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# The effects of parenteral or dietary omega-3 polyunsaturated fatty acids in rat models of spinal cord injury

Hall, Jodie Christine Elizabeth

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# The effects of parenteral or dietary omega-3 polyunsaturated fatty acids in rat models of spinal cord injury

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**A thesis submitted in accordance with the regulations for the degree of**

**Doctor of Philosophy**

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# Abstract

There is currently no effective treatment for spinal cord injury (SCI). Long chain omega-3 polyunsaturated fatty acids (PUFA) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) have beneficial effects in various neurological disorders. DHA and EPA have neuroprotective effects when given intravenously (i.v.) after SCI, but dietary enrichment with these fatty acids is less well-characterized. It is important to characterize the effect of these compounds after parenteral and oral administration, as both regimes could be used clinically. The aims of this thesis were to: i) characterize the inflammatory response in the rat after T12 compression SCI, ii) characterize the effects of acute i.v. injection of DHA or EPA on inflammation after SCI, iii) explore the effects of i.v. DHA in a rat contusion model of SCI, iv) assess the effects of dietary enrichment with DHA or EPA before and/or after SCI.

Compression SCI led to acute infiltration of neutrophils and delayed accumulation of macrophages/microglia in the spinal cord, and a systemic inflammatory response in plasma and liver. DHA i.v. injection reduced neutrophil infiltration to the epicentre and C-reactive protein in the plasma, whereas EPA had no significant effect. There was no effect of i.v. EPA or DHA on the increase in cytokines/chemokines following injury. Acute DHA restored stepping ability after contusion SCI, but there was no effect on histological markers. Dietary enrichment with EPA after compression SCI had a detrimental effect on recovery, but this was not correlated with changes in neurones, oligodendrocytes or macrophages/microglia. Dietary pre-treatment with DHA had no effect on locomotor outcome after compression SCI.

Therefore, the inflammatory response after SCI is not changed significantly by acute administration of EPA and DHA. The study did not reveal a beneficial prophylactic effect of dietary DHA, but highlighted a possible risk associated with dietary EPA after SCI.

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**AUTHOR STATEMENT**

All work in this thesis is original and all necessary acknowledgements have been made.

# Abbreviations

| | |
|---|---|
| AA | Arachidonic acid |
| ALA | α-linolenic acid |
| BBB | Basso, Beattie and Bresnahan score |
| BDNF | Brain-derived neurotrophic factor |
| CINC | Cytokine-induced neutrophil chemoattractant-1 |
| CNS | Central nervous system |
| COX | Cyclooxygenase |
| $CO_2$ | Carbon dioxide |
| $cPLA_2$ | Cytosolic phospholipase A2 |
| CRP | C reactive protein |
| CSF | Cerebrospinal fluid |
| CSPG | Chondroitin sulphate proteoglycan |
| CST | Corticospinal tract |
| DC | Dorsal columns |
| DH | Dorsal horn |
| DHA | Docosahexaenoic acid |
| DNA | Deoxyribonucleic acid |
| DRG | Dorsal root ganglion |
| EPA | Eicosapentaenoic acid |
| ER | Endoplasmic reticulum |
| ERK | Extracellular regulated kinase |
| FA | Fatty acid |
| FFA | Free fatty acid |
| FITC | Fluorescein isothiocyanate |
| GABA | γ-Aminobutyric acid |
| GAP43 | Growth associated protein 43 |
| GDNF | Glial cell line-derived neurotrophic factor |
| GFAP | Glial fibrillary acidic protein |
| H & E | Haematoxylin and Eosin |
| HETE | Eicosatetraenoic acid |
| HPEPE | Hydroxyeicosapentaenoic acid |

| | |
|---|---|
| HRP | Horse radish peroxidase |
| HSP | Heat shock protein |
| IFNγ | Interferon gamma |
| Iba1 | Ionized calcium binding adaptor molecule 1 |
| $IP_3$ | Inositol triphosphate |
| IL | Interleukin |
| iNOS | inhibitory Nitric oxide synthase |
| $K^+$ | Potassium |
| KCl | Potassium chloride |
| LA | Linoleic acid |
| LPS | Lipopolysaccharide |
| MAPK | Mitogen-activated protein kinase |
| MBP | Myelin basic protein |
| MCP | Monocyte chemoattractant protein |
| MHC | Major histocompatibility complex |
| MIP | Macrophage inflammatory proteins |
| MPO | Myeloperoxidase |
| MMP | Matrix metalloproteinase |
| MP | Methylprednisolone |
| mRNA | messenger ribonucleic acid |
| $Na^+$ | Sodium |
| NASCIS | National Acute Spinal Cord Injury Studies |
| NDS/NGS | Normal donkey/goat serum |
| NeuN | Neuronal nuclei |
| NF | Neurofilament |
| NFκ-B | Nuclear factor κ-light-chain-enhancer of activated B cells |
| NMDA | N-methyl-D-aspartate |
| NPD1 | Neuroprotectin D1 |
| NYU | New York University |
| OSU | Ohio State University |
| PB | Phosphate buffer |
| PBS | Phosphate-buffered saline |
| PC | Phosphatidylcholine |
| PFA | Paraformaldehyde |

| | |
|---|---|
| $PGE_2$ | Prostoglandin $E_2$ |
| $PLA_2$ | Phospholipase $A_2$ |
| PNS | Peripheral nervous system |
| PPAR | peroxisome proliferator-activated receptor |
| PS | Phosphatidylserine |
| PUFA | Polyunsaturated fatty acid |
| RBCs | Red blood cells |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| RXR | Retinoid X receptor |
| SCI | Spinal cord injury |
| TBI | Traumatic brain injury |
| TLR | Toll-like receptor |
| TNF-$\alpha$ | Tumor necrosis factor-$\alpha$ |
| TRAAK | TWIK-related arachidonic acid activated $K^+$ channel |
| TREK | TWIK-related $K^+$ channel |
| TRITC | Tetramethyl-rhodamine isothiocyanate |
| TWIK | Tandem P domain weakly inwardly rectifying $K^+$ channel |
| VLWM | Ventrolateral white matter |
| VH | Ventral horn |
| VWM | Ventral white matter |

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# Chapter 1: General Introduction

It is estimated that the worldwide annual incidence of spinal cord injury (SCI) is 22 per million of population, nearly 1000 new cases occurring in the UK each year (Grundy and Swain, 2002; Rossignol et al., 2007). There is currently no effective treatment. Although these numbers are not large compared to illnesses such as cancer or heart disease, people living with SCI are left with debilitating consequences, depending on the level and severity of the lesion. Paralysis, sensory loss, intractable pain, pressure sores, and urinary and other infections seriously diminish their quality of life. The cost of living with SCI can be immense. The average cost varies depending on the amount of care needed and the unemployment rate for people with SCI is high. Therefore, it is essential to pursue research into basic mechanisms involved in SCI in the hope of developing efficacious therapeutic strategies (Rossignol et al., 2007).

Recently, our laboratory has shown that omega-3 polyunsaturated fatty acids (PUFAs) have the potential to act as neuroprotective agents in SCI (King et al., 2006; Huang et al., 2007a; Lim et al., *in press*). The purpose of this doctoral research project is to further investigate the effects of treatment with omega-3 polyunsaturated fatty acids (PUFAs) in models of SCI, and in particular to explore whether they alter the inflammatory response following SCI. This work will be introduced with a review of SCI pathology and the inflammatory response following injury, followed by an overview of existing neuroprotective strategies and the field of PUFAs, finishing with an introduction to the methodology that has been used.

## 1.1 Gross anatomy of the spinal cord

The spinal cord is contained in the vertebral column and extends from the brainstem to the cauda equina (Fig. 1.1A). In the human, it can be divided into segmental levels by the pairs of nerves that arise from it, into cervical (C1-C8), thoracic (T1-T12), lumbar (L1-L5), sacral (S1-S5) and coccygeal (1 pair) regions. This makes up 31 spinal cord segments in the human, whereas in the rat there are 34: 8 cervical, 13 thoracic, 6 lumbar, 4 sacral and 3

coccygeal segments (Paxinos, 2004). The nerves have both dorsal and ventral roots and leave the vertebral column through the intervertebral foramina that lie adjacent to each vertebral body. The dorsal roots convey sensory information from the afferent axons of the spinal nerves and motor commands are sent via the ventral roots (Purves et al., 2001).

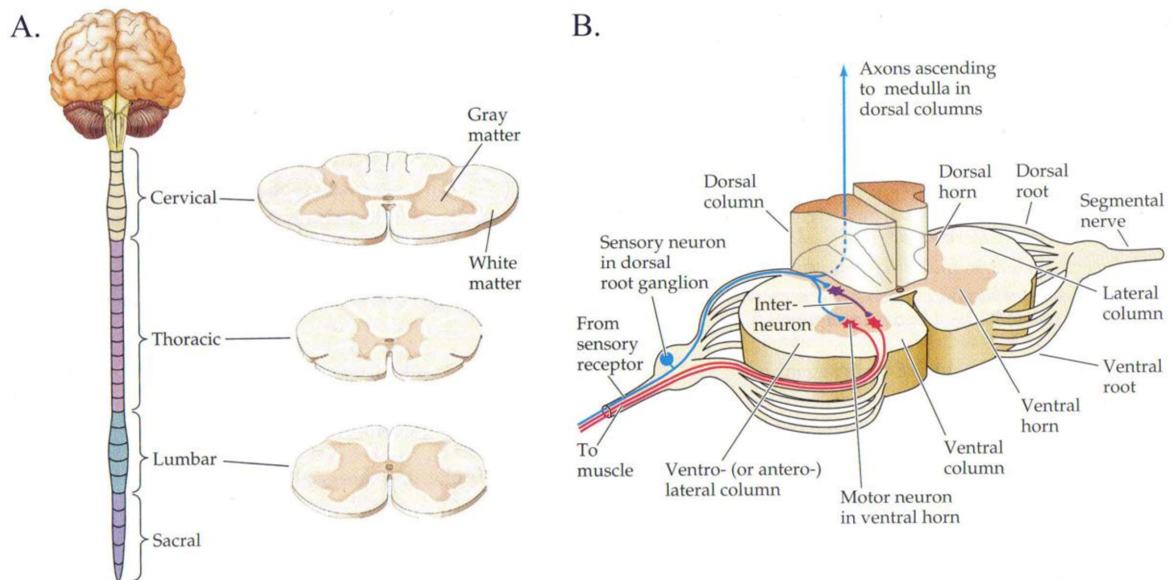



**Figure 1.1 Anatomy of the spinal cord. (A)** Transverse sections of the spinal cord, which can be divided into cervical, thoracic, lumbar and sacral regions and is made up of both gray and white matter. **(B)** Transverse section of the spinal cord showing the major ascending and descending tracts, which project to both the brain and the periphery (image reproduced from Purves, et al., 2001).

The regions of the spinal cord which innervate the limbs are enlarged to accommodate the greater number of nerve cells and connections. Thus, the cervical enlargement spans C5-T1 and corresponds to the arms; the lumbar enlargement spans L2-S3 and corresponds to the legs. The cauda equina is a collection of lumbar and sacral nerve roots that run for some distance in the vertebral canal before emerging (Purves et al., 2001).

Sensory dorsal root ganglia (DRG) lie adjacent and connect to the spinal cord. They send axons that constitute the **sensory** component of the peripheral nervous system (PNS). These axons end in specialized receptors that process information about a wide range of stimuli such as pain, temperature and pressure. The PNS is also divided into the **somatic motor** component, where peripheral motor axons innervate muscle to control skeletal movement

and most voluntary behaviour and the **autonomic** portion, which contains preganglionic visceral motor neurones in the spinal cord and brainstem that form synapses with post ganglionic motor neurones that lie in the autonomic ganglia. These ganglia innervate smooth muscle, glands and cardiac muscle, controlling most involuntary behaviour. There is a **sympathetic** division of the autonomic nervous system, where the ganglia are located along or in front of the vertebral column and send their axons to peripheral targets. In the **parasympathetic** division, the ganglia are located within the organs they innervate. The gut contains small ganglia scattered throughout its wall, making up the enteric nervous system (Purves et al., 2001).

*Internal anatomy of the spinal cord*

The spinal cord is a component of the central nervous system (CNS) and is made up of gray matter containing cell bodies, surrounded by white matter which consists of axonal tracts. In transverse sections, the gray matter is divided into dorsal, lateral and ventral 'horns' (Fig. 1.1B). The **dorsal horns** receive sensory information that enters the spinal cord via the dorsal roots. The **lateral horns** are largely found in the thoracic region and contain the preganglionic visceral motor neurones that project to the sympathetic ganglia. The **ventral horns** contain the cell bodies of motor neurones that send axons through the ventral roots to terminate in muscle tissue. The white matter is divided into dorsal, lateral and ventral columns. The axons of the **dorsal columns** carry ascending sensory information from somatic mechanoreceptors. The **lateral columns** carry information from the cerebral cortex in the brain to contact spinal motor neurones, also known as the **corticospinal tracts,** located in rodents beneath the dorsal columns as well as a minor ventral component. The **ventral** (and **ventrolateral**) **columns** carry ascending information such as pain and temperature, as well as descending motor information (Purves et al., 2001).

*Blood supply of the spinal cord*

Since this thesis will be dealing with the inflammatory response to SCI, it is pertinent that a brief overview of the bloody supply to the nervous system is given, as blood provides the

vehicle for systemic cells involved in the inflammatory reaction which are recruited to the injury site.

The entire blood supply of the brain and spinal cord originates from two sets of branches from the dorsal aorta (Fig. 1.2). The vertebral arteries arise from the subclavian arteries, and together with the medullary arteries that arise from segmental branches of the aorta, provide the primary vascularisation of the spinal cord (Purves et al., 2001). The medullary arteries join to form the **anterior** and **posterior spinal arteries**. If any of the medullary arteries are obstructed or damaged, for example during a surgical procedure, the blood supply to specific parts of the spinal cord may be compromised and result in neurological damage. A loss of posterior supply generally leads to loss of sensory functions, whereas loss of the anterior supply more likely causes motor deficits (Purves et al., 2001).

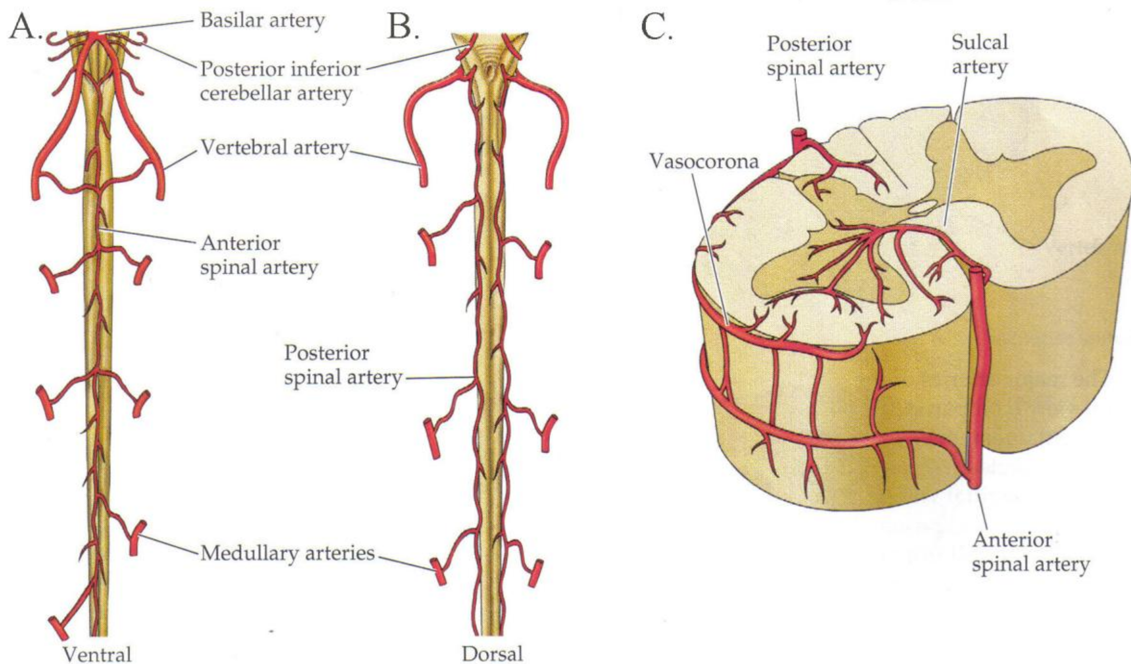



**Figure 1.2 Blood supply to the spinal cord.** **(A)** Ventral and **(B)** dorsal aspects of the spinal cord surface, displaying the major arteries that supply blood to the spinal cord. **(C)** Transverse section of the spinal cord showing the vascularisation of the grey and white matter (image reproduced from Purves, et al., 2001).

The main outcome following SCI that will be measured in the experiments that follow is the recovery of locomotor function. In some cases, measures of autonomic function of the

bladder and somatosensory tests, such as the development of allodynia/hyperalgesia will be carried out. Therefore, a brief description of the physiological control of these functions will follow.

*The locomotor system*

After SCI, one of the most dramatic impairments is the loss of locomotor function. In humans, the American Spinal Injury Association (ASIA) Impairment Scale is widely used to determine the level of SCI and assess motor function and pin-prick and light-touch sensory function. In clinical trials, an improvement in the ASIA scale is the main outcome measure for the efficacy of treatments and ranges from A-E, where A is a complete SCI (no motor or sensory function) and E is normal motor and sensory function. In rodent models of SCI, there are several tests that can be used to assess the recovery of sensory and motor function. These will be discussed in chapter 6. The main functional measurement that will be taken in this thesis is an assessment of locomotor recovery. Therefore, a brief overview of motor function and the systems involved in locomotion will be given here.

All movements produced by the skeletal musculature, whether reflexive or voluntary, are ultimately dependent on **lower motor neurones** in the ventral horn spinal cord gray matter that directly innervate skeletal muscle. Activation of lower motor neurones is determined by local circuits within the brain and spinal cord. Descending pathways comprised of **upper motor neurones** modulate the lower motor neurone circuitry. The cell bodies of upper motor neurones are located either in the cortex or in the brainstem, i.e. the vestibular nucleus, the red nucleus, or the reticular formation. The motor and ‘premotor’ areas of the frontal lobe are responsible for the planning and precise control of voluntary movements. The cerebellum controls movement by regulating the activity of the upper motor neurons. Finally the **basal ganglia**, a group of structures in the forebrain, suppress unwanted movements and prime upper motor neurone circuits for the initiation of movements. Disorders of the basal ganglia such as Parkinson’s disease and Huntington’s disease highlight the importance of such a process. The local circuit neurones also receive direct

input from sensory neurones. All of these inputs together provide coordination between the different muscle groups essential for organised movement (Purves et al., 2001).

The lower motor neurones form a pool that innervates a single muscle and their axons extend up and down one or more spinal cord segments. Two types of lower motor neurone are found in these neuronal pools: γ motor neurones and α motor neurones. The most medial part of the ventral horn contains lower motor neurone pools that innervate axial or proximal muscles of the limbs, whereas the lateral parts contain lower motor neurones that innervate the distal muscles of the limbs (Purves et al., 2001). When there is damage to lower motor neurones of the spinal cord, this is referred to as the '**lower motor neurone syndrome**'. This results in paralysis of the affected muscles, including a loss of reflexes, muscle tone and wasting of the affected muscles due to denervation and disuse. Damage to any of the descending motor pathways gives rise to the '**upper motor neurone syndrome**', characterised by spasticity of muscles, which is most severe in the arms and legs. This is preceded by an initial period referred to as **spinal shock,** which reflects the decreased activity of spinal circuits suddenly deprived of input from the motor cortex and brainstem (Purves et al., 2001).

The movement of a single limb during locomotion is a cycle consisting of a **stance** phase, during which the limb is extended and placed in contact with the ground to propel the body forward and a **swing** phase, during which the limb is flexed to leave the ground and then brought forward to begin the next stance phase. In quadrupeds, changes in locomotor speed are also accompanied by changes in the sequences of limb movements. At low speeds, there is back-to-front progression of leg movements, first on one side and then on the other. As the speed increases, the movements of the right forelimb and left hindlimb are synchronised with the left forelimb and right hindlimb. Incredibly, despite the precise coordination and timing of the movement of individual limbs required for locomotion, following transection of the thoracic spinal cord, a cat or rat's hindlimbs will still make coordinated locomotor movements if the animal is supported and placed on a moving treadmill (Pearson, 1976; Edgerton and Roy, 2002; Rossignol et al., 2002). The speed of locomotion is determined by the speed of the treadmill, suggesting that the movement may be merely a reflex response

to stretching the muscles. However, this was ruled out because when the dorsal roots were also sectioned there were still locomotor movements, although they were slower and less coordinated (Pearson, 1976). These experiments led to the discovery that the basic rhythmic patterns of each limb involved in locomotion are dependent on **central pattern generators** – a local circuit in the spinal cord responsible for the alternating flexion and extension of the limb. In the human, this ability to mediate rhythmic stepping is reduced and likely reflects the dependence for bipedal locomotion on an increased requirement of upper motor neurone pathways, such as those involved in postural control (Purves et al., 2001).

After SCI, considerable recovery of motor/locomotor function is generally achieved when the ventral and lateral white matter is preserved (Basso et al., 2002). Injuries to either the dorsal corticospinal tract (CST) or rubrospinal tract do not produce persistent and severe locomotion deficits, except deficits of fine paw and digit movements (Whishaw and Gorny, 1998; Muir and Whishaw, 1999, 2000; Metz and Whishaw, 2000; Loy et al., 2002). In contrast, the reticulospinal tract plays a crucial role in the initiation of locomotor function (Jordan, 1998). In the acute decerebrate cat, the pontomedullary medial reticular formation is the origin of long fast-conducting descending projections that travel in the ventrolateral funiculus, and this pathway must remain intact to elicit hindlimb locomotor activity in response to brainstem electrical stimulation (Steeves and Jordan, 1980; Noga et al., 1991; Whelan, 1996). The loss of propriospinal tracts in the white matter may also contribute to the loss of locomotor function after SCI. Propriospinal cells are involved in the coordination of locomotor information between cervical and lumbar enlargements and may not be essential for locomotor function under normal conditions (McClellan, 1994). However, in the injured spinal cord, propriospinal projections may play a part in compensating functions of damaged supraspinal projections (McClellan, 1994; Rouse and McClellan, 1997). In mice, propriospinal relay connections that bypass one or more injury sites are able to mediate spontaneous functional recovery and supraspinal control of stepping, even when there has been essentially total and irreversible interruption of long descending supraspinal pathways (Courtine et al., 2008). Since regeneration of short propriospinal tracts may be a more achievable goal compared to long-distance axonal

regeneration (Guest et al., 1997), their axonal regrowth across a SCI site may be very important for restoration of locomotor function.

Locomotion on a grid requires sensorimotor coordination that allows proper placement of the foot on the grid bar. Descending pathways modulate locomotor function mediated by the lumbosacral circuitry, such as the rubrospinal tract in the dorsolateral funiculus and the reticulospinal tract that is more diffusely distributed in the ventral and lateral white matter (Zemlan et al., 1984; Jones and Yang, 1985; Brosamle and Schwab, 1997). The grid test shows long-term disruption, with increased footfaults, after a dorsal hemisection lesion or contusion that includes the dorsal columns and the CST (Grill et al., 1997). Changes in descending serotonergic and noradrenergic pathways and in primary afferent pathways could also be expected to modify locomotor function, e.g. through projections to the interneuronal pools comprising the central pattern generator (Mitsui et al., 2005).

*The somatic sensory system*

After SCI, there is considerable loss of sensation and there are tests in models of SCI that can measure recovery of sensation. The somatic sensory system can be divided into two major components: the mechanosensory system, for the detection of mechanical stimuli such as light touch and pressure, and the pain and temperature sensory system for the detection of painful stimuli and temperature.

A diverse set of cutaneous and subcutaneous sensory receptors at the body's surface relay information to the CNS for interpretation and action. There are additional mechanoreceptors located in joints, muscle and other deep structures called proprioceptors, which relay information generated by the musculoskeletal system. Mechanosensory information is carried to the brain by several ascending pathways that run in parallel through the spinal cord, brainstem and thalamus, to reach the primary somatic sensory cortex in the brain. The primary ascending sensory pathways in the human are the dorsal column-medial lemniscus, carrying the majority of mechano and proprioceptor information, and the spinothalamic pathways, mediating pain and temperature sensation. In the rat brain,

a substantial amount of the somatic sensory cortex represents the large facial whiskers that provide a large part of the sensory input for rats and mice. In humans, a larger amount of somatic sensory cortex is devoted to the hands and face. Thus, the sensory input that is particularly significant to a species gets relatively more cortical representation (Purves et al., 2001).

The cutaneous and subcutaneous sensory receptors can be subdivided based on function into at least three groups: mechanoreceptors, nociceptors, and thermoceptors. The perception of innocuous mechanical stimuli is associated primarily with myelinated Aα and Aβ axons that have relatively rapid conduction velocities: 70-120 m/s and 30-70m/s respectively (Loeser and Bonica, 2001; Djourhi and Lawson, 2004). In contrast, the axons associated with nociceptors conduct relatively slowly, but there are fast (about 20 m/s) and slow (about 2 m/s) pathways, which are commonly made up of finely myelinated Aδ axons, and unmyelinated C fibres (Purves et al., 2001).

Upon entering the spinal cord, afferents from peripheral mechanoreceptors branch into ascending and descending branches, which then send collateral branches to several spinal segments. The majority of the incoming axons ascend ipsilaterally through the dorsal columns to the lower medulla, where they terminate in the gracile and cuneate nuclei (together called the dorsal column nuclei). Axons in the dorsal columns that convey information from lower limbs are in the medial subdivision of the dorsal columns, called the gracile tract, and information from the upper limbs, trunk and neck are in the lateral subdivision, called the cuneate tract. At the upper thoracic levels, the dorsal columns account for more than a third of the cross-sectional areas of the human spinal cord. Despite this, lesions to the dorsal columns of the spinal cord have only a modest effect on simple tactile tasks. The ability to initiate movements related to tactile exploration may be impaired. This relatively mild deficit is possibly due to the fact that some axons involved in mechanosensation also run in the spinothalamic tract (Purves et al., 2001).

*Pain*

The sensation of pain is initiated by nociceptors, which are mainly 'free nerve cell endings'. Like somatic sensory receptors, nociceptors arise from cell bodies in dorsal root ganglia, sending one axonal process to the periphery and the other into the spinal cord. A variety of stimuli trigger afferent signals via these nerve cells. Painful stimuli are usually associated with tissue damage, such as from cuts or bruises. These may be caused by the release of pro-inflammatory substances such a prostaglandins and histamine, which enhance or sensitise the responsiveness of nociceptor endings. This has led to analgesic treatments such as aspirin, which acts by inhibiting the synthesis of pro-inflammatory prostaglandins. However, in some cases of nerve injury, painful sensation can last far beyond the period of initial damage. Chronic or neuropathic pain is experienced by a significant number of people living with SCI and is rather vaguely defined as **"**pain initiated or caused by a primary lesion or dysfunction in the nervous system" (Merskey and Bogduk, 1994). Neuropathic pain can arise from inflammation (e.g. neuritis), injury to nerve endings (e.g. from amputation) or nerve invasion by tumours.

The main pathways that carry information about noxious stimuli to the brain are the spinothalamic and spinoreticular tracts and they ascend anterolaterally in the spinal cord white matter. Like the sensory neurones in DRGs, the centrally projecting axons of nociceptive neurones enter the spinal cord via the dorsal roots and branch into ascending and descending collaterals, called the dorsolateral tract of Lissauer. Axons in Lissauer's tract run up and down for one or two spinal cord segments and then penetrate the gray matter of the dorsal horn. Once within the dorsal horn, the axons branch and contact neurones in several laminae. Axons from neurones in these laminae cross the midline and project to the brainstem and thalamus in the ventrolateral quadrant of the contralateral half of the spinal cord, forming the spinothalamic tract.

Since the mechanosensory pathway ascends ipsilaterally in the cord, a unilateral spinal lesion will produce loss of touch, pressure and proprioception below the lesion on the same side. However, diminished sensation of pain below the lesion will be observed on the side

opposite to the mechanosensory loss. This helps define the level of the SCI. The complexity of the pathways and mechanisms involved in the perception of pain makes chronic or neuropathic pain difficult to treat, such that the currently prescribed analgesics are largely ineffective. Therefore, research focused on the reduction of chronic pain is essential, since this pain greatly affects the quality of life, and frequently leads to depression and suicide (Segatore, 1994; Cairns et al., 1996).

*The autonomic nervous system*

Individuals with SCI have identified the recovery of autonomic functions as a high priority for improving their quality of life (Anderson, 2004). Recovery of bladder function will be explored in this thesis and is discussed in more detail in chapter 6. Briefly, normal lower urinary tract function requires the coordinated activity of the sympathetic, parasympathetic and somatic nervous systems. SCI induces profound changes in bladder innervation, particularly afferent circuitry, morphology and structure (Beattie et al., 1993). If the SCI is below the thoracic level (a lower motor neurone injury) areflexia persists (Blaivas et al., 1981; Kaplan et al., 1991). If the lesion is to the thoracolumbar region, and involves sympathetic preganglionic neurons, the bladder neck may also become hypoactive. Suprasacral (supraconal) SCI (an upper motor neuron injury) typically produces hyperreflexia of the smooth (detrusor) muscle of the bladder and tonic activation of the striated urethral sphincter. Detrusor hyperreflexia and detrusor–sphincter dyssynergia result from both a loss of tonic supraspinal inhibition and the emergence of aberrant spinal reflexes (Inskip et al., 2009).

## 1.2 Pathology of SCI

Traumatic SCI leads to several pathological changes, extending from minutes to years after the injury. The response to SCI can be divided into three phases: the acute phase, a phase of secondary tissue loss and a chronic phase (Bareyre and Schwab, 2003). In the acute phase, directly following impact, there is immediate mechanical damage to neuronal tracts from shearing of neuronal and endothelial cell membranes, leading to a haemorrhagic zone of necrosis predominantly in the grey matter due to the abundant vascularisation of this region

(Tator, 1995; Profyris et al., 2004). Blood flow is reduced, creating necrotic ischaemic conditions. Oedema of the cord develops and there is accumulation of intra-neuronal $Ca^{2+}$ and extracellular $K^{+}$. These processes result in failure of normal neural function, vasospasm and spinal shock (Schwab and Bartholdi, 1996; Bareyre and Schwab, 2003).

In the secondary phase of pathological changes, minutes to weeks after injury, there is expansion of the haemorrhagic front rostral and caudal to the primary injury site, continuing up to 3 days. This is accompanied by increased release of lipids and eicosanoid production, increased production of free radicals, inflammatory reactions and excessive release of excitatory neurotransmitters such as glutamate, thus overstimulating glutamate *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and producing excitotoxic conditions. There is both necrotic and apoptotic cell death of neurons, oligodendrocytes and microglia. Chronic depolarization leads to massive influx of intracellular calcium, together with mobilization from intracellular stores, and activation of calcium-dependent enzymes, leading to cell death (Anderson and Hall, 1993; Bareyre and Schwab, 2003; Profyris et al., 2004).

Traumatic SCI provokes a significant inflammatory response, with the recruitment of peripherally derived immune cells 6 h to several weeks after the injury. Continual activation of the immune system drives persistent inflammation, which extends in the chronic phase from days to years after the trauma. Apoptotic cell death continues, channel and receptor functions are impaired, and scarring and demyelination accompany Wallerian degeneration and conduction deficits (Lawrence et al., 2002). This secondary period of pathology may be amenable to treatment.

### 1.2.1 The cellular inflammatory response after SCI

One the first aims of this thesis is to characterize the inflammatory response after SCI. An overview of what has been described in various SCI models is given below.

The CNS has historically been considered to be an 'immune privileged' environment due to its inability to mount an immune response and to process antigens. This view was

consistent with our understanding of the blood-brain barrier, a specialised endothelium with tight junctions, separating the brain parenchyma from the blood. This was initially considered a barrier to protect the CNS. Tissue grafts, bacteria and viruses were all observed to evade immune recognition when delivered to the brain parenchyma, but in the 1920's it was reported that rejection of a foreign tumour did occur in the brain if it approached the ventricles (Galea et al., 2007). Since then, the definition of 'immune privilege' of the CNS has become too ambiguous to hold up to the discoveries made in recent decades. We now know that the CNS has the ability to mount a well-organised immune response, specific to different regions of the brain and spinal cord (Trivedi et al., 2006; Galea et al., 2007).

*The innate immune response*

The inflammatory response to trauma in the CNS involves the innate immune response, which is non-specific and acts as the immediate, first line of defence (Lawrence et al., 2002; Trivedi et al., 2006). The cells responsible for the innate immune response are principally the white blood cells (leukocytes) and microglia, the resident macrophages of the CNS (Trivedi et al., 2006). All of these cells originate in the bone marrow from a common progenitor called the hematopoietic stem cell (Fig. 1.3).

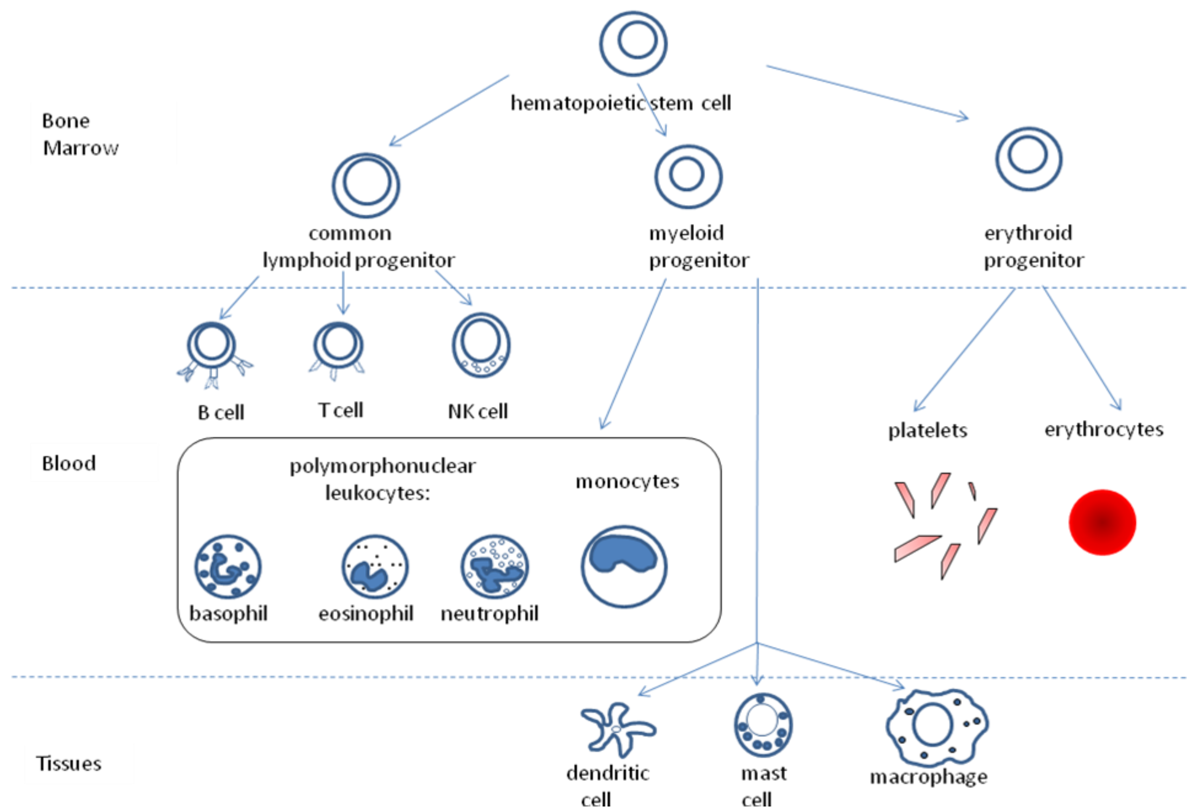



**Figure 1.3 Origin of inflammatory cells of the immune system.** Pluripotent hematopoietic stem cells divide and differentiate into progenitor cells that give rise to the lymphoid lineage, the myeloid lineage, and the erythrocyte lineage.

Blood granulocytes are the first cells to infiltrate into the injured parenchyma (Fig. 1.4), typically polymorphonuclear neutrophils. Approximately 100 billion neutrophils enter and leave the circulation daily in normal adults, outweighing production of erythrocytes by at least 2:1, and they usually live for only 1 or 2 days (Gallin and Snyderman, 1999). They are principally phagocytic and microbicidal cells, engulfing and killing microorganisms (Parham, 2000). They accumulate within hours in tissues and release their contents including myeloperoxidase (MPO), metalloproteases (MMPs), cytokines and chemokines, such as tumour necrosis factor-α (TNF-α), interleukins-1 and 8 (IL-1, IL-8) and transforming growth factor-β (TGF-β), which aid in loosening the extracellular matrix and activating resident macrophages (Profyris et al., 2004). They also generate reactive oxygen species that eliminate and digest invading organisms (Lawrence et al., 2002). Not long after releasing their contents, neutrophils enter the process of programmed cell death and

following apoptosis, their debris is phagocytosed by macrophages. Neutrophils are crucial for the first line of response and for summoning macrophages, as well as for the re-establishment of homeostasis. However, in the context of brain injury and SCI they are considered to play a significant role in lesion exacerbation, illustrated in studies showing that either depletion of circulating neutrophils, inhibition of neutrophil-related proteolytic enzyme activities, or inhibition of neutrophil adhesion, result in neuroprotection (Clark et al., 1996; Trivedi et al., 2006).

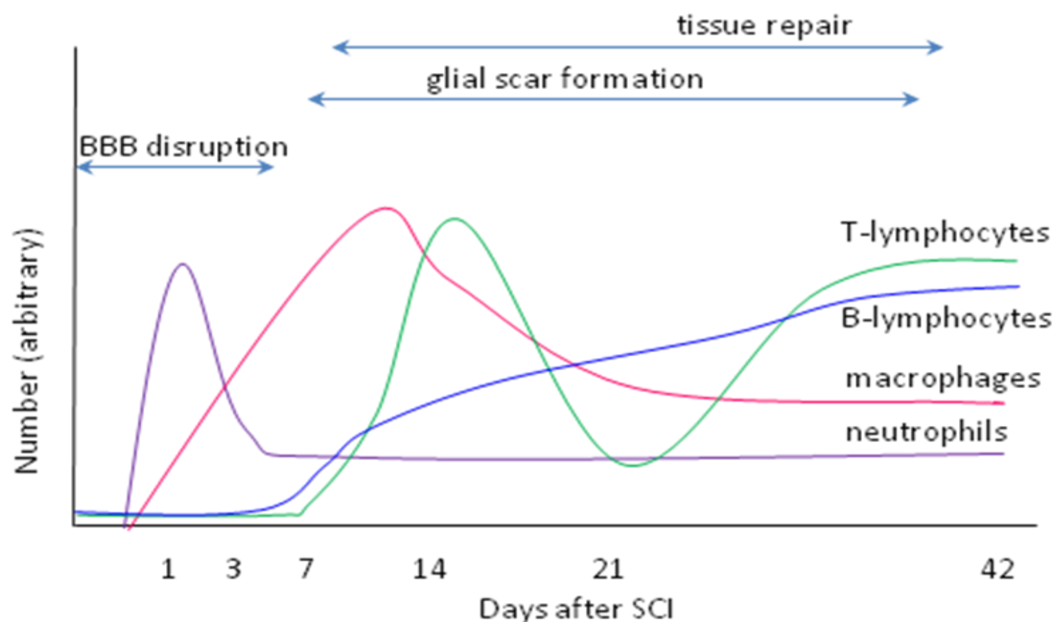



**Figure 1.4 Time course of acute inflammation after spinal cord injury.** This schematic illustrates the timing after SCI, of distinct cellular events as observed from animal studies to date (adapted from Trivedi et al, 2006)

By 48 h after injury, the recruitment of neutrophils declines (Profyris et al., 2004) and is followed by the entry of monocytes to the injured parenchyma, where they differentiate into macrophages (Popovich et al., 1997; Trivedi et al., 2006). The recruitment of monocytes after SCI is delayed, their numbers peaking between 7 and 14 days (Fig. 1.4). Macrophages are larger than neutrophils and are characterized by an extensive cytoplasm with numerous vacuoles (Gallin and Snyderman, 1999; Parham, 2000). Inflammatory macrophages proliferate and phagocytose debris. This further activates microglia, the resident macrophages in the nervous system (Gallin and Snyderman, 1999; Serhan and Savill,

2005). Activation of macrophages leads to secretion of inflammatory and potentially damaging mediators such as free radicals, eicosanoids, glutamate, cytokines and NOS, which drive leukocyte recruitment and activation. Macrophages are diverse in their response to injury and these same cells in non-neuronal tissues are usually self-limiting and considered to play a beneficial role by triggering the initiation of the resolution of inflammation by suppressing pro-inflammatory signalling. They can promote wound healing through the secretion of matrix metalloproteases (MMPs) that break down exudate, and of mediators that stimulate angiogenesis and even regeneration (Popovich et al., 1997; Lawrence et al., 2002; Serhan and Savill, 2005). In the injured spinal cord, macrophages accumulate chronically without any obvious benefit to recovery. Gensel et al. (2009) demonstrated that a single mode of activation (injection of zymosan), relevant to SCI, endows macrophages with the ability to simultaneously promote axon regeneration and kill cells. Acute non-specific depletion or inhibition of CNS macrophages is neuroprotective after SCI (Giulian and Robertson 1990; Blight 1994; Popovich et al. 1999; Mabon et al. 2000), but delayed implantation of macrophages stimulated by regenerating peripheral nerve segments is beneficial (Ralapino et al., 1998). Given the functional heterogeneity exhibited by CNS macrophages, selective therapies targeting these cells may only be feasible and safe clinically once we fully understand the mechanisms controlling CNS macrophage activation after SCI (Kigerl et al., 2007).

Glial cells, i.e. the microglia, astrocytes and oligodendrocytes, make up more than 70% of the total cell population in the CNS. Once thought of as merely a supportive system for neurons, glial cells are now regarded as key modulatory, trophic, and immune elements in the brain tissue. The main mediators of neuroinflammation are microglial cells and they migrate rapidly to the injury site (Banati et al., 1993; Kettenmann, 2007). Microglia express multiple phenotypes, with corresponding functions in tissue repair and/or damage (Schwartz and Yoles, 2006). In the adult CNS, they show three main phenotypes: 1) “resting”, with ramified processes extending in all directions from the soma; 2) activated, slightly larger, with retracted processes; and 3) reactive, or phagocytic, with swollen cell bodies and short cytoplasmic extensions (Flaris et al., 1993; Popovich et al., 1997). In the latter phenotype, microglia become difficult to distinguish from infiltrating macrophages

(Popovich et al., 1997; Schnell et al., 1999), which can make it difficult to interpret their potentially different roles in tissue.

After SCI, microglia are activated by various ligands released at the injury site and by antigen-presenting cells such as macrophages, via Toll-like receptors (TLRs), a family of receptors first discovered in Drosophila (Anderson et al. 1985) and located on the cell surface. Subsequently they produce growth factors, cytokines and chemokines, start to phagocytose debris and express major histocompatibility complex II (MHC II) molecules. They may present degraded peptides on their cell surface to helper T-lymphocytes as part of the adaptive immune response (discussed below). Activated microglia have been observed, using immunohistochemistry, by the first day after SCI, to increase in number by 7 days, then reach a plateau between 2 and 4 weeks (Fig. 1.4) (Popovich et al., 1997; Trivedi et al., 2006). Microglia have been implicated in necrotic and apoptotic neuronal cell death, through production of TNF-α, IL-1, reactive free radicals, and nitric oxide, each of which can be damaging to the spinal cord (Banati et al., 1993; Popovich, 2000; Profyris et al., 2004; Trivedi et al., 2006).

Glial-fibrillary-acidic protein (GFAP) content is increased in astrocytes after SCI, in a process termed reactive gliosis (Schnell et al., 1999). Astrocytes form a glial scar at the interface with the macrophage-infiltrated destruction zone (Klusman and Schwab, 1997) and upregulate chondroitin sulphate proteoglycans (CSPGs), extracellular matrix molecules which are inhibitory to neuronal growth, although the exact mechanisms remain to be clarified. Astrocytes release the proinflammatory cytokine IL-1, as well as mediators that promote CNS repair such as brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), in response to stimulation of macrophages and microglia, by IL-1 and TNFα (Profyris et al., 2004). Although scar formation provides a barrier for axonal regeneration, several recent studies have found that reduced astrocyte activity and astrogliosis after SCI worsened functional outcome (Faulkner et al., 2004; Herrmann et al., 2008; Renault-Mihara et al., 2008). Reactive astrocytes help seal off and isolate the necrotic core of the lesion site, re-establish the integrity of the blood-brain barrier, and detoxify molecules such as free radicals and glutamate (Sofroniew, 2005).

There are clear differences in the magnitude and timing of the innate inflammatory response, mainly involving monocytes, in peripheral tissues and the PNS compared to the CNS parenchyma, in response to the same stimulus. There are further differences between the brain and spinal cord, possibly due to differences in the amount of vascularisation (Andersson, 1992; Schnell et al., 1999). Leukocytes are noted to be preferentially recruited via the venous blood system and only occasionally from the arterial side. In the rat spinal cord, veins entering from the anterior median sulcus are almost twice as numerous as arteries, whereas in the rat brain there is a roughly equal proportion of veins and arteries (Schnell et al., 1999). The numbers of monocytes infiltrating the injury site after SCI is thought to be delayed and inadequate, and thus might mean there is inadequate removal of debris, leading to  can persistence of macrophages in the injury site after SCI without any improvement in recovery.

In summary, after SCI, prolonged presence of macrophages and activated microglia indicate a state of chronic neuroinflammation. The usual outcome of inflammation is successful resolution and repair of tissue damage, rather than the persistence of the inflammatory response, when there is scarring, loss of organ function and chronic disease, such as in the case of atherosclerosis and rheumatoid arthritis. Understanding why there is persistence of inflammation might help to explain the reasons why SCI leads to many irreversible destructive processes. It remains unclear to what extent the relative contributions of the opposing functions of macrophages affect progression of secondary damage. The beneficial events may be overshadowed by an excessive accumulation of toxic molecules produced by inflammatory cells that damage otherwise intact tissue (Trivedi et al., 2006; Farooqui et al., 1997b). Consequently, there has been a development of therapeutic strategies to modulate wound repair using macrophage secretory products.  If after SCI, manipulation of the immune response can aid in recovery, it is a worthwhile goal to optimise the potential anti-inflammatory properties of compounds such as omega-3 PUFAs (discussed later).

*The adaptive immune response*

There has been evidence of the involvement of the adaptive immune response after SCI from observations of lymphocyte infiltration to the injury site (Popovich et al., 1997; Schnell et al., 1999; Sroga et al., 2003). Compared to the innate immune response, the adaptive immune response is delayed but more specific, due to the processes of activation involved. It is not clear exactly how the adaptive immune system is activated after SCI, and there are differences between species in the amount and time course of lymphocyte infiltration. This will be discussed only briefly below, since the adaptive immune system will not form a major part of the work covered in this thesis.

Lymphocytes are smaller than leukocytes and have a life span of 1-2 months. They circulate in the blood and congregate in lymphoid tissues or organs such as the thymus, spleen, lymph nodes and adenoids. B lymphocytes (B cells) and T lymphocytes (T cells) are the two most important types. In mouse models, B cells have been found scattered throughout the site of SCI 1 – 7 days after injury (Schnell et al., 1999; Ankeny et al., 2006). In the area quantified, their numbers increased to several hundred by 42 days and B cells were found in large clusters resembling lymphoid follicles found in spleen/lymph nodes (Ankeny et al., 2006). There is very little known about the involvement of B cells in the injured cord. In a model of chronic neuroinflammation, i.e. experimental allergic encephalomyelitis, they are known to promote demyelination (Trivedi et al., 2006). B cells secrete antibodies – soluble forms of immunoglobulins that are released into the blood and extracellular fluid, and in particular autoantibodies which can bind CNS antigens and cause neuropathology in multiple sclerosis (Ankeny et al., 2006). All individuals have low numbers of autoantibodies present, but progression to autoimmune disease is rare (Popovich et al., 1996a). In contrast, in SCI, data from other laboratories has shown that B cells may help promote remyelination and axonal growth (Rodriguez and Lennon, 1990; Huang et al., 1999). These data have not been confirmed in human tissue.

Activated T cells are classified according to their function. Cytotoxic T cells typically express the molecule CD8 on their cell surface and kill tumour cells and infected cells. T

cells expressing the cell surface molecule CD4 activate the cells they recognise, such as macrophages and B cells, by secreting cytokines, in the lymph node or at the site of infection (Janeway et al., 2005; Trivedi et al., 2006). Using immunohistochemistry, Popovich et al. (1997), Sroga et al. (2003) and Schnell et al., (1999) have observed T lymphocytes in low numbers in the uninjured spinal cord. Recruitment to the injured parenchyma in rat tissue after SCI occurs within the first week, in parallel with microglial activation and infiltration of circulating macrophages. Since they are able to recognize specific antigens, such as myelin basic protein, and proliferate in response to those antigens, T cells have the potential for promoting more selective tissue damage than macrophages and neutrophils (Popovich et al., 1996a; Trivedi et al., 2006). In terminal human cases after SCI, very low numbers of CD8 T cells and even lower numbers of CD4 T cells have been found in the first 10 days and up to one year after SCI, but not B cells (Fleming et al., 2006). Some studies have demonstrated a neuroprotective role of autoimmune T cells (Hauben et al., 2000a; Schwartz and Hauben, 2002). Hauben (2000a) demonstrated the beneficial secretion of neuroprotective factors, remyelination, repair and recovery after SCI following immunization with T cells in rats. However, the timing, number and site of implantation of T cells is crucial (Schwartz and Yoles, 2006).

