Animal models of neuropathic pain after spinal cord injury

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Abstract

Approximately 70% of spinal cord injured patients suffer from pain and it is estimated that in 40-50% of these, the pain is of central neuropathic origin. This pain can be perceived to originate at, or below the level of injury and both evoked and spontaneous pain can occur. Neuropathic pain after spinal cord injury (SCI) is difficult to treat and often poorly controlled by the currently available analgesics so that development of better treatments is an important need. Current ideas about the treatment of SCI pain are that different approaches may be needed to treat the different types of pain (e.g. evoked and spontaneous, at level and below level) as they may have different mechanisms. However, this mechanistic approach to treatment is hampered by a poor understanding of the underlying mechanisms. This in turn depends on development of animal models and pain assessment techniques suitable for mechanistic studies. In this thesis, several rodent SCI models have been investigated using a range of assessment techniques some of which were developed in the course of the study.

Contusion injuries at a low thoracic level are currently the most popular model used to investigate central neuropathic pain in rodents. However this model and the assessments used with it are subject to a number of limitations. We therefore began by re-evaluating this model using a relatively severe (200 kdyn) injury since this is indicated in the literature as being necessary for the development of robust signs of neuropathic pain. We found that this model showed robust signs of tactile allodynia and thermal hyperalgesia of the forepaws and in addition by developing new tests, were able to demonstrate cold allodynia and hyperalgesia. Although the hindpaws also showed responses that would normally be interpreted as mechanical allodynia and thermal hyperalgesia, the absence of accompanying supraspinally mediated behaviours (including licking following heat stimuli) indicated that the enhanced responsiveness to these stimuli might not give rise to pain. Further investigation using operant testing supported this idea and tract tracing suggested that this may be due to substantial interruption of ascending nociceptive pathways. Testing over the back at locations confirmed electrophysiologically to involve sensory processing at, above and below the injury level supported the idea that increased sensitivity in this model developed at and above, but not below level. In addition, observations on the forepaws suggested evidence of spontaneous pain which has never been described in SCI models previously and provides an important opportunity for studying the underlying mechanisms.

Because the 200 kdyn low thoracic model proved unsuitable for the study of below level pain we next investigated whether a less severe injury at this level would provide a better model. Injuries of 150 kdyn were found to result in most of the same indicators of pain following forelimb testing as were seen following 200 kdyn injuries but all signs were less pronounced, in particular, indicators of evoked pain. Testing over the back led to increased sensitivity below level which had not been evident in the 200 kdyn model, providing an opportunity for below level testing. However, interpretation of hindpaw tests remained equivocal.

Because the low thoracic model showed features that suggested forelimb assessments were particularly useful for the assessment of above level pain of different modalities as well as spontaneous pain, we investigated the effect of moving the injury closer to the segments assessed by such tests. Injuries at the T3/T4 level were found to lead to enhancement of all of the behavioural signs seen in the 200 kdyn low thoracic injury animals, especially signs of spontaneous pain. This model may therefore be optimum for the assessment of above/at level pain.

The work presented in this thesis provides the clearest and most comprehensive data yet on the utility of models of SCI for the investigation of central neuropathic pain and represents a significant advance in the field. The finding that injuries at low thoracic levels may (depending on injury severity) be unsuitable for assessment of below level pain has implications for previous studies of the mechanisms of post SCI pain, many of which have used exclusively hindlimb assessments in these models. The hope is that an improved understanding of the models used here and an improved ability to investigate different modalities of evoked pain, and in addition spontaneous pain, will enhance the quality of future research in this area and lead to both a better understanding of central neuropathic pain mechanisms and the development of more effective analgesics for this type of pain.

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Dedication

This work is dedicated to my parents who passed away while I was busy in this research work.

Author's Declaration

I declare that the work presented in thesis is my own and that this thesis has not been submitted for a degree at another institute.

List of abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
AP	anteriorposterior
ASIA	American Spinal injury Association
ATP	adenosine triphosphate
BBB	Basso, Beattie, and Bresnahan
BL	base-line
C	cervical
C°	degree Celsius
Ca^{+2}	calcium
cAMP	cyclic adenosine monophsphate
CAT	cold avoidance time
CCL2	cysteine cysteine chemokine ligand 2
CCL21	cysteine–cysteine chemokine ligand 21
CCR2	cysteine cysteine chemokine ligand 2 receptor
CDP	cord dorsum potential
Cl^-	chloride
CNS	central nervous system
COX-2	cyclooxygenase-2
CREB	cyclic AMP responsive element binding protein
CSPGs	chondroitin sulphate proteoglycans
CTB	cholera toxin B
CVLM	caudal ventrolateral medulla
DRG	dorsal root ganglia
DV	dorsoventral
DWB	dynamic weight bearing
EAA	excitatory amino acid
EMG	electromyogram
ERK	extracellular signal related kinase
FG	fluorogold
FL	footlifting
fMRI	functional magnetic resonance imagining
FP	forepaw
GABA	gamma aminobutyric acid

Glu	glutamate
HP	hindpaw
HPC	heat, pinch and cold
5-HT	5-hydroxy tryptophan
IASP	International Association for Study of Pain
ICC	immunocytochemistry
IH	Infinite Horizon
IL-1β	interleukin 1beta
IR	infra red
ISCIP	International Spinal Cord Injury Pain
i.t.	intraperitoneal
i.v.	intravenous
JNK	c-Jun N-terminal kinase
K+	potassium
KCC2	K+-Cl- cotransporter 2
kHz	kilohertz
L	lumbar
LPB	lateral parabrachial
LRN	lateral reticular nucleus
µA	microampere
MAPKs	mitogen activated protein kinases
ML	mediolateral
mN	millinewton
mW/cm^2	milliwatts per centimetre squared
Na^+	sodium
NASCI	National Acute Spinal Cord Injury Study
Nav	sodium voltage gated channel
NBL	normal base-line
ND	no difference
NeuPSIG	Neuropathic Pain Special interest Group
NF-κB	nuclear factor kappa B
NGF	nerve growth factor
NKCC1	Na^+-K^+-Cl- cotransporter 1
NK1r	neurokinin 1 receptor
NMDA	N-methyl-D-aspartate
NOS	nitric oxide synthase

NYU	New York University
OSU	Ohio State University
PAG	periaqueductal gray
PBDS	double salt phosphate buffer
PEAP	place escape avoidance paradigm
PGE_2	prostaglandin E_2
PO	postoperative
Pre-op	preoperative
QUIS	quisqualic acid
R	rostral
ROS	reactive oxygen species
RVM	rostral ventrolateral medulla
S	sacral
S1	primary somatosensory cortex
SCI	spinal cord injury
SE	standard error
SEM	standard errors of means
SFL	spontaneous footlifting
SP	substance P
SP-SAP	substance P-saporin
SRD	subuncleus reticular dorsalis
T	thoracic
TCAs	tricyclic antidepressants
TNF-α	tumour necrosis factor alpha
TrkB	tropomyosin-receptor kinase B
TRPA1	ankyrin TRP
TRPM8	melastatin transient receptor potential
UV	ultra violet
vF	von Frey
WD	weight drop
WDR	wide dynamic range
WT	withdrawal test.

Chapter 1

Introduction

1.1 Spinal cord injury (SCI)

1.1.1 Epidemiology and causes

SCI is a devastating condition which leads to multifunctional loss below the injury level and many other complications which substantially reduce the quality of life. According to the geographical location, the annual incidence of SCI is reported to be about 10-80 patients per million population and its prevalence was estimated to be about 235-1800 victims per million inhabitants (Wyndaele and Wyndaele, 2006; Hagen et al., 2012).

Young men engaged in high-risk activities are the main victims of SCI (Couris et al., 2010; Varma et al., 2010); nevertheless, the mean of age has increased significantly with time. Automobile crashes as well as violence are a major cause of traumatic SCI which are the highest in South Africa and the lowest in Europe. Furthermore, diseases such as tumour, ischemia and were also reported as contributory factors in SCI (Jackson et al., 2004; Krause, 2004; Hagen et al., 2012).

1.1.2 Classification

Sensory and motor disorders are the main outcome after SCI. Based on preserved function below the injury level, SCI can be characterized clinically into "incomplete" or "complete". However, the human and animal post-mortem studies revealed that the spinal cord is rarely completely severed after blunt trauma (Kakulas, 1988, 1999; Bunge et al., 1993). As a result, the concept of '*discomplete lesion*' was introduced by Sherwood et al. (1992). The discomplete lesion was defined as *"the clinically complete lesion but which is accompanied by neurological evidence of residual brain influence on spinal cord function below the lesion"*. Later, the term '*sensory discomplete*' was utilized to describe those who have clinically complete SCI but preserved some sensations below level of the lesion (for review see Finnerup and Jensen, 2004). Terms such as quadriplegia or tetraplegia are also commonly used to denote paralysis of the four limbs and the lower limbs, respectively. Less severely, quadriparesis is used to describe weakness of all four limbs while paraparesis indicates weakness of lower limbs.

Among several examination scales (e.g. Frankel scale and Yale scale) for classification of patients with SCI, American Spinal injury Association (ASIA) is the most accepted by many authors (Jackson et al., 2004). This scale is rated from A to E base on criteria such as the neurological level (i.e. the first normal segment above the injury level), dermatome

normal responses to light touch and pinprick sensory tests and the strength of ten key muscles (i.e. elbow flexion " C5", wrist extension " C6", elbow extension " C7", finger flexion "C8", finger abduction " T1", hip flexion " L2", knee extension "L3", ankle dorsiflexion "L4", toe extension "L5", ankle plantar flexion "S1"). The absence of any sensory as well as motor functions in the lowest sacral segments (i.e. S4-S5 innervating anal sphincter) is indicated as a "complete" or grade A SCI. Grade B is referred as preserving sensory but not motor function below the neurological level (i.e. incomplete SCI), including the sacral segments S4-S5 (i.e. voluntary contraction of anus). Grade C (i.e. motor incomplete) is considered when motor function is preserved below the neurological level but too partially usable (i.e. more than half of key muscles below the neurological level have a muscle grade of less than 3 (i.e. active movement with less than the full range against gravity). When the muscle grade scored more than 3 on a scale from 0 to 5, the SCI is classified as grade D (i.e. motor incomplete) while E on the scale indicates "normal" where motor and sensory scores are normal.

The proportions of SCI at each level were varied between different publications. For instance, about 92 % (Dincer et al., 1992) and 50% (Jackson et al., 2004) of cases were reported to be cervical injuries in Turkey and the US, respectively. Comparatively, only 5% of the same level SCI was indicated in China (Li et al., 2011).

1.1.3 Complications

In addition to the sensory and motor loss, a number of complications can be experienced by SCI victims. For example, chest and abdominal muscle deficits with subsequent breathing difficulty are observed in patients affected at higher cervical levels of spinal cord (Grandas et al., 2005). By contrast, bowel, bladder and sexual dysfunctions are usually predominant among the SCI patients injured at lower levels of the spinal cord (Jamil, 2001; Liem et al., 2004). Additionally, spasticity (Levi et al., 1995) and autonomic dysreflexia (Rabchevsky, 2006), chronic neuropathic pain (Ehde et al., 2003), pressure ulcers (Liem et al., 2004), renal infection (Garcia Leoni and Esclarin De Ruz, 2003) and obesity (Buchholz and Bugaresti, 2005) are among the secondary consequences of spinal injuries.

1.1.4 Pathology

The pathological and degenerative processes post SCI can be classified into three main phases: acute, subacute (intermediate) and chronic. The acute phase is caused by the direct mechanical damage, characterized by an immediate haemorrhage and infiltration of several

types of blood cells, plasma-derived proteins, inflammatory mediators and fluids into spinal parenchyma leading to acute oedema followed by neuronal, glial and endothelial cell death (Kakulas, 1999; 2004). During the intermediate phase (minutes to weeks after SCI), a variety of secondary pathological cascades exacerbate the initial damage: hypoxia as a result of vascular disruption, excitotoxicity following calcium (Ca^{+2}) influx, lipid peroxidation subsequent to massive production of free radicals and inflammation, result in further degeneration (for review see Klussmann and Martina-Villalba, 2005). Further apoptosis with subsequent formation of cystic cavities due to removal of the debris by macrophages and scars of astrocytic and collagenous fibres (Kakulas, 2004; Sharma, 2008) are the main characters of the chronic phase of the SCI (Fig. 1-1). Additionally, regeneration of nerve roots as well as formation of new blood vessels and massive numbers of macrophages in the lesion area can be added to the features of this stage which tends to be more static (Sharma, 2007).

1.1.4.1 Haemorrhage, oedema, electrolyte imbalance and toxic mediators

At a very early stage, SCI leads to haemorrhage and leakage of fluid containing blood cells, serum proteins and electrolytes from the damaged capillaries (Aarabi et al., 2006). Oedema occurs within minutes and can cause direct cell death by the physical pressure while haemorrhage with localized thrombosis and vasospasm can result in an ischemic state (Sharma, 2006). The ischemic period can lead to insufficient oxygen and nutrient supply to neuronal and glial cells (Tanaka et al., 2005). As a result of insufficient energy production, the cell becomes unable to maintain the normal cation concentration gradients (for review see Klussmann & Martina-Villalba, 2005). In addition, ischemia triggers many pathological events such as production of reactive oxygen species (ROS) (Cayli et al., 2004; Usul et al., 2004). In addition to direct damage of cell membrane (contains transporters and ionic pumps) by the physical force, ROS can play a role in hydrolysis of cell membrane by membrane-lipid peroxidation (for review see Yezierski, 2005). These events can result in shifting of cations such as sodium (Na^{+}), potassium (K^{+}) and Ca^{+2} from extracellular to intracellular compartment (Goldman et al., 1983). Na^{+} and K^{+} can mediate cell death directly by electrolyte imbalance and osmosis while Ca^{+2} can alter cellular signalling pathways with subsequent apoptosis (Yezierski, 2005).

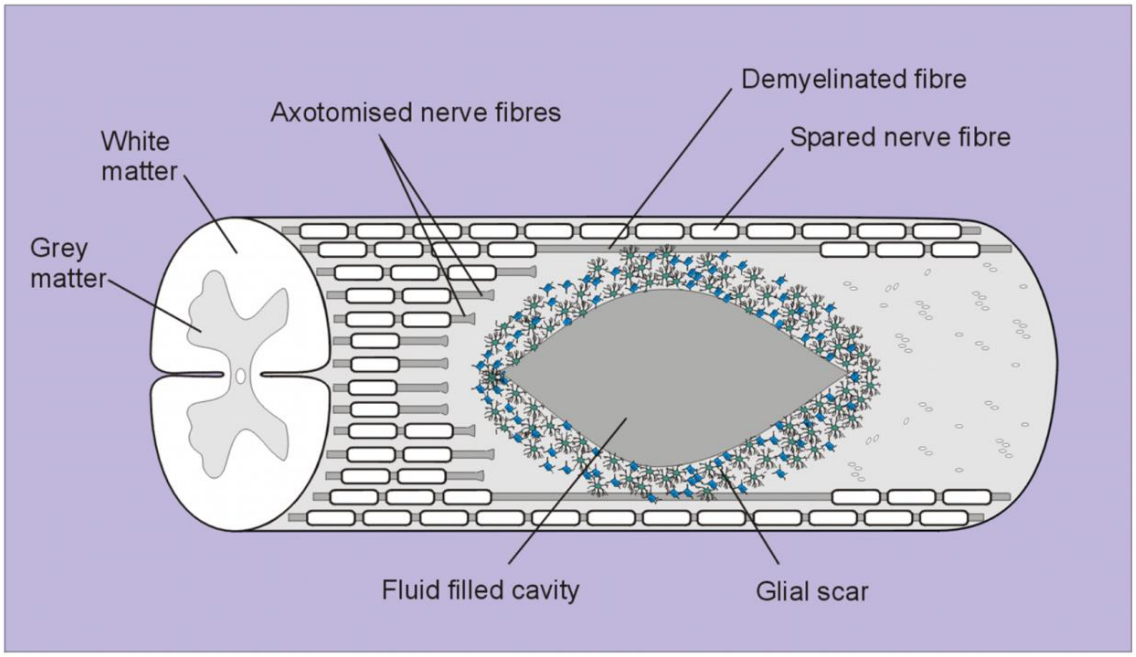

Fig. 1-1. Illustrates the pathological features of SCI. A contusion SCI leads to a massive cellular death and extensive damage to the most of the projections at the injury site and a minority of ascending axons at the periphery of the contusion can escape the complete interruption but some may suffer a certain degree of demyelination. A cavity surrounded by a dense glial scar is formed in a chronic phase.

The primary insult as well as disruption of synaptic reuptake and accumulation of Ca^{+2} in the intracellular compartment can lead to a massive increase in concentration of synaptic excitatory amino acids (EAAs) such as glutamate (Glu) (Liu et al., 1991, 1999; Hulsebosch, 2002). A further increase in the intracellular Ca^{+2} can be mediated by these EAAs with subsequent induction of cyclooxygenase-2 (COX-2) as well as nitric oxide synthase (NOS), formation of more ROS which collectively aggravate the cellular death (Kontos and Wei, 1986; Liu et al., 2002; Liu et al., 2004). The production of several potentially harmful mediators such as cytokines as well as activation of transcription factors such as nuclear factor kappa B (NF-κB) are also provoked at early time post SCI (for review see Yezierski, 2009).

1.1.4.2 Neuroinflammation

SCI triggers activation of both microglia (i.e. silent but sensing under basal conditions) and astrocytes (Crown et al., 2006; Gwak et al., 2008). Microgliosis and astrogliosis can be activated directly by a variety of mediators such as Glu, chemokines, adenosine triphosphate (ATP), ROS and neurotrophic factors which are released by injured neurons (Carbonell et al., 2005; Kurpius et al., 2006; Weisshaar et al., 2010; Pirttimaki et al., 2011). Microglia are chronically transformed into a more reactive state (Zhao et al., 2007a) through activation of metabotropic and ionotropic Glu receptors at early stage after SCI (reviewed in Gwak et al., 2012). The activated microglia release several mediators such as Glu and proinflammatory cytokines as well as ROS (Crown et al., 2008; Zheng et al., 2010) which, subsequently, triggers calcium signalling waves in astrocytes, leading to astrogliosis (Palygin et al., 2010). The activated astrocytes have a similar capability for synthesis and release of proinflammatory cytokines (Ji and Strichartz, 2004). At early stages after SCI, microgliosis and astrogliosis are responsible for some beneficial activities such as removal of myelin and axonal debris as well as the uptake of excess extracellular Glu and K^+ (Bethea and Dietrich, 2002; Hulsebosch et al., 2009). Activated glia become responsible for release an abundant amount of chemoattractants such as tumour necrosis factor alpha (TNF-α), interleukin 1beta (IL-1β) and 6 (IL-6) and chemokines (Moalem and Tracey, 2006; Hanisch and Kettenmann, 2007) which collectively attract more inflammatory immune cells (Rice et al., 2007).

1.1.4.3 Apoptosis and demyelination

Programmed cell death (apoptosis) through activated proteases (especially caspases and calpains) was first observed in rodent models (Shuman et al., 1997) and confirmed in

monkeys (Crowe et al., 1997). Apoptosis is limited to the site of trauma at early time points after SCI but remote places became also affected at the chronic stage (Li et al., 1999). In addition to neuronal loss, acute and chronic death of oligodendrocyte was also reported with subsequent disintegration and breakdown of myelin (Norenberg et al., 2004). In rat contusion model of SCI, primary demyelination of axons occurred initially at epicentre of the lesion due to the direct insult and over time, secondary demyelination progresses to other cranial and caudal sites of spinal cord (Totoiu and Keirstead, 2005). Human data suggests that in some case, the process of axonal demyelination can continue for more than a decade after SCI (Guest et al., 2005).

1.1.4.4 Scar

Glial cells contribute to a variety of supportive functions such as regulation of Glu, K^+ and other ions. After spinal lesion, activated astrocytes up-regulate production of chondroitin sulphate proteoglycans (CSPGs: Fitch et al., 1999; Jones et al., 2003). With oligodendrocyte precursors and microglia, this forms the outer zone of a dense glia scar while the core of the scar consists of meningeal cells and oligodendrocyte precursors (Cervos-Navarro and Lafuente, 1991). These two zones are separated by a basement membrane composed of collagen (Stichel and Muller, 1998). The scar is a major mechanical and chemical obstacle to axonal regeneration (Silver and Miller, 2004; Yiu and He, 2006) but it supports the fragile parenchyma and serves as a scaffold for re-vascularisation (Stichel and Muller, 1998; Okada et al., 2006).

1.1.4.5 Cavity

Over time, a cavitation is centrally formed at the lesion site by apoptosis of cells, inflammatory response and tissue degradation (Sandvig et al., 2004). Debris of macrophages, connective tissue and blood vessels fill the cavities (Norenberg et al., 2004). Usually, these gaps in the spinal parenchyma represent a final stage of the necrotic process and relatively stable after a period of weeks to months, characterizing by axotomized nerve fibres, a fluid-filled cavity surround by a glia scar and variable amount of intact tissue surrounding the lesion. In a minority of cases (4%), the cavities could be continuously progressed over years to eventually develop syringomyelia (Schurch et al., 1996).

1.1.5 Treatment

Several strategies have been investigated for treatment of SCI; however, neuroprotection as well as rehabilitation approaches are the most applicable in human. For purpose of neuroprotection, methylprednisolone is commonly used to limit death of spinal parenchyma in an acute stage after SCI. The National Acute Spinal Cord Injury Study (NASCI) performed a study on 500 patients to investigate the effect 30 mg/kg bolus over 15 min followed by a maintenance infusion of 5.4 mg/kg/hr for 24- 48 hr (Bracken et al., 1997). A marked improvement of neurologic outcomes up to one year after SCI was reported but the steroid must be critically administered in the first 8 hr post SCI. This regimen has been admitted recently by Bracken (2012) to be the only pharmacological therapy provides real efficacy in terms of motor improvement.

1.2 Clinical aspects of SCI pain

International Association for Study of Pain (IASP) defined pain as unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage (Merskey, 1979). This can be considered as an appropriate description for an evoked pain; however, pain may be also generated spontaneously following abnormal changes in the somatosensory system. For instance, pathological conditions such as multiple sclerosis, stroke, peripheral nerve lesions and SCI can lead to unpleasant sensations without activation of nociceptors (Wydenkeller et al., 2009). This type of pain is known as neuropathic pain.

Pain is considered by a majority of patients to be the worst complication following SCI. It is directly linked to the bad mood, sleep disturbance and interference with daily life activities (Putzke et al., 2002), leading to depression and even suicide (Widerstrom-Noga et al., 2001a). In addition to its negative physical and psychological impacts on the quality of social life, pain post SCI can slow down or even interrupt rehabilitation programs (Margot-Duclot et al., 2009).

1.2.1 Classification

Historically, there are more than 29 classifications dealt with pain post SCI based on criteria such as location (above or at and below level of the injury), pathophysiology (e.g. neuropathic or nociceptive pain) and pain origin (e.g. segmental pain, visceral pain and

phantom pain) (Hicken et al., 2002; Calmels et al., 2009). In this context, we will focus on the most popular and practical classifications.

1.2.1.1 Cardenas pain classification

This scheme involves two major categories for pain; namely neurologic and musculoskeletal pain. Neurologic pain is subcategorized into SCI pain, transition zone pain, radicular pain, and visceral pain whereas musculoskeletal pain was subdivided into mechanical spine pain and overuse pain (Cardenas et al., 2002a).

1.2.1.2 IASP pain classification

In this scheme, SCI pain was classified into three-tiered system according to the pathophysiology and topographical level (Siddall et al., 2000). Tier one involves pain of nociceptive and neuropathic natures. Subsequently, Tier II classifies nociceptive category into two subcategories; namely musculoskeletal and visceral pain while neuropathic pain includes three subgroups; known as above, at-level and below-level neuropathic pain. All of these subcategories can be further subclassified at Tier III according to the underlying causes of pathology. However, it is not always possible to demonstrate underlying pathology at the Tier III level (Bryce et al., 2007).

1.2.1.3 Bryce/Ragnarsson pain classification

This classification has been considered as a variant of the IASP scheme but it differs in reversal of tiers (Bryce and Ragnarsson, 2001). Instead of characterizing pain into nociceptive or neuropathic in the first step, this scheme classified pain according to its location into above, at and below the level of the SCI. Pain can be characterized as nociceptive or neuropathic based on its nature in a subsequent step while the final tier categorized pain according to the its etiology subtypes. In a later comment, Bryce et al. (2007) wrote "*IASP and Bryce/Ragnarsson schemes were useful in their present form but required more validation"*.

1.2.1.4 International Spinal Cord Injury Pain (ISCIP) classification (2012)

Similar to IASP classification, the current scheme has three tiers and deals with a variety of pains that related and unrelated to the SCI (Bryce et al., 2012a and b). In tiers one and two, pain has classified into types and subtypes, respectively, whereas tier three indicated the primary source and/or pathology (Table. 1-1).

In their comment, Finnerup and Baastrup (2012) have claimed that the term above in the older classifications could be misleading to the conclusion that this type of pain occurred above the injury level as direct consequence of SCI. As a result, the term above level SCI neuropathic pain has now become included in the other neuropathic pain category according to current taxonomy.

1.2.2 Prevalence and onset of SCI pain

Despite the high figures (up to 90%) for the prevalence of pain among SCI patients in the very early reports (Davis and Martin, 1947; Botterell et al., 1954), currently, it is widely accepted that two-thirds of SCI patients are vulnerable to pain with at least one third experiencing an intense pain (Siddall et al., 2001; Finnerup and Jensen, 2004; Baastrup and Finnerup, 2012). The onset for pain after SCI varies between patients. For example, 47% of 88 patients studied by Barrett et al. (2003) developed pain within the first year after SCI while and in the same study, 27% of cases experienced pain distress developed 10 or more years post injury. In a bigger sample (901 SCI case), pain was reported in 34% of patients immediately after the injury whereas 58% of patients showed a longer onset within the first year (Turner et al., 2001). Overall, it seems to be the first 12 months following the injury is the most often onset for development of pain (Nepomuceno et al., 1979; Tasker et al., 1992; Stormer et al., 1997; Turner and Cardenas, 1999; Falci et al., 2002; Rogano et al., 2003).

100 SCI patients were interviewed by Siddall et al. for presence or absence of unpleasant sensory disorders. At 6 months post SCI (Siddall et al., 1999a), 40% of victims suffered musculoskeletal pain while none had visceral pain. In addition, 36% and 19% of patients experienced at and below level neuropathic pain, respectively. In the long term (5 years after SCI) follow up (Siddall et al., 2003), musculoskeletal pain remained the most common, affecting 59% of the patients whereas 41% and 34% of the patients had at and below level neuropathic pain, respectively. A tiny minority (5%) of population mentioned visceral pain as a common complication of SCI. In addition, allodynia was only reported in 14% of those patients. When data from both studies were taken together and the mean time of onset was calculated for each type of pain, at level neuropathic pain (1.2 years) and musculoskeletal pain (1.3 years) had a similar onset while below-level neuropathic and visceral pain had later onsets of 1.8 and 4.2 years, respectively.

Tier One Pain type	Tier Two Pain subtype	Tier Three Primary pain source and/or pathology (write or type in)
Nociceptive pain	Musculoskeletal	• …….. • e.g. glenohumeral arthritis, lateral epicondylitis, comminuted femur fracture, quadratus lumborum muscle spasm,
	Visceral	• …….. • e.g. myocardial infarction, abdominal pain due to bowel impaction, cholecystitis
	Other nociceptive pain	• …….. • e.g. autonomic dysreflexia, headache, migraine headache, surgical skin incision
Neuropathic Pain	At level SCI pain	• ..... • e.g. Spinal cord compression, nerve root compression, and cauda equine compression
	Below level SCI pain	• ..... • e.g. Spinal cord ischemia, spinal cord compression
	Other neuropathic pain	• ..... • e.g. Carpal tunnel syndrome, trigeminal neuralgia, diabetic polyneuropathy
Other pain		• ..... • e.g. Fibromyalgia, Complex Regional Syndrome type I, interstitial cystitis, irritable bowel syndrome
Unknown pain		• .....

Table 1-1. International Spinal Cord Injury Pain classification (Bryce et al., 2012a).

1.2.3 Musculoskeletal nociceptive SCI pain

Traumatic SCI can lead to development of nociceptive pain as a result to damage of surrounding musculoskeletal structures such as bones, joints, ligaments, muscles, intervertebral discs, and facet joints (Nashold et al., 1981). Activation of nociceptors within these structures can result in unpleasant sensation perceived locally or radiating to other regions (Ragnarsson, 1997; Widerstrom-Noga et al., 2009). The ISCIP classification (Bryce et al., 2012a) described some of the characteristics of musculoskeletal pain: such pain is increased/decreased or otherwise changed by movement or a change in position. There may be tenderness of musculoskeletal structures on palpation and evidence of skeletal pathology on imaging. Musculoskeletal pain usually responds to anti-inflammatory and opioid drugs. This pain is usually ‘dull’ and ‘aching’ and tends to be chronic but less severe than neuropathic pain (Siddall et al., 2003).

1.2.4 Visceral nociceptive SCI pain

Visceral pain describes nociceptive pain located in the thorax, abdomen, or pelvis due to activation of nociceptors in visceral structures such as kidney, bowel or sphincters. The following criteria were proposed for diagnostic purposes (Bryce et al., 2012a): a temporal relationship to food intake or visceral function (e.g. constipation; Siddall et al., 1997), tenderness of visceral structures on palpation, and evidence of visceral pathology on imaging or testing (e.g. urinary tract infection or renal stone; Widerstrom-Noga et al., 2009). ‘dull’, ‘cramping’ and tender are common description by patients suffering visceral nociceptive pain (Finnerup and Jensen, 2004).

1.2.5 Neuropathic SCI pain

This type of pain is distinguished from nociceptive pain on basis of certain descriptors of the sensation such as “burning”, “electrical”, or “shooting” and “tingling” (Putzke et al., 2002). It is severe, persistent and hard to treat (Siddall et al., 2003). Neuropathic pain can be perceived as spontaneous (i.e. continuous or paroxysmal) and/or evoked (allodynia or hyperalgesia). The continuous spontaneous pain have qualities such as ‘hot-burning’ or ‘painful cold’, ‘pins and needles’, ‘tearing’, ‘bursting’, ‘sequeezing’ and ‘pressure’ while the paroxysmal type can be experienced as ‘electrical shocks’, ‘stabbing’ and‘throbbing’ sensation (Defrin et al., 2001; Calmels et al., 2009). Evoked pain can be due to a stimulus which is not normally painful (i.e. allodynia) or experienced as an abnormally increased perception of a painful stimulus (i.e. hyperalgesia) (Widerstrom-Noga et al., 2009). There

is a third class of painless but unpleasant sensations referred to as paresthesia or ‘phantom pain’ (Siddall et al., 1999a). Paresthesia can be spontaneous or evoked and is commonly experienced early after SCI but may disappear later (Calmels et al., 2009).

1.2.5.1 At level neuropathic SCI pain (central and peripheral)

Pain that originates within the dermatomes corresponding to the neurological level or three dermatomes below this level characterized as at level (Siddall and Loeser, 2001; Widerstrom-Noga et al., 2008). At level pain is classically referred as segmental, transitional zone, border zone, and end zone or girdle zone pain and generally symmetrical in distribution (Widerstrom-Noga et al., 2009). It is usually associated with damage to the nerve root and spinal cord itself (i.e. spinal cord pathology) (De Miguel and Kraychete, 2009). If neuropathic pain occurs within “at level” distribution but without any obvious spinal cord and nerve root, it should be classified as other neuropathic pain type (see Table 1-1) (Bryce et al., 2012a). Nerve root damage is often characterized by unilateral pain exacerbated by movement (Widerstrom-Noga et al., 2009). At level neuropathic SCI pain is more common than below level and appears with faster onset, however, both are extremely severe and persistence (Siddall et al., 1999a, 2003).

1.2.5.2 Below level neuropathic SCI pain (central)

Below level neuropathic pain is attributed to pure central origin such as central sensitization (Vierck et al., 2000). It is less common than at level but generally more severe and late in onset (Siddall et al., 2003). Below level pain is defined as occurring at least four dermatomes below the neurological level (Bryce et al., 2012a). This type of pain in patients modified by mood or attention but unrelated to position or movement (Widerstrom-Noga et al., 2009). Additionally, below level neuropathic pain after SCI can be triggered by sudden noises or physical jarring and exacerbated by smoking or alcohol, emotional factors, fatigue and even weather changes (Davis, 1975; Widerstrom-Noga et al., 2009). In spite of the fact that spinothalamic lesion alone is not sufficient to produce this condition because such lesions may be pain free (Eide et al., 1996), an abnormal spinothalamic function with an altered sensitivity to temperature and pinprick are regarded as predisposing to below level neuropathic pain post SCI (Vestergaard et al., 1995; Bowsher, 1996).

1.2.6 Diagnosis of neuropathic SCI pain

Due to the fact that identification of neuropathic pain in SCI victims is not an easy task, a mixture of patient's interview, clinical history, magnetic resonance imaging (MRI), computed tomography, electrodiagnostic studies, and X-rays have been extensively used (Hansson and Haanpaa, 2007; Calmels et al., 2009; Haanpaa et al., 2011). In addition, careful neurological examinations have also been used for evaluation and quantification of evoked pain behaviours after SCI (Barrett et al., 2003; Siddall et al., 2003). The lowest intensity of a stimulus at which a subject experiences pain is defined as the pain threshold. For instance, the integrity of large fibre and dorsal column function can be assessed by testing sensory thresholds to the mechanical stimuli. On the other hand, evaluating thermal threshold to a heat stimulus can be of a great value in assessment of spinothalamic tract function (Nathan et al., 1986). Additionally, these measurements can be utilized for detection of allodynia, a painful response to normally nonpainful (i.e. innocuous) stimuli, while hypoalgesia and hyperalgesia are estimated as an increase and a decrease in pain threshold, respectively (for review see Bryce et al., 2007).

1.2.6.1 French DN4 questionnaire

Among the different questionnaires that have been used for diagnosis of neuropathic pain post SCI, the French DN4 questionnaire seems to be the most reliable (Bouhassira et al., 2005). This questionnaire involves ten items, with seven questions to be answered by the patient and three forms of examinations performed by the physician (Table 1-2). Each of the items is based on a comparative study between two groups of patients, one suffering neuropathic pain and the other arthritic pain. Positive responses for 4 out of 10 items lead to a diagnosis of neuropathic pain.

1.2.6.2 IASP mandatory and supportive criteria

More recently, the IASP has proposed two sets of criteria to be used as diagnostic tools for neuropathic pain following SCI (Treede et al., 2008). These are based on standards and measures such as the existence of a relevant anatomically defined lesion, the existence of pain symptoms at the level of the injury and eventually the areas around it (Table 1-3).

Answer the 4 questions below with yes or no each time:

Question 1: ***does your pain present one or more of the following characteristics:***

1. Pain feels like burning.
2. Sensation of painful cold.
3. Pain feels like electric shock.

Question 2: ***in the same area, is your pain associated to one or more symptoms:***

4. Tingling.
5. Prickling (picks and needles).
6. Numbness.
7. Itching.

Clinical Exam.

Question 3: ***is the pain located in an area where the exam unveils:***

8. Hypoesthesia to contact.
9. Hypoesthesia to pricking.

Question 4*: is the pain provoked or increased by:*

10. Brushing.

Table 1-2. Shows the DN4 questionnaire for diagnosis of neuropathic pain after SCI (threshold = 4/10)

Mandatory criteria for neuropathic SCI pain:

A. ***Criteria for SCI:***

1. A history of a relevant lesion or disease affecting the spinal cord and/or cauda equine.
2. At least one diagnostic test confirming a lesion or disease of spinal cord and/or cauda equine.

B. ***Criteria for neuropathic SCI pain*:**

1. The pain is located at or below the neurological level of SCI.
2. Negative or positive sensory signs in the area of pain compatible with spinal cord or root lesion.
3. Other causes of pain, such as nociceptive or peripheral neuropathic pain, excluded or considered highly unlikely.

Supportive criteria for neuropathic SCI pain:

1. The onset of pain within 1 year following the SCI.
2. No primary relation to movement, inflammation, or other local tissue damage.
3. Endorsement of one or more of the neuropathic SCI pain descriptors.
4. Allodynia or hyperalgesia within the pain distribution.

Table 1-3. Depicts criteria for neuropathic SCI pain (Treed et al., 2008). All points in A and B are mandatory for definite diagnosis of neuropathic SCI pain. If just the first point in each A and B plus 2/3 of the remaining scores (i.e. A2, B2 and B3) are present, this brings a probable presence for neuropathic SCI pain (Finnerup and Baastrup, 2012).

1.2.6.3 von Frey neurological examination

Von Frey (vF) filaments are a series of nylon monofilaments which are graded in stiffness and have different bending forces, depending on their diameter. A single filament can be brushed along the skin to generate a dynamic stimulus or applied statically at a single location to produce a static stimulus. For instance, pinprick test can be done utilizing a single high-threshold filament while sensory and pain thresholds can be assessed by using of a complete kit of these filaments spanning a range of forces. It is preferable to first examine these filaments on a normal reference site in the same patient before evaluating an affected area (Bryce et al., 2007).

1.2.6.4 Brush neurological examination

A brush or other similar material such as cotton wool can be utilized to detect tactile allodynia by light stroking of the skin. However, there are several limitations to obtaining consistent results with this method. For example, the brushing force and stroking velocity are all potential variables (Samuelsson et al., 2005).

1.2.6.5 Thermal neurological examination

Heat as well as cold stimuli are commonly used to assess thermal nociceptive (i.e. heat pain and cold pain) threshold by utilizing, for example, Peltier-type thermal device (Fruhstorfer et al., 1976). In this paradigm, pain threshold at unaffected skin areas of the same patient or a normative healthy subject is first investigated by gradual heating or cooling until the subject perceive hot or cold pain (i.e. method of limits). Subsequently, the affected area of skin is tested and then the results are compared to data from unaffected skin. According to the methods suggested by German Research Network on neuropathic pain, thermal stimuli are gradually applied in an ascending or descending manner at a velocity of 1°C/sec; from 32°C to 0°C or 50°C (Yarnitsky et al., 1995). Patient is initially trained for a standard use of the device by asking them to press a button when an unpleasant cold (i.e. cold pain perception threshold) or heat (i.e. heat pain perception threshold) sensation is perceived. If SCI-related paresis interferes with performing of the task, the patient can inform the examiner verbally about perception of any unpleasant sensation (Wasner et al., 2008).

1.2.7 Neuropathic SCI pain and severity of SCI

It might be difficult to draw a consistent and clear relationship between neuropathic pain and severity of SCI. For example, Siddall et al. (1999a) and Margot-Duclot et al. (2009) suggested that patients suffering from incomplete SCI are more common to develop allodynia and dysesthesia than those with complete SCI. In contrast, Ravenscorft et al. (1999) reported high prevalence of neuropathic SCI pain among cases with complete SCI while other reports denied both previous assumptions (Turner et al., 2001; Widerstrom-Noga et al., 2001a). The lack of a clear relationship between pain and completeness of SCI was also found by Werhagen and his team (2004) when 400 patients (from 1995 to 2000) were distributed into two groups; ASIA A and ASIA B-D and investigated for presence of neuropathic pain. Approximately, the same proportions (42% and 39%, respectively) of patient in the two cases experienced unpleasant sensory disorders.

1.2.8 Neuropathic SCI pain and location of SCI

Reports on the relationship between the level of injury and prevalence of neuropathic pain are not consistent. For example, some studies have suggested that thoracic and cauda equina injuries (Davis and Martin, 1947; Botterel et al., 1954; Burke et al., 1973; Nepomuceno et al. 1979) are more correlated with the prevalence of neuropathic pain. By contrast, several other reports (Richards et al., 1980; Summers et al., 1991; Stormer et al., 1997) failed to find a relationship between levels of SCI and prevalence of neuropathic SCI pain. Up to 6 months post SCI, Siddall et al. (1999a) did not observe any statistical significance between the prevalence of neuropathic SCI pain and occurrence of the spinal injury at cervical, thoracic, lumbar and sacral levels. However, the prevalence of allodynia was higher in patients with cervical lesions (39%) than in those with thoracic lesions (8%). In another study (Siddall et al., 2003), a relationship was noted between below level neuropathic pain and quadriplegia (50%) in comparison to paraplegia (18%). However, no relationship between neuropathic pain and cervical injuries was seen when Werhagen and associates (2004) evaluated a larger number of patients (about 400) with tetraplegia and paraplegia. This result was supported in following studies by De Miguel and Kraychete (2009) and Margot-Duclot et al (2009). In contradiction with the previous data, Ragnarsson (1997) reported more severe pain in SCI patients with paraplegia in comparison to those with quadriplegia.

1.2.9 Risk factors triggering neuropathic SCI pain episodes

Risk factors such as fatigue, infection, spasticity, pressure ulcers, constipation and urine retention were found to be significantly correlated with episodes of neuropathic SCI pain when independently investigated by Ravenscroft (2000) as well as Widerstrom- Noga and Turk (2004). Cruz-Almeida and others (2009) interviewed and examined about 150 mixed gender patients suffering SCI with different ASIA grades in order to investigate the effect of factors such as touch and cold on symptoms of neuropathic SCI pain. They reported aggravation of neuropathic SCI pain by cold weather in about two thirds of patients while just above one third of victims suggested touch as the main triggering factor. Last but not least, a relationship between mood dysfunction and pain following SCI was indicated by many authors (Richards et al., 1980; Summers et al., 1991; Cairns et al., 1996). This correlation was supported later by Siddall et al. (2003) where a strong link between presence of pain and poor mood was observed.

1.2.10 Treatment of neuropathic SCI pain

Due to a poor understanding of the underlying mechanisms of neuropathic SCI and also the fact that most of the current analgesics are based on data collected from peripheral models of neuropathic pain, treatment of neuropathic SCI pain is far from being achieved effectively. In addition to therapeutic strategy, a number of non-pharmacological methods management (e.g. rehabilitation and psychological support programmes) are also considered in order to cope with this unpleasant phenomenon. Surgical interventions such as spinal cord stimulation, dorsal rhizotomy, lateral spinothalamic tractotomy and spinal cordotomy can be a choice in intractable pain cases. Acupuncture was also indicated as a potential therapy (Dyson-Hudson et al., 2001).

Based on results of randomized clinical trials, the Neuropathic Pain Special interest Group (NeuPSIG) of the IASP published evidence-based guidelines for the pharmacological treatment of neuropathic pain in general (Dworkin et al., 2010) and this strategy is fully applicable to neuropathic SCI pain (Baastrup and Finnerup, 2012). The first-line in this approach includes tricyclic antidepressants (TCAs), dual reuptake inhibitors of serotonin and norepinephrine, calcium channel α_2-δ ligands (i.e. gabapentin and pregabalin), and topical lidocaine while opioids, including tramadol, were recommended as second-line of treatment.

Because they increase availability of 5-hydroxytryptophan (5-HT) in the central nervous system (CNS), authors such as Max et al. (1987) have proposed that TCAs have analgesic properties independent of their antidepressant activity, though this is controversial (Sandford et al., 1992). In a clinical study, Cardenas et al. (2002b) reported no improvement of painful symptoms in 84 SCI patients administered amitriptyline at a dose of 50 mg/day for 6 weeks. However, Attal et al. (2009) have argued that the dose used is too low. In another study, Rintala and colleagues (2007) conducted an eight-week trial to evaluate amitriptyline on 38 SCI patients (different severities and levels) with symptoms of neuropathic SCI pain and reported that amitriptyline at a relative high dose (150 mg/day) was more effective than placebo in relieving neuropathic pain at or below the level of SCI. However, the moderate improvement was mainly related to a subgroup of SCI patients with depressive behaviours and so might be linked to the antidepressants effect. In a randomized controlled study of 20 paraplegic patients (i.e. complete SCI at the thoracic and lumbar level) suffering at level and below level neuropathic pain, gabapentin at a dose of 3600 mg/day reduced the intensity as well as the frequency of neuropathic pain (Levendoglu et al., 2004). In support, pregabalin (150-600 mg/day) is reported to improve symptoms of neuropathic SCI pain and sleep quality regardless of the injury level or completeness (Siddall et al., 2006).

1.3 Animal models of SCI

Several experimental models have been developed for basic research aimed at achieving a better understanding of the basic mechanisms of neuropathic SCI pain. Rat and mouse are the most commonly used species because of the cost, accessibility, low susceptibility to infection and the consistency between the studies (Fernandez et al., 1991; Onifer et al., 2007; Rice et al., 2007). Methods used to produce injury vary from blunt contusion to sharp transection or ischemic and excitotoxic injury. Typically, these injuries are produced at a low thoracic level (i.e. T9/T10) to avoid the more severe adverse effects which can be associated with more rostral injuries and to spare normal motor activity in the forelimbs (Gensel et al., 2006).

1.3.1 Contusion SCI

Experimental contusion (i.e. traumatic SCI) can produce incomplete SCI and simulates early neuropathology and chronic evolutionary events reported in the clinical field (Noble and Wrathall, 1985; Hayes and KaKulas, 1997; Vaccaro et al, 1998). Additionally,

contusion leads to similar electrophysiological and functional outcomes as that observed in the human traumatic SCI (Metz et al., 2000). Contusion injury produces motor dysfunction below the injury level manifested by paresis or paralysis of the hindlimbs and urinary bladder dysfunction. Both show recovery with time progressed (reviewed in Yeizerski, 2005; Nakae et al., 2011). Furthermore, this type of SCI is commonly reported to result in a central neuropathic pain syndrome at (i.e. forepaw or trunk dermatomes) as well as below (i.e. hindpaw dermatomes) the injury level but different incidences of allodynia and hyperalgesia have been reported in different labs. For example, Siddall et al. (1995) and Yoon et al. (2004) in independent studies emphasized the importance of restricted spinal cord contusion (i.e. moderate severities) for generating allodynia following SCI. By contrast, Hubscher and Johnson (1999) as well as Lindsey et al. (2000) reported that animals with severe contusion injuries were more likely to develop hypersensitive responses to thermal and mechanical stimuli than those with restricted injury. The exact reason for this discrepancy is not well known, however, the difference in the contusion devices, rat strain and sex as well as the type of behavioural testing performed may have contributed (Yoon et al., 2004). Overall, mechanical allodynia (mostly based on reflexive behaviours) as well as abnormal sensitivity to thermal stimuli were evident in 85% to 90% of animals for several weeks or longer (Mills et al., 2001a; Nesic et al., 2005; Hall et al., 2010).

Devices for producing contusion injuries have evolved since Allen (1911) described one of the first weight drop model. Gruner (1992) used the same principles to develop the New York University (NYU) impactor where a 10-g rod guided through a tube is dropped perpendicularly onto the exposed dorsal surface of the spinal cord. Different vertical drops (6.25 or 12.5 or 25 or 50 mm) can be used to generate different injury severities. However, this device has some limitations such as bouncing of the rod and an inability to control the final velocity of the impactor (reviewed in Kwon et al., 2002).

The Ohio State University (OSU) impactor is a more advanced computer controlled device (Noyes, 1987b; Bresnahan et al., 1987; Stokes, 1992). This impactor is a displacement controlled (usually ranging from 0.8 to 1.1 mm) and following impact process, it is actively withdrawn to overcoming the main disadvantage of the NYU impactor (i.e. 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.

A more recent device introduced by Scheff and colleagues (2003) is Infinite Horizon (IH) impactor which is a force-feed back controlled device with a sensor attached to the impactor which measures the actual force applied to the spinal cord. As a results, the IH device avoids inconsistent injury severities due to movement of the target during impact events which is common in other devices producing a consistent contusion force without prior touching of the spinal cord and with mechanical retraction of the impactor which avoiding bouncing on the cord. The device provides graphical and numerical output of the actual force and displacement occurred which thus provides an immediate evaluation of success of the process.

1.3.2 Clip compression

This model was introduced by Rivilin and Talor in 1978 to model the compression component of traumatic SCI. Injuries of different severities are produced by compressing the spinal cord for variable durations (3 sec, 1 min, and 5 min) and/or adjusting the closing force of the clip (2-98 g) (Fehlings and Talor, 1995) or forceps (Blight, 1991). Following clip compression of the spinal cord, injured animals can suffer immediate and significant motor deficits in the hindlimbs which gradually improve over the next few weeks post SCI. low thoracic clip compression injury can produce mechanical as well as thermal (heat and cold) hypersensitivity at the plantar surface of the hindpaws and tactile allodynia over the trunk (Bruce et al., 2002; Hama et al., 2007).

1.3.3 Transection and hemisection SCI

The exposed spinal cord can be fully or partially transected by using microdissecting instruments such as spring ophthalmic microscissors or scalpel blade. In addition to study of axonal regeneration (Lopez-Vales et al., 2005; Steward et al., 2006), development of muscle spasm and musculoskeletal pain was reported in complete transection model (Edgley et al., 2006; Crone et al., 2007; Murray et al., 2010). Comparatively, hemisection offers several advantages such as a mild lesion and fast functional recovery postoperatively (Christensen et al., 1996; Christensen and Hulseboch, 1997). Below level of hemisection, injured animals become hemiplegic in ipsilateral side from 5 days to 4 weeks (Hains et al., 2000; Coronel et al., 2011) and may also suffer certain degree of contralateral weakness (kato et al., 1985; Saruhashi et al., 1996; Arvanian et al., 2009). In neuropathic SCI pain research, lower thoracic hemisection produce clear manifestations of neuropathic pain above (i.e. forepaws according to the old taxonomy), at (girdle region near SCI) and below (i.e. hindpaws) the injury level in contralateral as well as ipsilateral dermatomes

(Christensen et al., 1996; Bennett et al., 2000; Mills et al., 2001a; Gwak et al., 2004; Lee et al., 2010). However, Hubscher and Johnson (1999) did not observe any manifestations for development of at level tactile allodynia in dorsal and lateral hemisectioned rats while it was very marked in the severe contusion group.

1.3.4 Excitotoxic SCI

This model was mainly developed in order to simulate the pathophysiology of elevated levels of EAAs and their neurodegenerative and neuroinflammatory consequences following ischemic and traumatic SCI (Yezierski et al., 1993, 1998). Intraspinal injection of EAA receptors agonists such as quisqualic acid (QUIS) or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) can produce morphological changes such as cavitation, cystic formation and gliopathy, as seen in traumatic human SCI. Because of the uncontrolled extent of tissue damage produced in most of other models of SCI, excitotoxic SCI was introduced to produce confined gray matter damage and to spare some axons passing the lesion level. As a result, it would be possible to correlate abnormal sensations after SCI to certain neuronal damage.

Excitotoxic SCI produces clear signs of spontaneous pain behaviours such as overgrooming/autotomy as well as evoked pain signs such as mechanical allodynia and thermal hyperalgesia. Both pains were observed in ipsilateral as well as contralateral dermatomes representing segments at level (i.e. girdle area) or below level (i.e. hindpaws) of the lesion (Yezierski et al., 1998). Spontaneous pain behaviours can be seen in up to 90% of injured animals while evoked pain manifestations were reported in almost all cases (Yezierski et al., 1998). Moreover, nocifensive enhancement was reported in dermatomes at the level of SCI (Vierck et al., 2000).

1.3.5 Ischemic or photochemical SCI

This is a less frequently used model in which a vascular occlusion in the spinal cord is produced by intravenous (i.v.) injection (through the tail) of 32.5 mg/kg erthrosin B (i.e. photosensitizing dye) followed by an immediate irradiation (for 1, 5, 10 or 20 min) of the target segment with an argon ion laser at a wavelength of 514.5 nm (Watson et al., 1986; Hao et al., 1991). This process leads to a photochemical reaction which produces oxygen at the endothelial surface and forms a thrombosis in the vascular bed with subsequent ischemia and tissue necrosis in the spinal cord (Xu et al., 1992). Depending on the duration of the exposure to the laser (i.e. severity), the injured animals may or may not develop

motor deficits below the SCI (Prado et al., 1987; Hao et al., 1991). On the other hand, 75%-90% (depending on the severity) of the injured animal are reported to show a clear mechanical allodynia (i.e. vocalization in response to light touch) at dermatomes innervated by the ischemic spinal segments. This is especially marked in the first 24 hours (i.e. acute phase) and continues for several days to months (i.e. chronic phase) after the SCI (Hao et al., 1991; Xu et al., 1992). In addition, autotomy and spontaneous vocalization as well as hypersensitivity to cold stimuli were reported (Hanis and Vera-Portocarrero, 2010; Jaggi et al., 2011).

1.4 Sensory assessment of experimental SCI

Following SCI, abnormal sensory perceptions can be stimulus-independent (i.e. non-evoked or spontaneous) or evoked in response to stimuli (Merskey and Bogduk, 1994). Despite the fact that non-evoked SCI pain in rodents is extremely difficult to quantify, it can be recognized by development of behaviours such as overgrooming/autotomy (Siddall et al., 1995; Kauppila, 1998), continuous vocalization and aggression (Christensen et al., 1996). In addition, reductions in locomotion (Larsen and Arnt, 1985; Mills et al., 2001b) and weight (Siddall et al., 1995) have been suggested as additional indicators. By contrast, the stimulus-dependent (i.e. evoked) pain hypersensitivity can be assessed as a state of enhanced response to normally non-nociceptive (i.e. allodynia,) as well as normally nociceptive stimuli (i.e. hyperalgesia). These two behaviours are commonly observed in human patients with chronic pain symptoms (Bowsher, 1996). Two modalities of stimuli are commonly used for assessment of pain; thermal (i.e. heat or cold) or mechanical (brush, pinch and pressure).

In order to evaluate manifestations of evoked SCI pain, it is essential to utilize quantifiable and sensitive testing methods. A number of methods have emerged to fulfil these criteria, however, most are dependent on nocifensive behaviours such as the flexor withdrawal in response to certain stimuli (for review see Vierck et al., 2000; Sandkühler, 2009). In addition, some authors highlighted the importance of using addition behaviours such as paw licking, vocalization, biting, head orientation and escaping regarded as being processed supraspinally, as confirmative indicators for the perception of evoked pain (Siddall et al., 1995; Vierck et al., 2000; Lindsey et al., 2000). However, some of these evoked behaviours such as paw licking, vocalization and head orientation to the site of stimulus are preserved in decerebrated rats and may therefore brainstem reflexes (Carroll and Lim, 1960; Woolf, 1984; Berridge, 1989; Matthies and Franklin, 1992). In their

attempt to setup a method for pain assessment which meets the ideal requirements, Mauaderli and his colleagues (2000) described a modern version of the 'escape-test' (originally described by Warner, 1932 and modified by Bohus and Wied, 1967; Randall and Ricco, 1969). This paradigm involves a complicated process of escape from a preferred but noxious environment, to a non-preferred but safe one, which can be considered as a behaviour requiring supraspinal processing of the noxious stimulus by the tested animal.

SCI-unrelated factors may affect pain outcomes after SCI. For example, acute fear (Lewis et al., 1980) and anxiety (Ploghaus et al., 2001; Rhudy and Meagher et al., 2000) can mask (i.e. stress-induced hypoalgesia) and enhance (long-lasting hyperalgesia) pain perception, respectively. Long exposure to a cold environment was reported to produce a state of elevated response to painful mechanical stimuli (i.e. mechanical hyperalgesia) (Satoh et al., 1992) while chronic restraint stress can result in thermal hyperalgesia when the tail-flick assay is used (Gamaro et al., 1998). With respect to the diet, a tryptophan-poor corn diet can result in reduced levels of brain serotonin with subsequent development of diet-induced hyperalgesia (Lytle et al., 1975). Comparatively, mechanical hyperalgesia was observed in rats after 10 days on a magnesium-deficient diet (Dubray et al., 1997). Apart from the diet, age, strain and sex can also play an important role as determinant factors for development of allodynia and hyperalgesia. Interestingly, Mills and colleagues (2001a) assessed three commonly used outbred strains of rats (i.e. Long-Evans, Wistar, and Sprague-Dawley) for development of mechanical and thermal allodynia after contusion and hemisection SCIs. This team highlighted that the Sprague Dawley strain exhibits faster locomotor recovery than the other two strains and it is more vulnerable to development of mechanical and thermal allodynia, especially in the hemisection model of spinal injury. More recently (LaCroix-Fralish et al., 2005) and in comparison to the males, female Sprague Dawley and Long-Evans rats displayed more pain sensitivity following nerve root injury.

1.4.1 Paw von Frey sensory testing

The paw withdrawal reflex in response to mechanical stimulus applied to the plantar surface has been commonly used for assessment of mechanical sensitivity post SCI. In addition to the paw withdrawal reflex, responses such as licking, escaping and head orientation were also utilized as supraspinal indicators. In spite of limitations such as a large number of attempts needed to evaluate the mechanical threshold and problems related

to the method of application, a calibrated set of static hairs (i.e. vF filaments) is most commonly used in pain research for studying mechanical sensitivity (for review see Sanduhler, 2009). These are a set of 20 nylon calibrated monofilaments with graded and different binding forces (i.e. 0.008-300g) which originally designed by Maximilian von Frey (1850-1932). An electronic version of this filaments been also been developed (Vivancos et al., 2004).

To evaluate the mechanical sensitivity of the paw using classic vF hairs, rats are placed inside a clear plastic cage with a wire mesh bottom which allows full access to the plantar surface of the paw. After an initial period of acclimatization, monofilaments with graded bending strength are pressed perpendicularly against the globrous skin of the plantar surface of the paw with sufficient force to form a U-shape. Duration of a stimulus application varies between studies: 3-5 sec (Yoon et al., 2004), 5-6 sec (Lindsey et al., 2000), 6-8 sec (Chaplen et al., 1994). Brisk (sharp) paw withdrawal and also immediate flinching upon removal of the hair are considered as a positive response (Hao et al., 1991; Kim and Chung, 1992; Kupers and Gybels, 1993; Lewin et al., 1993; Chaplen et al., 1994; Choi et al., 1994; Mills et al., 2001a; Christensen and Hulsebosch, 1997; Yoon et al., 2004). Three main protocols have been extensively used to estimate mechanical sensitivity of the paw (Detloff et al., 2010). One of the most popular procedures is detection of the frequency of responses to a series of stimuli; known as the ascending vF testing procedure. In this method, a lighter hair of selected vF filaments is presented 10 times (with 30-60 sec interval to avoid windup phenomenon) and then the percentage of response is calculated in an all or none manner. In an ascending and consecutive manner, the next stiffer filament is used and so on until the 15.1 g hair or 100% response is reached and thereafter the percentages of responses can be plotted against the applied forces (i.e. in mN or g) (Chaplen et al., 1994; Mills et al., 2001a). In the second and less popular protocol, the lightest (mostly 2.0-6.0 g; predicted sub-threshold) vF hair is introduced three times with a 30-60 sec interval and the response is recorded in each case. The next stiffer filament is then applied in the same manner and so on until positive responses are achieved in 2/3 (66%) of cases and thereafter the tactile sensory threshold can be considered as the lowest stimulus intensity producing a positive response in 2 of 3 applications. This protocol was used by Detloff et al. (2010) for estimation of dorsal, instead of plantar sensitivity, during the acute early recovery phase after SCI (i.e. before weight support or trunk control recovery). The last protocol was uniquely introduced by Chaplen and colleagues (1994) which currently has become the most accepted method for estimation of mechanical

sensitivity. In this paradigm, 50% withdrawal threshold was determined according to the ***up and down*** method of Dixon (1980) (see section: 2.4.1).

1.4.2 Randall-Selitto test

This test was introduced by Randall and Selitto (1957) to assess the efficacy of analgesics in response to mechanical stimulation in inflamed tissue. This test is mainly based on quantification of withdrawal responses produced by graded applications of mechanical force to certain dermatomes (usually dorsal or plantar surface of a paw). Despite offering some advantages such as avoiding manual application of the force (i.e. main disadvantage in a classic vF testing), animal restraint is the main drawback of this test (Taiwo et al., 1989) due to possible triggering of endogenous antinociceptive mechanisms mediated via stress (Kelly and Frankli, 1984). The typical version of this procedure (Ferreira et al., 1978), a rat is held against a platform and the hindpaw is localized between the platform and a bar. By means of pressurized air, the force of the bar pressing against the paw is gradually and constantly increased until reaching the endpoint (paw withdraw). An electronic device (known as Randall-Selitto electronic analgesy meter) has been developed (Anseloni et al., 2003). This approach has been used recently by Santos-Nogueira et al. (2012) to investigate signs of neuropathic pain after lower thoracic (i.e. T8) spinal cord had been subjected to 100 and 200 kdyn contusion. In this study, the mechanical sensitivity of both plantar and dorsal surfaces of forepaws as well as hindpaws was assessed. The mechanical sensitivity of fore and hind paws were similar in control animals but the dorsal surface was more sensitive than the plantar for both paws. Fore and hind paws showed clear signs of mechanical hypersensitivity post SCI, but it was more marked in the plantar surface than the dorsal surface of hindpaws while the developed mechanical allodynia was similar for both surfaces of the forepaws.

1.4.3 Plantar heat test

Heat sensitivity has been extensively studied in different pain models as an indicator for neuropathic pain. Kerasidis et al. (1987) used the hot-plate device (originally described by Woolfe and MacDonald, 1944) but for motor evaluation purposes after contusion SCI and there was no direct discussion of pain data. This device was also recently used in the hemisection model of SCI and licking as well as jumping was used as end points (Giglio et al., 2006). To perform this test, the animal is placed on a pre-heated plate (at 50°C) within a plastic enclosure and cover. The time required to observe the endpoint is then recorded manually (usually cut off time is 20 sec). However, limitations of this assay include lack of

automated detection of the end point as well as stimulation of scaly and non-glabrous skin. In addition, both dermatomes above and below injury level are tested simultaneously under stressful conditions.

To overcome the above obstacles, Hargreaves and his colleagues (1988) introduced a new and sensitive heat test, known as the Plantar-Hargreaves assay, which can be used more efficiently for assessment of heat hyperalgesia. This method utilized an infra red (IR) beam as a source of radiant heat which is applied the plantar surface of a paw with an automated detection of the nociceptive threshold, expressed as thermal latencies (in sec) needed for a withdrawal response (see section: 2.4.2).

Dirig et al. (1997) have emphasized the importance of returning the paw temperature to the baseline on repeated testing and indicated a decrease in the responses latencies (i.e. an increase in thermal nociceptor activity) as a result of continual testing at intervals less than 5 min. In their evaluation for this paradigm, Dirig and associates (1997) highlighted the importance of maintaining a constant floor temperature at 30°C. More recently this idea has been taken into account by using a modified Hargreaves testing device which contains a glass floor maintained constantly at 30°C (lau et al., 2012). Moreover an enhancement in response latencies of naïve animals was reported when tested for 4 consecutive days (Kocevski and Tvrdeic, 2008). Behaviours such as licking and head turn are also utilized by some authors (Christensen et al., 1996) as indicators for supraspinal processing. The main advantages of this method of pain testing are that several responses to cutaneous heat stimulation can be evaluated in unrestraint conscious animals as well as automated detection of the endpoint. Additionally, this assay permits minimum handling of the animal, independent testing of either paw.

1.4.4 Tail flick test

This test was originally introduced by D'Amour and Smith (1941) in order to develop a method for assessment of the antinociceptive properties of analgesic drugs. In the prototypical procedure, radiant heat was focused 3-5 cm from the tip of the tail held into a groove. The heat source was activated and the latency for brisk twitch (i.e. spinally-mediated withdrawal reflex) of the tail was then measured in sec. Some publications using this assay in SCI research have started to emerge recently (Giglio et al., 2006; Roman et al., 2011; Hama and Sagen, 2011; Oudega et al., 2012).

1.4.5 Testing over the trunk

Testing over the trunk has been performed by different labs using varying protocols different in terms of tested dermatomes, stimulus intensity and types of positive responses. For instance, Hao et al. (1991) assessed mechanical sensitivity over the back rostrally and caudally to ischemic SCI and vocalization as well as escape behaviour was considered as positive responses. The same principles but in a contusion SCI were used by Siddall and others (1995). They investigated signs of tactile allodynia over the trunk by stroking the tested animal with the end of a pencil. Different scores were assigned for vocalization and escape behaviour. In addition, the vocalization threshold was assessed by using a set of vF filament. A slightly modified protocol was used by Christensen et al. (1996) in the hemisection. At different locations bilaterally over ventral, dorsal and lateral sides of the trunk, the number of vocalization evoked by 10 consecutive applications of a vF monofilament (i.e. 49.8 mN = 5 g, considered innocuous by those authors) were recorded. Interestingly, there was no sign for development of allodynia on the ventral side while it was clear and evident dorsally and laterally both above and below the injury site (see also Christensen and Hulsebosch, 1997). Hubscher and Johnson (1999) studied tactile sensitivity of the dorsolateral trunk in rats subjected to graded contusion and hemisection (lateral and dorsal) injuries at T8 segment. The results revealed enhanced responses (i.e. vocalization, vigorous head turning toward the stimulus and an effort to escape) to the mechanical stimuli (a blunt forceps) applied to rostral dermatomes (T6-T7) which was limited to the contusion group. This was matched by electrophysiological data (recording of extracellular neuronal spike) indicating an increase in the number of responsive neurons as well as enhanced firing properties of a subset of neurons in the brainstem (i.e. caudal medullary reticular formation) to low threshold mechanical stimuli applied rostral to the contusion level. A simple procedure was used by Bruce et al. (2002) where an innocuous mechanical stimulus (36 mN = about 3.67 g) was applied over the back and avoidance responses (i.e. biting, jumping away or escaping and vocalization) were tabulated out of 10 applications. Other avoidance responses which have been utilized in assessment of trunk hypersensitivity include grab/push of the stimulating instrument with the forepaws (Hubscher et al., 2008), aggression and trunk shakes (Oatway et al., 2004).

Oatway et al. (2004, 2005) attempted to correlate the localization of neuropathic pain over the trunk with the IASP pain classification of human “at level” pain which was suggested to be anywhere within a band of 2-4 segments rostral and caudal to the injury site (i.e. supposed to be the transitional zone between normal sensation and sensory loss) (Siddall et

al., 2000). In this study, an innocuous mechanical stimulus (i.e. vF filament with force of 15 mN = about 1.531 g) applied 10 times randomly at dorsal trunk dermatomes corresponding to T9-T11 while the injury area (T12/T13 dermatomes) was avoided. Each stimulus lasted for 3 sec and 5 sec was left as an interval. The number of avoidance responses (i.e. flinching, escaping, vocalization and abnormal aggression) was thereafter tabulated out of 10 applications.

Another approach for studying of hyperpathia (i.e. tactile allodynia) on the dorsolateral back of animals subjected to T8 contusions was suggested by Hulsebosch et al. (2000) and the same replicated by Crown et al. (2005, 2006). Simply, they made a map of the girdle zone which contained several testing points over the back rostral and caudal to the injury level. These were tested by application of a vF monofilament with a bending force of 26 g which did not produce vocalization in naïve animals. For each tested point, the percentage of vocalizations was characterized. Hulsebosch's group found that a region of 5-6 dermatomes rostral as well as 2 dermatomes caudal to the injury site was the most sensitive to application of the mechanical stimulus. The same protocol was utilized by Crown et al. (2012) accepted that escaping as well as biting were added to vocalization as indicative of evoked pain behaviours developed increased sensitivity in T5-T10 dermatomes (regarded as at level) after T10 contusions made using the IH device.

In a unique procedure, Lindsey's team (2000) classified the responses they observed as segmental responses (skin contraction or body flinch) or supraspinal responses (vocalization, orientation or biting the stimulus or escaping). They introduced a method that permits specific points on the back to be tested over time. Five days before surgery is performed, a grid of 30 points separated by 10 mm was drawn on the back. In order to determine the preoperative threshold, 6 selected points were tested on the first day using a vF hair with a bending force of 11.4 g. The same test but with next stiffer filaments (20.82 g) was repeated and responses were recorded. On the same day, this procedure was repeated until the same 6 points were tested with 50.45g and 108.28 hairs. In the next five days, the same procedure was repeated until 30 points become tested in the same previous manner. After SCI, an immediate increase in evoked pain behaviours were reported which lasted up to 10 weeks post T9/T10 contusion.

1.4.6 Testing of cold allodynia

Cold hypersensitivity is one of the important manifestations of neuropathic pain (Verdugo and Ochoa, 1992; Greenspan et al., 2004). In rodent models of neuropathic pain, there are a variety of testing methods for assessment of cold allodynia. Early tests introduced dipping the tail of restrained animals into a cool water bath and the latencies for tail flick response were counted (Pizziketti et al., 1985). However, animal restraint can trigger endogenous antinociceptive mechanics which can modulate the measured end point (Gamaro et al., 1998). In more recent tests, a source of cold stimuli which normally are not painful is lightly introduced to the glabrous plantar surface of the hindpaw of unrestrained animals. The cold stimuli used include a bubble of acetone (Choi et al., 1994) or ethyl chloride (Hao et al., 1996) formed at the end of a piece of small polyethylene tubing or a blunt needle. In addition, an ice probe in a 2 ml microcenterfuge tube with a wooden applicator stick has been also utilized (Casals-Diaz et al., 2009). In SCI models of neuropathic pain, acetone is most frequently used (Kim et al., 2003; Yoon et al., 2004; Hama and Segan, 2007; Jung et al., 2008; Baastrup et al., 2010; Coronel et al., 2011) followed by ethyl chloride (Hao et al., 1998; Yu et al., 1998; Yezierski et al., 1998; Wu et al., 2004) and ice probe (Lindsey et al., 2000). The responsiveness to cold has been estimated in various ways. For example, the cold stimulus may be applied 5 times (once every 5 min) to each paw and then the frequency of spinally-mediated response (i.e. number of paw withdrawal / 10 applications) is expressed as a percentage (Lindsey et al., 2000; Choi et al., 1994; Hao et al., 1998; Yoon et al., 2004; Rahman et al., 2006**;** Dias et al., 2007; Kim et al., 2009; Cornel et al., 2011).

