co-ordination or improvement of forelimb function after hESC-MSC transplants compared to control animals. Similarly, terminal electrophysiological experiments showed that the amplitudes of CDPs evoked by radial nerve stimulation were closely similar at all recording locations in both groups of animals. However, corticospinal evoked CDPs showed deterioration of function in the transplanted animals compared to the control group of animals. Histology indicated that hESC-MSCs survived in considerable numbers and were present in all transplanted animals at 7 weeks leading to solid infilling of the injury site and a reduction in injury site extent compared to the medium injected control group of animals.

In conclusion, the results in the first part of the thesis suggest that maximum damage after contusion injury occurs at the time of injury and spontaneous recovery of function largely mitigates the small amount of the secondary damage. Therefore, injury due to high velocity impact might not be amenable to neuroprotective therapies. Furthermore, evidence of modest spontaneous plasticity in the spared corticospinal system suggests some compensatory plasticity after the injuries.

Table of Contents

List of Tables

List of Figures

Dedication

I dedicate this thesis to my late beloved father, who would have been extremely happy to see me achieve this milestone and to my dear and caring mother whose continuous courage, prayers and support have kept me going in times of anguish.

Acknowledgement

I am extremely thankful to Almighty Allah who gave me patience, health and knowledge to complete this piece of work.

I would like to express my gratitude to my supervisor Dr. John Riddell for his full support, guidance and encouragement throughout my PhD studies. I am very grateful to him in helping me in every aspect of my research project. He is a great mentor and has guided me through the last 4 years.

I am very thankful to all my colleagues in the spinal cord group and especially indebted to my colleague Mr Andrew Toft for his support and help throughout my experimental work. I would also like to acknowledge Dr Che Anuar, Dr Jake Griffin and Dr Ahmed Emraja who were always around in times of need. Many thanks to all my colleagues in the spinal cord group, with whom it has been pleasure to work. I will miss you all when I am back home. I am also thankful to all the staff in CRF for their help.

I am grateful to Khyber Medical University, Peshawar, Pakistan and the Higher Education Commission of Pakistan for providing me with funding to pursue my higher studies. I am particularly indebted to Prof. HafizUllah, Vice Chancellor KMU for his continuous support throughout these years. I am also thankful to all KMU PhD scholars whose support and company in Glasgow was a memorable experience. The trips with the travellers group have provided me with memories I will cherish for the rest of my life.

I am deeply thankful to all my brothers, sisters and other family members for their continuous encouragement, prayers and support that have helped me throughout my PhD. I would also like to especially thank my dear wife who has been supportive through thick and thin during my stay in Glasgow.

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.

Abbreviations

ºC	Degrees Celcius
µ	Micro
Ω	Ohm
%	Percent
A	Ampere
gm	Gram
hr(s)	Hour(s)
i.m.	Intramuscular injection
i.v.	Intravenous injection
m	Milli (10^{-3})
min(s)	Minute(s)
mm	Millimeter
n	Numbers
V	Volt
s.c.	Subcutaneous
ANOVA	Analysis of variance
ASIA	American Spinal Cord Injury Association
BBB	Blood brain barrier
BBB	Basso, Beattie, Breshnan locomotor scale
BDA	Biotin Dextran Amine
BDNF	Brain-derived neurotrophic factor
CDP(s)	Cord dorsum potential(s)

CNS	Central nervous system
CSF	cerebro-spinal fluid
CSPGs	Chondroiton sulphate proteoglycans
CST	Corticospinal tract
Ctb	Cholera toxin b
DMEM	Dulbecco's modified eagle medium
ECM	Extracellular matrix
FBS	Fetal bovine serum
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
hESC	Human embryonic stem cells
hESC-MSC	Human embryonic stem cells derived mesenchymal stem cell
MAG	Myelin associated glycoprotein
MSCs	Mesenchymal stem cells
NF	Neurofilament
NGF	Nerve growth factor
NT-3	Neurotrophin 3
NT-4	Neurotrphin 4
OEC	Olfactory ensheathing cell
PBS	Phosphate buffered saline
SCI	Spinal cord injury
SEP	Somato-sensory evoked potential
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling

1 Introduction

1.1 Spinal cord injury

Spinal cord injury (SCI) is a complex and devastating condition that impacts on every aspect of an individual's life, ranging from the patient's ability to carry out everyday responsibilities to skilled tasks. It has a significant impact on the quality of life, life expectancy, and economic burden. The majority of spinal cord injuries lead to a permanent disability and there is a lifelong dependency (Thuret et al., 2006). This lifetime debility is due to the limited capacity of the spinal cord for repair and the lack of an effective treatment to overcome these detrimental consequences after injury or to reverse the damage (Di Giovanni, 2006).

The spinal cord is a caudal extension of the brain stem down into the vertebral column. It receives and processes sensory information from the muscles, joints, skin and viscera and sends motor signals to the body muscles. It also receives autonomic responses from different parts of the body. Traumatic injury to the spinal cord leads to disruption of these ascending and descending pathways leading to loss of sensory, motor and autonomic function. This has a number of consequences depending on the injury level and severity; paralysis being the most common along with other associated conditions due to immobility such as deep vein thrombosis, pressure ulcers and osteoporosis; autonomic dysreflexia and neuropathic pain are also common complications associated with SCI (Vawda et al., 2012). Moreover, patients and relatives experience social and emotional stress due to permanent immobility, along with financial burdens (Baptiste and Fehlings, 2007).

1.2 Epidemiology

It has been estimated that approximately 130,000 new cases of SCI occur worldwide each year (Illes et al., 2011). The incidence and aetiology differs demographically with a range of 10-83 per million population each year (Wyndaele and Wyndaele, 2006) and its prevalence is estimated to be around 235-1800 sufferers per million in different parts of the world (Hagen et al., 2012). Even in the UK and Ireland every year around 1000 individuals are paralyzed due to SCI, adding to the overall burden of 50,000 already affected. In the USA,

there are about 12,000 new cases annually, excluding those who die immediately after injury (National Spinal Cord Injury Stastitical Centre, 2014).

SCI most commonly affects young people involved in high risk activities (Couris et al., 2010, Varma et al., 2010) and especially in their 3rd decade of life (Baptiste and Fehlings, 2007). In the USA road traffic collisions (RTCs) contribute about 36.5%, 28.5% are a result of falls and 9.2% is due to sporting injuries (National Spinal Cord Injury Stastitical Centre, 2014). In the UK about 38.5% are due to RTCs and 41.7% falls (Apparelyzed, 2013). Sport related injuries are more common in developed countries whereas work related injuries are more common in developing countries and more recently injuries related to firearms are on the increase in some parts of the world.

1.3 Anatomy of the Spinal cord

1.3.1 General anatomy of spinal cord

The spinal cord is a caudal extension of the brain and it lies inside the vertebral column which supports and protects the cord from physical damage. It is covered by three membranes called meninges. The dura mater is the outer layer, arachnoid mater is the middle layer and innermost is called the pia mater. In addition, cerebrospinal fluid (CSF) which gives further cushioning to the cord runs in the subarachnoid space (space between arachnoid and pia mater) (Figure 1-1). CSF also maintains the metabolic environment and relative immunological protection to the CNS (Irani and Prow, 2007).

The spinal cord contains millions of neurons involved in the transmission of sensory, motor and autonomic information from trunk, limbs, viscera and other parts of the body to CNS and vice versa. Sensory information enters the cord through the dorsal nerve roots, which enter the dorsal aspect of the spinal cord. Motor nerve roots exit the ventral aspect of the cord and combine with sensory roots to form a spinal nerve.

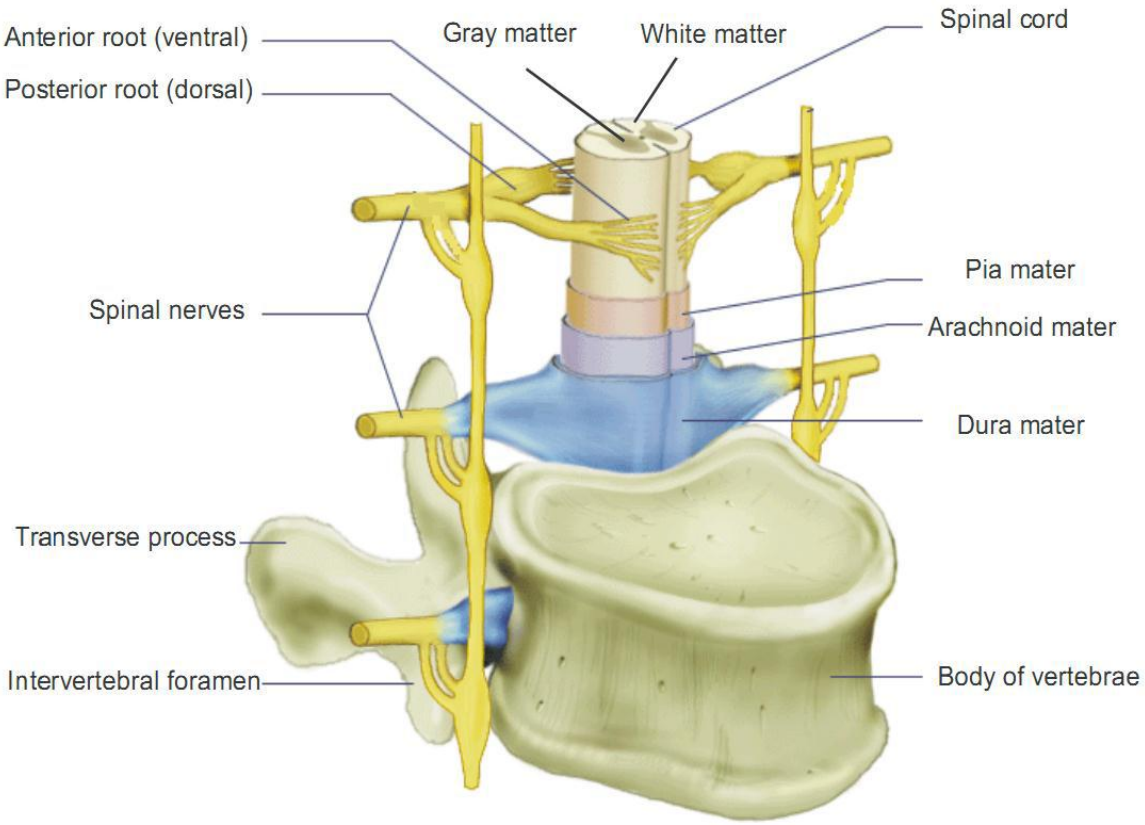

Figure 1-1. Organization of the spinal cord.

Diagram showing arrangement of the 3 layers of meninges (pia mater, arachnoid mater and dura mater) and the surrounding vertebral column, providing protection to the delicate spinal cord. The transverse section of the spinal cord shows the centrally located butterfly shaped grey matter surrounded by white matter. Grey matter is mainly composed of the neuronal and glial cell bodies, whereas white matter primarily contains axonal fibres. Each segment of the spinal cord consists of two anterior (ventral) and posterior (dorsal) roots which carry motor and sensory impulses respectively (Adapted from (Molson Medical Informatics, 2008).

1.3.1.1 Grey and white matter

The spinal cord is divided into a butterfly-shaped column called the grey matter which consists of neuronal cell bodies and is surrounded by white matter which contains nerve fibres (Afifi and Bergman, 2005). The grey matter is further divided into the dorsal horn, the intermediate grey, the central grey and the ventral horn. It can also be divided into 10 laminae from dorsal to ventral aspect each with different morphological and functional classes of neurons (Rexed, 1952). Grey matter is surrounded by the white matter containing

ascending and descending pathways and is further divided into dorsal, dorsolateral, ventrolateral and ventral funiculi (Molander and Grant, 1995). Different ascending and descending tracts run through different parts of the white matter. There are some anatomical differences between different species with respect to where the tracts are placed in the white matter. Comparison of different tracts running in the rat and human spinal cord are shown in the Figure 1-2.

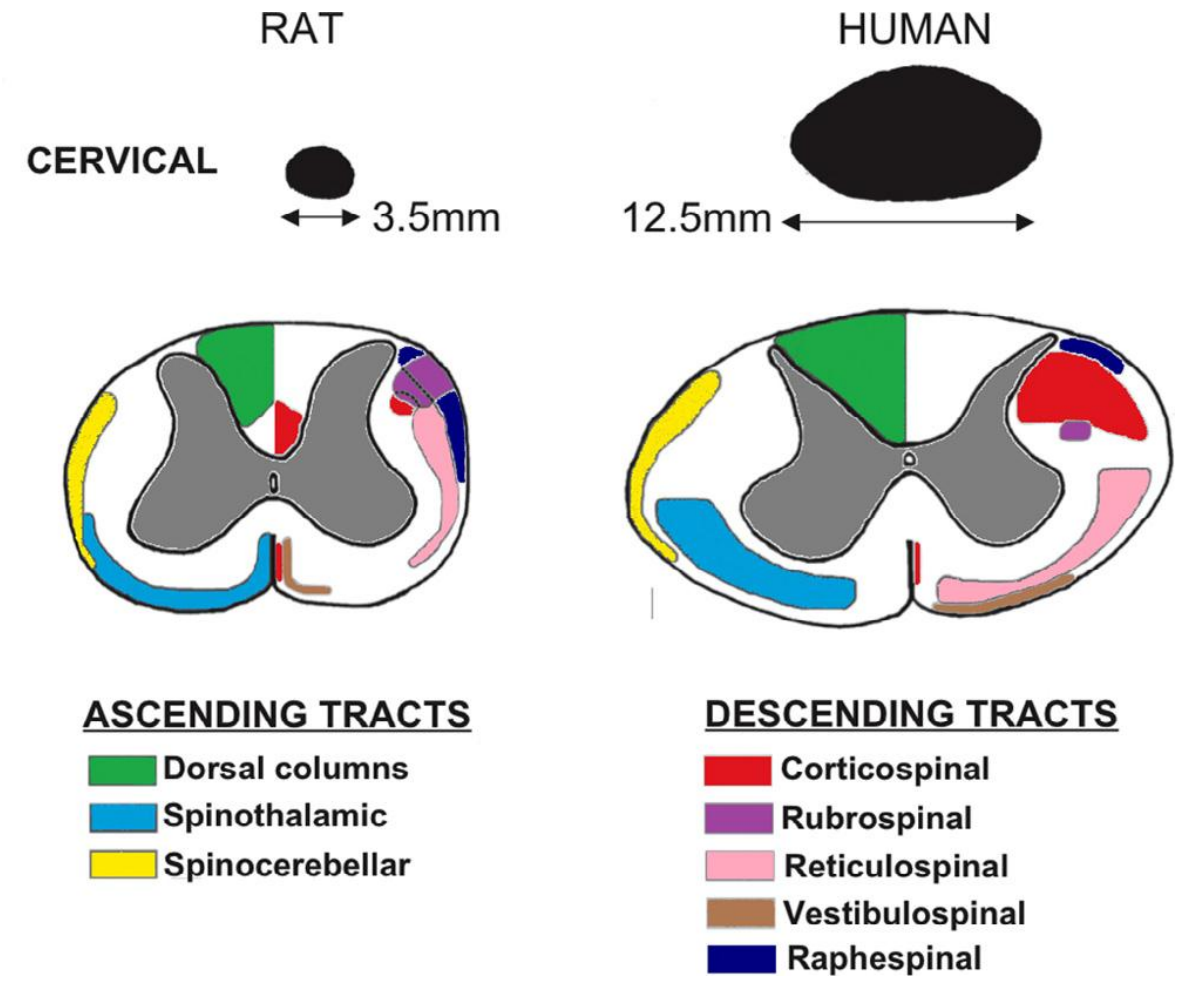

Figure 1-2. Comparison of rat and human spinal cord.

Comparison of size of the spinal cord and approximate location of different ascending and descending tracts are compared in rat and human spinal cord. Main component of CST in rats lies in the ventral part of the dorsal white column. Minor components are located in the lateral and ventral white matter. In humans main component is located in the lateral white matter and minor component in ventral white matter. Dorsal column pathway lies in the dorsal white matter in both rats and humans (Adapted from (Silva et al., 2014).

1.3.1.2 Segmental organisation

The length of the spinal cord is shorter than the vertebral column and thus spinal segments do not correspond to the overlying corresponding vertebral bones. The spinal cord diameter varies rostrocaudally and contains two enlargements at the mid cervical and lumbosacral regions, which extend from spinal segments C3 to T2 and L1 to S3 respectively. These enlargements represent the location of neurons that innervate the upper and lower limbs (Afifi and Bergman, 2005).

Human spinal cord: The human spinal cord occupies the upper two-thirds of the vertebral column and is divided into 31 segments comprising 8 cervical, 12 thoracic, 5 lumbar, 5 sacral and 1 coccygeal (Figure 1-3).

Rat spinal cord: The rat spinal cord consists of 34 segments and is divided into 8 cervical, 13 thoracic, 6 lumbar, 4 sacral and 3 coccygeal.

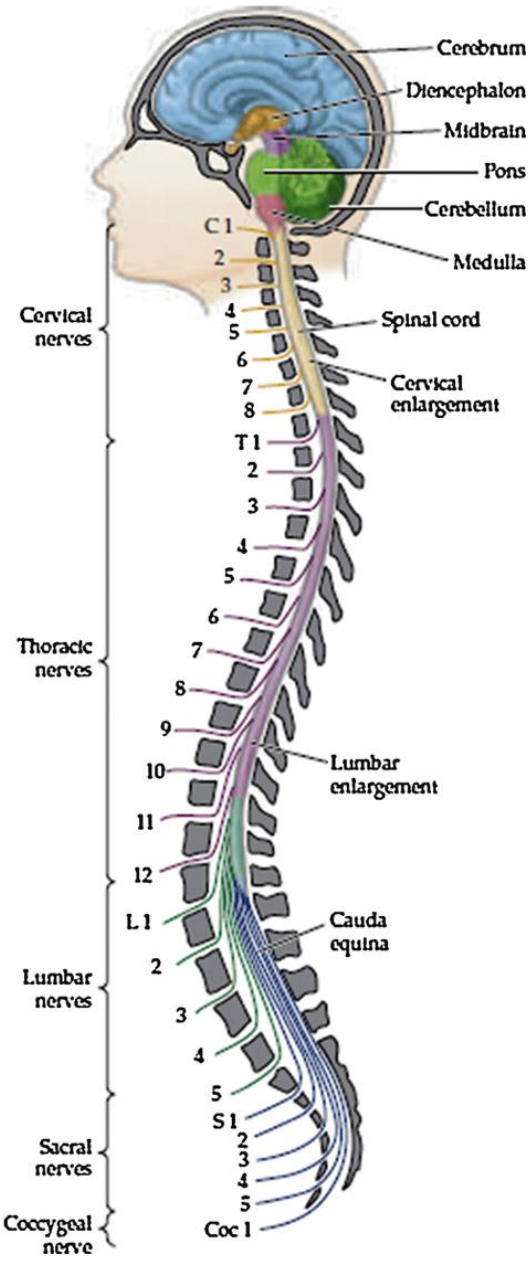

Figure 1-3. Schematic diagram of human spinal cord segments, vertebrae and respective roots.

There are 8 cervical, 12 thoracic, 5 lumbar, 5 sacral and 1 coccygeal nerve arising from the each side of the spinal cord. The spinal segments at birth correspond to the vertebral levels. However, the vertebral column grows much faster as compared to the spinal cord and thus the adult spinal cord occupies the upper two-third of the vertebral column. The terminal portion of the cord tapers to form the conus medullaris and is located at L1-2 vertebral regions. The spinal segments continue to exit from the corresponding vertebral foramina. The lumbar, sacral and coccygeal roots gets longer during the process of development within the vertebral column and the roots collectively with conus medullaris are known as the cauda equine. (Adapted from (Silva et al., 2014).

1.3.2 Ascending pathways in the dorsal column

Ascending pathways in the spinal cord carry information from the trunk and limbs to the brain. The ascending fibres in the dorsal column are divided into two main groups. 1) direct dorsal column pathway (DC pathway) 2) postsynaptic dorsal column pathway (PSDC pathway). Ascending pathways in the dorsal column have similar organisation both in human and rats (Figure 1-2).

1.3.2.1 Ascending pathways in the dorsal column of the rat spinal cord

The direct dorsal column pathway or dorsal column-medial leminiscus pathway is the main ascending pathway in the mammalian CNS. In addition, the DC pathway contains 25% propriospinal fibres. Cell bodies of the primary afferents of the DC pathway lie in the dorsal root ganglion. They send branches peripherally to innervate sensory receptors in the skin, muscle and viscera, and also centrally to the spinal cord via dorsal roots. In the spinal cord primary afferents bifurcate into ascending and descending fibres. Descending fibres generally do not maintain a well-organized topographical distribution and usually terminate within two segments from their site of entry. However, a small fraction of the ascending projecting afferents terminate in the dorsal column nuclei (Figure 1-4), while most of the other fibres terminate in various regions of the grey matter.

About 15% of the ascending fibres originating in the lumbar region reach the nucleus gracilis and up to 63% from the cervical region ascend all the way up to the nucleus cuneatus in the medulla. The ascending fibres have a topographical arrangement in the dorsal column of the spinal cord as seen in the somatosensory cortex. Fibres from the tail and hindlimb project close to the midline through the fusciculus gracilis, accumulating laterally as they travel rostrally, while fibres from the trunk and forelimb ascend laterally in the fasiculus cuneatus as they join the dorsal columns (Smith and Bennett, 1987). Axons travelling in the dorsal column send collaterals in the grey matter mainly in the dorsal horn, intermediate region and to a certain extent in the ventral horn (Willis and Coggeshall, 2004).

It is estimated that about 25% of the primary afferents in the dorsal column are comprised of unmyelinated fibres in the rat (Chung et al., 1987). These unmyelinated afferents carry information from nociceptors or receptors in viscera to the dorsal column nuclei (McNeill et al., 1988, Tamatani et al., 1989, Patterson et al., 1990).

The post synaptic dorsal column pathway is a polysynaptic pathway, relayed by neurons located in the nucleus proprius located ventral to the substantia gelatinosa in lamina III and IV. This pathway consists of axons arising from the neurons in the spinal cord which project to the dorsal column nuclei (Giesler et al., 1984). The axons from postsynaptic dorsal column neurons terminate at all levels of the gracile and cuneate nuclei and also in the external cuneate nucleus (de Pommery et al., 1984). All PSDC cells from the lumbar region project via dorsal columns but some of the PSDC from the cervical region go through the dorsal column and dorsolateral column.

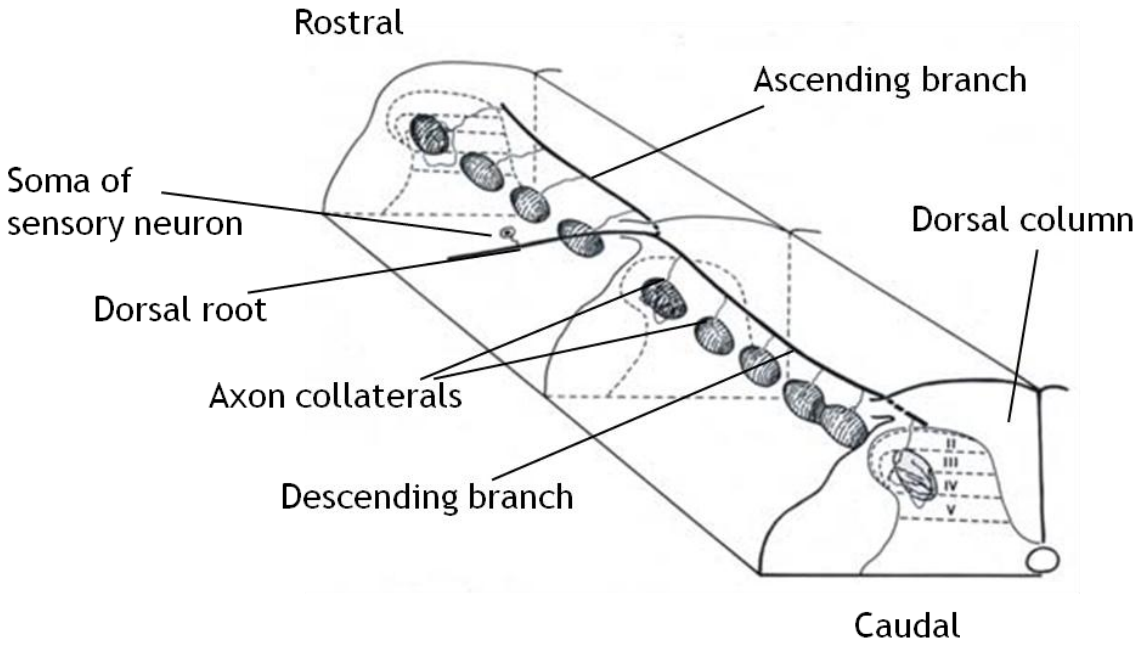

Figure 1-4. Schematic representation of primary afferent fibre organization in the spinal cord.

The somas of the primary sensory neurons are located in the dorsal root ganglia. The ganglion gives rise to peripheral and central axonal projections. The central axons from large myelinated sensory afferents enter the dorsal column via dorsal roots where these bifurcate into ascending

and descending branches. Primary afferents give off collaterals at regular interval which arborize in the dorsal horn. The descending branches terminate within one to two segments, while a proportion of the ascending fibres travel to the brain to terminate in the dorsal column nuclei. (Adapted from (Brown et al., 1981).

1.3.3 Corticospinal tract

The Corticospinal (CST) tract is one of the main descending pathways and found only in the mammalian CNS. It is an important descending pathway associated with fine motor control of hands in humans and other primates and is therefore one of the most investigated pathways in animal studies of SCI and repair. The distribution of corticospinal neurons in the cerebral cortex is similar in the human and rat, however, the organisation of corticospinal pathway in the spinal cord is different. In humans the main component of the CST is located in the lateral white matter and a minor component lies near the midline in the ventral column. Whereas, in the rat the main component of CST (dorsal CST), is located in the ventral aspect of the dorsal white matter below the dorsal column pathway and minor components lie in the ventral and lateral white matter (Figure 1-2).

1.3.3.1 Origin and course of CST in the rat

Corticospinal neurons, also called pyramidal neurons are located in layer V of the sensorimotor cortex. There is a considerable overlap between the somatosensory and motor cortex in the rat cerebral cortex. Pyramidal fibres have been shown to send collaterals to various brainstem nuclei including the dorsal column nuclei, the red nucleus, inferior olivary nuclei and pontine nuclei (O'Leary and Terashima, 1988).

The CST is divided into a crossed and uncrossed component. The majority of the fibres cross to the contralateral side in the medulla oblongata at the pyramidal decussation. The main crossed fibres then descend through the ventromedial aspect of the dorsal column of the spinal cord and comprise 90% of fibres. However, minor components are found in the ipsilateral dorsal, ipsilateral ventral and contralateral dorsolateral funiculi (Brosamle and Schwab, 1997).

The CST fibres terminate mainly within a region corresponding approximately to laminae III-V in the contralateral grey matter of the spinal cord. However, terminations are also found in lamina VI at cervical and lumbar enlargements. On the other hand, terminations have also been found within other laminae including ipsilateral laminae III-X, with an emphasis in laminae VII, VIII and X, contralateral laminae VI-X, and sparse projections to the more superficial laminae I and II (Casale et al., 1988, Brosamle and Schwab, 1997, 2000) (Figure 1-5).

Function: The CST is an important descending motor tract and it is involved in fine voluntary movements of the body; for example, forelimb movements as in the reaching task (Anderson et al., 2005). In rats, damage to either the main dorsal component (McKenna and Whishaw, 1999) of the CST or the motor cortex (Schrimsher and Reier, 1993) results in errors involving fine digital movements of the limbs.

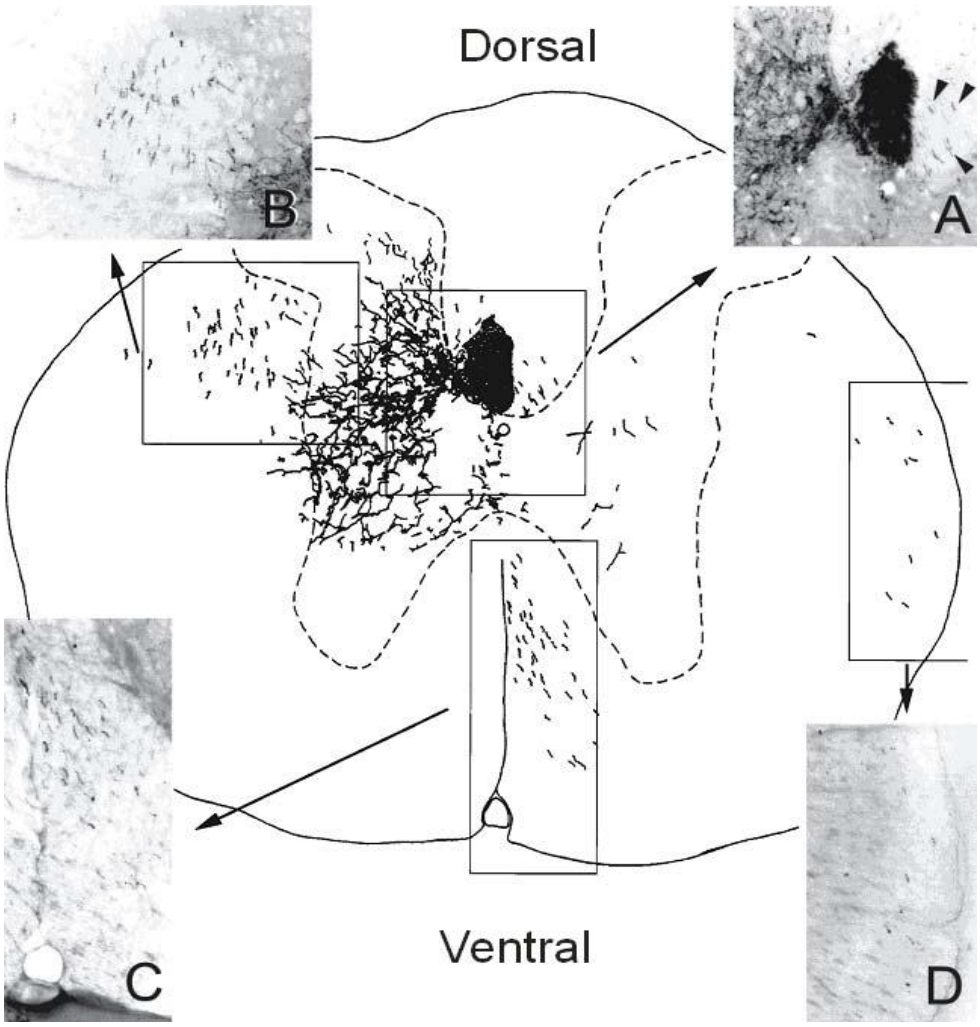

Figure 1-5. Schematic representation of Corticospinal Tract (CST) organization. Schematic cross section at mid thoracic level of the adult rat spinal cord.

Diagram illustrates Biotin Dextran Amine (BDA) tracing from the right hemicortex showing several components of the CST. Camera lucida reconstruction of different components of the CST photographed from A-D. A: The main contralateral component of CST in the ventral part of dorsal white matter (darker area) and ipsilateral dorsomedial funiculus (arrowheads). B: The contralateral minor component of CST running in the lateral white matter. C: Ipsilateral ventromedial funiculus running in the ventral white matter. D: Ipsilateral lateral white matter. The dash line demarcates the white and grey matter (taken from (Brosamle and Schwab, 1997).

1.4 Injury level and type

The overall outcome after SCI depends on the level and type of the injury. Depending on the preserved neurological function below the level of the injury, SCI can be characterized into complete and incomplete injuries. However, post

mortem studies reveal that the spinal cord is rarely completely severed after trauma (Norenberg et al., 2004). In a complete injury there is no sensation or voluntary motor output below the level of the injury. Incomplete injuries have some preservation of function below the level of the injury, which could be sensory or motor or both. This classification is defined by the lowest level that has normal neurological function.

Clinically, the American Spinal injury Association (ASIA) Impairment Scale (AIS) is the most widely accepted scheme for classifying patients according to the injury severity and deficits in neurological function (Kirshblum et al., 2011a).

The AIS is divided into 5 categories from A-E depending on injury level and testing of key muscle functions corresponding to 10 myotomes. The sensory level is determined as the most caudal level at which there is a normal response to both light touch and pinprick. The motor level is determined by normal strength in key muscles on both side of the body. Motor strength and spinal level of normal motor output are determined by performance of the following voluntary movements; 1) elbow flexion “C5” 2) wrist extension “C6” 3) elbow extension “C7” 4) finger flexion “C8” 5) finger abduction “T1” 6) hip flexion “L2” 7) knee extension “L3” 8) ankle dorsiflexion “L4” 9) toe extension “L5” and 10) ankle plantar flexion “S1”.

The classification of AIS used in grading the degree of impairment is as follows:

ASIA A: Also called “complete” SCI where there is complete loss of function. There are no motor or sensory outputs observed in the lowest sacral segment i.e. S4-S5.

ASIA B: categorised as sensory incomplete and sensory but not motor function is preserved below the neurological level and also includes sacral segments S4-S5.

ASIA C: is considered motor incomplete and motor function only is partially conserved below the level of the injury and more than half of the key muscles below the level of the injury have muscle power of less than grade 3.

ASIA D: also known as motor incomplete and here motor function is preserved and more than half of the key muscles have power of grade 3 or more.

ASIA E: indicates motor and sensory functions are normal.

The level of the injury in the spinal cord determines the deficits in parts of the body where function may be lost and impaired. Cervical level injuries mostly lead to quadriplegia in which all the four limbs are affected. Paraplegia occurs in injuries usually below the first thoracic level.

Cervical level SCI is the most common in Europe and is estimated to account for about 45% of all cord injuries; followed by thoracic injuries, approximately 29% and 23% for lumbar (Hasler et al., 2012).

Functional deficits with injuries at different levels: Functional deficits depend on the level and extent of the injury. There are more severe consequences as the level of the injury is higher up the spinal cord. Lumbar and sacral injuries result only in loss or decreased control of the lower limbs and pelvic region. Lower thoracic injuries between T9-T12 result in partial paralysis of lower body and legs, while injuries higher in the thoracic region (T1-T8) leads to poor control of trunk and abdominal muscles. People with C7-T1 injuries tend to have deficits in their digits and hand function whilst control of the remaining arm musculature is intact. Injury at the C6 level leads to loss of finger and hand function whilst wrist function remains intact. Patients with C5 injuries have loss of wrist function and are only able to control upper arm and shoulder, whereas C4 level pathology might include slight control over the shoulder and upper arm but this will be weaker compared to C5 level injury. Injury at C3 and above usually leads to loss of control of the diaphragm and ventilator support for breathing is needed in this case (Figure 1-6) (Thuret et al., 2006).

In addition to the above complications, regardless of the level of the pathology patients might suffer from secondary consequences of spinal injuries such as spasticity (Levi et al., 1995), neuropathic pain (Ehde et al., 2003), autonomic dysreflexia (Rabchevsky, 2006) and pressure ulcers (Liem et al., 2004).

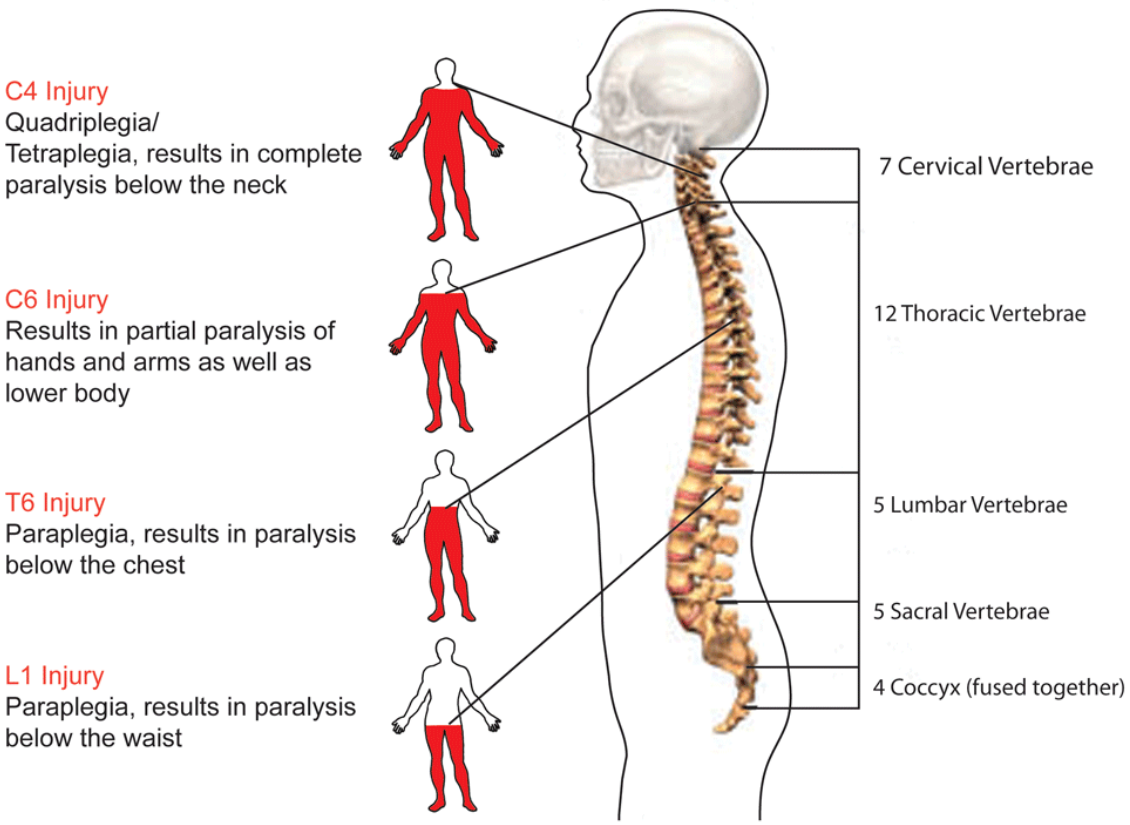

Figure 1-6. Diagram showing levels of SCI and functional deficits caused at each level.

The symptoms and deficits after SCI depend on the level and severity of the injury. Injury at cervical levels lead to quadriplegia e.g. injury at C4 spinal level results in quadriplegia and incomplete paralysis below the neck. Injury below the first thoracic level result in paraplegia e.g. T6 SCI cause paralysis in lower half of the body while maintaining function in upper extremities. Injury at L1 leads to paralysis below the waist line. (adapted from (Thuret et al., 2006).

1.5 Animal models of SCI

SCI has a complex pathology which is still not fully understood. In order to investigate the mechanisms and potential treatments different animal models of SCI have been developed. Although it is impossible to address all the features of human SCI in animals, different injury models have been utilised to assess different aspects of traumatic human SCI. However, it is known that there are differences in anatomical, functional and behavioural outcome in different animal strains and variations should be kept in mind while assessing the results in different injury models (Mills et al., 2001).

Both rodents and higher animals including dogs, cats and pigs, have been used to develop animal SCI models (Blight and Young, 1989, Jeffery et al., 2005, Navarro et al., 2012). However, rodents, including rats and mice, have been the most commonly used due to the relative ease of breeding, cost effectiveness, relatively low susceptibility to infections, consistency between studies and well established functional assessment methods.

1.5.1 Contusion

The contusion injury model in animals mimics incomplete SCI as seen in clinical cases of human SCI. Initial mechanical trauma is followed by a cascade of secondary responses which are considered to continue for months to years. Furthermore, contusion also leads to motor and sensory dysfunction below the injury, such as paralysis of the hindlimbs and bladder dysfunction in thoracic level contusion injuries and both conditions show some degree of functional recovery with time. Histological examination has revealed that grey matter is easily disrupted compared to white matter (Gensel et al., 2006, Ek et al., 2010, James et al., 2011) and leads to the formation of fluid filled cystic cavities which correspond with the findings observed in clinical settings (Edgar and Quail, 1994, Squier and Lehr, 1994). The contusion models are potentially useful for evaluating neuroprotective strategies, plasticity and demyelination, because of partial preservation of tissue. However, due to the incomplete nature of the injury and the complexity of spinal tracts it is not an ideal SCI model for investigation of regeneration.

The first well documented contusion injury model using a weight drop method was produced by Allen in 1911. Different severities of injuries were produced using the New York University (NYU) impactor by dropping a 10-g rod perpendicularly onto the dorsal surface of the exposed spinal cord. Different severity injuries can be generated by dropping the impactor from different heights (Gruner, 1992). However, this method has some limitations such as the bouncing of the rod causing multiple impacts and the length of time it stays in contact with the cord (dwell time) and the inability to control the final velocity of the impactor (reviewed in (Kwon et al., 2002).

A more advanced model, using a computer controlled device, is the Ohio State University (OSU) impactor (Bresnahan et al., 1987, Noyes, 1987, Stokes, 1992). This impactor controls the displacement of the rod and is actively withdrawn following the impact (usually ranging from 0.8 to 1.1 mm) and hence avoids bouncing. However, the impactor tip has to be placed in contact with the surface of the spinal cord (30 μm pre-set displacement) before operation.

Another device, the Infinite Horizon (IH) impactor is a force-feedback controlled device with a sensor attached to the impactor which measures the actual force applied to the spinal cord (Scheff et al., 2003). As a result, the IH device can optimize the consistency and precision of the mechanical forces applied during injury. It can also generate different degrees of injury severity and dwell times can be adjusted. The device also gives an instant graphical and numerical output of actual force and displacement for immediate evaluation of individual injury.

Contusion injuries lead to impairment of sensory and motor function, however, modest recovery is observed with time depending on the severity of the injury. Complete loss of locomotor ability in the hindlimbs is initially observed after severe thoracic contusion injury made with IH impactor. The BBB score drops to zero 1 day after injury followed by a gradual improvement in locomotor ability plateauing at 3 weeks at a score of 10 (Totoiu and Keirstead, 2005). Similarly, locomotor function is initially severely impaired with mean BBB score of 3.4 at 1 day after a mild contusion injury (150 kdyn) at T10. This is followed by spontaneous recovery with the BBB score reaching 13 at 3 weeks and remaining at this level into chronic post-injury stages (i.e. 6 months post-injury) (James et al., 2011). Similar findings are observed in cervical contusion models, where loss of gripping ability is seen immediately after the injury followed by a modest spontaneous recovery. Maximal improvement in the grip strength is reached at 2 weeks after the injury (Anderson et al., 2009b).

1.5.2 Compression

This injury model was developed in 1978 by Rivilin and Tator to produce static compression to the cord (Rivlin and Tator, 1978). It is used to simulate the ongoing compression secondary to the residual spinal column displacement.

Compression injuries can be produced using clips (Fehlings and Tator, 1995) or forceps to pinch the cord (Blight, 1991), applying sub dural inflatable balloons (Martin et al., 1992, Vanicky et al., 2001) or placing a certain amount of weight on the surface of the cord (Black et al., 1986, Nystrom et al., 1988, Huang et al., 2007). As for contusion models, different severity of injury can be produced by altering the duration and extent of displacement (Joshi and Fehlings, 2002). This model is valuable in understanding acute pathophysiology and studying neuroprotective therapies after SCI.

1.5.3 Transection

Partial or complete transections of spinal cord using microdissecting instruments have also been used frequently to model SCI. These can be performed with relative ease and consistency. Transection models are most useful for assessing axonal regeneration across the injury (Schrimsher and Reier, 1993, Webb and Muir, 2002, Steward et al., 2006) in long white matter tracts. In comparison, partial transection is less severe and offers several advantages such as fast postoperative recovery. Also a particular tract can be severed to gain specific information. Similarly unilateral transection can be done for comparison with the contralateral, unaffected side.

1.5.4 Chemically-mediated SCI

Different chemically- mediated SCI models also exist and these models are used to evaluate specific pathology involved in SCI. For example, excitotoxicity is produced by intraspinal injection of quisqualic acid causes release of excitatory amino acids and mimics glutamate excitotoxicity as seen after spinal trauma (Yezierski et al., 1993, Yezierski et al., 1998). Demyelination is another feature seen in SCI and this type of pathology can be triggered by injection of ethidium bromide into the spinal cord (Gilson and Blakemore, 2002). Similarly, vascular occlusion of spinal vessels by intravenous injection of Erythrosin B (photosensitizing agent) followed by irradiation with argon laser leads to ischemic necrosis of spinal tissue (Watson et al., 1986, Hao et al., 1991). Specific pathologies can be produced in chemically mediated SCI and hence specific feature can be studied in detail and therapeutic intervention targeting each pathological feature can individually be targeted.

1.6 Pathology following SCI

Most of the pathological information regarding SCI is obtained from animal studies, due to the paucity of information from clinical injuries. Traumatic SCI results in a cascade of events, starting immediately after the impact, which can continue for years (Schwab and Bartholdi, 1996). Events after SCI can be divided into an acute stage starting immediately after impact, followed by a secondary phase of tissue loss lasting for days and weeks and a chronic phase which can continue for months to years (Tator, 1995, Hulsebosch, 2002). Different pathological events following SCI are summarized in the Figure 1-7.

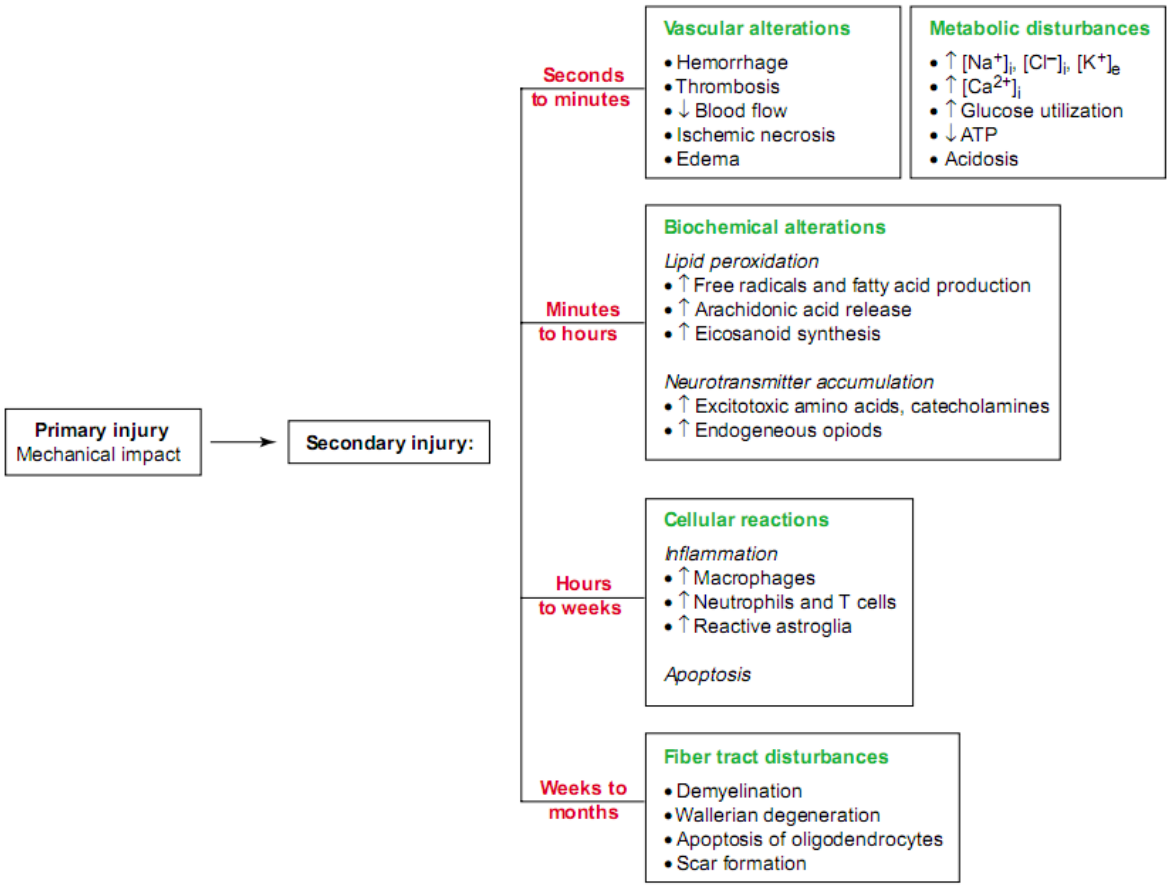

Figure 1-7. Summary of pathophysiological events after SCI.

SCI involves two stages of injury processes. Primary injury is due to the mechanical impact which results in local effects causing damage. This initial damage is followed by cascade of secondary processes starting minutes after injury and progresses for weeks and months (taken from (Bareyre and Schwab, 2003)).

1.6.1 Blood flow alterations

SCI disrupts blood vessels causing haemorrhage in the vicinity of the injury site. This is observed in both human and experimental animal models. These blood flow alterations are vital in the evolution of the secondary process. Disruption of blood flow leads to hypoxia causing a substantial ischemic necrosis (Dumont et al., 2001) and oedema develops due to intracellular calcium accumulation and an increase in extracellular potassium ion concentration (Schwab and Bartholdi, 1996). Vasospasm (Anthes et al., 1996) and thrombosis (Koyanagi et al., 1993) further aggravate the ischemia. Due to inadequate oxygen this translates into a general failure of neural function leading to a stage of spinal shock (Bach-y-Rita and Illis, 1993, Dumont et al., 2001), which last for a few days in animal models to several weeks in humans (Bareyre and Schwab, 2003).

Traumatic SCI has shown to lead to up-regulation of the expression of proteinases, in particular matrix metalloproteinases which leads to disruption of the blood-brain barrier (BBB). This breakdown of the BBB facilitates the influx of inflammatory cells into the CNS enhancing the inflammatory response. Haemorrhage usually starts centrally in the grey matter and later extends into the white matter (Mautes et al., 2000).

1.6.2 Oedema

Haemorrhage and leakage of fluid from the damaged capillaries into the normal fluid filled spaces contributes to local oedema. This occurs within minutes after trauma and is maximal in the first few hours (Griffiths and Miller, 1974). Oedema starts in the central portion of the cord and is seen to spread outwards into the white matter and is also seen to extend a considerable distance rostrally and caudally (Noble and Wrathall, 1989, Wang et al., 1993). The extent depends on the severity of injury. It is also suggested that it exerts its harmful effects by compressing the local tissue and vasculature, thus contributing to further necrosis (Griffiths and Miller, 1974).

1.6.3 Necrosis, apoptosis and excitotoxicity

Immediately after SCI, cellular necrosis starts at the injury epicentre, mainly as a result of local mechanical damage. This is associated with the initiation of inflammation due to the release of inflammatory cytokines from activated macrophages, microglia and astrocytes at the injury site (Dumont et al., 2001), along with severe depletion of energy stores.

The process of programme cell death (apoptosis) also ensues and is characterised by activation of proteases especially caspases and calpains, which leads to condensation and rupture of cells into their constituent parts (reviewed by (Beattie et al., 2000). Enzymatic action on the ionic channels leads to ionic imbalance which causes accumulation of intracellular Ca^{2+} (Iwata et al., 2004). This ionic imbalance alters the cellular signalling pathways leading to cellular death (Yezierski, 2005). Apoptosis was first observed in rodent models (Shuman et al., 1997) and later on was also confirmed in primates SCI (Crowe et al., 1997).

It has been shown that excitatory amino acids (EAAs) such as aspartate, glutamate, glutamine and aspargine reach toxic levels after SCI, and are responsible for causing excitotoxic cell death (Liu et al., 1991). Accumulation of these EAAs in the injured cord also increases the intracellular Ca^{+2}, with subsequent induction of cyclooxygenase-2 (COX-2) and nitric oxide synthase (NOS), leading to formation of reactive oxygen species (ROS), further potentiating the cellular death (Kontos and Povlishock, 1986, Bao and Liu, 2002, Liu et al., 2004).

Cellular death due to necrosis, apoptosis and excitotoxicity affects almost all cell types including neurons, astrocytes, microglia and oligodendrocytes.

1.6.4 Inflammatory response

SCI triggers a strong inflammatory response leading to the activation of glial cells (Crown et al., 2006, Gwak et al., 2008) and recruitment of leukocytes. Inflammation is known to have both destructive and constructive effects during the post-injury inflammatory episode (Bethea, 2000). This complex process

involves release of chemical mediators, epithelial damage, changes to vascular permeability, activation of microglia and recruitment of peripheral inflammatory cells.

Microglia are the first cells to be activated in the immune response after injury. They are stimulated within minutes after injury and start secreting pro-inflammatory cytokines and chemokines. Blood borne macrophages arrive at the injured spinal cord within 2-3 days and remain for weeks and contribute to the inflammatory process (Fleming et al., 2006). Activated microglia and infiltrating macrophages both produce other mediators of cellular death like ROS, nitric oxide and proteases. Activated glia release abundant amounts of chemo-attractants such as tumor necrosis factor-alpha (TNF-α), interleukin 1-beta (1L-1β) and interleukin 6 (1L-6), and attract more inflammatory immune cells (Rice et al., 2007, David and Kroner, 2011). These mediators are upregulated within an hour following injury and down regulated within 6-24 hours with a second peak occurring at 2 weeks after injury (Fleming et al., 2006).

At early stages microglia and astrocytes are also responsible for some beneficial effects like removal of myelin and axon debris along with uptake of excess extracellular glutamate and K+ (Fleming et al., 2006). In addition, releases of anti-inflammatory mediators like IL-10 and TGF-Beta have direct or indirect neuroprotective effects (David and Kroner, 2011). Reducing the inflammatory response has been shown to improve functional recovery (Stirling et al., 2004). However, the endogenous anti-inflammatory mediators are not sufficient to overcome the pro-inflammatory effects of macrophages. Similarly, neurotrophic factors likes BDNF are also released from the activated microglia/macrophages at the injury site which also helps in producing a limited healing response (Dougherty et al., 2000).

1.6.5 Demyelination & Axonal degeneration

Along with the neuronal loss after initial trauma, acute and chronic death of oligodendrocytes is also reported with subsequent disintegration and breakdown of myelin (Norenberg et al., 2004). The first phase of demyelination called primary demyelination is initiated by a primary injury response which involves

partial or complete destruction of the myelin sheaths at the injury epicentre while axons remain relatively intact (Griffiths and McCulloch, 1983). A secondary phase of demyelination is observed at 3 weeks which causes further damage to myelin sheaths surrounding the affected axons (Griffiths and McCulloch, 1983). This process of secondary demyelination is progressive and is reported at 450 days after SCI (Totoiu and Keirstead, 2005). Human studies suggest that this process can continue for more than a decade after SCI (Guest et al., 2005). Apart from demyelination at the injury site patches of demyelinating axons are also observed at areas away from injury epicentre in later stages of injury progression (Totoiu and Keirstead, 2005).

Axons may be disrupted due to initial trauma but some may still remain intact in the early stages and later on undergo anterograde degeneration. This process of anterograde degeneration is due to demyelination following oligodendrocyte death and this process is called Wallerian degeneration (Becerra et al., 1995). Wallerian degeneration is associated with disruption of spinal vasculature (Sinescu et al., 2010) and ionic imbalance resulting in calcium influx which induces apoptosis (Mattson and Chan, 2003).

1.6.6 Scar

Formation of the glial scar is a complex process and various cell types such as activated astrocytes, microglial cells, oligodendrocytes, meningeal fibroblasts and inflammatory cells are involved. Activated astrocytes undergo prolonged mitosis to form a dense network in conjunction with oligodendrocyte precursors and meningeal cells to form the glial scar (Faulkner et al., 2004). The glial scar is not only a mechanical barrier but also releases inhibitory factors that inhibit axonal regeneration and contribute to the inflammatory response. The main contributors to this hostile milieu are myelin associated growth inhibitors such as Nogo-A (Goldberg and Barres, 2000, GrandPre et al., 2000) myelin associated glycoprotein (MAG), tenascin R (Fawcett and Asher, 1999) and oligodendrocyte myelin glycoprotein (OMgp) (Wang et al., 2002b). These are found in white matter of the spinal cord and they inhibit axonal growth by stimulating growth cone collapse (Wang et al., 2002a). Other growth inhibitory factors such as chondroitin sulphate proteoglycans (CSPGs) which are markedly increased after

SCI, expressed in membranes of reactive astrocytes found in the glial scar and extracellular matrix, are also inhibitory to axonal growth (Silver and Miller, 2004).

Besides its detrimental effects, the glial scar helps to support the fragile parenchyma and acts as a scaffold for repairing the blood brain barrier (Stichel and Muller, 1998). It also helps restrict the degeneration and inflammation from spreading to the surrounding normal tissue (Faulkner et al., 2004).

1.6.7 The lesion cavity

Cavitation is observed after SCI and cavity formation tends to be initiated in the centre of the injury site (Dusart and Schwab, 1994, Fawcett and Asher, 1999). Cavities can extend rostro-caudally for several millimetres from the injury (Ek et al., 2010, James et al., 2011), with degradation of tissue due to apoptosis and inflammation (Sandvig et al., 2004). These lesion cavities are filled with extracellular fluid which contain residual macrophages, blood vessels and connective tissue (Norenberg et al., 2004). This extracellular fluid-filled space acts as another physical barrier to axonal regeneration. However, filled cavities with ECM provide a comparatively good substrate for regeneration of axons (Norenberg et al., 2004).

Lesion cavities are mostly seen as single cavities though multiple cavities are also observed. In approximately 4% of cases these cavities coalesce and give rise to the condition called syringomyelia (Schurch et al., 1996).

SCIs are relative stable after a period of weeks and months. The injury site is characterised by a fluid filled cavity surrounded by a glial scar and a rim of preserved tissue surrounding the lesion. The intact tissue surrounding the cavity may harbour spared fibres that might be responsible for the preservation of some function and spontaneous recovery after SCI.

1.7 Spontaneous recovery of function

It has been observed that a variable amount of spontaneous recovery can occur after SCI without any intervention in experimental animal models and humans

(Fouad and Pearson, 2004, Hagg and Oudega, 2006, Gulino et al., 2007). Though spontaneous regeneration and remyelination of injured fibres is very limited especially in adult CNS, a limited amount of functional improvement is nevertheless seen (Fawcett et al., 2007). The amount of recovery depends greatly on the severity and extent of injury.

1.7.1 Spontaneous recovery in humans

Spontaneous recovery of motor function in patients with motor-complete SCI (i.e. AIS A and AIS B) is limited and predictable (Brown et al., 1991, Fisher et al., 2005). However, the outcome and extent of recovery in cases with incomplete injuries as in AIS C and AIS D are highly variable (Fawcett et al., 2007). In the majority of cases with incomplete SCI, recovery is rapid in the first few months after trauma. Motor recovery usually reaches a plateau within 12-18 months when it is mostly considered stable. However, rarely it can continue for several years. Similarly, sensory recovery is considered to follow the same time course (McDonald and Sadowsky, 2002, Fawcett et al., 2007).

Steeves et al. (2011) carried out a retrospective analysis of spontaneous recovery of individuals with sensorimotor complete SCI at cervical level (C4-C7) who were registered in a European multicentre study about SCI clinical trial. They assessed change in the AIS grade and upper extremity motor score (UEMS) or motor level over the first year after SCI. Results showed about 70% of the individuals had spontaneously recovered at least one motor level and 30% recovered to 2 or more motor levels (Steeves et al., 2011). The motor level was determined by the lowest key muscle function in the myotome corresponding to the sensory level as described in the AIS (Kirshblum et al., 2011b). The average UEMS improved 10-11 motor points by the end of 1 year. However, the improvement in UEMS was not influenced by change in the AIS grade. Fawcett et al. (2007) reported that the majority of ASIA category A patients tend to remain as category A with approximately 10% down regulated to category B and 10% to ASIA C over the first year after SCI. Similarly, about 15-40% of ASIA B individuals were converted to ASIA C and up to 40% to ASIA D category. Conversion of ASIA C to D was between 60-80% with very few patients fully recovering to normal from ASIA D to ASIA E (Fawcett et al., 2007).

These finding suggests that almost all of the patients with SCI show some degree of spontaneous recovery below the ASIA injury level, however, recovery is highly dependent on the injury severity, as patients with less severe injury tend to show more robust recovery.

1.7.2 Spontaneous recovery in animals

A variable amount of functional recovery can be observed in animal models of SCI and there is also evidence of anatomical repair (McKenna and Whishaw, 1999, Weidner et al., 2001, Bareyre et al., 2004, Courtine et al., 2005, Ballermann and Fouad, 2006). A limited amount of spontaneous recovery is seen irrespective of the injury model and level of the injury, however, the extent of functional recovery depends on the severity of the injury (Noble and Wrathall, 1989).

Functional recovery in animal models is mostly assessed by behavioural outcome and electrophysiological methods. Different tests are carried out to evaluate different functions in SCI models. The Basso, Beattie, and Bresnahan open-field locomotor rating scale (BBB scale) is one of the most widely used for the assessment of locomotion in rat SCI models (Basso et al., 1995). Improvements in behavioural functional outcome using the BBB score are seen in different injury models including contusion, transection and hemisection models (Ballermann and Fouad, 2006, Collazos-Castro et al., 2006, Gwak et al., 2008). Similarly, recovery in cervical injury models with improvements in different forelimb function tests (e.g. grip strength, pellet retrieval, FLAS) are also observed regardless of the injury model (Gensel et al., 2006, Anderson et al., 2009a, Khaing et al., 2012).

Spontaneous recovery in behavioural assessments is also confirmed by electrophysiology, showing enhancements in motor evoked potentials (MEPs) and somatosensory evoked potentials (SSEPs) over time (Bazley et al., 2012). These findings were correlated with anatomical evidence of repair, tissue preservation and sprouting in different pathways including corticospinal (Rosenzweig et al., 2010), reticulospinal (Ballermann and Fouad, 2006) and propriospinal (Bareyre et al., 2004) systems.

Overall, it is observed that spontaneous recovery of function in most animal models is greater compared to humans suffering with comparable injuries. This enhanced recovery in animals may be partly because of the compensatory mechanisms, for example, recovery of gait in quadruped animals is due to adaptation of trunk, forelimb and hindlimb movements to compensate for the deficit caused by SCI (Rossignol et al., 2004, Ballermann and Fouad, 2006).

1.7.3 Processes underlying spontaneous recovery of function

Mechanisms which lead to spontaneous functional recovery are complex and not fully understood. As previously described axons have intrinsic ability to regrow but the hostile environment of the injured spinal cord inhibits this recovery. Although local neurotrophin expression is increased after SCI, it is not sufficient to overcome the influence of inhibitory molecules released at the injury site and sustain the regenerating ability of injured axons (Satake et al., 2000). Plasticity and remyelination are thought to be the main contributors to spontaneous recovery, and axonal regeneration can be excluded from the mechanisms involved in spontaneous functional recovery.

1.7.3.1 Plasticity

Plasticity is a generic term which indicates the ability of the nervous system to change. Neuronal plasticity was initially described as a change in the neuronal circuitry of brain in response to memory and learning (DeFelipe, 2006). It is now believed that a degree of spontaneous plasticity is present in most parts of the CNS and that it is enhanced after injury (Thuret et al., 2006). It can involve two mechanisms: ‘synaptic plasticity’ where there is modification of the synaptic connectivity (Gulino et al., 2007) and ‘anatomical plasticity’ which refers to formation of new circuits through axonal sprouting (Bareyre et al., 2004).

Spontaneous plasticity has been investigated in different animal models of SCI, including contusion (Hill et al., 2001), partial transection (Fouad et al., 2001) and ischemic injury (von Euler et al., 2002). Plasticity observed after SCI involves formation of new connections amid axonal sprouting from undamaged neurons and those left unaffected (Bareyre et al., 2004), along with modifications and changes in synaptic connections (Raineteau and Schwab, 2001,

Gulino et al., 2007). Apart from neuronal organization of the spinal cord rostral and caudal to the injury site, changes have also been observed in the somatosensory cortex (Raineteau and Schwab, 2001, Kaas et al., 2008, Fouad et al., 2011) and brainstem (McKenna and Whishaw, 1999) in humans and animals (Raineteau and Schwab, 2001, Fouad et al., 2011).

In addition to having beneficial effects, increased sprouting may lead to abnormal connections after SCI. It is thought it may be responsible for some adverse effects such as development of pain, autonomic dysreflexia and spasticity (Bradbury and McMahon, 2006).

1.7.3.1.1 Plasticity in the spinal cord after SCI

Injury-induced plasticity at the spinal cord level has been reported in experimental models of SCI. In a primate model spontaneous plasticity has been observed as the formation of new synaptic connections after hemisection injury (Aoki et al., 1986, Rosenzweig et al., 2010). This includes sprouting of axons across the midline from the non-injured contralateral side to the affected side. Almost 60% of the pre-lesion density of axons is observed below the injury and this is accompanied by improved behavioural outcomes (Rosenzweig et al., 2010). In rodents plasticity is seen in the form of extensive sprouting of axons from the ventral CST following interruption of the main dorsal component (Weidner et al., 2001). Descending propriospinal neurons have also been shown to be involved in the formation of new circuits which are reorganized after SCI (Bareyre et al., 2004, Cote et al., 2012). These new connections act as a relay between the cortex and their spinal target of origin. These anatomical observations are paralleled by observation of functional recovery (Bareyre et al., 2004).

Extensive rerouting and sprouting is also observed in ventrolateral bulbospinal pathways after injury of the upper cervical cord and this is correlated with respiratory improvements (Darlot et al., 2012). Reticulospinal fibres have also shown sprouting and formation of new connections caudal to a thoracic lateral hemisection (Ballermann and Fouad, 2006).

1.7.3.1.2 Plasticity at a cortical level after SCI

SCI can produce extensive long term functional and anatomical cortical reorganization (Jain et al., 1997, Ghosh et al., 2009), which is observed to begin immediately after injury (Aguilar et al., 2010). Cortical reorganization is seen in areas with intact peripheral connections which enlarge and invade the regions that have lost their connections as a result of injury. Extensive changes in the activation of cortical and subcortical areas of the brain are observed during hand movements in patients with SCI, irrespective of whether hand function is normal or impaired, suggesting reorganization of neuronal activity in these areas (Bruehlmeier et al., 1998). Readjustments in different areas of human motor cortex are reported such as adaptation of the hand area to a different body reference scheme (Bruehlmeier et al., 1998, Raineteau and Schwab, 2001). Similarly, massive cortical reorganization after dorsal column lesions in monkeys is observed (Jain et al., 1997). Similar observations have also been made in different rat models of SCI (Fouad et al., 2001, Bareyre et al., 2004).

1.7.3.2 Remyelination

A certain amount of spontaneous remyelination is observed within the spinal cord after injury which contributes to spontaneous functional recovery. Oligodendrocyte precursor cells (OPCs) (Gensert and Goldman, 1997, Keirstead and Blakemore, 1999) and Schwann cells (Salgado-Ceballos et al., 1998) are the main contributors in the process. OPCs are recruited to the site of SCI and differentiate into mature oligodendrocytes to form myelin (Gensert and Goldman, 1997, Yang et al., 2006). Schwann cells which normally myelinate the peripheral nervous system are also capable of migrating to the injury site after SCI and start myelinating the denuded axons (Takami et al., 2002a). This process of remyelination of demylinated axons in spinal cord is also correlated with improvements in behavioural assessment (foot print analysis and rotarod test) (Murray et al., 2001).

There are arguments regarding how long spontaneous remyelination takes place and how complete the process of remyelination is. Some authors have observed long term and robust remyelination after injury (Salgado-Ceballos et al., 1998, Lasiene et al., 2008, Powers et al., 2012). The most compelling evidence on the

amount of spontaneous remyelination comes from the study done by Powers et al. 2012. They calculated the number of remyelinated axons by measuring the internodal length that traversed the injury site (Powers et al., 2012). Axons with shorter internodal length are indicative of remyelination (Lasiene et al., 2008). Using the internodal length approach to calculate remyelination Powers and colleagues found that the majority of the spared descending fibres are remyelinated by 3 months. These remyelinated fibres were concentrated around the injury epicentre and axonal diameters were approximately 23% smaller compared to rostral and caudal zones. In addition it was also predicted that the conduction velocity at the injury epicentre would be reduced by 25% (Powers et al., 2012). Similarly, using electron microscopy, comparison of demyelinated axons around the injury perimeter one month after injury were 64%, in contrast to 30% demyelinated fibres observed 12 months post-injury, suggesting remyelination is a slow ongoing process (Salgado-Ceballos et al., 1998). However, in another study remyelination was reported to increase in chronic progressive fashion beginning 7 days after injury up till 120 days and then declined at 450 days after injury (Totoiu and Keirstead, 2005), suggesting that the process of spontaneous remyelination may be incomplete and abortive (Blight, 1989, Totoiu and Keirstead, 2005).

Apart from these positive results obtained through spontaneous plasticity it should also be kept in mind that recovery can be enhanced by providing locomotor training or other forms of rehabilitative therapy (Rossignol, 2000, Krajacic et al., 2010).

Much of the work done in the field of spontaneous plasticity and recovery after SCI has focussed on thoracic spinal cord injury models and assessment of functional outcome has focussed on the hindlimb. Reports using cervical injury models particularly contusion injuries are less common. This is despite cervical injuries being clinically more common and the prime importance of restoring hand function in quadriplegic patients (Anderson, 2004). Though considerable interest in cervical injury has developed over last the few years, there is still a need to evaluate functional changes in the spinal cord after contusion injury at the cervical level to assess the contributions of primary trauma and secondary transformations on the injury outcome over time.

1.8 Assessments of Function

Reliable evaluation of functional recovery is necessary in order to study spontaneous functional improvements over time after SCI. Assessment of function is also useful in evaluating the beneficial effects of a therapy used in SCI models. Therefore, proper selection of appropriate functional assessment methods in animal studies is crucial to properly interpret the results. The recovery of function is mainly assessed with behavioural testing and more rarely by electrophysiology.

1.8.1 Behavioural testing

Final functional outcome following SCI depends on the extent of neuronal damage, loss of white matter and reorganization of the remaining neural tissue. Behavioural assessments help to determine the lesion severity, location and extent of spontaneous recovery and also document improvements in response to therapeutic intervention. However, it is important to choose a suitable behavioural technique to match the relevant hypothesis of the experiment (Basso, 2004). Ideally the behavioural responses in animal models of SCI should be relevant to those seen in human patients with SCI (Sedy et al., 2008).

Some of these tests are simple and require little specialist training, while others need specialist training and sophisticated equipment. Muir and Webb divided behavioural tests according to the type of data collected. These are end point measures, kinematic measurements and kinetic measurements (for review see (Muir and Webb, 2000).

1) **End point:** these are measures in which animals are required to accomplish a particular goal and are scored on their ability to achieve it.

2) **Kinematic**: these describe qualitative measures where movements of the whole body and body segments relative to each other and/or an external frame of reference are measured and compared.

3**) Kinetic**: these tests help to quantify or describe the force produce by a limb or limbs, for example during weight support or force generated during pulling a

force transducer such as a grip strength meter (for review see (Muir and Webb, 2000).

Behavioural tests can also be divided into motor, sensory, sensory-motor, autonomic and reflex based test, according to the predominant sensorimotor systems involved in a particular behaviour. However, it is very difficult to differentiate one system involvement in a particular behavioural outcome since multiple systems normally contribute to all functions.

1.8.1.1 Motor tests

These tests primarily assess skeletal muscle functions that are not involved in locomotion. There are many tests that look at specific aspects like the inclined plane test which evaluates the animal's ability to maintain body position on a board that is continually raised by increasing the angle (Pearse et al., 2005). Similarly, the limb hanging test utilizes the grasping function of the paws (Pearse et al., 2005) and forelimb asymmetry tests assess asymmetries produced by a variety of spinal cord injuries (Gensel et al., 2006). Food pellet reaching tests evaluate the ability to use the forelimbs to reach, grasp and retrieve a food pellet after cervical injuries (Whishaw, 2000). Grip strength tests assess neuromuscular function by sensing the peak amount of force generated by animals grasping a bar (Pearse et al., 2005, Anderson et al., 2009a) and will be discussed in more detail in the third chapter of this thesis.

1.8.1.2 Sensory tests

Purely sensory function in animal models of SCI is difficult to evaluate. However, changes in sensory function indicative of pain after SCI can be evaluated by observing exaggerated responses in animal behaviour to normally innocuous stimuli. For example, von Frey's filament test is used to evaluate the degree of mechanical allodynia (Gris et al., 2004). In the von Frey test calibrated filaments of increasing force are applied to the plantar surface of the forelimb or hindpaw and a positive response is seen if the paw is briskly withdrawn (Gris et al., 2004). Increased sensitivity is indicated by withdrawal from less stiff filament than normal. In addition, use of a pre-heated plate for hot plate-based tests and noting the time of response in the form of a withdrawal can detect enhanced

sensitivity to thermal stimuli (Gale et al., 1985). Altered responses can be accompanied by licking, vocalization, overgrooming and aggression (Sedy et al., 2008).

1.8.1.3 Sensory-motor tests

These tests assess coordination and functional integration in sensory and motor systems. In the rope walking test animals are trained to run over the horizontal oriented rope (Kim et al., 2001) and in the beam walking test rats are trained to run over different diameter beams (Hicks and D'Amato, 1975, Metz et al., 2000). Animals are videotaped and the number of errors (slips and falls) made by the animal while crossing are counted. Similarly the horizontal ladder walking (grid walking) test is very sensitive in evaluating sensory-motor coordination (Behrmann et al., 1992, Metz et al., 2000). In the horizontal ladder walking test animals walk along a horizontal ladder with variable rung spacing (Metz and Whishaw, 2002, Chan et al., 2005). Runs are video recorded as for rope and beam walking and then the animals limb placement on the rungs evaluated. This will also be discussed in further detail later in the thesis.

1.8.1.4 Locomotor tests

Locomotor test are those in which the locomotor apparatus, the forelimbs and hindlimbs are tested after SCI. These tests are mostly in the form of an ordinal rating scale. The Basso, Beattie and Bresnahan locomotor rating scale (BBB scale) is the most commonly used test for assessing locomotion in spinal cord injured rats. It scores animals' hindlimb kinematic ability on a scale of 0 to 21, where 0 indicates no hindlimb motor function while 21 represents function in an uninjured rat (Basso et al., 1995). This scoring system does not require animal training, scoring can be learned quickly and reliably, and results can be compared with other labs (Basso et al., 1995). Similarly for forelimb locomotor assessment a forelimb assessment scale (FLAS) has been devised. The FLAS is a modification of the BBB score and it has a scoring from 0 showing no forelimb motor function to 17 for uninjured animal (Anderson et al., 2009a).

1.8.2 Electrophysiological testing

Electrophysiology is the study of the electrical properties of cells and tissues. *In vivo* electrophysiology represents a way of directly quantifying function and changes in motor and sensory pathways after SCI reliably. Also the electrophysiology can be focussed on a particular tract and fibres, and therefore changes in a single pathway can be examined. In addition, training of animals is not required as in behavioural assessment methods. In addition, electrophysiological methods are also used in evaluation of therapies used in SCI (Bradbury et al., 2002, Toft et al., 2007). However, these techniques require expensive instruments and extensive personnel training and an ability to interpret recordings at the time they are recorded to deal with any technical issues (Blight, 1992).

1.8.2.1 Cord dorsum potential

Cord dorsum potential (CDP) recording is a technique used to measure the postsynaptic electrical activity evoked in the spinal cord. CPDs reflect the depolarization of interneurons or primary afferents fibres in the dorsal horn (Willis and Coggeshall, 2004). The characteristics and amplitude of CDPs reflect the level of activation and strength in the local circuits due to stimulation of ascending pathways (if stimulation from a peripheral nerve) or due to descending pathways (for example stimulation of corticospinal system).These potentials can be recorded directly from the surface of the cord, from the skin overlying the vertebral column (Sedgwick et al., 1980) and also from the epidural space (Shimoji et al., 1977).

CDPs can be used to assess the functional abilities of spinal pathways after SCI (Sedgwick et al., 1980) and monitor the function perioperatively (Ahn and Fehlings, 2008). It provides a sensitive and reliable method for observing the outcomes of experimental SCIs and also for assessing the effects of potential therapies and is therefore useful in SCI research (Bradbury et al., 2002, Riddell et al., 2004, Toft et al., 2007). Using a crush injury model of the cervical cord of rats, application of chondroitinase ABC was shown to improve CDP potentials evoked by CST axons below the injury. This finding correlated with anatomical and behavioural evidence (Bradbury et al., 2002). Similarly after a dorsal column

lesion with application of OECs, improvements in CDPs evoked by stimulation of fibres in dorsal roots were recorded. This was attributed to a neuroprotective action (Toft et al., 2007). Hence, assessing CDPs in different SCI models can be very useful to evaluate changes in the circuitry in the vicinity of the lesion and can also help in evaluating the therapeutic effects of different interventions on the spinal circuits.

1.8.2.2 Somatosensory evoked potentials

Somatosensory evoked potentials (SEPs) are a series of waves that can be recorded from the surface of the somatosensory cortex. SEPs reflect the sequential activation of neural structures along the somatosensory pathway in response to electrical stimulation of peripheral nerve (Sedgwick et al., 1980). SEPs provide direct quantitative measurements of the functional ability of the ascending somatosensory pathways. They are used for the diagnosis and characterisation of neurological abnormalities in humans (Small et al., 1978, Dietz and Curt, 2006). SEPs are also used for monitoring the condition of the spinal cord intraoperatively (Grundy, 1983). This sort of direct feedback on the state of neural networks and pathways allows surgeons to safely perform complicated surgeries, such as resection of tumours and reconstructive procedures (Malhotra and Shaffrey, 2010). SEPs have been also used to assess the integrity of the spinal cord in animal models of SCI. Changes in the amplitudes and latencies of SEPs are correlated with injury severity and behavioural outcomes (Fehlings et al., 1989, Nashmi et al., 1997). Thus it can be another way of quantitatively analysing the effects of therapeutic interventions in SCI models.

1.9 Repair strategies

Historically, it was considered that adult neural tissue has no ability to regenerate after injury. However, Richardson and Aguayo, in the early 1980's showed that nerve fibres can regenerate through transplants of peripheral nerve placed into the injured spinal cord (Richardson et al., 1982). These findings were a massive breakthrough in the field of neuroscience and have been followed by numerous strategies which have been developed to induce an environment that promotes neuronal survival and axonal regeneration in an injured CNS.

Although as described previously, there is to some extent spontaneous recovery of function after SCI, it is not sufficient to recover normal function. As discussed above there are many factors that hinder recovery and might be targeted to promote recovery of function. For example, reducing oedema, reducing free radicals, controlling inflammation and preventing neural tissue dying off due to continued apoptosis. Similarly we need to promote repair of the injured demyelinated axons and also provide an environment suitable for neurite outgrowth to promote regeneration and plasticity.