Individuals with SCI are highly susceptible to infection (Cruse et al., 1993) and this immune suppression is level-dependent, since lymphoid organs are innervated by sympathetic preganglionic neurones distributed throughout the thoracolumbar spinal cord. Therefore, a T3 SCI has been shown to impair antibody synthesis when compared to T9 SCI, which did not (Lucin et al., 2009). Clearly, there is dysregulation of the adaptive immune system after SCI, but this is only recently being understood.

### 1.2.2 The inflammatory cytokine response after SCI

Cytokines are small cell-signalling proteins and can be divided into interleukins, interferons, the TNF family and chemokines (Janeway et al., 2005). Apart from exploring the cellular inflammatory response after SCI, it will be important to consider the cytokine response after SCI, which precedes and regulates inflammatory cell infiltration and which

may be amenable to treatment with omega-3 PUFAs. Some of the more well-studied cytokines that are upregulated after SCI are summarized in Table 1.1.

| Cytokine | DNA/mRNA/protein | Timepoint | Up/down (+/-) regulated |
|---|---|---|---|
| TNF-α | mRNA[2, 7, 9] | 2, 3 h | +, normal by 6 h |
| | protein[5] | 1, 6 h | + |
| TGF-β | mRNA[1, 3, 5, 7] | 24 h – 7 d, 35 d | + |
| | protein[5] | 24 h, 168 h | + |
| MCP-1 | mRNA[8 1] | acute - hours | + |
| MIP-1α | mRNA[2, 3] | 3, 24 h – 4 d, 7d | + |
| MIP-1β | mRNA[2, 3] | acute - hours | + |
| IL-6 | mRNA[1, 2, 3,7, 9] | 2, 3, 6 h | +, - by 24 h |
| CINC | mRNA[6, 8] | 6h | + |
| | protein[6] | 4, 8, 12 h | + |
| IL-1β | mRNA[1, 2, 3, 7, 9] | 2 h | +, - by 24 h |
| | Protein[4] | | |

**Table 1.1 Changes in cytokine mRNA and protein expression in the spinal cord following SCI**. [1] = Bareyre & Schwab (2003); [2] = Bartholdi and Schwab (1997), [3] = Aimone *et al.* (2004), [4] = Nesic *et al*.,(2001), [5] = Tyor *et al*., (2002), [6] = Tonai *et al*., (2001), [7] = Streit *et al*., 1998, [8] = McTigue *et al.,* 1998. [9]=Pan *et al.,* 2002.

The release of cytokines after SCI is triggered by the binding of various injury-induced ligands such as heat shock proteins (HSPs), proteolytic cleavage products and extracellular matrix, binding to cell surface Toll-like receptors (TLR), such as TLR 2 and TLR 4 (Ohashi et al. 2000; Asea et al. 2002; Termeer et al. 2002). Under pathological conditions in the CNS, TLRs are found predominantly on microglia and macrophages (Laflamme and Rivest 2001; Zekki et al. 2002). Following SCI, the mRNA for TLR1, 2, 4, 5, and 7 are increased (Kigerl, et al., 2007). Zymosan (a TLR2 agonist) or lipopolysaccharide (a TLR4 ligand)-mediated activation of CNS macrophages causes focal axon pathology and demyelination, with transient loss of motor function (Popovich et al. 2002; Felts et al. 2005). However, TLR4 mutant and TLR2 knockout mice have impaired locomotor recovery relative to SCI

wild-type controls, which demonstrates that TLR2 and TLR4 are important in events which lead to post- SCI recovery (Kigerl et al., 2007).

Cells migrate towards the site of injury along gradients of chemoattractants such as macrophage inflammatory proteins (MIP) α and β, and the CXC chemokine cytokine-induced neutrophil chemoattractant-1 (CINC-1) (Bartholdi and Schwab, 1997; Parham, 2000; Profyris et al., 2004). Blocking the interaction between specific molecules expressed on leukocytes which bind to the surface of endothelial cells, attenuates the inflammatory response in the injured spinal cord, resulting in decreased oxidative damage and lipid peroxidation, and leads to improved neurological function (Mabon et al,. 2000).

Using *in situ* hybridization Bartholdi and Schwab (1997) showed that after spinal cord trauma IL-1β, TNFα and IL-6 were found in most cell types at the injury site 3 and 6 h after injury. IL-1β and TNFα have been shown to stimulate the production of each other (Pan et al., 2002), reaching a peak several hours after injury, but becoming negligible by 24 h. Binding of IL-1β and TNFα to their receptors induces the nuclear factor κ B (NFκB) pathway in neurones, microglia and endothelial cells, within 30 min after SCI. Active NFκB stimulates the production of inflammatory mediators such as reactive oxygen species (ROS), cytokines such as TNF-α, IL-1β and IL-6, the respiratory burst enzyme inducible nitric oxide synthase (iNOS), prostaglandins, arachidonic acid, proteases and endothelial cell adhesion molecules (CAMs) (Allan and Rothwell, 2001; Profyris et al., 2004). SCI also induces expression of TGF-β, although its expression is delayed compared to the pro-inflammatory cytokines, becoming measurable from 24 h and reaching a peak at 7 days. The protein levels of TGF-β continue to increase during the first week following SCI, and this is attributed to leukocyte infiltration and secretion of cytokines at the site of injury (Tyor et al., 2002). TGF-β counteracts the effects of proinflammatory cytokines by down-regulating iNOS and decreasing endothelial CAMs (Profyris et al., 2004). Monocyte chemotactic protein-1 (MCP-1) is a chemokine that acts on monocytes and T cells and expression levels for this cytokine were found to be upregulated 3 and 24 h after SCI (Allan et al., 2001; Aimone et al., 2004). It has been found in astrocytes, endothelial cells, and macrophages of rodents subjected to brain trauma, together with MIP α and β, and CINC-1

(Glabinski et al., 1996). Close correlation has been found between macrophage infiltration and elevated levels of these chemokines (Bartholdi and Schwab, 1997; Campbell 1997, 2003; Parham, 2000; Profyris et al., 2004).

Cytokines induce vasodilation and increase the permeability of blood vessels. This slows the blood flow and aids the migration of leukocytes through the epithelium of blood vessels into the tissues (Fig. 1.5). Crossing the specialised endothelium that constitutes the blood brain barrier is kept to a low level under normal physiological conditions (Engelhardt et al., 2006). On exposure to inflammatory mediators such as TNF-α, selectins and intercellular adhesion molecule (e.g. ICAM-1) are mobilized and delivered to the endothelial cell surface and bind to selectin ligands expressed on leukocytes. These interactions facilitate reversible cell-cell interactions such as rolling and 'intravascular crawling' along the vascular endothelium (Woodfin, et al., 2010). Integrins mediate firm arrest of leukocytes (Parham, 2000; Trivedi et al., 1996). Diapedesis of leukocytes of the endothelium can occur intercellularly, through the endothelium cell-cell junctions. Real-time imaging of activated lymphocytes (Engelhardt et al., 2006) has allowed us to see that crossing the compromised blood brain barrier, for example in the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis, occurs intracellularly (Fig. 1.5) and is dependent on integrins such as alpha4-integrins. Cells then cross the extracellular matrix, a movement facilitated by proteases that break down the membrane, followed by migration towards the site of injury (Fig 1.5; Parham, 2000).

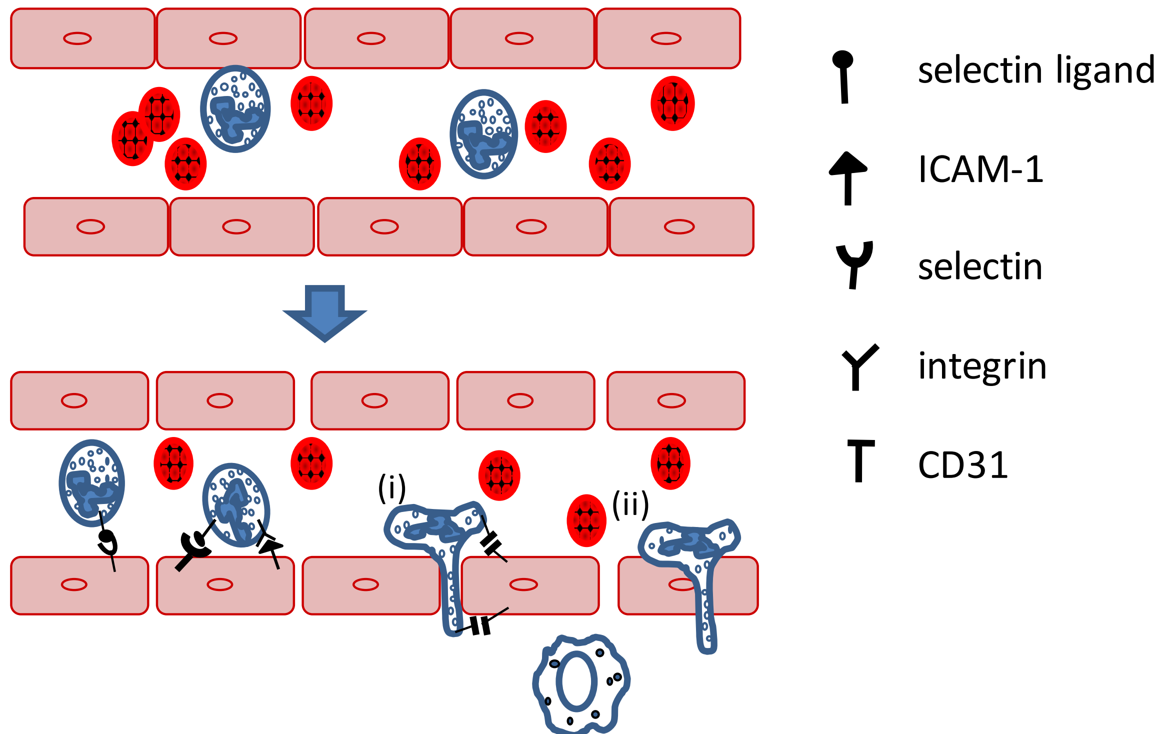



**Figure 1.5 Diapedesis of inflammatory cells.** Upon activation by inflammatory mediators, endothelial cells and leukocytes express receptors on their cell surface such as CD31 and selectins that bind to integrins and cell adhesion molecules (e.g. ICAM-1). The inflammatory cells in the blood are forced to slow and roll along the endothelial surface and then squeeze either i) intercellularly, or ii) intracellularly, through into the infected tissue.

The complement system will not be explored in this thesis, but it is also involved in the response to SCI. The complement system has been implicated in numerous CNS conditions, including traumatic brain injury (Kossmann et al., 1997; Stahel et al., 1998; Hicks et al., 2002; Rancan et al., 2003) and has recently been shown to be activated and play a role for at least 6 weeks after SCI (Anderson et al., 2004, Galvan et al., 2008; Beck et al., 2010). The three pathways of activation: classical, mannose-lectin binding and alternative, involve a series of plasma proteins which activate complement and the terminal pathway, i.e. the assembly of a lytic membrane complex, which can be bound to by phagocytic receptors on macrophages (Janeway et al., 2005). The three pathways can be activated by myelin (Morgan et al., 1995; Eggleton et al., 1998) and the therapeutic efficacy of complement inhibition has been demonstrated in stroke and brain ischemia models (Makrides, 1998; Barnum, 1999; Huang et al., 1999).

In summary, the cellular and molecular components of the inflammatory response after SCI are complex and remain to be fully understood. The findings described above offer some insight into how immunomodulation with anti-inflammatory treatments may be used to restore function after injury.

### 1.2.3 Systemic response to SCI

Cellular injury can release endogenous'damage'-associated molecular patterns (DAMPs; Matzinger, 1994). Zhang et al. (2010) have shown that injury releases mitochondrial DAMPs into the circulation. Thus, apart from the inflammatory response at the site of SCI, there is also a significant systemic influence, where acute-phase proteins (APPs) such as IL-1, IL-6 and TNF-α are secreted by hepatocytes in the liver and released into the circulation. The processes that are influenced by these APPs, such as leukocyte mobilization, fever, and changes in serum levels of glucocorticoids and cytokines, are part of the acute-phase response (APR) (Parham, 2000; Campbell et al., 2003). The systemic APR promotes a return to homeostasis in most cases. However, post-trauma recovery may be impeded by the development of multi-organ dysfunction syndrome and chronic disease (Campbell et al., 2003).

After SCI in the rat, the production of APPs is found to enhance the response to injury, increasing leukocyte recruitment, amplifying the inflammatory response in the damaged spinal cord and causing damage to the lungs and kidneys (Gris et al., 2008). Blocking this response using chemokine antagonists has been shown to lead to a corresponding decrease in the numbers of leukocytes recruited to the injured spinal cord and a reduction of damage to the blood brain barrier (Campbell et al., 2003, 2005; Davis et al., 2005). Although the hepatic chemokine response may not be specific to CNS injury, it opens the possibility for a new target for therapeutic intervention (Campbell et al., 2003). It will be interesting therefore, to also explore the systemic response in our model of SCI and changes after treatment with PUFAs.

## 1.3 Neuroprotective strategies following SCI

In the last decade there have not been any major developments in the treatment of SCI. However, there have been several promising advances in preclinical studies, some of which are in the process of being translated to the clinic and will be discussed below. Surgical intervention is usually the first approach made after SCI, in order to stabilise and decompress the spinal cord, followed by rehabilitative management, but these interventions will not be reviewed here. It has been shown that significant functional outcome after SCI could depend on the protection of a small number of axons. In rodents, the sparing of just 10–15% white matter can lead to recovery of stepping (Basso et al., 1996). Thus, neuroprotective strategies aim to attenuate the pathophysiological processes triggered after acute injury, thereby minimizing secondary damage and spread of damage. Studies which aim to enhance injury-induced adaptive changes (plasticity) rather than regeneration are also proving promising (Fouad et al., 2010). In addition, there are several novel methods of therapeutic delivery to the spinal cord that are being tested, such as viral vectors, nanoparticles and biomaterials. The following section will briefly highlight some of the most promising neuroprotective approaches that are being pursued.

Very recently, a selection of systematic reviews was published in the Journal of Neurotrauma (2010). The reviews were divided into three subjects and covered non-invasive pharmacologic treatments (Kwon 2010), cellular transplantation therapies (Tetzlaff 2010) and directly applied biologic therapies (Kwon BK, 2010). The review criteria were to select those with the most promising translational potential. A brief review of some of the most promising treatments will be given here.

Methylprednisolone (MP), a glucocorticoid, is the only approved drug therapy for SCI and has been shown to inhibit reactive oxygen-induced lipid peroxidation (Hall and Braughler, 1981), and decrease the number of neutrophils and macrophages in the injured spinal cord via the NFκB pathway (Bartholdi and Schwab, 1995; Klusman and Schwab, 1997; Profyris et al., 2004). Initial clinical results were promising, demonstrating a “modestly effective

therapy” when given within 8 h after SCI (Bracken et al., 1990). However, there remain serious concerns that the modest neurological improvements are not worth the associated side effects such as gastrointestinal bleeding and suppression of the immune system (Hall and Springer, 2004; Trivedi et al., 2006).

The main pharmacological interventions for SCI which are already in a form that can be used in the clinic include: erythropoietin, non-steroidal anti-inflammatory drugs (NSAIDs), anti-CD11d antibodies, minocycline, progesterone, estrogen, magnesium, riluzole, polyethylene glycol, atorvastatin, inosine, and pioglitazone. Some of these will be discussed below. A further treatment that has potential is systemic hypothermia (Jou et al., 2000; Yu et al., 2000; Morino et al., 2008; Lo et al., 2009).

Erythropoietin (EPO), a cytokine/hormone that regulates red blood cell production, has been shown to be neuroprotective in a variety of models of CNS and PNS injury such as stroke, peripheral nerve injury and contusion or compression SCI (Gorio et al., 2002; Grasso et al., 2006; King et al., 2007). Evidence in our laboratory has shown that derivatives of EPO such as carbamylated EPO (CEPO), that lack its erythropoietic effects, also show neuroprotective effects comparable to that of EPO, following a unilateral hemisection (King et al., 2007) and elsewhere following compression SCI (Leist et al., 2004).

Ibuprofen, a NSAID which reduces RhoA activation, has recently been found to promote both histological and behavioural improvements in thoracic contusion SCI models when administered 3 days post injury (Wang et al., 2009) or 7 days post injury (Fu et al., 2007).

The anti-CD11d antibody approach has been studied primarily by Dr. Lynne Weaver and colleagues (Gris et al., 2004; Saville, et al., 2004; Weaver, et al., 2005). The CD11d antibody is directed against a subunit of the CD11d/CD18 integrin that is expressed only by certain leukocytes such as neutrophils and monocytes. It reduces neutrophil and macrophage invasion, decreasing inflammation and lipid peroxidation, and improving tissue sparing. Behavioural outcomes revealed improved locomotion, decreased autonomic

dysreflexia, and reduced mechanical allodynia. Other CD11 integrins containing the subunits a–c have more widespread cellular distributions and antibodies directed against them have not been as effective as neuroprotective treatments. A 6-12 h post injury time window of intervention has been established, which is important with respect to clinical applicability.

The pathogenic influx of sodium into cells after CNS injury has led to research into the use of sodium channel blockers such as riluzole (Ates et al., 2007). Schwartz and Fehlings (2001) found that rats given riluzole after compression SCI had sparing of grey and white matter and recovery of behavioural function. Therapeutic neuroprotective efficacy has been reported with a delay in intervention of 15 min (Springer et al.,1997) and 30 min (Stutzmann et al., 1996). Mu and colleagues (2000b) reported that with a delay of 2 h after thoracic contusion SCI, riluzole alone did not have a beneficial effect either histologically or behaviorally, but it did when given in combination with methylprednisolone.

Minocycline, a second generation tetracycline and anti-inflammatory agent has been shown to provide significant neuroprotection and recovery of hindlimb function after contusion injury in rats. The drug inhibits mitochondrial cytochrome c release and reactive microgliosis (Teng et al., 2004). A recent study in a balloon-compression model of SCI in rats, showed that treatment with MC led to sparing of axons, but there was no locomotor recovery (Saganova et al., 2008). Other studies reported neuroprotective effects of minocycline (Wu et al., 2002; Wells et al., 2003). A recent study by Marchand and colleagues (2008) reported an attenuation of neuropathic pain after intraperitoneally administered minocycline. Presently, intravenously administered minocycline is under evaluation for the management of SCI in a phase I-II pilot study in Calgary, Alberta.

Labombarda and colleagues (2002) demonstrated that the hormone progesterone is neuroprotective. Progesterone is produced by neurones and glia in the central and peripheral nervous system of both male and female individuals. It has been tested worldwide in models of CNS trauma. However, of the two studies that utilized contusion SCI injuries, the results are contradictory. One reported no histological or behavioural

improvement in progesterone-treated animals (Fee et al., 2007), while the other reported more white matter sparing and significant behavioural improvement (Thomas et al., 1999).

Although pro-regenerative rather than neuroprotective strategies, the degradation of inhibitory chondroitin sulfate proteoglycans with chondroitinase ABC (Bradbury et al., 2002), the neutralization of myelin-mediated inhibition of neurite outgrowth with anti-Nogo (IN-1) antibodies (Schnell and Schwab 1990) and the inhibition of Rho activation with, for example, the Rho antagonist Cethrin® (Dergham, et al., 2002), have all been successfully attempted in pre-clinical studies, and some of them are at present in clinical studies. The positive effects of these treatments are thought to be due to regeneration of damaged axons but it is difficult to tell and equally possible that the behavioural improvements from these studies are due to compensatory sprouting of axons above the injury site. The main target of these agents is promotion of axonal growth/sprouting/plasticity by altering the inhibitory SCI environment or the response of axons to this environment. Yet most of the agents are tested in an acute injury paradigm, with immediate application at the time of injury. Separating the behavioral responses attributable to axonal growth versus those attributable to neuroprotection is difficult (Kwon et al., 2010). These studies need to be repeated with a clinically relevant therapeutic paradigm, along with the establishment of a clear and realistic time window of intervention before successful translation will be achieved.

Cellular transplantation therapies used in SCI research are promising and those studied have included Schwann cells (Lu et al., 2007; Zhang et al., 2007), olfactory ensheathing glial cells (Li et al., 1997), embryonic and adult neural stem/progenitor cells (Iwanami et al., 2005; Alexanian et al., 2006), fate-restricted neural/glial precursor cells (Han et al., 2004; Cao et al., 2005), and bone-marrow stromal cells (Ankeny et al., 2004). Many of these cell types when transplanted secrete trophic factors, which may have neuroprotective effects and/or promote plasticity in the spared spinal cord. Hence, it is difficult to attribute to one single mechanism the beneficial effects of these therapies. Nearly all cellular transplantation studies have been conducted in rodents, with the transplantations occurring 1-2 weeks after SCI, since transplantations performed immediately after injury generally

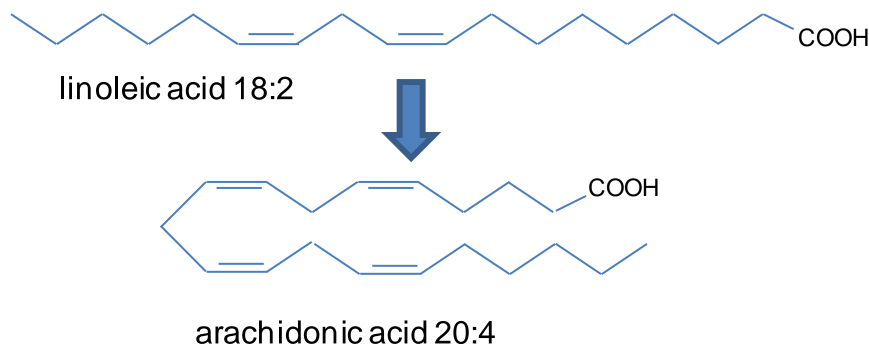
yielded poor results. This was mostly attributed to the initial phase of the inflammatory response. Treatments delivered in the chronic have been rare and have often failed to yield functional benefits (Tetzlaff et al., 2010).

There is an active search to provide better treatments for SCI patients, and although several novel and effective advances have been made in preclinical work, we have only just begun to see some of these treatments translated successfully into clinical trials in the human. One of the reasons for this is the complexity of the secondary degenerative response. Many treatments affect only one aspect of this response, and a successful treatment will probably have to target several of these mechanisms, i.e. possibly use a combination of the treatments described above. Combinatorial attempts have already been made in experimental models of SCI, such as the combination of cell transplants with neurotrophins (For review, Bunge and Pearse, 2003) or chondroitinase (Fouad et al., 2005). The main lesson that has been learned is that two or more treatments that are successful alone, when combined have complex interactions and may not act synergistically as expected.

### 1.4 Polyunsaturated Fatty Acids (PUFAs)

Fatty acids (FAs) consist of a carboxylic acid head and a long unbranched, carbon-hydrogen tail of varying lengths, which is either saturated or unsaturated. FAs required by mammals can be divided into three categories: i) saturated FAs, which do not have any double bonds, ii) monounsaturated FAs with a single double bond, and iii) polyunsaturated fatty acids (PUFAs) which contain multiple double bonds. The nomenclature for FAs is based on the number of carbon atoms and their saturation state. Thus, docosahexaenoic acid (DHA) contains 22 carbons and 6 double bonds and is therefore denoted 22:6; eicosapentaenoic acid (EPA) has 20 carbons and 5 double bonds and is therefore, 20:5. PUFAs can be further categorised according to the position of the first double bond from the methyl end of the acyl chain. **Omega-3** fatty acids such as alpha-linolenic acid (ALA, 18:3), EPA, and DHA contain the double bond at the third carbon position from the methyl group and **omega-6** fatty acids such as linoleic acid (LA, 18:2) and arachidonic acid (AA, 20:4) contain the double bond at position 6 from the methyl group (Fig. 1.6).

**Omega-6 PUFA**




**Omega-3 PUFA**

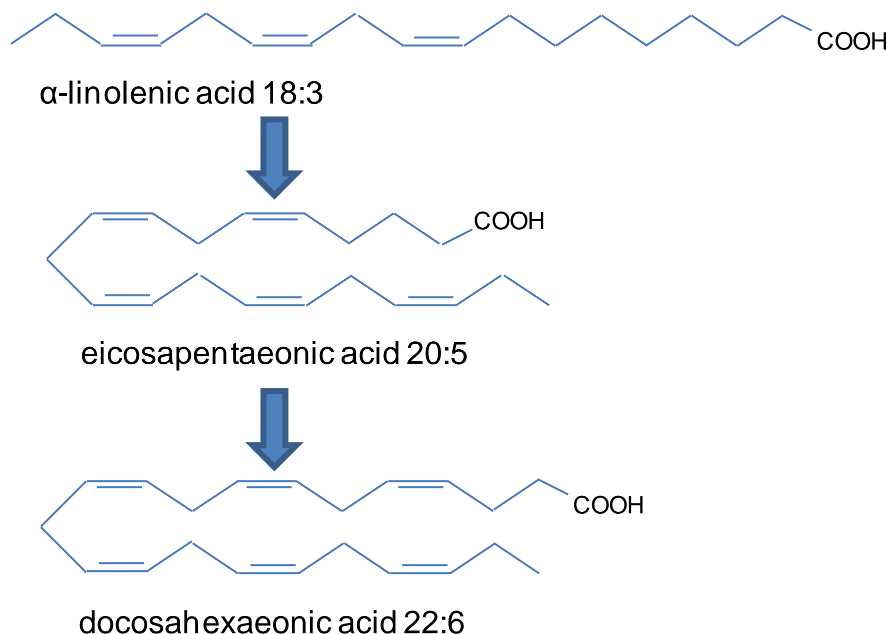



**Fig. 1.6 Structural formulae of common omega-3 and omega-6 PUFA**

Mammals are unable to synthesize PUFAs *de novo*, since they lack the necessary desaturase enzymes to introduce double bonds between carbon 1 and 9 (Salem Jr., 1989). Hence, they are primarily obtained directly from the diet or synthesized from the dietary precursors LA and ALA by a series of elongation and desaturation steps in the liver, and delivered by specific transporters to the brain. PUFAs such as DHA (to a lesser extent EPA) are located primarily in cell membranes as a structural component of phospholipids

and are released upon enzymatic action of phospholipases (Sprecher et al., 1995; Youdim et al., 2000; Igarashi et al., 2007a, b; for review, see Schwenk et al., 2010).

The CNS has the second highest concentration of lipids after adipose tissue, with a large proportion of brain lipids being comprised of PUFAs, particularly AA and DHA (Crawford et al., 1981; Salem Jr, 1989; Horrocks and Farooqui, 2004). Lipids constitute 70 – 75 % of the dry weight of myelin (Sastry, 1985). Normal brain function and structure has been proposed to depend on an optimal balance between omega-3 and omega-6 PUFAs and if this balance is disturbed, neurological deficits (Noaghiul, et al., 2003) and cognitive changes (Conquer, et al., 2000) may result, such as impaired learning in rodents (Enslen et al., 1991) and visual abnormalities in humans (Birch et al., 1992). Not surprisingly, therefore, the brain is highly resistant to dietary deficiency of PUFAs and of DHA in particular (Bourre et al., 1992; Rapoport et al., 2007).

Replacement of omega-6 with omega-3 fatty acids, such as EPA and DHA from enrichment in the diet, into structural phospholipids of neural membranes influences membrane fluidity, membrane vesicle formation and function, membrane permeability and gene expression, and decreases the transcriptional activation of many genes, including adhesion molecules, chemoattractants, and inflammatory cytokines (Stubbs and Smith, 1984; Stillwell and Wassall, 2003; De Caterina and Massaro, 2005). Lipid rafts have been proposed to float within the plasma membrane and influence signal transduction, trafficking and dynamics of receptors, enzymes and ion channels in the lipid bilayer (Tsutsumi et al., 1995; Yehuda et al., 2002; Isbilen et al., 2006; Farooqui et al., 2007a). Lipid rafts incorporate and are potentially modifiable by dietary fatty acids which can increase the clustering of lipid raft proteins (for review, see Yaqoob and Shaikh, 2010). By a direct interaction with the membrane and membrane-bound proteins, phospholipid-bound DHA can alter the speed of signal transduction and the formation of lipid rafts. PUFAs and DHA in particular, enhance neurite outgrowth, modulate neuronal signalling and neurotransmission and regulate gene expression (For review, see Farooqui et al., 2007a-c). Omega-3 PUFAs reduce neuronal excitability via background $K^+$ channels (Lauritzen et al., 2002, Chemin et al., 2005). PUFAs are agonists of transcription factors, including those of

the steroid/thyroid/retinoid superfamily, which includes the peroxisome proliferator-activated receptors (PPARs) and the retinoid-X-receptors (RXRs; for review, see Neerven and Mey, 2007). In the normal and contused spinal cord, RXR and PPARs have been shown using immunohistochemistry, to appear in the nuclei of reactive microglia and macrophages but the interpretation of changes in RXR and PPARs signalling after SCI remains to be clarified (Genovese et al., 2005; Schrage et al., 2006; Mucida et al., 2007).

### 1.4.1 PUFAs and Inflammation

Work in several laboratories has shown that DHA and EPA have potent anti-inflammatory properties on a wide range of immune functions, such as lymphocyte adhesion to endothelial cells (Khalfoun et al., 1996), antigen presentation (Hughes et al., 1997) and adhesion molecule expression (Harbige, 2003), and production of proinflammatory cytokines such as IL-1, IL-6 and TNF-α (Caughey et al., 1996; Wu and Meydani, 1998). DHA and EPA can inhibit the NFκB system, thus preventing the expression of pro-inflammatory cytokines including TNF-α and IL-1α and β, and can modulate leukocyte trafficking (De Caterina and Massaro, 2005; De Smedt-Peyrusse et al., 2008).

Human immune and inflammatory cells are typically rich in omega-6, with LA and AA comprising about 30% of fatty acids present (Gibney and Hunter, 1993; Yaqoob et al., 2000). This can affect pro-inflammatory signalling, since AA is oxidized into pro-inflammatory eicosanoids through three major metabolic routes: (i) the cyclooxygenase (COX) pathway to produce 2-series prostaglandins and thromboxanes, (ii) the lipoxygenase (LOX) pathway producing 4-series leukotrienes, hydroxyeicosatetraeneoic acids (HETEs) and lipoxins, and (iii) the cytochrome P450 (CYP) pathway to produce epoxyeicosatrienoic fatty acids (EETs) and hydroxylated HETEs, which are respectively primarily vasodilators or vasoconstrictors (Capdevila, et al., 2002a, b; Spector and Norris 2007). CYPs play a central role in the metabolism of endogenous eicosanoids (Capdevila et al., 1992; Oliw et al., 1982).

The intake of DHA and EPA, when provided in sufficient amounts, enriches the omega-3 content of leukocytes and can modify the functional responses of immune cells and modulate the expression of pro-inflammatory genes and the availability of lipid mediators (De Caterina and Massaro, 2005). EPA (VanRollins et al., 1988) and DHA (VanRollins et al, 1984) are metabolised into epoxidised and hydroxylated derivatives by CYPs from rat liver microsomes. EPA acts as a competitive substrate to AA for COX and generates the less inflammatory products, 3-series prostaglandins and thromboxanes, and 5-series leukotrienes. DHA metabolism by LOX produces docosatrienes, protectins, maresins and neuroprotectins (Hong et al., 2003; Marcheselli et al., 2003; Serhan et al., 2004, 2009; Serhan and Savill, 2005). At the same time, the metabolism of EPA and DHA by COX and LOX enzymes can generate anti-inflammatory and pro-resolving metabolites such as the resolvins of the E and D series, respectively, which antagonise the action of pro-inflammatory eicosanoids and are actively involved in the resolution of inflammation (Fig. 1.7) (for review, see Serhan et al. 2005, 2008; Farooqui et al., 2007b; Calder 2010). Thus, evidence suggests that the generation of omega-3 fatty acid metabolites from EPA and DHA may provide potent anti-inflammatory protective mechanisms for preventing neuronal damage after trauma.

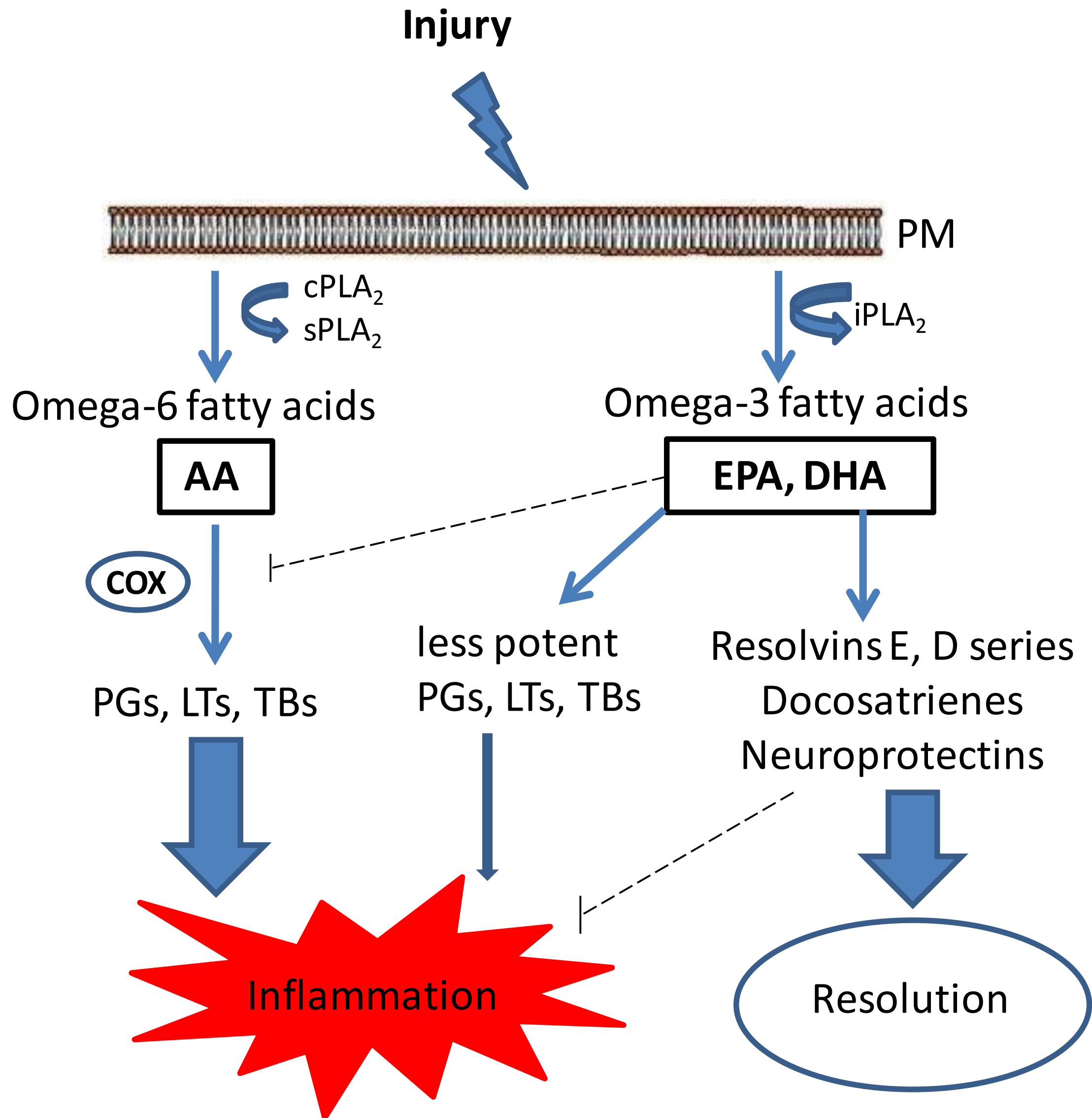



**Figure 1.7 Anti-inflammatory effects of DHA, EPA and their metabolites after injury.** Arachidonic acid (AA), via cyclooxygenase (COX) produces prostaglandins (PG), leukotrienes (LT), and thromboxanes (TB). DHA and EPA are metabolised into resolvins, docosatrienes and neuroprotectins. Together with DHA and EPA, these lipid mediators antagonise the effects of AA-derived PG and TB. Plasma membrane (PM); calcium-dependent cytosolic phospholipase A2 (cPLA2); calcium-independent (iPLA2); secretory phospholipase A2 (sPLA2). Adapted from Seki et al. (2009).

### 1.4.2 PUFAs, CNS injury and SCI

After SCI, there is fast release of free fatty acids (FFA), due to the activation of membrane phospholipases and lipases (Anderson and Hall, 1993). After impact injury to the spinal cord, significant elevation of total FFAs in grey matter can occur as early as 1 min. The increase in total FFAs in white matter occurs later and is less marked compared with that in grey matter. This correlates with the progression of spinal cord trauma, which after contusion or compression SCI extends from gray matter to white matter. Because of the specific phospholipid composition of neurones, AA is the primary fatty acid released. Levels of free AA in the injured spinal cord can exceed 20 times the control values, 6 h after the initial injury (Demediuk et al., 1985a, b, 1989; Murphy et al., 1994). Demediuk and colleagues (1989) demonstrated that a severe impact SCI resulted in decreased total phospholipid content for up to 3 days. However, there was no major class of phospholipids selectively hydrolyzed (Demediuk et al., 1985a; Demediuk et al., 1989; Yoshihara and Watanabe, 1990). Murphy et al. (1994) reported no detectable changes in phospholipid levels after a severe SCI to rat thoracic spinal cord. It was suggested that the release of FFAs and, in particular of AA, may not always involve general membrane degradation but rather an up-regulation and/or overstimulation of phospholipases (Murphy et al., 1994). Cytosolic phospholipase $A_2$ ($cPLA_2$) and secretory $PLA_2$ ($sPLA_2$; Fig. 1.7) may be the primary enzymes responsible for phospholipid hydrolysis and the AA release associated with SCI (Keyser and Alger, 1990). As discussed earlier, AA is the major source of eicosanoid synthesis and therefore the high levels released after SCI are likely play a significant influence in the subsequent inflammatory response.

In a model of transient spinal cord ischaemia in rats, the administration of an omega-3 fatty acid, α-linolenic acid (ALA) after injury, decreased neuronal loss and improved functional outcome (Lang-Lazdunski et al., 2003). In addition, DHA can counteract glutamate-induced excitotoxicity (Wang, et al., 2003). As excitotoxicity, inflammation and oxidative stress are part of the pathogenetic mechanisms involved in the secondary injury associated with SCI, this led King, Huang and colleagues (2006, 2007a) in our laboratory to explore the potential of using omega-3 fatty acids as neuroprotective agents after SCI. They found

that a single bolus intravenous injection of ALA, or DHA ( 250 nmol/kg), within 1 hour of hemisection SCI, or of DHA within 30 min of compression SCI, induced significant neuroprotection, by reducing neuronal and oligodendrocyte cell loss at 1 and 6 weeks and protection of myelin in the injury epicentre. Furthermore, locomotor function was improved, lipid peroxidation, protein oxidation and cyclooxygenase-2 levels were decreased at 24 h, and there was decreased macrophage recruitment to the spinal cord at 1 and 6 weeks (Huang et al., 2007a). In contrast, the omega-6 fatty acid AA administered after injury exacerbated injury, increased the size of the spinal cord lesion, decreased neuronal and glial cell survival and worsened the functional outcome. We have yet to explore whether the neuroprotection of neuronal and non-neuronal cells extends above and below the injury site and whether the effect is dose related.

Huang et al. (2007a) demonstrated that by adding DHA to the diet of the animals after injury for 6 weeks (400 mg/kg/day) led to additional improvements of the above outcome measures and amplified the effects of the DHA injection (Huang et al., 2007a). To explore the effects of increasing endogenous levels of omega-3 PUFAs, the *fat-1* transgenic mouse has been manipulated by researchers to express the Caenorhabditis elegans *fat-1* gene encoding an omega-3 fatty acid desaturase that does not exist in mammalian cells. This enzyme enables the generation of endogenous omega-3 PUFA by introducing a double bond, thus converting dietary omega-6 to omega-3 PUFA and leads to an abundance of omega-3 fatty acids in organs and tissues. Preliminary data from our laboratory suggests that the endogenously raised levels of omega-3 fatty acid confer resistance to injury in a mouse model of compression SCI. Recently, we have shown that intravenous EPA injection at 30 min after compression SCI in the rat is also neuroprotective, inducing tissue protection and better functional outcome (Lim et al., *in press*), although the effects appear to be weaker than those of DHA. We have not yet considered the effects of enrichment of EPA in the diet and it is important that we explore the effects of both EPA and DHA in other models of SCI, apart from compression and hemisection SCI.

In summary, omega-3 PUFAs are pleiotropic compounds and have been shown to regulate neuronal function by various mechanisms. The mechanisms are far from being fully

elucidated but it is evident that both structural and biochemical mechanisms play central roles in their actions. Alongside these studies, a considerable amount of research has revealed anti-inflammatory properties of PUFA and since there is a significant inflammatory component to SCI pathology, the potential interaction of this response with PUFA needs to be explored in more detail in SCI. Our laboratory previously found that DHA injection led to a reduction in the amount of macrophages in the epicentre after SCI, but we have not yet looked at neutrophils, activation of microglia and the additional cellular and molecular markers of inflammation.

## 1.5 Models of SCI

Before transfer into clinical trials, the efficacy and safety of new treatments for SCI must be investigated in animal models. Historically, the first experimental model of SCI, a weight-drop procedure in dogs, was introduced by Allen nearly a century ago (1911). Nowadays, rats and mice are most frequently used in SCI studies, as well as invertebrates, for developmental and regeneration studies. There are also various *in vitro* models of neuronal injury and SCI but these will not be reviewed here.

There are various *in vivo* models of SCI that can be used in preclinical research, and the choice of these depends mainly on the questions being addressed. For example, to explore the regeneration/sprouting of axons or a particular mechanism of neurone-glia interaction, a smaller, more reproducible, 'clean' lesion is preferred, such as a partial or full transection injury such as a dorsal column lesion, or a dorsal/lateral hemisection. On the other hand, in order to assess the efficacy of treatment for use in clinical trials, a more clinically relevant model of SCI should be used, such as the contusion (impact) or compression models of SCI. These models mimic many of the pathophysiological and morphological alterations observed in human SCI.

The modern contusion model induces instant mechanical deformation of the spinal cord by dropping a controlled weight onto it (discussed in chapter 5). Compression models of SCI involve a static injury to the spinal cord in which either a weight is placed on the spinal

cord (Nystrom et al., 1988; Holtz et al., 1989, 1990a), or the tissue is compressed using a modified aneurysm clip (Rivlin and Tator, 1978; Fehlings and Tator, 1995). Contusion and compression injuries are usually severe and this means a significant amount of time is involved in animal care, due to loss of autonomic function, requiring manual voiding of the bladder 2-3 times per day.

These models are not without their drawbacks. First, any SCI in an animal will be performed under anaesthesia, and animals will not receive the myriad of other drugs often required by acute trauma patients. A confounding factor to bear in mind in all of these studies is that there is spontaneous regeneration of the CNS in rodents, as well as other profound genetic and species differences in the pathology of SCI (Mills et al,. 2001; Basso et al, 2006; Kigerl et al., 2006).

Our laboratory has used the compression model of SCI to assess the effectiveness of various neuroprotective treatments, such as DHA and EPA (Fig. 2.1; King et al., 2006; Huang et al., 2007a, b). Although there are similarities in gross histolopathological changes, particularly in the ventral horns and grey matter, the compression model of SCI differs from the contusion SCI model in the amount and duration of neuronal loss (Huang et al., 2007b) and in some dynamics of the inflammatory response; this is discussed in more detail in chapter 5.

In summary, a substantial body of data supports the use of omega-3 PUFAs and their metabolites as neuroprotective and anti-inflammatory agents. The increased understanding of the involvement of neuroinflammation after SCI has attracted considerable interest in treatments which modulate its effects. It remains to be answered fully, whether treatment with omega-3 PUFAs has an impact on the inflammatory response after SCI. The use of PUFAs as treatment after SCI will be relatively easy to transfer to the clinic, due to their safety and minimal toxicity, but it would be extremely beneficial to have precise knowledge of the time course of entry of various inflammatory cells into the injured human spinal cord and activation of resident spinal cord cells such as microglia, and of their many actions, destructive or beneficial, that affect neural tissue in order to develop second generation, more selective treatments.

## 1.6 Aims

Omega-3 PUFAs have significant potential in the treatment of SCI. However, before translation to the clinic, and in order to minimise the risk of translational failure, it is important to i) gather more information on the impact of these compounds on the very early events after injury, a period characterised by complex inflammatory reactions, ii) validate the neuroprotective effects in additional species and models of injury and finally also to iii) explore in more depth the various regimes of administration that could be considered for treatment and/or prophylaxis, and define the optimum compound,

Thus, the studies that follow are divided into four chapters:

**I.** The characterisation, using immunohistochemistry, of the cellular inflammatory response in the spinal cord following rat compression SCI

**II.** The assessment of the effects of acute DHA or EPA injection on the acute inflammatory response following compression SCI

**III.** The assessment of the effects of acute DHA injection in rat contusion SCI.

**IV.** The assessment of the effects of dietary treatment with EPA and DHA in rat compression SCI.

# Chapter 2: Materials and Methods

## 2.1 Spinal cord injury

All experimental protocols were approved by the Animal Care Committee of Queen Mary, University of London, in accordance with the UK Animals Act 1986 and with international guidelines on animal use.

*Compression SCI (Chapters 3, 4, 6)*

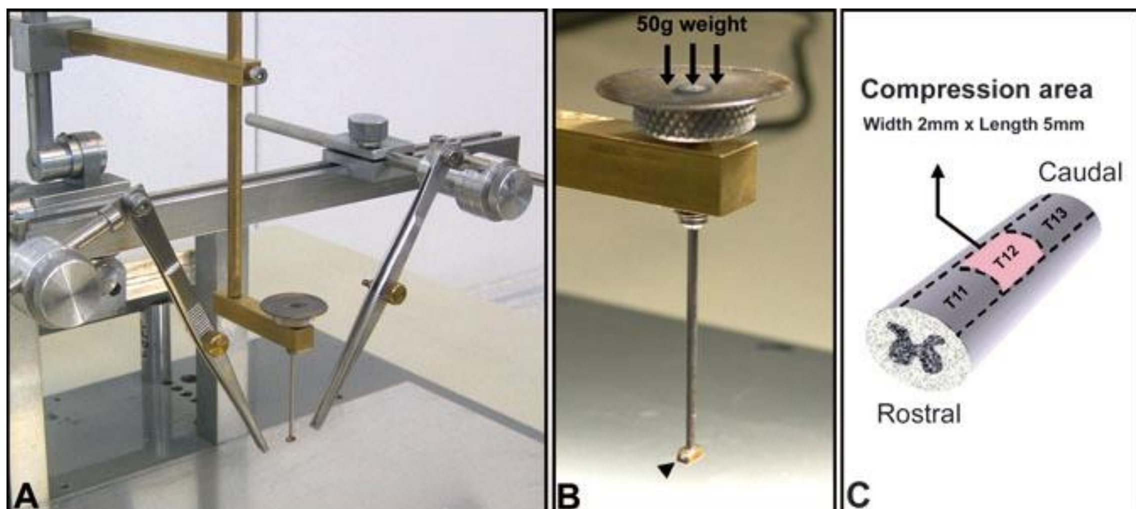



**Figure 2.1 The compression device. (A)** T11 and T13 vertebral processes are clamped in a frame and **(B)** the compression device is lowered onto the surface of T12 **(C)** followed by the addition of a 50 g weight for 5 min (image reproduced from Huang et al. 2007b).

Compression SCI was performed at vertebral level T12, following the methods described by Huang et al. (2007b) on adult female Sprague-Dawley rats (200-250 g; Charles-River, Margate, UK). Animals were deeply anaesthetized in a fume box with a mixture of 4% isoflurane (Meril, Essex, UK) in addition to a mixture of oxygen at a flow rate of 750–1000 mL/min. Subsequent anaesthesia throughout the procedure was maintained using 1.5–2% isoflurane with oxygen delivered through a nosepiece. After checking for absence of hindlimb and corneal reflexes, a midline incision was made with a scalpel through the skin and muscle overlying the spinal column. Vertebral level T12 was located by palpation of the spine and ribcage: the bottom of T12 is located at the position where the last rib intersects the spine. A retractor was put in and all muscle was removed and ligaments

incised around the T12 vertebra. A laminectomy was then performed on T12 using micro-rongeurs. Bone fragment and blood was removed using forceps and cotton buds, leaving the dura undisturbed. The T11 and T13 vertebral processes were left intact and clamped in a spinal compression frame (Fig. 2.1A), and compression was applied by suspending the base of the compression platform (area 2 x 5 mm, Fig. 2.1B) onto the exposed cord, under microscopic control. A weight of 50 g was applied statically to the platform for exactly 5 min. Tail flick confirmed good placement of the platform. The platform and retractor was then removed and the injury area checked for the development of a central bruise, or enlargement of the dorsal blood vessel (Fig 2.2). Absence of bruising and/or tail flick was noted (this could occur if the platform was resting on bone that had not been removed) and correlated with hindlimb movement (below). The muscle layers were sutured and the skin layers closed with wound clips. Laminectomy, i.e. removal of the T12 vertebral bone only, without compression, was performed in control rats. Following surgery, animals were given buprenorphine intramuscularly (10-20 mg/kg; Reckitt Benckiser, UK) and 5 ml saline (0.9% NaCl w/v) subcutaneously. The PUFA and saline control injections were delivered intravenously in a tail vein 30 min after SCI. Animals were checked up to 4 h after SCI to confirm absence of hind limb movement and removed from the study if there was extensive movement of any joint. For recovery studies longer than 24 h, manual bladder expression was performed 2-3 times a day for the first week and at least once daily thereafter, until the establishment of reflex voiding. Cages were changed at least every 2 days during the first week. Care sheets (Appendix 1) for each animal were kept with a record of their weight and bladder recovery throughout each experiment. The rats were given free access to food and tap water and maintained on a 12 h light/dark cycle at 21 °C.