Other approaches have included recording the number of elevations of the paw during 40 sec period after application of the cold stimulus Sakurai et al. (2009) or recording the time (sec) during which the animal licked the stimulated paw in period of 30 sec after spraying ethyl chloride Gustafsson and Sandin (2009).Flatters and Bennette (2004) combined graded on 0-4 point scale: no response (0); quick withdrawal (1); flick or stamp (2); prolonged withdrawal or repeated flick of the paw (3); repeated flicking of the paw with persistent licking (4). Another method involved observation for 5 min after acetone application and cold sensitivity was estimated as the total time of flinching, licking or biting of the limb (Vissers et al., 2005).

Interestingly, cold hypersensitivity has also been assessed over the trunk in an ischemic model of SCI. Hao et al. (1998) as well as Wu et al. (2004) assessed cold allodynia in dermatomes rostral to a photochemical SCI site. After spraying ethyl chloride on shaved

skin over the trunk, score of 0 indicated no response, 1 a transient skin twitch while 2 and 3 indicated transient and sustained vocalization, respectively.

In an advanced method of cold assessment, a cold plate can be used which offers advantages such as better control of the temperature delivered to the paw, providing surface on which the animal is freely moving, permitting the tester to observe of any response with least stress. Behaviours such as footlifting (FL) have been used as an indicator of cold hypersensitivity in models of peripheral neuropathic pain (Bennett and Xie, 1988; Hama and Sagen, 1993; Choi et al., 1994; Jasmin et al., 1998). FL can be assessed as an absolute number or as duration but the former has been considered more reliable (Chio et al., 1994; Jasmin et al., 1998) (see Discussion). A plate cooled by a bed of ice (5±0.5°C) was used by Brewer and Yezierski (1998) to assess an excitotoxic model of SCI and latencies for paw lifting and licking were measured to assess cold sensitivity. Allchorne and associates (2005) used a more sophisticated cold plate adjusted to temperature of -5°C to 25°C and based on their observations in naive adult male Sprague Dawley rats, suggested -5°C to 9°C as temperatures eliciting cold pain whereas 10°C or more were reported as innocuous, allowing testing of cold allodynia and cold hyperalgesia, respectively.

1.4.7 Operant thermal assays

Because of concern over using traditional reflexive behaviours as indicators for pain perception, Mauderli and colleagues (2000) introduced a novel assay based on cortical processing of pain due to thermal stimuli. This procedure is based on a simple fact (originally mentioned by Orgen, 1985) is stated that rodents have an innate aversion to light and preference for the dark. As a result, the animal is simply challenged to make a decision (involving cortical processing) between a preferred dark compartment where noxious stimuli are experienced and a less attractive light compartment free of noxious stimuli. Mauderli's team (2000) utilized a dark compartment with a floor that can be heated or cooled and a brightly lit but thermally neutral platform for escaping. The escape procedure was made more complicated by tilting floor of the neutral compartment toward the hanging septum separating the two compartments. To run an experiment, an animal was placed in the dark compartment with heated or cooled floor and the duration of time (latency in sec) before escaped to the brightly illuminated neutral platform was determined. In addition, the cumulative amount (e.g. out of 10 min trial) of time spent on the escape platform as well as the number of times the animal moved back and forth were also

determined at different temperatures. The same group (Vierck et al., 2005) used a similar approach to evaluate thermal sensitivity of rats in a model of peripheral neuropathic pain.

Klein et al. (2010) used a less aggressive protocol involving two chambers; one with a thermally noxious floor and the other with a thermally neutral floor. They were equally illuminated and separated with a simple partition allowing free movement of the animal between the compartments. Rats were placed in one of these chambers and the time spent in each compartment over 20 min test session was determined.

1.4.8 Operant mechanical or place escape avoidance paradigm (PEAP)

Based on similar principles to the thermal operant assay, the preference of rodents for darkness can be challenged by repeated but intermittent application of mechanical stimuli. The earliest one of this test was by LaBuda and Fuchs (2000a, b) in models of peripheral neuropathic pain and by there and others for inflammatory pain (Wilson et al., 2007; Uhelski and Fuchs, 2009; Boyce-Rustay et al., 2010). In an interesting study, La-Graize et al. (2004) investigated the preference to the dark after a lesion of anterior cingulate cortex in rats subjected to L5-spinal nerve ligation. Data revealed that a lesion to the anterior cingulate cortex can significantly increase the amount of time spent in the dark when the ipsilateral hindpaw was stimulated (for review see Fuchs and McNabb, 2012). The apparatus for this assay was simply two identical chambers with a mesh floor, one of them made black from outside and the other left transparent (or white), and both with an equal distribution of natural light. The two chambers were separated by a simple septum in the middle allowing free movement between the compartments (simple escape procedure). The rat was placed in the dark side and immediately tested with 476 mN (= about 48.571 g) noxious monofilament, applied each 15 sec to the plantar surface of the paw whenever the animal was in the preferred dark side. The percentage of time spent in each chamber was then calculated out of a 30 min long session. Slight modifications were added by Baastrup et al. (2010, 2011) in order to adapt this paradigm to assess signs of neuropathic pain in T9/T10 contusion model. vF hairs where bending forces of 0.69 g and 75.86 g were used for testing of the dorsolateral trunk (i.e. at level) and hindpaws (i.e. below level) dermatomes, respectively. Contradicting with data emerged from other assays, the injured and sham animals spent similar amount of time in dark when the PEAP perform in the hindpaws. Injured animals spent more time in the light than shams when stimuli applied to

the above, but there was no difference between animal groups when stimuli were applied to the hindpaws despite lower withdrawal threshold in injured animals.

More recently, modification of this approach was used to evaluate of mechanical sensitivity in rats subjected to T12 125 kdyn contusion using the IH impactor (Lau et al., 2012). The apparatus consisted of a dark box and brightly illuminated box connected by a rectangular track. The floor of the track can be scrapped between a smooth surface or one covered with metal spikes (0.4 mm tip diameter and with 10.0 mm between each). For 3 consecutive days, the rats were placed in the light area and trained to exit the illuminated box and a smooth floor to the dark side. The floor of the track was then replaced with respective 2 and 3 mm heights spiky surfaces. Subsequently, the rats were placed on the lighted area and responses were recorded. Under these conditions, each rat had been subjected to a great deal of challenge. He either stayed in the hated illuminated area or performed the escaping task but subjected to noxious mechanical stimulation by the spikes during a crossing the track to the dark side. According to those authors, the amount of time spent on the spiky track was conversely correlated with the evoked mechanical sensitivity.

1.5 Projection neurons

Lamina I is a major termination area for Aδ and C primary afferents carrying sensory information from thermoreceptors and nociceptors and projection neurons in this lamina can respond to cutaneous stimuli of different modalities (Christensen and Perl, 1970). Projection neurons in lamina I are mainly high-threshold neurons (nociceptive specific) and some are wide dynamic range (WDR) and low-threshold cells (cool or warm) (Dado et al., 1994; Bester et al., 2001). Comparatively, deep laminae receive collateral inputs from mainly pepidergic Aδ and C fibres (Lawson et al., 1997; Naim et al., 1997, Todd et al., 2000) as well as Aβ terminals (Willis and Coggeshall, 1991). Neurons of the deep laminae were reported to be sensitive to noxious stimuli of different modalities (Polgar et al., 2007a) as well as innocuous stimuli (Willis and Coggeshall, 1991). Collectively, superficial laminae are the main region for distribution of nociceptive-specific neurons whereas the deep laminae contain mostly WDR neurons (for review see D'Mello and Dickenson, 2008). This anatomical organization is supported by the extensive immunoreactivity of lamina I against neurokinin 1 receptor (NK1r) antibody while a lesser degree of NK1r staining was observed in deep laminae (Todd et al., 1998; Spike et al., 2003).

1.5.1 Lamina I projection neurons

In addition to glutamatergic excitatory or gamma aminobutyric acid (GABA)ergic and glycinergic inhibitory interneurons (Todd and Sullivan, 1990), 5-10% of the neuronal population in this lamina has projections which terminate in several brain centres (Bice and Beal, 1997; Zhang et al., 2006; Al-Khater et al., 2008). These neurons can be classified according to the shape of their cell bodies as well as the pattern of primary dendrites into three main morphological classes; fusiform, pyramidal and multipolar (or flattened) (Todd et al., 2002; Almarestani et al., 2007). Most projection neurons in lamina I (80%) show strong immunoreactivity against NK1r (Spike et al., 2003) which reveals their nociceptive nature (Todd et al., 2002). However, there is a small population of large multipolar projection neurons which are weakly stained by NK1r antibody and are termed as large gephyrin-coated cells (Puskár et al., 2001; Polgár et al., 2008). In addition, most of the NK1r positive cells exhibit internalization of NK1r receptors (Mantyh et al., 1995) as well as c-fos expression following noxious stimulation (Mantyh et al., 1997).

In the rat, the densest termination from lamina I neurons were in the lateral parabrachial (LPB) nucleus and medullary targets such as the caudal ventrolateral medulla (CVLM) (Todd et al. 2000; Spike et al., 2003; Polgár et al., 2010). Comparatively, fewer projections neurons are retrogradely labelled from periaqueductal grey (PAG) and thalamus (Al-Khater and Todd, 2009). Al-Khater and Todd (2009) estimated that there are 176 spino-LPB neurons in lamina I of the contralateral C7 segment and 335 at the L4 level. Approximately, the same numbers of cells were labelled at C7 and L4 after tracer injection into the CVLM (Spike et al., 2003; Polgár et al., 2010). However, 85%-100% of spino-CVLM cells can be retrogradely labelled from the LPB nucleus while only 80 to 90% of spino-LPB can be traced by targeting the CVLM (Polgár et al., 2010). This clearly shows an intensive collateralization from spino-LPB axons to CVLM area. Al-Khater et al. (2008) reported about 90 and 15 spinothalamic neurons in lamina I per side at C7 and L4 segments, respectively. Comparatively, about 83 spino-PAG cells per side reported in lamina I of C7 and a similar number at L4 (Al-Khater and Todd (2009). The same study revealed that 95% of spinothalamic neurons in lamina I can also be labelled form LPB while in the same lamina; only 5% of spino-LPB can be labelled from the thalamus. In addition, over 90% of lamina I neurons in the lumbar spinal cord that project to PAG were labelled from LPB or CVLM (Spike et al., 2003).

In an electrophysiological study, Bester et al. (2000) investigated the electrophysiological properties of lamina I spinoparabrachial cells in rat lumbar cord and characterized these cell into two main groups. The first is a nociceptive specific (75%) which responded to noxious mechanical and thermal stimuli (threshold was about 43°C). The second is a minor group (25%) of polymodal WDR cells which activated by noxious as well as innocuous mechanical and thermal stimuli. The heat threshold of these cells was indicated to be about 45°C while cold threshold extended from 15°C to 20°C. More recently, Andrew (2009) investigated the response spino-LPB neurons in lamina I to different modalities and intensities of stimuli in a chronic construction injury model of neuropathic pain. Five different categories of spino-LPB were reported. The majority of the tested neurons (more than 60 out of 95) were nociceptive specific which encoding noxious mechanical and heat but not cold stimuli while the rest classified as HPC (responded to noxious **h**eat, **p**inch and **c**old) and WDR. In addition, 3/95 neurons responded to cool stimuli but inhibited by warm stimuli (thremoceptive neurons) while a single unit (1/95) did not inhibit by stimuli between 36°C to 40°C and termed cool-specific.

In rat, many studies have implicated lamina I spinothalamic projection neurons in pain perception. Activation of spinothalamic neurons (WDR and nociceptive specific neurons) in lamina I by innocuous and noxious stimuli was reported at the cervical level (Dado et al., 1994). In this study, 25 of the 50 tested neurons (i.e. WDR) responded to both noxious and innocuous mechanical stimuli while 22/50 neurons (i.e. nociceptive) and 3/50 (i.e. low threshold) were sensitive to noxious and innocuous mechanical stimuli, respectively. In addition, the same report indicated that the vast majority of thermosensitive spinothalamic neurons at the cervical level are nociceptive heat specific neurons while 29% of tested cells were sensitive to cold, and a majority of them were WDR cells.

In cat, Craige et al. (2001) classified lamina I spinothalamic neurons into three categories according to their responses to thermal and mechanical stimuli. Nociceptive specific cells encoding noxious heat but insensitive to cold. These cells activated at 43°C (38% of the investigated population). The second type of spinothalamic neurons is polymodal HPC, encoding noxious heat, noxious pinch and noxious cold stimuli. These cells have median thresholds for heat and cold stimuli at 45°C and 24°C, respectively. In addition, these cells were reported to be inhibited by warm stimuli and may play an important role in heat and cold pain. The third class of spinothalamic neurons is thermoceptive cells encoding innocuous cool or warm stimuli which displayed the highest activation at 15°C.

1.5.2 Deep laminae projection neurons

While the smaller neurons in lamina III/IV are mainly interneurons, there is a subgroup of large NK1r immunereactive cells (about 20 and 16 cells in each side in L4 and C7, respectively) with projections which terminate in the brain at different levels (Todd et al., 2000, Todd, 2002, Al-Khater and Todd, 2009). These neurons have long dorsal dendrites which arborise in laminae I/II and also send projections to the CVLM, LPB nucleus, thalamus and PAG (Marshall et al. 1996; Todd et al. 2000).

Todd et al. (2000) targeted the PAG as well as other brainstem structures such as LPB, CVLM and dorsal caudal medulla with 1% cholera toxin B (CTB). 90-95% of the large NK1r-positive neurons in lamina III-IV on the contralateral side of L4 were labelled after targeting lateral reticular nucleus, while many (over 60%) of them were traced from the LPB nucleus. Comparatively, spino-PAG neurons were only a tiny minority of this population. A minority (29%) of the large NK1r-positive neurons were reported in the lumbar level after tracer injection in the thalamus (Naim et al., 1997; Al-Khater and Todd, 2009). At cervical level, about 75% and 85% of these neurons were labelled from the thalamus and the LPB nucleus, respectively, and about 65% contained tracers injected in the both targets (Al-Khater and Todd, 2009). In the same study, spino-PAG neurons were found to be 6% of the large NK1r positive cells at the cervical level and 8% at L4.

With reference to the deeper laminae, laminae V/VI have dense projections to the lateral reticular nucleus (LRN) and subuncleus reticular dorsalis (SRD) (together called caudal reticular nuclei) (Raboisson et al., 1996), the gigantocellular/lateral paragigantocellular reticular nuclei and parabrachial internal lateral subnucleus (Bernard et al., 1995). A fewer cells project from lamina V-VI neurons were regarded to thalamus (Marshall et al, 1996) and PAG (Bernard et al., 1995; Gauriau and Bernard, 2002).

1.6 Mechanisms of neuropathic SCI pain

The primary damage to spinal structures as well as secondary pathophysiological and biochemical changes of the surviving tissues can collectively lead to development of a state of abnormal sensory perception. In addition to the central sensitisations, hyperexcitability of peripheral nervous system can play a role. So far, these mechanisms are far from being comprehensively understood. The following context contains the main mechanisms of neuropathic pain after SCI classified according to the level of pain.

1.6.1 Mechanisms of above level neuropathic pain

Recently, peripheral sensitization was added as a new component participating in the development and maintenance of neuropathic pain above the injury level. For example, Carlton and associates (2009) have reported that sensitization of peripheral nociceptors in the median nerve is associated with development of mechanical allodynia and heat hyperalgesia in plantar surface of the forepaw after T10 contusion. Similarly, Bedi et al. (2010) reported spontaneous hyperexcitability of small neurons in cervical DRGs in rats that had received low thoracic contusion injuries. These rats were hyperesponsive to heat and mechanical stimuli applied to the plantar surface of the forepaws. Electrophysiological recording from C and Aδ primary afferents fibres showed that SCI led to spontaneous activity near the somas of the first order neurons. These peripheral changes at cervical level were associated with central sensitization of WDR neurons which indicates the hyperexcitability in the peripheral nervous system can be transmitted to the supraspinal structures (Carlton et al., 2009). During central sensitization, WDR neurons have increased background activity, expanded receptive fields, increased after discharges rates and increased responses to low threshold stimuli (Christensen and Hulsebosch, 1997; Hains et al., 2003). Neuroinflammation and gliopathy were also reported to play a role in central sensitisation where chronic activation of astrocytes and microglia were observed in the cervical enlargement after low thoracic contusion (Nesic et al., 2005). Another possible mechanism can be mediated by enhancement of the descending excitatory mechanism from On-cells in rostral ventrolateral medulla (RVM: Suzuki and Dickenson, 2005; Heinrich et al., 2009).

1.6.2 Mechanisms of at level neuropathic pain

Mechanisms of at level neuropathic pain after SCI can be broadly classified into two main categories. Firstly, spinal mechanisms lead to neuronal hyperexcitability near the injury site which are mainly mediated by excess EAAs, gliopathy and neuroinflammation, loss of GABA neurons and abnormalities of 5-HT system. Secondly, mechanisms mediated by changes occur supraspinally in brainstem and thalamic nuclei.

In a T13 contusion model, Drew et al. (2001) reported an abnormal increase in excitability of WDR in segments near the injury site in response to the applied stimuli to at level dermatomes. This may be mediated by direct loss of inhibitory GABAergic neurons as a result of the primary insult, resulting in neurotoxicity due to massive increase in concentration of EAAs at early stages after SCI (Yezierski et al., 1998; Drew et al., 2004).

A considerable reduction in the number of GABAergic neurons has been reported in different SCI models such as transient spinal cord ischemia (Zhang et al., 1994) and spinal cord contusion (Drew et al., 2004) as well as hemisection (Gwak et al., 2006; Gwak and Hulsebosch, 2011). Alternatively, reduction of GABAergic inhibitory tone may be attributed to an abnormality of chloride (Cl^-) homeostasis (abnormal expression Cl^- cotransporters) following SCI (Cramer et al., 2008; Lu et al., 2008; Hasbargen et al., 2010).

Another mechanism of neuronal hyperexcitability occurred in at level spinal segments may be mediated via gliopathy with subsequent altered interaction between neurons and activated glial cells (Gwak et al., 2008; Gwak and Hulsebosch, 2009). The hypertrophic glial cells are responsible for production of several proinflammatory cytokines (e.g. TNF-α and IL-β), ATP, EAAs and SP (Johnstone et al., 1999; Martin, 1992; Piani et al., 1992; Tanaka et al., 1994; Ferrari et al., 1997; Shafer and Murphy, 1997; Watkins and Maier, 2003). These mediators are strong candidates for enhancing neuronal excitability (Nedergaard, 1994; Zündorf et al., 2007; Roh et al., 2010) via triggering activation of a number of mitogen activated protein kinases (MAPKs). These include extracellular signal related kinase (ERK) as well as p38 MAPK which in turn lead to phosphorylation of cyclic adenosine mono phosphate (cAMP) responsive element binding protein (CREB) with subsequent modulation of gene transcription of NK1r, COX-2, c-fos, TrkB, prodynorphin (for review see Hulsebosch et al., 2008; Ji et al., 2009). In addition, ERK and pCREB can trigger neuronal hyperexcitability by phosphorylation of NR1/2 subunits of N-methyl-D-aspartate (NMDA) receptors (Hulsebosch et al., 2009). Crown and associates (2008) indicated a correlation between at level mechanical allodynia and an upregulation of phosphorylated p38 as well as ERK in microglia, neurons and astrocytes in spinal segments proximal to a T10 contusion. In a previous study, Crown et al. (2006) reported a marked reciprocal relationship between vocalization threshold estimated by application of vF hairs to the back of allodynic rats and expression of pCREB in the spinal segments located at level of excitotoxic SCI.

Sprouting of descending 5-HT projections can also occur near the injury site and modulate sensory transmission in at level segments (Bruce et al., 2002; Millan et al., 2002; Inman and Stewars, 2003). Interestingly, the mechanical sensitivity at the injury level was reported to be reversed by administration of 5-HT3 receptor antagonists (Oatway et al., 2004). Alternatively, at level mechanical allodynia after contusion injuries was reported to be correlated with development of neuronal hyperexcitability in medullary reticular

formation (Hubscher and Johnson, 1999) as well as in thalamic nuclei (Hubscher and Johnson, 2006).

1.6.3 Mechanisms of below level neuropathic pain

One simple explanation for development of below level pain following SCI is loss of descending supraspinal modulation (Hains et al., 2002). Other mechanisms which explained below level pain in terms of neuronal hyperactivity below the injury site are fairly complex. This is because the fact that hyperexcitability of these neurons may not be fully transmitted to the supraspinal structures due to the interruption of ascending pathways at the injury site. Indeed, deafferentation pain was reported in patients with ASIA A SCI and also cordotomy is not effective in treatment of central deafferentation pain which led to hypothesis that brain plays a central role in this condition (reviewed in Finnerup and Jensen, 2004). Unfortunately, almost all previous publications (see highlighted references in table 1-4) in the experimental field correlated the hyperexcitability of lumbar neurons and/or enhancement of the withdrawal reflex of hindlimbs to the applied stimuli with development of below level SCI pain after low thoracic SCI. It is difficult to accept that as a direct evidence for development deafferentation pain after SCI, instead, it can be a good evidence for development of spastic hyperreflexia. It might be important to consider the concept that below level or deafferentation pain is mainly driven by supraspinal components such as cortical and subcortical structures (for reviews see Finnerup and Jensen, 2004; Yezierski, 2005; Finnerup and Baastrup, 2012). If this is the case, our understanding for the mechanisms and related behavioural signs of below level pain should pay more attention to engage cortical and subcortical components rather than nociceptive and/or motor circuits at spinal level.

Reports of spontaneous central pain in patients with complete SCI (ASIA A) suggest that pain is generated at and/or above the injury site. Abnormal bursting activity in the thalamus has been reported in patients with below level central pain (Lenz et al., 1989, 1994, Radhakrishnan et al., 1999; Gorecki et al., 2010). In addition, changes in the metabolic activity in the thalamus of patients with deafferentation pain due to SCI suggest neuronal and/or glial dysfunction. The mechanism of these changes after deafferentation is not clear but may be caused by reduced GABA inhibition, imbalance between medial and lateral thalamus and disinhibited spinoreticulothalamic pathways (see Finnerup and Jensen, 2004). Another theory proposed that ectopic impulses are generated in the damaged spinothalamic tract or other ascending axons in patients with sensory discomplete SCI

(Wasner et al., 2008). This theory was supported by previous experimental studies which reported upregulation of a chemokine known as cysteine–cysteine chemokine ligand 21 (CCL21or Exodus-2 or 6-Ckine) in neurons near the SCI (Vela et al., 2002; Zhao et al., 2007b) and via spinothalamic tract, CCL21 can be anterogradely transported to the thalamus (de Jong et al., 2005; Hains et al., 2005; Zhao et al., 2007b). In the thalamus, it activates microglia and triggers phosphorylation of ERK1/2 and/or p38MAPK with subsequent release of PGE_2 which binds to its EP2 receptor on neurons and provokes neuronal hyperexcitability of thalamic nuclei (Zhao et al., 2007a).

By using functional MRI (fMRI), abnormal biochemical activity as well as cortical reorganization was also indicated in more rostral structures such as prefrontal cortex and anterior cingulate cortex (Stanwell et al., 2010). Flor et al. (1995) indicated a relationship between persistent deafferentation pain (i.e. phantom pain) in patients with arm amputation and reorganization in primary somatosensory cortex (S1). By using fMRI scanning, Wrigley and colleagues (2009) investigated 20 SCI patients (10 of them had below level neuropathic pain *vs.* 10 without pain) in order to study if the degree of S1 reorganization following SCI correlated with deafferentation neuropathic pain. The results showed a meaningful relationship between the degree of cortical reorganization and the intensity of neuropathic pain in deafferented dermatomes following SCI. In an experimental study, Yague et al. (2011) reported abnormal hyperexcitability of the S1cortex bilaterally following stimuli applied to the hindpaws after hemisection in. Chen et al. (2002) and Ding et al. (2005) proposed that sprouting of new neurons into the region of the deafferented cortex may play a role in the cortical reorganization. These changes were suggested to increase the excitatory tone while diminishing the descending inhibitory barrage (Jacobs et al., 1991; Chen et al., 2002).

1.7 Towards a standardization of neuropathic pain modelling and assessment after SCI

In order to understand neuropathic pain after SCI, animal models should provide molecular, biochemical, anatomical and functional consequences which contribute to development and maintenance of abnormal sensations after the injury. Selecting of valid and effective testing methods is the next challenging step because most of the current testing assays are relied on reflex-based responses which do not engage supraspinal structures such as thalamus and cortex. Indeed, nocifensive reflexes such as withdrawal response are present in spinalized animals.

1.7.1 Reliability of current models of below level neuropathic SCI pain

Contusion (Nesic et al., 2005), compression (Bruce et al., 2002), hemisection (Christensen et al., 1996) spinal cord injuries are commonly used to model below level pain. In spite of the fact that below level pain after SCI is currently considered to be mainly mediated by components located in the forebrain such as thalamus and cortex (see Finnerup and Jensen, 2004; Yezierski, 2005, 2009; Finnerup and Baastrup, 2012), most labs are still relied on hindpaw nocifensive measures (mainly withdrawal reflex) for evaluation of sensory changes below the injury level. Two issues that may potentially complicate interpretation of behavioural testing performed on the hindlimbs. This is 1) the development of spasticity with the associated changes in spinal cord circuits and uncertainty as to whether these are "upstream" or "downstream" of the components of dorsal horn circuitry signalling pain and 2) the possibility, depending on injury severity that nociceptive transmission to the brain may be interrupted. In view of these issues, it is reasonable to ask to what extent investigations so far conducted in the field have made use of hindlimb reflexive testing and whether the issues associated with these tests raise doubts regarding the validity of some of this work. Recently, Baastrup et al. (2010) reported an enhancement in withdrawal reflex of the hindpaws after low thoracic contusion but this was not associated with an increase in pain perception when hindpaws tested by operant test (i.e. PEAP). More intensive work is needed to be done in order to clarify the validity of different behavioural assessment methods used to investigate below level neuropathic pain after SCI. Furthermore, it is crucial to study the effect of the injury severity on the validity of these assays.

Table 1-4. provides information on a large cross section of literature reporting on the mechanisms which may underlie pain in SCI models. The table is organised according to some of the main mechanisms currently considered important. Each line of the table represents one study and shows the type of injury together with information about the testing that was used and the specific focus of the work. As can be seen from this table, in a very high proportion of studies, the only behavioural tests used to assess below level pain were tests directed at the hindpaws. Although this is not a comprehensive listing, it is extensive and it shows that about half of the studies rely exclusively on hindlimb nocifensive measurements. It can also be seen that many of the spinal cord injuries used are quite severe and in most cases are likely to interrupt nociceptive pathways originating from below level segments. It can therefore be concluded that most of those relying on hindlimb withdrawal assays cannot be assessing pain because the ascending pathways

necessary to convey nociceptive signals to the cortex for elaboration of signals into this sensory sensation are not intact. It could be argued that these nevertheless reflect nociceptive processing at a spinal level and that lower thresholds or shorter latencies reflect alterations in these. However, even this is not secure because of uncertainty as to the loci of the altered properties and whether they involve nociceptive spinal cord circuitry, more ventrally located circuits involved in motor control rather than sensory processing, or both. In many studies, behavioural testing has been used simply as a mean of demonstrating that the model used is appropriate as a model for below level neuropathic pain and that some phenomenon occurs in such a model. For example, Hains et al. (2003) showed that treatment with minocycline reduced the activity in dorsal horn neurons in the lumbar spinal cord which was associated with an increase in sensory threshold for hindpaw withdrawal response. Although this study depends on hindpaw testing for behavioural outcome, it at least has supportive evidence from the electrophysiological properties of spinal cord neurons. Even that is not secure enough because the transmission of the hyperexcitability from lumbar neurons to supraspinal level is compromised by interruption of nociceptive pathways at the injury site. In other cases, however, behavioural outcome is a more crucial measure and the work has sought to manipulate a mechanism which in turn modulates the signs of pain. These studies, highlighted in the table, are especially subject to potential criticism. In very few studies, stimuli were applied to below level locations over the back. However, these tests are less reliable because of the possibility of interruption of ascending pathways due to classic reliance on severe injuries. Moreover, some of the applied stimuli to what considered below level locations is actually processed in segment located above the injury level because tactile stimuli are not applied at enough secured distance below the injury site.

1.7.2 Reliability of current girdle assessments of at level pain

It is generally agreed that at level neuropathic pain after SCI is mainly linked to segmental hyperexcitability of spinal neurons as well as descending excitatory mechanisms from the brainstem (for review see Yezierski, 2005). In that sense, models such as excitotoxic (Yezierski et al., 1993, 1998) and ischemic (Hao et al., 1991; Xu et al., 1992) spinal cord injuries are frequently used to study at level SCI pain. In addition, at level pain can also be modelled using traumatic (Siddall et al., 1995; Hubscher et and Johnson, 1999; Lindsey et al., 2000; Hall et al., 2010) and hemisection (Christensen et al., 1996) spinal cord injuries. Grooming behaviours and vF testing over the trunk are generally used to assess this type of pain (30% of the studies; see table 4-1). Although this can be considered a reliable test of

at pain, most studies misjudge the testing locations and therefore mixed above and at level pains. As a result, one aim of this project is to clarify this confusion in terms of exact testing locations because each of these types of pain may be mediated by different mechanisms (see above).

1.7.3 Injury severity and neuropathic pain after SCI

Contusion injuries at low thoracic level are frequently reported to produce sensory changes in forepaws (i.e. above level), over the trunk proximal to the injury site (i.e. at level) and in hindpaws (i.e. below level). However, different labs failed to be agreed about the relationship between severity of SCI and development of neuropathic pain. For example, Siddall et al. (1995) and Yoon et al. (2004) reported more robust pain-like behaviours in rats with restricted contusion injuries while Hubscher and Johnson (1999) as well as Lindsey et al. (2000) reported that animals which received more severe contusion injuries were more likely to develop robust signs of neuropathic pain. This assumption was supported when Knerlich-Lukoschus et al. (2008) assessed sensory changes in rats subjected to 100, 150 and 200 kdyn using IH. As a result, it would be useful to investigate the relationship between development of pain-like behaviours and the severity of SCI at low thoracic level (T9). Moreover, the effect of contusion severity on validity of the behavioural outcomes from different levels needs further clarification.

1.7.4 Injury level and neuropathic pain after SCI

The clinical studies (Siddall et al., 1999a, 2003) as well as experimental molecular data (Siddall et al., 1999b; Drew et al., 2001; Crown et al., 2006) put forward claiming the notion that mechanisms of neuropathic pain are more robust spinal segments closer the injury site. It would be of a great value if these pathological mechanisms can be translated into robust pain-like behaviours by moving the injury site closer to the representative segments of the tested dermatome. Due to classic reliance no low thoracic models, tactile allodynia and thermal hyperalgesia following SCI have not been consistently observed in the forepaws and even under the same injury conditions, only a proportion of animal exhibited signs of evoked pain behaviours above SCI (Mills et al., 2001a). For such reason, many groups of researchers tended to test over the trunk within dermatomes close to the injury in order to approaching more sensitivity. However, trunk testing has some limitations such as approaching a quantitative mechanical measure (e.g. up and down method) over a hairy skin which seemed to be more sensitive where the animal still responded even to the lightest vF filaments. Additionally, it is not possible to test heat

sensitivity over the trunk. SCI at upper thoracic level may produce optimum signs of neuropathic pain in the plantar surface of the forepaws and hence provides a perfect solution.

1.8 Aim of the thesis

The general aim of the work presented in this thesis was 1) to provide a better understanding of current contusion models of SCI for the study of neuropathic pain, 2) to develop new or improved tests for investigating pain in SCI models and 3) to investigate the possibility of developing improved models of post SCI pain.

The appropriateness of classic low thoracic SCI to model different of neuropathic pain after SCI types (i.e. above, at and below) is needed to be investigated by using different injury severities (200 kdyn *vs* 150 kdyn) and valid behavioural assessments. Whether or not making the injury site at upper thoracic level (T3/T4 200 model) will optimize manifestations of neuropathic pain in the forepaws is another question required to be answered. Two low thoracic and one upper thoracic models are presented in three separate chapters as following:

1) T9 200 kdyn contusion.
2) T9 150 kdyn contusion.
3) T3/T4 200 kdyn contusion.

The justifications and aims of the investigation of each model are described in more details in the introduction to each chapter.

Gliopathy and neuroinflammation

Reference	SCI	Sensory testing	Testing locations	mechanism
Gwak et al., 2008	T13 hemisection	vF WT	HP	Propentofylline attenuate glial activation
Gwak & Hulsebosch 2009	T13 hemisection	vF + heat WT	HP	Glial activation
Zhao et al., 2007a	T9 WD (25mm)	vF + heat WT	HP	Spinal glial/neuronal interaction (PGE_2)
Zhao et al., 2007b	T9 WD (25mm)	vF + heat WT	HP	Thalamic glial/neuronal interaction(CCL21)
Hains and Waxman, 2006	T9 WD (25mm)	vF + heat WT	HP	Activation of lumbar microglia
Detloff et al., 2008	T8 OSU (0.5 & 1.1mm)	vF WT	HP	Activation of lumbar microglia
K-Lukoschus et al., 2008	T8 IH (100-200kdyn)	vF + heat WT	HP	Expression of CCL2/CCR2
Tan et al., 2009	T9 WD (25mm)	vF + heat WT	HP	Minocyclin inhibit activation of microglia
Gwak et al., 2009	T13 hemisection	vF + heat WT	HP	Remote activation of microglia and astrocytes
Choi et al., 2012	T9/T10 WD (25mm)	vF + heat WT	HP	ROS, P38 and ERK in microglia reduced by acupuncture
Marchand et al., 2009	T13 hemisection	vF WT	HP	Etanercept reduces glial activation and pain
Nesic et al., 2005	T10 IH (200kdyn)	vF WT	HP + FP	Upregulation of astrocytes
Rafati et al., 2008	T9 IH (150 & 200 kdyn)	vF WT	FP	Role of NF-κB in neuroinflammation
Crown et al., 2008	T10 IH (150kdyn/1sec)	Girdle	R	P38 & pERK in astrocytes

GABA dysfunctions

Reference	SCI	Sensory testing	Testing locations	mechanism
Cramer et al., 2008	T9 WD (12.5mm)	Heat WT	HP	Expression of NKCCI & KCC2
Gwak et al., 2006	T13 hemisection	vF WT	HP	Activation of GABA reduce SCI pain
Lu et al., 2008	T12 hemisection	vF WT	HP	Reduction of KCC2 after SCI
Hasbargen et al., 2010	T9 WD (12.5mm)	Heat WT	HP	Role of NKCC1 and KCC in SCI pain
Drew et al., 2004	T8 WD (2cm)	Girdle	R & C	Loss of GABA

Neuronal hyperexcitability

Reference	SCI	Sensory testing	Testing locations	mechanism
Zhang et al., 2005	L6/S1 transection	vF + heat WT	HP	Sensitization of WDR neurons
Hama & Segan 2011	T6-T7 compression/1min	vF + heat WT + TF	HP +tail	Blockade of Na^{+} channel
Hains et al., 2003	T9 WD (25mm)	vF + heat WT	HP	Changes in $Na_v1.3$
Hains et al., 2005	T9 WD (25mm)	vF + heat WT	HP	Changes in $Na_v1.3$
Gwak et al., 2007	T13 hemisection	vF WT	HP	AMPA Glu receptors
Bennett et al., 2000	T13 hemisection	vF + heat WT	HP+FP	NMDA Glu receptors
Mills et al., 2002a,b	T10 WD (12.5mm)	vF + heat WT	FP	Group II & III metabotropic Glu receptors
Hao et a., 2004	Ischemic SCI	Girdle	Mapped area	Neuronal hyperactivity
Drew wet al., 2001	T13 WD (5cm) “severe”	Girdle	R + C	Neuronal hyperexcitability
Crown et al., 2005	T10 IH (150kdyn/1sec)	Girdle	R	pCREB and neuronal hyperexcitability
Crown et al., 2012	T10 IH (150kdyn/1sec)	Girdle	R	Ca^{+2}- calmodullin activation
Hoheisel et al., 2003	T8, T10, L3-L6 WD (75mm)	Girdle	R	Neuronal hyperactivity
Hubscher and Johnson, 1999	T8 hemisection + compression (2 mm/5sec)	Girdle	R	Brainstem hyperexcitability

Serotonergic changes

Reference	SCI	Sensory testing	Testing locations	mechanism
Oatway et al., 2005	T12/T13 compression (35g/1min) “severe”	vF WT + Girdle	HP + R	Serotonergic fibres and inflammation
Chen et al., 2008	T12/T13 compression 35g/1min) “severe”	vF WT + Girdle	HP + R	$5\text{-}HT_3$ receptor
Oatway et al., 2004	T12/T13 compression (35g/1min) “severe”	Girdle	R	$5\text{-}HT_3$ receptor

Other mechanisms

Reference	SCI	Sensory testing	Testing locations	mechanism
Gwak et al., 2003	T13 hemisection	vF WT	HP	Role of NGF in development of SCI pain
Hutchinson et al., 2004	T8 OSU (1.1mm)	vF WT	HP	SCI pain reduced by exercise
Endo et al., 2009	T9 IH 200kdyn	vF WT	HP	C fiber sprouting
Clark et al., 2010	T10 IH 200kdyn	vF + heat WT	HP	Cathepsin induces SCI pain
Hall et al., 2010	T8 IH 200kdyn	Girdle	R	Asymmetrical damage needed for SCI pain
Carlton et al., 2009	T10 IH (150kdyn/1sec)	vF + heat WT	FP	Peripheral and central sensitization
Bedi et al., 2010	T10 IH (150kdyn/1sec)	vF + heat WT+ Girdle	HP + FP + R & C	Spontaneous activity in the DRG

Table 1-4. Literature survey on the use of hindlimb and other test sites in SCI models investigating pain. The table includes a large cross section of the literature encompassing a total of 41 previous studies. The reports are organised by topic according to some of the main mechanisms considered to play a role in central neuropathic pain. Each line represents one report. The columns from left to right indicate the first author, the SCI model used, the type of sensory tests conducted and the locations at which they were applied. Further details of the topic of the investigation is shown in the far right column and studies where a treatment was tested for its effect on behavioural signs of pain, i.e. studies where the behavioural testing is of particular importance, are shown highlighted. Abbreviations (alphabetical): AMPA= α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, C=caudal, $Ca^{+}2$=calcium, CCL2=cysteine cysteine chemokine ligand 2, CCL21= cysteine cysteine chemokine ligand 21, CCR2= cysteine cysteine chemokine ligand 2 receptor, DRG=dorsal root ganglia, ERK= extracellular signal-regulated kinase, FP=forepaw, GABA= γ-Aminobutyric acid, Glu=glutamate, HP=hindpaw, 5HT=5-hydroxy tryptophan, IH=infinite horizon, KCC2= K^{+}-Cl^{-} cotransporter 2, Nav=sodium voltage gated channel, Na^{+}=sodium, NF-κB= nuclear factor kappa B. NGF=nerve growth factor, NKCC1= Na^{+}-K^{+}-Cl- cotransporter 1, NMDA=N-methyl,D-aspartate, OSU= Ohio State University impactor, pCREB= phosphorylated cAMP response element binding protein, PGE_2=prostaglandin E_2, R=rostral, ROS=reactive oxygen species, SCI=spinal cord injury, T= thoracic, vF=von Frey, WD=weight drop impactor, WDR=wide dynamic range, WT=withdrawal test.

Chapter 2

Materials and methods

2 Materials and methods

All experiments described in this thesis were approved by the Ethical Review Process Applications Panel of the University of Glasgow and were performed in accordance with the European Community directive 86/609/EC and the United Kingdom Animals (Scientific Procedures) Act 1986.

2.1 Animals

Adult male Sprague Dawley rats (Harlan UK Ltd) were used in this project and they weighed 125-150 g and 200-275 g when arrived and at time of SCI, respectively. All animals were housed pair-wise in cages with 12 hours light/dark cycle with free access to food and water at all times. Before collection of preoperative data, these animals were handled and acclimatized to the test environment and apparatus for at least 1 hour per a day for about 7 days but with a break in the middle for the weekend. During these sessions the animals were subjected to the same handling and transfer between apparatus as in the real test situation (e.g. the animals were held by their tails, the vF filament was held over their back, they were subjected to the sound of the timer etc). By the end of a week of this type of handling, these animals showed no signs of fear or stress with minimum urination and defecation.

2.2 SCI procedure

All operations were carried out with aseptic precautions. Prior to the surgical procedures, animals were administered 1ml/kg saline (Baxter Healthcare, UK) as well as 0.3 mg/kg buprenorphin (vetergesic®; Alstoe Animal Health, UK) to control acute pain due to the surgical intervention. 4% and 1-2% isofluorane (Abbot Laboratories Ltd, Maidenhead, Berkshire, UK) was used for induction (in anaesthetics box) and maintenance (by mask on operation table) of anaesthesia, respectively. Vaseline was placed on both eyes in order to prevent dryness of cornea while the animal was being anesthetized. During surgery, body temperature was maintained at 37±1°C using a homeothermic heat pad controlled by a rectal probe. Injuries were performed at different segmental levels in different models and the target segments and surgical approach therefore differed. However, in general, hair overlying the target vertebrae was removed by shaving and the skin was treated with povidone-iodine solution (Betadine®; Seton Healthcare group Plc, Oldman, England). The skin was incised, and muscles overlying the vertebral column were bluntly dissected,

exposing the target vertebrae. Tissues were held in a parted position using a retractor. A dorsal laminectomy (exposing spinal cord but leaving the dura intact) was then performed on the target vertebra. The animal was then transferred to a frame and the vertebral column stabilized by clamping the vertebrae immediately rostral and caudal to the exposed spinal cord using Adson's forceps. Contusion injuries were performed using an Infinite Horizon device (Precision System & Instrumentation, LLC, Nottinghill, US). This is a microprocessor controlled force feedback device, capable of producing injuries of different severity according to the force set by the experimenter, with a high degree of consistency. During the contusion process, a tip of 2.5 mm diameter is precisely positioned 3-4 mm above over the exposed spinal cord by adjusting a 3 axis machine table on which the impactor is mounted. The impactor strikes the spinal cord and travels into the tissue until it reaches the preset force whereupon it is automatically and immediately withdrawn. Information on the actual force delivered, together with tissue displacement measured in µm, is provided graphically (Fig. 2-1). The graphs allow the user to assess any problems with the procedure such as inadvertent striking of bone rather than soft tissue. The wound was then closed using absorbable sutures on muscle layers and stainless steel wound clips on the skin. An identical procedure was followed for animals in sham groups except that the exposed spinal cord was not impacted. Postoperatively, rats received 50 mg/kg Carprofen (Rimadyl®; Pfizer Ltd, Kent, UK) as well as 1 ml/kg saline. Subsequently, the animals were placed in a warmed cabinet overnight at 37°C with soft food and water provided within easy reach. For the first week post-injury, the animals monitored for any signs of distress and bladders were manually emptied twice each day. In case of infection if any, 0.4 ml of 2.5% enrofloxacin (Baytril®, Bayer Plc, Newbury, Berkshire, UK) in 1 litre of tape water was administered. Body weight was measured every morning for the first 8 days post injury and then once per week. Diet supplements (fruit loop cereal) and regular food pellets were placed on the floor of each cage to provide easy access for the animals.

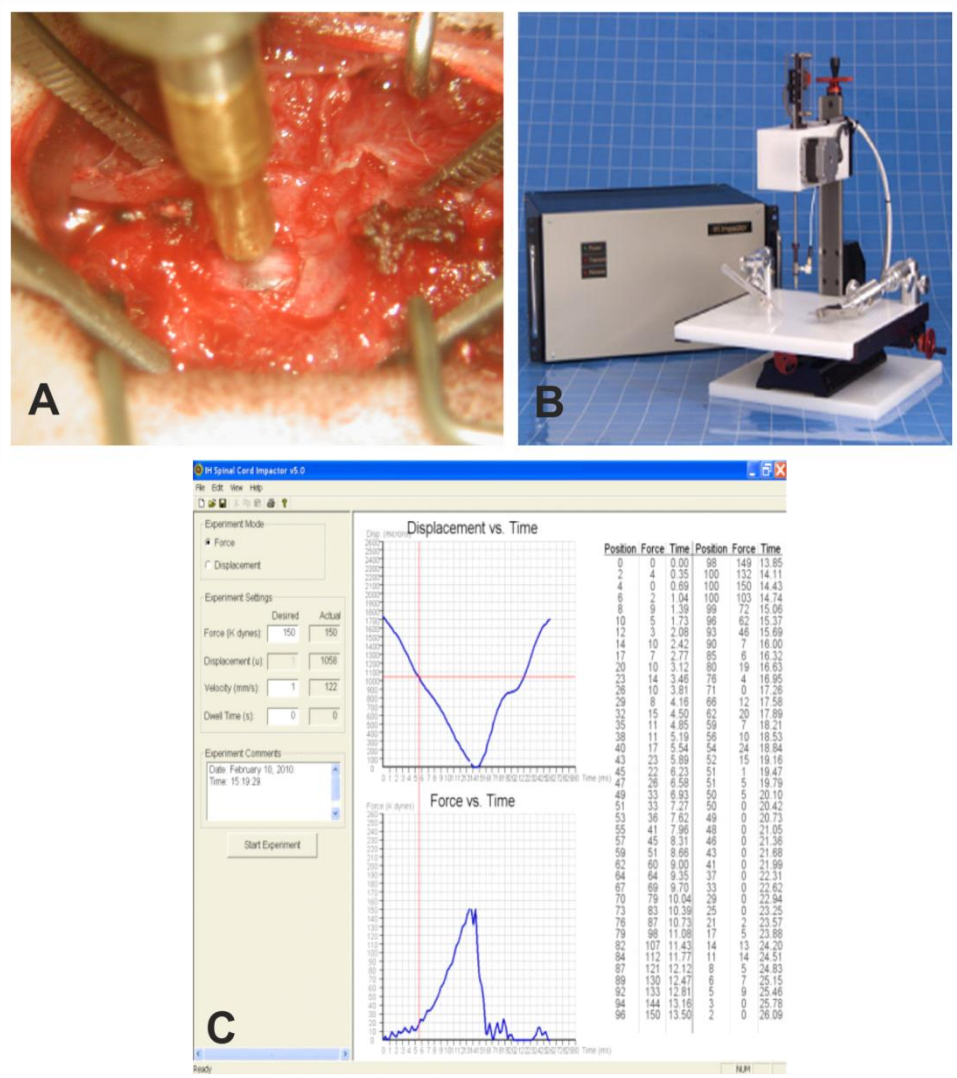

Fig. 2-1. Infinite Horizons instrument. A, shows a high velocity impactor positioned over an exposed spinal cord. **B**, depicts the two main parts of the instrument; the impactor unit and the controlling box. Parameters of the contusion process (displacement in µm/msec and force in kdyn/msec) are illustrated in **C**. These measurements provide an immediate evaluation of the contusion process and therefore ensuring highly consistent injuries.

2.3 Motor evaluation

Because behavioural testing for signs of pain involves a motor response, the animal must be capable of sufficient movement for these tests to be valid. The motor capacity of the animals following SCI was therefore assessed using a commonly employed open field locomotor function scale referred to as the Basso, Beattie, and Bresnahan (BBB) scale (Basso et al., 1995). Because of well documented insensitivities in the upper parts of this scale (i.e. scores above 14), a BBB subscore introduced by Lankhorst et al. (1999), was also used.

The BBB testing scale is a strict ruler which scores the appearance of movements in the order they have been observed to recover in rats after contusion SCI (Table 2-1). This rating scale runs from 0 through to 21: where 0 means no hindlimb movement while 21 points indicates consistent, coordinated gait with paralleled paw placement of the hindlimb and consistent trunk stability. Scores from 0 to 7 rank animals with isolated movements of three joints (i.e. hip, knee, and ankle). Animals exhibiting hindlimb sweeping (i.e. rhythmic extension and flexion of all 3 joints of hindlimb with or without plantar placement of hindpaw), plantar paw placement, hindquarter weight support (i.e. elevation of hindquarter from the floor) and forelimb/hindlimb coordination are scored from 8 to 14. Although they were not being used for the injured animals, scores from 15 to 21 are determined by whether there is toe clearance during the stepping phase, whether hindpaw position is predominantly rotated or parallel, whether the trunk is stable and whether the tail is raised. In any situation, terms such as occasional refers to $\leq$ 50% of time while frequent and consistent are descriptors for 51-94% and $\geq$95% of time, respectively.

The BBB subscore (Lankhorst et al 1999) consists of 7 points which derived from BBB locomotor rating scale (Table 2-2). This 7 point scale is an additive measure and is designed to provide greater sensitivity at the higher and of the scale i.e. at advanced stages of recovery and focuses on evaluation of toe clearance, paw position, trunk stability and tail position.

Data were collected simultaneously in both occasions by placing an animal in mid of circular enclosure (diameter = 88 cm) on a table with a smooth surface and observed for 4 min.

Description	Score
No observed hindlimb movement.	**0**
Slight movement of 1 or 2 joints, usually the hip and/or knee.	**1**
Extensive movement of 1 joint with or without slight movement of other joint.	**2**
Extensive movement of 2 joints.	**3**
Slight movement of all 3 joints.	**4**
Slight movement of 2 joints and extensive movement of the third.	**5**
Extensive movement of 2 joints and Slight movement of the third.	**6**
Extensive movement of all 3 joints oh the hindlimb.	**7**
Sweeping or plantar placement of paw but with no weight support in all occasions	**8**
Plantar placement of paw with weight support in stance only or dorsal steps with occasional, frequent, or consistent weight support	**9**
Plantar steps with occasional weight support + no forelimb/hindlimb coordination.	**10**
Plantar steps with frequent to consistent weight support + no forelimb/hindlimb coordination.	**11**
Plantar steps with frequent to consistent weight support + occasional forelimb/hindlimb coordination.	**12**
Plantar steps with frequent to consistent weight support + frequent forelimb/hindlimb coordination.	**13**
Plantar steps with consistent weight support + consistent forelimb/hindlimb coordination.	**14**
Consistent planter stepping and consistent forelimb/hindlimb coordination + no toe clearance or occasional toe clearance during forward limb advancement + predominantly paw position is parallel to the body at initial contact and rotated at lift off.	**15**
Same as a score 15 except toe clearance becomes frequent.	**16**
Same as a score 15 but toe clearance becomes frequent and predominantly paw position is parallel to the body at both initial contact lift off.	**17**
Same as a score 15 except toe clearance becomes consistent.	**18**
Same as a score 15 except toe clearance becomes consistent and predominantly paw position is parallel to the body at both initial contact lift off + tail is down.	**19**
Same as a score 19 but tail is consistently up while the trunk is instable.	**20**
Normal locomotion; Consistent planter stepping and consistent forelimb/hindlimb coordination + consistent toe clearance + predominantly paw position is parallel to the body at initial contact and at lift off + tail is consistently up + consistent stability of the trunk.	**21**

Table 2-1. Shows a 21 points BBB rating scale for evaluation of motor recovery after SCI (Basso et al., 1995). For each score, an operational definition is indicated.

Description	Subscore
Instable trunk during steps (i.e. lateral weight shift, causing waddling form side to side)	**+0**
Stable trunk during steps	**+1**
Tail is not consistently raised	**+0**
Tail consistently raised	**+1**
No toe clearance	**+0**
Occasional toe clearance	**+1**
Frequent toe clearance	**+2**
Consistent toe clearance	**+3**
Hindpaw is rotated outside of the body at initial contact and at liftoff	**+0**
Hindpaw is parallel to the body at initial contact but rotated outside at liftoff	**+1**
Hindpaw is parallel to the body at both initial contact and at liftoff	**+2**

Table 2-2. Shows BBB subscores and related descriptions for fine motor recovery after SCI (Lankhorst et al., 1999). A total of 7 points (summation) is needed for full recovery.

2.4 Sensory evaluations

A range of sensory assays were used to evaluate the effect of SCI on the sensitivity of animals to tactile and thermal stimuli, which are generally considered as indicators of evoked pain. In addition, we also attempted to assess signs of non-evoked pain or spontaneous pain.

2.4.1 Plantar von Frey (vF) test

This test was used to detect sings of tactile allodynia by assessing the 50% mechanical threshold for responses to stimuli applied to fore and hind paws. The mechanical sensitivity was evaluated using a set of 20 monofilaments made of nylon with different graded bending forces (0.008-300 g). To perform this test, rats were placed in a clear plaxiglass compartment (24 x 20 x 14 cm) with a wire mesh floor which allowed full access to the plantar surface of the paw (Fig. 2-2). Animals were allowed to acclimatise in the apparatus for about 15-20 min. After an initial period of exploration, the animals typically settled down in a corner, with all four limbs in a normal resting position and only occasional grooming. This is important to avoid misinterpretation of the test. In early pilot experiments, before an appropriate acclimatisation procedure had been devised, animals could adopt a “guarding or freezing” posture (i.e. with an arched trunk, head up, body weight shifted to the hindquarters and forepaws fully extended). Frequently this was accompanied by urination or defecation after stimulus application. Testing of animals under these conditions frequently led to false negative responses.

For each animal, the plantar (glabrous) surface of each paw (avoiding the keratinized pads) was tested independently by a slow (i.e. gradual rather than sudden) and perpendicular application of the vF hairs with sufficient force to cause slight bucking (U-shape) for about 6-8 sec (Chaplen et al., 1994). As far as possible, the filament was applied consistently to the same location, and the lower third of the foot was favoured as this appeared to be the most sensitive area when the filament was applied at different locations in pilot experiments (Fig. 2-3). An interval of 10 sec was left between successive applications of the filament. A brisk withdrawal of the paw was considered a positive response. If a positive response occurred, the animal was not tested again until the rat had resumed a stationary resting posture and was calm. Testing was avoided if the animal was moving, rearing, grooming, sleeping, or in the “guarding or freezing” posture described above. Some of

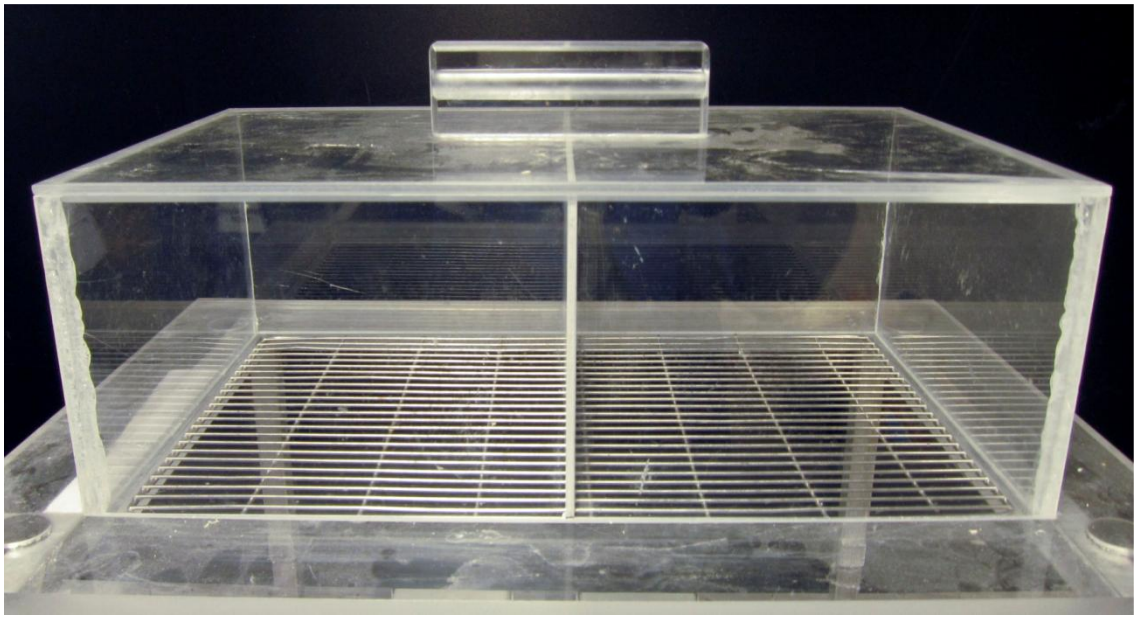

Fig. 2-2. A photograph shows the testing apparatus for plantar von Frey test. The mesh surface has hole's dimension of 10 x 50 mm and the thickness of the metal is 1.91 mm.

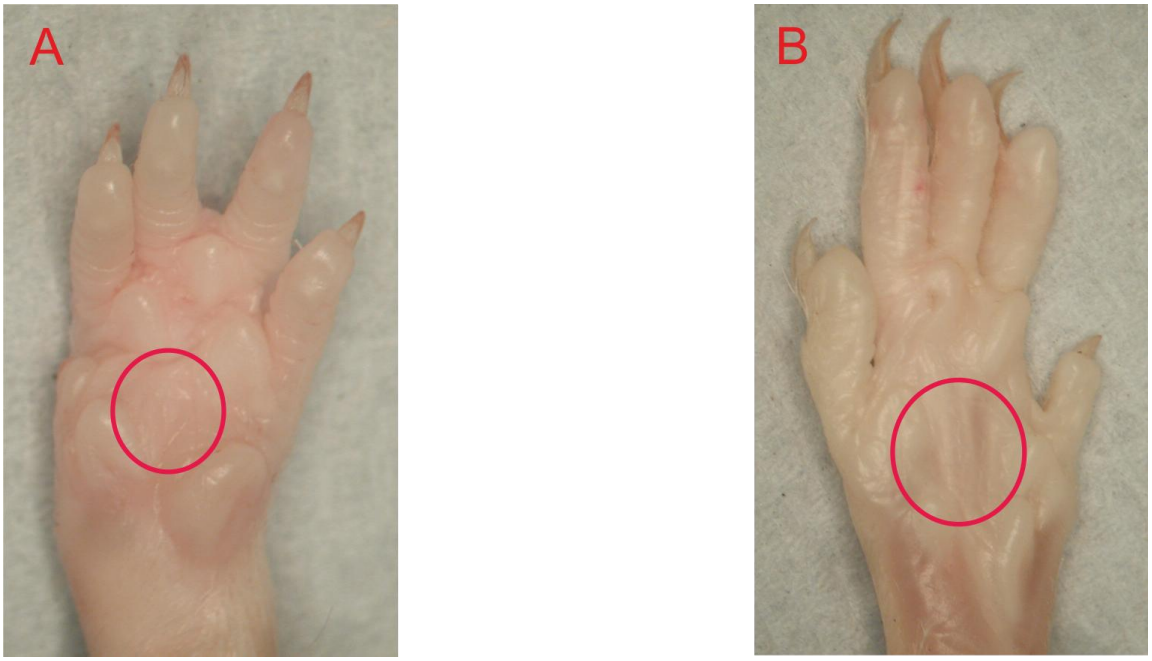

Fig. 2-3. Photographs illustrate the testing location in the paws. A, shows the targeted testing area (red circle) in the plantar surface of the forepaw while **B**, showing the same but in the hindpaw.

these “distracted” behaviours could occur if unexpected noise occurred near the testing environment.

In this paradigm, the 50% withdrawal threshold was calculated according to the ***up-down*** method of Dixon (1980). Briefly, a hair size of 4.56 (i.e. equivalent to 4 g; in the middle of the series) is initially applied to the plantar surface of the paw. In the absence of a response, the next stronger hair is presented whilst if a response is present, the next lighter hair is used. Testing is continued until 5 consecutive responses have been observed following the first crossing of threshold (change in outcome to stimulus presentation). Out of these 6 responses (i.e. including the one before crossing of the threshold), the 50% threshold is calculated using the following formula:

$$\textbf{50\% threshold (g)} = (10^{[X_f + \kappa\delta]}) / 10{,}000$$

Where X_f = value (in log units) of the final von Frey hair used; κ = a value from an appendix according to the pattern of positive and negative responses; δ = mean difference (in log units) between stimuli (i.e. 0.249 for our set of filaments).

Baseline (preoperative) data were collected after appropriate acclimatisation and normally within a few days of the SCI or sham operation. Postoperatively, testing was begun only once sufficient motor recovery (9 or more on BBB scale) had occurred. This was confirmed by locomotor evaluation. For animals with thoracic injuries, recovery was considered adequate 14 days following the injury. For animals with cervical level injuries, forelimb testing also commenced 14 days following injury, but the hindpaws, being less seriously affected, could be tested 7 days after injury (see Results). Thereafter, testing was performed on a weekly basis for the remainder of the survival period. For each test session, thresholds were expressed as an average of the right and left sides.

2.4.2 Plantar heat test

In order to test for signs of thermal hyperalgesia, the reaction time for paw withdrawal in response to a heat stimulus was investigated according to the protocol of Hargreaves et al. (1988). Animals were placed individually into plaxiglass chambers (each 22 x 17 x 14 cm) with a glass floor and allowed to acclimatize to the testing environment (about 10-15 min). After an exploratory period animals typically displayed limited locomotion and then adopted a resting posture (as described above). A movable IR radiant heat source was then precisely positioned underneath the glass floor in order to directly target the plantar surface

of the paw (avoiding the pads). A trial was commenced by switching on the IR beam which simultaneously starts an electronic timer.

Forepaws and hindpaws have different sensitivity to heat stimuli which may be attributed to some differences in ascending pain pathways from C7 and L4 segments. The spinothalamic pathway is denser at cervical than lumbar level in rat (Al-Khater et al., 2008) which may mean a greater representation of forepaws in comparison to the hindpaws in somatosensory cortex (Kaas, 1983; Remple et al., 2003) and hence more accurate stimulus localization in forepaws (Al-Khater and Todd, 2009). This assumption is supported in a series of preliminary experiment where the forepaws showed much more sensitivity than the hindpaws when subjected to the same intensity of heat stimuli. This resulted in latencies from forepaws were too short (5±1 sec in naïve animals) to allow accurate measurement of changes after the injury. As a result, the IR beam was adjusted so that the typical response latency was approximately 10±0.5 sec. For the hindpaws an appropriate setting was found to be 60 milliwatts per centimetre squared (mW/cm^2) while for the forepaws the setting was reduced to 45 mW/cm^2. A photoelectric cell (within the heat source) detects withdrawal of the paw and stops the timer. The withdrawal latency can then be read from the timer (accurate to 0.1 sec). A total of 5 latency measurements were made alternatively for each paw and values for both forepaws and hindpaws were averaged. An interval of 5 min was left between each measurement to avoid any potentiation of responses (Dirig et al., 1997).

Experience showed that a number of factors are important to obtain accurate results when performing this test. As with the vF test, testing should be avoided when the animal is moving, grooming, rearing or sleep. Any urination should be cleaned up and the animal allowed settling again. Since testing sessions were frequently of long duration (in many cases extended over 2 hours), the temperature within the testing units can build up (Dirig et al., 1997; Lau et al., 2012), so that ventilation of these units by occasional removal of the cover is crucially important. It was found that in preliminary experiments, ventilating the testing houses reduced the variability of response latencies by avoiding dramatic changes in floor temperature (Fig. 2-4).

By using an electronic thermometer (Bioseb in vivo research instrument, France, www.bioseb.com), 9 readings of the floor temperature (from outside) of the housing unit contained the animal were collected (room temperature = 23±0.5°C). These started at

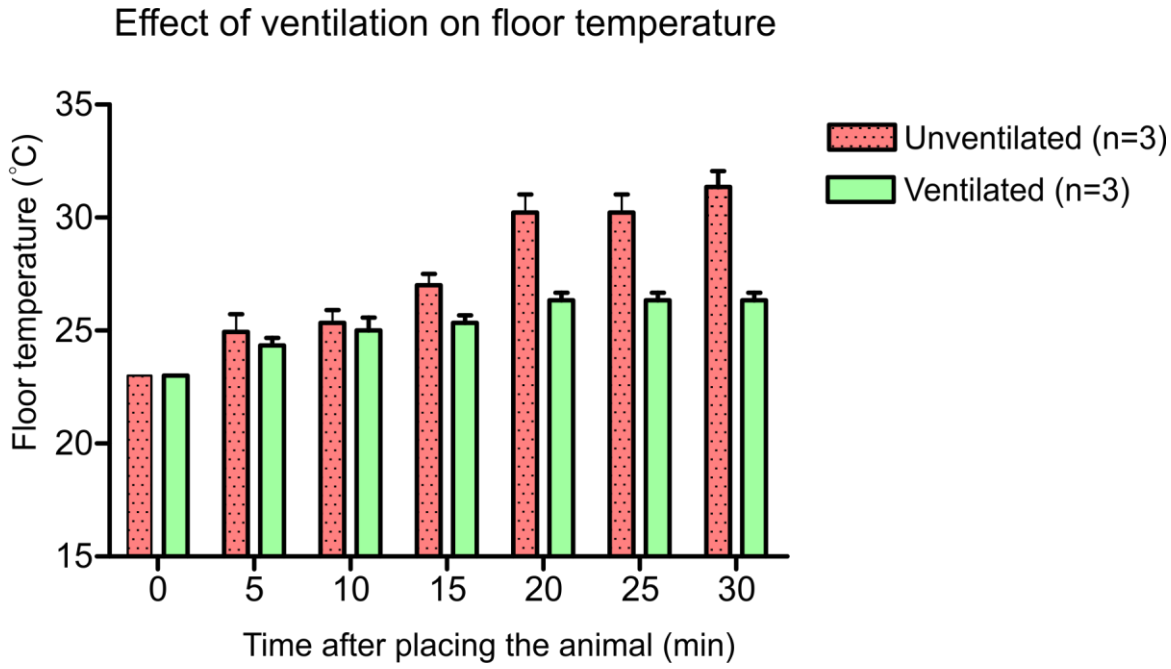

Fig. 2-4. Shows the effect of ventilation process on the temperature of the floor of the housing unit contained an animal for 30 min. Bars represent data collected from 3 animals and expressed as mean ± SE. For each animal, 9 readings (each 5 min) were collected from 9 different areas of the outside surface of the floor and thereafter a mean was taken.

the moment when the animal was placed in and continued at 5 min interval and then an average was taken at each time point

Furthermore, heating of the glass surface as a result of prolonged contact with the animal (i.e. the animal being stationary) tends to lead to a reduction in response latencies. This can be avoided by encouraging the animal to move at the same time as when ventilating the test cage. In some cases, the animals refused to move and tended to sleep regardless of ventilation or disturbance such as scratching the plaxiglass testing unit. On such occasions, the rat was moved back to its own cage and left for some time before being returned to the test apparatus and reacclimatized for resumption of testing.

In addition to the measuring withdrawal latencies, a note was made of whether the animal licked the tested paw. Results were expressed as the number of licking responses observed out of 10 observations (5 each on the left and right paw).

Testing was performed preoperatively and postoperatively as for plantar von Frey testing (see above) but the plantar heat and plantar von Frey tests were carried out on separated days in order to avoid any interaction between the two paradigms.

2.4.3 Footlifting (FL) test

Cold allodynia as well as ongoing pain are important features of neuropathic pain in humans following SCI (Frost et al., 1988). Both of these features were investigated in SCI animals by observation of the incidence of FL. To discriminate FL evoked by cold stimuli from FL that may be a reflection of spontaneous pain, tests were conducted on flat surfaces of different temperatures. In addition, since FL was observed when the animals were being tested for mechanical allodynia using the von Frey test (see above), observations of FL were also conducted while the animals were standing on the mesh platform used for this test.

To test for cold sensitivity, the animals were placed in plaxiglass chamber, (16.5 x 16.5 x 10 cm) the floor of which was formed by a cold plate (Bioseb in vivo research instrument, France, www.bioseb.com) maintained at 7.5°C. The size of the chamber allowed rats to move freely but the limited height helped to reduce rearing activity and thus maintain the paws in contact with the surface for most of the time. The forepaws and hindpaws were assessed separately. The animals were placed directly on the plate and left for 5 min. Starting at the second minute, the number of times each forepaw (or each hindpaw) was

lifted from the surface, over the next 4 min was counted (by using a tally counter). The animal was then immediately removed and returned to its cage.

This procedure was repeated three times for the forepaws and three times for the hindpaws and the average number of FL calculated. The interval between successive trials was 5 min in order to allow normal paw temperature to be re-established. FL associated with exploratory behaviours such as locomotion, repositioning of posture, head turning, rearing and grooming were excluded and only lifting during sitting or standing were counted. On some occasions, FL was accompanied by aversive behaviours such as foot shaking or licking. However, these behaviours were inconsistently seen and were not recorded.

To assess FL that may reflect spontaneous pain, the same procedure was used except that the hot/cold plate was maintained at a neutral temperature (30 ±1°C; Galbraith et al., 1993; Choi et al., 1994; Dirig et al., 1997; Allchorne et al., 2005).

To assess FL that might reflect mechanical allodynia, the same procedure was used except that animals were kept in the housing used for von Frey testing with a mesh floor.

These three tests were performed on 3 separate days. Extra care to maintain a calm and quiet environment (i.e. minimising distraction of the animals) during the conduct of this test was found to be important. On all occasions, video recordings of the test were collected and reviewed to confirm the accuracy of the live observations. Because the animals needed to have adequate balance to make observations reliable, FL data was collected only in the later stages of the experiment (in weeks 4, 5 and 6 PO).

2.4.4 Trunk von Frey test

2.4.4.1 Electrophysiological determination of dermatomes

The purpose of this paradigm was to assess mechanical allodynia over the trunk. It is necessary to be able to perform these tests at positions corresponding to what would be termed “at level” and “below level” in the clinical setting. However, in animals it is not possible to carry out sensory testing that established the dermatomal level of the injury in the clinical sense (i.e. as in the ASIA tests). In addition there is a lack of information in the literature on the location of dermatomes over the trunk and there relation to spinal segments (see Discussion). In order to be able to reliably select areas of hairy skin on the back corresponding to those where stimuli are processed in segments above the injury level

and below the injury level at T9, we therefore carried out electrophysiological experiments designed to identify these areas.