To address the above mentioned aspects, therapies are broadly divided into 4 main categories 1) therapies that help promote neuroprotection, which can minimize secondary damage and preserve residual function, 2) therapies that provide an environment permissive for promoting axonal regeneration of the axotomized pathways, 3) therapies that help to enhance remyelination and reduce the conduction deficits of the demyelinated and damaged axons and 4) therapies that augment plasticity by forming new connections and promoting axonal sprouting.

1.10 Therapeutic approaches

Research into possible treatments for SCI is rapidly progressing but up till now there is no efficient and reliable clinical treatment available for SCI patients. Although, numerous therapeutic interventions are currently in phase I and II clinical trials so far none have offered substantial beneficial effects or reached phase III clinical trial. The only strategy used in clinical settings is the stabilization and decompression of the spinal cord in combination with the early use of high dose methylprednisolone. Although, surgical decompression in animal models has shown improved functional recovery and minimizes secondary injury, human trials show controversial results especially in the case of early intervention and critically ill patients; as is the case with therapeutic intervention of methylprednisolone providing neuroprotection. The only other option for SCI patients is rehabilitative therapy, in order to optimize residual function and help prevent complications like infections, pressure sores, muscle wastage, poor ventilation and spasticity.

1.10.1 Neuroprotection

Neuroprotection refers to the action of different therapies which protect the spinal cord from harmful effects due to the secondary response after injury and therefore help preserve neural pathways and maintain residual function after SCI.

Methylprednisolone is one of the earliest neuroprotective agents to be used. It is considered to exert its neuroprotective effects by inhibiting free radical production and down regulating the expression of pro-inflammatory genes (Hall, 1992, Almon et al., 2002). Although it is widely used in clinical settings its therapeutic effects in SCI are debatable (Suberviola et al., 2008).

Erythropoietin is a hematopoietic growth factor which has been shown to exert a neuroprotective and trophic effect in the CNS. Administration of erythropoietin after SCI has been shown to have a neuroprotective effect by reducing inflammation, decreasing apoptosis and so increasing sparing of white and grey matter (Arishima et al., 2006, Brines and Cerami, 2008).

Similarly, an anti-inflammatory cytokine interleukin-10 is reported to be capable of reducing the inflammatory response of monocytes and macrophages and inhibiting the production of TNF-α by activated astrocytes. Systemic administration has been shown to reduce the cavity size by 50% and also to enhance functional recovery in animal models (Bethea, 2000). However, Takami and co-workers reported that IL-10 administration led to sparing of grey matter without functional improvement (Takami et al., 2002a). Therefore further preclinical trials are needed to confirm the efficacy of this approach.

Application of the broad spectrum antibiotic minocycline has been shown to lessen the degree of apoptosis observed in oligodendrocytes, inhibit microglial activation and inhibit the release of pro-inflammatory mediators. Minocycline treatment has also been reported to increase axonal sparing, reduce lesion size and improve functional outcome measured using the BBB scale and inclined plane test (Wells et al., 2003). Minocycline appears to exert its anti-inflammatory effects by modulating microglial activation with subsequent reduction in the release of cytokines, matrix metalloproteinases (especially

MMP-12), nitric oxide and lipid mediators of inflammations (Stirling et al., 2005). Minocycline treatment has been shown to reduce TNF-α mRNA levels after SCI and prevent the lipopolysaccharide –induced production of TNF-α in glial cells. It also reduces in caspase-1-mediated liberation of IL-1ß and IL-6 production. Minocycline also exerts its neuroprotective effects by increasing the release of anti-inflammatory cytokine IL-10 when administered immediately after SCI (Lee et al., 2003b). Furthermore, minocycline reduces inflammation by diminishing cell infiltration and migration through reduction of chemokine production and/or chemokine receptor expression (Stirling et al., 2005). Decreased levels of nitric oxide, prostaglandins (PGE-2) and caspase-1 and 3 are also observed after treatment with minocycline (Stirling et al., 2004).

The lipid lowering drug atorvastatin and the non-steroidal anti-inflammatory drugs (NSAIDs) like indomethacin and ibuprofen have also been tested in animal models with some neuroprotection and improvement in function after SCI (Pantovic et al., 2005, Wang et al., 2009).

1.10.2 Promoting/stimulating axonal growth/regeneration

Axonal regeneration and sprouting is necessary to re-establish connectivity across the lesion site and this is regarded as the ultimate way to restore lost function and the most desirable long term goal (Tuszynski and Steward, 2012). Axonal regeneration refers to the regrowth of transected nerve fibres and their re-connection with target neurons while axonal sprouting refers to formation of new fibre branches from spared non-injured fibres (Tuszynski and Steward, 2012).

1.10.2.1.1 Neurotrophic Factors

Trophic factors are associated with cellular growth, neurite guidance and survival of neuronal cells. Levels of neurotrophic factors decrease as development continues, though these factors persist throughout adult life in regions associated with functional plasticity like cortex and olfactory bulb (Maisonpierre et al., 1990). However, in the spinal cord there is a huge reduction of these neurotrophins from embryonic stages to adulthood (Maisonpierre et al., 1990).

Neurotrophins are a group of molecules which can stimulate axonal sprouting and regeneration in sensorimotor pathways after SCI. Nerve growth factor (NGF), neurotrophin-3 (NT-3) and glia cell line derived neurotrophic factor (GDNF) can promote regrowth of damaged axons across the dorsal root entry zone and formation of functional connections in the dorsal horn (Ramer et al., 2000). Similarly, brain-derived neurotrophin factor (BDNF) and NT-3 are reported to promote regeneration in reticulospinal, rubrospinal, raphespinal and proprioceptive sensory axons (Bregman et al., 1997, Bradbury et al., 1999, Liu et al., 1999, Kwon et al., 2002, Tuszynski et al., 2003). VEGF, fibroblast growth factor (FGF), leukaemia inhibitory factor (LIF) and neurotrophin-4, 5(NT-4/NT-5) have also been demonstrated to have beneficial effects in SCI models (Silva et al., 2014). Kwon and colleagues suggested that neurotrophic factors are less effective in chronic SCI due to loss of receptors in injured neurons (Kwon et al., 2004).

1.10.3 Overcoming inhibition/promoting plasticity

Plasticity, as discussed previously, could be desirable for the recovery of function and it is enhanced after injury. However, plasticity in terms of sprouting and synaptic remodelling is limited by a number of features in the hostile milieu of the injured CNS.

1.10.3.1.1 Myelin Inhibitors

Myelin, for instance, produces a number of inhibitory molecules that restrict axonal growth. The most commonly known myelin associated inhibitors are Nogo-A, myelin associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) (McKerracher et al., 1994, GrandPre et al., 2000, Wang et al., 2002a).

The administration of an antibody against Nogo-A (Anti-Nogo) has been shown to improve behavioural outcomes and lead to axonal sprouting and corticospinal regeneration (Caroni and Schwab, 1988). Furthermore, Nogo-A, MAG and OMgp have all been shown to exert their inhibitory effects by binding to a common receptor (Nogo-66 receptor) (Wang et al., 2002b). NEP 1-40 a Nogo receptor antagonist which binds with the Nogo-66 region of the Nogo receptor complex

inhibits these myelin inhibitors. The administration of NEP1-40 is also demonstrated to be effective in axonal regeneration and functional recovery (GrandPre et al., 2002).

1.10.3.1.2 Glial scar inhibitors

Another important feature in the natural response to injury is the formation of a glial scar. The lesion scar acts as a physical barrier to axonal regeneration. The glial scar is composed of reactive astrocytes and microglia/macrophages, which release various chondroitin sulphate proteoglycans (CSPGs) into the extracellular space. These CSPGs in the ECM act as potent inhibitors of endogenous regeneration (Morgenstern et al., 2002). CSPG expression has also been shown to increase following SCI, to peak within 2 weeks and persist for several months (Tang et al., 2003). Chondroitinase ABC (ChABC) is an enzyme that degrades the inhibitory chondroitin sulphate proteoglycan (CSPG) molecules present in the glial scar. ChABC produces a more permissive environment for axonal regeneration and helps restore post-synaptic activity below the injury site and so promotes functional recovery (Bradbury et al., 2002).

1.10.3.1.3 Growth cone collapse inhibitors

Rho pathway antagonists: The Rho pathway plays an important role in transducing extracellular signals that lead to changes in cytoskeletal proteins and depolymerisation of actin filaments causing growth cone collapse (Dickson, 2001). It is reported that inhibition of either Rho or Rho-associated kinase can have a beneficial effect on axonal regeneration and recovery of function. C3 transferase is an enzyme that inhibits the Rho pathway and thereby promotes neurite outgrowth (Jalink et al., 1994). A Phase 1 clinical trial in SCI patients was carried out using the Rho antagonist BA-210 and showed slight improvement in motor recovery without serious side effects (Fehlings and Vawda, 2011).

It is reported that neurotrophin binding to neuronal membranes via distinct tyrosine kinase receptors can lead to elevation of intracellular cAMP. In addition, cAMP elevation has also been shown to promote regeneration by inhibiting the growth cone collapse in the neurites. Therefore, it might be beneficial to increase the intracellular levels of cAMP. Molecules like Rolipram prevent cAMP hydrolysis and hence can be used to enhance axonal growth (Whitaker et al.,

2008). However, rolipram is shown to be more effective when used in combination with other cellular strategies like OECs or Schwann cells (Bretzner et al., 2010).

1.10.4 Promoting remyelination

Damage to the myelin sheath is a common pathological feature of traumatic SCI and it is reported that demyelination continues for months to years after SCI (Totoiu and Keirstead, 2005). Axonal demyelination leads to loss of normal neuronal functioning and it is a potential therapeutic target in SCI. Ongoing remyelination after SCI has been reported but the process is not sufficient to restore full function and does not always appear to be complete (James et al., 2011). Therefore, any therapy preventing loss of myelin or helping remyelination is likely to promote recovery. Rolipram has been shown to decrease oligodendrocyte death after injury and therefore indirectly may promote remyelination in injured tissue (Whitaker et al., 2008). Cell transplants are among the potential treatments which have been shown to promote remyelination in SCI. OPCs and Schwann cells have shown remyelination of demyelinated axons after being transplanted into SCI models (Xu and Onifer, 2009). OPCs were shown to differentiate into mature oligodendrocytes when transplanted into injured cord and promote remyelination of demyelinted axons (Rossi and Keirstead, 2009). In addition, NPCs and MSCs have also been reported to promote some degree of remyelination and improved functional outcome after SCI (Chopp et al., 2000, Hawryluk et al., 2014).

1.11 Cell transplantation

As discussed previously, it is well known that in many cases of SCI there is formation of a fluid filled cavity surrounded by a glial scar. Hence, treatments that fill this gap and replace damaged cells with functional cells that support the remaining parenchyma and also provide a scaffold for regenerating axonal tracts would be ideal. In addition there is a need to provide a milieu favourable for regeneration, enhanced plasticity and induce remyelination. Transplantation may be an appropriate approach to address many of these properties required of an effective therapy. Various cell types have been investigated in SCI models including Schwann cells (Duncan et al., 1981, Biernaskie et al., 2007), neural

stem/progenitor cells (Akiyama et al., 2001), macrophages (Rapalino et al., 1998), mesenchymal stem cells (Akiyama et al., 2002), olfactory glial cells (Riddell et al., 2004, Toft et al., 2007) and oligodendrocyte precursor cells (Bambakidis and Miller, 2004, Keirstead et al., 2005). For an extensive review on cellular transplants see Tetzlaff et al. (2011) (Tetzlaff et al., 2011).

1.11.1 Stem cells

Stem cells are cells with the capacity of self-renewal and for producing a progeny that can differentiate into different mature cell types. Stem cells have been identified in the embryo and in several organ systems in the adult. Stem cells have the ability to differentiate into cells from any germ cell line and have the capability to expand in quantities required for cell transplantation. These properties make stem cells potential candidates for transplant mediated repair in SCI (Rossi and Keirstead, 2009).

The benefits of stem cells have been well documented in pre-clinical scenarios and they offer several potential therapeutic benefits. The rationale for using stem cells in SCI includes replacement of lost neural tissue, secretion of trophic factors, prevention of cyst formation and regulation of the glial scar (Sahni and Kessler, 2010). However, the effects of stem cells are multifactorial and it is difficult to ascertain which mechanism contributes to the beneficial effects shown by different types of stem cells. Transplantation of different types of stem cells has been shown to promote recovery in rodent models of SCI as discussed below. Several types of stem cells have also been tested in safety studies in human SCI with modest improvement in function (Sahni and Kessler, 2010).

1.11.1.1 Embryonic stem cells

Embryonic stem cells (ESCs) are pluripotent cells derived from the inner cell mass of the blastocyst and have the ability to self-renew. Also ESCs have the ability to differentiate into cells derived from all three germ layers. ESCs were first derived from pre-implantation mouse embryos (Evans and Kaufman, 1981) and later also isolation from human embryos (Thomson et al., 1998). These cells are known to grow indefinitely in culture without undergoing senescence. ESCs

can be banked stably and also maintain a normal karyotype and differentiation potential for years. Currently hESCs show great potential for replacement therapies as these are commercially viable (Coutts and Keirstead, 2008).

ESCs are not normally directly transplanted into injury models due to ethical concerns surrounding the use of human embryos in order to isolate ESCs and secondly, due to safety concerns for their potential to form teratomas (Silva et al., 2014). These cells are usually pre differentiated into a desirable cell population *in vitro* before being transplanted. OPCs differentiated from ESCs have been transplanted into contusion SCI and demonstrated to improve locomotion and remyelination (Keirstead et al., 2005, Nistor et al., 2005). McDonald et al. (1999) used neurally differentiated mouse ESCs in rat spinal cord 9 days after injury and demonstrated cell survival and differentiation into astrocytes, oligodendrocytes and neurons along with functional improvement (McDonald et al., 1999).

1.11.1.2 Induced pluripotent stem cells

Induced pluripotent stem cells (iPSCs) have been obtained from mouse and human fibroblasts by introduction of four genes (Sox2, Klf4, Oct3/4 and c-Myc) into the cells (Takahashi and Yamanaka, 2006, Takahashi et al., 2007). These cells have been shown to have properties characteristic of ESCs such as differentiation potential, cell surface antigens, gene expression and telomerase activity (Takahashi et al., 2007). These cells offer advantages over hESCs, firstly, they circumvent ethical issues by allowing auto-transplantation and secondly they could reduce the risk of immunological rejection when used for autologous transplantation (Takahashi and Yamanaka, 2006, Takahashi et al., 2007).

Miura and colleagues (2009) demonstrated that these cells can be differentiated into functional neurons, astrocytes and oligodendrocytes (Miura et al., 2009). However they also reported they have the same potential for forming teratomas as ESCs (Takahashi and Yamanaka, 2006, Takahashi et al., 2007). In SCI, NSCs derived from iPSCs have been used in immunodeficient mouse contusion models showing differentiation into neurons, astrocytes and oligodendrocytes. They also lead to improvements in behavioural and electrophysiological recovery with no malignant transformation (Tsuji et al., 2010, Nori et al., 2011). Furthermore, it

is reported that transplantation of iPSCs which have been pre-differentiated into neuroepithelial like stem cells are able to support regeneration of CST pathways and promote functional recovery of hindlimbs in mice (Fujimoto et al., 2012).

1.11.1.3 Neural stem cells

Neural stem cells (NSCs) also called neural progenitor cells (NPCs) are multipotent cells which have the ability to differentiate into neurons, oligodendrocytes and astrocytes. These are endogenous cells found in the adult and developing CNS. These can be isolated from different regions and expanded *in vitro* as neurospheres (Gage, 2000). The rationale for using NPC is that their neural origin means they more readily differentiate into neural phenotypes and are less likely to become neoplastic compared to ESCs (Coutts and Keirstead, 2008). There is also evidence that they secrete neurotrophic factors like NGF, GDNF and BDNF (Lu et al., 2003). NSCs have also shown to protect against excitotoxic injury (Lu et al., 2003, Llado et al., 2004). However, in most instances transplanted NSCs in normal or injured spinal cord either remained undifferentiated or differentiate along a glial lineage limiting the efficacy of direct NSC transplantation (Shihabuddin et al., 2000, Cao et al., 2001). Nevertheless, several studies have reported functional improvements and evidence of axonal regeneration (Hofstetter et al., 2002, Tarasenko et al., 2007, Parr et al., 2008b). However, pre-differentiation into more committed lineages especially oligodendrocyte differentiation shows more promising outcomes (Hwang et al., 2009, Alexanian et al., 2011b).

Human NSCs are usually obtained from human cadavers and foetuses; this limits the potential supply (Coutts and Keirstead, 2008) and moreover makes it difficult from ethical aspects. Similarly, NSCs derived from adult sources are more difficult to expand into large quantities to be used in clinical settings (Morshead et al., 1998, Doetsch et al., 1999) and cells lose their differentiation potential with time in culture (Wright et al., 2006).

1.11.1.4 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are one of the most promising stem cell types for SCI. There is evidence that they promote functional recovery by providing a

neuroprotective and neuroregenerative environment after injection into injured spinal cord (Phinney and Prockop, 2007, Rossi and Keirstead, 2009). MSCs are multipotent cells with the capability of developing into many cell types including bone, muscle, cartilage and neural tissue (Minguell et al., 2001). They were first discovered by Alexander Friedenstein as spindle shaped cells in cultures derived from bone marrow (Friedenstein et al., 1974). In addition to their derivation from adult bone marrow, MSCs have been identified in several other tissues and organs of both fetal and adult origin (Campagnoli et al., 2001, Meirelles et al., 2006, Sessarego et al., 2008) and can easily be harvested from a variety of tissues including adipose tissue (Zuk et al., 2001), olfactory mucosa (Tome et al., 2009), umbilical cord blood (Erices et al., 2000) and embryonic tissue (Thomson et al., 1998, Lee et al., 2010). However, MSCs derived from mature differentiated tissue have more restricted potential for differentiation as compared to embryonic tissue. MSCs are particularly appealing because they are relatively easy to isolate and expand in culture with only subtle loss of potency (Jiao et al., 2011). They can be used for both autologus (Fehlings and Vawda, 2011) and allogenic transplants without having adverse immune reactions (Jiao et al., 2011). Moreover, they do not undergo neoplastic transformation (Lee et al., 2010) and have demonstrated safety in early clinical trials (Lazarus et al., 1995).

1.11.1.4.1 Therapeutic effects of MSCs

The beneficial effects of MSCs are believed to be due to their ability to modulate the detrimental effects of the SCI milieu (Uccelli et al., 2011). These cells have been shown to provide neuroprotection against glutamate excitotoxicity (Lu et al., 2011) and also secrete neurotrophic factors (Neuhuber et al., 2005, Crigler et al., 2006). In addition, there is evidence these cells can promote remyelination (Chopp et al., 2000, Liu et al., 2011) and regeneration (Sasaki et al., 2006, Sheth et al., 2008) and also function as axon guiding strands across the lesion cavity (Hofstetter et al., 2002). Furthermore, MSCs have been shown to reduce levels of pro-inflammatory molecules by modulating the immune system (Chidgey and Boyd, 2008). It is also reported that MSC may transdifferentiate into glial and neuron like cells at least *in vitro* (Chen et al., 2006), however, transdifferentiation *in vivo* is debatable.

1.11.1.4.2 MSCs survival in SCI models

Survival and proliferation of MSCs after transplantation into SCI models is quite variable. There are reports showing low survival of transplanted MSCs after SCI (Neuhuber et al., 2005, Himes et al., 2006, Sheth et al., 2008, Boido et al., 2014). In one study survival was as low as 1% after 4 weeks (Boido et al., 2014), while in another study gradual decline in the number of cells was observed over time with only 15% of cells surviving by 3 weeks (Wu et al., 2003). However, better survival with up to 50% of cells surviving at 11 weeks is also reported (Himes et al., 2006). Survival of MSCs in the studies were attributed to timing of transplant as improved survival of cells was observed in 1 week delayed transplant as compared to transplants done immediately after injury (Himes et al., 2006). This poorer survival after acute transplants is attributed to the hostile environment of the injured spinal cord immediately after injury.

1.11.1.4.3 Differentiation of MSCs

There are mixed reports on the differentiation ability of MSCs after transplantation into SCI models. Although, neural differentiation into oligodendrocytes, astrocytes and neurons reported, convincing evidence is lacking. In the majority of studies MSCs remained undifferentiated when transplanted into SCI models (Ankeny et al., 2004, Ohta et al., 2004, Yoshihara et al., 2006). However, there are reports of MSCs expressing neuronal and glial markers (Jendelova et al., 2004, Dasari et al., 2007). Dasari 2007 reported marked differentiation of BM-MSCs into neurons, oligodendrocytes and astrocytes (Dasari et al., 2007). In contrast, Lee et al. (2003) claimed possible astrocytic differentiation of the transplanted BM-MSCs, however, there was no evidence of neuronal differentiation using MAP2 or NeuN immunostaining (Lee et al., 2003a). In one study MSCs were shown to express neuronal markers *in vitro* but these cells did not show signs of impulse conduction with whole cell recordings (Hofstetter et al., 2002). In addition, MSCs expressing neuronal markers *in vitro* did not show any signs of differentiation *in vivo* (Lu et al., 2005). Regardless of whether or not MSCs differentiate into neural cells, MSC transplants in SCI have been reported to promote repair and improve functional outcome as discussed below.

1.11.1.4.4 Regeneration and remyelination after MSC transplantation

One of the beneficial effects attributed to MSCs is their ability to promote remyelination in the injured spinal cord. Extensive remyelination is reported in many studies (Chopp et al., 2000, Lu et al., 2007, Liu et al., 2011). Increased remyelination is noted by increased expression of MBP in one study (Liu et al., 2011). Furthermore, some investigations revealed Schwan cell myelination after MSC transplants (Lu et al., 2007) while myelination by MSCs themselves is reported by others (Akiyama et al., 2002). In contrast, no remyelination was observed in demyelinated axons after intralesional MSC transplantation (Hunt et al., 2008).

The axonal regeneration is also reported to be facilitated by MSCs and this is attributed to secretion of neurotrophic factors like BDNF by transplanted MSCs (Lu et al., 2005, Sheth et al., 2008, Sasaki et al., 2009). Extensive axonal regeneration has been claimed in some studies (Chiba et al., 2009). Chiba et al. used anterograde tract tracing with Fluoro-ruby and reported a high density of regenerating dorsal CST fibres in animals transplanted with MSCs. In addition they also claimed co-localization of neuronal markers like NeuN and MAP2 in the transplanted MSCs (Chiba et al., 2009). However, in the majority of the studies axonal regeneration beyond the transplant could not be demonstrated (Lu et al., 2005, Cao et al., 2007, Lu et al., 2007). In one of the study significant number of neurofilament positive and Ctb traced sensory axons were found at the lesion site penetrating through the glial scar in animals transplanted with MSCs as compared to control animals (Lu et al., 2007).

1.11.1.4.5 Anti-inflammatory effects after MSC transplantation

Anti-inflammatory effects of MSCs are also reported by many studies. MSCs are shown to reduce inflammation by decreasing microglia and macrophage reactivity (Neuhuber et al., 2005, Himes et al., 2006). It is thought that up-regulation of anti-inflammatory factors like TGF-ß1 by MSCs reduces the inflammatory response (Hawryluk et al., 2014). Secondly, these cells have also been shown to modify the macrophage phenotype from M1 (considered pro-inflammatory) to M2 (anti-inflammatory). This shift of macrophage activation favours axon and myelin sparing (Nakajima et al., 2012). This

immunomodulatory effect of MSCs further makes the conditions favourable for axonal regeneration and promotes tissue repair.

1.11.1.4.6 Tissue repair/cavity reduction

There are reports that MSC helps in the reduction of the lesion size and help preserve the surrounding normal tissue (Wu et al., 2003, Ankeny et al., 2004, Bakshi et al., 2006, Boido et al., 2014). This reduction is due to neuroprotective effects of transplanted MSCs via reduction of glial cavity and increased sparing of white matter. It is assumed that MSCs penetrate the glial cavity and reduce its volume by degrading the inhibitory molecules leading to reduction of the cavity (Boido et al., 2014). There is also evidence of secretion of laminin which contributes to reduced cavitation and supports nerve regeneration (Wu et al., 2003, Ankeny et al., 2004).

1.11.1.4.7 Functional outcomes after MSC transplantation

There are several studies which demonstrate functional improvements after MSCs transplantations in different animal models of SCI (Lee et al., 2007, Abrams et al., 2009, Osaka et al., 2010, Pal et al., 2010, Alexanian et al., 2011a, Kang et al., 2012, Boido et al., 2014). The improvement of function is observed in different injury models including contusion (Hofstetter et al., 2002), compression (Urdzikova et al., 2006) and transection (Kamada et al., 2005). Transplantation of adult rat bone marrow derived MSCs seven days after thoracic contusion injury is reported to lead to a significant improvement in BBB score (Chopp et al., 2000, Hofstetter et al., 2002, Pal et al., 2010). Similar results have been reported when adult human MSCs have been delivered intravenously (Cizkova et al., 2006a, Osaka et al., 2010) or injected directly into injury site (Himes et al., 2006). In contrast, there have been reports that MSCs have failed to show functional improvement after transplantation into SCI (Ankeny et al., 2004, Lu et al., 2005, Sheth et al., 2008, Sasaki et al., 2009). However, recently a meta-analysis showed a trend towards functional improvement in contusion and compression injury models. This analysis showed that significantly higher BBB scores were reported in transplanted animals compared to non-transplanted controls with none of the studies reporting any detrimental effects after MSC transplants (Oliveri et al., 2014). Improvements in electrophysiological

recordings of MEP and BBB scores have ever been reported in a completely transected injury model with axonal regeneration across the lesion site being claimed (Kang et al., 2012).

1.11.1.4.8 Effect of dose on functional outcome

In one study improvements in function even been reported to be dose-dependent suggesting enhanced recovery of function in animals transplanted with large amount of cells compared to smaller doses (Pal et al., 2010), however, there are findings that increasing the dose of MSCs does not have significant effect on functional improvements (Kang et al., 2012) or any effect on the tissue sparing and cavity reduction (Bakshi et al., 2006).

1.11.1.4.9 Effect of timing of transplantation on functional outcome

Similarly, there are also different reports on the effect of timing of transplantation on the functional outcome and tissue improvement in SCI model. Transplants performed sub-acutely have revealed more encouraging results (Chopp et al., 2000, Hofstetter et al., 2002, Cizkova et al., 2006a) as compared to transplants carried out immediately after SCI (Ankeny et al., 2004). Bakshi and colleagues performed MSCs transplants at different time points after contusion SCI to optimize a suitable time for MSC transplants. They found that cell survival was significantly better if transplants were performed before 2 weeks, however, they also observed MSCs survival in a 4 week delayed transplantation (Bakshi et al., 2006). Partially filled cavities and improvements in BBB score are also reported after MSC transplantation at 3 months post injury (Zurita and Vaquero, 2006).

Delaying the transplants after injury increases the chances of graft survival as the inflammatory responses after SCI tend to subside with time. Furthermore, improved graft survival can lead to robust secretion of growth factors and neurotrophins which can further enhance the regenerative capabilities of transplanted MSCs and hence improve functional outcome.

Although MSCs derived from bone marrow are the most widely studied in SCI models, promising results are also observed with the transplantation of human umbilical cord blood (Cao and Feng, 2009) and adipose tissue derived (Ra et al.,

2011) MSCs in experimental SCI models. These have been shown to reduce glial scar formation (Veeravalli et al., 2009), decrease cavity size and increased tissue sparing (Park et al., 2011, Shang et al., 2011) along with promotion of significant functional improvements (Lim et al., 2007, Lee et al., 2011).

1.11.1.4.10 MSCs transplant in SCI patients

Pre-clinical animal studies suggest that MSC transplants have translational potential in SCI. MSC transplantation has been attempted in clinical settings for human spinal cord injury but results obtained have not been promising. Slight improvement in motor score and electrophysiological recordings were observed in 3 out of 10 patients with ASIA A injury after transplantation of autologous MSCs from bone marrow in 6 months follow up (Park et al., 2012). In another study only one patient out of 13 transplanted with autologous bone marrow derived MSCs showed improvement from ASIA A to B (Bhanot et al., 2011). However, results from clinical trials have shown that MSC transplantation in SCI patients is safe with no major transplant related complications (Sykova et al., 2006, Bhanot et al., 2011, Park et al., 2012).

1.11.2 Other cell types

1.11.2.1 Olfactory ensheathing cells

Olfactory ensheathing cells (OECs) are a glial cell type present only in the olfactory mucosa. These cells have the ability of lifelong regeneration and act as support cells surrounding the olfactory axons. OECs can be obtained from nasal biopsies and although difficult to isolate and cultured to obtain sufficient cell numbers (Feron et al., 2005, Lima et al., 2006) can also be used for autologous transplantation. OECs have been used in experimental models of SCI focussing on regeneration, remyelination and functional recovery with variable results.

Some studies have reported long distance axonal regeneration (Li et al., 1997, Ramon-Cueto et al., 2000, Keyvan-Fouladi et al., 2003), while others have failed to observe this (Andrews and Stelzner, 2004, Ramer et al., 2004, Lu et al., 2007). There is evidence of functional improvement after transplantation of OECs in animal models of SCI both in acute and chronic injuries (Nash et al., 2002, Guest et al., 2008, Takeoka et al., 2011, Ziegler et al., 2011). However, a

large number of experiments failed to demonstrate any therapeutic improvements after transplantations in SCI models (Ramer et al., 2004, Riddell et al., 2004, Steward et al., 2006, Deumens et al., 2013). Tetzlaff et al. reviewed preclinical studies using OECs and concluded that about two-thirds of the experimental studies reported showed improvements in behaviour outcome (Tetzlaff et al., 2011). In addition to regeneration, OECs have also been shown to improve functional outcome by neuroprotective mechanisms (Takami et al., 2002b, Sasaki et al., 2006) and local plasticity (Chuah et al., 2004). It is suggested that the methods of obtaining and purifying cells along with their source could affect the ability of OECs to support neuronal survival and regeneration after SCI (Novikova et al., 2011).

1.11.2.2 Schwann cells

Schwann cells (SCs) are one of the most studied cell types in SCI transplantation. These cells produce the myelin sheath that surrounds PNS axons and play a key role in endogenous repair of peripheral nerve. Therapeutic effects of SCs are thought to be mainly due to their ability to produce a variety of growth factors, by expression of surface adhesive molecules and production of extracellular matrix molecules, which support axon growth (Mirsky et al., 2002, Oudega and Xu, 2006, Park et al., 2010a). Endogenous SCs also have the ability to proliferate, de-differentiate and infiltrate the site of a SCI (Xu et al., 1997, Weidner et al., 1999, Hill et al., 2006).

SCs are relatively easy to isolate (from peripheral nerves), purify and expand in culture to generate a considerable number of cells. These cells can potentially also be derived from different categories of stem cells (Park et al., 2010a). SCs transplanted into SCI models have demonstrated reduced cavity formation and sensory and spinal axon extension into grafts. Moreover, these regenerated fibres were myelinated and also led to improved locomotor function (Takami et al., 2002b). Axonal regeneration along with remyelination and improvement in electrophysiological function has also been reported in many studies with SC transplants (Li and Raisman, 1994, Tuszynski et al., 1998, Kohama et al., 2001). However, it has been shown that SCs aggravate the astrocytic reaction which limits less effective integration into the host spinal cord (Lakatos et al., 2000,

Shields et al., 2000). In addition, CST axons show poor and delayed regeneration after SC transplants (Keyvan-Fouladi et al., 2005) and the axons do not grow through the grafts (Pinzon et al., 2001). However, when used in combination with other neurotrophic factors and blockers of inhibitory factors improved therapeutic outcomes are reported (Chen et al., 1996, Chau et al., 2004).

1.11.2.3 Oligodendrocytes progenitor cells

Oligodendrocyte precursor cells (OPCs) are precursors to oligodendrocytes and when differentiated form the myelin sheath which supports and insulates central axons. The myelin sheath is essential for normal propagation of impulses in axons. As discussed previously, demyelination is a common feature in SCI and leads to loss of function of the affected axons. OPCs are therefore a potential candidate in SCI repair. OPCs can be derived from stem cells including human and animal embryonic stem cells.

OPCs injected into demyelinated spinal cord differentiate into oligodendrocytes and show myelination of affected axons (Rossi and Keirstead, 2009). OPCs are also shown to provide trophic support and repair the environment by up regulation of neurotrophic factors such as TGF-B2 and BDNF (Zhang et al., 2006). Limited axonal sprouting is also observed but this is often abortive due to insufficient trophic factors to overcome the inhibitory environment and therefore, a combinatory therapy might be a more appropriate for therapeutic outcome (reviewed in (Watson and Yeung, 2011). OPCs transplanted into thoracic contusion SCI seven days post-injury (Keirstead et al., 2005) or immediately after complete spinal cord transection (Erceg et al., 2010) led to remyelination and improvement in locomotor function (Keirstead et al., 2005, Erceg et al., 2010). Similarly, OPCs transplanted 7 days after cervical SCI in rats showed improvements in forelimb function, reduction in cavity formation, increased sparing of white and grey matter and altered gene expression (Sharp and Keirstead, 2009).

1.11.2.4 Activated macrophages

The immune system is known to contribute to healing of damaged tissue. However, in damaged CNS it is believed to have some harmful effects and

suppression of the immune response might be helpful in prevention of ongoing damage. Macrophages especially monocytes are capable of expressing different functional programs in response to environmental signals (Nagorsen et al., 2005). It is known that “classical” activation of macrophages turns them into potent effector cells that destroy microorganisms and tumour cells. On the other hand, “alternatively” activated macrophages are capable of producing growth factors, promoting angiogenesis and scavenging neurotoxins such as glutamate (Butovsky et al., 2006).

It is observed in animal models of SCI that activation of intrinsic macrophages by pro-inflammatory agents has negative effects on functional outcome (Popovich et al., 2002). However, depletion of macrophages led to significant improvement in overground locomotion, sparing of white matter and a decrease in tissue cavitation (Popovich et al., 1999). Schwartz and Yoles (2006) have reported that adequately activated macrophages can positively contribute to homeostasis in the CNS. However, the timing of treatment was found to be important for successful functional recovery. The optimal time for transplantation was found to be 1-2 weeks post-injury (Schwartz and Yoles, 2006).

1.12 Summary of study aims

The aims of this thesis were as follows:

1. The aim of work described in chapter 2 was to use a direct electrophysiological approach to investigate and characterise the effects on spinal cord function of a moderate contusion SCI at the cervical level. Local circuit function was assessed by recording CDPs immediately after injury and at different time points for up to 6 months after SCI in order to characterise the time course of damage contributed by primary and secondary processes and to determine any spontaneous recovery of function in the vicinity of the impact. It is hypothesized that high velocity injury would produce both primary and secondary damage that would be revealed by assessing function at different time points after injury.

2. The aim of work described in chapter 3 was to combine the injury model and electrophysiology used in chapter 2 with assessments of function in order to determine whether delayed transplantation of human embryonic stem cell derived mesenchymal stem cells (hESC-MSCs) promote improved recovery of function.

2 Responses of the local somatosensory pathways to cervical contusion injury

2.1 Introduction

Traumatic injury to the spinal cord leads to disruption of ascending and descending pathways leading to motor, sensory and autonomic dysfunction. Initial trauma causes primary injury and loss of function but this is followed by a cascade of secondary molecular and cellular events. These events include both degenerative and reparative mechanisms. Some of these processes are thought to contribute to mechanisms of progressive damage leading to further deterioration and loss of function. Concurrently, other mechanisms promote limited repair and spontaneous recovery of function over time.

As reviewed in chapter 1 (Introduction) several processes are postulated to contribute to the progression of the secondary damage. Briefly, there are vascular changes (haemorrhage, thrombosis, vasospasm) and disruption of blood brain barrier which causes oedema and ischemic necrosis of the surrounding tissue. In addition, formation of free radicals causes disruption of membranes leading to ionic imbalance and loss of functional integrity. Apoptosis, necrosis and excitoxicity is observed in all neural tissue. The inflammatory response from resident microglia and infiltrating macrophages further aid cellular death and also help phagocytose cellular debris leading to cavity formation. Astrocytes along with oligodendrocyte precursors and meningeal cells lead to glial scar formation, which surrounds the cavity. Demyelination and axonal degeneration can continue for months to years along with extension of the injury cavity.

The mechanisms behind spontaneous recovery of function are also not fully understood and it is suggested that injury induced plasticity or rearrangements in the nervous system may play a role in functional recovery after SCI (Raineteau and Schwab, 2001). Anatomical and synaptic plasticity in the injured and remaining un-injured axons have been observed in animal models of SCI. Merzenich et al. 1984 illustrated that non-functioning synapses and pathways become functional after injury (Merzenich et al., 1984). Sprouting is also observed in the spared axons and these may form new functional synapses (Weidner et al., 2001, Bareyre et al., 2004). Remyelination of the injured axons by the endogenous oligodendrocyte precursors and infiltrating Schwann cells has also been shown to improve functional outcome (James et al., 2011). Although it

is shown that adult neurons have the intrinsic ability to regrow over long distances (David and Aguayo, 1981), anatomical evidence suggests that this regrowth is inhibited by the hostile milieu after SCI. Therefore regeneration is unlikely to contribute to the spontaneous functional recovery.

Although it is known that both secondary injury processes and reparative processes are triggered by the initial injury, the interacting dynamics and net effects of these two processes are poorly understood and the time course with which they impact on spinal cord function is not clear. In fact, we do not know to what extent function in white matter tracts and local circuits in the grey matter are affected by the initial impact of a contusion injury. Similarly, it is unclear how much secondary injury mechanisms add to the initial loss of function which occurs in the first few hours after injury, and how long these processes continue to cause deterioration of function. Furthermore, we are not sure when the secondary processes resolve, and/or the endogenous repair processes outweigh the continuing deterioration leading to functional improvement. In addition the time over which functional improvements continue to occur, and the time point at which an injury can be considered chronic in a rodent contusion injury model, is also undetermined.

Most of our understanding of secondary processes comes from cellular, molecular and anatomical studies and loss of axons or neurons are used as indicators of functional loss. There has been so far no direct measurement of the effects of secondary mechanisms on spinal cord function. Similarly, recovery of function is regularly assessed by behavioural testing in animals, but these measure overall function and do not directly measure function in the spinal cord. Compensatory changes in the brain are likely to contribute to recovery of function (Jain et al., 1997, Ghosh et al., 2009) and it is therefore difficult to distinguish recovery due to compensatory plastic changes within the brain and restorative and reparative mechanisms in the spinal cord. Therefore, a method is required which can directly measure function in the local circuits and specific long tracts within the spinal cord.

2.2 Aims

The aim of the study described in this chapter was to use an electrophysiological approach to directly assess the functional changes in local spinal circuits in the vicinity of a spinal cord contusion injury.

The aim was to use this approach to investigate the following:

1) To assess how much damage is caused acutely by the contusion injury.

2) To determine the contribution made by secondary processes to loss of function.

3) To determine how long function in local spinal circuits and pathways continues after injury.

4) To establish the time point at which repair/functional improvements at the spinal level begin.

5) To determine if there is any plasticity in the spinal circuits/pathways.

6) To determine how long functional improvement in local circuits and pathways continues after injury and when the SCI has functionally stabilised.

To assess the functional changes in the ascending and descending pathways of the spinal cord, cord dorsum potentials (CDPs) evoked by stimulation of the radial nerve and CST stimulation at the level of the pyramids were recorded in the vicinity of the contusion injury from the surface of the cord. Comparison of these potentials were made in normal animals and in contusion injured animals at time points range from acute injury to 6 months following injury.

2.3 Materials and methods

2.3.1 Animals

All the experiments in this project were approved by the Ethical Review Process Application Panel of University of Glasgow and performed in accordance with the UK Animals (Scientific Procedure) Act, 1986.

A total of 158 male adult Lister Hooded rats (Harlan, Loughborough, UK) were used in this study. The Lister Hooded strain was used as these animals are good at performing reaching tasks which we had originally planned to carry out along with the electrophysiology. Among these, twenty-four (24) rats were used as non-injured controls for comparison with injured animals. One of the control animals died during the preparatory surgery for electrophysiology. All other animals (134) were subjected to bilateral contusion injuries of 175 kilo-dyne (Kdyn) severity. Injuries were intended to be performed at the 6^{th} cervical level of the spinal cord. Among the injured rats, 7 animals died during the post-operative recovery period and 2 animals developed scratching at the surgical wound and were euthanized. 13 animals died during the preparatory surgery for electrophysiology experiments. In addition, injuries were found to be erroneously located rostral or caudal to the C6 segment in 12 animals and these were excluded from the final analysis. Furthermore, in 10 of the non-injured and 7 injured animals pyramidal evoked CDPs could not be included either due to difficulty in obtaining correct placement of the stimulating electrode within the pyramids. Therefore, the findings in this chapter are based on a total of 124 animals for the radial nerve evoked CDPs and 107 for the pyramidal evoked CDPs. The number of animals for radial nerve and pyramidal evoked CDPs for each group of animals are shown in Table 2-1. All the animals were housed pair-wise in cages with a 12 hour light/dark cycle and bedding was changed every week.

2.3.2 Contusion injury

Contusion injuries in this project were performed by Dr. John Riddell and Mr Andrew Toft with assistance from myself and the animal house technical staff. It

would take me a long time to reach a level of consistency; all surgeries were performed by Dr Riddell and Mr. Toft.

2.3.2.1 Drugs

All the animals undergoing contusion injury routinely received pre-operative medications as required.

Buprenorphine (Vetergesic): 0.05 mg/kg was administered subcutaneously at the time of induction of anaesthesia for analgesia. A second dose was given the following morning.

Carprofen (Rimadyl): This non-steroidal anti-inflammatory drug was given at a dose of 5 mg/kg body weight subcutaneously at the time of surgery.

Saline: 0.2-0.4 ml/100 gm of body weight was administered subcutaneously two or three times daily for three days and as required afterwards.

Enrofloxacin (Baytril): This antibiotic was administered at a dose of 5 mg/kg subcutaneously twice daily for 7 days starting on the day of surgery.

Ampicillin (Amfipen): This antibiotic was administered at a dose of 22.5 mg/kg subcutaneously once pre operatively.

An ophthalmic ointment was applied to the animals eyes at the time of surgery to prevent drying.

2.3.2.2 Anaesthesia

Animals were anaesthetized with 5 % isoflurane in oxygen at the time of induction. Anaesthesia was maintained using 1-3 % isoflurane in oxygen for the rest of the surgery.

2.3.2.3 Contusion injury procedure

After induction of anaesthesia, the animal was placed in a stereotactic frame with a head holder. The T2 vertebra was identified and fixed with the help of a

clamp to straighten and position the cord horizontally. The vertebral column was exposed at the cervical level by a midline incision. The cervical spinal cord at C6 was exposed by performing a laminectomy to remove the C5-C6 laminae. The vertebral bodies of C4 and C7 were clamped with lockable Adson's forceps to stabilize the spine.

The Infinite Horizon Impactor™ (Precision Systems and Instrumentation, LLC, Lexington, KY, USA; Figure 2-1), a commercially available instrument was used to perform the contusion injury. The impactor tip was aligned with the midline of the exposed spinal cord, using the left and right-side C6 dorsal roots as a visual guide. The edge of the impactor tip was then aligned with the border between C5 and C6 dorsal roots (Figure 2-2). A force of 175 kdyn was set and a computer controlled stepper motor was activated. The impactor tip was lowered at approximately 120 m/s to contuse the exposed spinal cord until the user-defined force was reached, triggering automatic retraction of the tip. Graphs of force and displacement vs. time were generated by the IHI software and saved and stored for further analysis offline (Figure 2-3).

Graphs generated by the software provided an instant indication of whether the impactor tip was hitting the cord accurately with a desired force. Any graph with a blunted or a second peak on the ascending phase of force vs. time graph was suggestive of the impactor hitting the surrounding bone, along with reduced displacement of the cord. However, this rarely happened in this study.

For animals which were allowed to recover post injury, the muscle layers were closed using a 3-0 vicryl suture and the skin was closed with wound clips. Post-operative medications as described previously were administered routinely. Animals were allowed to recover and placed in a warm incubator (27-30 ºC) for 24 hours with easy access to food and water. Afterwards the animals were transferred to the normal housing conditions for a period of up to 6 months depending on the time course group allocated. The animals which were intended for acute CDPs recordings underwent electrophysiological recordings immediately after contusion injury.

2.3.2.3.1 Post-operative care

Animals were checked post operatively for recovery from anaesthesia and during the night for any signs of distress and dehydration. Bladder was manually palpated 2 times daily for 3-4 days until the normal voiding reflex has returned. Post-operative care was provided by the animal house technical staff in the morning and at night by myself or Andrew Toft.

2.3.3 Electrophysiological experiments

2.3.3.1 Anaesthesia

Anaesthesia was induced with 5 % isoflurane in oxygen and maintained afterwards with 1-3 % isoflurane in oxygen during the initial surgery. Subsequently, the anaesthesia was maintained with frequent intravenous doses of sodium pentobarbital (10 mg/kg) when required, after jugular venous cannulation (described in 2.3.3.4). The depth of anaesthesia was monitored by assessing pedal withdrawal reflexes, corneal reflexes, electrocardiogram and arterial blood pressure. Further doses of anaesthetic were administered if there was a withdrawal reflex due to pinching of the limb or blood pressure increased due to pinching or if the resting blood pressure was abnormally high.

Throughout the recording phase, animals were paralysed and artificially ventilated (explained below) and hence withdrawal reflexes were eliminated. During this time, anaesthetic was routinely given at regular intervals, consistent with the rate required before paralysing. The adequacy of this regime was checked by continuous monitoring of arterial blood pressure and its response to noxious stimuli and additional anaesthetic top ups could be given if required.

2.3.3.2 Pre-operative drugs

Before commencement of the surgery atropine sulphate (Martindale Pharmaceutical, UK) 0.05 mg/kg was administered subcutaneously to reduce bronchial constriction and salivary secretions.

Dexamethasone sodium phosphate (Faulding Pharmaceutical Plc, UK) was injected intramuscularly at a dose of 2-3 mg/kg to prevent swelling of brain and spinal cord.

2.3.3.3 Preparatory surgery

After induction of anaesthesia the animal was placed in a supine position on the operating table. Core body temperature was monitored with a rectal thermometer and maintained at 37°C using a heating blanket. The animal was secured with adhesive tapes attached to each limb and needle electrodes were placed in the skin of both forelimbs and right hind limb to continuously monitor the heart rate and electrocardiogram. Fur was removed from the relevant areas with the help of an animal fur clipper before making any skin incision. A dissecting microscope (Zeiss S5, Germany) with foot controller was used to aid fine surgery including cannulations, radial nerve dissection and laminectomy. To stop bleeding, an electrocautery (Eschmann Equipment, TD50, UK) was used.

2.3.3.4 Cannulations

The carotid artery, jugular vein and trachea were cannulated in order to monitor arterial blood pressure, administer intravenous drugs and artificially ventilate the animal after paralysing, respectively. A midline skin incision was performed overlying the trachea. The skin and muscles are retracted and with careful blunt dissection through the muscles and connective tissue about a 2 cm length of carotid artery was exposed and separated from the vagus nerve. The artery was ligated distally with 5-0 silk thread and clamped proximally. A small incision was made near the distal ligature and a cannula inserted in the direction of the heart and tied in place before removal of the clamp. The cannula was filled with heparinised saline (50 units/ml heparin in saline) to prevent blood clotting and maintain patency of the vessel for continuous monitoring of arterial blood pressure. The arterial cannula was attached to a force transducer to allow measurement of mean arterial blood pressure. The jugular vein was exposed and cannulated in a similar way, which permitted administration of intravenous drugs. Usually, the right carotid artery and jugular vein were cannulated.

For tracheal cannulation the muscles overlying the trachea was retracted and a small length of trachea was cleared from surrounding tissues and any bleeding staunched. A small opening was created using fine micro scissors and the tracheal cannula was inserted and tied in position. Secretions from the lungs and trachea were aspirated from time to time to maintain patency. All three cannulae were secured to skin by threads in order to prevent dislodging and the skin incision was closed using Michel clips.

2.3.3.5 Radial nerve dissection

For dissection of the radial nerve, the animal was put in a prone position; the left forelimb was extended laterally and fixed with adhesive tape and fur removed with a clipper. An incision was made on the dorsal aspect extending from the shoulder to the elbow joint. The tendon of the triceps brachii muscle was cut with the help of an electro-cautery to expose the superficial branches of the radial nerve. Approximately 2 cm of the superficial radial nerve was cleared of the surrounding tissue with careful blunt dissection and sectioned distally. A suture was attached to the distal end in order to lift the nerve without damaging it (shown in Figure 2-4). The exposed nerve was then covered with cotton soaked in saline to prevent it from drying.

2.3.3.6 Laminectomy

In order to expose the cervical spinal cord a laminectomy was performed from C2-T1 segments of the spinal cord. A midline incision was made on the dorsal surface extending from the skull to the upper thoracic region of the back. The skin was retracted; the cervicoauricular and trapezius muscles were separated and partially removed from both side of the vertebral column. With the help of bone rongeurs, the posterior arch was cautiously removed to expose the dorsal surface of the spinal cord. In lesioned animals there was already a partial laminectomy done previously at the time of contusion. The cervical spinal cord was exposed from C2-T1 segments and in the case of the injured animals, the injury cavity was obvious as a greyish area at the level of the C6 segment. For preventing bleeding from bone edges bone wax and gel foam were used. Extreme caution was exercised in order to avoid any damage to the cord which is crucial to retaining good function. To prevent the cord and dura from drying out

before making the recordings, cotton soaked in saline was placed over the exposed cord.

2.3.3.7 Transferring the animal to the recording frame

After the initial preparatory surgery, the animal was transferred to the recording frame. The animal's head was placed in the head holder and secured by an incisor bar and ear bars. The spinal cord was straightened and fixed with the help of a clamp that gripped the T2 dorsal bony process. Securing the animal with bars and clamps provided a steady horizontal position for recordings.

2.3.3.8 Artificial ventilation

In order to prevent movement when stimulating the radial nerve and CST at the level of the pyramids, it was necessary to paralyse the animal using a neuromuscular blocker. The animal was given Pancuronium Bromide (Sigma, UK) in a dose of 0.3 mg/kg i.v. with regular top up doses every 30-40 minutes or as required. Immediately afterwards, the animal was put on an artificial ventilator (Ventilator, UGO, Italy) and carbon dioxide levels were monitored in expired breath using a micro-capnometer (Columbus Instruments, USA). The CO_2 levels were maintained around 4% throughout the experiment by adjusting the ventilation rate and stroke volume. The tracheal cannula was cleared of any fluid accumulation due to secretions from lungs.

2.3.3.9 Craniotomy

In order to insert a stimulating electrode into the brain to stimulate the CST bundle in the pyramid a small craniotomy was performed in the skull. The CST fibres within the pyramids were stimulated by a dorsal approach. Co-ordinates for stimulation of the CST were calculated before making the craniotomy (see below).

A bipolar electrode was used to maximise the current density close to the tip and reduce stimulus spread. The electrode was mounted on a Kopf U-frame at an angle of 20 degree and the tip of the electrode was calibrated before the experiment with an ear bar set in the frame. Calculation were made from a stereotaxic atlas of the rat brain and the following co-ordinates were chosen

(medio-lateral: 0.7 mm to the right; anterio-posterior: slightly rostral to intra-aural line, depth approximately 1 mm below the intra-aural line) with the aim of targeting the middle of the right pyramidal tract (Paxinos and Watson, 1998). The positions of the co-ordinates on the Vernier scales of the electrode carrier were noted. During the experiment the electrode was lowered with animal's head fixed in the head holder the electrode was lowered and the point where the tip would be touching the skull was noted. A small craniotomy was then created allowing room for the electrode to be moved a few mm lateral or medial if needed in order to reach the middle of the pyramidal bundle.

2.3.3.10 Paraffin pools

Paraffin pools were created around the posterior side of the left forelimb and the laminectomy using flaps of incised skin. These were secured with threads passed through the skin incision edges tied firmly to the recording frame. The pools were filled with liquid paraffin maintained at close to 37 ºC using a radiant heat source. This insulation prevented current spread to the surrounding tissue when stimulating the peripheral nerves. Paraffin pools also helped to prevent tissue from drying and heat loss. A small strip of mm paper was put alongside the cord and the C5/C6 border was identified to provide a reference point for positioning of the recording electrode.

2.3.3.11 Stimulation of the radial nerve

The radial nerve was carefully mounted on a bipolar silver stimulating electrode immersed in the liquid paraffin pool. Care was taken to avoid any contact between the nerve and the surrounding tissue. Cathodal stimuli were applied by positioning the cathode of the stimulating electrode proximally and the anode distally. Once the nerve was in position, the electrode was secured firmly in position. The nerve was stimulated with single square wave electrical pulses of 0.2 ms duration and up to 50-100 μA.

2.3.3.11.1 Technical considerations for supramaximal stimulation of the radial nerve

In order to obtain a quantitative measure of the strength of CDPs evoked by stimulation of the primary afferent pathways which can be comparable between

animals it is essential to activate the same pathways to an equal extent in each animal. To obtain maximal activity in myelinated cutaneous A-α and A-β fibres, a supra-maximal stimulus intensity was applied to the radial nerve. Recordings were made at a point 2 mm rostral to the C5/C6 border, where maximum radial evoked CDPs were obtained in an injured animal. For each animal, a supramaximal stimulus intensity was determined by finding the threshold for activation and then increasing the stimulus intensity until a maximal CDP amplitude was seen (i.e. maximum A-α and A-β postsynaptic response).This was typically between 20-50 μA and supra-maximal intensity used for stimulation was typically set at 50-100 μA.

2.3.3.12 Stimulation of the corticospinal tract

The corticospinal tract was activated by stimulating corticospinal bundles within the pyramids using a dorsal approach. A concentric bipolar microelectrode was used to maximise the current density close to the tip of the electrode and reduce stimulus spread. The electrode shaft diameter was 0.25 mm with a 50 mm length (SNE-100X, Kopf instruments, Tujunga, CA, USA). The impedance and insulation of each electrode was checked before each experiment (typically less than 100-200 kΩ).

After adjusting the sterotaxic co-ordinates and securing the holder the electrode was lowered through a small opening made in the dura into the brain. It was lowered to a position 1 mm above the inter-aural line. Initial electrical stimuli of 0.2 ms duration and 500 μA amplitude were applied until the optimum position of the electrode was found while monitoring the CDPs and examining the recruitment characteristics. Typically, the final stimulation amplitude was between 200-500 μA. The usual initial location for recording CDPs while examining recruitment was 4 mm rostral to the C5/C6 border. The electrode was initially lowered in 0.5 mm steps, followed by 0.2 and 0.1 mm steps while monitoring the size of the CDP and determining the threshold. Sometimes the base of the skull was reached without seeing optimum conditions for eliciting pyramidal evoked CDPs. In that situation the electrode was withdrawn to the surface and after moving the electrode either medial or lateral 0.5 mm, the electrode was lowered again to find the most accurate position.

2.3.3.12.1 Technical considerations for CST stimulation

To be sure that only the right CST bundle was activated, a stimulus recruitment curve for pyramidal-evoked CDPs was collected. This was done firstly by finding the minimum strength of current at which the pyramidal-evoked CDP could be detected (usually equal to or less than 10 μA). Recordings of the CDP were then made while increasing the intensity of the stimuli applied within the pyramids in graduated steps from threshold to values that were supramaximal. When the electrode was appropriately placed the stimulus-recruitment curve would show a sharp rise in CDP response, with increasing stimulus intensity, followed by a plateau. The value producing a maximal CDP response was determined with this procedure and a supramaximal value was then chosen for that particular experiment. In cases where the stimulating electrode was too close to the midline, the stimulus-response curve would show a consistent rise with increasing stimulus intensity. In cases where the electrode was too far lateral, the maximal CDP response would likely be low but the threshold high.

A further reason for the reduced amplitudes of CDPs in the injured spinal cord may be that the current flow produced by synaptic activity is conducted away more rapidly through the fluid in the injury cavity than in the solid spinal cord tissue which may have greater resistance.

2.3.3.13 Recording of cord dorsum potentials

In order to assess spinal cord function in the vicinity of the injury, CDPs evoked by stimulation of the radial nerve and corticospinal tract were recorded. A bipolar silver ball electrode was used to make these recordings by placing one pole on the dorsal surface of the cord. The indifferent pole was placed on the nearby muscles. CDP recordings were made at 1 mm intervals, from 8 mm rostral to 8 mm caudal of the C5/C6 border (Figure 2-5). The electrode was aligned according to the mm paper strip placed alongside the exposed cord. At each position the electrode was placed just medial to the dorsal root entry zone on the left side.

CDP recordings were made by either stimulating the radial nerve or CST fibres within the pyramids with a stimulus repetition rate of approximately 3 Hz. An

average of 25 sweeps were recorded for radial nerve stimulation, while 50 sweeps were averaged for the smaller CDPs collected during pyramidal stimulation. A typical CDP trace for radial and pyramidal CDPs is shown in Figure 2-8.

During the recordings, great care was taken to remove any cerebrospinal fluid accumulation on the surface of the cord. CSF accumulation tended to short circuit the recording electrode and results in smaller amplitude potentials which is a potential source of inaccuracy. Therefore, the fluid was removed regularly between the recordings after moving the electrode to new a recording position, before making the recordings and also during the recordings if CDPs tended to decrease in amplitude.

The recordings of all electrical potentials were digitised using a CED 1401+ interface (Cambridge Electronic Design, Cambridge, UK) and stored on a computer at a sampling rate of 26 kHz. Signal software version 2.1 (Cambridge Electronic Design, Cambridge, UK) was used for offline analysis to average and measure the amplitudes of electrical potentials. A schematic of the radial CDP recording procedure is shown in Figure 2-6.

2.3.3.14 Recording of cord dorsum potentials immediately after injury (acute experiment group)

After the initial set up for the electrophysiology experiment, up to the point of making the paraffin pools (without adding the paraffin) the procedure for recording immediately after injury in the acute time point group was the same as described above. At this point the cord was firmly fixed in position using Adson forceps prior to injury. The injury procedure was identical to that for contusion injuries in the animal house. The injury was made using the IHI impactor at 175 kdyn force, with the dura intact. The exact time at which the impact occurred was noted; then the Adson forceps were removed and the dura was opened. The laminectomy pool was filled with paraffin, the CDP recording electrode was placed in position and the pyramidal stimulating electrode was inserted into the brain through the craniotomy. The position of the pyramidal electrode was then optimised (as described above in 2.3.3.12).

The time required to complete this procedure was close to 20 minutes. Therefore after the initial 2 experiments it was decided to standardise the start of the first recordings at 20 minutes post-injury. Each recording map (+8 to -8) took about 20-30 minutes and therefore it was feasible to record every 30 minutes after injury. This was continued for approximately 4 hours. Both pyramidal and radial recordings were obtained for each recording location. A schematic diagram showing the arrangement for recording of pyramidal evoked CDPs is shown in Figure 2-7.

2.3.4 Perfusion fixation

At the end of the experiment animals were transcardially perfused with mammalian Ringer's solution followed by paraformaldehyde solution. Animals were perfused by inserting a 20 g needle into the left ventricle and perfusing with mammalian Ringer's solution containing 0.1 % lidocaine in order to dilate the blood vessels. This was followed by 1 litre of 4 % paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The cervical spinal cord and brain stem were then carefully dissected out for post-fixation and cryoprotection. This was achieved by placing the tissue overnight in the same paraformaldehye solution with 30 % sucrose added.

2.3.5 Histological processing

2.3.5.1 Histological processing of brainstem CST stimulation site

After washing in 0.1 M PB solution, the electrode track was identified and a tissue block was prepared carefully. The block was notched at the ventral aspect of the spinal cord on the left side (opposite to the site of insertion of the electrode). The blocks were sectioned transversely at 100 µm using a freezing microtome (Ernst Leitz Wetzlar, Germany). Sections with electrode tracks were then mounted on plain glass slides in vectashield mounting medium (Vector laboratories, UK). These were then covered with a glass cover-slip, sealed with nail varnish and stored at -20 ºC.

2.3.5.2 Histological processing of spinal cord injury site

The spinal cord tissue was washed with 0.1 M PB and the injury site was identified and cut into a 6 or 7 mm long block. Dorsal and ventral roots were carefully trimmed using fine scissors without damaging the lesion site. The block was notched at the ventro-caudal aspect (for orientation purposes when sectioned) and the block was then cut into 60 µm parasagital sections using a Leica CM1850 cryostat (Leica Microsystems GmbH, Wetzlar, Germany) at -20 ºC. Occasionally, when cutting through the cavity at the point where the cavity was very extensive, some of the fragile sections would break into pieces and could not be recovered for analysis. This happened rarely. Afterwards, sections were washed in 0.3 M PBS double salt (PBSD) twice for 10 minutes and incubated in 50 % ethanol for 30 minutes. The sections were then washed three times for 10 minutes each in 0.3 M PBS and treated with different primary antibodies. Sections were incubated with anti-NF200 (1:1000, Sigma)/anti-GFAP (1:1000, Dako) and anti-Laminin (1:100)/anti-GFAP (1:1000) primary antibodies for 72 hours at 4 ºC, diluted in 0.3 M PBS plus 0.3 % triton detergent to label axons/astrocytes and basal lamina/astrocytes respectively. This was followed by appropriate species specific secondary antibodies conjugated to fluorophores; Alexa 488 (1:500) and Rhodamine (1:100) diluted in 0.3 M PBS plus 0.3 % triton detergent, for 3-4 hours at room temperature and covered to protect from sunlight. These were then washed with 0.3M PBS three times for 10 minutes each, followed by mounting on plain glass slides in anti-fade medium (Vectashield). Sections were coverslipped, the edges sealed with nail varnish and stored at -20 ºC in darkness.

2.3.6 Cortical tracer injection

In order to investigate the effects of the injury on the integrity of the corticospinal pathway 3 animals were subjected to injury in the same way as the animals prepared for electrophysiological recording. Cortical tracer injection using biotinylated dextran amine (BDA; 10,000 MW, product no. D-1956, Life Technologies) was performed 2 weeks after the C6 contusion injury in order to label the CST. The tracer was prepared as 20 % BDA in 0.1 M phosphate buffer with 2 % fast green dye (R.A. Lamb supplies, UK; product 42053) in order to detect spillage of tracer during injection into the cortex. BDA cortical injections

were performed by Mr. Andrew Toft with my assistance. The scalp was incised and craniotomy was performed through the skull to expose the right sensorimotor cortex using bregma as a reference point. A glass micropipette of 30-40 µm tip diameter was filled with BDA tracer and mounted on a stereotaxic manipulator. Injections were performed at 10 sites 1 mm apart in a grid pattern ranging from 1 mm rostral of bregma and 2 mm caudal and up to 4 mm laterally. Approximately 300 nl of BDA was injected at each injection site from 1.8 mm below the surface up to a depth of 1.0 mm. The scalp incision was sutured closed. Animals were recovered and allowed to survive for 2 week after the injections to allow the time needed for movement of tracer along the CST fibres.

2.3.7 Microscopy

2.3.7.1 Verification of stimulation site in the pyramids

Sections containing stimulating electrode tracks were reviewed under a light microscope (Zeiss Axioplan 2 Imaging) to confirm the appropriateness of the location of the electrode in the pyramids.

2.3.7.2 Verification of contusion injury

Sections containing the injury cavity were viewed by phase contrast light microscopy to determine the cavity perimeters and the relationship between the grey and white matter boundaries. In general all sections with the injury site were also examined under the epifluorescence microscope (Zeiss Axioplan 2 Imaging) to observe neurofilament/GFAP and laminin/GFAP immunolabelling and parasagittal sections passing through the injury area close to the midline were selected for confocal scanning and illustration. Selected sections were scanned with a Zeiss LSM 710 confocal system using X20 objective lenses. Laser excitation wavelengths used for scanning were 488 nm fluorescent (Alexa 488) and 561 nm fluorescent (Rhodamine). Tissue sections were scanned as a tiled composite of multiple fields to cover the whole section. All sections were scanned through a 14-18 µm thickness of the stained tissue to accumulate a series of optical sections at 2 µm intervals. Stacked images were projected into 2D builds (maximum intensity projection image) using Zeiss Zen software (Zeiss,

Germany). Images were exported to Adobe Photoshop CS3 (Adobe Systems, USA) and prepared for illustration by making minor changes to brightness and contrast.

2.3.7.3 Quantification of cavity dimensions

To investigate difference in the extent of the cavity between 7 weeks and 6 months survival animals, calculations of cavity length and width were carried out. For calculation of the cavity length all parasagittal sections containing the injury site were examined under epiflourescence with X2.5 objective lenses, projected on the monitor connected to the microscope. Most rostral and caudal points in the cavity were noted and distances between the 2 points were measured using a ruler calibrated with the graticule. Most rostral and caudal borders of the injury cavity identified and the distance was measured with the ruler. Similarly, for the width of the cavity all the sections with cavities were counted under the microscope and multiplied by 60 (each section has 60 µm thickness) to get measurement in micrometres. Cavity length and width were expressed in mm.

2.3.8 Off-line analysis of electrophysiological data

Averaged records of CDPs were examined offline to measure the amplitude of CDPs with the help of Signal software V2.1. The maximum amplitude at each location was measured and data collected in a Microsoft Excel spread sheet before being transported into GraphPad Prism 4 software (GraphPad Software Inc, USA), to create graphs.

2.3.9 Statistical analysis

The statistical analysis was performed using GraphPad Prism 4 software. One-way Analysis of variance and post-hoc Tukey's multiple comparison tests were performed for each recording location (+8 to -8) among all the experimental groups in the study to reveal any differences among the groups in each recording location. A paired Student's t-test was applied to compare the cavity length and width between 7 weeks and 6 month survival animals.

Time point investigated	Total number of animals (n)	Animals for radial recordings (n)	Animals for pyramidal recordings (n)
Normal	24	23	13
Acute	17	14	12
3 days	15	15	14
2 weeks	24	15	13
4 weeks	21	15	15
7 weeks	14	13	12
3 months	19	14	14
6 months	23	15	14
Total (n)	158	124	107

Table 2-1. Table showing number of animals used at each time point.

Total number of animals assigned to different groups at each time point is shown on the left. Numbers from which radial-nerve evoked CDP data was obtained are shown in the middle and numbers from which pyramidal-evoked CDP data was obtained are shown on the right.

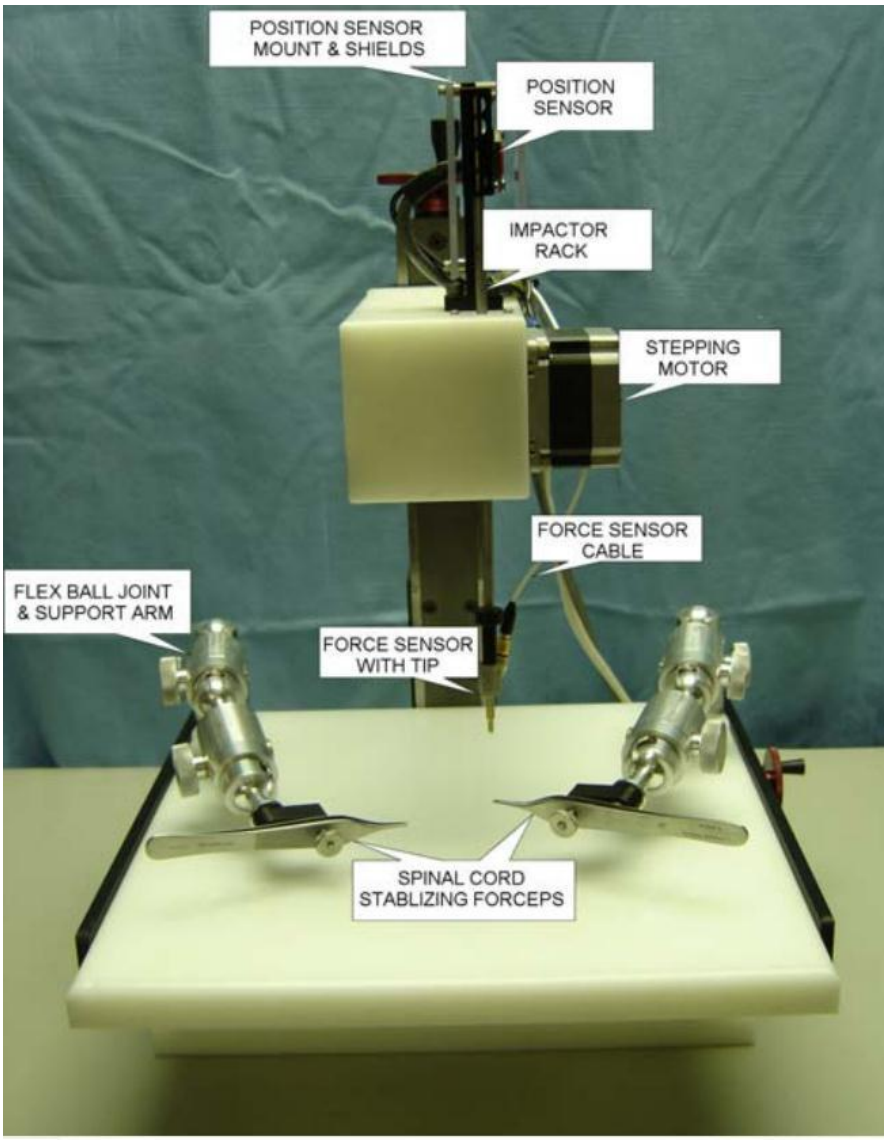

Figure 2-1. The Infinite Horizon Impactor.

The adjustable support arms were used to position the adson forceps. The forceps were lockable and could be used to stabilize the animal. The impactor tip was then lowered and moved over the exposed spinal cord to be positioned over the C6 segment. After positioning, the computer controlled stepping motor was activated which drove down the impactor rack. The force and position sensor were connected to the lower end of the impactor rack and signals were passed to the control unit via cable connected to the control unit (image taken from the PSI-IH Impactor user manual 2007).

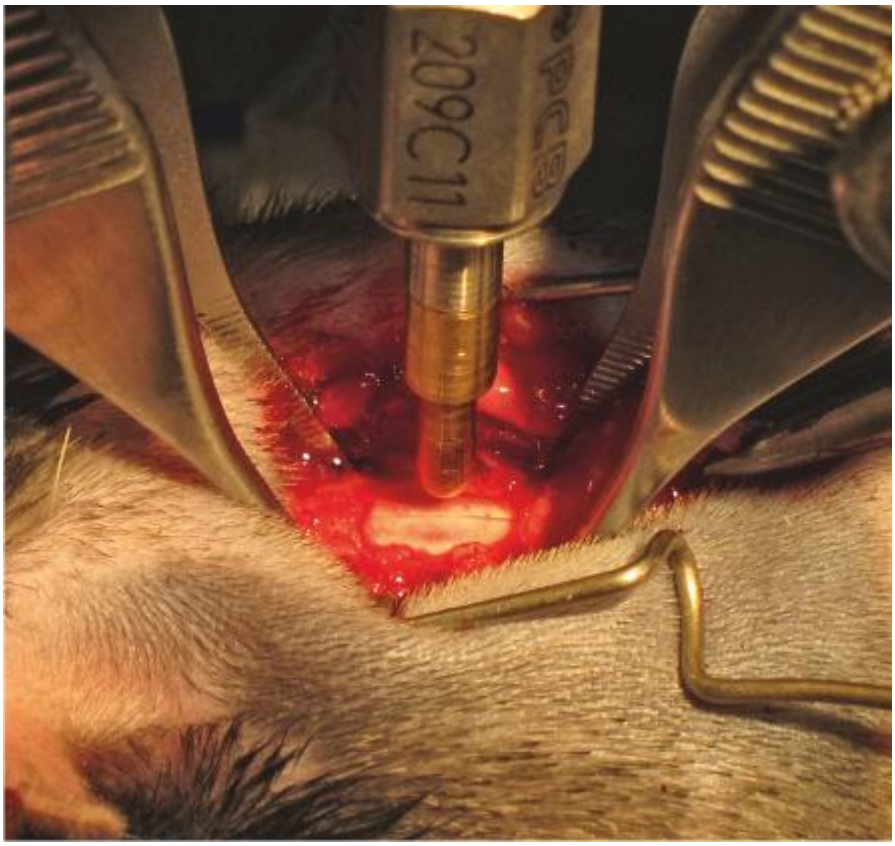

Figure 2-2. Contusion injury set up.

The C6 contusion injury involved performing a laminectomy between C5-C7 vertebrae and firmly fixing the C5 and C7 vertebrae with Adson forceps. The Infinite Horizon Imapctor was then positioned over the exposed C6 spinal cord segment and a computer controlled contusion of 175 Kdyn force was performed (Image taken from Mohamad 2014).

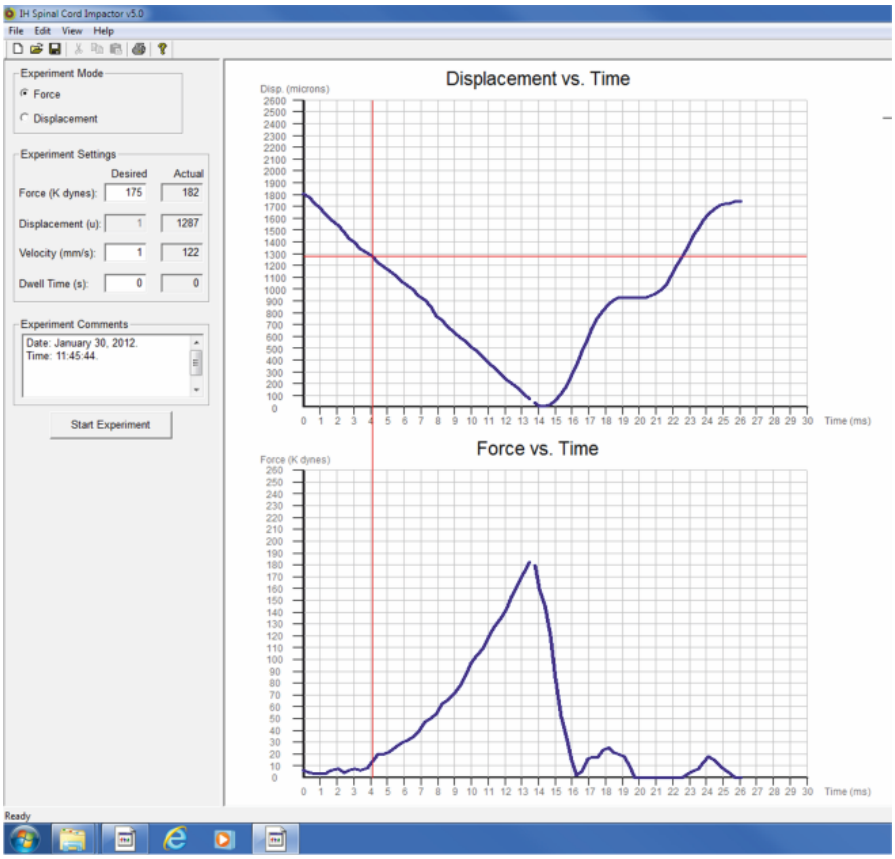

Figure 2-3. Screen shot of the Infinite Horizon Impactor software.

Example of the information provided by the IHI software immediately after each contusion. The upper graph shows the displacement vs. time and lower graph shows force vs. time for a 175 Kdyn impact. The actual displacement and force are shown numerically in the boxes on the left of the window.

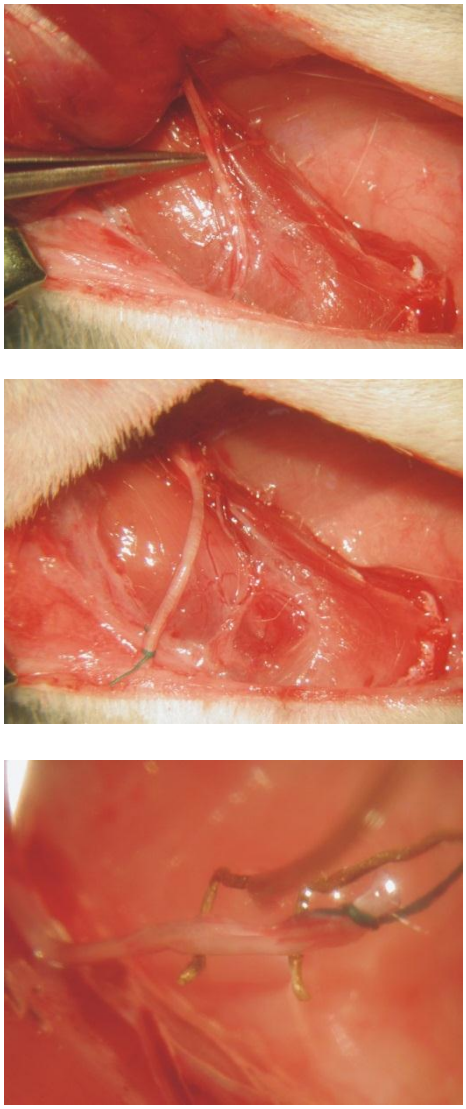

Figure 2-4. Radial nerve dissection and stimulation.

This set of images shows the stages in dissection and presentation of the radial nerve for electrical stimulation. The upper image shows the exposed superficial branches of the radial nerve. The middle image shows the branches of the radial nerve dissected out, sectioned, and with a suture loop attached to the distal end. The lower image shows the radial nerve placed on bipolar silver wire electrodes immersed in a paraffin pool (Image adapted from (Meng, 2009).

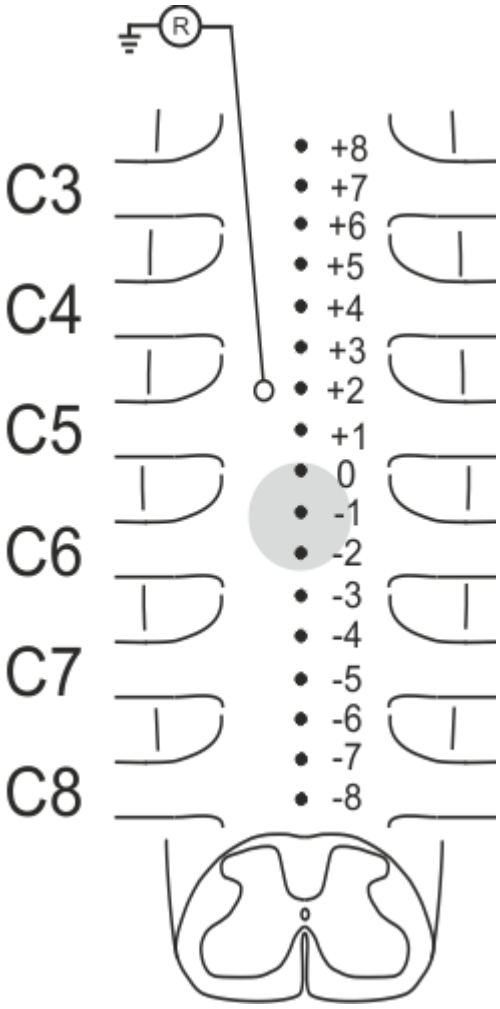

Figure 2-5. Schematic diagram showing locations for CDP measurements.