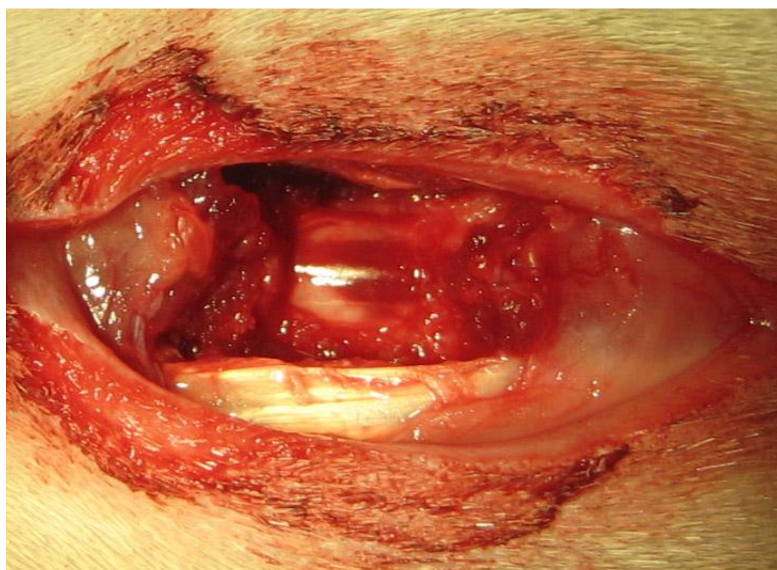


**Figure 2.2 The injury site following compression.** Development of a bruise was confirmed after each operation.

All of the compression SCI surgery used in this thesis was performed by the author, except for the day 14 and day 21 tissue used in the characterisation of the baseline inflammatory response (Chapter 3), and the day 7 tissue used for whole section analysis for ED1 (Chapter 4), which was performed by Dr. Wenlong Huang or Dr. Rachael Ward. The cryosectioning and staining of the day 7 tissue used for whole section histological assessment of ED1 was done by Dr. Siew-Na Lim and the analysis by Liying-Li. The day 21 tissue was cut by Mathuri Sakathinatasan. All surgery, sectioning and analyses were performed blinded to the treatment groups.

*Contusion SCI (Chapter 5)*

28 adult female Sprague-Dawley rats (205–222 g; Harlan, Houston, TX) were anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), and a dorsal laminectomy was performed at the T8 vertebral level. Rats then received a moderate spinal contusion injury using the Infinite Horizons device (Precision Systems and Instrumentation, Lexington, Kentucky, USA) with a preset force of 200 kDynes (actual forces were 200–214 kDynes). The muscles overlying the spinal cord were then sutured and the skin was closed by using surgical clips. Animals were given 5 ml of saline and placed into warm recovery cages. Postsurgical care included 5 days of treatment with antibiotics (gentomicin, 5 mg/kg) and 5

ml saline daily for the first week to maintain hydration, and twice-a-day manual bladder expression until spontaneous voiding returned. All procedures conformed to NIH and The Ohio State University (OSU) animal care guidelines.

The surgery was performed by two surgeons blinded to the treatment groups. The injections were delivered intravenously in a tail vein 30 min after SCI by the author. Miss. C. Amy Tovar, along with other trained OSU researchers, assisted the author with behavioural testing. Tissue was harvested at OSU and sent to our laboratory for histological analysis. All researchers, including the author, were blinded to the treatment groups throughout all experimental procedures and tissue analysis.

## 2.2 Treatment with omega-3 PUFAs

*Acute i.v. injection*

On the day of surgery, solutions of DHA or EPA were prepared in sterile saline (NaCl, 0.9% w/v) and adjusted to pH 7.4, from 5 µl concentrated stock aliquots kept in light sensitive, airtight glass containers (Agilent, Stockport, UK) at -20°C to prevent oxidation. The 1 M stock aliquots of fatty acids (Sigma, Dorset, UK) were prepared in a plastic airtight atmos bag (Sigma, Dorset, UK) under 100% $N_2$ and were made up in absolute ethanol. Glass pipettes and containers were used where possible throughout to prevent interaction of fatty acids with, and adsorption onto, plastic surfaces.

*Acute DHA/EPA compression study (Chapter 4)*

Animals received the following treatments via a tail vein under brief isoflurane anaesthesia (2%): an injection of (i) saline (vehicle) (n=46; 0.9% NaCl), (ii) DHA (n=29; 250 nmol/kg), or (iii) EPA (n=26; 250 nmol/kg), 30 min after injury. The injection volume was 5 ml/kg. Animals were perfused and tissue processed as described below. Treatment was allocated randomly and the surgeon was blinded to the treatment groups throughout the surgery and the behavioural testing period.

*Acute DHA contusion study (Chapter 5)*

Animals received the following treatments via a tail vein whilst still under ketamine and xylazine anaesthesia: an injection of (i) saline (vehicle) (n=10; 0.9% NaCl), (ii) DHA (n=10; 250 nmol/kg), (iii) DHA (n=8; 500 nmol/kg) 30 min after injury. The injection volume was 5 ml/kg and all researchers were blinded to the treatment group as described above.

*Dietary enrichment*

Prior to beginning the studies, the animal's average daily food intake was assessed over a two week period by providing 30 g food per day, and weighing how much was left. From these observations, the daily intake was 25-30 g. Between one and two weeks prior to the start of the studies the animals were weighed and randomly divided into groups, and the chow was changed from the standard pelleted form to the ground form, to allow habituation to the different food texture.

The control diet consisted of 5KB3 Certified EURodent diet 14 % (IPS Product Supplies Limited, London; Appendix 2). The omega-3 PUFA-enriched diet used in Chapter 6 consisted of the 5KB3 diet to which oil enriched in DHA or EPA (Incromega DHA700E SR; or Incromega SE7010R; both Croda Healthcare, UK) was added. The DHA oil contained 70–75% DHA and 10 % EPA. The EPA oil contained 70 % EPA, and 5-10 % DHA. The remaining percentages were made up of AA (< 5%) and other fatty acids. The volume of DHA or EPA preparation added to the chow calculated correspondingly, so that it would result in a target daily dose of 400 mg fatty acid per kilogram of animal body mass (Appendix 6). Oil-enriched food was prepared fresh each week under $N_2$ and stored in separate bags for each day (-20 °C). The oils used for enrichment were stored at -20 °C and each time the bottle of stock EPA was opened, it was flushed with $N_2$ to prevent oxidation. Previous measurements in our laboratory confirmed that this method of diet preparation retained high concentration of PUFA for several weeks, without oxidation (unpublished, Dyall). 30 g was allocated per rat, per day. It was changed daily between 10:00 am and 12:00 noon, and any left-over food was discarded. Food consumption was monitored before

and after SCI. There were 3-4 rats per cage and the experimental groups were mixed. There was no significant difference in the amount of diet consumed between any of the experimental groups, but animals were found to consume less during the first week following SCI (60-70 % less). Therefore, the full dose received was recalculated based on these findings. The average amount of food consumed over 28 days after SCI in the EPA study dropped to approximately 11-13 g, i.e. a dose of 150-170 mg/kg per day throughout the 28 day survival period. In both DHA pretreatment studies animals consumed approximately 25 g before SCI (330 mg/kg). In the first DHA study, animals consumed approximately 20- 24 g, i.e. 270-320 mg/kg per day after SCI, respectively.

## 2.3 Behavioural analysis

*Open field test of locomotion:*

The Basso, Beattie, Bresnahan (BBB) score is a way of measuring locomotor function in hindlimbs following SCI (Basso et al., 1996). As long as the method is carried out consistently, the BBB score can be used to compare results between laboratories and to compare the effectiveness of different treatments. The scale ranges from the highest score of 21(uninjured animals with normal locomotion) to 0 (complete paralysis). Typically following SCI, a curve is obtained which reaches a plateau at various scores, characteristic of injury severity. A subscore can further be calculated from the data collected from the BBB score (Lankhorst et al., 1999). This can be informative when a BBB recovery curve reaches an artificial plateau at the time where stepping and coordination is regained, although recovery of finer aspects of paw placement may be overlooked.

Animals were allowed to acclimatise preoperatively to the testing environment in an open field, which was a circular plastic enclosure (Fig. 2.3; width 88 cm, depth 30 cm; Water Features On-Line, Tyne & Wear, UK). This was considered to be an optimum size for at least four continuous steps at the time when the animals began walking, to give an accurate assessment of coordination. Animals were introduced to the open field on at least three separate occasions, either in cage groups or individually. This was essential for the avoidance of fear behaviour (urination, freezing, crouching during locomotion and dragging

hind limbs) especially at later recovery time points after SCI, which could give an inaccurate assessment of locomotor recovery. During testing, each rat was allowed to walk continuously in the open field for 4 min, while characteristics of hind limb movement (ankle, knee and hip) were marked on a sheet (Appendix 3). The rat was then removed from the open field, which was then wiped with 50 % ethanol solution and allowed to dry before the next rat was introduced. This was to help prevent the spread of any urinary infections and to remove any 'fear scent' that might have been present in the urine and could affect behaviour of the animal (Basso, 2004). A BBB score was assigned for each hind limb using the locomotor rating scale (Appendix 4) and the score for both hind limbs was averaged. Following surgery, the animals' locomotor function was monitored every day using the open field test for the first 7 days and then at 10, 14, 21 and 28 days post-operation.

**Figure 2.3 Open field test.** Animals were allowed to acclimatise to the testing environment before SCI. Each animal was observed for 4 min and movements related to recovery of hind limb and locomotor functions were recorded.

### 2.3.1 Measurement of bladder function

Data on bladder function were collected on the care sheets (Appendix 1). Each day for 7-10 days following SCI, it was noted whether manual expression was required and whether the abdomen was wet, indicating incontinence. Bladder width and length measurements were

taken post-mortem after perfusion. The bladder was dabbed with paper towel and measurements taken with a ruler. The area was calculated from these.

*Ultrasound*

Bladder volume was measured using a high resolution portable digital ultrasound system (Sonosite® MicroMaxx®; BCF Innovative Imaging, Livingston, Scotland, UK) with a SLA/13-6 MHz 26 mm linear array transducer as described by Al-Izki et al. (2009). The ultrasound system acquires a high-resolution two-dimensional image from which volumetric calculations were made. For bladder measurements, the abdomens were shaved and an ultrasound gel (Alpha tube ultrasound scanning gel; BCF Innovative Imaging) was applied. The baseline reading was taken while the animal was anaesthetized preceding the SCI surgery. Following SCI, on days 3, 5 and 8, the awake animal was gently restrained, and the transducer was first placed longitudinally against the animal to capture the maximum bladder length and depth (Fig. 2.4). Next; the transducer was rotated 90° to capture the maximum width of the bladder. The volume of bladder urine was automatically calculated by the ultrasound imaging software.

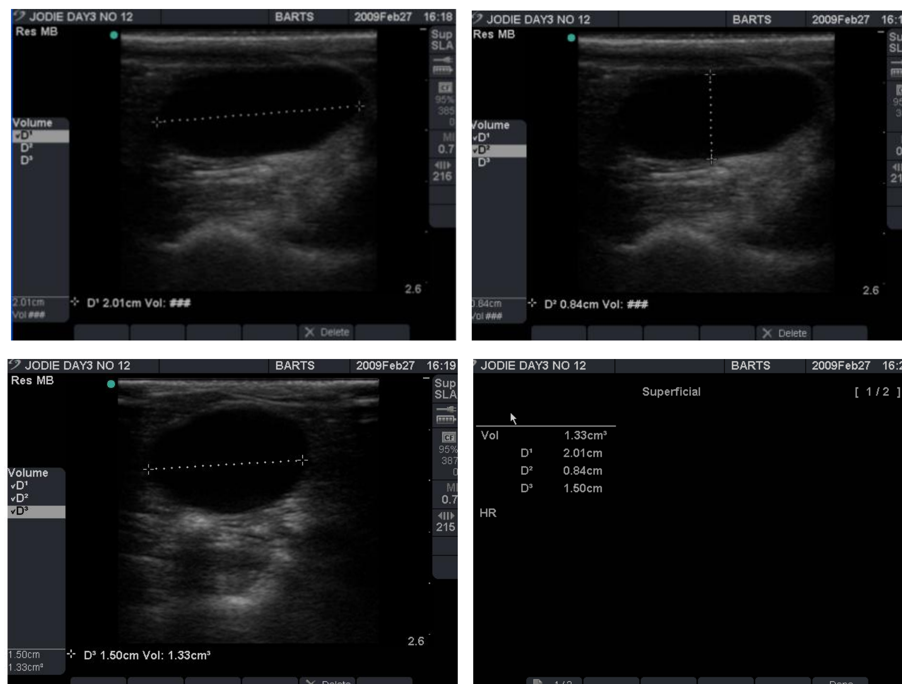



**Figure 2.4 Images used to calculate bladder volume.** The dotted line indicates the width ($D^1$) depth ($D^2$) and width ($D^3$) measurements used to calculate the volume.

### 2.3.2 Measurement of mechanical hypersensitivity

For behavioural assessment of tactile hypersensitivity readings were taken using an automated plantar aesthesiometer (‘von Frey’: Ugo Basile, Italy). This consists of a movable actuator that lifts a blunt probe at a controlled force rate onto the plantar paw surface (Fig. 2.5). This rate was set at 1.7 grams/second with a cut off weight of 50 grams and latency of 30 seconds. This experimental set-up benefits from the removal of observational bias, human error, and variable force applied by the investigator using manual von Frey hairs. The mechanical aesthesiometer has greater accuracy (to nearest 0.1 gram) and the weight incurred on the plantar paw increases at a linear rate from 0 to 50 g, whereas manual hair numbers have an exponential force increase, with large force differences at higher hair values between adjacent hairs. Rats were acclimatised and a baseline reading taken, and then tested post operatively. The probe made contact with the same plantar area, and increased force until the foot was voluntarily withdrawn. A positive response was determined as a single voluntary flexion withdrawal of the foot, with or without foot licking or biting, from the meshed flooring during the increasing weight of the probe. Each hind limb was tested 3 times, with an interval of 5 minutes to prevent habituation to the stimulus. Post-operative tests were performed on days 7, 14, 21 and 28.

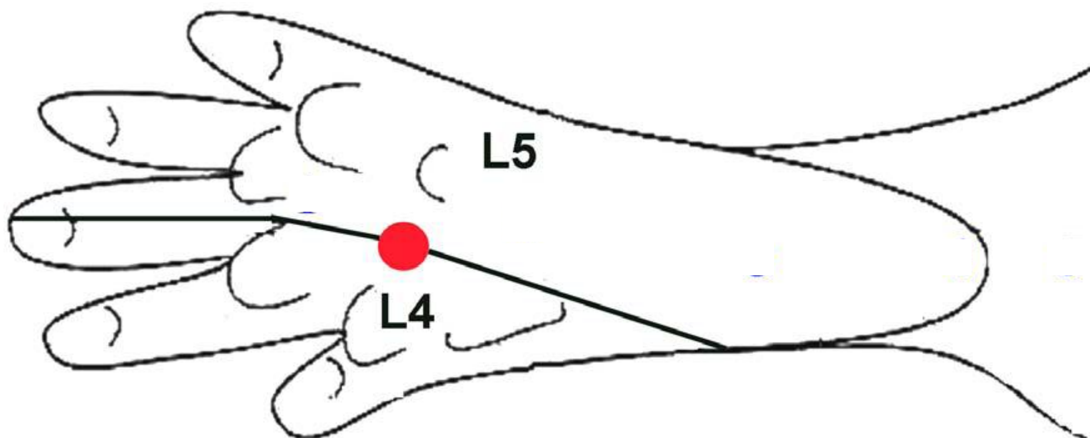



**Figure 2.5 Behavioural testing of the rat plantar hind paw**. Diagram modified from Takahashi *et al.* 2003. The area for mechanical testing is at the border of the L4 and L5 dermatomes, avoiding paw pads, as indicated by the red circle.

## 2.4 Tissue harvesting for immunohistochemical analysis

*Perfusion fixation and embedding of rat tissue*

At specific time points following injury, animals were euthanised using $CO_2$ asphyxiation and placed on a plastic board inside a fume hood. Once the absence of corneal or forepaw reflexes was confirmed, a midline incision was made along the chest. Scissors were used to cut the abdominal wall, from mid abdomen to the xiphisternum. The xiphisternum was then grasped with forceps and the diaphragm trimmed away from the ribcage. The ribs were cut on either side of the chest cavity, exposing the heart. The sternum and ribs were then held back with forceps to allow easy access to the heart. The fixative delivery system was gravity-fed and the tap opened slightly to allow a slow flow of saline (0.9% NaCl). A cannula was then inserted into the left ventricle and clamped in place. The tap was opened to allow full flow of saline and the right atrium cut. Once all the blood had been washed from the animal, judged by the lightening of the liver to a pale red-yellow colour, the tap was switched over to allow ~300 mL fixative (4% paraformaldehyde, pH 7.4) to flow through.

Tissue was dissected and post fixed for 2 hr, then cryoprotected in 20% sucrose (in phosphate buffer, 0.1M pH 7.4) for at least 3 days. A segment of liver tissue of approximately 1$cm^3$ and 5 mm segments containing the spinal cord lesion site, rostral and caudal segments, and the equivalent levels in naïve and laminectomy groups were embedded in OCT Embedding Medium (VWR, Lutterworth), frozen on dry ice, and stored at −80 ºC for subsequent processing for immunohistochemistry.

*Cryosectioning of Embedded Rat Tissue*

At least 10 minutes before use, the Leica CM 1900 cryostat (Leica, Milton Keynes, U.K.) was set to hold both the chamber and chuck at –12-20 °C and the temperature left to stabilise. Tissues were moved from the -80 ºC freezer and left in the cryostat chamber for 5-10 minutes to warm up to -12-20 ºC before being mounted on the chuck. Rat spinal cord or liver sections were mounted onto the chuck using OCT Embedding Medium (VWR, Lutterworth, U.K.), which was left to freeze for 5-10 minutes before sectioning. 15 µm

thick transverse sections were cut for spinal cord, 10 µm for liver and transferred onto Superfrost slides (VWR, Lutterworth, U.K), and left to dry for at least 20 min. Sections were cut serially so that each slide contained a selection of sections throughout the lesion and were stored in cryoprotectant (120 ml ethylene glycol, 200 ml PBS, and 200 g sucrose) at -20 ºC until required for general histological staining or immunohistochemistry (IHC).

### 2.5 Immunohistochemistry on cryosections

Cryostat spinal cord and liver sections kept in cryoprotectant were removed from the -20 ºC freezer and the chosen slides were washed with gentle agitation in PBS (5 x 5 min). After 1 h incubation in 10% normal donkey serum or normal goat serum, they were incubated overnight with the primary antibodies (Table 2.1). Preceding the application of the serum and SMI32 antibody, slides were immersed in ice-cold methanol and then washed in PBS. The next day, sections were washed and incubated for 1-2 h with secondary antibodies tagged either with i) fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate (both 1:400), or ii) biotin, amplified with ABC and revealed with the di-aminobenzidine (DAB) method. During the course of this research, the protocol was modified slightly to include the use of the second generation fluorescence labelled Alexa Fluor® goat anti-mouse or rabbit 594 or 488 secondary antibodies (1:1000-2000; Invitrogen, Paisley, UK ). Slides labelled with NeuN, APC, or JT1 were then counterstained with Hoechst (0.2 µg/ml) and then were mounted in glycerol:PBS (1:10) or in DPX after dehydration in the case of DAB sections, and cover slipped.

**Table 2.1: Primary antibodies used**

| *Abbreviation* | *Concentration and species* | *Distributor* | *Specificity* |
|---|---|---|---|
| ED-1 | 1:1000 Mouse | Serotec, UK | CD68 macrophages, monocytes, microglia |
| GFAP | 1:1000 Mouse | Chemicon, UK | Glial fibrillary acidic protein in astrocytes |
| OX-42 | 1:500 Mouse | Serotec, UK | CD11b alpha subunit of Type-3 complement receptor (CR3) on resting and activated microglia/macrophages/monocytes/neutrophils |
| Iba1 | 1:500 – 1:1000 Rabbit | Wako, Germany | Allograft Inflammatory Factor-1 (AIF1) or ionized calcium-binding adaptor molecule 1- a Ca2+-binding peptide produced by activated monocytes and microglial cells |
| OX-1 | 1:100 Mouse | Serotec, UK | CD45 or LCA-leukocyte common antigen on B lymphocytes |
| OX19 | 1:100 Mouse | Serotec, UK | CD5 molecule on T cells |
| W3/25 | 1:100 Mouse | Serotec, UK | CD4 molecule on helper T cells |
| OX-8 | 1:100 Mouse | Serotec, UK | CD8 alpha on T cells, mostly thymocytes and majority of NK cells |
| JT-1 | 1:1000 - 1:2000 Rabbit | Gift from Prof H. Perry, Southampton University | Raised against rat neutrophils |
| NeuN | 1:1000 Mouse | Chemicon.Chandlers Ford. U.K. | Mature neuronal cell bodies |
| SMI32 | 1:1000 Mouse | Sternberger Monoclonals, Leeds, UK | Non-phosphorylated neurofilament |
| APC | 1:200 Mouse | Calbiochem, USA | Adenomatous polyposis coli tumour suppressor protein on oligodendroctyes |

### 2.6 Quantification of histological markers

All analyses were performed blinded. Regions from at least three sections per animal were used for quantification or measured and data expressed as means ± S.E.M.

*Neutrophils*

Tissue sections were viewed on a Nikon Eclipse 80i fluorescence microscope using a 40x objective. The quantitative analysis of neutrophils in all groups and time points in spinal cord tissue was conducted by counting all labelled cells within the field of view in areas of the dorsal horn (DH) and ventral horn (VH), dorsal columns (DC) and ventrolateral white matter (VLWM; Fig. 2.1). The equivalent spinal cord region was identified on sections for each animal for accurate comparison. All cells within and touching the edge of the field of view were included. In the liver, a grid was placed over three sections per animal in Photoshop; three random areas were selected and then identified under the 20x objective and labelled cells counted.

*ED1 and Iba1 - macrophages/microglia*

Images were taken using a MBF CX9000 camera and Stereo Investigator software. Figures were prepared using Adobe Photoshop. For the time course study, the ED1 immunoreactivity of macrophages/microglia in the epicentre was quantified in the areas of the spinal cord shown in Fig. 2.6 using the Q-Win software. A measuring frame (ED1: 360 x 288 µm; Iba1: 288 x 216 µm) was placed over the area of interest and a binary image representing areas of immunoreactivity was then created. Any areas covered by significant tears or holes were measured and eliminated from the total area measured. The level of immunoreactivity was expressed as the percentage of the area of the measuring frame that contained immunoreactivity (field detected area, FDA).

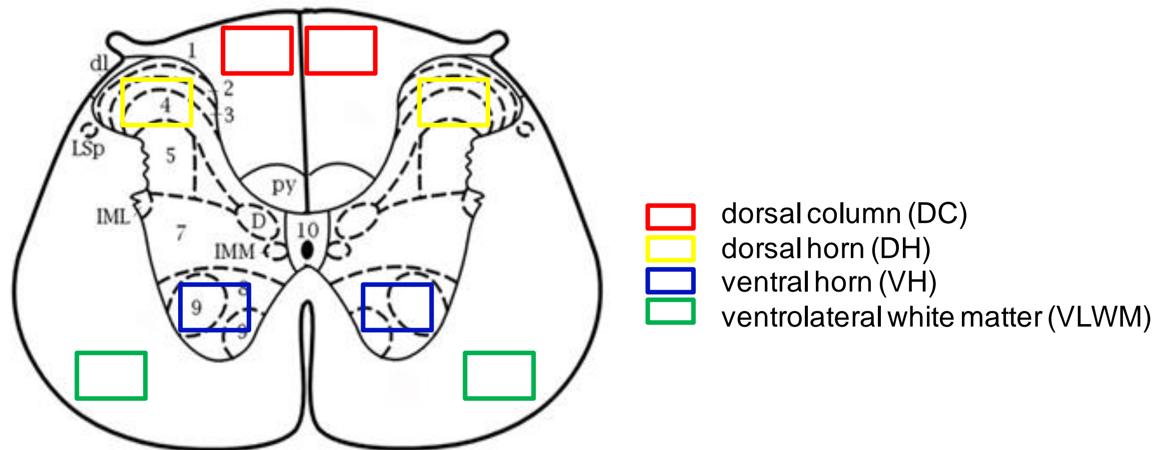



**Figure 2.6 Diagram illustrating areas where ED1 and Iba1 quantitative analysis was performed** (image outline adapted from Paxinos and Watson, 1997)

Due to the observation of unequal localisation of isolated ED1-labelled clusters of cells, the analysis in the subsequent chapters was performed in the epicentre and rostral segments with Q-Win using the whole section as shown in Fig. 2.7. An outline was drawn around the section, and a binary image created. The level of immunoreactivity was expressed as the percentage of the area of the outline that contained immunoreactivity (field detected area, FDA). This method has been identified as one of the best to represent the amount of macrophages in the injury site after SCI (Donnelly et al., 2009).

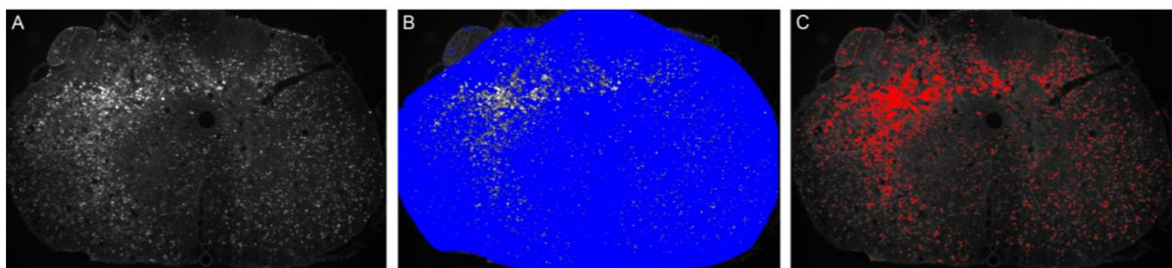



**Figure 2.7 Q-Win whole section analysis of ED1 immunoreactivity. (A)** Montages of transverse section of the spinal cord labelled with ED1 were created. **(B)** In the Q-Win programme, an outline was drawn around the section and the images converted to a binary mode **(C)** The level of immunoreactivity was set, shown in red, and this information converted to the percentage labelling of the image outline.

*NeuN neurones and APC oligodendrocytes*

Various automated methods were attempted to count NeuN- and APC- labelled cells. However, the automated method poorly reflected the numbers from a manual count in the boxed areas of interest. A manual method was therefore used. High power images (20 x objective) were taken in the areas shown in Fig. 2.6 and 2.8. To maintain consistency with previous work in the laboratory, boxes of specific sizes were used. For NeuN: a 216 x 173 µm box in the DH and 360 x 288 µm box in the VH (Fig 2.6) was placed over the image as shown. For APC: 360 x 288 µm box in the DC, LWM and VWM areas, and a 216 x 360 µm box in the CST (Fig. 2.8) was used. Inclusion criteria were any NeuN- or APC-labelled cells containing Hoechst-stained nuclei within the box or touching the bottom and left hand boundary of the box, whereas cells in contact with the other two boundaries were excluded. Where possible, counts were expressed as a percentage of the equivalent naïve animal tissue counts in the same areas at an equivalent spinal level.

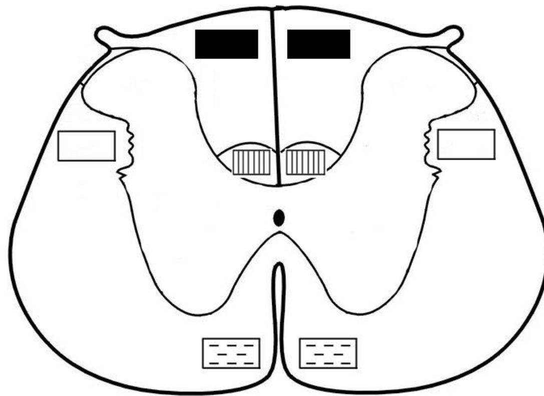


**Figure 2.8 Quantification of APC-labelled oligodendrocytes**. High power images of the white matter were taken in the boxed areas shown and counts were obtained of labelled cells containing a nucleus.

*SMI32 neurofilament*

High power images (40 x objective) were taken in specific areas (Fig 2.9A). A 360 x 288 µm box DC, VWM and VLWM and a 80x80 µm box was placed over the CST (due to the different size of particles in this area, see chapter 6) areas. In the Image J programme (Fig. 2.9 B), these images were converted to a set threshold for all images (Fig. 2.9C) and then

counted automatically using the ‘analyse particles’ programme. Any sections with high background noise to signal ratio were given an altered threshold to best represent the staining seen. The treatment groups were blinded until the analysis was complete.

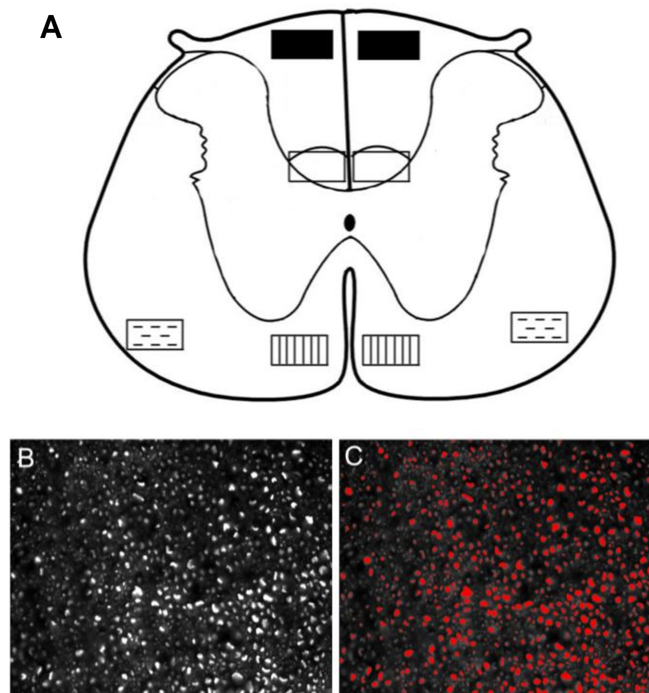



**Figure 2.9 Image J analysis of SMI32-labelled axons.** High power images were taken in the areas shown in **(A)**. In the Image J programme, these images were converted to a set threshold for all images **(B, C)** and then counted automatically using the ‘analyse particles’ programme.

## 2.7 Tissue harvesting for protein analysis

Animals were given an overdose of sodium pentobarbital (Euthatal, Merial Animal Health Ltd, Essex). This method was used in preference over $CO_2$ asphyxiation, in order to have more time for the collection of blood from the animal before death. It was found that blood coagulated too quickly when collecting it after $CO_2$ asphyxiation. Once the absence of corneal or forepaw reflexes was confirmed, the heart was exposed as described above. Blood was collected using a needle (21G) from the left ventricle into a 5 ml syringe containing sodium citrate, transferred to a plastic tube and placed on ice until centrifugation. A segment of liver of approximately $1cm^3$, and 5 mm tissue segments

containing the spinal cord lesion site or equivalent level in naïve animals and laminectomy only animals, were rapidly dissected and frozen on dry ice. Blood was spun at 12 000 g for 15 min at 4°C. The plasma layer was collected and transferred to fresh microfuge tubes and stored at -20 °C. Plasma samples were used for cytokine analysis (see below). The red blood cell pellets were sent to the University of Stirling (Prof. Gordon Bell) for lipid analysis.

*Protein Extraction*

The liver tissue and spinal cord epicentres were homogenized on ice in 700 µl lysis buffer (1% Nonidet-p40, 200 mM HEPES, pH 7.4, 100 mM NaCl, 100 mM NaF, 1 mM $Na_3VaO_4$, 5 mM EDTA with 1x protease inhibitor cocktail; Roche Diagnostics, Burgess Hill, UK). Homogenates were centrifuged for 15 min at 12 000 g at 4°C and the supernatants were retained and assayed for protein content using the bicinchoninic acid (BCA) protein assay kit (Pierce, Thermo-Fisher Scientific, Cramlington, UK), against bovine serum albumin standards.

*Cytokine Multiplex Assay*

All reagents were purchased from MesoScale Discovery (MSD, Gaithersburg, USA) unless otherwise stated and the assays were performed as described in the manufacturer's instructions (sensitivity: 12.5 pg/ml). On the day of the analysis reagents were brought to room temperature. 25 µl of recombinant calibrators were diluted in Rat Serum Cytokine Assay Diluent, to generate a standard curve from 2500 ρg/ml followed by 4-fold serial dilution. The same homogenate buffer that was used to prepare the spinal cord and liver samples was used to dilute the standards. 25 µl of Rat Serum Cytokine Assay Diluent was dispensed into the bottom of each well of a 96-well MULTI-SPOT MSD plate containing capture antibodies, in order to cover the entire bottom. The plate was sealed with parafilm and incubated for at least 30 minutes with vigorous shaking on an agitator at room temperature. 25 µl of either calibrator or sample solution was dispensed into a separate well of the MSD plate. The plate was sealed and incubated with vigorous shaking for 2 hours at room temperature. The plate was washed 3 times with PBS + 0.05% Tween-20. 150 µl of

Read Buffer was added to each well of the MSD plate taking care to avoid bubbles in the fluid, which could interfere with reliable reading of the MSD plate. The plate was read and analysed on the SECTOR$^{TM}$ Imager. Once the range of the standard curves used for calculating relative values was confirmed to be linear for each cytokine/chemokine, raw readings were converted to pg/ml of serum or pg/mg total protein for liver and brain.

*C-Reactive Protein (CRP) Measurement*

The quantitative measurement of CRP in the rat plasma was performed using a commercial rat CRP ELISA kit (BD Biosciences, Oxford, UK). Plasma samples were diluted to 1:4000 and 100 µl of each sample was allowed to react with antibodies coated on specially treated microplate wells for 30 min. The wells were then washed to remove unreacted plasma proteins, and an enzyme-labeled rabbit anti-rat CRP (conjugate) was then added to react with and tag the antigen-antibody complexes for 30 min. The wells were then washed again to remove unreacted conjugate. A urea peroxide substrate with tetramethylbenzidine (TMB) as chromogen was added to initiate colour development. Development of a blue colour indicated a positive reaction while negative reactions appeared colorless or with a trace of blue. The reaction was interrupted with a stop solution that turned the blue positive reactions to yellow. Negative reactions remained colourless or with a hint of yellow. Colour intensity (absorbance) was read at a wavelength of 450 nm on a spectrophotometer. Quantification of absorbance was accomplished using a standard curve generated by measuring twofold dilutions of the standard provided.

*Myeloperoxidase (MPO) Assay*

The extent of neutrophil infiltration/activation was estimated by measuring the MPO activity. During the first 72 h after SCI, neutrophils constitute the greatest portion of MPO-positive cells (Taoka et al. 1997; Saville et al. 2002). For measurement of MPO activity, the spinal cord homogenates from the tissue collected as described above, were allowed to thaw on ice and 25 µl samples from each animal were assayed individually in duplicate in a 96-well plate. The reaction was started by adding 0.1 ml of sample and 100 µl TMB (Sigma, UK). Absorbance was measured at 620 nm at 15 and 60 min and MPO activity was

determined by comparing the reaction rate in the samples with the rate of reaction plotted against the standard human leukocyte MPO curve from serial dilutions (0-12.5 U/ml in homogenate buffer; Sigma, UK). Results are expressed as units per gram of tissue.

### 2.8 Statistical Analysis

All statistical tests were performed using GraphPad Prism version 5 (GraphPad Software Inc., San Diego, CA, USA). The data are presented as means and standard error of the means. BBB data were analyzed using one-way or two-way analysis of variance (ANOVA) with Tukey's or Bonferroni's post hoc comparisons, or independent *t* tests. Differences were considered significant when $p < 0.05$. The Fisher Exact Test was performed using a Fisher Exact Test online calculator.

# Chapter 3: Results I. Characterisation of the cellular inflammatory response following compression spinal cord injury

## 3.1 Introduction

One of the first characterisations of the inflammatory response in the CNS following injury was made by Perry *et al*. in 1987, where a comparison was made between the responses of the optic nerve in the CNS versus the sciatic nerve in the PNS, to a forceps crush injury. The contribution of the inflammatory response to axonal pathology began to be questioned at this point and the idea of inflammation as a bystander event was challenged. The debate over the relevance of the role of the immune system in recovery after CNS injury began to take shape in the 1990's and has become more and more complex, with the emergence of divergent roles, particularly attributed to macrophages.

Most descriptive characterizations of the inflammatory response have been carried out in rodent models of SCI such as crush or contusion (Popovich et al., 1997; Zhang et al., 1997; Sroga et al., 2003). It quickly became clear that the inflammatory response following SCI involves a dynamic interaction between endothelium, blood vessels, neurones and glia, as well as the infiltration of blood-derived (haematogenous) monocytes. More recently, the mobilisation of blood-derived cells from the bone marrow has been shown to be influenced by a systemic response to injury in the liver and enhanced by signalling from the injury epicentre (Campbell et al., 2003, 2005). Differences were discovered between species and even between strains of the same species, primarily in the timing of the entry and composition of cell types in the injury epicentre at acute and chronic time points after SCI. Specifically, neutrophils accumulate within the lesion over the course of hours to days, and in most species are rapidly cleared during the first week post-injury. In mice, elevated numbers of neutrophils may persist in the lesion for months (Kigerl et al., 2006). In rats, lymphocytes start to infiltrate the lesion alongside monocytes 3-7 days post-injury. In contrast, lymphocyte entry is delayed in humans and mice, with peak numbers evident after a delay of months post-injury (Sroga et al., 2003; Kigerl et al., 2006). Unique to mice is the

formation of a dense connective tissue matrix in the lesion in parallel with lymphocyte accumulation (Popovich et al., 1997; Fleming et al., 2006; Kigerl et al., 2006; Sroga et al., 2003; Donnelly et al., 2007). We need to understand the importance of these differences and the relative contributions of each cell type in order to develop effective treatments.

### 3.1.1 The acute neutrophil response (minutes to hours)

There appears to be agreement between laboratories that following SCI there is an immediate up regulation of pro-inflammatory cytokines and chemokines, e.g. TNFα and IL-1β by damaged neurones and glia at the injury epicentre, followed by the rapid entry of neutrophils (Bartholdi and Schwab, 1997; Bareyre and Schwab, 2003; Aimone et al., 2004). The molecular cytokine and chemokine response will be covered in more detail in the next chapter. Neutrophils are important for the removal of debris but regarded as cytotoxic following CNS injury. Perhaps due to this, their actions are short-lived and most neutrophils undergo programmed cell death within 48 h (Lawrence et al., 2002). Several laboratories have attempted to block neutrophil infiltration with the use of nitrogen mustard (Taoka et al., 1997), or antibodies directed against integrins such as CD11d (Bao et al., 2004, 2005; Fleming et al., 2008, 2009) or adhesion molecules such as P-Selectin (Taoka et al., 1997). Such molecules are expressed by neutrophils and aid in their diapedesis of the blood-brain-barrier. All of these studies found reduced neutrophil infiltration along with improved motor outcome. The consensus seems to be that reducing neutrophil infiltration is advantageous after SCI.

### 3.1.2 The chronic macrophage/microglial inflammatory response (hours to days)

The infiltration of neutrophils is followed by the delayed entry of monocytes in the injured parenchyma around 3 days, where they differentiate into macrophages and numbers peak between 7 and 14 days (Popovich et al., 1997; Trivedi et al., 2006). Macrophages persist at the site of injury and are functionally heterogeneous and diverse in their response to injury. Their actions in non-neuronal tissues are usually self-limiting and have been considered to play a beneficial role, which includes triggering the initiation of resolution of inflammation

by suppressing pro-inflammatory signalling (Serhan and Savill 2005). Interestingly, it has been demonstrated that implantation of activated macrophages that have been exposed to peripheral nerves leads to regrowth of the transected optic nerve and partial recovery from spinal transection (Lazarov-Spiegler et al., 1996; Rapalino et al., 1998). These experiments originated from the theory that early and robust invasion by macrophages may be one of the reasons why axonal regeneration is more effective in the PNS than in the CNS. It has been shown in pre-clinical models of SCI that blocking or reducing acute macrophage accumulation at the injury site using chlodronate or integrin antibodies is beneficial to functional recovery (Blight et al., 1994; Popovich et al., 1999; Gris et al., 2004; Bao et al., 2004, 2005; Fleming et al., 2008, 2009).

The divergent effects of the experiments described above can be explained in part by manipulation of different macrophage subsets. It has recently been shown that neurotoxic "classically-activated" pro-inflammatory (M1) macrophages dominate the lesion within one week following SCI and that these cells overwhelm a comparatively smaller and transient "alternatively-activated" (M2) macrophage response (Kigerl et al., 2009). The M1 macrophage phenotype persists at the injury site for several weeks. One possibility is that depletion or alteration of the M1 subset is responsible for the improved recovery reported in some studies. *In vitro*, the M1 and M2 macrophages exert distinct effects on neurone survival and axon growth. In the presence of media conditioned by M1 or M2 macrophages (MCM), dorsal root ganglion (DRG) neurones exhibit distinctive growth characteristics. M1 macrophages stimulate a sprouting growth response that is often associated with pain *in vivo*, whereas M2 macrophages stimulate long distance axon growth. The mechanisms that control these divergent macrophage functions at sites of injury are not understood (Kigerl et al., 2009).

Microglia, the resident macrophages of the CNS, have been shown to respond immediately to SCI and migrate rapidly to the injury site (Banati et al., 1993; Kettenmann, 2007). It has also been shown that infiltrating monocytes from bone-marrow differentiate into microglia (Soulet and Rivest, 2008). Microglia express multiple phenotypes with corresponding different functions in tissue repair and/or damage (Schwartz and Yoles, 2006). In the adult

CNS, they show three main phenotypes: 1) "resting", with ramified processes extending in all directions from the soma; 2) activated, slightly larger, with retracted processes; and 3) reactive, or phagocytic, with swollen cell bodies and short cytoplasmic extensions (Flaris et al., 1993; Popovich et al., 1997). As mentioned previously, in the latter phenotype, microglia become difficult to distinguish from infiltrating macrophages (Popovich et al., 1997; Schnell et al., 1999). Thus, it is not clear what the ratio is of blood-derived versus resident macrophages that remain in the epicentre and spread above and below the initial injury site at later time points. Also, their exact contribution to toxicity or to wound healing remains to be fully understood.

Attempts have been made to isolate the haematogenous derived macrophages from resident microglia/macrophages. Recently, Schecter et al. (2009) used irradiation-induced bone marrow chimeric mice, with GFP-labelled bone marrow specific to monocytes. This allowed labelled blood-derived leukocytes to be identified at later time points after SCI. They found that the spatial organization of the infiltrating myeloid progenitor cells around the lesion site has a direct impact on functional recovery. In another study, this group have shown that infiltrating monocyte-derived cells mediate a function essential for repair that cannot be provided by resident microglia during SCI (Shechter et al., 2009). It is clear that the macrophage response after SCI is heterogeneous, and that a more in-depth analysis will be required when assessments of the impact of treatments on the SCI inflammatory response are made.

### 3.1.3 The chronic lymphocyte immune response

Research in multiple sclerosis has consistently shown the pathological potential of neuroantigen-reactive T and B lymphocytes, particularly those that recognize and mount reactions against myelin proteins (e.g. myelin basic protein; MBP). These are autoimmune responses which amplify the CNS macrophage response, resulting in blood-brain barrier injury, toxicity to oligodendrocytes and neurones and loss of function (Benveniste et al., 1997; Schroeter and Jander, 2005; Donnelly et al., 2007). There is a growing body of evidence in animal models and human SCI supporting the idea that traumatic or ischemic

CNS injury can trigger pathological autoimmunity (Popovich et al., 1996a; Jones et al., 2002; Fee et al., 2003; Gonzalez et al., 2003; Ankeny et al., 2006). Using transgenic mice and rats vaccinated to expand MBP-reactive T cells, Jones et al. (2002) have shown that autoimmune reactions exacerbate demyelination and axonal pathology, increasing the size of the contusion lesion and causing loss of supraspinal neurones. Mice and rats without T-lymphocytes (RAG knockout and athymic nude rats) have attenuated neuropathology after TBI and SCI (Fee et al., 2003; Potas, et al., 2006). Also, antibody-mediated blockade of lymphocyte chemokines inhibits T cell infiltration and attenuates secondary injury after SCI (Gonzalez et al., 2003). In parallel to T cell activation in the adaptive immune response, there is usually activation of B cells and both generate antibodies. Some of these are autoantibodies against CNS antigens. The presence of autoantibodies directed against CNS antigens has been confirmed in human serum samples from chronic SCI individuals (Hayes et al., 2002) and in a mouse model of contusion SCI (Ankeny et al., 2006). Schwartz and colleagues have challenged the view that autoreactive T cells are destructive to neurones, stating that autoreactive T cells, specifically those responding to myelin proteins, have the potential to control the pathological immune response following CNS injury, but do so inefficiently (Moalem, et al., 1999; Hauben et al., 2000a; Schwartz and Kipnis 2001, 2002). They have supported this concept by demonstrating that passive or active immunization with autoimmune T cells directed against the MBP antigen limits secondary neurodegeneration in injured optic nerve (Moalem, et al., 1999 and contused spinal cord (Hauben et al., 2000a). As a result, they propose therapeutic vaccines to treat SCI and TBI (Schwartz, 2001; Donnelly et al., 2007). Clearly, our understanding of lymphocyte functions in the injured nervous system is incomplete.

Most studies on the inflammatory response following SCI have been in the hemisection, or contusion models of SCI. Differences have been found between strains of rat and mouse, and between models. For example, the peak timing of the accumulation of macrophages varies from 3 to 7 days in a hemisection or contusion model in the rat (Table 3.1), whereas the peak of neutrophil infiltration remains similar at 1 day. There are further differences in the timing of lymphocyte infiltration (Table 3.1). Therefore, it is essential that a characterisation is carried out in our model of SCI, in order to establish a ‘baseline’ for

infiltration of inflammatory cells and define the optimum time line in which to assess the effects of fatty acids.

**Table 3.1 Timing of infiltration of inflammatory cells to the spinal cord after SCI in different models**

| | | | dpi peak: | | |
|---|---|---|---|---|---|
| **study** | **species** | **model** | **neutrophils** | **macrophages/ microglia** | **lymphocytes** |
| Kigerl (2006)* | mice (4 strains) | T9 contusion | 7 | 14&42 | 14&42 |
| Bao (2005) | rats (Wist) | T4 clip compression | 1 | 3 | - |
| Carlson (1998) | rats (L.E.) | T10 contusion | 1 | 2 | - |
| Dusart & Schwab (1994) | rat | T9 dorsal hemisection | 1 | 3 | 3 |
| Popovich (1997)** | Rat (SD & Lew) | T8 contusion | - | 7 | 7 (SD), 3 (Lew) |
| Schnell (1999) | mice (C57/BL6) | T8 dorsal hemisection | 1 | 4 | 2 |
| Sroga (2003) | rat (Lew) & | T8 contusion | - | 7 | 1 |
| | mice (C57/BL6) | T9 contusion | - | 7 | 56 |
| Zhang (1997) | rat (SD) | T8 crush | 1 | 3 | - |

*One strain of mouse had a significantly more reduced and delayed lymphocyte & macrophage infiltration than the 3 other strains.
** The Lewis strain had a prolonged increase in macrophages/microglia at 28dpi AND 4-8 mm rostral and caudal to the epicentre, when the amount dropped in the SD strain at the same time points.

dpi: days post SCI; Wist: Wistar; L.E.: Long Evans; SD: Sprague Dawley; Lew: Lewis; (-) not quantified.

### 3.1.4 The systemic response following SCI

In parallel to the inflammatory response at the site of SCI, there is also a significant systemic influence from increased levels of proinflammatory cytokines such as IL-1, IL-6 and TNF-α in the circulation, which are collectively termed acute-phase proteins (APPs) and are secreted by hepatocytes in the liver. The changes that are influenced by these APPs, such as leukocyte mobilization, fever, and changes in serum levels of glucocorticoids and cytokines, are part of the acute-phase response (APR) (Parham, 2000; Campbell et al., 2003). As a result, post-trauma recovery may be impeded by the development of multi-organ dysfunction syndrome and chronic disease (Campbell et al., 2003, 2005; Davis et al., 2005; Gris et al., 2008).

After SCI or brain injury in the rat, the production of certain chemokine APPs such as CINC-1 and CCL2 is found to enhance the response to injury, increasing leukocyte recruitment. Blocking this response using chemokine antagonists has been shown to lead to a corresponding decrease in the numbers of leukocytes recruited to the injured spinal cord or brain. This has challenged the dogma that local chemokines at the site of injury attract a circulating population of phagocytes from the blood up a concentration gradient (Campbell et al., 2005). Although the hepatic chemokine response may not be specific to CNS injury, it opens the possibility for a new target for therapeutic intervention. It will be interesting therefore, to also explore the systemic response in our model of SCI and changes after treatment with omega-3 PUFAs.