Initially, animals were placed in an anaesthetic chamber and anaesthesia was induced using isoflurane (5% in O_2). They were then transferred to a mask on an operating table for cannulation of a carotid artery and jugular vein for purpose of blood pressure recording (by attaching the cannula to a pressure transducer) and i.v. administration of drugs, respectively. In addition, the trachea was cannulated for later application of artificial ventilation. Following cannulation, anaesthesia was switched to sodium pentobarbital (10 mg/kg i.v., Sigma, UK), given as required for maintenance of anaesthesia (typically every 20 to 30 minutes). The depth of anaesthesia was assessed by monitoring the pedal withdrawal reflexes, the corneal reflex and blood pressure. A midline skin incision was made over the back and a laminectomy performed to expose the spinal cord at various segments from T4 to T13. The vertebrae at the rostral and caudal ends of each laminectomy were stabilised using clamps. The dura was opened and the spinal cord covered in warm mineral oil in order to prevent drying and to isolate electrical recordings. Before starting electrical stimulation and recording, neuromuscular transmission was blocked with pancuronium bromide (Sigma, UK; 0.1mg i.v. at 60 min intervals) and animals were artificially ventilated at a rate and tidal volume appropriate to maintain end-tidal pCO2 close to 4.0%. During this period of paralysis, regular doses of anaesthetic were given at a frequency commensurate with that required before paralysis and the adequacy of anaesthesia monitored by continuously recording blood pressure and its response to noxious stimuli. Core temperature was maintained close to 38°C and mean blood pressure 100-120 mm Hg.

Electrical stimulation of the skin was performed using a bipolar electrode manufactured from two clinical monopolar needle electrodes (Oxford Instruments Medical, UK). For purpose of mapping the effect of stimuli applied to the dorsal trunk, the skin was stimulated using 0.2 msec rectangular current pulses of 500 microampere (µA) at multiple sites: 2 cm lateral from midline (left side) and between 1 cm rostral and 5 cm caudal of the spinal contusion site, in 5 mm steps. Recordings of the cord dorsum potentials (CDPs) were made from the surface of the spinal cord by using a silver ball electrode which was moved in 1 mm steps along the exposed spinal cord, close to the left side dorsal root entry zone. Recordings were digitised and stored on a computer at a sampling rate of 20 kilohertz (kHz) without filtering using Signal v3.0 (Cambridge Electronic Design, UK).

Averaging (up to 50 individual potentials per recording) and measurements were performed off-line using Signal v3.0.

2.4.4.2 Sensory testing

Based on the results obtained from the electrophysiological study, sensory testing was performed using stimuli applied over the trunk were characterized at three different locations; 1 cm above the segmental level of the SCI, 2 cm below the segmental level and 5 cm below the segmental level (Fig. 2-5). In all these cases, the testing areas were 2 cm lateral to the midline which offers two advantages. Firstly, the intended dermatomes 1 cm above the injury site was at save distance from the incised skin. Secondly, our preliminary studies revealed that the animals were more sensitive laterally than medially.

In this assay, a monofilament (size 2.44 generates force of 0.04 g) was applied to these sites on the dorsolateral surface of the trunk. In preliminary experiments this filament was shown to consistently evoke 1 to 3 responses from the animal out of 10 applications. A stronger filament evoked responses that were too frequent and a lighter filament sometimes elicited no response. Prior to the testing session, rats were acclimatized to a plaxiglass cage (42 x 26 x 12 cm) for 20 min. After an initial exploratory period, the animals typically settled to a stationary position in the corner of the cage with some occasional grooming and were then ready for testing. The filament was applied perpendicularly to the hairy skin with sufficient force to cause slight buckling. The filament was applied 10 times (5 times each on the right and left sides), and was maintained in contact for about 7 sec. Each application was separated by an interval of 20 sec.

Several aspects of the test require care in order to obtain reliable results. For example, it helps if the experimenter holds the filament just above the animal, hovering for some time until the animal becomes accustomed to this and no longer pays attention to the filament. The filament should be applied using a slow approach to the animal to avoid drawing attention to its approach and any associated alarm. In addition, the least sensitive sites should be tested first to reduce anticipation of the stimulus. For example, after SCI, when some sites become very sensitive, locations inducing activity processed below the injury level were tested was the first, followed by sites inducing activity at the level of the injury or well above the injury. As with other testing, occasions when animals were moving or showing behaviours such as rearing, sleep, grooming or where the animal was distracted

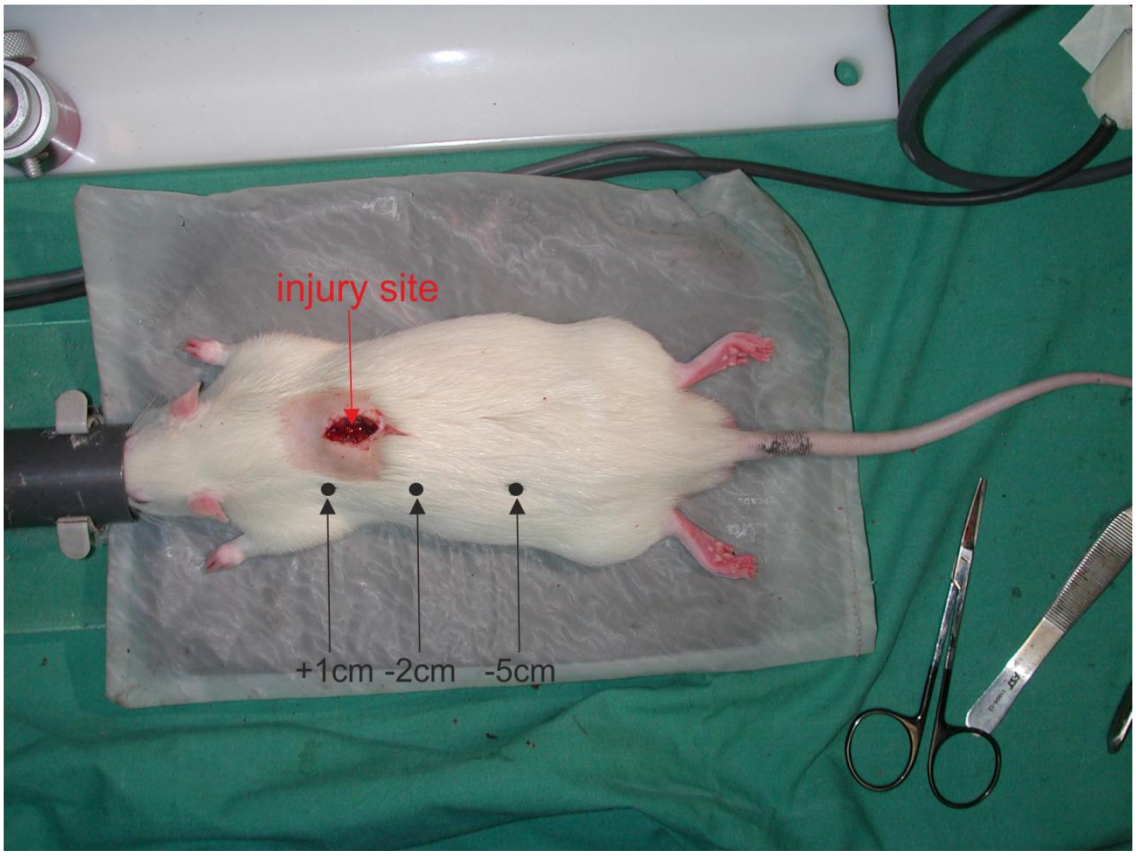

Fig. 2-5. A photograph shows the testing locations over the dorsolateral trunk. According to the electrophysiology, 1 cm above the injury site represents the above level dermatomes while 2 cm and 5 cm below the SCI are testing areas for at and below level, respectively.

by noises within or external to the room, were avoided. If the animal showed freezing (e.g. guarding position with urination or defecation) when the stimulus is applied, it should be returned to the home cage for 5 min and then returned to the testing arena. Following positive responses, animals should only be tested again when they are once more stationary and calm. The number of stimuli eliciting the following types responses was recorded:

- Escape
- Orientation/ head turning
- Scratching/ licking
- Flinching/ shaking
- Biting/vocalization

The results expressed as a total incidence of response out of 10 applications of the filament. Because biting was commonly seen after the surgery in the inured group, it was also recorded and plotted versus each of the testing time points.

Preoperative data was collected a few days before SCI or sham surgery and resumed 7 days following surgery. A shorter recovery than for tests on the limbs was considered adequate because the responses to tests at this level do not depend as critically on motor recovery of the limbs. Results were obtained at weekly intervals for 6 weeks after SCI.

2.4.5 Place escape avoidance paradigm (PEAP) test

This test was performed in order to determine whether the increased sensitivity to stimuli seen using various behavioural tests, including some which could be produced entirely by reflex mechanisms, was processed at a cerebral level i.e. consciously perceived as pain (LaBuda and Fuchs, 2000a, b, 2001; LaGraize et al., 2004; Baastrup et al., 2010, 2011). This test was adapted from one recently used by Baastrup et al. (2010) in a SCI model. In this test, the animal is given a choice of two environments. The plaxiglass enclosure is divided into two compartments (each of 24 x 20 x 14 cm) divided by a partition with an opening allowing the animal to move between the two halves of the chamber. One half of the chamber was covered on the outside with white card (light area) and the other half was enclosed by darkened walls (black card covering the plaxiglass). Ambient light was equal over both chambers which had either no cover, a transparent cover or a darkened cover depending on the test (see below).

The general principle of the test is that the animal is allowed to move freely between the light and dark compartments. Normal animals prefer the dark chamber and will spend most of their time in this chamber. However, each time the animal is moved to the dark side of the chamber, stimuli are applied to the back or the hindpaws using vF filaments or cold plate. The time that animal spends in the dark chamber is then recorded.

Four variations of this test were performed using different forms or sites of stimulation: 1) application of vF filament to the dorsolateral trunk +1 cm above the injury with the animal standing on a smooth plastic floor and no cover over the chamber (see Fig. 2-6A), 2) application of vF filament to the dorsolateral of the hairy back at -5 cm below the injury with the animal standing on a smooth plastic floor and no cover over the chamber, 3) application of a vF filament to the hindpaws while the animal stood on a mesh surface (as used for von Fey testing of the paws, see Fig. 2-6B) and the chamber had a transparent cover and 4) a cooled plate forming the floor of the darkened side adjacent to a floor at room temperature forming the floor of the light side of the chamber, with a darkened cover over darkened compartment.

To perform the test using vF stimuli, animals were placed in the darkened side with the partition closed off and habituated for 3 min. Thereafter the partition was opened and animals allowed moving freely between the two compartments for an additional 2 min, making the pretesting period 5 min in total. Observations were then made for a further 30 min period and the time spent by the animal in the dark compartment, together with the number of times the animal passed through the partition from one side to the other were recorded and expressed as a percent of the total testing time (10 min and 30 min epochs). During this period, each time the animal moved to the dark compartment, the foot or the back (depending on the test) was stimulated using a vF filament. The stimuli were applied every 15 sec whilst the animal remained in this compartment. For the plantar surface of the hindpaws the filament used was size 5.07 (10 g) and for the hairy skin of the back, the filament used was size 2.44 (0.04 g). The weight of the filaments used was chosen in pilot tests according to the filament which resulted in the sham animals spending approximately 75% of their time on the darkened side. For the cold plate variation of this test, the plate was maintained at 7.5°C (for further details see chapter 5).

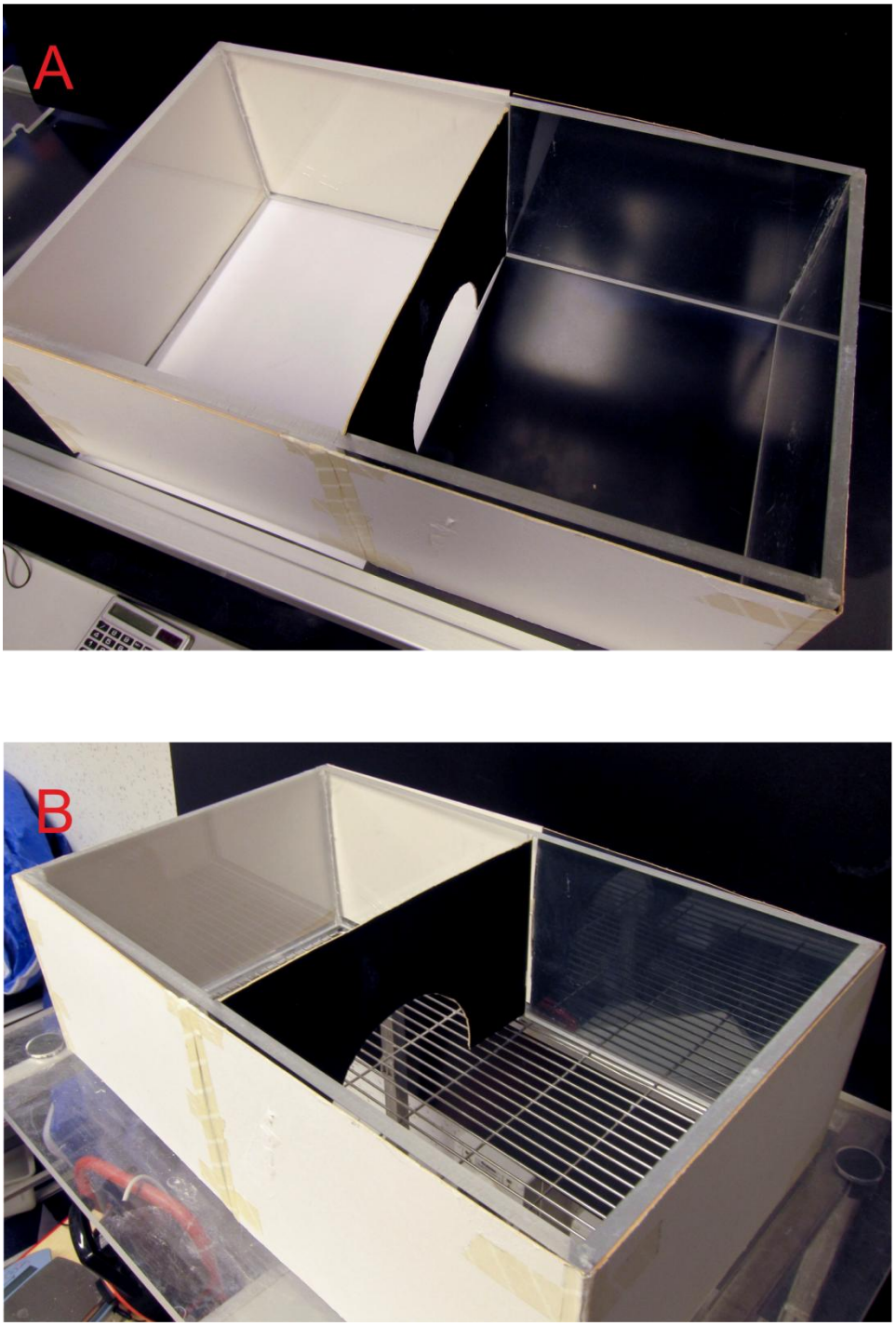

Fig. 2-6. Photographs show the testing apparatus used for PEAP performed over the trunk (A) and in the hindpaws (B).

In order to avoid any influence of learning on the results, this test was carried out on no more than two occasions on the same animal, always separated by at least 14 days (typically week 4 and week 6 post injury) and on a day when no other tests were performed.

2.5 Dynamic weight bearing (DWB) test

In order to investigate whether developed pain behaviours in fore and hindpaws can lead to any changes in postural equilibrium of the animals, paw weight distribution was assessed by using a DWB device (model version: 1.4.1.23, http://www.bioseb.com, Bioseb BP 32025, 13845 Vitrolles cedex France). Briefly, this device consists of a plaxiglass enclosure (24 x 24 x 32 cm) with a floor covered with matt containing calibrated force transducers (Fig. 2-7). These measure the pressure (weight) which each paw of the animal exerts on the floor. This information, together with a video recording showing the animals position within the cage is fed to a PC for acquisition and subsequent off-line analysis. To perform the test, rats were placed in the enclosure and allowed to move around freely or remain at rest for 4 min while data was continuously acquired. The data was then analysed using software provided with the device. The first stage of this involves reviewing the software's automatic determination of the position of the animal against the video recording and correcting any instances of mis-orientation or examples of the tail being mis-identified as a paw. On completion of this validation step the software calculated the weight placed on each paw averaged over time, excluding those periods when the animal was in motion. The weight distribution was separately calculated for those periods when all four paws were in contact with the ground, when only 3 paws were in contact and when only both forepaws and hindpaws were in contact. The DWB test was used to identify the percentage of weight paw distribution in 40 normal rats (16+24). The injured and sham animals were tested at different time points after surgery which varied for different models depending on the degree of motor recovery. The weight bearing data for each paw was expressed as a percentage of the animal's body weight.

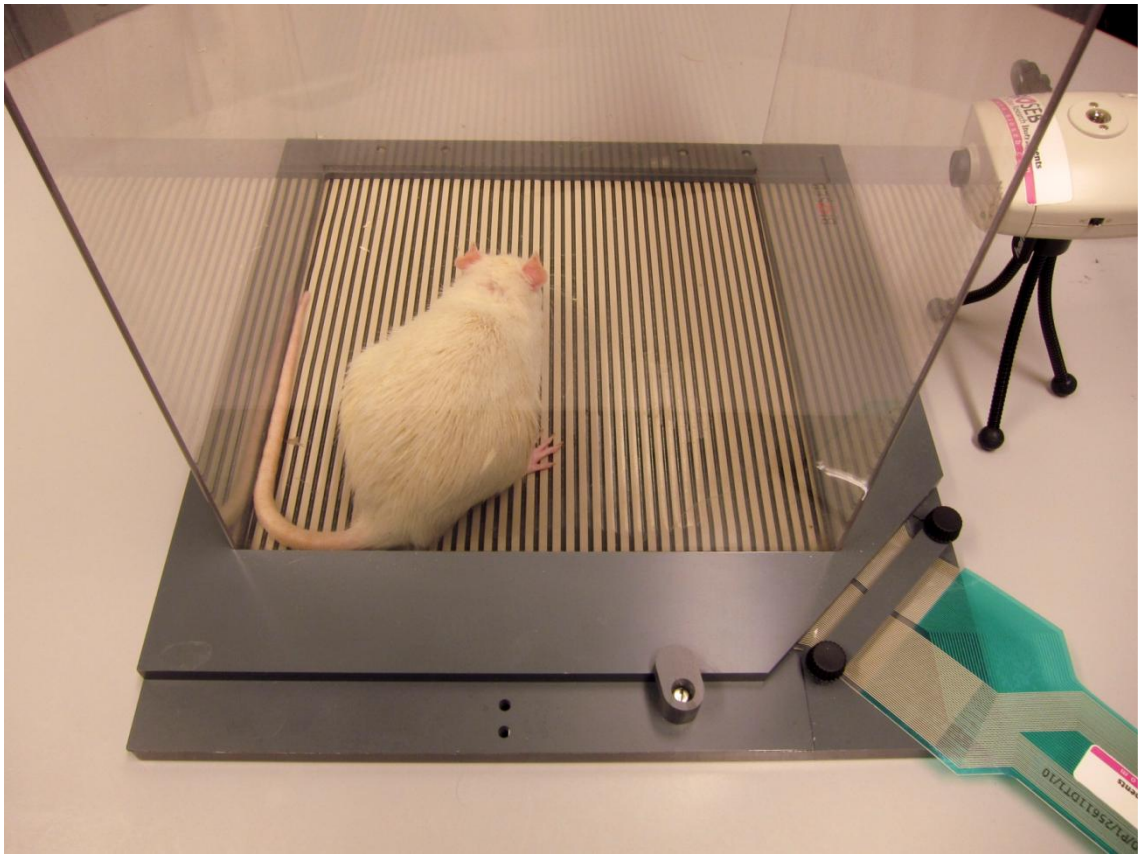

Fig. 2-7. A photograph depicts the dynamic weight bearing device. A plastic enclosure included an animal's stood on a floor contains a mechanosensitive transducer measuring the amount of the weight distributed to each paw. In the upper left side, a digital camera capturing synchronized video tracks for analysis.

2.6 Brain injections

Projections from the dorsal horn play a critical role in conveying nociceptive information to the higher centres (Christensen and Perl, 1970; Bester et al., 2000). The majority of neurons forming these ascending tracts terminate in the LPB and CVLM (Spike et al., 2003; Al-Khater and Todd, 2009; Polgár et al., 2010). Contusion SCI can damage the cell bodies of these neurons in the gray matter and their projections in the white matter due to the primary assault and secondary degenerative process (Dumont et al., 2001; Sekhon and Fehlings, 2001; McDonald and Sadowsky, 2002). Theoretically, this could interfere with the conveyance of sensory information from areas below the SCI to higher centres. To determine the extent to which this occurs in our models, we used tract tracing of the spino-LPB and spino-CVLM projections and assessed the numbers of neurons at different segmental levels of spinal cord in normal animals and in animals with a contusion injury. In contusion injured animals, retrograde labelling of ascending tract neurons was performed 6 weeks after the injury.

Tract tracing procedures were carried out using the same aseptic precautions, methods of anaesthesia and peri-operative care procedures as described for the surgeries carried out to set up contusion injured and sham animals (see section: 2.2). After induction of anaesthesia, the animal's heads were fixed in a stereotaxic head holder (David Kopf instrument, CA, USA) by mean of two horizontal ear bars and an incisor bar. The pipette to be used for injection was then attached to the manipulator of the stereotaxic apparatus and the tip placed at mediolateral and horizontal 0 and the midline by alignment to the point of one of the ear bars set to this position (ear bar zero). Readings were taken from the Vernier scales on the manipulator and these were noted. The appropriate settings for positioning the tip of the pipette within the LPB nucleus or CVLM were then calculated. The targets for these sites were determined from the rat brain atlas of Paxinos and Watson (2005). For LPB injections, the target coordinates were: anteroposterior equal to - 0.4 mm; dorsoventral equal to +3.2 mm; mediolateral equal to +2.1 mm. For CVLM injections the target co-ordinates were: anteroposterior equal to - 4.7 mm; dorsoventral equal to +/- 0.0 mm; mediolateral equal to +2.1 mm.

Skin over the skull and underlying fascia were incised and reflected laterally to expose the skull in the region of lambda. A burr hole was then created in the skull at a point appropriate for the intended target, using a dental drill. Occasional bleeding from the skull bone was controlled with bone wax. The pipette was then filled with tracer, moved to the

injection site and advanced into the brain to the appropriate depth. An injection of 50 nl of 4% Fluorogold (FG: Proteus Biocop, USA) was made using pressure pulses (a pulse every10-20 msec) applied using a pico injector (World Precision Instrument, USA). At the end of the injection, the pipette was kept in place for a further 5 min to allow tracer to dissipate from the injection site and so minimize leakage of the tracer back along the pipette track. Finally, the procedure was completed by closing the skin wound with 3/0 absorbable sutures. After brain injections, rats were allowed to survive for three days to allow transport of the tracer and were then fixed by perfusion.

2.7 Perfusion and tissue processing

Most animals used in the study were perfusion-fixed in order to examine the injury site histologically and all animals used for tracer injections were perfusion fixed. Anaesthesia was induced with isoflurane in an anaesthetic chamber as described previously and were then given a lethal dose (300 mg) of sodium pentobarbital (Euthatal® 200mg in ml; Vericore Ltd, Dundee, UK) administered intraperitoneally (i.p.). The animal was then transferred to a ventilated bench for the process of intracardial perfusion. This involved opening the thorax to expose the heart and thereafter a cannula was inserted into the left ventricle while a cut was made in the upper anterior part of the right atrium to allow blood to drain. Ringer's solution was first introduced and allowed to circulate for 5 sec in order to rinse blood from the body and then one litre of freshly depolymerized 4% formaldehyde in 0.1 phosphate buffer (pH = 7.4) was introduced to stabilizing proteins and make them insoluble (i.e. tissue fixation). The perfusion solutions were introduced under pressure by suspending the receptacles in which they were contained approximately 1.5 meter above the animal. Following fixation the relevant parts of the spinal cord were removed from the animal by dissection and when required for the confirmation of injections sites, the brain was removed by craniotomy

In order to confirm the identity of specific segments, certain landmarks were used in conjunction with dissection of dorsal roots. The spinal segments can be determined from the top by identifying the first cervical roots (C1) at the junction of the spinal cord with the medulla oblongata and then counting caudally. Alternatively, the fifth lumbar spinal dorsal roots can be identified as the L5 DRG is just caudal to the rostral extent of the hip bones (ilia) and the L4 DRG lies just rostral to this landmark. These roots can be followed rostrally to identify the corresponding segment and adjacent segments determined by

counting successive roots. In addition, the T13 segment should have dorsal roots originating from DRGs located at the intervertebral foramen just beneath the last rib.

After removal, the spinal cords and brains were post-fixed overnight in 4% paraformaldehyde containing 30% sucrose in order to cryoprotect the tissue before cutting on a freezing microtome. Prior to sectioning the spinal cord was divided into blocks (typically 6-8 mm long) which were notched in order to allow identification of the section orientation. For blocks to be cut transversely, the notch was made by slivering off a part of the ventral funiculus along the length of the block, while for blocks to be cut parasagittal, the notch was made by cutting one end of the block obliquely in the dorso-ventral plane. Blocks were mounted on the freezing microtome using O.C.T. (Sakura Finetek, Netherland) and either transverse or parasagittal sections cut at 70 µm thickness.

Brains were trimmed to blocks encompassing tissue 1mm rostral and caudal to the injection site and notched on the ventral quadrant of one side. Coronal sections were cut at 100 µm.

Sections were collected into tubes containing 0.1 M phosphate buffer, ready for performing immunocytochemistry (ICC).

2.8 Immunocytochemistry

The immunoflueresence processing was started by incubation of tissue section in 50% ethanol for 30 min at room temperature. This enhances penetration of the antibodies by disruption of cell membrane phospholipids and the formation of pores. Sections were then rinsed twice in 0.3 M double salt phosphate buffer (PBDS) for 10 min at room temperature. This process is very effective in reducing nonspecific binding of the primary antibodies to non-target proteins. PBDS has a strong ionic buffering property which reduces binding ability of all protein types but because the target protein has high affinity for its primary antibody, this binding is not affected. Sections were then incubated with a primary antibody cocktail diluted in 0.3 M PBSD and 0.3 M triton detergent for 48 to 72 hours at 4°C. The triton detergent enhances penetration of the primary antibody. Following this incubation, sections were washed twice in 0.3 M PBSD for 10 min at room temperature in order to remove the excess of the antibody cocktail and prevent nonspecific staining. The tissue sections were then incubated with a cocktail of appropriate secondary antibodies diluted in 0.3 M PBSD and 0.3 M triton detergent for 4 hours at 4°C and

protected from light by wrapping in tin-foil. At the end of this incubation, the tissue sections were rinsed in 0.3 M PBSD for 10 min at room temperature in order to remove the excess secondary antibody cocktail and then given a further rinse in 0.1M PB. Finally, the tissue sections were mounted in an anti-fade mounting medium (vectashield "Vector Laboratories, UK") on glass slides and covered by using a coverslip. The edges of the coverslips were sealed with nail varnish to prevent evaporation of the anti-fade medium and drying out of the tissue sections. The slides are subsequently stored at -20°C in darkness.

The antibodies used are show in Table 2-3. The primary antibodies used were directed against specific antigens including FG molecules, the NK1 receptor, neuronal nuclei. Secondary antibodies conjugated to fluorochromes were used for staining of the spinal sections. The flourophores included DL649 which was selective for FG molecules while Rhodamine and Alexa 488 were used for visualization of NK1r and neuronal nuclei, respectively, as it shown in the following table:

Primary Antibody	Secondary Antibody	Specificity
guinea-pig FG [1:1000] (Chemicon, CA, USA)	guinea pig DL649 [1:500] (Jackson Immunoresearch)	FG molecules: to detect labelled neurons
rabbit NK1r [1:10,000] (Sigma, UK)	rabitt Rhodamine [1:100] (Jackson Immunoresearch)	NK1r: to detect NK1r expressing neurons
mouse NeuN [1:1000] (Sigma, UK)	mouse Alexa 488 [1:500] (Molecular Probes)	Strong labelling of neuronal nuclei and light labelling of cytoplasm

Table 2-3. Primary and Secondary Antibodies. Antibodies used for immunocytochemistry and their dilutions are shown and the labelled structures are indicated.

No antibodies were used for staining of the brain sections as FG can be visualized (as gold yellow colour) directly under epifluorescence using a UV filter block.

2.9 Visualization and analysis

2.9.1 Spinal sections

Immunolabelled tissue was examined using a Bio-rad Radiance 2100 confocal system (Bio-Rad, Hemel Hempstead, UK). The dorsal half of the gray matter on the left side of the spinal cord was targeted in each tissue section analysed. Sections were scanned

sequentially under a x20 dry lens, using 488, 543 and 637 nm laser lines or channels in steps of 2 µm through the full thickness of stained tissue. The shortest laser wavelength (i.e. 488 nm) was absorbed by Alexa 488 and re-emitted as green light while 543 nm channel was used for excitation of Rodamine with subsequent red colour emission and finally 637 nm laser was selective for LD 637 displayed by software as blue. Multiple x20 fields of view (usually 4-9) were required to cover the area of interest in each tissue section. Z-series stacks of these scans were exported to ImageJ v1.44o (NIH Image, Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2007) for quantification.

Z-series stacks were opened and examined using the "Cell counter" plugin within ImageJ. This allowed cells to be identified, marked and counted throughout the scanned stack. Stereological principles were followed to ensure representative numbers of cells were counted. Neurons with cell bodies partially sectioned at the top of the image stack were excluded but those with cell bodies partially sectioned at the bottom of the image stack were counted. The cell counts from all x20 fields of view scanned from a single section were summed to calculate the total number of cells per section. Five to ten sections were analysed per spinal segment and the mean number of cells per section calculated for each segment. This "animal mean value" was calculated for each animal in each experimental group and these were averaged to calculate a "group mean" for each experimental group.

2.9.2 Brain sections

Serial sections through the brain injection sites were examined to verify the accuracy of each injection. Sections were examined using a Nikon Eclipse E600 epifluorescence microscope with dark field condenser. Under dark field, the general anatomy of the tissue could be discerned to verify that injections were made within the correct structures. Under epifluorescence imaging, using an ultra violet (UV) filter block, the injected FG tracer could be visualised to verify that the injection encompassed the targeted structures.

Digital photographs of representative injection sites were acquired using a Zeiss Axiocam digital camera controlled with Axiovision v3.1 software (Carl Zeiss Microimaging, Germany). Complete sections were photographed using dark field and fluorescence optics by imaging several fields of view at x4 magnification and a resolution of 1300 x 1030 pixels. Individual fields of view were exported to Adobe Photoshop CS3 where they were

montaged to reconstruct complete sections and processed for minor brightness and contrast adjustments for presentation.

2.10 Statistical analysis

All statistical analysis were applied to data expressed as means ± standard errors of means (SEM) by using Prism version 4, GraphPad Software Inc, San Diego CA, www.graphpad.com. Before any statistical treatment, data were analysed for their distributions around means (i.e. parametric or nearly parametric vs. nonparametric) by using the following normality tests: *Kolmogorov-Smirnov, D'Agostino and Pearson omnibus* and *Shapiro-Wilk*. Almost all behavioural data showed *Gaussian* or nearly *Gaussian* distributions except results collected from BBB/BBBsubscore in the T9 150 kdyn model where moderately *non-Gaussian* shapes were estimated.

In thoracic models, measures collected from plantar vF, trunk vF, plantar heat assays as well as BBB/BBB subscore methods in T3/T4 200 kdyn and T9 200 kdyn models were assessed by two-way analysis of variance (i.e. *multifactorial ANOVA*) in order to analyze the effects of SCI at each testing point (i.e. SCI *vs*. sham at each time point) as well as the effects of time in each isolated group (i.e. SCI or sham). In all of these cases, analysis was followed by multiple comparison (i.e. SCI *vs*. sham) using the *Bonferroni post-hoc test*. Despite the fact that this test has advantage of being easy to understand, it is a relatively too conservative test that can lead to *P values* that are too high and confidence intervals that are less wide. In the case of small group comparisons (i.e. < 5 columns), this is a minor concern but the chance of a type I error is increased when larger numbers of comparisons are made (i.e. >5 columns) (Scheff et al., 2002). As a result, the traditional *alpha level* (*P value*) resulted from *Bonferroni post-hoc test* was modified in order to reject the *null hypothesis* (i.e. getting a significant difference by chance). In this process which known as *Bonferroni correction*, the traditional *P value (i.e. 0.05)* was divided by the number of comparisons to be made (i.e. 0.05/number of time points) and the resultant number became our *corrected P value* determining a significant difference (Scheff et al., 2002). The approach resolved differences in multiple comparisons between different groups only i.e. SCI *vs.* sham. In addition, therefore, repeated measure *one-way ANOVA* was run to compare the means of each group at different time points, followed by *Tukey's post-hoc test* with the confidence interval set to larger than 95%. In the case of the T9 150 kdyn model, plantar vF, trunk vF and plantar heat assays were analysed in the same way, while a different method was applied for BBB/BBBsubscore. A *multifactorial ANOVA* was

used (as above), followed by the *Kruskal-Wallis non-parametric* version of *ANOVA* (in order to analyse the effects of time) using *Dunn's post-hoc comparisons* between time points *(P<0.05)*.

Data collected from FL assays were assessed in a similar manner. FLs collected over the grid and cold plates as well as neutral plate (i.e. 30ºC) were analysed firstly by *two-way ANOVA* followed by *Bonferroni post-hoc multiple comparisons* using the normal *alpha value (i.e. P<0.05)*. In addition, repeated measures *one-way ANOVA* was used to clarify the effects of time (as well as temperature range in T9 200 kdyn model) in each group, followed by *Tukey's post-hoc test (P<0.05)*. Uniquely (two testing time points) for analysis of FL on the cold plate in T3/T4 200 model, *paired t-test* and *unpaired t-test with Welch's correction (P<0.05)* were used. For analysis of SFL over 25°C, 30°C, 35°C in T3/T4 200 kdyn, standard one-way ANOVA was used because of unequal sample sizes. *Tukey-Kramer post-hoc test* was also used to account for unequal sample sizes *(P<0.05)*.

In all models, means collected from licking responses were analysed by three different ways. Firstly, SCI group data was compared to shams using *two-way ANOVA* followed by *Bonferroni post-hoc test (P<0.05)* as described above. Secondly, *repeated measures one-way ANOVA* with *Tukey's post-hoc test (P<0.05)* was used to analyse licking data at different time points. Finally, a *one sample t-test (P<0.05)* was used to estimate the departure of data in each group from the base line.

Two-ways ANOVA followed by *Bonferroni post-hoc multiple comparisons (P<0.05)* were used for all variables collected from various PEAPs. In spite of the fact that this method will compare data into two directions (SCI *vs.* sham and 10 min *vs.* 30 min), P value will be only calculated for means of SCI *vs.* sham at each time epoch (i.e. comparison of rows). If there is an effect for time, it will be only referred without any justification for alpha value. In order to estimate P value for data at 10 min *vs.* 30 min (differences in the same group), further analysis was done by using *paired t-test*. Crossing was also subjected to statistical analysis. In case of equal sample sizes, crossing at each epoch (10 or 30 min) was compared by *unpaired t-test (P<0.05)* while in case of unequal samples, *Welch's correction* was added. In both occasions, distribution of values was *Gaussian* or nearly *Gaussian*.

Because different models have different recovery patterns, assessments of the postures were performed at different time points in each case. In T3/T4 200 kdyn model, DWB data

was collected in week 6 PO and a direct comparison between SCI and sham animals were carried out using an *unpaired t-test (Gaussian distribution)* with *Welch's correction* because of unequal group sizes of SCI and sham animals. In addition, *one sample t-test (P<0.05)* was used to compare each group mean to the base line. This provided clear information about how much deviation in each group from the baseline of the normal animals (SCI *vs.* normal and shams *vs.* normal). Regarding of T9 200 kdyn, data from operated animals were collected in weeks 3 and 6. Because there was no enough motor recovery in two SCI animals (missing values at week three), comparisons between SCI and sham animals was made using *Standard one-way ANOVA* followed by *Tukey-Kramer post-hoc test*. *One sample t-test* was used for testing for differences between each group and base line means of the normal animals. The same principles were used to analyse data from the T9 150 kdyn model using *Standard one-way ANOVA* but followed by *Tukey's post-hoc test (P<0.05)* because of equal sample sizes at all testing time points.

The independent readings collected from % of CAT in T3/T4 200 kdyn model was analysed by using an *unpaired t-test* with *Welch's correction (P<0.05)* because the SCI group had a larger sample size.

Finally, the same principles were used for comparison of SCI animals from different experimental groups (e.g. T3/T4 200 kdyn *vs.* T9 200 kdyn).

Chapter 3

T9 200 kdyn model

3 T9 200 kdyn model

3.1 Introduction

Because the vast majority of SCIs in humans are traumatic contusion injuries, use of rodent contusion injury models is becoming increasingly popular for the investigation of mechanisms underlying central pain after SCI. Currently, the lower thoracic (T9/T10) injury level is almost exclusively used whether studying behavioural changes considered to reflect mechanisms above the injury level (in forepaws) or below the contusion site in hindpaws. However, there are several potential limitations of the models as currently used and the work in this first chapter was aimed at clarifying these. The issues which these experiments aimed to address were as follows:

1. Validity of using hindpaw tests to assess below level pain. The appropriateness of SCI models for the investigation of below level pain has been questioned. There are two potential areas of concern. One is that the SCI may interrupt the ascending pathways responsible for conveying nociceptive information to the brain so that pain is not an outcome when stimuli are applied below the injury level. This potential problem is compounded by the fact that SCI leads to spasticity in muscles below the level of the injury. This spasticity is due to changes that occur in reflex circuitry and some of the elements of this reflex circuitry are used in the behavioural responses to stimuli that are used as a measure correlating with pain. Taken together, these issues mean that there is a concern about whether certain tests performed on the hindlimbs (those that are or can be produced entirely by reflex mechanisms) actually reflect changes in nociceptive circuitry on route to the brain and that even if they do the resulting signals may not reach the brain. In other words, there is doubt as to the validity of using certain tests applied to the hindlimbs as a measure of below level pain following SCI. These issues remain to be clarified.

2. Reliability of girdle tests for at level and below level pain. Because of the complications of below level pain testing using the hindlimbs, some groups have used tests applied to the back as an alternative. There has been some variability in the results obtained using this approach and some variability in the sites to which stimuli have been applied relative to the injury level. Generally however, the location of stimuli have been described in relation to the physical level of the injury and anything applied above this level considered at or above level pain and anything applied below the injury level

considered below level. However, this may be problematic on two counts. Firstly, the terminology for above, at and below level pain is borrowed from the clinical literature which is not easily transferred to the rodent model. The clinical terminology has a quite specific meaning which is not directly related to the physical level of the injury but rather to the level as defined by sensory testing of the dermatomes. Thus the level of an injury is considered to be the last dermatome with normal sensation which will necessarily be rostral to the level of the injury. The second issue which compounds this problem is that there is a lack of information on the dermatomes of the thoracic region of the rat and it may not even be safe to assume that all stimuli applied to hairy skin below the physical level of an injury will be processed in spinal cord segments below the injury. This issue therefore needs to be clarified as stimuli which are consider "below level" may in fact be "at level".

3. Tests of cold allodynia. Cold allodynia is one of the main symptoms of which SCI patients with neuropathic pain complain (Defrin et al., 2001; Finnerup et al., 2003; Cruz-Almeda et al., 2009) and can be considered of greater importance because the fact that cold stimuli can difficult to avoid. However, the tests commonly used for this such as the acetone test or ice probe test are crude, can activate tactile receptors as well as thermoreceptors and the temperatures reached at the point of stimulation are not known and poorly controlled. An improvement on the available tests for cold allodynia is therefore highly desirable.

4. Testing pain *vs.* nociception – cortical processing. Many of the tests used in the assessment of pain depend upon the observation of behaviours which can be shown to occur in spinalised animals (Schouenborg et al., 1992; Kauppila et al., 1997, 1998; Wienecke et al., 2010) or in decorticated animals (Woolf, 1984) and it could therefore be argued that they are tests of nociception and that the extent to which they correlate with the perception of pain is therefore uncertain. For this reason, it is desirable to include within the testing paradigms assessments where it is certain that cortical processing of the nociceptive signal and the sensory experience of a painful sensation must be involved. Such tests have not been commonly employed so far in SCI pain research.

5. Modelling spontaneous pain. Following SCI, spontaneous pain can be a more common symptom even than evoked pain. However, so far there have been only incidental reports of phenomena (e.g. scratching) which might reflect spontaneous pain in animal models of SCI. Since this type of pain is highly relevant to human SCI pain, it will be important to develop more quantitative methods for evaluating spontaneous pain if possible.

3.2 Methods

In this part of this study, a total of 93 adult male Sprague Dawley rats (200-350 g) were used. Twenty five animals were subjected to a contusion injury (T9 200 kdyn) while 15 animals were operated on to perform a laminectomy but no contusion injury was performed (sham operated animals). Forty nine naive (i.e. normal unoperated) animals were used. Nine of these were used as control animals for a tract tracing study while 40 animals were used for obtaining baseline data in the DWB test. Four naive rats were used for an electrophysiological study.

3.2.1 Behavioural testing

3.2.1.1 BBB rating scale and BBB subscore

The BBB rating and BBB subscore assays were performed preoperatively and at weekly intervals after surgery. Fourteen contusion injured and 14 sham animals were assessed.

3.2.1.2 Dynamic weight bearing test

Body weight distribution on the paws was evaluated in 40 normal animals. Five contusion injured animals and 6 sham animals were evaluated at week 3 postoperatively while 8 SCI and 6 sham animals were evaluated at week 6.

3.2.1.3 Forepaw von Frey and heat tests

Preoperatively, the base line data for plantar von Frey and plantar heat (response latencies) assays were collected from 18 animals distributed equally into SCI (n=9) and sham (n=9) groups. Starting two weeks after the surgery, these animals were tested weekly until week 6 after SCI. Observations on the incidence of licking after each test were made on 6 animals in each group.

3.2.1.4 Hindpaw von Frey and heat tests

Hindpaw tests were identical to forepaws test accept that fourteen SCI animals were used to estimate response latencies in the heat test.

3.2.1.5 Footlifting test

Forepaw and hindpaw lifting were evaluated while 8 SCI rats and 6 shams stood over a plate adjusted at 7.5°C in weeks 4, 5 and 6 after the injury. In the same weeks but on different days, a similar number of shams were assessed at 30°C whereas only 6 contusion injured rats were tested. During week 6 after the surgery, FL was assessed on a plate controlled at a range of graded temperatures (7.5°C, 15°C, 20°C, 25°C, 30°C and 35°C) in 6 SCI rats and 6 shams. Eleven contusion injured animals and eleven sham animals were evaluated for forepaw and hindpaw lifting while stood on a metal grid in weeks 4, 5 and 6 after surgery.

3.2.1.6 Electrophysiological determination trunk dermatomes

This is described in detail in section 2.4.4.1. For purpose of identification of appropriate testing locations on the dorsolateral trunk, 4 normal rats were utilized and 6 SCI rats were also investigated in week 7 after injury.

3.2.1.7 Trunk von Frey test

Data for this test were collected preoperatively and on 6 occasions on a weekly basis after surgery. 14 contusion injured rats and 9 shams were used to investigate above level stimuli (1 cm above), 11 SCI and 9 sham animals were utilized to assess at level stimuli (2 cm below) and 8 contusion injured and 6 operated sham rats were used to investigate below level stimuli (5 cm below).

3.2.1.8 PEAP test

In this model, three von Frey-related PEAP tests were performed using the time at which responses to von Frey testing showed maximal changes as a guide. Above level PEAP test was performed in week 4 PO while below level or hindpaw PEAP test was performed in week 6 after the contusion injury. Sixteen rats were divided equally into contusion injured and sham groups and used for the 1cm above level PEAP test while 12 rats (6 SCI *vs.* 6 shams) were used for the 5 cm below PEAP test. Eight SCI animals and 6 shams were used to investigate cortical processing when stimuli were applied to the hindpaws (hindpaw PEAP test).

3.2.2 Tract tracing of projection neurons

In order to investigate the effect of a severe contusion at the lower thoracic spinal level on projection neurons, a series of tract tracing experiments were performed 6 weeks after the injury.

3.2.2.1 CVLM

For purpose of investigating projections from spinal cord to the CVLM, tract tracing was carried out in 6 normal and 7 contusion injured rats. Spinal bocks for sectioning were prepared from segments C7 (6 normal and 4 SCI), T8 (2 normal and 2 SCI) and L4 (3 normal and 2 SCI rats). Projection neurons retrogradely labelled in sections from the CVLM traced animals were quantified throughout the whole dorsal horn.

3.2.2.2 LPB nucleus

Brain injections were used also to target the LPB nucleus followed by investigation of labelled neurons in lamina I in sections taken from L4 segments. These segments were prepared from two groups of SCI and normal animals, each containing 3 rats.

3.3 Results

3.3.1 Contusion parameters

The weights of all animals at the time of surgery are shown in Fig. 3-1A. Investigations were carried out using batches of 3 to 6 animals. The contusion device provides information on the actual force delivered and the displacement of the impactor tip relative to the surface of the spinal cord. The values for each of the animals are plotted in Fig. 3-1B. Most of the impacts were close to the target force of 200 kdyn and resulted in displacements of 740 to 1358 µm (Fig. 3-1C). The force-time graphs were also inspected at time of injury for any indication that bone rather than soft tissues may have been struck. This is signalled by a sharper (i.e. shorter duration) force record or by an interruption to what should be a smoothly increasing force profile. Animals with this type of profile invariably showed little or no motor deficits on recovery (see BBB, below) and were omitted from the study.

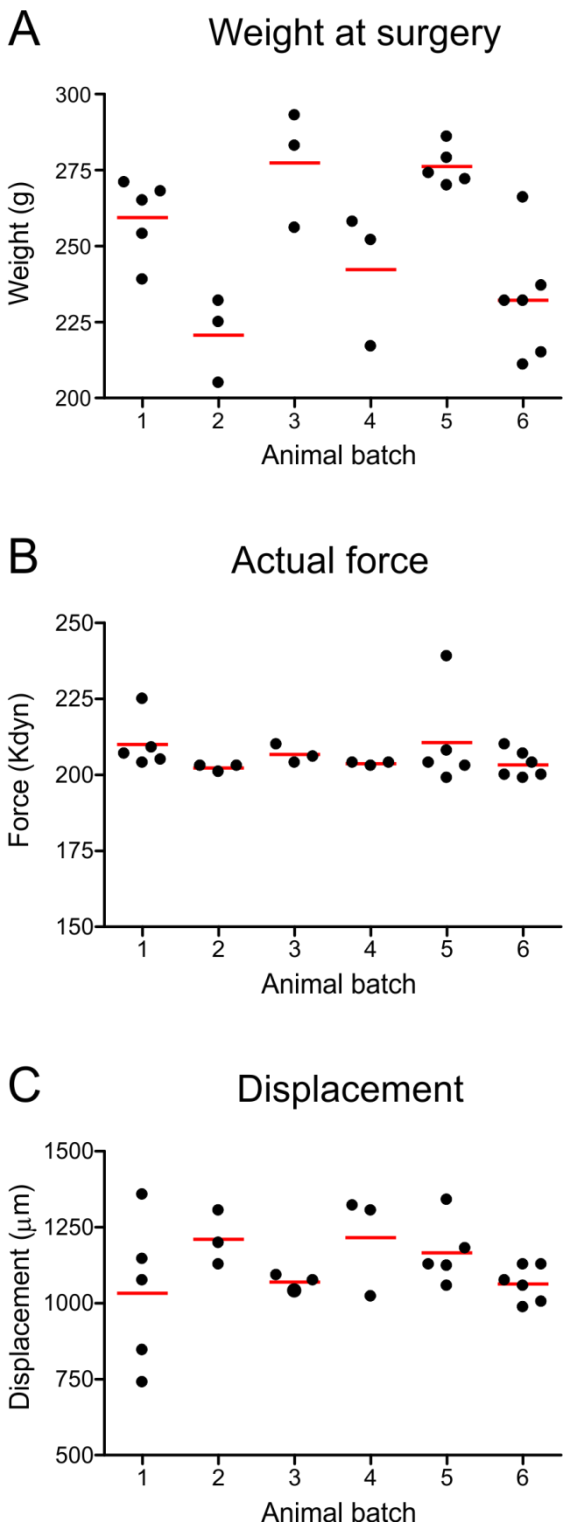

Fig. 3-1. Contusion injury data. A represents weight of animals at surgery. **B** is showing actual force delivered by the impactor and measured by the devise during the impact. **C** illustrates actual displacement of the impactor i.e. distance that the impactor tip travelled from the surface of the spinal cord into the cord before withdrawing. The graphs show scatter plots for the values measured for each of 25 animals subjected to contusion injury.

3.3.2 Histology of the injury site

Examples of sections from the injury are showing the extent of damage following 200 kdyn injuries is shown in Fig. 3-2. The injury site was extensively damaged and therefore very fragile so that only limited histological processing was possible and this was successful in only a few animals. However, it is evident from the example in Fig. 3-2 that the damage involved extensive parts of the gray and white matter with white matter around the outer rim of the spinal cord tending to be best preserved. Despite the impacted surface being dorsal, the injury involved tissue both above and below the central canal and frequently resulted in a fluid filled cavity at the injury site.

3.3.3 Motor evaluation

Assessment of the effect of the injury on the movement capacity of the animals and the pattern of recovery of movement was carried out using the BBB locomotor rating scheme (Basso et al., 1995). This served three purposes. Firstly, it provides confirmation that the injuries performed were consistent and had a closely similar outcome. Secondly, it enables a comparison with the injury severity of other studies, for those where the same or similar scoring system has been used. Thirdly, it provided a guide as to the ability of the animals to respond to stimuli and therefore an appropriate time point to commence testing.

Fig. 3-3A shows that all animals had normal 21 scores of 21 before the contusion and sham operations. One week after the SCI, animals showed BBB scores of about 6, at which point, the hindpaws show neither plantar placement nor weight support. This improved markedly in the second week to a score of about 10. The group as a whole shows a slower progressive improvement but only to a maximum of point 12 on the scale (corresponding to: *frequent to consistent weight supported plantar steps and occasional forelimb/ hindlimb coordination).* Recovery did not progress further on the BBB scale because the animals never showed *frequent to consistent forelimb/hindlimb coordination* which is the next step in recovery as stimulated in the BBB scoring system. However, animals did show some aspects of recovery that are included in higher scores of the BBB scale. For example, animals already showed occasional toe clearance as early as week 3. This does not appear on the BBB scale until point 15. Our animals did not therefore conform to the precise order of recovery of locomotor characteristics required for application of the BBB scoring in these upper parts of the scale. This is a problem that has been encountered by others and has led to the introduction of a BBB subscore to improve assessment of recovery of the

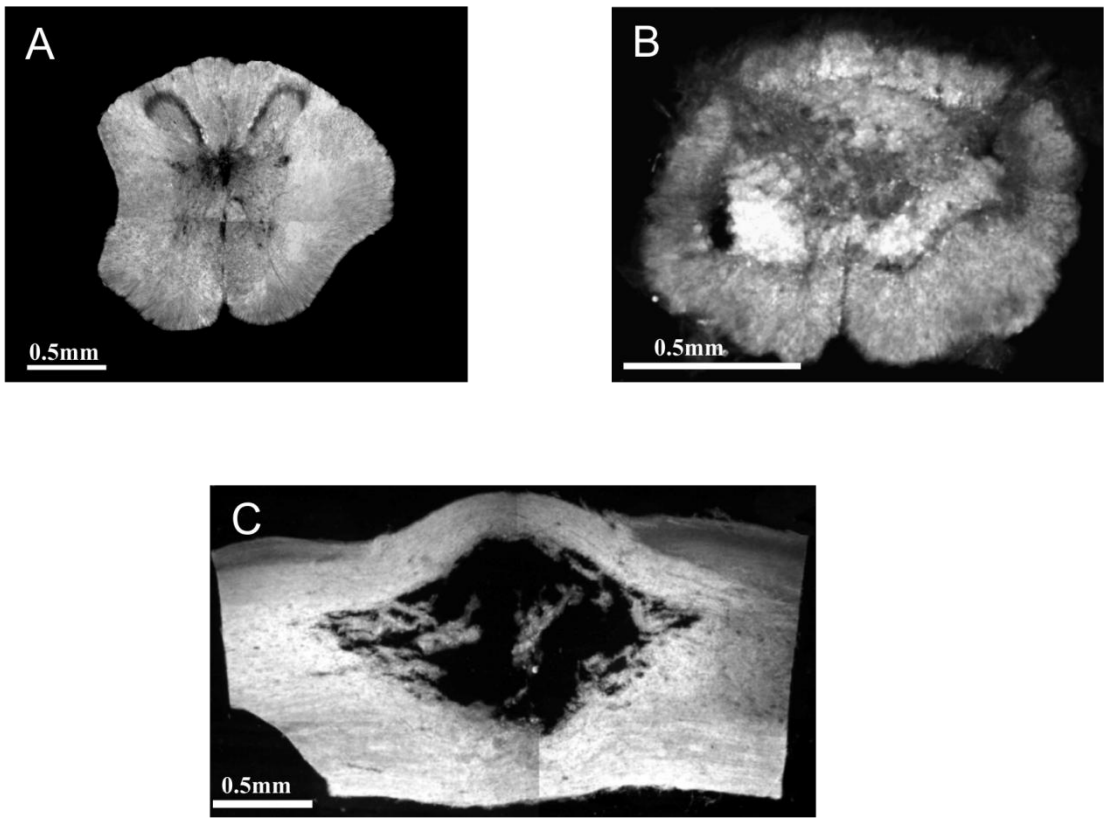

Fig. 3-2. Shows the injury area before and after exposure to T9 200 kdyn contusion injuries. A, B and **C** show composite images taken by using the dark field condenser and a Zeiss digital camera attached to a Nikon Eclipse E600 microscope under x20 magnification (scale bar = 0.5 mm). A transverse section prepared from normal and injured T9 segments appear in A and B, respectively. C illustrates a parasagittal view of epicentre damage to T9 segment by force of 200 kdyn.

finer aspects of locomotor recovery (Lankhorst et al., 1999). The results of this subscore method are show in Fig. 3-3B. A progressive improvement was seen from week 2 onwards reflecting mainly improvement in toe clearance, tail posture and body stability. Paw angle never returned to normal in these animals. The sham surgery had no effect on the locomotor scores of animals in this group even at one week after the surgery.

A BBB score of 10 equates to plantar stepping of the hindpaws with occasional weight support on hindlimbs but an absence of forelimb and hindlimb coordination. Most previous studies have considered a score above 8 (i.e. *plantar placement with some weight support on hindlimbs*) compatible with sensory testing. The BBB scoring of the animals in this study therefore shows that the animals had recovered more than adequate capacity to perform motor responses to sensory tests by the start of testing. Testing with stimuli applied over the back, responses to which did not depend on the full recovery of the hindlimbs began in week 1 and for stimuli applied to the paws, testing began in week 2.

3.3.4 Dynamic weight bearing test

A dynamic weight bearing test was used to evaluate the effect of SCI on the postural support of the animals as indicated by the distribution of body weight among the four paws. The results from this test are shown in (Fig. 3-4). In normal animals (n=40), 33% of the total body weight is distributed to the forelimbs (Fig. 3-4A) with the hindlimbs bearing around 67% of body weight (Fig. 3-4B). In other words, in naive rats, body weight is distributed more on the hindpaws than the forepaws. In animals subjected to sham surgery (n = 6), there was no change in this weight distribution pattern at 3 weeks or at 6 weeks following surgery. There was no statistical difference between the weight distribution on forepaws and hindpaws between sham operated and normal animals (*P>0.05, one sample t-test)*. However, in SCI animals there was a clear redistribution of weight, with more of the weight being distributed to the forepaws than in the naive animals at week 3 after the injury (an increase from 33% to 44%) and a concomitant reduction in the weight distributed to the hindpaws (a decrease from 67% to 54%; Fig. 3-4C and D). This change was statistically different in comparison to both normal animals *(P<0.05, one sample t test)* and sham animals *(P<0.05, Tukey-Kramer post-hoc test).*The redistribution of weight after SCI did not recover with time since there was no statisitccal differences between data collected from the injured animals at weeks 3 and weeks 6 time points (unequal sample size; *unpaired t test, P>0.05*) (Fig. 3-4E and F).

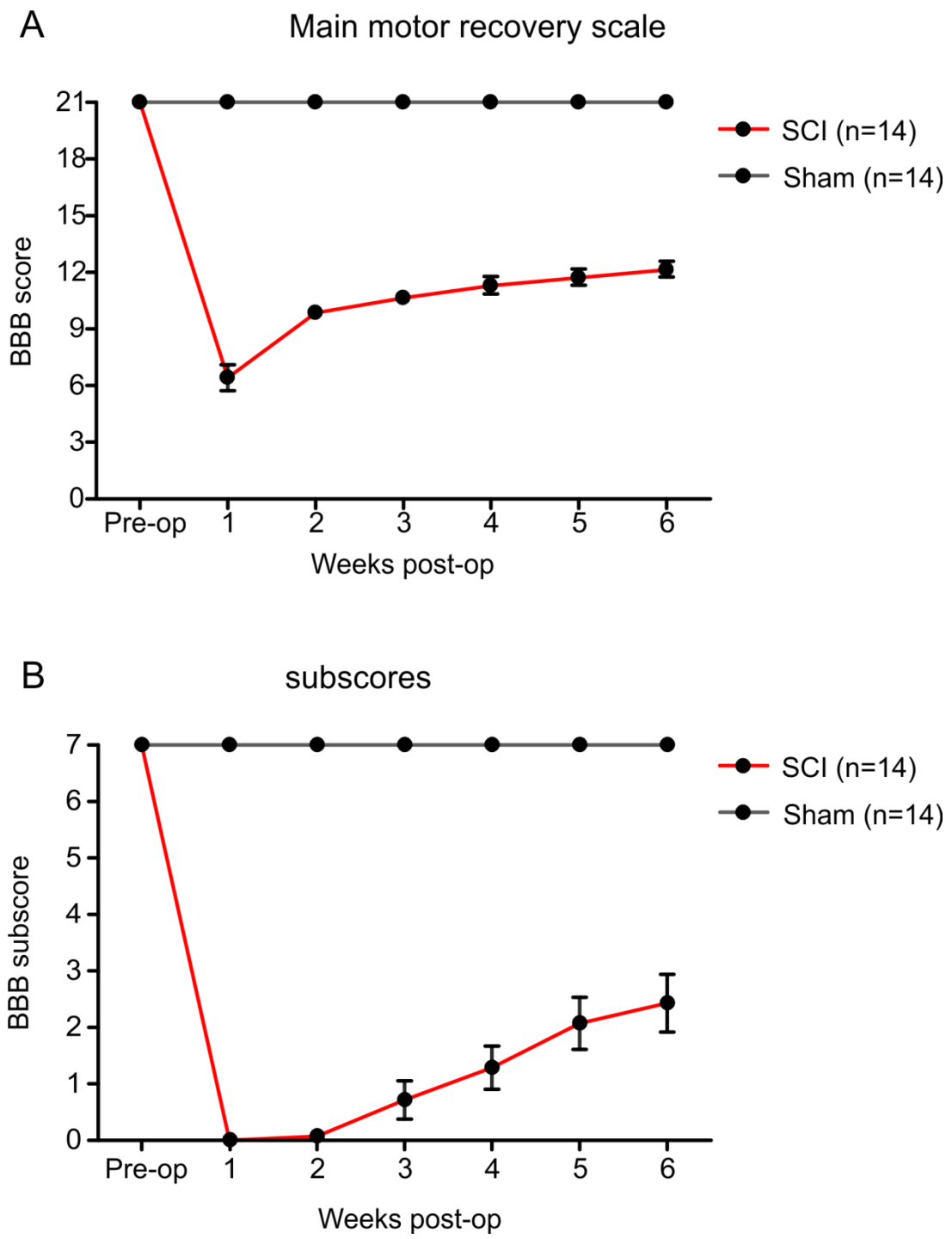

Fig. 3-3. BBB rating scale and subscore for locomotor activity of SCI and sham animals over the course of the study. A shows the main motor recovery (i.e. BBB rating scale) from preoperative and weekly (1 to 6) tests performed on T9 200 kdyn contusion injured animals (n=14) and sham operated control animals (n=14). **B** illustrates the motor recovery subscore for the same animals over the same time period. The values shown are mean ± SE.

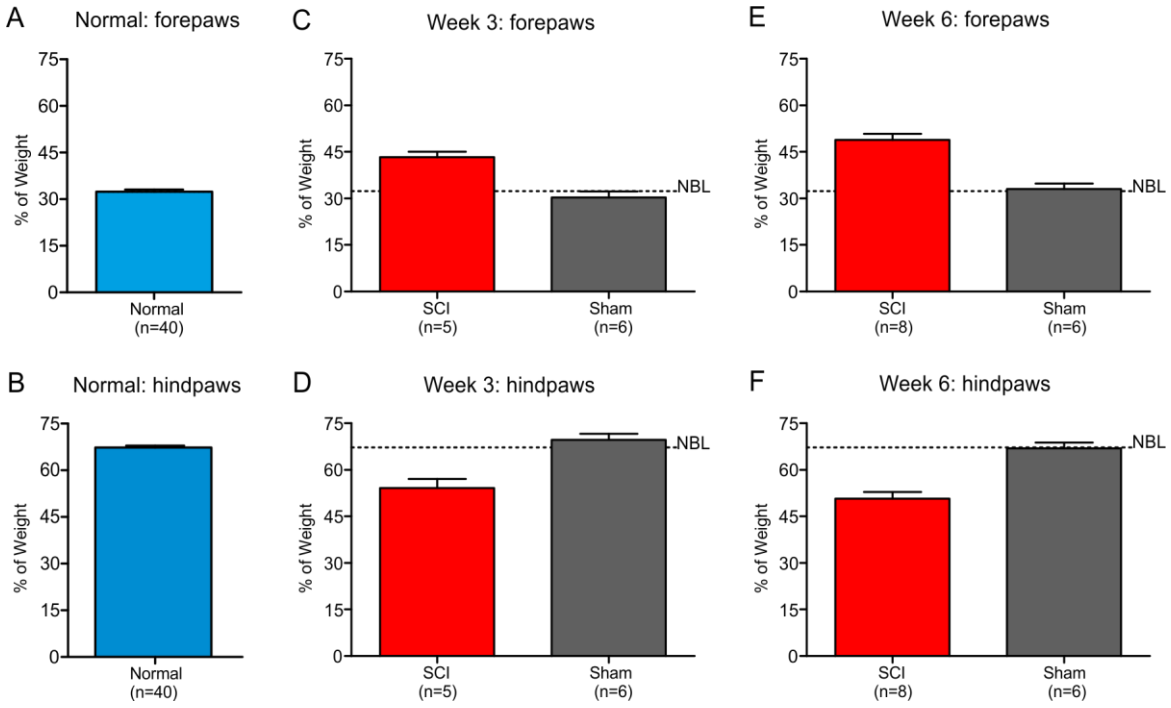

Fig. 3-4. DWB data collected from forepaws and hindpaws. A and **B** show the percentage of body weight distributed to the forepaws and hindpaws, respectively, in normal animals (n=40). **C** and **D** show the percentage of body weight distributed to the forepaws and hindpaws, respectively, in injured (n=5) and sham (n=6) animals at 3 weeks post SCI. **E** and **F** show the percentage of body weight distributed to the forepaws and hindpaws, respectively, in injured (n=8) and sham (n=6) animals at 6 weeks post SCI. The dotted lines represent the percentage of paw-weight distribution averaged in naive animals (n=40). The dotted lines (normal base line "NBL") represent the average percentage hindpaw-weight distribution in naive animals (n=40). All histograms represent mean ± SE.

3.3.5 Responses of forepaws to applied stimuli

3.3.5.1 Plantar von Frey test

The forepaws were tested for signs of increased sensitivity to tactile stimuli (vF hairs). The effect of the injury on the withdrawal response to tactile stimuli applied to the forepaws is shown in Fig.3-5. A decrease in the threshold for eliciting withdrawal was evident at the first test session 2 weeks after the injury. The threshold continued to decrease in subsequent weeks reaching a maximum at around week 4 to 5 and plateaued at week 6. The difference in threshold between SCI and sham animals was statistically significant from week 2 to 6 *(P<0.01-0.001, Bonferroni post-hoc test)*. Sham operated animals showed little change in threshold in the same tests. There was no statistical difference between preoperative data and postoperative data for sham animals at any time point *(P>0.05, Tukey's post-hoc test)*. These observations are indicative of tactile allodynia in the SCI but not sham animals.

3.3.5.2 Plantar heat test

Responses to the plantar heat test are shown in Fig. 3-6. A reduction in latency was evident at week 3 and this became statistically significant by week 4 *(P<0.001Bonferroni post-hoc test)*. The reduction was maximal at 5 weeks and plateaued at 6 weeks (Fig. 3-6A). The response latency for the sham group of animals did not change in the same test period *(*preoperative data *vs*. postoperative data at each time point: *P>0.05, Tukey's post-hoc test)*. In addition to measuring response latency to the heat stimulus, animals were also carefully observed to determine whether or not they licked the paw to which the heat stimulus had been applied. The frequency with which a licking response was seen following withdrawal is shown in Fig. 3-6B. Before the injury, animals licked the stimulated paw following 7 or 8 of the 10 stimulus applications (5 to each forepaw). The contusion injuries or sham operations had no effect on this licking response, which was seen with a similar frequency in all subsequent test sessions with no significant difference at any time point (*P>0.05* for each group *vs*. the base line, *one sample t-test*). These observations are indicative of heat hyperalgesia in the SCI but not sham animals and the consistent presence of licking response indicates supraspinal processing of the increased responsiveness to thermal stimuli.

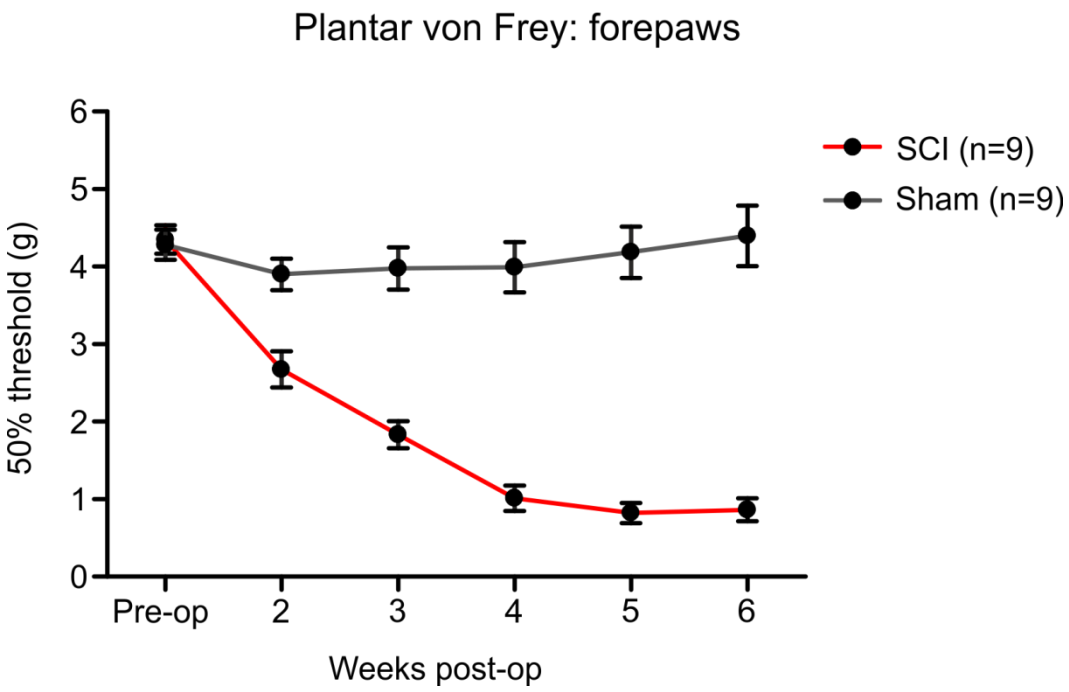

Fig. 3-5. Plantar von Frey testing of the forepaws. The graph shows the 50% threshold for the force of vF filament eliciting a withdrawal response on application to the plantar surface of the forepaws. The test was performed preoperatively and on a weekly basis (2 to 6) after the surgery. Data for both forepaws of each animal was averaged and the plotted data points represent the mean ± SE for contusion injured (n=9) and sham (n=9) operated groups.