A silver ball electrode was positioned just medial to the dorsal root entry zone. Recordings were made in 1 mm steps rostral and caudal to injury location with C5/C6 border taken as 0 reference point. Positive sign indicates rostral while negative sign indicates caudal to C5/C6 border. (Image adapted from original images from Dr John Riddell).

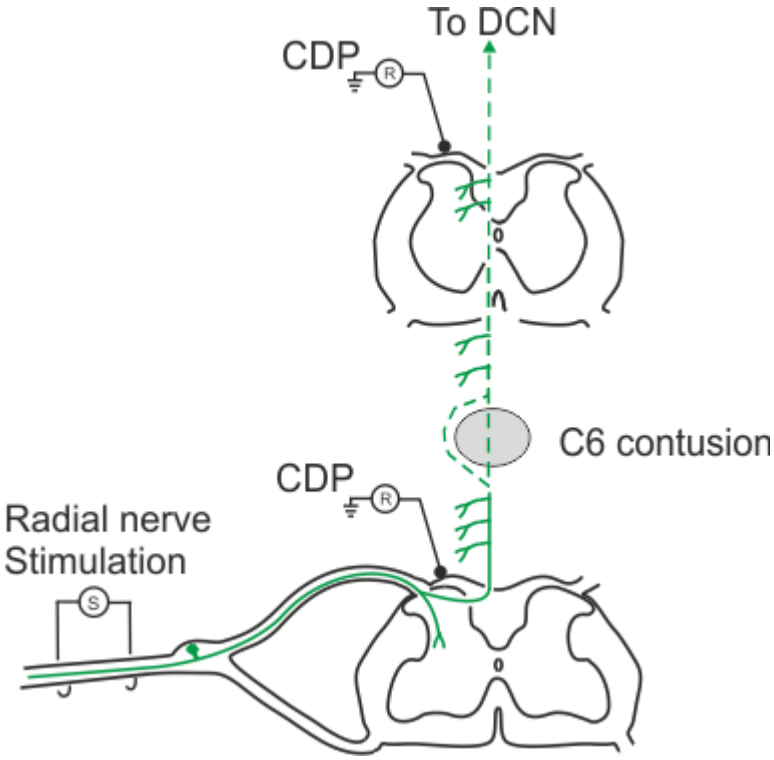

Figure 2-6. Schematic diagram showing the set up for recording of radial evoked CDPs.

The radial nerve was stimulating using a bipolar silver electrode and CDP recordings made from the dorsal surface of the spinal cord. (Image adapted from original images from Dr John Riddell).

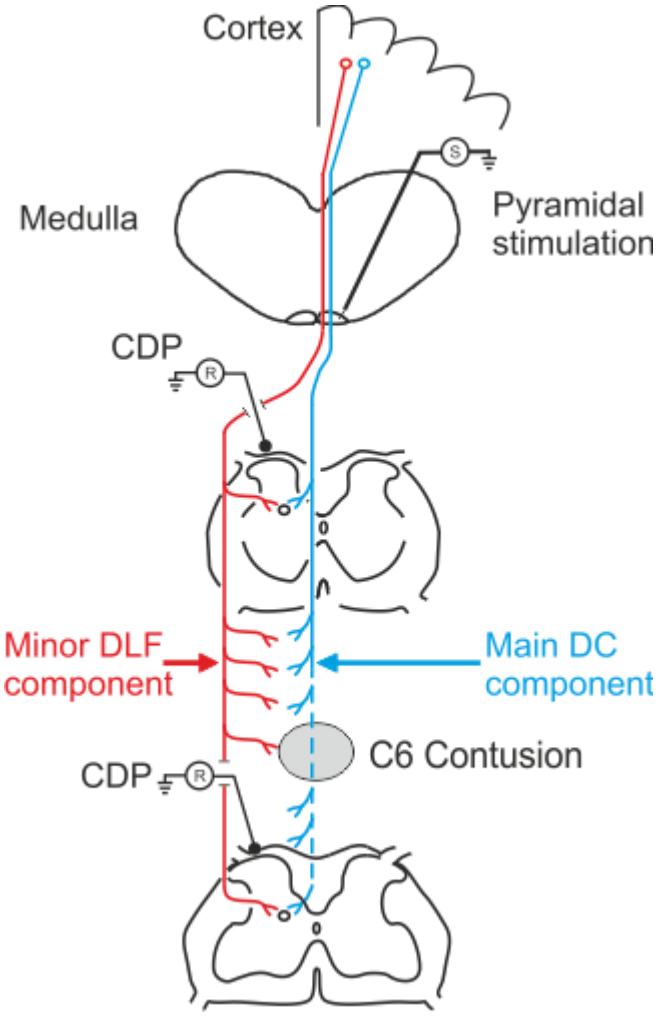

Figure 2-7. Schematic diagram showing set up for recording of pyramidal evoked CDPs.

Pyramidal bundles at the level of the pyramid were stimulated using a concentric bipolar electrode. The electrode was inserted stereotaxically by a dorsal approach through a hole made in the back of the skull. (Image adapted from original images from Dr John Riddell).

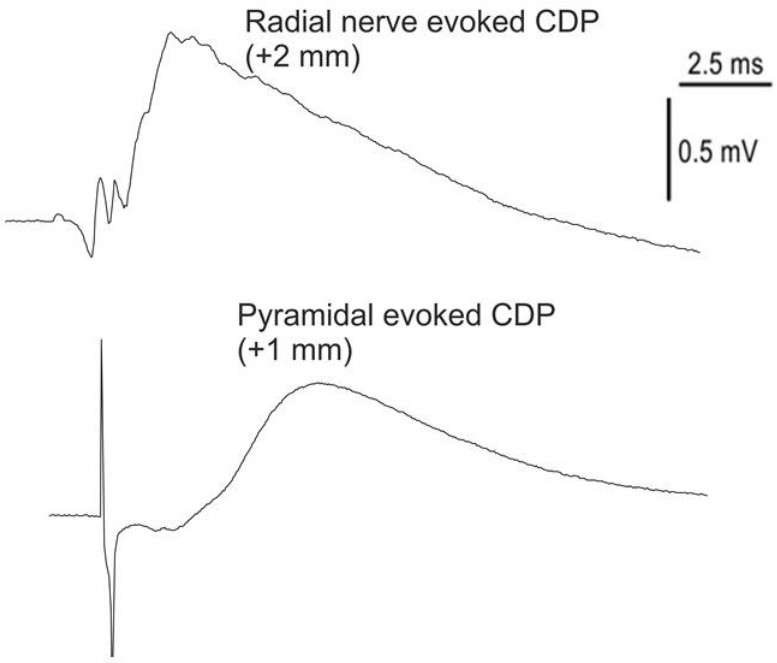

Figure 2-8. Examples of typical CDP traces.

Upper trace shows CDP evoked by pyramidal stimulation. Lower trace shows CDP evoked by radial nerve stimulation.

2.4 Results

2.4.1 Electrophysiological assessment of local circuit functions following contusion injury

In this study, cord dorsum potentials (CDPs) evoked by stimulating the radial nerve and CST stimulation within the pyramids were used to assess function in the cervical spinal cord. Recordings made from normal animals (n=23) and animals with a contusion injury (n=101) at the C6 level of the spinal cord were investigated at different time points. The different experimental groups and the number of animals used in each group are summarized in Table 2-1.

2.4.1.1 Comparison of body weights and injury severities

It was essential to limit the variable parameters in all the experiments, in order to ensure that any differences observed in the electrophysiological recordings were only due to differences in the survival time. Animals from the different experimental groups investigated at different survival times received the same injuries. A moderate severity injury was selected and a force of 175 kdyn was used for all animals. All the injuries were made with the IHI device (as described previously) and the injury parameters provided by IHI software were recorded and graphed for comparison, so that any atypical animals could be excluded from the study. IHI software is capable of recording the actual force pushing on the cord at the time of impact and the displacement of the impactor tip. These recordings were used to confirm that all animals received similar injuries. These comparisons for actual force and displacement were graphed and are shown in Figure 2-12 and Figure 2-13 respectively.

However, contusion injuries of the same impact force with the same sized impactor tip produce different degrees of damage depending on the size of the cord. The weights of animals used in most of the groups was broadly similar at the time of the injury (see Figure 2-9). An exception to this was the animals used to investigate the effect of acute injuries. The animals used in this group were larger and more closely matched those of the normal animals and the animals in the other groups at the time of the electrophysiological recording (see Figure 2-10).

The size of the animals at the time of the electrophysiology is also relevant as it is important that the length of the cord and hence the recording locations (which were at 1 mm intervals for 8 mm above and below the injury) should be the same relative to segmental location for animals in each of the groups. The weights of the animals at electrophysiology are shown in Figure 2-10. Because of the different survival times of each of the groups there are differences in the weights of the animals in each group. The lowest weights are for the animals in the 3 day group because this is the shorter survival time and because the animals lose weight in the first few days after the injury operation. However, because all of the animals are fully mature adults these differences in weight are not associated with significant differences in spinal cord length. This is shown in Figure 2-11 where the segmental location corresponding to the -8 recording location is indicated for each of the groups. This is the recording location farthest from the 0 mm reference point and should therefore show the largest discrepancy. As can be seen the segmental location of this recording position in each group shows a very similar distribution and all lay between rostral T1 and caudal C8.

Minimising the variables such as the weights, injury severities and cord displacements, mean that any changes in the electrophysiological recordings seen in the different groups can be attributed to genuine effects of survival time of the animal after injury.

2.4.1.2 Assessment of radial nerve evoked CDPs after contusion injury

Radial evoked CDP recordings were made from normal animals (n=23), acutely injured animals (n=14), 3 day survival animals (n=15), 2 week survival animals (n=15), 4 week survival animals (n=15), 7 week survival animals (n=13), 3 month survival animals (n=14) and 6 month survival animals (n=15) (Table 2-1).

2.4.1.2.1 Components of surface recordings evoked by radial nerve stimulation

Potentials evoked by radial nerve stimulation were recorded with a silver ball electrode positioned on the dorsal surface of the spinal cord at a 1 mm intervals, from 8 mm rostral to 8 mm caudal to the C5/6 border (Figure 2-5). Different components of a CDP recording are shown in Figure 2-14. Electrical stimulation

of the radial nerve produces an afferent volley followed by a distinct negative CDP. The afferent volley is the first (shortest latency) deflection in the surface recording and is a short duration wave which represents action potentials travelling along the afferent fibres of the radial nerve. The afferent volley is seen clearly at locations close to the segment of entry of the stimulated root and it becomes dispersed at locations more rostral and caudal. The afferent volley is followed by a longer duration wave called the CDP. CDPs represent the synaptic activity generated by connections between the collaterals of the primary afferent fibres and spinal cord neurons and these provide a measure of the strength of these connections. The height of the CDP from the start of the volley is the amplitude of the CDP and is measured using Signal software. Examples of radial evoked CDPs recorded from a normal animal and from animals at 4 weeks and 6 months after injury are shown in Figure 2-15.

In order to test the contribution of each spinal root to the CDPs evoked by the radial nerve stimulation (i.e. the level of entry of radial nerve afferents producing the CDPs), dorsal roots were sequentially sectioned in 4 animals, while recording the CDPs from a fixed position. The CDP amplitudes after sectioning the roots were measured and expressed as a percentage of the CDP amplitudes before dorsal root cutting. The contribution of afferents from the radial nerve to the CDPs evoked at different locations is shown in Figure 2-16. The main contribution is from afferents entering through the C6 root (87.1 – 50.3 %) followed by afferents entering via the C7 root (31.4 – 6.5 %). There is minimal contribution from afferents entering more rostrally via the C5 root. This means that the afferents generating radial nerve CDPs enter at or below the level of the contusion injury.

2.4.1.2.2 Radial evoked CDP recordings from normal animals

The CDPs evoked by radial nerve stimulation were investigated in 23 normal animals. Examples of CDPs recorded from a normal animal are shown in Figure 2-15. The rostro-caudal distribution of CDPs for the entire normal animal group is shown in Figure 2-17, where the mean CDP amplitude at each recording location is plotted. In normal animals the largest CDPs were recorded at around 2 mm above and 2 mm below the C5/6 border. This region represents the C6 spinal segment and most of the input of the primary afferents from the radial

nerve enter via the C6 root so that, as expected, the amplitudes are greatest close to where the sensory fibres enter the cord. The mean maximal amplitudes in this region were around 0.95 mV. CDPs further rostral and caudal of the C5/6 border gradually declined in amplitude but could still be detected even at the most rostral and caudal recording locations (i.e. +8 and -8 mm).

2.4.1.2.3 Acute effects of contusion injury on radial nerve evoked CDPs

Mapping of the distribution of the radial evoked CDPs was performed in these animals at 30 minute intervals. The first mapping was started 20 minutes after injury and the last mapping was performed at 3 hours 50 minutes after injury. Examples of CDPs recorded from one acutely injured animal at 8 different time points after injury from 20 minutes to 3 hours 50 minutes are shown in

Figure 2-18. Recordings at the earliest time point after injury (20 minutes) showed that the contusion injury had a substantial effect on the amplitudes of radial nerve evoked CDPs when compared to those recorded from normal animals. Figure 2-19 shows a plot of CDP amplitudes for recordings from acute animals at 20 minutes compared to those recorded from the group of normal animals. As can be seen, the contusion leads to a reduction in amplitude of the CDPs at most of the recording locations with the most substantial reduction being produced in the region of impact (approximately 0 mm to -2 mm). Rostral and caudal to this region of impact the CDPs are much less affected. Figure 2-20 shows plots of the amplitudes of CDPs recorded from acute animals at 5 locations, expressed as a percentage of normal CDP amplitude at the same location. This shows that the amplitudes of CDPs at the centre of the impact (-1 mm) were reduced to less than 20 % of normal (a highly significant difference), while at locations 2 mm either side of the injury epicentre (i.e. at -3 or +1), CDPs were reduced to 60 – 70 % (also highly significant difference). Amplitudes 5 mm away from the centre were less affected still being 70 % (significant difference, $p < 0.05$) at +4 and 95 % at -6 mm (non-significant).

The fact that potentials rostral to the injury are affected similarly compared to potentials below the injury, suggests that the main sensory axons in the dorsal column which carry sensory impulses past the level of the injury are not substantially affected. This in turn suggests that most of the reduction in the

amplitudes is due to damage to neurons and axons collaterals at the injury centre.

Figure 2-21 shows plots of CDP amplitudes for all of the CDP mapping from acutely injured animals which has commenced at 30 minute intervals throughout the period of recording. Figure 2-22 shows selected plots from the beginning (20 minutes), middle (2 hours 50 minutes) and end (3 hours 50 minutes) of the recording period. By recording every 30 minutes it could be seen that the amplitudes of CDPs continued to show changes with time after injury. These affected mainly the potentials recorded at the margins of the injury both rostrally (+1 to +3) and caudally (-3 to -5). The amplitudes of the CDPs at the centre of the impact (-1), which were already strongly affected by the initial impact, showed little further change. Figure 2-20 shows plots of the amplitudes of CDPs recorded from acute animals at 5 locations at the beginning, middle and end of the recording period (as in Figure 2-22), expressed as a percentage of control. As can be seen there is little change in the amplitudes of the potentials at -1 (injury centre) but a progressive reduction in the amplitude at +1 and -3, either side of the injury epicentre. Between the first (20 minute) and last (3 hours 50 minutes) CDP mapping there was further reduction in amplitudes. At the +1 site this was further declined by approximately 17 % and at -3 it was 20 % of normal. However, none of these changes in amplitude in the course of the acute experiment reached significance (i.e. there were no significant differences at any recording location).

2.4.1.2.4 Assessment of radial nerve evoked CDPs 3 days after injury

The distribution of radial evoked CDPs was mapped in fifteen animals at 3 days after contusion injury, the earliest time point investigated in recovery experiments. Figure 2-23 shows plots of radial nerve evoked CDPs where those recorded from 3 day survival animals are compared with those recorded at the beginning (20 minute) and end (3 hours 50 minutes) of the acute experiments. Compared to the last recording made in the acute experiments (approximately 4 hours after injury) there was a further reduction in CDP amplitudes at 3 days but this was only rostral to the injury.

CDPs at the injury centre (-1) were also reduced in amplitude in 3 day animals compared to the last recording in the acute animals while CDPs caudal to the injury (-4 to -8) were larger in amplitude and those farthest from the injury centre (-5 to -8) were restored to the same amplitude as seen in animals immediately following the injury. Figure 2-24 shows plots of amplitudes of CDPs recorded at 5 locations from 3 day survival and acute injury animals. Above the injury at +4 and +1, CDPs were reduced in amplitude by approximately a further 20 % of normal in 3 day animals compared to recording made 20 minutes after injury while at the injury centre the decrease was much more modest (5 % at -1). The reduction in CDP amplitudes above the injury were significant at +2 ($p < 0.05$), +1 ($p < 0.001$) and 0 ($p < 0.01$). In contrast, at the location -6 which is well caudal to the injury, the CDP amplitude increased by approximately 25 % of normal compared to the latest acute recording time point. Therefore the CDP amplitude here was restored to that seen in normal animals.

The reduction in CDP amplitudes above the injury could be explained by reduced conduction in the main sensory axons which carry impulses from the entry level of radial afferents (C6 and C7) past the injury to C5 and C4. This might reflect a secondary injury mechanism such as demyelination of the radial nerve fibres in the dorsal columns. In contrast, the increase in CDP amplitudes seen below the injury suggests that some reparative process has occurred within the first few days of injury.

2.4.1.2.5 Assessment of radial nerve evoked CDPs 2 weeks after injury

The distribution of radial evoked CDPs was mapped in fifteen animals at 2 weeks after contusion injury. Figure 2-25 shows a comparison of radial nerve evoked CDPs recorded from 3 day and 2 week survival animals and immediately following injury in the acute experiments. There was a substantial increase in CDP amplitudes at 2 weeks compared to 3 days after injury. The increase was particularly marked at rostral locations where CDP amplitudes had deteriorated at 3 days (0 to +8 locations). The increase in amplitudes was such that at most locations CDPs were returned to values closely similar to those recorded immediately after injury (20 minute recordings in acute animals).

Figure 2-26 shows a plot in which the amplitudes of CDPs recorded 2 weeks after injury at 5 locations are compared to those recorded at 3 days and immediately after injury in the acute animals. At location rostral to the injury (+4 and +1) the increase in amplitude between 3 day and 2 week survival animals is about 30 % (+4) and 25 % (+1). The increase in CDP amplitudes above the injury at 2 weeks compared to 3 days is significant at +3 ($p < 0.05$), +2 ($p < 0.05$) and +1 ($p < 0.01$). There was a modest improvement of approximately 5 % at the injury epicentre (-1) and caudally at -3. In contrast, at location -6, which is well caudal to the injury, the amplitudes were not affected and remained similar to those recorded at 3 days.

The return in CDP amplitudes rostrally is most likely explained by a repair mechanism affecting the main sensory axons, possibly remyelination of the affected axons.

2.4.1.2.6 Assessment of radial nerve evoked CDPs between 4 weeks and 6 months

To investigate changes in function from sub-acute out to chronic time points, the distribution of radial evoked CDPs was mapped in groups of animals maintained for 4 weeks (n=15), 7 weeks (n=13), 3 months (n=14) and 6 months (n=15) after injury. Figure 2-27 shows plots of CDPs recorded 4 weeks and at the time points between 3 months and for comparison the CDP mapping performed 2 week after the injury. The CDP amplitudes recorded at each of these time points were very similar. CDP amplitudes recorded at 2 weeks change very little at 4 weeks and 7 weeks indicating that function in local circuits has largely stabilized by 2 weeks after the injury. At 3 months, however, there is a tendency for CDP amplitudes below the injury to be smaller than at 7 weeks but this was not significant.

Figure 2-28 shows the distribution of CDPs mapped at 6 months compared to 2 weeks and 3 months after injury. Above the injury CDPs remained remarkably stable between 2 weeks and 6 months after injury. However, below the injury, the trend towards a reduction in amplitude seen in the recordings made at 3 months had progressed further and was clearly evident in the animals investigated at 6 months.

Figure 2-29 shows a plot in which the amplitudes of CDPs recorded 6 months after injury at 5 locations are compared to those recorded at 2 weeks and 3 months after the injury. The bar graphs show the trend for a small (5-10 %) reduction in amplitude between 2 weeks and 6 months after injury which is insignificant at most locations. However, caudal to the injury at -3 mm, CDPs are reduced by 20 % of normal. There is therefore a localized deterioration in function below the injury level at the 6 month time point. However, this trend was not statistically significant.

2.4.1.3 Assessment of pyramidal-evoked CDPs after contusion injury

Pyramidal-evoked CDP recordings were made from normal animals (n=13), acutely injured animals (n=12), 3 day survival animals (n=14), 2 week survival animals (n=13), 4 week survival animals (n=15), 7 week survival animals (n=12), 3 month survival animals (n=14) and 6 month survival animals (n=14) (Table 2-1).

2.4.1.3.1 Components of CDPs evoked by pyramidal stimulation

A concentric bipolar stimulating electrode was used to stimulate the pyramids (as described in methods) and potentials were detected with a recording electrode on the surface of the spinal cord. Recordings were made with the same silver ball electrode as for the radial nerve evoked CDPs and recordings were made on the dorsal surface at 1 mm intervals, from 8 mm rostral to 8 mm caudal of the C5/6 segmental border (Figure 2-5). Examples of pyramidal evoked CDPs recorded from a normal animal and from animals injured at 4 weeks and 6 months after injury are shown in Figure 2-30. CDPs consist of a stimulus artefact which indicates the onset of stimulation, followed by a small afferent volley which represents the action potentials travelling along the CST fibres. This is much less distinct that for radial nerve stimulation and is only seen at locations close to the stimulation site and becomes dispersed further away from the stimulation site. A CDP follows the afferent volley. The height of the CDP from the start of the afferent volley is the amplitude of the CDP. This represents the strength of the synaptic activity generated by the connections between collaterals of the CST fibres and spinal neurons in the grey matter.

2.4.1.3.2 Pyramidal evoked CDP recordings from normal animals

CDPs evoked by pyramidal stimulation were investigated in 13 normal animals. The rostro-caudal distribution of CDPs for the entire normal animal group is shown in Figure 2-31. Pyramidal evoked CDPs are smaller in amplitude than those evoked by radial nerve stimulation and are more evenly distributed over the length of the cervical spinal cord. The largest potentials occur close to the C5/6 border where mean maximal amplitudes were around 0.35 mV. Amplitudes declined above and below this but the decline is more marked caudally probably because the descending volley becomes more dispersed the farther caudally it has to travel.

2.4.1.3.3 Acute effects of contusion injury on pyramidal evoked CDPs

Mapping of the distribution of the pyramidal evoked CDPs was performed at 30 minutes intervals (as carried out for radial nerve evoked CDPs). The first mapping was started 20 minutes after injury and the last mapping was performed at 3 hours 50 minutes after injury. Recordings at the earliest time point after injury (20 minutes) showed that the contusion injury had a substantial effect on the amplitudes of pyramidal evoked CDPs when compared to those recorded from normal animals. Figure 2-32 shows a plot of CDP amplitudes for recordings from acute animals at 20 minutes compared to those recorded from the group of normal animals. As can be seen, the contusion leads to a reduction in amplitude of CDPs at most of the recording locations with most substantial reduction being produced in the area caudal (-2 to -8) to the region of the impact, followed by those in the region immediately rostral to the injury centre (0 to +2). However, farther rostral to the impact area (+3 to +8) CDPs were largely unaffected. Figure 2-33 shows a plot of the amplitudes of CDPs recorded from acutely injured animals at 20 minutes after the injury at 5 locations, expressed as a percentage of normal CDP amplitude at the same location. The CDP amplitudes at caudal locations (-4 and -6) were reduced to less than 8 % of normal and at injury centre (-1) to approximately 20 % of normal. CDPs rostral to the injury epicentre at +4 mm and +6 mm were less affected with a modest reduction of approximately 3 % and 10 % of normal, respectively. The reductions in amplitude at +1 mm and more caudally were all highly significant.

The fact that potentials caudal to the injury are substantially reduced by the injury, suggests the main component of the CST (located in dorsal columns) on which these potentials largely depend is extensively damaged immediately after the injury. The smaller amplitude CDPs evoked caudal to the injury are considered mainly due to stimulation of the minor CST components (the lateral and ventral CST). This is consistent with anatomical findings (see 2.4.3).

Figure 2-34 shows plots of CDP amplitudes for all of the CDP mapping commenced at 30 minute intervals throughout the period of recording from acutely injured animals. By recording every 30 minutes it could be seen that the amplitudes of CDPs remained almost unchanged throughout the acute recording period (20 minutes to 3 hours 50 minutes) at most of the recording locations after the injury.

2.4.1.3.4 Assessment of pyramidal evoked CDPs 3 days after injury

The distribution of CDPs evoked by pyramidal stimulation was mapped in fourteen animals 3 days after contusion injury, the earliest recording time point examined in recovery experiments. Figure 2-35 shows plots of pyramidal evoked CDPs where those recorded from 3 day survival animals are compared with those recorded at the beginning (20 minute) and end (3 hours 50 minutes) of acute experiments. Compared to the recordings made in the acute experiments there was a further reduction in CDP amplitudes at 3 days, which was more marked rostral to the injury where potentials were not changed immediately after injury. This worsening of CDPs suggests reduced conduction in the intact CST fibres rostral to the injury and might reflect some contribution by secondary injury mechanism such as demyelination or die back of these fibres in the dorsal column.

Comparison of CDPs recorded at 5 locations from 3 day survival animals and acute injury animals at 20 minutes is shown in Figure 2-33. Compared to the acute injury animals, CDPs recorded from the 3 day survival animals were reduced by a further 20 % of normal above the injury at +6 mm and +4 mm and by 15 % at the injury centre (-1 mm). CDPs below the injury were also reduced in amplitude in the 3 day animals compared to the acute. The reductions were only a small proportion of normal (approximately 2 %) because they were already

markedly affected by the injury in the acute animals. However, the changes here were significant between +2 and -1 when compared between the two groups of injured animals.

2.4.1.3.5 Assessment of pyramidal evoked CDPs 2 weeks after injury

The distribution of pyramidal evoked CDPs was mapped in thirteen animals at 2 weeks after contusion injury. Figure 2-36 shows plots of pyramidal evoked CDPs where those recorded from 2 week animals are compared to 3 day animals and immediately following the injury in acute experiments. There was a substantial increase in CDP amplitudes at 2 weeks compared to 3 days after injury such that at most locations CDPs were returned to values closely similar to those recorded immediately after injury at 20 minute. An exception to this was the region at the most rostral extent of the mapped region (+6 to +8 mm).

Figure 2-37 shows a plot in which the amplitudes of CDPs recorded 2 weeks after injury at 5 locations are compared to those recorded at 3 days and immediately after injury in the acute animals. The restoration of CDP amplitudes in 2 week animals to those seen acutely can be seen at all locations except +6 where the CDP amplitudes remain closer to that seen in the 3 day survival animal. However, the change was only significant at +1 mm.

2.4.1.3.6 Assessment of pyramidal evoked CDPs between 4 weeks and 6 months after injury

The distribution of pyramidal evoked CDPs was investigated in animals surviving for 4 weeks (n=15), 7 weeks (n=12), 3 months (n=14) and 6 months (n=14) after the injury. Figure 2-38 shows a comparison of CDPs recorded from the animal groups investigated between 2 weeks and 3 months after injury. At the injury centre and above the injury there was very little change in CDPs with amplitudes remaining remarkably stable between 2 weeks and 3 months. However, below the injury (-3 to -7) there was clear evidence of an increase in the strength of corticospinal actions with an increase in CDP amplitudes being evident in the 7 week survival animals and a further clear increase in the 3 month survival group.

Figure 2-39 shows the CDP distributions with 6 month survival animals added. From this it can be seen that there is little change between 3 months and 6 months after the injury.

Figure 2-40 shows a plot in which amplitudes of CDPs recorded 3 months after injury at 5 locations are compared to those recorded at 2 weeks and 6 months after the injury. Caudal to the injury at -4 and -6, CDP amplitudes increased at the 3 month time point to approximately 10 % of normal compared to recordings at 2 weeks after injury as a proportion of the amplitudes at these locations of 2 weeks this represents an increase of 110 %. However, none of these increases reached significance. However, the amplitudes at injury centre (-1) and rostral to the injury remained approximately similar to 2 weeks surviving animals.

2.4.2 Injury site

The appropriate location of the contusion injury was confirmed in all injured animals at the end of the electrophysiological experiments included in this study.

Examples of sections containing lesion cavities from different animal groups are shown in Figure 2-41 to Figure 2-47. Typically between 20 and 25 parasagittal sections were found to contain areas of damaged tissue when examined under the light microscope.

In acutely injured animals, the damage to the spinal cord tissue was mostly observed at the core. Examples of parasagittal sections obtained from animals perfused approximately 4 hours after the injury are shown in Figure 2-41. Sections near the midline of the injury show extensive disruption and damage to the spinal cord parenchyma and loss of variable amount of tissue at the central part of the section. This loss of tissue occurred during tissue processing due to the area of damaged parenchyma being extremely delicate and lacking normal integrity.

Injury sites at 3 days after injury showed more consistent damage to the spinal tissue extending several mm from the centre both in rostral and caudal directions. Examples of parasagittal sections 3 day after injury are shown in

Figure 2-42. Some loss of tissue at the core of the sections may be due to loss of badly disrupted tissue during processing.

Histology from the 2 weeks (Figure 2-43) survival animals showed consistent cavities which in some cases were partly filled with laminin and in some sections there was clear up regulation of GFAP associated with the glial scar around the injury.

At later time point the cavitation become progressively more extensive and variable in morphology. At 4 weeks (Figure 2-44) some animals showed single continuous cavities while others showed multiple cavities separated by fine septae that tended to be rich in laminin.

At seven weeks (Figure 2-45) and at 3 months (Figure 2-46) the cavities were more elongated than at 4 weeks and only their septae and matrix infilling tended to occur in the most ventral part of the injury site.

At six months (Figure 2-47) the cavities were further expanded in the rostro-caudal direction.

2.4.2.1 Injury extent

Results obtained from radial CDP measurements suggest that there might be some on-going degeneration late in the course of the injury. To determine whether this might be due to an expansion of the injury cavity measurements of the cavity extent in rostro-caudal and lateral direction were performed. For this purpose tissue from survival animals of 7 weeks and 6 months groups were selected. This selection was based on electrophysiology data as the amplitudes of CDPs evoked by radial nerve stimulation had stabilized by 7 weeks but then showed further deterioration at 6 months suggesting a late phase of tissue loss.

Cavity length and width was determined from the histology from seven animals in the 7 week group and eight animals in the 6 month group. 7 weeks and 6 months animals are shown in Table 2-2. Comparison of cavity extent in rostro-caudal (length) and lateral (width) directions is shown in Figure 2-48. Paired Student’s t-test analysis showed that there was a significant increase in the

cavity length in 6 month animals compared to the 7 week animals. However, no difference was observed in cavity width formation. This suggests increased cavity length and further loss of neuronal tissue at this chronic time point is responsible for late deterioration in the amplitudes of the CDPs.

2.4.3 BDA labelling of CST fibres

Figure 2-49 shows BDA labelled CST fibres above and below the level of the injury. The main CST located in the dorsal column was intact above the injury along with the minor lateral and ventral components of the CST evident from the BDA labelled axons. However, caudal to the injury level there is no labelling of fibres in the dorsal column indicating the main CST fibres are completely interrupted. However, BDA labelled minor components of CST can be observed in the lateral and ventral white matter similar to those observed rostral to the injury.

Cavity length (mm)		Cavity width (mm)	
7 weeks	**6 months**	**7 weeks**	**6 months**
4.33	4.33	2.10	2.34
3.36	4.16	2.46	2.04
4.53	5.20	2.34	2.34
2.50	4.83	2.22	2.34
3.46	5.16	2.58	2.46
3.73	5.13	2.64	2.52
4.03	3.66	2.70	1.92
	5.83		3.00
Mean (3.71)	Mean (4.79)	Mean (2.43)	Mean (2.37)

Table 2-2. Table showing the maximum cavity length and width in 7 weeks and 6 month animal groups.

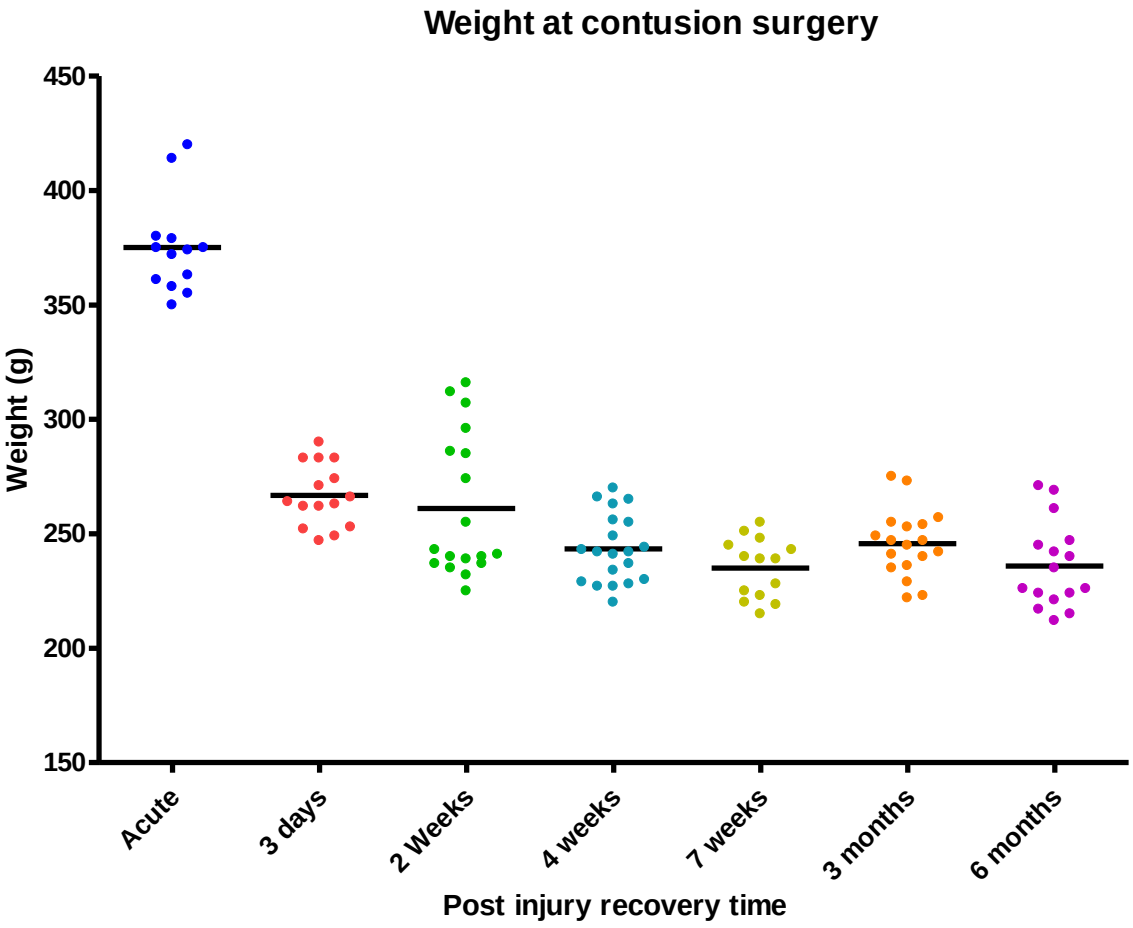

Figure 2-9. Body weights of individual animals at the time of contusion injury.

Scatter plot showing body weights of individual animals at the time of contusion injury in the groups of animals used to investigate the effects of injury on spinal cord function at different survival times post-injury. Horizontal bars represent the mean weight in each group.

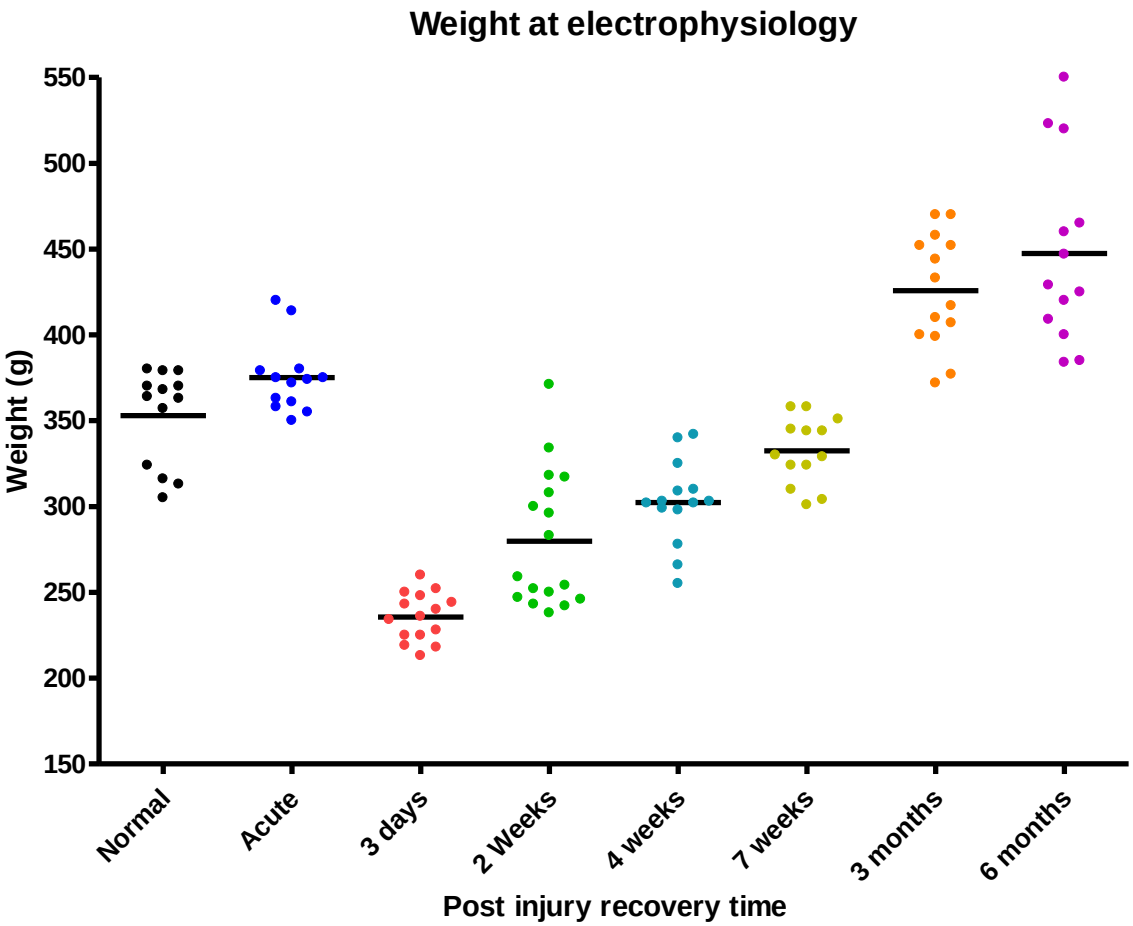

Figure 2-10. Body weights of individual animals at the time of electrophysiology.
Scatter plot showing body weights of individual animals at the time of electrophysiology in the normal group and different post injury recovery groups. Horizontal bars represent the mean weight in each group.

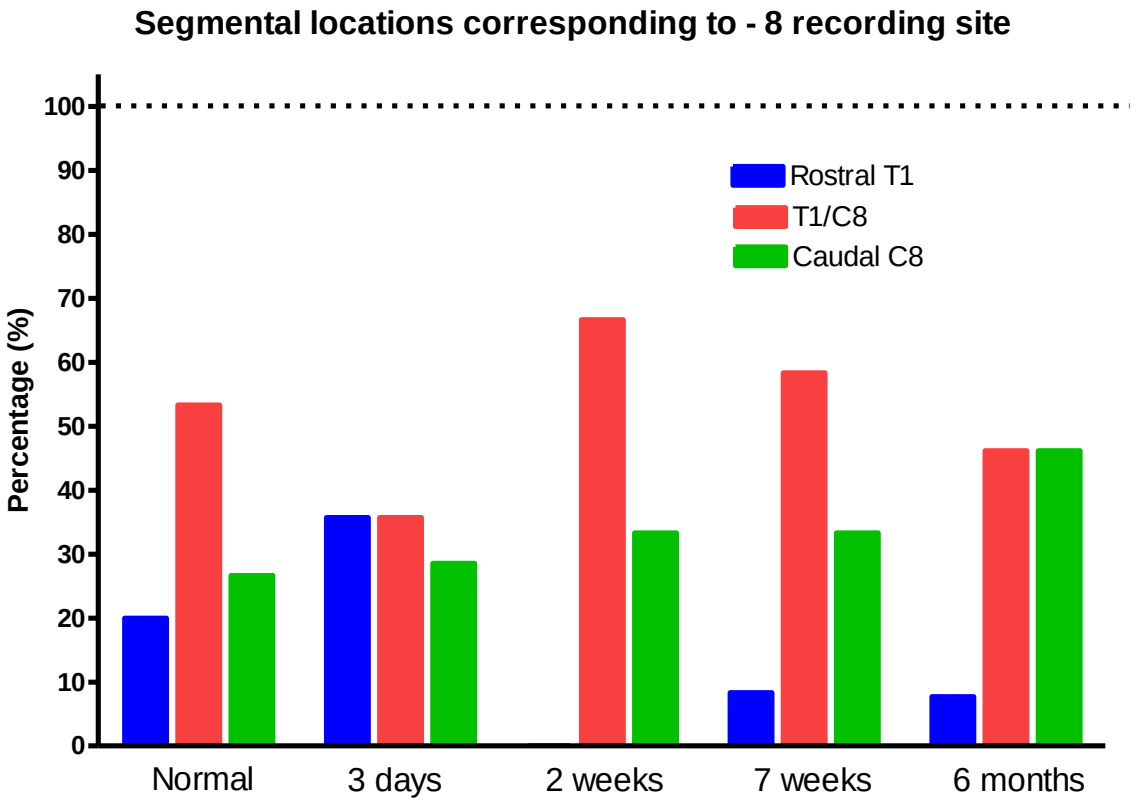

Figure 2-11. Comparison of segmental locations corresponding to - 8 recording location in different animal groups.

Plot showing the segmental locations corresponding to the - 8 recording location in normal animals and the groups of animals investigated at 3 days, 2 weeks, 7 weeks and 6 months after injury. In all animals the - 8 location was either rostral T1, T1/C8 or caudal C8.The bar plots show the percentage of recording positions corresponding to each of these segmental location.

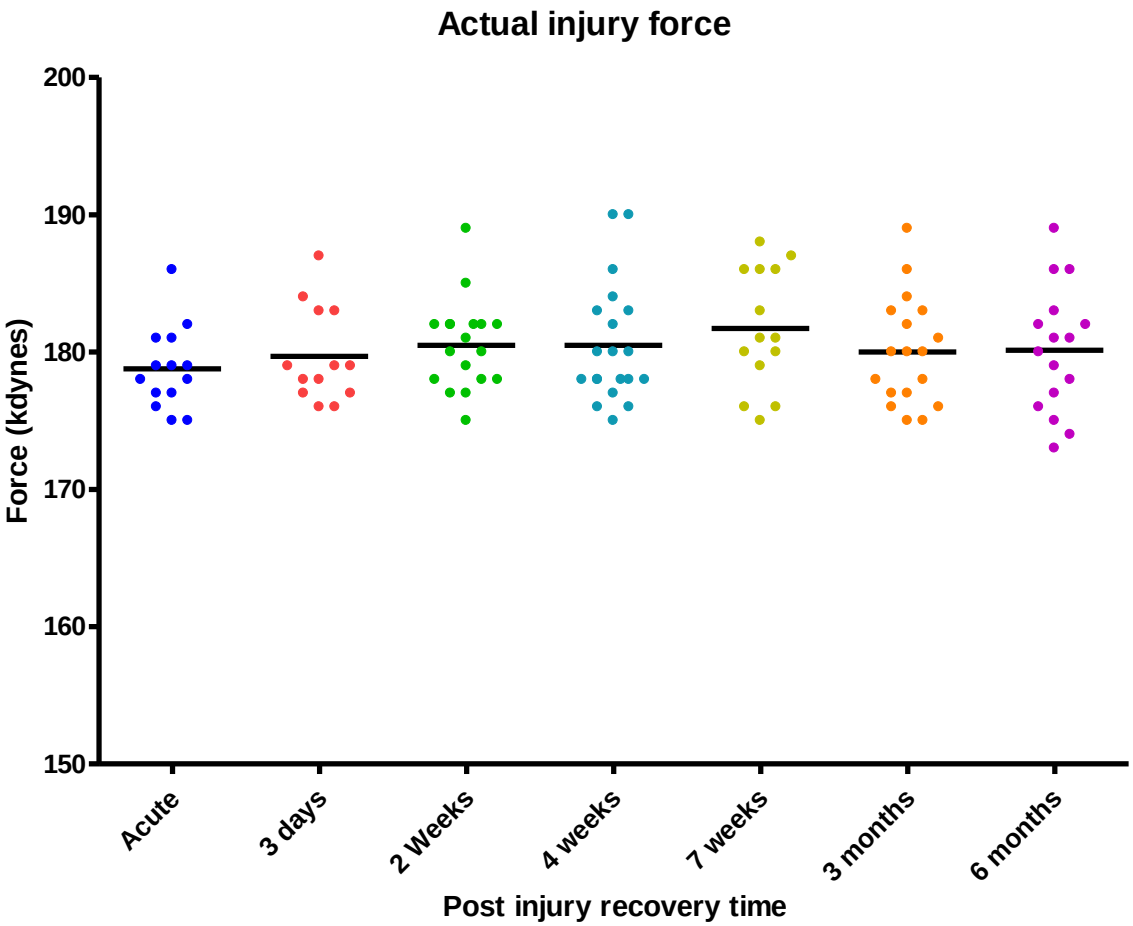

Figure 2-12. Actual force of contusion injury in each of the different post injury recovery groups.

Scatter plot showing the actual force produced by the IH impactor set to deliver 175 kdyn force. There was no significant difference between the mean force for any of the groups (one-way ANOVA with Tukey's post-hoc test). Horizontal bars represent the mean force in each group.

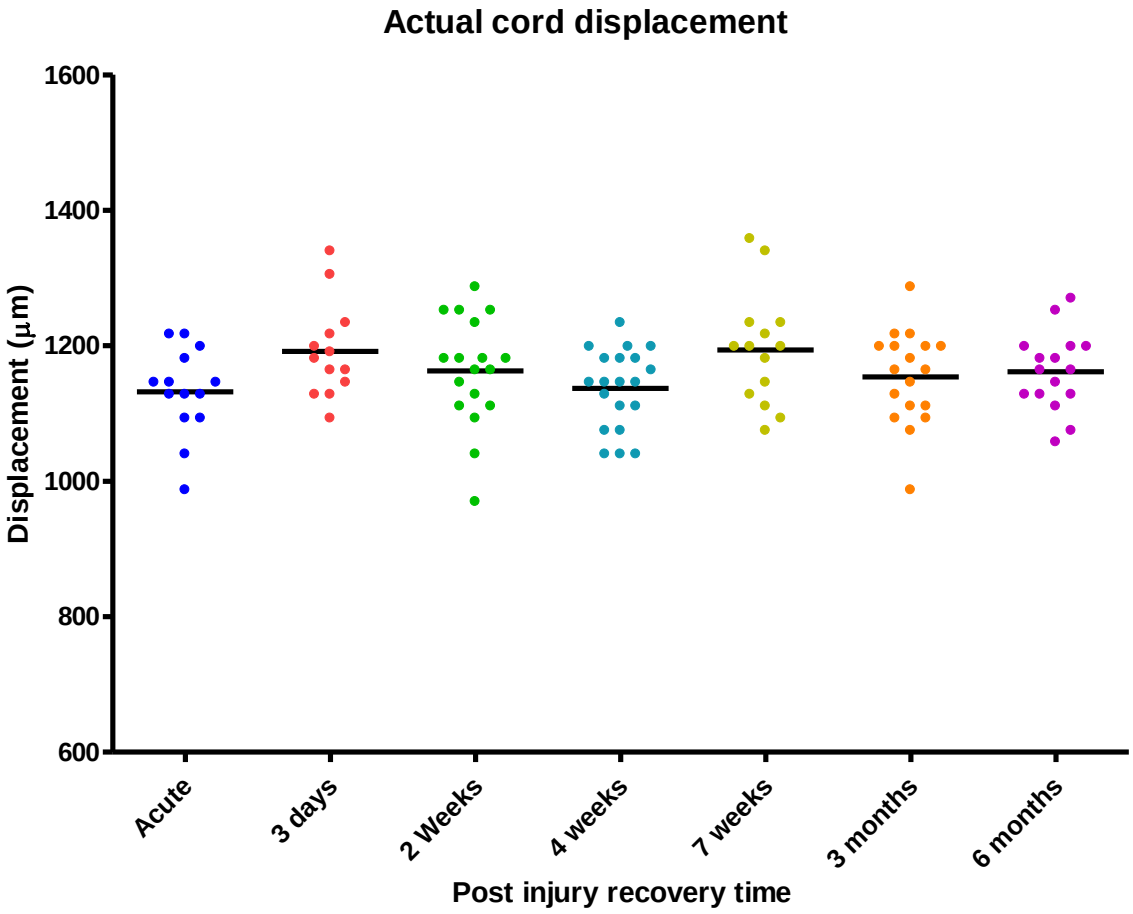

Figure 2-13. Actual cord displacement as a result of 175 kdyn contusion injury in each of the post injury groups.

Scatter plot showing the displacement of the impactor tip into the cord tissue from the surface. There was no significant difference between the mean displacement for any of the groups (one-way ANOVA with Tukey's post-hoc test). Horizontal bars represent the mean displacement in each group.

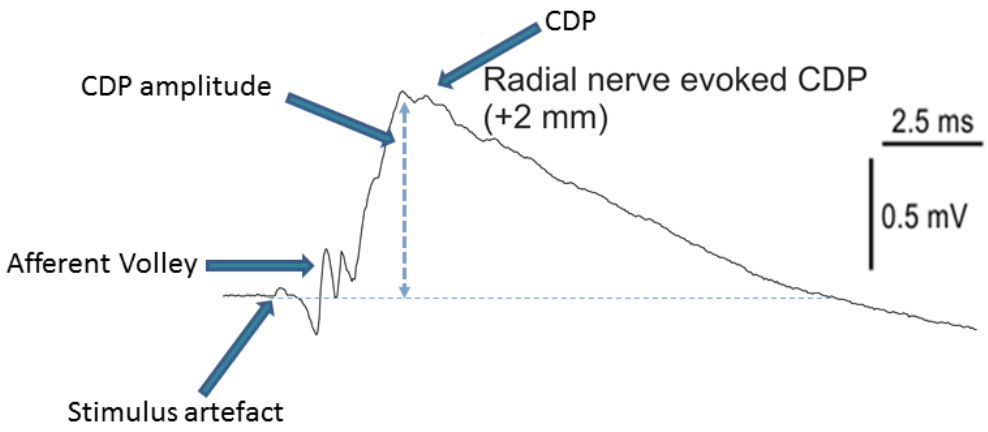

Figure 2-14. Different components of the surface potentials evoked by radial nerve stimulation.

Example of a CDP trace generated in response to radial nerve stimulation and recorded from the surface of the cervical spinal cord. The recording is an average of 25 sweeps. The afferent volley represents action potentials travelling along afferent fibres. The CDP represents the synaptic activity generated by connections between the collaterals of the primary afferent fibres and spinal cord neurons and provides an indication of the strength of these connections. The CDP amplitude was measured as shown by the vertical arrow using Signal software.

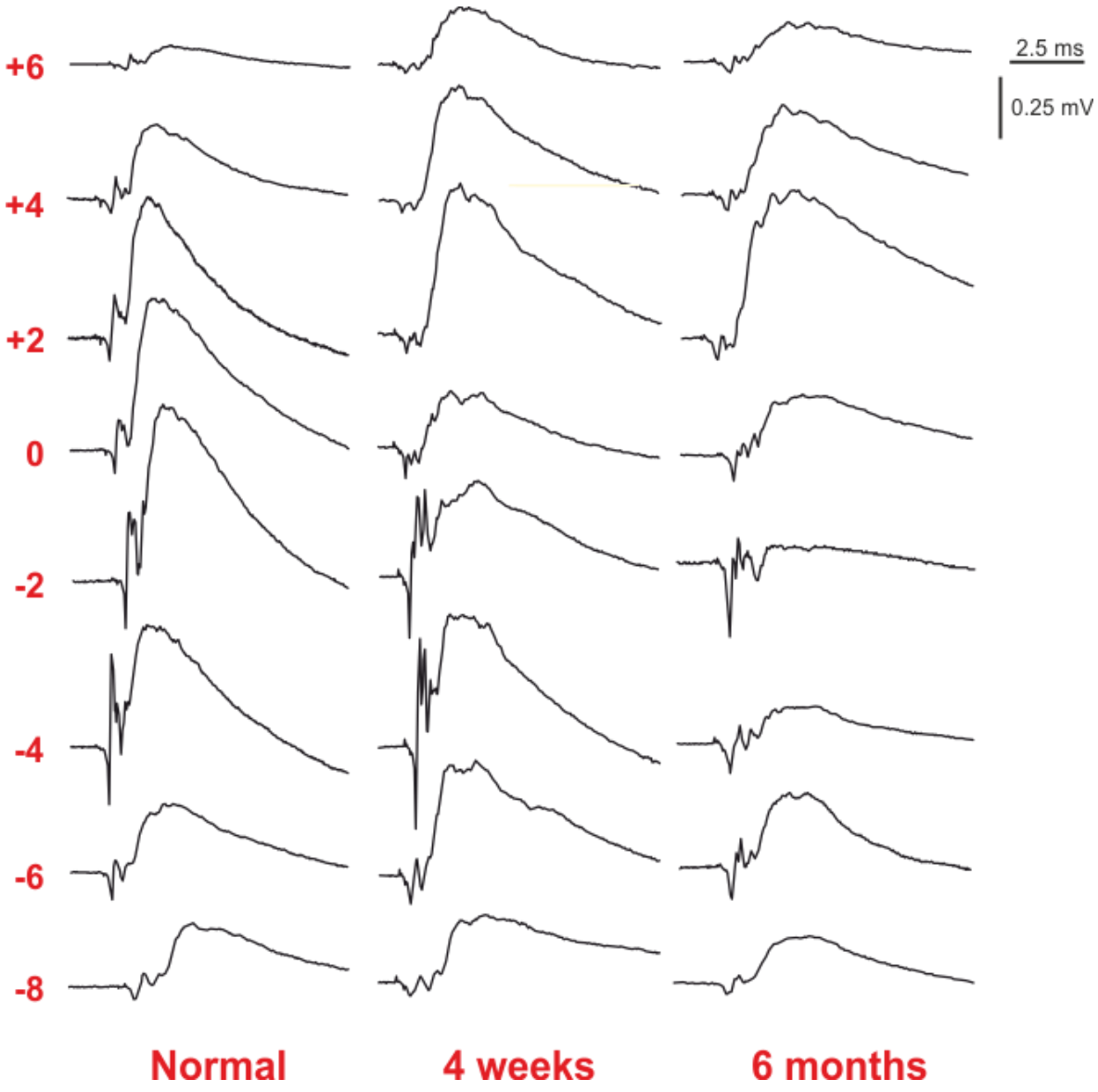

Figure 2-15. Examples of radial evoked CDP traces recorded from a normal animal and from injured animals 4 weeks and 6 months after contusion injury.

Each column of traces shows recordings from one animal. On the left the recording is from a normal animal and recordings in the middle and on the right are from 4 week and 6 month survival animals respectively. Recordings were made at 1 mm intervals from 8 mm rostral (+8) to 8 mm caudal (-8) to C5/6 border. Only every other trace is shown for clarity. Traces are averages of 25 sweeps and the calibration applies to all traces.

A

Roots sectioned	Percentage of amplitude (%)
Intact	100
C5	94.6
C5 + C7	88.1
C5 + C7 + C6	1

B

Roots sectioned	Percentage of amplitude (%)
Intact	100
C5	96.1
C5 + C7	82.1
C5 + C7 + C6	7.5

C

Roots sectioned	Percentage of amplitude (%)
Intact	100
C5	96.7
C5 + C7	65.3
C5 + C7 + C6	15.1

D

Roots sectioned	Percentage of amplitude (%)
Intact	100
C5	98
C5 + C7	89.3
C5 + C7 + C6	3.2

Figure 2-16. Contribution of spinal dorsal roots to radial evoked CDPs.

To test the contribution of each spinal root to the overall amplitude of radial evoked CDPs, dorsal roots were sequentially sectioned in 4 animals while CDPs were recorded. The amplitudes following root sectioning are expressed as a percentage of the amplitude before sectioning. In A and B recordings were made at +2 mm location, while in C and D recordings were made at -4 mm location. The most substantial reduction in amplitude occurred when the C6 root was cut followed by the C7 root. The C5 root contributed very little to the CDP in any of the 4 animals.

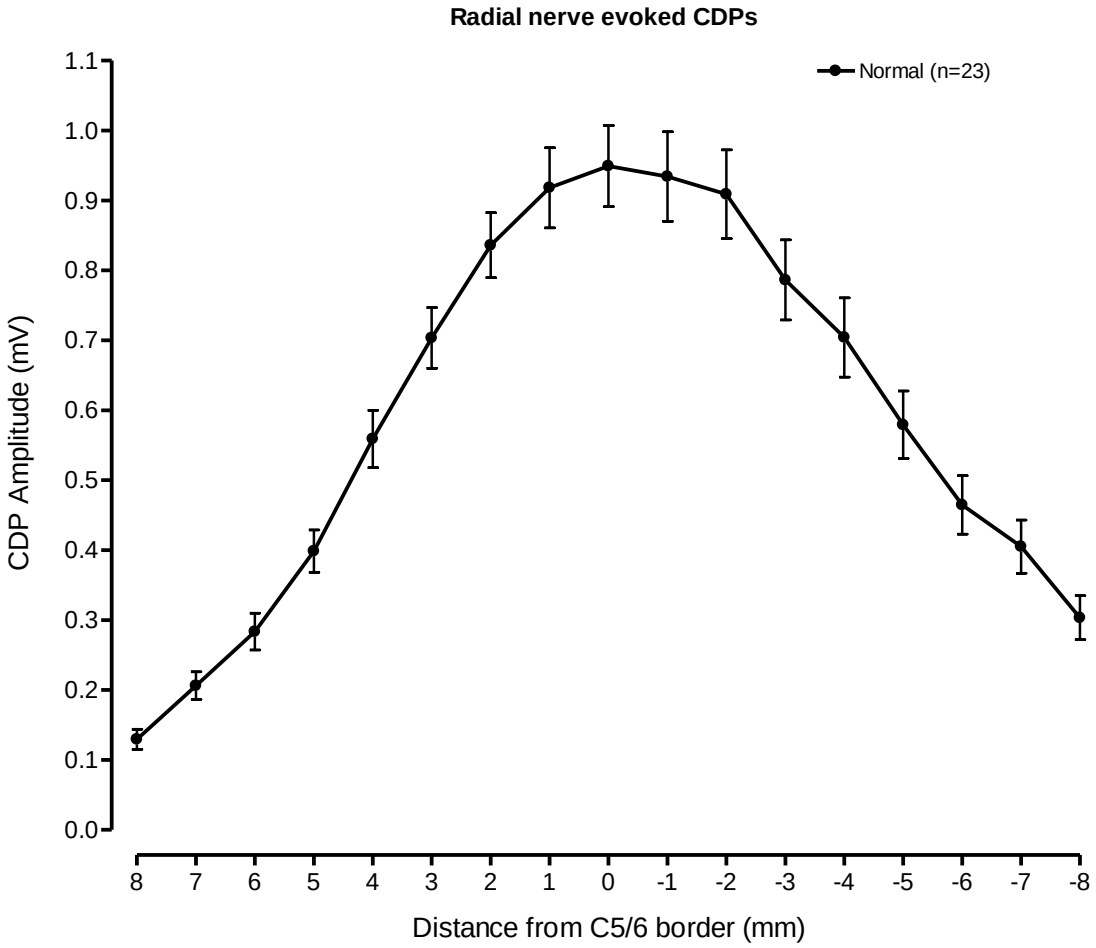

Figure 2-17. Amplitudes of radial nerve evoked CDPs recorded in normal animals.

Each data point is the mean CDP amplitude for all animals in the normal (non-injured) group. The error bars show +/- SEM. The recordings were made over the cervical spinal cord. Numbers on the x-axis represents the recording locations on the surface of the cord relative to the C5/6 border (0mm). In normal animals, the biggest radial evoked CDPs were recorded around 2 mm above and below the C5/6 border. The mean amplitude of the biggest CDPs were around 0.95 mV. CDPs decreased in amplitude more rostrally and caudally.

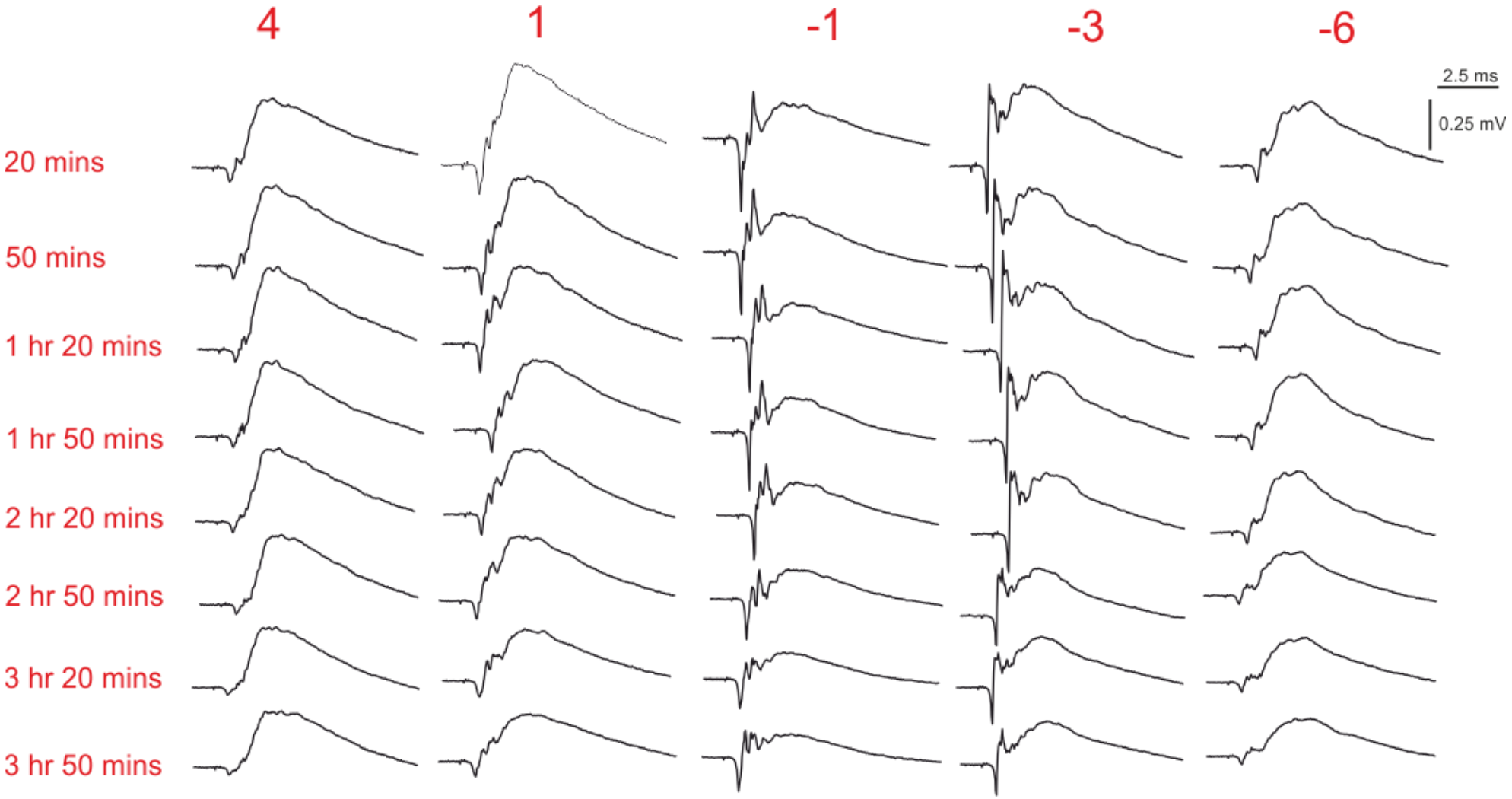

Figure 2-18. Examples of radial evoked CDPs traces recorded in an acutely injured animal.

Each column of traces shows recordings from one location, relative to C5/6 border (0 mm). On the left of the recordings then time after injury when recordings were made are shown. The mappings were made at 30 minutes intervals with first mapping made 20 minutes after contusion injury. Traces from only 5 locations are shown for clarity. Traces represent averages of 25 sweeps and the calibration applies to all traces. Note that CDPs in each consecutive recording are of progressively smaller amplitudes (largest CDPs at 20 minutes compared to smallest at 3 hours 50 minutes).

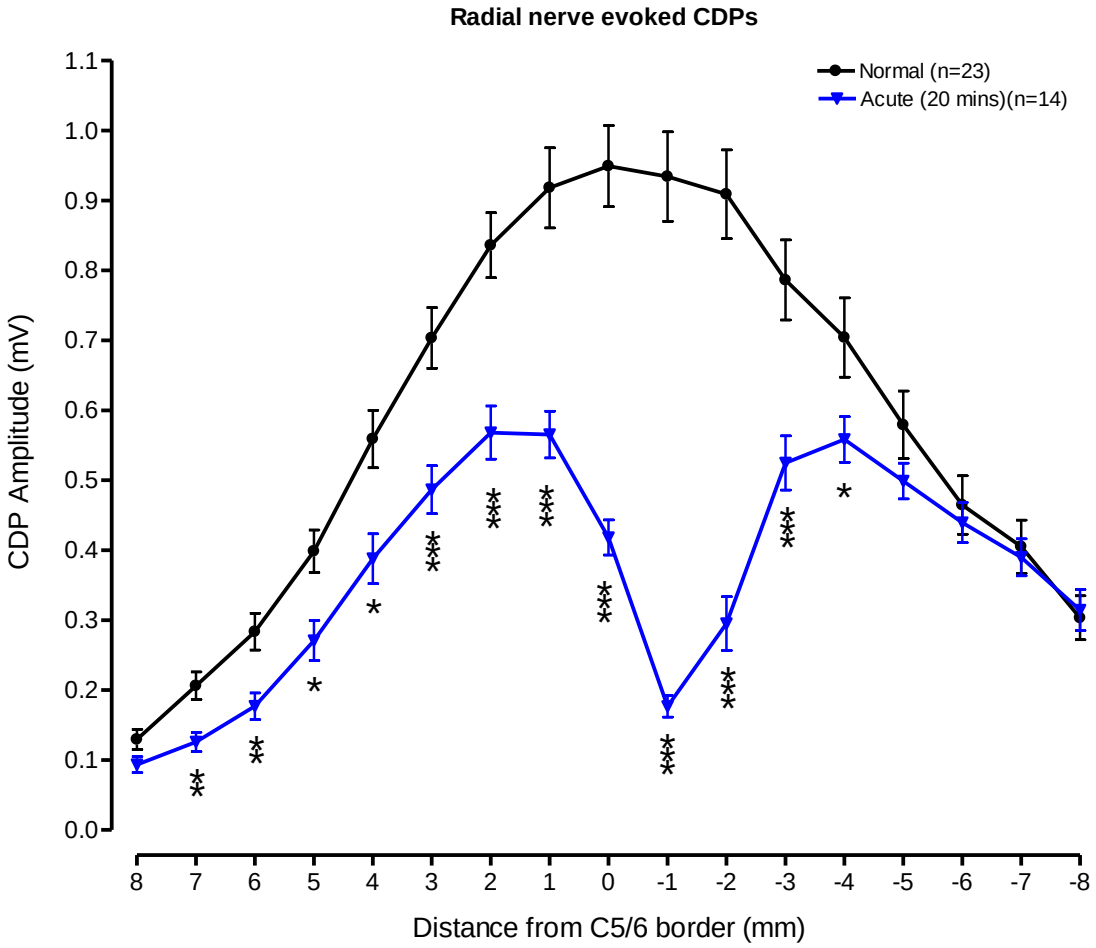

Figure 2-19. Amplitudes of radial nerve evoked CDPs recorded in acutely injured animals at 20 minutes after injury.

The plot shows the mean amplitudes of CDPs recorded 20 minutes after injury from animals in the acute injury group (n=14). The plot for normal animals is also shown for comparison. Each data point is the mean CDP amplitude for all animals in the group. The error bars show +/- SEM. The contusion injury was carried out at C6 segment of the cord with the rostral edge of the impactor tip aligned with C5/6 border (0 mm). The recordings were made over the cervical spinal cord. Numbers on the x-axis represents the recording locations on the surface of the cord relative to C5/6 border (0 mm). After injury, CDPs at the lesion epicentre (-1 mm) and around the vicinity of the injury (+2 mm to -3 mm) declined significantly compared to those seen in normal animals. The greatest decline was observed at the lesion epicentre (-1 mm). The asterisks (*) represent the statistical significance of the difference between CDP amplitudes recorded at 20 minutes compared to normal (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

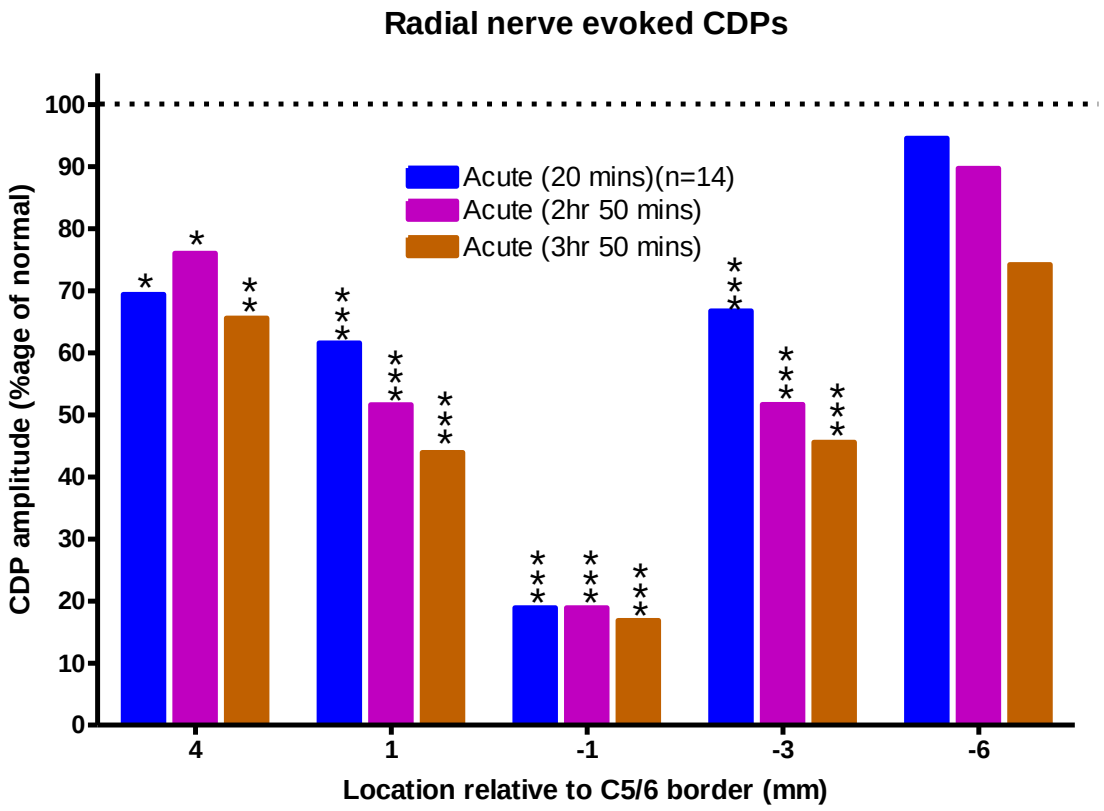

Figure 2-20. Changes in amplitude of radial nerve evoked CDPs at different time points after acute injury.

Plot showing the mean amplitudes of CDPs recorded in acutely injured animals at 20 minutes, 2 hours 50 minutes and 3 hours 50 minutes after injury at 5 different locations. The CDP amplitudes are plotted as a percentage of the mean CDP amplitude in normal animals. Normal CDP amplitudes are represented as a dotted line (100 %). The asterisks (*) represent the statistical significance of the difference between CDP amplitudes recorded at different stages in the acute experiment compared to normal (* = $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$). There were no significant differences between recordings made at 20 minutes and other acute time points at any of the recording locations.

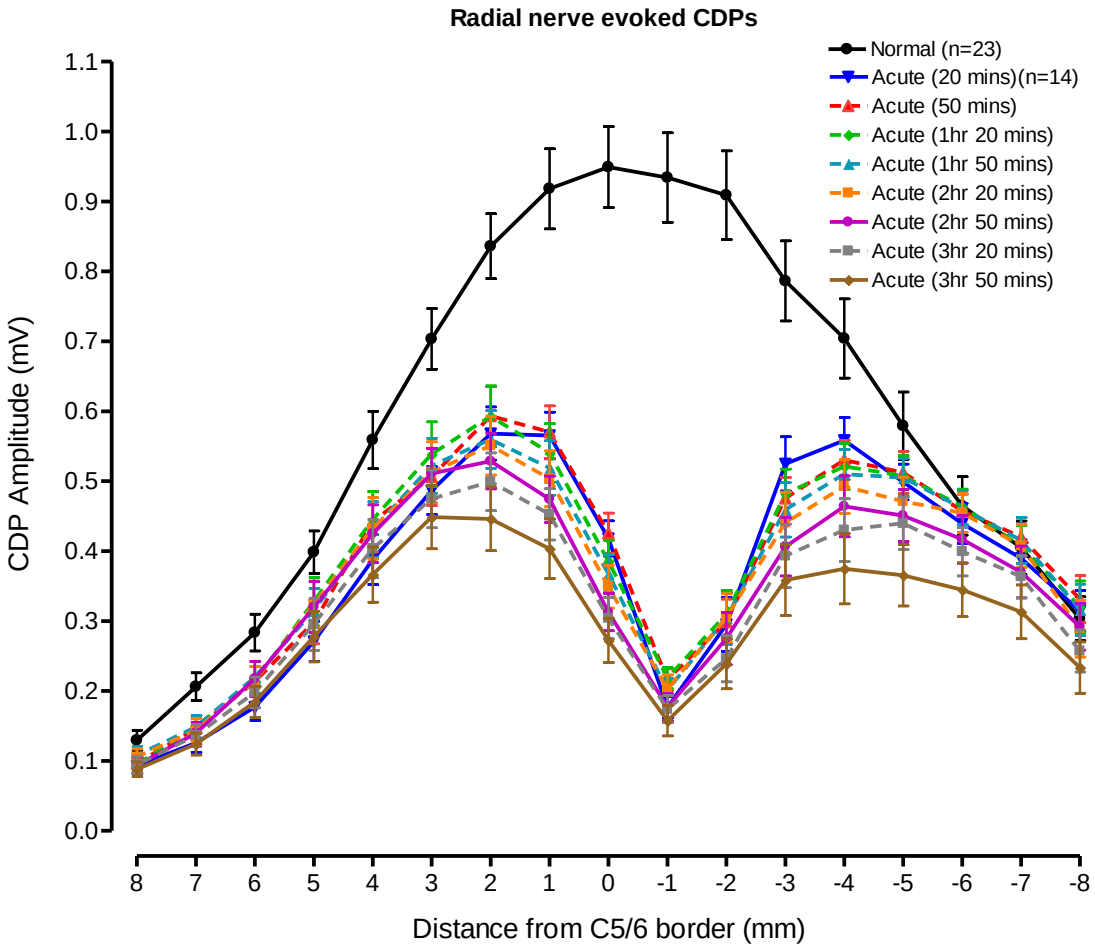

Figure 2-21. Amplitudes of radial nerve evoked CDPs recorded in acutely injured animals at all acute recording time points.

The plot shows the mean amplitudes of CDPs recorded at 30 minute intervals from 20 minutes to 3 hour 50 minutes after injury. The plot for normal animals is also shown for comparison. Each data point is the mean CDP amplitude for all animals in the group (n=14 for all acute data points shown). The error bars show +/- SEM. The contusion injury was carried out with the rostral edge of the impactor tip aligned with C5/6 border (0 mm). The recordings were made over the cervical spinal cord. Numbers on the x-axis represents the recording locations on the surface of the cord relative to C5/6 border (0 mm). CDPs at the lesion epicentre (-1 mm) in all acute recording time points remained almost the same. However, the amplitudes of those in the vicinity of the injury both rostrally (0 mm to +4 mm) and caudally (-2 mm to -5 mm) showed a gradual decline in amplitude with each successive recording time point. The maximum decline was seen at the latest time point (3 hour 50 minutes).