### 3.1.5 Aims

The aims for this chapter were to characterize the inflammatory response in our compression model of SCI over several time points and to compare our findings to the published literature. We hoped to identify the most accurate methods of quantification of various parameters of the inflammatory response in order to assess the effects of fatty acid treatment on SCI inflammation in subsequent chapters. This would help us to identify specific actions of fatty acids and also help us understand the contribution of the inflammatory response to recovery after SCI.

## 3.2 RESULTS

### 3.2.1 Neutrophils infiltrated the epicentre 4 h after SCI

The most commonly used way to quantify neutrophils is to use a general cellular marker/stain, for example cresyl violet and to identify and count cells containing a lobed nucleus, characteristic of these leukocytes. However, this stain marks all cell nuclei and it is difficult to obtain an accurate estimate. Alternatively, the amount of myeloperoxidase (MPO) released by neutrophils in the tissue can be quantified by western blot. This is a good indicator of neutrophil activity; however it may not be the most accurate quantification or specific for CNS neutrophils in a heterogeneous homogenate. We have been able to obtain a more specific polyclonal rabbit antiserum against murine neutrophils, generated in Professor H. Perry's laboratory. The epitope has not yet been characterized but has the same staining characteristics as MBS-1 (Cunningham, et al., 2005; Teeling et al., 2007). Western blot analysis on spinal cord homogenates consistently reveals a dominant band of 65kDa (Fig 3.1).

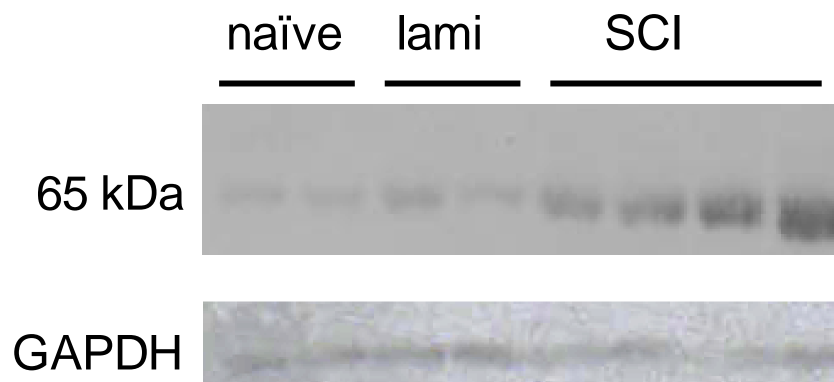



**Fig. 3.1 Western blot analysis of the JT1 antibody in the SCI epicentre at 24 h.** There was a dominant band of 65 kDa, which increased in density following SCI.

In order to validate this neutrophil marker (JT1), high power confocal images were taken to distinguish between cells with lobed nuclei or cells with a rounded, single nucleus. Fig. 3.2 shows neutrophils in blood vessels in a section of the spinal cord 4 h after SCI (A, C) and penetrating the meningeal layer and parenchyma at the site of SCI (D).

The JT1 antibody was optimised using a biotinylated secondary antibody, ABC amplification and the DAB method, and similar profiles of neutrophils were seen in adjacent sections labelled with immunofluorescence (IF). However, in 4 h and 1 d tissue, because of haemorrhage into the injury epicentre from damaged blood vessels, the presence of red blood cells made it very difficult to distinguish them from the slightly larger neutrophils (Fig. 3.2B). Therefore, the immunofluroescence method was chosen and quantification was performed on tissue labelled with JT1.

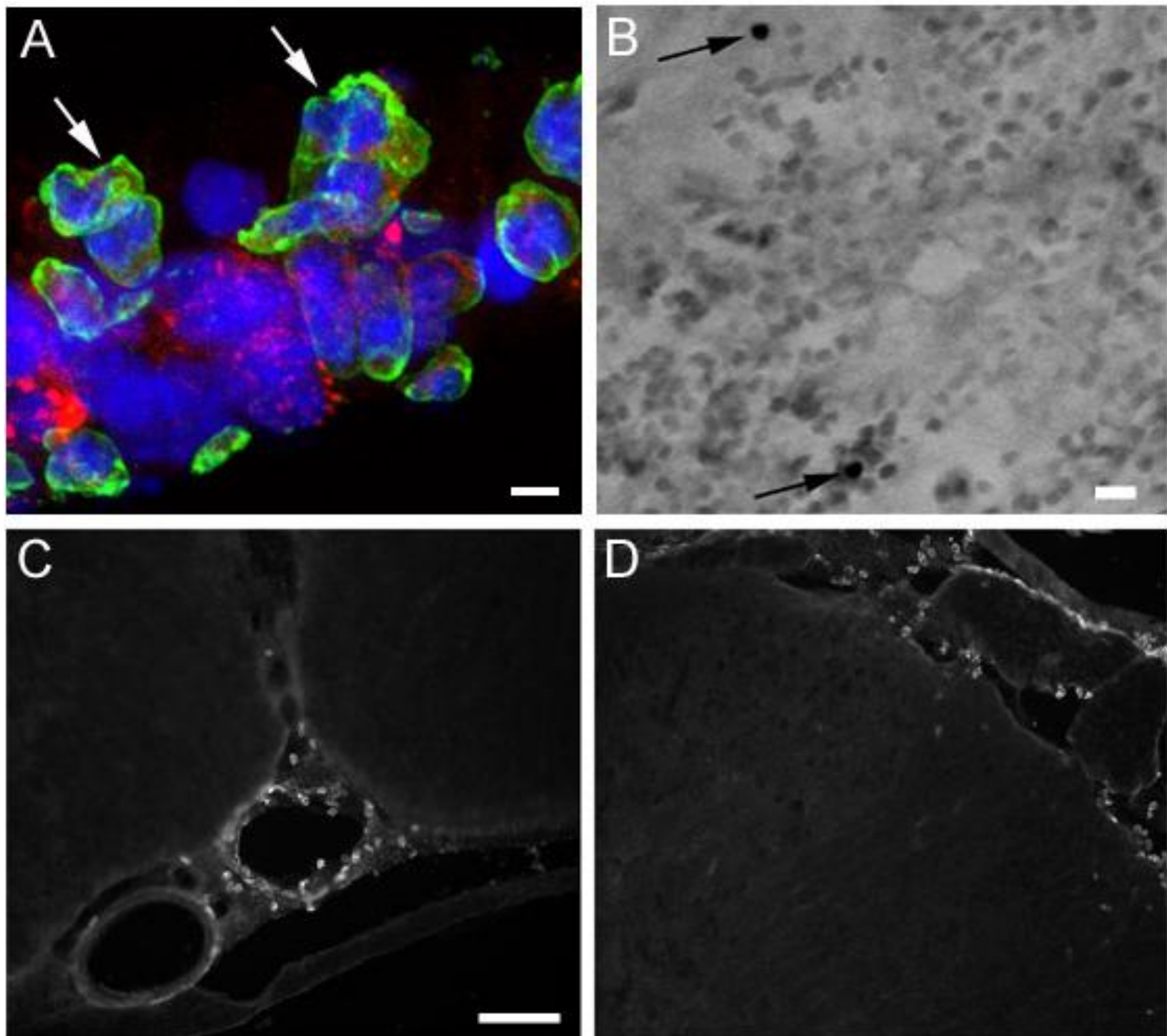



**Figure 3.2 Neutrophils in the epicentre at 4 h.** Only cells with lobed nuclei are labelled with JT1 (green, arrows, **A),** whereas other mononuclear cells are labelled with the macrophage marker ED1 (red**, A**). Using the DAB method it was difficult to distinguish labelled neutrophils (arrows) at this time point from red blood cells in the background **(B).** Using immunofluroescence, neutrophils were seen attached to blood vessels (**C**) and traversing into the injury site (**D**)**.** Scale bar = 5 µm **(A**), 20 µm **(B**), 100 µm **(C, D**).

Examples of representative sections of SCI and sham laminectomy control tissue in the ventral horn area of the epicentre are shown in Fig. 3.3A-D. The inset in Fig. 3.3C shows an example of a typical neutrophil with multilobed nucleus. Detailed quantification of neutrophils in the epicentre (Fig. 3.4) revealed a significant influx of neutrophils to the dorsal horn and dorsal columns within 4 h, spreading to the ventral horns within 1 day, the response reaching a peak at 1 day. Only 1 or 2 neutrophils were found 7, 14, 21 and 28 days after SCI, as well as in control laminectomy or naïve tissue. 2 way ANOVA revealed a significant time (F=23.64) but not region (F=1.67) effect.

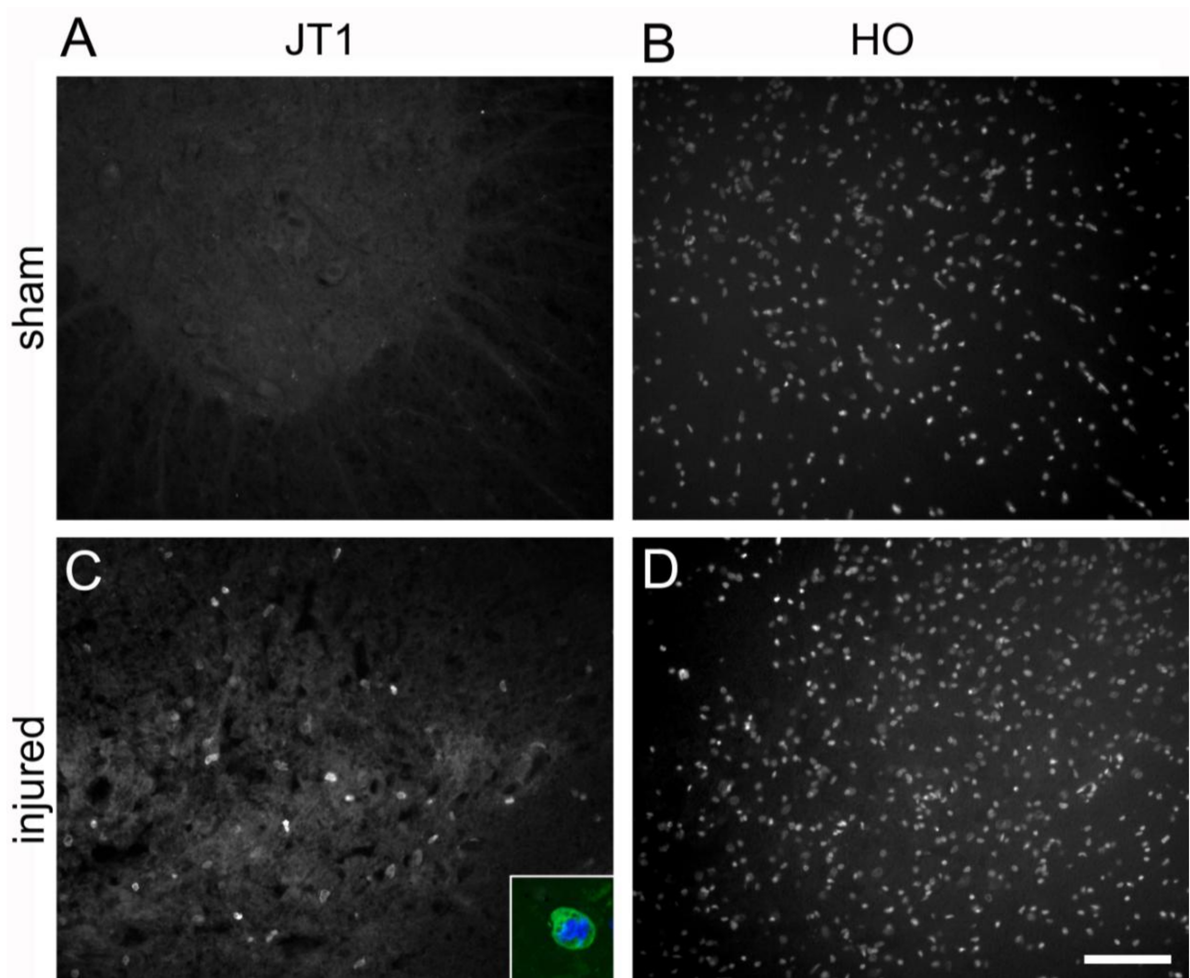



**Figure 3.3 Neutrophil infiltration in the ventral horn**. **(A)** A sham control section of the spinal cord, with absence of neutrophils (JT1 staining) representative of all time points. (C) Neutrophils distributed extensively in the same area of the spinal cord at 24 h. Inset shows high power confocal image of a neutrophil (JT1, green) with characteristic multilobed nucleus (HO-Hoechst, blue). (B & D) show respective Hoechst-labelled nuclei on the same sections as A & C. Scale bar = 100 µm. Figure reproduced from Hall et al., (*in submission).*

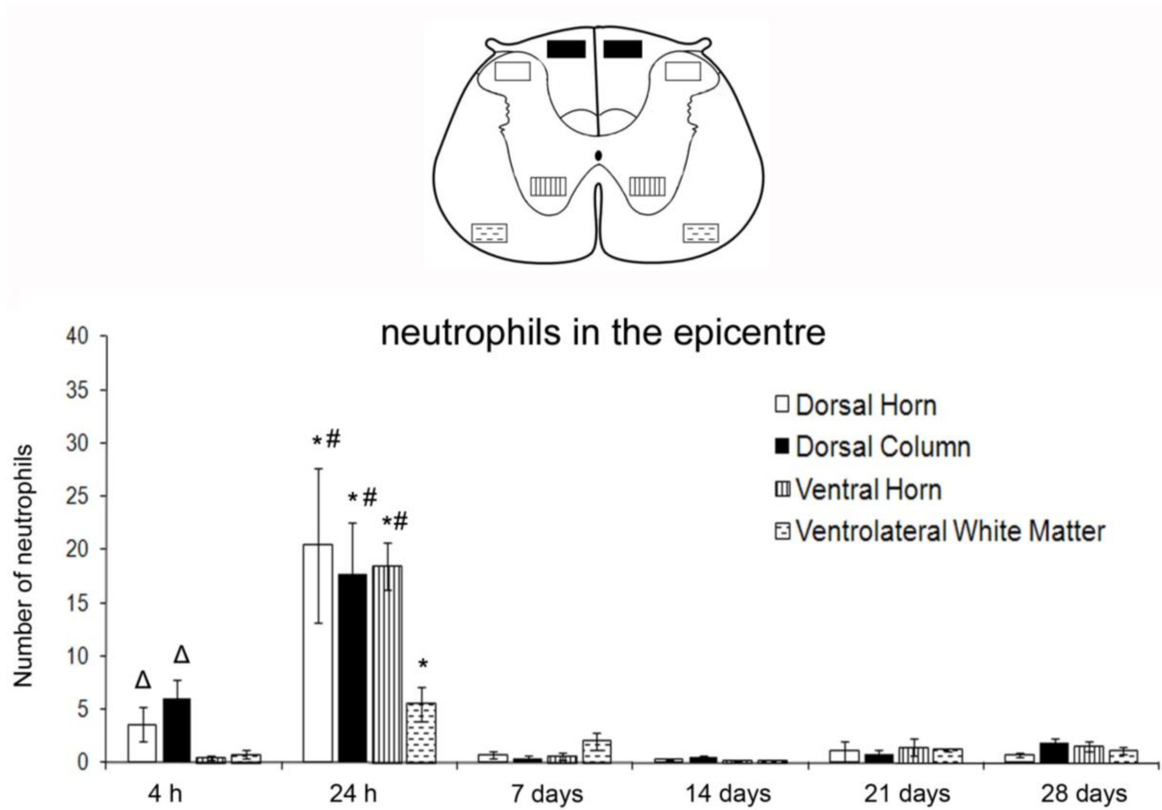



**Figure 3.4 Time course of neutrophil infiltration**. Detailed quantitative analysis of neutrophils in the epicentre. At 4 h neutrophils were seen mostly in the dorsal horns and dorsal columns (Δ $p<0.05$ compared to VH and VLWM and all other timepoints). Maximum infiltration was at 24 h in the dorsal columns and grey matter (* $p<0.05$ compared to all other time points; # $p<0.05$ compared to VLWM). Numbers returned to control levels at 7 days. Results are expressed as mean ± SEM of the number of animals; n=3-4 per group. Data reproduced from Hall et al., (*in submission).*

### 3.2.2 Macrophages filled the epicentre 7 – 28 days after SCI

ED1 (CD68) is an intracellular marker, packaged in vesicles and labels monocytes, activated and phagocytic macrophages/microglia. It is one of the most commonly used antibodies to label macrophages. Fig. 3.5 shows infiltration of DAB labelled ED1 monocytes into the injury epicentre and accumulation of macrophages over several time points. ED1 labelling was negligible or absent in laminectomy control tissue (not shown). Disruption of the blood brain barrier was highlighted by the presence of red blood cells (RBCs) seen in 4 h and 1 day tissue (* in Fig. 3.5). There were no RBCs present in spinal cord tissue at 7 days

Detailed quantitative analysis of the epicentre revealed only 1 or 2 labelled cells infiltrating the injured epicentre within the first 24 h (Fig. 3.6). By 7 days and up to 28 days a significant increase in ED1 labelled macrophages were distributed throughout the grey and white matter covering 7-12% of the measured fields. 2 way ANOVA revealed a significant time ($F=19.89$) and region ($F=5.53$) effect. Overall, comparatively fewer macrophages were seen in the ventrolateral white matter but this was only significant at 14 days compared to the dorsal columns and ventral horns. There was a significant drop in the amount of macrophages in the dorsal columns at 14 days. Levels of ED1 staining in the grey matter of the dorsal and ventral horns reflect areas that were found at later time points to contain large cystic cavities filled with macrophages (shown later, Fig. 3.12).

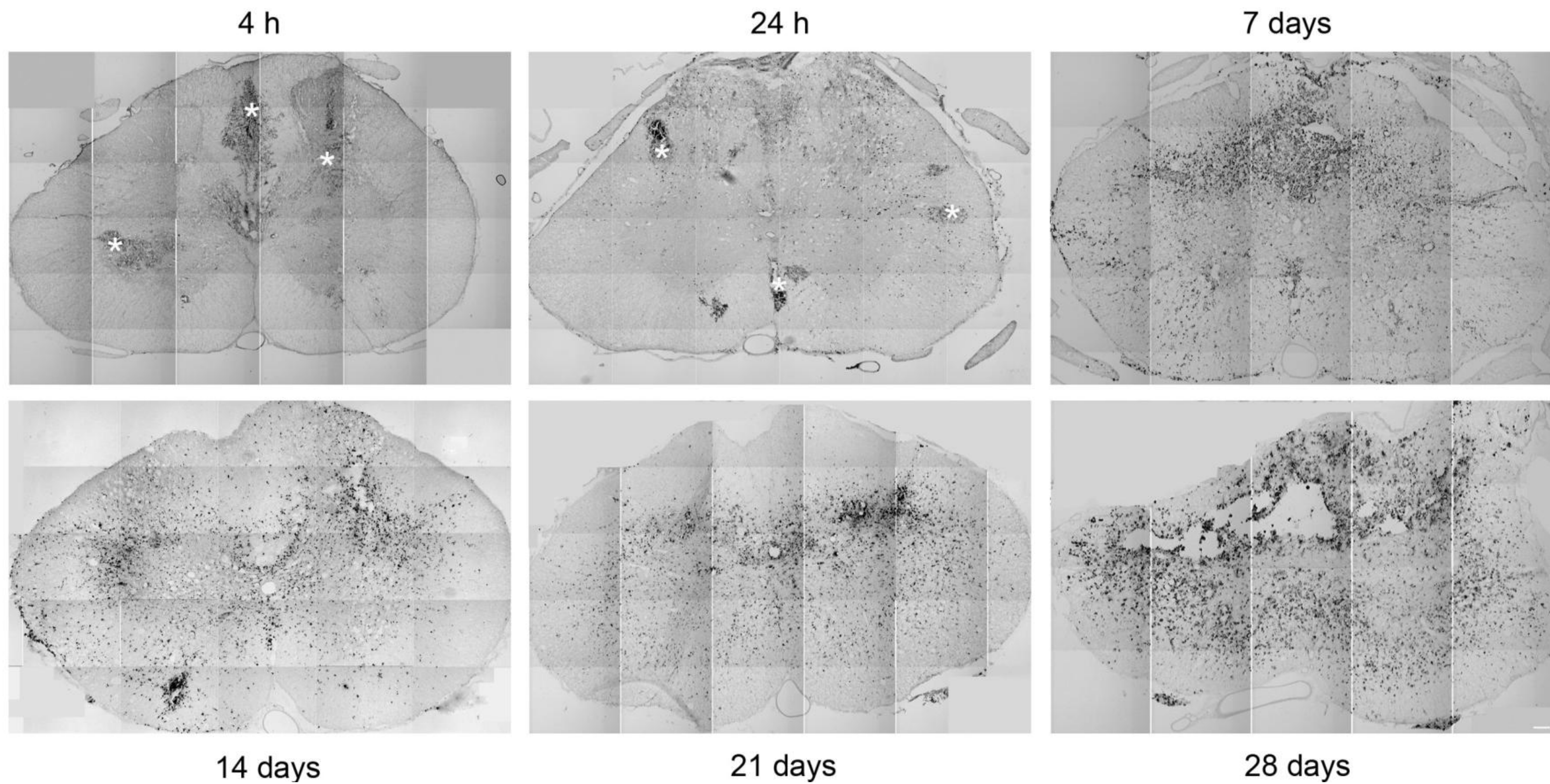
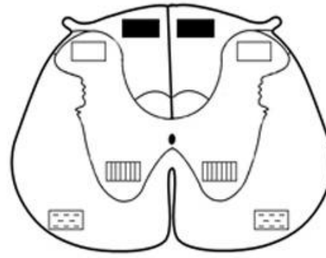



**Figure 3.5 Time course of macrophage (ED1) infiltration.** At 4 and 24 h, macrophages were absent or small and few in number, but this was masked by the presence of red blood cells from ruptured blood vessels (shown by *). Macrophages initially appeared in the dorsal half of the cord (24 h) but over 7-28 days became swollen and filled the entire area of the section. In some cases, cavities were also visible. Scale bar = 100 µm.



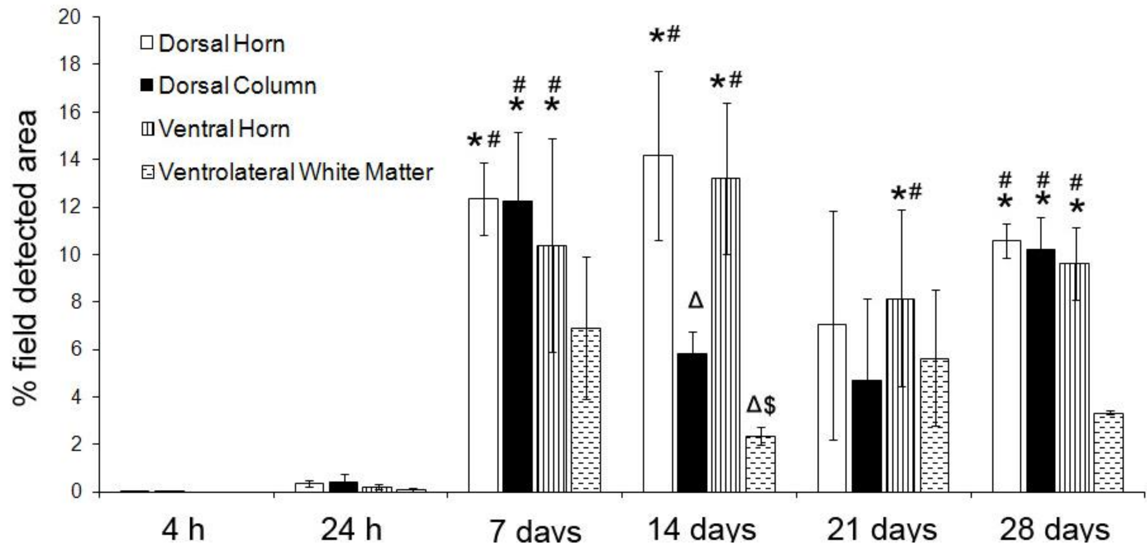



**Figure 3.6 Quantification of macrophage (ED1) infiltration.** Detailed quantification revealed no or few macrophages at 4 and 24 h. Maximal amounts were found at 7 to 28 days after SCI (* $p<0.05$ compared to 4 h; # $p<0.05$ compared to 24 h). Highest levels were seen in the dorsal columns and grey matter. A relatively smaller amount was seen in the ventrolateral white matter at all time points but this was only significant at 14 days (Δ $p<0.05$ compared to dorsal columns and $ $p<0.05$ compared to ventral horn). Results are expressed as mean ± SEM; n=3-4 per group.

### 3.2.3 Activated microglia/macrophages were visible 7-28 days after SCI

OX42 (CD11b) and Iba1 (ionized calcium binding adaptor molecule 1) are two antibodies commonly used to identify resting and activated microglia in rat CNS tissue. Both antibodies showed similar labelling of cells in laminectomy control tissue at all time points, with uniform labelling of cells in the white and grey matter (Fig. 3.7A, B). However, in injured tissue OX42 showed low signal to noise ratio using either DAB or

immunofluorescence methods and poor detection of ramified processes (Fig. 3.7C), whereas the Iba1 antibody labelled both ramified microglia and rounded cell types in grey and white matter (Fig. 3.7D). Therefore, in subsequent chapters the Iba1 antibody was used to detect resting and activated microglia.

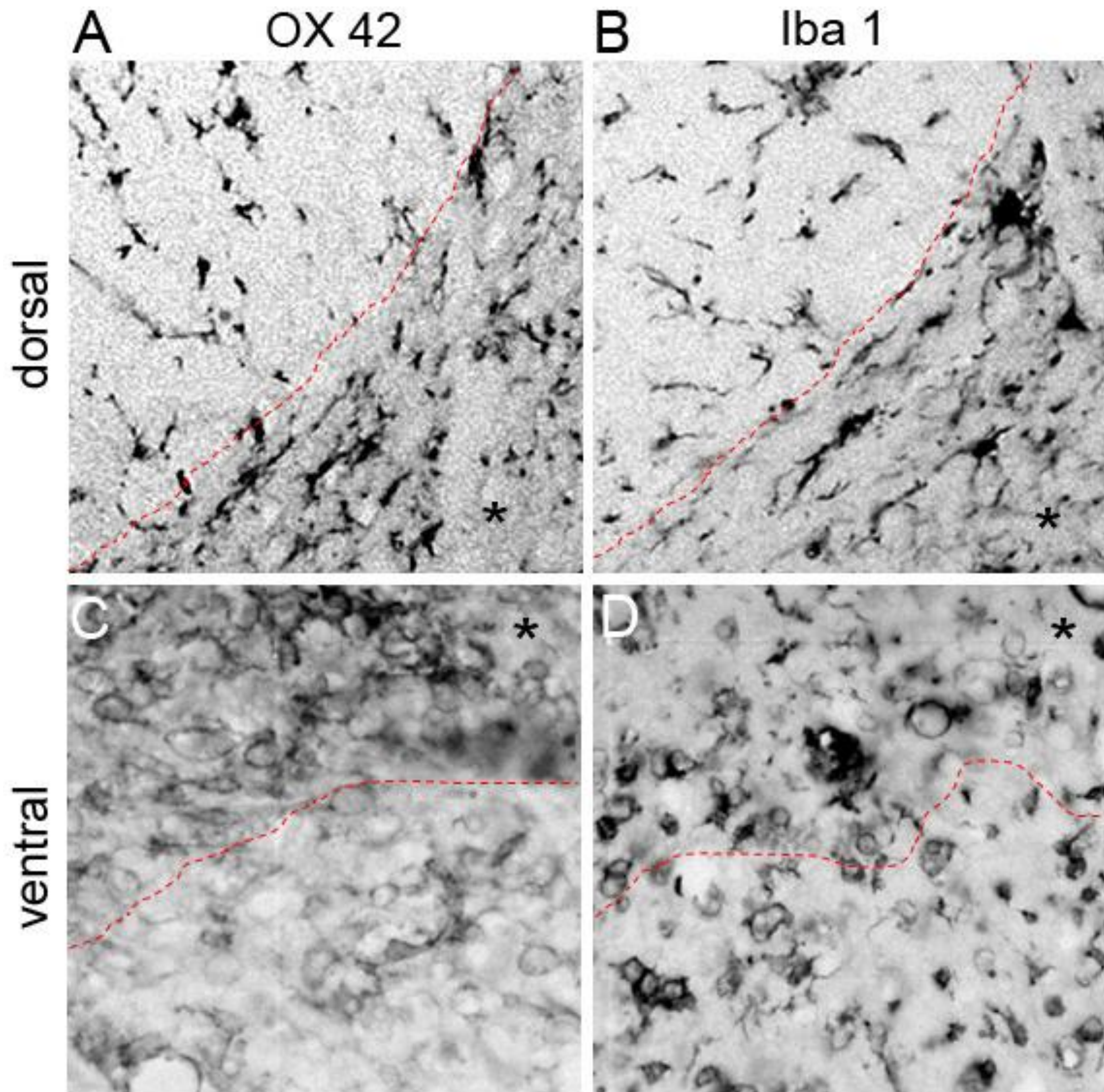
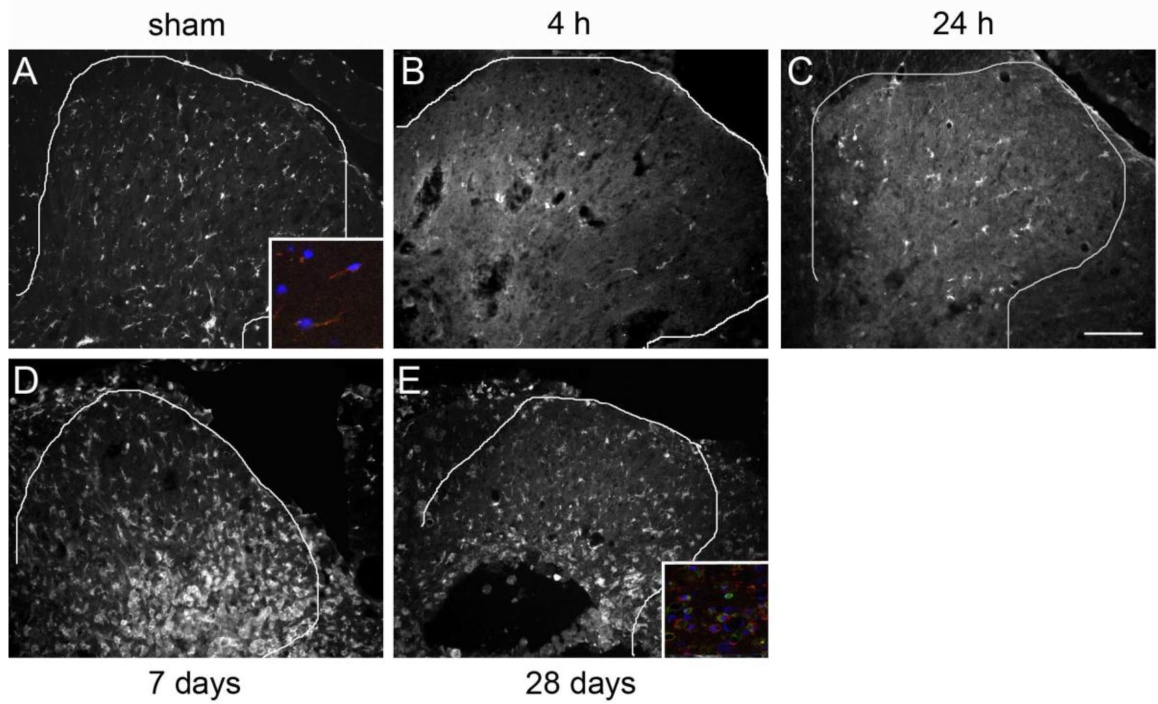



**Figure 3.7 Microglial markers. (A, B)** Ramified microglia (OX42, Iba1) in the dorsal horn and dorsal column of adjacent sections of laminectomy control spinal cord representative of all time points analysed (red dotted line marks the boundary between white and grey matter; (*) denotes the grey matter side). Labelling was uniform in the white and grey matter regions. **(C, D)** Swollen microglia in the ventral horn and ventrolateral white matter of adjacent sections of injured spinal cord 28 days after SCI (OX42, Iba1). Iba1 labelled more cells than OX42 in the white matter of all sections.

Figure 3.8A-E shows representative Iba1-labelled sections of SCI and sham laminectomy control tissue in the dorsal horn area of the epicentre at several time points. Iba1 labelling on 4 and 24 h tissue appeared to be less than in sham tissue, possibly due to loss of signal or a higher signal to noise ratio. However, 7 and 28 day tissue revealed a substantial increase in size and number of Iba1 cells. The two insets (Fig. 3.8A, E) show examples of the different phenotypes of microglial cells mentioned in the introduction; i) small, with elongated processes (Fig. 3.8A) and ii) enlarged and rounded (Fig. 3.8E). Detailed quantification (Figure 3.8F) confirmed the histological observations of low signal at 4 and 24 h and a significant increase of microglial labelling at later time points. 2 way ANOVA revealed a significant time ($F=19.89$) and region ($F=8.19$) effect. There was no remaining tissue available at the 14 and 21 day time points for Iba1 analysis. Laminectomy (sham) tissue was taken from all time points ($n = 2\text{-}3$ per group) and pooled, since there was no significant difference between time points.



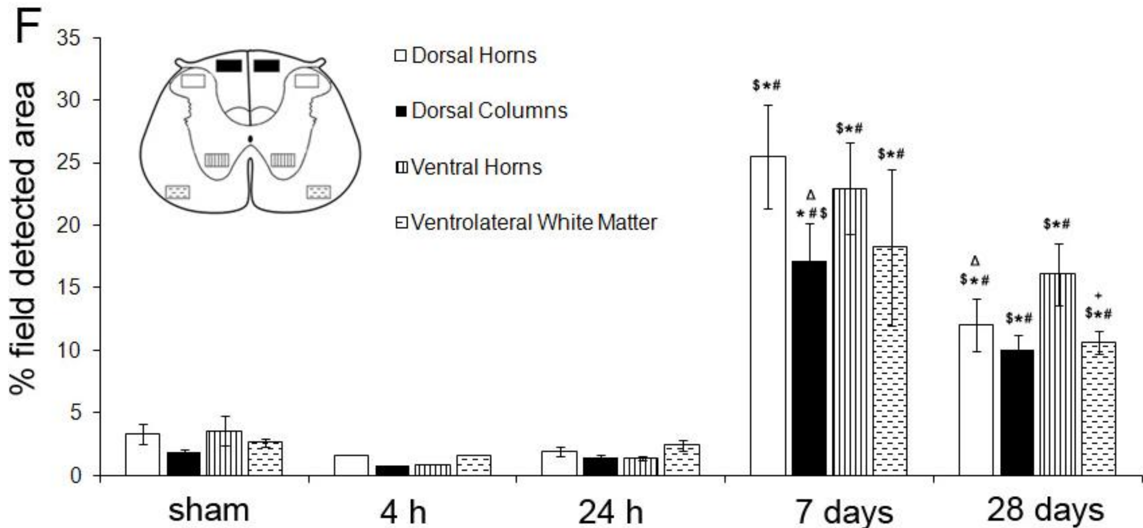




**Figure 3.8 Time course of microglia response.** Images show representative microglia (Iba1) labelling in the dorsal horn **(A-E)**. Cells became swollen and filled several areas of the epicentre. Insets **(A and E)** show high power confocal images of microglia labelled with Iba1 and Hoechst. Scale bar = 100 µm. **(F)** Detailed quantitative analysis of Iba1 supports a significant microglial reaction seen at 7 and 28 days. Results are expressed as mean ± SEM; n=3-4 per group.$*# $p<0.05$, compared to sham, 4 h and 24 h. + $p<0.05$ compared to 7 day VLWM, Δ $p<0.05$ compared to 7 day DC.

### 3.2.4 Infiltrating and resident macrophages (microglia) share the same phenotypic markers

ED1 and Iba1 both label resident phagocytic or activated microglia as well as infiltrating macrophages. This means that once blood-derived (haematogenous) macrophages occupy the epicentre, it is not possible to distinguish between them and resident microglia. It has not been documented to date, whether all Iba1 labelled cells co localise with ED1, or vice versa, or if a percentage of each marker co-localise as a proportion of total macrophages. Using confocal microscopy, we found a heterogeneous population of microglia at 28 days: some that were Iba1+/ED1+ and others that were Iba1+/ED1- (Fig. 3.9). Several apparent hypertrophic Iba1+/ED1+ cells were discovered that were larger (15-20 μm) than the ED1 cells seen at 1 day. Some of the Iba1+ cells also showed punctate ED1 labelling localised on the cellular processes.

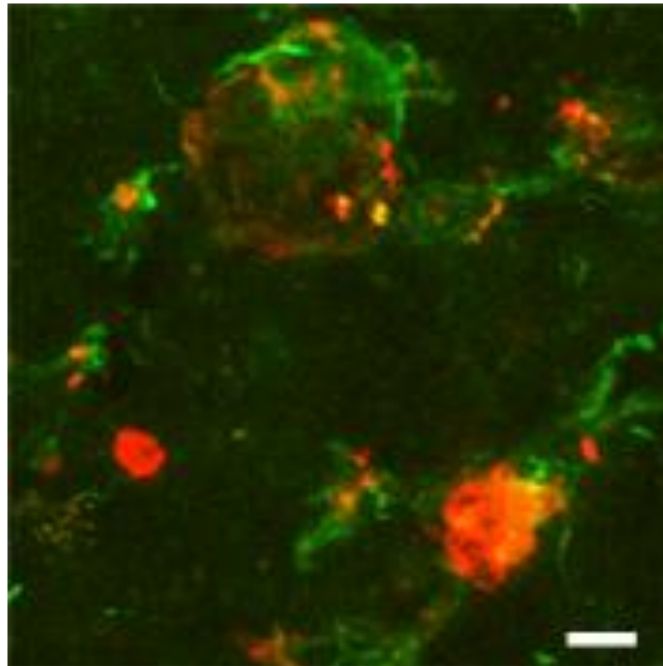


**Figure 3.9 Microglia/macrophages 28 days after SCI**. Large, swollen cells were labelled with both ED1 (red) and Iba1 (green) in the epicentre. Scale bar = 5μm.

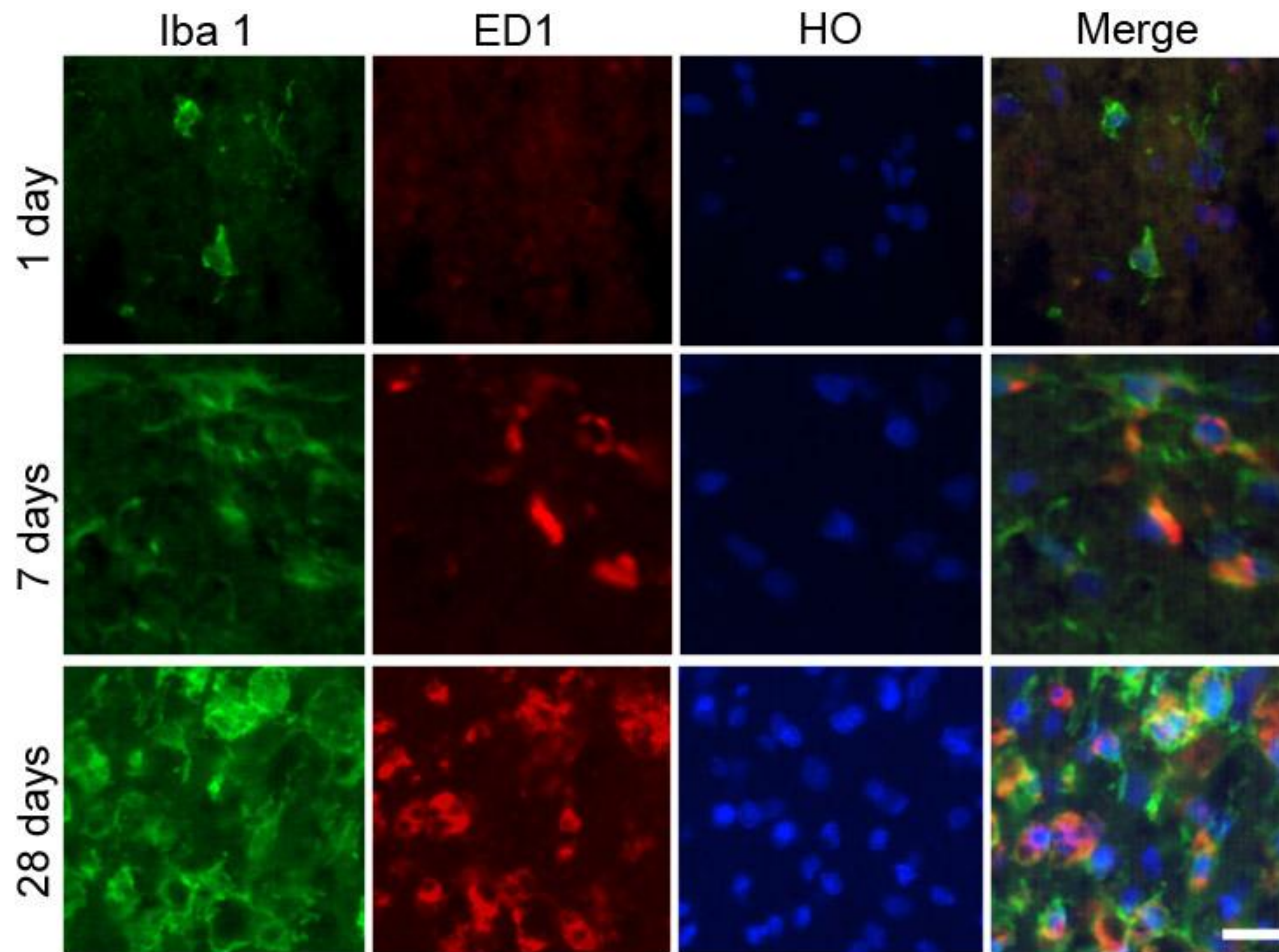



**Figure 3.10 Time course of ED1/Iba1 microglia/macrophage staining.** Iba1 single labelled cells were predominantly visible at 1 day. At 7 days, Iba1 labelled cells were mostly distinct from the rounded ED1 labelled cells. At 28 days many large, Iba1 and ED1 double labelled cells were seen. Scale bar = 20 μm.

Figure 3.10 shows in more detail how the two cell types varied over time. At 1 day after SCI, Iba1-labelled microglia were dominant. At 7 days, the two populations were quite distinctive and Iba1 cells were mostly separate from rounded ED1 labelled cells, although there was co-localisation of some cells. At 28 days a mixture of large, Iba1 and ED1 cells was observed.

### 3.2.5 Lymphocyte numbers were negligible after SCI

Using the cell surface markers CD4, CD8, CD45 and CD5 to identify B and T lymphocytes, validation of the specificity of these markers was first performed in spleen tissue. CD45 labelled cells in germinal zones typical of B lymphocytes (Fig. 3.11A) whereas CD4, CD8 and CD5 labelled T cells in areas surrounding arterioles. In the spinal cord epicentre, only one or two B cells were seen in one or two animals 7 d after SCI (Fig. 3.11B). Low numbers of CD4, CD8 and CD5 T cells were seen at 7 d and they were associated with blood vessels (Fig. 3.11C). T lymphocytes were absent in a preliminary assessment of 28 day tissue.

### 3.2.6 Reactive astrocytes formed a boundary around macrophages

Using an antibody that labels glial fibrillary acidic protein in astrocytes (GFAP), a comparison was made between laminectomy and injured spinal cord tissue (Fig. 3.12A, B). Laminectomy tissue showed GFAP labelling of astrocytes throughout the grey and white matter, whereas 28 days after SCI an astrocytic barrier was found to wall off intact white matter from an area filled with macrophages and reactive microglia (Fig 3.12C).

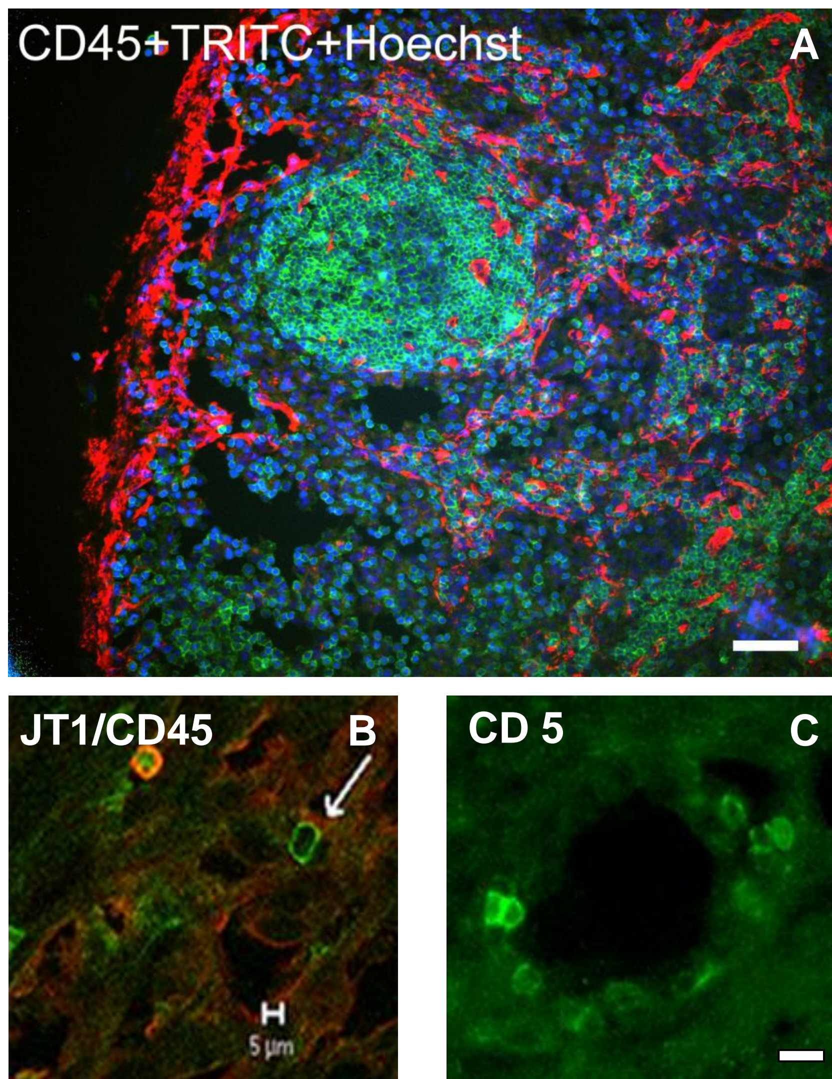



**Figure 3.11 Lymphocytes (A)** B lymphocytes (CD45, green) in a germinal zone found in naïve spleen tissue. Nuclei of other cells are visible in blue (Hoechst) and vascular labelling is in red (GAP43). Scale bar = 100 µm. **(B)** Spinal cord epicentre 7 days: a neutrophil (red) and a B lymphocyte (CD45, green, arrow). **(C)** At 7 days a few T lymphocytes (CD5, green) were seen in the epicentre grey matter associated with a blood vessel. Scale bar = 10 µm.

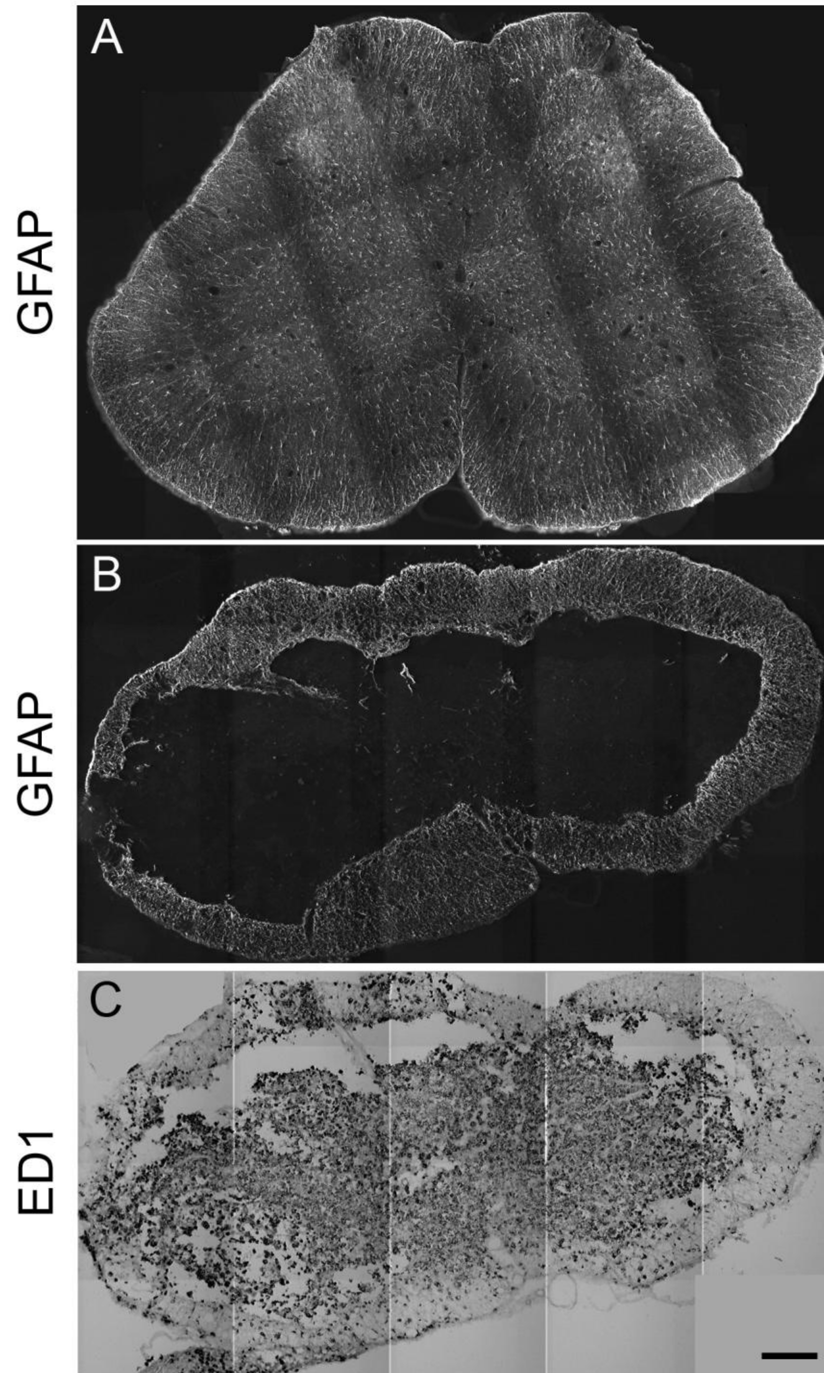



**Figure 3.12 Astrocytes (A)** Astrocyte (GFAP) labelling in the spinal cord after laminectomy. **(B)** 28 days after SCI, an astrocytic (GFAP) barrier walls off an area in the epicentre. (C) An adjacent section showing that the walled-off area is filled by macrophages (ED1). Scale bar represents 90µm (A-C).