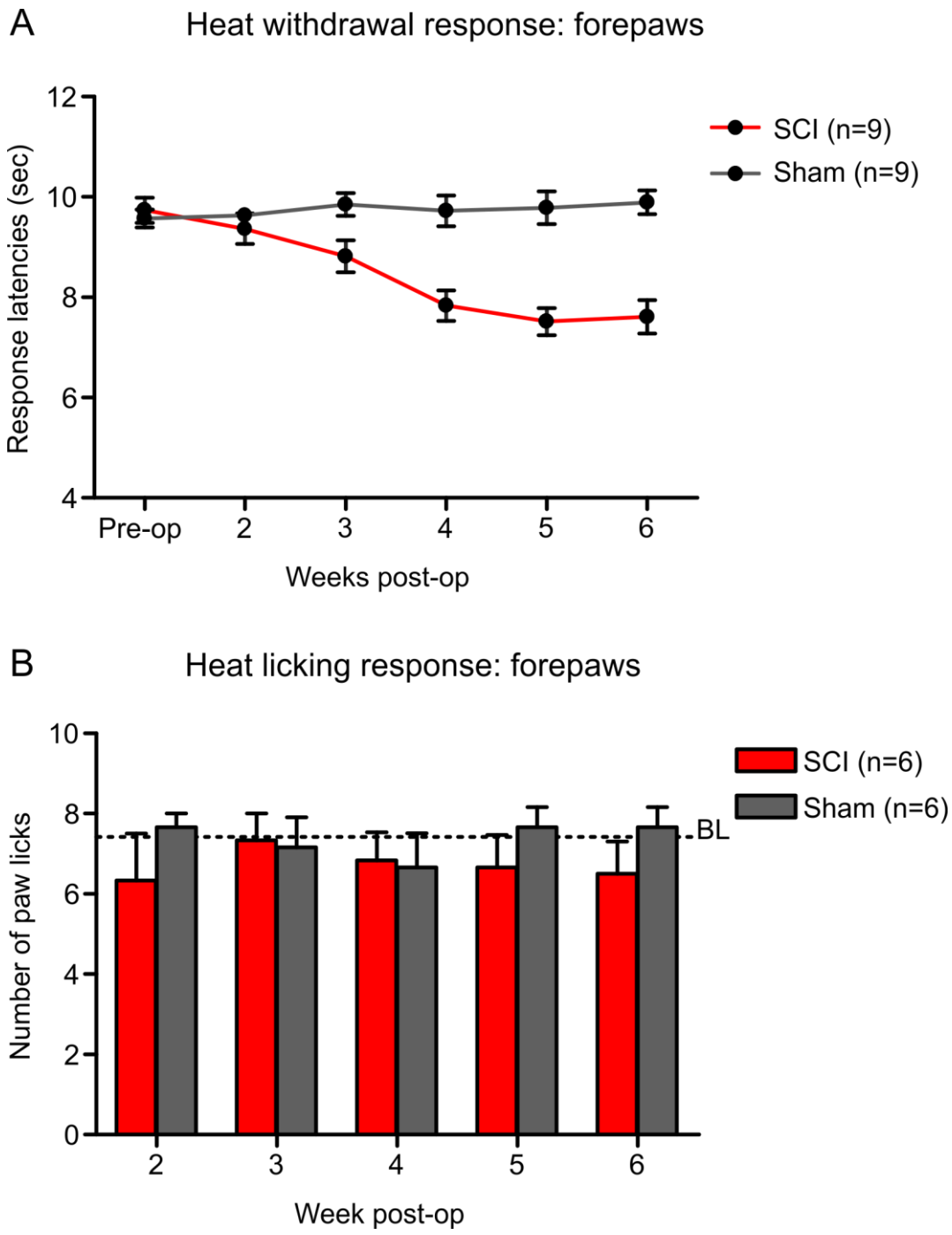

Fig. 3-6. Behavioural responses to heat stimuli applied to the forepaws. A is a graph showing latencies for withdrawal of the forepaws in response to radiant heat applied to the plantar surface. Data for both forepaws of each animal was averaged for SCI (n=9) and sham (n=9) groups. Bars in **B** are showing the frequency with which licking was seen following 10 applications of heat to the plantar surface of the forepaws. Tests were performed on both paws and data averaged for each injured (n=6) and sham (n=6) group. The dotted line (base line "BL") shows the average of incidence of licking observed in all animals before the injury or sham surgery. The observations in A and B were made preoperatively and at 2, 3, 4, 5 and 6 weeks after the injury. All observations are shown as the mean ± SE.

3.3.6 Responses of hindpaws

3.3.6.1 Plantar von Frey test

Contusion SCI had a similar effect on the withdrawal response of the hindlimb to tactile stimuli as for stimuli applied to the forepaws as shown in Fig. 3-7. Both the time course and the percentage reduction in threshold from preoperative values showed a closely similar pattern. However, the absolute threshold in grams was higher from the outset, reflecting the lower sensitivity of the hindpaw plantar skin. Sham operated animals showed little change in threshold in the same tests. The decrease in threshold in injured animals was very significant from week 2 ($P < 0.001$, *Bonferroni post-hoc test*). Such observations have frequently been considered indicative of tactile allodynia developing in the hindpaws of SCI animals (but see discussion).

3.3.6.2 Plantar heat test

The effect of SCI on the withdrawal latencies of the hindpaws differed in several respects from that seen for the forepaws. Although, a reduction in withdrawal latency occurred for the hindpaws as in the forepaws, the reduction occurred earlier and was more marked than for the forepaws (Fig. 3-8A). A reduction in latency was very marked from the first test following injury at 2 weeks *(*shams *vs.* SCI: *P<0.001, Bonferroni post-hoc test)*, becoming maximal by 4 to 5 weeks. The response latency for the sham group of animals did not change in the same test period. The most significant difference seen between the forepaws and the hindpaws was in the licking response following the application of heat stimuli (Fig. 3-8B). When heat stimuli were applied preoperatively to the hindpaw, licking of the paw was seen with a similar frequency to that for testing of the forepaw. However, when tested postoperatively, although the sham animals continued to show a licking response with a similar frequency throughout the remainder of the test period, the licking response was virtually abolished in the SCI animals. A reduction in the latency of withdrawal responses to heat stimuli are generally considered indicative of thermal hyperalgesia but the abolition of licking suggests that neural signalling associated with heat stimuli applied to the hindpaw may not be processed supraspinally after the SCI.

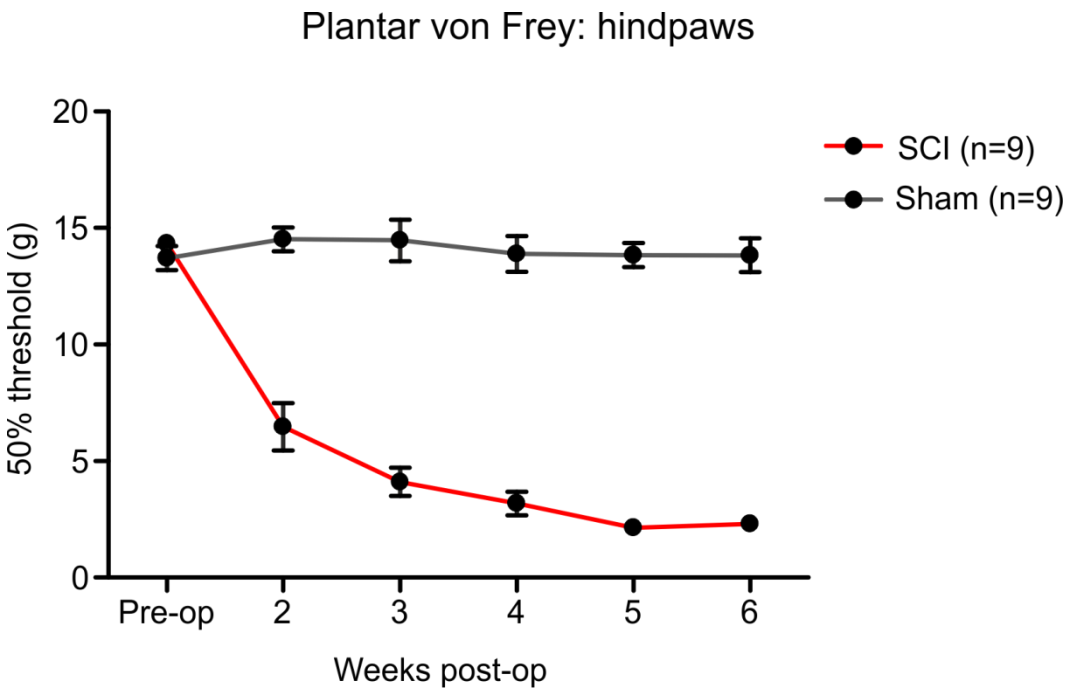

Fig. 3-7. Plantar von Frey testing of the hindpaws. The graph shows the 50% threshold for the force of vF filament eliciting a withdrawal response on application to the plantar surface of the hindpaws. The test was performed preoperatively and on a weekly basis (2 to 6) after the surgery. Data for both hindpaws of each animal was averaged and the plotted data points represent the mean ± SE for contusion injured (n=9) and sham (n=9) operated groups.

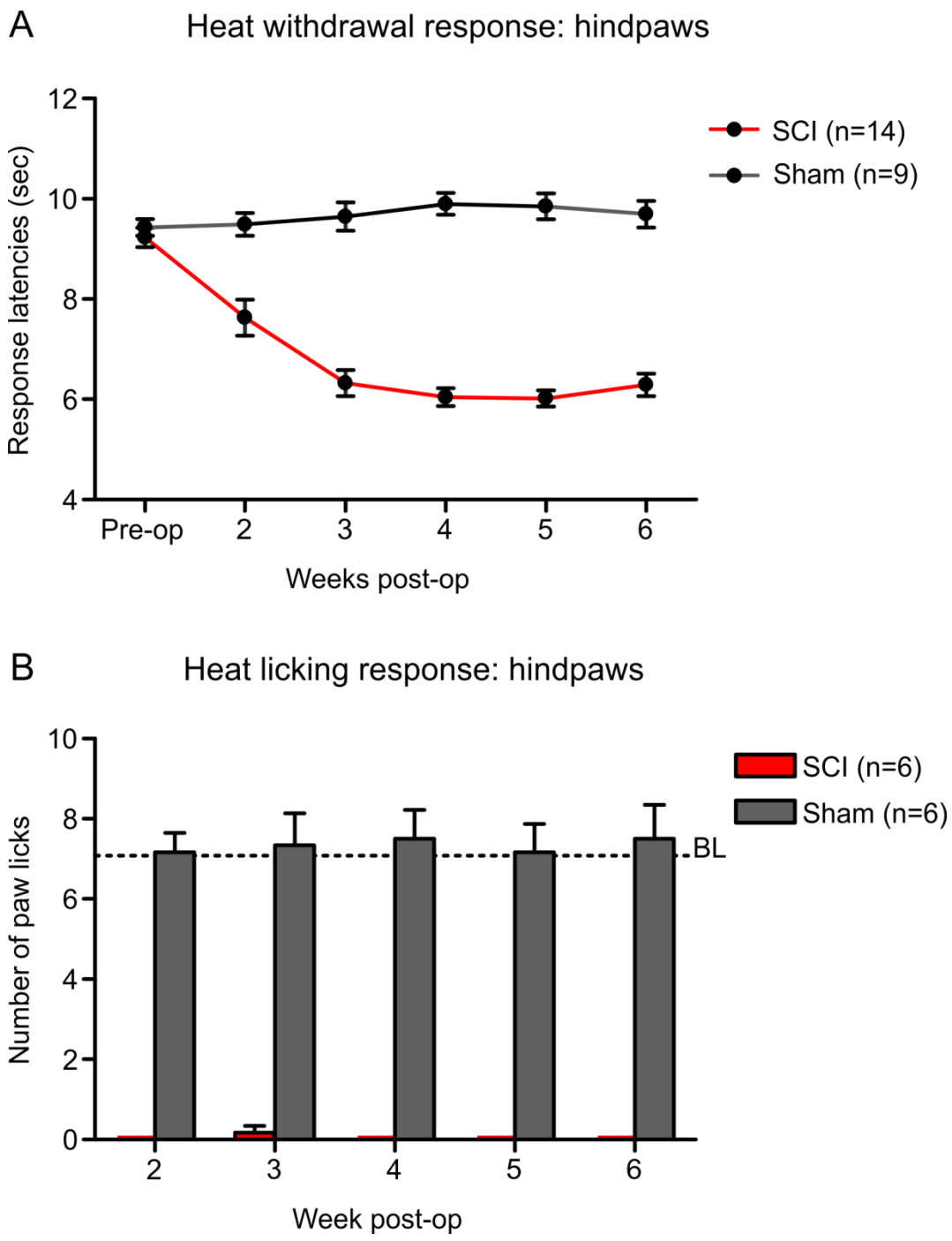

Fig. 3-8. Behavioural responses to heat stimuli applied to the hindpaws. A is a graph showing latencies for withdrawal of the hindpaws in response to radiant heat applied to the plantar surface. Data for both hindpaws of each animal was averaged for SCI (n=14) and sham (n=9) groups. Bars in **B** are showing the frequency with which licking was seen following 10 applications of heat to the plantar surface of the hindpaws. Tests were performed on both paws and data averaged for each injured (n=6) and sham (n=6) group. The dotted line (base line "BL") shows the average of incidence of licking observed in all animals before the injury or sham surgery. The observations in A and B were made preoperatively and at 2, 3, 4, 5 and 6 weeks after the injury. All observations are shown as the mean ± SE.

3.3.7 Forepaw lifting data

3.3.7.1 Responses on the cold plates

When observed on the cold plate maintained at a temperature (7.5°C), both SCI and sham animals showed lifting of the forepaws from the platform which, on some occasions, was associated with aversive behaviours such as licking and shaking, indicating the noxious nature of this temperature. The incidence of FL and the difference between contusion injured and sham operated animals was highly consistent and very marked when tested at 4 weeks, 5 weeks and 6 weeks after the contusion injury or sham operations. These were statistically different at all three time points tested *(P<0.01, Bonferroni test post-hoc)* (Fig. 3-9A). In each group, there was no significant differences in FL investigated at different time points *(P>0.05, Tukey's post-hoc test)*. These observations are indicative of cold hyperalgesia in the SCI animals.

When tested on a plate cooled to 15°C or 20°C, sham animals showed no aversive behaviours, suggesting innocuous cold nature of the plate. Although shams showed lifting of the forepaws from the platform, this was only noticed in very few occasions (1to 2 FLs during 4 min). The incidence of FL was highly consistent and very frequent in the contusion injured animals in comparison to the shams when tested at 6 weeks after the injury and this difference was highly significant *(P<0.001, Bonferroni post-hoc test)* (Fig. 3-9C). These observations are clear evidence for the development of cold allodynia in the SCI animals.

3.3.7.2 Responses on the thermally neutral plates

Contusion injured animals also showed some FL when observed on a smooth and thermally neutral plate maintained at 30°C, but the incidence was approximately two thirds that seen on the cold plate (Fig. 3-9B). Sham animals, which showed a low incidence of FL on the cold plates, showed no FL on the neutral plate. These observations were seen consistently without significant differences at weeks 4, 5 and 6 time points *(P>0.05, Tukey-Kramer post-hoc test)*. At week 6 after the surgery, animals were also tested at 25°C and 35°C (Fig 3-9C). The SCI but not the sham animals showed similar degrees of FL to that seen at 30°C (no significant difference between SCI animals at 25°C *vs.* 30°C *vs.* 35°C: *P>0.05, Tukey-Kramer post-hoc test)*. In addition, further comparisons versus 7.5°C revealed no significant difference (*P>0.05, Tukey-Kramer post-hoc test*). FL observed at 25°C, 30°C and 35°C are suggestive of spontaneous pain in the forepaws.

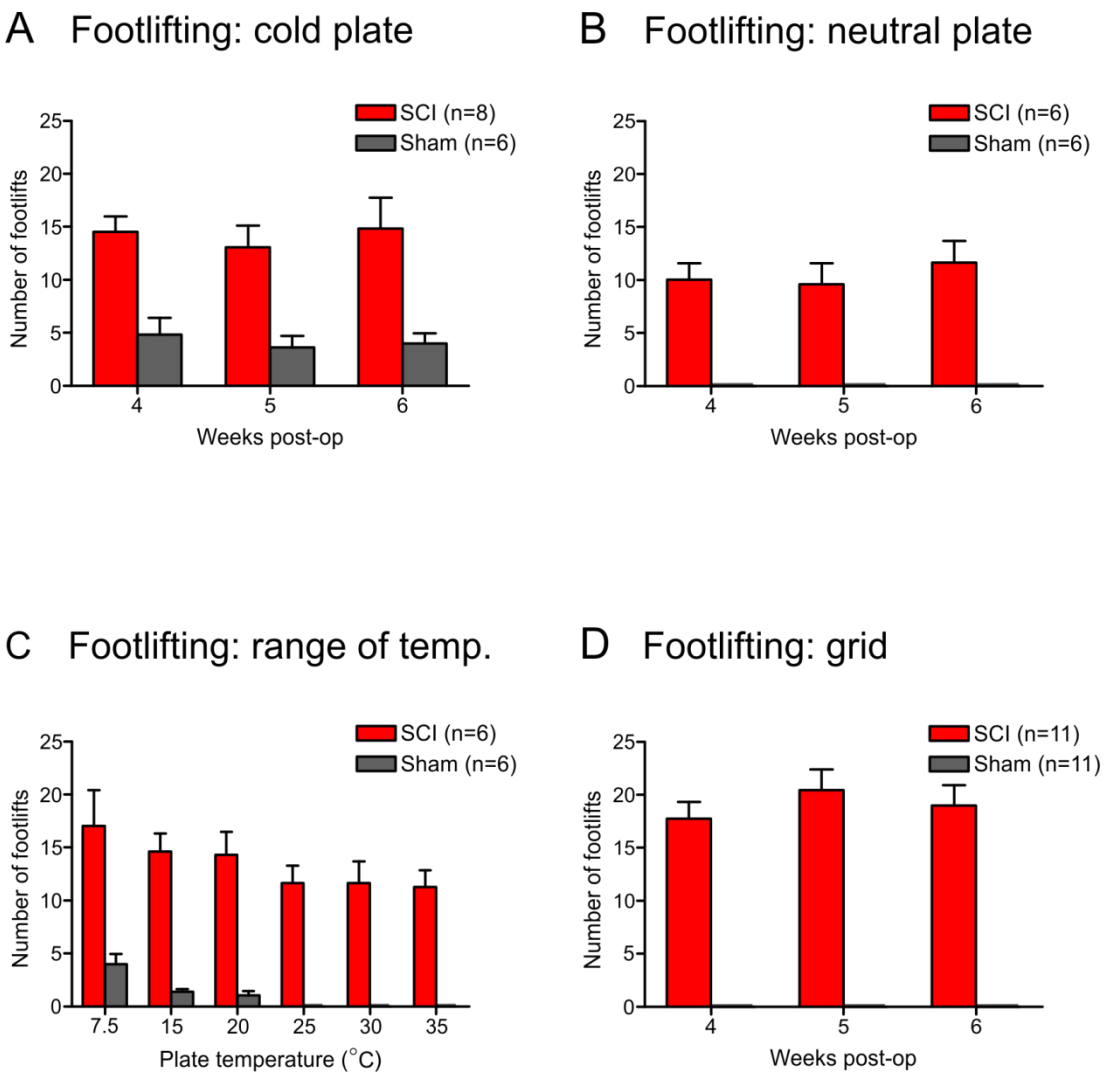

Fig. 3-9. Observations on the frequency of forepaw lifts on different floors. Each of the graphs (A-D) show the number of footlifting episodes (averaged for both forelimbs) observed during a 4 min period. **A** shows results for 8 contusion injured and 6 sham operated animals standing on a cold plate adjusted at 7.5°C. **B** shows data for 6 SCI rats and 6 shams standing on a neutral plate at 30ºC. Histograms in **C** indicate number of footlifting episodes observed while SCI (n=6) and sham (n=6) animals stood on a plate adjusted to a range of temperatures (7.5°C, 15°C, 20°C, 25°C, 30°C and 35°C). **D** indicates results collected from 11 SCI and 11 sham rats standing on a mesh grid. Histograms in A, B and D represent mean ± SE of results collected in weeks 4, 5 and 6 following the SCI and sham surgery while assessments on a plate adjusted to different temperatures (C) were carried out only in week 6 after the injury.

3.3.7.3 Responses on the metal grid

When animals were observed on the metal grid that was used for carrying out the vF testing, the contusion injured animals showed a high and similar incidence of FL at weeks 4, 5 and 6. These observations were completely absent in the sham controls (Fig. 3-9D). This suggests that mechanical allodynia leads to discomfort resulting from the force exerted by the narrow metal rungs of the grid on the forepaws. The response to the mechanical stimuli of the grid presumably occurs against the background of spontaneous FL seen on a thermally neutrally flat surface.

3.3.8 Hindpaw lifting data

The same set of observations as described above for the forepaws were made for the hindpaws.

3.3.8.1 Responses on the cold plates

When observed on the cold plate maintained at a temperature (7.5°C), both SCI and sham animals showed occasional lifting of the hindpaws from the platform (Fig. 3-10A). The incidence was similar to that for forepaw lifting in sham animals. However, whereas the incidence of forepaw lifting was much higher in SCI animals compared to shams, there was no difference in the incidence of hindpaw lifting between SCI and sham animals *(P>0.05, Bonferroni post-hoc test)*. These observations suggest that, unlike in the forepaws, cold hyperalgesia does not develop in hindpaws of SCI animals, or that if the spinal mechanisms occur, then the associated changes in neural signalling are not conveyed supraspinally and do not therefore lead to altered behaviour.

When observed on a plate cooled to 15°C or 20°C, the incidence of FL was significantly higher in the contusion injured than the sham group (at 15°C : *P< 0.05;* at 20°C: *P<0.001, Bonferroni post-hoc test*) (Fig. 3-10C). The observations suggest development of cold allodynia in hindpaws of the SCI group (see discussion).

3.3.8.2 Responses on the thermally neutral plates

When the hindpaws were observed with the animals on thermally neutral plates (maintained at 25°C or 30°C or 35°C), no evidence of FL was seen in either SCI or sham groups in any of the sessions (Fig. 3-10B and C). This is in contrast to the FL behaviour

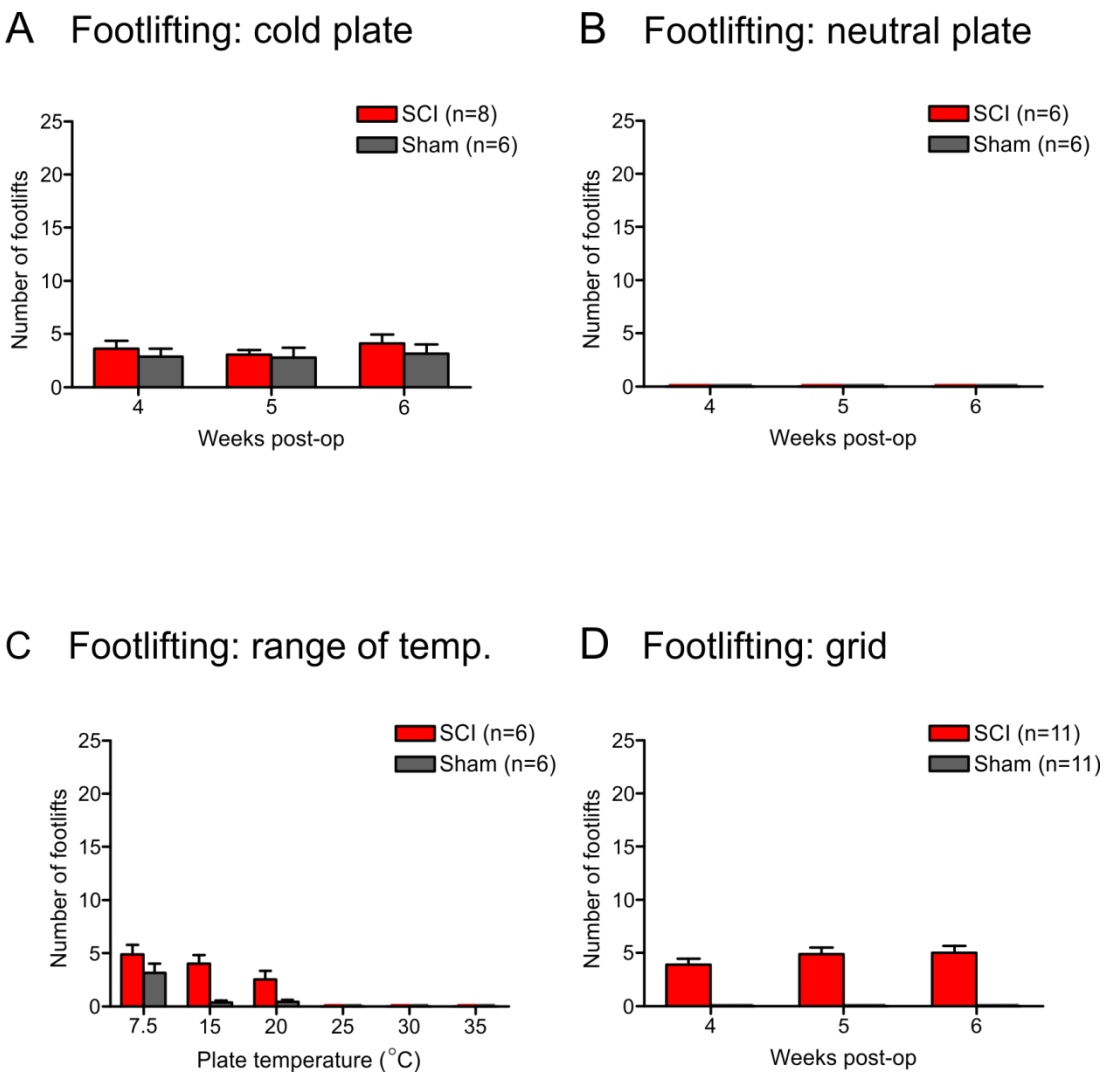

Fig. 3-10. Observations on the frequency of hindpaw lifts on different floors. Each of the graphs (A-D) show the number of footlifting episodes (averaged for both hindlimbs) observed during a 4 min period. **A** shows results for 8 contusion injured and 6 sham operated animals standing on a cold plate adjusted at 7.5°C. **B** shows data for 6 SCI rats and 6 shams standing on a neutral plate at 30°C. Histograms in **C** indicate number of footlifting episodes observed while SCI (n=6) and sham (n=6) animals stood on a plate adjusted to a range of temperatures (7.5°C, 15°C, 20°C, 25°C, 30°C and 35°C). **D** indicates results collected from 11 SCI and 11 sham rats standing on a mesh grid. Histograms in A, B and D represent mean ± SE of results collected in weeks 4, 5 and 6 following the SCI and sham surgery while assessments on a plate adjusted to different temperatures (C) were carried out only in week 6 after the injury.

seen in the forepaw of SCI animals on the same surface. These observations suggest that whereas there may be spontaneous pain in the forepaws, it is absent in the hindpaws.

3.3.8.3 Responses on the metal grid

When animals were observed on the metal grid that was used for carrying out the von Frey testing, the contusion injured animals showed occasional lifting of the hindpaws, but this was much less frequent than for the forepaws in the same test session (Fig. 3-10D). The incidence of this FL was very consistent for test sessions at 4 weeks, 5 weeks and 6 weeks post the injury. At no point was hindpaw lifting seen in sham animals. It is possible that these responses reflect increased spinal reflex sensitivity and responses to the force distribution of the narrow metal rungs of the grid representing a tactile stimulus which in the SCI animals with hyperreflexia is sufficient to result in a flexion response. The same stimuli may be below threshold for producing such responses in the sham animals which have normal tactile sensitivity and normal reflex excitability.

3.3.9 Assessment of responses to stimuli applied to the trunk

3.3.9.1 Electrophysiological identification of appropriate sites on the trunk for application of stimuli processed by spinal cord segments at, above and below the injury level

The pain experienced by spinal cord injured patients can be classified as being perceived to originate above, below or at the injury level. Identification of these different types of pain depends on determination of the neurological level using sensory tests to identify the most caudal dermatome with normal sensation. This is not possible in animal models. Furthermore, while the injury is performed at a known level in animal models, information on the location of the dermatomes that relate to different segments of the thoracic spinal cord is not available as far as we are aware. We therefore carried out electrophysiological experiments to determine locations on the skin of the back where application of tactile stimuli evokes sensory input processed in segments above, below and at the level of the T9 contusion injury.

Preliminary establishment of topography:

Experiments were first performed in 3 normal animals to obtain an indication of skin areas providing input to the different thoracic segments from T4 to T13. Because the length of

cord spanned by segments T4 to T13 is too long to expose in the same experiment, data for different regions was obtained in different animals. Fig. 3-11A shows a diagrammatic representation of the experimental arrangement. Electrical stimuli activating large diameter sensory fibres were applied to skin of the back while recording CDPs at different segmental levels. Recordings were made using a surface electrode which was moved along the spinal cord over each of the thoracic segments in turn. Stimuli were applied along a rostro-caudally oriented line, parallel to, and 2 cm lateral to, the midline. At each recording site, the stimulating electrode was moved in a rostral to caudal direction along this line in 5mm steps and the amplitude of the CDP evoked at each stimulus location was measured. Fig. 3-11B shows plots of the segmental locations where the largest CDPs were evoked by stimuli applied between the T9 injury level and 5 cm caudal to this level. The results show that there is a caudal shift in the representation of skin over the back relative to the spinal segments processing sensory input. This information was used to guide the choice of 3 positions for stimulation of skin over the back suitable for assessment of above level, at level and below level pain. The chosen locations were: 1) Above level stimulus site: 1cm above the T9 injury (+ 1cm). 2) At level stimulus site: 2 cm caudal to the T9 injury (-2 cm). 3) Below level stimulus site: 5 cm caudal to the injury (-5 cm). These locations are illustrated diagrammatically on Fig. 3-11A.

Investigation of chosen stimulation sites:

A further set of experiments was then carried out, in both normal and T9 contusion injured animals, to investigate the responses to stimuli applied at these 3 specific locations in more detail. The segmental distribution of CDPs evoked from these stimulus sites was established by mapping CDP amplitudes while stimulating at each of the three sites. In addition, the dorsal roots by which sensory input from these regions of skins reach the spinal cord was determined by cutting dorsal roots in turn while recording CDPs. Examples of CDPs evoked by stimuli at these three locations are shown in Fig. 3-12A and B. Plots showing the distribution and maximum of CDPs produced by stimulation at the locations are shown in Fig. 3-12C.

1) Above level stimulus site (+1 cm). Stimulation here results in sensory input which is processed rostral to the main area examined in the previous experiments and was shown to lead to CDPs in segments T4 to T6 with maximal CDPs occurring in rostral T5 (one normal *vs.* 2 injured animals). Sectioning dorsal roots at this level showed that almost all inputs travel through the T4 dorsal root were abolished.

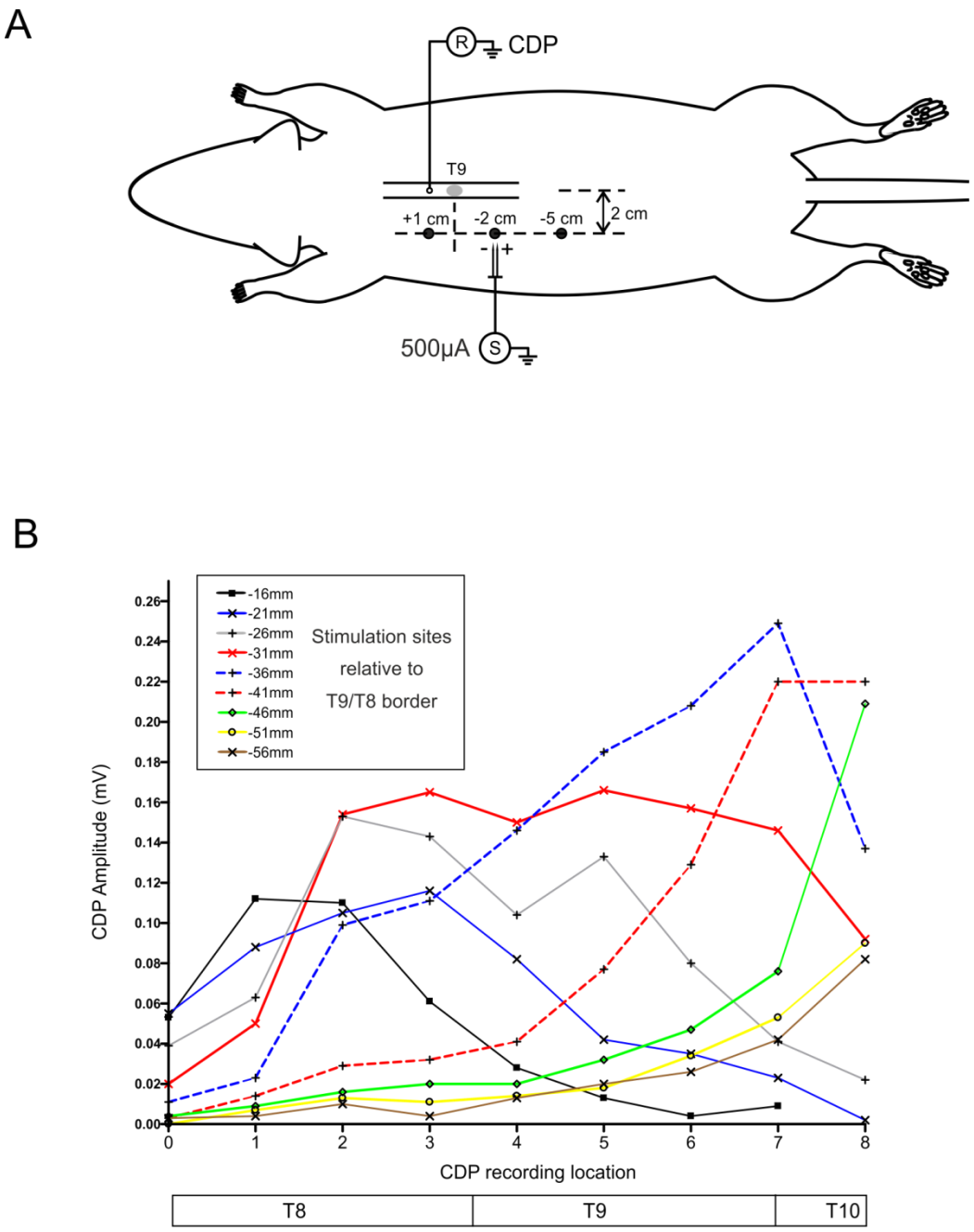

Fig. 3-11. Electrophysiological mapping of the relationship between stimulation sites and segmental processing level for stimuli applied over the back. A is a schematic diagram of the experimental arrangement. The diagram depicts electrodes for applying stimuli to the skin (S) and an electrode for recording cord dorsum potentials (CDPs) from the surface of the spinal cord (R). **B** shows plots of the amplitudes of CDPs recorded from a normal animal. Each plot represents the distribution of CDPs evoked while stimulating at a single location as indicated in the key, between 16 mm and 56 mm caudal to the T8/T9 border which was used as a reference.

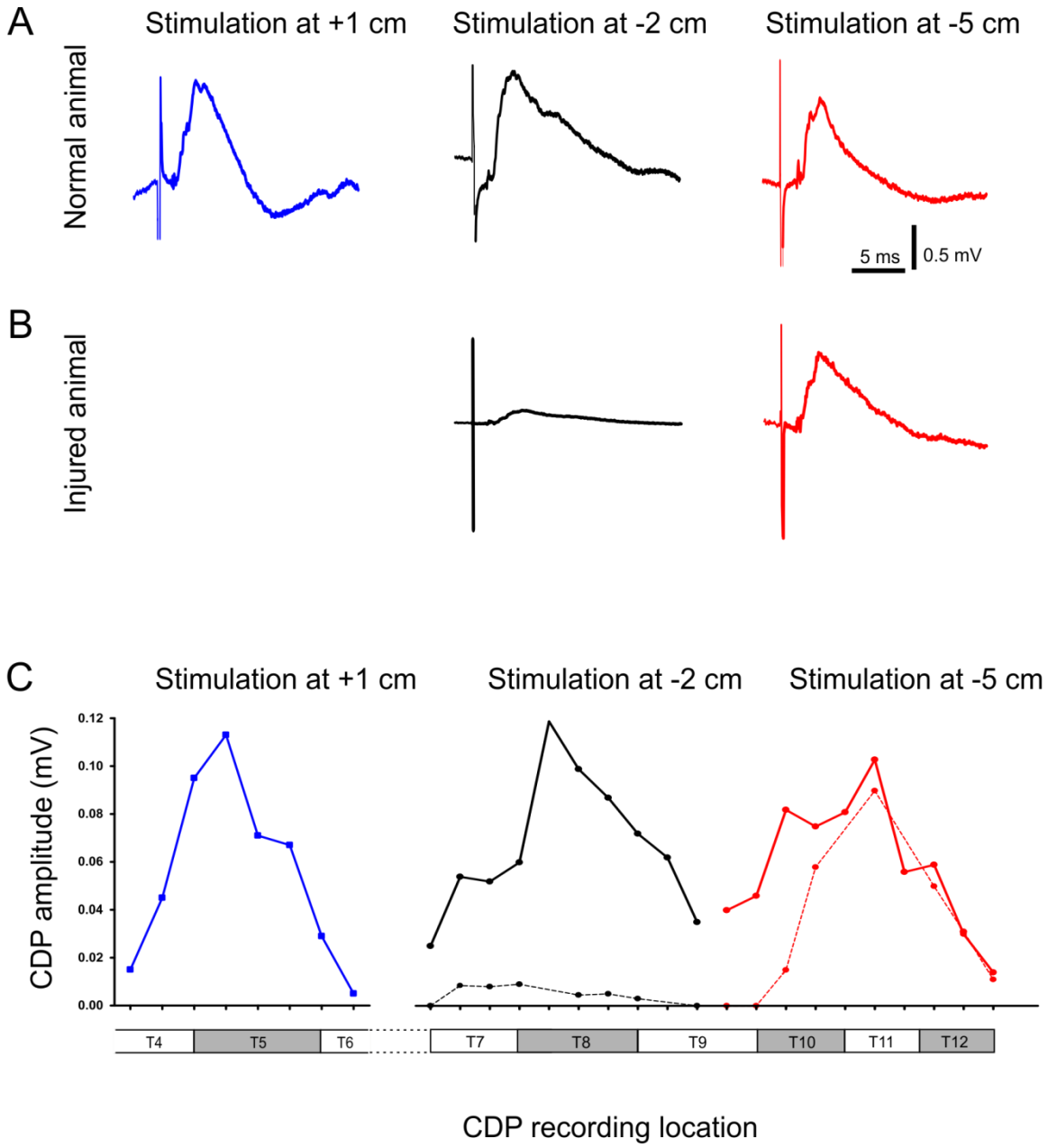

Fig. 3-12. Distribution of CDPs evoked by stimulation at specific locations. A and **B** are examples of maximal CDPs for each stimulation site (+1 above, -2 cm below and -5 cm below the injury level) in normal (A) and injured (B) animals. Plots in **C** are showing the distribution of CDPs evoked by stimulation at +1 cm, -2 cm and -5 cm. The solid lines represent data normal animals while the dotted lines show equivalent data obtained from animals with a 200 kdyn T9 injury.

2) At level stimulus site (-2 cm). Stimulation at this site results in CDPs from T7 to T9 with the maximal CDP occurring in T8 (2 normal *vs.* 5 injured animals). The majority of the input producing these CDPs travelled through the T8 dorsal root.

3) Below level stimulus site (-5 cm). Stimuli applied here result in CDPs distributed from T9 to T12 with a maximal response around mid T11 (2 normal *vs.* 6 injured animals). Much of the input from this position is carried through the T11 dorsal root but some also travels through adjacent T10 and T12 dorsal roots.

Responses to stimuli applied to these locations were also investigated in 6 animals with T9 200 kdyn contusion injuries. In these animals, stimuli applied 2 cm caudal to the injury produced CDPs in the same segments, but with amplitudes very substantially smaller (less than 10%), than those recorded from normal animals (Fig. 3-12C middle plot). In contrast, CDPs evoked by stimulation 5 cm caudal to the T9 injury were of almost normal amplitude except at the rostral extent of their distribution in the T9 segment (Fig. 3-12C right hand plot). Since CDPs evoked in the segments immediately below the T9 injury (stimulation at -5 cm) are minimally affected by the injury, the much diminished response seen in the segments immediately above the injury (stimulation at -2 cm) is most likely to be due to damage to the dorsal roots that carry sensory input to these segments rather than damage to the spinal cord itself (see also tract tracing results, below).

3.3.9.2 Responses to tactile stimuli applied to the trunk at, above and below the injury level

In this section the terms “above level”, “at level” and “below level” are used to refer to stimuli applied to the back at 1 cm above, 2 cm below and 5 cm below the T9 injury respectively. In the previous section we provide information on the segmental levels at which sensory input from these stimulus locations is processed within the spinal cord and thus the physical relationship of these to the injury site at T9. The same terminology is used clinically but in this case the levels are determined by sensory testing so that the terminology is not directly equivalent (see discussion).

Figures 3-13, 3-14 and 3-15 show the results obtained when testing the effect of SCI on the sensitivity of these three sites to the vF filament selected in preliminary experiments (see methods) which was applied on 10 occasions to each site. Before SCI, responses to stimuli were similar at each of the test locations. The majority of animals responded to only 1-3 of

the 10 stimuli, on the remaining occasions the stimulus was completely ignored. The most common type of response before injury was slight escape or head turning. The low incidence of response suggests that the chosen stimulus is innocuous in nature and the similarity of response at different locations suggest that the sensitivity to such stimuli is similar over a large area of the back.

Responses to above level stimuli

The incidence of responses to stimuli applied at +1 cm (above level) is shown in Fig. 3-13. The incidence of any of the recognised types of response to the stimuli (Fig. 3-13A) more than doubled at the first test session after injury (1 week) and continued to increase in subsequent weeks, reaching a maximum of about 9/10 at 4 weeks. The incidence remained at this elevated level for the subsequent two weeks of the testing period, though some animals reached the maximal (10/10) response level so that this apparent plateauing of the response may reflect saturation of the test. The incidence of attempted biting of the filament (Fig. 3-13B), behaviour rarely if ever seen before injury, also increased progressively following the injury. This behaviour continued to increase throughout the 6 week post injury test period, becoming the most common type of response by week 5. In comparison, the sham animals showed only the slightest suggestion of an increased sensitivity *(*non significant: *P>0.05, Tukey's post-hoc test)* and only in the first week following injury. These observations are a clear indication for the development of tactile allodynia over the back to above level stimuli.

Responses to at level stimuli

The incidence of responses to stimuli applied at -2 cm (at level) is shown in Fig. 3-14. The incidence of any of the recognised types of response to the stimuli (Fig. 3-14A) was little changed at the first test session after injury (1 week) but doubled by the second week and continued to show a gradual progressive increase throughout subsequent weeks. This did not, however, reach saturation level during the 6 weeks of postoperative testing. The incidence a biting response also differed for above level and at level stimuli. Whereas the incidence of attempted biting of the filament for above level stimuli showed an increase soon after injury (Fig. 3-14B), this type of behaviour remained quite rare in response to at level stimuli, with only a suggestion of an appearance developing towards the very end of the test period. In comparison, the sham animals showed no suggestion of an increased sensitivity (non significant: *P>0.05, Tukey's post-hoc test)*. These observations are a clear

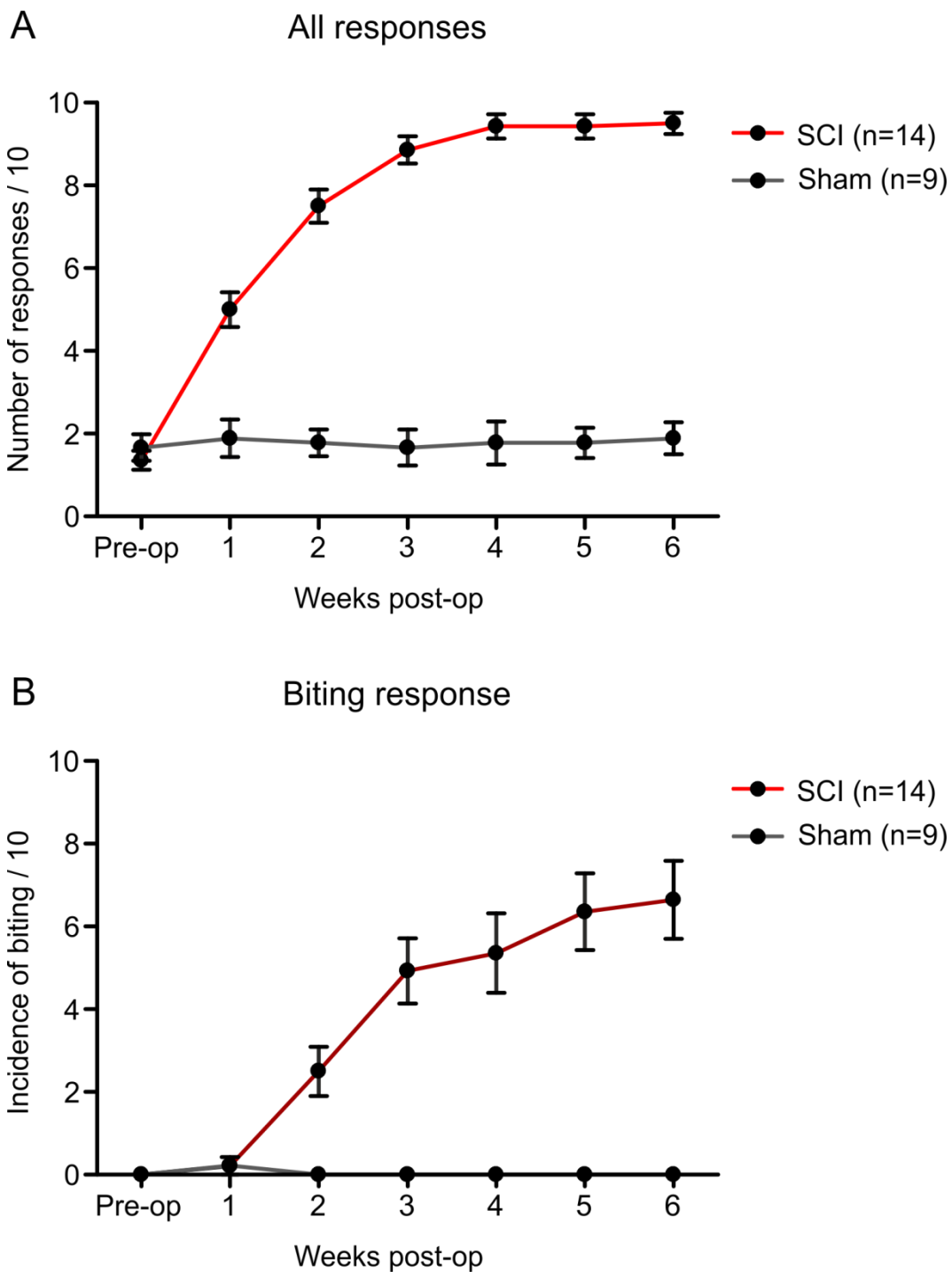

Fig. 3-13. Behavioural testing using stimuli applied at +1 cm (above level) over the back. **A** graph showing the incidence of any of the recognised types of response to tactile stimuli applied over the back. **B** shows just the incidence of a biting response. The plots show the mean ± SE for responses observed in SCI (n=14) and sham (n=9) groups in each test session performed preoperatively and then on a weekly basis after the surgery until week 6.

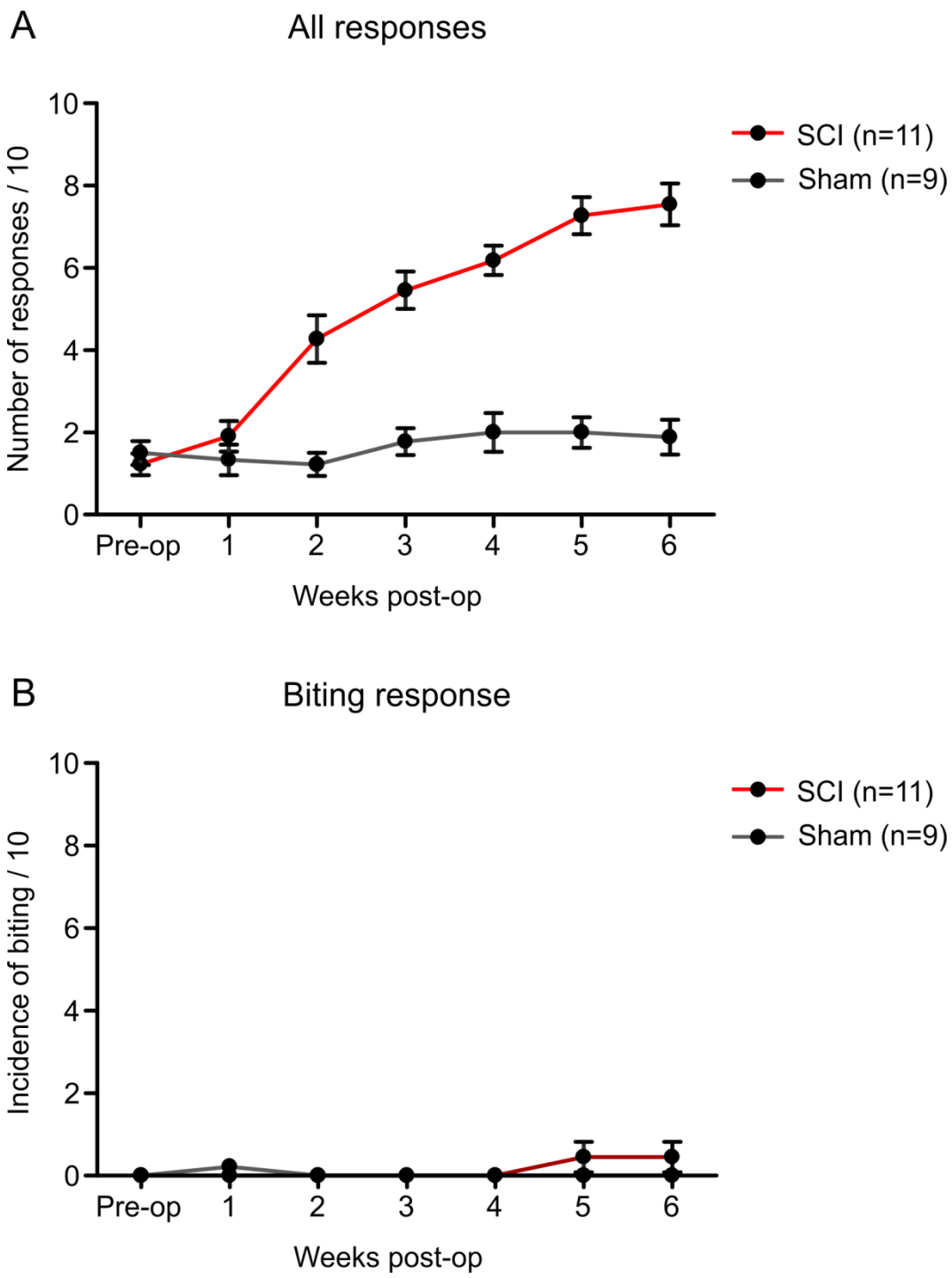

Fig. 3-14. Behavioural testing using stimuli applied at -2 cm (at level) over the back. A is a graph showing the incidence of any of the recognised types of response to tactile stimuli applied over the back. **B** illustrates just the incidence of a biting response. The plots show the mean ± SE for responses observed in SCI (n=11) and sham (n=9) groups in each test session performed preoperatively and then on a weekly basis after the surgery until week 6.

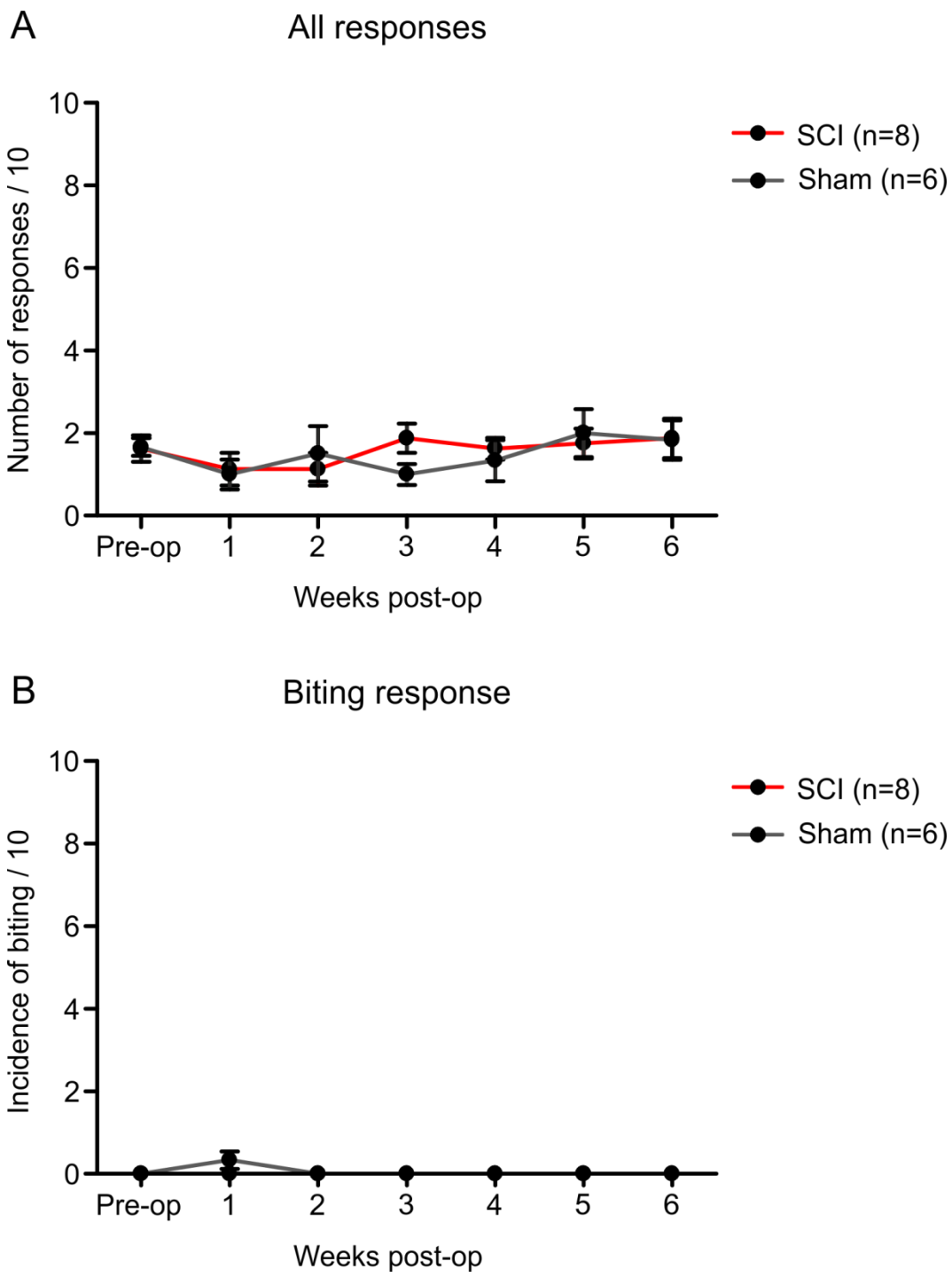

Fig. 3-15. Behavioural testing using stimuli applied at -5 cm (below level) over the back. **A** is a graph showing the incidence of any of the recognised types of response to tactile stimuli applied over the back. **B** illustrates just the incidence of a biting response. The plots show the mean ± SE for responses observed in SCI (n=8) and sham (n=6) groups in each test session performed preoperatively and then on a weekly basis after the surgery until week 6.

indication for the development of tactile allodynia to at level stimuli over the back but suggest that this is less marked than for the above level stimuli.

Responses to below level stimuli

The results obtained when applying stimuli to the back at -5 cm (below level) are shown in Fig. 3-15. In contrast to the results obtained in this model when applying stimuli processed above level and at level, SCI had no effect on the response to stimuli applied below level. The progressive increase in sensitivity seen at the more rostral stimulus locations was not observed here but neither did the injury abolish the baseline response. Animals continued to respond to approximately 2 out of the 10 stimulus applications throughout the duration of the testing. The incidence of a biting response was also unchanged apart from the transient appearance in a minority of animals at the first test session one week after surgery.

3.3.10 PEAP test

This test was used to confirm cortical processing of nociceptive signals. During preliminary experiments we arranged the test conditions such that normal animals showed a clear preference for the dark half of the cage, spending on average about 75% of their time in this environment even though stimuli were continuously applied to animals while they were located on this side of the housing. We then tested groups of sham operated animals and injured animals while applying stimuli using vF filaments applied to three different sites: 1) over the back at +1 cm (above level), 2) over the back at -5 cm (below level) and 3) to the hindpaws.

3.3.10.1 Above level stimuli applied to the back

The results obtained when applying stimuli to the back above the injury level are shown in Fig. 3-16. Sham operated animals (n=8) showed a clear preference for the dark side of the cage spending an average of 75% of the time in the black compartment. This did not differ between the first 10 minutes of the test and the total 30 minute duration of the test (Fig. 3-16A). Exploratory behaviour i.e. frequency of crossing between the light and dark halves of the cage were consistent over time with the number of crossings in the 30 minute test period as a whole being about 12 (Fig. 3-16B).

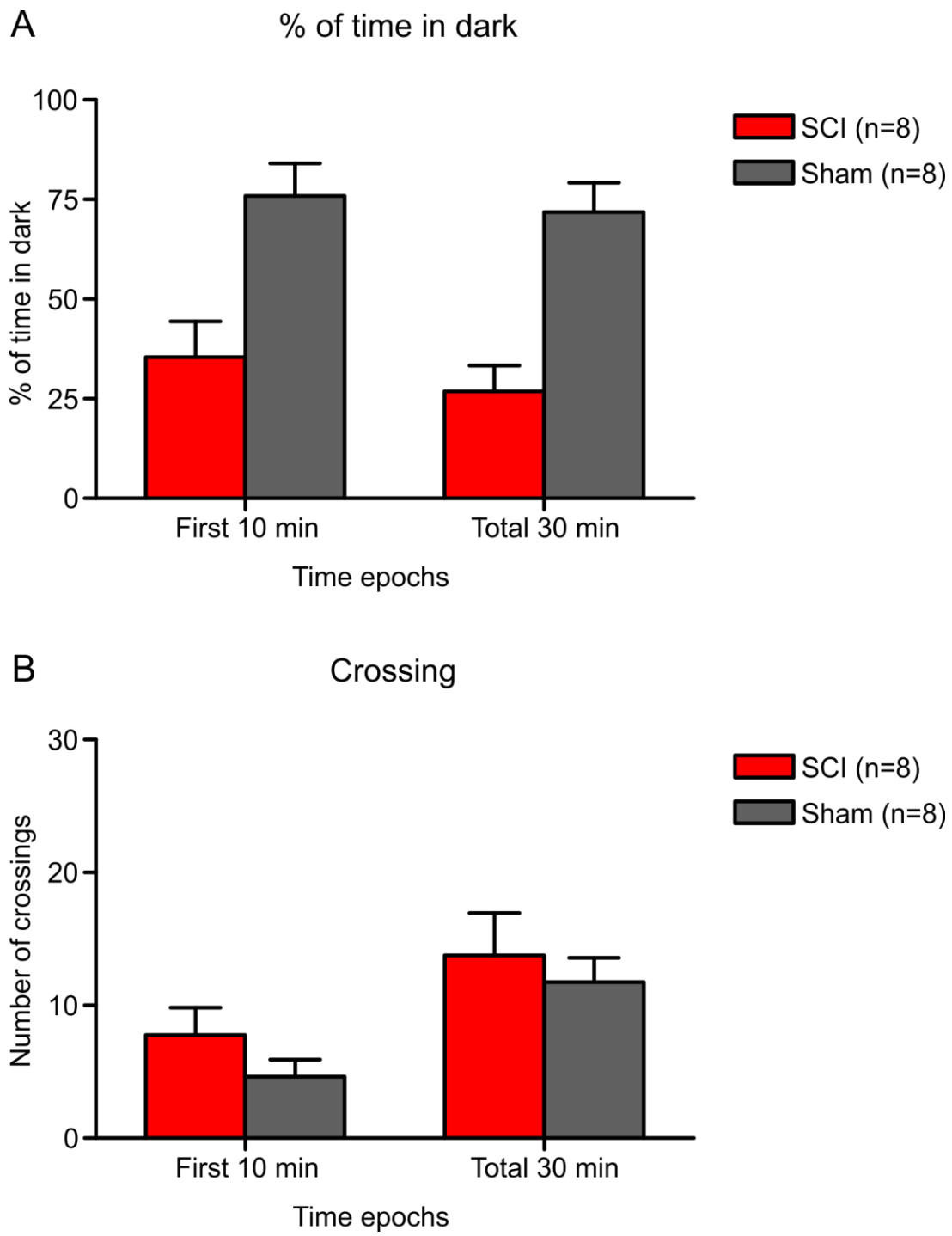

Fig. 3-16. Results from PEAP test using vF stimulation of the back at +1 cm above the injury site. A indicates the percentage of the test time that the animals spent on the dark side of the test chamber. **B** shows the number of times that the animals crossed between the light and dark sides of the chamber. The bars represent the mean values ± SE for the SCI (n=8) and sham (n=8) groups of animals. Data (collected in week 4 PO) for the first 10 min of the test session and full 30 min of the session is shown separately.

On the other hand, under the same conditions and with the same stimuli, SCI animals (n=8) spent less time than the sham animals on the dark side of the cage and this behaviour was similar in the first 10 minutes of testing as in the whole of the 30 minute test period (Fig. 3-16A). On average, the SCI animals spent 27 % of their time on the dark side compared to 72% for the sham operated animals over the 30 minutes of the test, a difference which is highly significant *(P<0.001, Bonferroni post-hoc test).* There was no difference in the frequency with which SCI and sham animals crossed between the light and dark sides of the cage *(P>0.05, unpaired t-test*). These observations suggest that the SCI animals perceive the stimuli as more unpleasant than sham animals and that the stimuli therefore result in a greater modification of the behaviour of the SCI animals than the shams. For this modification in behaviour to occur, neural activity resulting from the stimuli must be processed at a conscious level.

3.3.10.2 Below level stimuli applied to the back

Fig. 3-17 shows data collected from the same operant test when stimuli were applied to the back below the injury level (at the -5 cm stimulus location). In contrast to the marked difference in behaviour between sham and SCI animals seen using above level stimuli, with below level stimuli the time spent on the dark side by sham and SCI animals was very similar (Fig. 3-17A; 77% and 81 % respectively for the 30 minute session; which is not significantly different, *P>0.05, Bonferroni post-hoc test).* In addition, they showed similar crossing behaviour between the light and dark halves of the cages (Fig. 3-17B). This suggests no difference in the perception of the stimuli applied at -5cm in the sham and SCI animals. This is consistent with the absence of any increased sensitivity to stimuli applied at this location in contrast to the marked increased in sensitivity when applied above level.

3.3.10.3 Stimuli applied to the hindpaws

The results obtained using this test when a vF filament was applied to the hindpaws of sham and SCI animals is shown in Fig. 3-18. As with stimuli applied to the back below level, there was little difference in behaviour between sham and SCI animals when stimuli were applied to the hindpaws, both groups of animals spending more time on the dark side of the cage (58% for sham and 71% for SCI which is not significantly different; *P>0.05, Bonferroni post-hoc test)* (Fig. 3-18A). However, the contusion injured animals displayed more crossing activity between the light and dark sides than the sham animals (Fig. 3-18B), however, this difference between the two groups was statistically insignificant at the

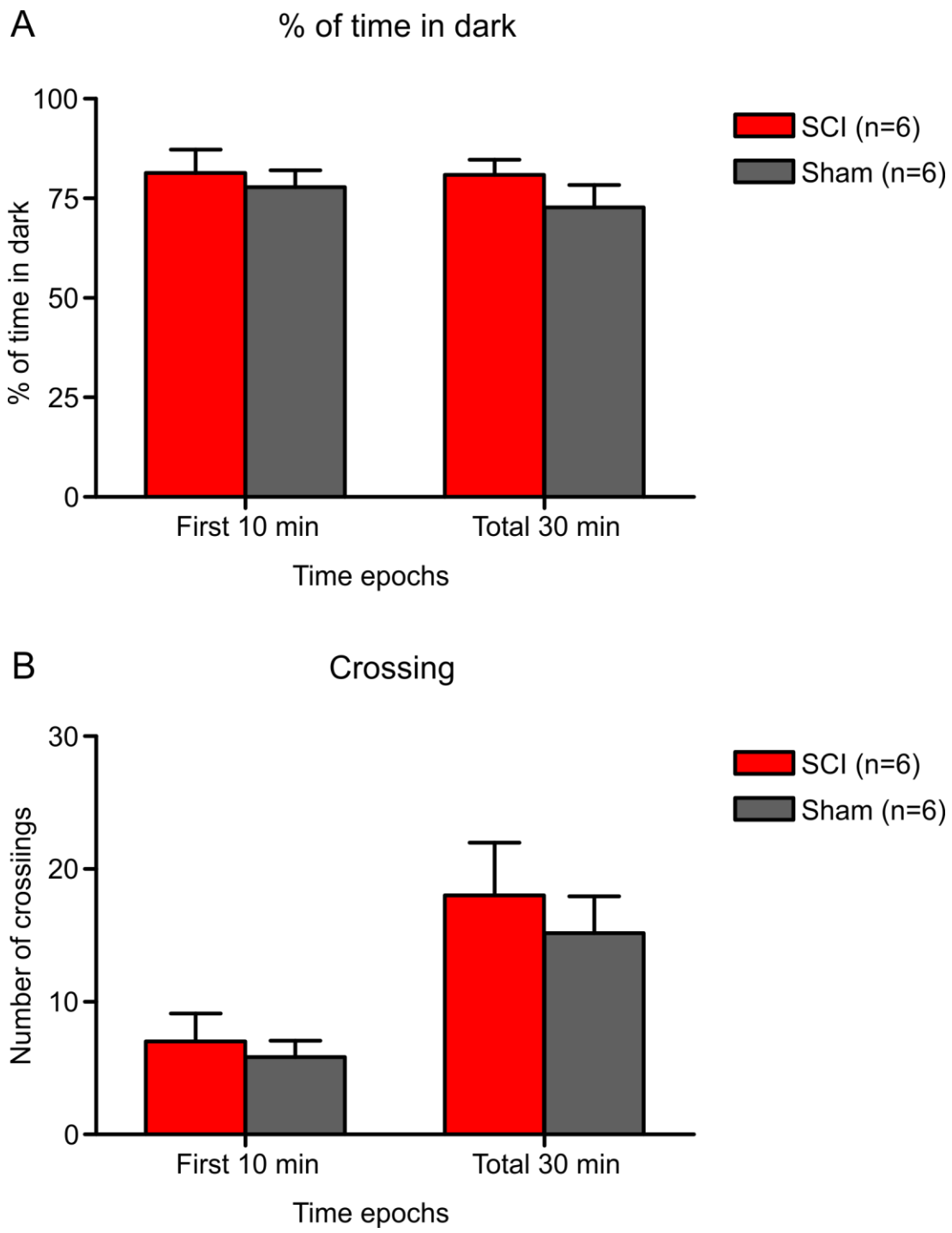

Fig. 3-17. Results from PEAP test using vF stimulation of the back at -5 cm below the injury site. A indicates the percentage of the test time that the animals spent on the dark side of the test chamber. **B** shows the number of times that the animals crossed between the light and dark sides of the chamber. The bars represent the mean values ± SE for the SCI (n=6) and sham (n=6) groups of animals. Data (collected in week 6 PO) for the first 10 min of the test session and full 30 min of the session is shown separately.

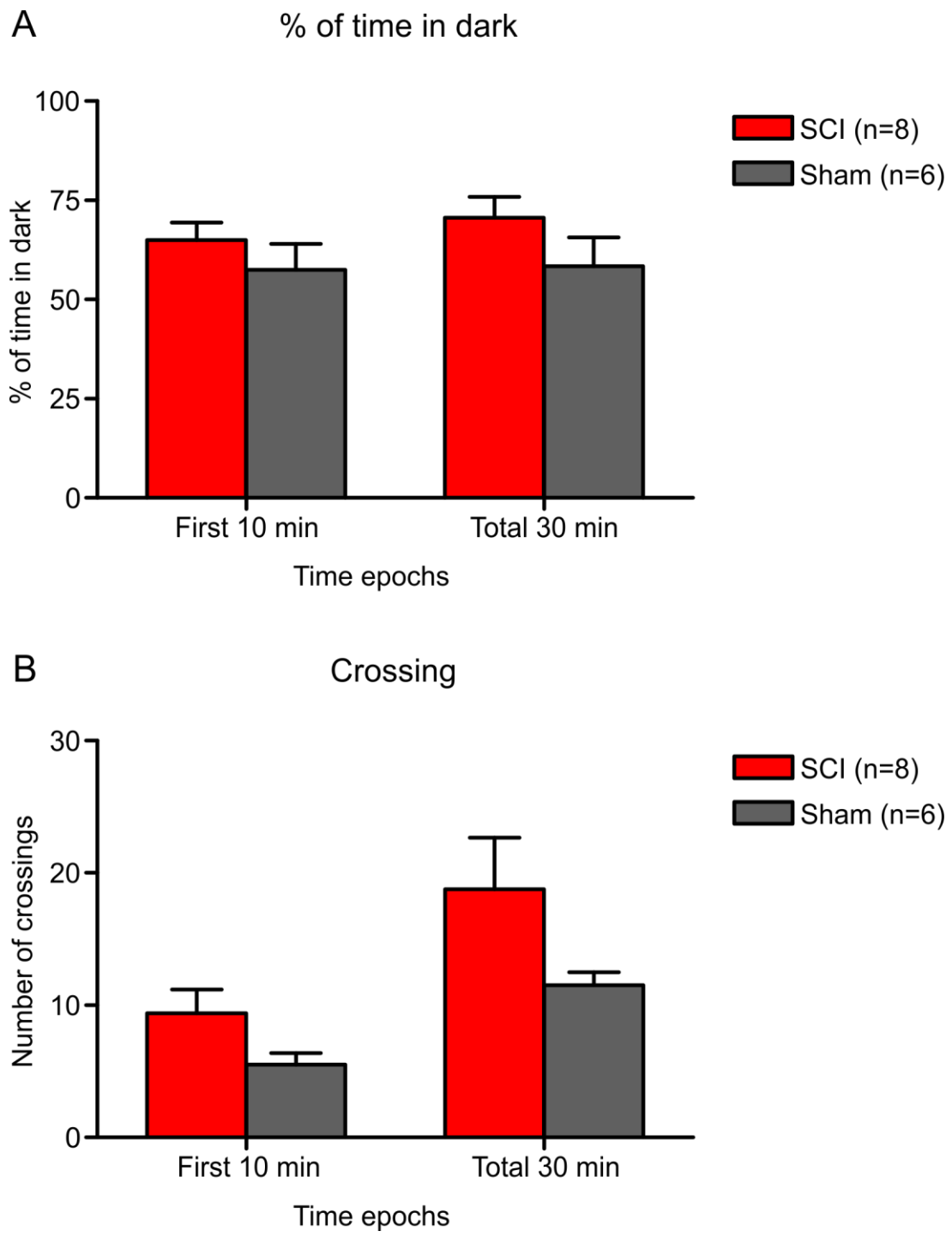

Fig. 3-18. Results from PEAP test using vF stimulation of the hindpaws. A indicates the percentage of the test time that the animals spent on the dark side of the test chamber. **B** shows the number of times that the animals crossed between the light and dark sides of the chamber. The bars represent the mean values ± SE for the SCI (n=8) and sham (n=6) groups of animals. Data (collected in week 6 PO) for the first 10 min of the test session and full 30 min of the session is shown separately.

two testing epochs *(P>0.05, Bonferroni post-hoc test)*. The lack of difference between sham and injured animals with stimuli applied to the hindpaws is in contrast to the increased sensitivity of the hindpaws in injured but not sham animals seen during the plantar vF test and plantar heat test.