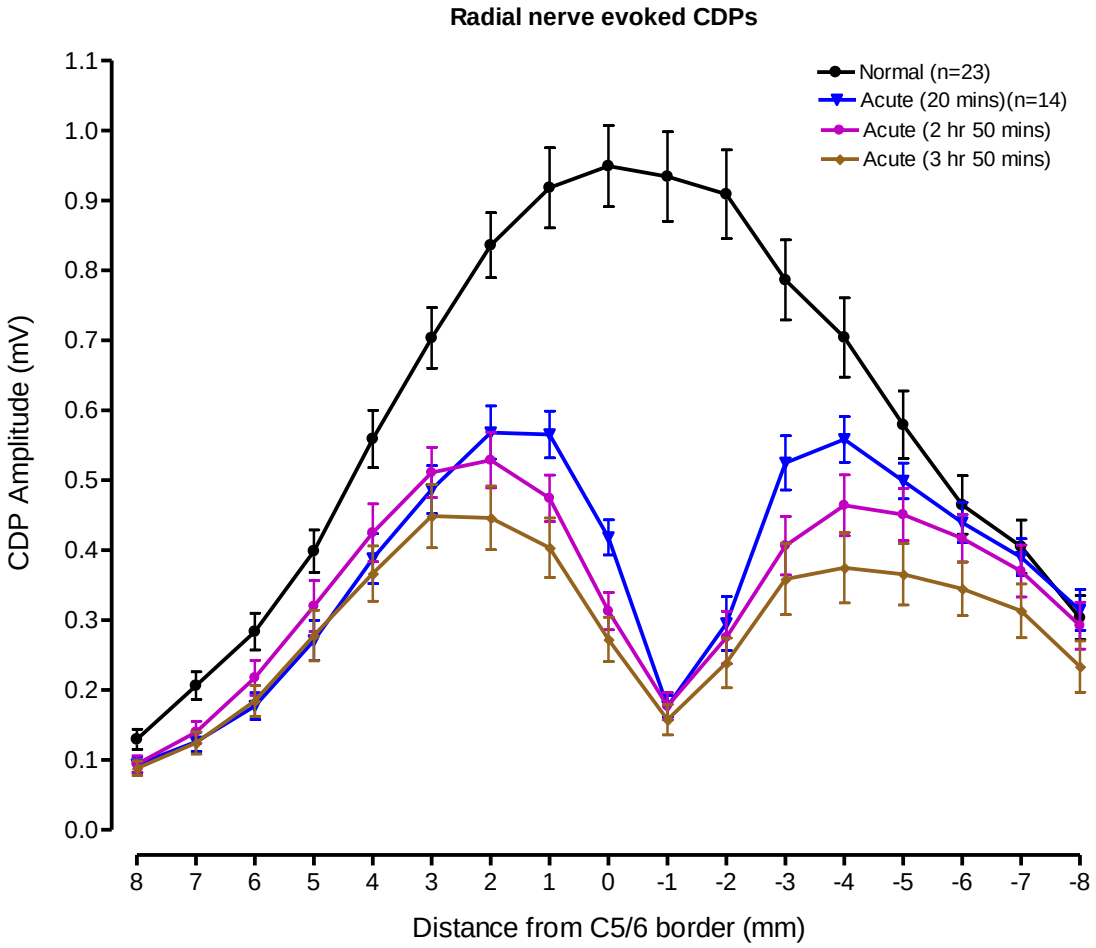

Figure 2-22. Amplitudes of radial nerve evoked CDPs recorded in acutely injured animals at 20 minutes, 2 hours and 50 minutes and 3 hours and 50 minutes after injury.

The plot shows a comparison of the mean amplitudes of CDPs recorded at 20 minutes, 2 hours and 50 minutes and 3 hours and 50 minutes after injury. The plot for normal animals is also shown for comparison. Each data point is the mean CDP amplitude for all animals in the group (n=14). The error bars show +/- SEM. The contusion injury was carried out with the rostral edge of the impactor tip aligned with C5/6 border (0 mm). The recordings were made over the cervical spinal cord. Numbers on the x-axis represents the recording locations on the surface of the cord relative to C5/6 border (0 mm). CDPs at the lesion epicentre (-1 mm) remained almost unchanged. Those closed to the vicinity of the injury both rostrally (0 mm to +2 mm) and caudally (-3 mm to -5 mm) showed a gradual decline from 20 minutes to 3 hours 50 minutes. There were no significant differences between CDPs recorded at the different time points.

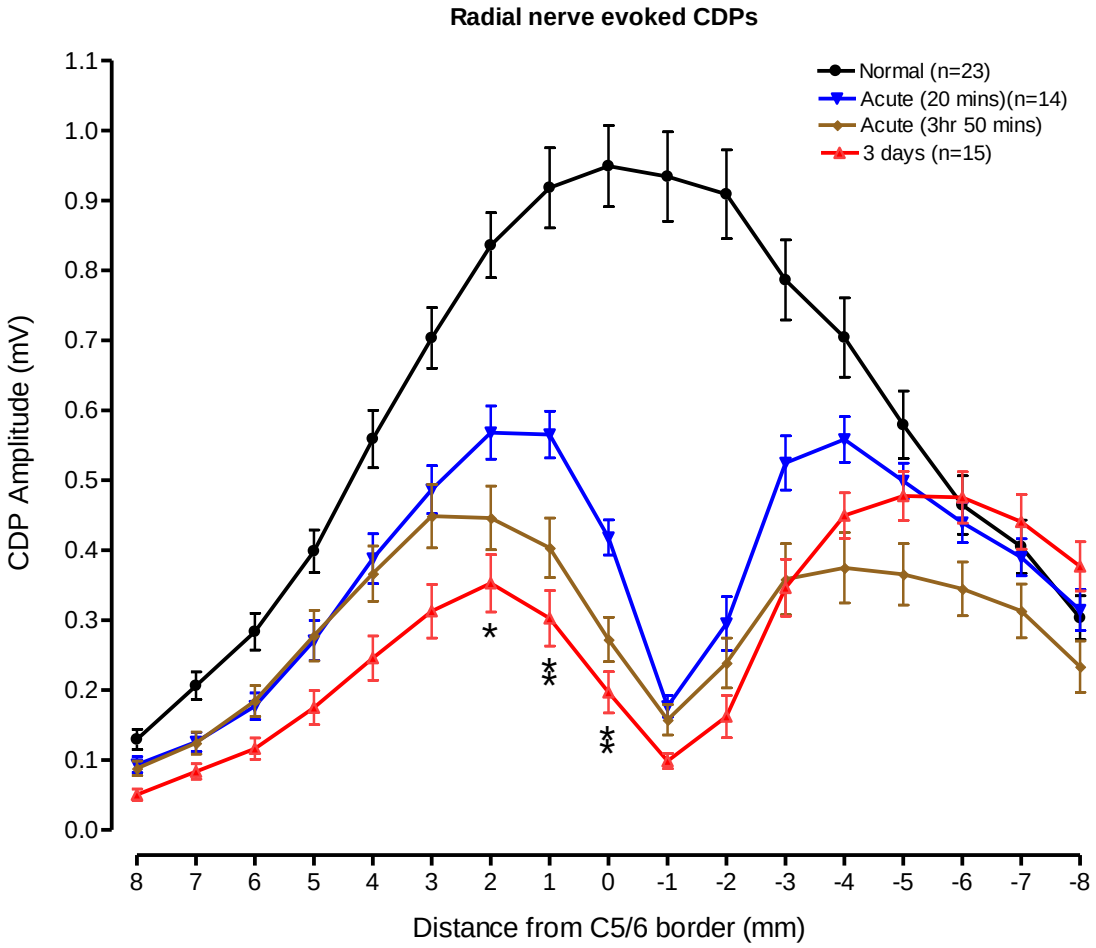

Figure 2-23. Amplitudes of radial nerve evoked CDPs recorded at the beginning and end of the acute experiments and from animals surviving 3 days after injury.

The plot shows a comparison of the mean amplitudes of CDPs recorded in acutely injured animals at 20 minutes (n=14) and 3 hours and 50 minutes (n=14) and at 3 days (n=15) after injury. The plot for normal animals is also shown for comparison. Each data point is the mean CDP amplitude for all animals in the group. The errors bar show +/- SEM. The contusion injury was carried out with the rostral edge of the impactor tip aligned with C5/6 border (0 mm). The recordings were made over the cervical spinal cord. Numbers on the x-axis represents the recording locations on the surface of the cord relative to C5/6 border (0 mm). In the 3 day survival animals CDPs at the lesion epicentre (-1 mm) and rostral to the injury (0 mm to +8 mm) declined in amplitude compared to those recorded in the acutely injured animals at 20 minutes and 3 hours 50 minutes after injury. Below the injury (-3 mm to -8 mm) CDPs recorded from the 3 day survival animals showed recovery of amplitudes almost equal to those seen acutely at 20 minutes after the injury. The asterisks (*) represent the statistical significance of the difference between CDP amplitudes recorded acutely at 20 minutes compared to 3 days (* = $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$).

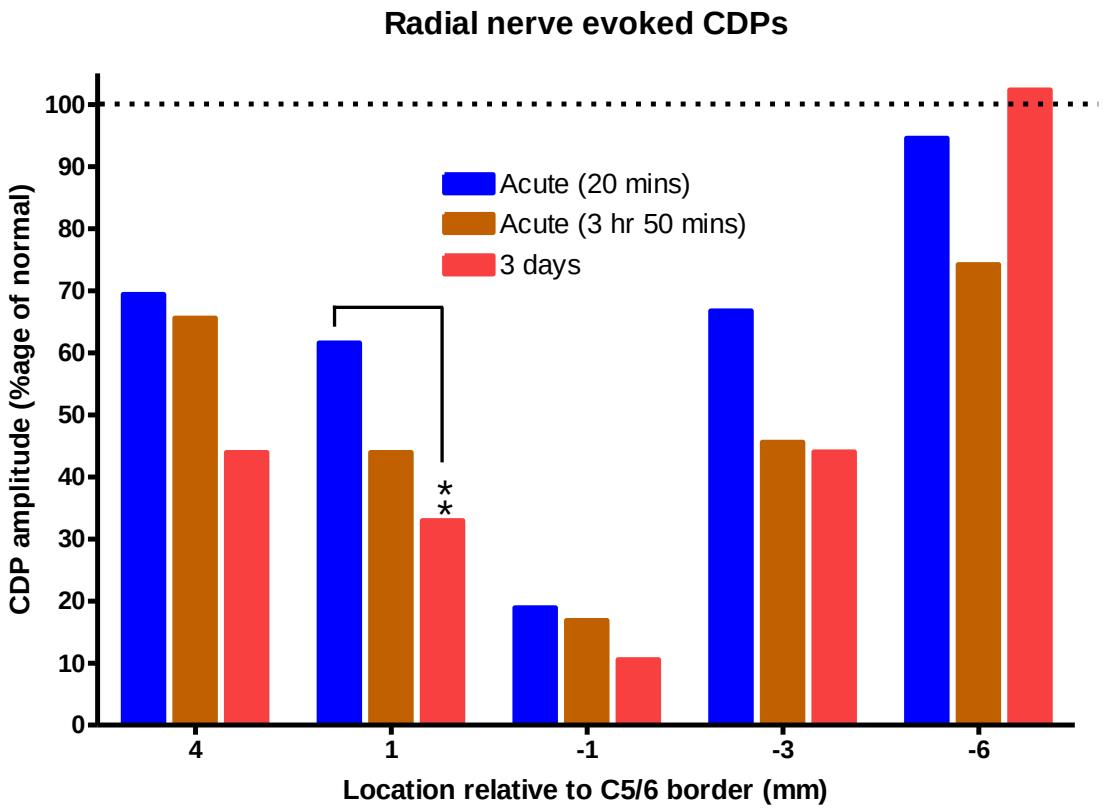

Figure 2-24. Changes in amplitude of radial nerve evoked CDPs in acutely injured animals and 3 day survival animals.

Plot showing the mean amplitudes of radial nerve evoked CDPs recorded in acutely injured animals at two time points; 20 minutes and 3 hours and 50 minutes and in 3 days survival animals after injury at 5 different locations. The CDP amplitudes are plotted as a percentage of the mean CDP amplitude in normal animals. Normal CDP amplitudes are represented as a dotted line (100 %). * = p <0.05, **= p <0.01, ***= p < 0.001. The asterisks (*) represent the statistical significance of the difference between CDP amplitudes recorded acutely at 20 minutes compared to 3 days (* = p <0.05, **= p <0.01, ***= p < 0.001).

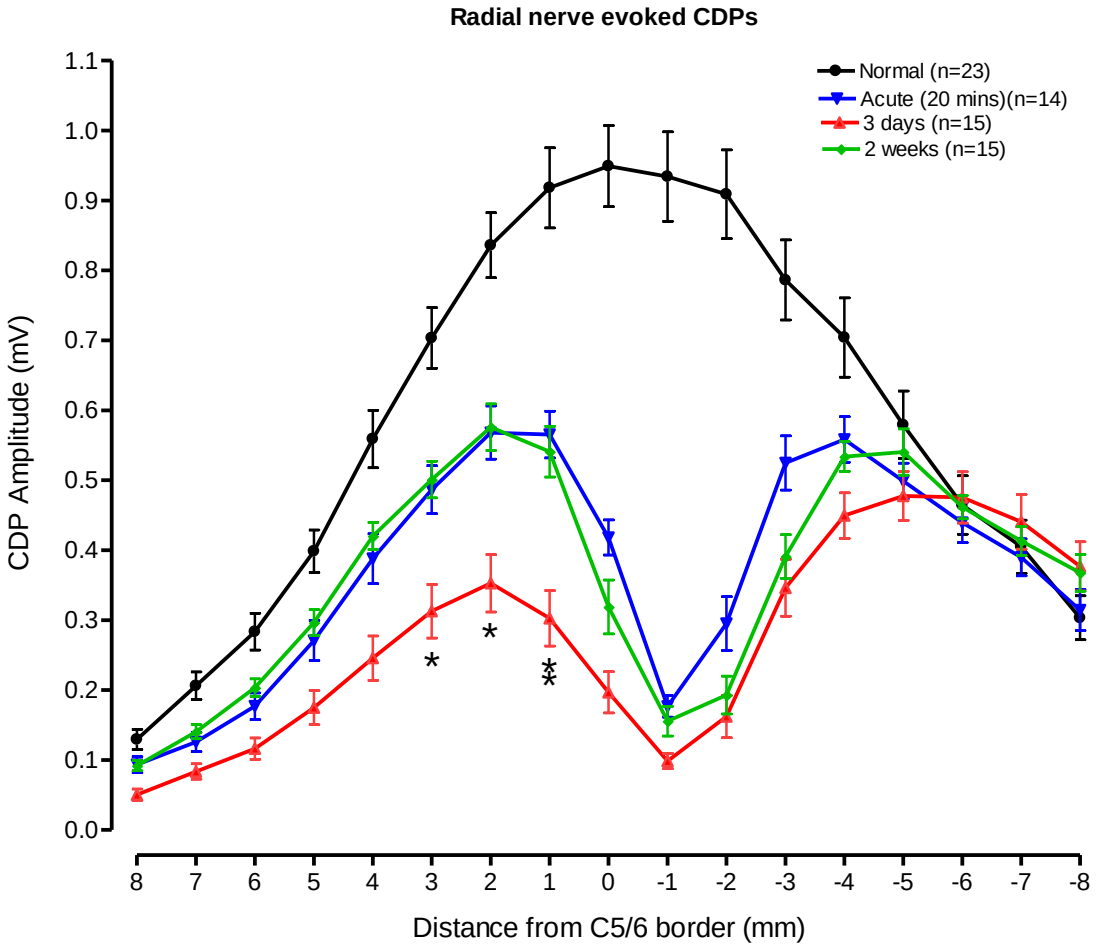

Figure 2-25. Amplitudes of radial nerve evoked CDPs recorded in acutely injured animals at 20 minutes and from animals surviving 3 days and 2 weeks after injury.

The plot shows a comparison of the mean amplitudes of CDPs recorded in acutely injured animals at 20 minutes (n=14) and surviving animals at 3 days (n=15) and 2 weeks (n=15) after injury. The plot for normal animals is also shown for comparison. Each data point is the mean CDP amplitude for all animals in the group. The error bars show +/- SEM. The contusion injury was carried out with the rostral edge of the impactor tip aligned with C5/6 border (0 mm). The recordings were made over the cervical spinal cord. Numbers on the x-axis represent the recording locations on the surface of the cord relative to C5/6 border (0 mm). In the 2 week survival animals CDPs at the injury epicentre (-1 mm), rostral (0 mm to +8 mm) and caudal (-4 mm to -8 mm) to the injury showed recovery of CDP amplitudes almost equal to those seen in acutely injured animals at 20 minutes after injury. The asterisks (*) represent the statistical significance of the difference between CDP amplitudes recorded at 3 days compared to 2 weeks (* = $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$).

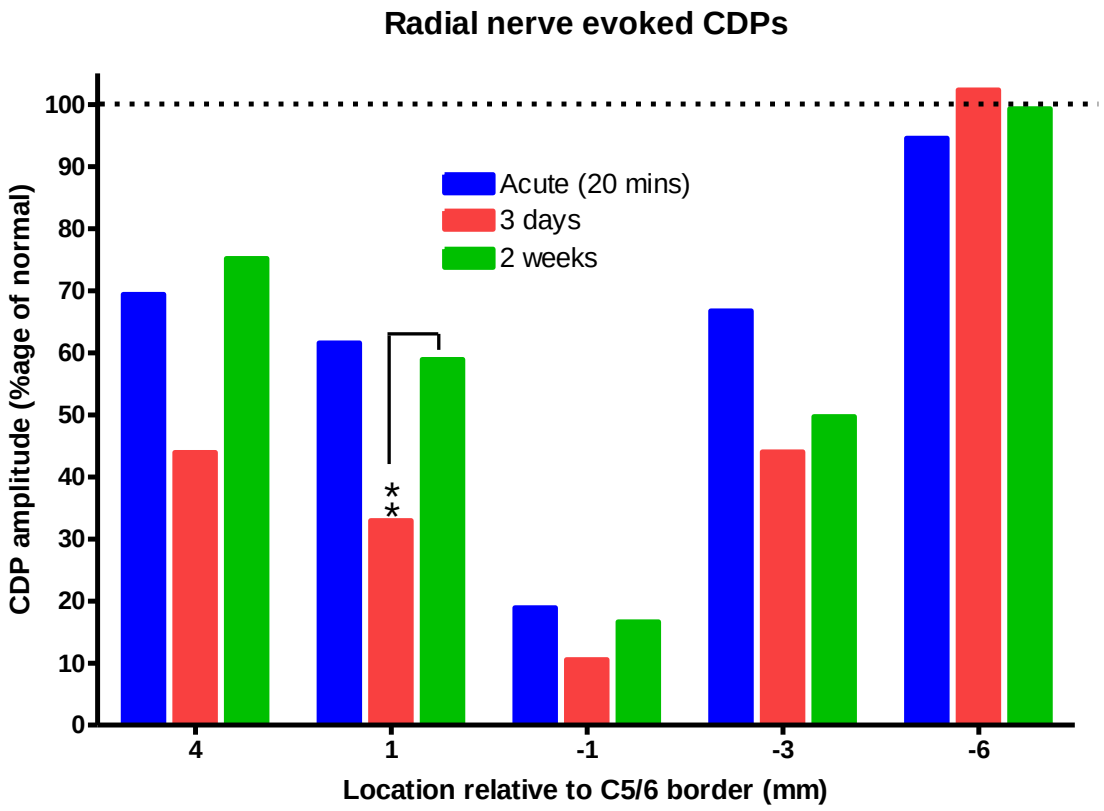

Figure 2-26. Comparison of the amplitudes of radial nerve evoked CDPs in acutely injured animals and animals surviving 3 days and 2 weeks after injury.

Plot showing the mean amplitudes of radial nerve evoked CDPs recorded 20 minutes after an acute injury compared to 3 days and 2 weeks after injury. The plots show amplitudes at 5 different locations as a percentage of the mean CDP amplitude in normal animals. Normal CDP amplitudes are represented as a dotted line (100 %). The asterisks (*) represent the statistical significance of the difference between CDP amplitudes recorded at 3 days compared to 2 weeks (* = $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$).

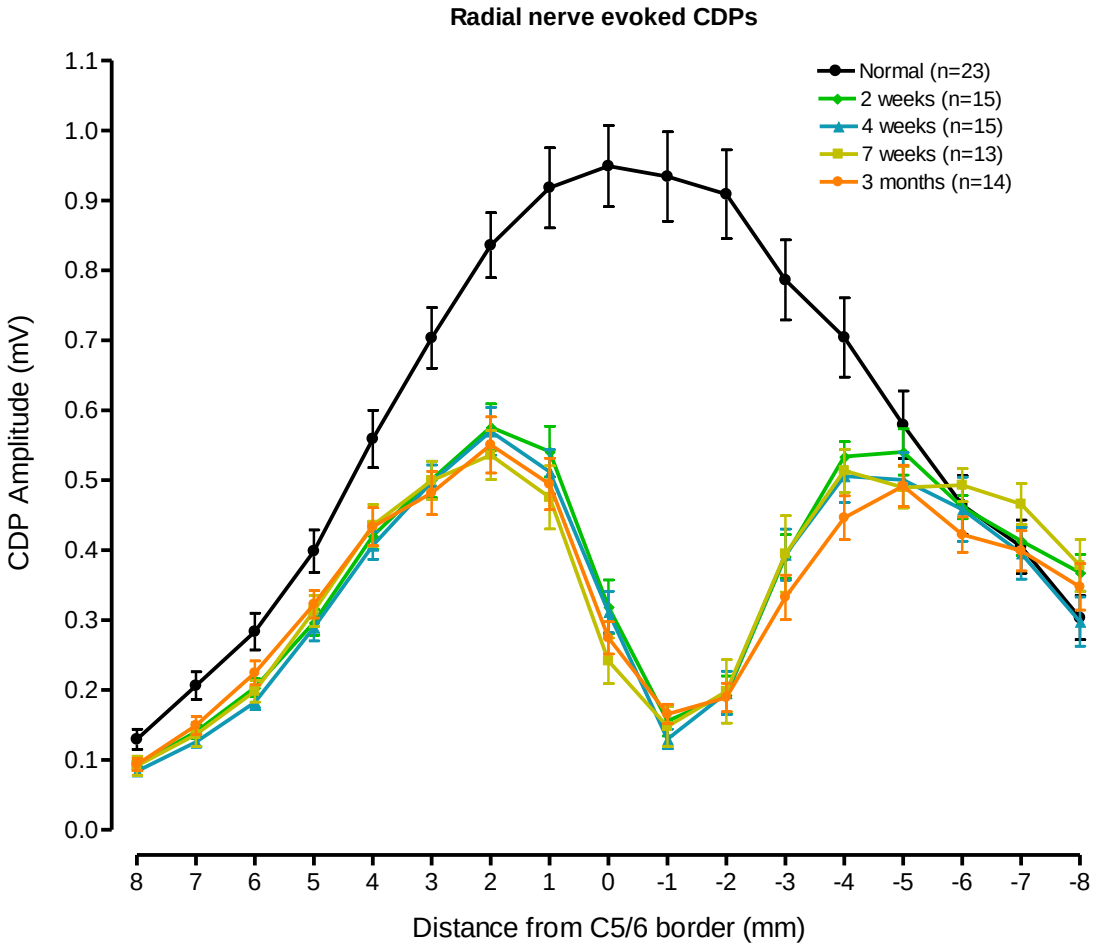

Figure 2-27. Amplitudes of radial nerve evoked CDPs recorded from animals surviving 2 weeks, 4 weeks, 7 weeks and 3 months after injury.

The plot shows a comparison of the mean amplitudes of CDPs recorded from the groups of animals investigated at 2 weeks (n=15), 4 weeks (n=15), 7 weeks (n=13) and 3 months (n=14) after injury. The plot for normal animals is also shown for comparison. Each data point is the mean CDP amplitude for all animals in the group. The error bars show +/- SEM. The contusion injury was carried out with the rostral edge of the impactor tip aligned with C5/6 border (0 mm). The recordings were made over the cervical spinal cord. Numbers on the x-axis represent the recording locations on the surface of the cord relative to C5/6 border (0 mm). CDPs remained almost stable between 2 weeks and 3 months after injury. There were no significant differences between the groups at any recording location (+8 mm to -8 mm). There were no significant differences between CDPs recorded at the different time points.

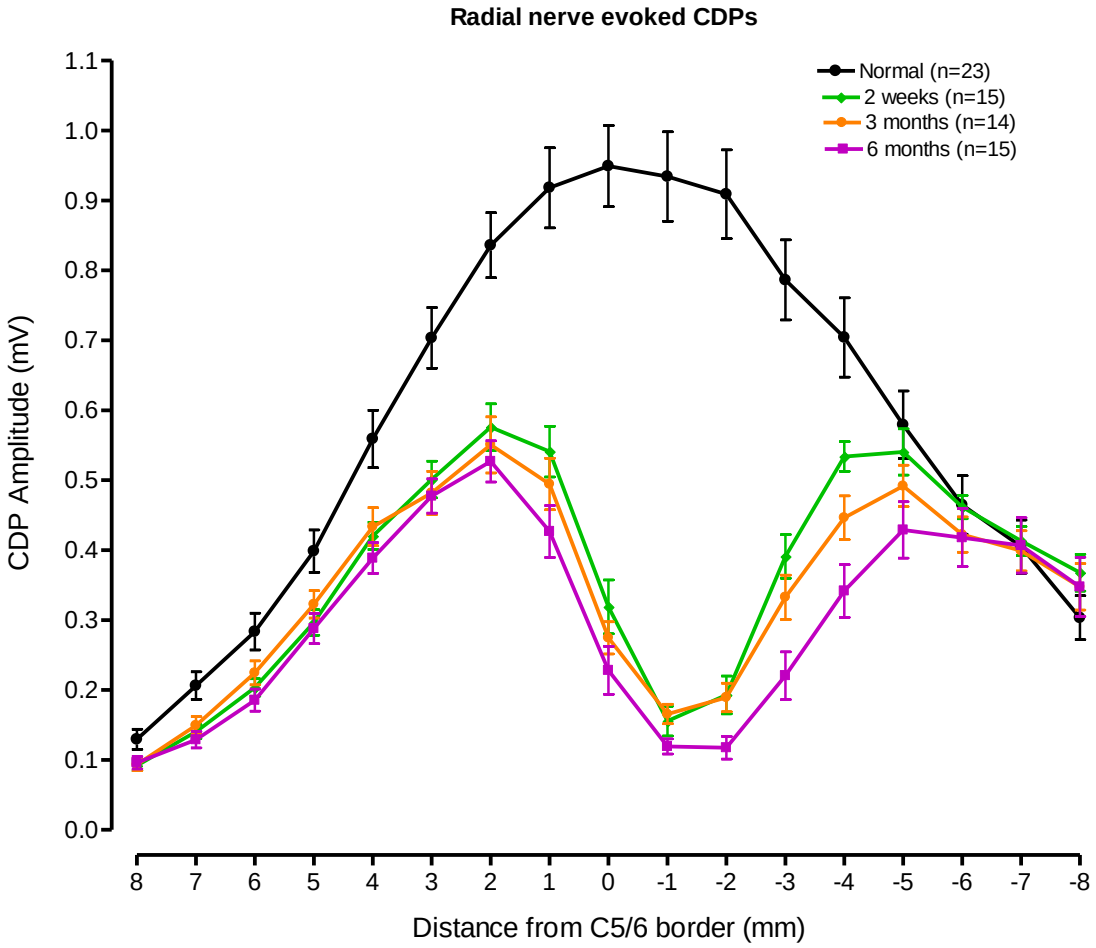

Figure 2-28. Amplitudes of radial nerve evoked CDPs recorded from animals investigating 2 weeks, 3 and 6 months after injury.

The plot shows a comparison of the mean amplitudes of CDPs recorded from the animal groups investigated 2 weeks (n=15), 3 months (n=14) and 6 months (n=15) after injury. The plot for normal animals is also shown for comparison. Each data point is the mean CDP amplitude for all animals in the group. The error bars show +/- SEM. The contusion injury was carried out with the rostral edge of the impactor tip aligned with C5/6 border (0 mm). The recordings were made over the cervical spinal cord. Numbers on the x-axis represent the recording locations on the surface of the cord relative to C5/6 border (0 mm). In the 6 month survival animals a decrease in CDP amplitudes was see mainly caudal to the injury (-1 mm to -5 mm) (compared to 2 weeks and 3 months survival animals). There were no significant differences between CDPs recorded at the different time points.

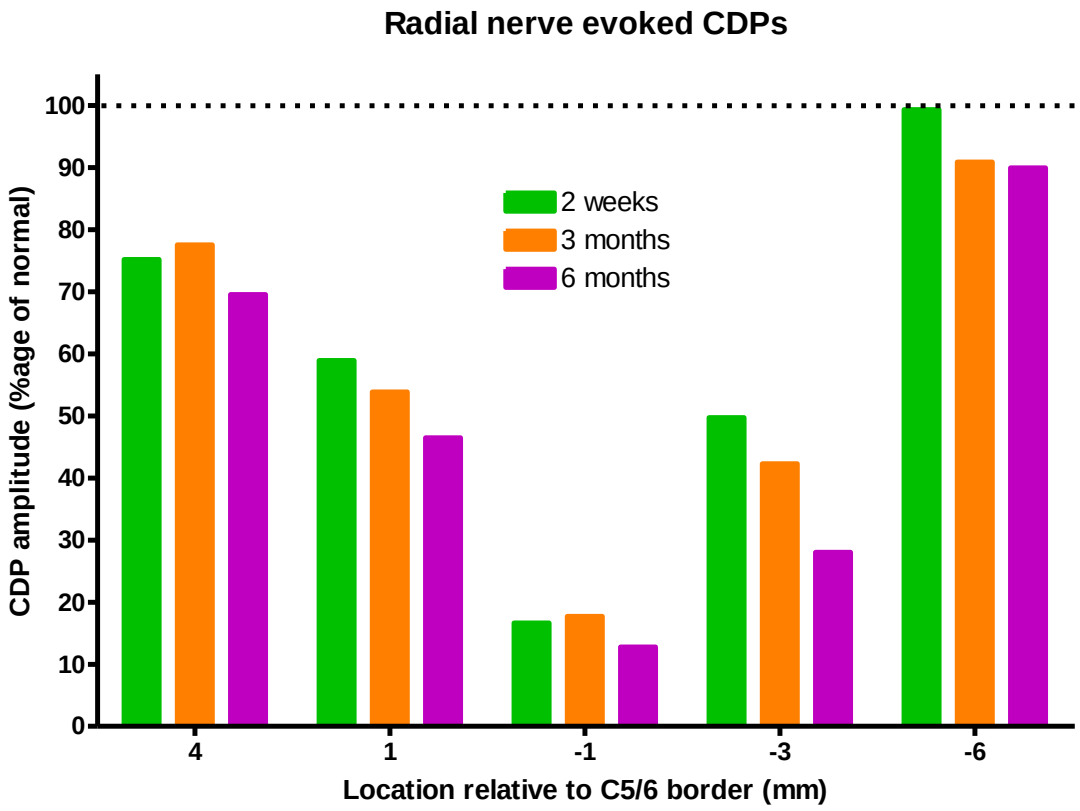

Figure 2-29. Changes in amplitude of radial nerve evoked CDPs in animals investigated 2 weeks, 3 months and 6 months after injury.

Plot showing the mean amplitudes of radial nerve evoked CDPs recorded from the animal groups investigated at 2 weeks, 3 months and 6 months after injury. The plot shows CDP amplitudes recorded at 5 different locations expressed as a percentage of the mean CDP amplitude in normal animals. Normal CDP amplitudes are represented as a dotted line (100 %). There were no significant differences between CDPs recorded at the different time points.

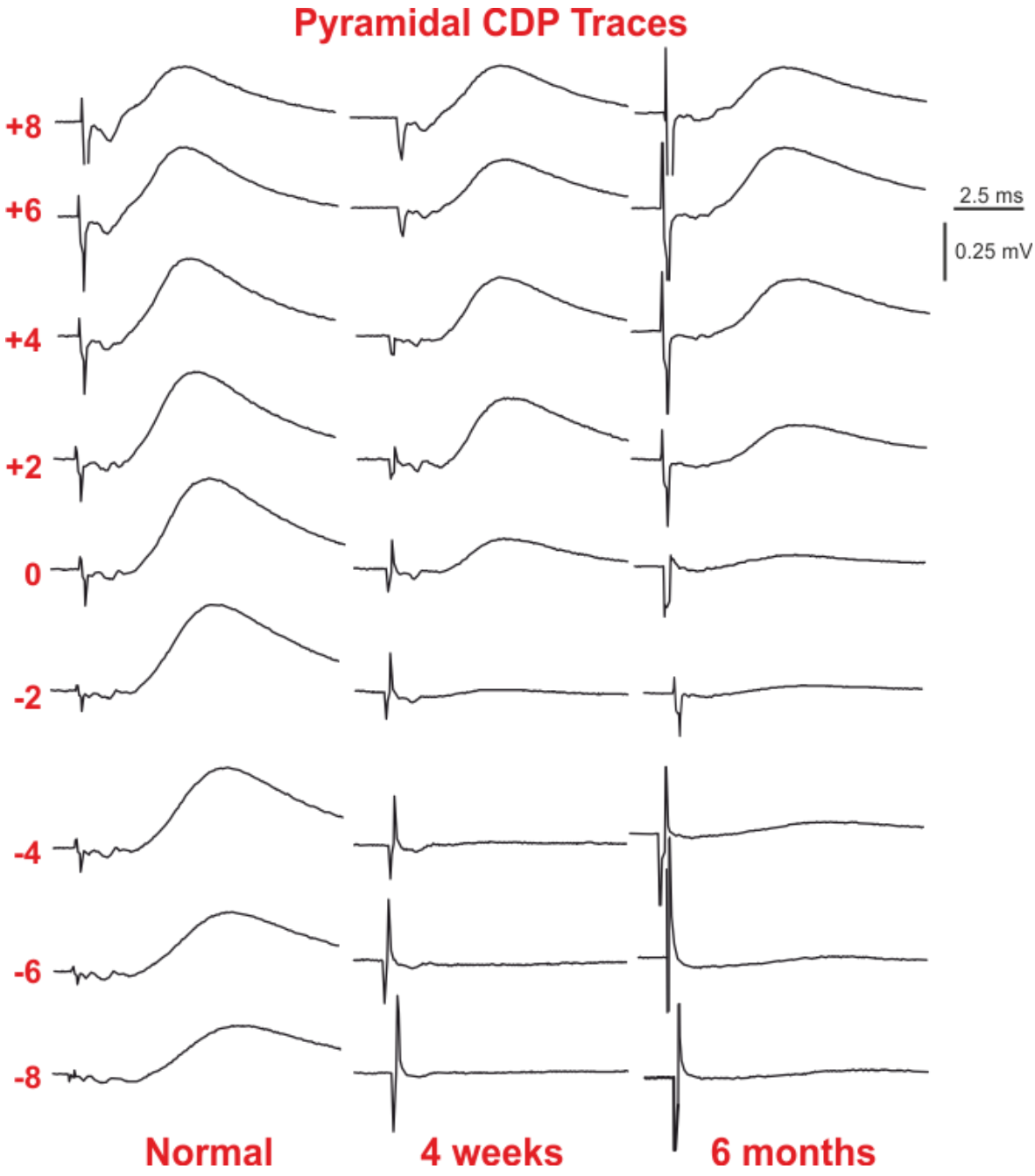

Figure 2-30. Examples of pyramidal evoked CDP traces recorded from a normal animal and from injured animals 4 weeks and 6 months after contusion.

Each column of traces shows recordings from one animal. On the left the recording is from a normal animal and recordings in the middle and on the right are from animals investigated at 4 weeks and 6 months after injury respectively. Recordings were made at 1 mm intervals from 8mm rostral (+8) to 8 mm caudal (-8) to C5/6 border. Only every other trace is shown for clarity. Traces represent averages of 25 sweeps and the calibration applies to all traces.

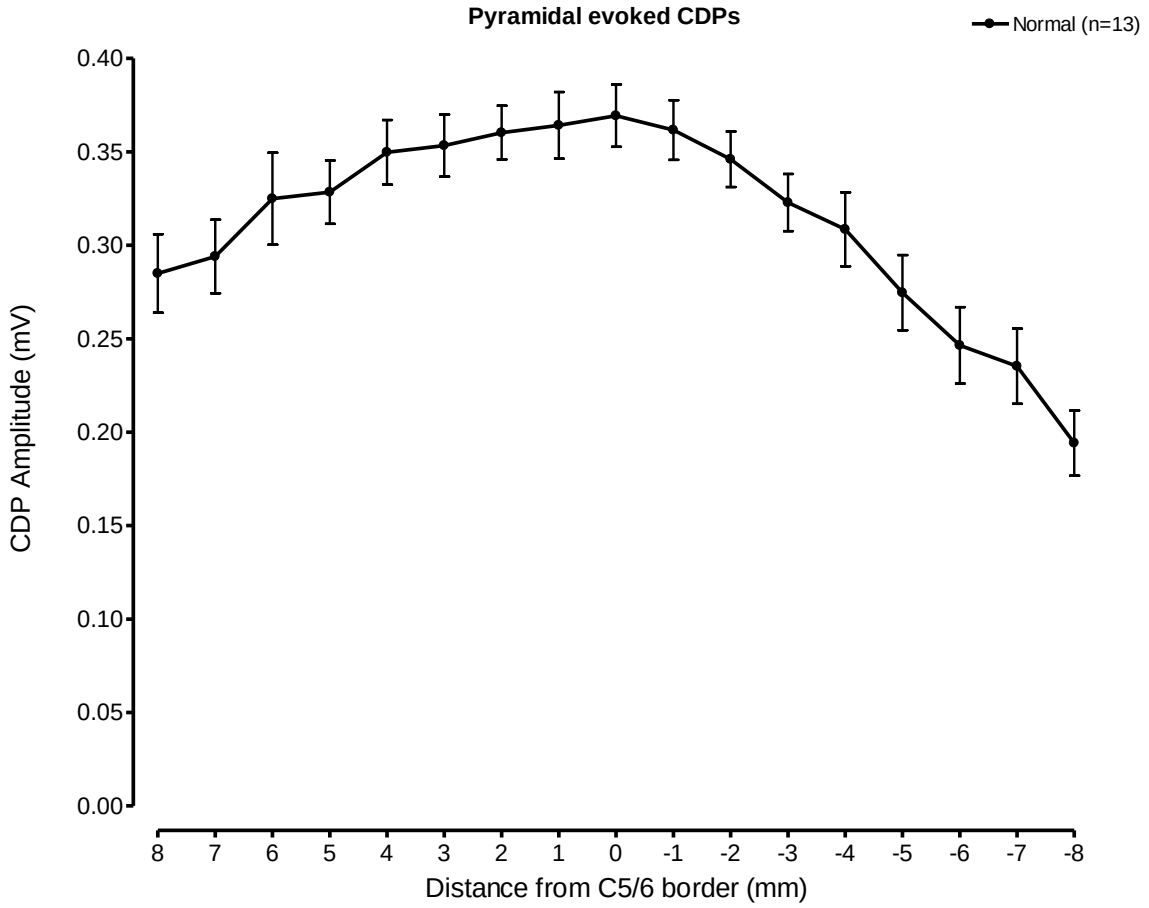

Figure 2-31. Amplitudes of pyramidal evoked CDPs recorded in normal animals.

Each data point is the mean CDP amplitude for all animals in the normal (non-injured) (n=13) group. The error bars show +/- SEM. The recordings were made over the cervical spinal cord. Numbers on the x-axis represents the recording locations on the surface of the cord relative to the C5/6 border (0 mm). In normal animals, the biggest pyramidal evoked CDPs were recorded around 4 mm above to 3 mm below the C5/6 border. The mean amplitudes of the largest CDPs were around 0.37 mV (at 0 mm and -1 mm). CDPs at the caudal end (-5 mm to -8 mm) were smaller in amplitude compared to the rostral end (+6 mm to +8 mm).

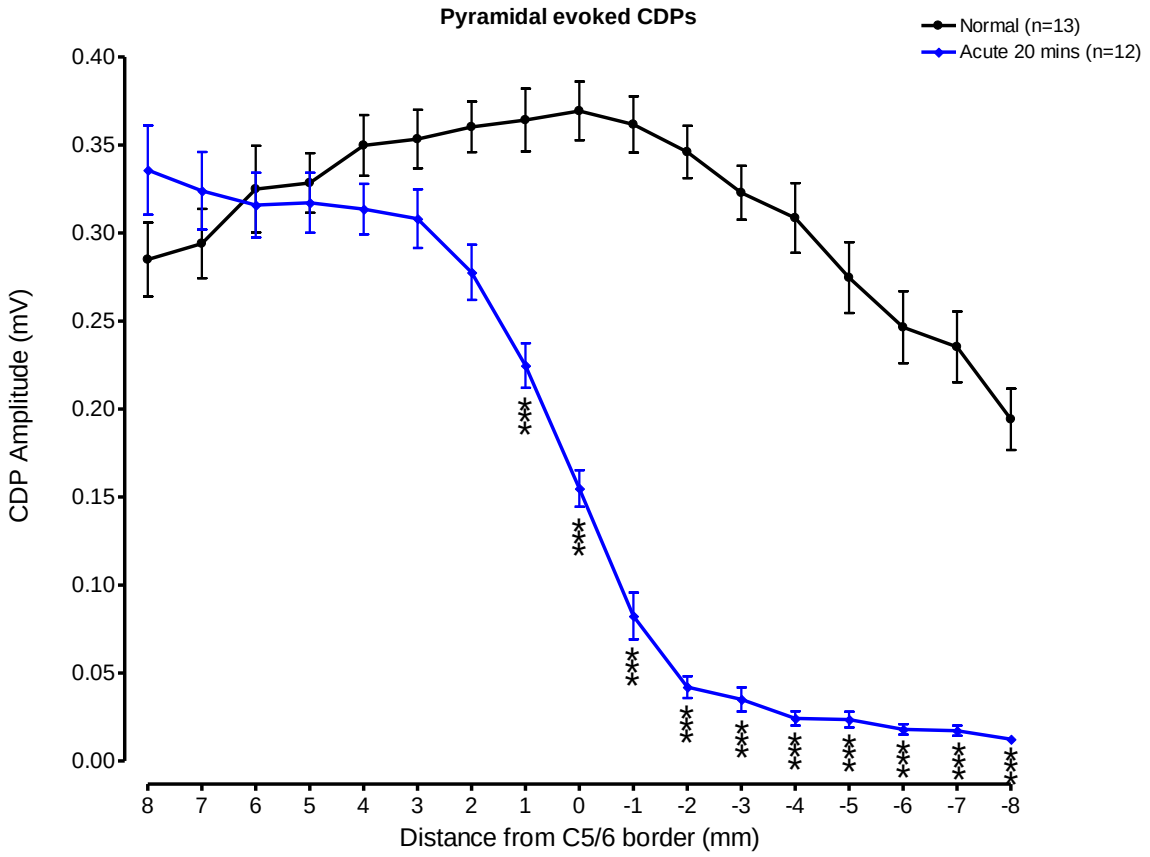

Figure 2-32. Amplitudes of pyramidal evoked CDPs recorded in acutely injured animals at 20 minutes after injury.

The plot shows the mean amplitudes of CDPs recorded 20 minutes after injury from animals in the acute injury group (n=12). The plot for normal animals is also shown for comparison. Each data point is the mean CDP amplitude for all animals in the group. The error bars show +/- SEM. The contusion injury was carried out with the rostral edge of the impactor tip aligned with C5/6 border (0 mm). The recordings were made over the cervical spinal cord. Numbers on the x-axis represent the recording locations on the surface of the cord relative to C5/6 border (0 mm). Twenty minutes after injury CDP amplitudes below the lesion epicentre (-2 mm to -8 mm) were virtually absent, while those above the injury (+3 mm to +8 mm) were largely unaffected. The asterisks (*) represent the statistical significance of the difference between CDP amplitudes recorded at 20 minutes compared to normal (* = $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$).

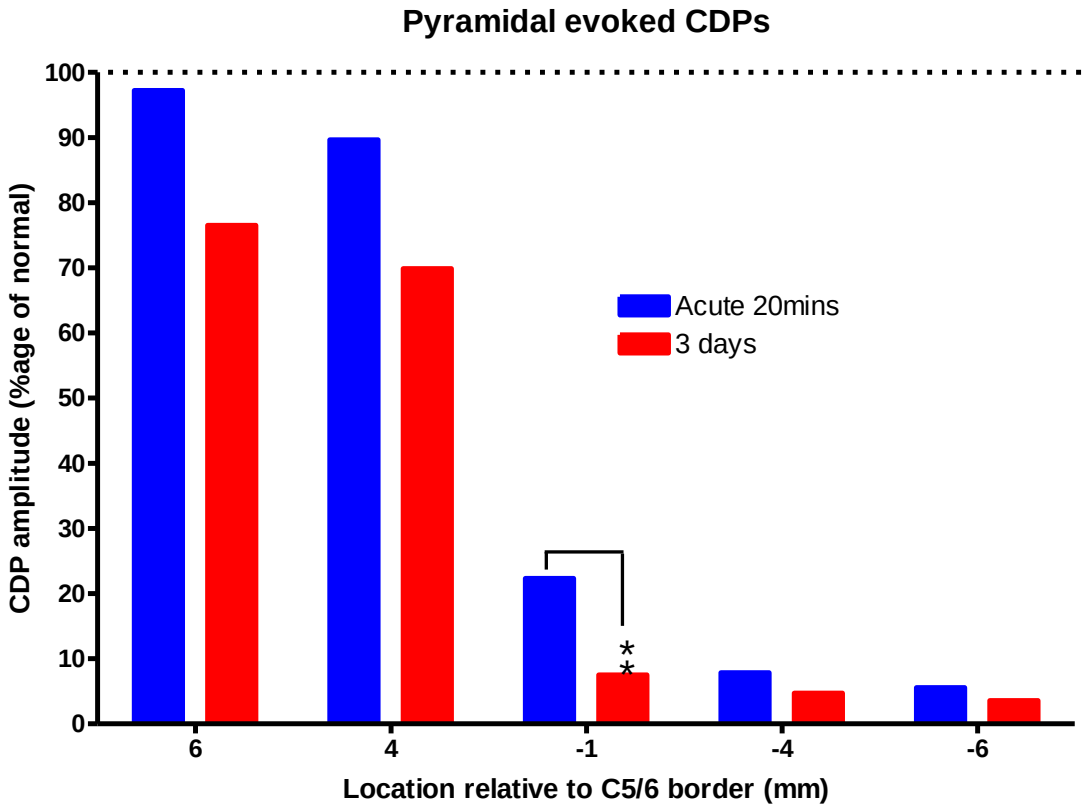

Figure 2-33. Changes in amplitude of pyramidal evoked CDPs in acutely injured animals and 3 day survival animals.

Plot showing the mean amplitudes of pyramidal evoked CDPs recorded in acutely injured animals at 20 minutes and in 3 day survival animals. The plots show amplitudes at 5 different locations plotted as a percentage of the mean CDP amplitude in normal animals. Normal CDP amplitudes are represented as a dotted line (100 %). The asterisks (*) represent the statistical significance of the difference between CDP amplitudes recorded acutely at 20 minutes compared to 3 days (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

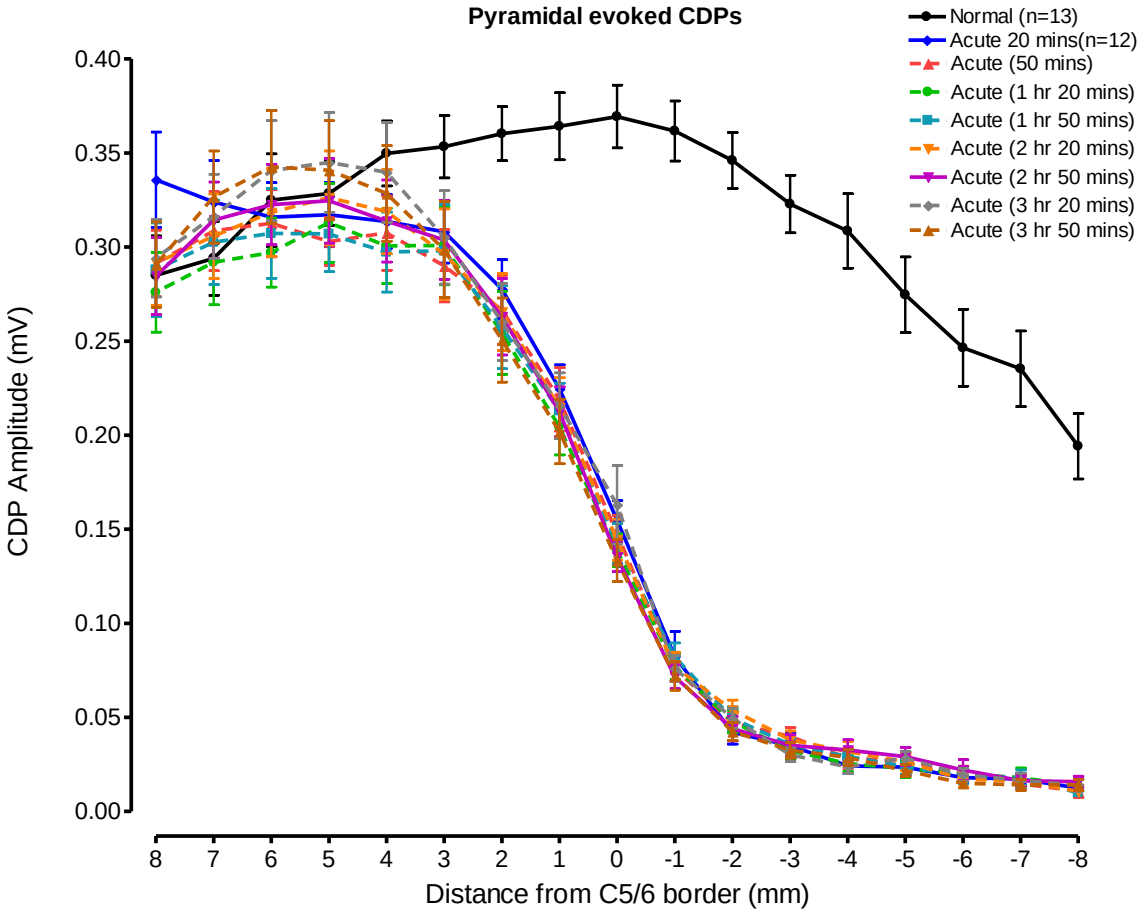

Figure 2-34. Amplitudes of pyramidal evoked CDPs recorded in acutely injured animals at all acute time points.

The plot shows the mean amplitudes of CDPs recorded at 30 minute intervals from 20 minutes to 3 hours 50 minutes after injury. The plot for normal animals is also shown for comparison. Each data point is the mean CDP amplitude for all animals in the group (n=12 for acute data points shown). The error bars show +/- SEM. The contusion injury was carried with the rostral edge of the impactor tip aligned with C5/6 border (0 mm). The recordings were made over the cervical spinal cord. Numbers on x-axis represent the recording locations on the surface of the cord relative to C5/6 border (0 mm). There was little change in the CDPs recorded at the start (20 minutes after injury) and end (3 hours 50 minutes) of the recordings made after acute injury.

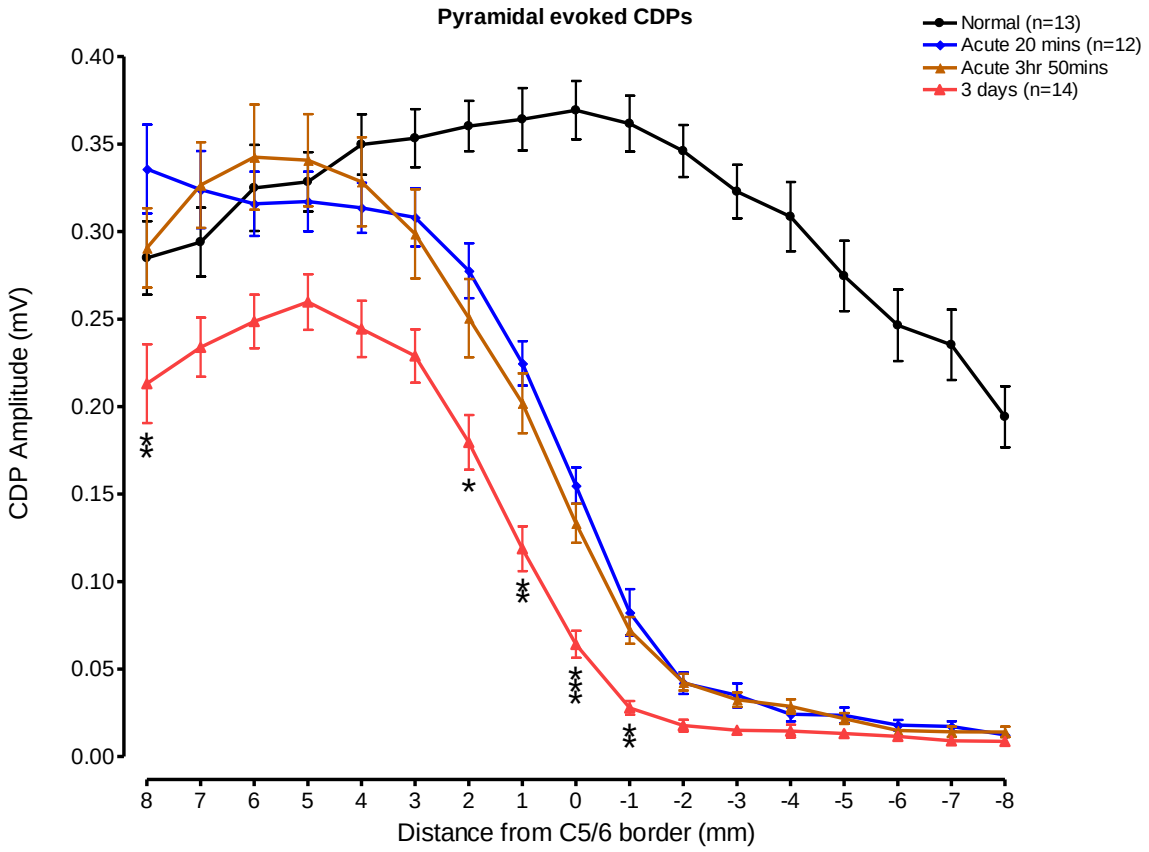

Figure 2-35. Amplitudes of pyramidal evoked CDPs recorded at the beginning and end of the acute experiments and from animals surviving 3 days after injury.

The plot shows a comparison of the mean amplitudes of CDPs recorded in acutely injured animals at 20 minutes (n=12) and 3 hours 50 minutes (n=12) and in animals 3 days (n=14) after injury. The plot for normal animals is also shown for comparison. Each data point is the mean CDP amplitude for all animals in the group. The error bars show +/- SEM. Contusion injury was carried out with the rostral edge of the impactor tip aligned with C5/6 border (0 mm). The recordings were made over the cervical spinal cord. Numbers on x-axis represent the recording locations on the surface of the cord relative to C5/6 border (0 mm). CDP amplitudes were smaller in the 3 day survival animals compared to acutely injured animals. The asterisks (*) represent the statistical significance of the difference between CDP amplitudes recorded acutely at 20 minutes compared to 3 days (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

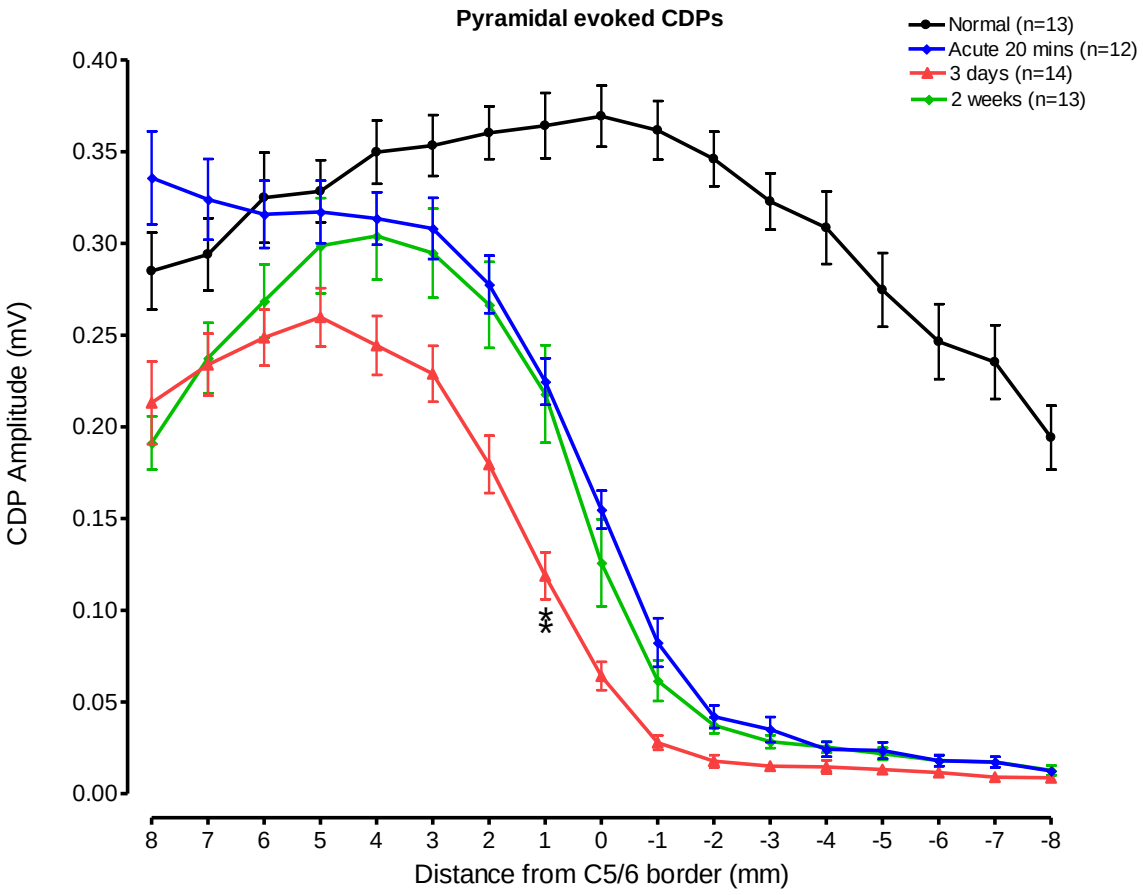

Figure 2-36. Amplitudes of pyramidal evoked CDPs recorded in acutely injured animals and from animals investigated 3 days and 2 weeks after injury.

The plot shows a comparison of the mean amplitudes of CDPs recorded in acutely injured animals at 20 minutes (n=12) and in animals investigated at 3 days (n=14) and 2 weeks (n=13) after injury. The plot for normal animals is also shown for comparison. Each data point is the mean CDP amplitude for all animals in the group. The error bars show +/- SEM. Contusion injury was carried out with the rostral edge of the impactor tip aligned with C5/6 border (0 mm). The recordings were made over the cervical spinal cord. Numbers on x-axis represent the recording locations on the surface of the cord relative to C5/6 border (0 mm). In the 2 week survival animals CDPs are restored at most locations almost to the amplitudes seen in acutely injured animals at 20 minutes after injury. The asterisks (*) represent the statistical significance of the difference between CDP amplitudes recorded at 3 days compared to 2 weeks (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

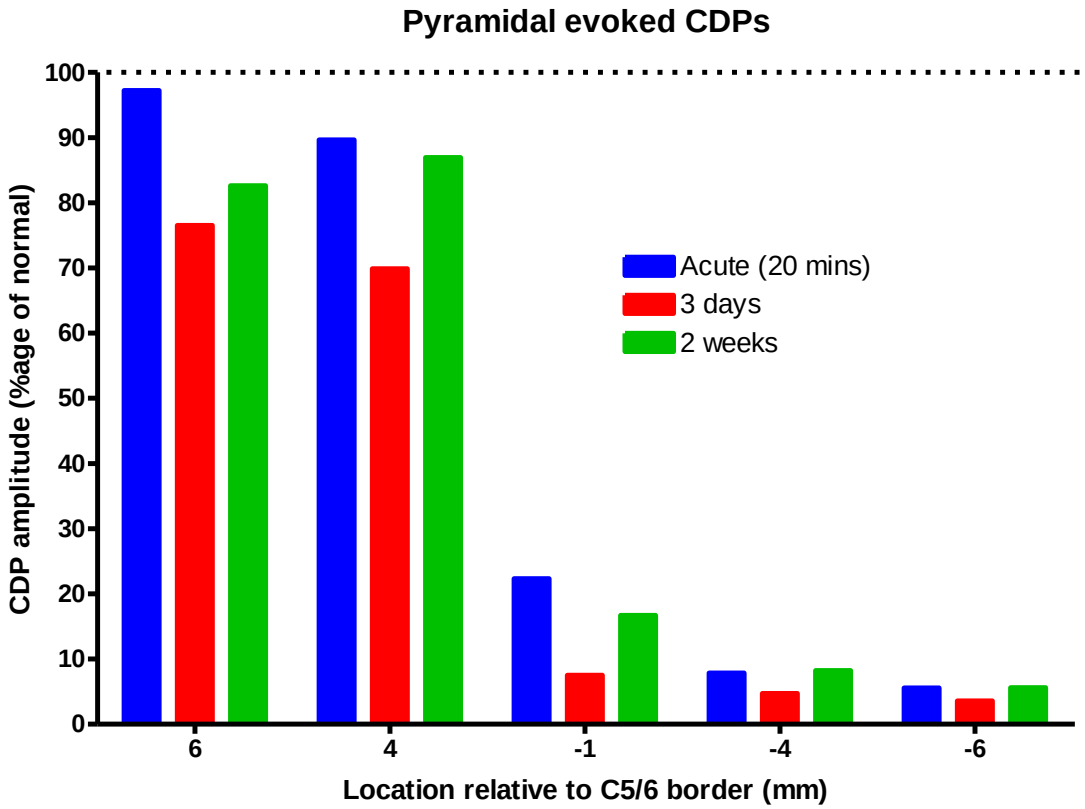

Figure 2-37. Comparison of the amplitudes of pyramidal evoked CDPs in acutely injured animals and animals investigated at 3 days and at 2 weeks after injury.

Plot showing the mean amplitudes of pyramidal evoked CDPs recorded 20 minutes after an acute injury compared to animals recorded from at 3 days and at 2 weeks after injury. The plot shows amplitudes at 5 different locations expressed as a percentage of the mean CDP amplitude in normal animals. Normal CDP amplitudes are represented as a dotted line (100 %). There were no significant differences between CDPs recorded at the different time points.

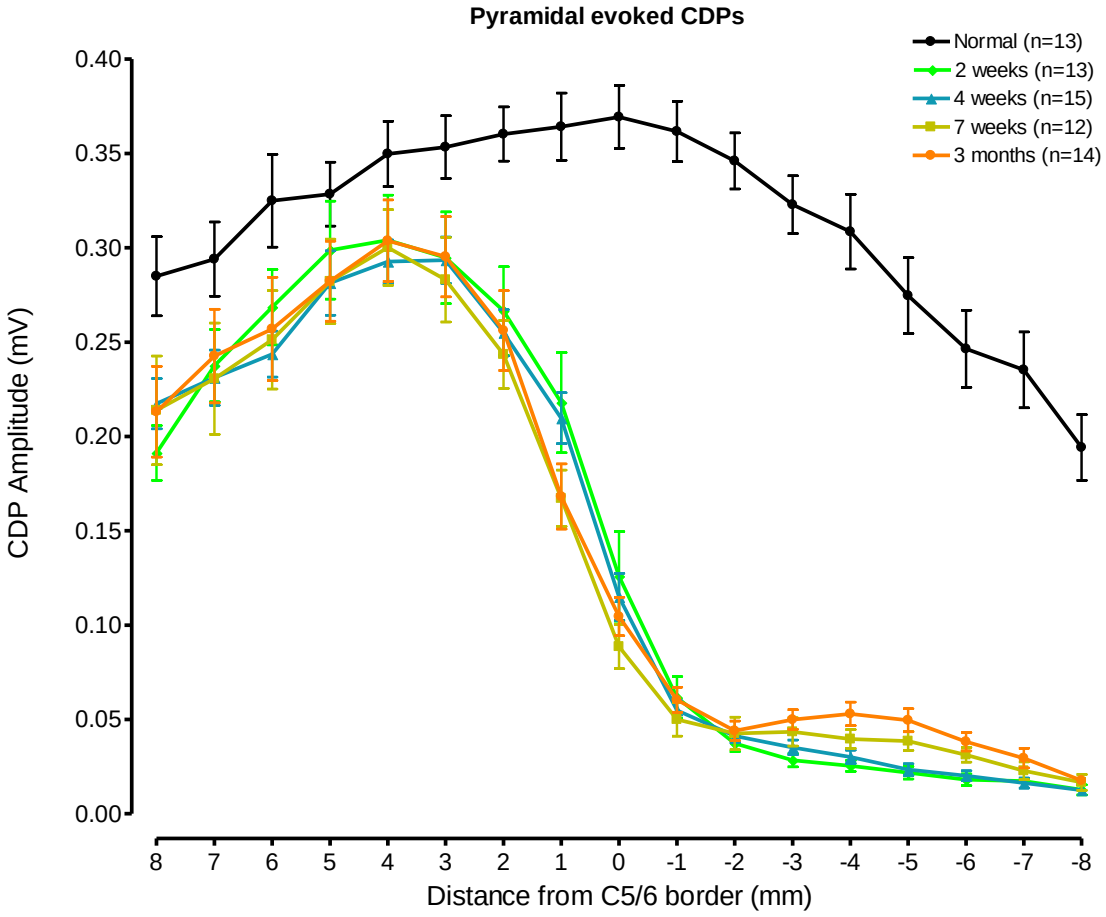

Figure 2-38. Amplitudes of pyramidal evoked CDPs recorded in surviving animals at 2 weeks, 4 weeks, 7 weeks and 3 months after injury.

The plot shows a comparison of the mean amplitudes of CDPs recorded from the groups of animals investigated at 2 weeks (n=13), 4 weeks (n=15), 7 weeks (n=12) and 3 months (n=14) after injury. The plot for normal animals is also shown for comparison. Each data point is the mean CDP amplitude for all animals in the group. The error bars show +/- SEM. Contusion injury was carried out with the rostral edge of the impactor tip aligned with C5/6 border (0 mm). The recordings were made over the cervical spinal cord. Numbers on x-axis represent the recording locations on the surface of the cord relative to C5/6 border (0 mm). CDPs rostral to the injury and at the injury epicentre remained almost stable between 2 weeks and 3 months after injury, however, a slight increase in CDP amplitudes was observed over time caudal (-3 mm to -6 mm) to the injury. There were no significant differences between CDPs recorded at the different time points.

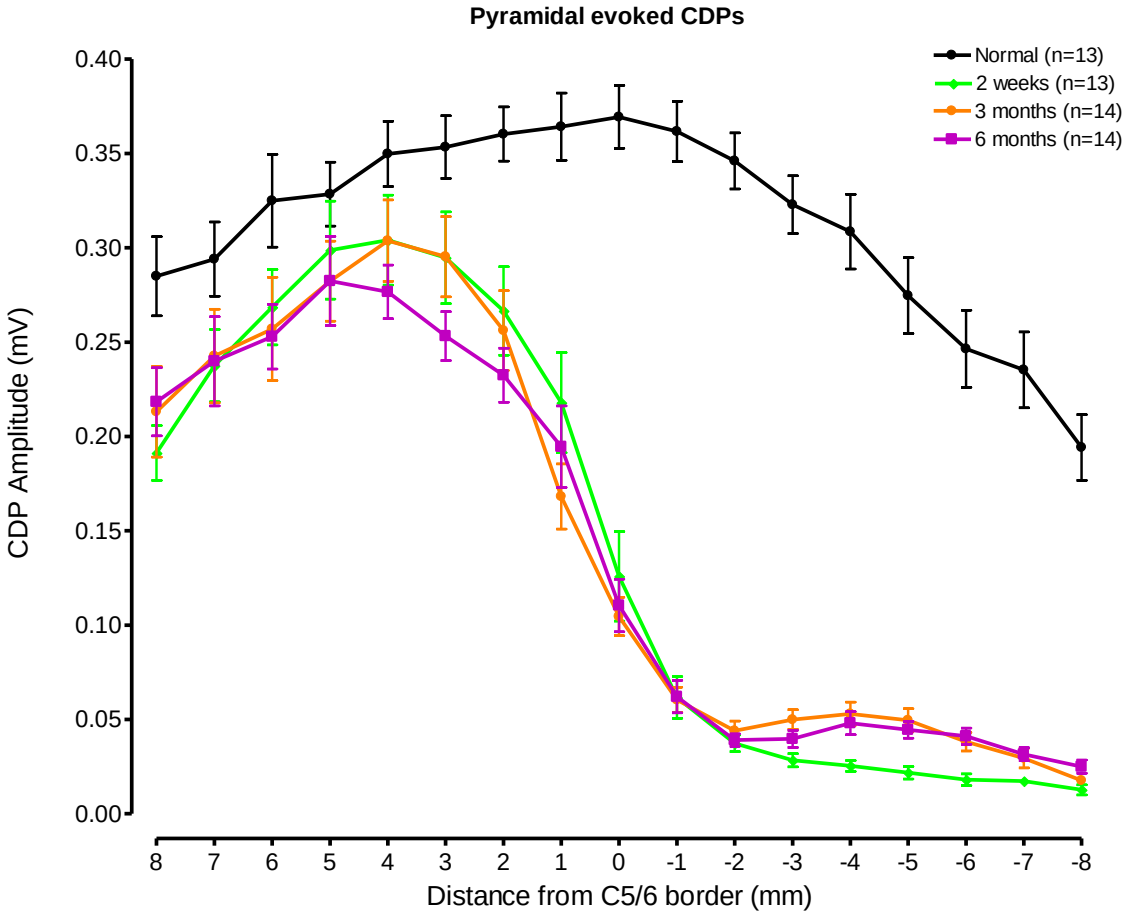

Figure 2-39. Amplitudes of pyramidal evoked CDPs recorded from animals investigated 2 weeks, 3 months and 6 months after injury.

The plot shows a comparison of the mean amplitudes of CDPs recorded from animal groups investigated 2 weeks (n=13), 3 months (n=14) and 6 months (n=14) after injury. The plot for normal animals is also shown for comparison. Each data point is the mean CDP amplitude for all animals in the group. The error bars show +/- SEM. Contusion injury was carried out with the rostral edge of the impactor tip aligned with C5/6 border (0 mm). The recordings were made over the cervical spinal cord. Numbers on x-axis represent the recording locations on the surface of the cord relative to C5/6 border (0 mm). In the 6 month survival animals CDPs below the injury (-3 mm to -7 mm) showed increased amplitudes compared to the 2 week survival animals but were similar to those recorded from the 3 month group. There were no significant differences between CDPs recorded at the different time points.

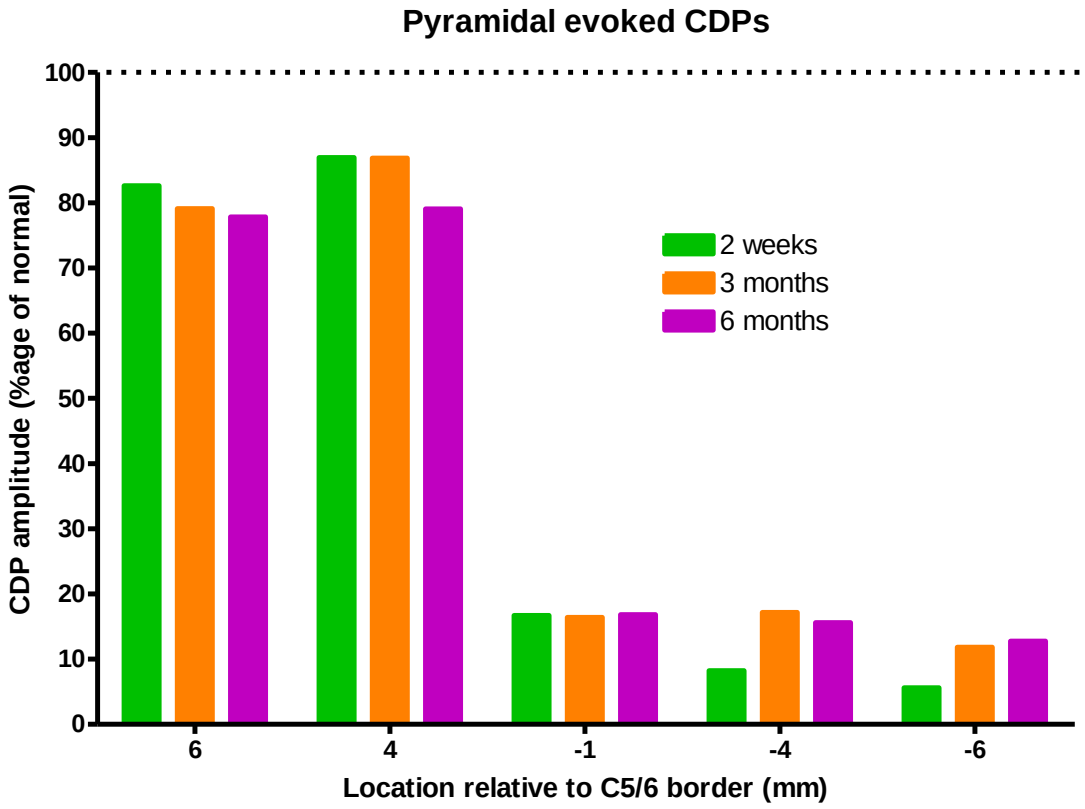

Figure 2-40. Changes in amplitude of pyramidal evoked CDPs in animals investigated 2 weeks, 3 months and 6 months after injury.

Plot showing the mean amplitudes of pyramidal evoked CDPs recorded in animals investigated at 2 weeks, 3 months and 6 months after injury. The plot shows CDP amplitudes recorded at 5 different locations expressed as a percentage of the mean CDP amplitude in normal animals. Normal CDP amplitudes are represented as a dotted line (100 %). There were no significant differences between CDPs recorded at the different time points.

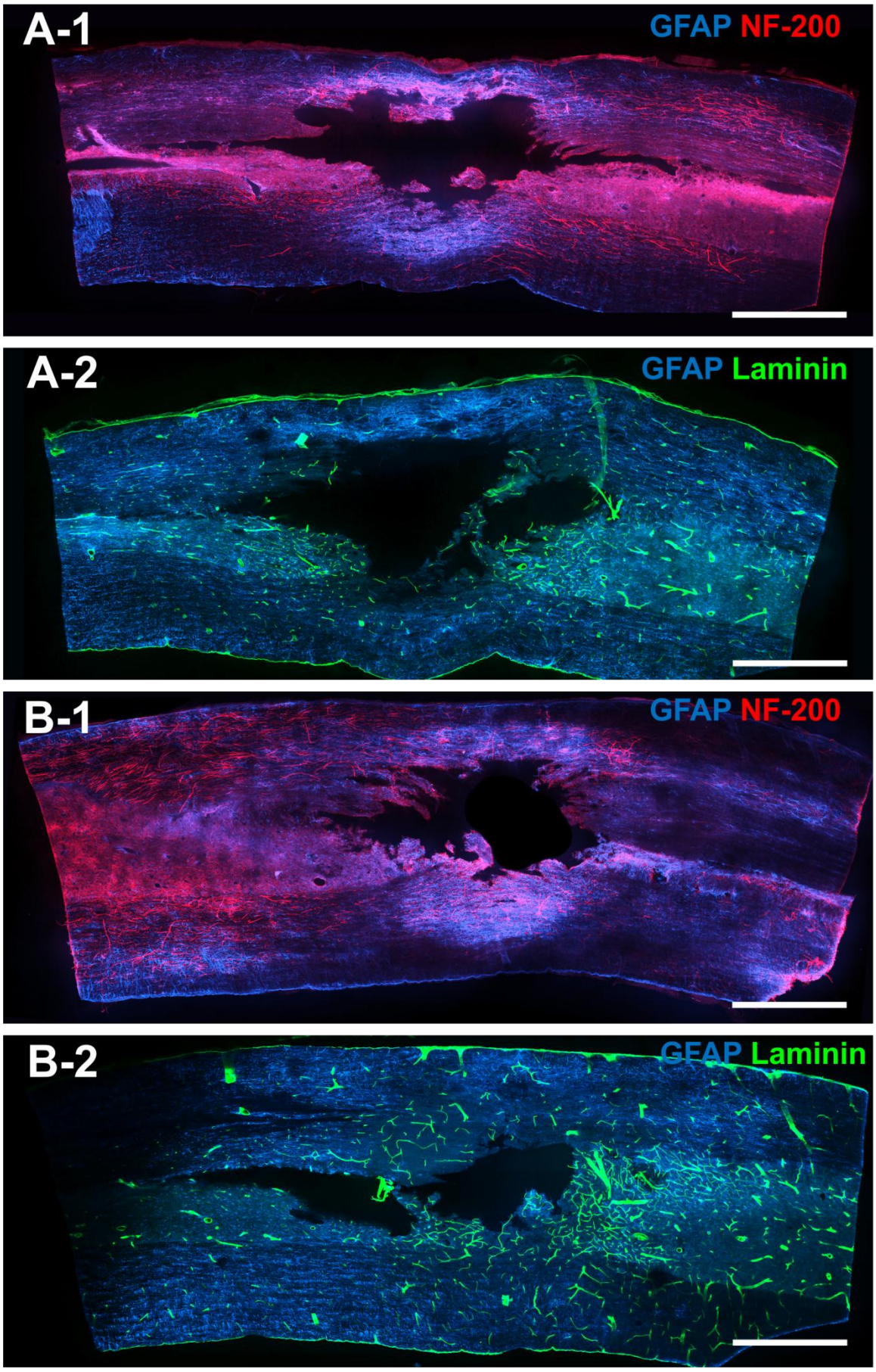

Figure 2-41. Examples of injury site histology at an acute time point.

The panels show confocal microscope images of the injury site approximately 4 hours after injury. Each panel is an example obtained from a parasagittal section passing through the injury area close to the midline. The top two panels (A-1 and A-2) show sections from one animal and the bottom two panels (B-1 and B-2) show sections from a second animal. All images show the distribution of GFAP immunolabelling (blue). A-1 and B-1 show NF 200 immunolabelling in addition while A-2 and B-2 show laminin distribution. The micrographs are projections of 7-9 optical sections and composed from multiple x20 fields of view. All scale bars are 1 mm.