### 3.2.7 The inflammatory response expands beyond the injury epicentre

As work progressed during this doctoral study, it was found that in experiments assessing chronic inflammatory markers, tissue in the epicentre contained several cavities. In sets of tissue where there were cavities, it was not possible to obtain an accurate quantification of inflammatory, neuronal or other non-neuronal cell markers at the epicentre. Therefore, in some cases, we analysed tissue rostral to the epicentre, where there were fewer cavities and there was spared tissue.

Since neutrophils were found to be largely absent in the epicentre from 7 days, we used primarily ED1 and Iba1macrophage/microglia markers to explore the inflammatory response at times of 7 days and beyond.

Figure 3.13A-C shows representative transverse sections of tissue from the epicentre and sections spanning 5 mm directly rostral and caudal to the epicentre. Due to the unpredictable variation in the location of areas of ED1 staining in rostral and caudal sections, the analysis made was based on % ED1 labelling of the whole section and an average of three sections was used for each animal (Fig. 3.13D). As expected, the highest amount of ED1 labelling was seen in the epicentre and about half of that amount in the rostral tissue, but this was not significant ($p>0.05$). There was also staining in caudal regions, but this was significantly less ($p<0.05$) and will therefore not be considered in the following chapters.

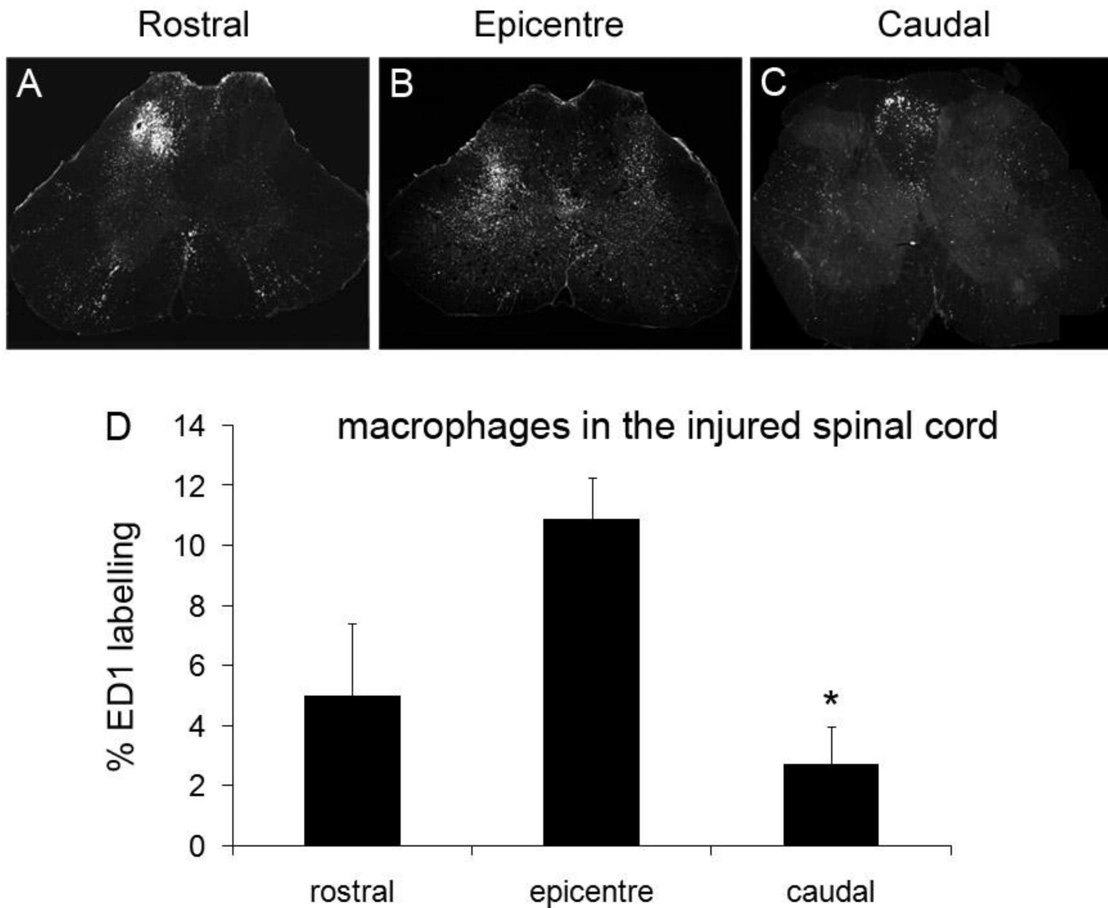



**Figure 3.13 Distribution of ED1 labelling rostral and caudal to the injury epicentre at 7 days.** Analysis of the whole transverse section was carried out, as described in the Methods chapter. There was significantly less ED1 labelling in the caudal segment compared to the epicentre (* $p<0.05$). Results are expressed as mean ± SEM; n=5 per group.

### 3.2.8 The systemic inflammatory response to compression SCI

*Neutrophils were recruited to the liver 4 h after SCI*

Parallel to the inflammatory events observed in the spinal cord after SCI, activation of resident macrophages and infiltration of neutrophils was studied in the liver (examples shown in Fig. 3.14). This is because we planned to assess the effects of omega-3 PUFAs delivered systemically and it is possible that they have effects on cytokines or leukocytes involved in the acute phase response in the liver.

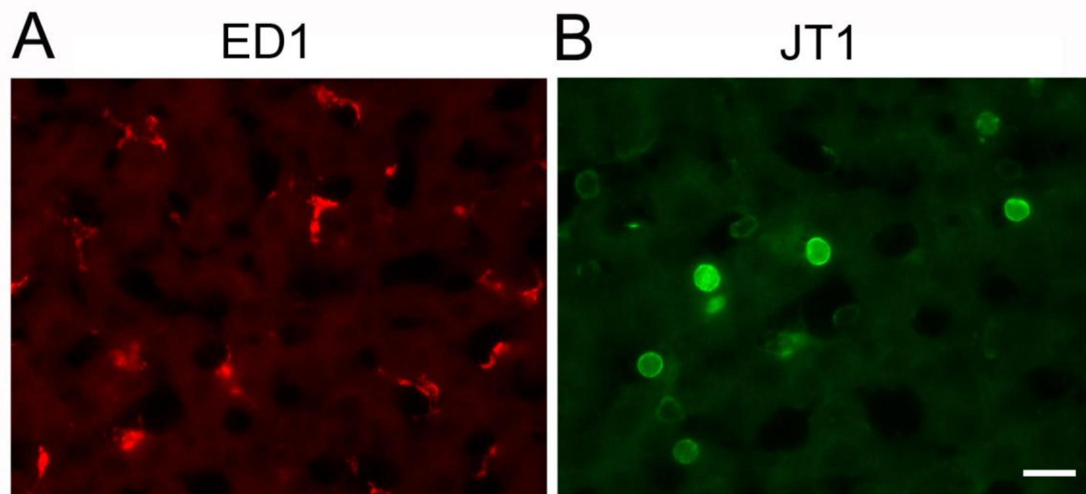



**Figure 3.14 Inflammatory response in the liver after SCI. (A)** Kupffer cells (ED1, red) in the liver and **(B)** neutrophils (JT1, green) in the same representative section following compression SCI. Scale bar = 15 µm. Figure reproduced from Hall et al., (*in submission).*

Quantification (Fig. 3.15) revealed that a few neutrophils were found scattered throughout the liver of naïve animals. One way ANOVA and post-hoc tests revealed that 4 and 24 hours following SCI, the amount of neutrophils found infiltrating the liver tripled significantly ($F=11.06$, $p<0.05$). In laminectomy control tissue there was a similar significant trauma related increase in hepatic neutrophils ($p<0.05$). At 28 days following SCI, neutrophil numbers remained elevated in SCI and this also appeared to be the case in laminectomy tissue (it was only possible to quantify two animals in the laminectomy group at this time point).

ED1 Kupffer cells were also seen in the liver (Fig. 3.14A). Their morphology was not rounded, as that of mature monocytes and macrophages found in the spinal cord, but elongated and probably located in the lumen of sinusoids, anchored to the sinusoidal endothelium of hepatic lobules. After several attempts, using various protocols, we found that the labelling of these cells with ED1 was inconsistent between animals and we therefore did not assess this marker in subsequent work.

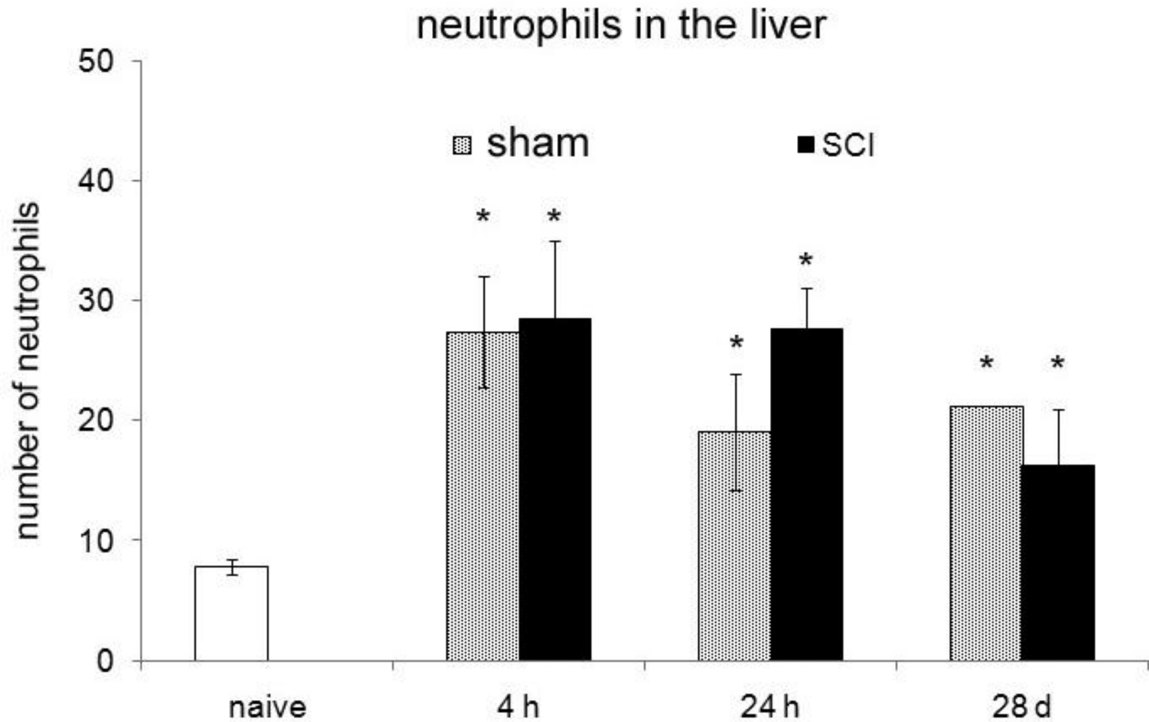



**Figure 3.15 Time course of liver neutrophil infiltration.** Detailed quantification revealed low numbers of neutrophils in naive liver tissue. Following sham surgery or SCI there was a significant increase in hepatic neutrophils compared to the naïve group and the numbers remained elevated 28 days after SCI (* $p<0.05$). Results represent mean ± SEM. n = 2-4 animals per group. Data reproduced from Hall et al., (*in submission).*

## 3.3 DISCUSSION

### 3.3.1 Neutrophils acutely infiltrated the injury epicentre

Following compression SCI there was an acute infiltration of neutrophils to the epicentre at 4 h and 24 h. The observation of peak neutrophil infiltration to the spinal cord at 1 day correlates with that reported by other laboratories, summarised in Table 3.1 (Dusart and Schwab, 1993; Zhang et al., 1997; Carlson et al., 1998; Schnell et al., 1999). We observed initial infiltration of neutrophils to the dorsal horns and dorsal columns, spreading at the peak to all areas of the injured parenchyma. A similar regional analysis has not been carried out (Popovich et al., 1997; Sroga et al., 2003), or was quantified in a different plane, so a comparison cannot be made (Zhang et al., 1997; Schnell et al., 1999). The methods used to quantify neutrophils also varied between studies. For example, MPO activity was used by Carlson et al. (1998), a general H & E stain was used by Zhang et al. (1997), and an in-house marker - HB 199 by Schnell et al. (1999). Bao et al. (2005) used an antibody for gp91$^{Phox}$, a membrane component of the enzyme NADPH oxidase expressed by phagocytes. However, this marker is also expressed by macrophages. We saw negligible numbers at later time points, in contrast to observations in mouse tissue where numbers persisted up to a month after contusion SCI (Kigerl et al., 2006). However, in a mouse hemisection model the numbers declined after 1 day (Schnell et al., 1999). Again, in these two studies different antibodies were used to label the neutrophils. It is possible that the JT1 antibody used here did not label the entire neutrophil population, but after comparison with other commercially available antibodies and histological images in the literature it was the most favourable choice. A preliminary analysis was made using this antibody on epicentre homogenates by Western blot. A consistent band was found, at 65 kDa, which increased in density at 4 and 24 h after SCI. It will be essential in future to fully characterise the epitope, in order to gain more information as to the nature of the cellular component detected. In particular, it appears to be localised to the cell surface and some cells were labelled less intensely than others. This could be due to down regulation of a receptor which could be representative of a functional characteristic such as release of myeloperoxidase, or initiation of phagocytosis, which could help to identify important intra-cellular signalling pathways. It would be

extremely useful in future, when assessing anti-inflammatory treatments, if we could distinguish between different neutrophil phenotypes and subpopulations.

### 3.3.2 Macrophages/microglia dominated the injury epicentre at later time points

The macrophage/microglia response was delayed, in comparison to the neutrophil response, not peaking until 7 days and remained elevated in the gray matter until 28 days. Our observations are similar to reports that have been published of the morphology of large cells, containing abundant lipid vacuoles, in sites of tissue destruction (Klusman and Schwab, 1997; Sroga et al., 2003). The results from the quantification of ED1 macrophages/microglia from 7 days after compression SCI were difficult to interpret, due to large variations between animals and areas analysed. It appeared that the response in the dorsal columns was reduced by half at 14 days. A drop in the overall amount of ED1 labelled cells in the epicentre has been reported elsewhere after 7 days, but this was followed by a plateau in the amount of ED1, not another increase as seen here. A similar extensive analysis in the different areas of the spinal cord has not been quantified (Dusart and Schwab, 1994; Zhang et al., 1997; Carlson et al., 1998; Sroga et al., 2003; Bao et al., 2005; Popovich et al., 2007).

Carlson et al. (1998) and Popovich et al., (1997) reported the highest density of macrophages in the grey matter and dorsal funiculus of the injury epicentre and we also saw highest levels of macrophages in these areas. This regional specificity of macrophage location occurred alongside similar areas of breakdown/permeability of the blood-brain barrier to horseradish peroxidase observed by Schnell et al. (1999) after SCI. They reported widespread blood-brain barrier breakdown at one day, continuing up to 14 days in a smaller region, which appeared to be confined to the grey matter and dorsal funiculus. Also relevant, is the fact that there is a larger microvascular network within the spinal gray matter, relative to the white matter (Popovich et al., 1997) and almost twice the number of veins entering the spinal cord, from where leukocytes are recruited, compared to arteries (Koyanagi et al., 1993). There are also differences in expression of adhesion molecules (Schnell et al., 1999) and chemokines, which may contribute to the regional characteristics of cellular infiltration and localisation described here (Popovich et al., 1997).

The Iba1 macrophage/microglia quantification cannot be compared directly with the ED1 macrophage/microglia quantification since the levels of immunoreactivity and image analysis will vary for each antigen. In particular, the Iba1 epitope labels an antigen on the cell surface, whereas ED1 labels a cytoplasmic antigen. Some authors have attempted to correct for this limitation by using a 'fill target' function (Popovich et al., 1997), but this was not considered necessary for the purposes of the analysis made here. Our aim was to obtain a quantitative estimate of changes in the magnitude of the cellular infiltrate. This method does not necessarily reflect changes in cell number. So an increase in ED1 immunoreactivity may not necessarily reflect changes in actual cell numbers but does indicate an increased state of activation for the cell types under investigation in this study.

Despite the technical issues, the Iba1 signal in the injury epicentre followed a similar pattern to the ED1 time course. Thus, the increase in Iba1 labelling was delayed compared to neutrophils and was elevated at 7 and 28 days. The amount of Iba1 labelling in the boxed area at 7 days appeared to be double the percentage of ED1 labelled cells. This could reflect the additional labelling of the population of resident microglia along with the infiltrating cells. At 28 days, the amount of Iba1 staining was similar to the levels of ED1 labelling in the epicentre at this time point. This reflects what was seen histologically, that nearly all macrophages/microglia in the epicentre at 28 days were large, rounded cells and there were few or no 'resting' microglia present. Activated microglia have been quantified by others, using immunohistochemistry, already by the first day after SCI, increasing in number by 7 days, then plateauing between 2 and 4 weeks (Popovich et al., 1997; Trivedi et al., 2006). The increase at 1 d was not obvious in our model and may be partly due to limitations in the sensitivity of the method of quantification. At these earlier timepoints, there was an increased amount of background noise compared to the signal (i.e. decreased signal to noise ratio), which therefore made it difficult to detect the thickening of the fine processes of microglia. Other methods have been used to quantify macrophages/microglia, such as Western blot analysis (Bao et al., 2005). However, this method means it is not possible to observe the location of the macrophages in the spinal cord. In other studies, counts have been carried out of macrophages/microglia (Roggendorf et al., 1996; Felts et al., 2005). However, it would not have been possible here, since it was difficult to visualise individual cells in dense clusters in the areas of damage after SCI. A comparison of various methods to quantify

macrophages by Donnelly et al. (2009) concluded that the least variable and most time efficient way of analysing macrophages/microglia after SCI was to use a densitometric thresholding method, similar to that used here.

In future work, it will be interesting to see if there are also differences between ED1 and Iba1 staining in tissue rostral to the injury epicentre. In addition, in SCI models it might yield more meaningful data if an analysis is done in areas where there is tissue sparing, rather than where there is already extensive tissue destruction and cavitation. Here, a comparison was made between ED1 labelling in sections rostral and caudal to the epicentre. A considerable amount (half of that seen in the epicentre) of macrophages was found to spread by 7 days as far as 5 mm above the epicentre, fewer below, and was found to be concentrated in one or two discrete areas, such as the dorsal funiculus, presumably in areas containing degenerating fibre tracts, as seen by Popovich et al., (1997). Macrophages can persist in areas of Wallerian degeneration for several months (Dusart and Schwab, 1994; Schwab and Bartholdi, 1996). Since our exploration of the tissue sections rostral and caudal to the epicentre revealed a heterogeneous distribution of macrophages, with both isolated clusters and widespread scattering of macrophages of different morphologies and sizes, this led us to critically evaluate our method of quantification. The method used in the epicentre, restricting measurement to specific discrete regions of the cord, meant that it was possible to miss the small areas of accumulations of macrophages. Although balanced by a slight loss in resolution quality, we found that to get a more accurate representation it was necessary to measure the immunoreactivity of the entire section.

Easily distinguishing between infiltrating and resident macrophages remains a challenge at present. The data reported here have shown that not all Iba1 labelled microglia stain for the ED1 marker. One study has compared ED1 with OX42 labelling, another marker of activated microglia (Popovich et al., 1997). A higher amount of OX42 labelling than ED1 labelling was found at all time points, the peak of OX42 slightly preceding ED1 at 7 days in Sprague-Dawley and Lewis rats, compared to 14 days post-SCI. Another study has compared ED1 and Iba1 in contusion SCI (Wu et al., 2005). According to their analysis, there was less Iba1 than ED1 overall. Co localisation staining was performed, similar to what was observed here, but quantification was not performed.

Macrophages have been found to persist for several months to years in the injury site after SCI in human (Fleming et al., 2006). Unlike neutrophils, macrophages do not undergo apoptosis in the first 1-2 days after entry to the injury site (Parham, 2000). In models of inflammation that reach resolution, the phagocytosis of neutrophils and debris by macrophages reduces the inflammatory reaction, which terminates upon their departure from the site of injury (Serhan and Savill, 2005). It is possible that blood derived macrophages infiltrate the spinal cord after SCI, departing in the same process as described above and that the macrophages found at chronic time points in the spinal cord are, in fact microglia that remain in an activated state. However, there are several experiments that found blocking the infiltration of leukocytes to the injury site was beneficial, which would suggest that blood-derived macrophages do accumulate in the epicentre. The beneficial effect of blocking macrophage infiltration is challenged by the research of Schechter et al. (2009), who found that the hematogenous derived macrophages perform an essential role that microglia are unable to carry out alone. The above observations could reflect differing phenotypes in a similar way to the M1 and M2 phenotypes described by Kigerl et al. (2009). Although the markers have not been characterised in rat tissue, it may be possible to use markers such as CD86 and iNOS that have been used to define the M1 population, and arginase 1 and CD206 for the M2 population, in combination with a pan-CNS macrophage marker. A heterogeneous population of microglia has also been demonstrated based on the Toll-like receptors (TLRs) present on their cell surfaces (Flaris et al., 1993).

Importantly, alongside the increase in macrophages/microglia in the epicentre, there appears to be an inverse correlation with locomotor recovery. The behavioural analysis was not included in this chapter, but in our model of SCI, locomotor function improves dramatically in the first 10 days post-SCI and then reaches a plateau, without full recovery. This means that recovery can occur despite the progression of the early inflammatory response, but at the later time points full recovery might not be reached due to the persistence of activated macrophages/microglia. Since full recovery is possible in the spinal cord in smaller, less severe lesions, it is possible perhaps, that ‘boosting’ the beneficial part of the inflammatory response after severe SCI towards the reparative

phenotype and towards a resolution of the inflammatory reaction is required in order for full recovery to be obtained.

### 3.3.3 Astrocytes walled off damaged areas of tissue

We found extensive astroctye labelling in uninjured as well as injured spinal cord tissue where, at later time points, they appeared to “wall off” cystic cavities containing macrophages. Astrocytes support neurones in the brain tissue and release several factors, including AA, DHA, and prostaglandins. During ischemic injury, deprivation of oxygen induces activation, proliferation, and hypertrophy of microglial cells and astrocytes (Farooqui et al., 2007b). After SCI astrocytes upregulate inhibitory molecules such as chondroitin sulphate proteoglycans (CSPGs) which are repellent to neurite outgrowth (Bareyre and Schwab 2003; Profyris et al., 2004). Both activated microglial cells and activated peritoneal macrophages have been shown to have striking morphological effects on astrocytes (Fitch et al., 1999). Therefore, the presence of macrophages may have prevented the entry of astrocytes into the necrotic injury site. Since it was not possible to define and study subpopulations of astrocytes, their roles in the inflammatory response as a whole remains to be fully investigated.

### 3.3.4 Lymphocyte numbers were negligible

We found very few lymphocytes in the epicentre at any of the time points investigated after compression SCI. Reports of infiltration of B and T lymphocytes to the injury site have been made after rat (Dusart and Schwab, 1994) or mouse hemisection (Schnell et al., 1999) and rat or mouse thoracic contusion (Hauben et al., 2000a; Sroga et al., 2003; Ankeny et al., 2006). In mice there appears to be a more robust and protracted response at later time points (14-56 days) (Kigerl, 2006; Sroga) than in rats, where there appears to be an early recruitment (1-7 days) in low numbers (Popovich et al., 1997; Sroga et al., 2003, Dusart and Schwab, 1994). The fact that neither B lymphocytes nor CD4 and CD8 phenotypes of T lymphocytes have been quantified in rat tissue in the literature to date, must illustrate their sparse appearance (Popovich et al., 1997; Sroga et al., 2003). Our observations are in agreement with Fleming et al., (2006) who found very low numbers in human spinal cord following SCI.

Attempts were made to optimize the lymphocyte markers used here by increasing their concentration or using amplification with tyramide but it may be that the strain and species differences that are found in the time course of lymphocyte infiltration meant that the optimal time points were missed. Therefore, we would need to look in future at further time points. For the purposes of this doctoral study, the numbers were considered too low to make an extensive analysis possible.

In classical immunology, the presence of lymphocytes is an indication of activation of the adaptive response of the immune system and has important implications for autoimmune disorders. It has been suggested that there are sufficient numbers of autoreactive lymphocytes to CNS antigens to create an autoimmune response (Ankeny et al., 2006). Activated T cells can modulate macrophage function and antibody production by B lymphocytes and others believe that they modulate wound healing through the release of cytokines, e.g. TGF-β and/or neurotrophins (Moalem et al., 1999; Hauben et al., 2000a). Together with the conflicting reports describing the role of lymphocytes after SCI it is clear that it would be important to follow up this part of the inflammatory response in future work (Shwartz and Hauben, 2002; De Caterina and Massaro, 2005; Ankeny et al., 2006).

### 3.3.5 Systemic response

We found that compression SCI is accompanied by a hepatic response. Evidence reported by Davis et al. (2005) and Campbell et al.(2003, 2005) demonstrates that the APR of the liver stimulates additional recruitment of leukocytes to the injury site following a mild compression SCI at thoracic level 8, and exacerbates damage. Such observations open up the possibility of reducing the inflammatory response and damage to the CNS by blocking the APR response in the liver. An increase in neutrophils was found at all time points considered after SCI, partly supporting the work reported in the literature. Interestingly, no difference was found in the number of neutrophils recruited to the liver after injury compared to laminectomy surgery alone, indicating that the APR response of the liver is not specific to injury of the spinal cord in our model and is a generic response

to tissue trauma. The APR response of a laminectomy group was not described in the studies described above (Campbell et al., 2005; Davis et al., 2005).

### 3.3.6 Conclusions

The results in this characterisation study demonstrate that the inflammatory response to compression injury is very similar to the response reported in contusion injury. Neutrophils are dominant in the injury epicentre at one day and then disappear, to be replaced at later time points (7-28 days) by macrophages/microglia, surrounded by an astrocytic barrier. There were very few lymphocytes present in the cord at the time points studied. There was a systemic response to trauma and compression SCI, marked by elevation in neutrophil numbers in the liver. From 7 days, there was a spread of macrophages to specific areas rostral and caudal to the epicentre of the spinal cord. The distinct timings and location of the different cell types are probably related to levels of cytokines/chemokines released from damaged blood vessels and cells in the injury site. An analysis of cytokines/chemokines will be described in the following chapter.

# Chapter 4: Results II. Comparison of the effects of the acute intravenous injection of DHA and EPA on the early inflammatory response

## 4.1 Introduction

There have been several promising approaches to the treatment of SCI that target the acute inflammatory response but none are yet used clinically. The literature to date reflects that it is widely believed by many researchers that the initial inflammatory response after SCI, i.e. the increase in pro-inflammatory signalling, along with infiltration of activated neutrophils, is detrimental to recovery from SCI. However, there is also evidence to the contrary, and this controversy is discussed in more detail later. If inflammation is damaging, reducing this early component of the inflammatory response would improve outcome. The cellular inflammatory response after SCI has already been introduced in chapters 1 and 3.The following introduction will expand on the cytokine/chemokine inflammatory response described in chapter 1 and consider why current acute anti-inflammatory treatments have not been successful. The remainder of the chapter will explore the effects of acute treatment with omega-3 PUFAs on the early CNS inflammatory response.

### 4.1.1 The inflammatory cytokine/chemokine response after SCI

The entry of inflammatory cells to the injury site after SCI is not random. As noted in the previous chapter, neutrophils enter earliest, followed by monocytes. One theory explaining the selective leukocyte entry into CNS regions of inflammation is that it is influenced by the prior differential expression of specific cytokines (Glabinski et al.1995, 1996a, b; Adams and Lloyd, 1997; Ransohoff, 1997; Ransohoff and Tani, 1997; McTigue, 1998)(refer to Table 1.1 of cytokines/chemokines in the general introduction, Chapter 1).

Cytokines are immunomodulating signalling molecules and are classified into five major groups: interleukins, growth factors, interferons, chemokines, and tumor necrosis factor

(TNF). IL-6, IL-1β and TNFα are the most well-documented and have been found in most cell types at the CNS injury site, at 3 and 6 h after injury (Bartholdi and Schwab, 2007). IL-1β and TNF-α have been shown to stimulate reciprocally their secretion (Pan et al., 2002), their amount reaching a peak several hours after injury but becoming negligible by 24 h. IL-1β, IL-6 and TNF-α are known to be mediators of inflammation and sustained elevations of TNF-α and IL-1β cause neuronal and oligodendrocyte death (Hermann et al., 2001; Shamash et al., 2002; Cai et al., 2003). Over-expression of IL-6 in the spinal cord enhances leukocyte infiltration, decreases axonal growth and impairs locomotor recovery (Lacroix et al., 2002).

Chemokines are a specific family of small cytokines (approximately 8-10 kilodaltons), or chemotactic cytokines. Their name is derived from their ability to induce directed chemotaxis in nearby responsive cells. They are classified according to shared structural characteristics, such as the presence of four cysteine residues in conserved locations that are key to forming their three-dimensional shape. One major subgroup is known as α-chemokines, including human interleukin-8 (IL-8). These are typically chemoattractive activators for neutrophils (Adams and Lloyd, 1997; Rollins et al., 1997). The expression of chemokines is regulated by the inflammatory cytokines TNF-α, IL-1β and interferon-γ (Hurwitz et al., 1995; Majumder et al., 1996; Ping et al., 1996; Sun et al., 1997; Streit et al., 1998). Cytokine-induced neutrophil chemo-attractant 1 (CINC-1) is the rat equivalent of IL-8 and growth-regulated oncogene (GRO) protein in humans. Elevated expression of the CINC proteins has been reported in the rodent CNS following stroke (Yamagami 1999). Blocking the interaction of chemokines such as macrophage inflammatory protein (MIP) with its receptor by using an infusion of the antagonist vMIPII (Ghirnikar et al., 2000) has been shown to limit the inflammatory response and provide significant neuroprotection after contusion SCI in the rat (Eng and Lee, 2003 for review).

Not all cytokines are neurotoxic. A growing number of cytokines have been found to exert beneficial effects on neurones and oligodendrocytes *in vitro* and *in vivo* (Lindholm et al., 1987; Martinou et al.,1990; Schwartz et al., 1991; Hama et al., 1991; Ikeda,et al., 1996; Kossmann,et al., 1996).Thus, a better understanding of the production of these

factors in the injured CNS is needed if we want to exploit this process with the aim to improve neurone survival after injury.

C-reactive protein (CRP) is an extensively studied marker of systemic inflammation in humans and rats. It is an acute-phase protein produced in the liver, thought to be regulated by IL-6, IL-1 and TNF-α and binds specifically to phosphocholine (Thompson et al., 1999), a component of microbial capsular polysaccharide, and participates in the innate immune response. Previous studies indicate that a chronic low-grade inflammation is involved in the pathogenesis of atherosclerosis, and an elevated CRP level is a risk factor for coronary artery disease (Ridker, 2003; Sattar et al., 2003). Elevated levels in adults with chronic SCI have been reported and provide evidence suggesting a state of chronic inflammation post-SCI (Manns et al., 2005; Gibson et al., 2008).

### 4.1.2 Acute anti-inflammatory strategies for SCI

Some of the anti-inflammatory strategies being explored to date, such as minocycline and integrin antibodies, have been introduced in Chapter 1. There are additional anti-inflammatory strategies currently being researched.

Cyclooxygenases (COX), lipoxygenases (LOX) and iNOS exert detrimental effects after SCI. COX enzymes exacerbate the inflammatory response by synthesising proinflammatory eicosanoids. Many studies have reported that a decrease in iNOS and COX leads to neuroprotection. Administration of selective inhibitors for iNOS and COX inhibitors has been tested in a model of SCI (Resnick et al., 1998; Hains et al., 2001) alone or in combination with grafts of olfactory ensheathing cells, and the results showed that treatment with COX and iNOS inhibitors improved locomotor ability and led to tissue sparing (López-Vales et al., 2006).

Blocking the proinflammatory cytokines TNF-α or IL-1β confers neuroprotection in rodent models of SCI (Nesic et al., 2001; Sharma et al., 2003; Genovese et al., 2005) and traumatic brain injury (Sanderson et al., 1999). Systemic treatment with the anti-inflammatory cytokine IL-10 limits secondary neurodegeneration and improves

locomotor recovery in some but not all SCI studies in rodents (Bethea et al., 1999; Brewer et al., 1999; Takami et al., 2002). Taurine is an organic acid and can act as an osmoregulator, antioxidant, inhibitory neuromodulator, and regulator of intracellular $Ca^{2+}$ flux. Injecting taurine intraperitoneally within 30 min after SCI decreases IL-6 and myeloperoxidase (MPO) activity, and significantly decreases motor dysfunction compared to controls (Nakajima et al., 2010).

Statins are the most commonly prescribed class of lipid-lowering drugs, and recently, it has been recognized that statins also have powerful immunomodulatory and anti-inflammatory effects. Treatment with simvastatin and atorvastatin appeared to offer remarkable improvements histologically and behaviourally (Pannu et al., 2005; 2007). Although initial results were promising, administration of simvastatin and atorvastatin after thoracic spinal cord contusion injury by an independent laboratory failed to replicate the effect when given with a 1 h delay (Mann et al., 2010). Discrepancies of efficacy between these two laboratories may be at least in part due to the different models used, severity and segmental level of SCI.

The time and expense needed to establish *de novo* pharmacological or biological therapies for acute SCI has encouraged the development of neuroprotective treatments based on drugs that are already in clinical use, like most of those described above. They offer the advantage of a well-characterized safety and pharmacokinetic profile in humans. Omega-3 PUFAs are well tolerated compounds and have potent neuroprotective effects. They are therefore, a good candidate for translation into SCI clinical trials.

### 4.1.3 PUFAs, SCI and neuroinflammation

Apart from their important structural and physiological roles, after injury, omega-6 and omega-3 PUFAs are important potential regulators of the onset and resolution of the inflammatory response (Farooqui et al., 2007b; Serhan et al., 2008), as described in Chapter 1.

After SCI, the enzyme phospholipase A2 is up-regulated in microglia, neurons and oligodendrocytes (Liu et al., 2006). This enzyme releases free fatty acids (FFA; Anderson and Hall, 1993) from cellular membranes. Arachidonic acid (AA), an omega-6 PUFA, is highly enriched in the membrane phospholipids of neurones and is one of the major species of fatty acids released (Demediuk et al., 1985a, b, 1989; Murphy et al., 1994). Cyclooxygenases and lipoxygenases play a key role in converting precursors of omega- 6 PUFAs such as AA to proinflammatory eicosanoids such as prostaglandins, thromboxanes and leukotrienes (Smith and DeWitt, 1996), leading to further proinflammatory signalling of cytokines and chemokines which enhance vascular permeability and attract leukocytes to the injury site. Omega-3 fatty acids such as EPA act as a competitive substrate for cyclooxygenases and generate less inflammatory eicosanoids such as 3-series prostaglandins and thromboxanes, 5-series leukotrienes, as well as anti-inflammatory metabolites such as resolvins and protectins (for review, see Calder 2009). Conversion of DHA by a lipoxygenase-like enzyme produces anti-inflammatory and pro-resolving chemical lipid mediators collectively called docosanoids (Serhan, 2006). DHA and EPA metabolites have been shown to antagonize the effects of proinflammatory eicosanoids, decrease proinflammatory cytokine production in glial cells and attenuate neutrophil migration and tissue injury in peritonitis and ischemia-reperfusion injury models (Hashimoto et al., 1999; Hong et al., 2003; Marcheselli et al., 2003; Serhan et al., 2004; Spite et al., 2009).

Work in other laboratories has shown that DHA is a potent inhibitor of lymphocyte adhesion to endothelial cells. DHA and EPA can inhibit the NFκB signalling system (De Caterina and Massaro, 2005; De Smedt-Peyrusse et al., 2008), in turn preventing the expression of pro-inflammatory cytokines including TNF-α and IL-1α and β and modulating leukocyte trafficking. Thus, evidence suggests that the generation of omega-3 fatty acid metabolites from EPA and DHA may provide potent anti-inflammatory protective mechanisms for preventing neuronal damage after trauma.

Work in our laboratory has shown that the omega 3 polyunsaturated fatty acids (omega-3 PUFAs) possess potent neuroprotective effects. In particular, DHA injection alone and in combination with DHA dietary intervention leads to a significantly improved outcome

after SCI (Huang et al. 2007a; King et al., 2006). We also have some evidence that another omega-3 PUFA, eicosapentaenoic acid (EPA), could be neuroprotective when given as an acute bolus treatment following SCI (Lim et al., *in press*). Macrophage infiltration and cyclooxygenase-2 protein levels at the epicentre were reduced by DHA (Huang et al. 2007a). Therefore, it is possible that part of the neuroprotective effect of these agents is due to their anti-inflammatory properties. Their effect on the acute infiltration of other leukocytes such as neutrophils has not yet been characterised after SCI.

### 4.1.4 Aims

In this chapter we will explore the effects of an early (30 min post-SCI) DHA or EPA bolus injection on neutrophil (4 and 24 h) and macrophage (7 days) infiltration in the spinal cord and in the liver. In addition, we will measure cytokines and chemokine in the injury epicentre and systemically, and make an assessment of the impact of DHA or EPA bolus injection on their levels.

## 4.2 RESULTS

The three experimental groups used in this chapter will be referred to as the following:

Saline/vehicle – one bolus **intravenous** injection of saline (0.9 % NaCl, pH 7.4) injection, 30 min after compression SCI
DHA (250 nmol/kg) – one bolus **intravenous** injection, 30 min after compression SCI
EPA (250 nmol/kg) – one bolus **intravenous** injection, 30 min after compression SCI

### 4.2.1 DHA, but not EPA, significantly reduced neutrophil infiltration at the epicentre

In the previous chapter we found that high levels of neutrophils influx into the injury epicentre at 4 and 24 h after SCI. Therefore, in this study we evaluated the effects of intravenous omega-3 PUFA injection on neutrophil infiltration at 4 and 24 h. There appeared to be fewer neutrophils in the ventral horns following DHA injection. Representative immunohistochemical labelling of neutrophils in the epicentre 24 h after SCI and intravenous injection of saline, DHA or EPA is shown in Figure 4.1. Detailed quantification of JT1 cells in specific areas of the epicentre revealed a significant decrease in the ventrolateral white matter (VLWM) at 4 h following DHA injection compared to saline injection (-71%, $*p<0.05$; Fig. 4.2A). There was a similar trend towards a decrease in neutrophil infiltration in the dorsal columns 4 h after DHA and EPA treatment, but this was not significant. At 24 h after SCI, the number of neutrophils in the epicentre increased three to twenty-fold in the saline group compared to 4 h, and there were significantly fewer neutrophils in the ventral horns of the DHA-treated group than the saline treated group (-40%, $*p<0.05$; Fig 4.2B). There was a trend towards a reduction in the number of neutrophils in most areas of the injury epicentre after treatment with EPA but this did not reach significance.

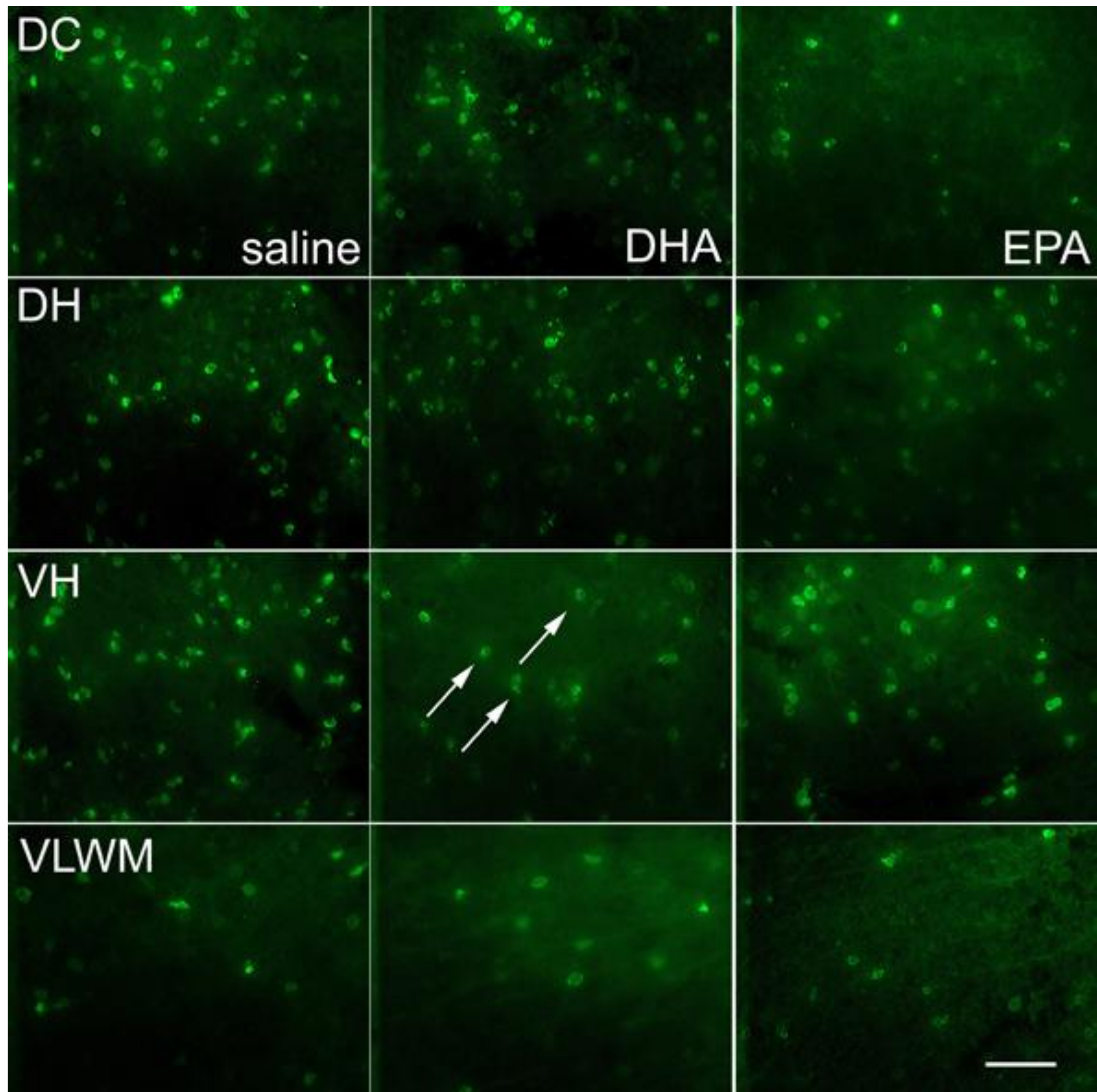



**Figure 4.1**. **Representative immunohistochemical labelling of JT1 neutrophils in specific areas of the epicentre 24 h after SCI and one intravenous injection of saline, DHA (250 nmol/kg) or EPA (250 nmol/kg) 30 min after SCI**. There appeared to be fewer neutrophils (arrows) in the ventral horns following DHA injection, and this was confirmed by quantitative analysis (Fig X). Dorsal columns (DC), dorsal horns (DH), ventral horns (VH), ventrolateral white matter (VLWM). Scale bar = 50 µm. Figure reproduced from Hall et al., (*in submission).*

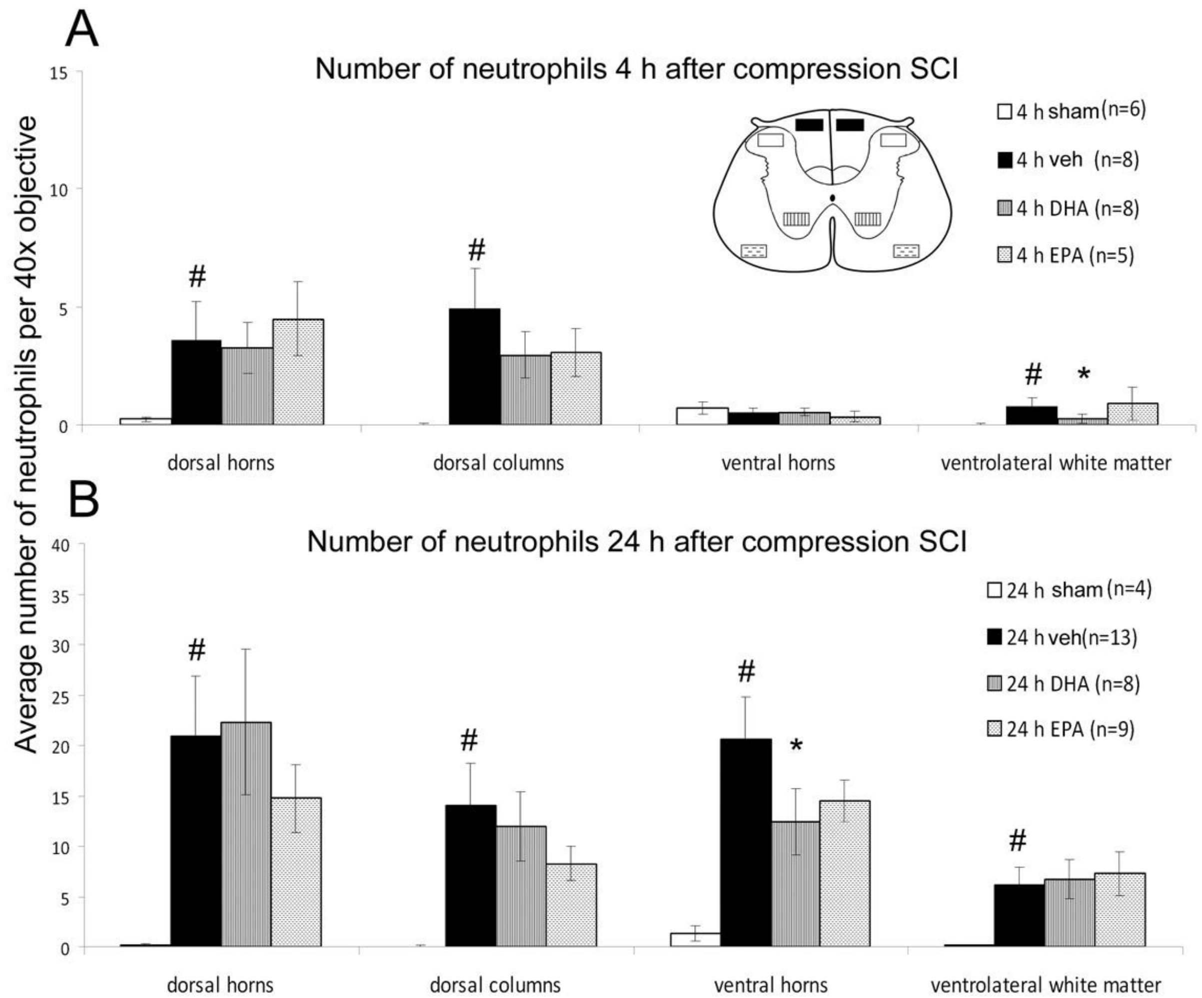



**Figure 4.2**. **Quantification of neutrophils in specific areas of the injury epicentre, 4 h and 24 h after SCI and one intravenous injection of DHA (250 nmol/kg), EPA (250 nmol/kg), or vehicle (veh) 30 min after SCI. (A)** JT1 immunostaining revealed that there was a significant increase in neutrophils in the dorsal columns (DC), dorsal horns (DH) and ventrolateral white matter (VLWM) of the vehicle group compared to sham (#p<0.05). There were significantly fewer neutrophils in the VLWM of the DHA-treated group than the vehicle group (*p<0.05). There was a trend towards a reduction in the DC following treatment with DHA and EPA but this was not significant. **(B)** 24 h after SCI the number of JT1 immunoreactive neutrophils in the epicentre increased three to twenty-fold in the vehicle group compared to 4 h and was significantly greater than sham (#p<0.05). There were significantly fewer neutrophils in the ventral horns of the DHA treated group than the vehicle treated group (*p<0.05). There was a trend towards a reduction in the number of neutrophils in most areas of the injury epicentre after treatment with EPA, but this did not reach significance. Results are mean ± SEM number of animals in brackets. Data reproduced from Hall et al., (*in submission).*

### 4.2.2 Myeloperoxidase activity

In parallel with the reduction of neutrophils in the DHA-treated group we quantified the levels of myeloperoxidase (MPO) activity, an enzyme released by neutrophils, in the injury epicentre, to see whether they were also reduced. MPO activity (Fig. 4.3) was significantly increased 2-3 fold (#$p<0.05$) at 4 and 24 h after SCI compared to animals that received laminectomy surgery, but was not attenuated by treatment with DHA or EPA (Fig. 4.3).