3.3.11 Assessment of damage to ascending nociceptive pathways

The sensory tests described above show that responses of a type that require supraspinal processing in the brain stem or cortex are absent or unchanged when stimuli are applied below the level of the injury. This may be because injury does not lead to an enhanced pain state in segments below the injury or alternatively, it may be due to interruption of ascending pathways conveying nociceptive information to the brain. To investigate the latter possibility we therefore carried out a retrograde labelling study of spinal projection neurons which are of established importance in pain processing. The analysis focused on neurons labelled by injections of FG into the CVLM and LPB nucleus. Correct targeting of the tract tracer to the intended structures was checked by inspecting sections of the brain from the injection site under a fluorescence microscope. Representative images were collected by confocal microscopy and examples are shown in Fig. 3-19A (CVLM) and 3-19B (LPB nucleus).

Numbers of retrogradely labelled neurons in the dorsal horn of segments C7, T8 and L4 were quantified after CVLM injections using confocal microscopy and image analysis. Injections were made into the CVLM of 6 normal animals and 7 SCI animals Accurate targeting of the injection sites was confirmed in all animals included in the study and an example of a CVLM injection site is shown in Fig. 3-19A. Estimates were made of the numbers of retrogradely labelled neurons in an area encompassing the dorsal horn which included all grey matter dorsal of the central canal (Fig. 3-20A). Typical examples of retrogradely labelled neurons in the T8 and L4 segments of normal animals are illustrated in Fig. 3-20C and E, respectively, while Fig. 3-20B, D, and F show example of section scanned from segments C7, T8 and L4 of contusion injured animals. Fig. 3-20G, H and I show magnified zones for selected areas containing neurons labelled with FG.

Estimates of the numbers of projection neurons retrogradely labelled from the CVLM are shown in Fig. 3-21. The numbers of neurons in the dorsal horn C7 were closely similar in normal (n=6) and in SCI (n=4) animals (Fig. 3-21A). The mean number of dorsal horn neurons in normal animals was 16.07 ± 1.8 per section (31 sections examined) while the

mean for sections from SCI animals was 16.98 ± 0.7 (15 sections examined). In both cases, projection cells were mainly distributed in lamina I and the lateral spinal nucleus while scattered to a lesser extent in deeper laminae and around the central canal. We assume that these neurons, which are far from the injury site are not affected by the injury and that the similarity in the numbers of labelled neurons can therefore be taken as an indication that the injections led to labelling of a comparable proportion of projection neurons in both groups of animals. The mean number of labelled neurons in T8 sections from normal animals (n=2) was 13.72 ± 0.1 (13 sections examined) which is fewer than in C7. The mean number in T8 of SCI animals (n=2) was 9.15 ± 0.5 (11 sections examined) which is considerably less than that in normal animals (Fig. 3-21B). This presumably reflects a direct death of projection neurons in the T8 segment as a result of the injury in T9. The mean number of retrogradely labelled neurons in the dorsal horn of sections from L4 segment in normal animals (n=3) were 36.31 ± 5.7 (10 sections examined) compared to 3.05 ± 0.5 (11 sections examined) in SCI animals (n=2) (Fig. 3-21C).

The CVLM is one of the main targets of lamina I neurons (approximately 80% to 90%, Polgár et al., 2010) but some neurons project to the LPB nucleus and not the CVLM. In order to check that these lamina I neurons were also interrupted, FG injections were also made into the LPB nucleus of 3 normal and 3 SCI animals. Accurate targeting of the injection sites was confirmed in all animals included in the study and an example of a LPB injection site is shown in Fig. 3-19B. Typical examples of retrogradely labelled neurons in lamina I at the L4 segment of a normal animal are illustrated in Fig. 3-22A. Labelled neurons were counted in lamina I of the L4 segment. In 11 sections (70 µm) from 3 normal animals, the mean number of labelled cells was 9.97 ± 0.87 (Fig. 3-22B) while none of the sections from L4 segment of 3 contusion injured animals contained any lamina I labelled neurons, confirming the observations made for injections into the CVLM (Fig. 3-22C).

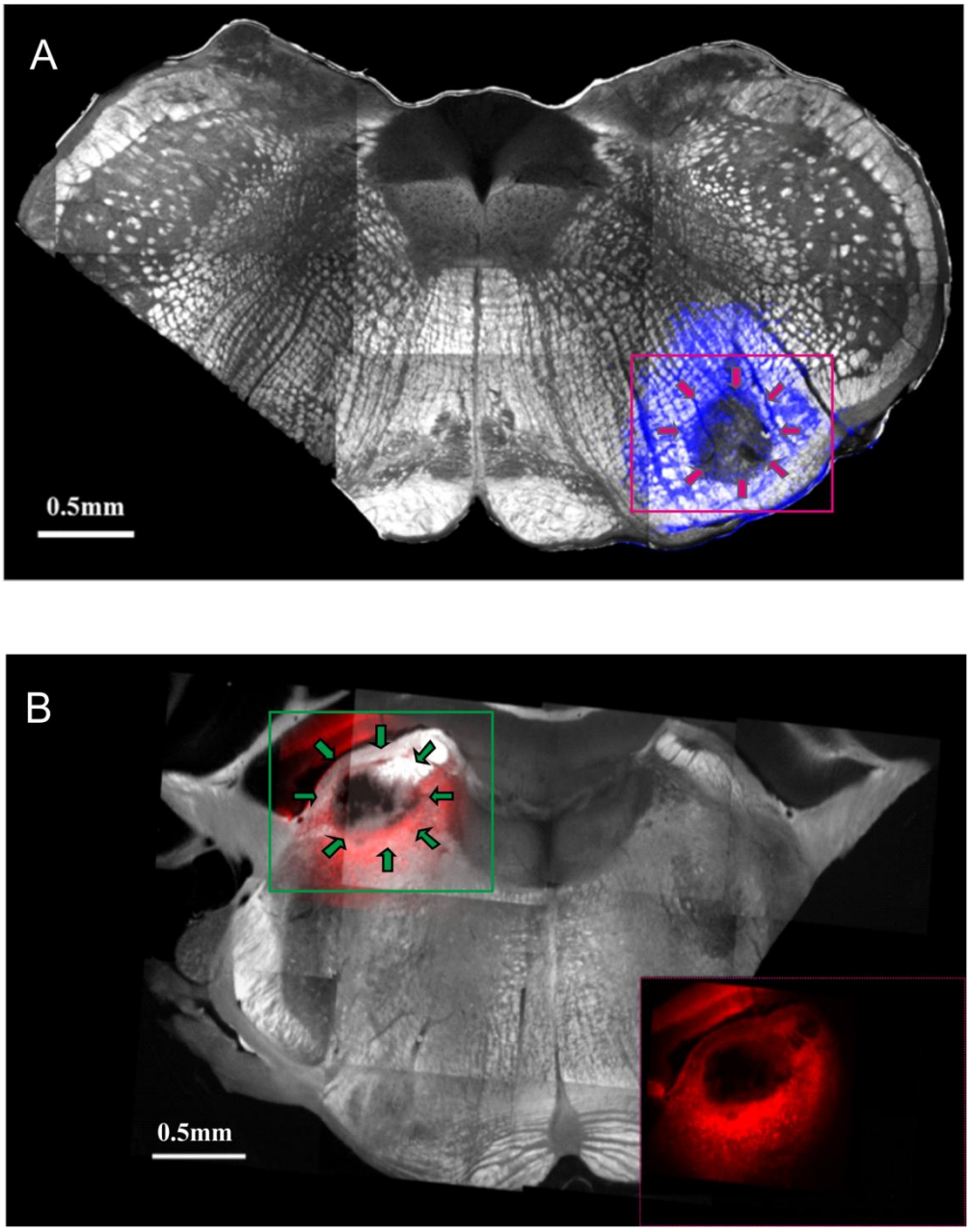

Fig. 3-19. Examples of injection sites from the tract tracing study. A and **B** show montages of confocal microscope scans of coronal sections through the brain. For CVLM site, injections targeted anteriorposterior (AP) -4.7 caudal, mediolateral (ML) 2.1 lateral and dorsoventral (DV) +/-0 above in relation to ear bar zero. For LPB nucleus, injections were done at approximately AP -0.4 caudal, ML 2.1 lateral and DV+3.2 above in relation to ear bar zero. The arrows indicate the sites where injections of the retrograde tract tracer FG were made into the CVLM (A) or LPB nucleus (B). Scale bar = 0.5 mm.

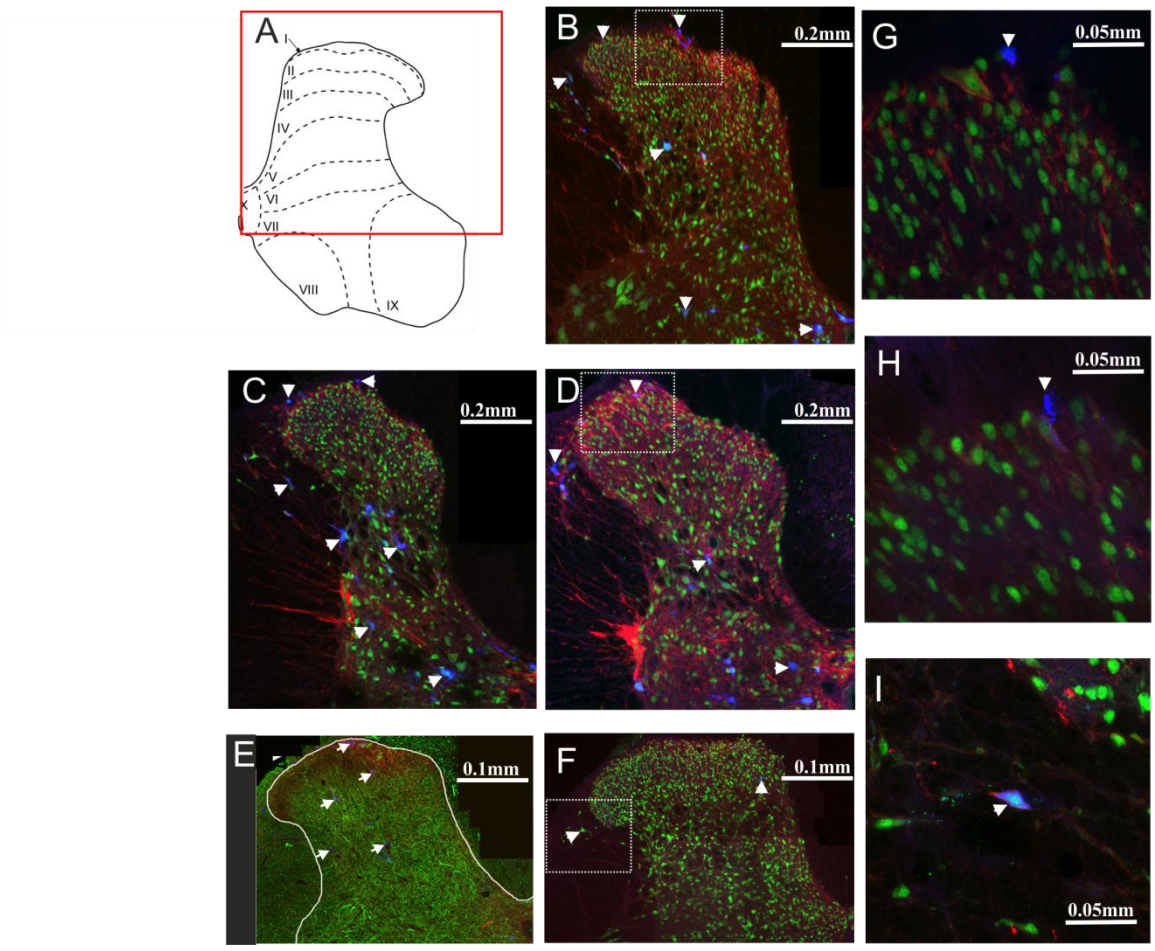

Fig. 3-20. Retrograde labelling of neurons projecting to the CVLM. The red box in **A** illustrates the dorsal horn targeted for quantification of projection neurons (image adapted from Todd, 2010). **B** is confocal image showing normal distribution of projection neurons in the dorsal horn obtained from a C7 segment of an injured animal (scale bar = 0.2 mm). **C** and **D** montage of confocal microscope scans of the dorsal horn obtained from sections of the T8 segment in normal (C) and SCI (D) animals (scale bar = 0.2 mm). **E** and **F** montage of confocal microscope scans of dorsal horn taken from sections of the L4 segment in normal (E; scale bar = 0.1mm) and injured (F; scale bar = 0.1 mm) animals. **G**, **H** and **I** represent boxed area in B, D and F (scale bar= 0.05 mm). Neurons retrogradely labelled with FG in blue, Neu N green and Nk1 red. Arrows indicate retrogradely labelled neurons.

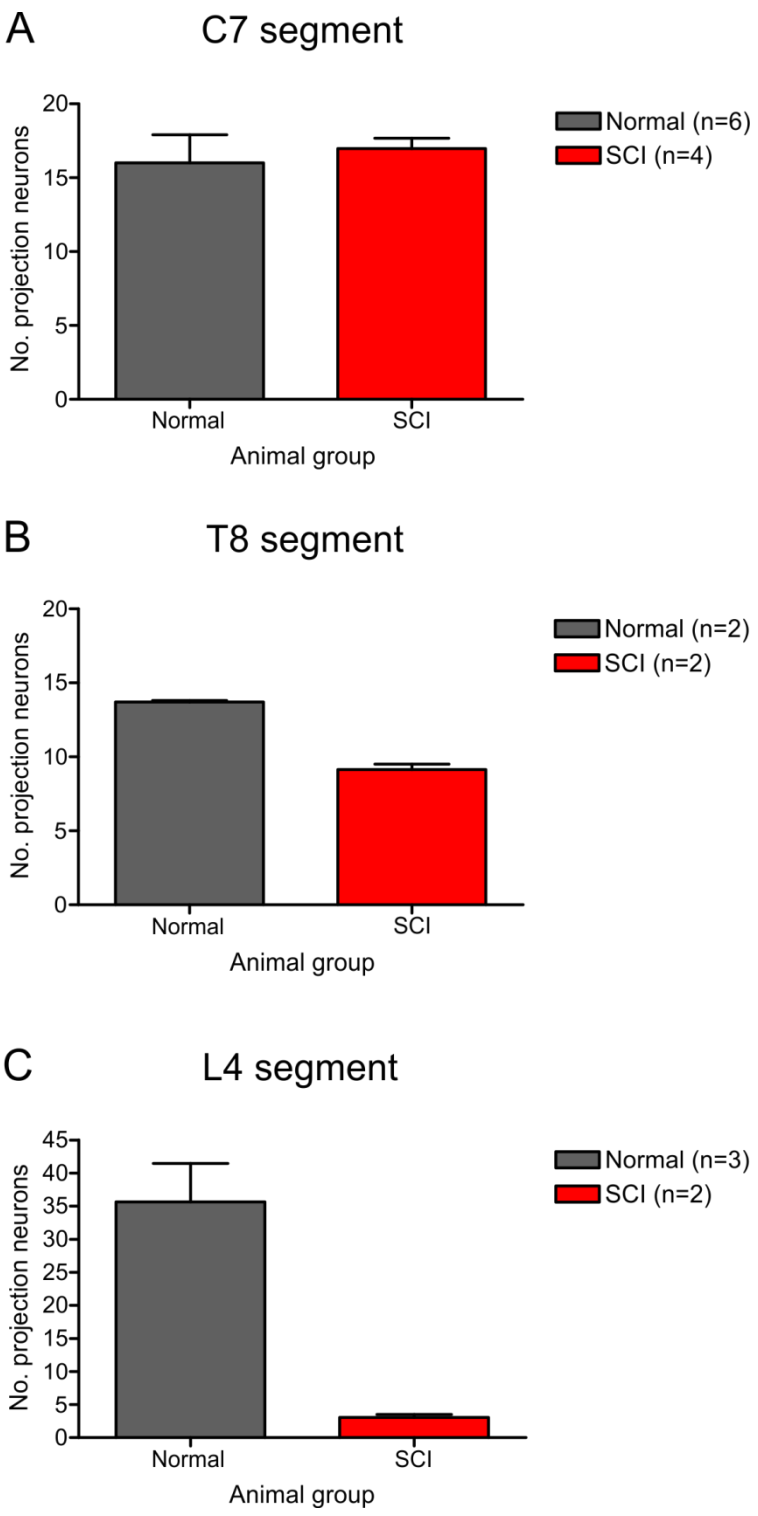

Fig. 3-21. Comparison of numbers of spino-CVLM projection neurons in normal and SCI animals. Histograms showing estimates of the numbers of retrogradely labelled neurons in the whole dorsal horn of the C7 (**A**), T8 (**B**) and L4 (**C**) segments of normal and injured animals. For each segment, bars show the mean numbers ± SE in the dorsal horn per 70 µm sections.

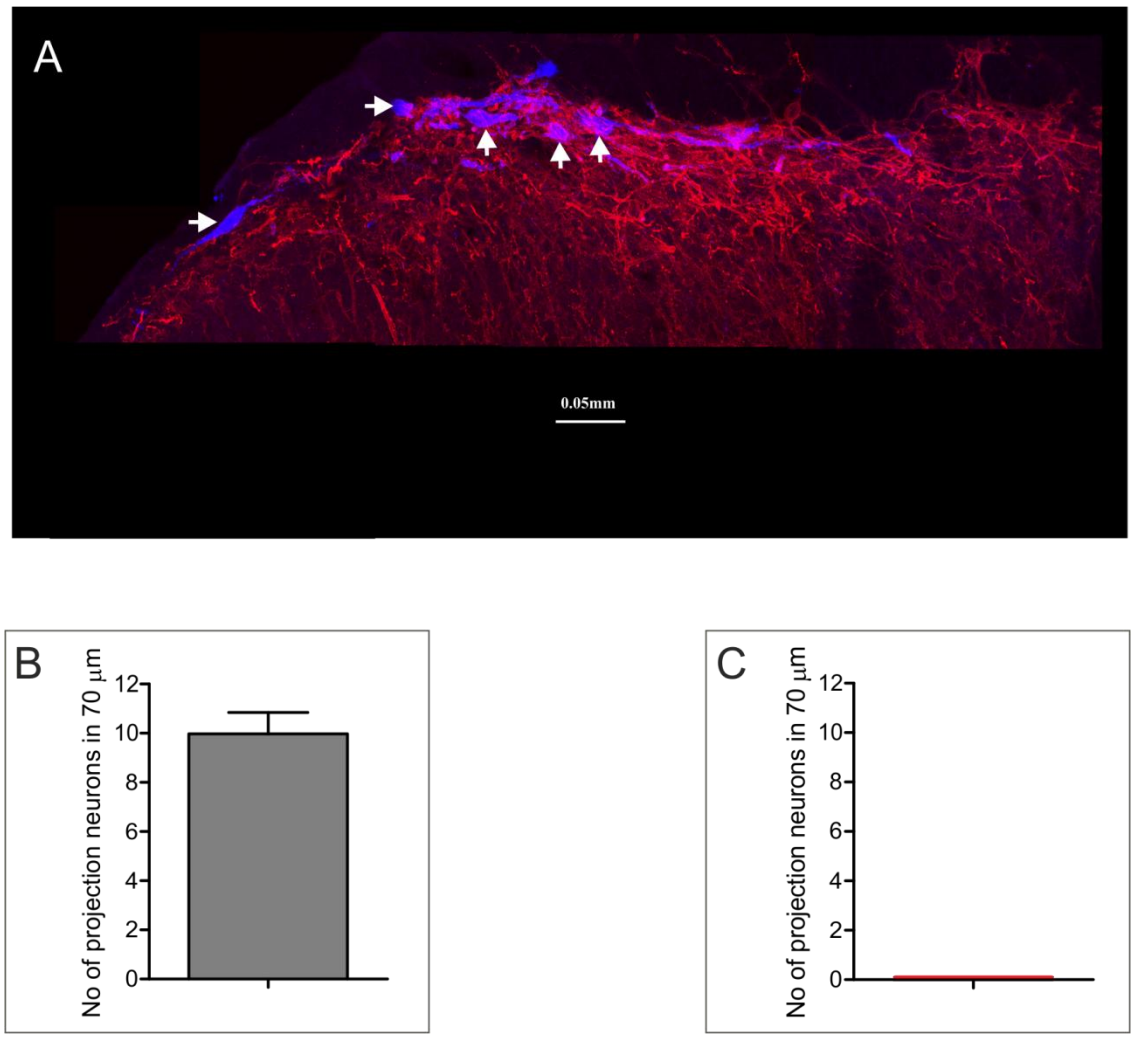

Fig. 3-22. Retrograde labelling of projection neurons in L4 segment from the LPB nucleus. A shows montage of confocal microscope scan of dorsal horn taken from the L4 segment in normal animal illustrates projection neurons in lamina I (scale bar = 0.05 mm). Projection neurons retrogradely labelled from the LPB nucleus with FG in blue while NK1r in red. Arrows indicate retrogradely labelled projection neurons. **B** and **C** illustrate the number of projection neurons in lamina I per 70 µm sections taken from L4 segment in normal (n=3) and injured animals (n=3), respectively. Bars in the graphs expressed as mean ± SE.

3.3.12 Summary of the results

The main findings in this chapter are summarized in table 3-1: 1) Enhancement of the withdrawal response to mechanical and heat stimuli when applied to forepaws and hindpaws. 2) Intact licking response in the forepaws but not in the hindpaws. 3) Development of tactile allodynia when assessed over the trunk above and at the level dermatomes but not in well below level areas. 4) Mechanical and cold sensitivities were evident in fore and hind (but not cold hyperalgesia) paws as assessed by foot lifting test. 5) Frequent lifting of the forepaws was seen on smooth and thermally neutral surface. 6) Data obtained from the operant tests showed an enhancement of cortical processing for sensory inputs applied to dermatomes located above but not below the injury site. 7) Tract tracing study revealed no differences in number of projection neurons at C7 segment while there was a slight reduction at T8 segment. Projection neurons were massively reduced at L4 segment.

A

Type of the test	Forepaws	Hindpaws
DWB	↑	↓
Plantar vF test	↓ Threshold	↓↓ Threshold
Plantar heat test	↓ Latencies	↓↓ Latencies
Licking response	ND	Abolished
FL on cold plate (7.5°C)	↑↑	ND
FL on cold plate (15 and 20 °C)	↑	↑
FL over neutral plates	↑	Not seen
FL over grid	↑↑↑	↑
PEAP	Not tested	ND

B

Type of the test	Above level	At level	Below level
Trunk vF test	↑↑↑	↑	ND
Trunk PEAP	++	Not tested	ND

C

Spinal segment	C7	T8	T12
Projection neurons	ND	↓	↓↓↓↓

Table 3-1. Summary of the results of T9 200 kdyn. A shows the summary of data collected from fore and hind paws while **B** shows the summary of trunk von Frey and PEAP testing. Data of tract tracing are summarized in **C**. ND indicates no difference.

3.4 Discussion

3.4.1 Forepaw vF data

3.4.1.1 Comparison to the results of others labs

Our data indicates that that all injured animals show a clear reduction in the 50% threshold which develops two weeks after injury. This becomes most marked between four and six weeks after injury.

Comparison with the results of previous studies is complicated by the different methods used to produce the SCI, different severities of injury, different levels of injury, different testing protocols and different animal strains. Even studies using contusion injuries have used various devices. While a direct comparison is possible with studies that have used the IH device, comparison with studies using other devices is more problematic but can be attempted by comparing the effects of the injury on locomotor capacity (BBB scores for example), where this is usually reported alongside the pain data, since this should provide an indication of injury severity.

Although forepaw testing has been performed much less frequently than hindlimb testing, there are several reports in different models, including studies using the IH device. Two studies used a contusion force of 200 kdyn, one at the T8 level (Santos-Nogueira et al., 2012) and the other at the T10 level (Nesic et al., 2005). These injuries are therefore closely comparable to ours. Nesic et al. used the vF test but assessed the frequency of response and reported tactile allodynia in 85%-90% of SCI animals with an onset of 4 weeks or more. The lower incidence and later onset compared to our data might be due to the different assessment method. Santos-Nogueira et al. (2012) used a pressure test (Randall-Selitto method) to assess paw sensitivity at 2, 4 and 6 weeks. They reported increased sensitivity which was evident at 2 weeks and remained at 6 weeks. These results confirm that a 200 kdyn injury performed using the IH device produces robust signs of tactile allodynia in dermatomes well above the injury site.

In two further studies using the IH device, a T10 injury was produced using a force of 150 kdyn but with a dwell time of 1 sec (Carlton et al., 2009; Bedi et al., 2010). In a preliminary study, we investigated injuries of 150 kdyn with a 1sec dwell and from the BBB scores (see Fig. 3-23) concluded that the sustained compression of the dwell produces a more severe contusion than an injury of 200 kdyn force without any dwell. Both studies

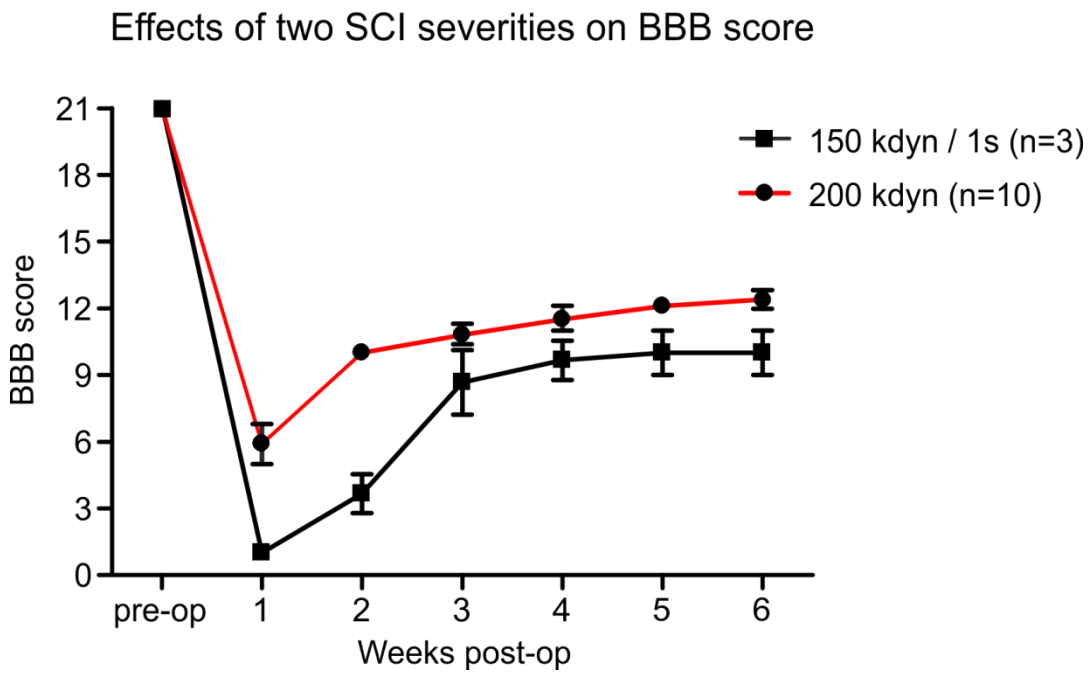

Fig. 3-23. Comparison of BBB scores in animals subjected to 150 kdyn / 1sec and 200 kdyn contusion injuries at T9 segment.

(Carlton et al., 2009; Bedi et al., 2010) reported that this injury can produce a clear drop in the 50% threshold of the plantar surface of the forepaw when tested by the up down method. However, sensory testing was performed at only limited time points in these studies.

Forelimb sensitivity has also been examined in weight drop contusion models. Two severities of injury have been investigated. Lindsey et al. (2000) subjected rats to an injury at the lower thoracic level produced by weight drop from 25 mm. They also performed BBB testing and their reported scores suggest that this injury is comparable to that produced by the IH device set at 200 kdyn force. These animals developed clear and marked signs of tactile allodynia in forepaws when assessed by the up down method. These developed at three weeks after the injury and persisted for at least 10 weeks. The incidence of this tactile allodynia is not reported but in another study by Mills et al. (2001a), where a less severe weight drop injury was studies (12.5 mm height), mechanical allodynia was seen in only 60% of Sprague Dawley animals. However, the mechanical sensitivity was assessed by the vF hairs according to frequency of response protocol. The BBB data from the study showed that this contusion injury was less severe injury (correlated well with 150 kdyn). In a similar injury but at T8, Hulsebosch et al. (2000) reported a clear sign of tactile allodynia in forepaws which developed at much later onset (28 days) after SCI.

Two studies have assessed forepaw sensitivity after lateral hemisection at the T13 level. Interestingly, Christensen and others (1996) found an increase in forepaw sensitivity using the withdrawal frequency protocol. Tactile allodynia occurred after 2 to 3 weeks and became most marked at 5 weeks. In an identical model, using the same testing method, Mills et al. (2001a) reported tactile allodynia in 75% of injured Sprague Dawley rats at 7 days post injury which became most marked at 35 days after the hemisection. In the same study, a lower incidence of tactile allodynia was reported in the Wistar (i.e. 60%) and Long Evans (40%) rats.

3.4.1.2 Issues in vF testing

Several protocols have been used to assess tactile sensitivity with von Frey hairs. The most popular are 1) estimation of the frequency of the response (i.e. number of positive responses out of certain number of filament application) and 2) the up down method of Chaplan et al. (1994). The up-down method has the advantage that the test involves the

fewest number vF hair applications (i.e. 4-9 trials for each paw). This minimizes the possibility that repetitive testing contributes to the development of mechanical sensitivity in the plantar surface of the paws. This is confirmed in our study where we did not observe any change in sensitivity of the sham group, indicating that the observed changes were directly linked to the affect of SCI.

Despite the fact that when used for baseline testing of intact animals nociceptors might be activated as a stiff bristle is often required to elicit a response, a reduction in threshold or increase in sensitivity is generally considered to reflect tactile allodynia. This is mainly based on the fact that after SCI, the stimulus intensities eliciting the withdrawal response are reduced to less than 1.43 g. Based on electrophysiological recording data from peripheral nerves (sural and plantar), there is some evidence suggesting that vF hairs with bending force less than 1.43 g are not able to stimulate cutaneous nociceptors and instead, stimulate receptors served by Aβ fibres (Chung et al., 1992; Leem et al., 1993). In addition, there is clear evidence that impulses generated in the large myelinated Aβ fibres contribute to development of tactile allodynia in animal models of neuropathic pain (for review see Sandkuhler, 2009). For example, innocuous (low threshold) electrical stimulation in the Aβ range can lead to expression of pERK (normally expressed only after activation of nociceptive fibres) in dorsal horn neurons after peripheral nerve injury (for review, see Ji et al., 2009). In rats with neuropathic pain, activation of Aβ fibres by light touch is reported to trigger excitation of neurons in superficial laminae (Schoffnegger et al., 2008).

There are certain technical issues that apply to this assay specifically in the context of its use to assess animals with a SCI. Because these animals have poor balance and tend to shift their weight from hindpaws to forepaws, there is the potential for this to affect the performance of tests involving movement of the forepaws. To greater dependence on the forelimbs for weight support might lead to collection of some false negative data if animals are tested too soon after SCI. To avoid this issue in our study, the tested started at 2 weeks after the injury when animals showed sufficient motor recovery to minimize interference with this test. By 14 days after SCI, our injured rats had a mean score of 9 on BBB rating scale and regained plantar placement of the hindpaws and body weight support. As a result, the injured rats became less dependent on the forelimb for weight support and if the vF stimulus is applied with sufficient duration, there should no concern about collection of false negative results. This is confirmed in all literatures. Another technical point was that animals frequently developed spontaneous forepaw lifting when on the von Frey testing

grid and this sometimes made it difficult to access the plantar surface of the paws to apply the filament. Since the interval between tests is not critical, this need not affect the results. However, patience is required to complete the tests in animals showing this behaviour.

Throughout this study we have used mainly the Sprague Dawley strain of animals. However, other strains also develop increased sensitivity in the forepaws after SCI. Mills et al. (2001a) compared Long Evans, Wistar and Sprague Dawley strains after thoracic contusion injury. Long Evans rats were reported to be the most sensitive while Sprague Dawley and Wistar rats showed a similar degree of tactile allodynia (Mills et al., 2001a).

3.4.2 Forepaw plantar heat data

3.4.2.1 Comparison to the results of other labs

Thermal hyperalgesia became evident in 200 kdyn contusion injured animals 4 weeks after injury and reached a maximum by 4 to 6 weeks after the injury. These results are broadly consistent with the limited observations made in previous studies. Carlton et al. (2009) investigated animals with more severe injuries (150 kdyn / 1 sec dwell) made with the IH impactor and reported a significant heat hyperalgesia at the onetime point tested (35 days). However, they provide no information on the incidence among their animals. In a further study (Bedi et al., 2010) in which the same injury was used, thermal hyperalgesia was observed when the forepaws were tested at one and five months after injury.

Similar results have been reported in studies using the weight drop method of SCI. Mills et al. (2001a) reported forepaw heat hyperalgesia in 87% of animals subjected to weight drop from 12.5 mm (moderate injury) at the T10 level. Reductions in latency became evident during the first three weeks after injury and peaked in the fifth week. Using the same injury at the T8 level, Hulsebosch et al. (2000) reported heat hypersensitivity in the forepaws with an onset of 4 weeks post injury.

Heat hyperalgesia of the forepaw has also been reported after lateral T13 hemisection injuries. Christensen et al. (1996) reported heat hyperalgesia that developed during the first two weeks after injury and peaked by week 3. These authors do not indicate the frequency of incidence but Mills et al. (2001a) reported forepaw heat hyperalgesia in 100% of animals subjected to this type of injury. Finally, in an excitotoxic model with neurotoxin

injected at the T12-L2, a clear reduction in withdrawal response latencies to heat stimuli was reported (Yezierski et al., 1998).

3.4.3 Interpretation of forepaw data

The marked drop in the 50% threshold and response latencies of the withdrawal response is interpreted as evidence for the development of increased sensitivity of the plantar surface of the forepaws. This in turn is considered a correlate of tactile allodynia and heat hyperalgesia, although it is known that a withdrawal response to tactile and heat stimuli can be produced largely by spinal reflex mechanisms but to some extent, the supraspinal mechanisms can modulate the response (Schomburg et al., 1990; Kalliomaki et al., 1992; Schouenborg et al., 1994; Kauppila et al., 1998). Above the injury level, the withdrawal response might be enhanced by spinal or peripheral mechanisms due to affect of SCI on cervical segments (Carlton et al., 2009). Another possibility reported in other models of neuropathic pain is development of abnormal activation of on-cells in RVM which would promote descending excitatory (or disinhibitory) influence onto spinal cord (see Suzuki and Dickenson, 2005; Heinricher et al., 2009).

In the heat test, the withdrawal response was associated with licking of the stimulated paw in about of 70% of the cases. This was observed in both injured and sham animals and was not changed by SCI. The licking response is considered to involve supraspinal mechanisms (see below) while the withdrawal may involve, at least in part, a spinal reflex mechanism.

Heat hyperalgesia was not clearly established until the fourth week post SCI. This is later than the onset of tactile allodynia in the forepaws and also later than the onset of the reduced latency to heat stimuli applied to the hindpaw (see below). This might be attributable to motor impairment since DWB data revealed that the injured rats tended to shift their body weight to the forelimbs which could compromise the forepaw response. However, this seems unlikely given the time course of development of tactile allodynia.

In reliability terms, the increased evoked pain behaviours in dermatomes far above (forepaws) the thoracic level of the injury could be a concern about accuracy of this model because this sort of pattern is not seen in humans. However, this is not necessary the case. If the mechanisms by which pain develops involve physical process such as diffusion then this will occurred under the same principles in human and rodents and it will be

responsible for transmission of pathological molecules to the same distance of tissue when measured in mm without scaling down in proportion to anatomically different body size. A spread of a segment or so in the human spinal cord which would be at level, would be several segment in the rat. In other words, the mechanistic spread could be absolute rather than relative and would explain why there are greater parts of the body involved in the rodent model. All in all, pain mechanisms which in man find expression in altered sensitivity mainly associated with dermatomes corresponding to segments close the injury site might, in rodent be reflected by more widespread changes affecting a larger part of the body (more dermatomes and more spinal segments) in rodents. Development of evoked pain behaviours in above level forepaws can be viewed as being an experimental advantage because it provides the possibility of testing of the paws.

3.4.4 Hindpaws vF data

3.4.4.1 Comparison to the results of other labs

Von Fey testing of the hindpaws showed a clear reduction in 50% threshold for the withdrawal response with an early onset. This was evident in all injured animals, but not in sham animals and became most marked 6 weeks after SCI.

This test has been widely used on the pain field and is the most common test used in studies of pain in SCI models. Tactile allodynia has consistently been reported in these models but the time course and incidence of its occurrence vary. Several studies have been performed using the IH device. For example, Knerlich-Lukoschus et al. (2008) used 200 kdyn but at the more rostral T8 level and tactile allodynia was assessed by the up down method. Their BBB data is similar to ours, and they also reported an early onset of mechanical sensitivity which peaked similarly to our results at 6 weeks PO. Santos-Nogueira et al. (2012) studies the same injury but tested for tactile allodynia using the Randall-Selitto test and reported increased sensitivity to pressure at 2 weeks which was maximal at 6 weeks. Neither study reported the incidence of sensitivity. In a further study using the IH device (Nesic et al., 2005), rats were subjected to 200 kdyn T10 contusions and investigated for tactile sensitivity by the frequency protocol. Approximately, 85-95% of injured animals developed mechanical sensitivity at 4 weeks PO. A lowering of withdrawal thresholds to vF stimulation has also been reported in studies using low thoracic 200 kdyn contusion injuries where the time course was not studies and the incidence among the animals not reported (Endo et al., 2009; Clark et al., 2010).

Increased tactile sensitivity of the hindpaws has also been reported in studies using the OSU impactor which is a displacement controlled device. Kloos et al. (2005), investigated the effect of injuries of a range of severities, produced by impactor displacements ranging from 0.3 to 1.3 mm. BBB locomotor testing was performed alongside sensory testing and comparison of their reported BBB scores with our results suggested that a 200 kdyn contusion injury produced with the IH device is similar in effect in terms of locomotor outcome to an injury produced using 0.9 mm displacement on the OSU device. However, surprisingly, when tested using the up down method, this injury did not lead to any detectable enhancement of sensitivity. In the same study, injuries produced by displacements of at least 1.1 mm were needed to observe a clear reduction in 50% threshold of the withdrawal response. Other studies using a displacement of 1.1 mm have confirmed increased hindpaw tactile sensitivity (Hutchinson et al., 2004; Detloff et al., 2008). Neither reports the incidence but the onset was at 2-3 weeks and maximal effects at 3-4 weeks post SCI.

In rats subjected to T9/T10 contusion by weight drop from 25 mm, a significant drop in 50% threshold of the withdrawal response developed in the second week PO and persisted for 10 weeks PO (Lindsey et al., 2000). Comparatively, less robust sensitivity was reported when rats were subjected to a less severe weight drop injury (12.5 mm height; Yoon et al., 2004). After the same injury at T8, Hulsebosch et al. (2000) were able to observe a marked increase in the number of withdrawal responses to application of vF hairs but not until 4 weeks after injury while a further study using this injury (Mills et al., 2001a) produced highly variable results.

SCI produced by various other methods, including T12/T13 compression (Bruce et al., 2002), T13 hemisection (Christensen et al., 1996) and excitotoxic damage (T12-L2) (Yezierski et al., 1998) are also reported to evoke mechanical sensitivity in the hindpaws.

3.4.5 Hindpaw plantar heat data

3.4.5.1 Comparison to the results of other labs

In our study, all rats subjected to 200 kdyn at lower thoracic showed an early reduction in the withdrawal response latencies when heat stimuli applied to hindpaws and these manifestations became most marked 4-6 weeks after the injury.

Despite, there are more studies performed on hindpaws, they did not report the incidence of heat sensitivity among the injured animals. When Mills et al. (2001a) tried to do so, they suffered high variability and discarded the results obtained from the hindpaws in the heat test. However, their rats were injured by using weight drop contusion.

Our results are mostly consistent with data delivered by other labs using IH impactor. For example, Knerlich-Lukoschus et al. (2008) investigated manifestation of heat sensitivity in hindpaws of rats subjected to 200 kdyn at T8 segment by using IH impactor. This study showed an enhanced withdrawal response to heat stimuli applied to hindpaws but with later onset (significantly differed from the base line data only at 6 weeks PO) in comparison to our study (2 weeks PO). In other study, Clark et al. (2010) used a similar force of contusion but at T10 segment and the most significant reduction in response latencies to the heat stimuli was reported in hindpaws 30 days post SCI. Heat sensitivity in hindpaws was also reported when a more severe injury (150 kdyn / 1 sec) at T10 was used (Bedi et al., 2010).

By using other contusion device such as weight drop, Hulsebosch et al. (2000) reported a delayed (4 weeks PO) but clear and significant drop in withdrawal response latencies when tested the hindpaws. Heat sensitivity of the hindpaws was also reported in other different models of SCI. For example, T13 lateral hemisection as well as excitotoxic SCI performed at T12-L2 SCI were suggested to produce a clear manifestation of heat hyperalgesia in hindpaws (Christensen et al., 1996; Yezierski et al., 1998).

3.4.6 Interpretation of hindpaw data

Although both the plantar von Frey and heat tests indicate an increased sensitivity of the hindpaws to these stimuli, how this relates to pain perception is unclear because of two complicating factors. The first of these is that it is well established that SCI can lead to spasticity in muscles below the injury. This is the result of changes in spinal reflex circuitry in segments below the injury and these changes in motor circuits could influence the responses to the tests performed on the hindpaws since it is likely that the same circuits are required for limb withdrawal responses. The second potential factor complicating interpretation of the hindlimb tests is the possibility of damage to ascending pathways conveying nociceptive signals to the brain. The later raise the question of whether any enhancement of nociceptive signalling at the spinal cord level is processed supraspinally and leads to altered pain perception. The absence of a licking response in the 200 kdyn

injury animals, which is in clear contrast to the sham operated animals, suggests the possibility of interrupted pain nociceptive transmission since licking required supraspinal processing. This issue is discussed further below, together with other relevant evidence.

3.4.6.1 Spasticity and alterations in reflex circuits below SCI

It is well established that acute spinalisation leads to changes in reflex responses below the transaction. In acutely spinalised animals, there are exaggerated withdrawal reflexes and reflex pathways investigated electrophysiologically are also found to be released from descending inhibitory control from the brain. This has been extensively described in cats (Sherrington and Sowton 1915) and rats (Schouenborg et al., 1992; Kauppila, 1997, 1998; You et al., 2009). In chronically spinal cord injured animals, spastic hyperreflexia have been described not only following complete transection (Bertman and Advokat, 1995; Wienecke et al., 2010) but also in other models of SCI including incomplete injury such as contusion injuries. For example, Baastrup and colleagues (2010), 42 of 43 rats subjected to T12-T13 contusion injury showed clear signs of spasticity. The symptoms included abnormal tail and hindlimb postures, flicking movements or flexion of the tail or hindlimb in response to pinch, spontaneous and evoked (tail pinch) spasms of the hindlimb or clonus. Spasticity has also been studied by measuring torque at the ankle and electromyogram (EMG) of ankle extensors during imposed movements at different velocities in wake animals. In this study increased torque and peak EMG activity was seen following contusion injuries (Bose et al., 2002).

In our study, we did not attempt to examine spasticity directly. However, some incidental observations were made that is likely to reflect spasticity. Some aspects of the responses of injured animals to hindpaw stimuli differed from those normally seen in other pain models (e.g. peripheral neuropathic pain models). Stimulation of the plantar surface of the hindpaws with the lightest filament in the vF series (size 1.65= 0.008g) led to a rapid series of flexion and extension movements, such that the paw was rapidly lifted from and then replaced on the floor several times in quick succession. These occurred spontaneously in the sense that they were seen in the absence of stimuli intentionally applied by the experimenter but we cannot rule out the possibility that they were triggered by contact of the paw with, for example, bedding material on the cage floor.

Several authors have raised the question of whether changes in spinal reflex pathways that lead to spasticity affect the behavioural responses used to assess pain sensitivity of the

hindpaws (Christensen and Hulsebosch, 1997; Verick et al., 2000; Hultborn, 2003; Yezierski, 2005; Mogil, 2009; Baastrup et al., 2010; Hains and Vera-Portocarrero, 2010, Nakae et al., 2011). At the very least, the presence of changes in motor circuits related to spasticity complicate interpretation of altered responses of the hindlimbs to tactile and heat stimuli and lead to uncertainly as to the extent to which these responses indicate changes in motor circuits unrelated to nociception versus changes in nociceptive circuits which could, if transmission to the brain were intact lead to altered pain perception. The two are not, of course, mutually exclusive. The initial processing of sensory input that subsequently feeds via interneuronal circuits onto motoneurons is likely to be shared with nociceptive circuits leading to pain.

3.4.7 Consistency of neuropathic signs

Our observations show changes in the sensitivity of the forepaws and hindpaws to tactile and thermal stimuli and these occurred with a high degree of consistency i.e. in all animals investigated. Not all studies report the consistency with which animals show clear signs of pain but those that do often refer to a proportion of animals in which sensitivity to stimuli is not affected by the injury. For example, Mills et al. 2001b studied a weight drop contusion and reported forepaw tactile allodynia and heat hyperalgesia in 60% and 85% of animals, respectively. These authors did not report data for the hindpaws because they found the result to be too variable. Nesic et al. (2005) reported an increased sensitivity of the forepaws and hindpaws to mechanical stimuli with an incidence of 85%-90% in animals with contusion injuries produced using the IH device at 200 kdyn.

There are two likely reasons for these differences in consistency. The first is variation in the severity of the injury due to differences in the type of device, injury parameters or injury level employed. With the IH device the force feedback control should optimize injury consistency and the graphs provide an indication of failed injuries due to bone strikes. A second potential reason is differences in the assessment method used. Our observations on animals with 150 kdyn injuries (see below) suggests that differences in the assessment method may be more important than variations in injury severity. Both of the studies referred to above used a protocol for assessment of mechanical sensitivity in which withdrawal responses to vF filaments were counted as a positive response only when accompanied by a behaviour considered to require supraspinal processing (e.g. as paw licking and head turning, biting and avoidance). This approach was used in order to avoid the potential problem of tests that did not reflect pain perception because of damage to

ascending pathways (Christensen et al., 1996). It is possible that variation in the injury and therefore the degree of interruption of ascending pathways explains the inconsistent results obtained by Mills et al (2001a) when testing the hindpaws. However, this does not explain variation in the responses to tests performed on the forepaws. In addition, there is a potential problem with this approach since it assumes that the threshold for supraspinally mediated behaviours are always the same or lower than that of the withdrawal response. If, however, the supraspinally mediated responses have a higher threshold then this could lead to false negative data. In fact, Baastrup et al. (2010) have recently reported that after contusion injury, hindlimb withdrawal responses show a marked reduction in threshold while there is no change in threshold for brainstem evoked responses so that their threshold are around 15 g greater than for the withdrawal response.

3.4.8 Footlifting behaviour

Although FL behaviour has been used in the field of pain research for assessment of pain in animals, it has not yet been used in models of SCI. In this project, we have used it to study cold and mechanical sensitivity as well as spontaneous pain. In comparison to shams, rats with 200 kdyn low thoracic injuries showed frequent paw lifting behaviour on a plate cooled to noxious or innocuous temperatures and when standing on a mesh (evoked FL). In addition, FL was frequently observed on a flat, thermally neutral surface (spontaneous FL). The evoked FL was displayed much more frequently by the forelimbs than the hindlimbs while spontaneous FL was observed only in the forelimbs.

3.4.8.1 Assessing sensitivity to cold stimuli

Studies in the field of pain research have relied mostly on the use of the acetone and less frequently on application of ice probes or ethyl chloride for the assessment of cold sensitivity (Kupers et al., 1998; Jang et al., 2005; Rahaman et al., 2006; Dias et al., 2007; Gustafsson and Sandin, 2009; Lindsey et al., 2000; Kim et al., 2003; Wu et al., 2004, Yoon et al., 2004). In the contusion model of SCI, Lindsey et al. (2000) reported an increased frequency and reduced latency of withdrawal responses on application of an ice probe to the hindpaw. Yoon et al. (2004) also investigated a contusion injury model and reported an increase in the frequency of withdrawal responses. Similar observations were made by Kim et al. (2003) in a hemisection model. Attempts have also been made to assess cold sensitivity over the girdle but only in photo-ischemic models of SCI (Wu et al., 2004; Gao et al., 2012).

The application of cooling substances has a number of limitations. Firstly, there is no control over the temperature and the actual temperatures reached at the skin are not known. Secondly, the effect of cold stimuli applied by these methods (i.e. ice probe or ethyl chloride spray) may not be limited to stimulation of thermoreceptors but might also activate mechanoreceptors (e.g. Kupers et al., 1998; Lindsey et al., 2000; Gustafsson and Sandin, 2009). Finally, the use of withdrawal responses has the same limitations regarding spinal versus supraspinal mechanisms as the von Frey and plantar heat tests.

Because of these limitations other methods involving placement of the animal on a cooled surface have been adopted in some studies (Bennett and Xie, 1988; Choi et al. 1994; Jasmine et al., 1998). This allows testing with a known temperature which can be adjusted as required. In the sciatic nerve construction injury model, Bennett and Xie (1988) evaluated cold sensitivity with animals on a plate cooled to 4°C. They found the frequency rather than the duration of withdrawal responses was the more consistent measure. In a spinal nerve ligation model of pain, Choi et al. (1994) assessed cold sensitivity over a plate at 5°C and reported that the injured rats displayed consistent cold sensitivity based on the duration of FL. More recently, Jasmine and colleagues (1998) investigated models of peripheral neuropathic and inflammatory pain using a range of temperatures (5°C to 20°C). They found that the frequency of FL was increased at 5°C and 10°C in the affected hindlimb and suggested that number of foot lifts is less likely than the duration of FL to be affected by motor weakness and development of spasticity.

3.4.8.2 Technical issues in cold testing

In our tests employing the cold plate, several precautions were found to be necessary to optimize the reliability of the results. When tested with the plate chilled to 7.5°C, the animals displayed much rearing-like activity, even when height of the cage lid was reduced to 10 cm. To take account of this, the time that the paws were in contact with the plate was recorded independently for the fore and hind paws to ensure that each was in contact with the plate for 4 min. Secondly, also at this temperature (7.5°C) there were occasions when contact of the scrotum with the cold plate lead to a sudden response involving most of the hindquarters rather than just a lifted paw but which could be confused with hindpaw lifting. This was overcome by using a video recording for final confirmation of the live scored results. A further potential problem raised by Allchorne et al. (2005) is that long duration test sessions may lead to hyposensitivity to cold due to desensitization or numbness and hence a reduction of the cold perception. This was avoided in our procedure

by limiting the test duration to a maximum of 5 min. Finally, this test was particularly affected by noisy conditions, with any type of distraction leading to a risk of false negative results.

3.4.8.3 Rational for noxious and innocuous cold temperatures

In this model, we evaluated cold sensitivity by counting the number of times animals lifted their paws when standing on a plate cooled to three temperatures (7.5°C or 15°C or 20°C). Normal paw skin temperature is about 30°C (Galbraith et al., 1993). There is not a unanimous view on the point at which cooling becomes noxious. In some studies (Bennett and Xie, 1988; Hama and Segan, 1993; Choi et al.1994; Jasmine et al., 1998) paw lifting is reported to be absent when naïve rats are tested at 4°C to 5°C while Ro and Jacobs (1993) reported a withdrawal reaction when paws were placed in a 6°C bath and considered this temperature to represent noxious cold. In a more recent study, Allchorne and colleagues (2005) investigated cold sensitivity by measuring the latency of paw withdrawal when naïve rats were placed on a plate at temperatures ranging from -5°C to 25°C. They suggested that temperatures in the range -5°C to 9°C to be noxious based on a significant reduction in the latency for withdrawal of the hindpaws compared to the latency at 15°C. The differences between the reports may be attributed to factors such as testing conditions, duration of assessment and the index used (e.g. number *vs.* duration of FL). In human, higher temperatures (10°C to 15°C) have been considered noxious (Chery-Croze, 1983; Abbadie et al., 1994; Chen et al., 1996), however, this may be related to the fact that humans can verbally express discomfort before a withdrawal response is elicited (Jasmine et al., 1998). Interestingly, Simone and Kajander (1996) recorded electrophysiologically from Aδ and C fibres innervating the hindpaws of rats and determined their responsiveness to a range of cold temperatures. Thresholds were found to vary widely and were often below 0°C, especially for Aδ fibres. Bester and other (2000) investigated physiological response of spino-PB neurons in lamina I of rat lumbar cord to a wide range of temperatures and identified a group of WDR spino-PB cells which responded to cold stimuli with thresholds ranging from 15°C to 20°C but with maximal firing rates at much lower temperatures. Approximately, a similar data was indicated for nociceptive HPC cells (activated by noxious thermal and mechanical stimuli) by Andrew (2009). In cat (Craig et al. 2001), two populations of lamina I spinothalamic neurons have been reported to respond to cold, polymodal HPC cells with a median threshold for cold stimuli at 24°C and population of thermoceptive specific cells which are activated by nonnoxious thermal

stimuli (range between 34°C to 15°C). This study reported that cold pain is mediated by polymodal HPC nociceptive cells in lamina I.

In our study, shams displayed only very occasional FL of forepaws and hindpaws at 15°C and 20°C but this became significantly more frequent at 7.5°C suggesting that in our test paradigm this temperature is noxious. In agreement with this, aversive behaviours (e.g. licking and shaking) were also observed when the forepaws were assessed at 7.5°C but not at temperature above this (i.e. 15°C and 20°C). These aversive behaviours were only observed in forepaws and this might be related to the fact that the spinothalamic projection from cervical segments is much more numerous than from the lumbar cord leading to greater pain discrimination of the forepaws (Al-Khatar and Todd, 2009).

3.4.8.4 Interpretation of FL at 7.5°C, 15°C and 20°C

After 200 kdyn T9 injuries FL of the forepaws was very considerably and similarly increased at all of the temperatures tested (7.5°C, 15°C and 20°C) compared to that seen in sham operated controls. This suggests a marked increase in cold sensitivity which comprises both cold hyperalgesia and cold allodynia. In comparison, FL of the hindpaw was much less frequent and while there was a significant difference between SCI and sham animals at 20°C and 15°C there was no difference at 7.5°C. This suggests that there is also an increase in the sensitivity of the hindpaws to cold stimuli but that this involves cold allodynia but not hyperalgesia. However, FL in the hindpaws was a very rare event (less than once per minute) and interpretation of these observations is complicated by evidence of interruption of ascending pathways by the injury.

Tract tracing showed that axons of projection neurons in the L4 segment are markedly interrupted at injury site. However, it is possible that the few neurons with intact axons remaining in the deeper laminae may be enough to carry some cold information from the hindpaws and hence explain some remaining cold sensitivity in the hindpaws.

3.4.8.5 Rational for neutral temperatures

In this model, paw lifting was investigated on a flat surface maintained at temperatures 25°C, 30°C and 35°C which can be considered thermally neutral on the basis of the following evidence. Normal paw temperature measured in 85 Sprague Dawley rats was reported to be 30°C to 31°C (Galbraith et al., 1993). Klein and others (2010) tested the thermal preference of naive rats to 30°C *vs.* 35°C using two chambers with separate

temperature controlled floors and found that animals spent similar amounts of time in both compartments. Our observations are in full agreement with the evidence above because our sham animals did not display any abnormal behaviour when tested at 25°C, 30°C and 35°C. There is evidence suggesting that thermoreceptors for cool or cold such as the melastatin transient receptor potential (TRPM8) and ankyrin TRP (TRPA1) only become activated at temperatures less than 23°C (Decosterd and Woolf, 2000; Story et al., 2003) and 18°C (Kim and Chung, 1992), respectively.

3.4.8.6 Interpretation of FL at 25°C, 30°C and 35°C

Paw lifting over a neutral flat surface maintained at 25°C, 30°C or 35°C was only observed in the SCI animals. This was only evident in the forepaws and suggests development of above level spontaneous pain in this model of SCI.

Paw lifting has not so far been described in SCI models but previous studies of peripheral neuropathic pain have interpreted FL seen on a surface at 30°C as a sign of spontaneous pain. Paw lifting from a plate at 30°C has been reported in both the chronic constriction injury (Bennett and Xie, 1988) and spinal nerve ligation (Choi et al., 1994) models of peripheral nerve injury. To confirm that the FL was a spontaneous behaviour Choi et al. (1994) showed that denervation of the paw (sectioning the sural, superficial peroneal, plantar and saphenous nerves) had no effect on the FL behaviour.

Previous attempts to quantify spontaneous pain after SCI have used reductions in locomotion (Larsen and Arnt, 1985; Mills et al., 2001b), weight loss (Siddall et al., 1995) and excessive scratching or grooming leading to skin lesions (Yezierski et al., 1998). However, the relationship of these indicators to spontaneous pain is not clear. In this sense, if our method for quantification of FL over a plate adjusted at neutral temperature can be clearly linked to spontaneous pain then it would provide the first direct method for measurement of spontaneous pain in a SCI model.

Spontaneous activity is known to develop in primary afferent neurons in different models of peripheral neuropathy (Burchiel et al., 1985; Kajander and Benette, 1992; Amir and Devor, 1993; Boucher et al., 2000; Liu et al., 2000; Wu et al., 2001) and may be behind the induction and maintenance of central sensitization and pain (Gracely et al., 1992; Zhang et al., 2000; Sukhotinsky et al., 2004; Xie et al., 2005; Pitcher and Henry, 2008). In some studies, spontaneous FL has been correlated with development of abnormal spontaneous

activity in the DRGs (Schafers et al., 2003; Djouhri et al., 2006). Peripheral spontaneous activity (i.e. nociceptors of median nerve) has also been seen in SCI models (Carlton et al., 2009). In Sprague Dawley rats subjected to a severe contusion at T10 using the IH device, Bedi and colleagues (2010) reported an increase in spontaneous firing of DRG neurons (including nociceptive populations) at lumbar and cervical levels. Spontaneous activity of nociceptive neurons in the DRGs is one possible explanation for development of chronic spontaneous pain in the forepaws in our study.

In humans, spontaneous pain can be perceived as below level (as well as at level). However, we did not see any evidence of hindpaw lifting on a flat thermally neutral surface. This means either that below level spontaneous pain does not develop in our model (despite being present for the forepaws) or that the behavioural signs, for some reason, cannot be expressed. Bedi and colleagues (2010) reported an increase in spontaneous firing in lumbar of DRG neurons, as well as those from cervical segments after a T10 contusion injury (IH impactor, 150 kdyn / 1 sec). If this is the mechanism underlying spontaneous FL behaviour then we might expect it to occur in the hindpaws as well as the forepaws. However, various evidence suggest that in our model, the pathways conveying nociceptive signals from lumbar segments to the brain have been substantially interrupted. If spontaneous activity in DRG neurons is the main mechanism responsible for spontaneous FL, then the failure of this activity to reach supraspinal levels may explain the absence of FL behaviour in the hindlimbs. The importance of lamina I in processing of spontaneous pain has been highlighted in previous studies (for review see Yezierski, 2005). In the excitotoxic model, injured animals displayed spontaneous grooming behaviours (Yezierski et al., 1998). Ablation of lamina I NK1r expressing projection neurons (80% of projection neurons in lamina I, Spike et al., 2003) by administration of the neurotoxin saporin conjugated to SP (SP-SAP) (Mantyh et al., 1997) led to a marked reduction in onset and severity of at level spontaneous grooming behaviours (Yezierski et al., 2004).

On the other hand, spontaneous below level pain occurs in patients with a complete SCI and can occur in neurologically complete (i.e. ASIA A) patients. This might suggest that this spontaneous pain is due to mechanisms operating supraspinally (for example spontaneous activity in the thalamus, Lenz et al., 1987; Pattany et al., 2002; Gerke et al., 2003), in which case it does not occur or is not detectable in our model. Alternatively, it is possible that the classification of patients as neurologically complete, which is based on a

pin prick test, is not sufficient to detect some intact ascending pathways that convey primarily other modalities of nociceptive input (Wasner et al. 2008).

3.4.8.7 Interpretation of FL on the metal grid

Injured rats but not the sham group displayed FL while standing on a metal grid. This involved both the fore and hind paws but was much more frequent in the forepaws. Because FL was not observed in shams, the grid is considered to be innocuous under normal circumstances and is the standard grid used for von Frey testing. Lifting of the forepaws was also observed on a flat thermally neutral surface (as described above) but almost doubled in frequency when animals were placed on the grid. This increase in forepaw lifting is interpreted as a sign of tactile allodynia, consistent with the dramatic drop in the mechanical threshold detected by the von Frey test. When standing on the grid, the animal's weight will be concentrated on the limited area of the paws in contact with the wires making up the grid. This presumably becomes uncomfortable for animals with SCI which have developed tactile allodynia in the forepaws.

In the hindpaws, FL, which was not observed on a thermally neutral flat surface, appeared when the animals were placed on the grid. However, the frequency was very low – about 25% of that seen in the forepaws. This FL is also probably related to the lowering of mechanical threshold in the hindpaws and the low incidence may reflect the significant interruption of ascending nociceptive signals from segments below the injury. Alternatively, it is possible that this occasional FL behaviour is entirely reflex mediated.

3.4.9 Dynamic weight bearing

In models of peripheral neuropathic pain, rodents that show mechanical allodynia of the affected limb using the von Frey test also show a redistribution of weight bearing such that less weight is born on the affected paw. This has been demonstrated using a DWB device (Mogil et al., 2010; Tetréault et al., 2011; Robison et al., 2012; Doré-Savard et al., 2012). We used the same device to determine whether there was a redistribution of weight after SCI. Since animals showed robust signs evoked pain in the forepaws indicating tactile allodynia, while evidence suggests that signalling from the hindpaws is significantly interrupted, we expected that animals would shift their body weight to the hindpaws. i.e. that weight on the forepaws would become uncomfortable leading the animals to adjust their posture so that more of their weight was on the hindpaws. Instead, the opposite was

seen. SCI animals showed a clear redistribution of weight from the hindpaws onto the forepaws compared to naive animals and sham controls. This was the same at both 3 weeks and 6 weeks post injury. This shifting of weight towards the forepaws is most likely a compensatory response to the loss of function in pathways providing postural control below the injury level. Since we were not able to investigate weight distribution in our animals at an early time point due to lack of weight support in the hind quarters, e.g. 1 week PO, before the development of tactile allodynia in the forepaws, it is not possible to come to a firm conclusion as to whether this influences weight distribution at the later time points or whether weight distribution entirely reflects postural changes as a result of the effect of the injury on motor control. However, a further group of animals subjected to a 150 kdyn injury (see next chapter) were tested at weeks 1, 3 and 6 and the results from these animals which also developed tactile allodynia in the forepaws showed the same pattern of weight redistribution and this did not differ at week 1 from the later weeks.

3.4.10 Brainstem behaviours elicited over the trunk

3.4.10.1 Taxonomy and characterization of testing levels

In the clinical literature on SCI, the location of the pain perceived is described in relation to the injury using the terms “above level”, “at level” and “below level”. These terms on based, not on an identification of the physical (i.e. segmental) location of the injury itself, but rather on the neurologically determined level of the injury (see introduction). This rests on identification of the lowest dermatome in which there is normal sensation and may therefore be several segments above the area of actual spinal cord damage. As a result, it is difficult to apply these terms directly to the rodent model where such neurological tests, which depend on patient co-operation, cannot be performed. Because of this, most previous studies have aimed to apply stimuli in dermatomes corresponding to segments above or below the physical level (i.e. segmental level) of the injury and to refer to this as above level, at level or below level testing. However, a problem associated with this approach is the lack of information about how the dermatomes of the thoracic segments in the rat relate to spinal cord segments. In other words, maps for the thoracic dermatomes have not (to our knowledge) been reported. As a result, it has often been assumed that the dermatomes involve the skin of the back immediately adjacent (i.e. at the same level) as the corresponding spinal cord segment. Our electrophysiological investigations of this show that this is not the case and we used the electrophysiological information to choose specific stimulation sites where the segmental level of processing was known.

3.4.10.2 Technical limitations

Repetitive mechanical stimuli applied can lead to learning and anticipation process. To minimise this, the number of the stimulus applications was limited to five on each side of the animal. The evidence that this avoided any problem is that the frequency of response in the sham operated animals remained constant over the whole testing period. Stimuli applied to the back activate hair receptors as well as potentially activating the same types of receptor as are found in the glabrous skin of the footpads. This was reflected in the fact that responses were elicited in normal animals by filaments much lighter than those at threshold for forepaw withdrawal. We therefore had to avoid shaving areas to be tested at the operations. Any natural variations introduced by changes in hair density with time would be controlled for by use of the sham animals. Since there is a possibility that different behaviours have different thresholds and/or are affected differently by SCI, although we assessed a range of response types we also analysed the response to biting individually, since this is a response that was very rarely seen before injury or in sham animals.

3.4.10.3 Comparison with other studies

In comparison to pre-injury and sham controls, animals with SCI developed clear signs of tactile allodynia to stimuli applied 1 cm rostral to the injury and also, to a lesser degree, to stimuli applied 2 cm caudally. In contrast, there was no change in responses to mechanical stimuli applied 5 cm caudally. The changes in response to stimuli at +1 cm and -2 cm were evident from the first week after the injury and become most marked between 4 and 6 weeks after injury.

There have been several previous reports using the infinite horizon device. In animals subjected to 200-250 kdyn injuries at the T8 segment Hubscher and colleagues (Hall et al., 2010; Hubscher et al., 2010) observed tactile allodynia in response to stimuli applied just above the injury level which was more prevalent in males (about 70%; Hall et al., 2010) than females (about 30% ;Hubscher et al., 2010) . This was referred to as “at level” pain though from our electrophysiological experiments it is likely to involve segments well above the injury level. These authors concluded that the increased sensitivity was associated with an asymmetrical injury. Bedi et al. (2010) investigated the incidence of vocalisation at several levels over the back extending from above to below the injury in animals with a T10 contusion injury produced by in impact of 150 kdyn with a 1 sec dwell.

The incidence of vocalization was most marked above the injury level while no significant change was reported below the injury. In the same injury model, Crown et al. (2008, 2012) tested above the injury site and reported early allodynia (14 days PO) becoming most marked at 35 days post SCI in about 90% of animals.

Sensitivity over the back has also been investigated in animals with injuries produced using weight drop devices. Siddall and colleagues (Siddall et al., 1995; Drew et al., 2001, 2004) investigated animals with T13 injuries mild enough to allow recovery of normal walking. They reported a lowered threshold for vocalisation in response to a range of vF filaments applied to the back above the level of the injury. However, they also reported an increased threshold for vocalisation below the level of the injury.

Two further studies used the NYU weight drop device (with a drop height of 25 mm) and performed extensive mapping of locations covering most of the back from above to below the injury site bilaterally. Lindsey et al. (2000) investigated animals with an injury at T9/T10 (resulting in a BBB score of about 9) and reported robust signs of allodynia, mainly at locations rostral to the injury site. These were evident at 3 weeks and maximal at 10 weeks post SCI. Baastrup et al. (2010) investigated animals with injuries at T12/T13 level (using a weight drop from 25 mm) and reported that 59% of injured animals developed allodynia in an area at the level of the injury. In a study by Hulsebosch et al. (2000), animals subjected to a less severe injury at T8 segment from height of 12.5 mm. Allodynia was reported at girdle zone extended over the dorsolateral trunk from the injury level to cervical region but with a slower onset (4 weeks after SCI) in comparison to our data.

Hubscher and Johnson (1999, 2006) investigated rats with T8 injuries made with the OSU device (relatively severe; 0.9-2.0 mm displacement). They reported mechanical hypersensitivity (judged by responses such as orientation, escape and vocalisation) in 65%-70% of animals when stimuli were applied to dermatomes located close to injury site. They also reported (Hubscher and Johnson, 2006) that sensitivity did not change when assessed below the injury site but it did not indicate the exact location.