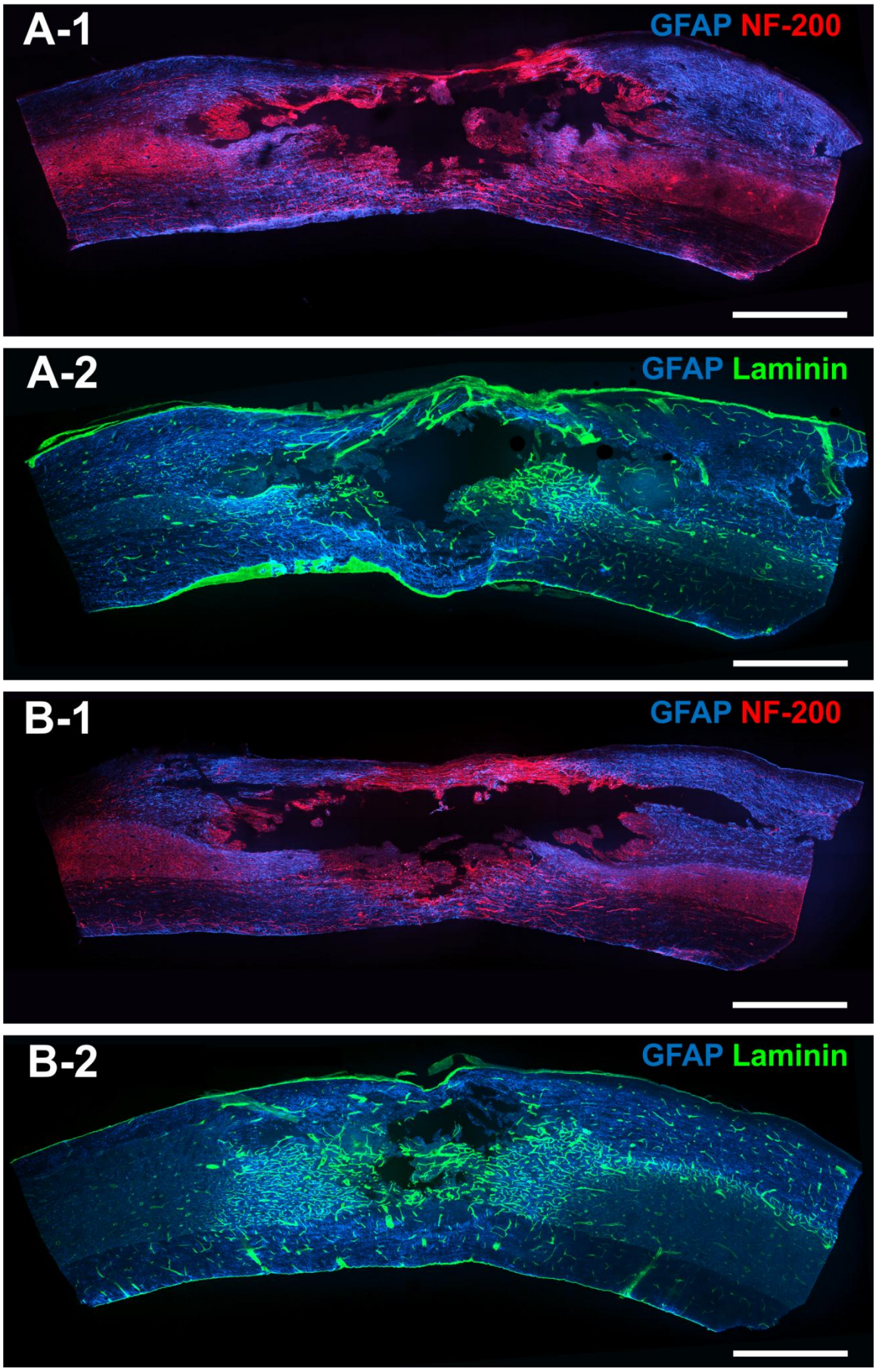

Figure 2-42. Examples of injury site histology at 3 days after injury.

The panels show confocal microscope images of the injury site in 3 days survival animals after injury. Each panel is an example obtained from a parasagittal section passing through the injury area close to the midline. The top two panels (A-1 and A-2) show sections from one animal and the bottom two panels (B-1 and B-2) show sections from a second animal. All images show the distribution of GFAP immunolabelling (blue). A-1 and B-1 show NF 200 immunolabelling in addition while A-2 and B-2 show laminin distribution. The micrographs are projections of 7-9 optical sections and composed from multiple x20 fields of view. All scale bars are 1 mm.

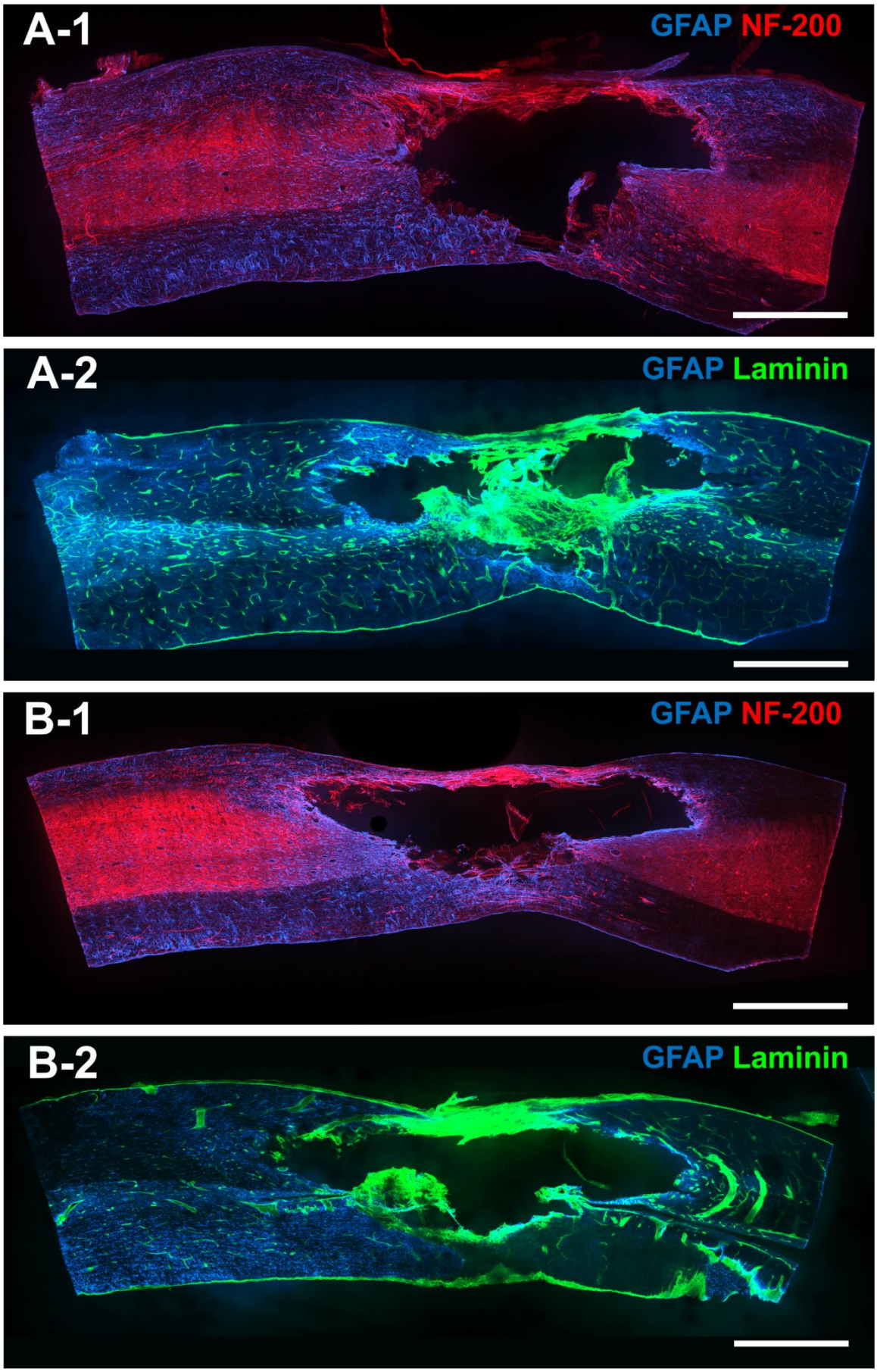

Figure 2-43. Examples of injury site histology at 2 weeks after injury.

The panels show confocal microscope images of the injury site in 2 weeks survival animals after injury. Each panel is an example obtained from a parasagittal section passing through the injury area close to the midline. The top two panels (A-1 and A-2) show sections from one animal and the bottom two panels (B-1 and B-2) show sections from a second animal. All images show the distribution of GFAP immunolabelling (blue). A-1 and B-1 show NF 200 immunolabelling in addition while A-2 and B-2 show laminin distribution. The micrographs are projections of 7-9 optical sections and composed from multiple x20 fields of view. All scale bars are 1 mm.

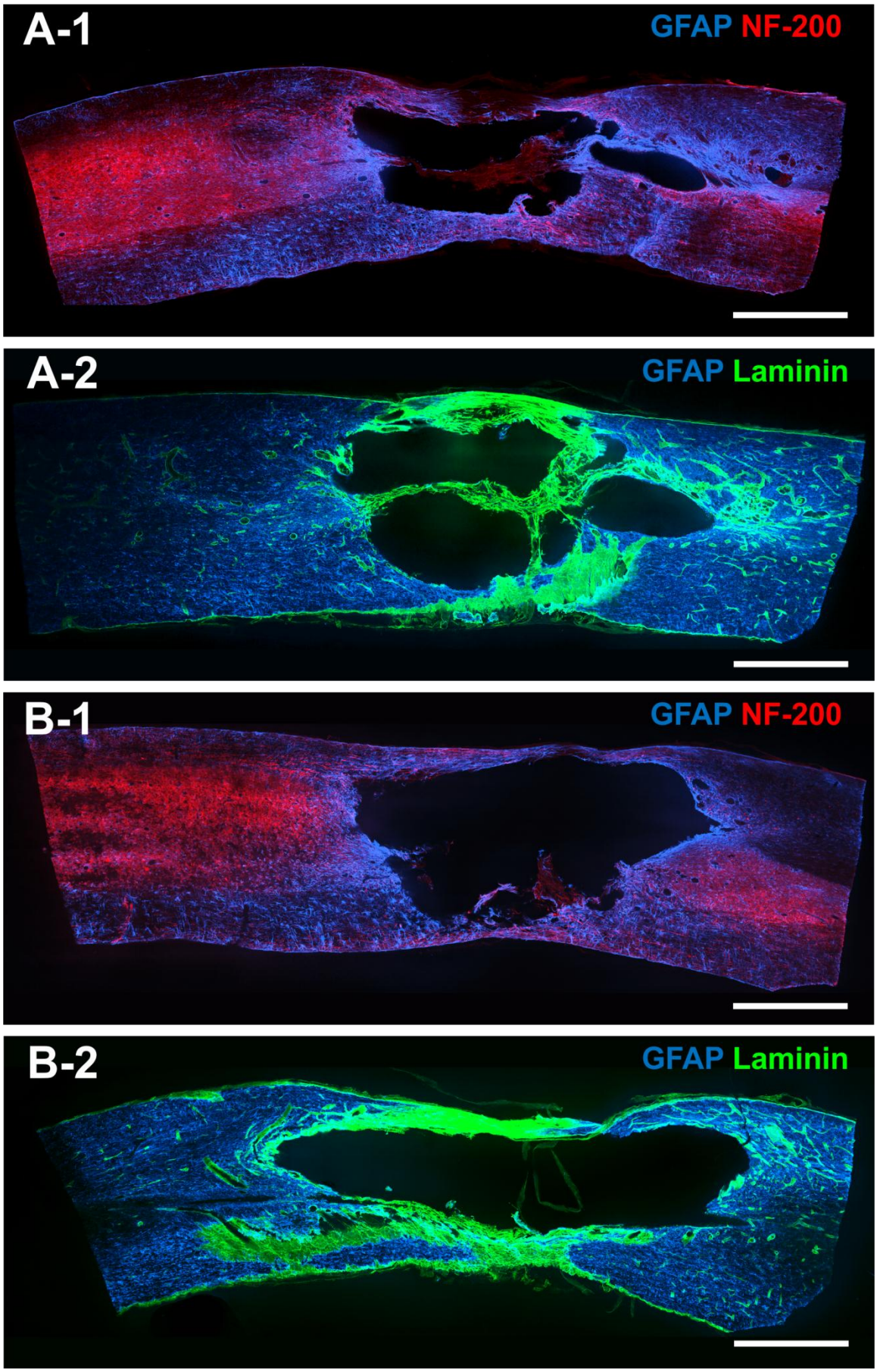

Figure 2-44. Examples of injury site histology at 4 weeks after injury.

The panels show confocal microscope images of the injury site in 4 weeks survival animals after injury. Each panel is an example obtained from a parasagittal section passing through the injury area close to the midline. The top two panels (A-1 and A-2) show sections from one animal and the bottom two panels (B-1 and B-2) show sections from a second animal. All images show the distribution of GFAP immunolabelling (blue). A-1 and B-1 show NF 200 immunolabelling in addition while A-2 and B-2 show laminin distribution. The micrographs are projections of 7-9 optical sections and from multiple x20 fields of view. All scale bars are 1 mm.

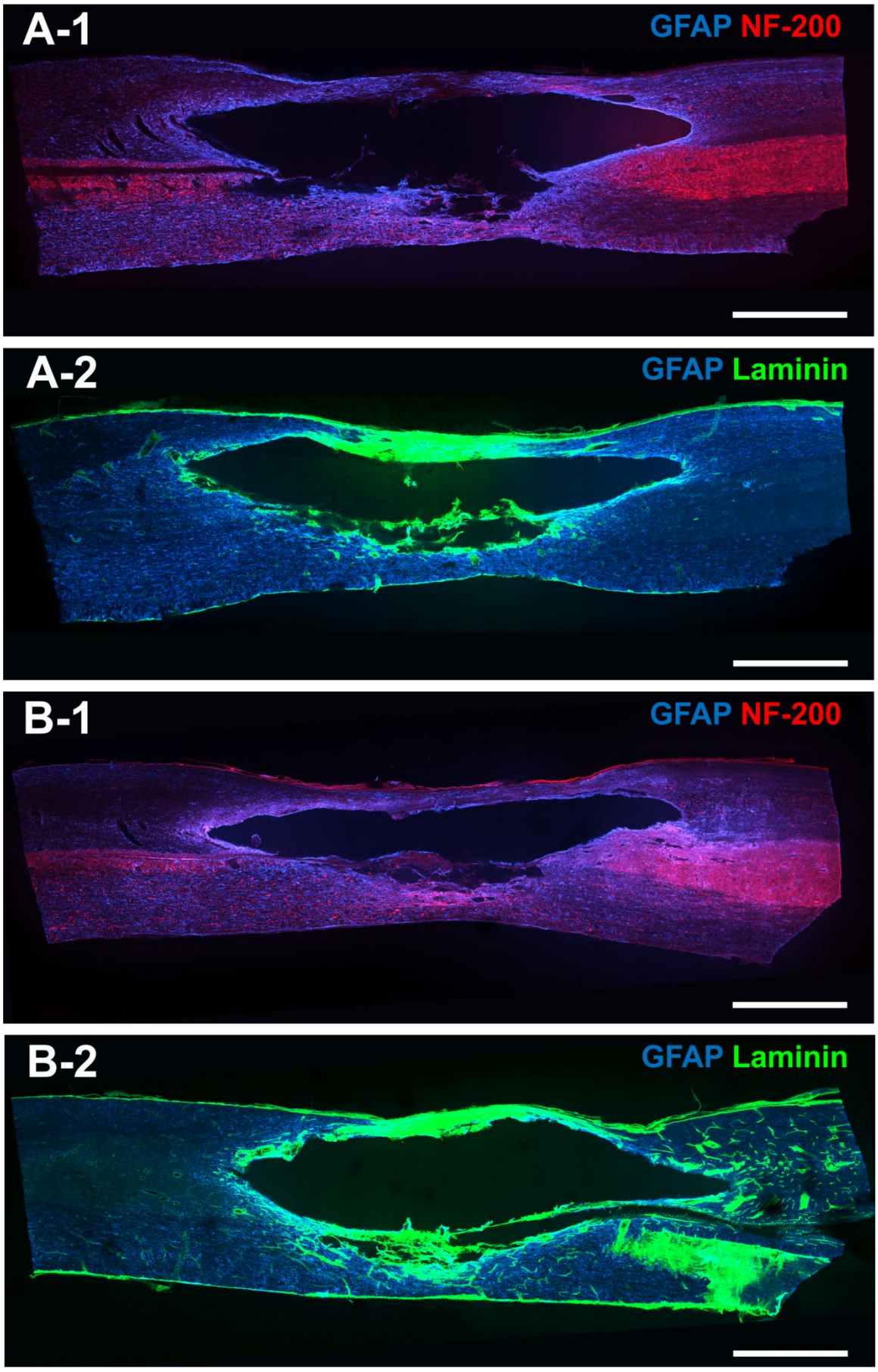

Figure 2-45. Examples of injury site histology at 7 weeks after injury.

The panels show confocal microscope images of the injury site in 7 weeks survival animals after injury. Each panel is an example obtained from a parasagittal section passing through the injury area close to the midline. The top two panels (A-1 and A-2) show sections from one animal and the bottom two panels (B-1 and B-2) show sections from a second animal. All images show the distribution of GFAP immunolabelling (blue). A-1 and B-1 show NF 200 immunolabelling in addition while A-2 and B-2 show laminin distribution. The micrographs are projections of 7-9 optical sections and composed from multiple x20 fields of view. All scale bars are 1 mm.

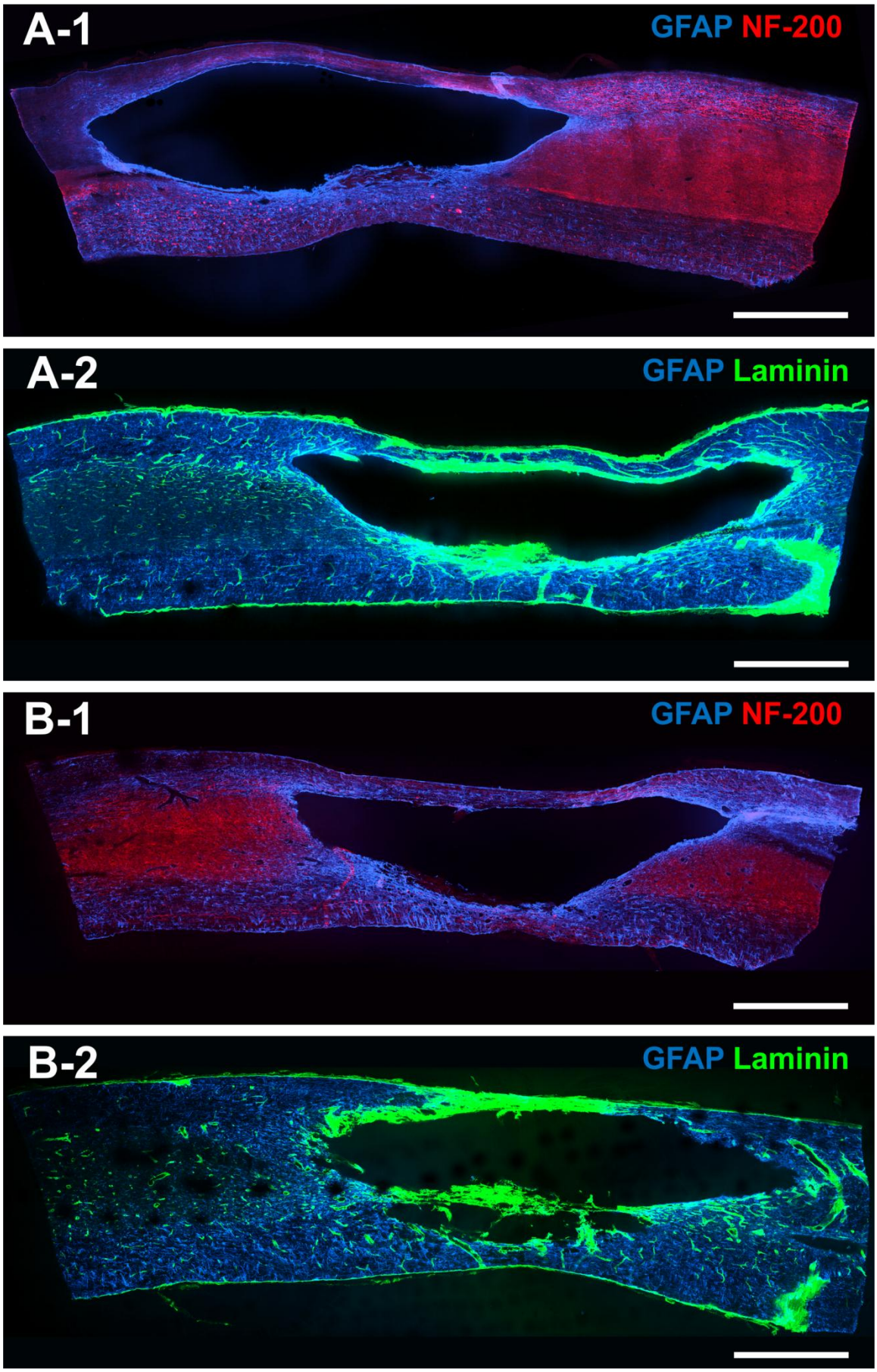

Figure 2-46. Examples of injury site histology at 3 months after injury.

The panels show confocal microscope images of the injury site in 3 months survival animals after injury. Each panel is an example obtained from a parasagittal section passing through the injury area close to the midline. The top two panels (A-1 and A-2) show sections from one animal and the bottom two panels (B-1 and B-2) show sections from a second animal. All images show the distribution of GFAP immunolabelling (blue). A-1 and B-1 show NF 200 immunolabelling in addition while A-2 and B-2 show laminin distribution. The micrographs are projections of 7-9 optical sections and composed from multiple x20 fields of view. All scale bars are 1 mm.

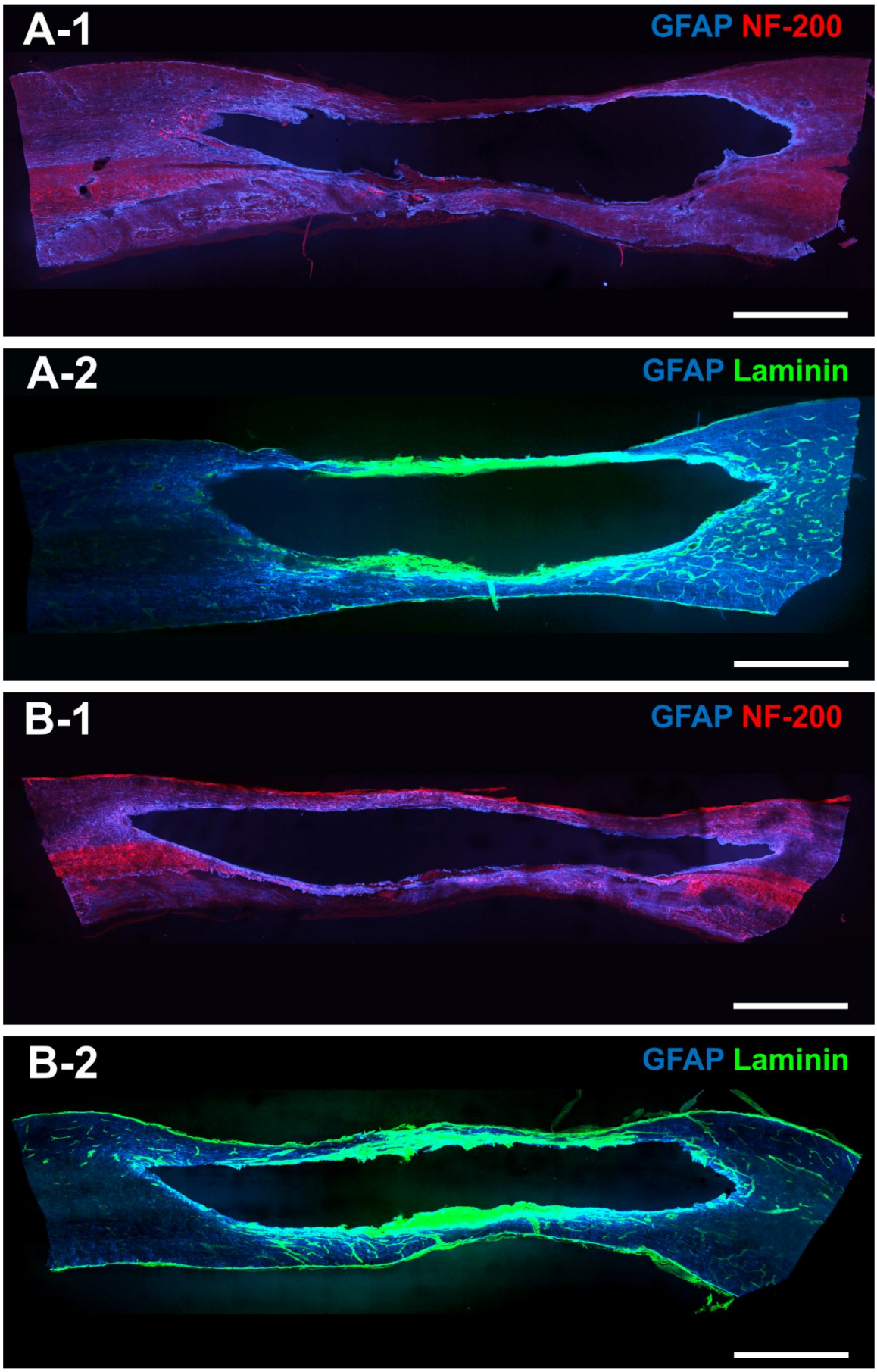

Figure 2-47. Examples of injury site histology at 6 months after injury.

The panels show confocal microscope images of the injury site in 6 months survival animals after injury. Each panel is an example obtained from a parasagittal section passing through the injury area close to the midline. The top two panels (A-1 and A-2) show sections from one animal and the bottom two panels (B-1 and B-2) show sections from a second animal. All images show the distribution of GFAP immunolabelling (blue). A-1 and B-1 show NF 200 immunolabelling in addition while A-2 and B-2 show laminin distribution. The micrographs are projections of 7-9 optical sections and composed from multiple x20 fields of view. All scale bars are 1 mm.

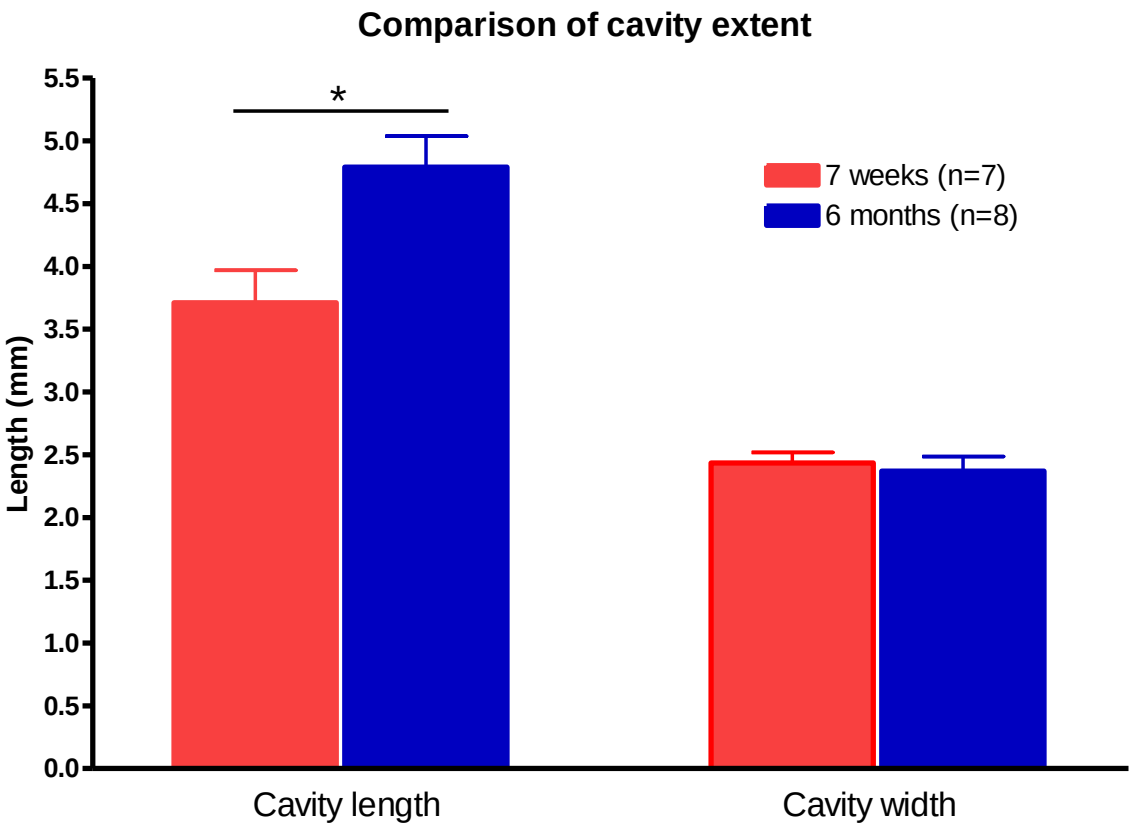

Figure 2-48. Comparison of injury cavity extent in animals at 7 weeks and 6 months after injury.

The bar plots represent the total rostro-caudal (length) and lateral (width) extent of the injury cavity measured from histology of the injury site from the groups of animals investigated 7 weeks (red) after injury and 6 months (blue) after injury. The error bars represent standard error of the mean (Fujimoto et al.). The cavity was significantly longer in the 6 month animals than the 7 week group. Statistical analysis used was students t-test and asterisk (*) indicates p value of <0.05.

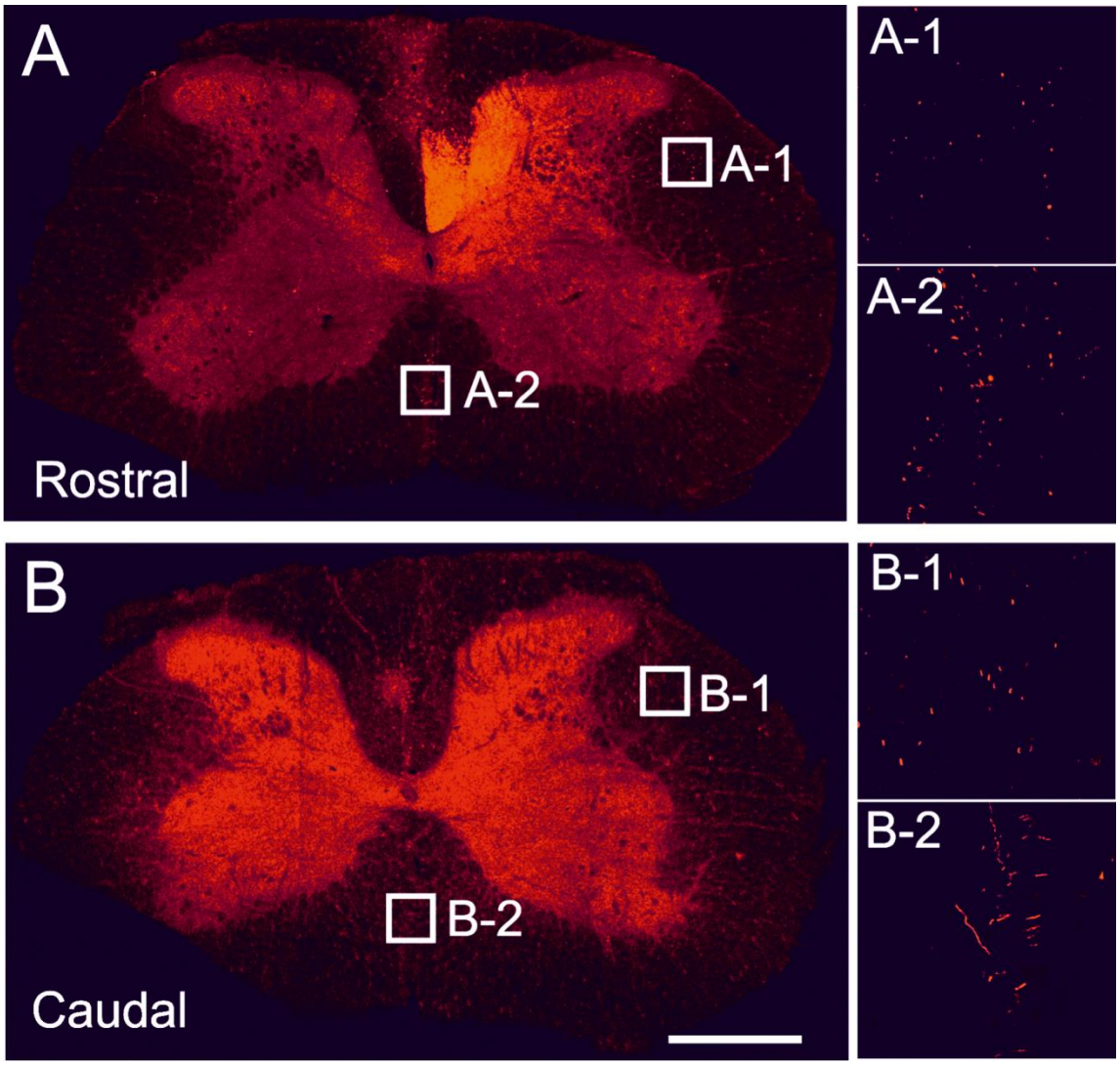

Figure 2-49. Example of BDA-labelled corticospinal tract after C6 contusion.

The panels show confocal images of anterograde labelling of left CST 4 weeks after contusion injury. Panels represent section rostral (A) and caudal (B) to the injury site. A-1 and B-1 show BDA labelled CST fibres in the lateral white matter, while A-2 and B-2 show the ventral CST fibres (scale bar=0.5 mm). BDA labelled CST fibres are absent in the dorsal column suggests complete axotomy after contusion injury.

2.5 Discussion

2.5.1 Rationale for using cord dorsum potentials as a measure of function in the spinal cord

In this chapter an electrophysiological approach has been used to investigate the time course of functional changes in grey matter of the spinal cord following a contusion injury. The main approach has been to maximally stimulate the sensory afferent fibres from the radial nerve and descending corticospinal pathways and record the surface potentials generated by these pathways in the vicinity of the injury. Measuring the surface potentials in the form of cord dorsum potentials provides a sensitive method to measure the global activity produced at connections within the spinal cord in order to quantify the efficacy of these pathways. Therefore, such recordings can be used to directly investigate functional changes in the spinal cord following injury. However, CDP measurements are biased towards detection of activity in the dorsal grey matter as this is closer to the electrode on the dorsal surface. This makes them particularly suitable for measuring the synaptic connections of sensory afferents and the corticospinal tract which terminate predominantly in the dorsal grey matter (Casale et al., 1988, Willis and Coggeshall, 2004).

Impact to the dorsal surface of the cord causes damage to axons in the dorsal column and neurons and their connections in the dorsal grey matter. However, the midline injuries cause damage to axons located more medially in the white matter and those located more laterally are spared. Our model of C6 injury is made at the centre of the cord length where stimulation of sensory afferents of the radial nerve produces CDPs. The CDPs at this location represent the synaptic current generated at the connections between the sensory afferents from the radial nerve and the neurons within the grey matter of the spinal cord. Measurements of the amplitudes of CDPs therefore provide a measure of the strength of the connections between the sensory afferents and the neurons in the spinal cord. Hence CDP amplitudes provide a direct measure of function at the level of the spinal cord and loss of connections caused by the injury at the C6 level can be expected to lead to a decrease in CDP amplitudes.

2.5.2 Loss of sensory function at the site of impact immediately after contusion injury

Investigation of the recordings made immediately after the contusion injury show that there is profound loss of sensory potentials localised to the area of the impact which is evident by a dip in the distribution of CDPs evoked by radial nerve stimulation compared to normal. In this injury model of midline contusion, CDPs can still be evoked at close to normal amplitudes both above and below the injury. The relatively normal amplitudes of CDPs at positions farthest from the injury and especially of those above the injury suggests that the main sensory dorsal columns axons from the radial nerve are not substantially damaged. The radial afferents enter mainly at the C6 and C7 levels and if they were damaged then the potentials above the injury in particular would be reduced in amplitude or abolished as following a wire knife injury to the dorsal columns (Toft et al., 2007). Since there is no evidence that dorsal column axons are damaged in the immediately acute phase of the injury. This in turn suggests that significant decline in the CDP amplitudes at the injury epicentre is mainly due to disruption of the neurons and local axon collaterals by which these potentials are generated. Acute recordings show that most of the damage or functional loss at the centre of the impact occurs at the time of the impact but to each side of the injury there is gradual worsening of function with each consecutive recording made at 30 minute intervals for the first 4 hours.

The fact that parent dorsal column axons from the radial nerve are spared in the midline cervical injury is probably due to the fact that sensory axons in the dorsal columns are topographically organised so that sensory afferents from the forelimbs, including those from the radial nerve, occupy locations that lie laterally (fasiculus cuneatus). In comparison dorsal column axons from hindlimbs (e.g. sciatic nerve) are located in the medial portion (fasiculus gracilis) of the dorsal column. Thus by centering the impactor tip on the midline of the C6 segment of the spinal cord the forelimb afferents would be relatively spared compared to those from the hindlimbs. In addition, Anderson and colleagues (2009) reported more severe impairment of sensory function (by using von Frey test) in the hindlimbs compared to forelimbs following a midline cervical contusion (Anderson et al., 2009b). However, these findings have not been

confirmed with anatomical methods and electrophysiological techniques. Confirmatory anatomical evidence to show that primary afferents from the radial nerve are spared in this injury model could be provided by injecting Cholera toxin b (Ctb) tracer into the forelimb nerves. In addition, electrophysiological evidence that primary afferents from the forelimbs projecting in the dorsal column are not affected by the midline cervical injury could be obtained by recordings sensory evoked potentials (SEPs) on stimulation of forelimb (i.e. radial) nerves.

This loss of function attributed to disruption of neurons at the injury centre correlates with observations by Liu et al. (1997) who reported electron microscopic evidence of disappearance of cytoplasmic Nissl substance at the injury epicentre 5 minutes after a weight drop injury at the thoracic level and that there is formation of a clear boundary between damaged and normal neurons in the grey matter (Liu et al., 1997). Extensive haemorrhage with marked disruption of neuropil evident by swollen cell processes, ruptured cell and basement membranes and degenerative changes in neurons is observed within 10 minutes after weight drop injuries in cats (Griffiths et al., 1978). In addition, immediately after a contusion to the cervical cord there is reported to be damage to grey matter vasculature and disruption to the membranes of neuronal cell bodies and axons localized within 1 mm of the lesion epicentre (Choo et al., 2007). Furthermore, TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) positive neurons are first evident 4 hours after injury and were primarily restricted to the gross lesion area (Liu et al., 1997). Immediate disruption of membranes and appearance of apoptotic cell markers acutely after injury provides an indirect assessment of loss of functional integrity in neurons at the site of impact. However, to our knowledge this is the first time that electrophysiological evidence has been obtained for loss of function due to damaged neurons after a contusion injury. In addition, using a weight drop injury model at thoracic level maximum neuronal loss is observed 8 hours after impact and apoptotic neurons are no longer evident 24 hours after injury (Crowe et al., 1997, Liu et al., 1997). Furthermore, in cervical contusion injuries made with Ohio State University device, it has been reported that maximum grey matter damage occurs at the injury epicentre and this is not progressive between 3 days and 9 weeks after injury (Pearse et al., 2005). This

suggests that neuronal death occurs within the first few hours after injury and is not an ongoing process in contusion injury due to a high velocity impact. This supports our findings of a similar level of function at the injury epicentre at all the recording time points studied (i.e. immediately after injury up to 6 months). The gradual decline of CDP amplitudes within the first few hours either side of the injury reflects reduction of function in neurons located close to the injury centre. However, this impairment of function is likely transient as CDP amplitudes below the injury recover at the 3 day time point to levels seen acutely. This implicates neurons in the vicinity of the injury which loss transient function is not a therapeutic target in contusion injury model. In contrast, above the injury function worsens by 3 days and recovers more slowly possibly reflecting secondary injury mechanism affecting oligodendrocytes followed by repair of parent axon myelination is discussed below.

2.5.3 Contribution of secondary injury mechanisms to loss of sensory function

Results from the group of animals investigated at 3 days after injury show additional loss of function compared to within hours of injury. This loss of function was particularly marked in the recordings rostral to the injury site. As this decrease in function was observed selectively above the injury it suggests that it may mainly involve the parent axons in the dorsal columns. For example it may reflect demyelination of these axons. Weight drop injuries can lead to complete or partial destruction of myelin sheath while axons are relatively intact and mixture of demyelinated and normal axons are observed at 1 and 3 days after injury (Griffiths and McCulloch, 1983). Quantitative analysis using electron microscopy has shown that maximum numbers of demyelinated axons are observed 1 day after thoracic contusion injuries made with Infinite Horizon Impactor apparatus. The demyelinated axons are mostly concentrated around the injury site and they reduce in number by 7-14 days (Totoiu and Keirstead, 2005). Similarly maximum numbers of TUNEL positive glial cells are observed 24 hours after injury as reported by Liu et al. (1997) and would be expected for demyelination of the spared axons. Although, myelin pathology has not been studied extensively in cervical injuries, Siegenthaler *et al,* (2007) have reported that there is no significant difference in the number of demyelinated axons

caused by cervical and thoracic contusion injuries. Injuries were produced using IHI device in their study (Siegenthaler et al., 2007).

2.5.4 Repair and stability in sensory pathways

CDP recordings at 2 weeks after injury show that potentials in the sensory system have largely stabilized and the amplitudes of the potentials above the injury have been restored to the levels observed acutely after injury. This reflects a repair process in the sensory system and this is possibly remyelination of the parent axons that were affected due to secondary damage. In addition, restoration of amplitudes to the levels observed immediately after injury suggest that this process would appear to be very efficient and almost complete (maximal) by 2 weeks after injury. The fact that CDPs are largely of stable amplitude from 2 weeks onwards suggests that most of the secondary damage observed at 3 days has subsided by 2 weeks.

The process of remyelination in SCI models has been reported in many studies (Salgado-Ceballos et al., 1998, Totoiu and Keirstead, 2005, Powers et al., 2012). Remyelination is evident after thoracic contusion in rats (Totoiu and Keirstead, 2005) and also observed after chemically-induced demyelination in mice (Jeffery and Blakemore, 1995) within 7 days after injury. Both Schwann cells and oligodendrocytes are reported to accomplish spontaneous remyelination in injured spinal cord (Beattie et al., 1997, Totoiu and Keirstead, 2005). The process of remyelination is reported to be ongoing after injury (Salgado-Ceballos et al., 1998) and it is also suggested that remyelination increases in a chronic progressive manner (Totoiu and Keirstead, 2005) and maximal remyelination is observed by 3 to 4 months (Totoiu and Keirstead, 2005, Powers et al., 2012). Furthermore, James et al. (2011) reported spontaneous improvement of conduction in ascending dorsal column fibres after thoracic contusion injury. The improvement of conduction in the ascending fibres plateaued approximately 4 weeks after injury. This was also correlated behaviourally with improvement in ladder walk test performance (James et al., 2011). In our study we have observed restoration of function within 2 weeks after injury and no additional functional improvement afterwards. This in turn suggests that the process of

ongoing remyelination reported in previous studies may not be adequate to promote further recovery of function in the local circuits of the spinal cord.

2.5.5 Late deterioration of function in sensory pathways

Although function in sensory circuits appears mainly stable from 2 weeks after injury, a late phase of decline in function has been observed. This is evident by decrease in CDP amplitudes at the most chronic time point investigated in this study (i.e. 6 months after injury). This deterioration of function is seen caudal to the injury centre and possibly reflects further loss of neural tissue. This is supported by evidence of an extension in cavity dimensions in animals surviving 6 months after injury compared to cavities in animals at 7 weeks after injury. However, it is not clear why this should affect potentials below the injury but not above. The observation on cavity expansion are in contrast to the findings reported in a thoracic contusion model where no change in cavity extent was observed between 14 weeks and 8 months survival animals (Hill et al., 2001). A decrease in afferent terminal density is observed caudal to the injury after Ctb tracer injections into the forelimb nerves (radial, ulnar and median) and also a decrease in neuronal density in animals surviving 6 months after injury compared to 7 weeks survival animals (unpublished data from the lab). Byrnes and colleagues studied the inflammation related gene up to 6 months after thoracic weight drop SCI and found that these genes are up regulated chronically and may contribute to further tissue loss in chronic SCI (Byrnes et al., 2011).

2.5.6 Loss of function in corticospinal pathways immediately after contusion injury

The response of CST to the contusion injury could be examined by stimulating the CST fibres within the pyramids and recordings CDPs along the length of the cervical segments. Recordings pyramidal evoked CDPs immediately after the injury showed that propagation of impulses in the main component of the corticospinal tract below the injury are acutely disrupted. The amplitude of CDPs caudal to the injury were significantly reduced and did not return during the first 4 hour hours of recording after injury. The main CST component located in the ventral part of the dorsal column comprises 90 % of the CST fibres (Hicks and D'Amato, 1975, Miller, 1987) and pyramidal evoked CDPs are mainly

dependant on inputs from the dorsal CST. Substantial reduction in CDP amplitudes below the injury level suggests that the dorsal component fibres are completely damaged after injury. This is supported by the evidence that BDA labelled CST fibres are completely absent in the dorsal column below the injury. It was also reported by Anderson and colleagues that the CST in the dorsal columns is completely severed after cervical contusion injury using IHI device (Anderson et al., 2009b) and in a thoracic weight drop injury by Hill et al. (Hill et al., 2001).

2.5.7 Contribution of secondary injury mechanisms to loss of corticospinal function

At 3 days after the injury corticospinal evoked CDPs show a modest deterioration of function. This deterioration of function is more marked above the injury and may reflect secondary injury mechanism such as demyelination or die back of CST fibres. However, corticospinal CDPs above the injury improved at 2 weeks though amplitudes remained smaller than normal, especially close to the injury site. This suggests that the proximal end of the severed CST axons in the dorsal column may undergo retrograde degeneration and could be responsible for the reduction in CDP amplitudes close to the injury site. It should be possible to confirm this using tracer injection and quantification of the remaining CST fibres rostral to the injury. It has been observed that in contusion injury CST fibres terminate in retraction bulbs as far as 5 mm rostral to the injury site, with the majority of retraction bulbs within 1-2 mm of the rostral edge of the cavity, suggesting die back of some CST fibres which then sprout and grow back up to the lesion margins (Hill et al., 2001).

2.5.8 Evidence of plasticity in the corticospinal pathways below the injury

At 2 weeks after the injury corticospinal CDPs recorded above the injury are quite stable over time but there is a gradual increase in the CDP amplitudes below the level of the injury. This suggests some plasticity occurs in the spared corticospinal projections reaching levels below the injury (i.e. spared by the injury) and that this plasticity leads to strengthening of corticospinal actions below the injury. This plasticity is most likely from the fibres of the minor

component of the CST located in the lateral white matter as these are known to connect with neurons in the dorsal horn in a similar region to fibres of the main component.The ventral CST fibres project ipsilaterally and they may form connections more ventrally nearer to the motonuclei and synaptic activity produced by these fibres is less likely to be detected with a dorsally placed recording electrode. Furthermore, tracer injections into the sensorimotor cortex revealed a higher density of BDA labelled axons projecting caudal to the injury in 6 months surviving animals compared to 4 weeks after the injury (unpublished data from the lab). Extensive sprouting from the minor components of CST following interruption of the main dorsal component of CST is observed by many investigators in hemisection and transection models of SCI (Weidner et al., 2001, Steward et al., 2008, Rosenzweig et al., 2010). In a contusion injury model CST sprouting in the main dorsal component rostral to the injury is reported to be initiated 3 weeks after injury and extensive sprouting is observed by 14 weeks (Hill et al., 2001). This suggests that following the contusion injury CST fibres are amenable to spontaneous plasticity as observed in other models. To our knowledge this is the first time that enhancement in amplitudes of CDPs evoked by corticospinal stimulation due to plasticity in the minor components has been reported after a contusion injury.

2.5.9 Implications for understanding SCI pathology and developing therapies

The findings in this chapter suggest that most of the damage and functional loss in the spinal cord occurs at the time of initial injury. This is evident from the substantial decline in CDP amplitudes produced at the time of injury at the injury epicentre and the finding that these potentials remain almost constant at all the time points investigated. Function either side of the injury worsens within the first few hours after injury. This decline in function is not persistent and returns to levels close to those seen immediately after contusion but the pattern of recovery is different above and below the injury. Function below the injury returns to levels close to those seen immediately after contusion within 3 days. However, above the injury, further deterioration of function is observed at 3 days suggesting an additional effect of secondary injury mechanism i.e. demyelination of the primary afferents in the dorsal column. At 2 weeks

improvement of function is observed above the injury where function returns to levels close to those seen acutely, indicating a repair process i.e. remyelination. This process seems to be very efficient and complete in this injury model.

These findings suggest functional loss due to a high velocity impact as produced by the IHI device is greatest at the centre of the injury, and that it is maximal at the time of primary trauma and is irreversible at this location. Function above and below the injury deteriorates soon after the initial trauma but returns to levels seen acutely within a relatively short period of time (2 weeks). This indicates neurons close to the injury centre (i.e. immediately above and below) show only transient impairment of function and may also not benefit from treatment. It is possible that remyelination is part of the repair process and if so the return of function to acute levels suggests that demyelination does not persist permanently in this injury model and this is consistent with previous suggestions by Plemel et al. (2014) that demyelination is not an important therapeutic target in SCI (Plemel et al., 2014).

Conversely, compression injury models cause a more progressive neuronal loss after injury (Huang et al., 2007). It is reported that there is approximately 30 % additional neuronal loss between 1 and 3 days after compression to the thoracic spinal cord applied for 5 minutes (Huang et al., 2007). The ongoing neuronal loss suggests that secondary mechanisms are more pronounced in compression injury compared to contusion injury. Many neuroprotective therapies have been tested with the hope of mitigating secondary damage after SCI and preserving the spared neurologic tissue in both contusion and compression injury models (Kwon et al., 2011). Erythropoietin has been shown to have a neuroprotective effect mainly by reducing apoptosis, inflammation, lipid peroxidation and preserving tissue in both weight drop contusion and compression injury models at the thoracic level (Gorio et al., 2002). There were also significant improvements in the behavioural assessments using BBB scores which returned approximately to normal by 28 days in both injury models compared to controls, although the pattern of recovery was slightly slower in contusion injured animals (Gorio et al., 2002). However, replication of this study using similar injury models in order to evaluate the efficacy for clinical translation failed to find any beneficial effects in either model (Pinzon et al., 2008a). Mann and colleagues have also

failed to show any therapeutic beneficial effects of erythropoietin or its derivative darbepoetin administered 1 hour after contusion injury made with the Ohio State University apparatus (Mann et al., 2008). Similarly, the broad spectrum antibiotic minocycline has been shown to inhibit microglial activation and inhibit the release of pro-inflammatory mediators. In addition, it can lead to increased axonal sparing, reduced lesion area and improved function in behavioural assessments (i.e. BBB and inclined plane test) when administered after thoracic compression (Wells et al., 2003). However, the previously reported beneficial effects of Minocycline in a weight drop contusion model using the NYU device in rats (Lee et al., 2003b) has not been replicated in studies with a similar injury model and dosage (Pinzon et al., 2008b). The negative outcome after administration of neuroprotective therapies further supports the hypothesis that in contusion injury due to a high velocity impact the maximum damage is seen due to the primary insult and there is minimal contribution from the secondary injury mechanisms. The fact that the contribution from secondary damage is minimal in the contusion injury model should be taken into consideration in future when investigating neuroprotecitve therapies. Furthermore, as suggested by Huang et al. (2007) the ongoing damage due to secondary processes in a compression injury model may be more amenable to neuroportective therapies (Huang et al., 2007). However, direct electrophysiological evidence is needed to assess whether ongoing neuronal loss in compression injury is also associated with a continuing reduction of function.

2.5.10 Technical considerations

2.5.10.1 Anaesthetic use in contusion injury surgery

Isoflurane anaesthetic was used for all groups of animals during the contusion injury surgery with the exception of the acutely injured group of animals, where pentobarbital was used during the contusion injury. This was because the acute animals were prepared for the electrophysiological experiment in order to carry out CDP measurement immediately after injury. In addition, rats were anaesthetised for a longer duration during preparatory surgery for the electrophysiology before the contusion. Therefore the duration and use of different anaesthetics may affect the outcome of the injury and its effect on functioning at the level of the spinal cord. It has been reported that different

anaesthetic usage during injury surgery has effects on recovery after injury. Behavioural functional outcome is reported to show better recovery of locomotion after administration of isoflurane compared to animals injured under pentobarbital anaesthesia (Nout et al., 2012). Therefore it is necessary to consider that the functional outcome in the acute group of animals may be greater than it would have been had isoflurane been used as the anaesthetic as in all other group of animals.

2.5.10.2 Electrophysiology

During electrophysiological experiments efforts were made to minimise variability in the collection of the electrophysiology data. Therefore continuous monitoring of the animals physiological condition was carried out including blood pressure, tidal expired CO_2 and core body temperature. Electrophysiological recordings are very sensitive to level of the anaesthesia and therefore efforts were made to maintain this at the same level throughout by continually monitoring the withdrawal reflexes and changes in blood pressure and administering anaesthetic accordingly during paralysis. Observations in the lab suggest that pentobarbital anaesthesia has minimal effects on CDP amplitudes if maintained at a steady level. CDP measurements are very sensitive to the accumulation of CSF around the recording electrode which leads to reduction in the amplitudes of CDPs, therefore fluid was removed regularly. Supramaximal stimulation of the afferent fibres and the corticospinal fibres were used in order to activate all of the neurons or fibres comprising the system and avoiding the stimulus spread to the surrounding structures.

3 Investigation of human embryonic stem cell derived mesenchymal stem cells (hESC-MSC) as a potential therapy for spinal cord injury

3.1 Introduction

MSCs are multipotent cells with the capability to self-replicate and develop into many cell types including bone, muscle, cartilage and neural tissue (Minguell et al., 2001). As reviewed in chapter 1 (Introduction), the use of MSCs in SCI treatment is considered promising as they have been shown to promote repair and functional recovery. Also, the relative ease with which these cells can be isolated and expanded in culture with only subtle loss of potency makes them a promising candidate for use in the treatment of degenerative diseases.

MSCs have been identified and can be isolated from several tissues and organs of both fetal and adult origin (Campagnoli et al., 2001, Meirelles et al., 2006). MSCs obtained and harvested from several tissues have been investigated in SCI model including those obtained from adipose tissue (Zuk et al., 2001), umbilical cord blood (Erices et al., 2000), olfactory mucosa (Tome et al., 2009, Lindsay et al., 2013) and embryonic tissue (Thomson et al., 1998, Lee et al., 2010). MSCs obtained from bone marrow are the most widely studied (Tetzlaff et al., 2011). However, autologous transplantation of bone marrow derived MSCs (BM-MSCs) has several disadvantages which may limit their use in the clinical setting. These include the need for an invasive procedure for obtaining the cells and variability in cell quality and quantity. There can also be limitations as a result of slow proliferation, limited life span and gradual loss of stem-like properties during in vitro expansion (Ringe et al., 2002).

However, among the potential sources of these cells is the possibility of differentiating them from human embryonic stem cells (hESCs). It has been demonstrated that MSCs can be successfully derived from hESCs. Using a modified version of the process reported by Olivier et al. (2006) MSCs have been successfully derived in several sets of experiments in our lab (from a hESC line) with similar properties to BM-MSCs .

These hESCs derived MSCs were transplanted in animal models of traumatic SCI and showed good survival within the injury site and usually filled the injury area resulting in a reduction in the size of the injury cavity, when compared to non-transplanted animals. Numerous blood vessels were found at the injury site in transplanted animals, as judged by laminin and smooth muscle actin staining. In

addition, the transplants supported regeneration of ascending dorsal column but not corticospinal fibres though this regeneration was limited to the transplant region. The regenerating fibres also showed signs of acquiring myelin sheaths with positive immunolabelling for CASPR being detected at the centre of the transplants (Mohamad, 2014).These effects are similar to those reported for MSCs of bone marrow origin and suggest that transplants of these cells may help repair the injured spinal cord.

3.2 Aim

Although numerous studies have investigated bone marrow derived MSCs and some have suggested that these cells can help improve function after SCI, hESC derived MSCs have not yet been investigated. We hypothesized that since these cells appear anatomically to behave similarly to bone marrow MSCs when transplanted into a contusion injury that they may also improve functional outcome. The aim of this study was therefore to transplant hESC derived MSCs in a clinically relevant model of SCI, to investigate their ability to improve impaired function. However, it is difficult to compare the SCI model in rodents to injuries seen in humans. Contusion injury is considered to be a clinically relevant model. The 3 week time point after injury in rodents has been considered chronic and could mimic chronic injury in humans. Therefore, the aim of this chapter was to combine electrophysiological and behavioural approaches to determine whether hESC-MSC transplants promote repair and improvement of function after a delayed transplant in a contusive injury model.

3.3 Material and Methods

3.3.1 Experimental Design

A summary of the experimental design for the experiments reported in this chapter is shown in the Figure 3-1. Animals were handled and trained daily to perform behavioural tests for 2 weeks prior to a contusion injury (C6 175 kdyn) and baseline data was collected 1 day prior to contusion injury (C6 175 kdyn). The tests were then performed each week after injury for 9 consecutive weeks. hESC-MSCs transplantation were performed at 3 weeks after the injury, a time point which is clinically relevant and when the injury has stabilised as indicated by the results reported in chapter 2. Immunosuppression with cyclosporine A (Sandoz, UK) 20 mg/kg body weight s.c. was started 2 days prior to transplant of either hESC-MSC or medium for the rest of the study. At 7 weeks post-transplant, animals were subject to electrophysiology experiments under general anaesthesia with pentobarbital. After electrophysiological recordings, each animal was perfused and histological processing was carried out to assess cell survival and the morphology of the injury site using immunochemistry and epiflourescence/confocal microscopy.

3.3.2 Animals

All of the experiments in this project were approved by the Ethical Review Process Application Panel of the University of Glasgow and performed in accordance with the UK Animals (Scientific Procedures) Act 1986 (ASPA).

Findings in this chapter are based on a total of forty-five (44) adult male Sprague Dawley rats (Harlan, Loughborough, UK). SD rats are used in this project because they are good in performing grip strength and ladder task and reaching task was not planned in this project. All of the animals were subjected to 175 kdyn contusion injury at the C6 level of the spinal cord. Of these, sixteen (16) were transplanted with hESC-MSC at 3 weeks post injury and thirteen (13) were injected with media into the injury site at the same time point. All the animals injected with cells or medium were allowed to survive for 7 weeks and their functional abilities were assessed weekly using behavioural tests. Electrophysiological assessment was performed at the end of the study using the

same protocol as described in chapter 2. A further 3 animals were perfused at 3 weeks post injury to assess the nature of the cavity at the time of transplant.

In addition, 6 animals were transplanted with hESC-MSCs in order to assess the outcome of transplantation performed after a short delay following injury. Three animals were transplanted 5 days after contusion injury and 3 animals were transplanted 7 days after contusion injury. Animals with these sub-acute transplants were perfused 3 weeks after transplantation. Similarly in order to assess the nature of the injury cavity at 5 days and 7 days after contusion injury (the time points at which acute transplants were performed) 6 animals were injured with 175 kdyn contusion injury. 3 of these animals were perfused after 5 days and 3 after 7 days survival.

3.3.3 Cell culture

All of the cell culture was performed by Dr. Che Anuar Che Mohamed in Dr. Jo Mountford's laboratory (based in the Davidson building), Institute of Cardiovascular and Medical Sciences, University of Glasgow. For full details of cell culture procedure see Che Anuar PhD thesis 2014.

3.3.3.1 Culture of hESC

Briefly the Wisconsin hESC line H1 (Thomson et al., 1998) was used as the starting population to derive MSCs. The method for derivation of MSCs from hESCs was based on the method described by (Olivier et al., 2006) which does not require any feeder layer of animal origin. The population of cells was grown and maintained under feeder free conditions to minimise the involvement of animal related products. These cells were initially expanded in culture in order to be used as a starting point for differentiation into MSCs.

hESCs were grown on murine embryonic fibroblast (MEF) cells when originally acquired from the source. They were switched to feeder free conditions after 28 passages and cultured on 6 well plates pre-coated with extracellular matrix (ECM; CellStart, Life Technologies) in StemPro hESC HFM media. These cells were further passaged 10 to 20 times in feeder-free conditions in order to ensure the removal of MEF cells at which point they are suitable as a starting population for MSC differentiation. The cells were fed daily with StemPro hESC HFM media

until they were 90% confluent. Afterwards, the cells were mechanically detached and vigorously pipetted to dissociate the remaining cell clumps. These dissociated cells were re-plated and cells from each 6 well plate were split into a further 6 wells for maintenance and expansion. All the cultures were maintained at 37° C in an incubator with 5% CO_2 and 95% O_2.

3.3.3.2 Differentiation of hESCs to MSCs

In order to differentiate hESCs into MSCs, 2 confluent wells of hESCs were selected from a 6 well plate and used as a starting population for differentiation. The cells were mechanically detached, dissociated by pipetting vigorously and divided into 4 equal portions. Each portion was transferred into a T25 flask to obtain 4 flasks from each well. These were kept in an acidic environment (7.5% CO_2) at 37ºC in an incubator for approximately 28 days in D10 (DMEM+FBS 10%NEAA+P/S) media. The medium was changed regularly every 7 days. After 28 days, the cells were dissociated by adding TrypLE Select and incubated for 2-4 hours.

The MSC like cells derived after 28 days differentiation were initially plated in a T25-cm^2 flasks in D10 media and left to attain 90% confluency, which usually took 5-10 days. These cells were then dissociated with 2 ml TrypLE Select, returned to the incubator for 5 minutes and replated at 1 x 10^4 cells/cm^2 density in a T25 flask. The cells were maintained in D10 media at 37° C and 5% CO_2. The flasks were examined by light microscopy daily until 90% confluent (usually taking 3-4 days). These cells were replated again into T25 flasks and the media changed every 2-3 days. After 3 passages sufficient numbers of cells were obtained to provide a stock for cryopreservation. To preserve the cells they were counted using a haemocytometer (Hawksley BS.748 improved Neubauer counting chamber) and 2 x10^6 cells/ml were re-suspended in 1 ml of MSC media and 1 ml of this suspension (cells and medium) transferred to a CryoTube™ and placed into liquid nitrogen for long term storage.

3.3.3.3 hESC-MSC characterization

The hESC derived MSCs were characterised for cell surface markers, gene expression profiles, and growth and differentiation protocols by Mohamad. The

protocols used were similar to those described elsewhere in the literature (Olivier et al., 2006, Chamberlain et al., 2007, Uccelli et al., 2008).

3.3.3.3.1 Morphology

At the end of differentiation period i.e. by 28 days cells showed a heterogeneous spindle shaped morphology under the microscope which is characteristic of MSCs. In addition 3-5 days after re-plating following differentiation cells were strongly attached to the plastic surface of the culture flasks.

3.3.3.3.2 Surface markers

Surface marker expression was analysed using flow cytometry in order to examine MSC related markers. The differentiated cells were highly positive (70-90 %) for CD44, CD73, CD105, HLA ABC, CD13 and CD166 which are markers characteristic of MSCs. There was low expression of other MSC markers like CD71 and CD90 (35-50 %) and CD271, CD106 and Stro-1 (5-20% of cells). Less than 2 % of cells expressed the haematopoietic markers, CD45 and CD34.

3.3.3.3.3 Gene expression

Quantitative real time PCR (RT-PCR) was performed in order to look for changes in the mRNA expression levels of genes specific for MSCs at different stages of the differentiation protocol. At day 0 of hESC differentiation pluripotency genes *OCT4* and *Nanog* were highly expressed. Their expression levels were significantly down-regulated at 28 days. Expression of the mesenchymal genes *COL1A1, CD105* and *Twist1* significantly increased by day 28. Expression of Early mesodermal lineage markers *Goosecoid* (GSC) and *Brachury* was minimal after differentiation. There was also extremely low or no expression of the haematopoietic markers CD31 and CD45.

3.3.3.4 Expansion and preparation of hESC-MSCs for transplantation

hESC-MSCs were thawed approximately 3 weeks before transplantation. A cryotube containing 1 x10^6 cells was taken out of liquid nitrogen and placed in a water bath at 37° C. Once the cells were thawed they were carefully removed, transferred into a 15 ml falcon tube and 10 ml of culture media was added. The cells were centrifuged and the supernatant discarded; the cells were then re-suspended in culture media and divided equally into 2 T25 flasks. At 24 hours

after the first passage cells were transduced with lentivirus expressing a gene that encodes green fluorescent protein (GFP) in order to allow *in vivo* tracking after transplantation. The volume of virus was calculated from the titre derived multiplicity of infection and number of cells to be transduced. The cells were incubated with virus for 72 hours. The GFP labelled MSCs were then kept at 37° C and 5% CO_2 and were repeatedly passaged (usually 5 times) to obtain the required number of cells for transplantation. Depending on the cell count, the cells were re-suspended in 50-100 µl of fresh medium on the day of transplantation and transferred into a sterile 200 µl eppendorf tube. These were then transported to the operation theatre on ice. The cell suspension was concentrated to approximately 200,000 cells/µl prior to filling injection pipettes.

3.3.4 Contusion Injury

All the contusion injuries and cell/media transplant surgeries in this project were carried out by Dr. John Riddell. I provided assistance with the surgery and the filling of the cell injection pipettes with hESC-MSC/media for transplants and also injections into the injury cavity using the pico-pump.

The C6 spinal cord segment was subjected to a 175 kdyn contusion injury using the same protocol as described previously in chapter 2. A 10/0 silk (Ethicon) marking suture was placed in the dura over the injury site for identification during subsequent cell transplantation surgery. Peri-operative care and medications were the same as described in chapter 2.

3.3.5 Immunosuppression

In order to reduce the chances of cellular transplant rejection by the host immune system, the animals were immunosuppressed with daily injection of cyclosporine A. This was started 2 days prior to cell transplantation and continued for the duration of the experiment. Cyclosporine was administered at a dose of 20 mg/kg body weight subcutaneously. Control animals injected with media were also immunosuppressed using the same regime in order that any differnces between transplanted and control animals could be attributed solely to the transplantation of hESC-MSCs. Administration of cyclosporine by injection

was performed by animal house technical staff on weekdays and by myself or Dr Anuar over the weekends.

3.3.6 Delayed cell transplantation

Transplantation was performed 3 weeks after injury to coincide with a realistic delayed transplantation time-point in a clinical setting after human injury. The delayed protocol also helped in minimizing the chances of transplant rejection due to the elevated immune response immediately after injury.

3.3.6.1 Cell injection pipettes

Glass capillary tubes (GC 100T-10, Harvard Apparatus Ltd, UK) were used to prepare pipettes with a suitable diameter for injecting cells. These tubes were pulled using a vertical puller (Narishige PE-2, Narishige, Japan) with a controllable heating element that melts the glass into fine, tapered tips. Tips were cut with blunt micro-scissors to an internal diameter of approximately 60-70 µm. After measuring the diameter under a microscope the tip was then sharpened with the help of a rotating beveller. Pipettes were manually marked with a series of permanent marker graduations 1.5 mm apart, graduations being made from where the pipettes tapers towards its tip. Each division approximated to about 1 µl and these were used to keep track of the volume of cell suspension injected.

3.3.6.2 Transplantation of hESC-MSCs into the injury site

The hESC-MSCs were transplanted directly into the injury cavity that is formed after contusion. The cells were transported to the operating theatre in a PCR tube on ice, and were spun down in a mini centrifuge to separate out the media and cells. The media that accumulated on the top was removed with the help of the glass pipette in order to make a thick, concentrated population of cells. A glass micro-pipette was filled with cells by creating negative pressure in the pipette by applying suction using a syringe connected by flexible tubing to the pipette. The filled pipette was then mounted on a stereotaxic manipulator and inserted into the lesion cavity through a slit in the dura. The tip of the pipette was lowered to a depth of approximately 1200 µm and cells were injected by applying brief pressure pulses of 20-40 ms (Picoinjector, WPI, Sarasota FL, USA)

while the tip was slowly raised dorsally, to a depth of approximately 300 µm. The cells were injected until there was overflow from the injection site which usually required around an average of 40 µl of cell suspension, corresponding approximately to 8 x 10^6 cells per animal. If no overflow was seen then the injection was stopped after the introduction of 50 µl of cell suspension. Wounds were then closed in layers and the animal was allowed to recover in a warm environment with free access to food and water.

3.3.6.3 Media injection into the injury site

To provide a control group of animals without treatment an approximately equal number of injured animals to those receiving transplants of cells were injected with the same media which was used to suspend the cells. D10 Media was used containing D-MEM (Dulbecco's modified eagle medium) with FBS (Fetal Bovine Serum) (HyClone, Thermo Scientific, UK), NEAA (Non Essential Amino Acids) and antibiotics (penicillin and streptomycin). Glass micro-pipette was filled with media and injected using the same procedure as were used for cellular transplants described above.

3.3.7 Behavioural testing

All the transplanted and control animals were assessed for functional improvements using behavioural assessments on a weekly basis for the whole period of the experiment. The naive rats were initially acclimatised to the experimenter and the testing environment and this was followed by training on the equipment before performing the experiment. Grip strength and a horizontal ladder walking test were used to assess changes in the rats abilities to perform sensorimotor tasks before and after injury, and subsequently after transplantation of hESC-MSCs or media.

3.3.7.1 Animal handling and acclimatisation

All the animals selected were house pair-wise in cages with a 12 hour light/dark cycle. Before collection of pre-operative data each animal was handled for at least 10-15 minutes daily in the morning for 5-6 days in order to acclimatise them to general handling. During these sessions animals were also subjected to test the environment and positioned to acclimatise for the grip strength test. At

the end of 5-6 days animals showed no obvious signs of fear and stress with minimum urination and defecation. Animals were then trained on the behavioural apparatus for the next 5-6 days. The animals were allowed to run across the ladder and to grab the bar of the grip strength meter in order to become familiar with the testing environment and equipment.

All the behavioural tests were performed on the same day each week during the morning for the whole period of the experiment. The testing environment was maintained with similar conditions of temperature, light/dark cycle, and access to water, food and bedding.

3.3.7.1.1 Selection of animals for behavioural testing

Animals were only subjected to contusion injuries if they could be successfully trained to perform the behavioural tests. In order to ensure that the different experimental groups were composed of animals that were as comparable as possible in every respect, behavioural performance in the grip strength test was taken into account. The grip strength scores for all animals at 2 weeks after injury were plotted in descending order and alternate animals were assigned to the transplant and control groups.

3.3.7.2 Grip strength test

A grip strength meter (Andilog, Centor v3.22 apparatus (Figure 3-2) was used to assess the force generated by the animals when gripping the force sensor bar and while resisting being pulled from the bar (Figure 3-3). The apparatus was attached firmly to the edge of the bench with the help of F clamps and a grasping bar fixed to the force meter. The force measurements were recorded in grams (the final measurements were expressed as a ratio of force to body weight).

Animals body was supported with the right hand held below the forelimbs in such a way that both forelimbs were moved forward and with the experimenter’s left hand holding the rat’s tail; the animal was allowed to grasp the bar with both forepaws. Immediately after grasping the bar, the animal was pulled away from the apparatus, generating increasing force until the grip was broken, releasing the bar at which point peak or maximum force was produced. The peak force

recorded by the force meter at this point was noted and the animal was allowed to rest for at least 1 minute before repeating the same procedure. A total of 5 successful tests were performed with each animal and measurements recorded. The highest and lowest measurements were ignored and the middle 3 measurements were averaged. At the end of the experiment the weights of animals were recorded and the individual average forces were then expressed as the force exerted per gram of bodyweight.

3.3.7.2.1 Exclusion of data

Data from the animals at some points in the study were excluded when they were either unable to grasp the bar properly or if they displayed signs of spasticity in one or both forepaws (this happened occasionally after the contusion injury or transplant operation and it usually resolved within 1-2 weeks).

3.3.7.3 Ladder walk test

In the ladder walk test, animals walked along a horizontal ladder with variable rung spacing while being videotaped. The videotapes were then viewed and analysed using GOM Player software (version 2.2.64). Animals were allowed to cross a 1 meter long grid with the middle part of the grid (40 cm) arranged with irregular rung spacing. Figure 3-4 shows the arrangement of irregular spacing with a gap of 1 to 3 cm between the rungs. Animals started the test at a loading area which allowed them to increase speed before crossing the analysed area of the ladder. In addition transparent plastic barriers found both side of the grid to restrict the animals to a straight path. The ladder apparatus is shown in Figure 3-4. A camera was placed on a tripod level with the ladder and focussed on the area of the ladder with irregular spacing (analysis part of the ladder) in order to record clearly forepaw placements on the grid. Animals were trained during the acclimatisation and training period to run across the ladder when placed at one end of the platform. The home cage of the animal, containing its cage mate, was placed at the other end of the platform, which provided a motivational incentive to cross the ladder. The animals were placed at the left end of the ladder and video recorded while crossing the ladder. A total of 5 good runs where animals showed minimal hesitation in crossing the ladder and did not attempt to turn back and/or explored the sides were normally recorded. Animals

were allowed to rest for at least 1 minute in between trials. If animals were too hesitant to cross they were provided with a longer rest before each trial. All the measurements were made on the same day each week in as consistent a way as possible.

3.3.7.3.1 Analysis of videos

The best 3 out of the 5 runs recorded were chosen for final analysis on the basis on minimal hesitation while crossing. The videos were then analysed frame by frame using the GOM Media Player software (version 2.2.64). The paw placement was carefully observed in each frame and recorded for different errors and normal placements. Characteristics of different steps used are summarized in Table 3-1. These characteristics are also shown in Figure 3-5.

The characteristics of paw placements in the ladder walk test are categorised as:

A) Hit: when rat completed the step using the **plantar surface** of the paw to weight-bear while crossing the ladder and there was no disruption of gait.

B) Sub-optimal: when rat used **digits** of the paw to bear weight while crossing the ladder and there was no disruption of gait.

C) Slip: when rat placed paw on the rung and the paw then slipped off the rung leading to a disruption of gait.

D) Miss: when the rats paw missed a rung and fell right through between the rungs and below their level, but without this leading to a time delay and without any disruption of gait.

E) Clenched paw: when the rat completed the step using the **dorsal surface** of the clenched paw and did not fully bear weight on this limb.

F) Partial step: when the rat positioned a paw on a rung and did not fully weight-bear on this limb before repositioning the paw on another rung.

The hit and sub-optimal categories of paw placement were considered normal steps while the slip, miss, clenched paw and partial step categories were considered errors.

The number of errors and normal steps were counted for each animal and percentage errors for each limb calculated. The percentage errors for forelimbs and hindlimbs were averaged separately and expressed as forelimb or hindlimb errors.

In addition, the time taken by the animal to cross the analysed area during each trial was calculated from the time difference between first and the last step within the analysed area. Stride frequency was calculated by counting the number of steps made during the time taken to cross the ladder.

3.3.8 Electrophysiology

3.3.8.1 Anaesthesia

All the animals were anaesthetised with the same protocol as described in chapter 2.

3.3.8.2 Preparatory surgery

Preparatory surgery for electrophysiological assessment was the same as described previously in chapter 2.

3.3.8.3 Recordings of cord dorsum potentials

To assess spinal cord function in the vicinity of the lesion after transplantation of hESC-MSC or injection of media, CDPs evoked by stimulation of the radial nerve and CST within the pyramids were recorded using the same protocol as explained in chapter 2. The potentials were stored and analysed in similar manner as previously described in chapter 2.

3.3.9 Perfusion fixation

All the animals were perfusion-fixed at the end of the electrophysiology experiment as described in chapter 2.

3.3.10 Histological processing

Approximately 6 mm of the spinal cord centred on the middle of the injury site was dissected out of each animal. The dissected cords were cut in the parasagittal plane to preserve information on the extent of the injury site. Blocks were cut into 60 µm sections and processed as described in chapter 2. Sections were incubated with different combinations of anti-GFP (1:1000), anti-GFAP (1:1000), anti-NF200 (1:1000), anti-Laminin (1:100), anti-NeuN (1:1000), anti-nestin (1:400) and anti-CASPR(1:500) primary antibodies to label transplanted cells, astrocytes, axons, basal lamina, neuronal nuclei and paranodal junctions respectively. Appropriate species specific antibodies conjugated to flourophores (Alexa 488 (1:500), Rhodamine (1:100) and DL649 (1:500)) were used to label the specific primary antibodies.

3.3.11 Microscopy

The sections were examined using epiflourescence and confocal microscopy in a similar manner to that described previously in chapter 2. The only difference was the use of 3 channels for the confocal scanning at wavelength of 488 nm (Alexa 488), 561 nm (Rhodamine) and 633 nm (DL649).

3.3.12 Measurement of the injury cavity size

In order to quantify the injury dimensions of the transplanted and control animals at 7 weeks post-transplant or medium injection respectively, all sections from each animal were examined using a fluorescence microscope. The section from the middle (near the midline) with the largest injury cavity in control animals and the largest injury site as judged by the glial scar in case of the transplanted animals was selected for confocal imaging. The section chosen was scanned as a tiled scan at 4X magnification through 2-3 z sections. The length of the injury cavity/site was then calculated using photoshop software with a known scale factor. The rostral and caudal margins of the injury site were judged by GFAP immunolabelling and lack of normal tissue integrity and the measurement was carried out using a ruler tool in photoshop. This length was multiplied by the scale factor to determine the extent of the cavity. In addition, the width of cavity was calculated by counting the number of sagittal sections

where the injury tissue was observed and then multiply this by 60 μm, the section thickness.

3.3.13 Off-line analysis of CDPs

Off-line analysis of electrophysiological recordings was performed as explained in chapter 2.

3.3.14 Statistical analysis

Statistical analysis was performed using Graph Pad Prism software version 4.0. Results from the grip strength test and horizontal ladder test from each week were analysed using two-way ANOVA and Bonferroni post hoc analysis. Electrophysiological data from all recording locations for both the radial nerve and pyramidal evoked CDPs in control and transplanted groups was also analysed using 2-way ANOVA and Bon-ferroni post hoc tests. A paired students t-test was applied to compare the cavity length and width between transplanted and control animals.

Type of Step	Characteristics	Normal or Error
Hit	Completes the step using optimal paw position, no disruption of gait	Normal
Sub-optimal	Completes the step using digits for weight-bearing, no disruption of gait	Normal
Slip	Places paw on the rung then slips off, gait disrupted	Error
Miss	Paw misses a rung and falls right through, no time delay	Error
Clenched paw	Completes the step using clenched paw, no disruption of gait	Error
Partial step	Positions paw on a rung, does not fully weight-bear on this limb and repositions paw on another rung	Error

Table 3-1. Classification scheme used to define step types made during the ladder rung walking test.