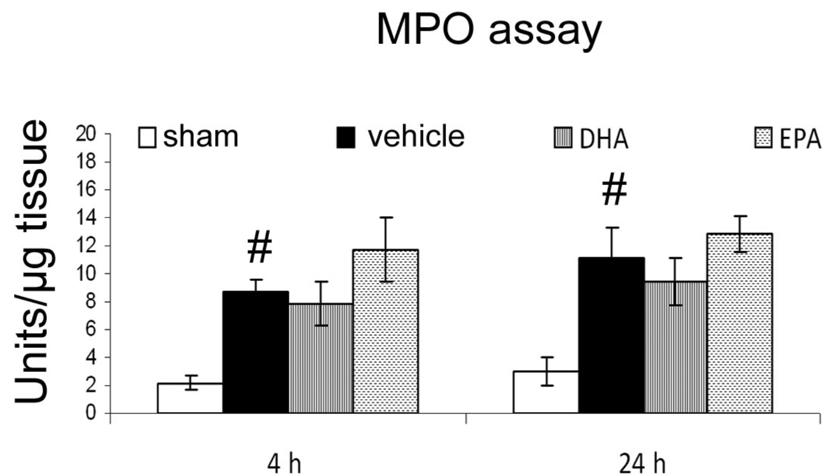



**Figure 4.3 Quantification of MPO in the epicentre, 4 h and 24 h after SCI and one intravenous injection of DHA (250 nmol/kg), EPA (250 nmol/kg), or vehicle (veh) 30 min after SCI.** MPO activity was significantly increased (#$p<0.05$) in the injury epicentre after SCI compared to sham but was not attenuated by treatment with DHA or EPA. Values are the mean ± SEM of 4 and 24 h sham (both n=4); 4 h vehicle and EPA (n=5), 4 h DHA (n=4); 24 h vehicle, DHA and EPA (all n=5). Data reproduced from Hall et al., (*in submission).*

### 4.2.3 Cytokine and chemokine changes in the injury epicentre

Since it was the first time that cytokine and chemokines were to be measured in spinal cord tissue using the electrochemiluminescence method in our laboratory, a preliminary validation study was carried out on a 7-plex plate using a 24 h plasma sample. The only cytokine/chemokine 7-plex plate available at the time for rat tissue contained the following antibodies: IFN-γ, IL-1β, IL-4, IL-5, IL-13, KC/GRO/CINC, TNF-α. Levels of most of these factors were undetectable or at very low levels in plasma one day after SCI. Some of the wells on the plate were used to measure levels in spinal cord tissue. A significant increase ($p<0.001$) was detected in all 7 of the cytokines and chemokines in

the epicentre after SCI compared to naive and sham groups (data not shown). Following these preliminary results, we designed a custom 4-plex plate containing a select group of factors: IL-6, KC/GRO/CINC, IL-1β and TNF-α, which are reported to be increased after SCI and are related to neutrophil infiltration (Bareyre and Schwab, 2003; Bartholdi and Schwab, 1997; Aimone et al., 2004; Tonai et al., 2001; Streit et al., 1998; McTigue et al., 1998; Pan et al., 2002).

Following measurement of spinal cord, liver and plasma samples on the 4-plex plates detailed above we found that SCI led to a significant increase ($p<0.001$; Fig. 4.4) in the levels of IL-6, KC/GRO/CINC, IL1-β and TNF-α in the epicentre 4 h after SCI. Cytokines and chemokines returned to baseline levels at 24 h. Treatment with DHA (Fig. 4.4) or EPA (Fig. 4.5) did not affect the increase in levels of these cytokines and chemokines after SCI. The data for EPA and DHA in the spinal cord is shown separately because the two treatments were assessed in a separate set of animals, with new controls and separate plates for each experiment.

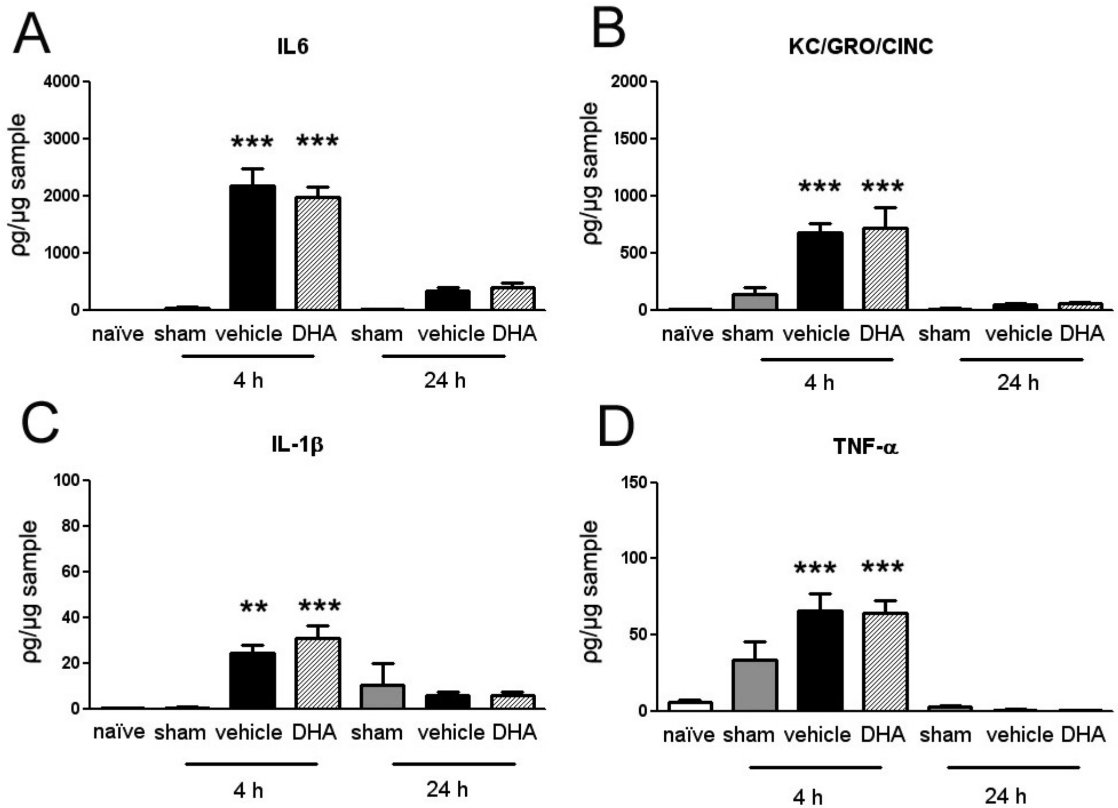



**Figure 4.4**.**Quantification of cytokines/chemokines in the injury epicentre 4 h and 24 h following SCI and one intravenous injection of DHA (250 nmol/kg) treatment 30 min after SCI. (A-D)** SCI led to a significant increase (**p<0.01, ***p<0.001) in the levels of IL-6 (A), KC/GRO/CINC (B), IL1-β (C) and TNF-α (D) in the epicentre 4 h after SCI compared to naïve and sham control. Cytokines and chemokines returned to baseline levels at 24 h. Treatment with DHA did not affect the levels of any of these cytokines and chemokines after SCI. Results are mean ± SEM number of animals (n=4-7 per group). Data reproduced from Hall et al., (*in submission).*

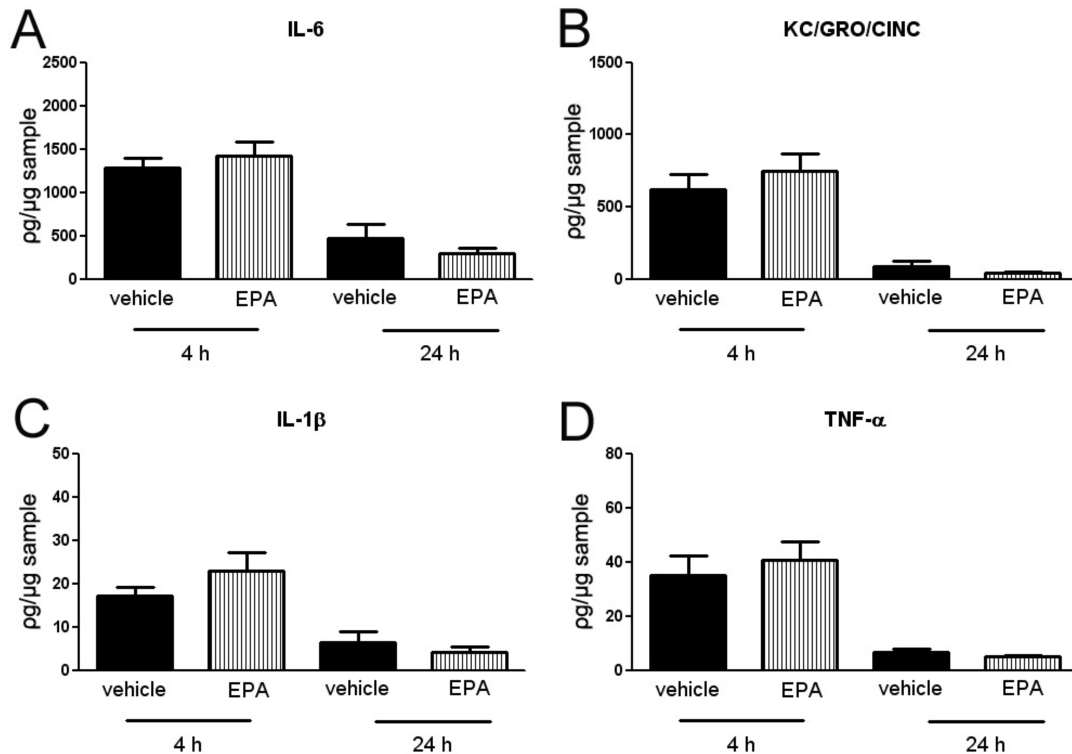



**Figure 4.5 Quantification of cytokines/chemokines in the injury epicentre 4 h and 24 h following SCI and one intravenous injection of EPA (250 nmol/kg) 30 min after SCI.** **(A-D)** Treatment with EPA did not alter the levels of IL-6 **(A)**, KC/GRO/CINC **(B)**, IL-1β **(C)** or TNF-α **(D)** after SCI. Results are mean ± SEM number of animals (n=5-6 per group). Data reproduced from Hall et al., (*in submission)*.

### 4.2.4 Effects of acute omega-3 PUFA injection on systemic inflammatory markers

4 h and 24 h after SCI and laminectomy surgery there was a trauma-related increase in the number of neutrophils recruited to the liver (Fig. 4.6A). Treatment with DHA or EPA did not reverse this increase.

The same 4-plex plates detailed in the previous section were also used to measure cytokines and chemokines in liver tissue and circulating levels in the plasma. Circulating plasma (Fig. 4.6B) and hepatic levels (Fig, 4.6C) of KC/GRO/CINC were significantly increased 4 h following laminectomy and compression SCI ($p<0.05$ and $p<0.01$, respectively). Levels returned to baseline 24 h after surgery. Treatment with DHA or EPA did not reverse this increase. A similar pattern was apparent for IL-6, IL1-β and

TNF-α, but the levels in many samples were close to the limit of detection (data not shown).

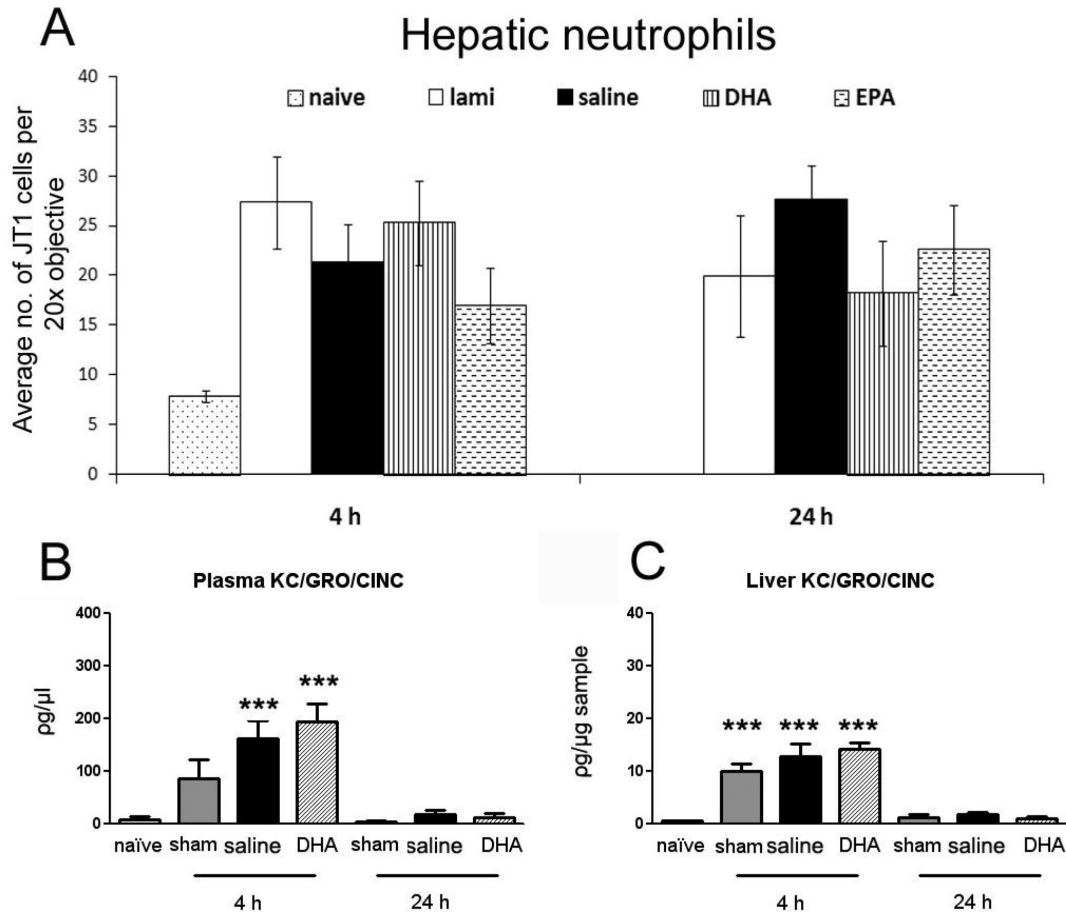



**Figure 4.6 Quantification of systemic neutrophils, cytokines and chemokines after SCI and one intravenous injectionof DHA (250 nmol/kg), EPA (250 nmol/kg), or saline 30 min after SCI. (A)** 4 and 24 h after SCI there was a trauma related increase in the number of neutrophils recruited to the liver. Treatment with DHA or EPA did not reverse this increase. Results are mean ± SEM of naïve (n=3); 4 and 24 h sham (both n=4); 4 h vehicle (n=11); 24 h vehicle (n=7); 4 and 24 h DHA (both n=8); 4 h EPA (n=6); 24 h EPA (n=9).Circulating plasma **(B)** and hepatic levels **(C)** of KC/GRO/CINC were significantly increased 4 h following compression SCI compared to naive levels (***p<0.001) and additionally in the sham group (***p<0.001). Levels returned to baseline values 24 h after surgery. Treatment with DHA or EPA did not reverse this increase. Results are mean ± SEM number of animals (n=3-7 per group). Figure reproduced from Hall et al., (*in submission).*

*C - reactive protein (CRP)*

Control levels of CRP in the 4 h plasma samples from sham or naïve animals were 384 ± 32 µg/ml (Fig. 4.7). Following SCI, there was a significant increase in the vehicle (saline)

treated group to 471 ± 20 µg/ml. DHA significantly reduced the CRP levels to control levels (392 ± 10 µg/ml; $p<0.05$), whereas EPA had no significant effect (507 ± 23 µg/ml; $p>0.05$).

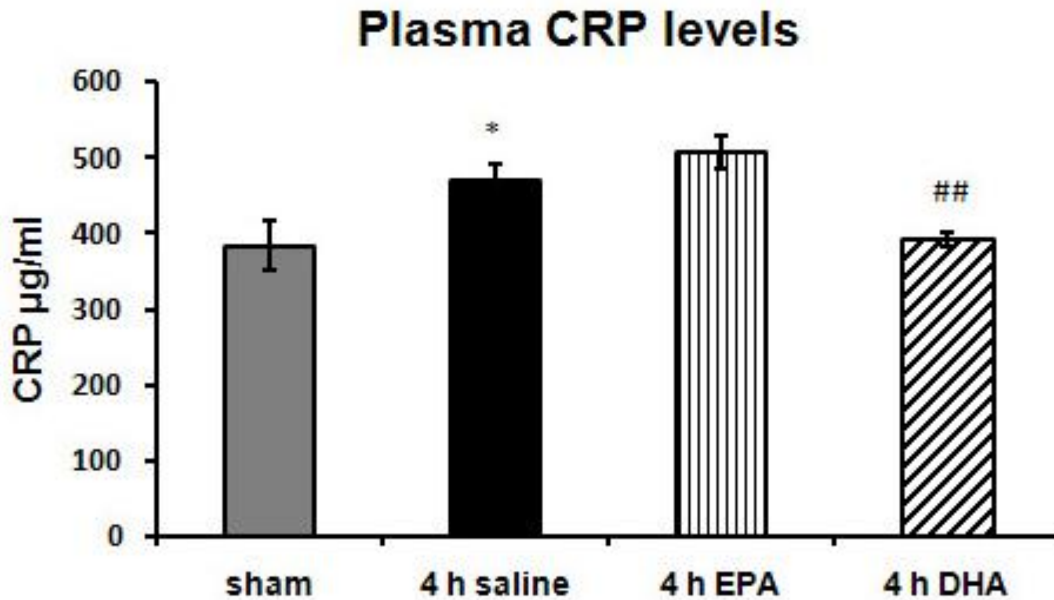
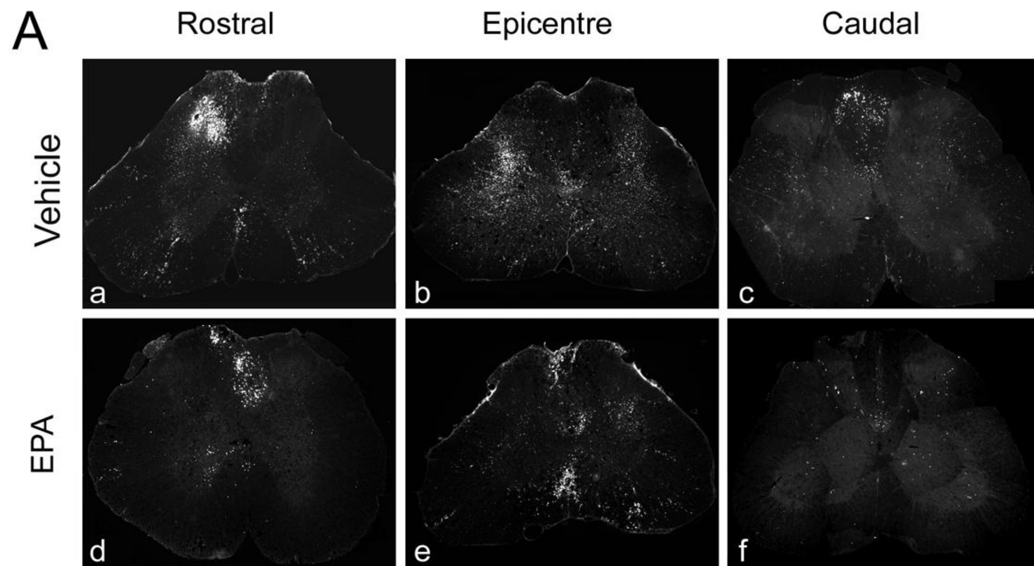



**Fig. 4.7 CRP plasma levels 4h after SCI and one intravenous injectionof DHA (250 nmol/kg), EPA (250 nmol/kg), or saline 30 min after SCI.** ELISA revealed a significant increase in the vehicle (saline) treated group ($p<0.05$). DHA significantly reduced the CRP levels to control levels ($p<0.05$), whereas EPA had no significant effect ($p>0.05$). Data reproduced from Hall et al., (*in submission).*

#### 4.2.5 Effects of acute EPA injection on macrophage infiltration

Our laboratory has previously assessed the effect of DHA injection on macrophage infiltration. A significant reduction was found at 7 and 28 days (Huang et al., 2007a). Detailed quantification in the rostral, epicentre and caudal sections revealed a trend towards a reduction of 28% in the epicentre of ED1 labelled macrophages at 7 days following acute EPA injection (Fig. 4.8B). However, this did not reach significance ($P > 0.05$). Similarly, in rostral and caudal sections there was no statistically significant decrease in the amount of macrophages in the EPA treated group ($P > 0.05$).



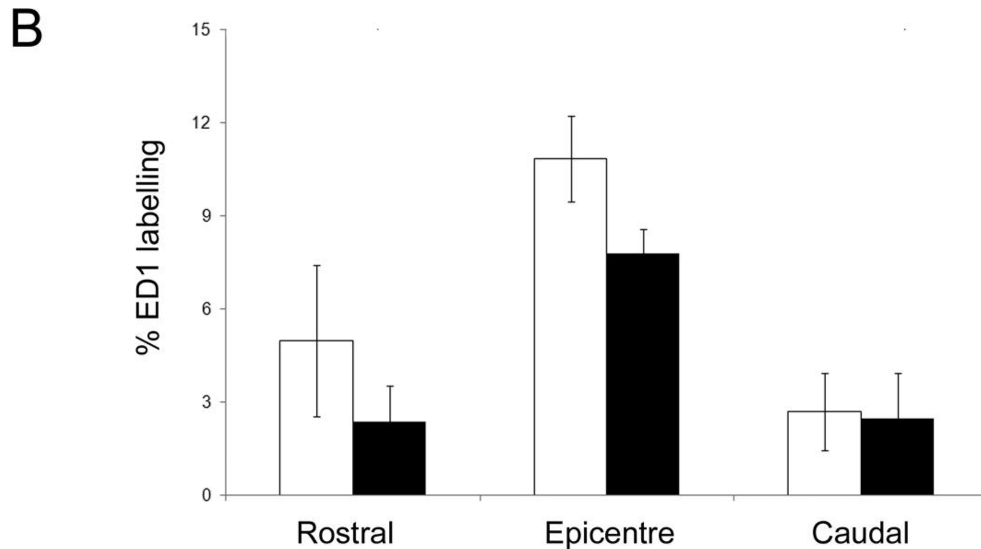




**Figure 4.8 ED1 labelling of macrophages and activated microglia 7 days following SCI and one intravenous injection of EPA (250 nmol/kg), or saline 30 min after SCI.** Following treatment with EPA, there appeared to be a decrease in the amount of ED1 labelling in the epicentre **(Ae versus Ab).** Detailed quantitative analysis **(B)** revealed a trend towards a decrease in the amount of ED1 labelled macrophages in the epicentre. However, this did not reach significance ($P > 0.05$). Similarly, in rostral and caudal sections **(Ad versus Aa; Af versus Ac)** there was no significant decrease in the amount of macrophages in the EPA treated group ($P > 0.05$). Data represents mean ± SEM; n=4 or 5 in each group. Figure reproduced from Lim et al., 2010 but created and data analysed by JH.

## 4.3 DISCUSSION

In the current chapter, we have explored the effects of DHA and EPA injection on the acute local and systemic inflammatory response following compression SCI. An i.v. injection of DHA (250 nmol/kg), 30 min after SCI, significantly reduced the amount of neutrophils in some areas of the injury epicentre, whereas the EPA (250 nmol/kg) injection had no significant effect, although there was a trend towards a reduction. Further experiments showed a robust increase in the injury epicentre of MPO and cytokines and chemokines and CRP in the plasma, at 4 h after SCI. DHA significantly returned CRP levels to control values, whereas EPA did not. DHA and EPA did not change the myeloperoxidase (MPO) activity in the tissue epicentre, nor did they reduce the levels of pro-inflammatory cytokines and chemokines, systemically or locally, associated with neutrophil migration.

### 4.3.1 Effects of DHA and EPA on the local neutrophil response to compression SCI

A reduction in the number of neutrophils of 71% in the ventrolateral white matter (VLWM) at 4 h and 40% in the ventral horns at 24 h was found in the injured epicentre after treatment with DHA. DHA could be targeting events in the endothelia, preventing chemotaxis and extravasation of neutrophils, as demonstrated by others (Sperling et al., 1993; Lee et al., 1985). It is not clear why the effect of DHA was confined to only two regions of the epicentre. We cannot directly compare the impact of treatment on neutrophils to the work of Fleming et al. (2008, 2009) since their quantification was not based on a cell count, but the percentage of immunoreactivity of MPO positive neutrophils. Also, a much larger sample box (0.5 cm x cord width) than ours was analysed on longitudinal sections. The improved behavioural and sensory outcome that they observed following their clip compression model of SCI was correlated with at least a 50% reduction in neutrophil infiltration at 3 days, but no improvement was seen after delayed treatment and a reduction of only 36%. Despite a decrease in our model of up to 71 % in the VLWM at 4 h, the smaller reduction in other areas of the epicentre would dilute the effect if the counts were pooled to reflect a larger total area and there would have been a modest reduction overall. It is therefore very unlikely that the behavioural

improvement associated with omega-3 injection found elsewhere (Lim et al., *in press*; King, et al., 2006; Huang, et al. 2007a) is solely due to the modest reduction seen in neutrophil numbers.

DHA and EPA have been reported to differ in their regulation of inflammatory processes. For example, DHA is more effective than EPA in reducing acute inflammatory processes such as infiltration of lymphocytes and expression of cytokines (Tomobe et al., 2000), whereas others suggest the opposite (Sierra et al., 2008; Terano et al., 1987). Some of these differences can be attributed to the different inflammation models used, such as controlled *in vitro* models of inflammation with activation of specific receptors such as toll-like receptor 4 (TLR) 4 by lipopolysaccharide. There are further differences in the amount, delivery and form (free fatty acid or ester) of PUFAs used. The concentration of DHA required for some of these reported effects is beyond the physiological range of DHA, which can reach for example 6.7 μM in murine serum in animals fed on a diet enriched with fish oil (Kim et al., 2010). Furthermore, studies that reported a modification in the composition of fatty acids in leukocyte membranes after fatty acid intake were based on chronic dietary enrichment (Sperling et al., 1993; De Deckere et al., 1999; Healy, et al., 2000; Wang et al., 2006; Bousquet et al., 2008).

A reduction in neutrophil number has usually been found to be associated with a parallel MPO decrease (Bao et al., 2004; Fleming et al., 2008) which was not the case here. It is possible that any effect on MPO in our study was masked as a result of analyzing a mixed homogenate from the entire epicentre section. The neutrophil quantification was carried out on specific areas, so was possibly a more sensitive analysis. Although samples are measured against a standard curve created using purified MPO, it is not clear whether the assay is specific for MPO or in fact measures all endogenous peroxidase activity. The increase in MPO that was seen here at 4 and 24 h correlates with the work of Bao et al. (2004), who looked at 6, 24, 72 h and 1 week and found elevated levels up until 72 hours, with a return to control levels at 1 week. The amount of MPO that they found is not directly comparable with our results due to the differences in SCI model, assay protocol, storage of samples, activity between the purified MPO standard, use of TMB substrate and plate to plate variation, but it would appear that we found considerably

more activity in our samples at 24 h (100 fold; 0.1 U/μg compared to our 10 U/μg). Taoka et al. (1997) found a larger peak of MPO activity (~0.7 U/μg) at 3 h after SCI, in male Wistar rats. In addition, Xu et al. (2009) reported a significant increase at 24 h (0.5 U/μg), but these were both still a lower activity than what we found. We did encounter some technical difficulties with the assay while optimising the standard curve and thought that the enzyme activity was lower than expected. Therefore, we increased the concentration of the enzyme for the standard curve, but this should not have affected the detection of enzyme activity in the samples.

We chose to examine the effects of omega-3 fatty acid injection during the peak of the neutrophil response, at 4 and 24 h after SCI. It is possible that the effects of treatment on neutrophil numbers and MPO activity at these time points were masked by saturation of the response. Typically, neutrophils undergo programmed apoptosis around 24 h after entering inflamed tissue (Gallin and Snyderman, 1999) and metabolites of DHA and EPA can enhance the removal of neutrophils and resolution of inflammation (discussed below; see also Serhan and Savill, 2005). It would be interesting to examine whether DHA or EPA accelerate this process after SCI. In our model, the timing of peak neutrophil infiltration (one day) correlates with doubling of neuronal (NeuN) loss from 43% at 1 day to 73% at 3 days. The death of neurones at 3 days is unlikely to be due to the initial injury alone (Huang et al., 2007b). Whether the number of neutrophils in the epicentre remains high at 3 days, or if they undergo apoptosis at this time point has not yet been examined in our model.

A reduction in neutrophils is not always associated with improved outcome. Stirling et al. (2009) found that treatment with an antibody against Ly6G (a major antigen on mature neutrophils) 2 and 24 h after SCI in mice, reduced circulating neutrophils by >90%, but this was accompanied by reduced spared white matter and worsened behavioural outcome. The authors alluded to a novel hypothesis, that neutrophils after SCI might be involved in the wound healing response by promoting the astrocyte response. Reactive astrocytes help seal off and isolate the necrotic core of the lesion site, help to re-establish the integrity of the blood-brain barrier, and detoxify molecules such as free radicals and glutamate (Sofroniew, et al., 2005). This scar formation provides a barrier for axonal

regeneration, however recent studies have found that reducing astroctye activity and astrogliosis after SCI worsened functional outcome (Faulkner et al., 2004; Herrmann et al., 2008; Renault-Mihara et al., 2008). Clearly, further work is needed to confirm this hypothesis of a beneficial role of neutrophils after SCI.

### 4.3.2 Effects of DHA and EPA on the cytokine/chemokine response to compression SCI

In parallel with the effects of the DHA injection on neutrophil infiltration, we considered whether there might be a related effect on signalling molecules associated with SCI pathology and neutrophil infiltration. Active tissue invasion by inflammatory cells requires appropriate adhesion molecules and chemokines, such as cytokine-induced neutrophils chemoattractant-1 (CINC-1), or IL-8 to attract cells to their targets. It has already been mentioned above that the use of omega-3 PUFAs has been associated with a reduction in the amounts of these molecules.

Until recently, it has only been possible to measure one protein at a time using enzyme-linked immunosorbent assay (ELISA). Another widely used method to quantify levels of cytokines is to measure mRNA levels using reverse transcriptase polymerase chain reaction (RT-PCR). However, changes in mRNA levels will only have an impact if the increases in transcription result in changes in the levels of the encoded bioactive proteins. Recent technology has now made it possible to measure multiple molecules at a time in one sample. We designed a custom plate containing a select group of factors: IL-6, KC/GRO/CINC, IL-1β and TNF-α, related to SCI pathology and neutrophil infiltration. Discussion with the manufacturer helped avoid the use of a combination of cytokines/chemokines whose amounts could cover entirely different ranges. We saw a robust increase at 4 h in all of these factors followed by decrease to control levels at 24 h, in agreement with the report on the mRNA expression of these cytokines in contusion SCI by Streit et al. (1998) and by others (Yakovlev and Faden, 1994; Wang et al., 1997; Lee et al., 2000). It would be expected that mRNA changes would precede protein levels, and if we had looked at additional time points between 4 and 24 h this might have been elucidated. We also might have seen further differences such as a slightly later peak of

IL-6 (6 h, Streit et al., 1998) compared to TNF-α and IL-1β (1 h). Elevated IL-1β and TNF-α mRNA have also been detected in the injured spinal cord within 1 hr post-injury (Yakovlev and Faden, 1994; Wang et al., 1997; Lee 1999). Levels remained high at day 1, returned to low levels by day 3, and were not detected by day 5. Similar dynamics were seen in rat brain in stroke models (Taupin et al., 1993; Wang et al., 1998), along with increased CINC concentration (Yamagami et al., 1999) but a smaller increase in IL-6 and TNF-α and a smaller, later increase of IL-1β was seen in axotomized facial nerve (Streit et al., 1998). Delayed and reduced infiltration of leukocytes to the brain has been associated with the delayed increase in cytokine levels (Schnell et al., 1999).We found no difference in the levels of pro-inflammatory cytokines and chemokines in the epicentre after DHA or EPA injection. This was surprising, since treatment with omega-3 PUFAs has been shown to indirectly reduce a variety of inflammatory cytokines in other models of inflammation (Tomobe et al., 2000; De Caterina and Massaro 2005), possibly by blocking activation of NF-κB (De Smedt-Peyrusse et al., 2008).

The antibodies were attached to the **bottom** of each well, rather than coating the entire surface of the well as in the ELISA method, which can reduce noise/background levels. Using the method of electrochemiluminescence detection rather than the ELISA method also meant that background signals were minimal, because the stimulation mechanism (electricity) is decoupled from the signal (light). We did not find significant well to well variation between replicates and therefore measuring samples in duplicate was sufficient. It is the first time that spinal cord homogenates have been measured using this method and the increase in cytokines/chemokines in all treatment groups at 4 h compared to the control group would suggest that the method was satisfactory.

The short duration of cytokine/chemokine increase after SCI, indicates a self-limited process. It is likely that the early increase in levels of cytokines/chemokines originates from cells endogenous to the spinal cord, probably astrocytes or microglia, since these cells have been shown to produce IL-6 *in vitro* and monocytes do not infiltrate the epicentre until later time points (Sawada et al., 1992; Suzumura et al., 1996;). In addition, IL-6 knockout mice show marked attenuation of glial cell activation (Klein et al., 1997). Multi-labelling analysis has revealed that microglia and astrocytes, 30–45 minutes post SCI in the mouse, were the two major sources of IL-1β and TNF-α at these times (Pineau

et al., 2007). Colocalization studies revealed that all populations of CNS resident cells, including neurones, synthesized cytokines between 3 and 24 hours post-SCI. In addition, Pineau et al. (2009) demonstrated that astrocytes distributed throughout the spinal cord initially contributed to early neuroinflammation by rapidly synthesizing monocyte chemoattractant protein-1 (MCP-1), KC, and MIP-2, from 3 up to 12 h post-SCI.

The early transient expression also suggests that these cytokines/chemokines play a primary role in the acute phase of the injury response, and that they are not directly involved in causing additional, secondary tissue damage. Dramatic upregulation of IL-1β and TNF-α transcripts does not occur following CNS injuries in which the blood-brain barrier remains intact, i.e., the axotomized facial nucleus (Streit et al., 1998). It is therefore likely that breakdown of the blood-brain barrier, which is a common occurrence in traumatic and ischemic injuries, contributes to the induction of the transcription of these cytokines. It has been reported that direct injection of IL-1β or TNF-α into normal CNS caused widespread leukocyte margination, but little or no diapedesis, suggesting that the normal CNS is resistant to leukocyte diapedesis (Andersson  et al., 1992).

Our treatment with omega-3 PUFA did not attenuate the increase in cytokine/chemokines. This would suggest that the reduction in neutrophil number by DHA was independent of the cytokine/chemokines that we measured, although it could be that the modest effect we saw was diluted by analysing the entire spinal cord homogenate. Methylprednisolone and dexmedetomidine treatment have been shown to significantly decrease TNF-α and IL-6 levels at 24 h along with a reduction in neutrophils (Can et al., 2009). Also, Xu et al. (2009) found that in combination dexamethasone and aminoguanidine reduced TNF-α or IL- 1β increase along with a neutrophil reduction (measured by MPO assay), but did not have this effect when given separately. Application of antiserum to TNF-α locally following an incision of the dorsal horn at T10/11was neuroprotective, but the time points of intervention were not clinically viable (30 min preceding injury, and up to 10 min post-SCI) (Sharma et al., 2003). Etanercept is a TNF blocker and used to treat inflammatory disorders. It has not been shown to improve locomotor outcome but can reduce the development of neuropathic pain (Marchand et al. 2009). Blocking cytokines/chemokines in a limited number of studies

has led to improved locomotor outcome, but these treatments have not progressed to clinical trials. It could be that manipulating these molecules is not as effective compared to changing the levels of those molecules involved in the physical mechanisms of leukocyte transmigration, such as the integrins, which appear to have had more success preclinically.

### 4.3.3 Effect of omega-3 PUFA injection on systemic inflammatory markers

Injury causes a systemic inflammatory response syndrome (SIRS) that is clinically much like sepsis (Bone et al., 1992) and consists of the acute phase response (APR), where cytokines and chemokines such as IL-6 and Il-1β are manufactured by the liver and released into the circulation. Evidence reported by Davis et al. (2005) and Campbell et al. (2003; 2005) demonstrates that the acute phase response (APR) of the liver stimulates additional recruitment of leukocytes to the injury site following a mild compression SCI at thoracic level 8, and exacerbates damage (Campbell et al., 2005).

We found that compression SCI was accompanied by a sustained systemic response, marked by accumulation of neutrophils and resident macrophages in the liver and pro-inflammatory cytokine and chemokines in circulating plasma and the liver. Our results partly support the work of Campbell et al. (2005), although the response of a laminectomy group was not described in that study. We found no difference in the number of neutrophils recruited to the liver after SCI or after laminectomy surgery alone, indicating that the APR of the liver is not specific to SCI in our model and is a generic response to tissue trauma. Cellular injury can release endogenous 'damage'-associated molecular pattern molecules (DAMPs) that activate innate immunity (Matzinger, 1994; Zhang et al., 2010) and can elicit neutrophil-mediated organ injury. However, it remains unclear why the increase in neutrophil number in the liver persists at 28 days. Our hypothesis was that omega-3 PUFA treatment by i.v. injection could influence the systemic response and therefore open the possibility of a parallel therapeutic benefit. However, neither DHA nor EPA injection had an impact on this systemic response.

Interestingly, there were differences between the effects of EPA and DHA on CRP levels in the plasma. DHA significantly reduced levels of CRP in the plasma after SCI, whereas EPA did not. The amount of CRP in plasma in the control group fell within the range reported by other studies, which is a much higher constitutive level in the rat than in humans (350-400 mg/l vs. 1.1-1.5 mg/l; Madsen et al., 2003). Elevated CRP levels have been measured in adults with chronic SCI (>3 mg/l) (Manns et al., 2005; Gibson et al., 2008) but have not been documented acutely after SCI. There is no direct interaction that has been proposed for PUFA with CRP, but it is likely to be an indirect mechanism. In some studies of healthy individuals, there have been reports in the literature, mainly correlative, where an inverse relationship has been found between the intake of omega-3 PUFAs and levels of CRP (Madsen et al., 2001; Niu et al., 2006; Poudel-Tandukar et al., 2009).

We found relatively higher levels of KC/GRO/CINC and IL-6 in plasma compared to the liver samples at 4 h. This indicates that the peak of their production, if this was in the liver, would have occurred at an earlier time point between <1-4 h. However, it might be that local production of these markers at the injury site, where higher levels were found in this case, was more important in contributing to their elevated levels at this time point. We found very low levels of TNF-α and IL-1β, close to the limit of background levels in plasma and liver samples (not shown here). Wang et al., (1996) could not detect TNF in the CSF or serum collected from rats both with and without SCI. However, this was measured using a cytotoxic assay and cannot be directly compared with our results. The authors suggested that TNF is produced locally in the spinal cord following traumatic injury, in agreement with our findings.

### 4.3.4 EPA injection showed a trend towards reducing macrophage infiltration at 7 days

Treatment with DHA (250 nmol/kg) after compression SCI has been shown previously in our laboratory to reduce the amount of macrophage infiltration to the epicentre, 7 days and 6 weeks after injury (Huang et al., 2007a). However, another study using a hemisection model of SCI found that there was no difference in the macrophage levels between the DHA (250 nmol/kg) and saline treated groups 7 days after SCI (King et al.,

2006). Here, despite a strong trend towards a reduction, along with significant neuronal and oligodendrocyte protection and improved behavioural outcome (not shown here, Lim et al., *in press*), the same dose of EPA did not significantly alter the levels of macrophages at 7 days. The low numbers in each group limited the power of statistical analysis in this case, and it is probable that increasing group size could have led to a significant difference. It is clear from our collection of data that there is not a straightforward relationship between the presence of tissue macrophages in the epicentre (identified with the ED1 marker) and behavioural outcome.

It was obvious from looking at the tissue sections in all groups that the injury epicentre was extensively damaged and perhaps saturated with the accumulation of inflammatory cells. We therefore also analysed sections rostral or caudal to the epicentre, to explore whether some differences between the two groups in the amount of macrophage infiltration would be seen along with increased sparing of tissue. However, although the amount of macrophages was less overall and there was still a trend towards a reduction, there was still no statistically significant effect of the EPA treatment. Phagocytic microglia are also labelled by ED1, but it would be useful to extend the analysis to include activated as well as phagocytic microglia. This could be done using Iba1 and would label additional activated microglial cells that might not have been picked up by the ED1marker alone.

Reducing numbers of macrophages after SCI may not be an ideal intervention. A significant number of findings show that immunosuppression caused by trauma itself sets in days to weeks after the injury. A greater loss of immune function after SCI could seriously compromise recovery. High dose methylprednisolone has been criticised for causing widespread and prolonged effects on monocytes/macrophages and neutrophils, that lead to extended suppression of monocyte/macrophage function and prolonged actions of intraspinal neutrophils (Dayyani et al., 2003) due to the lack of clearing by macrophages. The immune response following SCI has been shown to be important for aiding regeneration (Schwartz et al., 1999). Implantation of macrophages (Lazarov-Spiegler 1996; Rapalino, 1998) and passively transferred activated T cells specific to a CNS antigen, myelin basic protein (MBP; Moalem, et al., 1999) have been shown to protect neurons from secondary degeneration. Therefore, it may be more desirable to

preserve or enhance the neuroprotective properties of macrophages and the infiltration of T cells at later time points following SCI.

### 4.3.5 The omega-3 PUFA injection may be converted into active anti-inflammatory metabolites or directly interact with cellular targets

It is not yet clear what proportion of the free DHA and EPA that is injected in our experiments is simultaneously incorporated into phospholipids, taken up by binding proteins, directly bound to intracellular or extracellular receptors, or rapidly converted into anti-inflammatory metabolites, thus indirectly influencing recruitment of neutrophils. Proinflammatory eicosanoids derived from AA such as the 2-series prostaglandins and thromboxanes, and 4-series leukotrienes stimulate the production of lipoxins (Levy et al., 2001), also derived from AA which are significantly involved in reducing the entry of new neutrophils to sites of inflammation (Serhan et al., 1995) and reperfusion injury (Chiang et al., 1999) and macrophages to ingest and clear apoptotic neutrophils (Takano et al., 1998; Godson et al., 2000). These events coincide with the biosynthesis of less inflammatory products from EPA, the 3-series prostaglandins and thromboxanes, and 5-series leukotrienes, and from both EPA and DHA, the anti-inflammatory and pro-resolving metabolites such as the resolvins of the E and D series, respectively, which are actively involved in the resolution of inflammation (for review, see Serhan et al. 2005, 2008; Farooqui et al., 2007b; Calder 2010). At nanomolar levels, one of the metabolites of EPA, resolvin E1, dramatically reduces dermal inflammation, peritonitis, dendritic cell migration, and interleukin IL-12 production (Arita et al. 2005). These metabolites have been shown to antagonize the effects of AA-derived prostaglandins and thromboxanes, decrease proinflammatory cytokine production and attenuate neutrophil migration and tissue injury in peritonitis and ischemia-reperfusion injury models (Hashimoto et al., 1999; Serhan et al., 2002; Hong et al., 2003; Marcheselli et al., 2003; Spite et al., 2009). The D resolvins derived from DHA decrease leukocyte–endothelial interactions *in vivo* and neutrophil recruitment by direct modulation of leukocyte adhesion receptor expression. In addition, they block TNF-α-induced interleukin (IL)-1β transcripts and are potent regulators of neutrophil infiltration in brain (Serhan et al. 2004; Spite et al., 2009). The pro-resolving metabolites derived from EPA and DHA are a log order more potent

than their parent compounds, and interestingly, there is some indication that protectin D1 may have overall a better pro-resolving profile than resolvin E1 (Serhan et al., 2006, 2008). The conversion of DHA and EPA into their metabolites may occur very fast. It has been shown that both long chain omega-6 and omega-3 fatty acids have a rapid disappearance in plasma (Zhou et al. 2002; Rapoport et al 2007). After intravenous injection, the fatty acids will very likely be rapidly incorporated into phospholipids, but a fraction of the injected dose could be converted into metabolites. It will be interesting to quantify the metabolites of DHA and EPA such as resolvins and protectins in future work.

Since we have found a rather modest effect of DHA on the acute inflammation after SCI, we need to consider other mechanisms to account for the previously documented neuroprotective effects of omega-3 PUFAs (King, et al., 2006; Huang et al., 2007a) One possibility may involve action on retinoid X receptors (RXRs), one of several members of the retinoic acid receptor family. These ligand-regulated transcription factors are required to mediate the retinoic acid signalling cascade and are activated and expressed by microglia after spinal cord contusion injury (Mey et al., 2005). Minor changes were observed in the quantitative expression of retinoid receptors after SCI, but their cellular location appeared to move from the cytosol of motoneurones and glia in uninjured tissue, to the nucleus of macrophages and surviving neurones close to the injury site (Schrage et al., 2006). A recent paper has demonstrated that fenretinide, a semisynthetic analogue of retinoic acid, significantly reduced tissue damage, proinflammatory gene levels and improved locomotor recovery when administered after SCI. Interestingly, this was accompanied by a significant decrease in AA and increase in DHA levels in plasma and injured spinal cord tissue (López-Vales et al., 2010). It is possible that the DHA bolus could modulate retinoid signalling after SCI.

DHA and EPA also bind to and activate a second group of transcription factors, called peroxisome proliferator-activated receptors (PPARs).Three isoforms have been described, PPARα, PPARγ and PPAR-β/δ. All possess anti-inflammatory activity and are known to occur in neural tissues (Drew et al., 2005). PPARα was demonstrated to be involved in recovery in a murine knockout model of lung inflammation (Cuzzocrea et al., 2008). PPARγ has been shown to regulate inflammatory signalling in neural cells

(Park et al., 2003; Welch et al., 2003). Furthermore, PPAR-β/δ agonists reduce inflammatory activity and exert beneficial effects in a mouse model of SCI (Ding et al., 2006; Paterniti, et al. 2010).

Another possible target is the potassium channel TREK-1, which is a key component in shaping the characteristics of neuronal excitability. TREK-1 is mechano-sensitive and can also be activated by PUFAs, including DHA. Models of epilepsy and ischemia have shown that the marked neuroprotection induced by omega-3 PUFAs is abolished in mice with a disrupted TREK-1 gene (Lauritzen et al., 2000; Heurteaux et al., 2004). TREK-1 is not directly involved in inflammation. Since we have found modest or no effect of DHA and EPA on acute inflammation after SCI, it is possible that activation of TREK-1 could account for the neuroprotective effects of omega-3 PUFA previously documented (King, et al., 2006; Huang et al., 2007a). The involvement of all these specific targets could be tested in future experiments using selective antagonists and transgenic animals.

### 4.3.6 Conclusions

The purpose of the work in this chapter was to focus on the early phase of inflammation after SCI and to assess whether it is affected by an acute administration of omega-3 fatty acids. A modest reduction in neutrophil number was seen in the epicentre after DHA, but the reduction did not reach significance after EPA treatment. No other parameter was affected by the fatty acids. The dose of fatty acid that we used in this study confers strong histological protection and markedly improves locomotor recovery (Huang et al. 2007a; King *et al.*, 2006). The role of inflammatory processes in the outcome and recovery following SCI is controversial. It remains to be established, using other markers, whether omega-3 PUFAs have a major impact on the specific early inflammatory response after SCI, and to what extent the modulation of inflammation is a determinant of their neuroprotective effect.

# Chapter 5: Results III. The effects of the acute intravenous administration of DHA in a contusion model of SCI in the rat

## 5.1 Introduction

The models that are considered most clinically relevant for spinal cord injury are the compression and contusion models, rather than the laceration-type injuries (Tetzlaff et al., 2010). The contusion device was first described by Allen (1911) as a simple weight that is dropped from a varying height. This basic method has now been optimized through precision controlled devices such as the MASCIS/NYU impactor, designed by Wise Young, John Gruner, and Carl Mason at New York University Medical Centre in 1991, the Ohio State University (OSU) electromagnetic SCI device (Stokes et al., 1992), which is a displacement-driven model, and the force-driven Infinite Horizons Contusion (IHC) device (Scheff et al., 2003; Precision Systems and Instrumentation, Lexington, KY).