Bruce et al. (2002) subjected animals to SCI using a clip compression (35g and 50g) method at junction of T12/T13 which were comparatively severe. By week 4 after SCI, BBB assessments showed mean scores of about 10 and 5 for 35g group and 50g group, respectively. Trunk sensitivity was tested before and after (week 3 and 4) SCI using

innocuous vF filament. In both SCI groups, a similar degree of tactile allodynia developed on the trunk rostral to the injury as judged using avoidance responses such as escaping, biting and vocalization. More recently, 35g clip compression was also used by Oatway et al. (2004, 2005) and tactile allodynia was reported on the back immediately rostral and proximal to the lesion. In an extended behavioural study, Gris et al. (2004) used the same injury model and assessed manifestations of at level allodynia for 12 weeks after the injury. A marked increase in incidence of avoidance response was reported at week 4 after SCI and remained relatively the same until week 10 thereafter it maximized at week 12 after the injury. The trunk hypersensitivity to tactile stimuli was also reported in hemisection (Christensen et al., 1996) and ischemic models (Hao et al., 1991) of SCI.

3.4.10.4 Interpretation of the results

Stimuli of sufficient intensity applied over the back in normal animals can elicit a variety of responses such as head turning, generalized escape behaviours, scratching, licking. biting and vocalization. These responses have been shown to be present even in chronically decorticate or decerebrate rats in which structures rostral to the mesencephalon including thalamus have been removed (Woolf, 1984). This shows that an intact brainstem is sufficient for their expression. While it is possible that in intact animals, these behaviours are mediated entirely by processing in the brainstem, it is likely that more rostral structures are also involved (see discussion of the PAEP test, below).

Above level. Our results show that there could be a clear sensitization of these responses after SCI but their expression depended on the stimulus location in relation to the injury site. SCI animals showed a marked increase in incidence of responses to stimuli applied to the back at +1 cm. Since the electrophysiology shows that sensory input from this location is processed in T4, this indicates changes in sensory processing in segments well above the injury level and probably rostral to what would be considered “at level” in human subjects. This is consistent with the observation of changes in the sensitivity of the forepaws.

At level. The increased sensitivity to stimuli applied at -2 cm indicates changes in the processing of sensory inputs to the T8 segment and therefore changes most likely representative of “at level” pain in humans. The increased sensitivity was slower in onset and less marked than at +1 cm. However, the difference in sensitivity at the two locations could not be accurately estimated from the collective avoidance behaviours because animals responded to every stimulus at +1 cm from a few weeks after injury. To overcome

this issue, the incidence of biting responses was quantified. This became the most common response to stimuli applied at +1 cm but remained very rare in response to stimuli applied at -2 cm. The less marked increase in sensitivity for stimuli probably reflects several factors. Although intuitively it might be expected that the pathological mechanisms leading to sensitization might occur earlier and to a greater extent in segments immediately rostral to the injury (T8) rather than several segments away (T4), we saw evidence of two types of damage as a consequence of the injury which are likely to mitigate the development of increased sensitivity. Firstly electrophysiological recording showed that inputs (CDP amplitudes) to this segment from low threshold cutaneous afferents were much diminished in the injured compared to normal rats probably because of damage to the dorsal roots carrying inputs from the dermatome 2 cm below the injury level. Secondly, tract tracing (discussed below) showed that the projection neurons in the T8 segment were considerably reduced in number. The sensitization to tactile stimuli at this level therefore occurs despite this direct damage to the signalling pathway caused by the contusion injury.

Below level. In contrast to the clear change in responses to stimuli applied at +1 cm and -2 cm, no change in tactile sensitivity was seen at -5 cm and no difference was seen between sham control and SCI animals. The electrophysiology showed that sensory signals from the skin at -5 cm are processed in segments below the level of the injury (i.e. T10 to T12) and also confirmed that the input from low threshold afferents is normal (i.e. there is no damage to dorsal roots entering at this location). However, tract-tracing (discussed below) shows that the number of projection neurons belonging to nociceptive pathways which have intact axons reaching supraspinal levels is dramatically reduced below the injury level in the SCI animals. This later conclusion is supported data from the PEAP test (discussed below).

3.4.11 PEAP test

SCI rats spent less time on the dark side of the cage than the sham controls when stimuli were applied to the back above the injury level. However, there was no difference in the time spent on the dark side of the cage for SCI and sham animals when stimuli were applied below the injury level, either to the back or the hindpaws.

This assay was used recently by Baastrup and colleagues (2010) to investigate cortical processing of pain in a SCI model. These authors reported that stimuli applied to the back at the injury level (and therefore most likely processed above the injury level), resulted in a

modification of the animals behaviour. They concluded that this reflected cortical processing of the signals leading to escape/avoidance behaviours to such stimuli and that the difference between SCI and sham animals reflected enhanced sensitivity to these stimuli. In our experiments, we were able to replicate these results when we applied stimuli over the back at a position (+1 cm) which we had shown electrophysiologically is processed in segments well above the injury level. Because we used the same vF filament in this test as when performing von Frey testing of the trunk, the observations from these two tests are directly comparable. This suggests that, when applied above level, stimuli eliciting such behaviours as licking, biting and head turning, which can be performed by decorticated rats, lead to the sensation of pain and that such observations are therefore a valid measure of pain.

Baastrup et al. (2010) originally introduced the PEAP test in order to determine whether the reduced threshold for withdrawal of the hindpaws in response to mechanical stimuli was accompanied by an increased sensation of pain. Since they saw no evidence for cortical processing when this test was performed on the hindpaws, they concluded that the changes in reflex behaviours were a reflection of spasticity rather than a reflection of pain experienced by the animal. We also found in our experiments after T9 injuries of 200 kdyn that stimulation of the hindpaws did not influence SCI animals any differently from the sham animals despite clear reductions in threshold for the withdrawal response. These observations suggest that stimulation of the hindpaws does not lead to a sensation of pain in these animals. However, our interpretation of the reasons for this differ from those of Baastrup et al. (2010).

In contrast to the altered behaviour elicited by stimuli applied above level (+ 1cm) over the back, stimuli applied below level (-5 cm) did not differ in their effect on SCI and sham animals. This correlates with the absence of increased sensitivity to the same stimuli assessed from the frequency of brainstem behaviours. Both observations strengthen indications that nociceptive information from below the injury level may not be reaching the brain (see below).

3.4.12 Tract tracing

Because several types of behavioural data suggested that signals from below the injury level were not reaching the brain, tract tracing of known nociceptive pathways was carried

out. Tracer was injected into two of the main targets of ascending nociceptive projection neurons, which for neurons in the lumbosacral segments are the LPB nucleus and CVLM.

Histological examination of the brain injection site was used assess correct targeting of injection and this was further confirmed for the CVLM injected animals by quantifying retrogradely labelled ascending tract neurons in dorsal horn of the C7 segment. This segment is well away from the injury site and should not be affected by the injury. The numbers of labelled neurons in normal and injured animals were shown to be similar. In addition, the numbers of spino-LPB neurons in lamina I of the L4 segments of normal animals was similar to that previously reported (Spike et al., 2003; Al-Khater and Todd, 2009; Polgár et al., 2010).

The number of neurons labelled from the CVLM in the dorsal born of the T8 segment immediately above the injury level were quantified in normal and SCI animals and were reduced by about one third following injury. The fact that ascending tract neurons survive even in segments immediately adjacent to the injury is consistent with the increased sensitivity to stimuli applied at -2 cm (a location shown by electrophysiology to involve processing in the T8 segment) as assessed by behavioural responses that require processing in the brain. The reduction in the numbers of labelled neurons may partly explain why the development of sensitivity to stimuli processed at this level is delayed and less marked that for stimuli processed in more rostral segments despite being closer to the injury pathology and potentially therefore the triggering mechanisms.

Numbers of CVLM projecting neurons in the dorsal horn and of LPB neurons in lamina I of the L4 segment, well below the injury level, were also quantified. The results showed a very substantial reduction in labelled neurons projecting to the CVLM and virtually complete absence of lamina I projection neurons projecting to the LPB nucleus. Because the L4 segment is well below the injury level, the cell body is not likely to be affected directly by the injury in the same way as for neurons in the T8 segment, we attribute the reduction in labelled neurons to interruption of the axons in the white matter at the level of the injury. However, this assumes that complete interruption is the only mechanism that prevents retrograde labelling of ascending tract neurons. The extensive reduction in number of labelled neurons at lumbar level is consistent with behavioural observations showing the absence of licking of the hindpaws in response to plantar heat stimuli and the lack of effect of tactile stimuli applied to the hindpaw in the PEAP test. In this model, we therefore consider the reductions in threshold for hindlimb withdrawal responses to be

unreliable as a measure of pain because signalling between the brain and spinal cord below the injury is compromised. Whether the alterations in these responses reflect changes in nociceptive circuits which would be reflected in an alteration in pain perception if the ascending tracts were intact is difficult to know. The presence of mechanisms allied to spasticity leave this in some doubt.

Chapter 4

T9 150 kdyn model

4 T9 150 kdyn model

4.1 Introduction

The significant disruption of ascending nociceptive pathways which is caused by low thoracic injuries of 200 kdyn is a clear disadvantage for the assessment of below level post-SCI pain. On the other hand, the likelihood of observing robust signs of central neuropathic pain is thought to depend on the severity of the injury and more severe models may more reliably lead to pain-like behaviours (Hubscher and Johson, 1999; Lindsey et al., 2000; Knerlich-Lukoschus et al., 2008). The next chapter describes experiments using a less severe T9 contusion injury made using an impact of 150 kdyn. The rationale for this was to see it was possible to reduce the injury severity to a level where ascending nociceptive pathways were preserved but which still triggered the pathological processes leading to central neuropathic pain. If this were possible then it may provide a model for investigating below level pain.

4.2 Methods

Fifty-seven adult male Sprague Dawley rats (200-350 g) were used in experiments reported in this chapter. Fourteen of these animals were subjected to contusion injury (T9 150 kdyn) while 14 underwent sham surgery. In addition, 24 normal animals were used for obtaining baseline of weight distribution information while five animals were used only for tract tracing studies.

4.2.1 Behavioural testing

Full details of the behavioural tests used in this chapter are provided in the general methods.

4.2.1.1 BBB rating scale and BBB subscore

The motor recovery of the hindlimbs was assessed preoperatively and weekly after the injury using the BBB rating scale and BBB-subscore. Fourteen contusion injured and 14 sham animals were used in the main BBB score while 10 animals in each group were investigated using the BBB subscoring method in addition.

4.2.1.2 Dynamic weight bearing test

The percentages of body weight born on each paw were estimated in 24 normal animals using the DWB device. At one week, three and six weeks post SCI, 6 operated shams were evaluated while 8 contusion injured rats were tested at weeks 1 and 6 and 6 SCI rats were tested at week 3.

4.2.1.3 Forepaw von Frey and heat tests

The base line data for plantar von Frey and plantar heat (response latencies) assays were collected preoperatively from 7 contusion injured rats and 9 operated shams. The same animals were tested weekly from 2 to 6 weeks after the injury. A licking response was assessed at the same in 7 SCI rats and 6 shams.

4.2.1.4 Hindpaw von Frey and heat tests

Hindpaw tests were carried out at same time points as the forepaw testing. Results for the plantar von Frey test was collected from 7 SCI rats and 9 sham controls. Response latencies to heat stimuli were investigated in 11 SCI and 9 sham animals while licking responses were studied in 7 SCI and 6 sham operated animals.

4.2.1.5 Footlifting test

Footlifting assays were carried out at 4, 5 and 6 weeks after injury. Eight contused and 8 sham rats were tested on the cold (7.5°C) and neutral (30°C) plates. Ten contused and 10 sham animals were assessed on a mesh grid.

4.2.1.6 Trunk von Frey test

The trunk von Frey test was used to investigate sensitivity above and below the injury site preoperatively and once a week for 6 consecutive weeks after the injury. Above level tests (+1 cm above the injury) were performed on 11 injured and 9 sham animals. Below level assessments (-5 cm below the injury) were performed on 7 contused rats and 6 sham controls.

4.2.1.7 PEAP test

The PEAP test was carried out in two different ways to investigate the degree to which nociception below the injury level leads to the perception of pain. Eight contusion injured

animals and 6 shams were evaluated while mechanical stimuli were applied to the dorsolateral trunk 5 cm below the SCI. In a second protocol, the results were collected from the hindpaws of 7 SCI animals and 6 sham operated rats. Because both tests were carried out at 6 weeks after injury, different animals were used in each case to avoid any possibility for learning.

4.2.2 Tract tracing of projection neurons

To determine whether a milder force at low thoracic level resulted in greater sparing of projections from neurons below injury level, 4% FG was injected into CVLM of 5 normal and 2 injured rats at week 7 after SCI. Three days later, all rats were perfused and the C7, T12 and L4 segments processed as described previously for quantification of the labelled cells in the dorsal horn.

4.3 Results

4.3.1 Contusion parameters

The weights of all animals at the time of surgery are shown in (Fig. 4-1A). The contusion injuries were made using the IH instrument. Scatter plots in Fig. 4-1B shows the actual force delivered in kdyn. Most of the impacts were close to the target force of 150 kdyn and resulted in displacements of 705 to 1005 µm (Fig. 4-1C). Any animals for which there were indications of impact with bone were omitted.

4.3.2 Motor evaluation

Assessment of the effect of the injury on the locomotor capacity of the animals and the pattern of recovery of movement was carried out using the BBB locomotor rating scheme (Basso et al., 1995) and BBB subscore system (Lankhorst et al., 1999).

In comparison to the previous 200 kdyn data, 150 kdyn injuries had a milder effect on motor activity of the hindquarters. Fig. 4-2A shows that one week after the injury, animals were already displaying *plantar steps with frequent to consistent weight support and occasional forelimb/hindlimb coordination* (about mean score of 12 on BBB rating scale). This improved gradually during the subsequent weeks to a score of 14 (i.e. *plantar steps with consistent weight support and consistent forelimb/hindlimb coordination*) in week 6 after SCI. The subscore shows marked improvement in the first 4 weeks after the injury but

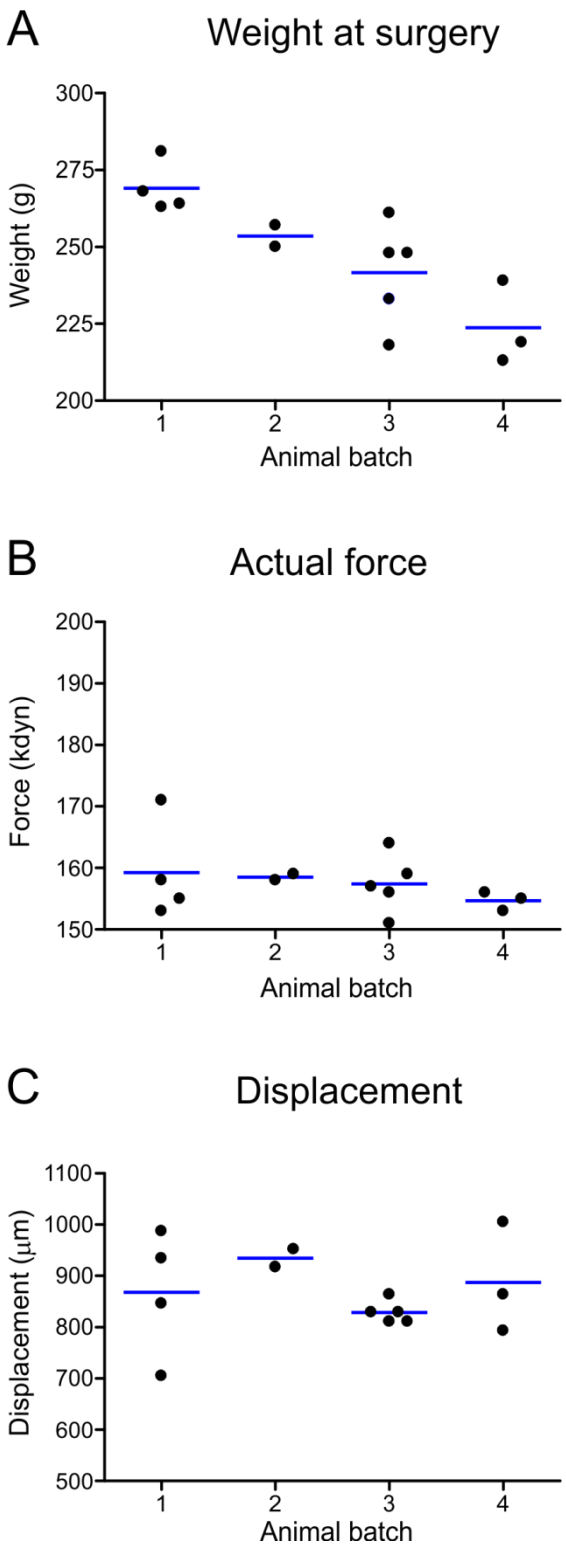

Fig. 4-1. Contusion injury data. A shows weight of animals at the surgery. **B** illustrates actual force delivered by the impactor and measured by the devise during the impact. **C** represents actual displacement of the impactor i.e. distance that the impactor tip travelled from the surface of the spinal cord into the cord before withdrawing. The graphs show scatter plots for the values measured for each of 14 animals subjected to contusion injury.

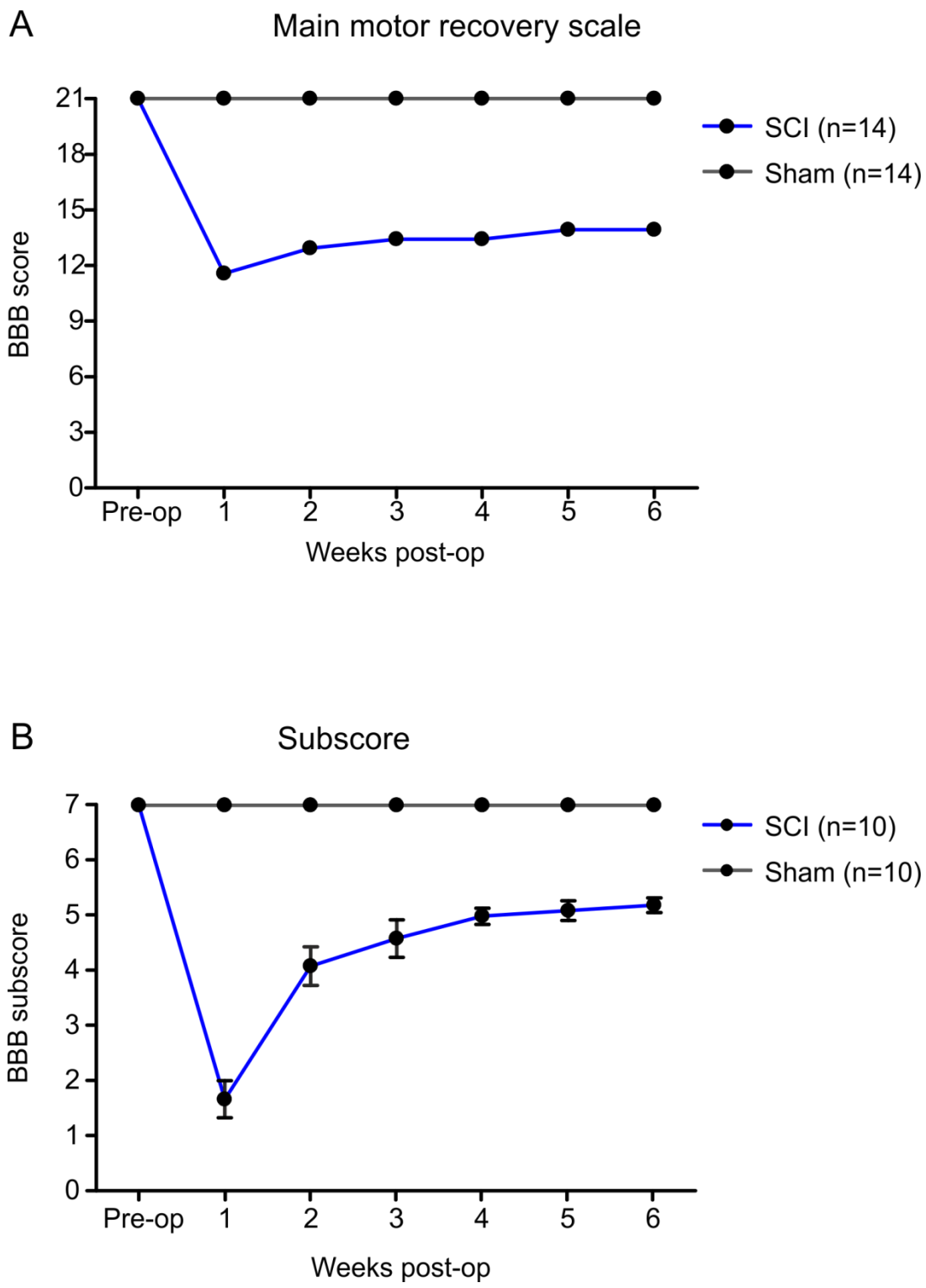

Fig. 4-2. BBB rating scale and subscore for locomotor activity of SCI and sham animals over the course of the study. A shows the main motor recovery (i.e. BBB rating scale) from preoperative and weekly (1 to 6) PO tests performed on T9 150 kdyn contusion injured (n=14) and sham operated control animals (n=14). **B** shows the motor recovery subscore for SCI (n=10) and sham (n=10) animals over the same time period. The values shown are mean ± SE.

only a slight recovery of fine motor activity was seen between the fourth and the sixth week post SCI because the angle of the hindpaws never became parallel to the body in most injured animals (Fig. 4-2B). There was a high degree of consistency in the rating for each of the animals. The sham surgery had no effect on the locomotor scores of animals in this group even at one week after the sham operation.

4.3.3 Dynamic weight bearing test

Dynamic weight bearing test was used to evaluate the effect of SCI on the postural support of the animals as indicated by the distribution of body weight on the four paws. Fig. 4-3 shows the percentage body weight distributed to the forepaws in naive (normal), sham and SCI animals. In normal animals (n=24), 32.5% of the total body weight is distributed to the forelimbs (Fig. 4-3A). In animals subjected to sham surgery (n = 6), there was no change in this weight distribution pattern at 1 (Fig. 4-3B), 3 (Fig. 4-3C) and 6 (Fig. 4-3D) weeks following surgery. There was no statistical difference between the weight distribution on forepaws between sham operated and normal animals (at any testing point, sham *vs.* dotted line: *P>0.05, one sample t-test)*. This indicates that posture does not change as the animal grew up and gained weight. However, in SCI animals there was a clear redistribution of weight, with more of the weight being distributed to the forepaws than in the naive animals at week 1 after the injury (an increase from 32.5% to 42.5%). This change was statistically different in comparison to both normal animals *(P<0.05, one sample t test)* and sham animals *(P<0.05, Tukey-Kramer post-hoc test).* This redistribution did not recover with time and remained the same during week 3 and 6 after SCI (Fig. 4-3C and D).

The percentages of body weight distributed to the hindpaws in normal, sham and SCI animals are illustrated in Fig. 4-4. In normal animals (n=24), 67.39% of the total body weight is distributed to the hindlimbs (Fig. 4-4A). In animals subjected to sham surgery (n=6), there was no change in this weight distribution pattern at 1 (Fig. 4-4B), 3 (Fig. 4-4C) and 6 (Fig. 4-4D) weeks following surgery. There was no statistical difference between the weight distribution on hindpaws between sham operated and normal animals (at any testing point, sham *vs.* dotted line: *P>0.05, one sample t-test).* However, in SCI animals less weight being distributed to the hindpaws than in the naive animals at week 1 after the injury (a decrease from 67.39% to 57.38%). This change was statistically different in comparison to both normal animals *(P<0.05, one sample t test)* and sham animals *(P<0.05, Tukey-Kramer post-hoc test)*. This redistribution did not recover with time and remained the same during weeks 3 and 6 after SCI (Fig. 4-4C and D).

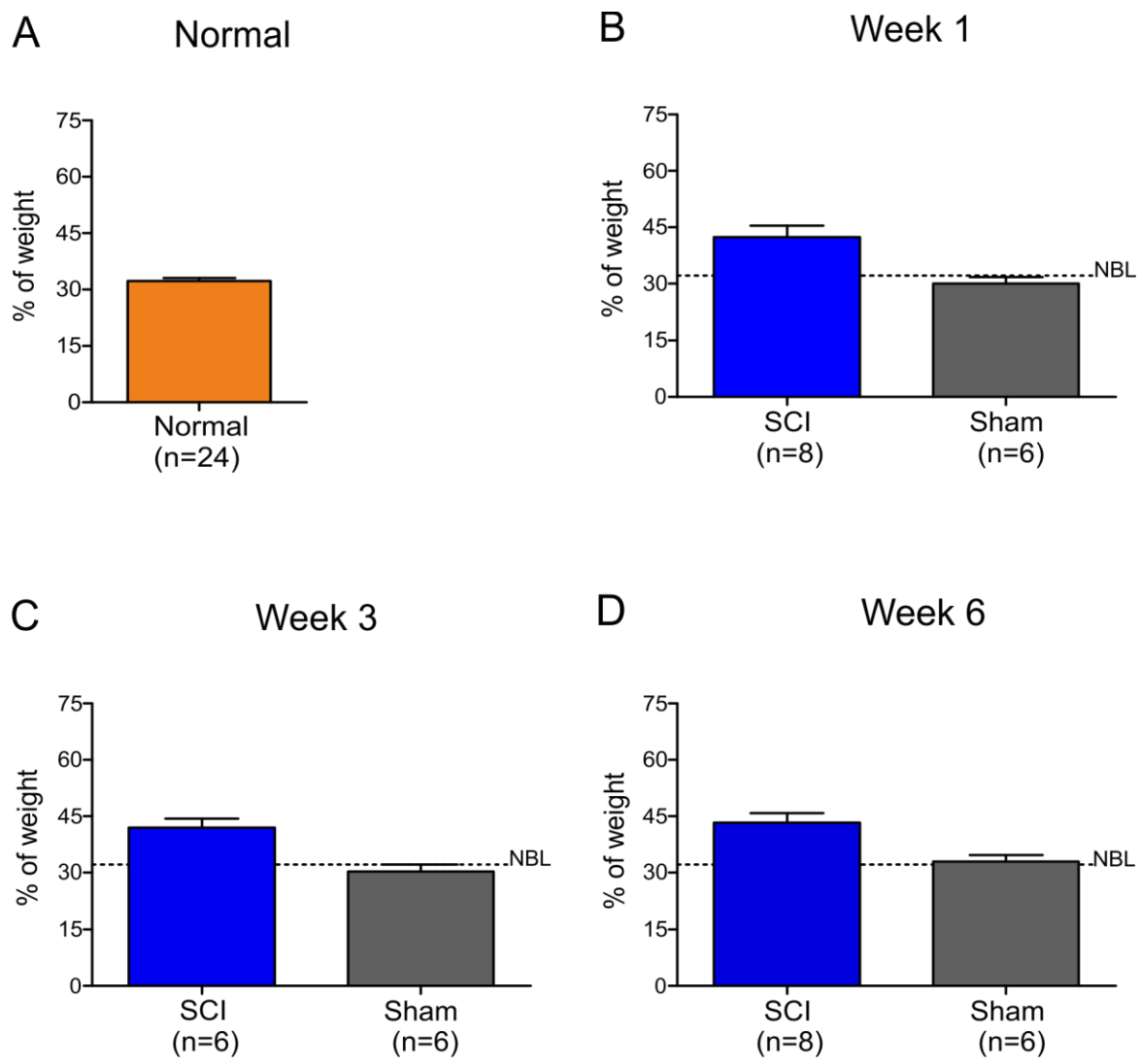

Fig.4-3. DWB data collected from forepaws. A shows the percentage of body weight distributed to the forepaws in normal animals (n=24). **B, C** and **D** represent the percentage of body weight distributed to the forepaws at 1, 3 and 6 weeks post SCI, respectively. In B and D, data were collected from 8 contusion injured and 6 sham rats while 6 rats in each group were assessed in week 3 after SCI. The dotted lines (normal base line "NBL") represent the average percentage forepaw-weight distribution in naive animals (n=24). All bars represent mean ± SE.

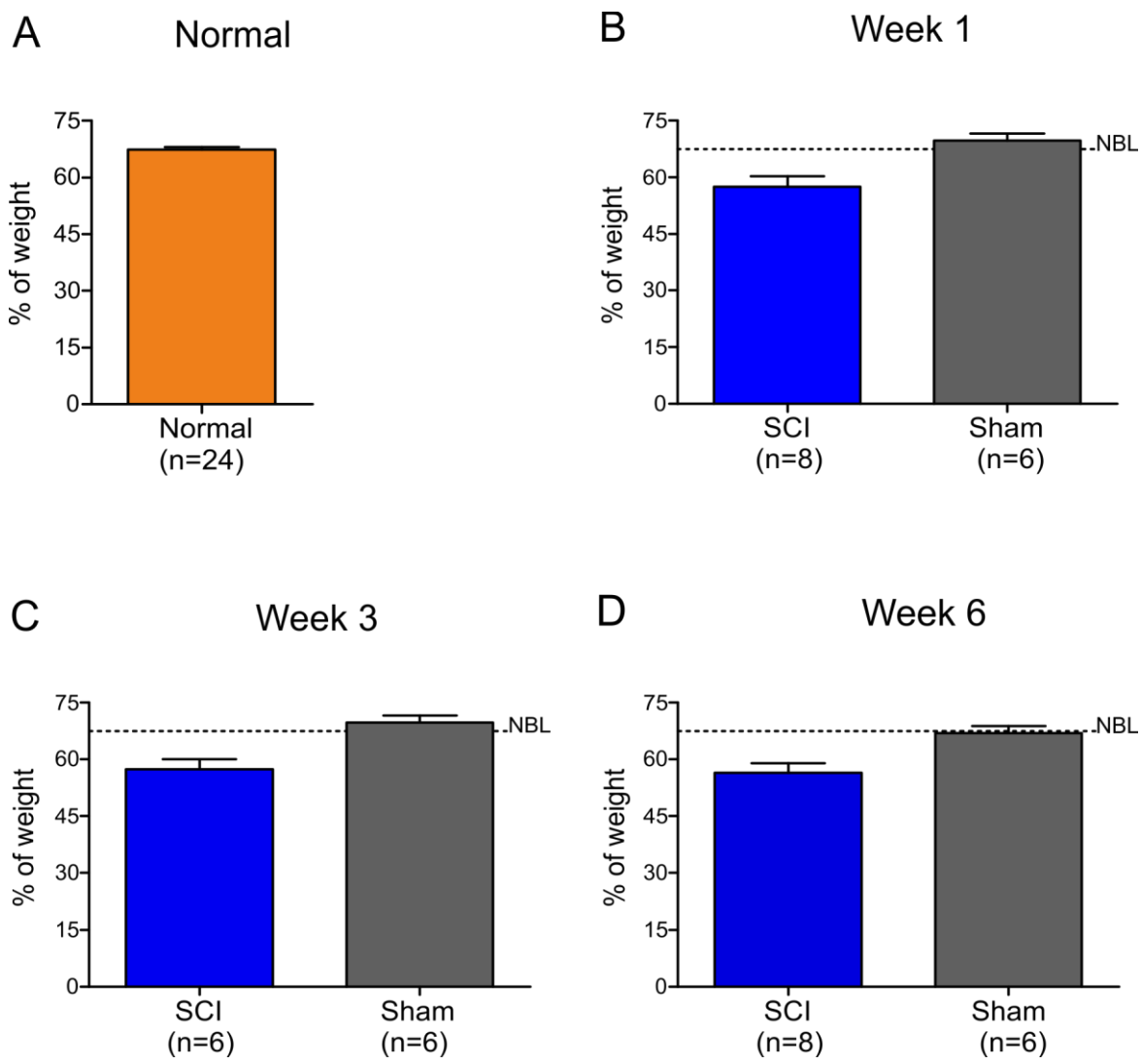

Fig. 4-4. DWB data collected from hindpaws. A shows the percentage of body weight distributed to the hindpaws in normal animals (n=24). **B, C** and **D** represent the percentage of body weight distributed to the hindpaws at 1, 3 and 6 weeks post SCI, respectively. In B and D, data were collected from 8 contusion injured and 6 sham rats while 6 rats in each group were assessed in week 3 after SCI. The dotted lines (normal base line "NBL") represent the average percentage hindpaw-weight distribution in naive animals (n=24). All bars represent mean ± SE.

4.3.4 Responses of forepaws

4.3.4.1 Plantar von Frey test

The effect of the injury on the withdrawal response to tactile stimuli (vF hairs) applied to the forepaws is shown in Fig. 4-5. A decrease in the threshold for eliciting withdrawal was evident at the first test session (2 weeks) after the contusion injury. The threshold continued to decrease in subsequent weeks reaching a minimum at around week 4 and plateauing at weeks 5 and 6. The difference in threshold between SCI and sham animals was statistically significant from week 2 to 6 *(P<0.001, Bonferroni post-hoc test)*. Sham operated animals showed little changes in threshold in the same tests. There was no statistical difference between preoperative data and postoperative data for sham animals at any time point *(P>0.05, Tukey's post-hoc test)*.

4.3.4.2 Plantar heat test

Responses to the plantar heat test are shown in Fig. 4-6. There were no significant differences in the response latencies between SCI and sham animals over the course of the study *(insignificant Bonferroni alpha value according to the correction concept)* (Fig. 4-6A). In each group, there are only minor changes in the response latencies collected preoperatively and after the surgery at each test point *(P>0.05, Tukey's post-hoc test)*. In addition to measuring response latency to the heat stimulus, animals were also carefully observed to determine whether or not they licked the paw to which the heat stimulus had been applied. The frequency with which a licking response was seen following withdrawal is shown in Fig. 4-6B. Before the surgery, animals licked the stimulated paw following 7 or 8 of the 10 stimulus applications (5 to each forepaw). The contusion injury or sham operation had no effect on this licking response, which was seen with a similar frequency in all subsequent test sessions with no significant difference at any time point (*P>0.05* for each group *vs*. the base line, *one sample t-test*). These observations are suggestive of a similar sensitivity to the heat stimulus by both groups before and after the surgery which indicates that a T9 150 kdyn contusion injury does not produce heat hyperalgesia in forepaws.

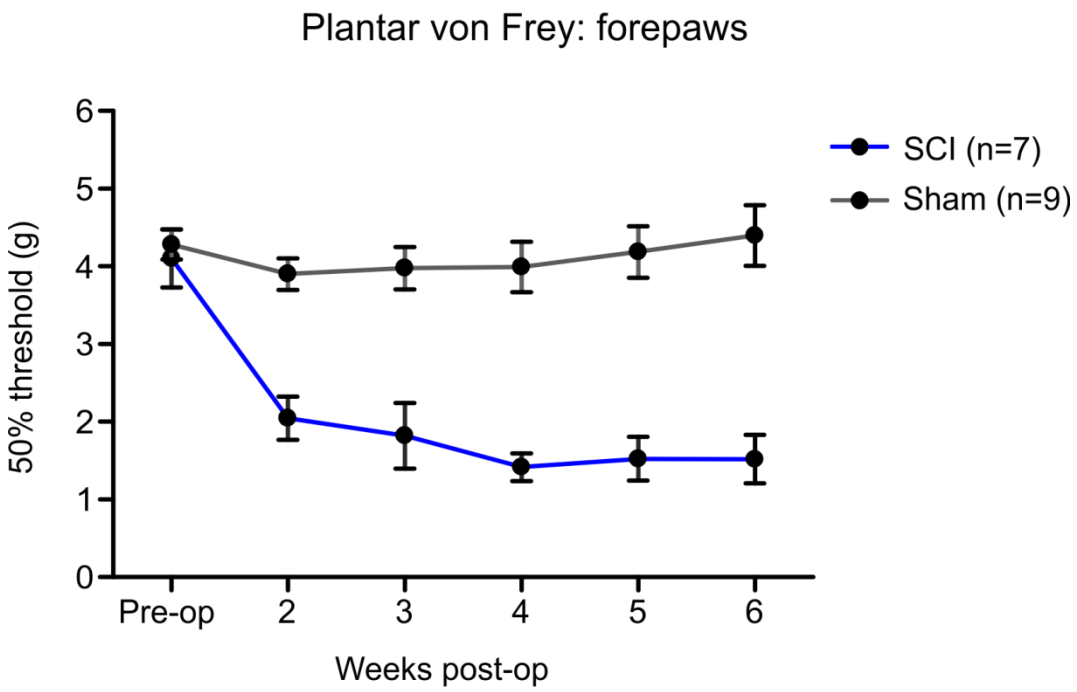

Fig.4-5. Plantar von Frey testing of the forepaws. The graphs show the 50% threshold for the force of vF filament eliciting a withdrawal response on application to the plantar surface of the forepaws. The test was performed preoperatively and on a weekly basis (2 to 6) after the surgery. Data for both forepaws of each animal was averaged and the plotted data points represent the mean ± SE for contusion injured (n=7) and sham (n=9) operated groups.

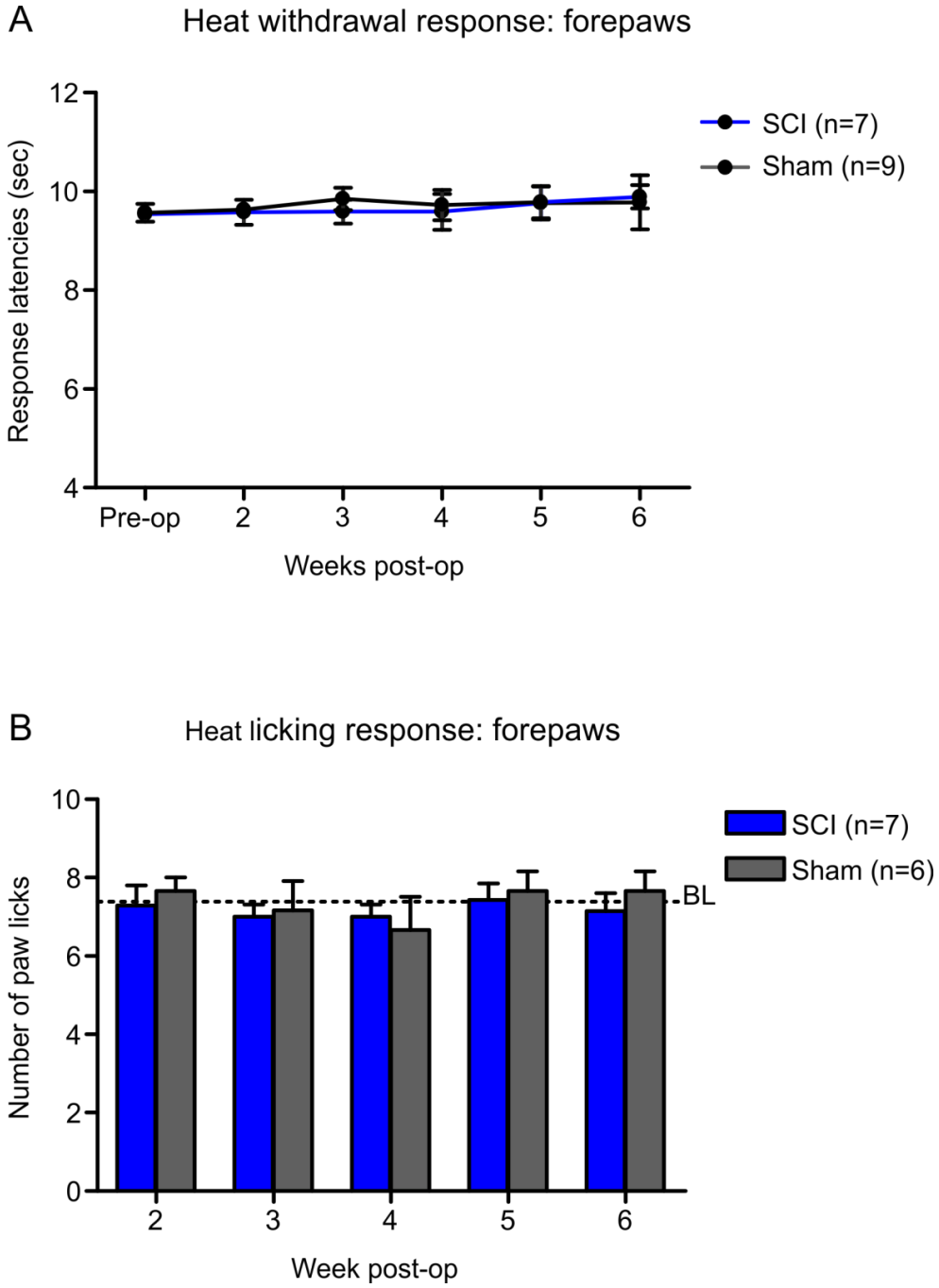

Fig. 4-6. Behavioural responses to heat stimuli applied to the forepaws. A is a graph showing latencies for withdrawal of the forepaws in response to radiant heat applied to the plantar surface. Data for both forepaws of each animal was averaged for SCI (n=7) and sham (n=9) groups. Bars in **B** represent the frequency with which licking was seen following 10 applications of heat to the plantar surface of the forepaws. Tests were performed on both paws and data averaged for each injured (n=7) and sham (n=6) group. The dotted line (base line "BL") shows the average incidence of licking observed in all animals before the SCI or sham surgery. The observations in A and B were made preoperatively and at 2, 3, 4, 5 and 6 weeks after the injury. All observations are shown as the mean ± SE.

4.3.5 Responses of hindpaws

4.3.5.1 Plantar von Frey test

Contusion injury had a similar effect on the withdrawal response of the hindlimb to tactile stimuli as for stimuli applied to the forepaws as shown in Fig. 4-7. Both the time course and the percentage reduction in threshold from preoperative values showed a closely similar pattern. However, the absolute threshold in grams was higher from the outset, reflecting the lower sensitivity of the hindpaw plantar skin. Sham operated animals showed little changes in threshold in the same tests. The decrease in threshold in injured animals was very significant from week 2 ($P < 0.001$, *Bonferroni post-hoc test*).

4.3.5.2 Plantar heat test

Responses to the plantar heat test are shown in Fig. 4-8. In comparison to the sham group, SCI animals showed a marked reduction in withdrawal latencies in response to heat stimuli at the first test session (2 weeks) after the contusion injury (Shams *vs.* SCI: $P<0.001$, *Bonferroni post-hoc test)*. A maximum reduction was reached in week 4 and this did not change over the following two weeks (Fig. 4-8A). The response latency for the sham group of animals did not change in the same test period. When heat stimuli were applied preoperatively to the hindpaw, licking of the paw was seen with a similar frequency to that for testing of the forepaw. When tested postoperatively, the sham animals continued to show a licking response with a similar frequency throughout the remainder of the test period. However in the SCI animals, licking was initially substantially reduced but not abolished (Fig. 4-8B). In the first two testing sessions after SCI, licking of the hindpaw was seen following 20% of stimulus applications while this increased to about 50% at weeks 4 to 6 after injury. None of the contusion injured animals showed the normal degree of licking displayed before the surgery.

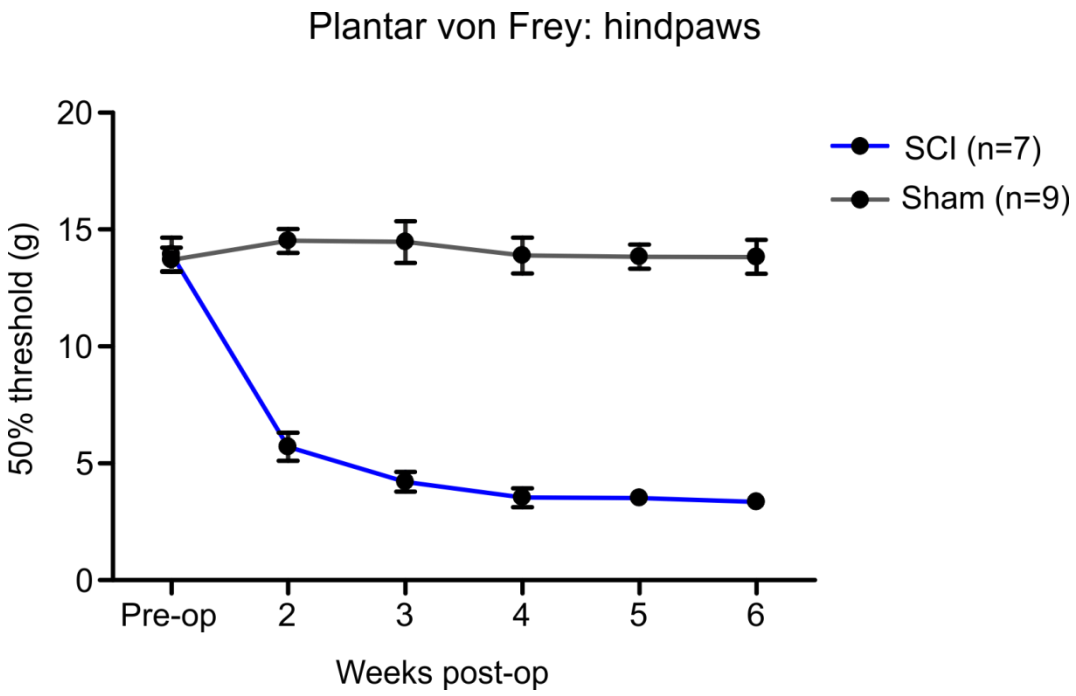

Fig. 4-7. Plantar von Frey testing of the hindpaws. This graph shows the 50% threshold for the force of vF filament eliciting a withdrawal response on application to the plantar surface of the hindpaws. The test was performed preoperatively and on a weekly basis (2 to 6) after the surgery. Data for both hindpaws of each animal was averaged and the plotted data points represent the mean ± SE for contusion injured (n=7) and sham (n=9) operated groups.

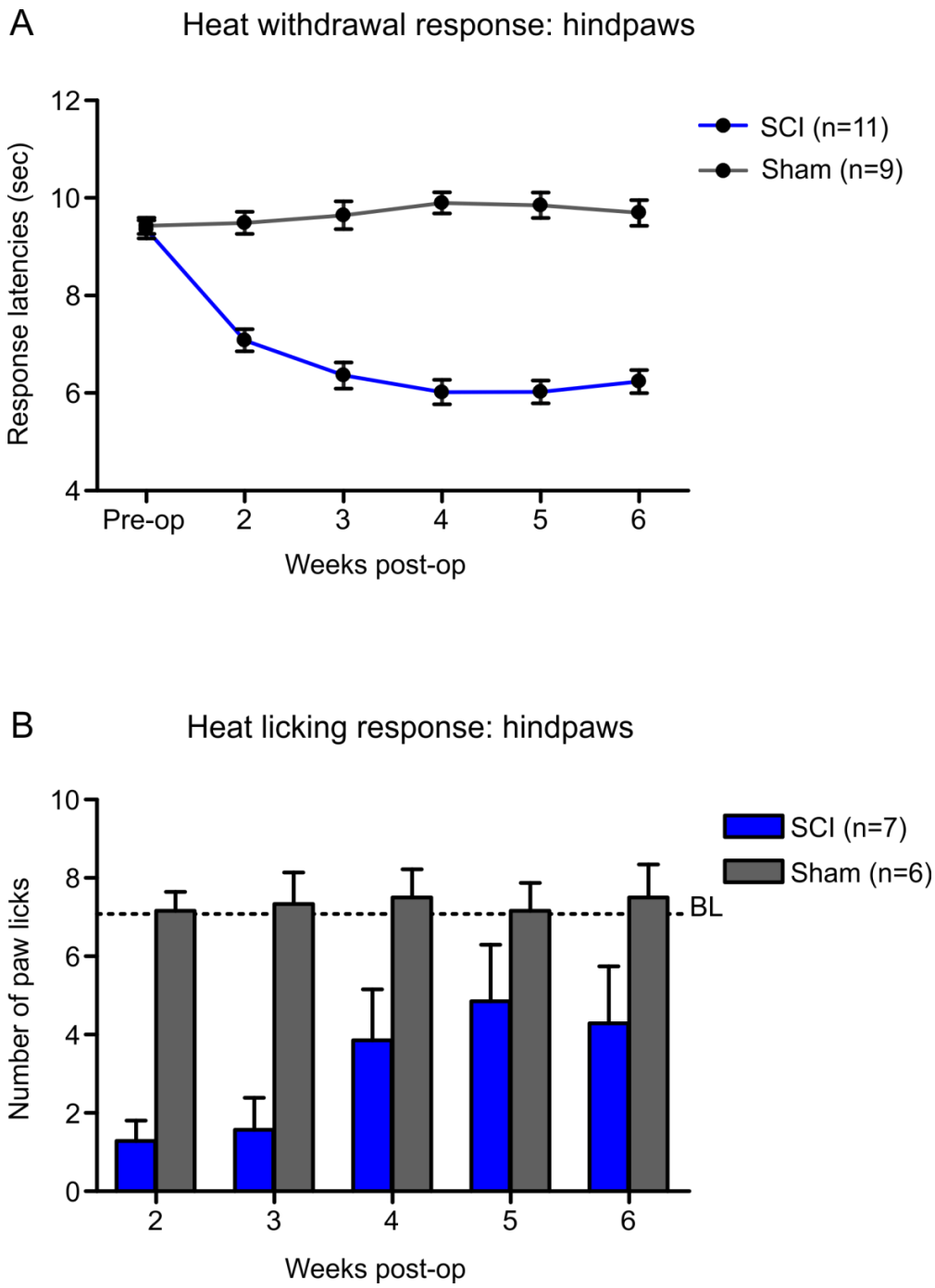

Fig. 4-8. Behavioural responses to heat stimuli applied to the hindpaws. A is a graph showing latencies for withdrawal of the hindpaws in response to radiant heat applied to the plantar surface. Data for both hindpaws of each animal was averaged for SCI (n=11) and sham (n=9) groups. Bars in **B** represent the frequency with which licking was seen following 10 applications of heat to the plantar surface of the hindpaws. Tests were performed on both paws and data averaged for each injured (n=7) and sham (n=6) group. The dotted line (base line "BL") shows the average of incidence of licking observed in all animals before the injury or sham surgery. The observations in A and B were made preoperatively and at 2, 3, 4, 5 and 6 weeks after the injury. All observations are shown as the mean ± SE.

4.3.6 Forepaw lifting data

4.3.6.1 Responses on the cold plate

When observed on the cold plate maintained at a temperature (7.5^{o}C), both SCI and sham animals showed lifting of the forepaws from the surface which, on some occasions, was associated with aversive behaviours such as licking and shaking of the paw. In comparison to the shams, the incidence of FL in the contusion injured group was more frequent but not statistically different during week 4 after SCI *(P>0.05, Bonferroni post-hoc test)*. During the fifth and sixth week PO, SCI animals showed more marked forepaw lifting on the cold stimuli than the sham group. These were statistically different at weeks 5 and 6 after injury (*P<0.01, Bonferroni test post-hoc)* (Fig. 4-9A). In each group, there was no significant differences in FL investigated at different time points *(P>0.05, Tukey's post-hoc test)*.

4.3.6.2 Responses on the thermally neutral plate

Contusion injured animals also showed some FL when observed on a smooth and thermally neutral plate maintained at 30^{o}C, but the incidence was approximately half that seen on the cold plate (Fig. 4-9B). Sham animals, which showed a low incidence of FL on the cold plates, showed no FL on the neutral plate. These observations were seen consistently in SCI animals without significant differences at 4, 5 and 6 weeks after SCI *(P>0.05, Tukey's post-hoc test)*. Like FL data collected form T9 200 kdyn animals, the behaviours observed at 30°C is suggestive of spontaneous pain in the forepaws.

4.3.6.3 Responses on the metal grid

When animals were observed on the metal grid, the contusion injured animals showed a high and similar incidence of FL at weeks 4, 5 and 6 PO which was completely absent in the sham controls (Fig. 4-9C).

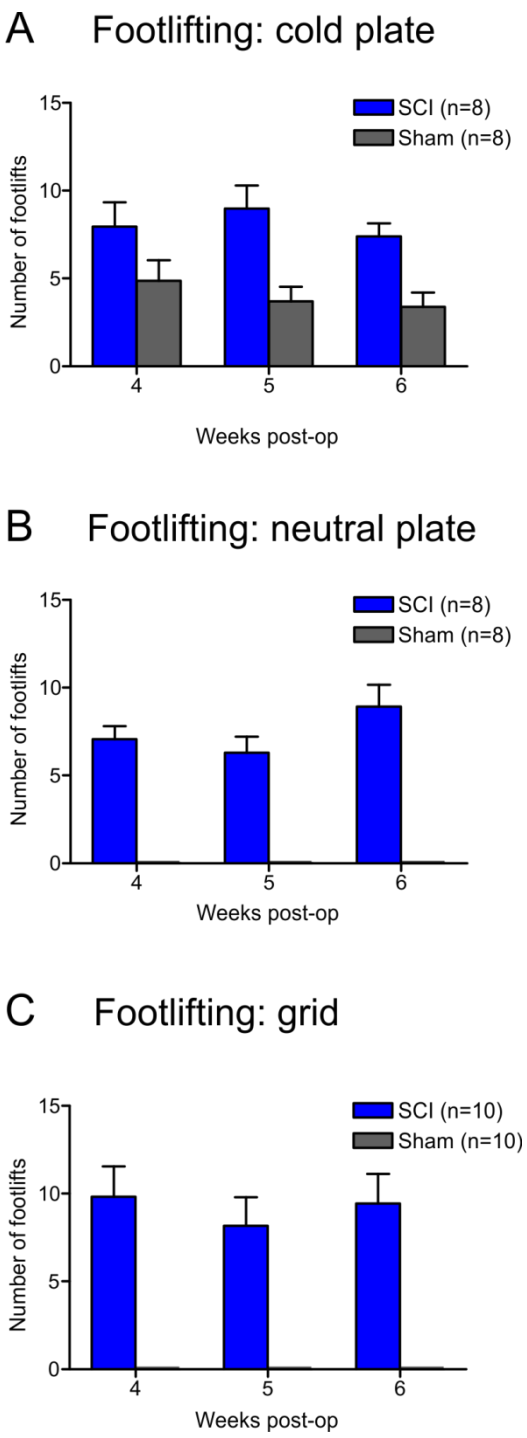

Fig. 4-9. Observations on the frequency of forepaw lifts on different surfaces. Each of the graphs (A-C) show the number of footlifting episodes (averaged for both forelimbs) observed during a 4 min period. **A** shows results for 8 contusion injured and 8 sham operated animals stood on a cold plate adjusted to 7.5°C. **B** shows data for 8 SCI rats and 8 shams standing on a neutral plate at 30°C. The histograms in **C** indicate the number of footlifting episodes observed while SCI (n=10) and sham (n=10) animals stood on a mesh grid. Columns in A, B and C represent mean ± SE of results collected in weeks 4, 5 and 6 following the SCI and sham surgery.

4.3.7 Hindpaw lifting data

The same set of observations as described above for the forepaws were made for the hindpaws.

4.3.7.1 Responses on the cold plate

When observed on the cold plate maintained at a temperature (7.5°C), both SCI and sham animals showed lifting of the hindpaws from the platform but without any aversive behaviours (e.g. licking and shaking of the paw). In comparison to sham controls, SCI animals displayed a significantly more hindpaw lifts in weeks 5 and 6 *(P<0.01, Bonferroni post-hoc test)* after the surgery (Fig. 4-10A). Unlike T9 200 kdyn data, these observations suggest that cold hyperalgesia was developed in hindpaws of animals subjected to 150 kdyn injuries at the T9 segment.

4.3.7.2 Responses on the thermally neutral plate

When the hindlimbs were observed with the animals on a thermally neutral plate (maintained at 30^{o}C), no evidence of FL was seen in either SCI or sham groups during week 4 and 5 after the injury (Fig. 4-10B). In the week 6 after the surgery, some animals in SCI group displayed a very few lifts (less than 3). Because the sham group did show any hindpaw lifting, this difference is statistically significant *(P<0.001)* according to *Bonferroni post-hoc multiple comparisons*. However, we should bear in mind that these paw lifts was only seen in a few occasions by some animals.

4.3.7.3 Responses on the metal grid

When animals were observed on the metal grid that was used for carrying out the von Frey testing, the contusion injured animals showed occasional lifting of the hindpaws, but this was much less frequent than for the forepaws in the same test session (Fig. 4-10C). The incidence of this FL was very consistent for test sessions at 4 weeks, 5 weeks and 6 weeks post injury. At no point was hindpaw lifting seen in sham animals.

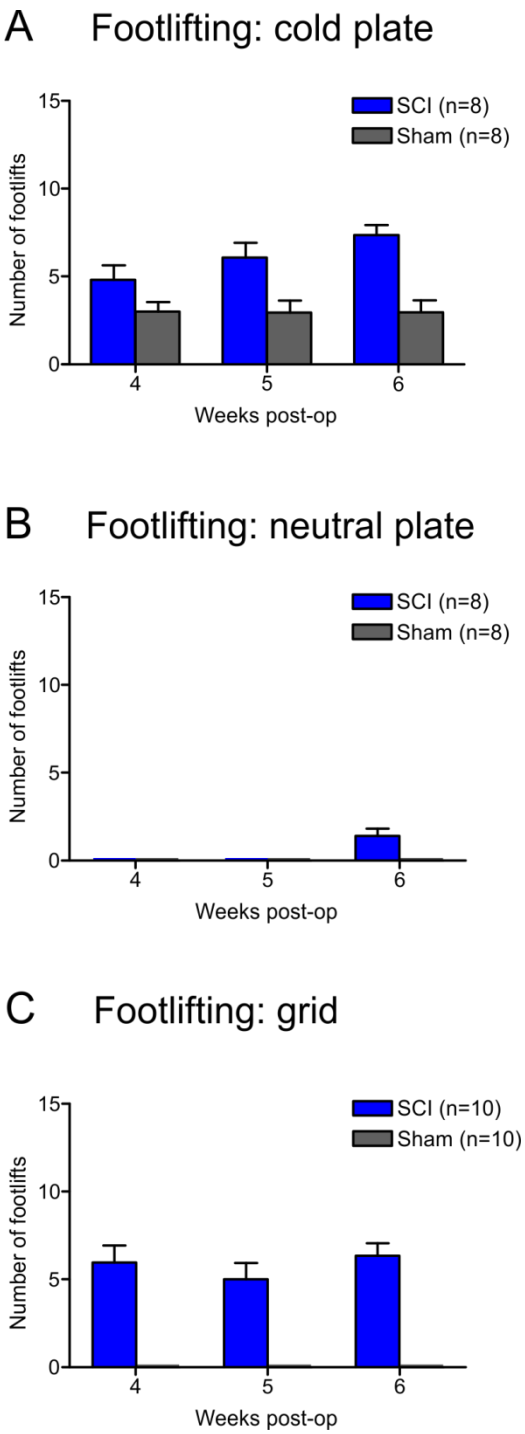

Fig. 4-10. Observations on the frequency of hindpaw lifts on different surfaces. Each of the graphs (A-C) show the number of footlifting episodes (averaged for both hindlimbs) observed during a 4 min period. **A** shows results for 8 contusion injured and 8 sham operated animals stood on a cold plate adjusted to 7.5°C. **B** shows data for 8 SCI rats and 8 shams stood on a neutral plate at 30°C. The histogram in **C** indicates the number of footlifting episodes observed while SCI (n=10) and sham (n=10) animals stood on a mesh grid. Columns A, B and C represent mean ± SE of results collected in weeks 4, 5 and 6 following the SCI and sham surgery.

4.3.8 Trunk von Frey test

4.3.8.1 Responses to above level stimuli

The incidence of responses to stimuli applied at +1 cm (above level) is shown in Fig. 4-11. The incidence of any of the recognised types of response to the stimuli (Fig. 4-11A) showed an immediate and sharp increase at the first test session after injury (1 week) and continued to increase, reaching a maximum of about 9/10 as soon as week 2 after the injury. The incidence remained at this elevated level for the subsequent weeks of the testing period, though most animals reached the maximal (10/10) response level so that this apparent plateaued response may reflect saturation of the test. The incidence of attempted biting of the filament (Fig. 4-11B), behaviour very rarely if ever seen before injury, also increased progressively following injury. This behaviour continued to increase throughout the 6 week post injury test period, becoming most common type of response by week 4. In comparison, the sham animals showed only the slightest suggestion of an increased sensitivity *(*non significant: *P>0.05, Tukey's post-hoc test)* and only in the first week following injury. These observations are a clear indication for the development of tactile allodynia over the back to stimuli applied to above level dermatomes.

4.3.8.2 Responses to below level stimuli

The results obtained when applying stimuli to the back at -5 cm (below level) are shown in Fig. 4-12. Unlike T9 200 kdyn animals, rats subjected to the 150 kdyn contusion injuries showed a sharp increase in the incidence of response during week 1 after SCI. This trend increased gradually over the period between weeks 4 and 6 but never reached the maximal level as seen when stimuli were applied above level (Fig. 4-12A). The responses of SCI animals were frequently observed as head turnings, slight escapes and orientations to the stimulus application side. In comparison, the sham animals showed only minor changes in tactile sensitivity over the course of the study *(*non significant: *P>0.05, Tukey's post-hoc test).* In both groups, biting response was never seen either before or after the surgery (Fig. 4-12B). Taken together, these observations are a clear indication for the development of tactile allodynia over the back -5 cm below the injury level.

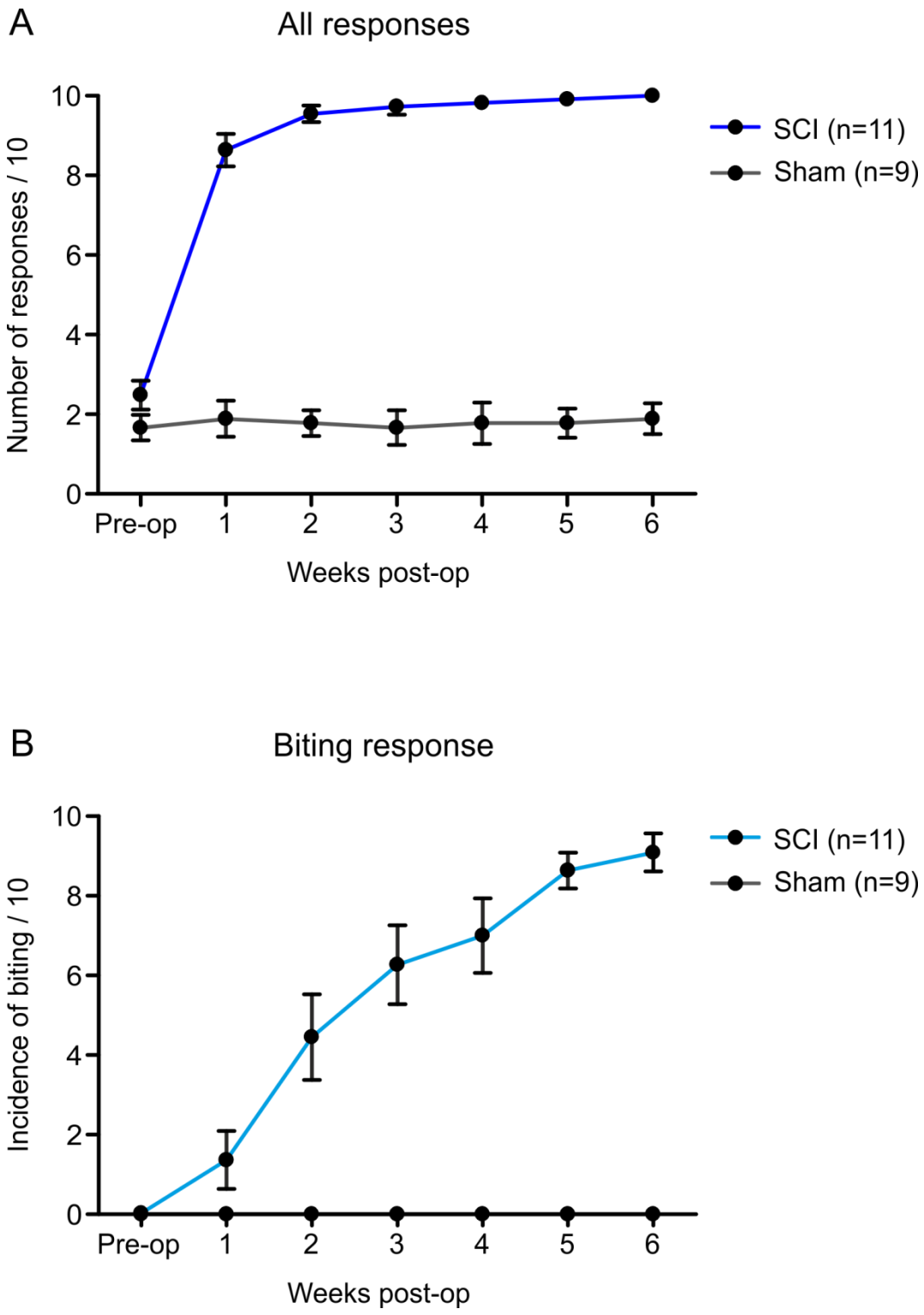

Fig. 4-11. Behavioural testing using stimuli applied at +1 cm (above level) over the back. A graph showing the total incidence of any of the recognised types of response to tactile stimuli applied over the back. **B** shows just the incidence of the biting response. The plots show the mean ± SE for responses observed in SCI (n=11) and sham (n=9) groups in each test session performed preoperatively and then on a weekly basis after the surgery until week 6.

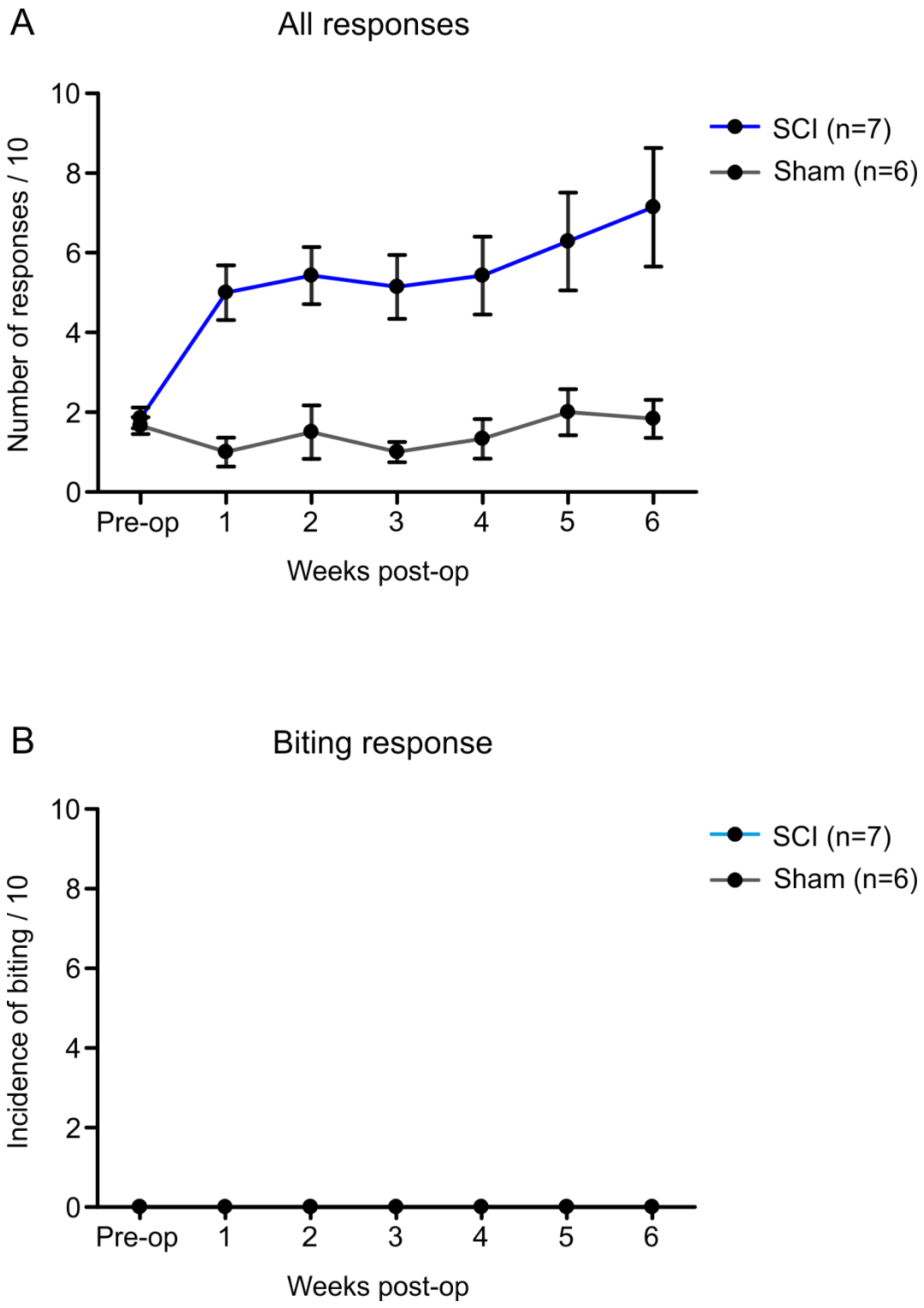

Fig. 4-12. Behavioural testing using stimuli applied at -5 cm (below level) over the back. **A** graph showing the total incidence of any of the recognised types of response to tactile stimuli applied over the back. **B** shows just the incidence of the biting response. The plots show the mean ± SE for responses observed in SCI (n=7) and sham (n=6) groups in each test session performed preoperatively and then on a weekly basis after the surgery until week 6.