Each step was classified as one of six categories, based on the characteristics observed during frame-by-frame analysis. Hit and sub-optimal were categorised as ‘normal’, whereas slip, miss, clenched paw and partial step were counted as ‘errors’.

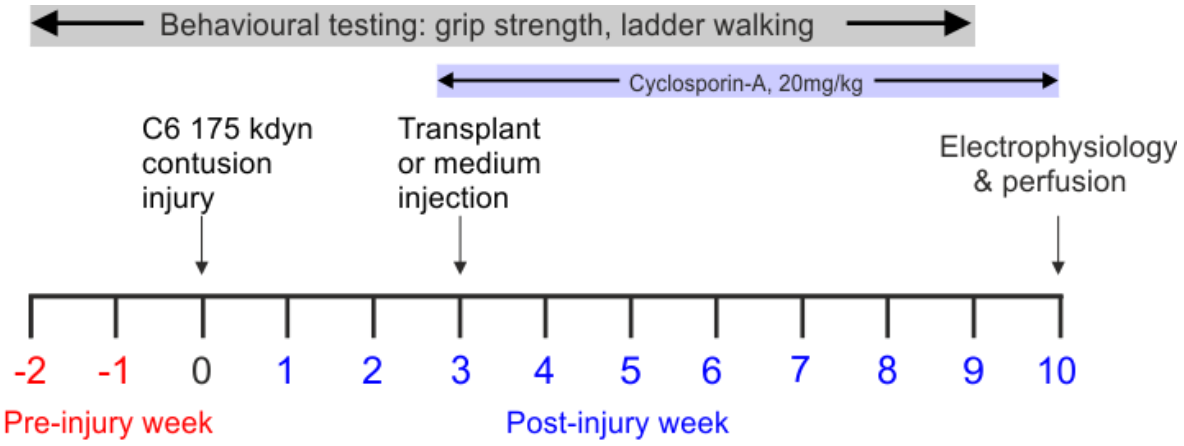

Figure 3-1. Schematic diagram summarising the experimental design.

Schematic diagram to show the overall plan of the experiment. The timing of individual experimental procedure is indicated relative to the contusion injury (Week 0). Behavioural testing was carried out 2 weeks before the injury to 9 weeks post injury. Cyclosporin injections were started 2 days before hESC-MSC tranplants or medium injection (3 weeks post injury). Electrophysiology and perfusion were performed 10 weeks post injury.

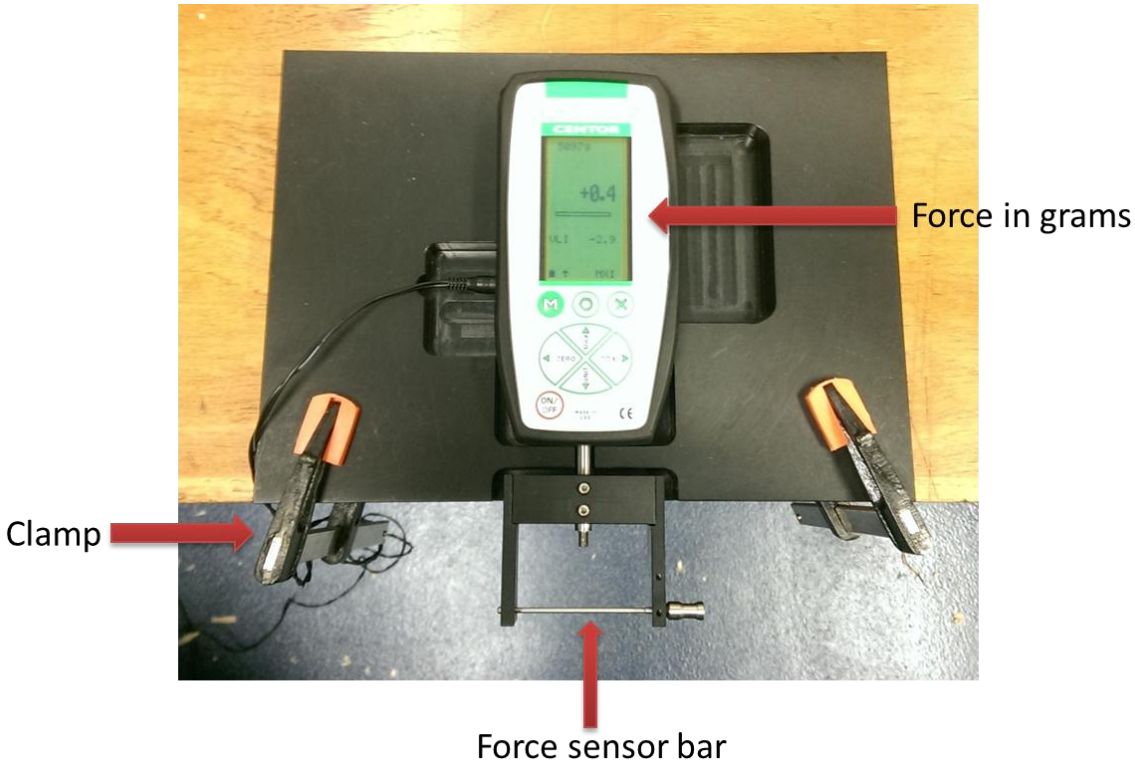

Figure 3-2. The grip strength meter (GSM) (Andilog, Centor v3.22) apparatus.

The figure shows different parts of the GSM apparatus. The GSM apparatus is fixed firmly to the edge of the table with the help of clamps. The animal grips the force sensor bar with both forepaws. Force applied to the force sensor bar is recorded and displayed on the screen of the apparatus.

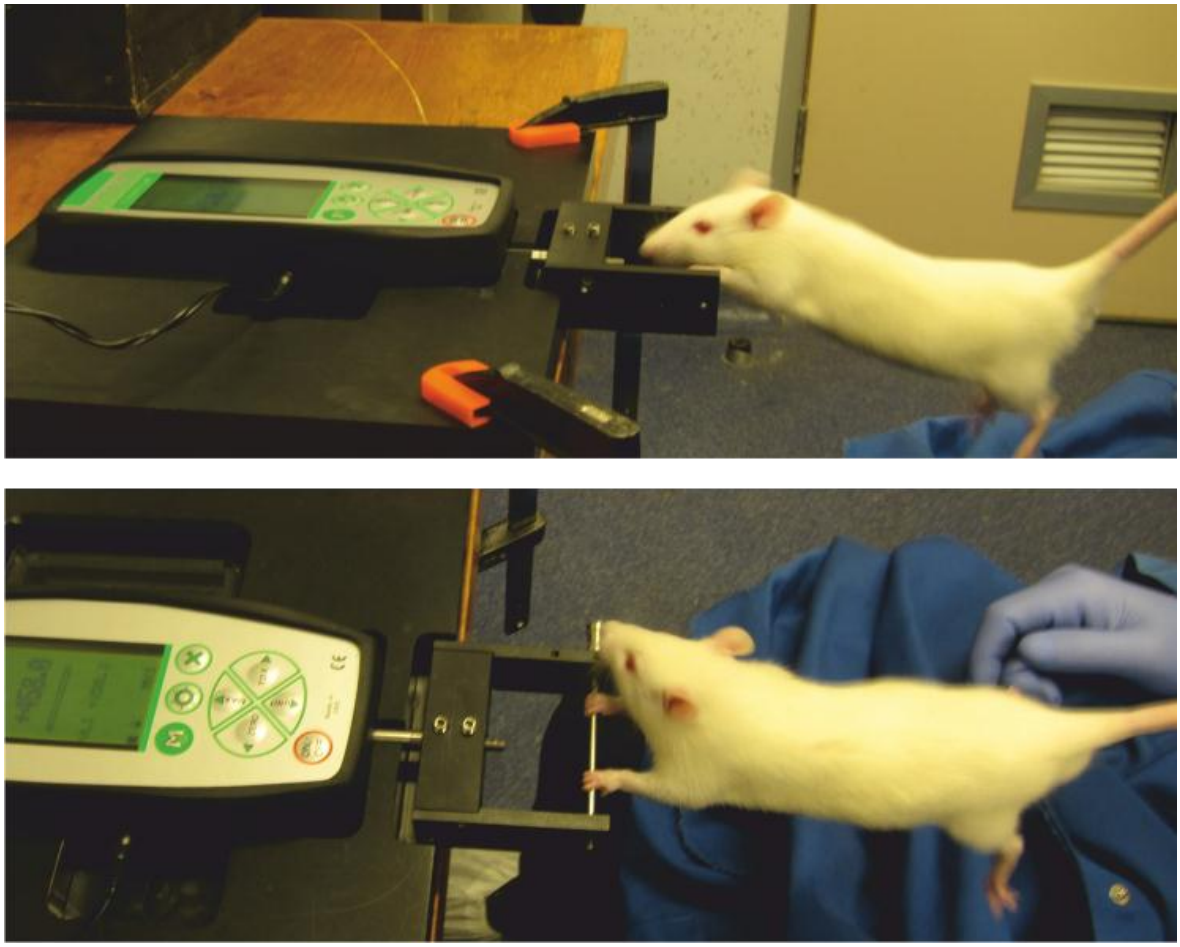

Figure 3-3. Images showing animal gripping the force sensor bar of the GSM apparatus.

The animal is placed so that it grips the bar and then pulled back by the tail in hanging position (upper image). The lower image shows the position of both forepaws gripping the sensor bar while force generated is displayed on the screen. The animal is retracted away from the apparatus generating maximum force until the grip is broken and the bar released.

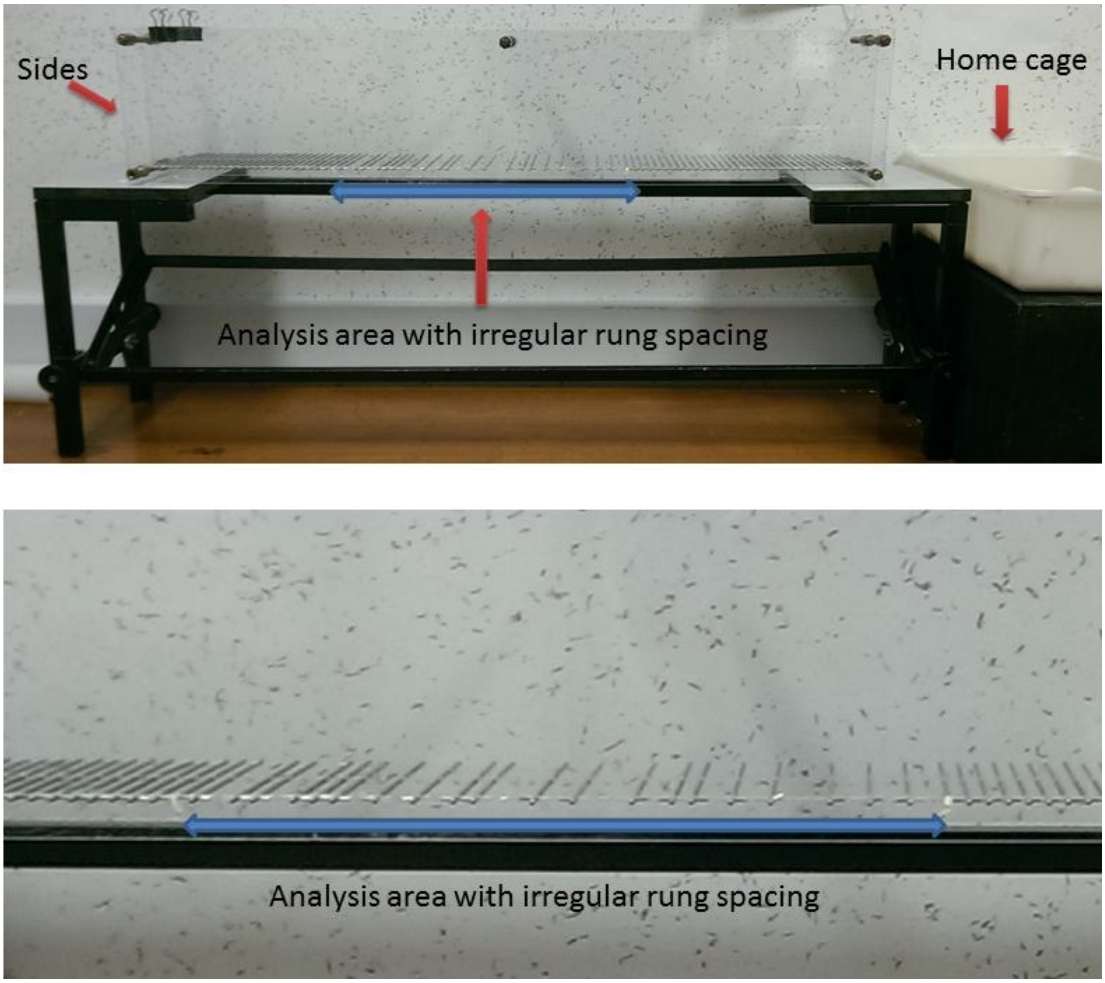

Figure 3-4. Horizontal ladder walk apparatus.

Image showing the setup of the ladder apparatus (upper image) and the analysis area with irregular rung spacing (lower image) for the ladder walk test. The ladder apparatus is placed on a stand so that the ladder is suspended above the table. The area analysed lies in the middle part of the ladder and is setup with irregular rung spacing (lower image) ranging from 1 to 3 cm. Plastic walls are present on both sides of the ladder. The animal is placed between them on the left side to cross the ladder. The home cage is placed at the other end of the ladder. A camera is placed on a tripod level with the ladder in order to videotape the animals in the analysis area (with irregular rung spacing).

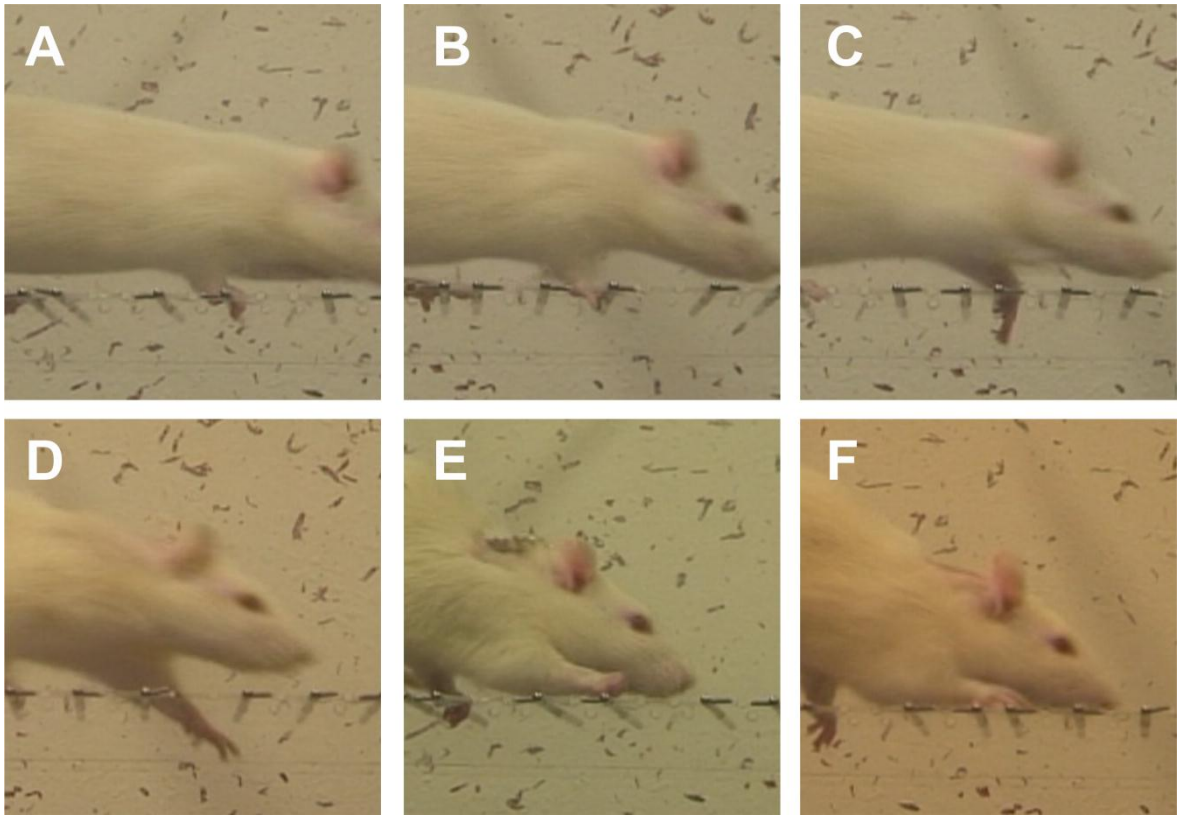

Figure 3-5. Categorization of paw placement in horizontal ladder walk test.

Images showing the different types of placement of the forepaws seen while animals cross the ladder. Paw positions were categorized as either normal (A, B) or errors (C, D, E, F). **A. Hit:** The animal completes the step using optimal paw position with the digits gripping around the rung. **B. Sub-optimal:** The animal completes the step using digits for weight-bearing. **C. Slip:** The animal places forepaw on the rung then slips off. **D. Miss:** The animals forepaw misses a rung and falls right through. **E. Clenched:** The animal forepaw completes the step using its clenched paw and the dorsal aspect in contact with the ladder. **F. Partial:** The animal places forepaw on rung but did not fully bear weight on this limb before repositioning on another rung.

3.4 Results

3.4.1 Assignment of animals to hESC-MSC transplanted and control groups

In order to investigate whether hESC-MSC transplants improve functional outcome, 48 animals were trained to perform the grip strength and ladder walking tests. All of these animals received 175 kdyn contusion injuries at the C6 level of the spinal cord. One animal did not recover from this operation. One week before the transplantation surgery animals were assigned to the transplant or control groups on the basis of the grip strength score. The grip strength scores at 2 weeks after injury were plotted in descending order and the first 36 animals out of the 47 (i.e. those with the highest grip strength score) were divided equally into the transplant and control groups. However, after the transplants 1 animal in the transplant group died post operatively. One more transplanted animal and 2 control animals were euthanized in the course of the study due to autotomy of a paw. The remaining 16 transplanted animals and 16 control animals completed the behavioural assessments up to 9 weeks after the injury. One of the transplanted animals and 3 of the control animals died during the preparatory surgery for electrophysiological experiments. In addition, injuries were found to be erroneously located rostral to the C6 segment in 3 control animals and these were therefore excluded from the study. Electrophysiological data was therefore obtained from 15 transplanted and 10 control animals. Although 16 transplanted and 16 control animals completed behavioural assessment, the 3 control animals which were found to have an incorrect injury level were not included in the analysis. Therefore behavioural data for the ladder walking test was based on 16 transplanted and 13 control animals. For the ladder walking test for most time points data was obtained from all animals, however, the exceptions were one animal at the pre-operative time point and two animals at 1 week after transplantation, where no data could be obtained. Although all 16 transplanted and 13 control animals were subjected to the grip strength test at each time point, at most time points 3 animals in the transplant group were unable to perform the test and these are not included in the final analysis. Therefore the data for the grip strength test was based on 13 transplanted and 13 control animals. It is impossible to be blinded because of

the effect of the injury and the surgical site is exposed at the time of electrophysiology experiment.

Retrospectively the injury parameters from the IHI software recorded at the time of the contusion injury were graphed for comparison between the two groups. These recordings were used to confirm that animals assigned to each group (transplant and control) received similar injuries. Graphs showing these comparisons for actual force and displacement are shown in Figure 3-6 and Figure 3-7 respectively. Figure 3-8 shows comparisons of body weights of animals at the time of the contusion injury, transplant/medium injections and electrophysiological experiments for both groups. The weights in both animal groups were closely similar at the time of the contusion injury, so that they would have similar sized cords and therefore be similarly affected by the injury. The weights at the time of electrophysiology were also closely similar and therefore the electrophysiological data was obtained in similarly sized animals for both groups.

In addition, to improve the survival of the transplanted cells, animals were immunosuppressed with daily injections of Cyclosporine at a dose of 20 mg/kg body weight. Therefore, for optimisation purposes the same daily dose of Cyclosporine was given to the control animals.

Monitoring and minimising any differences in variables such as weight, injury severity or behavioural performance between the transplanted and control groups of animals should mean that any changes in functional outcome between the groups can more confidently be attributed to the hESC-MSC transplants.

3.4.2 Behavioural testing

3.4.2.1 Grip strength test

All thirteen hESC-MSC transplanted and thirteen control animals included were able to perform the grip strength test before the contusion injury. The grip strength test was performed on a weekly basis and scores were calculated as the ratio of maximum force (g) generated by the animal to its weight (g). Comparison of the grip strength scores for the transplanted and control groups of animals for each week of testing are shown in Figure 3-9.

3.4.2.1.1 Assessment of grip strength score prior to contusion injury

Normal animals can generate a force of around 3-4 times their body weight with the forelimbs. Before the contusion injury, the mean grip strength score was approximately 3.6 in animals assigned to both transplant and control groups.

3.4.2.1.2 Effects of contusion injury on grip strength score

Contusion injury lead to a significant decline in the grip strength score in all animals tested one week after the injury. The mean grip strength score reduced to 2.1 in both groups of animals which represents a marked reduction in the gripping ability immediately after the injury. There was a modest improvement at 2 weeks after the injury with mean scores increasing to about 2.65 in both groups. However, this improvement did not persist and there was a decline in mean grip strength score at 3 weeks in both transplant and control groups. At this point animals were assigned to control or transplant groups on the basis of their grip strength score as described above.

3.4.2.1.3 Effects of the delayed hESC-MSC transplants or medium injections on grip strength score

hESC transplants or medium injections were performed 3 weeks after the contusion injury. Grip strength measurements were obtained 1 week after the transplants/medium injections i.e. 4 weeks after injury. At week 4 there was a modest decline in the grip strength score in the transplanted animals in comparison to scores observed before transplantations, the mean score reduced to 2.09 from 2.23 observed at week 3 (Figure 3-9). In contrast the mean score in the control animals improved to 2.6 after medium injections suggesting that the surgery itself might have no detrimental effect on the gripping ability of the animals. At week 5 the grip strength score showed improvement in both the transplanted and control groups, however, the mean values in the transplanted (2.5) animals were still smaller than controls (2.95). Grip strength in transplanted animals showed further improvement at week 6 with mean scores rising to 2.85, a score comparable to controls (2.86) at this time point. However, testing performed at week 7 and 8 showed that the improvement was not consistent as the mean grip strength score in both transplanted and control groups declined again to between 2.39-2.59. At the last assessment point i.e. week 9 the grip strength score again showed enhancements in gripping ability in

both groups and was comparable to the mean score at week 6. There was no statistically significant difference between the transplanted and control groups at any time after the transplantations/medium injections.

This shows that the grip strength after the contusion injury shows moderate spontaneous improvement with time but that a significant functional deficit persists. No functional enhancements in the hESC-MSC transplanted group were observed in the grip strength test over the 6 weeks examined after the transplantations.

3.4.2.2 Ladder walk test

The ladder walk test was performed weekly and useful data was obtained from 16 transplanted and 13 control animals. Forelimb and hindlimb function were assessed by counting the number of errors as described previously in the Methods and expressed as a percentage of the total steps taken. Stride frequency and crossing time for individual animals were also calculated as described previously.

3.4.2.3 Assessment of the forelimb in ladder walk test

Figure 3-10 shows a comparison of forelimb errors in the transplant and control group of animals for ladder testing performed each week.

3.4.2.3.1 Assessment of forelimb function in ladder walk test prior to contusion injury

All sixteen animals subsequently transplanted and all but one of the thirteen control animals performed the ladder walk test prior to injury, making very few errors in forelimb stepping and crossing the ladder with minimal hesitation. The forelimb errors in the animals assigned to the transplant and control groups were closely similar with mean errors of 16.79 % and 16.44 % respectively.

3.4.2.3.2 Assessment of forelimb function in ladder walk test after contusion injury

Contusion injury had a substantial effect on forelimb function in the ladder test. The mean errors significantly increased 1 week after the injury in all the animals assigned to transplant and control groups, however all animals were able to cross the ladder. Mean forelimb error rate increased by approximately 20 % in

both groups, the mean error rate rising to 36.3 in the transplant and 35.2 in control group of animals. The deficit in the forelimbs improved by approximately 6 % at week 2 in both groups but a modest increase in errors was observed in the animals assigned to the transplant group at 3 weeks compared to the control group. However, there was no significant difference in scores of transplant and control groups of animals at either time point (two-way ANOVA with post hoc bonferroni tests).

3.4.2.3.3 Effects of delayed hESC-MSC transplants or medium injections on forelimb function in ladder walk test

hESC transplants or medium injections were performed 3 weeks after the contusion injury and ladder walk test data obtained 1 week after this i.e. week 4 after injury. Two animals were unable to perform the test at this time point. Compared to the errors before the transplant/medium injections, a modest increase in errors by a further 3 % and 7 % was observed in the transplanted and control groups of animals, respectively (Figure 3-10). This deterioration of forelimb function may relate to the transplant/medium injection surgery procedure leading to a slight detrimental effect as also observed for transplanted animals in the grip strength test. At week 5 and 6 after injury a trend towards improved forelimb function was observed in both groups. There was a modest reduction in errors by approximately 7 % in the transplanted and 3 % in control groups of animals at week 6 compared to those at week 4 after injury. However, this trend was not consistent. At the last assessment, 9 weeks after injury, deficits persisted in the forelimbs for both groups to a similar extent. There were no significant differences between the forelimb errors in the transplanted and control groups of animals at any time point after transplant/medium injection (2-way ANOVA and Bonferroni post-hoc analysis).

These results show that forelimb function decreases markedly after contusion injury with modest spontaneous recovery with time. However, a significant functional deficit persists for up to 9 weeks after the injury and hESC-MSC transplants did not improve forelimb function.

3.4.2.4 Assessment of the hindlimb in ladder walk test

Figure 3-11 shows a comparison of hindlimb errors in the transplant and control groups of animals for ladder testing performed each week. In non-injured animals the errors were trivial in the hindlimbs in comparison to those observed in the forelimbs. The mean scores in animals assigned to the transplant and control groups were very similar before injury and were 2.47 and 3.48 respectively. The errors remained almost similar throughout the experiment suggesting the hindlimbs are not affected by the injury and that the modest and variable trend for increased errors after injury may be secondary to errors observed in the forelimbs.

3.4.2.5 Crossing time

The time taken by animals to cross the analysis area (area with irregular rung spacing) was calculated for each trial and averaged to obtain the mean crossing time. Figure 3-12 shows a comparison of mean crossing time in the transplant and control groups of animals for ladder testing performed each week.

All animals were able to cross the ladder rapidly without any hesitation and delay prior to the contusion injury. The mean time for crossing the 40 cm analysis area before the injury was 2.47 seconds for animals assigned to the transplant and 2.20 seconds for the control group. One week after the injury some of the animals were hesitant or slow to walk, however, all were able to cross the ladder. The mean crossing time increased to more than twice that seen before the injury in both the transplant (5.23 sec) and the control (5.22 sec) groups of animals (Figure 3-12). The crossing time fluctuated but remained nearly constant around a mean of 5 seconds throughout the remainder of the experiment in both groups. The crossing time was also not affected after the hESC-MSC transplants or medium injections performed at 3 weeks.

3.4.2.6 Stride frequency

Stride frequency is a measure of the average number of forelimb or hindlimb steps taken per unit time. Changes in the stride frequency were calculated separately for forelimbs and hindlimbs. Figure 3-13 shows a comparison of

forelimb and hindlimb stride frequency in transplant and control groups of animals for ladder testing performed each week.

In normal (non-injured) animals the mean forelimb stride frequency was approximately 4 steps/sec in animals assigned to transplant and control groups of animals. The forelimb stride frequency declined significantly after the injury to a mean of approximately 3 steps/sec in both groups of animals. There was a tendency for a further gradual decline over the remainder of the experiment and this was approximately similar for transplant and control groups of animals. From week 3 till the last assessment time point at week 9 after injury the mean stride frequency was between 2.1 and 2.6 steps/sec. There was no difference in the hESC-MSC transplanted or control groups of animals (2-way ANOVA and Bonferroni post-hoc analysis).

The hindlimb stride frequency was around 3.0-3.5 steps/sec in normal animals (similar to the forelimb) and dropped significantly after injury to a mean of approximately 1.5 steps/sec. Thereafter the hindlimb stride frequency remained closely similar to this over the rest of the post-injury period and there were no significant difference in the transplant and control groups of animals at any time point (2-way ANOVA and Bonferroni post-hoc analysis).

Figure 3-14 shows a comparison of the number of forelimb and hindlimb steps taken to cross the ladder in transplant and control groups of animals for tests performed each week. In normal animals the forelimb and hindlimb steps were approximately 4.7 steps/run and 3.8 steps/run respectively. Immediately after the injury the average number of forelimb steps taken significantly increased while the number of hindlimb steps did not change. There was modest reduction in the number of forelimb steps at 2 weeks after injury which remained similar throughout the remainder of the experiment. There was no significant difference between transplant and control groups of animals at any time point.

Stride frequency provides a measure of the degree of co-ordination between forelimbs and hindlimbs. Stride frequency is closely similar for forelimbs and hindlimbs before the injury. That is to say, the forelimbs and hindlimbs are co-ordinated. Injury causes lack of co-ordination between the forelimbs and hindlimbs.

Despite the increase in the number of forelimb steps accompanying the increase in crossing time, there is also a decrease in stride frequency or a decrease in the number of steps per unit time. The increase in the crossing time was approximately 220 % after injury and therefore the expected decrease in stride frequency would be to 45 % of that observed before the injury (i.e 1.8 steps/sec for the forelimbs and 1.4 steps/sec for the hindlimbs) as the crossing distance is fixed. The decrease in stride frequency for the forelimbs is 3 steps/sec (down to 75 % of that before injury) which is not actually as much as expected (1.8 steps/sec) given the increase in crossing time. The decrease in stride frequency for the hindlimbs is 1.5 steps/sec approximately as expected (i.e. 1.4 steps/sec) given the increase in crossing time. This can be observed in the graph showing forelimb and hindlimb stride frequency.

The number of steps taken by the forelimbs and hindlimbs before the injury are approximately the same as expected for co-ordinated forelimb and hindlimb stepping, so step length should also be similar. The step length in the hindlimb does not change because the number of steps in crossing the fixed distance does not change before and after the injury, however, it decreases for the forelimbs as the number of steps made by forelimbs in crossing the same area increased after injury.

There is a small reduction in the discoordination at 2 weeks after injury but in general there is a little change for the remainder of the study. None of the parameters differs significantly at any stage between control and transplanted animals (2-way ANOVA and Bonferroni post-hoc analysis).

3.4.3 Electrophysiological assessment of local circuit function of the spinal cord in the hESC-MSC transplanted animals

CDPs evoked by stimulating the radial nerve and pyramidal tract were used to assess function in the local grey matter of the spinal cord as described in the previous chapter (chapter 2). The electrophysiological data included recordings from 15 animals transplanted with hESC-MSCs and 10 medium injected control animals. The electrophysiological assessments were carried 10 weeks after the contusion injury with transplantation or medium injections performed 3 weeks after the injury.

3.4.3.1 hESC-MSC transplants and their effects on radial nerve evoked CDPs

CDPs evoked by radial nerve stimulation were recorded with a sliver ball electrode on the dorsal surface of the spinal cord at 1 mm intervals from 8 mm rostral to 8 mm caudal to the C5/6 border as described in chapter 2 in both the transplanted and control groups of animals. Figure 3-15 shows a comparison of the rostro-caudal distribution of CDPs recorded from the transplanted and control groups of animals.

Contusion injury at the C6 level produces a marked reduction in the amplitudes of CDPs centred on the contusion site due to loss of neurons in the grey matter and their input connections as described previously in chapter 2 and this takes the form of a dip in the CDP distribution localised to the injury site as shown in Figure 3-15. The CDP mappings show no difference in the amplitudes at most of the recording locations in the transplanted and control group of animals except at the most caudal end i.e. -6 mm to -8 mm, where amplitudes in the transplanted animals were comparatively smaller. However, there were no significant differences at any recording location from +8 to -8 which suggest hESC-MSC transplantations have no effect on radial evoked CDPs after injury.

3.4.3.2 hESC-MSC transplants and their effects on pyramidal evoked CDPs

CDPs evoked by pyramidal stimulation were recorded at 1 mm intervals from 8 mm rostral to 8 mm caudal to the C5/6 border, in both the transplanted and control group of animals (as described in chapter 2). The rostro-caudal distribution of pyramidal evoked CDPs in the transplanted and control groups of animals is shown in Figure 3-16.

The general distribution showed the same pattern in transplanted and control groups of animals. The CDPs show greater amplitudes at locations above the C5/6 border and markedly smaller amplitudes below the C5/6 border which is caused by damage to the main CST located in the dorsal column as described in chapter 2. However, CDPs at most of the recording locations were larger in amplitude in the control group of animals compared to transplanted animals. The CDPs rostral to the C5/6 border i.e. from +8 to +1, show significantly higher amplitudes, which may be improvements of connections in the grey matter in the control group of animals or in contrast, greater deterioration of connections

in the transplanted animals. In addition, CDP amplitudes below the injury (-3 mm to -8 mm) were also greater in the control group of animals compared to the transplanted animals, although this difference was non-significant. CDP comparisons in the transplanted and control animals suggest transplanted cells have some unfavourable effect on the CST fibres which may be responsible for the reduction in the CDP amplitudes.

3.4.4 Histology

3.4.4.1 Injury site 3 weeks after contusion

Three animals were perfused 3 weeks after injury and spinal blocks containing the injury site were removed in order to assess the injury site at the time of transplantation. Examples of histology from the injury site are shown in Figure 3-17. The injury site showed cavities which encompassed large parts of both white and grey matter. Sometimes these consisted of more than one fluid filled cavities and sometimes these were partly filled with laminin. A glial scar formed around the cavities with dense GFAP immunolabelling observed at the injury perimeters compared to areas beyond the injury.

3.4.4.2 Injury site 7 weeks after hESC-MSC transplants

Spinal cord blocks containing the injury site were removed from twelve animals transplanted with hESC-MSCs and processed for anatomical observations. Figure 3-18 shows examples of the GFP-labelled hESC-MSCs at the injury site 7 weeks after transplantation. Sections from all the transplanted animals showed the presence of GFP-labelled cells at the injury site. The distribution of cells was variable. In some animals cells were spread throughout the whole of the injury area (Figure 3-18B & C) while in other animals surviving cells occupied only part of the injury site (Figure 3-18A). However, none of the animals had injury cavities. In all animals either transplanted cells or a mixture of cells and extracellular matrix (ECM) completely fill the injury site. In a few sections transplanted cells were seen beyond the injury site. In areas outside the injury site where transplanted cells were observed, GFAP immunolabelling was largely absent suggesting neuropil loss at sites where hESC-MSCs migrated.

3.4.4.3 Injury site 7 weeks after medium injection

Spinal cord blocks from nine control animals injected with medium were removed and processed for comparison with transplanted animals. Examples of histology from medium injected control animals is shown in Figure 3-19. Section from all control animals showed extensive cavities and these encompassed large parts of both white and grey matter, particularly in the sections close to the midline where the cavity is greatest in extent. In some cases cavities partially filled with ECM, which was rich in laminin (Figure 3-19A & C). A glial scar was formed around the cavities as indicated by denser GFAP immunolabelling.

3.4.5 Injury cavity size of hESC-MSC transplant and medium injection

To study the effects of hESC-MSCs on the morphology of the contusion injury, the injury site/cavity dimensions were calculated in all animals processed for histology in the transplanted (n=12) and control (n=9) groups of animals. Cavity length and width were determined as described previously in Methods. The data is shown in Table 3-2 and Table 3-3 for transplanted and control groups of animals, respectively. Figure 3-20 shows a graphical comparison of injury extent in both groups of animals. The rostro-caudal length of the injury site was less in the transplanted animals than in the non-transplanted control animals and the difference was highly significant ($p < 0.001$). The width of the injury was also reduced in the transplanted animals and this also reduced significantly ($p < 0.05$). The reduction in extent of the injury site suggests that hESC-MSC transplants help preserve surrounding tissue and this may be attributed to the neuroprotective nature of MSCs as reported in literature.

3.4.6 Acute transplants

As described above, transplants of hESC-derived MSCs performed 3 weeks after injury had no observable beneficial effect on functional outcome. Since one possible reason for this is that transplants are performed too late after the injury to be effective, we performed additional experiments with transplants at earlier time point to determine how well the cells survive and to examine the histological appearance of the injury site.

3.4.6.1 Injury site at 5 and 7 days after injury

In order to assess the nature of the injury site at the time points when acute hESC-MSC transplants were performed, 3 animals were perfused at 5 days after injury and 3 animals 7 days after injury and spinal blocks containing the injury site were removed and processed. Figure 3-21 shows examples of injury site histology from these animals.

Tissue sections at 5 days after the injury showed extensive damage extending several mm from the centre in both rostral and caudal directions. Some loss of tissue at the core of the sections may be due to badly disrupted tissue that is lost during processing as shown in Figure 3-21(A). Similarly the injury site in 7 day survival animals showed widespread damage and a modest increase in GFAP immunolabelling around the injury perimeter suggesting initiation of cavity formation as shown in Figure 3-21(B).

3.4.6.2 Acute hESC-MSC transplantation at 5 and 7 days

Three animals transplanted 5 days after injury were perfused three weeks after transplantation and spinal cord blocks containing the injury site were removed and processed as described in Methods. Examples of injury site in animals transplanted at 5 days after injury are shown in Figure 3-22.

There were no evidence of cavities and all of the sections containing the injury site were filled with ECM and transplanted cells, usually a mixture of both but predominantly ECM in sections passing through the middle of the injury. Although there was evidence of cell survival cells did not extend throughout the whole of the injury site. Most of the cells were found at the lateral edges of the injury particularly in those sections passing through the very centre of the injury. For sections passing through more lateral aspects of the injury where the injury site is therefore smaller, a larger proportion of the injury area is occupied by transplanted cells. Area of the injury in the transplanted injury site was largely devoid of GFAP immunlabelling and in some sections particularly those near the midline where transplanted cells have tracked rostrally or caudally, GFAP is largely absent. This displacement of the host astrocytes suggest transplanted cells causing neuropil disintegration.

Three animals transplanted at 7 days after injury were perfused 3 weeks after transplantation and spinal cord containing the injury site processed for histology. Examples of injury site histology are shown in Figure 3-23. The main features of the injury site were generally similar to those observed for transplantations performed 5 days after injury. The transplanted injury site was noticeably devoid of GFAP immunolabelling. GFP labelled cells were present mainly at the rostral and caudal end of the injury but the larger part of the injury was filled with matrix. There was no evidence of cavities and in some cells had tracked rostrally and caudally leading to the displacement of glia in those regions. In more lateral sections a larger proportion of the injury site contained transplanted cells, reflecting the tendency for cells to survive better at the perimeter of the injury.

Animal ID	Length (mm)	Width (mm)
R19013	2.728	1.26
R19113	1.899	1.02
R18413	2.747	1.2
R9713	2.718	1.5
R10713	2.33	1.44
R10313	2.907	1.8
R10113	2.921	1.98
R10913	2.896	1.44
R26013	2.607	1.92
R26313	2.671	1.86
R27013	2.187	1.86
R27113	3.031	1.56
Mean	2.637	1.57

Table 3-2. Table showing injury site/cavity dimensions in transplanted animals.

Quantification of the maximal length and width of injury area in individual animals in hESC-MSC transplants group.

Animal ID	Length (mm)	Width (mm)
R26213	3.306	2.34
R26413	3.3	2.04
R26513	3.982	1.92
R11113	4.047	1.92
R11213	3.587	1.92
R18513	3.252	1.98
R18713	3.672	1.98
R26813	3.509	2.04
R9913	3.192	1.68
Mean	3.539	1.98

Table 3-3. Table showing injury site/cavity dimensions in control animals.

Quantification of the maximal length and width of injury area in individual animals in control group

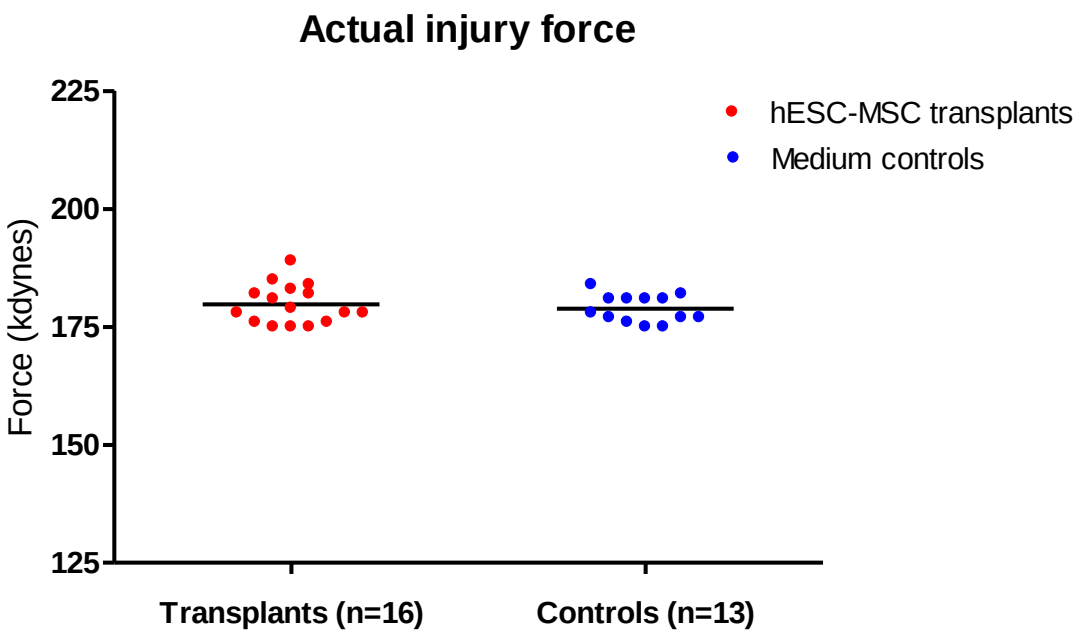

Figure 3-6. Comparison of actual contusion injury force in transplanted and control groups of animals.

Scatter plot showing the actual force applied by the IH impactor set to deliver 175 kdyn force. There was no significant difference between the mean force for any of the groups (students t-test). Horizontal bars represent the mean force in each group.

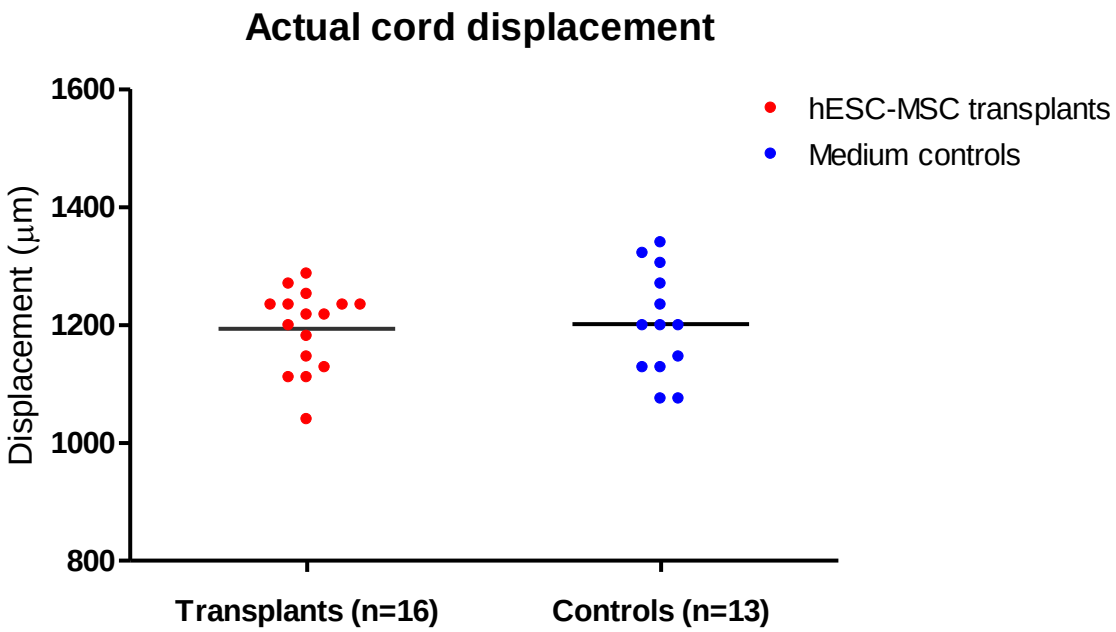

Figure 3-7. Comparison of actual cord displacement as a result of 175 kdyn contusion injury in transplanted and control animals.

Scatter plot showing the displacement of the impactor tip into the cord tissue from the surface. There was no significant difference between the mean displacement for any of the groups (students t-test). Horizontal bars represent the mean force in each group.

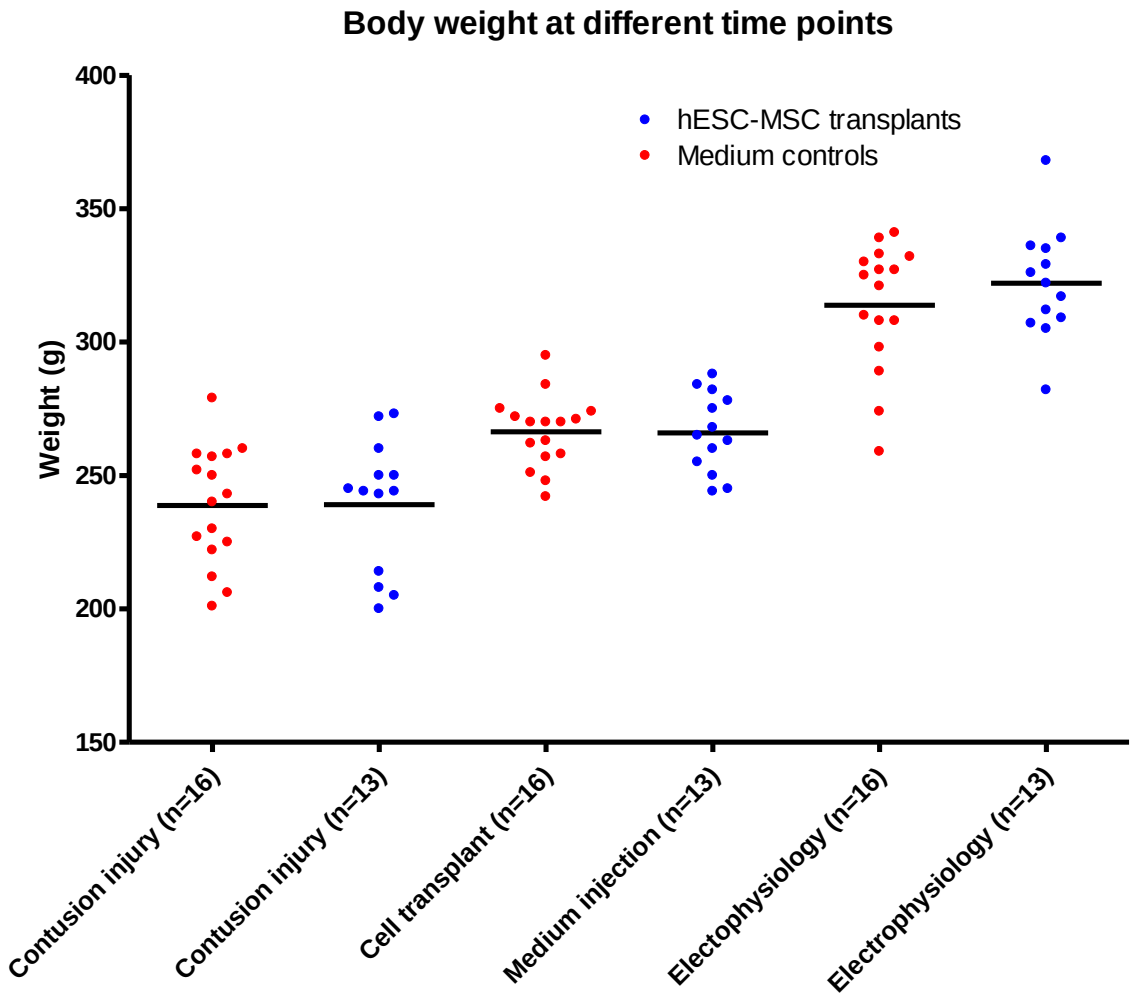

Figure 3-8. Comparison of individual body weights of transplanted and control animals at different points in the study.

Scatter plot showing body weights of individual animals in hESC-MSC transplanted and medium control group of animals at the time of contusion injury, cells/medium injection and electrophysiology experiments. Horizontal bars represent the mean weight. There was no significant difference between the mean weights of transplanted and control animals at any point (students t-test, $p>0.05$ at all points).

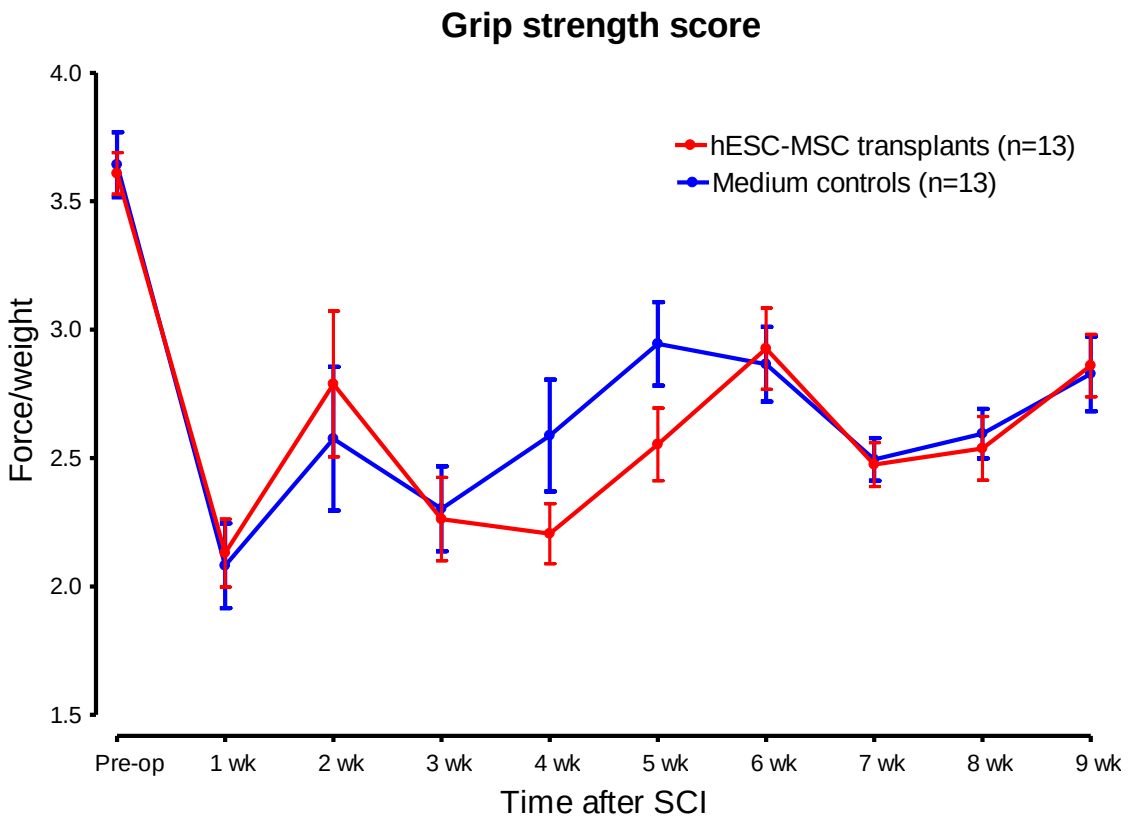

Figure 3-9. Comparison of grip strength scores for transplanted and control animals.

Plots of grip strength for hESC-MSCs transplanted (red) and medium injected control (blue) group of animals. Grip strength is plotted as a ratio of force to body weight. Each data point is the mean score for all animals in the group tested in each week. Error bars represent the standard errors of the mean (Fujimoto et al.) within the group. There was no significant difference between the groups at any point (2-way ANOVA with Bon-ferroni post hoc analysis, all p values > 0.05). n=13 in transplants except week 5 (n=12) and n=13 in controls except week 1 (n=12).

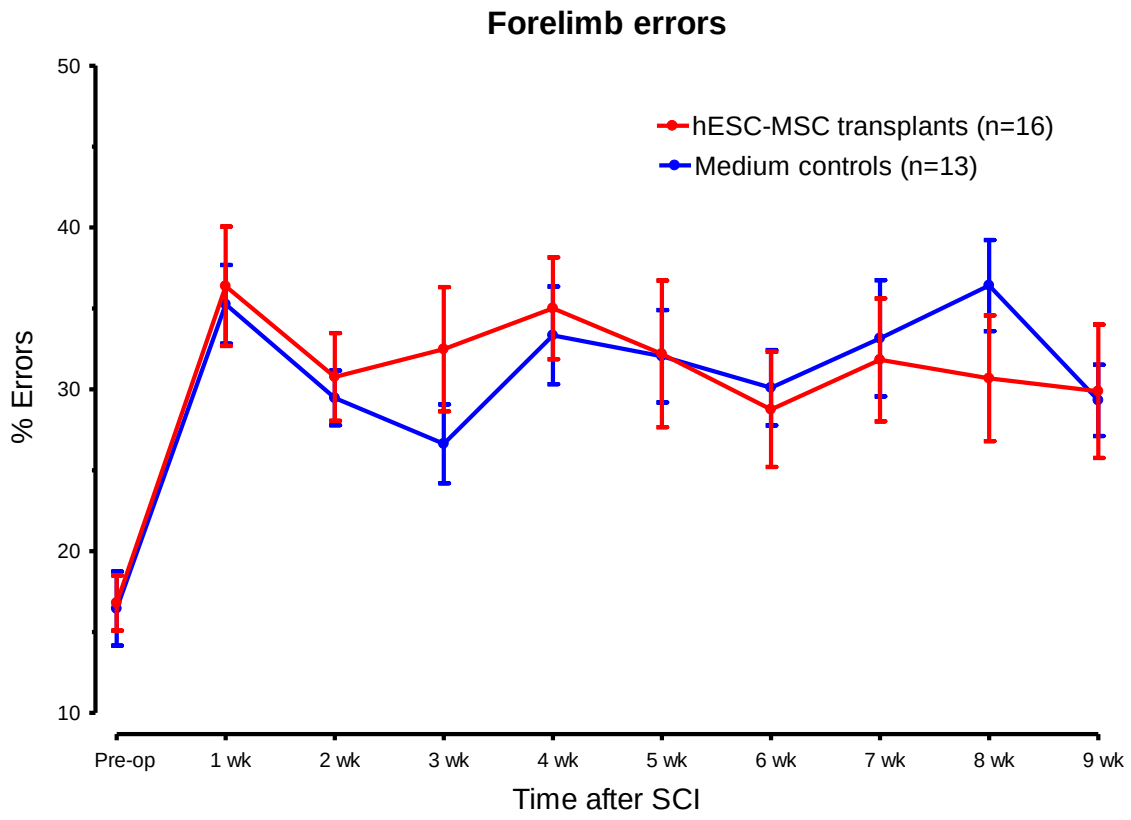

Figure 3-10. Comparison of forelimb errors in ladder walk test for transplanted and control animals.

Plots of forelimb errors for hESC-MSCs transplanted (red) and medium injected control (blue) group of animals. Forelimb errors are plotted as a percentage of the total number of steps. Each data point is the mean of all forelimb errors for all animals in the group tested each week. Error bars represent the standard errors of the mean (Fujimoto et al.) within the group. There was no significant difference between the groups at any point (2-way ANOVA with Bon-ferroni post hoc analysis, all p values > 0.05). n=16 in transplants except week 4 (n=14) and n=13 in controls except pre-op (n=12).

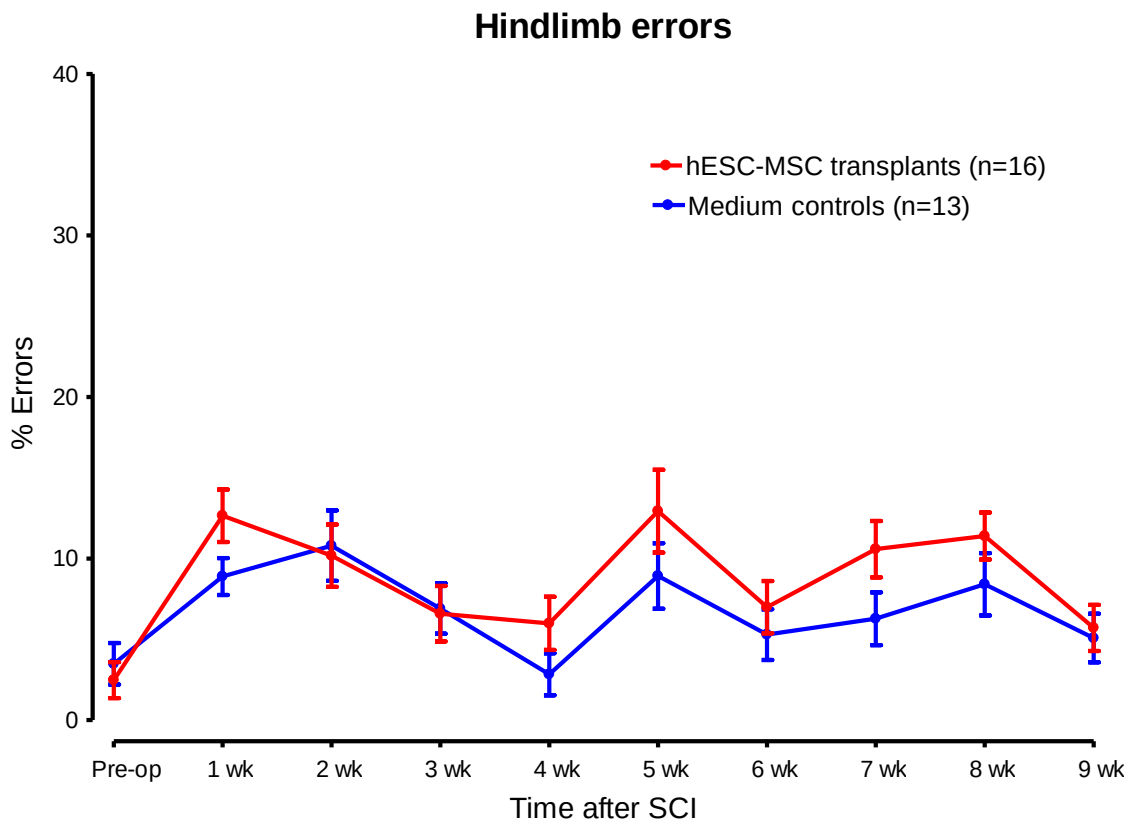

Figure 3-11. Comparison of hindlimb errors in ladder walk test for transplanted and control animals.

Plots of hindlimb errors for hESC-MSCs transplanted (red) and medium injected control (blue) groups of animals. Hindlimb errors are plotted as a percentage of the total number of steps. Each data point is the mean of all hindlimb errors for all animals in the group tested in each week. Error bars represent the standard errors of the mean (Fujimoto et al.) within the group. There was no significant difference between the groups at any point (2-way ANOVA with Bon-ferroni post hoc analysis, all p values > 0.05). n=16 in transplants except week 4 (n=14) and n=13 in controls except pre-op (n=12).

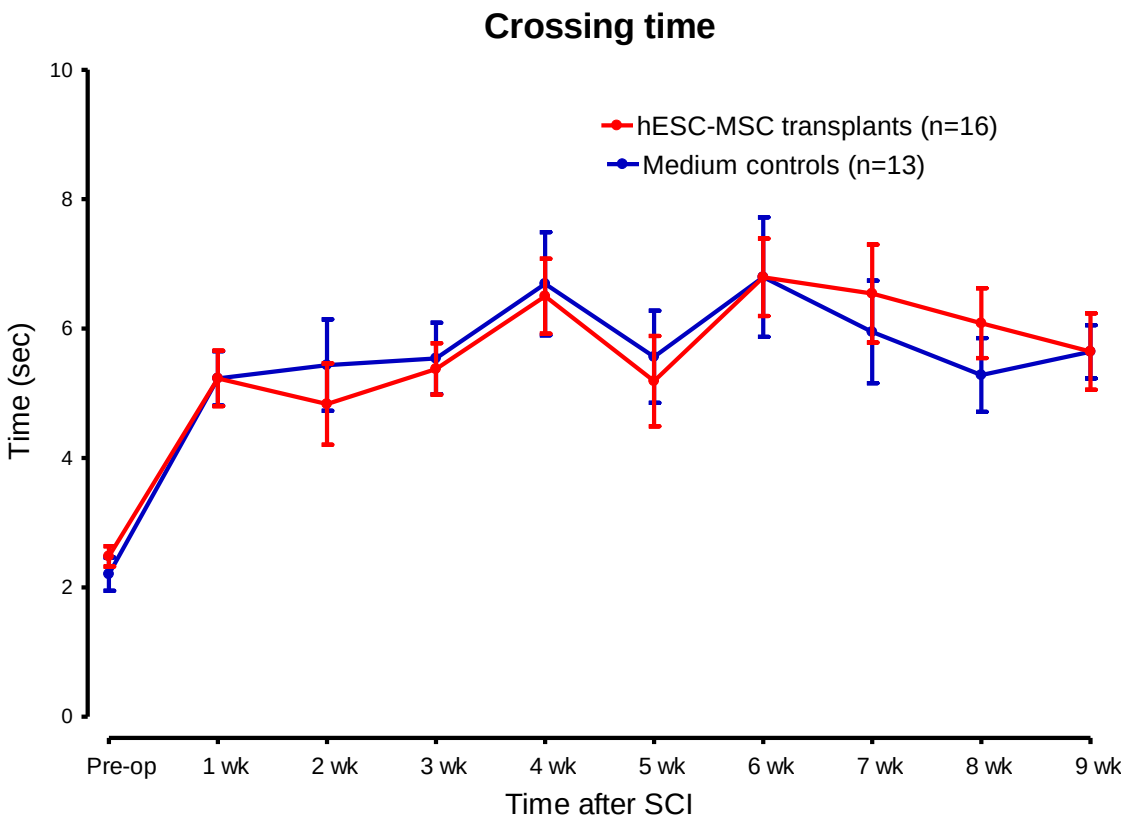

Figure 3-12. Comparison of crossing time in ladder walk test for transplanted and control animals.

Plots of crossing time for hESC-MSCs transplanted (red) and medium injected control (blue) groups of animals. Crossing time is plotted as the average time taken by to cross the analysis area of the ladder. Each data point is the mean crossing time for all animals in the group tested in each week. Error bars represent the standard errors of the mean (Fujimoto et al.) within the group. There was no significant difference between the groups at any point (2-way ANOVA with Bon-ferroni post hoc analysis, all p values > 0.05). n=16 in transplants except week 4 (n=14) and n=13 in controls except pre-op (n=12).

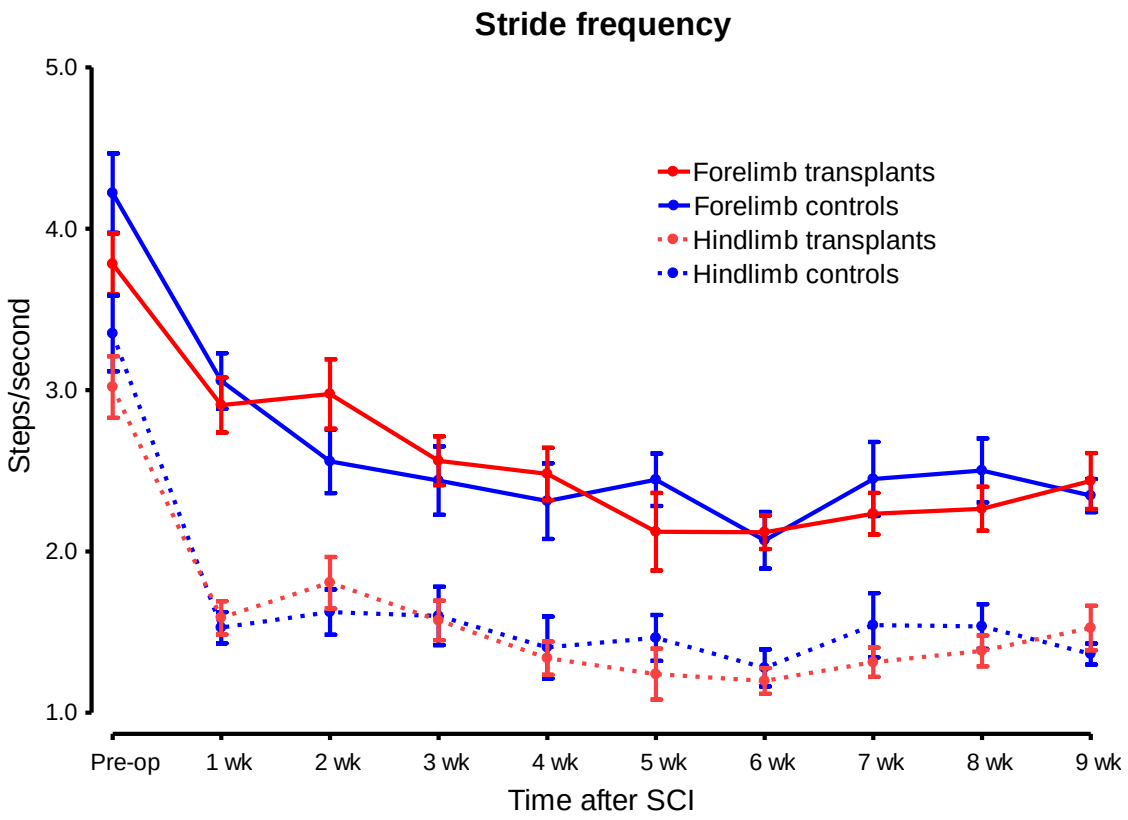

Figure 3-13. Comparison of stride frequency in ladder walk test for transplanted and control animals.

Plots of forelimb and hindlimb stride frequency for hESC-MSCs transplanted (red) and medium injected control (blue) groups of animals. Stride frequency is plotted as the number of steps per unit time (sec). Each data point is the mean stride frequency for all animals in the group tested in each week. Error bars represent the standard errors of the mean (Fujimoto et al.) within the group. There was no significant difference between the groups at any point (2-way ANOVA with Bon-ferroni post hoc analysis, all p values > 0.05). n=16 in transplants except week 4 (n=14) and n=13 in controls except pre-op (n=12).

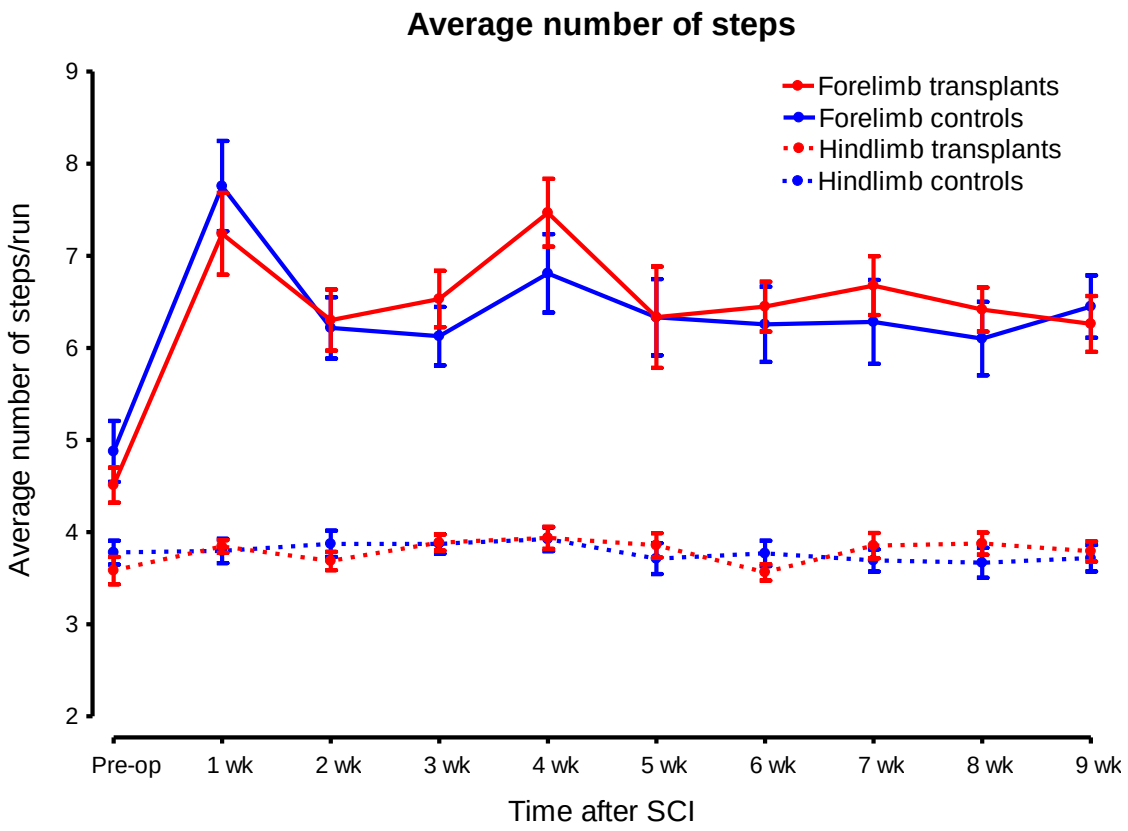

Figure 3-14. Comparison of number of steps in ladder walk test for transplanted and control animals.

Plots of average number of forelimb and hindlimb steps for hESC-MSCs transplanted (red) and medium injected control (blue) groups of animals. Number of steps is plotted as average number of steps per run. Each data point is the mean steps for all animals in the group tested in each week. Error bars represent the standard errors of the mean (Fujimoto et al.) within the group. There was no significant difference between the groups at any point (2-way ANOVA with Bon-ferroni post hoc analysis, all p values > 0.05). n=16 in transplants except week 4 (n=14) and n=13 in controls except pre-op (n=12).

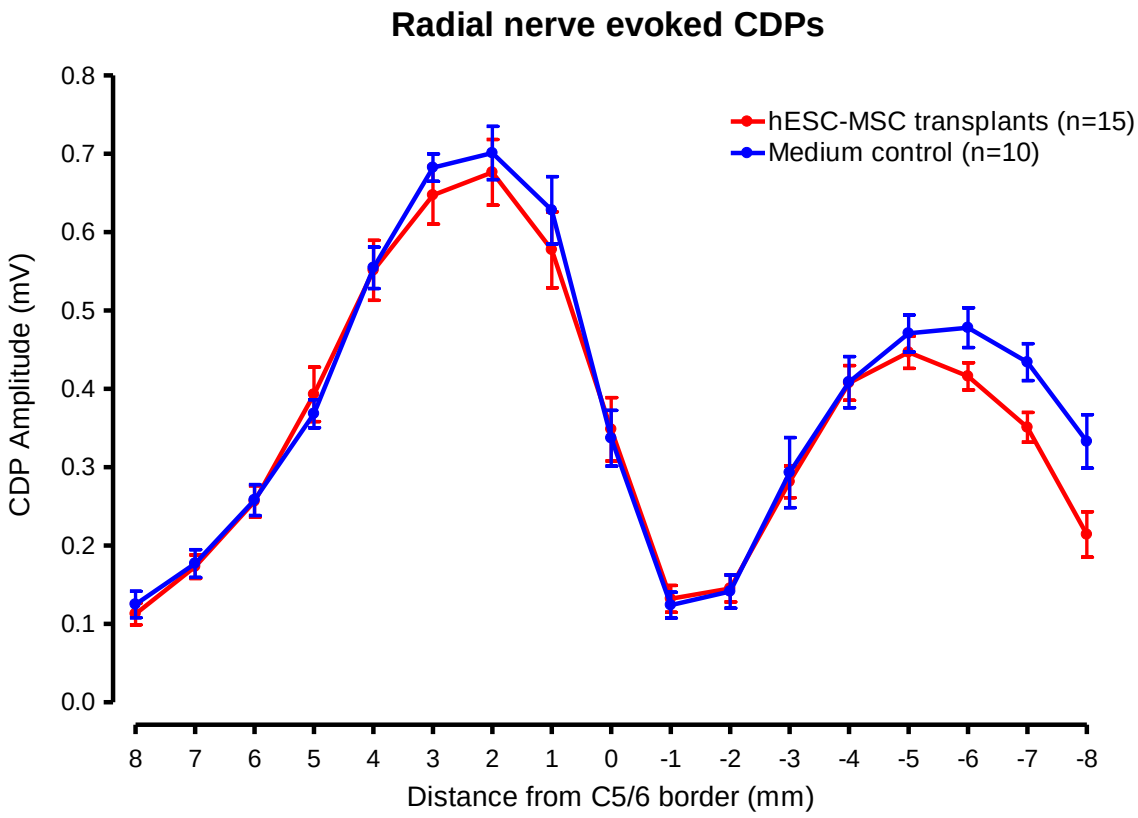

Figure 3-15. Amplitudes of radial nerve evoked CDPs recorded in transplanted and control animals.

The plot shows a comparison of the amplitudes of CDPs recorded in hESC-MSCs transplanted and medium injected control groups of animals. Electrophysiological experiments were carried out 10 weeks after injury (7 weeks after transplants/medium injections). Each data point is the mean CDP amplitude for all animals in the group. The contusion injury was carried out with the rostral edge of the impactor tip aligned with C5/6 border (0 mm). The recordings were made over the cervical spinal cord. Numbers on the x-axis represent the recording locations on the surface of the cord relative to C5/6 border (0 mm). Error bars represent the standard errors of the mean (Fujimoto et al.) within the group. There was no significant difference between the groups at any point (2-way ANOVA with Bon-ferroni post hoc analysis, all p values > 0.05).

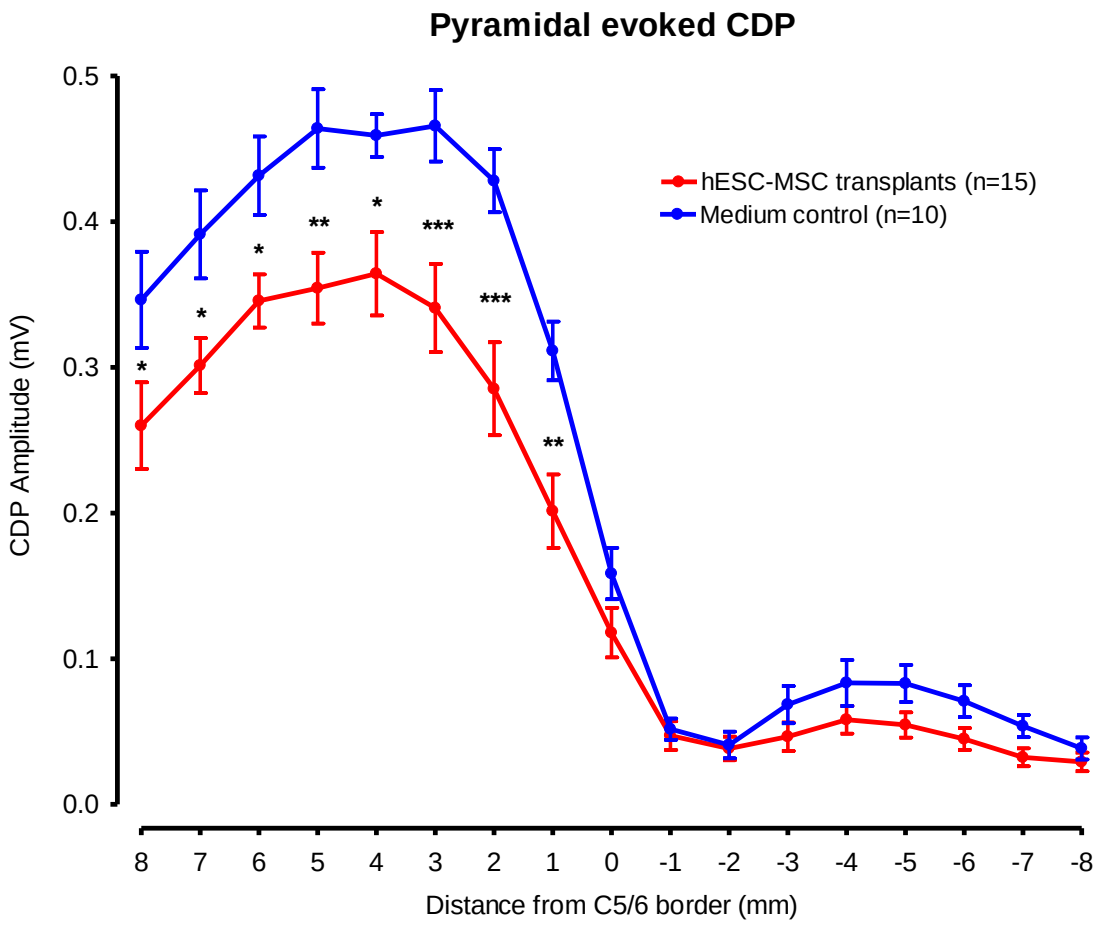

Figure 3-16. Amplitudes of pyramidal evoked CDPs recorded in transplanted and control animals.

The plot shows a comparison of the amplitudes of CDPs recorded in hESC-MSCs transplanted and medium injected control groups of animals. Electrophysiological experiments were carried out 10 weeks after injury (7 weeks after transplants/medium injections). Each data point is the mean CDP amplitude for all animals in the group. The contusion injury was carried out with the rostral edge of the impactor tip aligned with C5/6 border (0 mm). The recordings were made over the cervical spinal cord. Numbers on the x-axis represent the recording locations on the surface on the cord relative to C5/6 border (0 mm). Error bars represent the standard errors of the mean (Fujimoto et al.) within the group. 2-way ANOVA with Bon-ferroni post hoc analysis was applied for statistical analysis (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The error bars show +/- SEM.