### 5.1.1. Comparison between the contusion and compression models of SCI

The NYU impactor precisely measures the movement of a 10-gram rod onto the dorsal surface of the thoracic cord. The height of the rod dropped can be accurately controlled. After impact, a computer readout gives information on impact trajectory, impact velocity, cord compression distance and time. The OSU device is used frequently for mouse experiments (Jones et al. 2002; Sroga et al. 2003; Ankeny et al., 2006; Basso et al., 2006; Kigerl et al., 2006). The IHC instrument enables the application of standard-force injuries through electronically controlled impaction, and force levels can be varied accordingly. The IHC device is usually applied to the lower thoracic spinal levels (T8-T10). However, the device has also been used to create a cervical hemicontusion injury (Siriphorn et al., 2010). By altering the impact force, a force of 150 kDyne in rats relates to a moderate-mild SCI, with a plateau to a recovery of locomotor function of 15 on the BBB test (Rabchevsky et al., 2003; Cao et al., 2005). This equates to consistent plantar stepping and coordination but with some dragging of the hindlimbs.

Development of the contusion model of SCI was carried out with the NYU impactor device, alongside the establishment of the open field test of locomotion (BBB score; Basso et al., 1996). The OSU device was characterised in the mouse (Ma et al., 2001) and the IHC device was characterised by Scheff et al. (2003) and Cao et al. (2005), along with the development of the mouse open field test of locomotion (BMS score; Basso et al., 2006). A comparison was made between the NYU and IH contusion devices by Rabchevsky (2003), where a similar amount of gray matter damage was found, while the NYU device rendered a greater loss of white matter, reflected in more severe hind limb functional deficits.

The main difference between the contusion and compression models of SCI, is the initial impact force, which is less, but more prolonged following compression. Contusion is likely to cause greater and immediate shear stress at the site of injury and possibly more cell death. Maximal neuronal loss after contusion SCI has been shown to occur at 8 h after injury (Liu et al., 1997), with no further loss at later time points (Grossman et al., 2001). In contrast, following compression SCI there is a protracted period of neuronal loss, which extends rostral and caudally to the injury site up to a month after the injury, as shown in our laboratory (Huang et al., 2007b). These differences may be due to the pathogenesis of the secondary insults such as ischemia, inflammation and free radical production.

The inflammatory response follows a similar pattern in the contusion and compression models of SCI and has been discussed in the previous chapters. In both models in the rat, there is an initial (within 24 h) infiltration of neutrophils, followed by the chronic accumulation of phagocytic macrophages/microglia at later time points (within 7 days), spreading above and below the initial injury site.

### 5.1.2 Treatments that have been assessed in more than one model of SCI

Several promising preclinical treatments in SCI have failed to be translated successfully to the clinic. In order to increase the probability of successful translation to the clinic, it is important to show efficacy in more than one species and/or model of SCI and ideally to

replicate the success of the treatment in more than one independent laboratory. Although this is an important exercise, it is done at the expense of a study that might not be published. The "Facilities of Research-Spinal Cord Injury" is part of a recent NIH initiative which supports the independent replication of published studies and some of these will be described below.

Targeting myelin inhibition, or Nogo, is one of the longest-studied therapeutic strategies in SCI. The anti-Nogo (IN-1) antibody, developed in Martin Schwab's laboratory has been tested in rat and primate species using more than one model of SCI (Schnell and Schwab, 1990; Thallmair, 1998; Merkler et al., 2001). Intrathecally applied anti-Nogo IgG treatment has been demonstrated to stimulate regeneration/sprouting of axons and lead to functional improvements (Liebscher et al., 2005; Freund et al., 2007, 2009; Wannier-Morino et al., 2008). Another approach is the intrathecal or subcutaneous injection of the competitive antagonist of the Nogo receptor with a synthetic Nogo-66 (1-40) peptide (Nogo extracellular peptide, residues 1-40, or NEP1-40). Histological improvements, i.e. stimulation of sprouting/regeneration of axons and modest behavioral improvements have been demonstrated with acute intervention (Grandpre et al., 2002; Atalay et al., 2007; Cao et al., 2008) and with a delay of treatment up to 7 days post-SCI (Li and Strittmatter, 2003). Together, the two approaches have provided compelling evidence to support the pursuit of their translation but the majority of investigation has resided within single laboratories. A formal, NIH-funded replication study failed to reproduce the significant benefits of NEP1-40, although there was a weak suggestion that NEP1-40 ‘‘created a situation that was slightly more conducive to axon regeneration’’ (Steward et al., 2008). There is need for further investigation to characterize the robustness of this intervention. The anti-Nogo antibody treatment has not undergone a similar replication study, possibly due to commercial intellectual property issues, but has been translated into human clinical trials, with a European and Canadian trial being initiated in 2007.

Minocycline has been evaluated relatively extensively in thoracic contusion SCI (Lee et al., 2003; Teng et al., 2004; Festoff et al, 2006), balloon compression (Saganová et al., 2008), clip compression (Wells et al., 2003), and transection models (Stirling et al., 2004; Labombarda et al., 2006; Gonzalez et al., 2009). A range of doses was administered, the

most common dose used was 90 mg/kg, given intraperitoneally immediately after, or within 1 h of SCI and led to improved locomotor outcome. Non-behavioural outcomes included tissue sparing, decreased cytochrome c release, decreased caspase 3 positive oligodendrocytes, and macrophages/microglia. Pinzon and colleagues (2008) made an NIH-sponsored attempt to reproduce the positive effects of minocycline that had been demonstrated by others in contusion SCI, but failed to reveal any benefit to either intraperitoneally or intravenously administered minocycline at the 90mg/kg dose with no clear explanation for the contradictory results from this attempted reproduction. Intravenously administered minocycline is however, under evaluation as a neuroprotective compound for the management of SCI in a phase I/II pilot study in Calgary, Alberta.

Erythropoietin is another example of a promising treatment for SCI that has been studied in more than one model of SCI. Neuroprotective effects have been reported after unilateral hemisection (King at al., 2007) and improved behavioural outcome after thoracic contusion SCI, when delivered within 30-60 min (Gorio et al., 2002; Boran et al., 2005). However, Mann and colleagues (2008) examined both erythropoietin and darbepoetin, a more bioavailable version of EPO, but found neither to have any neuroprotective efficacy. An NIH-sponsored study by Pinzon and colleagues (2008) failed to reproduce the positive effects of EPO described previously, particularly the studies by Gorio and colleagues. Like minocycline, it is unclear how to interpret these contradictory reports on the efficacy of EPO in acute SCI.

### 5.1.3 Aims

Our laboratory has demonstrated so far the neuroprotective effect of DHA in the hemisection and compression models of SCI, and more recently, in a mouse model of compression SCI (Lim, et al., *in press*). The aim of the study presented in this chapter was to assess in a pilot study the effect of an acute i.v. administration of DHA in a rat contusion model of SCI. We carried out a T8 contusion SCI (200 kDyne force) and we used the same treatment paradigm as that used in compression SCI (Huang et al., 2007a). We studied the effect of the treatment on locomotor function and also on tissue injury markers.

## 5.2 RESULTS

### 5.2.1 Locomotor recovery

The control group recovered to a BBB score of 10 ± 0.3 by day 14, where the majority of the group regained occasional weight supported plantar steps, but no forelimb-hindlimb coordination (Fig. 5.1). All groups recovered to a BBB score of 11 by 28 days (saline: 11.1 ± 0.1; DHA 250 nmol/kg: 11.2 ± 0.3; DHA 500 nmol/kg: 11.4 ± 0.3). A 2 way RM ANOVA revealed that there was no significant effect of treatment ($F=2.024$; $p>0.05$) and time ($F=315.5$; $p>0.05$). The subscore analysis at 28 days following SCI showed a difference between groups, with the DHA 500 nmol/kg group having the highest score (4.9 ± 1.03) compared to the control group (2.7 ± 0.82), but this was not statistically significant (one way ANOVA; $p>0.05$; Fig. 5.2).

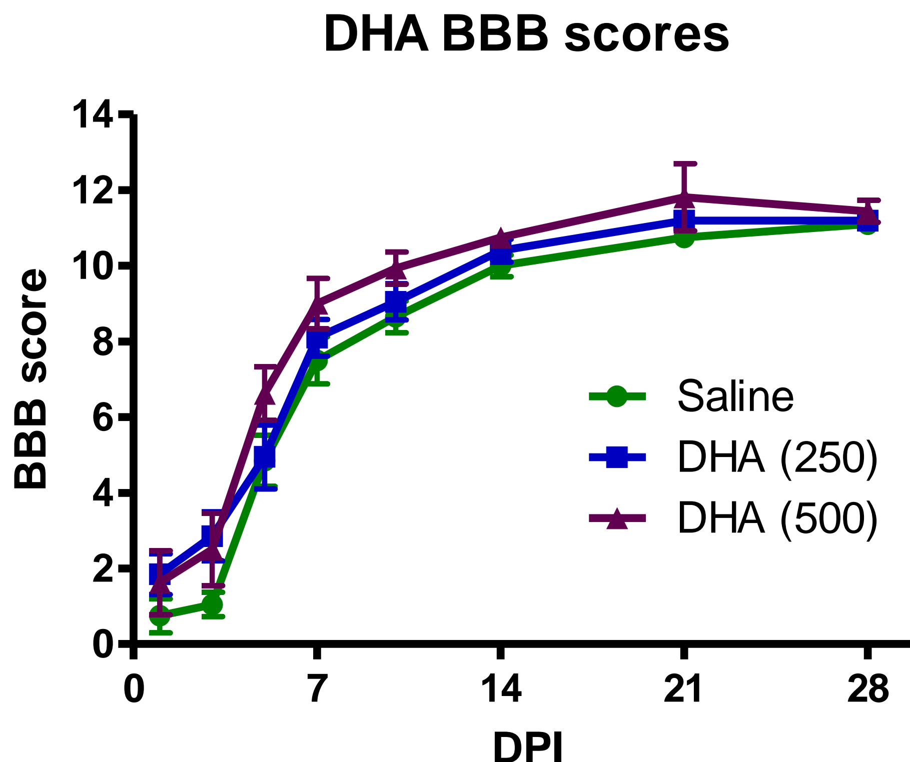



**Figure 5.1 Effect of the acute administration of DHA on the BBB score after rat contusion SCI.** Animals received i.v. saline, 250 or 500 nmol/kg DHA, 30 min after SCI, and were tested for 28 days post-injury. There was no significant difference between the three treatment groups. Error bars represent SEM, n = 8-10 animals per group.

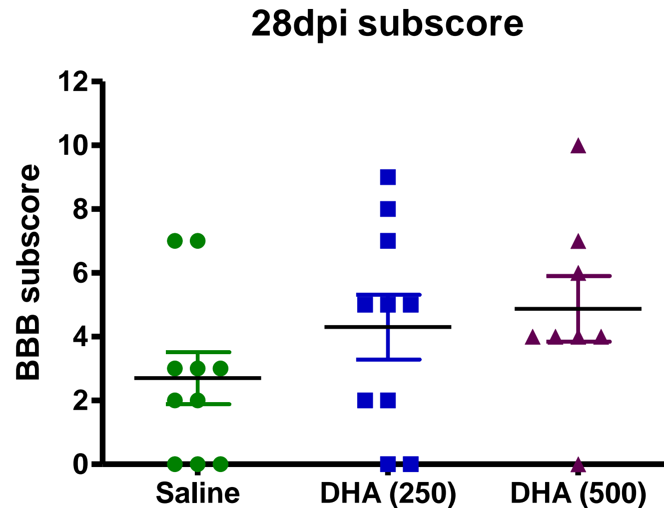



**Figure 5.2 Effect of the acute administration of DHA on the BBB subscore after rat contusion SCI.** Animals received i.v. saline, 250 or 500 nmol/kg DHA 30 min after SCI, and were tested post-injury. There was no significant difference between the three treatment groups. Error bars represent SEM, n = 8-10 animals per group.

When the groups were analysed based on the frequency of stepping at 28 days post-SCI, there was a clear difference between the groups, with 30 % of the control saline-injected group stepping frequently/consistently compared to 60 % in the DHA 250 group and 88 % in the DHA 500 nmol/kg group. Statistical analysis using the Fischer's exact test revealed that the DHA 500 nmol/kg, but not the DHA 250 nmol/kg group, had significantly more frequent/consistent steppers than the saline-injected group ($p<0.05$; Table 5.1).

**Number of frequent or consistent steppers**

| Group | # frequent/consistent steppers |
|---|---|
| Saline | 3/10 |
| DHA 250 | 6/10 |
| DHA 500 | 7/8 |

**Table 5.1 Effect of the acute administration of DHA on the recovery of stepping function after rat contusion SCI.** Animals received i.v. saline, 250 or 500 nmol/kg DHA 30 min after SCI. Significantly more rats in the DHA 500 nmol/kg treated group recovered frequent or consistent stepping compared to the saline-treated group ($p<0.05$). n = 8-10 animals per group.

### 5.2.2 Histology

#### 5.2.2.1 Epicentre

Similar to compression SCI tissue, contusion produced a central area of tissue destruction at the epicentre, with a spared peripheral rim (Fig. 5.3A, B). Because of the limited time available, only 7-8 animals from the saline and DHA 500 nmol/kg groups were used for tissue analysis.

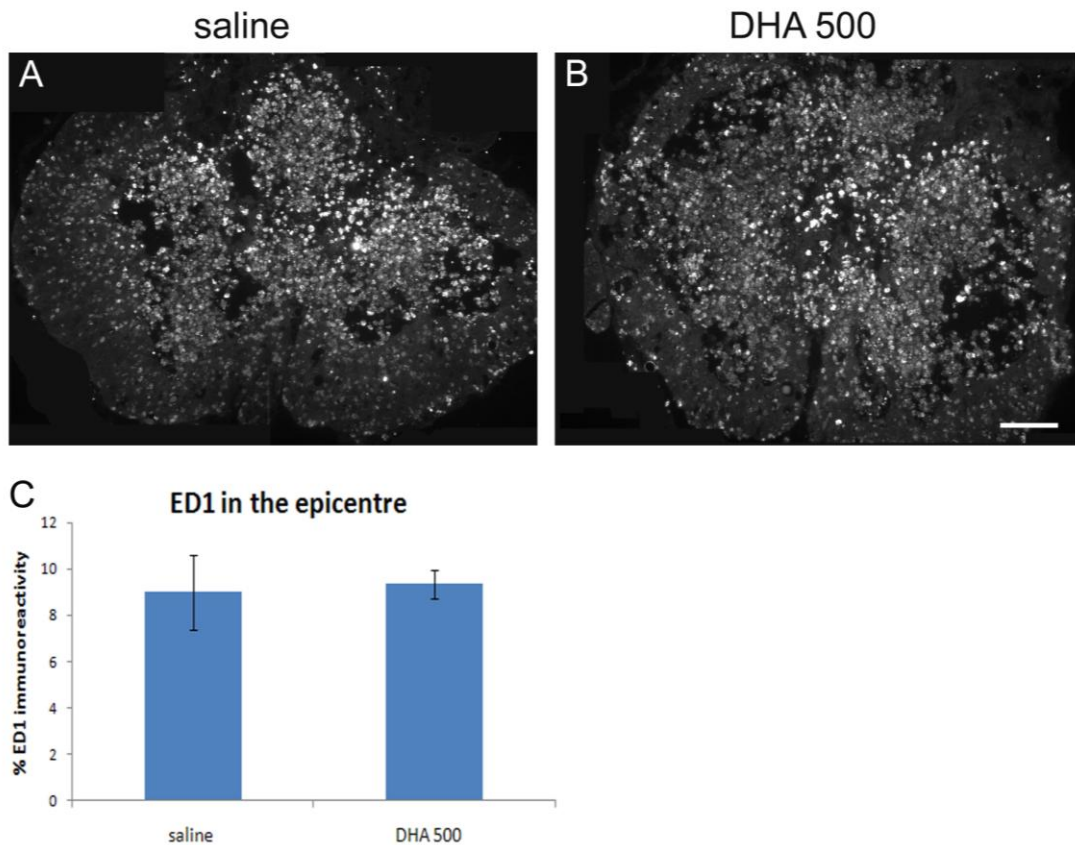



**Figure 5.3 Effect of the acute administration of DHA on ED1 staining at the injury epicentre after rat contusion SCI.** Animals received i.v. saline or 500 nmol/kg DHA 30 min after SCI and the tissue was analysed 28 days post-injury. The analysis of the area covered by macrophages (ED1) revealed no significant difference between groups. Results represent mean ± SEM; n = 7-8 animals per group. Scale bar = 200 µm.

*Macrophages/microglia*

A large cystic cavity filled with macrophages was observed in regions previously occupied by the gray matter. Macrophages also occupied the rim of preserved tissue surrounding the cystic cavity (Fig. 5.3A, B). The quantitative analysis showed this to be 9

± 1.6% of the whole transverse section in the control saline-injected group; Fig. 5.3). There was no significant difference in the amount of macrophages (ED1) present in the saline and DHA 500 nmol/kg groups ($p>0.05$).

*Neuronal survival*

A small region of gray matter was spared in the dorsal horns of the injury epicentre, but only in a minority of animals (Fig. 5.4). Therefore, quantitative analysis of neurones was extended to a region 5 mm rostral to the injury epicentre (Figs. 5.7 and 5.8 below).

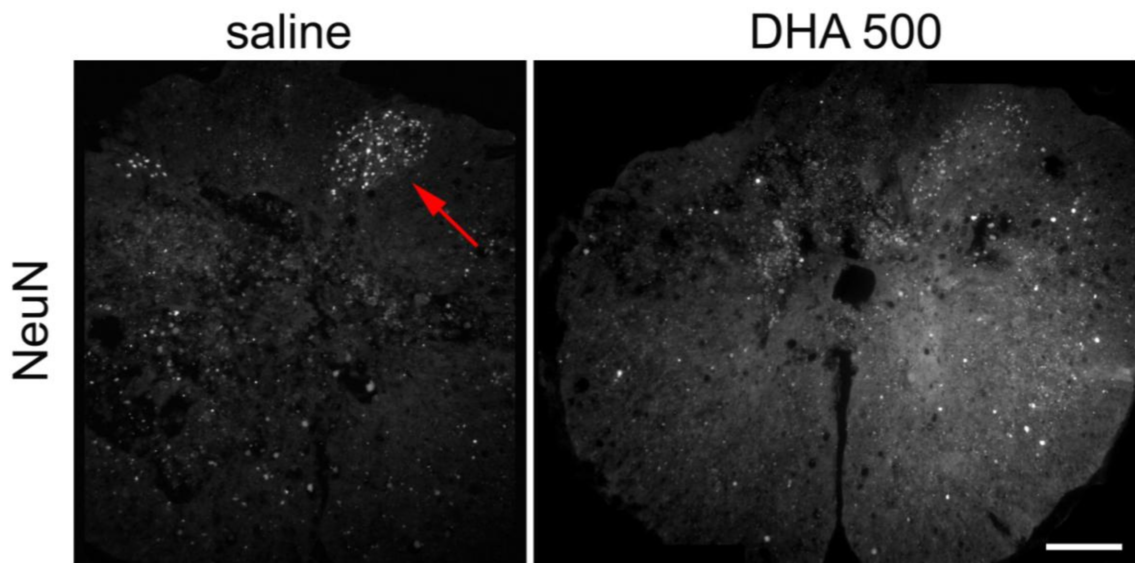



**Figure 5.4 Effect of the acute administration of DHA on NeuN staining at the injury epicentre after rat contusion SCI.** Animals received i.v. saline or 500 nmol/kg DHA 30 min after SCI, and the tissue was analysed 28 days post-injury. There was little preservation of neurones in the grey matter of the injury epicentre. A few neurones could be seen in the dorsal horns (red arrow). Scale bar = 200 µm.

### 5.2.2.2 Rostral

*Macrophages/microglia*

The tissue was relatively intact 5mm rostrally, compared to the injury epicentre. Macrophages (ED1) were found confined to the dorsal columns, as well as scattered throughout the white matter (Fig. 5.5A-B). Quantification confirmed a smaller amount of macrophages at this distance compared to the epicentre (1.6 ± 0.2 % compared to 9 ± 1.6 % in the control group in the epicentre; Fig. 5.5C). However, there was no significant difference in the amount of macrophages between the saline-injected and the DHA 500 nmol/kg treated groups ($p>0.05$).

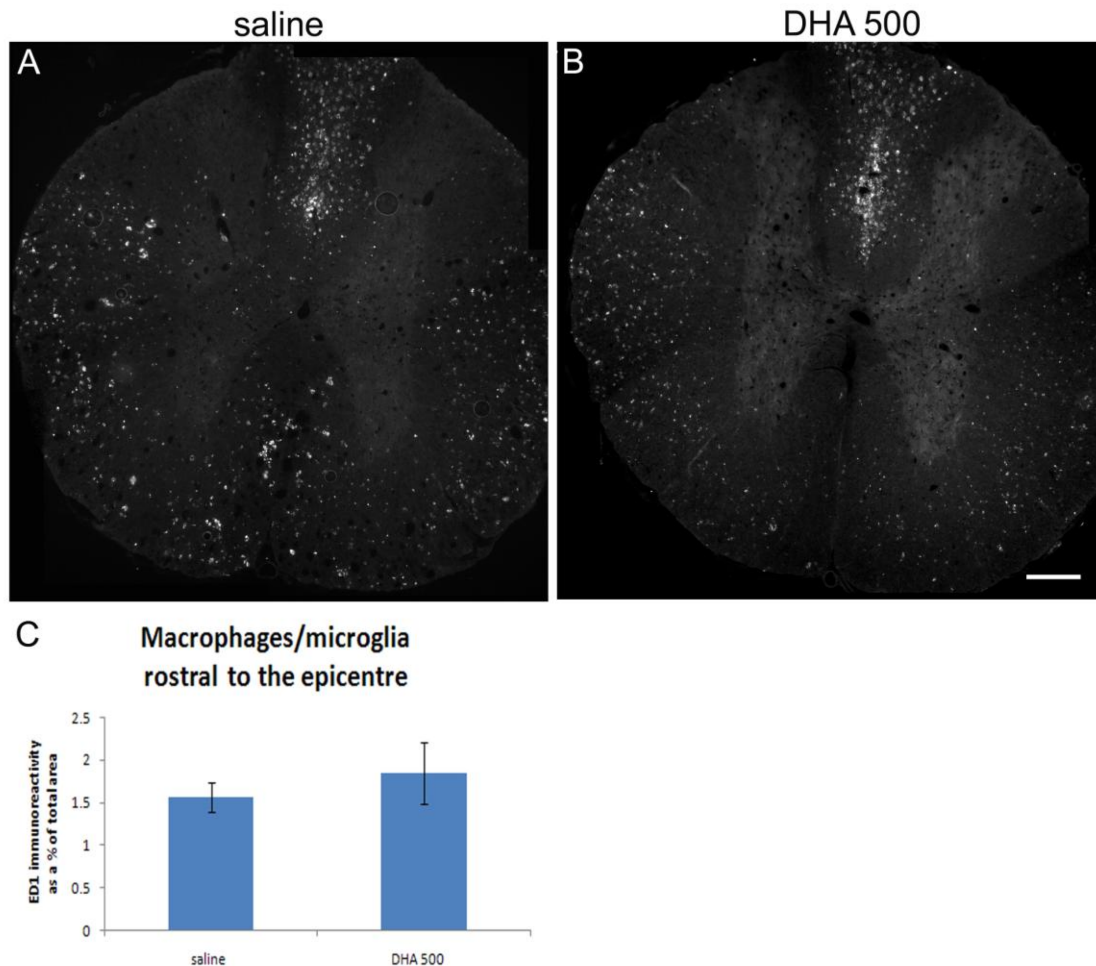
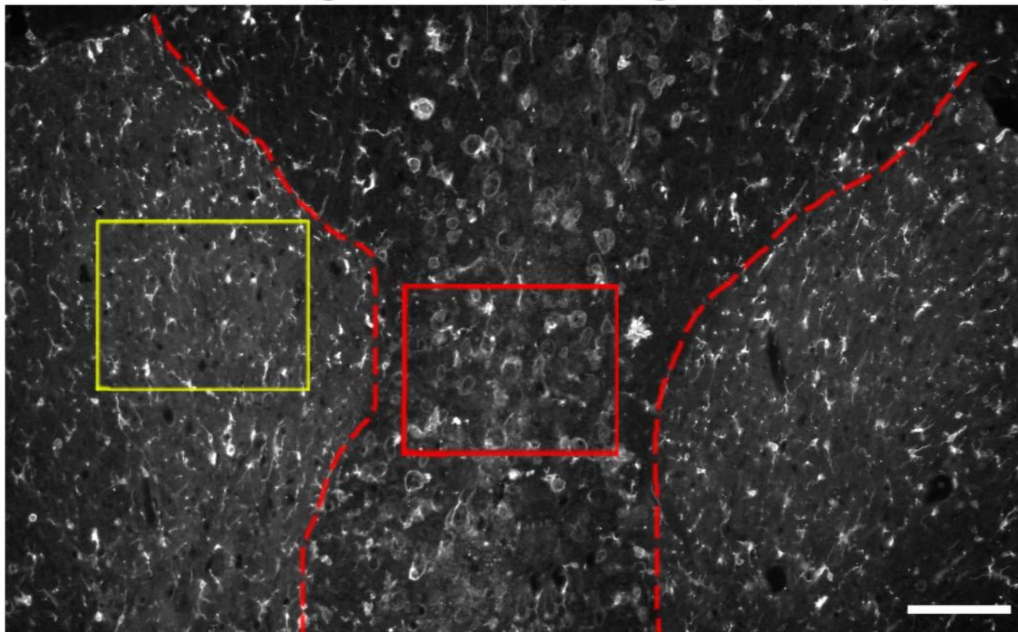



**Figure 5.5 Effect of the acute administration of DHA on ED1 staining rostral to the injury epicentre after rat contusion SCI**. Animals received i.v. saline **(A)** or 500 nmol/kg DHA **(B)**, 30 min after SCI, and the tissue was analysed 28 days post-injury. Quantification **(C)** revealed that there was no significant difference ($p>0.05$) between the saline and DHA 500 nmol/kg treated groups. Results represent mean ± SEM; n = 7-8 animals per group. Scale bar = 200 μm.

Both microglia/macrophages of the phagocytic/activated phenotype, as well as the resting/ramified phenotype were labelled with the Iba1 antigen (Fig. 5.6). There was an area of phagocytic/activated microglia/macrophages confined to the dorsal columns, similar to the ED1 labelled cells, with additional Iba1-labelled cells displaying the resting/ramified phenotype throughout the remainder of the section. Quantification was performed on the phagocytic/ activated phenotype in the dorsal columns (6.8 ± 0.9 % of the analysis box in the control group; Fig. 5.6B) and on the resting/ramified phenotype in the dorsal horns (3.0 ± 0.45 % of the analysis box in the control group; Fig. 5.6C). There was no significant difference between the saline and DHA-treated groups in either of these areas ($p>0.05$ %), therefore the analysis was not extended to further areas.

*Neuronal survival*

Quantification of neurones was carried out by counting NeuN labelled cells in specific areas of the grey matter as described in the Methods chapter (Fig. 5.7A-C). Compared to counts on uninjured control tissue at approximately the T10/11 level (see Chapter 6), there was little neuronal loss at approximately the T7/8 level after T9 contusion (17.5 % in the dorsal horn and 6.7 % in the ventral horns, data not shown). There was no significant difference in the amount of neuronal survival at this level between the saline- and DHA-treated groups in either the dorsal horns ($p>0.05$; Fig. 5.7C), or ventral horns ($p>0.05$; Fig. 5.8C).



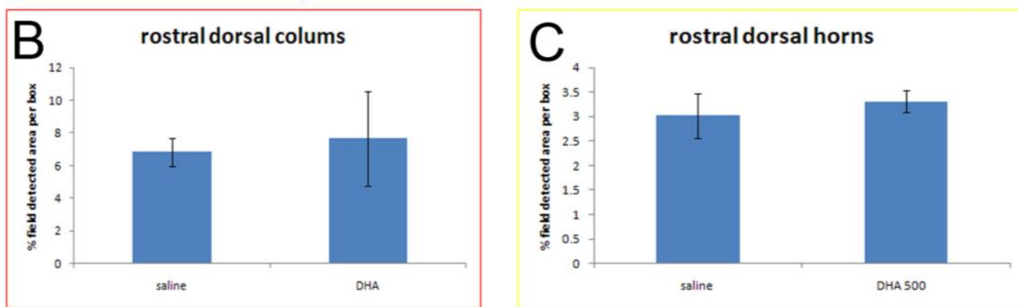




**Figure 5.6 Effect of the acute administration of DHA on the microglia/macrophages (Iba1 staining) rostral to the injury epicentre after rat contusion SCI.** Animals received i.v. saline or 500 nmol/kg DHA 30 min after SCI, and the tissue was analysed 28 days post-injury. Quantification of microglia (Iba1) in the dorsal columns (red box and graph, **B**) and dorsal horns (yellow box and graph, **C**) revealed that there was no significant difference between the saline-injected and DHA-injected groups ($p>0.05$). Results represent mean ± SEM; n = 7-8 animals per group. Scale bar = 100 µm.

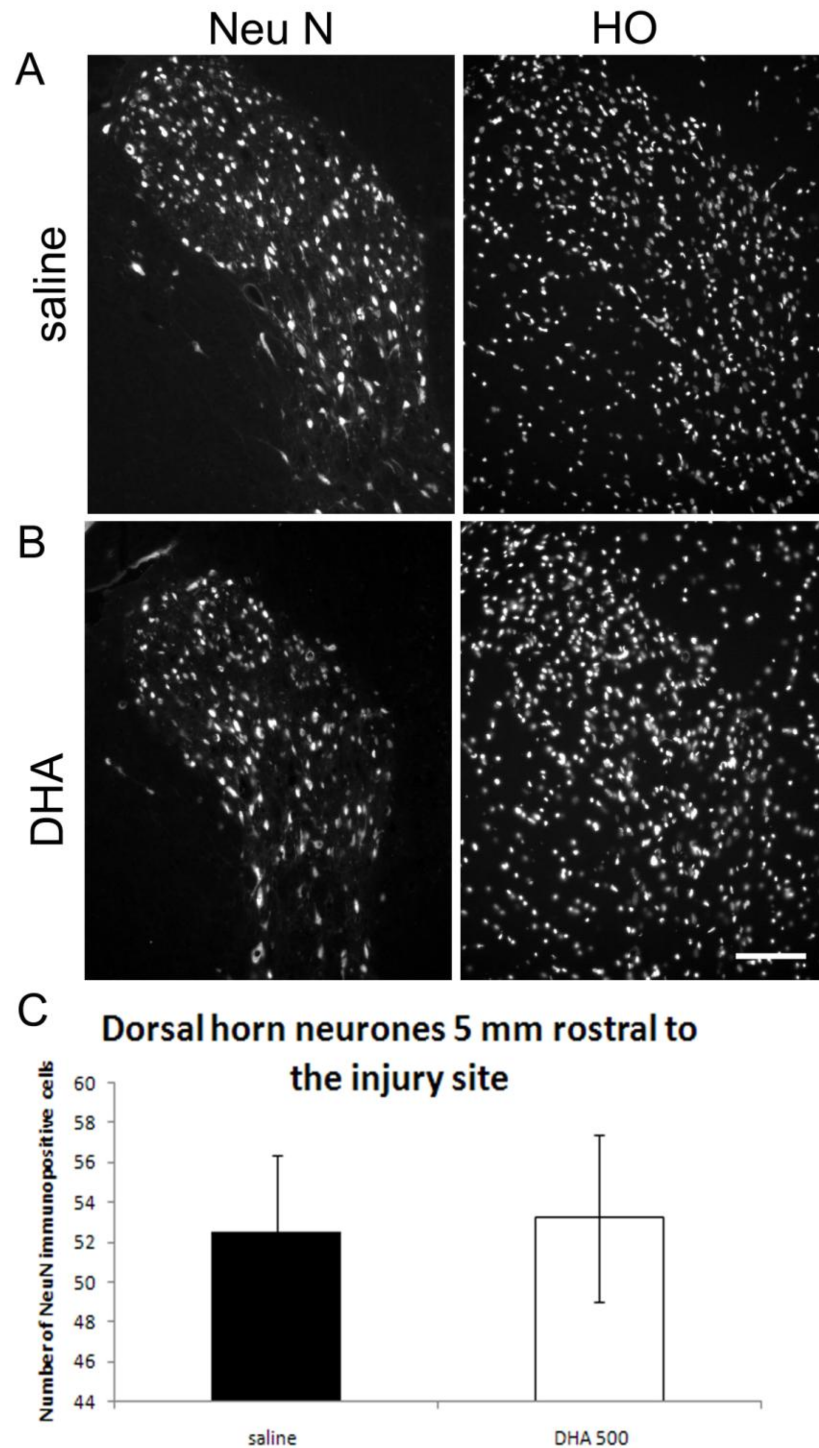



**Figure 5.7 Effect of the acute administration of DHA on NeuN staining in the dorsal horns rostral to the injury epicentre after rat contusion SCI. (A-B)** Representative sections showing NeuN labelled neurones in the dorsal horn. Animals received i.v. saline **(A)** or 500 nmol/kg DHA **(B)**, 30 min after SCI, and the tissue was analysed 28 days post-injury. The same sections are counterstained with Hoechst on the right. Scale bar = 100 µm **(C)** Quantification revealed no significant difference in the amount of neuronal survival at this level between the two groups ($p>0.05$). Results represent mean ± SEM; n =7-8 animals per group.

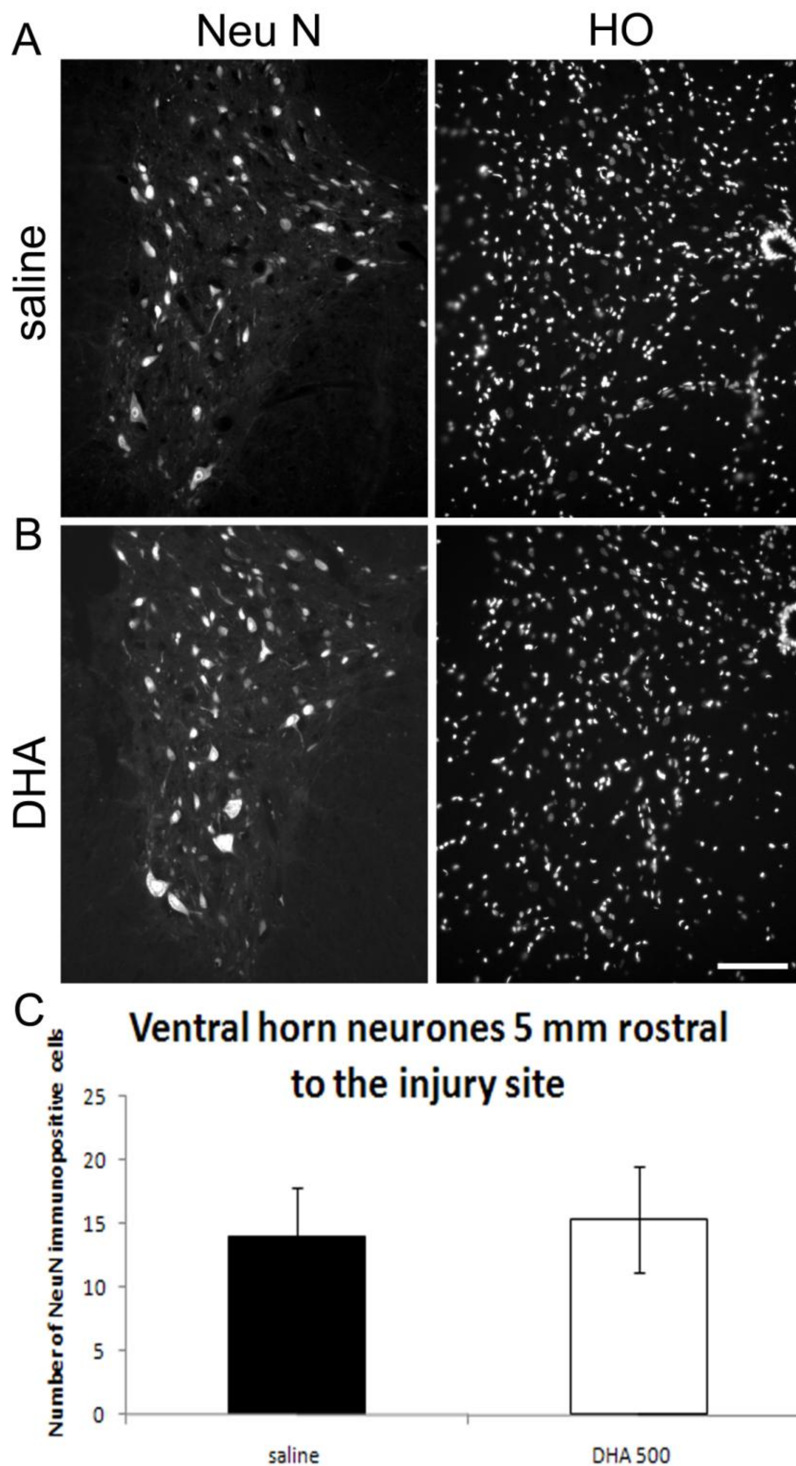



**Figure 5.8 Effect of the acute administration of DHA on NeuN staining in the ventral horns rostral to the injury epicentre after rat contusion SCI. (A-B)** Representative sections showing NeuN labelled neurones in the ventral horn. Animals received i.v. saline **(A)** or 500 nmol/kg DHA **(B)**, 30 min after SCI, and the tissue was analysed 28 days post-injury. The same sections are counterstained with Hoechst on the right. Scale bar = 100 µm. **(C)** Quantification revealed no significant difference in the amount of neuronal survival at this level between the two groups ($p>0.05$). Results represent mean ± SEM; n =7-8 animals per group.

*White matter pathology*

Using the non-phosphorylated neurofilament marker SMI32, an analysis was carried out on immunoreactivity in specific areas of the white matter (Fig 5.9). Following SCI, swollen particles of various sizes were seen in a haphazard distribution (Fig. 5.9). In the control and DHA (500 nmol/kg) groups (Fig. 5.10), the numbers of SMI32-labelled particles in the areas analysed were: DC (732 ± 46 vs.856 ± 131), CST tract (417 ± 45 vs.442 ± 32), VWM (1202 ± 70 vs.1233 ± 35) and VLWM (1053 ± 80 vs. 1021 ± 39) respectively. There was no significant difference between the two treatment groups ($p>0.05$).

Normal, uninjured spinal cord at a similar thoracic level was not available for this study. However, in the subsequent chapter, SMI32 labelling in uninjured spinal cord tissue can be seen as numerous, regular-sized punctate particles which were evenly distributed (see Fig. 6.14 in Chapter 6).

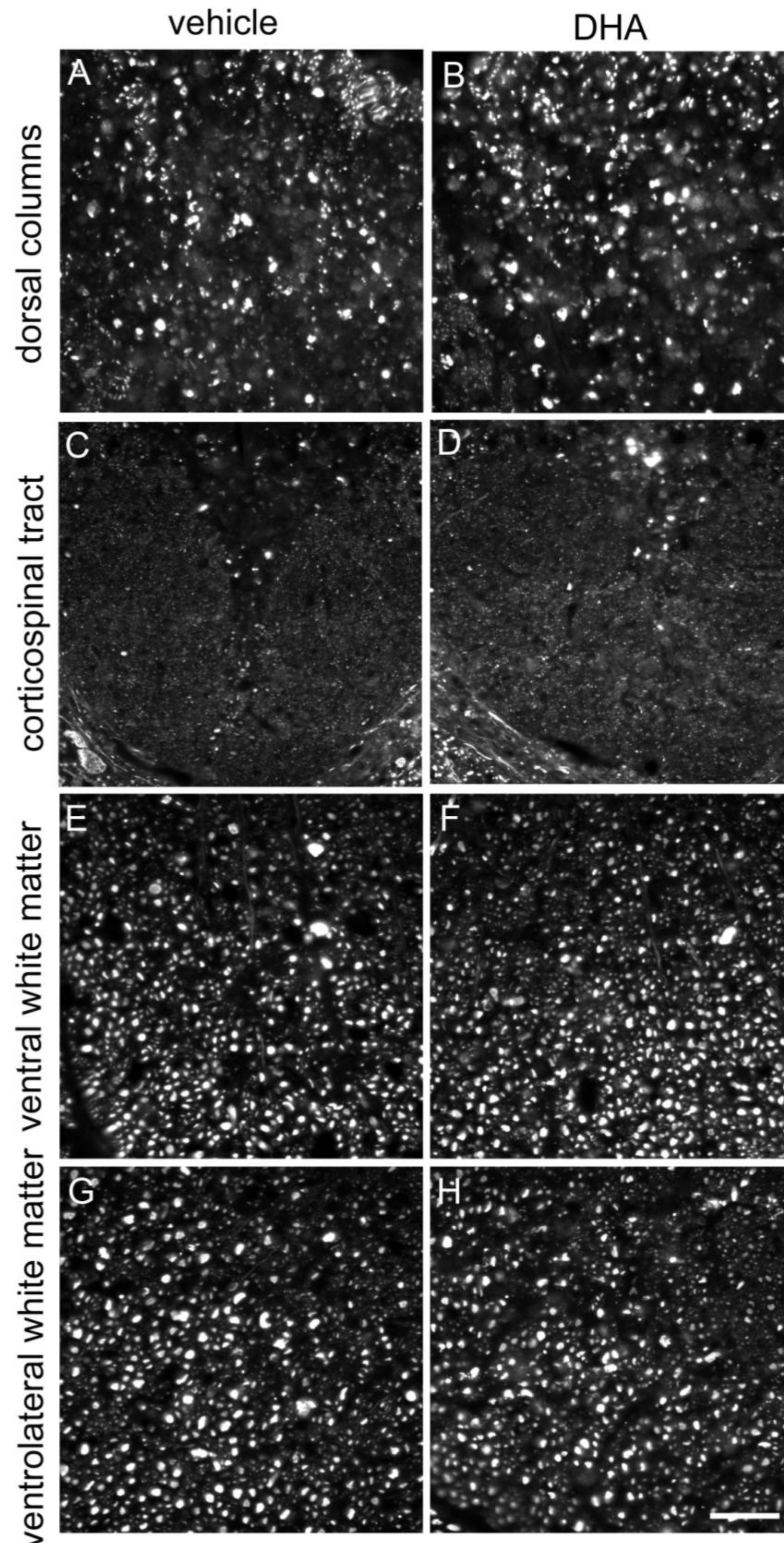



**Figure 5.9 Effect of the acute administration of DHA on the SMI32 staining rostral to the injury epicentre 28 days after rat contusion SCI. (A-I)** Representative sections showing SMI32 labelling in the DC **(A, B)**, CST **(C, D)**, VWM **(E, F)** and VLWM **(G, H)**. SCI-animals received vehicle (i.v. saline, **A, C, E, G**) or 500 nmol/kg DHA **(B, D, F, H)**, 30 min after SCI. Scale bar = 50 µm.

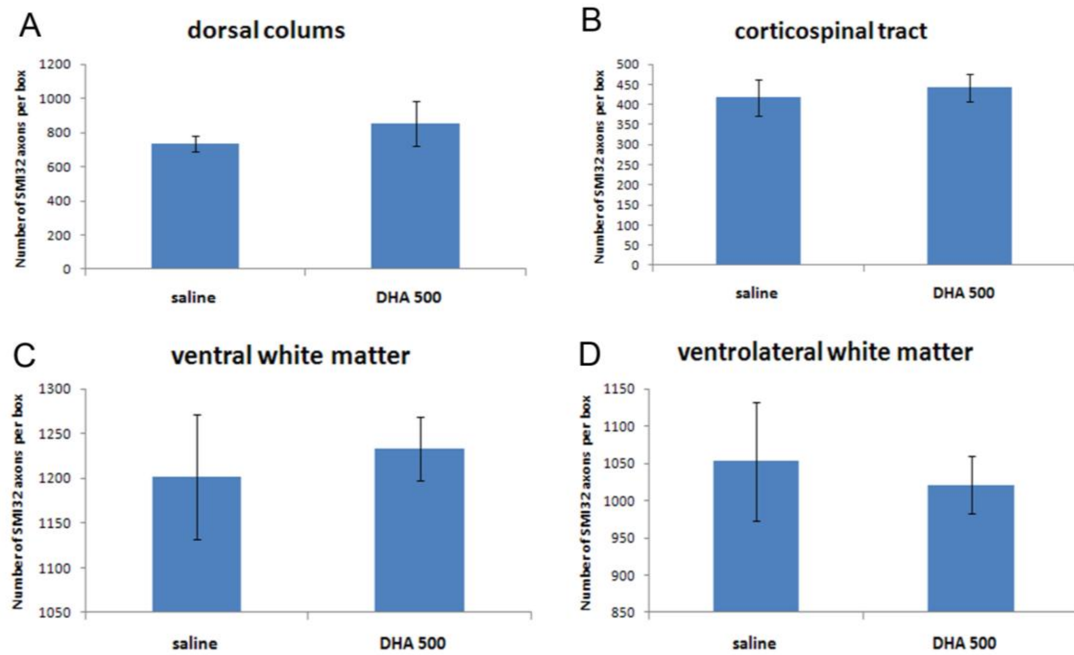



**Figure 5.10 Effect of the acute administration of DHA on the SMI32 staining rostral to the injury epicentre after rat contusion SCI. (A-D)** Animals received i.v. saline or 500 nmol/kg DHA 30 min after SCI, and the tissue was analysed 28 days post-injury. Quantification in the DC **(A)**, CST **(B)**, VWM **(C)** and VLWM **(D)** revealed no significant difference between the two treatment groups. Results represent mean ± SEM; n = 7-8 animals per group.

## 5.3 DISCUSSION

In this study we have shown that an i.v. injection of DHA, delivered within 30 min of contusion SCI, leads to a significant improvement in the recovery of stepping, but not in the BBB score or sub score. We saw no differences between control and DHA 500 nmol/kg-treated animals in markers of neuronal survival, axonal integrity, or in the microglia/macrophage presence at the injury site or rostral to the epicentre.

The T8 contusion injury revealed some differences in the functional outcome as well as in the histopathology compared to our T12 50 g static compression model. The force of 200 kDyne was chosen to lead to a similar injury severity as that obtained in our compression model. Locomotor recovery reached a plateau of a BBB score of 11 at 28 days post-contusion, compared to a slightly higher score of 12-13 in the compression SCI (King et al. 2006; Huang et al., 2007a). This could be an important difference in injury severity, since it represents the start of coordinated walking.

Previously in the compression model, we found that 250 nmol/kg DHA was an effective dose which led to significant functional and histological improvement (Huang et al., 2007a). Here, the dose of DHA 500 nmol/kg, but not the dose of 250 nmol/kg led to a significant improved locomotor outcome, i.e. recovery of frequent/consistent stepping. However, DHA did not affect the main BBB score. There have been other studies which found no difference in BBB score, but improvement in other aspects of locomotor recovery. For example, in their macrophage depletion study, Popovich et al. (1999) reported a significant and continuous improvement in more refined parameters of the step cycle after treatment, which was not evident in the BBB scale. The different dose efficacies in the two SCI models could reflect the differences in the severity, as mentioned above, and the differences in the evolution of the injury and tissue pathology between the two models. For example, if the neuroprotection effect derived from the DHA injection is mainly associated with a reduction in apoptotic or necrotic cell death (King et al., 2006; Huang et al., 2007a) and there is more cell death in the first 24 h following contusion than compression SCI, this may explain the need for a higher dose of DHA following contusion SCI, and therefore a shift to the right of the dose-response curve. This will be further explored in more extensive dose-response studies in future.

### 5.3.1 Histology

*Cavitation and macrophages/microglia*

A notable difference between the contusion and compression injury epicentres was that rather than several large, cystic cavities following compression SCI, such as those seen at 28 days by Huang et al. (2007 a, b), there was one large central cavity in the contusion epicentre, filled with macrophages. Interestingly, contusion injuries delivered by the NYU impactor have been shown to result in multiple cavities in the rat, with septa or fibrous bands of tissue in-between them (Basso, et al., 1996). The relevance of these differences is unknown, but the septa could provide a supportive substrate to spared axons and explain the better locomotor outcome in compression SCI. A very simple analysis method was used to estimate the area of cavitation after contusion injury. A more accurate method to measure the area of cavitation, volume, or in fact the amount of tissue sparing, would be to collect sagittal or horizontal sections spanning the injury site and to label spared white matter, with a myelin stain such as eriochrome cyanine. Serial sections could then be measured throughout the injury epicentre and expressed as the amount of myelin as a percentage of the total area (Han et al., 2010).

Greater motor deficits are related to greater SCI severity and less tissue sparing (Noble and Wrathall, 1989; Basso et al., 1995, 1996; Fehlings and Tator, 1995; Kloos et al., 2004). Thus, better tissue sparing may lead to better locomotor outcome. Consistent plantar stepping has been shown to depend on 10% white matter sparing, whereas coordination requires 25% white matter sparing (Kloos et al., 2005). It could be that the DHA 500 nmol/kg treatment, which led to significantly more frequent stepping, may have protected the tissue more, but this was too subtle to be detected using the approach used here, especially with the markers we used, and this was not quite enough to preserve the amount of tissue required for coordination. Central pattern generators (CPG) are important for the rhythmic generation of stepping movements; this is also the case in humans. However, in humans, supraspinal control is also essential for the performance of locomotion (Edgerton et al., 2001; van Hedel, et al., 2010). It is thought that recovery of forelimb–hindlimb coordination is dependent upon communication between the cervical

and lumbar enlargements (Ankeny et al., 2004), i.e., axonal regeneration or improved conduction across the lesion site. It is unknown whether communication across the lesion was improved here.