4.3.9 PEAP test

This test was used to confirm cortical processing of nociceptive signals. During preliminary experiments, we arranged the test conditions such that normal animals showed a clear preference for the dark half of the cage, spending on average about 75% of their time in this environment even though stimuli were continuously applied to animals while they were located on this side of the housing. We then tested groups of sham operated animals and injured animals while applying stimuli using vF filaments applied to two different sites: 1) over the back at -5 cm (below level) and 3) to the hindpaws.

4.3.9.1 Stimuli applied to below level over the back

The results obtained when applying stimuli to the back below the injury level are shown in Fig. 4-13. Sham operated animals (n=6) showed a clear preference for the dark side of the cage spending an average of about 75% of the time in the black compartment. This did not differ significantly between the first 10 minutes of the test and the total 30 minute duration of the test (Fig. 4-13A). Exploratory behaviour i.e. frequency of crossing between the light and dark halves of the cage were consistent over time with the number of crossings in the 30 minute test period as a whole being about 15 times (Fig. 4-13B).

On the other hand, under the same conditions and with the same stimuli, SCI animals (n=8) spent less time than the sham animals (n=6) on the dark side of the cage and this behaviour was similar in the first 10 minutes of testing as in the whole of the 30 minute test period (Fig. 4-13A). On average, the SCI animals spent 22% of their time on the dark side compared to 75% for the sham operated animals over the 30 minutes of the test, a difference which is highly significant *($P<0.001$, Bonferroni post-hoc test)*. There was no difference in the frequency with which SCI and sham animals crossed between the light and dark sides of the cage *($P>0.05$, unpaired t-test*) (Fig. 4-13B). These observations suggest that the SCI animals perceive the stimuli as more unpleasant than sham animals and that the stimuli therefore result in a greater modification of the behaviour of the SCI animals than the shams. For this modification in behaviour to occur, neural activity resulting from the stimuli must be processed at a conscious level.

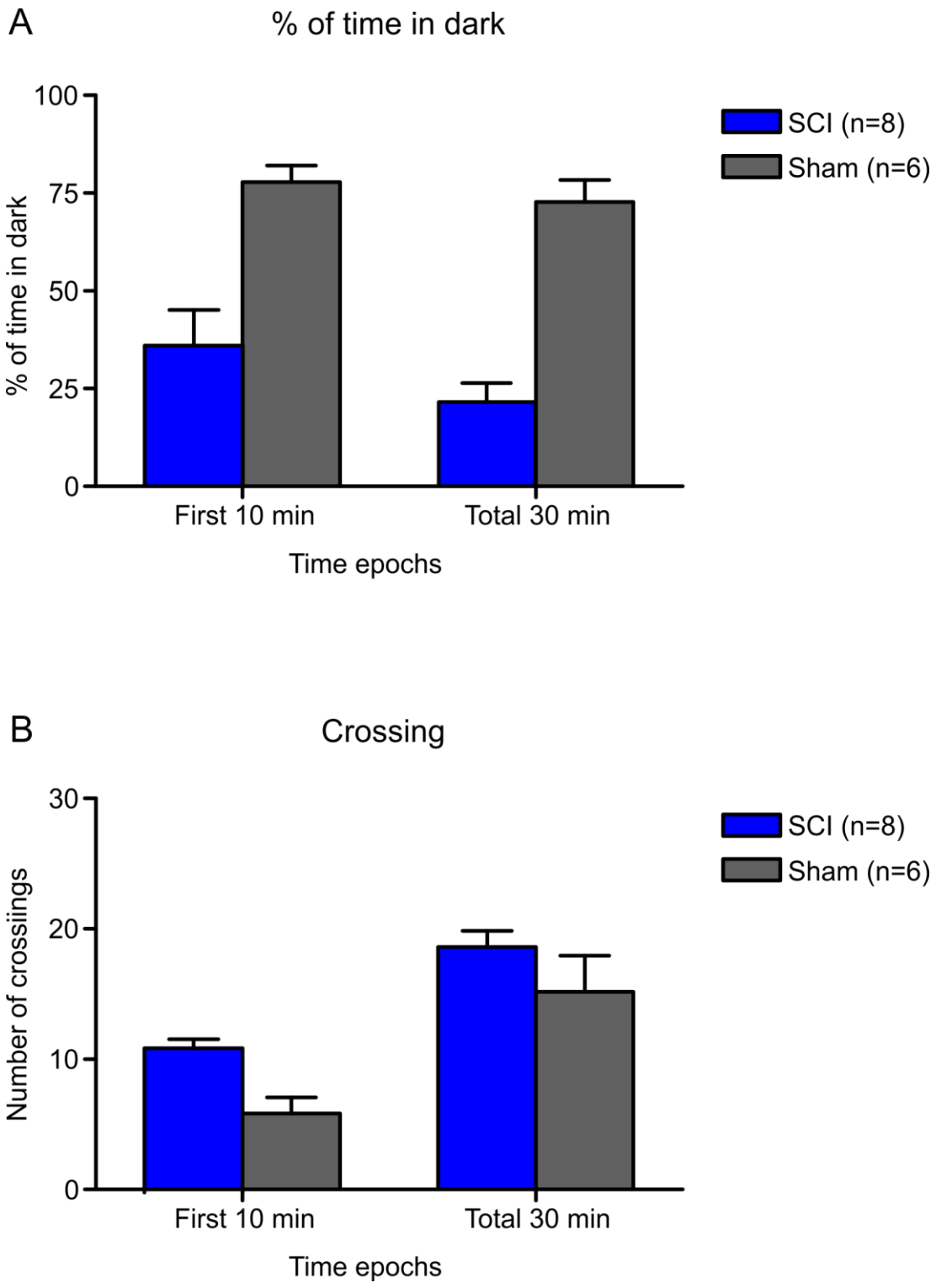

Fig. 4-13. Results from PEAP test using vF stimulation of the back at -5 cm below the injury site. A indicates the percentage of the test time that the animals spent on the dark side of the test chamber. **B** shows the number of times that the animals crossed between the light and dark sides of the chamber. The bars represent the mean values ± SE for the SCI (n=8) and sham (n=6) groups of animals. Data (collected in week 4 PO) for the first 10 min of the test session and full 30 min of the session is shown separately.

4.3.9.2 Stimuli applied to the hindpaws

The results obtained using this test when a vF filament was applied to the hindpaws of sham (n=6) and SCI animals (n=7) is shown in Fig. 4-14. Unlike results with stimuli applied to the back below level, there was a little difference in behaviour between sham and SCI animals when stimuli were applied to the hindpaws, both groups of animals spending more time on the dark side of the cage (Fig. 4-14A) (58% for sham and 70% for SCI which is not significantly different; *P>0.05, Bonferroni post-hoc test)*. However, the contusion injured animals displayed more crossing activity between the light and dark sides than the sham animals (Fig. 4-14B) which was statistically significant when the 30 min test period as a whole is considered *(P=0.0257, unpaired t-test with Welch's correction)*. This unpredicated result may be related to development of tactile allodynia in the forepaws and hence increased sensitivity to the grid floor used in this test. As a result, the tested animals confused to choose the safe compartment because both dark and while sides became uncomfortable.

4.3.10 Assessment of damage to ascending nociceptive pathways

Some of sensory testing shows that responses of a type that require supraspinal processing in the brainstem or cortex are present when stimuli are applied below the level of the injury. In comparison to T9 200 kdyn model, T9 150 kdyn contusion may be sparing more ascending pathways conveying nociceptive information to the brain. To investigate the later possibility, we therefore carried out a retrograde labelling study of spinal projection neurons of established importance in pain processing. In CVLM injections, numbers of retrogradely labelled neurons in the dorsal horn of segments C7, T12 and L4 were quantified using confocal microscopy and image analysis. Injections were made into the CVLM of 5 normal animals and 2 SCI animals. Accurate targeting of the injection sites was confirmed in all animals included in the study. Estimates were made of the numbers of retrogradely labelled neurons in an area encompassing the dorsal horn which included all gray matter dorsal of the central canal.

Typical examples of retrogradely labelled neurons in the T12 segments from normal animal are illustrated in Fig. 4-15A and C while those from animal subjected to contusion injury are shown in Fig. 4-15B and D. Fig. 4-15E and G illustrate examples of confocal scan for sections from L4 segment in normal animal while Fig. 4-15F and H show the same for an animal subjected to 150 kdyn contusion injury.

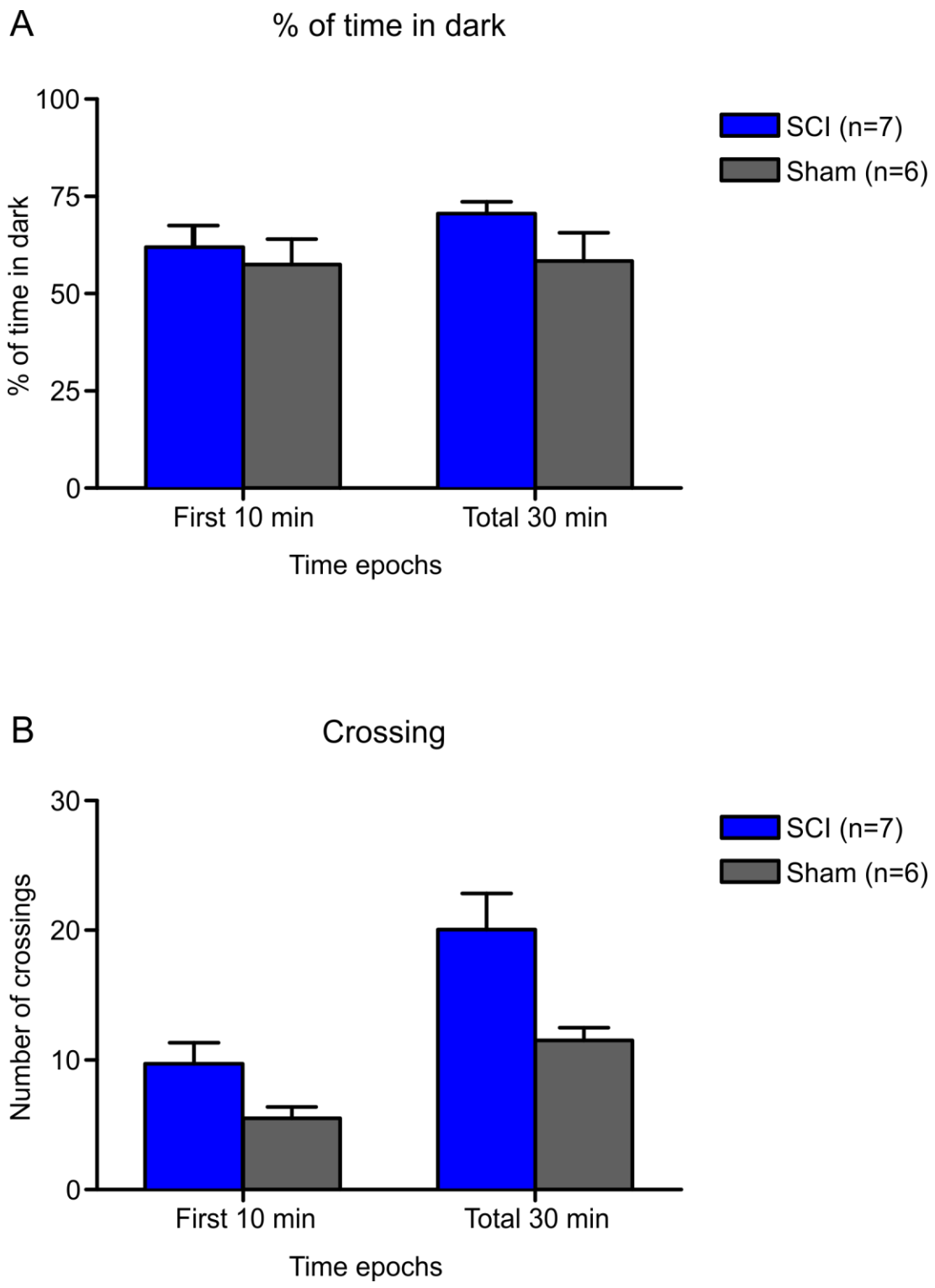

Fig. 4-14. Results from PEAP test using vF stimulation of the hindpaws. A indicates the percentage of the test time that the animals spent on the dark side of the test chamber. **B** shows the number of times that the animals crossed between the light and dark sides of the chamber. The bars represent the mean values ± SE for the SCI (n=7) and sham (n=6) groups of animals. Data (collected in week 6 PO) for the first 10 min of the test session and full 30 min of the session is shown separately.

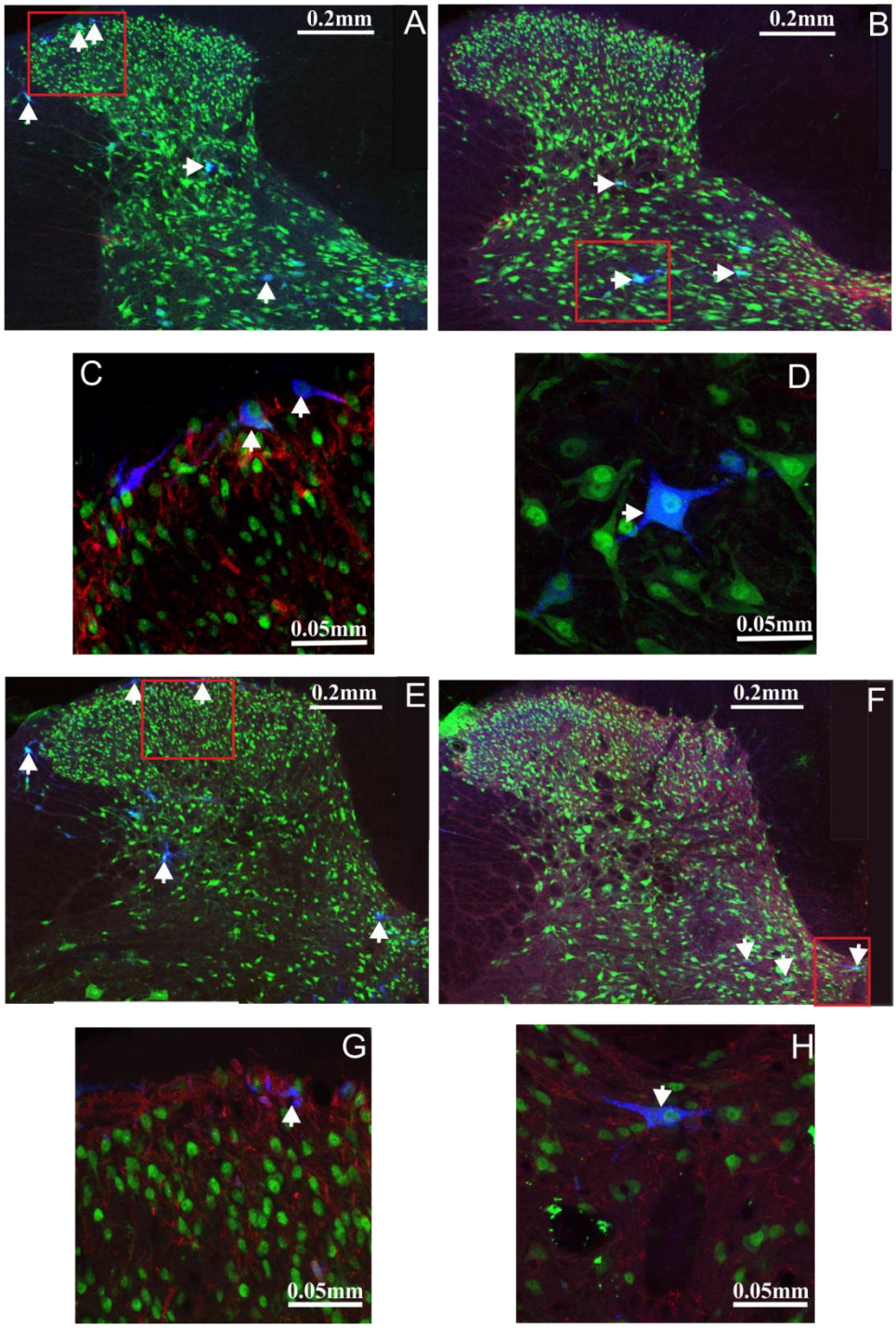

Fig. 4-15. Retrograde labelling of neurons projecting to the CVLM. A confocal image showing projections neurons in the dorsal horn of the T12 segment of a normal animal (scale bar = 0.2 mm). **B** montage of confocal microscope scans of the dorsal horn obtained from sections of the T12 segment in an injured animal (scale bar = 0.2 mm). **C** and **D** (scale bar= 0.05 mm) are representing boxed fields in A and B, respectively. **E** and **F** montage of confocal microscope scans of dorsal horn taken from sections of the L4 segment in normal (E; scale bar = 0.2 mm) and injured (F; scale bar = 0.2 mm) animals. **G** and **H** (scale bar= 0.05 mm) illustrate the red boxes in E and F, respectively. Neurons retrogradely labelled with FG in blue, Neu N green and Nk1 red. Arrows indicate retrogradely labelled projection neurons.

Estimates of the numbers of projection neurons retrogradely labelled from the CVLM are shown in Fig. 4-16. The numbers of neurons in the dorsal horn of C7 were relatively similar in normal (n=2) and in injured (n=2) animals (Fig. 4-16A). The mean number of dorsal horn neurons in normal animals was 14.9 ± 0.9 per section (8 sections examined) while the mean for sections from injured animals was 19.1 ± 4.9 (8 sections examined). The mean number of labelled neurons in T12 sections from normal animals (n=2) was 17.6 ± 4.6 (8 sections examined) (Fig. 4-16B). The mean number in T12 of contusion injured animals (n=2) was 4.0 ± 2.2 (8 sections examined) which is considerably less than that in normal animals. In sections from SCI animals, the labelled neurons were mainly observed in the lateral spinal nucleus and deep dorsal horn and none of these cells were seen in lamina I. This presumably reflects considerable damage to ascending axons at injury level. The mean number of retrogradely labelled neurons in the dorsal horn of sections from L4 in normal animals (n=5) was 28.43 ± 5.45 (19 sections examined) and 6.5 ± 3.0 in SCI animals (8 sections examined from 2 animals) (Fig. 4-16C). The distribution of labelled cells from SCI animals was nearly similar to that seen at T12 segment from the same animals. Interestingly, the labelled neurons at L4 segment were about double of that seen in animals subjected to 200 kdyn at the same level which is consistent with the observed responses during the behavioural testing in comparison to T9 200 kdyn models (see discussion).

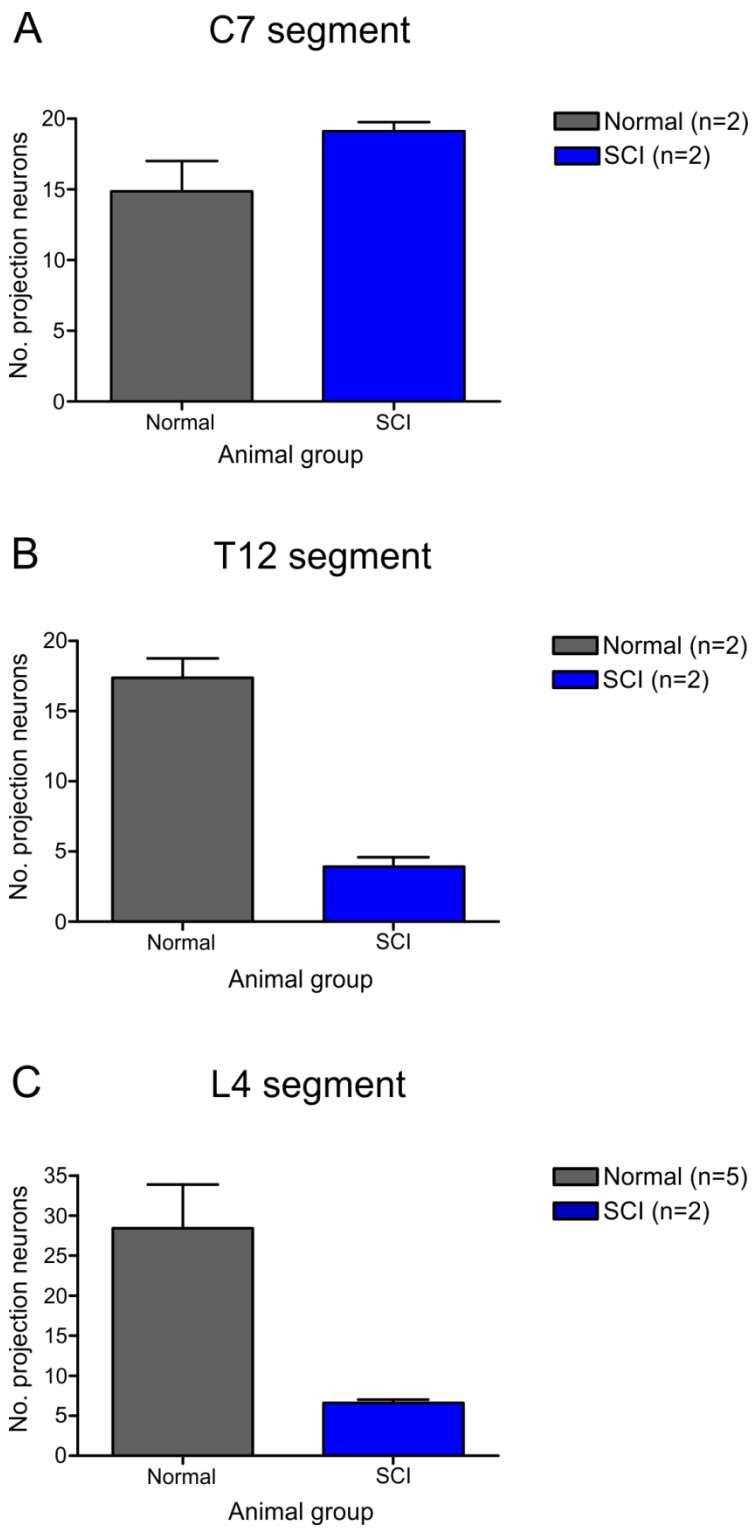

Fig. 4-16. Comparison of numbers of spino-CVLM projection neurons in normal and SCI animals. Histograms showing estimates of the numbers of retrogradely labelled neurons in the whole dorsal horn of the C7 (**A**), T12 (**B**) and L4 (**C**) segments of normal and injured animals. For each segment, bars show the mean numbers ± SE in the dorsal horn per 70 µm sections.

4.3.11 Summary of the results

The main findings in this model are summarized in table 4-1: 1) Enhancement of the withdrawal response to mechanical stimuli when applied to forepaws and hindpaws. 2) According to plantar heat assay, heat sensitivity was only evident in the hindpaws but not in forepaws. 3) Intact licking response in forepaws but, to a lesser extent, in the hindpaws. 4) Mechanical and cold sensitivity (both allodynia and hyperalgesia) was evident in fore and hind paws as assessed by footlifting test. 5) In addition, the same test revealed development of spontaneous pain in the forepaws. 6) Clear evidence for development of mechanical hypersensitivity in above level area over the trunk and to less extent in below level dermatomes. 7) Positive PEAP data was collected from below level over the back but not from the hindpaws. 8) Tract tracing study revealed no differences in number of projection neurons at C7 segment while there were massive reductions in numbers of projection neurons at T12 and L4 segment.

A

Type of the test	Forepaws	Hindpaws
DWB	↑	↓
Plantar vF test	↓ Threshold	↓↓ Threshold
Plantar heat test	ND	↓↓ Latencies
Licking response	ND	↓
FL over cold plate (7.5°C)	↑↑	↑
FL over neutral plate	↑	↑
FL over grid	↑	↑
PEAP	Not tested	ND

B

Type of the test	Above level	At level	Below level
Trunk vF test	↑↑↑	Not tested	↑
Trunk PEAP	++	Not tested	+

C

Spinal segment	C7	T12	L4
Projection neurons	ND	↓↓↓	↓↓↓

Table 4-1. Summary of the results of T9 150 kdyn. A shows the summary of data collected from fore and hind paws while **B** shows the summary of trunk von Frey and PEAP testing. Data of tract tracing are summarized in **C**. ND indicates no difference.

4.4 Discussion

4.4.1 Forepaw data

4.4.1.1 Comparison to the results of others labs

All animals subjected to 150 kdyn contusion injuries showed a significant reduction in mechanical threshold for paw withdrawal while sham animals showed no significant change. A significant change was evident at the first testing session (two weeks after the injury) and the maximum change developed by 4 weeks. This is similar to the pattern change seen in animals subjected to the more severe 200 kdyn injuries although these animals developed lower thresholds than in the 150 kdyn animals, suggesting that the more severe injury leads to a greater degree of sensitivity in the withdrawal response. In contrast to the 200 kdyn SCI animals, the 150 kdyn injury produced no significant change in the response latencies to heat stimuli applied to the forepaws.

Sensory testing of the forepaws has been employed relatively infrequently, especially in studies on mild injury models. There has been one previous study using the IH impactor. Rafati et al. (2008) tested animals with 150 kdyn injuries at the T10 level using the von Frey test at 10 weeks after injury and reported a decrease in threshold compared to the preoperative base line data. This is in agreement with our observations in this SCI model. Three studies using forepaw assessments have been carried out in the weight drop model with injuries (drop height 12.5mm) roughly comparable to 150 kdyn injuries made with the IH device. Hulsebosch et al. (2000) investigated both tactile allodynia and heat hyperalgesia. Both were reported to be evident by 4 weeks after the injury. Mills et al. (2000a) reported tactile allodynia and heat hyperalgesia in forepaws of 60% and about 85% of the SCI animals, respectively. Lindsey et al. (2000) assessed the plantar surface of forepaws by plantar von Frey test (by up down protocol) but saw no sign of tactile allodynia. The thermal hyperalgesia reported in the first two of these weight drop studies is in contrast to our results with the IH device and may be due to differences in the injury.

4.4.2 Hindpaw data

4.4.2.1 Comparison to the results of others labs

All animals subjected to 150 kdyn contusion injuries showed a dramatic reduction of mechanical threshold when evaluated by the plantar von Frey test applied to the hindpaws.

The reduction in threshold was seen at the first test, two weeks after injury and maximal by 4 weeks. These changes were virtually identical to those seen in animals with 200 kdyn injuries. In addition, in contrast to the forepaws in this model, a very marked reduction in the withdrawal latencies to heat stimuli were seen. These were evident from week 2 (the first test session) and maximal by week 4. The reduction in these response latencies was very similar to that seen in the 200 kdyn animals but there was one notable difference: whereas the plantar heat stimuli were never accompanied by licking in the 200 kdyn model, in the 150 kdyn a licking response (which is frequent pre-injury in sham animals) gradually returned.

Knerlich-Lukoschus et al. (2008) using a 150 kdyn injury at T8 reported reduced thresholds to mechanical stimuli but not reduced latencies to heat stimuli applied to the hindpaws at 7, 15 and 42 days after SCI. Several studies using the weight drop method to produce moderate severity injuries at low thoracic levels (see above), have reported marked reductions in mechanical threshold in the hindpaws (Lindsey et al., 2000; Hulsebosch et al., 2000; Yoon et al., 2004). In addition, heat hyperalgesia has been reported in the hindpaws (Hulsebosch et al., 2000).

The enhanced responses to the mechanical and heat stimuli, could normally be considered to reflect tactile allodynia and heat hyperalgesia, but are complicated by the SCI. However, while the 200 kdyn injured animals, showed no licking response to thermal stimuli, the 150 kdyn animals showed some recovery of the licking response. This suggests that some of the nociceptive signal related to the heat stimulus reached supraspinal levels. Although this indicates a degree of functional integrity of ascending nociceptive pathways, some doubt must remain as to whether the enhanced sensitivity to mechanical and thermal stimuli reflects enhanced pain or even nociception, since the potential complication of spastic hyperreflexia remains a possibility in these animals.

4.4.3 Footlifting behaviours

In this model, FL was assessed while animals stood over a flat cold surface (7.5°C), a flat thermally neutral plate (30°C) and while standing on a grid surface used for the plantar von Frey test. In comparison to sham controls, rats with 150 kdyn contusion injuries showed frequent paw lifting behaviour on a plate cooled to a noxious temperature (7.5°C), and when standing on a mesh (evoked FL). In addition, FL was frequently observed on a flat, thermally neutral surface (spontaneous FL). The evoked FL was displayed much more

frequently by the forelimbs than the hindlimbs while spontaneous FL was observed only in the forelimbs.

4.4.3.1 Interpretation of FL on the cold plate (7.5°C)

The increased frequency of FL seen at a temperature of 7.5°C is a clear indication for the development of cold hyperalgesia in forepaws and hindpaws. The cold hyperalgesia in the forepaws was less pronounced following 150 kdyn injuries than 200 kdyn injuries. However, hindpaw lifting was significantly increased following 150 kdyn injuries compared to sham controls whereas there was no difference following 200 kdyn injuries. This again may be related to the degree of sparing of ascending nociceptive projections from below the injury level.

4.4.3.2 Interpretation of FL on the neutral plate (30°C)

On a flat thermally neutral surface paw lifting was seen in 150 kdyn SCI animals but not sham controls and was only evident in the forepaws. This FL behaviour is assumed to reflect spontaneous pain. The incidence was similar to that seen in animals with a more severe 200 kdyn injury. This is in agreement with some human data reporting no direct relationship between the completeness of SCI and development of spontaneous pain (Turner et al., 2001; Widerstrom-Noga et al., 2001a; Werhagen et al., 2004).

4.4.3.3 Interpretation of FL on the mesh grid

Sham controls showed no abnormal behaviours while standing on the grid, but on the same surface injured rats displayed frequent lifting of forelimbs and to a lesser extent hindlimbs. Forepaw lifting was less frequent in 150 kdyn injured animals than 200 kdyn injured animals. This suggests greater mechanical sensitivity of the forepaws in the 200 kdyn compared to 150 kdyn injured animals and this is consistent with a greater reduction in mechanical threshold detected by the von Frey test in the 200 kdyn animals. Hindpaw lifting occurred with a similar frequency in 150 kdyn injured animals as the 200 kdyn injured animals (no statistical difference; *P>0.05, Bonferroni post-hoc test*).

Interpretation of this result is complex. Both groups of injured animals showed a similar reduction in hindpaw withdrawal thresholds. The 150 kdyn injured animals showed some behavioural evidence of supraspinal transmission of nociceptive information (licking of the hindpaw after thermal stimuli) and also showed a greater number of retrograde labelling

projection neurons on the L4 segment On the other hand, spastic hyperreflexia is a potential complicating factor in both groups of animals and neither showed a positive response when the PEAP test was performed using stimuli to the hindpaws.

4.4.4 Dynamic weight bearing

SCI animals showed a clear redistribution of weight from the hindpaws onto the forepaws compared to naive animals and sham controls. Similar to 200 kdyn injured animals, the posture changes observed in 150 kdyn injured group soon after the injury did not recover towards normal even at the later time points tested. The weight shift towards the forepaws is most likely a compensatory response to the loss of function in pathways providing postural control below the injury level since it occurs at early and later testing weeks. The redistribution of weight was very similar in 150 kdyn and 200 kdyn injured animals. As with the 200 kdyn animals, there is no clear evidence that pain influences this redistribution. In the 150 kdyn animals the situation is, if anything less clear than for the 200 kdyn animals since it is uncertain as to whether the animals experience pain from the hindpaws.

4.4.5 Trunk von Frey test

4.4.5.1 +1 cm above level

A similar increase in sensitivity to stimuli applied above the injury level was seen in both 150 and 200 kdyn injured animals. Both showed the same degree of increase in sensitivity and in both cases biting became a main response. However, the increased sensitivity occurred earlier in the less severe injury. These observations are consistent with a previous study in animals subjected to contusion injuries using the IH impactor (T10 150 kdyn) (Crown et al., 2005) and a further study using a weight drop injury of comparable severity (12.5 mm) (Hulsebosch et al., 2000).

4.4.5.2 -5 cm below level

In contrast to the 200 kdyn injured animals which showed no change in sensitivity to stimuli applied below the injury level, 150 kdyn injured animals showed a degree of enhanced sensitivity at this location, though less than above the injury level.

These observations indicate development of tactile allodynia below the injury in these animals and since the behaviours involve supraspinal processing, they indicate that ascending nociceptive pathways originating below the injury are at least partially intact. These behaviours are not complicated by the possibility of spasticity in the same way as withdrawal responses in the hindlimb, although it is possible that brainstem behaviours such as biting and head turning can be enhanced due to increased excitability of spinobulbospinal reflexes and may not therefore correlate precisely to pain perception (for review see Yezierski, 2005 and Mogil, 2009). However, we also saw a clear influence of stimuli applied at -5 cm in the PEAP test which indicates cortical processing from this level.

4.4.6 PEAP test

Since in the 150 kdyn injury model we saw evidence of increased sensitivity to stimuli applied below level, both to the back and to the hindpaws, the PEAP test was performed at both of these locations to look for evidence of pain perception.

4.4.6.1 -5 cm PEAP test

When the PEAP test was performed using tactile stimuli applied to the back below the injury level, all SCI animals spent significantly less time on the dark side of the cage in comparison to the sham controls. This clearly indicates that SCI animals perceived tactile stimuli at this location as painful. This is in contrast to the results obtained when the PEAP test was performed at this location in 200 kdyn injured animals and is consistent therefore with the results of testing on the back showing increased sensitivity to tactile stimuli at this location in 150 kdyn but not 200 kdyn animals.

4.4.6.2 Hindpaw PEAP test

In contrast to the PEAP test using stimuli applied to the back, there was no difference in the behaviour of SCI and sham animals when stimuli were applied to the hindpaws. These results may appear to be inconsistent with the reduced thresholds of the withdrawal response to tactile stimuli. It is unlikely that this is explained by nociceptive signals failing to reach the brain since there are several pieces of evidence that nociceptive transmission from levels below the injury is at least partially intact. These include licking of the hindpaws in response to heat, lifting of the hindpaws in response to noxious cold and on a mesh grid and anatomical observations from tract tracing (see below). Another possibility

is that there is no change in nociceptive circuits feeding into ascending nociceptive pathways and that the change in the withdrawal reflex reflects represents spastic hyperreflexia. A final possibility is that the hindlimb stimulation does not modify the behaviour of the animals because they are already experiencing pain as a result to the grid mesh on which they are standing (which is the same on both light and dark sides of the cage) and the hindpaw stimulation does not add significantly to this.

4.4.7 Tract tracing

The numbers of retrogradely labelled neurons in the T12 and L4 segments of animals with 150 kdyn SCI were reduced compared to normal animals but were still present at both locations. The presence of labelled neurons in the T12 segment is consistent with the increased sensitivity to the testing on the back and PEAP test performed using stimuli applied at -5 cm in the 150 kdyn animals. More labelled neurons were seen in the L4 segment of 150 kdyn injured animals than in animals with 200 kdyn injuries, in which neurons projecting from this segment were almost completely interrupted. This is consistent with licking of the hindpaws in response to heat, lifting of the hindpaws in response to noxious cold and on a mesh grid.

Chapter 5

T3/T4 200 kdyn model

5 T3/T4 200 kdyn model

5.1 Introduction

The commonly used low thoracic level injury model produces SCI which is many segments distant from those segments responsible for processing of sensory input from the forepaws and hindpaws. Clinically, post-SCI pain is generally more frequent at segmental levels close to the injury site (see Siddall et al., 1999a, 2003). There is also some evidence from animal studies to suggest that the pathologic mechanisms of neuropathic pain are more robust in the spinal segments close the SCI site and can be translated into evoked pain behaviours in the corresponding dermatomes. For example, Siddall et al. (1999b) used animals subjected to T9 SCI by weight drop method and investigated levels of c-fos in segments located at graded distance from the injury site after application of tactile stimuli to the back. The data showed that c-fos upregulation was most marked in segments adjacent to the injury site in the allodynic rat. The same conclusion was reached by Crown et al. (2006) where activation of different cascades of MAPKinases (pERK, pP38 and pJNK) and phosphorylation of CREB (downstream of pERK pathway) were most marked at segments just proximal to the SCI level in rats showing signs of mechanical allodynia over the back.

Since the T9 200 kdyn model showed a number of pain-like behaviours using tests applied to the forepaws, allowing investigation of several evoked pain modalities as well as potential measures of spontaneous pain, we next investigated whether moving the injury site closer to the segments processing sensory input from the forepaws would enhance these behavioural signs of pain. Injuries of 200 kdyn were made at the T3/T4 level while sensory input from the forepaws is processed mainly in segments C5 to C7 (Takahashi and Nakajima, 1996).

5.2 Methods

In this chapter, 61 adult male Sprague Dawley rats weighting between 200 and 350 g were used. Eighteen rats were subjected to a 200 kdyn contusion injury at upper thoracic level (i.e. T3/T4 segment) while 9 rats underwent sham surgery. Further normal 34 rats used only for obtaining base line DWB.

5.2.1 Behavioural testing

More details of the behavioural tests used in this chapter are provided in the general methods.

5.2.1.1 BBB rating scale and BBB subscore

The same previously described assays for evaluation of motor recovery were used. The BBB rating scale and BBB subscore (14 injured rats *vs.* 9 shams) were performed preoperatively and then on a weekly basis after the injury.

5.2.1.2 Dynamic weight bearing test

DWB data was obtained from normal animals (n=40) and results from operated animals (11 SCI *vs.* 6 sham rats) at week 6 post injury.

5.2.1.3 Forepaw von Frey and heat tests

Preoperatively, forepaw data for plantar von Frey and heat tests were collected from 14 SCI and 9 sham rats. These tests were subsequently carried out in week 2 post injury and then on a weekly basis until the end of the behavioural study (week 6 PO). The licking response was assessed at the same time in 11 contused and 9 sham animals.

5.2.1.4 Hindpaw von Frey and heat tests

Hindpaw tests were carried out at the same time points as the forepaw testing. Six sham controls were utilized in von Frey and heat tests while 9 SCI animals were used for estimation of 50% mechanical threshold as well as for investigation of heat hyperalgesia. The licking response was investigated in 6 contusion injured and 6 sham operated animals.

5.2.1.5 Footlifting test

Nine SCI and 6 sham animals were tested on a cold plate adjusted to 7.5°C at 4 and 5 weeks after injury. Paw lifting was also investigated over a flat surface maintained at 30°C in 8 SCI and 6 shams during weeks 4, 5 and 6 after contusion and sham operations. SCI animals (n=6 to 8) were further assessed while standing on a plate controlled at 25°C or 35°C in a week 6 PO for purposes of comparison of the spontaneous behaviour (SFL) at

different graded neutral temperatures. Lifting of the paws was also tested on a metal grid (11 SCI rats *vs.* 9 shams) in weeks 4, 5 and 6 PO.

5.2.1.6 Trunk von Frey test

Testing over the back was carried out in an identical way to that described for the 150 kdyn model. Above level tests (+1 cm above injury level) were performed on 14 SCI and 9 sham operated animals while 9 SCI and 9 sham animals were utilized for below level (-5 cm) assessments.

5.2.1.7 PEAP test

Two different types of PEAP tests were carried out in this model: +1 cm above level (in week 4 PO) and cold PEAP (in week 6 PO).

In the above level PEAP test (8 SCI rats *vs.* 6 shams), the mechanical stimuli were applied 1 cm above the injury site and the amount of time spent in the dark as well as number of crossings were recorded.

The Cold PEAP was performed in a slightly different way but according to the same general principles. In this test, white and black compartments were identical in size (16.5 x 16.5 x 10 cm) but the black side was designed to have a cold floor adjusted to 7.5°C and a dark cover whereas the floor of the white compartment was kept at room temperature (23 ± 0.5) with a transparent cover. The two sides were separated by a septum containing an opening, permitting the free movement of the tested animal between the two compartments. To perform this test, each animal was placed on the dark side and one min was allowed for acclimation. Then the amount of time spent by the animal on the dark side was measured during 10 min (first 5 min epoch and total 10 min epoch). The total time spent in the noxious compartment was expressed as a percentage of the total evaluation time (i.e. 5 and 10 min). Additionally, crossings were also recorded for the both testing epochs. Data for this test were collected from 6 SCI and 6 sham animals.

5.2.1.8 Cold avoidance test

A new assay called the cold avoidance test (CAT) was performed in week 6 PO. This paradigm was designed to quantify the cold sensitivity of the forepaws. To perform this test, each animal in the two groups (7 SCI *vs.* 6 shams) was evaluated over the cold plate

adjusted to 7.5°C but instead of counting FL, the amount of time when both forepaws were held off the cold floor was measured. Three sessions were run for each animal and each session was of 5 min duration but the avoidance time was assessed from the second min, so that the actual evaluation time was 4 min. Between sessions, the tested animal was returned to its original home cage in order to restore paw temperatures. Finally, the mean avoidance time was calculated and expressed as percentages (% of CAT) of the total evaluation time.

5.3 Results

5.3.1 Contusion parameters

The weights of all animals at the time of SCI are shown in Fig. 5-1A. The contusion injuries were made using the IH instrument. Scatter plots in Fig. 5-1B show the actual force delivered in kdyn. Most of the impacts were close to the target force of 200 kdyn and resulted in displacements of 934 to 1658 µm in spinal tissue of T3/T4 segments (Fig. 5-1C). Any animals for which there were indications of impact with bone were omitted.

5.3.2 Motor evaluation

Assessment of the effect of the injury on the movement capacity of the animals and the pattern of recovery of movement was carried out using the BBB locomotor rating scheme (Basso et al., 1995).

Fig. 5-2A shows that all animals had normal scores of 21 before the operations. After the operations, the motor activity of the hindquarters was severely affected in the contused group but remained unaffected in sham animals. At one week post the surgery, SCI animals were already showing BBB scores of about 9 (corresponding to *plantar placement of paw with weight support in stance only or dorsal steps with occasional, frequent, or consistent weight support*). This improved gradually during the subsequent weeks and most SCI animals displayed *plantar steps with consistent weight support and consistent forelimb/hindlimb coordination* (BBB score of about 14) in week 6 after SCI. There was a high degree of consistency in the rating for each of the animals. The subscore data shows marked and progressive improvement between 1 and 6 weeks after injury. These subscores reflect mainly improvement in toe clearance, tail posture and body stability. Paw angle never became normal in these animals (Fig. 5-2B). The sham surgery had no effect on the locomotor subcores of animals in this group even at one week after the surgery.

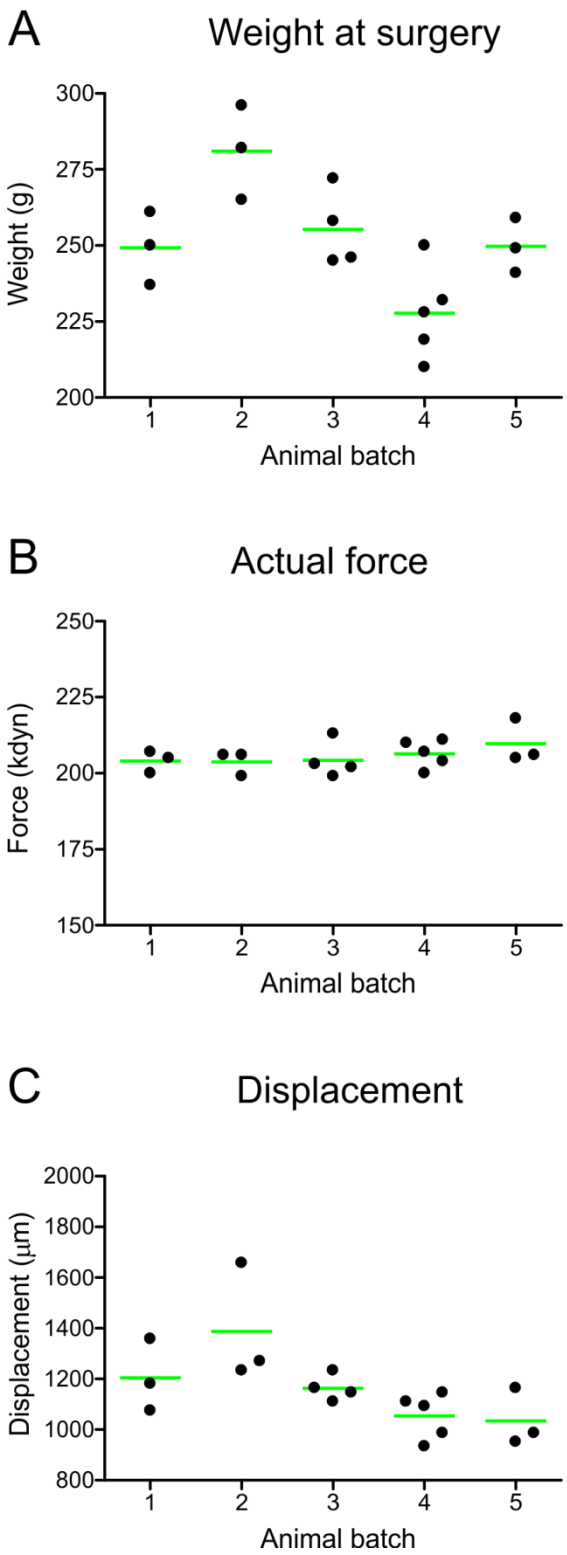

Fig. 5-1. Contusion injury data. A weight of animals at the surgery. **B** actual force delivered by the impactor and measured by the devise during the impact. **C** actual displacement of the impactor i.e. distance that the impactor tip travelled from the surface of the spinal cord into the cord before withdrawing. The graphs show scatter plots for the values measured for each of 18 animals subjected to contusion injury.

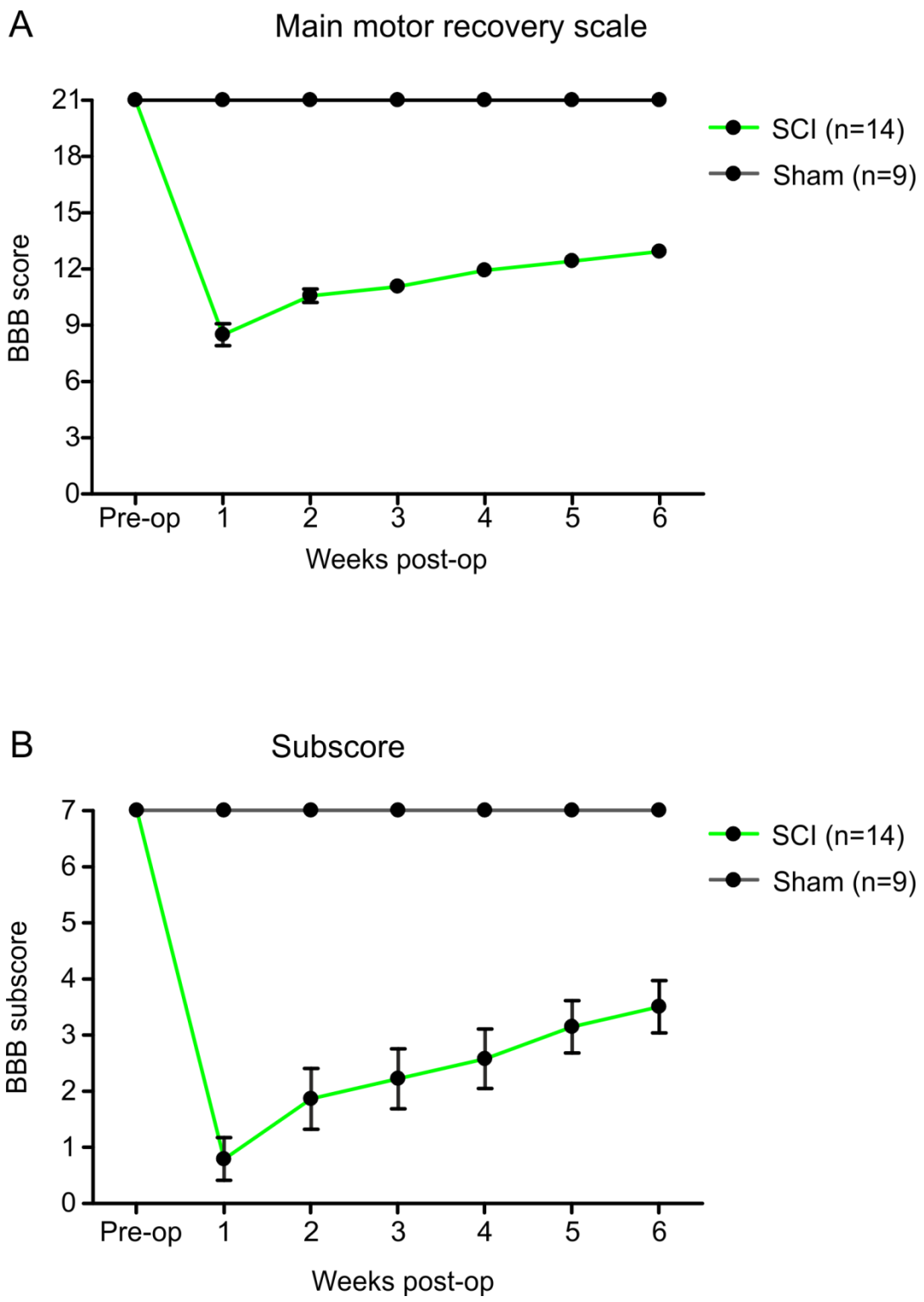

Fig. 5-2. BBB rating scale and subscore for locomotor activity of SCI and sham animals over the course of the study. A shows the main motor recovery (i.e. BBB rating scale) from preoperative and weekly post operative tests performed on T3/T4 200 kdyn contusion injured animals (n=14) and sham operated control animals (n=9). **B** shows the motor recovery subscore for the same animals over the same time period. The values shown are mean ± SE.

5.3.3 Dynamic weight bearing test

A dynamic weight-bearing test was used to evaluate the effect of SCI on the postural support of the animals as indicated by the distribution of body weight on the four paws. The results from this test are shown in (Fig. 5-3). In normal animals (n=40), 33% of the total body weight is distributed to the forelimbs (Fig. 5-3A) with the hindlimbs bearing around 67% of body weight (Fig. 5-3B). In other words, in naive rats, body weight is distributed more on the hindpaws than the forepaws. At week 6 after the surgery, the weight distribution pattern in sham controls (n=6) was not statistically different from that seen in normal animals *(P>0.05. one sample test)*. In SCI animals (n=11) there was a clear redistribution of weight, with more of the weight being distributed to the forepaws than in naive animals at week 6 after the injury (an increase from 33% to 40%) and a concomitant reduction in the weight distributed to the hindpaws (a decrease from 67% to 60%; Fig. 5-3C and D). These changes were statistically different in comparison to both normal animals *(P<0.05, one sample t test)* and sham animals *(P<0.05, unpaired t-test with Welch's correction).*In comparison to T9 200 kdyn animals, a less deficit in posture equilibrium was noticed in T4 200 kdyn animals despite using of the same contusion force but at different thoracic levels.

5.3.4 Responses of forepaws

5.3.4.1 Plantar von Frey test

The effect of the injury on the withdrawal response to tactile stimuli (vF hairs) applied to the forepaws is shown in Fig. 5-4. A decrease in the threshold for eliciting withdrawal was evident and consistent at the first test session (2 weeks) after the injury. The threshold continued to decrease in subsequent weeks reaching a maximum reduction at week 5 and plateaued at week 6. The difference in threshold between SCI and sham animals was statistically significant from week 2 to 6*)*. Sham operated animals showed little changes in threshold in the same tests. There was no statistical difference between preoperative data and postoperative data for sham animals at any time point *(P>0.05, Tukey's post-hoc test)*. In comparison to T9 200 kdyn animals, rats subjected to 200 kdyn at T3/T4 developed a faster tactile sensitivity (significant difference at weeks 2; *P<0.001, Bonferroni post-hoc test*). These observations are indicative for development of tactile allodynia in the forepaws of SCI but not sham animals.

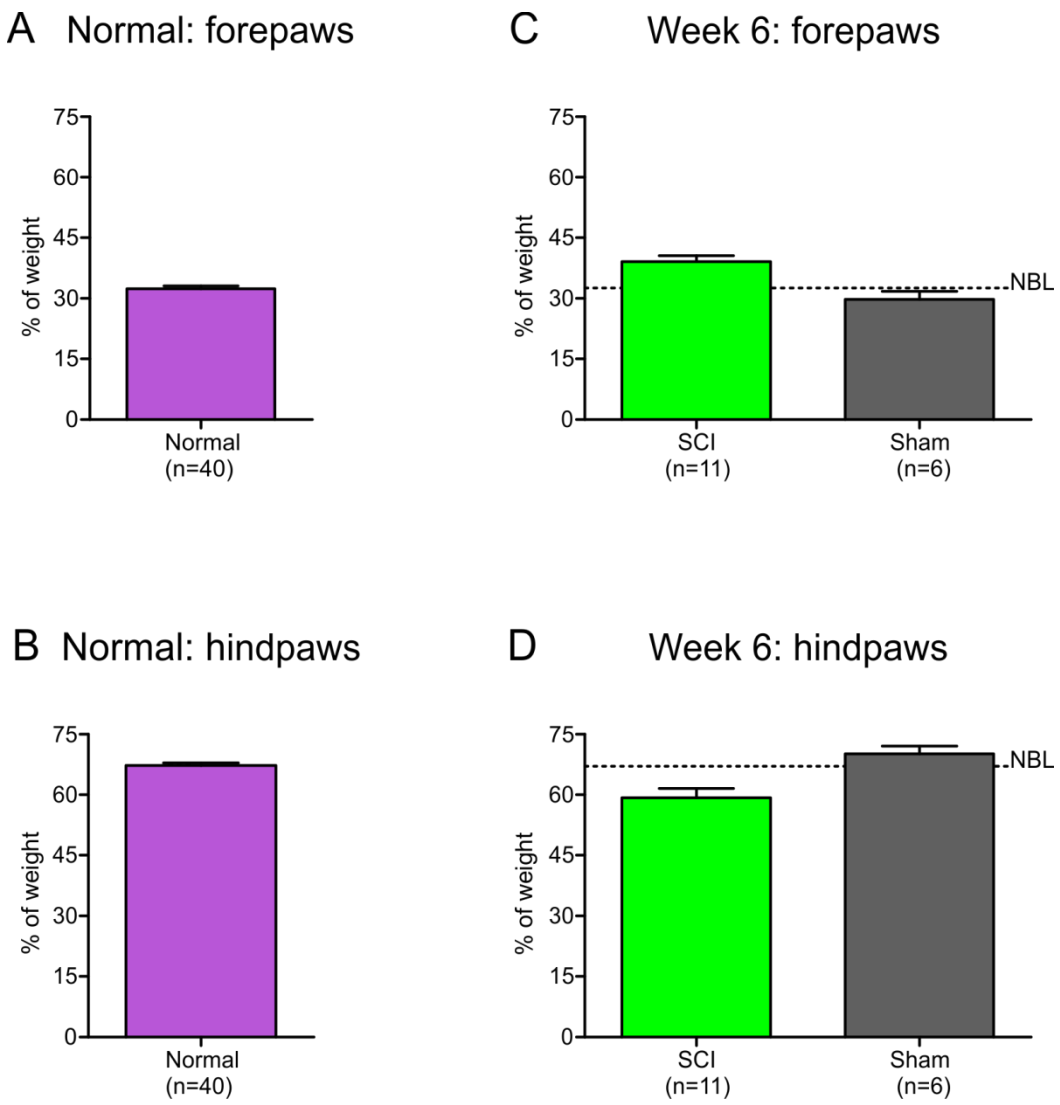

Fig. 5-3. DWB data collected from forepaws and hindpaws. In normal animals (n=40), **A** and **B** show the percentage of body weight distributed to the forepaws and hindpaws, respectively. **C** and **D** show the percentage of body weight distributed to the forepaws and hindpaws, respectively, in 11 contusion injured and 6 sham operated animals at 6 weeks post SCI. The dotted lines (normal base line "NBL") represent the percentage of paw weight distribution averaged in naive animals (n=40). All bars represent mean ± SE.

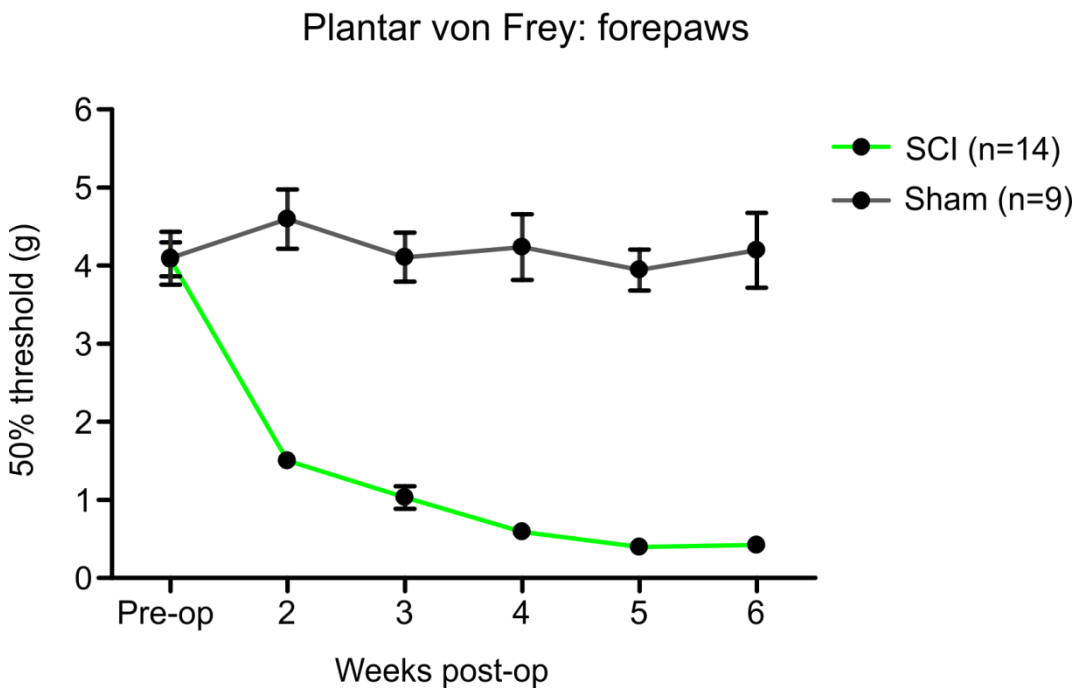

Fig. 5-4. Plantar von Frey testing of the forepaws. The graph shows the 50% threshold for the force of vF filament eliciting a withdrawal response on application to the plantar surface of the forepaws. The test was carried out preoperatively and at 2, 3, 4, 5 and 6 weeks after the surgery. Data for both forepaws of each animal was averaged and the plotted data points represent the mean ± SE for contusion injured (n=14) and sham (n=9) operated groups.

5.3.4.2 Plantar heat test

Responses to the plantar heat test carried out in the forepaws are shown in Fig. 5-5. T3/T4 200 kdyn animals showed an immediate (since the first test session in week 2 after SCI) drop in the withdrawal response latencies to heat stimuli which was statistically different in comparison to sham response latencies *(P<0.001, Bonferroni post-hoc test)*. In addition, heat hyperalgesia was more marked in T3/T4 200 kdyn animals when compared to rats subjected to T9 200 kdyn at week 2 *(P<0.01, Bonferroni post-hoc test)* and to less extent at week 3 *(P<0.05, Bonferroni post-hoc test)* after SCI. The reduction in latencies continued in subsequent testing sessions to be maximal at 6 weeks (Fig. 5-5A). The response latency for the sham group of animals did not change in the same test period *(*preoperative data *vs*. postoperative data at each time point: *P>0.05, Tukey's post-hoc test)*. In addition to measuring response latency to the heat stimulus, animals were also carefully observed to determine whether or not they licked the paw to which the heat stimulus had been applied. The frequency with which a licking response was seen following withdrawal is shown in Fig. 5-5B. Before the injury, animals licked the stimulated paw following 7 or 8 of the 10 stimulus applications (5 to each forepaw). The sham operations had no effect on the licking response, which was seen with a similar frequency in all subsequent test sessions with no significant difference at any time point (*P>0.05* for each group *vs*. the base line, *one sample t-test*). On the other hand, the SCI group displayed slightly fewer licking responses to heat stimuli during the first two test sessions which may be related to postural changes (i.e. weight shifted to the forepaws) after SCI. During the remaining period of the study, licking returned to the normal base line. These observations are indicative of heat hyperalgesia in the forepaws of SCI but not sham animals and the consistent presence of licking response indicates supraspinal processing of the increased responsiveness to thermal stimuli.

5.3.5 Responses of hindpaws

5.3.5.1 Plantar von Frey test

Contusion SCI had a similar effect on the withdrawal response of the hindlimb to tactile stimuli as for stimuli applied to the forepaws as shown in Fig. 5-6. Both the time course and the percentage reduction in threshold from preoperative values showed a closely similar pattern. The decrease in threshold in injured animals was very significant from week 2 in comparison to shams (*P < 0.001, Bonferroni post-hoc test*) but not to T9 200 kdyn animals *(P >0.05, Bonferroni post-hoc test)*.

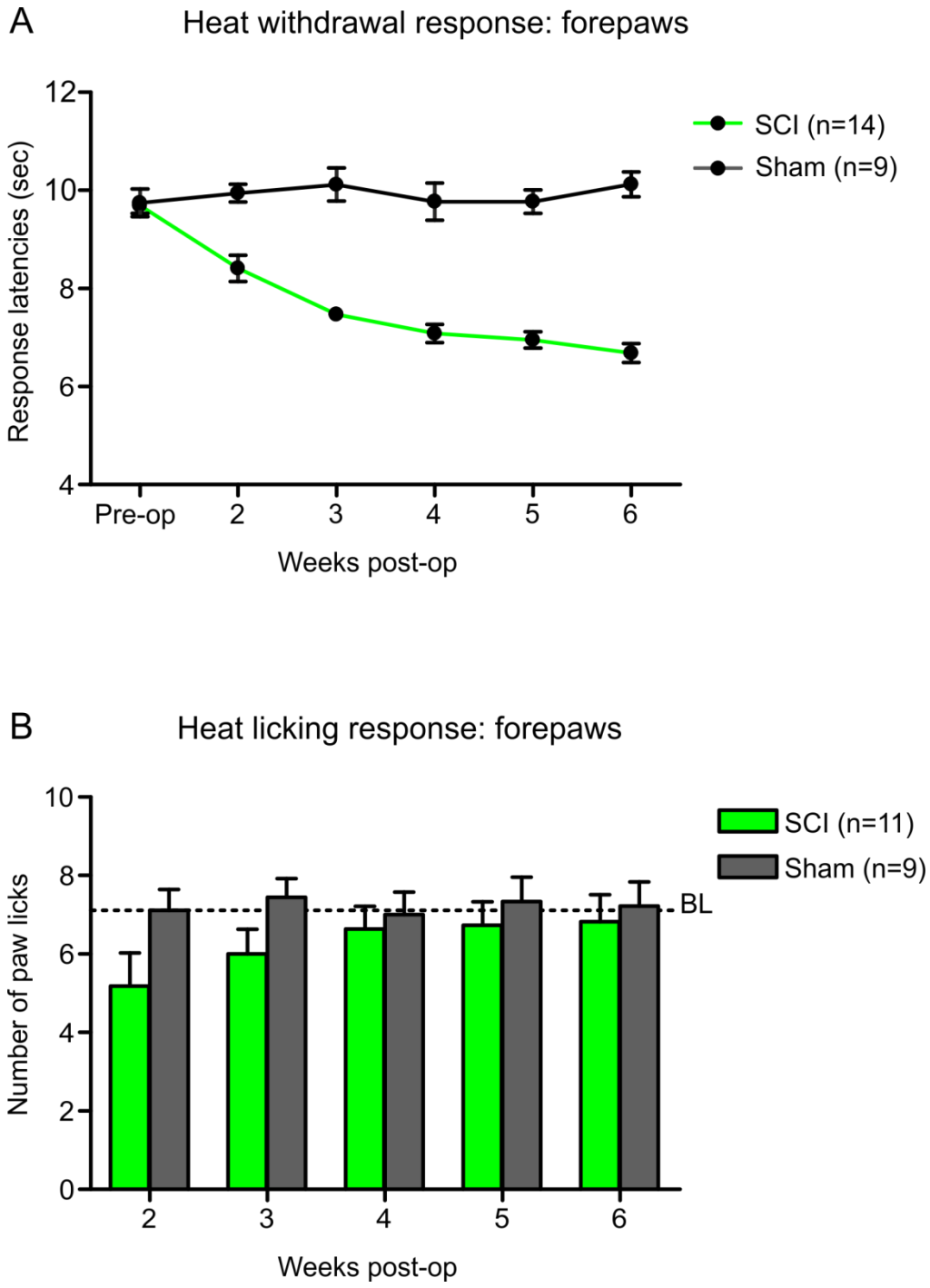

Fig. 5-5. Behavioural responses to heat stimuli applied to the forepaws. A graph showing latencies for withdrawal of the forepaws in response to radiant heat applied to the plantar surface. Data for both forepaws of each animal was averaged for SCI (n=14) and sham (n=9) groups. **B** bars showing the frequency with which licking was seen following 10 applications of heat to the plantar surface of the forepaws. Tests were performed on both paws and data averaged for each injured (n=11) and sham (n=9) group. The dotted line (base line "BL") shows the average incidence of licking observed in all animals before the injury or sham surgery. The observations in A and B were made preoperatively and at 2, 3, 4, 5 and 6 weeks after the injury. All observations are shown as the mean ± SE.

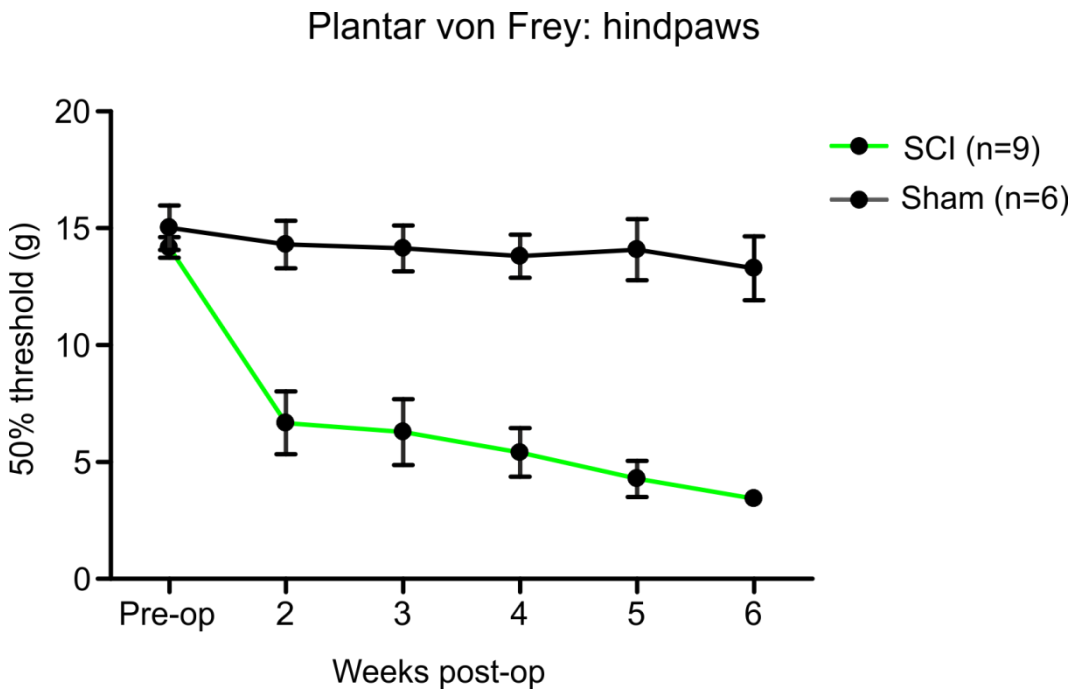

Fig. 5-6. Plantar von Frey testing of the hindpaws. The graph shows the 50% threshold for the force of vF filament eliciting a withdrawal response on application to the plantar surface of the hindpaws. The test was carried out preoperatively and at 2, 3, 4, 5 and 6 weeks after the surgery. Data for both hindpaws of each animal was averaged and the plotted data points represent the mean ± SE for contusion injured (n=9) and sham (n=6) operated groups.

5.3.5.2 Plantar heat test

The effect of SCI on the hindpaw withdrawal latencies was similar to that seen for the forepaws in terms of the onset and degree of reduction (Fig. 5-7A). A marked reduction was evident from the first test following injury at 2 weeks *(*shams *vs.* SCI: *P<0.001, Bonferroni post-hoc test)*, becoming maximal by 4 to 5 weeks and plateauing at week 6. Sham operated animals showed little changes in threshold in the same test period. There was no statistical difference between preoperative data and postoperative data for sham animals at any time point *(P>0.05, Tukey's post-hoc test)*. The most significant difference seen between the forepaws and the hindpaws was in the licking response following the application of heat stimuli (Fig. 5-7B). When heat stimuli were applied preoperatively to the hindpaw, licking of the paw was seen with a similar frequency to that for testing of the forepaw. However, when tested postoperatively, although the sham animals continued to show a licking response with a similar frequency throughout the remainder of the test period, the licking response was almost abolished in the SCI animals. In comparison to T9 200 kdyn injury, T3/T4 200 kdyn animals showed approximately similar data *(*no significant difference between the two models when analysed using *Bonferroni post-hoc test)*.

5.3.6 Forepaw lifting data

5.3.6.1 Responses on the cold plate

Data from this test was collected at two time points, weeks 4 and 5 but not week 6 PO. This was because the animals developed a strategy for minimising contact of the forepaws with the cold plate. This involved placing their forepaws on the wall of the enclosure instead of frequent lifting of the paws and paw lifting could not therefore be assessed at this time point. This required a change in testing strategy (see CAT).