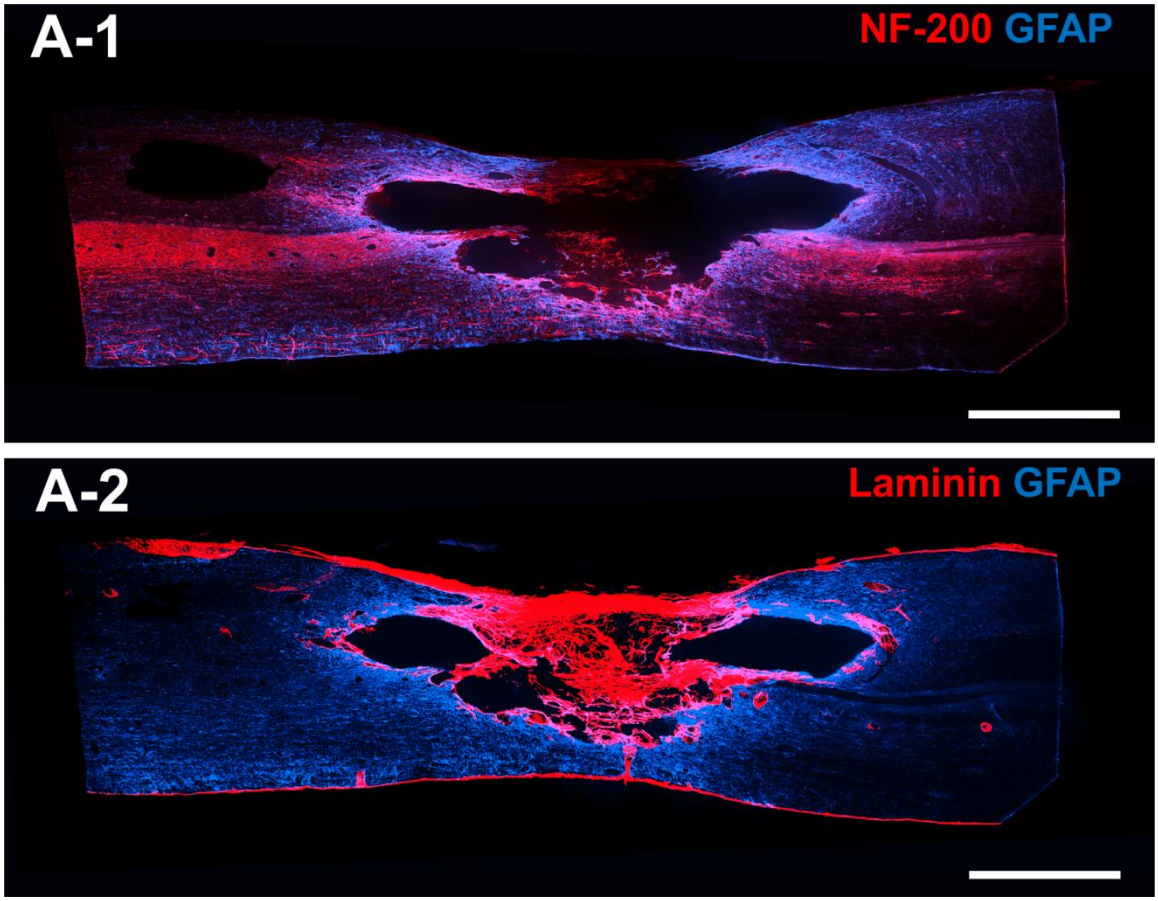

Figure 3-17. Examples of Injury site at the time of delayed hESC-MSC transplants or medium injections into the spinal cord.

The panels show confocal microscope images of the injury site 3 weeks after injury, the time point when transplants were performed. Each panel is an example obtained from a parasagittal section passing through the injury area close to the midline. Both panels (A-1 and A-2) show the distribution of GFAP (blue) immunolabelling. A-1 also shows NF 200 (red) and A-2 shows Laminin (red). The micrographs are projections of 7-9 optical sections and composed from multiple x20 fields of view. All scale bars are 1 mm.

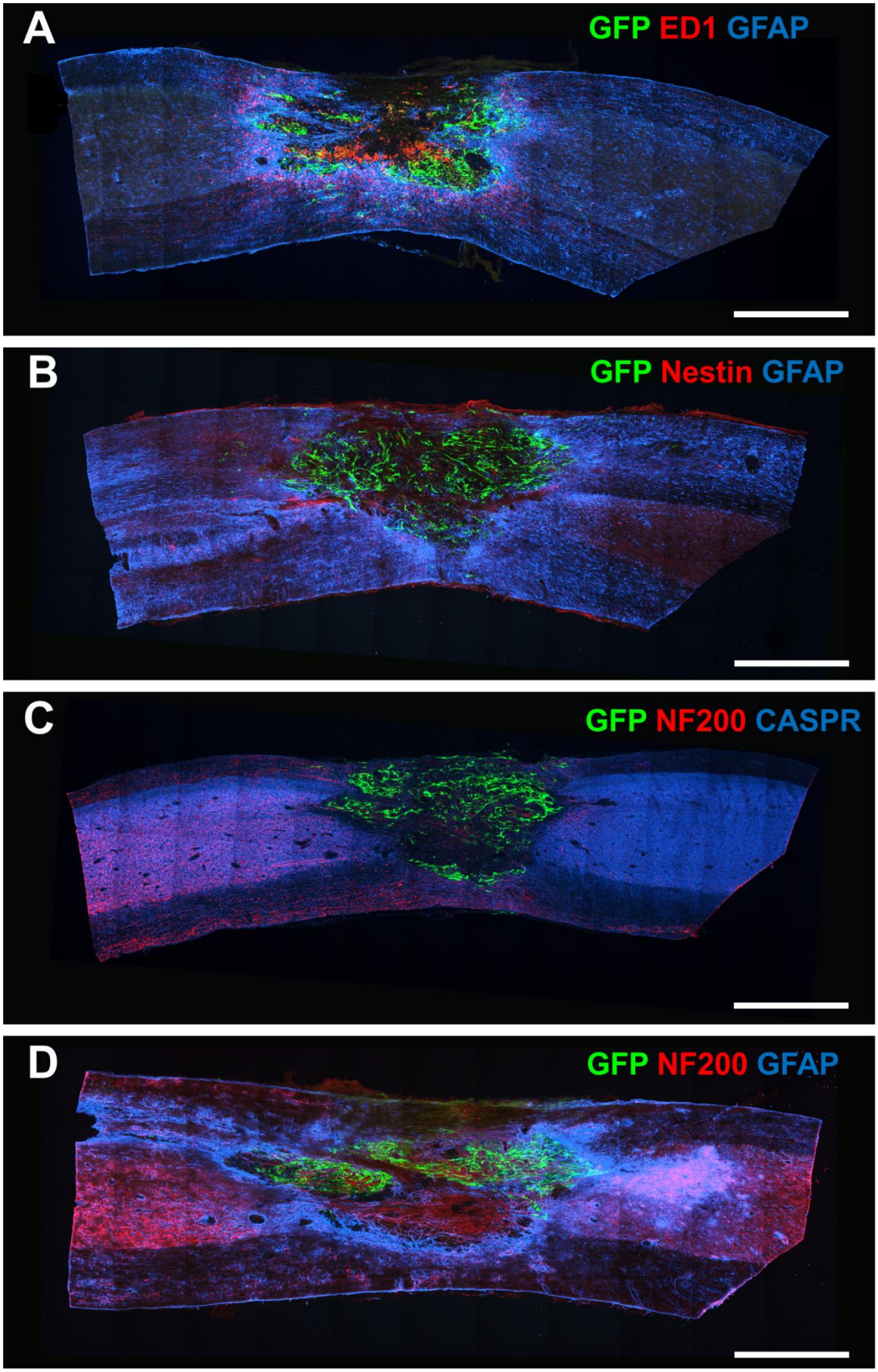

Figure 3-18. Examples of injury site histology from hESC-MSC transplanted animals.

The panels show confocal microscope images of the injury site 7 weeks after hESC-MSC transplants (10 weeks after injury). Each panel shows sections from a different animal. Transplants were performed 3 weeks after contusion injury. All images show the distribution of GFP-labelled hESC-MSCs (green). In addition, panel A also shows ED1 (red) and GFAP (blue) immunolabelling, panel B shows Nestin (red) and GFAP (blue) immunolabelling, panel C shows CASPR (blue) and NF 200 (red) immunolabelling and panel D shows NF-200 (red) and GFAP (blue) immunolabelling. The micrographs are projections of 7-9 optical sections and composed from multiple x20 fields of view. All scale bars are 1 mm.

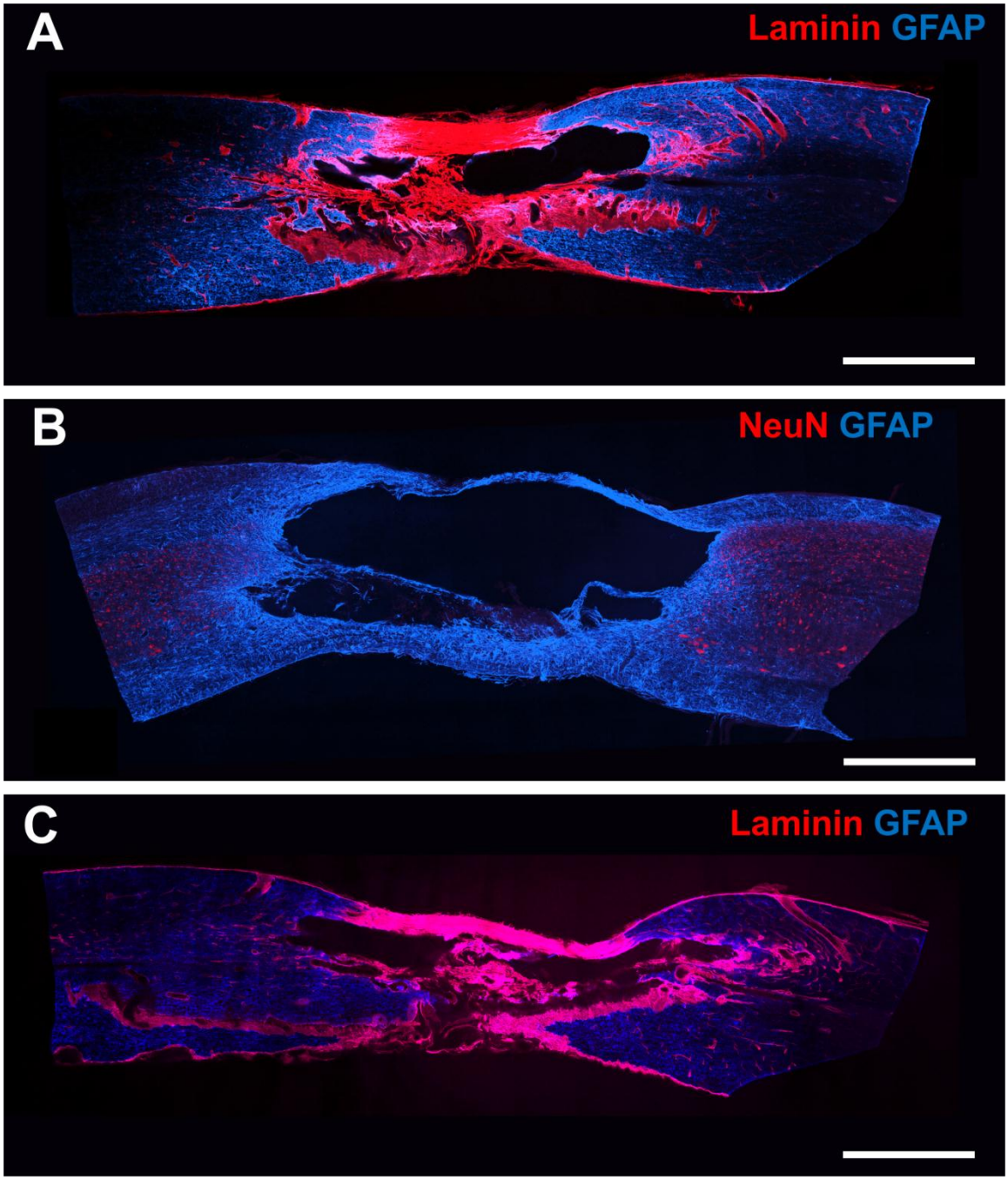

Figure 3-19. Examples of injury site histology from medium injected control animals.

The panels show confocal microscope images of the injury site 7 weeks after medium injections (10 weeks after injury). Each panel shows sections from a different animal. Medium injections were performed 3 weeks after contusion injury. Panel A shows GFAP (blue) and laminin (red) immunolabelling, panel B shows NenN (red) and GFAP (blue) immunolabelling and panel C shows Laminin (red) and GFAP (blue) immunolabelling. The micrographs are projections of 7-9 optical sections and composed from multiple x20 fields of view. All scale bars are 1 mm.

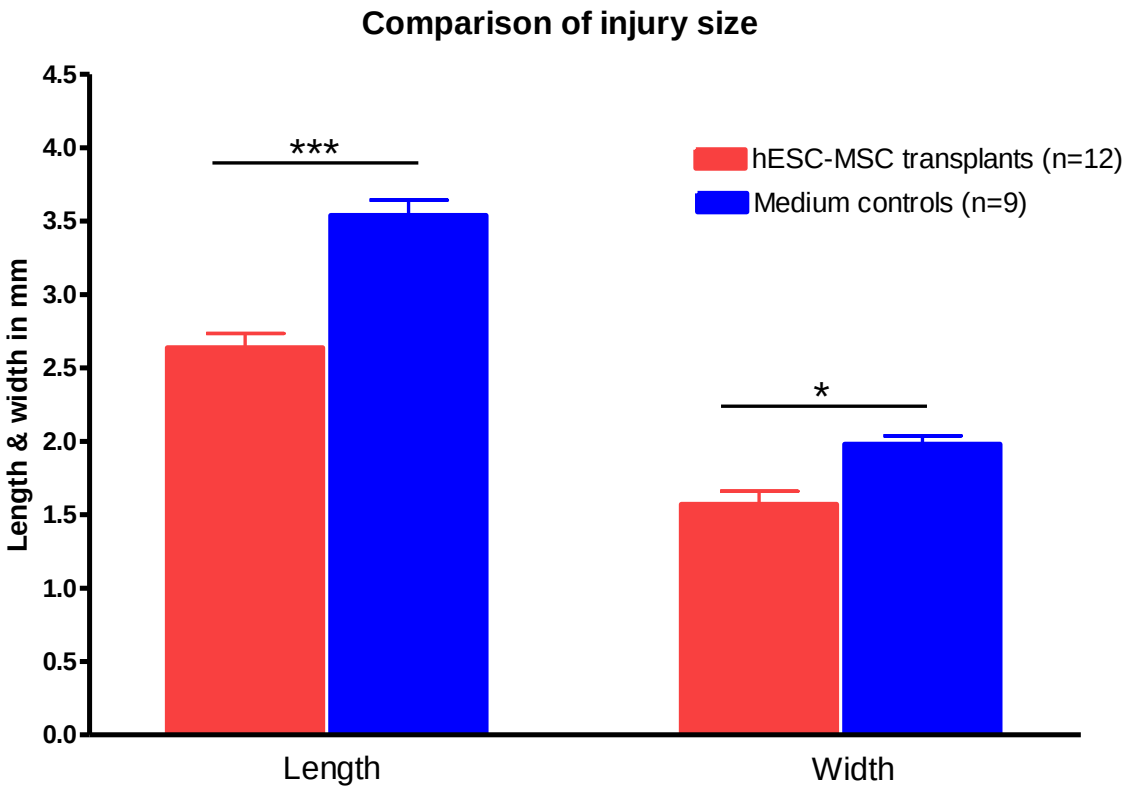

Figure 3-20. Comparison of injury dimensions in transplanted and control animals.

The bar plot shows the total rostro-caudal (length) and lateral (width) extent of the injury site for hESC-MSCs transplanted (red) and medium injected control (blue) groups of animals. The injury site in transplanted animals was estimated with GFAP (glial scar) and GFP (transplanted cells) immunoreactivity. The injury site in medium injection animals was determined from the edge of the cavity which formed in these animals. Injury site length was measured directly from histological section while the width was estimated from the number of 60 µm thick sagittal sections containing injured tissue. The error bars represent the standard errors of the mean (Fujimoto et al.). Statistical analysis used was students t-test (* $p < 0.05$, *** $p < 0.001$).

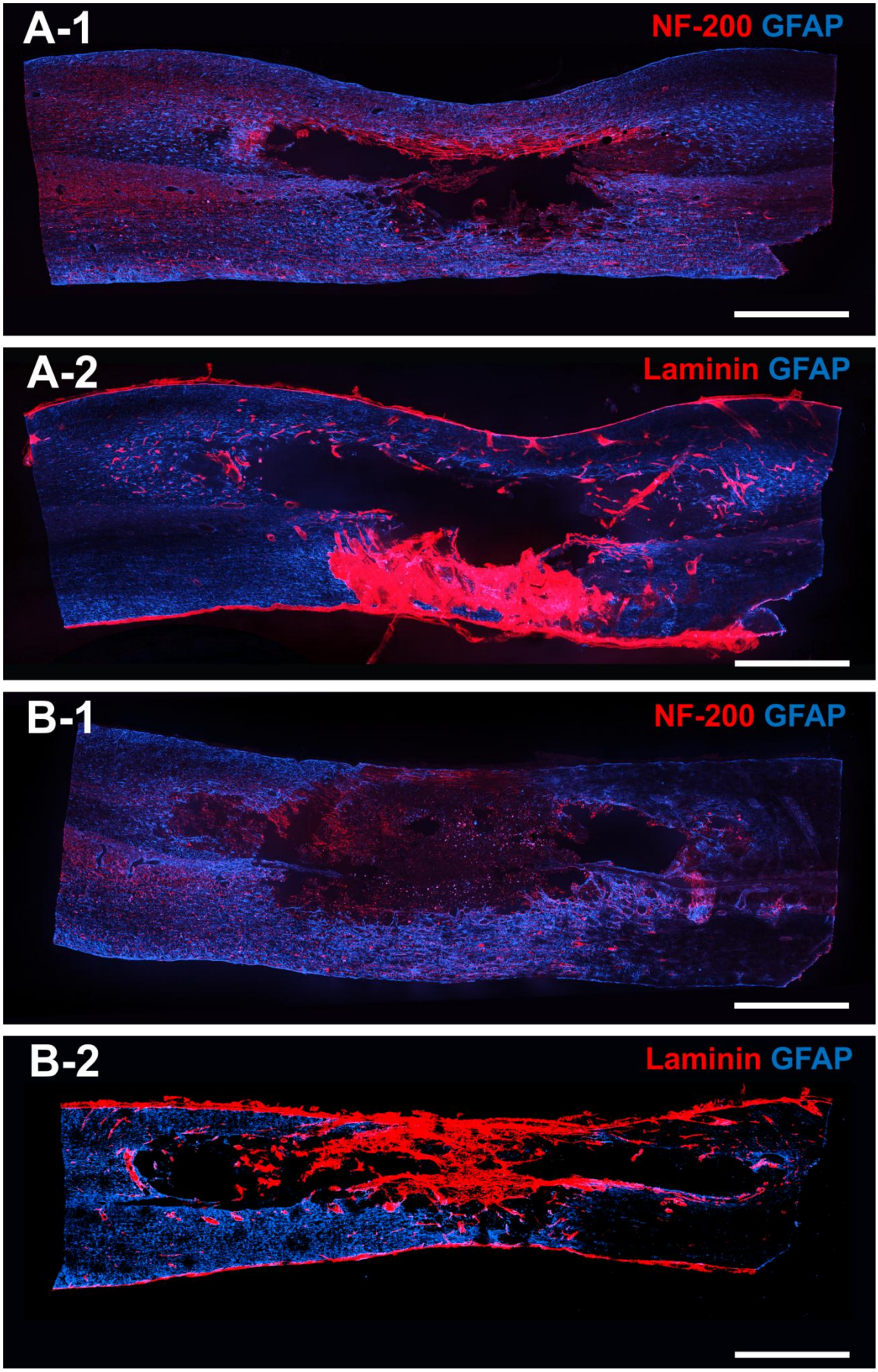

Figure 3-21. Examples of injury site histology at the time of sub-acute transplantations.

The panels show confocal microscope images of the injury site 5 days (A-1 and A-2) and 7 days (B-1 and B-2) after injury. Each panel is an example obtained from a parasagittal section passing through the injury area close to the midline. The top two panels (A-1 and A-2) show sections from animal perfused 5 days after injury and the bottom two panels (B-1 and B-2) show sections from animal perfused 7 days after injury. Panels A-1 and A-2 show sections from the same animal (5 day after injury), however, panels B1 and B-2 are from different animals (7 day after injury). All images show the distribution of GFAP immunolabelling (blue). A-1 and B-1 show NF 200 immunolabelling in addition while A-2 and B-2 show laminin distribution. The micrographs are projections of 7-9 optical sections and composed from multiple x20 fields of view. All scale bars are 1 mm.

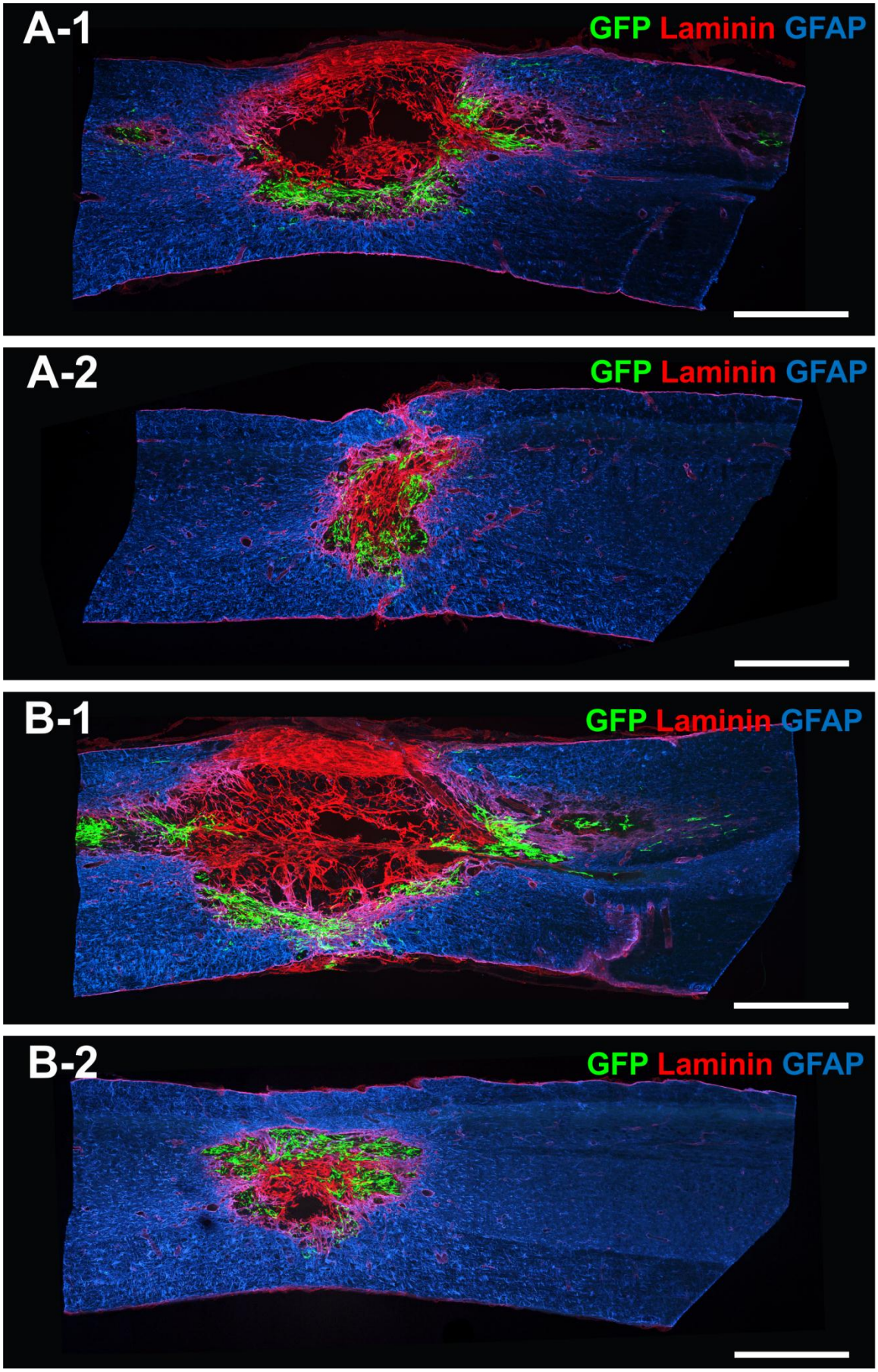

Figure 3-22. Examples of injury site histology from animals receiving sub-acute transplants (5 days after injury) of hESC-MSCs.

The panels show confocal microscope images of the injury site 3 weeks in a sub-acute transplant (5 days) of hESC-MSCs. The top two panels (A-1 and A-2) show sections from one animal and the bottom two panels (B-1 and B-2) show sections from a second animal. All images show the distribution of GFP immunolabelling (green), GFAP (blue) and Laminin (red). A-1 and B-1 are examples of injury site with cells from a parasagittal section close to the midline while A-2 and B-2 are examples of injury site with cells from the lateral part of the injury. The micrographs are projections of 7-9 optical sections and composed from multiple x20 fields of view. All scale bars are 1 mm.

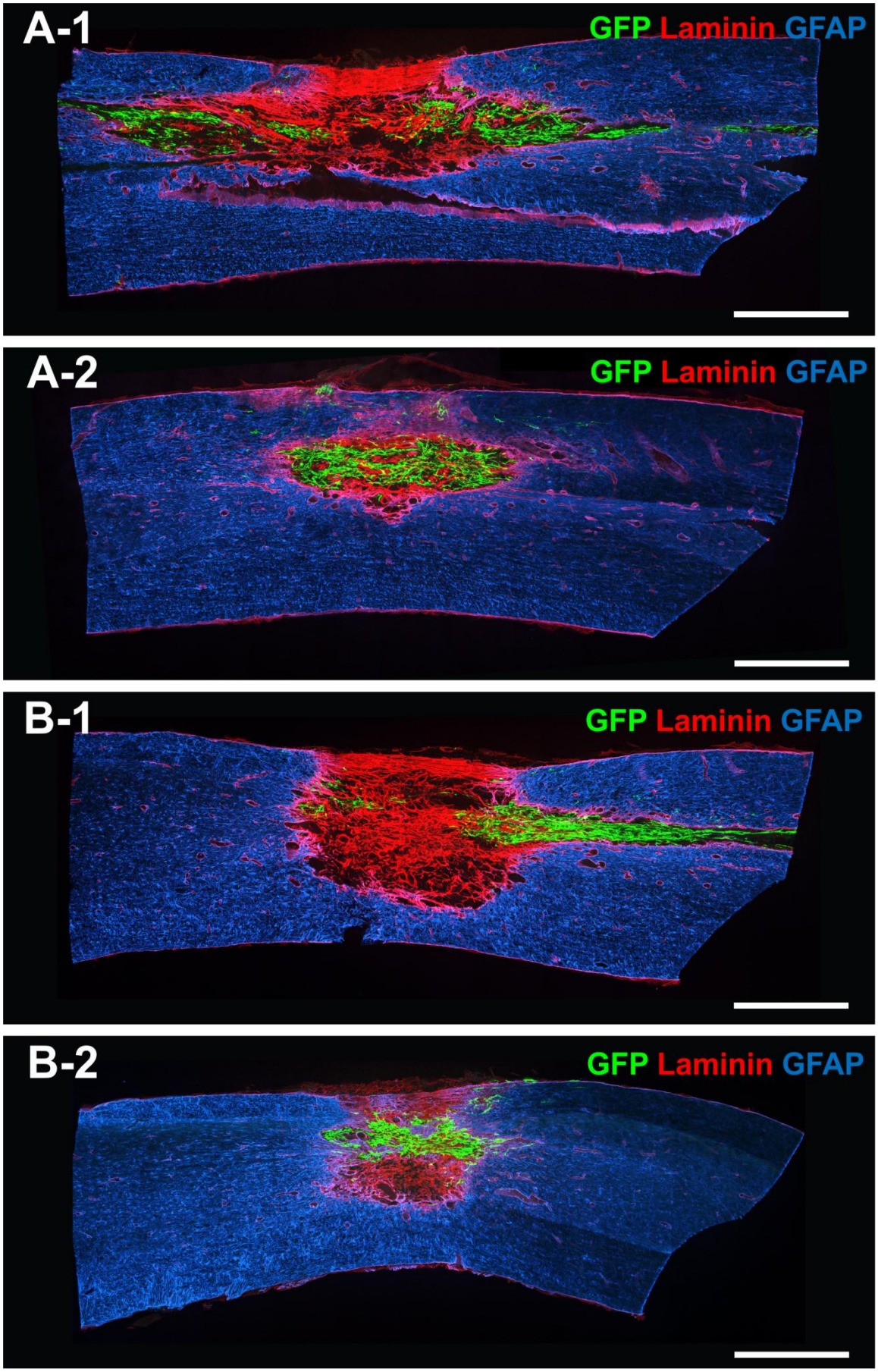

Figure 3-23. Examples of injury site histology from animals receiving sub-acute transplants (7 days after injury) of hESC-MSCs.

The panels show confocal microscope images of the injury site 3 weeks after sub-acute transplants (7 days) of hESC-MSCs. The top two panels (A-1 and A-2) show sections from one animal and the bottom two panels (B-1 and B-2) show sections from a second animal. All images show the distribution of GFP immunolabelling (green), GFAP (blue) and Laminin (red). A-1 and B-1 are examples of injury site with cells from a parasagittal section close to the midline while A-2 and B-2 are examples of injury site with cells from the lateral part of the injury. The micrographs are projections of 7-9 optical sections and composed from multiple x20 fields of view. All scale bars are 1 mm.

3.5 Discussion

3.5.1 Rationale for using hESC-MSCs in SCI

Mesenchymal stem cells (MSCs) from different sources have been reported to promote functional recovery in SCI. As well as the sources described previously MSCs can also be obtained from hESCs and have been successfully derived in our lab from a hESC line (Mohamad, 2014). This process of producing MSCs from hESCs was repeated several times and the quality of cells obtained were comparable within different sets of experiments, confirming these to be of consistent reproducibility. In addition, hESC-MSCs resembled adult bone marrow derived MSCs according to several criteria including morphology, plastic adherence, cell surface markers, gene expression and their ability to differentiate into osteoblasts and adipocytes. It has also been demonstrated that cryoprotection has minimal effect on the properties of these cells. The ability to generate a large amount of cells with a similar reproducible phenotype and minimal effect after cryoprotection on phenotypes indicates that these cells could be used as an “off the shelf” product for therapeutic interventions.

These cells offer several advantages over adult sources as they can be prepared in larger quantities with more consistency than MSCs obtained from bone marrow and adipose tissues. Cells obtained from patients have been shown to vary in consistency, quality and therapeutic potential (Minaire et al., 1984, Wright et al., 2008). In addition, hESC-MSCs can be produced in large amounts without the need for an invasive procedure such as that required to obtain MSCs from the bone marrow. Furthermore, hESC-MSCs have been transplanted in animal models of SCI in our lab and have been shown to promote anatomical aspects of repair as already discussed. These morphological features of repair indicate that these cells might be able to improve function after SCI and we therefore transplanted them into a clinically relevant model to assess their therapeutic potentials using functional outcome measures.

3.5.2 Injury model and dosing of hESC-MSCs

The contusion injury model is considered to be a clinically relevant model and human SCI resulting from trauma is thought to show many of the same pathological mechanisms. Most SCIs now occur at the cervical level of the spinal

cord (Anderson, 2004). It is difficult to compare the time course of SCI in rodents to that in human clinical scenarios in terms of what should be considered an acute and what constitutes a chronic time point. However, in this study we have chosen a 3 week time point and consider this to represent a time point in the rodent model and comparable to a chronic injury in humans. Part of the justification for this is that findings from chapter 2 showed that function at the level of the spinal cord stabilizes by 2 weeks and a time point beyond 2 weeks might therefore be regarded as chronic. In clinical settings it will be difficult to intervene with any cellular therapy early after the injury due to associated co-morbid injuries and it will be an advantage to delay transplantation in order to increase the survival of the transplanted cells by avoiding the period when the injury environment is most inhospitable in part due to the host immune response. It is therefore more feasible and advantageous to intervene at a chronic time point when the injury milieu has stabilised. It has been reported that transplantation performed sub-acutely is associated with better cell survival and improved functional outcome (Ankeny et al., 2004) and improvements in functional outcome have been reported in studies where transplantation was performed 3 months after contusion injury in rodents (Vaquero et al., 2006).

Pal *et al*. (2010) found in a pre-clinical study of MSCs transplanted into a contusion injury that functional outcome is dose dependant, demonstrating better results in MSC transplants with larger numbers of injected cells (5 million cells/kg) compared to a smaller number of cells (2 million cells/kg) (Pal et al., 2010). There is a large degree of biological variability in the morphology of injury cavities formed after contusion injuries. In part this is due to infilling by extracellular matrix which varies from animal to animal. In addition, previous studies of hESC-MSCs transplanted *in vivo* showed that they do not proliferate after transplantation. The variability in cavity formation combined with the lack of proliferation of the transplanted cells makes it difficult to optimise the dose of transplantations for all animals. In order to overcome this problem we used the approach of completely filling the injury cavity with cells. hESC-MSCs were injected into the injury cavity, until the cells were seen to overflow from the injection site. On average we would inject about 40 µl of cell suspension which would be around 8 million cells (200,000 cells/µl of suspension), into each animal. However, pressure injections of large volumes of cells in SCI models may

have a damaging effect because tissue tolerance may be exceeded by raised intraparenchymal pressure leading to hydrodynamic dissection and possible cord ischemia (Guest et al., 2011). We therefore observed the spinal cord during injection to see that there were no obvious signs of swelling or of reduced blood flow through pial blood vessels. The amount of cells used in our study represent quite a high dose compared to those used by others (e.g. 0.3 million cells; (Ankeny et al., 2004), 0.25 million cells; (Chopp et al., 2000), although these examples are for thoracic contusion injuries where cavities are probably smaller.

3.5.3 Interpretation of behavioural assessments

The first aim of the experiments reported in this chapter was to evaluate the functional outcome in terms of the behavioural assessment and correlate it with the electrophysiological findings. Previous transplantation studies have most commonly been carried out on thoracic injury models and most of the behavioural methods in use assess hindlimb function. Methods like the BBB scoring and open field locomotion are well recognised and widely used. In the case of cervical injuries and forelimb function assessments such as the forelimb locomotor scale (FLS) can be used as an alternative to BBB tests. It consists of an 18 point scale from 0 to 17, 0 representing no movement of the forelimb and 17 indicating continuous plantar stepping (Cao et al., 2008). However, previous findings in the lab using the cervical injury model suggested that rats recovered to pre-injury FLS scores within 3 weeks and therefore that this test does not show persistent functional deficits and might not be useful in the long term assessment of therapeutic effects (Ahmed *et al*. unpublished data). There are other tests to assess forelimb function such as pellet retrieval test (Metz and Whishaw, 2000), cylinder test (Liu et al., 1999) and sticker removal task (Schrimsher and Reier, 1992). However, the grip strength and ladder walk test provide a quantitative and convenient measure of the strength and co-ordination of the musculature of the digits and forelimbs. The grip strength and ladder walk test requires little expertise to perform and interpret the results. In addition, our injury model produces lesion of the main CST in the dorsal column and damage to CST has been reported to impair skilled motor movements such as the placing response and grasping movements of the distal forelimbs but does not affect the overall reaching ability of the proximal forelimb (Schrimsher and Reier, 1993, Whishaw et al., 1993). It has also been shown that when there is

damage to the main CST in the dorsal column there is no recovery of function in the digits (Schrimsher and Reier, 1992).

Grip strength measurement is one of the methods used in this study to assess the neuromuscular function of the forelimbs. It is a simple, reproducible and provides quantitative measure for evaluating flexor and extensor function of the digits. In addition, it measure muscle functions that are likely to be related to the corticospinal tract i.e. digital flexors (Anderson et al., 2005, Anderson et al., 2009b). Complete lateral hemisection injuries at the C5 segment are reported to cause a permanent loss of gripping and partial lesions sparing part of CST revealed residual gripping ability (Anderson et al., 2005). The compromised function is due to loss of descending input to motor neuron pools supplying the digital flexors of the forelimb which are located in C6 to T1 segment of the spinal cord (McKenna et al., 2000). In addition, the grip strength is highly relevant test as people living with cervical SCI report restoration of arm and hand function is of major importance to their daily activities (Anderson, 2004).

Animals sometimes develop spasticity in their forelimbs after injury with the forepaw often becoming stiff and held in flexed position. The animals can still passively hook onto the bar but the force generated is due to stiffness of the paw and does not show active gripping and therefore animals with these signs of spasticity were not included.

Results obtained from the grip strength test show that there is a significant decrease in gripping ability immediately after injury. A modest spontaneous recovery was observed afterwards but a significant deficit persists. A similar pattern of increase in gripping ability has been observed previously 2 weeks after cervical contusion injuries (Anderson et al., 2009b). Transplantation of hESC-MSCs did not show any further improvement in the gripping ability compared to controls suggesting no advantage of hESC-MSC transplants.

The horizontal ladder walking test is a sensitive test for evaluating sensory-motor co-ordination and the descending motor control of the limbs and can reveal deficits that are not obvious during normal locomotion or gait. The correct placement of the paw on the rungs, the weight bearing on each limb and forelimb-hindlimb co-ordination is required to successfully cross the ladder (Metz

and Whishaw, 2002, Webb and Muir, 2005, Metz and Whishaw, 2009). This task requires integrity of multiple systems including propiospinal, corticospinal, rubrospinal and dorsal column sensory fibres (Metz et al., 2000, Metz and Whishaw, 2002, Pearse et al., 2005). Our injury model shows complete axotomy of the main CST component in the dorsal column and the deficits or errors observed after injury might be mainly due to loss of CST control.

In this study an irregular rung pattern was used in order to make the task more demanding (Metz and Whishaw, 2009). It has been reported that gait velocity and stress can influence the outcome of the task and more errors can result when animals cross the ladder faster or without care and attention (Metz et al., 2000). Similar observations were made in our study where some animals crossed the ladder slowly and cautiously making few errors compared to those crossing faster. One strategy to overcome this problem in future studies would be to take into consideration the crossing approach while scoring the animals. Animals frequently used partial steps after injury and occasionally used the dorsal surface when the paw show signs of spasticity suggesting compensatory mechanisms in order to complete the task. Similar compensatory strategies have been reported with the use of wrist and forearm in the ladder walk test in severe cervical contusion injuries (Soblosky et al., 2001).

There was a significant increase in the forelimb errors after injury with modest improvement over time and the hindlimbs were not affected by injury. There was also loss of forelimb/hindlimb co-ordination with an increase in forelimb stepping frequency and crossing time. Transplantation of hESC-MSCs did not promote any improvement in comparison to controls throughout the assessment period. Similar observations are reported for the ladder walk test for hindlimb analysis after a thoracic contusion injury where MSC transplantation showed no improvement in comparison to controls (Himes et al., 2006). In contrast, there was improvement in hindlimb function revealed by the ladder test after transplantation of human bone marrow derived MSCs in a thoracic injury model but this was not accompanied by improvement in BBB scores (Park et al., 2010b).

3.5.4 Interpretation of electrophysiology assessment

The other aim of the experiments reported in this chapter was to test whether delayed hESC-MSC transplants lead to enhancement of corticospinal and radial nerve evoked CDPs after SCI. That is, to see if the presence of hESC-MSCs within the injury cavity can improve function at the injury epicentre and vicinity of the injured spinal cord by inducing plasticity of the spared fibre tracts and therefore lead to the improvements of CDP amplitudes in the transplanted animals compared to controls. However, the results obtained suggest that the presence of hESC-MSCs had no detectable effect on the amplitudes of CDPs in comparison to the control animals. These observations suggest that hESC-MSCs were not able to promote plasticity and/or improvement of function in sensorimotor pathways. In contrast, a reduction in the amplitudes of the CST evoked CDPs was observed in the transplanted animals suggesting a worsening of function compared to control animals.

There is a possibility that the electrophysiological assessment method is not sensitive enough to detect improvements in function. This seems unlikely because findings in the previous chapter showed that this technique is able to detect minor changes in amplitude of radial nerve evoked CDPs late in the course of the injury (i.e. reduction in CDP amplitudes below the level in the 6 month survival animals) and also evidence of plasticity in the minor component of the CST. This suggests that this method is sensitive enough to detect minor changes in the circuitry at the level of the spinal cord. Furthermore, enhancements in CDP amplitudes after OEC transplants have previously been reported after bilateral dorsal column lesions at the lumbar level (Toft et al., 2007). It is therefore reasonable to assume that if there was hESC-MSCs induced plasticity it would have been detected with the electrophysiological approach used.

3.5.5 Potential reasons for absence of functionally detectable improvements after hESC-MSC transplants

One possibility that might explain the current findings is that cells might not survive in sufficient numbers to promote robust plasticity in order to compensate for the functional deficit due to tissue loss in the grey and white

matter. However, this seems unlikely since we found that considerable numbers of the transplanted cells survived 7 weeks after transplantation. Although the number of transplanted cells remaining were not quantified, GFP-labelled cells were present in all the transplanted animals in the injury site. Furthermore, functional improvements have been reported with only 15 % of the transplanted MSCs surviving in a thoracic contusion injury 3 weeks after acute transplantation (Wu et al., 2003) and 1 % survival with improved function is reported in a compression injury model in mice (Boido et al., 2014).

To improve cell survival and make the host tissue more receptive to cells immunosuppression was given by administering cyclosporine daily by injection throughout the course of the study, starting 2 days prior to the transplantation of cells. Previous observations in the lab showed that without immunosuppression transplanted cells die within days of transplantation. However, a daily injection of 20 mg/kg body weight of cyclosporine significantly improved survival and was well tolerated by the animals (Mohamad, 2014). This is in agreement with reports that high doses of cyclosporine improve survival of transplanted neural stem progenitor cells after SCI (Parr et al., 2008a). However, immunosuppression might not be very efficient in longer studies as the number of transplanted cells surviving reduces with time (Mohamad, 2014); equally, long-term immunosuppression might not be desirable in clinical settings.

Transplantation of human cells into animal model (xenografting) may well exacerbate problems relating to graft survival. One possible strategy to avoid this could be the use of nude rats i.e. rats that are genetically altered so they do not express full complement of immune cells. This approach has been used in other xenotransplantation studies for example human MSCs (Hodgetts et al., 2013), glial restricted-progenitors (Jin et al., 2011) and OECs (Gorrie et al., 2010) transplantations in immunocompromised rats models of SCI. Because of the reduced immune response survival of the cells is generally good. However, the disadvantage of this approach is that it is less translationally relevant because clinical use of these cells will invariably require immunosuppressive treatment. In addition, to carry out behavioural testing in animals that are immunocompromised and should be maintained in a sterile environment.

As large cavities are already present at the time of transplantations there is also a possibility that transplanted cells present in the centre of the cavity are not able to get enough nutrients and metabolic support to survive, and die off in the course of time.

Because Cyclosporine administration has been shown to offer neuroprotective effects (Ibarra and Diaz-Ruiz, 2006) together with the enhancement of function in SCI models (McMahon et al., 2009), to be sure that any improvement of function could be attributed to the transplanted cells the same dose of cyclosporine was also administered to the control animals.

The timing of transplantation after injury also appears to have a crucial role in determining functional outcome as the neuroprotective and immune modulatory actions of MSC transplants may be of greatest benefit in the early stages after injury. There have been several studies reporting to improved function when MSC transplantations were performed either immediately or with shorter delay after injury (Chopp et al., 2000, Hofstetter et al., 2002, Cizkova et al., 2006b, Dasari et al., 2007). Therefore, it is a possibility that in chronic injuries the neuroprotective actions are less effective and that this is one of the reasons for lack of functional improvement in this study with transplants performed 3 weeks after injury. However, no direct association between locomotor recovery and the timing of MSC transplants has been observed in a meta-analysis of animal studies reported by (Oliveri et al., 2014). Osaka and colleagues (2010) studied the effects of i.v. administration of MSCs at different time points on functional outcome and reported significant improvements with injections of cells performed in the first 3 days after contusion injury (Osaka et al., 2010). Despite promising results in the use of MSCs to treat acute SCI, there are fewer studies in chronic injury models. Zurita *et al.* (2006) have reported some degree of functional improvement in paraplegic rats after administration of MSCs 3 months after thoracic contusion injury. They also claimed differentiation of transplanted MSCs into neural tissue one year after transplantation (Zurita and Vaquero, 2006).

It has been reported that MSCs promote functional improvement by mechanisms other than axonal regeneration and proliferation, such as improved blood supply, secretion of trophic factors that promote plasticity, remyelination and

immunomodulation of the injury (reviewed by (Forostyak et al., 2013). Observations in our lab have shown that hESC-MSCs, like other sources of MSCs, promote laminin production, myelination and angiogenesis at the injury site. However they are not able to promote bridging axonal regeneration and there is no evidence of proliferation or differentiation (Mohamad, 2014). In this study we have also not observed that these cells promote plasticity. In addition, neuroprotective mechanisms offered by transplanted cells are likely to decrease with time after injury and the therapeutic window for ameliorating the consequences of injury may be very narrow. In this study transplants were performed 3 weeks after injury and findings from previous chapter show there is no secondary damage and injury has stabilized, therefore neuroprotection is unlikely to be beneficial in this model of SCI. ECM rich in laminin at the injury site provides a good substrate for axonal regeneration and improved blood supply through angiogenesis may be important in survival of the transplanted cells but may not be enough to promote in functional improvement. Similarly, the immune response after trauma develops in first few days and anti-immune effects of MSCs would less likely benefit at this stage.

3.5.6 Are MSCs derived from hESC different from other sources of MSCs?

The International Society for Cellular Therapy (ISCT) has proposed three minimal criteria to define MSCs. These include plastic adherence, *in vitro* differentiation potential, the presence of CD73, CD90 and CD105 surface markers and absence of haematopoietic antigens such as CD14, CD34, CD45 or CD11b, CD79α or CD19 and HLA class II (Dominici et al., 2006). The hESC derived MSCs used in this study shown to meet the criteria of plastic adherence and in vitro differentiation into osteoblasts and adipocytes. They were also shown to express most of the markers described for other sources of MSCs. The hESC-MSCs were 70-90 % positive for the majority of the proposed markers i.e. CD44, CD73, CD105, CD166 and HLA-ABC but relatively low for CD90 (Mohamad, 2014). The *in vitro* analysis of these cells also showed decreased transcription of genes for pluripotency specifically expressed in hESCs (*OCT4* and *NANOG*) as differentiation of the cells into mesenchymal cells progressed. In addition, *COL1A1, Twist1* and *CD105* gene expression was upregulated which supports the commitment towards a mesenchymal lineage (Mohamad, 2014). The surface markers, gene expression

and *in vitro* differentiation all indicate that hESC-MSCs are very similar to MSCs derived from other sources and therefore it is reasonable to assume that they would behave similarly *in vivo*. However, secretome of the hESC-MSCs used in this study has not been fully investigated. It is possible that the cells fail to secrete molecules that are critical for the beneficial effects which some reports have claimed for MSCs or that they secrete factors which have a negative effect.

The transplanted cells have been shown to displace the resident glial cells in areas where cells have tracked outside the injury site. Similar observations have been made with bone marrow MSCs, OECs and Schwann cell transplantations (Lu et al., 2006, Toft et al., 2013). This intense reactivity to the host tissue is also observed when hESC-MSCs are transplanted into normal cords (Mohamad, 2014). This undesirable property of transplanted cells may be another possibility for the poor functional outcome in this study.

3.5.7 Transplanted cells fill the injury site and reduce the extent of injury

Animals transplanted with the hESC-MSCs showed solid filling of the lesion area in contrast to the medium injected control animals which showed extensive cavitation and tissue disruption. Solid infilling of the injury site was a consistent feature in all the transplanted animals and the injury area was occupied by a mixture of GFP labelled cells and endogenous matrix. hESC-MSC transplantation has been shown to encourage production of ECM rich in laminin throughout the injury site and profuse blood vessel formation in the area surrounding the injury site (Mohamad, 2014).

In addition, assessment of the extent of the injury by calculating injury size from the margins of the glial scar around the area filled with cells in transplanted animals and comparing this to the size of the cavity in controls indicated a reduction in injury size in transplanted animals at 10 weeks. Reduction in the cavity formation is in accordance with other studies using adult sources of MSCs (Wu et al., 2003, Ankeny et al., 2004) although in these studies transplants were done after a shorter delay after injury as compared to 3 weeks in this study. However, reduction of the injury size and prevention of cavity formation with

secretion of ECM rich in laminin, appears not to be sufficient alone to improve functional outcome.

We considered the possibility that a chronic injury may not be amenable to the therapeutic effects of hESC-MSC transplantations. We therefore investigated the feasibility of transplantations after a shorter delay. Results revealed cell survival, migration of transplanted cells, lack of cavities and filling of the injury predominantly with laminin. Survival of cells was found mostly at the margins of the injury site which suggest that cells at the core may die off due to lack of adequate metabolic support. Furthermore, absence of host astrocytes in areas where transplanted cells have tracked suggest an undesirable effect on the host tissue. This effect has also been observed after injection of hESC-MSCs in normal cords (Mohamad, 2014). These findings suggest hESC-MSC transplants performed earlier after SCI are feasible but may not have any added advantage in terms of cell survival and tissue preservation compared to 3 week delayed transplants.

ED1 immunolabelling was not used systematically but in the example (Figure 3-18) it provided clear evidence of luminous macrophages in the vicinity of the injury. These may be involved in phagocytosis of cellular debris from ongoing death of transplanted cells.

3.5.8 Comparison with previous studies

It is difficult to make direct comparisons with our behavioural observations as there are no previous studies assessing the effects of MSCs in cervical contusion injuries. However, several studies have reported investigation of MSCs in thoracic contusion and compression injury models. Recently a systematic review with meta-analysis suggested that MSC transplantations have a substantial beneficial effect on locomotor recovery in rat SCI models (Oliveri et al., 2014). In addition, there has not been any study reporting any detrimental effects with administration of MSCs and no correlation has been reported between locomotor recovery and the timing of administration of MSCs (Oliveri et al., 2014). Furthermore, there are studies which correspond with the results reported in this chapter indicating that MSC transplants can reduce cavity formation but without any corresponding improvement in the functional outcome (Ankeny et

al., 2004, Sheth et al., 2008) or only transient improvements with no consistent long term benefits compared to controls (Himes et al., 2006).

Pre-clinical investigations have used a variety of MSCs from different sources using different purification protocols and routes of administration and this may therefore explain some of the vastly different functional outcomes reported. In addition, the injury level and severity along with behavioural assessments are not standardized for and this heterogeneity between studies makes direct comparison difficult. All studies have demonstrated that MSC transplantation is safe without any detrimental effect regardless of recovery of function. However, there is a lack of studies using cervical level injury and this complicates the translation of these results into human SCI where traumatic injuries at the cervical level are most common. There is therefore need as for further evaluation of MSCs as a therapeutic intervention using cervical contusion injury models.

This study had been designed to investigate the therapeutic potential of hESC-MSCs in a clinically relevant model with the aim of having an off the shelf cellular transplant strategy for the treatment of SCI. This is the first time these cells have been used *in vivo* to assess functional outcome measures although, no functional improvements are observed with transplantation of hESC-MSCs. Further investigation with the aim of exploring the *in vitro* and *in vivo* trophic factor secreting properties of these cells would be desirable to better understand the potential therapeutic value of these cells.

4 General discussion and future directions

4.1 General discussion

4.1.1 Electrophysiological approaches for investigating function after spinal cord injury

In this project we have demonstrated that CDP recording can provide a sensitive method to detect changes within the grey matter of the spinal cord. We have used this approach to provide for the first time complete and detailed information on the time course of functional changes in circuits of the grey matter of the spinal cord after a contusion injury.

The work in the first part of the thesis provides an accurate picture of the temporal progression of functional changes in the spinal cord starting immediately after contusion injury and at different time points up to 6 months. Other electrophysiological techniques such as field potential recordings could be used to further explore functional changes in the spinal cord. However, recording of field potential may be difficult when there is cavity formation as observed after contusion injury.

In vivo electrophysiology is the only way to provide direct information on what is happening in the local circuits and pathways in the spinal cord after injury and treatments in experimental models of SCI. It has been demonstrated in this project that with this approach it is possible to observe changes in the sensory pathways after stimulation of peripheral nerves and the descending corticospinal system in the vicinity of a SCI. Other methods like behavioural testing can provide evidence of improved functional performance in the ability of the animal to fulfil certain sensorimotor tasks. However, the improvement of behavioural function cannot provide information on whether improvement occurs due to compensatory changes at supraspinal or spinal levels. Furthermore, most studies combine behavioural testing with anatomical assessments and draw conclusions based on correlation between these two approaches whether function improves or worsens after SCI and/or therapeutic strategy. However, an electrophysiological approach has the advantage that it can evaluate the function of specific pathways and changes in the functional connections within the target pathway. Hence *in vivo* electrophysiology is the

only method that can provide information on changes in function at the spinal cord level.

4.1.2 Limitation of electrophysiological approach

A limitation of the electrophysiological approach is that it can only be used to evaluate the function of certain pathways. This is because the pathways to be investigated need to be in a relatively accessible anatomical location. In addition, only certain pathways can be maximally activated which is essential in order to make quantitative comparisons among different groups of animals. In rats the corticospinal and the dorsal column pathways, which have been used in this thesis, are the most convenient for study as it is possible to stimulate them selectively and record the synaptic activity generated by these pathways in the form of CDPs.

Another disadvantage of the electrophysiological assessment approach is that it is not able to differentiate between strengthened connections that could be functionally and those that could have a detrimental effect. Plasticity in certain pathways might be beneficial and lead to improvement of function or maladaptive leading to problems such as pain or autonomic dysfunction. Therefore behavioural assessments are desirable along with electrophysiology to correlate the electrophysiological changes with behavioural outcomes. However, it is difficult to make specific correlations between changes in a single pathway like the corticospinal tract and behavioural outcomes as behaviour depends on the interaction of many different systems in the brain and spinal cord. These problems are not likely to impact on assessing contributions of primary and secondary damage and neuroprotective action (i.e. preservation of existing connection) but interpretation of the functional significance of plasticity is difficult with electrophysiology alone.

Another limitation of *in vivo* electrophysiological technique is that it requires immense expertise and extensive personnel training in order to conduct these experiments consistently. Similarly, expertise is needed for the interpretation of the recordings and dealing with any technical issues that arise during the experiments. The cost, time and sacrifice of large number of animals required to reach an adequate level of competency are issues in this kind of work.

4.1.3 Future directions

This thesis has provided useful information on using an *in vivo* electrophysiological approach to assess function in the grey matter after a contusion injury due to a high velocity impact to the cervical spinal cord. A similar approach has been used in animals with a dorsal column lesion to assess the effects of OEC transplants after SCI (Toft et al., 2007). However, so far there have been no investigations of the effects on the local circuit function of a compression or crush injury. The same electrophysiological method could provide valuable information on functional changes over time in grey matter in the vicinity of these injury models. It has previously been reported that there is gradual neuronal loss after compression injury rostral and caudal to the injury centre (Huang et al., 2007). Neuronal loss assessed by NeuN immunostaining at the core of the injury increased to 73 % at 3 days compared to 44 % 1 day after the compression injury (Huang et al., 2007). In contrast, neuronal loss is not evident after 24 hours in contusion injury models (Crowe et al., 1997, Liu et al., 1997). This suggests that secondary processes may be more pronounced in compression injury compared to the contusion injury model where the higher velocity impact may lead to greater neuronal loss at the time of injury. It could be valuable to know this for testing neuroprotective strategies and has implications for understanding how secondary damage caused in compression injury could be diminished.

As discussed previously in Chapter 2 deterioration of function in the local sensory system was observed at 3 days in fibres above the injury which is possibly due to demyelination of the parent axons past the injury. Furthermore, restoration of function has been observed over the same locations in 2 week survival animals attributed to spontaneous remyelination of the demyelinated axons. The process of demyelination and remyelination of the sensory axons have not been confirmed using anatomical techniques. Confirmatory evidence for these findings can be obtained with electron microscopy in future studies. In addition, electron microscopic investigation will provide information on the proportion of demyelinated, normally myelinated and remyelinated axons after injury confirming and evaluate the extent of damage and spontaneous repair to the sensory axons. Also information on peripheral versus central type myelin that lead to recovery of function in the affected axons can be obtained.

4.1.4 hESC derived MSCs as a potential therapy for SCI

There are numerous reports that transplants of mesenchymal stem cells derived from various sources have produced a positive outcome in pre-clinical models of SCI. MSCs derived from a hESC line resemble MSCs derived from other sources and offer several advantages. Firstly, hESC-MSCs can be expanded efficiently to produce large volumes of cells with a consistent phenotype. Secondly, cryopreservation has been shown minimal effect on the properties of these cells. Also cryopreserved cells would be easier to transport compared to cells in culture. Thirdly, with the use of these cells invasive procedures to obtain cells from the donors or patients themselves can be avoided. In addition, it provides an opportunity for an "off the shelf" product for early therapeutic interventions in SCI and other neurological conditions. However, as observed in this thesis delayed transplantation of hESC-MSCs in a contusion injury model did not lead to functional improvements and as discussed previously in Chapter 3, there are several potential reasons for these findings. However, the most likely of these is that the majority of previous studies were of the effects of transplants performed acutely or within a relatively short time (a few days) after SCI. The aim of a cellular transplant therapy for chronic stage injuries would be different from acute SCI and may necessitate reconstruction of the injured spinal cord via cellular replacement, glial scar modification, functional axonal regeneration and filling of the cavities that may form. Furthermore, SCI is a multifaceted problem and it is anticipated that a combination therapy will be a more effective way to promote recovery than a single therapy alone.

In fact, cellular transplants or even a mixture of cell therapies alone are not capable of reversing the consequences of SCI to any significant degree (Silva et al., 2014). Although, engineered cells might help, to achieve axonal regeneration a number of different strategies will need to be applied in combination. Lu et al. (2012) reported that combinatorial treatment can support axonal regeneration of the reticulospinal tract into and beyond either a partial cervical hemisection or complete thoracic spinal transection. They performed injections of cAMP into the reticular formation to stimulate endogenous growth of reticulospinal fibres. Secondly, they grafted bone marrow stromal cells into the lesion sites to provide a permissive bridge for axonal growth and thirdly, brain derived neurotrophic factor was applied to the spinal cord caudal to the

injury site in order to stimulate distal growth of motor axons. The severed reticulospinal fibres regenerated through the injury and also formed synapses on motor neuron below the lesion. Despite functional synapses below the lesion the behavioural outcome worsened in the treated animals (Lu et al., 2012). This highlights the complexity of reconstructing the injured spinal cord. Although with combinatorial therapy it is possible to achieve functional axonal regeneration a desirable functional outcome can only be achieved with formation of appropriate connections between regenerating axons and target neurons. Therefore looking at the complex nature of the SCI pathophysiology a mixture of therapies needs to be applied in a timely sequence with optimum dosing and route of administration to achieve functional improvement. In addition, intensive rehabilitation therapy may enhance functional recovery. Further pre-clinical studies using combination therapies are needed in other models of SCI before translating these strategies into clinics.

Gene therapies hold great promise for the treatment of many neurodegenerative disorders and traumatic injuries in the CNS. Advancement in the field of genetic research in SCI might lead to the identification of genes and their biomolecular targets that have potential to induce robust improvements through axonal regeneration and formation of functional connection. Furthermore gene therapies may provide transgene expression for longer duration and sustained production of desired factors, for example, sustained release of BDNF, NT-3/4 etc. This will be advantageous over cellular therapy where cells die off with the passage of time and steady levels of desired factors (e.g. neurotrophins/growth factors) cannot be maintained. Another advantage of gene delivery is that multiple therapeutic genes can be delivered in tandem from the same delivery systems including vectors and biomaterials. In order to promote repair in SCI researchers are exploring gene delivery as a mechanism to reduce inflammation, decrease the glial scar, improve cell survival, promote axonal regeneration and plasticity and promote differentiation/engraftment of transplanted stem/progenitor cells (Walthers CM 2015). Gene therapy therefore provides another therapeutic approach along with the combinatorial therapies which can be translated into clinics and can be beneficial for SCI patients.

4.1.5 Conclusion

This thesis provides a valuable insight into the changes in function that occur in spinal cord circuits in the vicinity of a contusion injury. It also provides a useful cervical model of SCI together with electrophysiological assessment to test therapies. Based on our results we conclude that injury due to a high velocity impact as made by IHI device shows that maximal damage or functional loss occurs at the time of primary trauma and secondary mechanisms have a minimal role in this injury model. This has implication that therapeutic window for a therapy aiming at neuroprotection that does not provide functional axonal regeneration may be very small to ameliorate the damage caused by contusion injury. The finding in this thesis has not been previously taken into consideration and it would have implication in terms of translation of a particular therapy into clinics and assigning patients that would benefit from a specific treatment. For example the mechanics of the injury may play a role in determining the appropriateness of specific treatment. SCI patients with a high velocity impact injury may need a different therapy compared to those with a more pronounced compression injury. This consideration has not been taken into account in clinical trials and may be one of the reasons for failure of improvement of function in SCI patients. Furthermore, delayed hESC-MSC transplants were not shown to provide any advantage in improvement of functional outcome. In addition, single therapy might not be sufficient to overcome the multifaceted pathology of SCI.

List of References

Abrams MB, Dominguez C, Pernold K, Reger R, Wiesenfeld-Hallin Z, Olson L, Prockop D (2009) Multipotent mesenchymal stromal cells attenuate chronic inflammation and injury-induced sensitivity to mechanical stimuli in experimental spinal cord injury. Restorative neurology and neuroscience 27:307-321.

Afifi AK, Bergman RA (2005) Functional Neuroanatomy. United States of America: Lange Medical Books/McGraw-Hill, Medical Publishing Division.

Aguilar J, Humanes-Valera D, Alonso-Calvino E, Yague JG, Moxon KA, Oliviero A, Foffani G (2010) Spinal cord injury immediately changes the state of the brain. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:7528-7537.

Ahn H, Fehlings MG (2008) Prevention, identification, and treatment of perioperative spinal cord injury. Neurosurgical focus 25:E15.

Akiyama Y, Honmou O, Kato T, Uede T, Hashi K, Kocsis JD (2001) Transplantation of clonal neural precursor cells derived from adult human brain establishes functional peripheral myelin in the rat spinal cord. Experimental neurology 167:27-39.

Akiyama Y, Radtke C, Honmou O, Kocsis JD (2002) Remyelination of the spinal cord following intravenous delivery of bone marrow cells. Glia 39:229-236.

Alexanian AR, Fehlings MG, Zhang Z, Maiman DJ (2011a) Transplanted neurally modified bone marrow-derived mesenchymal stem cells promote tissue protection and locomotor recovery in spinal cord injured rats. Neurorehabilitation and neural repair 25:873-880.

Alexanian AR, Svendsen CN, Crowe MJ, Kurpad SN (2011b) Transplantation of human glial-restricted neural precursors into injured spinal cord promotes functional and sensory recovery without causing allodynia. Cytotherapy 13:61-68.

Almon RR, DuBois DC, Brandenburg EH, Shi W, Zhang S, Straubinger RM, Jusko WJ (2002) Pharmacodynamics and pharmacogenomics of diverse receptor-mediated effects of methylprednisolone in rats using microarray analysis. Journal of pharmacokinetics and pharmacodynamics 29:103-129.

Anderson KD (2004) Targeting recovery: priorities of the spinal cord-injured population. Journal of neurotrauma 21:1371-1383.

Anderson KD, Gunawan A, Steward O (2005) Quantitative assessment of forelimb motor function after cervical spinal cord injury in rats: relationship to the corticospinal tract. Experimental neurology 194:161-174.

Anderson KD, Sharp KG, Hofstadter M, Irvine KA, Murray M, Steward O (2009a) Forelimb locomotor assessment scale (FLAS): novel assessment of forelimb dysfunction after cervical spinal cord injury. Experimental neurology 220:23-33.

Anderson KD, Sharp KG, Steward O (2009b) Bilateral cervical contusion spinal cord injury in rats. Experimental neurology 220:9-22.

Andrews MR, Stelzner DJ (2004) Modification of the regenerative response of dorsal column axons by olfactory ensheathing cells or peripheral axotomy in adult rat. Experimental neurology 190:311-327.

Ankeny DP, McTigue DM, Jakeman LB (2004) Bone marrow transplants provide tissue protection and directional guidance for axons after contusive spinal cord injury in rats. Experimental neurology 190:17-31.

Anthes DL, Theriault E, Tator CH (1996) Ultrastructural evidence for arteriolar vasospasm after spinal cord trauma. Neurosurgery 39:804-814.

Aoki M, Fujito Y, Satomi H, Kurosawa Y, Kasaba T (1986) The possible role of collateral sprouting in the functional restitution of corticospinal connections after spinal hemisection. Neuroscience research 3:617-627.

Apparelyzed SCIPS (2013) Spinal Cord Injury Statistics.

Arishima Y, Setoguchi T, Yamaura I, Yone K, Komiya S (2006) Preventive effect of erythropoietin on spinal cord cell apoptosis following acute traumatic injury in rats. Spine 31:2432-2438.

Bach-y-Rita P, Illis LS (1993) Spinal shock: possible role of receptor plasticity and non synaptic transmission. Paraplegia 31:82-87.

Bakshi A, Barshinger AL, Swanger SA, Madhavani V, Shumsky JS, Neuhuber B, Fischer I (2006) Lumbar puncture delivery of bone marrow stromal cells in spinal cord contusion: a novel method for minimally invasive cell transplantation. Journal of neurotrauma 23:55-65.

Ballermann M, Fouad K (2006) Spontaneous locomotor recovery in spinal cord injured rats is accompanied by anatomical plasticity of reticulospinal fibers. The European journal of neuroscience 23:1988-1996.

Bambakidis NC, Miller RH (2004) Transplantation of oligodendrocyte precursors and sonic hedgehog results in improved function and white matter sparing

in the spinal cords of adult rats after contusion. The spine journal : official journal of the North American Spine Society 4:16-26.

Bao F, Liu D (2002) Peroxynitrite generated in the rat spinal cord induces neuron death and neurological deficits. Neuroscience 115:839-849.

Baptiste DC, Fehlings MG (2007) Update on the treatment of spinal cord injury. Progress in brain research 161:217-233.

Bareyre FM, Kerschensteiner M, Raineteau O, Mettenleiter TC, Weinmann O, Schwab ME (2004) The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats. Nature neuroscience 7:269-277.

Bareyre FM, Schwab ME (2003) Inflammation, degeneration and regeneration in the injured spinal cord: insights from DNA microarrays. Trends in neurosciences 26:555-563.

Basso DM (2004) Behavioral testing after spinal cord injury: congruities, complexities, and controversies. Journal of neurotrauma 21:395-404.

Basso DM, Beattie MS, Bresnahan JC (1995) A sensitive and reliable locomotor rating scale for open field testing in rats. Journal of neurotrauma 12:1-21.

Bazley FA, Hu C, Maybhate A, Pourmorteza A, Pashai N, Thakor NV, Kerr CL, All AH (2012) Electrophysiological evaluation of sensory and motor pathways after incomplete unilateral spinal cord contusion. Journal of neurosurgery Spine 16:414-423.

Beattie MS, Bresnahan JC, Komon J, Tovar CA, Van Meter M, Anderson DK, Faden AI, Hsu CY, Noble LJ, Salzman S, Young W (1997) Endogenous repair after spinal cord contusion injuries in the rat. Experimental neurology 148:453-463.

Beattie MS, Farooqui AA, Bresnahan JC (2000) Review of current evidence for apoptosis after spinal cord injury. Journal of neurotrauma 17:915-925.

Becerra JL, Puckett WR, Hiester ED, Quencer RM, Marcillo AE, Post MJ, Bunge RP (1995) MR-pathologic comparisons of wallerian degeneration in spinal cord injury. AJNR American journal of neuroradiology 16:125-133.

Behrmann DL, Bresnahan JC, Beattie MS, Shah BR (1992) Spinal cord injury produced by consistent mechanical displacement of the cord in rats: behavioral and histologic analysis. Journal of neurotrauma 9:197-217.

Bethea JR (2000) Spinal cord injury-induced inflammation: a dual-edged sword. Progress in brain research 128:33-42.

Bhanot Y, Rao S, Ghosh D, Balaraju S, Radhika CR, Satish Kumar KV (2011) Autologous mesenchymal stem cells in chronic spinal cord injury. British journal of neurosurgery 25:516-522.

Biernaskie J, Sparling JS, Liu J, Shannon CP, Plemel JR, Xie Y, Miller FD, Tetzlaff W (2007) Skin-derived precursors generate myelinating Schwann cells that promote remyelination and functional recovery after contusion spinal cord injury. Journal of Neuroscience 27:9545-9559.

Black P, Markowitz RS, Cooper V, Mechanic A, Kushner H, Damjanov I, Finkelstein SD, Wachs KC (1986) Models of spinal cord injury: Part 1. Static load technique. Neurosurgery 19:752-762.

Blight AR (1989) Effect of 4-aminopyridine on axonal conduction-block in chronic spinal cord injury. Brain research bulletin 22:47-52.

Blight AR (1991) Morphometric analysis of a model of spinal cord injury in guinea pigs, with behavioral evidence of delayed secondary pathology. Journal of the neurological sciences 103:156-171.

Blight AR (1992) Spinal cord injury models: neurophysiology. Journal of neurotrauma 9:147-149; discussion 149-150.

Blight AR, Young W (1989) Central axons in injured cat spinal cord recover electrophysiological function following remyelination by Schwann cells. Journal of the neurological sciences 91:15-34.

Boido M, Garbossa D, Fontanella M, Ducati A, Vercelli A (2014) Mesenchymal stem cell transplantation reduces glial cyst and improves functional outcome after spinal cord compression. World neurosurgery 81:183-190.

Bradbury EJ, Khemani S, Von R, King, Priestley JV, McMahon SB (1999) NT-3 promotes growth of lesioned adult rat sensory axons ascending in the dorsal columns of the spinal cord. The European journal of neuroscience 11:3873-3883.

Bradbury EJ, McMahon SB (2006) Spinal cord repair strategies: why do they work? Nature reviews Neuroscience 7:644-653.

Bradbury EJ, Moon LD, Popat RJ, King VR, Bennett GS, Patel PN, Fawcett JW, McMahon SB (2002) Chondroitinase ABC promotes functional recovery after spinal cord injury. Nature 416:636-640.

Bregman BS, McAtee M, Dai HN, Kuhn PL (1997) Neurotrophic factors increase axonal growth after spinal cord injury and transplantation in the adult rat. Experimental neurology 148:475-494.

Bresnahan JC, Beattie MS, Todd FD, 3rd, Noyes DH (1987) A behavioral and anatomical analysis of spinal cord injury produced by a feedback-controlled impaction device. Experimental neurology 95:548-570.

Bretzner F, Plemel JR, Liu J, Richter M, Roskams AJ, Tetzlaff W (2010) Combination of olfactory ensheathing cells with local versus systemic cAMP treatment after a cervical rubrospinal tract injury. Journal of neuroscience research 88:2833-2846.

Brines M, Cerami A (2008) Erythropoietin-mediated tissue protection: reducing collateral damage from the primary injury response. Journal of internal medicine 264:405-432.

Brosamle C, Schwab ME (1997) Cells of origin, course, and termination patterns of the ventral, uncrossed component of the mature rat corticospinal tract. The Journal of comparative neurology 386:293-303.

Brosamle C, Schwab ME (2000) Ipsilateral, ventral corticospinal tract of the adult rat: ultrastructure, myelination and synaptic connections. Journal of neurocytology 29:499-507.

Brown AG, Fyffe RE, Rose PK, Snow PJ (1981) Spinal cord collaterals from axons of type II slowly adapting units in the cat. The Journal of physiology 316:469-480.

Brown PJ, Marino RJ, Herbison GJ, Ditunno JF, Jr. (1991) The 72-hour examination as a predictor of recovery in motor complete quadriplegia. Archives of physical medicine and rehabilitation 72:546-548.

Bruehlmeier M, Dietz V, Leenders KL, Roelcke U, Missimer J, Curt A (1998) How does the human brain deal with a spinal cord injury? The European journal of neuroscience 10:3918-3922.

Butovsky O, Ziv Y, Schwartz A, Landa G, Talpalar AE, Pluchino S, Martino G, Schwartz M (2006) Microglia activated by IL-4 or IFN-gamma differentially induce neurogenesis and oligodendrogenesis from adult stem/progenitor cells. Molecular and cellular neurosciences 31:149-160.

Byrnes KR, Washington PM, Knoblach SM, Hoffman E, Faden AI (2011) Delayed inflammatory mRNA and protein expression after spinal cord injury. Journal of neuroinflammation 8:130.

Campagnoli C, Roberts IA, Kumar S, Bennett PR, Bellantuono I, Fisk NM (2001) Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. Blood 98:2396-2402.

Cao FJ, Feng SQ (2009) Human umbilical cord mesenchymal stem cells and the treatment of spinal cord injury. Chinese medical journal 122:225-231.

Cao Q, Ding P, Lu J, Dheen ST, Moochhala S, Ling EA (2007) 2', 3'-Cyclic nucleotide 3'-phosphodiesterase cells derived from transplanted marrow stromal cells and host tissue contribute to perineurial compartment formation in injured rat spinal cord. Journal of neuroscience research 85:116-130.

Cao QL, Zhang YP, Howard RM, Walters WM, Tsoulfas P, Whittemore SR (2001) Pluripotent stem cells engrafted into the normal or lesioned adult rat spinal cord are restricted to a glial lineage. Experimental neurology 167:48-58.

Cao Y, Shumsky JS, Sabol MA, Kushner RA, Strittmatter S, Hamers FP, Lee DH, Rabacchi SA, Murray M (2008) Nogo-66 receptor antagonist peptide (NEP1-40) administration promotes functional recovery and axonal growth after lateral funiculus injury in the adult rat. Neurorehabilitation and neural repair 22:262-278.

Caroni P, Schwab ME (1988) Antibody against myelin-associated inhibitor of neurite growth neutralizes nonpermissive substrate properties of CNS white matter. Neuron 1:85-96.

Casale EJ, Light AR, Rustioni A (1988) Direct projection of the corticospinal tract to the superficial laminae of the spinal cord in the rat. The Journal of comparative neurology 278:275-286.

Chamberlain G, Fox J, Ashton B, Middleton J (2007) Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. Stem cells 25:2739-2749.

Chan CC, Khodarahmi K, Liu J, Sutherland D, Oschipok LW, Steeves JD, Tetzlaff W (2005) Dose-dependent beneficial and detrimental effects of ROCK inhibitor Y27632 on axonal sprouting and functional recovery after rat spinal cord injury. Experimental neurology 196:352-364.

Chau CH, Shum DK, Li H, Pei J, Lui YY, Wirthlin L, Chan YS, Xu XM (2004) Chondroitinase ABC enhances axonal regrowth through Schwann cell-seeded guidance channels after spinal cord injury. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 18:194-196.

Chen A, Xu XM, Kleitman N, Bunge MB (1996) Methylprednisolone administration improves axonal regeneration into Schwann cell grafts in transected adult rat thoracic spinal cord. Experimental neurology 138:261-276.

Chen Y, Teng FY, Tang BL (2006) Coaxing bone marrow stromal mesenchymal stem cells towards neuronal differentiation: progress and uncertainties. Cellular and molecular life sciences : CMLS 63:1649-1657.

Chiba Y, Kuroda S, Maruichi K, Osanai T, Hokari M, Yano S, Shichinohe H, Hida K, Iwasaki Y (2009) Transplanted bone marrow stromal cells promote axonal regeneration and improve motor function in a rat spinal cord injury model. Neurosurgery 64:991-999; discussion 999-1000.

Chidgey AP, Boyd RL (2008) Immune privilege for stem cells: not as simple as it looked. Cell stem cell 3:357-358.

Choo AM, Liu J, Lam CK, Dvorak M, Tetzlaff W, Oxland TR (2007) Contusion, dislocation, and distraction: primary hemorrhage and membrane permeability in distinct mechanisms of spinal cord injury. Journal of neurosurgery Spine 6:255-266.

Chopp M, Zhang XH, Li Y, Wang L, Chen J, Lu D, Lu M, Rosenblum M (2000) Spinal cord injury in rat: treatment with bone marrow stromal cell transplantation. Neuroreport 11:3001-3005.

Chuah MI, Choi-Lundberg D, Weston S, Vincent AJ, Chung RS, Vickers JC, West AK (2004) Olfactory ensheathing cells promote collateral axonal branching in the injured adult rat spinal cord. Experimental neurology 185:15-25.

Chung K, Langford LA, Coggeshall RE (1987) Primary afferent and propriospinal fibers in the rat dorsal and dorsolateral funiculi. The Journal of comparative neurology 263:68-75.

Cizkova D, Rosocha J, Vanicky I, Jergova S, Cizek M (2006a) Transplants of human mesenchymal stem cells improve functional recovery after spinal cord injury in the rat. Cellular and Molecular Neurobiology 26:1167-1180.

Cizkova D, Rosocha J, Vanicky I, Jergova S, Cizek M (2006b) Transplants of human mesenchymal stem cells improve functional recovery after spinal cord injury in the rat. Cellular and molecular neurobiology 26:1167-1180.

Collazos-Castro JE, Lopez-Dolado E, Nieto-Sampedro M (2006) Locomotor deficits and adaptive mechanisms after thoracic spinal cord contusion in the adult rat. Journal of neurotrauma 23:1-17.

Cote MP, Detloff MR, Wade RE, Jr., Lemay MA, Houle JD (2012) Plasticity in ascending long propriospinal and descending supraspinal pathways in chronic cervical spinal cord injured rats. Frontiers in physiology 3:330.

Couris CM, Guilcher SJ, Munce SE, Fung K, Craven BC, Verrier M, Jaglal SB (2010) Characteristics of adults with incident traumatic spinal cord injury in Ontario, Canada. Spinal cord 48:39-44.

Courtine G, Roy RR, Raven J, Hodgson J, McKay H, Yang H, Zhong H, Tuszynski MH, Edgerton VR (2005) Performance of locomotion and foot grasping following a unilateral thoracic corticospinal tract lesion in monkeys (Macaca mulatta). Brain : a journal of neurology 128:2338-2358.

Coutts M, Keirstead HS (2008) Stem cells for the treatment of spinal cord injury. Experimental neurology 209:368-377.

Crigler L, Robey RC, Asawachaicharn A, Gaupp D, Phinney DG (2006) Human mesenchymal stem cell subpopulations express a variety of neuro-regulatory molecules and promote neuronal cell survival and neuritogenesis. Experimental neurology 198:54-64.

Crowe MJ, Bresnahan JC, Shuman SL, Masters JN, Beattie MS (1997) Apoptosis and delayed degeneration after spinal cord injury in rats and monkeys. Nature medicine 3:73-76.