When observed on the cold plate maintained at a temperature (7.5°C), both SCI and sham animals showed lifting of the forepaws from the platform which, on some occasions, was associated with aversive behaviours such as licking and shaking of the paw, indicating the noxious nature of this temperature. The incidence of FL and the difference between contusion injured and sham operated animals was highly consistent and very marked when tested at 4 weeks and 5 weeks after the contusion or sham surgeries. These were statistically different at the two testing time points *(P<0.0001, unpaired t-test with Welch's correction)* (Fig. 5-8A). In each group, there was no significant difference in FL between

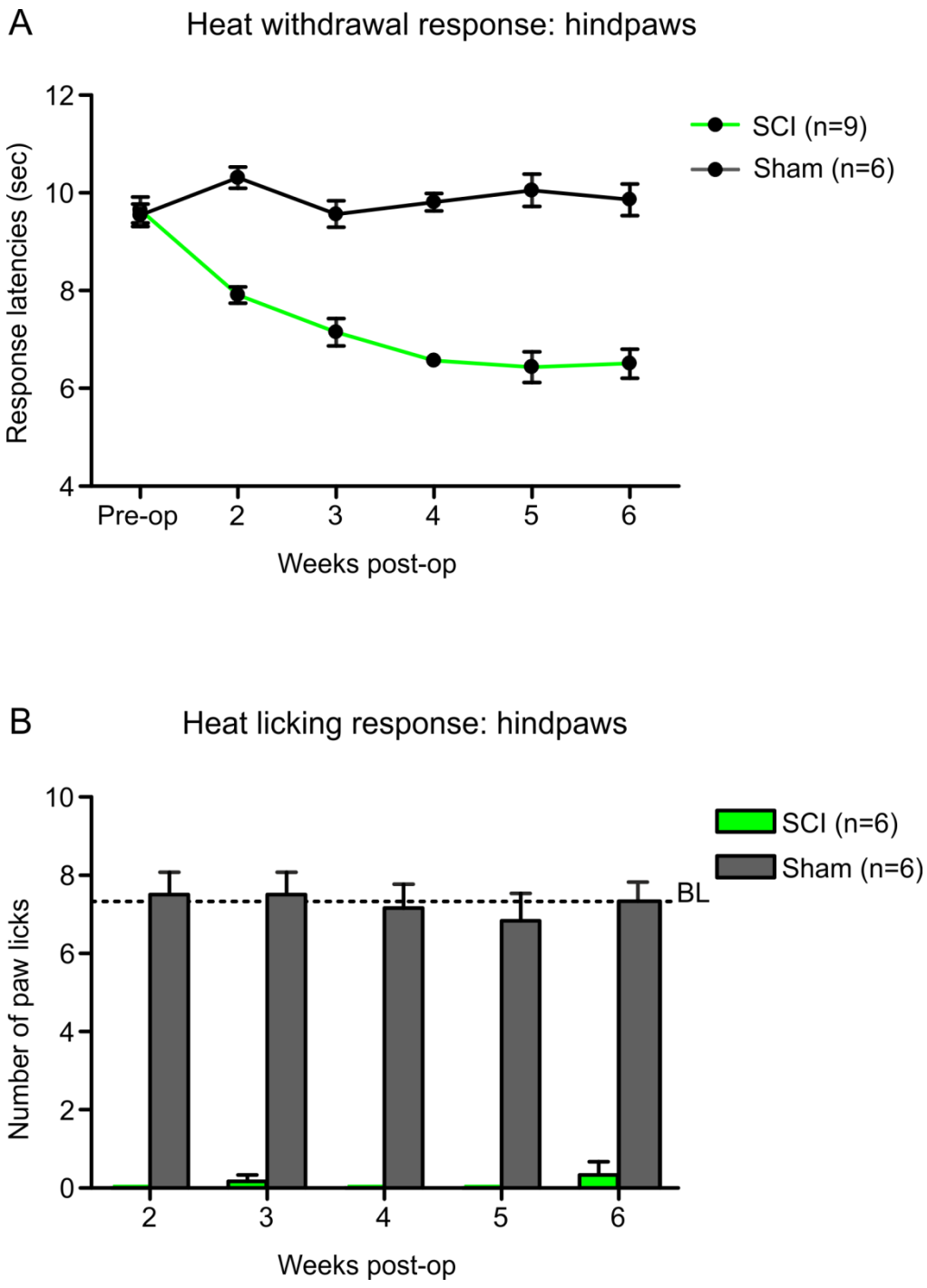

Fig. 5-7. Behavioural responses to heat stimuli applied to the hindpaws. A graph showing latencies for withdrawal of the hindpaws in response to radiant heat applied to the plantar surface. Data for both hindpaws of each animal was averaged for SCI (n=9) and sham (n=6) groups. **B** bars showing the frequency with which licking was seen following 10 applications of heat to the plantar surface of the hindpaws. Tests were performed on both paws and data averaged for each injured (n=6) and sham (n=6) group. The dotted line (base line "BL") shows the average incidence of licking observed in all animals before the injury or sham surgery. The observations in A and B were made preoperatively and at 2, 3, 4, 5 and 6 weeks after the injury. All observations are shown as the mean ± SE.

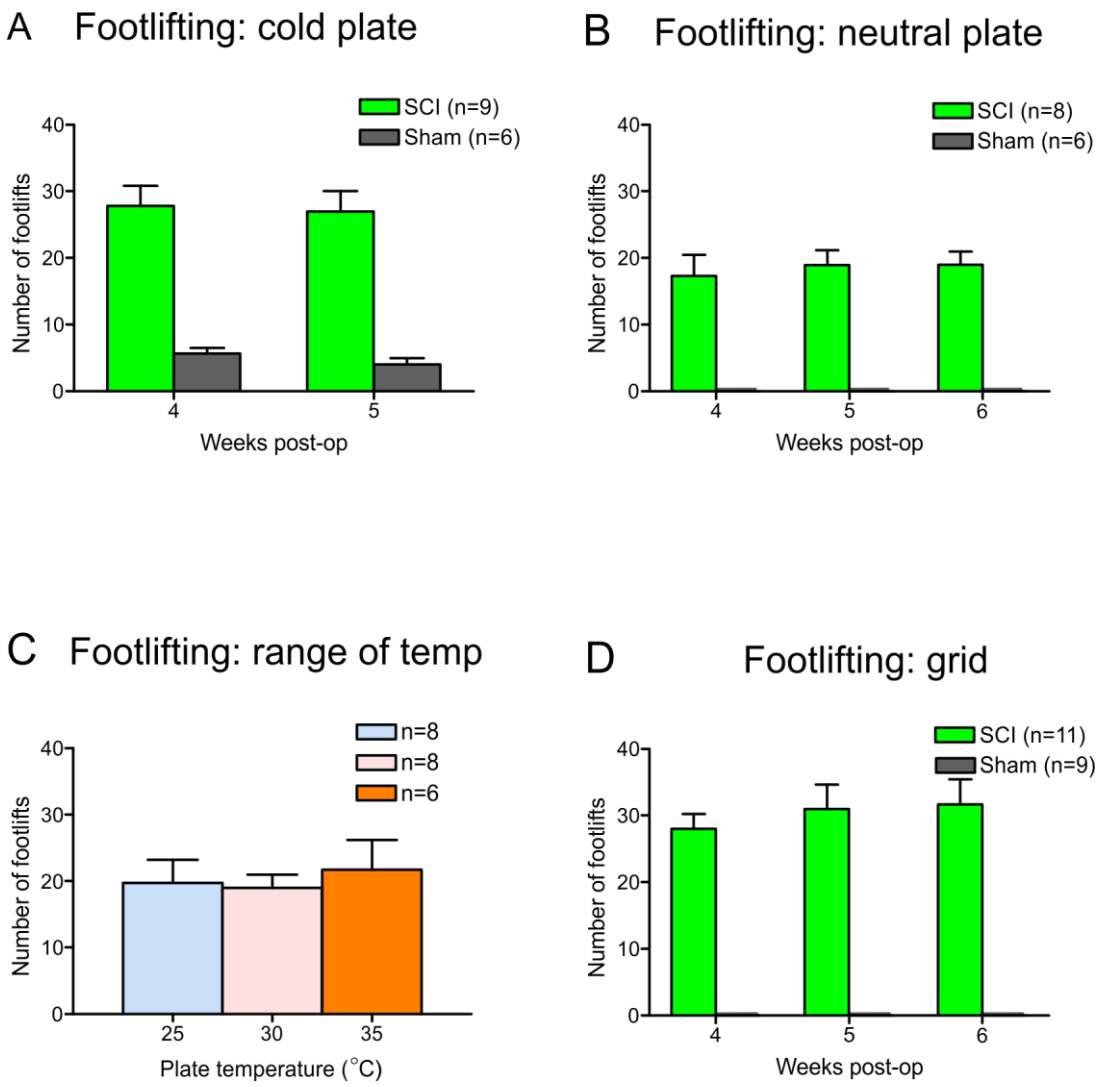

Fig. 5-8. Observations on the frequency of forepaw lifts on different surfaces. Each of the graphs (A-D) show the number of footlifting episodes (averaged for both forelimbs) observed during a 4 min period. **A** shows results for 9 contusion injured and 6 sham operated animals standing on a cold plate adjusted to 7.5°C. **B** shows data for 8 SCI rats and 6 shams standing on a neutral plate at 30°C. The histograms in **C** indicate the numbers of footlifting episodes observed while SCI animals stood on a neutral plate adjusted to 25°C (n=8) or 30°C (n=8) or 35°C (n=6). **D** indicates results collected from 11 SCI and 9 sham rats stood on a mesh grid. A comparison between SFL collected at different neutral temperatures (°C) was performed only in week 6 PO. All histograms represent means ± SE.

the different time points *(P>0.05, paired t-test)*. These observations indicate development of cold hyperalgesia in the forepaws of SCI animals which was more marked than that seen in T9 200 kdyn animals at weeks 4 and 5 PO *(P<0.01, Bonferroni post-hoc test)*.

5.3.6.2 Responses on the thermally neutral plates

Contusion injured animals also showed frequent FL when observed on a flat thermally neutral plate maintained at 30°C (Fig. 5-8B) and the incidence was approximately 100% more than that counted in T9 150 kdyn and 200 kdyn animals. Sham animals showed no FL on the neutral plate. These observations were seen consistently without significant differences between 4, 5 and 6 week time points *(P>0.05, Tukey's post-hoc test)*. At week 6 after the surgery, animals were also tested at 25°C and 35°C (Fig. 5-8C). SCI animals showed a similar degree of FL to that seen at 30°C (no significant difference at 25°C *vs.* 30°C *vs.* 35°C: *P>0.05, Tukey-Kramer post-hoc test)*. FL observed at 25°C, 30°C and 35°C are suggestive of spontaneous pain in the forepaws.

5.3.6.3 Responses on the metal grid

When animals were observed on the metal grid which was used for carrying out the von Frey testing, the contusion injured animals showed a high and similar incidence of FL at weeks 4, 5 and 6. These observations were completely absent in the sham controls (Fig. 5-8D). This suggests that mechanical allodynia leads to discomfort resulting from the force exerted by the narrow metal rungs of the grid on the forepaws. The response to the mechanical stimuli of the grid presumably occurs against the background of spontaneous FL seen on a thermally neutrally flat surface.

5.3.7 Hindpaw lifting data

The same set of observations as described above for the forepaws were made for the hindpaws.

5.3.7.1 Responses on the cold plate

When observed on the cold plate maintained at a temperature (7.5°C), both SCI and sham animals showed occasional lifting of the hindpaws from the platform (Fig. 5-9A). The incidence was similar to that for forepaw lifting in sham animals. However, whereas the incidence of forepaw lifting was much higher in SCI animals compared to shams, there

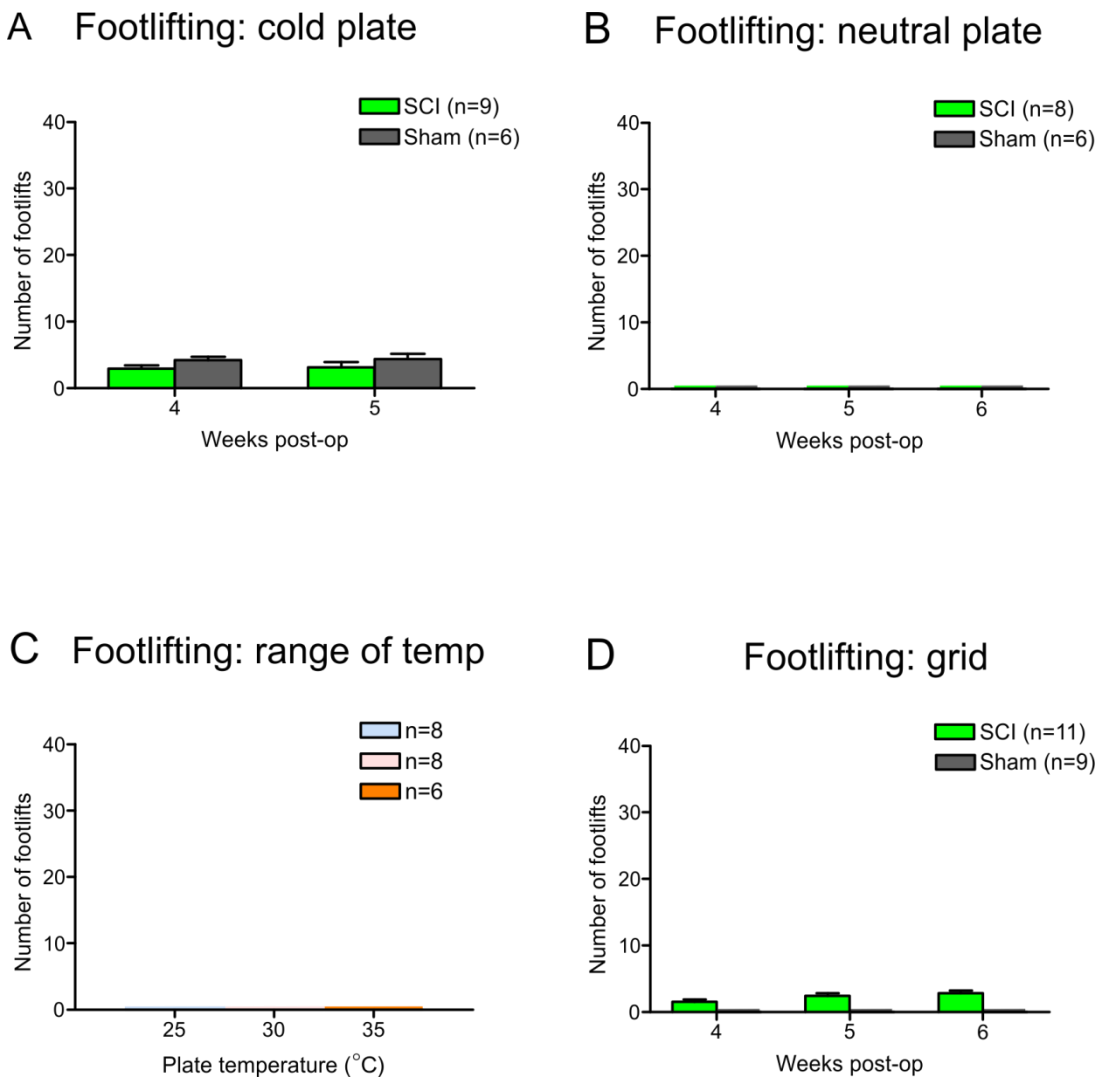

Fig. 5-9. Observations on the frequency of hindpaw lifts on different floors. Each of the graphs (A-D) show the number of footlifting episodes (averaged for both hindlimbs) observed during a 4 min period. **A** shows results for 9 contusion injured and 6 sham operated animals standing on a cold plate adjusted to 7.5°C. **B** shows data for 8 SCI rats and 6 shams standing on a neutral plate at 30°C. The histograms in **C** indicate the numbers of footlifting episodes observed while SCI animals stood on a neutral plate adjusted to 25°C (n=8) or 30°C (n=8) or 35°C (n=6). **D** indicates results collected from 11 SCI and 9 sham rats stood on a mesh grid. A comparison between SFL collected at different neutral temperatures (°C) was performed only in week 6 PO. All histograms represent means ± SE.

was no difference in the incidence of hindpaw lifting between SCI and sham animals *(P>0.05, unpaired t-test with Welch's correction)*. These observations suggest that, unlike in the forepaws, cold hyperalgesia does not develop in hindpaws of SCI animals, or that if the spinal mechanisms occur, then the associated changes in neural signalling are not conveyed supraspinally and do not therefore lead to altered behaviour.

5.3.7.2 Responses on the thermally neutral plates

When the hindpaws were observed with the animals on thermally neutral plates (maintained at 25°C or 30°C or 35°C), no evidence of FL was seen in either SCI or sham groups in any of the sessions (Fig. 5-9B and C). This is in contrast to the FL behaviour seen in the forepaw of SCI animals on the same surface. These observations suggest that whereas there may be spontaneous pain in the forepaws, it is absent in the hindpaws.

5.3.7.3 Responses on the metal grid

When animals were observed on the metal grid that was used for carrying out the von Frey testing, the contusion injured animals showed occasional lifting of the hindpaws, but this was much less frequent than for the forepaws in the same test session (Fig. 5-9D). The incidence of this FL was very consistent for test sessions at 4 weeks, 5 weeks and 6 weeks post injury. At no point was hindpaw lifting seen in sham animals. It is possible that these responses reflect increased spinal reflex sensitivity and responses to the force distribution of the narrow metal rungs of the grid representing a tactile stimulus which in the SCI animals with hyperreflexia is sufficient to result in a flexion response. The same stimuli may be below threshold for producing such responses in the sham animals which have normal tactile sensitivity and normal reflex excitability.

5.3.8 Trunk von Frey test

5.3.8.1 Responses to above level stimuli

The incidence of responses to stimuli applied at +1 cm (above level) is shown in Fig. 5-10. In the first test session after injury (one week), the incidence of any of the recognised types of responses to the stimuli was nearly tripled and continued to increase in subsequent weeks, reaching about 9 responses out of the 10 applications at 3 and 4 weeks (Fig. 5-10A). The incidence remained at this elevated level for the subsequent two weeks of the testing period, though some animals reached the maximal (10/10) response level so that

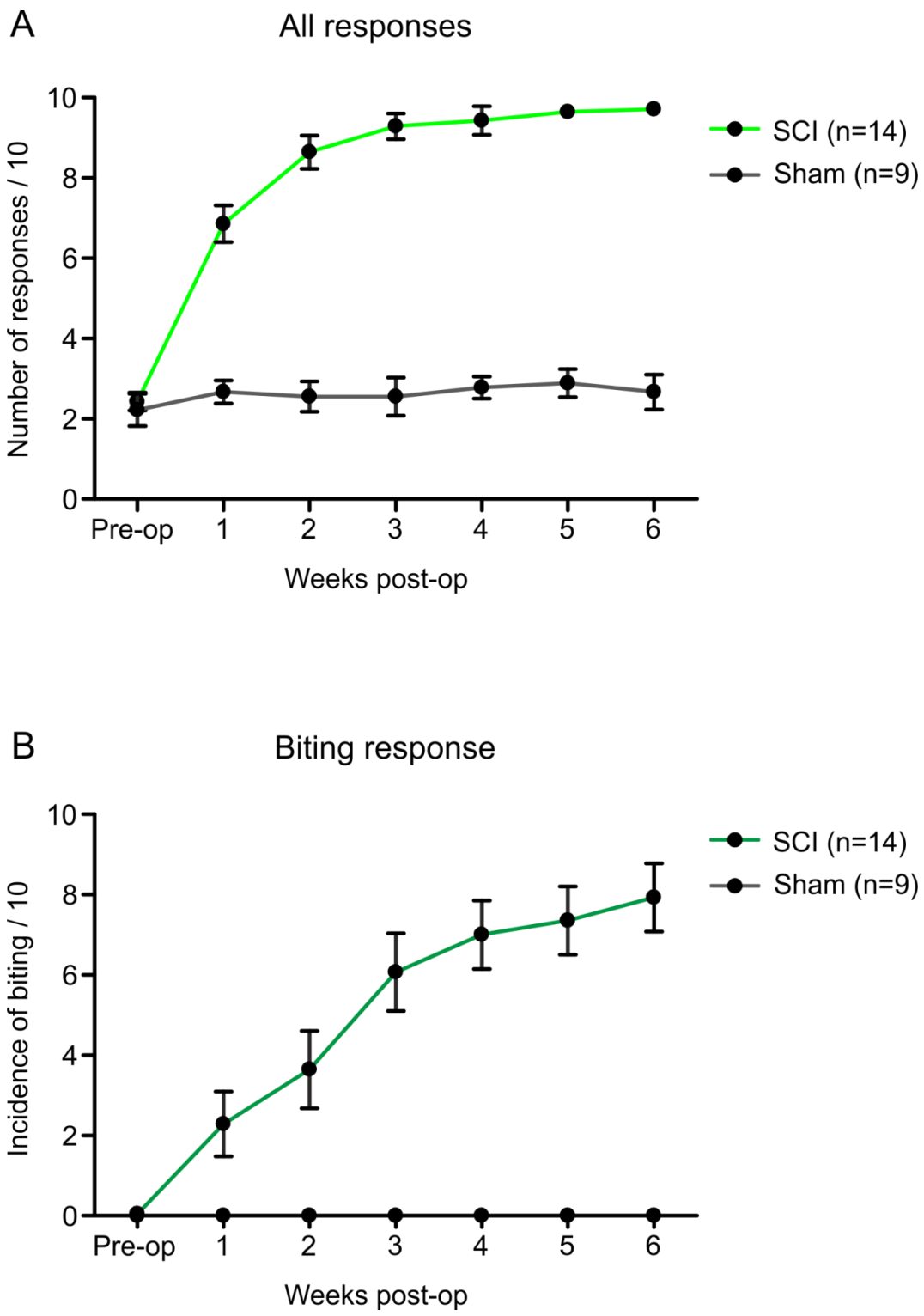

Fig. 5-10. Behavioural testing using stimuli applied at +1 cm (above level) over the back. The test was performed preoperatively and weekly from 1 to 6 weeks after the injury. **A** graph showing the total incidence of any of the recognised types of response to tactile stimuli applied over the back. **B** shows just the incidence of a biting responses. The plots show the mean ± SE for responses observed in SCI (n=14) and sham (n=9) groups.

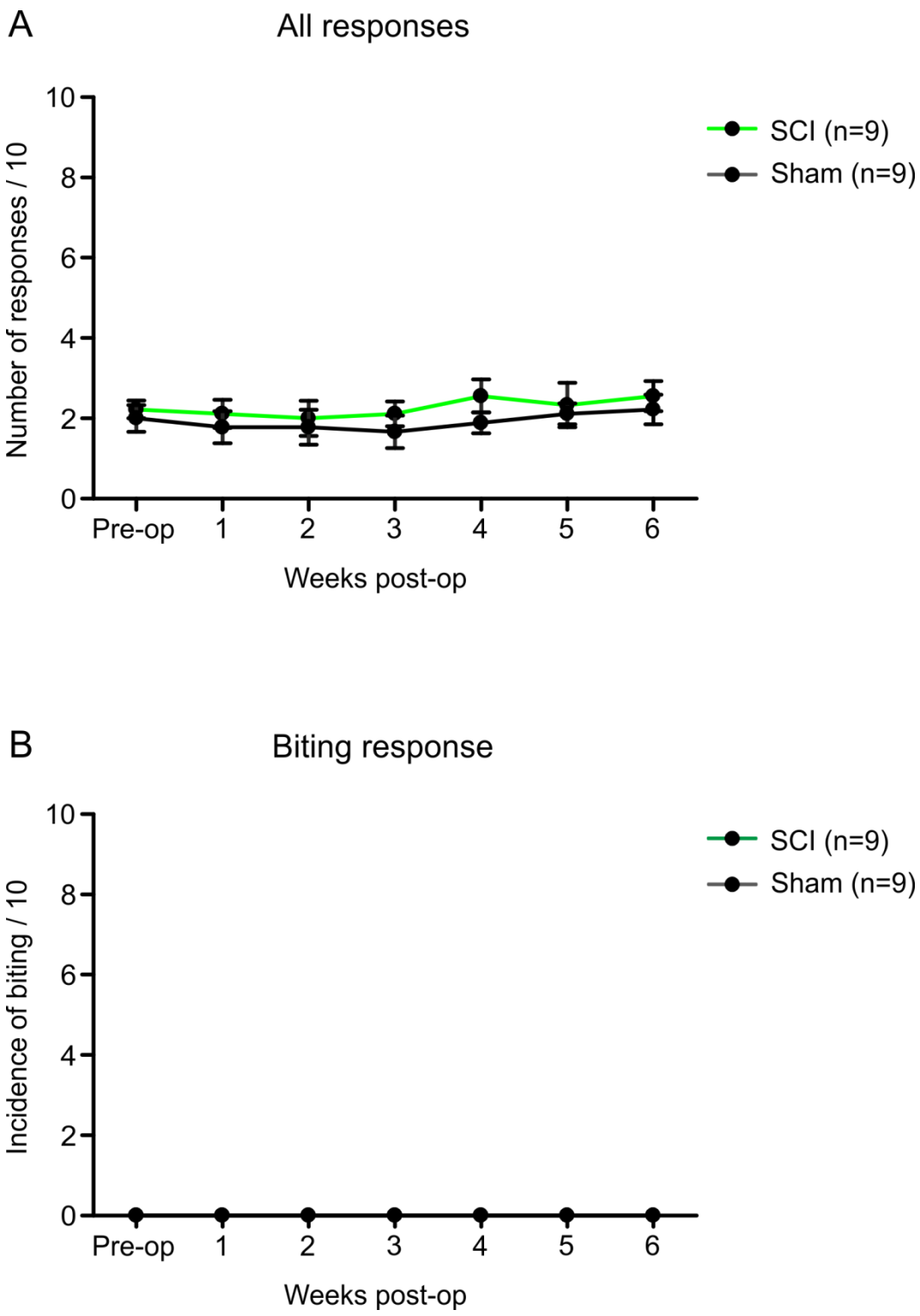

Fig. 5-11. Behavioural testing using stimuli applied at -5 cm (below level) over the back. The test was performed preoperatively and weekly from 1 to and 6 weeks after the injury. **A** graph showing the total incidence of any of the recognised types of response to tactile stimuli applied over the back. **B** shows just the incidence of a biting responses. The plots show the mean ± SE for responses observed in SCI (n=9) and sham (n=9) groups.

this apparent plateauing of the response may reflect saturation of the test. The incidence of attempted biting of the filament, a behaviour rarely if ever seen before injury, also increased progressively following injury (Fig. 5-10B). This behaviour continued to increase throughout the 6 weeks post injury test period, becoming the most common type of response by week 4. In comparison, the sham animals showed little change in sensitivity to tactile stimuli following the surgery *(*non significant: *P>0.05, Tukey's post-hoc test)*. These observations are a clear indication for the development of tactile allodynia over the back to +1 cm (above level) stimuli.

5.3.8.2 Responses to below level stimuli

The results obtained when applying stimuli to the back at -5 cm (below level) are shown in Fig. 5-11. In contrast to the results obtained in this model when applying stimuli processed above level, SCI had no effect on the response to stimuli applied below level (Fig. 5-11A and B). The progressive increase in sensitivity seen at the more rostral stimulus location was not observed here but neither did the injury abolish the baseline response. Animals continued to respond to approximately 2 out of the 10 stimulus applications throughout the duration of the testing. The incidence of a biting response was also unchanged after surgery.

5.3.9 PEAP test

Two versions of PEAP tests were performed in this model. In the first PEAP test (in week 4 PO), tactile stimuli were applied to the dorsolateral trunk at +1 cm above the injury while in the second version, cold PEAP test was carried out in week 6 after the surgery.

5.3.9.1 Above level PEAP test

The results obtained when applying stimuli to the back above the injury level are shown in Fig 2-50. Sham operated animals (n=6) showed a clear preference to the dark side of the cage spending an average of 85% and 75% of the time in the black compartment during the first 10 min and total 30 min of the test, respectively (Fig. 5-12A). Exploratory behaviour i.e. frequency of crossing between the light and dark halves of the cage were consistent over time with the number of crossings in the 30 minute test period as a whole was about 12 (Fig. 5-12B).

On the other hand, under the same conditions and with the same stimuli, SCI animals (n=8) spent less time than the sham animals (n=6) on the dark side of the cage and this behaviour was similar in the first 10 minutes of testing as in the whole of the 30 minute test period (Fig. 5-12A). On average, the SCI animals spent 25% of their time on the dark side compared to 75% for the sham operated animals over the 30 minutes of the test, a difference which is highly significant *(P<0.001, Bonferroni post-hoc test)*. In addition, contusion injured animals displayed more crossing activity between the light and dark sides than the sham animals (Fig. 5-12B) which was statistically significant at 10 min as well as 30 min testing epochs *(P<0.05, unpaired t-test with Welch's correction)*. These observations suggest that the SCI animals perceived the stimuli as more unpleasant than sham animals and that the stimuli therefore result in a greater modification of the behaviour of the SCI animals than the shams. For this modification in behaviour to occur, neural activity resulting from the stimuli must be processed at a conscious level.

5.3.9.2 Cold PEAP test

The results obtained from cold PEAP are shown in Fig. 5-13. Sham operated animals (n=6) showed a clear preference for the dark side of the cage spending an average of 68% and 75% of the time in the black compartment (with cold floor) during the first 5 min and total 10 min of the test, respectively (Fig. 5-13A). Exploratory behaviour i.e. frequency of crossing between the light and dark halves of the cage were consistent over time with the number of crossings in the 30 minute test period as a whole was about 8 (Fig. 5-13B).

Under the same testing conditions, SCI animals (n=6) spent less time than the sham animals on the dark side of the cage and this behaviour was similar in the first 10 minutes of testing as in the whole of the 30 minute test period (Fig. 5-13A). On average, the SCI animals spent 43.5% of their time on the dark side compared to 75% for the sham operated animals over the 30 minutes of the test, a difference which was significant *(P<0.01, Bonferroni post-hoc test)*. There was no difference in the frequency with which SCI and sham animals crossed between the light and dark sides of the cage *(P>0.05, unpaired t-test*) (Fig. 5-13B). These observations suggest that the SCI animals perceived the cold stimuli as more painful than sham animals and that the stimuli therefore result in a greater modification of the behaviour of the SCI animals than the shams. For this modification in behaviour to occur, neural activity resulting from the stimuli must be processed at a conscious level. According to data collected from FL test performed on the same plate

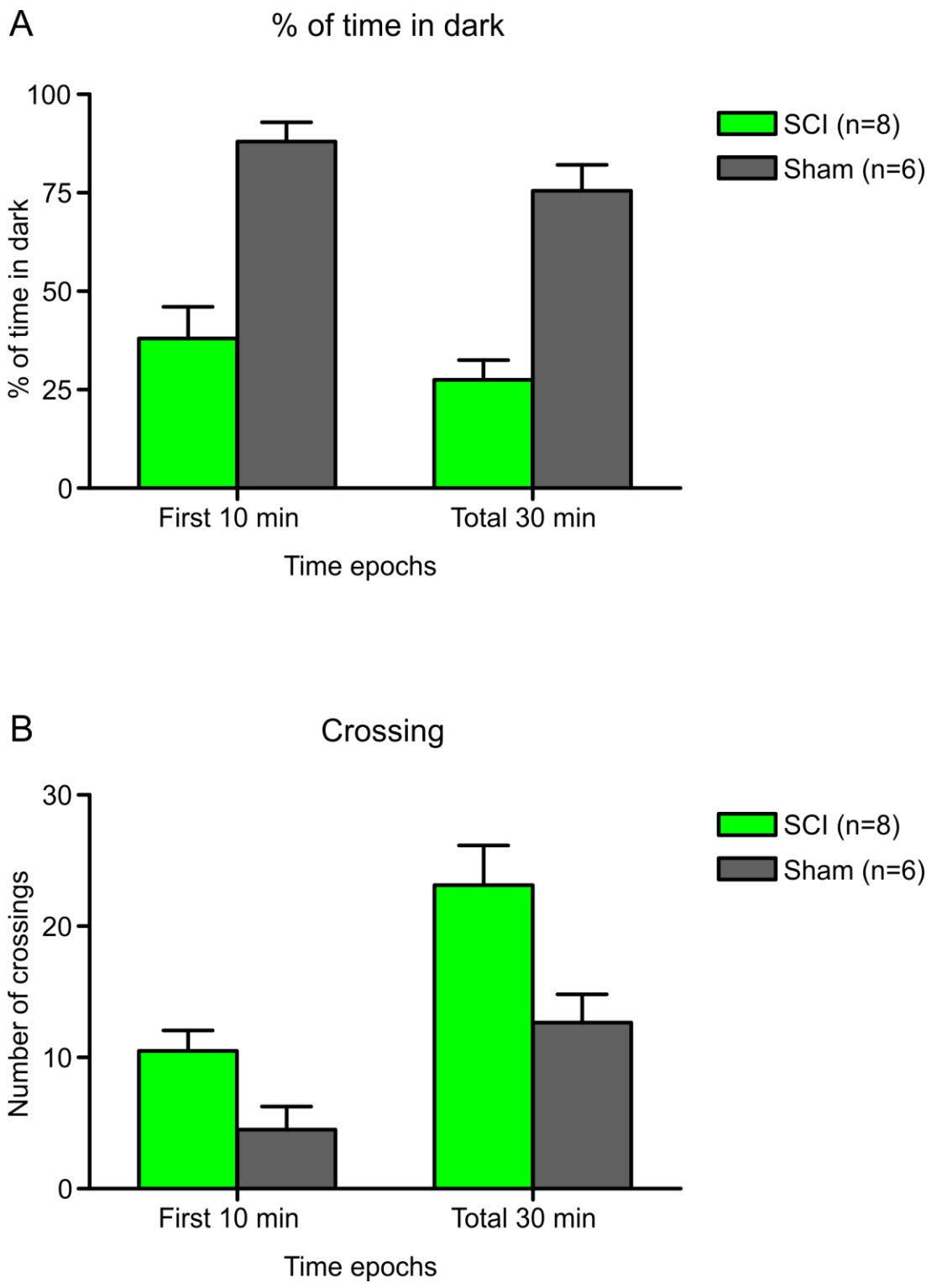

Fig. 5-12. Results from PEAP test using vF stimulation of the back at +1 cm above the injury site. A indicates the percentage of the test time that the animals spent on the dark side of the test chamber. **B** shows the number of times that the animals crossed between the light and dark sides of the chamber. The bars represent the mean values ± SE for the SCI (n=8) and sham (n=6) animals. Data (collected in week 4 PO) for the first 10 min of the test session and full 30 min of the session is shown separately.

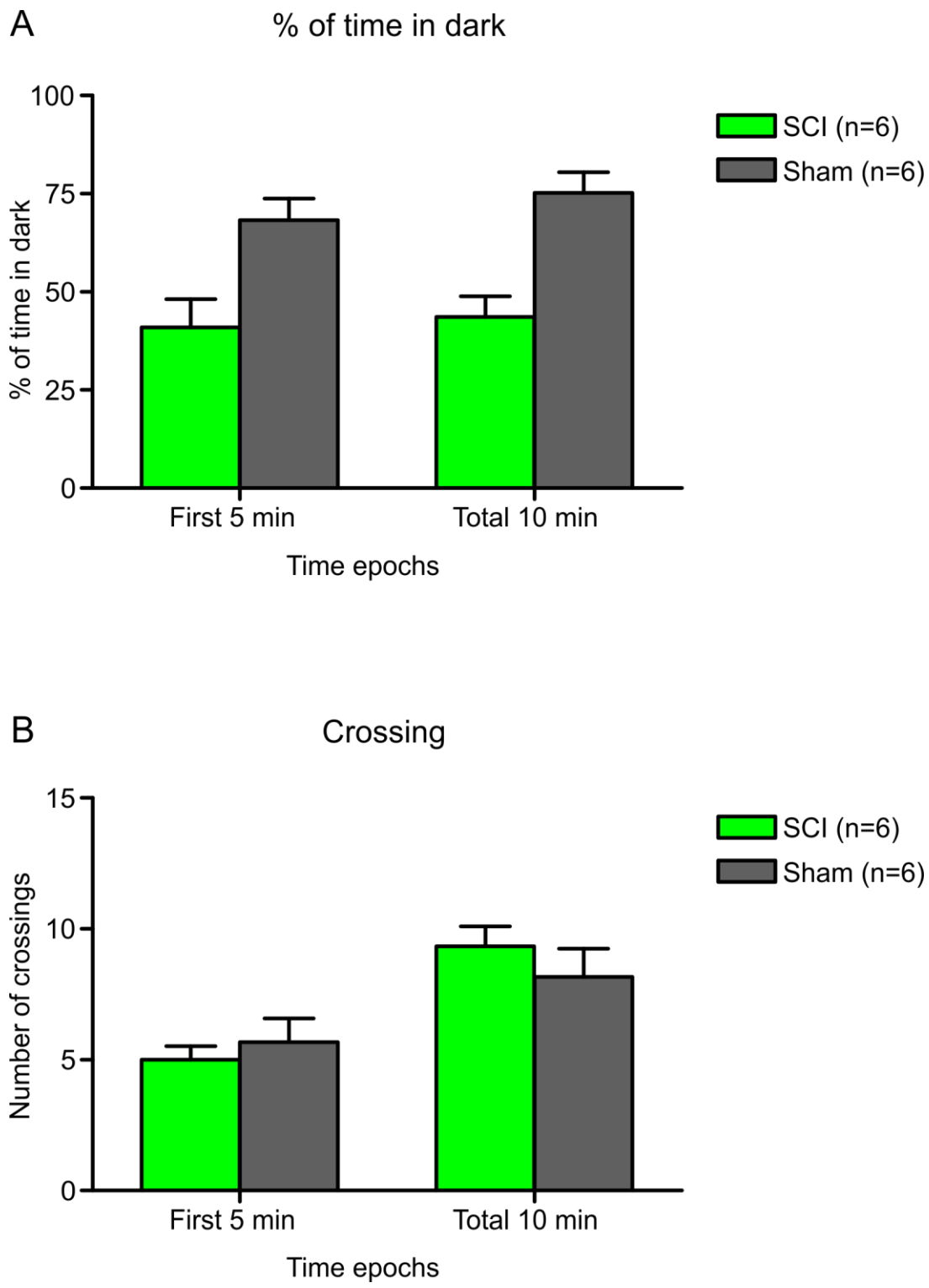

Fig. 5-13. Results from PEAP test using cold plate stimulation. A indicates the percentage of the test time that the animals spent on the dark side of the test chamber. **B** shows the number of times that the animals crossed between the light and dark sides of the chamber. The bars represent the mean values ± SE for the SCI (n=6) and sham (n=6) animals. Data (collected in week 6 PO) for the first 5 min of the test session and full 10 min of the session is shown separately.

(*vide supra*), this may relate to development of cold hyperalgesia in the forepaws. Indeed, hindpaw lifting data was similar in SCI and sham animals when tested at 7.5°C.

5.3.9.3 Cold avoidance test (CAT)

Results collected from cold avoidance test are depicted in Fig. 5-14. When shams were tested using this assay, they showed some signs of discomfort for just under 25% of the testing time, i.e. the forepaws were not in contact with the cold floor. However, some of this "avoidance" time was due to normal rearing behaviour. On the other hand, the contusion injured group showed behaviours which differed in quantitative and qualitative terms. SCI animals were extremely motivated to avoid touching the cold surface with the forepaws and sat on their hindpaws with the forepaws resting on the cage wall. This behaviour was observed for approximately 75% of the testing time which is very significantly longer than for the sham controls (*$P<0.0001$, unpaired t-test with Welch's correction)*. In some cases, these animals slept while the forepaws were on the thermally neutral walls of the testing chamber. This indicates that the forepaws were extremely sensitive to cold (i.e. cold hyperalgesia) while the hindpaws were not. Secondly, finding the cold floor noxious, animals quickly found a strategy for avoiding contact of the forepaws with the cold surface. This behaviour requires a degree of cerebral activity and the CAT is therefore a test of pain perception.

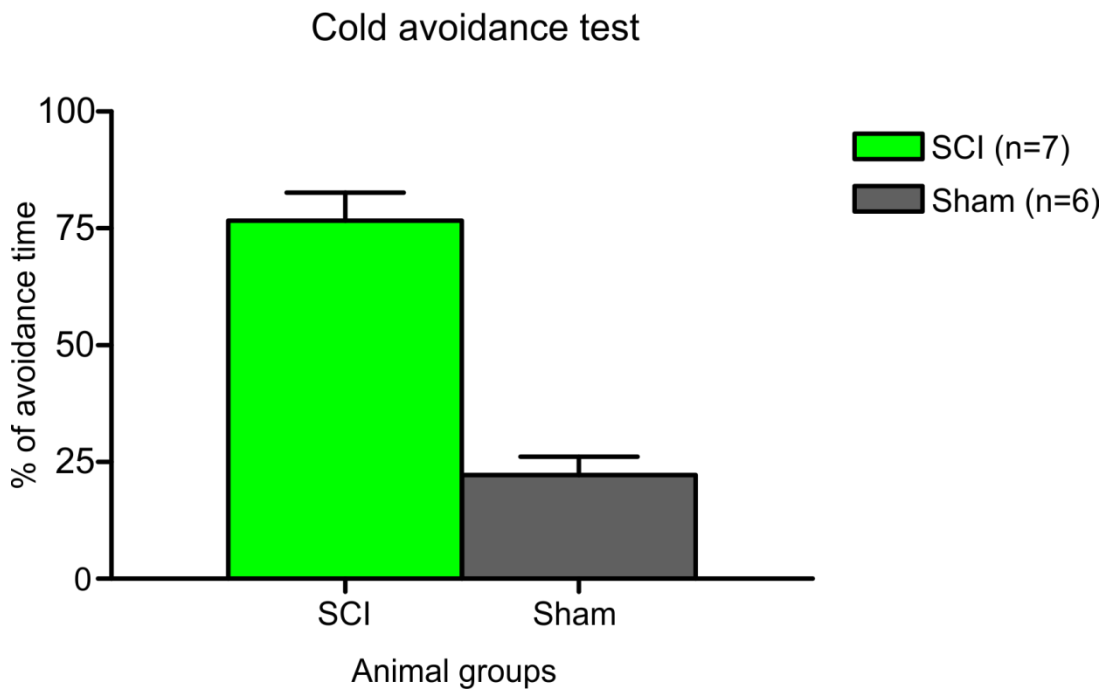

Fig. 5-14. Results from cold avoidance test. Histograms represent percentages of time that the forepaws were off the cold plate (7.5°C) during 3 testing periods of 4 min duration. The test was performed in week 6 after injury and data expressed as mean ± SE were collected from 7 contusion injured and 6 sham operated animals.

5.3.10 Summary of the results

The main findings in this model are summarized in table 5-1: 1) Robust manifestations of tactile allodynia and heat hyperalgesia in the forepaws. 2) Enhanced withdrawal response to applied mechanical and heat stimuli in hindpaws. 3) Intact licking response in the forepaws but not in the hindpaws. 4) Frequent lifting of the forepaws and occasion lifting the hindpaws when assessed on cold surface and over the grid. 5) Robust manifestation of spontaneous pain in forepaws when assessed by footlifing paradigm. 6) There was a strong indication for development of tactile sensitivity on the back above but not below the injury level. 7) Positive PEAP data were collected from application of vF filament over the trunk above the injury level as well as positive results were also seen in cold PEAP test. 8) CAT also showed a strong indication for development cold hyperalgesia.

A

Type of the test	Forepaws	Hindpaws
DWB	↑	↓
Plantar vF test	↓↓ Threshold	↓↓ Threshold
Plantar heat test	↓↓ Latencies	↓↓ Latencies
Licking response	ND	Abolished
FL over cold plate	↑↑↑	ND
FL over neutral plates	↑↑	Not seen
FL over grid	↑↑↑↑	ND
Cold PEAP	+	-
CAT	↑↑↑	-

B

Type of the test	Above level	At level	Below level
Trunk vF test	↑↑↑	↑	ND
Trunk PEAP	+	Not tested	Not tested

Table 5-1. Summary of the results of T3/T4 200 kdyn. A shows the summary of data collected from fore and hind paws while **B** shows the summary of trunk von Frey and PEAP testing. ND indicates no difference.

5.4 Discussion

High thoracic level injury models have been much less frequently studied than low thoracic models (Ramsey et al., 2010) and have mainly been used in the study of autonomic dysreflexia (Gris et al. 2004; Rabchevsky et al., 2010). The work described here is therefore the first study of neuropathic pain in forepaws after T3/T4 contusion.

5.4.1 Interpretation of forepaw data

In the plantar von Frey test, all animals subjected to T3/T4 injuries showed a very marked reduction in mechanical threshold of the forepaws. In comparison to the T9 200 model, the reductions in threshold tended to be earlier and more marked in the T3/T4 model. At week 2 of testing, the threshold was significantly lower in T3/T4 animals compared to T9 200 kdyn animals. In the plantar heat test, T3/T4 injured animals showed an early and clear reduction in withdrawal latencies. In the T3/T4 model thresholds in the injured animals were significantly different from shams at the first test session while in the T9 200 kdyn animals a significant difference was not reached until the 4 week test session. This suggests a more marked thermal hyperalgesia in T3/T4 animals compared to the T9 200 kdyn model.

5.4.2 Interpretation of hindpaw data

T3/T4 injured animals showed a marked decrease in mechanical threshold and latency of response to thermal stimuli. The licking response to thermal stimuli was virtually absent after injury. The results in the T3/T4 model are very closely similar to those in the T9 200 kdyn model. As discussed in relation to the lower thoracic models, interpretation of these evoked behaviours is complicated by spasticity below the injury level and damage to ascending pathways. In this sense, this model does not offer any advantage over lower thoracic injury models for the evaluation of below level pain.

5.4.3 Footlifting behaviours

5.4.3.1 Interpretation of FL on the cold plate

When tested on the cold plate (7.5°C), T3/T4 injured animals showed substantially more frequent forepaw lifting than sham controls at 4 and 5 weeks following injury. In comparison to the T9 200 kdyn animals, the T3/T4 injured animals exhibited

approximately double the number of foot lifts on the cold plate, which together with the avoidance behaviour demonstrated at 6 weeks after injury, indicates development of a much more intense cold hyperalgesia in the forepaws of the high thoracic injury animals. By contrast, hindlimb foot lifting was infrequent and was similar in both injured and sham animal groups. This suggests that cold hyperalgesia does not develop in the lumbar segments or that transmission to supraspinal levels from these segments is compromised. These results are similar to those observed for the hindlimbs of the T9 200 kdyn animals where ascending pathways were shown to be severely interrupted, contrasting with the T9 150 kdyn animals where more hindlimb FL was seen in injured compared to sham animals and where ascending pathways were more intact.

5.4.3.2 Interpretation of FL on the thermally neutral plates

Forelimb FL was also seen when T3/T4 injured animals were placed on a thermally neutral flat surfaces (25°C, 30°C and 35°C) but was never observed in sham animals. These observations suggest development of spontaneous pain in the forepaws of animals T3/T4 injured animals. In comparison to animals with a T9 200 kdyn injury, forelimb FL in T3/T4 injured animals was almost twice as frequent. This may be due to the closer proximity of the injury to the relevant cervical segments in T3/T4 injured animals. In contrast, this model failed to show signs of below level spontaneous pain. This is similar to observations in the lower thoracic injury models.

5.4.3.3 Interpretation of FL on the metal grid

T3/T4 contusion injured rats displayed frequent lifting of forelimbs and to a lesser extent hindlimbs when standing on the mesh grid. Forepaw lifting is interpreted as a sign of development of mechanical allodynia and is consistent with the reduced mechanical threshold to tactile stimuli seen in the von Frey test. Interpretation of the much less frequent hindlimb FL is complicated by the possibility of compromised ascending pathways. Compared to T9 200 kdyn injured animals the forepaw lifting was significantly more frequent indicating a more marked tactile allodynia in the T3/T4 model.

5.4.4 Dynamic weight bearing test

T3/T4 SCI animals showed a clear tendency to redistribute weight bearing from the hindpaws to the forepaws after injury. However, this redistribution was less marked than for animals with lower thoracic level injuries (both 150 kdyn and 200 kdyn). This may be

explained either by a difference in the effect of the injuries at the different levels on motor pathways providing control of posture, or it might reflect the influence of development of greater tactile allodynia in the forepaws of the T3/T4 injured animals. This could temper the animals tendency to bear more weight on the forepaws.

5.4.5 Trunk von Frey test

Testing over the trunk was performed at 1 cm above and 5 cm below the injury level. Above the injury level, all T3/T4 injured animals developed enhanced sensitivity to tactile stimuli. This was comparable to the enhanced sensitivity to stimuli at this level shown by animals with a T9 200 kdyn injury except that a biting response to the stimuli appeared earlier in animals with a T3/T4 level injury. In contrast, there were no change in tactile sensitivity to stimuli applied below the injury level and no difference between sham controls and SCI animals. This is the same result as seen in T9 200 kdyn animals. In both models, the absence of a change in sensitivity is likely due to interruption of ascending pathways since it contrasts with the clearly increased sensitivity at this level in T9 150 kdyn injured animals.

5.4.6 PEAP test

Assessment of the cortical processing of pain was performed by application of tactile stimuli over the back at 1cm above the injury site. When tactile stimuli were applied to the back above the injury level, all SCI animals spent significantly less time on the dark side of the cage in comparison to the sham controls. This clearly indicates that SCI animals perceived these tactile stimuli as painful and this is in full agreement with data from the T9 models.

Because of the marked sensitivity of the forepaws to cold in T3/T4 injured animals, we also developed a PEAP test involving the cold plate. In this test, T3/T4 injured animals were found to spend more time on the light side of the cage (with a thermally neutral floor) than the sham operated animals, indicating that the injury leads to a greater aversion to a cold surface. This is consistent with the development of a forepaw lifting and, at later test sessions, avoidance behaviour in the injured animals. Since the same behaviour was not seen in the hindpaws, we assume that the modification of behaviour seen in T3/T4 injured animals in the cold plate PEAP is driven by the increased sensitivity of the forepaws (see below).

5.4.7 Cold avoidance test (CAT)

Because the T3/T4 injured animals modified their behaviour on the cold plate at later testing time points and FL was no longer a useful assessment of cold sensitivity, we developed an alternative approach. Since animals were seen to modify their behaviour by supporting their weight on their hindquarters whilst resting their forepaws on the wall of the enclosure (a behaviour assumed to reflect avoidance of forepaw contact with the cold surface), the time spent performing this behaviour was quantified. The duration of this behaviour in T3/T4 injured animals was found to be 3 times as long as that in sham operated animals. The altered behaviour is presumably driven by this increased sensitivity of the forepaws to cold but also indicates that the same increase in sensitivity does not occur for the hindpaws. An advantage of this test is that since it involves a modified behaviour requiring increased effort, it must involve cortical processing and therefore is a test involving pain perception.

Chapter 6

General discussion

6 General discussion

6.1 Can SCI pain be adequately modelled using animals?

6.1.1 The ideal model

The ideal animal model would reproduce all of the categories of symptoms seen clinically in patients with SCI suffering from central neuropathic pain and also show these in a way that allows reliable and quantitative assessment of each of the symptoms. The model needs to allow investigation of above level, at level and below level pain since these may have different underlying mechanisms (for review see Finnerup and Jensen, 2004; Yezierski, 2009). It needs to model each of the types of symptoms that can occur involving different modalities and it needs to model spontaneous pain which is a common feature of pain following SCI. It also needs to bring about the state of neuropathic pain by pathological mechanisms which mirror those contributing to central pain following human traumatic SCI.

6.1.2 Are motor behaviours a reasonable way of assessing post SCI pain?

The limitations of our ability to study some aspects of post SCI pain pre-clinically is not just an issue of the appropriateness of the models but also to do with our ability to devise appropriate sensitive and reliable means of assessing those symptoms that are expressed in the model. The main obstacle to obtaining reliable information is the absence of co-operation of the subject. This forces us to make use of motor responses to sensory stimuli. An additional difficulty in studying pain in a SCI model is that the injury itself will inevitably compromise motor function to some extent and care has therefore to be taken to ensure that motor deficits do not interfere significantly with the test. Tests involving the limbs and in particular the hindlimbs are most susceptible to these problems while tests involving head orientation are least affected (for review see Yezierski, 2005). Even the forelimbs may be affected if performance of the task involves postural changes i.e., if after injury, weight support is more dependent on the forelimbs. However, our results suggest that it is possible to produce injuries which lead to clear changes in behaviour suggestive of pain but which do not appear to compromise motor function to an extent that affects performance of these tests, provided that an adequate period (2 weeks) is allowed for recovery from the worst effects of the injury on locomotor performance. Our results

suggest that other effects of the injury, directly on sensory pathways, are a more important concern with respect to sensory testing involving parts of the body below the injury.

6.1.3 Are contusion injuries the appropriate SCI model?

We have not compared different injury types in this project. Instead the focus has been on contusion model. The main justification for this is two-fold. Firstly, this type of injury is best reproduces the most common type of clinical SCI which is the result of an impact to the spinal cord i.e. a traumatic injury (Noble and Wrathall, 1985; Hayes and KaKulas, 1997; Vaccaro et al, 1998). It is therefore most likely to reproduce the pathological features that occur in human injuries. Secondly, this model also has the important advantage that the injury can be performed using sophisticated force controlled apparatus that maximises the consistency of the injury from animal to animal and provides user control over the severity of the injury. It should also be reproducible from lab to lab allowing for a direct comparison of results between different labs.

The results we have obtained in this project using the IH device support the idea that contusion injuries produced with this device provide a good model of SCI related pain. All of the animals which were injured, after exclusion of any where graphical data from the injury device suggested bone strikes, showed signs of neuropathic pain. The model therefore shows a very high degree of consistency which may in large part be due to the reproducibility of the injury under the control of the IH device. It is also possible that the greater consistency in development of pain signs seen in our study compared to some others (Mills et al., 2001a; Nesic et al., 2005) is due to the systematic way in which we devised the assessments. For example, considerable variation has been reported with respect to tests applied to the back (Siddall et al., 1995; Hubscher et al., 2010) and it is possible that we avoided some of this by carefully determining the correspondence between the topography of the skin and its segmental representation within the spinal cord. This showed that quite different responses may be obtained on stimulation of the back above the injury compared to stimulation at the injury level and that depending on the injury used, there may be no response to stimuli below the injury. Some of the previous variation may be explained by a failure to carefully limit stimulation to appropriate regions of the skin.

Other models may also have validity and the clamp model for example adds a static or compression component (Hubscher and Johnson, 1999; Oatway et al., 2004, 2005) to the

injury and could be argued to better reflect those injuries where there is continued pressure on the cord beyond the initial impact. The significance of compression for development of pain mechanisms is not clear but it is not essential for the expression of central neuropathic pain. Lateral hemisection injuries also appear to provide a robust model of post SCI pain (Christensen et al., 1996) but laceration injuries are rare clinically and because of the complexity of nociceptive pathways, limiting the injury to one side of the cord does not provide much advantage in terms of exploring the underlying mechanisms of pain development. Pain signs occur similarly on both sides of the animal (though are most often assessed using the hindpaws.

6.1.4 Are rodents an adequate species for pain studies?

There is also good evidence that rodents are a reasonable model organism to use in that there are many similarities in the basic neural circuitry of the primate and rodent in terms of basic sensory functions, particularly at the spinal cord level. It is likely that spinal cord mechanisms of nociception are similar in rodents and other species but there are some known differences. Rats differ in terms of the ascending pathways carrying nociceptive signals, for example. The spinothalamic tract is much less well developed in the rat than it is in primates and a greater proportion of the projection neurons from the lumbar segments of the spinal cord in particular project to other brain centres (for review see Todd, 2010) with a denser projection to targets serving the more vegetative aspects of the response to nociception rather than the affective aspects of pain (for review see Gauriau and Bernard, 2002, 2004). There will be much more extensive differences in the higher level processing of nociceptive signals and their elaboration into the sensation of pain. For example, there are some difference between the two species in cingulate cortex but both human and rat have an area 24b in anterior cingulate cortex which is important in pain processing (for review see Vogt and Vogt, 2004). Interestingly, La Garcia et al. (2004) reported that lesion of cingulate cortex in rats alleviates neuropathic pain signs (assessed by PEAP test) produced by L5 spinal nerve ligation. Overall, the difference between the two species may not have a great bearing on the gross behavioural tests performed in these studies, even though they are important in the clinical management of patients with pain after SCI.

6.2 Can different modalities of pain be modelled and assessed in rodents?

One advantage of the use of the plantar surface of the paws for the application of test stimuli, is that it allows the testing of different sensory modalities whereas only sensitivity to tactile stimuli can be readily tested with stimuli over the back. Use of the forepaws for plantar vF and heat tests, though more challenging than when applied to the hindpaws, provides reliable data for tactile and heat modalities. In addition, in the work in this thesis we describe new tests for investigating sensitivity to cold. These show that, as reported clinically (Defrin et al., 2001; Finnerup et al., 2003, Cruz-Almeida et al., 2009), increased sensitivity to cold is present above level in rodent SCI models. Although indications for this have been seen previously using the acetone or ethyl chloride test for example, this is the first time that sensitivity to precisely known temperatures has been investigated and it shows that there is both cold hyperalgesia and cold allodynia.

6.3 Can spontaneous pain be modelled and assessed in rodents?

It would be a great advantage to be able to model, assess and investigate spontaneous pain in animals since this is one of the major symptoms of which SCI patients complain. Our studies show that a paw lifting behaviour can be observed which might correspond to spontaneous pain. The FL occurs in the absence of any intentionally applied stimuli and it occurs while the animal is resting on an entirely smooth surface which is maintained at a thermally neutral temperature. Because of this, the paw lifting is considered to reflect a spontaneous behaviour in the sense that it is assumed to be provoked by activity that arises spontaneously in the nervous system (in the absence of any evoking stimulus) (Choi et al., 1994; Djouhri et al., 2006). Drawing together observations from across the models, FL was a feature of both high and low thoracic models but was clearer in the high thoracic model. It was also seen in the forepaws but was not obvious in the hindpaws in any of the models. Although we would like to argue that the paw lifting behaviour seen in the forepaws is a reflection of spontaneous pain, the absence of this behaviour below the injury level is puzzling. It could also be considered a weakness in the argument since clinically it appears that spontaneous pain can occur below level even in complete injuries (Ravenscorft et al., 1999, Werhagen et al., 2004). It will clearly be interesting in future experiments to determine what mechanisms underlie the paw lifting behaviour and whether treatments that reduce signs of evoked pain have any influence on paw lifting behaviour.

6.4 Time course of symptoms

The onset of behaviourally observable alterations in the responses to stimuli in the animal models was in some cases clear even 1 week after injury (the earliest time at which tests were performed post injury). This is much earlier that the onset of symptoms attributable to central neuropathic pain mechanisms in human patients which typically appear months to years after SCI (Siddall et al., 1999a, 2003).

More rapid dynamics are evident for other aspects of the rodent SCI model. For example, the spontaneous locomotor recovery that occurs following injury is much more rapid in rodents than in humans as well as being much more extensive. It is therefore unclear how the relative timing of the animal model and human injury should be viewed and it remains uncertain why, if the underlying cellular and molecular mechanism of the pathology that leads to pain in both species is similar, such a disparity in timing should arise. In humans, once neuropathic pain develops, it normally remains with the patient for the remainder of their life (Siddall et al., 1999a, 2003). We did not look at the full duration of symptoms in our animal models. We saw no evidence of a decrease in any of the indicators of pain we assessed over the 6 weeks which these were systematically studied. In some preliminary experiments a few animals were studied over a longer time period (12 weeks after SCI) and these showed persistent manifestations of tactile allodynia and hyperalgesia.

Others have reported that behavioural signs in animal models may persist for several weeks (Crown et al., 2005, 2008, 2012; Endo et al., 2009; Cark et al., 2010) or even months (Bedi et al., 2010).

6.5 Can a single model provide optimum requirements of all categories of neuropathic SCI pain?

A better understanding of the basic mechanisms of neuropathic SCI pain is likely based on development of suitable animal models which provide the fundamental requirements for modelling of different categories of neuropathic pain after SCI. In this thesis, we characterized three thoracic SCI models in rats (i.e. T9 200 kdyn, T9 150 kdyn and T3/T4 200 kdyn) for modelling of neuropathic pain above, at and below the injury site. However, the work presented in this project showed that different models may be required to see optimum signs of neuropathic SCI pain at each level in rodents. In other words, a standard model presenting all types of neuropathic pain after SCI may not be currently available.

6.5.1 Which model is optimum for studying above neuropathic SCI pain (T9 200 kdyn *vs.* T9 150 kdyn *vs.* T3/T4 200 kdyn)?

Low thoracic severe contusion (T9 200 kdyn) produced clear evidence for development of mechanical and thermal hypersensitivity in the forepaws which was associated with an indication for supraspinal processing when thermal stimuli were used. In addition, this injury model led to development of spontaneous pain in above level dermatomes (i.e. forepaws). This is despite the fact that the representative segments of the forepaws are located at considerable distance rostrally from the injury site where the pathological mechanisms are more robust. However, our results from the upper thoracic model (T3/T4 200 kdyn) provide clear evidence that making the injury site closer to the processing segments of the tested dermatome (i.e. forepaws) can optimize manifestations of evoked pain above level of the injury. Indeed, the amount of evoked above level pain was approximately doubled in some behavioural assays by moving the injury site to a more rostral location (i.e. from T9 to T3/T4 segments). Similarly, the upper thoracic severe contusion led to stronger and more robust manifestations of non-evoked pain in the forepaws which made it a perfect model for investigating the effect of analgesics on above level neuropathic pain (both evoked and non-evoked) after SCI.

In comparison to both T9 200 kdyn and T3/T4 200 kdyn models, less severe injury (150 kdyn) at low thoracic level (T9) seems to be less efficient to produce evoked sensory changes in the forepaws. This conclusion is mainly based on the observation that this model failed to produce signs of heat hyperalgesia in the planar surface of the forepaws. However, it produced approximately the same degree of non-evoked pain in the forepaws that seen in T9 200 kdyn. Considering the two observations, we can conclude that manifestations of evoked pain in the forepaws are highly dependent on the severity of the injury but this not the case for spontaneous pain which seems to be less affected by the severity of SCI.

When the trunk was tested above the injury site (+1 cm) using tactile stimuli, a robust and comparable increase in sensitivity appears to occur following injuries of different severity (e.g. 150 and 200 kdyn low thoracic) and irrespective of the level of the injury (high thoracic or low thoracic). However, sensory testing over the trunk has its own limitations such as certain modalities of nociception (i.e. thermal) cannot be studies at these dermatomes which show-off the importance of the forepaws.

6.5.2 Which model is optimum for studying at level neuropathic SCI pain (T9 200 kdyn)?

Despite that fact that at level pain was only investigated (-2 cm trunk von Frey testing) in the classic low thoracic model (T9 200 kdyn), there is clear evidence that this model can be considered an acceptable model for studying at level neuropathic pain after SCI using tactile stimuli applied over the back. Although the use of stimuli to the back to investigate pain has been described before, this is the first time that the topographical correspondence of the back dermatomes to the thoracic segments has been determined. As a result, stimulus locations correspond to what can be considered at level dermatomes has been accurately identified. This standardization of the locations provides a clear characterization of at level from above level pain. Indeed, almost all previous publications have considered above level as at level pain due to the misconception about the topographical relationship between the back dermatomes and spinal segments. This can be misleading because each of above and at level SCI pain is driven by its own mechanisms (see introduction). If our standards for testing locations are used, we think T9 200 kdyn can be considered as a good model for investigation of at level pain. However, we cannot hide the fact that under the current circumstances there is only a limited chance to assess different modalities (mainly mechanical) of evoked pain at level of the injury.

6.5.3 Which model is optimum for studying below level neuropathic SCI pain (T9 200 kdyn *vs.* T9 150 kdyn)?

In this thesis, there is more than one evidence showing that severe injuries at low thoracic levels which currently are used in most other labs may not appropriate for studying below level pain after SCI. Extensive interruption of the ascending pain pathway and development spasticity are one side of this assumption while the other side of this postulation is related to the fact that these labs are still relied on behavioural assays based on nocifensive reflexes such as withdrawal response. Such reflexive behaviour is well known to be processed spinally and therefore there is no engagement for the main mechanisms of below level pain which are mediated mainly by changes in cortical and subcortical structures (see introduction).

Our results put forward claiming that mild to moderate injuries at low thoracic level should be considered for better modelling of below level pain. This assumption is based on a direct comparison between different aspects of 200 kdyn and 150 kdyn models since both injuries performed by same device at a similar level (T9). Although changes in sensitivity

to tactile and thermal stimuli applied to the hindpaw were evident in all of our models investigated, there is a high probability that at least a proportion of the altered responses observed in these assessments (i.e. reduction in threshold and latency for nocifensive reflex of the hindlimb) could involve mechanisms affecting motor circuitry and associated with spasticity. The similarity of withdrawal data collected from the hindlimbs in the two models highlight that development of spastic hyperreflexia might mask any differences in nocifensive reflexes between 200 kdyn and 150 kdyn models. This complicates interpretation of reflexive outcomes from tests performed at hindlimbs because it is then uncertain what proportion, if any, of the change is attributable to changes in nociceptive circuitry which feed onto projection neurons and contribute to the perception of pain. In addition, there is a major concern about integrity of ascending pathways after severe contusion injuries since licking response was virtually abolished in T9 200 kdyn animals when heat stimuli was used. Since this behavioural response is mediated supraspinally, this suggests that nociceptive signals from the hindlimbs do not reach the brain and put more stress on reliability of using withdrawal reflex for assessment of below level pain in severe injury models. The use of stimuli applied to the back does however provide an alternative mean of assessing below level pain if two conditions are met. Firstly, the model must not involve an injury severe enough to extensively interrupt the ascending nociceptive pathways: the test can therefore be used in a 150 kdyn low thoracic contusion injury model but not after a 200 kdyn injury. Secondly, care is required to apply the stimuli in an appropriate location. This was found to require application of stimuli several cm below the physical level of the injury.

Application of tactile stimuli to the hairy back below the injury level did not produce major changes in nociceptive responses of animals subjected to 200 kdyn. Lack of pain perception below the injury level in this model is supported by the data collected from PEAP test performed in the hindpaws and over the trunk at well below level locations. The appropriateness of 200 kdyn injury for investigation of below level pain is also questioned by the tract tracing data which showed that projections originating from neurons located in below level spinal segments are severely interrupted at the injury site. Comparatively, reduction of the contusion force to 150 kdyn spared approximately a doubled number of ascending axons from L4 segment and therefore more routes for transmission of the generated impulses in the below level locations to the supraspinal centres. Unfortunately, hindpaw PEAP test produced a similar data in both models, however, this may not be directly linked to the degree of sensory changes in the hindpaws and it would rather be

related to uncomfortable (to the forepaws) mesh surface of the testing arena (see discussion of T9 150 kdyn). In support of this explanation, the two models showed contrast data (positive results from T9 150 kdyn) when assessments were made over the trunk but 5 cm below the injury level using paradigms based on supraspinally-mediated behaviours (-5 cm trunk vF and PEAP tests). Applied stimuli to these locations were shown to be processed in T10 to T12 segments (see electrophysiological data). The projections from these thoracic segments are expected to be more affected by T9 SCI than those originating from L4 segment. If this is the case, the negative results from hindpaw PEAP cannot be directly correlated with lack of pain perception in the hindpaws. This conclusion is supported by observation of licking response to the applied heat stimuli in T9 150 kdyn animals but not in the more severe injury. Moreover, animals subjected to milder injuries showed clear signs for development of cold hyperalgesia in the hindpaws which never seen in those with more severe injuries. All in all, the less severe injury (T9 150 kdyn) may provide a more reliable model for studying below level pain after SCI.

6.6 Recommendations for future investigations of SCI pain mechanisms.

The heavy reliance on hindpaw testing in the field at best means that data on mechanisms may be biased towards below level evoked pain and at worst could mean that our understanding of post SCI pain is based on less secure information from animal models than previously thought. The problems with hindpaw testing suggest that these should never be used as the only tests in a study. Tests involving stimuli applied to the back provide a robust means of testing above or at level pain and if appropriately placed can also provide a test of below level pain but only in models where projection pathways are sufficiently intact. This means that if the aim is to investigate below level pain, the injury model has to be mild enough to preserve sufficient communication with the brain. Although back testing is a robust option for testing above/at level pain, it is limited to tactile stimuli. Testing of the forepaws offers the possibility of investigating other modalities. In particular, our investigations confirm the development of heat hyperalgesia and provide a clear indication, for the first time using controlled temperatures, for cold allodynia and cold hyperalgesia. These tests of cold sensation, and also testing over the back, have the advantage of involving supraspinal processing. That this is elaborated to the sensation of pain is confirmed by the results of operant testing (the place escape avoidance paradigm) applied to these test stimuli. Finally, new observations on FL behaviour suggest the possibility of being able to model spontaneous pain after SCI.

The work in this thesis also shows that the location of the injury is another factor that should be considered in the choice of animals model. Moving the injury level to a high thoracic level increases the intensity of the pain signs in the forelimbs. This may therefore be the optimal model in which to investigate mechanisms underlying above level pain. It may especially be advantageous in the study of spontaneous pain since FL behaviour was notably more obvious in the T3/T4 SCI model. Models where the pain is particularly clear and robust may be particularly advantageous for the investigation of mechanisms and testing of analgesics, while the models in which signs of pain are milder may be useful in identifying treatments or procedures that exacerbate pain conditions as is suspected for some stem cell treatments and neurotropic factors tested for spinal cord repair.

The work presented in this thesis provides the clearest and most comprehensive data yet on the utility of models of SCI for the investigation of central neuropathic pain and represents a significant advance in the field. The hope is that an improved understanding of these models will enhance the quality of future research in this area and lead to both a better understanding of central neuropathic pain mechanisms and the development of more effective analgesics for this type of pain.

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