Crown ED, Ye Z, Johnson KM, Xu GY, McAdoo DJ, Hulsebosch CE (2006) Increases in the activated forms of ERK 1/2, p38 MAPK, and CREB are correlated with the expression of at-level mechanical allodynia following spinal cord injury. Experimental neurology 199:397-407.

Darlot F, Cayetanot F, Gauthier P, Matarazzo V, Kastner A (2012) Extensive respiratory plasticity after cervical spinal cord injury in rats: axonal sprouting and rerouting of ventrolateral bulbospinal pathways. Experimental neurology 236:88-102.

Dasari VR, Spomar DG, Cady C, Gujrati M, Rao JS, Dinh DH (2007) Mesenchymal stem cells from rat bone marrow downregulate caspase-3-mediated apoptotic pathway after spinal cord injury in rats. Neurochemical research 32:2080-2093.

David S, Aguayo AJ (1981) Axonal elongation into peripheral nervous system "bridges" after central nervous system injury in adult rats. Science 214:931-933.

David S, Kroner A (2011) Repertoire of microglial and macrophage responses after spinal cord injury. Nature reviews Neuroscience 12:388-399.

de Pommery J, Roudier F, Menétrey D (1984) Postsynaptic fibers reaching the dorsal column nuclei in the rat. Neuroscience letters 50:319-323.

DeFelipe J (2006) Brain plasticity and mental processes: Cajal again. Nature reviews Neuroscience 7:811-817.

Deumens R, Van Gorp SF, Bozkurt A, Beckmann C, Fuhrmann T, Montzka K, Tolba R, Kobayashi E, Heschel I, Weis J, Brook GA (2013) Motor outcome and allodynia are largely unaffected by novel olfactory ensheathing cell grafts to repair low-thoracic lesion gaps in the adult rat spinal cord. Behavioural brain research 237:185-189.

Di Giovanni S (2006) Regeneration following spinal cord injury, from experimental models to humans: where are we? Expert opinion on therapeutic targets 10:363-376.

Dickson BJ (2001) Rho GTPases in growth cone guidance. Current opinion in neurobiology 11:103-110.

Dietz V, Curt A (2006) Neurological aspects of spinal-cord repair: promises and challenges. The Lancet Neurology 5:688-694.

Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A (1999) Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell 97:703-716.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8:315-317.

Dougherty KD, Dreyfus CF, Black IB (2000) Brain-derived neurotrophic factor in astrocytes, oligodendrocytes, and microglia/macrophages after spinal cord injury. Neurobiology of disease 7:574-585.

Dumont RJ, Okonkwo DO, Verma S, Hurlbert RJ, Boulos PT, Ellegala DB, Dumont AS (2001) Acute spinal cord injury, part I: pathophysiologic mechanisms. Clinical neuropharmacology 24:254-264.

Duncan ID, Aguayo AJ, Bunge RP, Wood PM (1981) Transplantation of rat Schwann cells grown in tissue culture into the mouse spinal cord. Journal of the Neurological Sciences 49:241-252.

Dusart I, Schwab ME (1994) Secondary cell death and the inflammatory reaction after dorsal hemisection of the rat spinal cord. European Journal of Neuroscience 6:712-724.

Edgar R, Quail P (1994) Progressive post-traumatic cystic and non-cystic myelopathy. British journal of neurosurgery 8:7-22.

Ehde DM, Jensen MP, Engel JM, Turner JA, Hoffman AJ, Cardenas DD (2003) Chronic pain secondary to disability: a review. The Clinical journal of pain 19:3-17.

Ek CJ, Habgood MD, Callaway JK, Dennis R, Dziegielewska KM, Johansson PA, Potter A, Wheaton B, Saunders NR (2010) Spatio-temporal progression of grey and white matter damage following contusion injury in rat spinal cord. PloS one 5:e12021.

Erceg S, Ronaghi M, Oria M, Rosello MG, Arago MA, Lopez MG, Radojevic I, Moreno-Manzano V, Rodriguez-Jimenez FJ, Bhattacharya SS, Cordoba J, Stojkovic M (2010) Transplanted oligodendrocytes and motoneuron progenitors generated from human embryonic stem cells promote locomotor recovery after spinal cord transection. Stem cells 28:1541-1549.

Erices A, Conget P, Minguell JJ (2000) Mesenchymal progenitor cells in human umbilical cord blood. British journal of haematology 109:235-242.

Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154-156.

Faulkner JR, Herrmann JE, Woo MJ, Tansey KE, Doan NB, Sofroniew MV (2004) Reactive astrocytes protect tissue and preserve function after spinal cord injury. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:2143-2155.

Fawcett JW, Asher RA (1999) The glial scar and central nervous system repair. Brain research bulletin 49:377-391.

Fawcett JW, Curt A, Steeves JD, Coleman WP, Tuszynski MH, Lammertse D, Bartlett PF, Blight AR, Dietz V, Ditunno J, Dobkin BH, Havton LA, Ellaway PH, Fehlings MG, Privat A, Grossman R, Guest JD, Kleitman N, Nakamura M, Gaviria M, Short D (2007) Guidelines for the conduct of clinical trials for spinal cord injury as developed by the ICCP panel: spontaneous recovery after spinal cord injury and statistical power needed for therapeutic clinical trials. Spinal cord 45:190-205.

Fehlings MG, Tator CH (1995) The relationships among the severity of spinal cord injury, residual neurological function, axon counts, and counts of retrogradely labeled neurons after experimental spinal cord injury. Experimental neurology 132:220-228.

Fehlings MG, Tator CH, Linden RD (1989) The relationships among the severity of spinal cord injury, motor and somatosensory evoked potentials and spinal cord blood flow. Electroencephalography and clinical neurophysiology 74:241-259.

Fehlings MG, Vawda R (2011) Cellular treatments for spinal cord injury: the time is right for clinical trials. Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics 8:704-720.

Feron F, Perry C, Cochrane J, Licina P, Nowitzke A, Urquhart S, Geraghty T, Mackay-Sim A (2005) Autologous olfactory ensheathing cell transplantation in human spinal cord injury. Brain : a journal of neurology 128:2951-2960.

Fisher CG, Noonan VK, Smith DE, Wing PC, Dvorak MF, Kwon BK (2005) Motor recovery, functional status, and health-related quality of life in patients with complete spinal cord injuries. Spine 30:2200-2207.

Fleming JC, Norenberg MD, Ramsay DA, Dekaban GA, Marcillo AE, Saenz AD, Pasquale-Styles M, Dietrich WD, Weaver LC (2006) The cellular inflammatory response in human spinal cords after injury. Brain : a journal of neurology 129:3249-3269.

Forostyak S, Jendelova P, Sykova E (2013) The role of mesenchymal stromal cells in spinal cord injury, regenerative medicine and possible clinical applications. Biochimie 95:2257-2270.

Fouad K, Krajacic A, Tetzlaff W (2011) Spinal cord injury and plasticity: opportunities and challenges. Brain research bulletin 84:337-342.

Fouad K, Pearson K (2004) Restoring walking after spinal cord injury. Progress in Neurobiology 73:107-126.

Fouad K, Pedersen V, Schwab ME, Brosamle C (2001) Cervical sprouting of corticospinal fibers after thoracic spinal cord injury accompanies shifts in evoked motor responses. Current biology : CB 11:1766-1770.

Friedenstein AJ, Chailakhyan RK, Latsinik NV, Panasyuk AF, Keiliss-Borok IV (1974) Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. Transplantation 17:331-340.

Fujimoto Y, Abematsu M, Falk A, Tsujimura K, Sanosaka T, Juliandi B, Semi K, Namihira M, Komiya S, Smith A, Nakashima K (2012) Treatment of a mouse model of spinal cord injury by transplantation of human induced pluripotent stem cell-derived long-term self-renewing neuroepithelial-like stem cells. Stem cells 30:1163-1173.

Gage FH (2000) Mammalian neural stem cells. Science 287:1433-1438.

Gale K, Kerasidis H, Wrathall JR (1985) Spinal cord contusion in the rat: behavioral analysis of functional neurologic impairment. Experimental neurology 88:123-134.

Gensel JC, Tovar CA, Hamers FP, Deibert RJ, Beattie MS, Bresnahan JC (2006) Behavioral and histological characterization of unilateral cervical spinal cord contusion injury in rats. Journal of neurotrauma 23:36-54.

Gensert JM, Goldman JE (1997) Endogenous progenitors remyelinate demyelinated axons in the adult CNS. Neuron 19:197-203.

Ghosh A, Sydekum E, Haiss F, Peduzzi S, Zorner B, Schneider R, Baltes C, Rudin M, Weber B, Schwab ME (2009) Functional and anatomical reorganization of the sensory-motor cortex after incomplete spinal cord injury in adult rats. The Journal of neuroscience : the official journal of the Society for Neuroscience 29:12210-12219.

Giesler GJ, Jr., Nahin RL, Madsen AM (1984) Postsynaptic dorsal column pathway of the rat. I. Anatomical studies. Journal of neurophysiology 51:260-275.

Gilson JM, Blakemore WF (2002) Schwann cell remyelination is not replaced by oligodendrocyte remyelination following ethidium bromide induced demyelination. Neuroreport 13:1205-1208.

Goldberg JL, Barres BA (2000) Nogo in nerve regeneration. Nature 403:369-370.

Gorio A, Gokmen N, Erbayraktar S, Yilmaz O, Madaschi L, Cichetti C, Di Giulio AM, Vardar E, Cerami A, Brines M (2002) Recombinant human erythropoietin counteracts secondary injury and markedly enhances neurological recovery from experimental spinal cord trauma. Proceedings of the National Academy of Sciences of the United States of America 99:9450-9455.

Gorrie CA, Hayward I, Cameron N, Kailainathan G, Nandapalan N, Sutharsan R, Wang J, Mackay-Sim A, Waite PM (2010) Effects of human OEC-derived cell transplants in rodent spinal cord contusion injury. Brain research 1337:8-20.

GrandPre T, Li S, Strittmatter SM (2002) Nogo-66 receptor antagonist peptide promotes axonal regeneration. Nature 417:547-551.

GrandPre T, Nakamura F, Vartanian T, Strittmatter SM (2000) Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. Nature 403:439-444.

Griffiths IR, Burns N, Crawford AR (1978) Early vascular changes in the spinal grey matter following impact injury. Acta neuropathologica 41:33-39.

Griffiths IR, McCulloch MC (1983) Nerve fibres in spinal cord impact injuries. Part 1. Changes in the myelin sheath during the initial 5 weeks. Journal of the neurological sciences 58:335-349.

Griffiths IR, Miller R (1974) Vascular permeability to protein and vasogenic oedema in experimental concussive injuries to the canine spinal cord. Journal of the neurological sciences 22:291-304.

Gris D, Marsh DR, Oatway MA, Chen Y, Hamilton EF, Dekaban GA, Weaver LC (2004) Transient blockade of the CD11d/CD18 integrin reduces secondary damage after spinal cord injury, improving sensory, autonomic, and motor function. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:4043-4051.

Grundy BL (1983) Intraoperative monitoring of sensory-evoked potentials. Anesthesiology 58:72-87.

Gruner JA (1992) A monitored contusion model of spinal cord injury in the rat. Journal of neurotrauma 9:123-126; discussion 126-128.

Guest J, Benavides F, Padgett K, Mendez E, Tovar D (2011) Technical aspects of spinal cord injections for cell transplantation. Clinical and translational considerations. Brain research bulletin 84:267-279.

Guest JD, Herrera L, Margitich I, Oliveria M, Marcillo A, Casas CE (2008) Xenografts of expanded primate olfactory ensheathing glia support transient behavioral recovery that is independent of serotonergic or corticospinal axonal regeneration in nude rats following spinal cord transection. Experimental neurology 212:261-274.

Guest JD, Hiester ED, Bunge RP (2005) Demyelination and Schwann cell responses adjacent to injury epicenter cavities following chronic human spinal cord injury. Experimental neurology 192:384-393.

Gulino R, Dimartino M, Casabona A, Lombardo SA, Perciavalle V (2007) Synaptic plasticity modulates the spontaneous recovery of locomotion after spinal cord hemisection. Neuroscience research 57:148-156.

Gwak YS, Crown ED, Unabia GC, Hulsebosch CE (2008) Propentofylline attenuates allodynia, glial activation and modulates GABAergic tone after spinal cord injury in the rat. Pain 138:410-422.

Hagen EM, Rekand T, Gilhus NE, Gronning M (2012) Traumatic spinal cord injuries--incidence, mechanisms and course. Tidsskrift for den Norske laegeforening : tidsskrift for praktisk medicin, ny raekke 132:831-837.

Hagg T, Oudega M (2006) Degenerative and spontaneous regenerative processes after spinal cord injury. Journal of neurotrauma 23:264-280.

Hall ED (1992) The neuroprotective pharmacology of methylprednisolone. Journal of neurosurgery 76:13-22.

Hao JX, Xu XJ, Aldskogius H, Seiger A, Wiesenfeld-Hallin Z (1991) Allodynia-like effects in rat after ischaemic spinal cord injury photochemically induced by laser irradiation. Pain 45:175-185.

Hasler RM, Huttner HE, Keel MJ, Durrer B, Zimmermann H, Exadaktylos AK, Benneker LM (2012) Spinal and pelvic injuries in airborne sports: a retrospective analysis from a major Swiss trauma centre. Injury 43:440-445.

Hawryluk GW, Spano S, Chew D, Wang S, Erwin M, Chamankhah M, Forgione N, Fehlings MG (2014) An examination of the mechanisms by which neural precursors augment recovery following spinal cord injury: a key role for remyelination. Cell transplantation 23:365-380.

Hicks SP, D'Amato CJ (1975) Motor-sensory cortex-corticospinal system and developing locomotion and placing in rats. The American journal of anatomy 143:1-42.

Hill CE, Beattie MS, Bresnahan JC (2001) Degeneration and sprouting of identified descending supraspinal axons after contusive spinal cord injury in the rat. Experimental neurology 171:153-169.

Hill CE, Moon LD, Wood PM, Bunge MB (2006) Labeled Schwann cell transplantation: cell loss, host Schwann cell replacement, and strategies to enhance survival. Glia 53:338-343.

Himes BT, Neuhuber B, Coleman C, Kushner R, Swanger SA, Kopen GC, Wagner J, Shumsky JS, Fischer I (2006) Recovery of function following grafting of

human bone marrow-derived stromal cells into the injured spinal cord. Neurorehabilitation and neural repair 20:278-296.

Hodgetts SI, Simmons PJ, Plant GW (2013) Human mesenchymal precursor cells (Stro-1(+)) from spinal cord injury patients improve functional recovery and tissue sparing in an acute spinal cord injury rat model. Cell transplantation 22:393-412.

Hofstetter CP, Schwarz EJ, Hess D, Widenfalk J, El Manira A, Prockop DJ, Olson L (2002) Marrow stromal cells form guiding strands in the injured spinal cord and promote recovery. Proceedings of the National Academy of Sciences of the United States of America 99:2199-2204.

Huang WL, George KJ, Ibba V, Liu MC, Averill S, Quartu M, Hamlyn PJ, Priestley JV (2007) The characteristics of neuronal injury in a static compression model of spinal cord injury in adult rats. The European journal of neuroscience 25:362-372.

Hulsebosch CE (2002) Recent advances in pathophysiology and treatment of spinal cord injury. Advances in physiology education 26:238-255.

Hunt DP, Irvine KA, Webber DJ, Compston DA, Blakemore WF, Chandran S (2008) Effects of direct transplantation of multipotent mesenchymal stromal/stem cells into the demyelinated spinal cord. Cell transplantation 17:865-873.

Hwang DH, Kim BG, Kim EJ, Lee SI, Joo IS, Suh-Kim H, Sohn S, Kim SU (2009) Transplantation of human neural stem cells transduced with Olig2 transcription factor improves locomotor recovery and enhances myelination in the white matter of rat spinal cord following contusive injury. BMC neuroscience 10:117.

Ibarra A, Diaz-Ruiz A (2006) Protective effect of cyclosporin-A in spinal cord injury: an overview. Current medicinal chemistry 13:2703-2710.

Illes J, Reimer JC, Kwon BK (2011) Stem cell clinical trials for spinal cord injury: readiness, reluctance, redefinition. Stem cell reviews 7:997-1005.

Irani DN, Prow NA (2007) Neuroprotective interventions targeting detrimental host immune responses protect mice from fatal alphavirus encephalitis. Journal of neuropathology and experimental neurology 66:533-544.

Iwata A, Stys PK, Wolf JA, Chen XH, Taylor AG, Meaney DF, Smith DH (2004) Traumatic axonal injury induces proteolytic cleavage of the voltage-gated sodium channels modulated by tetrodotoxin and protease inhibitors. The

Journal of neuroscience : the official journal of the Society for Neuroscience 24:4605-4613.

Jain N, Catania KC, Kaas JH (1997) Deactivation and reactivation of somatosensory cortex after dorsal spinal cord injury. Nature 386:495-498.

Jalink K, van Corven EJ, Hengeveld T, Morii N, Narumiya S, Moolenaar WH (1994) Inhibition of lysophosphatidate- and thrombin-induced neurite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein Rho. The Journal of cell biology 126:801-810.

James ND, Bartus K, Grist J, Bennett DL, McMahon SB, Bradbury EJ (2011) Conduction failure following spinal cord injury: functional and anatomical changes from acute to chronic stages. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:18543-18555.

Jeffery ND, Blakemore WF (1995) Remyelination of mouse spinal cord axons demyelinated by local injection of lysolecithin. Journal of neurocytology 24:775-781.

Jeffery ND, Lakatos A, Franklin RJ (2005) Autologous olfactory glial cell transplantation is reliable and safe in naturally occurring canine spinal cord injury. Journal of neurotrauma 22:1282-1293.

Jendelova P, Herynek V, Urdzikova L, Glogarova K, Kroupova J, Andersson B, Bryja V, Burian M, Hajek M, Sykova E (2004) Magnetic resonance tracking of transplanted bone marrow and embryonic stem cells labeled by iron oxide nanoparticles in rat brain and spinal cord. Journal of neuroscience research 76:232-243.

Jiao J, Milwid J, Yarmush M, Parekkadan B (2011) A mesenchymal stem cell potency assay. Methods in molecular biology 677:221-231.

Jin Y, Neuhuber B, Singh A, Bouyer J, Lepore A, Bonner J, Himes T, Campanelli JT, Fischer I (2011) Transplantation of human glial restricted progenitors and derived astrocytes into a contusion model of spinal cord injury. Journal of neurotrauma 28:579-594.

Joshi M, Fehlings MG (2002) Development and characterization of a novel, graded model of clip compressive spinal cord injury in the mouse: Part 1. Clip design, behavioral outcomes, and histopathology. Journal of neurotrauma 19:175-190.

Kaas JH, Qi HX, Burish MJ, Gharbawie OA, Onifer SM, Massey JM (2008) Cortical and subcortical plasticity in the brains of humans, primates, and rats after

damage to sensory afferents in the dorsal columns of the spinal cord. Experimental neurology 209:407-416.

Kamada T, Koda M, Dezawa M, Yoshinaga K, Hashimoto M, Koshizuka S, Nishio Y, Moriya H, Yamazaki M (2005) Transplantation of bone marrow stromal cell-derived Schwann cells promotes axonal regeneration and functional recovery after complete transection of adult rat spinal cord. Journal of neuropathology and experimental neurology 64:37-45.

Kang KN, Kim da Y, Yoon SM, Lee JY, Lee BN, Kwon JS, Seo HW, Lee IW, Shin HC, Kim YM, Kim HS, Kim JH, Min BH, Lee HB, Kim MS (2012) Tissue engineered regeneration of completely transected spinal cord using human mesenchymal stem cells. Biomaterials 33:4828-4835.

Keirstead HS, Blakemore WF (1999) The role of oligodendrocytes and oligodendrocyte progenitors in CNS remyelination. Advances in experimental medicine and biology 468:183-197.

Keirstead HS, Nistor G, Bernal G, Totoiu M, Cloutier F, Sharp K, Steward O (2005) Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. The Journal of neuroscience : the official journal of the Society for Neuroscience 25:4694-4705.

Keyvan-Fouladi N, Raisman G, Li Y (2003) Functional repair of the corticospinal tract by delayed transplantation of olfactory ensheathing cells in adult rats. The Journal of neuroscience : the official journal of the Society for Neuroscience 23:9428-9434.

Keyvan-Fouladi N, Raisman G, Li Y (2005) Delayed repair of corticospinal tract lesions as an assay for the effectiveness of transplantation of Schwann cells. Glia 51:306-311.

Khaing ZZ, Geissler SA, Jiang S, Milman BD, Aguilar SV, Schmidt CE, Schallert T (2012) Assessing forelimb function after unilateral cervical spinal cord injury: novel forelimb tasks predict lesion severity and recovery. Journal of neurotrauma 29:488-498.

Kim D, Schallert T, Liu Y, Browarak T, Nayeri N, Tessler A, Fischer, Murray M (2001) Transplantation of genetically modified fibroblasts expressing BDNF in adult rats with a subtotal hemisection improves specific motor and sensory functions. Neurorehabilitation and neural repair 15:141-150.

Kirshblum SC, Burns SP, Biering-Sorensen F, Donovan W, Graves DE, Jha A, Johansen M, Jones L, Krassioukov A, Mulcahey MJ, Schmidt-Read M, Waring W (2011a) International standards for neurological classification of spinal cord injury (revised 2011). The journal of spinal cord medicine 34:535-546.

Kirshblum SC, Waring W, Biering-Sorensen F, Burns SP, Johansen M, Schmidt-Read M, Donovan W, Graves D, Jha A, Jones L, Mulcahey MJ, Krassioukov A (2011b) Reference for the 2011 revision of the International Standards for Neurological Classification of Spinal Cord Injury. The journal of spinal cord medicine 34:547-554.

Kohama I, Lankford KL, Preiningerova J, White FA, Vollmer TL, Kocsis JD (2001) Transplantation of cryopreserved adult human Schwann cells enhances axonal conduction in demyelinated spinal cord. The Journal of neuroscience : the official journal of the Society for Neuroscience 21:944-950.

Kontos HA, Povlishock JT (1986) Oxygen radicals in brain injury. Central nervous system trauma : journal of the American Paralysis Association 3:257-263.

Koyanagi I, Tator CH, Lea PJ (1993) Three-dimensional analysis of the vascular system in the rat spinal cord with scanning electron microscopy of vascular corrosion casts. Part 2: Acute spinal cord injury. Neurosurgery 33:285-291; discussion 292.

Krajacic A, Weishaupt N, Girgis J, Tetzlaff W, Fouad K (2010) Training-induced plasticity in rats with cervical spinal cord injury: effects and side effects. Behavioural brain research 214:323-331.

Kwon BK, Liu J, Oschipok L, Teh J, Liu ZW, Tetzlaff W (2004) Rubrospinal neurons fail to respond to brain-derived neurotrophic factor applied to the spinal cord injury site 2 months after cervical axotomy. Experimental neurology 189:45-57.

Kwon BK, Okon E, Hillyer J, Mann C, Baptiste D, Weaver LC, Fehlings MG, Tetzlaff W (2011) A systematic review of non-invasive pharmacologic neuroprotective treatments for acute spinal cord injury. Journal of neurotrauma 28:1545-1588.

Kwon BK, Oxland TR, Tetzlaff W (2002) Animal models used in spinal cord regeneration research. Spine 27:1504-1510.

Lakatos A, Franklin RJ, Barnett SC (2000) Olfactory ensheathing cells and Schwann cells differ in their in vitro interactions with astrocytes. Glia 32:214-225.

Lasiene J, Shupe L, Perlmutter S, Horner P (2008) No evidence for chronic demyelination in spared axons after spinal cord injury in a mouse. The Journal of neuroscience : the official journal of the Society for Neuroscience 28:3887-3896.

Lazarus HM, Haynesworth SE, Gerson SL, Rosenthal NS, Caplan AI (1995) Ex vivo expansion and subsequent infusion of human bone marrow-derived stromal progenitor cells (mesenchymal progenitor cells): implications for therapeutic use. Bone marrow transplantation 16:557-564.

Lee EJ, Lee HN, Kang HJ, Kim KH, Hur J, Cho HJ, Lee J, Chung HM, Cho J, Cho MY, Oh SK, Moon SY, Park YB, Kim HS (2010) Novel embryoid body-based method to derive mesenchymal stem cells from human embryonic stem cells. Tissue engineering Part A 16:705-715.

Lee J, Kuroda S, Shichinohe H, Ikeda J, Seki T, Hida K, Tada M, Sawada K, Iwasaki Y (2003a) Migration and differentiation of nuclear fluorescence-labeled bone marrow stromal cells after transplantation into cerebral infarct and spinal cord injury in mice. Neuropathology : official journal of the Japanese Society of Neuropathology 23:169-180.

Lee JH, Chung WH, Kang EH, Chung DJ, Choi CB, Chang HS, Hwang SH, Han H, Choe BY, Kim HY (2011) Schwann cell-like remyelination following transplantation of human umbilical cord blood (hUCB)-derived mesenchymal stem cells in dogs with acute spinal cord injury. Journal of the neurological sciences 300:86-96.

Lee KH, Suh-Kim H, Choi JS, Jeun SS, Kim EJ, Kim SS, Yoon do H, Lee BH (2007) Human mesenchymal stem cell transplantation promotes functional recovery following acute spinal cord injury in rats. Acta neurobiologiae experimentalis 67:13-22.

Lee SM, Yune TY, Kim SJ, Park DW, Lee YK, Kim YC, Oh YJ, Markelonis GJ, Oh TH (2003b) Minocycline reduces cell death and improves functional recovery after traumatic spinal cord injury in the rat. Journal of neurotrauma 20:1017-1027.

Levi R, Hultling C, Nash MS, Seiger A (1995) The Stockholm spinal cord injury study: 1. Medical problems in a regional SCI population. Paraplegia 33:308-315.

Li Y, Field PM, Raisman G (1997) Repair of adult rat corticospinal tract by transplants of olfactory ensheathing cells. Science 277:2000-2002.

Li Y, Raisman G (1994) Schwann cells induce sprouting in motor and sensory axons in the adult rat spinal cord. The Journal of neuroscience : the official journal of the Society for Neuroscience 14:4050-4063.

Liem NR, McColl MA, King W, Smith KM (2004) Aging with a spinal cord injury: factors associated with the need for more help with activities of daily living. Archives of physical medicine and rehabilitation 85:1567-1577.

Lim JH, Byeon YE, Ryu HH, Jeong YH, Lee YW, Kim WH, Kang KS, Kweon OK (2007) Transplantation of canine umbilical cord blood-derived mesenchymal stem cells in experimentally induced spinal cord injured dogs. Journal of Veterinary Science 8:275-282.

Lima C, Pratas-Vital J, Escada P, Hasse-Ferreira A, Capucho C, Peduzzi JD (2006) Olfactory mucosa autografts in human spinal cord injury: a pilot clinical study. The journal of spinal cord medicine 29:191-203; discussion 204-196.

Lindsay SL, Johnstone SA, Mountford JC, Sheikh S, Allan DB, Clark L, Barnett SC (2013) Human mesenchymal stem cells isolated from olfactory biopsies but not bone enhance CNS myelination in vitro. Glia 61:368-382.

Liu D, Liu J, Sun D, Wen J (2004) The time course of hydroxyl radical formation following spinal cord injury: the possible role of the iron-catalyzed Haber-Weiss reaction. Journal of neurotrauma 21:805-816.

Liu D, Thangnipon W, McAdoo DJ (1991) Excitatory amino acids rise to toxic levels upon impact injury to the rat spinal cord. Brain research 547:344-348.

Liu WG, Wang ZY, Huang ZS (2011) Bone marrow-derived mesenchymal stem cells expressing the bFGF transgene promote axon regeneration and functional recovery after spinal cord injury in rats. Neurological research 33:686-693.

Liu XZ, Xu XM, Hu R, Du C, Zhang SX, McDonald JW, Dong HX, Wu YJ, Fan GS, Jacquin MF, Hsu CY, Choi DW (1997) Neuronal and glial apoptosis after traumatic spinal cord injury. The Journal of neuroscience : the official journal of the Society for Neuroscience 17:5395-5406.

Liu Y, Kim D, Himes BT, Chow SY, Schallert T, Murray M, Tessler A, Fischer I (1999) Transplants of fibroblasts genetically modified to express BDNF promote regeneration of adult rat rubrospinal axons and recovery of forelimb function. The Journal of neuroscience : the official journal of the Society for Neuroscience 19:4370-4387.

Llado J, Haenggeli C, Maragakis NJ, Snyder EY, Rothstein JD (2004) Neural stem cells protect against glutamate-induced excitotoxicity and promote survival of injured motor neurons through the secretion of neurotrophic factors. Molecular and cellular neurosciences 27:322-331.

Lu P, Blesch A, Graham L, Wang Y, Samara R, Banos K, Haringer V, Havton L, Weishaupt N, Bennett D, Fouad K, Tuszynski MH (2012) Motor axonal regeneration after partial and complete spinal cord transection. The Journal of neuroscience : the official journal of the Society for Neuroscience 32:8208-8218.

Lu P, Jones LL, Snyder EY, Tuszynski MH (2003) Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury. Experimental neurology 181:115-129.

Lu P, Jones LL, Tuszynski MH (2005) BDNF-expressing marrow stromal cells support extensive axonal growth at sites of spinal cord injury. Experimental neurology 191:344-360.

Lu P, Jones LL, Tuszynski MH (2007) Axon regeneration through scars and into sites of chronic spinal cord injury. Experimental neurology 203:8-21.

Lu P, Yang H, Culbertson M, Graham L, Roskams AJ, Tuszynski MH (2006) Olfactory ensheathing cells do not exhibit unique migratory or axonal growth-promoting properties after spinal cord injury. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:11120-11130.

Lu S, Lu C, Han Q, Li J, Du Z, Liao L, Zhao RC (2011) Adipose-derived mesenchymal stem cells protect PC12 cells from glutamate excitotoxicity-induced apoptosis by upregulation of XIAP through PI3-K/Akt activation. Toxicology 279:189-195.

Maisonpierre PC, Belluscio L, Friedman B, Alderson RF, Wiegand SJ, Furth ME, Lindsay RM, Yancopoulos GD (1990) NT-3, BDNF, and NGF in the developing rat nervous system: parallel as well as reciprocal patterns of expression. Neuron 5:501-509.

Malhotra NR, Shaffrey CI (2010) Intraoperative electrophysiological monitoring in spine surgery. Spine 35:2167-2179.

Mann C, Lee JH, Liu J, Stammers AM, Sohn HM, Tetzlaff W, Kwon BK (2008) Delayed treatment of spinal cord injury with erythropoietin or darbepoetin--a lack of neuroprotective efficacy in a contusion model of cord injury. Experimental neurology 211:34-40.

Martin D, Schoenen J, Delree P, Gilson V, Rogister B, Leprince P, Stevenaert A, Moonen G (1992) Experimental acute traumatic injury of the adult rat spinal cord by a subdural inflatable balloon: methodology, behavioral analysis, and histopathology. Journal of neuroscience research 32:539-550.

Mattson MP, Chan SL (2003) Calcium orchestrates apoptosis. Nature cell biology 5:1041-1043.

Mautes AE, Weinzierl MR, Donovan F, Noble LJ (2000) Vascular events after spinal cord injury: contribution to secondary pathogenesis. Physical therapy 80:673-687.

McDonald JW, Liu XZ, Qu Y, Liu S, Mickey SK, Turetsky D, Gottlieb DI, Choi DW (1999) Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. Nature medicine 5:1410-1412.

McDonald JW, Sadowsky C (2002) Spinal-cord injury. Lancet 359:417-425.

McKenna JE, Prusky GT, Whishaw IQ (2000) Cervical motoneuron topography reflects the proximodistal organization of muscles and movements of the rat forelimb: a retrograde carbocyanine dye analysis. The Journal of comparative neurology 419:286-296.

McKenna JE, Whishaw IQ (1999) Complete compensation in skilled reaching success with associated impairments in limb synergies, after dorsal column lesion in the rat. The Journal of neuroscience : the official journal of the Society for Neuroscience 19:1885-1894.

McKerracher L, David S, Jackson DL, Kottis V, Dunn RJ, Braun PE (1994) Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. Neuron 13:805-811.

McMahon SS, Albermann S, Rooney GE, Moran C, Hynes J, Garcia Y, Dockery P, O'Brien T, Windebank AJ, Barry FP (2009) Effect of cyclosporin A on

functional recovery in the spinal cord following contusion injury. Journal of anatomy 215:267-279.

McNeill DL, Chung K, Carlton SM, Coggeshall RE (1988) Calcitonin gene-related peptide immunostained axons provide evidence for fine primary afferent fibers in the dorsal and dorsolateral funiculi of the rat spinal cord. The Journal of comparative neurology 272:303-308.

Meirelles LDS, Chagastelles PC, Nardi NB (2006) Mesenchymal stem cells reside in virtually all post-natal organs and tissues. Journal of cell science 119:2204-2213.

Meng T (2009) Response of sensorimotor pathways of the spinal cord to injury and experimental treatments. In: Faculty of Biomedical and Life Sciences, vol. PhD United Kingdom: University of Glasgow.

Merzenich MM, Nelson RJ, Stryker MP, Cynader MS, Schoppmann A, Zook JM (1984) Somatosensory cortical map changes following digit amputation in adult monkeys. The Journal of comparative neurology 224:591-605.

Metz GA, Merkler D, Dietz V, Schwab ME, Fouad K (2000) Efficient testing of motor function in spinal cord injured rats. Brain research 883:165-177.

Metz GA, Whishaw IQ (2000) Skilled reaching an action pattern: stability in rat (Rattus norvegicus) grasping movements as a function of changing food pellet size. Behavioural brain research 116:111-122.

Metz GA, Whishaw IQ (2002) Cortical and subcortical lesions impair skilled walking in the ladder rung walking test: a new task to evaluate fore- and hindlimb stepping, placing, and co-ordination. Journal of neuroscience methods 115:169-179.

Metz GA, Whishaw IQ (2009) The ladder rung walking task: a scoring system and its practical application. Journal of visualized experiments : JoVE.

Miller MW (1987) The origin of corticospinal projection neurons in rat. Experimental brain research 67:339-351.

Mills CD, Hains BC, Johnson KM, Hulsebosch CE (2001) Strain and model differences in behavioral outcomes after spinal cord injury in rat. Journal of neurotrauma 18:743-756.

Minaire P, Edouard C, Arlot M, Meunier PJ (1984) Marrow changes in paraplegic patients. Calcified tissue international 36:338-340.

Minguell JJ, Erices A, Conget P (2001) Mesenchymal stem cells. Experimental biology and medicine 226:507-520.

Mirsky R, Jessen KR, Brennan A, Parkinson D, Dong Z, Meier C, Parmantier E, Lawson D (2002) Schwann cells as regulators of nerve development. Journal of physiology, Paris 96:17-24.

Miura K, Okada Y, Aoi T, Okada A, Takahashi K, Okita K, Nakagawa M, Koyanagi M, Tanabe K, Ohnuki M, Ogawa D, Ikeda E, Okano H, Yamanaka S (2009) Variation in the safety of induced pluripotent stem cell lines. Nature biotechnology 27:743-745.

Mohamad CAC (2014) Human embryonic stem cell-derived mesenchymal stem cells as a therapy for spinal cord injury. In: Institute of Neuroscience and Psychology, vol. PhD United Kingdom: University of Glasgow.

Molander C, Grant G (1995) Spinal cord cytoarchitecture.

Molson Medical Informatics (2008), vol. 2014.

Morgenstern DA, Asher RA, Fawcett JW (2002) Chondroitin sulphate proteoglycans in the CNS injury response. Progress in brain research 137:313-332.

Morshead CM, Craig CG, van der Kooy D (1998) In vivo clonal analyses reveal the properties of endogenous neural stem cell proliferation in the adult mammalian forebrain. Development 125:2251-2261.

Muir GD, Webb AA (2000) Mini-review: assessment of behavioural recovery following spinal cord injury in rats. The European journal of neuroscience 12:3079-3086.

Murray PD, McGavern DB, Sathornsumetee S, Rodriguez M (2001) Spontaneous remyelination following extensive demyelination is associated with improved neurological function in a viral model of multiple sclerosis. Brain : a journal of neurology 124:1403-1416.

Nagorsen D, Deola S, Smith K, Wang E, Monsurro V, Zanovello P, Marincola FM, Panelli MC (2005) Polarized monocyte response to cytokine stimulation. Genome biology 6:R15.

Nakajima H, Uchida K, Guerrero AR, Watanabe S, Sugita D, Takeura N, Yoshida A, Long G, Wright KT, Johnson WE, Baba H (2012) Transplantation of mesenchymal stem cells promotes an alternative pathway of macrophage activation and functional recovery after spinal cord injury. Journal of neurotrauma 29:1614-1625.

Nash HH, Borke RC, Anders JJ (2002) Ensheathing cells and methylprednisolone promote axonal regeneration and functional recovery in the lesioned adult

rat spinal cord. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:7111-7120.

Nashmi R, Imamura H, Tator CH, Fehlings MG (1997) Serial recording of somatosensory and myoelectric motor evoked potentials: role in assessing functional recovery after graded spinal cord injury in the rat. Journal of neurotrauma 14:151-159.

National Spinal Cord Injury Stastitical Centre (2014) Facts and Figures at a Glance. Birmingham: Spain Rehabilitation Center 515.

Navarro R, Juhas S, Keshavarzi S, Juhasova J, Motlik J, Johe K, Marsala S, Scadeng M, Lazar P, Tomori Z, Schulteis G, Beattie M, Ciacci JD, Marsala M (2012) Chronic spinal compression model in minipigs: a systematic behavioral, qualitative, and quantitative neuropathological study. Journal of neurotrauma 29:499-513.

Neuhuber B, Timothy Himes B, Shumsky JS, Gallo G, Fischer I (2005) Axon growth and recovery of function supported by human bone marrow stromal cells in the injured spinal cord exhibit donor variations. Brain research 1035:73-85.

Nistor GI, Totoiu MO, Haque N, Carpenter MK, Keirstead HS (2005) Human embryonic stem cells differentiate into oligodendrocytes in high purity and myelinate after spinal cord transplantation. Glia 49:385-396.

Noble LJ, Wrathall JR (1989) Correlative analyses of lesion development and functional status after graded spinal cord contusive injuries in the rat. Experimental neurology 103:34-40.

Norenberg MD, Smith J, Marcillo A (2004) The pathology of human spinal cord injury: defining the problems. Journal of neurotrauma 21:429-440.

Nori S, Okada Y, Yasuda A, Tsuji O, Takahashi Y, Kobayashi Y, Fujiyoshi K, Koike M, Uchiyama Y, Ikeda E, Toyama Y, Yamanaka S, Nakamura M, Okano H (2011) Grafted human-induced pluripotent stem-cell-derived neurospheres promote motor functional recovery after spinal cord injury in mice. Proceedings of the National Academy of Sciences of the United States of America 108:16825-16830.

Nout YS, Beattie MS, Bresnahan JC (2012) Severity of locomotor and cardiovascular derangements after experimental high-thoracic spinal cord injury is anesthesia dependent in rats. Journal of neurotrauma 29:990-999.

Novikova LN, Lobov S, Wiberg M, Novikov LN (2011) Efficacy of olfactory ensheathing cells to support regeneration after spinal cord injury is influenced by method of culture preparation. Experimental neurology 229:132-142.

Noyes DH (1987) Electromechanical impactor for producing experimental spinal cord injury in animals. Medical & biological engineering & computing 25:335-340.

Nystrom B, Berglund JE, Bergquist E (1988) Methodological analysis of an experimental spinal cord compression model in the rat. Acta neurologica Scandinavica 78:460-466.

O'Leary DD, Terashima T (1988) Cortical axons branch to multiple subcortical targets by interstitial axon budding: implications for target recognition and "waiting periods". Neuron 1:901-910.

Ohta M, Suzuki Y, Noda T, Ejiri Y, Dezawa M, Kataoka K, Chou H, Ishikawa N, Matsumoto N, Iwashita Y, Mizuta E, Kuno S, Ide C (2004) Bone marrow stromal cells infused into the cerebrospinal fluid promote functional recovery of the injured rat spinal cord with reduced cavity formation. Experimental neurology 187:266-278.

Oliveri RS, Bello S, Biering-Sorensen F (2014) Mesenchymal stem cells improve locomotor recovery in traumatic spinal cord injury: systematic review with meta-analyses of rat models. Neurobiology of disease 62:338-353.

Olivier EN, Rybicki AC, Bouhassira EE (2006) Differentiation of human embryonic stem cells into bipotent mesenchymal stem cells. Stem cells 24:1914-1922.

Osaka M, Honmou O, Murakami T, Nonaka T, Houkin K, Hamada H, Kocsis JD (2010) Intravenous administration of mesenchymal stem cells derived from bone marrow after contusive spinal cord injury improves functional outcome. Brain research 1343:226-235.

Oudega M, Xu XM (2006) Schwann cell transplantation for repair of the adult spinal cord. Journal of neurotrauma 23:453-467.

Pal R, Gopinath C, Rao NM, Banerjee P, Krishnamoorthy V, Venkataramana NK, Totey S (2010) Functional recovery after transplantation of bone marrow-derived human mesenchymal stromal cells in a rat model of spinal cord injury. Cytotherapy 12:792-806.

Pantovic R, Draganic P, Erakovic V, Blagovic B, Milin C, Simonic A (2005) Effect of indomethacin on motor activity and spinal cord free fatty acid content after experimental spinal cord injury in rabbits. Spinal cord 43:519-526.

Park HW, Lim MJ, Jung H, Lee SP, Paik KS, Chang MS (2010a) Human mesenchymal stem cell-derived Schwann cell-like cells exhibit neurotrophic effects, via distinct growth factor production, in a model of spinal cord injury. Glia 58:1118-1132.

Park JH, Kim DY, Sung IY, Choi GH, Jeon MH, Kim KK, Jeon SR (2012) Long-term results of spinal cord injury therapy using mesenchymal stem cells derived from bone marrow in humans. Neurosurgery 70:1238-1247; discussion 1247.

Park SS, Byeon YE, Ryu HH, Kang BJ, Kim Y, Kim WH, Kang KS, Han HJ, Kweon OK (2011) Comparison of canine umbilical cord blood-derived mesenchymal stem cell transplantation times: Involvement of astrogliosis, inflammation, intracellular actin cytoskeleton pathways, and neurotrophin. Cell transplantation.

Park WB, Kim SY, Lee SH, Kim HW, Park JS, Hyun JK (2010b) The effect of mesenchymal stem cell transplantation on the recovery of bladder and hindlimb function after spinal cord contusion in rats. BMC neuroscience 11:119.

Parr AM, Kulbatski I, Wang XH, Keating A, Tator CH (2008a) Fate of transplanted adult neural stem/progenitor cells and bone marrow-derived mesenchymal stromal cells in the injured adult rat spinal cord and impact on functional recovery. Surgical neurology 70:600-607; discussion 607.

Parr AM, Kulbatski I, Zahir T, Wang X, Yue C, Keating A, Tator CH (2008b) Transplanted adult spinal cord-derived neural stem/progenitor cells promote early functional recovery after rat spinal cord injury. Neuroscience 155:760-770.

Patterson JT, Coggeshall RE, Lee WT, Chung K (1990) Long ascending unmyelinated primary afferent axons in the rat dorsal column: immunohistochemical localizations. Neuroscience letters 108:6-10.

Paxinos G, Watson C (1998) The rat brain in stereotaxic coordinates. Sydney: Academic Press.

Pearse DD, Lo TP, Jr., Cho KS, Lynch MP, Garg MS, Marcillo AE, Sanchez AR, Cruz Y, Dietrich WD (2005) Histopathological and behavioral characterization of

a novel cervical spinal cord displacement contusion injury in the rat. Journal of neurotrauma 22:680-702.

Phinney DG, Prockop DJ (2007) Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair--current views. Stem cells 25:2896-2902.

Pinzon A, Calancie B, Oudega M, Noga BR (2001) Conduction of impulses by axons regenerated in a Schwann cell graft in the transected adult rat thoracic spinal cord. Journal of neuroscience research 64:533-541.

Pinzon A, Marcillo A, Pabon D, Bramlett HM, Bunge MB, Dietrich WD (2008a) A re-assessment of erythropoietin as a neuroprotective agent following rat spinal cord compression or contusion injury. Experimental neurology 213:129-136.

Pinzon A, Marcillo A, Quintana A, Stamler S, Bunge MB, Bramlett HM, Dietrich WD (2008b) A re-assessment of minocycline as a neuroprotective agent in a rat spinal cord contusion model. Brain research 1243:146-151.

Plemel JR, Keough MB, Duncan GJ, Sparling JS, Yong VW, Stys PK, Tetzlaff W (2014) Remyelination after spinal cord injury: is it a target for repair? Progress in neurobiology 117:54-72.

Popovich PG, Guan Z, McGaughy V, Fisher L, Hickey WF, Basso DM (2002) The neuropathological and behavioral consequences of intraspinal microglial/macrophage activation. Journal of neuropathology and experimental neurology 61:623-633.

Popovich PG, Guan Z, Wei P, Huitinga I, van Rooijen N, Stokes BT (1999) Depletion of hematogenous macrophages promotes partial hindlimb recovery and neuroanatomical repair after experimental spinal cord injury. Experimental neurology 158:351-365.

Powers BE, Lasiene J, Plemel JR, Shupe L, Perlmutter SI, Tetzlaff W, Horner PJ (2012) Axonal thinning and extensive remyelination without chronic demyelination in spinal injured rats. The Journal of neuroscience : the official journal of the Society for Neuroscience 32:5120-5125.

Ra JC, Shin IS, Kim SH, Kang SK, Kang BC, Lee HY, Kim YJ, Jo JY, Yoon EJ, Choi HJ, Kwon E (2011) Safety of intravenous infusion of human adipose tissue-derived mesenchymal stem cells in animals and humans. Stem Cells and Development 20:1297-1308.

Rabchevsky AG (2006) Segmental organization of spinal reflexes mediating autonomic dysreflexia after spinal cord injury. Progress in brain research 152:265-274.

Raineteau O, Schwab ME (2001) Plasticity of motor systems after incomplete spinal cord injury. Nature reviews Neuroscience 2:263-273.

Ramer LM, Richter MW, Roskams AJ, Tetzlaff W, Ramer MS (2004) Peripherally-derived olfactory ensheathing cells do not promote primary afferent regeneration following dorsal root injury. Glia 47:189-206.

Ramer MS, Priestley JV, McMahon SB (2000) Functional regeneration of sensory axons into the adult spinal cord. Nature 403:312-316.

Ramon-Cueto A, Cordero MI, Santos-Benito FF, Avila J (2000) Functional recovery of paraplegic rats and motor axon regeneration in their spinal cords by olfactory ensheathing glia. Neuron 25:425-435.

Rapalino O, Lazarov-Spiegler O, Agranov E, Velan GJ, Yoles E, Fraidakis M, Solomon A, Gepstein R, Katz A, Belkin M, Hadani M, Schwartz M (1998) Implantation of stimulated homologous macrophages results in partial recovery of paraplegic rats. Nature medicine 4:814-821.

Rexed B (1952) The cytoarchitectonic organization of the spinal cord in the cat. The Journal of comparative neurology 96:414-495.

Rice T, Larsen J, Rivest S, Yong VW (2007) Characterization of the early neuroinflammation after spinal cord injury in mice. Journal of neuropathology and experimental neurology 66:184-195.

Richardson PM, McGuinness UM, Aguayo AJ (1982) Peripheral nerve autografts to the rat spinal cord: studies with axonal tracing methods. Brain research 237:147-162.

Riddell JS, Enriquez-Denton M, Toft A, Fairless R, Barnett SC (2004) Olfactory ensheathing cell grafts have minimal influence on regeneration at the dorsal root entry zone following rhizotomy. Glia 47:150-167.

Ringe J, Kaps C, Burmester GR, Sittinger M (2002) Stem cells for regenerative medicine: advances in the engineering of tissues and organs. Die Naturwissenschaften 89:338-351.

Rivlin AS, Tator CH (1978) Effect of duration of acute spinal cord compression in a new acute cord injury model in the rat. Surgical neurology 10:38-43.

Rosenzweig ES, Courtine G, Jindrich DL, Brock JH, Ferguson AR, Strand SC, Nout YS, Roy RR, Miller DM, Beattie MS, Havton LA, Bresnahan JC, Edgerton VR,

Tuszynski MH (2010) Extensive spontaneous plasticity of corticospinal projections after primate spinal cord injury. Nature neuroscience 13:1505-1510.

Rossi SL, Keirstead HS (2009) Stem cells and spinal cord regeneration. Current opinion in biotechnology 20:552-562.

Rossignol S (2000) Locomotion and its recovery after spinal injury. Current opinion in neurobiology 10:708-716.

Rossignol S, Brustein E, Bouyer L, Barthelemy D, Langlet C, Leblond H (2004) Adaptive changes of locomotion after central and peripheral lesions. Canadian journal of physiology and pharmacology 82:617-627.

Sahni V, Kessler JA (2010) Stem cell therapies for spinal cord injury. Nature reviews Neurology 6:363-372.

Salgado-Ceballos H, Guizar-Sahagun G, Feria-Velasco A, Grijalva I, Espitia L, Ibarra A, Madrazo I (1998) Spontaneous long-term remyelination after traumatic spinal cord injury in rats. Brain research 782:126-135.

Sandvig A, Berry M, Barrett LB, Butt A, Logan A (2004) Myelin-, reactive glia-, and scar-derived CNS axon growth inhibitors: expression, receptor signaling, and correlation with axon regeneration. Glia 46:225-251.

Sasaki M, Hains BC, Lankford KL, Waxman SG, Kocsis JD (2006) Protection of corticospinal tract neurons after dorsal spinal cord transection and engraftment of olfactory ensheathing cells. Glia 53:352-359.

Sasaki M, Radtke C, Tan AM, Zhao P, Hamada H, Houkin K, Honmou O, Kocsis JD (2009) BDNF-hypersecreting human mesenchymal stem cells promote functional recovery, axonal sprouting, and protection of corticospinal neurons after spinal cord injury. The Journal of neuroscience : the official journal of the Society for Neuroscience 29:14932-14941.

Satake K, Matsuyama Y, Kamiya M, Kawakami H, Iwata H, Adachi K, Kiuchi K (2000) Up-regulation of glial cell line-derived neurotrophic factor (GDNF) following traumatic spinal cord injury. Neuroreport 11:3877-3881.

Scheff SW, Rabchevsky AG, Fugaccia I, Main JA, Lumpp JE, Jr. (2003) Experimental modeling of spinal cord injury: characterization of a force-defined injury device. Journal of neurotrauma 20:179-193.

Schrimsher GW, Reier PJ (1992) Forelimb motor performance following cervical spinal cord contusion injury in the rat. Experimental neurology 117:287-298.

Schrimsher GW, Reier PJ (1993) Forelimb motor performance following dorsal column, dorsolateral funiculi, or ventrolateral funiculi lesions of the cervical spinal cord in the rat. Experimental neurology 120:264-276.

Schurch B, Wichmann W, Rossier AB (1996) Post-traumatic syringomyelia (cystic myelopathy): a prospective study of 449 patients with spinal cord injury. Journal of neurology, neurosurgery, and psychiatry 60:61-67.

Schwab ME, Bartholdi D (1996) Degeneration and regeneration of axons in the lesioned spinal cord. Physiological reviews 76:319-370.

Schwartz M, Yoles E (2006) Immune-based therapy for spinal cord repair: autologous macrophages and beyond. Journal of neurotrauma 23:360-370.

Sedgwick EM, el-Negamy E, Frankel H (1980) Spinal cord potentials in traumatic paraplegia and quadriplegia. Journal of neurology, neurosurgery, and psychiatry 43:823-830.

Sedy J, Urdzikova L, Jendelova P, Sykova E (2008) Methods for behavioral testing of spinal cord injured rats. Neuroscience and biobehavioral reviews 32:550-580.

Sessarego N, Parodi A, Podesta M, Benvenuto F, Mogni M, Raviolo V, Lituania M, Kunkl A, Ferlazzo G, Bricarelli FD, Uccelli A, Frassoni F (2008) Multipotent mesenchymal stromal cells from amniotic fluid: solid perspectives for clinical application. Haematologica 93:339-346.

Shang AJ, Hong SQ, Xu Q, Wang HY, Yang Y, Wang ZF, Xu BN, Jiang XD, Xu RX (2011) NT-3-secreting human umbilical cord mesenchymal stromal cell transplantation for the treatment of acute spinal cord injury in rats. Brain research 1391:102-113.

Sharp J, Keirstead HS (2009) Stem cell-based cell replacement strategies for the central nervous system. Neuroscience letters 456:107-111.

Sheth RN, Manzano G, Li X, Levi AD (2008) Transplantation of human bone marrow-derived stromal cells into the contused spinal cord of nude rats. Journal of neurosurgery Spine 8:153-162.

Shields SA, Blakemore WF, Franklin RJ (2000) Schwann cell remyelination is restricted to astrocyte-deficient areas after transplantation into demyelinated adult rat brain. Journal of neuroscience research 60:571-578.

Shihabuddin LS, Horner PJ, Ray J, Gage FH (2000) Adult spinal cord stem cells generate neurons after transplantation in the adult dentate gyrus. The

Journal of neuroscience : the official journal of the Society for Neuroscience 20:8727-8735.

Shimoji K, Matsuki M, Shimizu H (1977) Wave-form characteristics and spatial distribution of evoked spinal electrogram in man. Journal of neurosurgery 46:304-313.

Shuman SL, Bresnahan JC, Beattie MS (1997) Apoptosis of microglia and oligodendrocytes after spinal cord contusion in rats. Journal of neuroscience research 50:798-808.

Siegenthaler MM, Tu MK, Keirstead HS (2007) The extent of myelin pathology differs following contusion and transection spinal cord injury. Journal of neurotrauma 24:1631-1646.

Silva NA, Sousa N, Reis RL, Salgado AJ (2014) From basics to clinical: a comprehensive review on spinal cord injury. Progress in neurobiology 114:25-57.

Silver J, Miller JH (2004) Regeneration beyond the glial scar. Nature reviews Neuroscience 5:146-156.

Sinescu C, Popa F, Grigorean VT, Onose G, Sandu AM, Popescu M, Burnei G, Strambu V, Popa C (2010) Molecular basis of vascular events following spinal cord injury. Journal of medicine and life 3:254-261.

Small DG, Matthews WB, Small M (1978) The cervical somatosensory evoked potential (SEP) in the diagnosis of multiple sclerosis. Journal of the neurological sciences 35:211-224.

Smith KJ, Bennett BJ (1987) Topographic and quantitative description of rat dorsal column fibres arising from the lumbar dorsal roots. Journal of anatomy 153:203-215.

Soblosky JS, Song JH, Dinh DH (2001) Graded unilateral cervical spinal cord injury in the rat: evaluation of forelimb recovery and histological effects. Behavioural brain research 119:1-13.

Squier MV, Lehr RP (1994) Post-traumatic syringomyelia. Journal of neurology, neurosurgery, and psychiatry 57:1095-1098.

Steeves JD, Kramer JK, Fawcett JW, Cragg J, Lammertse DP, Blight AR, Marino RJ, Ditunno JF, Jr., Coleman WP, Geisler FH, Guest J, Jones L, Burns S, Schubert M, van Hedel HJ, Curt A, Group ES (2011) Extent of spontaneous motor recovery after traumatic cervical sensorimotor complete spinal cord injury. Spinal cord 49:257-265.

Steward O, Sharp K, Selvan G, Hadden A, Hofstadter M, Au E, Roskams J (2006) A re-assessment of the consequences of delayed transplantation of olfactory lamina propria following complete spinal cord transection in rats. Experimental neurology 198:483-499.

Steward O, Zheng B, Tessier-Lavigne M, Hofstadter M, Sharp K, Yee KM (2008) Regenerative growth of corticospinal tract axons via the ventral column after spinal cord injury in mice. The Journal of neuroscience : the official journal of the Society for Neuroscience 28:6836-6847.

Stichel CC, Muller HW (1998) The CNS lesion scar: new vistas on an old regeneration barrier. Cell and tissue research 294:1-9.

Stirling DP, Khodarahmi K, Liu J, McPhail LT, McBride CB, Steeves JD, Ramer MS, Tetzlaff W (2004) Minocycline treatment reduces delayed oligodendrocyte death, attenuates axonal dieback, and improves functional outcome after spinal cord injury. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:2182-2190.

Stirling DP, Koochesfahani KM, Steeves JD, Tetzlaff W (2005) Minocycline as a neuroprotective agent. The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry 11:308-322.

Stokes BT (1992) Experimental spinal cord injury: a dynamic and verifiable injury device. Journal of neurotrauma 9:129-131; discussion 131-124.

Suberviola B, Gonzalez-Castro A, Llorca J, Ortiz-Melon F, Minambres E (2008) Early complications of high-dose methylprednisolone in acute spinal cord injury patients. Injury 39:748-752.

Sykova E, Homola A, Mazanec R, Lachmann H, Konradova SL, Kobylka P, Padr R, Neuwirth J, Komrska V, Vavra V, Stulik J, Bojar M (2006) Autologous bone marrow transplantation in patients with subacute and chronic spinal cord injury. Cell transplantation 15:675-687.

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861-872.

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663-676.

Takami T, Oudega M, Bates ML, Wood PM, Kleitman N, Bunge MB (2002a) Schwann cell but not olfactory ensheathing glia transplants improve

hindlimb locomotor performance in the moderately contused adult rat thoracic spinal cord. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:6670-6681.

Takami T, Oudega M, Bethea JR, Wood PM, Kleitman N, Bunge MB (2002b) Methylprednisolone and interleukin-10 reduce gray matter damage in the contused Fischer rat thoracic spinal cord but do not improve functional outcome. Journal of neurotrauma 19:653-666.

Takeoka A, Jindrich DL, Munoz-Quiles C, Zhong H, van den Brand R, Pham DL, Ziegler MD, Ramon-Cueto A, Roy RR, Edgerton VR, Phelps PE (2011) Axon regeneration can facilitate or suppress hindlimb function after olfactory ensheathing glia transplantation. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:4298-4310.

Tamatani M, Senba E, Tohyama M (1989) Calcitonin gene-related peptide- and substance P-containing primary afferent fibers in the dorsal column of the rat. Brain research 495:122-130.

Tang X, Davies JE, Davies SJ (2003) Changes in distribution, cell associations, and protein expression levels of NG2, neurocan, phosphacan, brevican, versican V2, and tenascin-C during acute to chronic maturation of spinal cord scar tissue. Journal of neuroscience research 71:427-444.

Tarasenko YI, Gao J, Nie L, Johnson KM, Grady JJ, Hulsebosch CE, McAdoo DJ, Wu P (2007) Human fetal neural stem cells grafted into contusion-injured rat spinal cords improve behavior. Journal of neuroscience research 85:47-57.

Tator CH (1995) Update on the pathophysiology and pathology of acute spinal cord injury. Brain pathology 5:407-413.

Tetzlaff W, Okon EB, Karimi-Abdolrezaee S, Hill CE, Sparling JS, Plemel JR, Plunet WT, Tsai EC, Baptiste D, Smithson LJ, Kawaja MD, Fehlings MG, Kwon BK (2011) A systematic review of cellular transplantation therapies for spinal cord injury. Journal of neurotrauma 28:1611-1682.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. Science 282:1145-1147.

Thuret S, Moon LD, Gage FH (2006) Therapeutic interventions after spinal cord injury. Nature reviews Neuroscience 7:628-643.

Toft A, Scott DT, Barnett SC, Riddell JS (2007) Electrophysiological evidence that olfactory cell transplants improve function after spinal cord injury. Brain : a journal of neurology 130:970-984.

Toft A, Tome M, Barnett SC, Riddell JS (2013) A comparative study of glial and non-neural cell properties for transplant-mediated repair of the injured spinal cord. Glia 61:513-528.

Tome M, Lindsay SL, Riddell JS, Barnett SC (2009) Identification of nonepithelial multipotent cells in the embryonic olfactory mucosa. Stem cells 27:2196-2208.

Totoiu MO, Keirstead HS (2005) Spinal cord injury is accompanied by chronic progressive demyelination. The Journal of comparative neurology 486:373-383.

Tsuji O, Miura K, Okada Y, Fujiyoshi K, Mukaino M, Nagoshi N, Kitamura K, Kumagai G, Nishino M, Tomisato S, Higashi H, Nagai T, Katoh H, Kohda K, Matsuzaki Y, Yuzaki M, Ikeda E, Toyama Y, Nakamura M, Yamanaka S, Okano H (2010) Therapeutic potential of appropriately evaluated safe-induced pluripotent stem cells for spinal cord injury. Proceedings of the National Academy of Sciences of the United States of America 107:12704-12709.

Tuszynski MH, Grill R, Jones LL, Brant A, Blesch A, Low K, Lacroix S, Lu P (2003) NT-3 gene delivery elicits growth of chronically injured corticospinal axons and modestly improves functional deficits after chronic scar resection. Experimental neurology 181:47-56.

Tuszynski MH, Steward O (2012) Concepts and methods for the study of axonal regeneration in the CNS. Neuron 74:777-791.

Tuszynski MH, Weidner N, McCormack M, Miller I, Powell H, Conner J (1998) Grafts of genetically modified Schwann cells to the spinal cord: survival, axon growth, and myelination. Cell transplantation 7:187-196.

Uccelli A, Benvenuto F, Laroni A, Giunti D (2011) Neuroprotective features of mesenchymal stem cells. Best practice & research Clinical haematology 24:59-64.

Uccelli A, Moretta L, Pistoia V (2008) Mesenchymal stem cells in health and disease. Nature reviews Immunology 8:726-736.

Urdzikova L, Jendelova P, Glogarova K, Burian M, Hajek M, Sykova E (2006) Transplantation of bone marrow stem cells as well as mobilization by

granulocyte-colony stimulating factor promotes recovery after spinal cord injury in rats. Journal of neurotrauma 23:1379-1391.

Vanicky I, Urdzikova L, Saganova K, Cizkova D, Galik J (2001) A simple and reproducible model of spinal cord injury induced by epidural balloon inflation in the rat. Journal of neurotrauma 18:1399-1407.

Vaquero J, Zurita M, Oya S, Santos M (2006) Cell therapy using bone marrow stromal cells in chronic paraplegic rats: systemic or local administration? Neuroscience letters 398:129-134.

Varma A, Hill EG, Nicholas J, Selassie A (2010) Predictors of early mortality after traumatic spinal cord injury: a population-based study. Spine 35:778-783.

Vawda R, Wilcox JT, Fehlings MG (2012) Current stem cell treatments for spinal cord injury. Indian journal of orthopaedics 46:10-18.

Veeravalli KK, Dasari VR, Tsung AJ, Dinh DH, Gujrati M, Fassett D, Rao JS (2009) Human umbilical cord blood stem cells upregulate matrix metalloproteinase-2 in rats after spinal cord injury. Neurobiology of Disease 36:200-212.

von Euler M, Janson AM, Larsen JO, Seiger A, Forno L, Bunge MB, Sundstrom E (2002) Spontaneous axonal regeneration in rodent spinal cord after ischemic injury. Journal of neuropathology and experimental neurology 61:64-75.

Wang KC, Kim JA, Sivasankaran R, Segal R, He Z (2002a) P75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp. Nature 420:74-78.

Wang KC, Koprivica V, Kim JA, Sivasankaran R, Guo Y, Neve RL, He Z (2002b) Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. Nature 417:941-944.

Wang R, Ehara K, Tamaki N (1993) Spinal cord edema following freezing injury in the rat: relationship between tissue water content and spinal cord blood flow. Surgical neurology 39:348-354.

Wang X, Budel S, Baughman K, Gould G, Song KH, Strittmatter SM (2009) Ibuprofen enhances recovery from spinal cord injury by limiting tissue loss and stimulating axonal growth. Journal of neurotrauma 26:81-95.

Watson BD, Prado R, Dietrich WD, Ginsberg MD, Green BA (1986) Photochemically induced spinal cord injury in the rat. Brain research 367:296-300.

Watson RA, Yeung TM (2011) What is the potential of oligodendrocyte progenitor cells to successfully treat human spinal cord injury? BMC neurology 11:113.

Webb AA, Muir GD (2002) Compensatory locomotor adjustments of rats with cervical or thoracic spinal cord hemisections. Journal of neurotrauma 19:239-256.

Webb AA, Muir GD (2005) Sensorimotor behaviour following incomplete cervical spinal cord injury in the rat. Behavioural brain research 165:147-159.

Weidner N, Blesch A, Grill RJ, Tuszynski MH (1999) Nerve growth factor-hypersecreting Schwann cell grafts augment and guide spinal cord axonal growth and remyelinate central nervous system axons in a phenotypically appropriate manner that correlates with expression of L1. The Journal of comparative neurology 413:495-506.

Weidner N, Ner A, Salimi N, Tuszynski MH (2001) Spontaneous corticospinal axonal plasticity and functional recovery after adult central nervous system injury. Proceedings of the National Academy of Sciences of the United States of America 98:3513-3518.

Wells JE, Hurlbert RJ, Fehlings MG, Yong VW (2003) Neuroprotection by minocycline facilitates significant recovery from spinal cord injury in mice. Brain : a journal of neurology 126:1628-1637.

Whishaw IQ (2000) Loss of the innate cortical engram for action patterns used in skilled reaching and the development of behavioral compensation following motor cortex lesions in the rat. Neuropharmacology 39:788-805.

Whishaw IQ, Pellis SM, Gorny B, Kolb B, Tetzlaff W (1993) Proximal and distal impairments in rat forelimb use in reaching follow unilateral pyramidal tract lesions. Behavioural brain research 56:59-76.

Whitaker CM, Beaumont E, Wells MJ, Magnuson DS, Hetman M, Onifer SM (2008) Rolipram attenuates acute oligodendrocyte death in the adult rat ventrolateral funiculus following contusive cervical spinal cord injury. Neuroscience letters 438:200-204.

Willis WD, Coggeshall RE (2004) Sensory mechanisms of the spinal cord: Ascending sensory tracts and their descending control. New York: Kluwer academic/Plenum publishers.

Wright KT, Masri WE, Osman A, Roberts S, Trivedi J, Ashton BA, Johnson WE (2008) The cell culture expansion of bone marrow stromal cells from

humans with spinal cord injury: implications for future cell transplantation therapy. Spinal cord 46:811-817.

Wright LS, Prowse KR, Wallace K, Linskens MH, Svendsen CN (2006) Human progenitor cells isolated from the developing cortex undergo decreased neurogenesis and eventual senescence following expansion in vitro. Experimental cell research 312:2107-2120.

Wu S, Suzuki Y, Ejiri Y, Noda T, Bai H, Kitada M, Kataoka K, Ohta M, Chou H, Ide C (2003) Bone marrow stromal cells enhance differentiation of cocultured neurosphere cells and promote regeneration of injured spinal cord. Journal of neuroscience research 72:343-351.

Wyndaele M, Wyndaele JJ (2006) Incidence, prevalence and epidemiology of spinal cord injury: what learns a worldwide literature survey? Spinal cord 44:523-529.

Xu XM, Chen A, Guenard V, Kleitman N, Bunge MB (1997) Bridging Schwann cell transplants promote axonal regeneration from both the rostral and caudal stumps of transected adult rat spinal cord. Journal of neurocytology 26:1-16.

Xu XM, Onifer SM (2009) Transplantation-mediated strategies to promote axonal regeneration following spinal cord injury. Respiratory physiology & neurobiology 169:171-182.

Yang H, Lu P, McKay HM, Bernot T, Keirstead H, Steward O, Gage FH, Edgerton VR, Tuszynski MH (2006) Endogenous neurogenesis replaces oligodendrocytes and astrocytes after primate spinal cord injury. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:2157-2166.

Yezierski RP (2005) Spinal cord injury: a model of central neuropathic pain. Neuro-Signals 14:182-193.

Yezierski RP, Liu S, Ruenes GL, Kajander KJ, Brewer KL (1998) Excitotoxic spinal cord injury: behavioral and morphological characteristics of a central pain model. Pain 75:141-155.

Yezierski RP, Santana M, Park SH, Madsen PW (1993) Neuronal degeneration and spinal cavitation following intraspinal injections of quisqualic acid in the rat. Journal of neurotrauma 10:445-456.

Yoshihara H, Shumsky JS, Neuhuber B, Otsuka T, Fischer I, Murray M (2006) Combining motor training with transplantation of rat bone marrow stromal

cells does not improve repair or recovery in rats with thoracic contusion injuries. Brain research 1119:65-75.

Zhang YW, Denham J, Thies RS (2006) Oligodendrocyte progenitor cells derived from human embryonic stem cells express neurotrophic factors. Stem cells and development 15:943-952.

Ziegler MD, Hsu D, Takeoka A, Zhong H, Ramon-Cueto A, Phelps PE, Roy RR, Edgerton VR (2011) Further evidence of olfactory ensheathing glia facilitating axonal regeneration after a complete spinal cord transection. Experimental neurology 229:109-119.

Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue engineering 7:211-228.

Zurita M, Vaquero J (2006) Bone marrow stromal cells can achieve cure of chronic paraplegic rats: functional and morphological outcome one year after transplantation. Neuroscience letters 402:51-56.

Appendix

Appendix-A. Statistical analysis of differences between radial nerve evoked CDPs in different animals groups at each recording location.

Results of ANOVA and Tukey's post-hoc multiple comparison tests performed on mean CDP amplitudes at each recording location for each experimental group included in the study.

	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8
One way ANOVA	0.1659	0.4729	0.3355	0.0205	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	0.0005	P<0.0001	P<0.0001	0.0053
Tukey's Multiple Comparison Test																	
Normal vs Acute 20mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.01	P < 0.05	P > 0.05	P < 0.01	P < 0.01	P > 0.05
Normal vs 50 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.01	P < 0.01	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Normal vs 1hr 20 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.01	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Normal vs 1hr 50 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.05	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Normal vs 2hr 20mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.01	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Normal vs 2hr 50 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.01	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Normal vs 3hr 20 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P > 0.05	P > 0.05	P > 0.05	P < 0.05	P > 0.05
Normal vs Acute 4hrs	P > 0.05	P > 0.05	P > 0.05	P < 0.01	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.01	P > 0.05	P < 0.05	P < 0.01	P > 0.05
Normal vs 3 dys	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001
Normal vs 2wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.01	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Normal vs 4wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.05	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.01	P > 0.05	P > 0.05	P < 0.05	P < 0.01	P > 0.05
Normal vs 7wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.05	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.01	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Normal vs 3mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.01	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Normal vs 6mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.05	P > 0.05	P < 0.05	P < 0.05	P > 0.05
Acute 20mins vs 50 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 20mins vs 1hr 20 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 20mins vs 1hr 50 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 20mins vs 2hr 20mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 20mins vs 2hr 50 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 20mins vs 3hr 20 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 20mins vs Acute 4hrs	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 20mins vs 3 dys	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.01	P < 0.01	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 20mins vs 2wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 20mins vs 4wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 20mins vs 7wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 20mins vs 3mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 20mins vs 6mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.05	P < 0.001	P > 0.05	P > 0.05	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
50 mins vs 1hr 20 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
50 mins vs 1hr 50 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
50 mins vs 2hr 20mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
50 mins vs 2hr 50 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
50 mins vs 3hr 20 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05

50 mins vs Acute 4hrs	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
50 mins vs 3 dys	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.01	P < 0.01	P < 0.01	P < 0.05	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
50 mins vs 2wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
50 mins vs 4wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
50 mins vs 7wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
50 mins vs 3mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
50 mins vs 6mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.01	P > 0.05	P > 0.05	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 20 mins vs 1hr 50 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 20 mins vs 2hr 20mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 20 mins vs 2hr 50 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 20 mins vs 3hr 20 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 20 mins vs Acute 4hrs	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 20 mins vs 3 dys	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.05	P < 0.05	P < 0.01	P < 0.01	P < 0.05	P < 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 20 mins vs 2wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 20 mins vs 4wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 20 mins vs 7wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 20 mins vs 3mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 20 mins vs 6mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.01	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 50 mins vs 2hr 20mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 50 mins vs 2hr 50 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 50 mins vs 3hr 20 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 50 mins vs Acute 4hrs	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 50 mins vs 3 dys	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.05	P < 0.05	P < 0.05	P < 0.05	P > 0.05	P < 0.05	P < 0.05
1hr 50 mins vs 2wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 50 mins vs 4wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 50 mins vs 7wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 50 mins vs 3mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 50 mins vs 6mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 20mins vs 2hr 50 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 20mins vs 3hr 20 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 20mins vs Acute 4hrs	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 20mins vs 3 dys	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.05	P < 0.05	P < 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 20mins vs 2wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 20mins vs 4wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 20mins vs 7wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 20mins vs 3mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 20mins vs 6mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 50 mins vs 3hr 20 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 50 mins vs Acute 4hrs	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 50 mins vs 3 dys	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 50 mins vs 2wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 50 mins vs 4wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05

2hr 50 mins vs 7wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 50 mins vs 3mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 50 mins vs 6mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
3hr 20 mins vs Acute 4hrs	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
3hr 20 mins vs 3 dys	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
3hr 20 mins vs 2wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
3hr 20 mins vs 4wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
3hr 20 mins vs 7wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
3hr 20 mins vs 3mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
3hr 20 mins vs 6mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 4hrs vs 3 dys	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 4hrs vs 2wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 4hrs vs 4wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 4hrs vs 7wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 4hrs vs 3mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 4hrs vs 6mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
3 dys vs 2wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.01	P < 0.05	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
3 dys vs 4wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.05	P < 0.05	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
3 dys vs 7wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
3 dys vs 3mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.05	P < 0.05	P < 0.05	P > 0.05	P > 0.05
3 dys vs 6mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2wks vs 4wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2wks vs 7wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2wks vs 3mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2wks vs 6mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
4wks vs 7wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
4wks vs 3mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
4wks vs 6mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
7wks vs 3mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
7wks vs 6mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
3mths vs 6mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05

Appendix-B. Statistical analysis of differences between pyramidal evoked CDPs in different animals groups at each recording location.

Results of ANOVA and Tukey's post-hoc multiple comparison tests performed on mean CDP amplitudes at each recording location for each experimental group included in the study.

	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8
One way ANOVA	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	0.0322	0.0647	0.1562	0.0009	0.0002	P<0.0001
Tukey's Multiple Comparison Test																	
Normal vs Acute 20mins	P < 0.001	P < 0.001	P < 0.05	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Normal vs 50 mins	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Normal vs 1hr 20 mins	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Normal vs 1hr 50 mins	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Normal vs 2hr 20mins	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Normal vs 2hr 50 mins	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Normal vs 3hr 20 mins	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Normal vs Acute 4hrs	P < 0.001	P < 0.001	P < 0.01	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.01	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Normal vs 3 dys	P < 0.001	P < 0.001	P < 0.01	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.01	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Normal vs 2wks	P < 0.001	P < 0.001	P < 0.01	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Normal vs 4wks	P < 0.001	P < 0.001	P < 0.01	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.01	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Normal vs 7wks	P < 0.001	P < 0.001	P < 0.05	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.01	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Normal vs 3mths	P < 0.001	P < 0.001	P < 0.01	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.01	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Normal vs 6mths	P < 0.001	P < 0.001	P < 0.01	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 20mins vs 50 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 20mins vs 1hr 20 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 20mins vs 1hr 50 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 20mins vs 2hr 20mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 20mins vs 2hr 50 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 20mins vs 3hr 20 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 20mins vs Acute 4hrs	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 20mins vs 3 dys	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.01	P < 0.001	P < 0.01	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.01
Acute 20mins vs 2wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.001
Acute 20mins vs 4wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.01
Acute 20mins vs 7wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.05
Acute 20mins vs 3mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.01
Acute 20mins vs 6mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.05
50 mins vs 1hr 20 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
50 mins vs 1hr 50 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
50 mins vs 2hr 20mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
50 mins vs 2hr 50 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
50 mins vs 3hr 20 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05

50 mins vs Acute 4hrs	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
50 mins vs 3 dys	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.01	P < 0.01	P < 0.01	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
50 mins vs 2wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
50 mins vs 4wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
50 mins vs 7wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
50 mins vs 3mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
50 mins vs 6mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 20 mins vs 1hr 50 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 20 mins vs 2hr 20mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 20 mins vs 2hr 50 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 20 mins vs 3hr 20 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 20 mins vs Acute 4hrs	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 20 mins vs 3 dys	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.01	P < 0.01	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 20 mins vs 2wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 20 mins vs 4wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 20 mins vs 7wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 20 mins vs 3mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 20 mins vs 6mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 50 mins vs 2hr 20mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 50 mins vs 2hr 50 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 50 mins vs 3hr 20 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 50 mins vs Acute 4hrs	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 50 mins vs 3 dys	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.05	P < 0.01	P < 0.01	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 50 mins vs 2wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 50 mins vs 4wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 50 mins vs 7wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 50 mins vs 3mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
1hr 50 mins vs 6mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 20mins vs 2hr 50 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 20mins vs 3hr 20 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 20mins vs Acute 4hrs	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 20mins vs 3 dys	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.01	P < 0.01	P < 0.01	P < 0.01	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 20mins vs 2wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 20mins vs 4wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 20mins vs 7wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 20mins vs 3mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 20mins vs 6mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 50 mins vs 3hr 20 mins	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 50 mins vs Acute 4hrs	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 50 mins vs 3 dys	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.05	P < 0.05	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 50 mins vs 2wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 50 mins vs 4wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05

2hr 50 mins vs 7wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 50 mins vs 3mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2hr 50 mins vs 6mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
3hr 20 mins vs Acute 4hrs	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
3hr 20 mins vs 3 dys	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.05	P < 0.01	P < 0.001	P < 0.01	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
3hr 20 mins vs 2wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
3hr 20 mins vs 4wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
3hr 20 mins vs 7wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
3hr 20 mins vs 3mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
3hr 20 mins vs 6mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 4hrs vs 3 dys	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.05	P < 0.05	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 4hrs vs 2wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 4hrs vs 4wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 4hrs vs 7wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 4hrs vs 3mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Acute 4hrs vs 6mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
3 dys vs 2wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.01	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
3 dys vs 4wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.01	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
3 dys vs 7wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
3 dys vs 3mths	P > 0.05	P > 0.05	P > 0.05	P < 0.01	P < 0.01	P < 0.01	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
3 dys vs 6mths	P > 0.05	P > 0.05	P > 0.05	P < 0.05	P < 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2wks vs 4wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2wks vs 7wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2wks vs 3mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
2wks vs 6mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
4wks vs 7wks	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
4wks vs 3mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
4wks vs 6mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
7wks vs 3mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
7wks vs 6mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
3mths vs 6mths	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05