The amount of macrophages in the epicentre cannot be compared directly to the compression model in the study by Huang et al. (2007), due to differences in the method of analysis. In addition, the macrophage distribution in the epicentre looked notably different here compared to the study by Huang et al. (2007). Here, the macrophages filled the grey matter area in the epicentre, whereas in the compression study, there were multiple cavities and macrophages seemed to be distributed primarily in the white matter surrounding the grey matter, and were very sparse in the grey matter. However, in rostral segments, as seen by Popovich at al. (1997), the macrophage distribution in contusion tissue appeared similar to compression tissue (see Chapter 6), i.e. it was confined primarily to the dorsal columns, but also scattered throughout the white matter. No differences were found in the amount of macrophages/microglia between treatment groups after contusion in the epicentre or the rostral segment, in contrast to what was found by Huang et al. (2007a), who reported a significant decrease in ED1-labelled macrophages after DHA bolus treatment in specific areas of the epicentre at 6 weeks in the compression SCI model. The significance of this is not clear. Interestingly, no difference was seen in the amount of macrophages after acute i.v. DHA treatment in another, rat hemisection model of SCI at 7 days, despite a marked reduction in the lesion size and a significant improvement in locomotor function (King et al., 2006). These differences reflect the complexity of the inflammatory response after SCI and likely the limitations of assessment of this response using the ED1 staining as index. In future, it will be necessary to analyse the response of macrophages in much more detail. For example, it would be more informative to look at a change in the amounts of the various phenotypes of macrophages, such as the M1 or M2 types, as documented by Kigerl et al. (2009) and mentioned in Chapters 1 and 2. For example, if it can be assumed that the same markers used in mouse tissue would apply in the rat following SCI, one could look at an increase in arginase or CD206 expression, which is characteristic of the M2 phenotype, whereas CD86, CD16/32 or iNOS are indicative of the M1 phenotype and has been demonstrated to dominate the lesion core at later time points (Kigerl at al., 2009).

Also, it is important to be able to assess the functionality of macrophages, such as their expression of proinflammatory cytokines/chemokines such as TNF-α and IL-1β after activation. This could be performed in an *in vitro* assay on isolated macrophages (see Future work section, Chapter 7).

*Neuronal survival*

In the epicentre, we observed a small region of spared gray matter in the superficial dorsal horns, as characterised before in rat contusion injured tissue using the NYU impactor (Basso et al., 1996). There appeared to be more survival of neurones in the grey matter of the epicentre after compression SCI. Huang et al. (2007a, b) noted approximately 10 % survival of neurones in the ventral horns and 30 % in the dorsal horns of the epicentre at 6 weeks compared to uninjured tissue. Also in contrast to contusion tissue, more loss and therefore spread of damage to the grey matter (20 % vs. 18 % in the dorsal horn and 40 % vs. 7 % in the ventral horns, see chapter 6) was seen 5 mm rostral to the injury site after compression SCI than contusion SCI, in agreement with Huang et al., (2007b), who documented a protracted neuronal loss after compression SCI compared to contusion SCI. This might mean that the analysis in the rostral location here was outside the zone of neuronal loss surrounding the epicentre and could explain why there was no difference between the two groups.

It has been suggested that NeuN might not reflect neuronal loss (McPhail et al., 2004), but rather a down regulation of the antigen in intact neurones. However, Huang et al (2007b) performed counts of ventral horn neurons stained with toluidine blue, which showed a very similar pattern of loss, suggesting that the NeuN loss seen after the compression injury does represent actual neuronal loss. It would be interesting to analyse other markers for neurones in the grey matter such as the microtubule associated protein-2 (Map-2), which forms part of the cytoskeletal network and is specific for neurones and their dendrites (Bernhardt and Matus, 1984; Bloom et al., 1985; Binder et al., 1986; Matus, 1994). This marker has been shown to be down-regulated in the epicentre after compression SCI (Ward et al., 2010), the loss of MAP-2 extending a substantial distance (approximately 3 mm) rostrally and caudally from the injury site (Springer et al., 1997).

*White matter pathology*

In order to examine axonal pathology, a neurofilament marker was used (SMI32). The neurofilament protein is a component of the cytoskeleton and is present in neuronal cell bodies and axons in its phosphorylated and non-phosphorylated form. Ward et al., (2010) showed a decrease in the non-phosphorylated component following compression SCI. A loss of non-phosphorylated neurofilament compared to uninjured control was not quantified after contusion in this study since tissue was not available at the same thoracic level. However, compared to uninjured spinal cord at approximately T10/11 (not shown), there was an apparent overall loss after SCI, and the greatest loss appeared to be in the dorsal columns. There was no significant difference between treatment groups, which is in contrast to what was seen after the DHA 250 nmol/kg bolus injection in the compression model at the injury epicentre (King et al., 2006; Huang, et al., 2007a; Ward et al., 2010). Again, this difference could be due to the difference in injury severity between the two models (discussed above). The method of quantification was also performed slightly differently, although images were taken at the same magnification and similar areas. A manual count was performed previously. The different size of particles was obvious in the contused tissue here, and a gross count of all particles may not be as informative as a more extensive analysis based on particle size. If the larger particles are likely to be swollen, dystrophic axons, it might be interesting to set a cut off point and see if the treatment had any effect on the amount of abnormal axons of a certain diameter. This could be performed upon reanalysis of the sections, using the Image J software, this time collecting the raw data on particle size for each section. A programme can then divide the data for each section into bins of different sizes and then once collated, the data could be analysed for a shift in particle size.

### 5.3.2 Mechanisms

As discussed in the previous chapter, it is likely that the acute DHA injection is targeting early events such as reducing oxidative stress and lipid peroxidation (King et al., 2006; Huang et al. 2007a), which occur after SCI (Hall and Braughler,1986; Xu et al., 2005) and lead to dysfunction of mitochondria and subsequent neuronal death (Sullivan et al.,

2005). DHA might also reduced glutamate-induced excitotoxicity (Hogyes et al., 2003; Wang et al., 2003), which is known to be one of the triggers of apoptosis after SCI (Xu et al., 2004). The dose of DHA remains in a physiological range and is compatible with the affinity of this fatty acid for targeting of TREK-1 channels or retinoid receptors, which may be activated by the free DHA (de Urquiza, et al., 2000). DHA has anti-inflammatory actions and has been shown to reduce COX-2 expression after SCI (Huang et al., 2007a). The DHA in the injection may have had some anti-inflammatory effect, such as a reduction in the amount of neutrophil infiltration to the injury site as shown in the previous chapter, although this remains to be determined after the contusion injury. Also, the injected DHA may have led to production of anti-inflammatory downstream metabolites such as neuroprotectin D1 (Mukherjee et al., 2004). Finally, a small percentage of DHA might have been retro-converted to EPA, which can inhibit pro-inflammatory signalling directly, or through its downstream metabolites such as resolvin E1.

Various laboratories have attempted to validate alternative strong candidates for translation such as erythropoietin, minocycline and the anti-Nogo antibody in more than one model of SCI (For review, see Chapter 1 and Kwon et al., 2010). All have shown efficacy in more than one model of SCI and despite problems with one or two replication studies (Pinzon et al., 2008a, b; Steward et al., 2008), have demonstrated sufficient potential to be carried forward to SCI clinical trials.

In conclusion, this pilot study in contusion injury demonstrated that a higher dose of DHA (500 nmol/kg) injection was required compared to compression SCI for improved locomotor outcome (stepping function). There was no significant effect on the markers of tissue damage. As was concluded in the previous chapter, a higher and/or prolonged dose of the long-chain omega-3 PUFA may be necessary in contusion SCI, in order to see significant changes in the tissue response to injury and especially in the inflammatory markers after SCI. However, the improved functional outcome in the animals at the highest dose of DHA we have tested suggests that, in absolute terms, neuroprotection in this model of higher SCI severity is not unrealistic.

# Chapter 6: Results IV.The effects of dietary PUFA on the response to compression SCI

## 6.1 Introduction

Sir David Cuthbertson recognised that the inflammatory response is a key central player in the metabolic response to injury and discussed the future of parenteral and enteral nutrition (1982). He suggested that artificial nutrition could be used to modulate the activity of the immune system, anticipating the current situation of a range of nutrient components that are used in parenteral nutrition, including various lipids, although the optimisation of those components still remains uncertain today.

Addition of EPA or DHA to the diet is a way of increasing endogenous omega-3 PUFA levels, thus changing fatty acid composition in cell membranes and, indirectly, modifying signaling events, including those underlying anti-inflammatory effects. From our experience with DHA in rat compression SCI, boosting levels of DHA in the diet after the injury appears to enhance the neuroprotective effects of the bolus injection (Huang et al., 2007a). Pilot observations also indicated that an EPA bolus i.v. injection after SCI also shows some neuroprotective effects (Lim et al., *in press*); although overall they appear to be more modest than those of DHA. The effects of an EPA-enriched diet after SCI have not yet been explored. Therefore, this chapter will first provide a review of the treatments that have been given as dietary supplementation after SCI. This will be followed by an investigation of the effects of an EPA enriched diet after SCI. The chapter will close with our observations on the dietary prophylactic supplementation with DHA prior to injury.

### 6.1.1 Dietary alterations and SCI

It is considered that the present average Western diet does not include a sufficient intake of omega-3 PUFAs (Calder et al., 2009 for review). The typical American consumes 0.7-1.6 g of omega-3 PUFA per day, equivalent to approximately 0.2-0.7% of total calories (Kris-Etherton et al., 2000). Most of this is as ALA, the plant omega-3 PUFA. Intake of fish-derived long chain omega-3 PUFA (i.e., EPA and DHA) is reported to be less than

0.1-0.2 g per day. In comparison, the Greenland Inuit were reported to consume levels of 6–14 g/day, which corresponds to 2.7–6.3% of daily energy (Feskens et al., 1993). Similarly, traditional Japanese diets contain 1–2% of daily energy as long chain omega-3 PUFA (Okuyama et al., 1996; Nagata et al., 2002). With respect to physiological relevance, this range is similar to that seen in human clinical studies with these compounds, i.e. 1-9 g/day (0.45-4% of calories) of omega-3 PUFA, mainly in the form of EPA and/or DHA (Kelley et al., 1998; Kelley et al., 1999; Thies et al., 2001; Rees et al., 2006). Clinical studies have found that increased consumption of long-chain omega-3 PUFA either in the form of oily fish or fish oil supplements, compared to omega-6 PUFA is beneficial for coronary heart disease (Stone et al., 1996; Bucher et al., 2002), cancer (Caygill et al., 1995; Augustsson et al., 2003), immune disease (Mehta et al., 2009) and prevention or management of neurological disorders such as Alzheimer's disease (Calon et al., 2004; Cole et al., 2004).

Although there is a wealth of data looking at the long-term benefits of various diets on overall health and prevention of neurological diseases such as stroke, as well as reduction in risk for neurodegenerative disorders such as dementia, and alleviation of inflammatory conditions such as rheumatoid arthritis, there have been few studies that have looked at the neuroprotective effects of dietary modifications after SCI.

Questionnaires given to adults with chronic SCI have found evidence of inadequate dietary intake (Walters et al., 2009). Although the percentages of daily energy for men and women were within an acceptable macronutrient distribution range, inadequate intakes for men and women were found for vitamin A, magnesium, folate, zinc, vitamin C, thiamine, vitamin B12, riboflavin and vitamin B6. Another questionnaire collated by Opperman et al., (2010) recorded dietary intake, including supplements in people with SCI. 71% of the sample reported using supplements at least once, with 50.6% being classified as consistent supplement users. The top three supplements consumed were multivitamins (25%), calcium (20%) and vitamin D (16%). It is important to be aware of the use of supplements in the SCI community when treating chronic SCI.

Some SCI studies have looked at the impact of dietary intervention on muscle strength and bladder activity. Nash et al. (2007) found that whey and carbohydrate ingestion for five consecutive days immediately following a walking to fatigue test increased ambulation distance, time, and caloric expenditure in patients with incomplete SCI. Dietary glycine (1% to 3%) has been shown to inhibit bladder and urethral activity, and improve detrusor hyperreflexia and detrusor-sphincter dyssynergia in rats, following 4 weeks supplementation, commencing after SCI (Miyazato et al., 2005). It has been shown that rats fed for 4 weeks with a creatine supplemented diet before undergoing a moderate spinal cord contusion had a reduction in the spread of secondary injury (Hausmann et al., 2002).

It is well-established that dietary restriction (DR) increases longevity across many species, even if started in adulthood (Weindruch andWalford, 1982). Interestingly, therapeutic DR started after SCI has been shown to provide neuroprotection, anatomical plasticity and improve functional recovery (Plunet et al., 2008). The authors suggested a broad spectrum effect, and found that blood β-hydroxybutyrate levels, a ketone known to be neuroprotective, were increased 2–3-fold on the fasting days. In addition, Davis et al. (2008) found that fasting animals for 24 hr, but not 48 hr, after traumatic brain injury, resulted in a significant increase in tissue sparing, a decrease in biomarkers of oxidative stress and calcium loading, and increased mitochondrial oxidative phosphorylation in mitochondria isolated from the site of injury. Again, they showed that the underlying mechanism appeared to involve ketosis rather than hypoglycaemia.

### 6.1.2 Dietary enrichment with omega-3 PUFAs affects the cells involved in the inflammatory response

Fish oil, rich in long chain omega-3 PUFAs, is widely used as a dietary or nutritional supplement for its anti-inflammatory effect, for conditions such as atherosclerosis (Mori and Beilin, 2004). In the Western diet there is a high ratio of omega-6: omega-3 PUFAs, believed to be quite different from that of our ancestors (Simopoulos, 1999).

Fatty acids that are delivered in the diet are known to be incorporated into the cell membrane phospholipids of the liver, brain and leukocytes in a time- and dose-dependent manner (Salem et al., 2001; Calder et al., 2003, 2004, 2009; Bailes et al., 2010). Time-course studies have indicated that the incorporation of EPA and DHA into human immune cells reaches its peak within 4 weeks of commencing increased dietary intake (Healy et al., 2000; Yaqoob et al., 2000; Thies et al., 2001; Rees et al., 2006) and dose-response studies indicate that incorporation occurs in a manner that is highly correlated with the amount of the fatty acid consumed (Healy et al., 2000; Rees et al., 2006). Based on an example by Sperling et al. (1993), where omega-3 PUFAs supplements (9.4 g EPA, 5 g DHA,) were delivered to healthy volunteers for 10 weeks, fatty acids are taken up by the major classes of phospholipids including phosphatidylinositol, phosphatidylcholine and phosphatidylethanolamine, by 3 weeks.

Once fatty acids have been taken up into cell membranes, the composition may affect the balance of various biochemical reactions. For example, EPA and DHA inhibit AA metabolism due to direct competition for the same enzymes such as COX2. Thus, production of AA-derived eicosanoids is decreased by these long chain omega-3 fatty acids. This process can lead to changes in the expression of pro-inflammatory genes and the availability of pro-inflammatory lipid mediators, thus modulating the intensity and duration of neuroinflammation (Calder et al., 2009). In addition, as mentioned previously, EPA is also able to act as a substrate for both cyclooxygenase and 5-lipoxygenase, giving rise to eicosanoids with a slightly different structure to those formed from AA. Thus, fish oil supplementation of the human diet has been shown to result in increased production of $LTB_5$, $LTE_5$ and 5-hydroxyeicosapentaenoic acid by inflammatory cells, which play roles in the chemotaxis of neutrophils and monocytes (Goetzl & Pickett, 1980; Lee et al., 1984, 1985; Kragballe et *al.*1987; Endres, et al., 1989; Sperling et al., 1993). Through their effects on membranes, cell signalling, gene expression and lipid mediator profiles, omega-3 fatty acids influence aspects of innate immunity (Calder et al., 1990), and alter the production of inflammatory cytokines by endothelial cells, monocytes and macrophages (see Table 6.1). Omega-3 PUFAs have been shown to affect the inflammatory signalling of microglia in culture by inhibiting MMP 9 production (Liuzzi et al., 2007)

**Table 6.1 Anti-inflammatory effects of dietary treatment or pre-treatment with omega-3 PUFA**

| Study | Inflammatory model | Species | PUFA | Effects |
|---|---|---|---|---|
| Lo et al., 1999 | LPS-stimulated macrophage cell line | mouse | EPA pre-stimulation incubation | ↑ COX-2 mRNA & protein expression<br>↓ $PGE_2$ production |
| Novak et al, 2003 | LPS-stimulated macrophage cell line | mouse | Omega-3 emulsion (DHA + EPA) pre-stimulation incubation | Inhibited IκB, ↓NFκB<br>↓ TNFα mRNA and protein expression |
| Zhao et al., 2004 | Hypercholesterolemic subjects | human | ALA diet | ↓ CRP, ↓ ICAM-1, ↓ VCAM-1, ↓ E-selectin |
| de Caterina et al., 1994a, b | Subjects with chronic renal disease | human | 6 week DHA + EPA TG or ethyl ester diet | Both ↓ platelet generated thromboxane B2 prolongation of the bleeding time from ethyl esters |
| de Caterina et al., 1994 | IL-1, TNF, IL-4 or LPS stimulated endothelial cells | human | DHA or EPA | DHA ↓ ICAM-1,VCAM-1, E-selectin, IL-6 &IL-8 and ↓ adhesion of monocytes |
| Renier et al., 1993 | LPS-stimulated macrophages from atherosclerosis-susceptible mice | mouse | 6 & 15 week fish oil diet (DHA + EPA) | ↓ TNFα & IL-1β mRNA expression at both time points<br>↓ prostaglandin production |

**Table 6.1 Anti-inflammatory effects of dietary treatment or pre-treatment with omega-3 PUFA (continued)**

| Study | Inflammatory model | Species | PUFA | Effects |
|---|---|---|---|---|
| Endres, et al., 1989 | Endotoxin stimulated peripheral-blood mononuclear cells | human | 6 week fish oil diet (DHA + EPA) | ↓ IL-1β synthesis |
| Babcock et al., 2002 | LPS-stimulated macrophages | mouse | Omega-3 emulsion (DHA + EPA) pre-stimulation incubation | ↓ TNFα protein production |
| Abbate et al., 1996 | LPS-stimulated mononuclear cells | human | 18 week omega-3 PUFA ethyl ester diet (DHA + EPA) | ↓ procoagulant activity<br>↓ IL-6 |
| Trebble, et al., 2003a | Peripheral blood mononuclear cell | human | 4 week fish-oil diet (DHA + EPA) | ↓ TNFα & IL-6 production |
| Trebble, et al., 2003b | Lymphocytes | human | 4 week fish-oil diet (DHA + EPA) | ↓ PGE2 production<br>↑ IFN-γ production & lymphocyte proliferation |
| Wallace et al., 2003 | LPS-stimulated mononuclear cells | human | 12 week fish-oil capsules (DHA + EPA) | ↓ IL-6 production<br>no difference in TNFα, IL-1β, IL-2, IL-4, IL-10 or IFN-γ |
| Liuzzi et al., 2007 | LPS-stimulated microglia | rat | Fish oil (DHA + EPA) incubation simultaneous to activation | ↓ MMP-9 production |

**Table 6.1 Anti-inflammatory effects of dietary treatment or pre-treatment with omega-3 PUFA (continued)**

| Study | Inflammatory model | Species | PUFA | Effects |
|---|---|---|---|---|
| Lee et al., 1984 | Ionophore-activated neutrophils | human | EPA or DHA | EPA ↓ LTB4, DHA did not |
| Sperling et al., 1993 | LTB4, PAF, zymosan or calcium ionophore-stimulated neutrophils | human | 3-10 week DHA + EPA tablets | ↓ LTB4- stimulated IP3 formation<br>↓ calcium ionophore-stimulated LTB4 & LTB5<br>↓ LTB4- & PAF- stimulated chemotaxis |
| Ly et al., 2006 | Anti-CD3 and anti-CD28 stimulated splenic CD4(+) T-cells | mice | 14 d DHA or EPA diet | DHA & EPA ↓ proliferation<br>EPA but not DHA ↑ CTLA-4 |
| Tomobe et al., 2000 | Contact hypersensitivity reaction in the ear sensitized with 2,4-dinitro-1-fluorobenzene | mice | DHA or EPA ethyl ester diet | DHA, not EPA ↓ ear swelling, infiltration of CD4+ T cells, IFN-γ, IL-6, IL-1β, and IL-2 mRNA expression |
| Mickleborough et al., 2009 | LPS-stimulated asthmatic alveolar macrophages | human | DHA or EPA pre-stimulation incubation | EPA & DHA ↓ TNFα & IL-1β mRNA expression,<br>↓ LTB4, $PGE_2$, TNFα & IL-1β production<br>The EPA reduction was significantly more than DHA |
| Terano ***et** al*., 1987 | Patients with various thrombotic diseases | human | 16 week EPA diet | ↓ thromboxane formation, platelet aggregation, retention & whole blood viscosity<br>↑ red blood cell deformation & prolongation bleeding |

ICAM- intercellular cell adhesion molecule, VCAM - vascular cell adhesion molecule, TG – triglycerides, $LTB_4$ - leukotriene B4, PAF - platelet-activating factor, $IP_3$ – phosphoinositide, CTLA-4 - cytotoxic T-lymphocyte antigen 4

Clinically, in patients with septic shock in the intensive care unit (ICU) given an omega-3 emulsion enriched in EPA and DHA intravenously for a 5 or 10 day period there was modulation of several inflammatory events (Mayer et al., 2003a, b). Stimulation of neutrophils (Mayer et al., 2003a) or monocytes (Mayer et al., 2003b) from the septic patients, *ex vivo*, demonstrated the generation of less inflammatory 5-series leukotriene production and suppression of the proinflammatory cytokines TNF-α, IL-1β, IL-6, and IL-8. In addition, studies of an enteral feed including fish oil in combination with γ-linolenic acid given to acute respiratory distress syndrome (ARDS) patients for 4-7 days, showed a reduction in IL-8 in bronchoalveolar lavage fluid and higher than normal levels of oxygenation, measured by increased partial pressure of oxygen in Arterial Blood / Fraction of Inspired Oxygen (Pacht et al., 2003). Clinical improvement such as fewer days of ventilatory support, reduction of organ failure and decreased length of stay in the intensive care unit was seen in patients with ARDS or acute lung injury who were given an infusion of EPA with gamma-linolenic acid (GLA) for 4-7 days (Gadek et al., 1999; Pontes-Arruda et al., 2006; Singer et al., 2006). The above benefits were seen within days.

Sperling et al. (1993) isolated neutrophils and monocytes from eight healthy volunteers for biochemical investigation 4 weeks before starting dietary supplementation with omega-3 PUFA, and after 3 and 10 weeks of supplementation with 20 g of 'Super-EPA' (Phenmacaps, Elizabeth, NJ, USA) daily, providing 9 g EPA and 5 g DHA. Increases in the EPA content of all the major phospholipids of neutrophil membranes, including the phosphocholine, phosphoethanolamine, and phosphatidylinositol pools, were observed after 3 and 10 weeks of dietary omega-3 PUFA supplementation. In parallel, the AA content of the phosphocholine and phosphoethanolamine pools of neutrophil membranes declined substantially, with the exception of the phosphatidylinositol pool. The number of neutrophils did not change significantly but chemotaxis was significantly inhibited.

Since fatty acids given in the diet are also incorporated into monocytes, it is possible that there are influences on macrophage activation and phenotype. The view that macrophages could aid in recovery after SCI has been supported by the demonstration that macrophages in *in vitro* and *in vivo* models of inflammation switch to an anti-

inflammatory phenotype, through down regulation of pro-inflammatory signals such as TNF-α and IL-1β and up regulation of anti-inflammatory signals such as TGF-β, secretion of growth factors and neurotrophic factors such as BDNF, GDNF, NGF and NT-3 (Elkabes et al., 1996; Kerschensteiner et al., 1999; Nakajima et al., 2001; Huynh, et al., 2002; Nakajima and Kohsaka, 2004; Hashimoto et al., 2005b). It is possible therefore, that chronic treatment with PUFAs either by i.v. infusion or in the diet could have a dramatic influence on the inflammatory cell composition, also modifying the inflammatory environment in which macrophages become activated and function after SCI.

### 6.1.3 Dietary effects of PUFA after trauma and SCI

There is already evidence that lipid emulsion preparations enriched in omega-3 fatty acids have beneficial effects in critically ill patients (Calder, 2003). A study in which a mixed group of over 650 patients received parenteral nutrition containing fish oil (i.e. EPA and DHA), found a significantly lower rate of infections, fewer complications, and shorter length of hospital stay in the post-surgery patients receiving fish oil-containing emulsions compared with those receiving the control emulsions (Heller et al., 2006). Mortality was significantly decreased in those patients who received more than 0.1 g fish oil/kg per day. In stroke patients, lower proportions of serum PUFAs (which reflects the dietary intake of saturated and unsaturated fats) and higher proportions of saturated fatty acids have been found compared with controls (Iso et al., 2002). Furthermore, a higher content of serum omega-3 PUFAs in patients is associated with a decrease in the risk of stroke (Simon et al., 1995).

Our laboratory has shown that in combination with DHA injection, animals maintained on a DHA-enriched diet after compression SCI had an improved recovery after injury, increased neuronal and oligodendrocytes survival and axonal preservation (Huang et al., 2007a; Ward et al. 2010 in press). This suggests that oral supplementation with omega-3 PUFA can enhance neuroprotection. In contrast, i.v. acute treatment with AA after spinal cord hemisection in rats had detrimental consequences (King et al., 2006). Along with the evidence that enrichment of omega-6 PUFA in leukocytes may influence pro-

inflammatory signalling, this suggests that parenteral preparations with a considerable omega-6 component, such as soybean oil-containing preparations, would not be beneficial after SCI (Calder, 2003, 2009, 2010; Michael-Titus, 2007). Thus, PUFAs could be considered for dietary supplementation after SCI, as well as a preventative role, in populations at high risk of neurotrauma, such as military personnel, as suggested in a recent study on head injury (Bailes et al., 2010). Troops going into areas of conflict, where risk of traumatic injury is high, could supplement their dietary intake with long chain omega-3 PUFAs, thus providing the potential of reducing neurological sequelae following traumatic brain injury or SCI.

Apart from the acute neuroprotective effect at the injury site, it is also important to explore other potential benefits of omega-3 PUFAs for people living with chronic SCI. It has been shown that omega-3 PUFA supplementation for 6 months could contribute to an improvement in functional capabilities such as strength endurance in the arm (Javierre et al., 2006). The parameters used in this study were a timed repetition task and the time taken to cover a 90 m track with 6 % slope. Individuals with SCI have identified the recovery of autonomic functions as a high priority for improving their quality of life (Anderson, 2004). Due to the neuroprotective effects of DHA and EPA at the injury site and the reduction of axonal pathology (King et al., 2006; Huang et al., 2007a; Ward et al., 2010; Lim et al., *in press*), which could be related directly or indirectly to functions other than motor recovery, it is important to look at the impact of omega-3 PUFAs on outcome measures such as loss of bladder control and the development of neuropathic pain, which has a high incidence in the SCI community and remains a challenge to treat.

EPA and DHA in fish oil are mainly in the form of triacylglycerols. However, EPA and DHA may also be ingested as purified ethyl esters or free fatty acids. The advantages of using purified fish oil products is that compounds such as other fatty acids, cholesterol and other environmental contaminants can be removed. Kim et al. (2010) suggested a 4% (wt/ wt) fish oil or 1% purified omega-3 PUFA ethyl esters as a reasonable dose for animal feeding studies designed to probe the biological properties of omega-3 PUFA relevant to humans. This level of intake delivers 2.4% of total energy as omega-3PUFA, which is within the range consumed by humans and used in human clinical trials.

Previous studies in our laboratory have demonstrated structural changes in cell membranes in the spinal cord after 12 weeks of omega-3 PUFA dietary supplementation (270 mg/kg/day of EPA and DHA; ratio 1.5:1; Dyall et al., 2007) and significant improvement in outcome after SCI (Huang et al., 2007a) using an average daily dose of 300-400 mg/kg of DHA (0.3 %) in the ethyl ester form, although this was only assessed in addition to an i.v. bolus of DHA (250 nmol/kg) 30 min after SCI. A target dietary dose of maximum 400 mg/kg long-chain omega-3 PUFA will be used in the studies that follow. This dose is comparable to the present recommended daily total amount of EPA and DHA in healthy adults.

### 6.1.4 Aims

The aim of this chapter was to explore in more depth the impact of dietary manipulation with omega-3 PUFA on SCI. In the first part of the chapter, we present the results obtained in compression SCI with a dietary intervention after SCI based on EPA, preceded by an acute i.v. bolus of EPA or saline (vehicle). In the final part of the chapter, we present a pilot study on the dietary supplementation with DHA in the compression model of SCI.

## 6.2 RESULTS

The four experimental groups used in the study in the first part of the chapter will be referred to as the following:

1. **IV saline, control diet or control group**: SCI, followed by i.v. saline injection at 30 min and control diet for 4 weeks.
2. **IV EPA, control diet**: SCI, followed by i.v. EPA injection (250 nmol/kg) at 30 min, followed by control diet for 4 weeks.
3. **IV saline, EPA diet**: SCI, followed by i.v. saline injection, followed by EPA diet (approximately 150 mg/kg/day) for 4 weeks.
4. **IV EPA, EPA diet**: SCI, intravenous EPA injection as above, followed by EPA diet (approximately 150 mg/kg/day) for 4 weeks.

### 6.2.1 I: Effect of EPA on the locomotor recovery following compression SCI

The IV EPA and control diet group had the best locomotor outcome overall (BBB= 12.9 (Fig. 6.1) but this was not significantly different from the control group (IV saline, control diet; BBB = 10.8). Animals treated with an EPA-enriched diet following i.v. saline injection, had a significantly worse BBB score compared to control diet animals receiving i.v. saline (BBB = 5, day 28; $p<0.05$). This was already apparent within the first week after SCI (Fig. 6.1) and the group remained the worst out of the four groups in terms of locomotor recovery. The EPA injection appeared to counteract the detrimental effect of the EPA- enriched diet (BBB = 11.6; $p<0.001$), thus leading to a locomotor score similar to the control group. 2Way RM ANOVA revealed that there was a significant effect of time ($F=71.96$, $p<0.05$), the interaction time and treatment was significant ($F=2.43$, $p<0.05$), but overall the effect of treatment with EPA (acute i.v. and dietary) was not ($F=2.34$, $p>0.05$).

There was no significant difference in the sub scores between groups at 28 days ($p>0.05$; One-way ANOVA; Fig. 6.2). An assessment of animals that regained frequent or consistent stepping, showed that none in the IV saline, EPA diet group regained stepping

function, compared to 4 out of 6 in the control group, 3 out of 6 in the IV EPA, EPA diet group, and 5 out of 6 in the acute EPA injection and control diet group (Table 6.2)

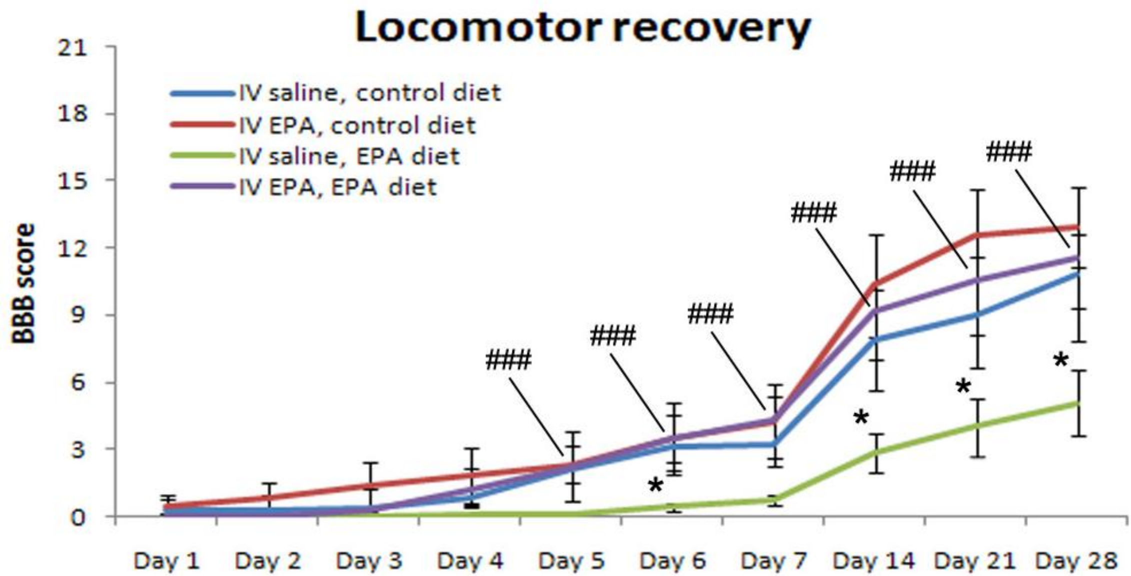



**Figure 6.1 The effect of treatment with dietary EPA on locomotor recovery after compression SCI.** The animals received saline or EPA (250 nmol/kg), 30 min after SCI and were then maintained on a control diet or EPA-enriched diet (150-170 mg/kg) for 28 days. Bonferroni's post-hoc analysis revealed that recovery in the IV saline, EPA diet group was significantly worse than the control group (IV saline, control diet; *p<0.05) and also worse than the IV EPA, EPA diet group (###p<0.001). Error bars represent SEM, n=6 per group.

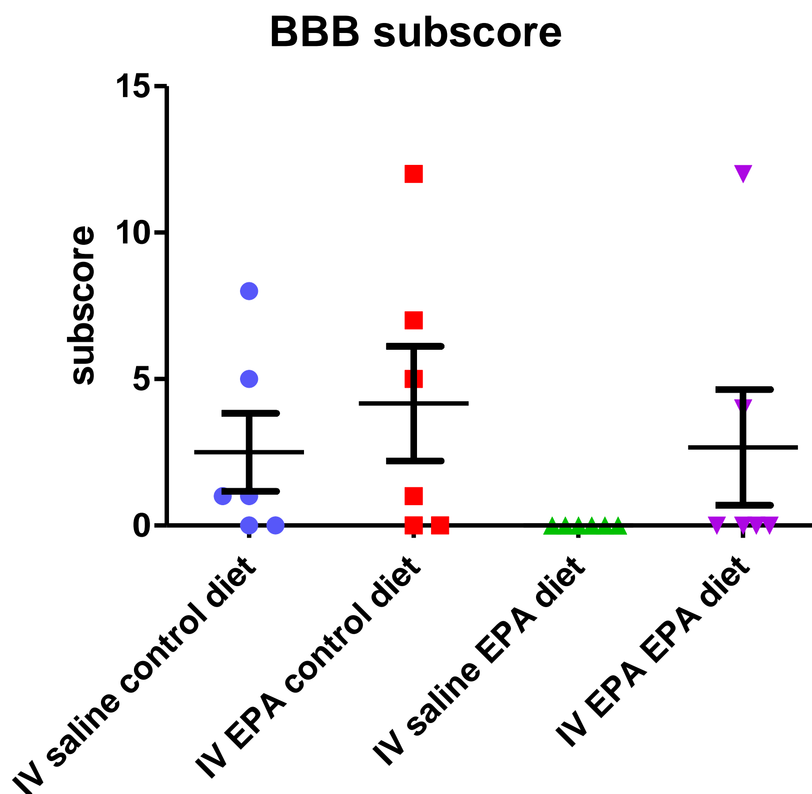



**Figure 6.2 The effect of treatment with dietary EPA on BBB subscore after compression SCI.** The animals received saline or EPA (250 nmol/kg), 30 min after SCI and were then maintained on a control diet or EPA-enriched diet (150-170 mg/kg) for 28 days. There was no significant difference in the BBB sub score between treatment groups at 28 days ($p>0.05$; one-way ANOVA). Error bars represent SEM, n=6 per group.

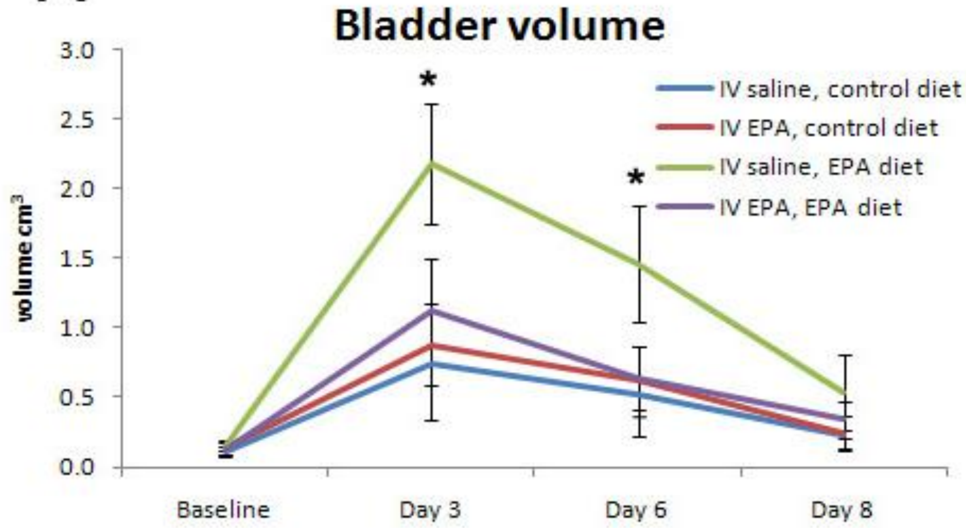
**Number of frequent or consistent steppers after SCI and dietary EPA treatment**

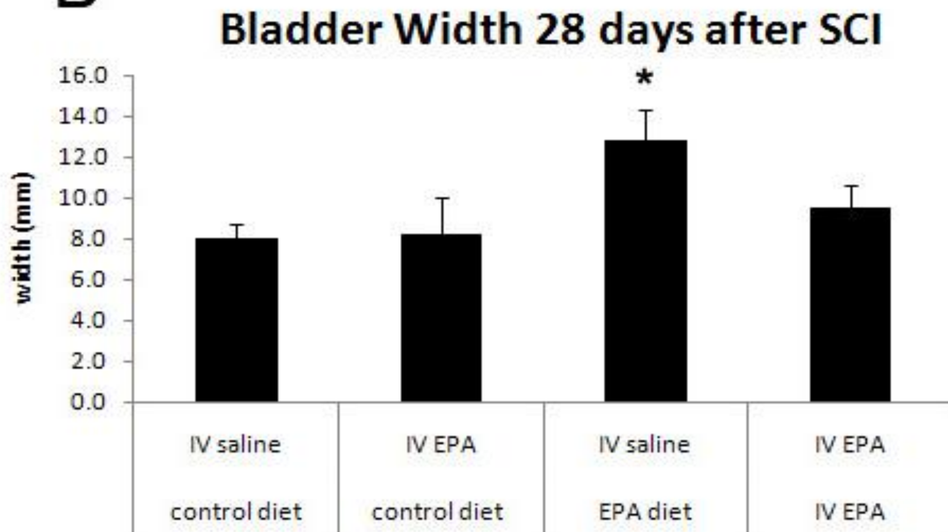
| Group | Number of frequent/consistent steppers |
|---|---|
| IV saline, control diet | 4/6 |
| IV EPA, control diet | 5/6 |
| IV saline, EPA diet | 0/6 |
| IV EPA, EPA diet | 3/6 |

**Table 6.2 The effect of treatment with dietary EPA on stepping after compression SCI.** The animals received saline or EPA (250 nmol/kg), 30 min after SCI and were then maintained on a control diet or EPA-enriched diet (150-170 mg/kg) for 28 days. 4 out of 6 rats in the IV saline, control diet group recovered frequent or consistent stepping compared to 0 out of 6 in the IV saline, EPA diet group. Fischer exact test revealed no significant difference.

### 6.2.2 Effect of EPA treatment on bladder function

On the day of surgery and days 3, 6 and 8 following SCI, bladder volume was measured using an ultrasound device as described in the Methods chapter. The volume in uninjured anaesthetised rats preceding surgery was 0.1 ± 0.03 ml (n=24). All groups had a transient increase in urine retention at day 3 post-surgery, with the IV saline, EPA diet group having the largest significant increase (2.18 ± 0.43 ml) compared to the control diet, saline injection group (0.74 ± 0.41 ml; Fig. 6.3A). By day 8, bladder volume was not significantly different from baseline (0.23 ± 0.11 ml in the control group). Despite the recovery in bladder function, in the IV saline, EPA diet group, there was a permanent increase in the size of the bladder width (Fig. 6.3B) found post mortem at 28 days compared to the other groups, which was significant compared to the control group (12.8 ± 1.1 mm vs. 8.0 ± 2.0 mm; $p<0.05$). Data was also collected regarding the day of resolution, or spontaneous voiding, i.e. the day preceding at least 3 days of an empty bladder (absence of urinary retention), or the day preceding at least 3 days of a dry abdomen (absence of incontinence; Fig. 6.3C). Most animals were dry around 3 days after SCI and did not need manual bladder expression after 8-10 days. There was no significant difference in resolution of spontaneous voiding between groups, although there was a trend towards a delayed recovery in the IV saline, EPA diet group.


A
Bladder volume
volume cm³
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
IV saline, control diet
IV EPA, control diet
IV saline, EPA diet
IV EPA, EPA diet
Baseline
Day 3
Day 6
Day 8


B
Bladder Width 28 days after SCI
width (mm)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
*
IV saline
control diet
IV EPA
control diet
IV saline
EPA diet
IV EPA
IV EPA

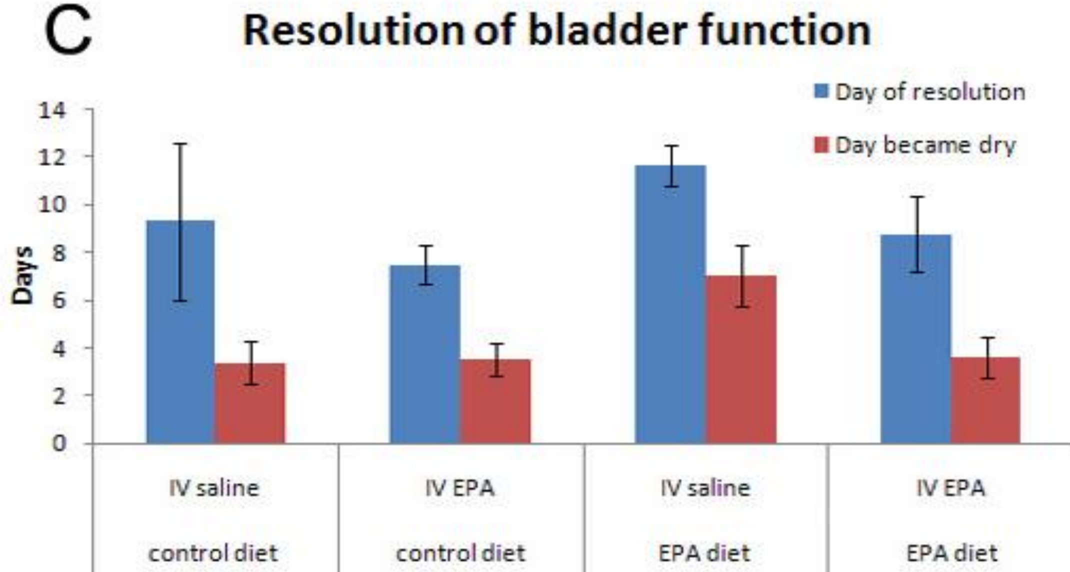
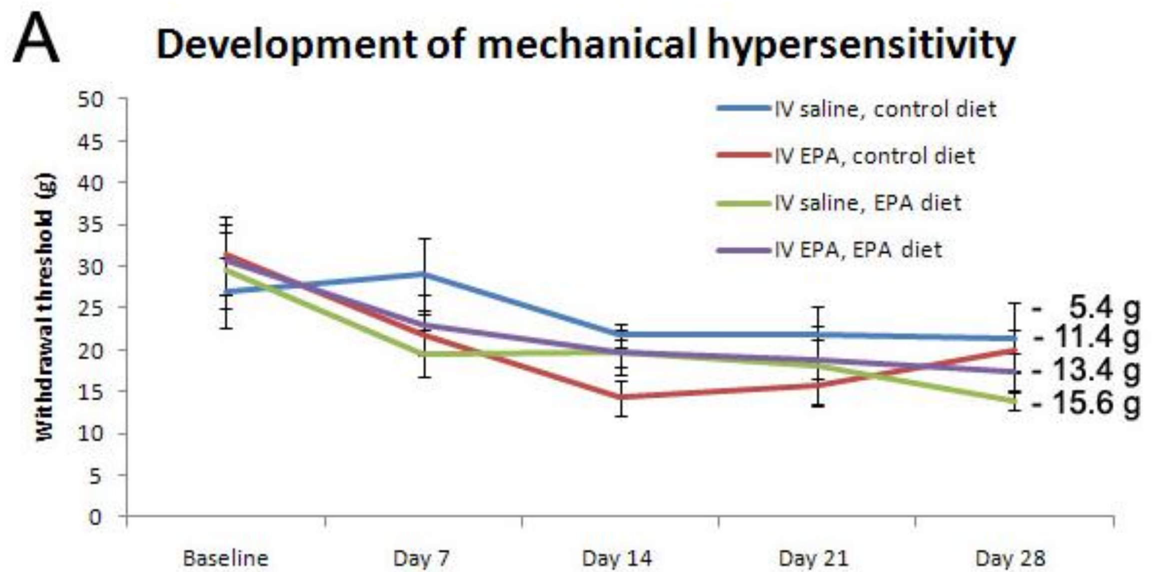

C
Resolution of bladder function
Day of resolution
Day became dry
Days
14
12
10
8
6
4
2
0
IV saline
control diet
IV EPA
control diet
IV saline
EPA diet
IV EPA
EPA diet

**Figure 6.3 The effect of treatment with dietary EPA on the bladder after compression SCI**. The animals received saline or EPA (250 nmol/kg), 30 min after SCI and were then maintained on a control diet or EPA-enriched diet (150-170 mg/kg) for 28 days. **(A)** Calculation of bladder dimensions from ultrasound readings revealed that there was a significantly larger increase in bladder volume, or the amount of urine retention in the IV saline, EPA diet group compared to the control group (*p<0.05, Bonferrroni post-hoc test) **(B)** Measurement of the bladder size at the termination of the experiment revealed a chronic significant increase in bladder width in the IV saline, EPA diet group compared to the control group (*p<0.05) **(C)** Additional records were collected throughout the experiment regarding the day of resolution (i.e. presence of an empty bladder); and the 'day became dry' (i.e. absence of incontinence). This revealed no significant difference between groups (*p>0.05, one-way ANOVA). Results represent the mean and error bars represent SEM; n=6 per group.

### 6.2.3 Effect of EPA treatment on the development of mechanical hypersensitivity

The baseline withdrawal threshold assessed as described in the Methods chapter, preceding SCI was approximately 30 g. This threshold decreased significantly ($p<0.01$; $F=8.4$, 2 way RM ANOVA) in all groups, and by approximately 50 % in the IV saline, EPA diet group at 28 days, but this was not statistically significant compared to the control group receiving saline and the control diet ($p>0.05$; $F=1.5$; Fig. 6.4A). It was not possible to obtain a threshold measurement until establishment of plantar placement; therefore not all rats were assessed at each time point. Furthermore, not all rats developed hypersensitivity. In order to distinguish between those rats which did not develop hypersensitivity, a further analysis was carried out, with an arbitrary value of 10% decrease from individual baseline score designated as a cut-off. Based on these criteria, additional differences were noted in some animals between left and right hind paw hypersensitivity (Fig. 6.4B).




**B**

**Number in each group that developed a ≥10 % decrease in threshold from the individual's baseline score:**

| Group | Right hind paw | Left hind paw | Both paws |
|---|---|---|---|
| IV saline, control diet | 4/5 | 4/5 | 3/5 |
| IV EPA, control diet | 5/6 | 5/6 | 5/6 |
| IV saline, EPA diet | 4/5 | 3/5 | 3/5 |
| IV EPA, EPA diet | 4/6 | 3/6 | 2/6 |

**Figure 6.4 The effect of treatment with dietary EPA on mechanical hypersensitivity after compression SCI.** The animals received saline or EPA (250 nmol/kg) 30 min after SCI and were then maintained on a control diet or EPA-enriched diet (150-170 mg/kg) for 28 days. **(A)** Mechanical withdrawal thresholds decreased from baseline values at 7, 14, 21 and 28 days after SCI. The number assessed in each group varied at each time point depending on re establishment of plantar placement. Results represent mean ± SEM. **(B)** The number per group that developed hypersensitivity based on an arbitrary value of 10% decrease from the individual's baseline score as a cut-off. Not all animals developed hypersensitivity based on these criteria. The analysis was further divided into right, left or both hind paws, highlighting the differences compared to when the values were combined.

### 6.2.4 Effect of EPA treatment on the fatty acid composition of red blood cells

Red blood cells (RBCs) were harvested at the time of tissue collection at 28 days and sent to the University of Stirling (Prof. Gordon Bell) for lipid analysis. In the group that received the EPA supplemented diet, a significant increase (1-2 fold; $p<0.05$) was noted in the total omega-3 content of RBCs, particularly of EPA, where the amount increased by 8-fold compared to the control diet group (Table 6.3). There was a small, but significant ($p<0.05$) increase in DHA content (less than one-fold) and no observed change in the total amount of saturated or monounsaturated fatty acids between the groups. A small, but significant decrease in total omega-6 fatty acids, including arachidonic acid, docosapentaenoic acid and docosatetraenoic acid was noted. There was also a significant increase in 18:1 dimethylacetal.

### 6.2.5 Effect of EPA treatment on tissue markers of injury after SCI

#### 6.2.5.1 Injury epicentre

Toluidine blue staining on epicentre sections revealed extensive damage and cavitation in all groups, surrounded by a small amount of tissue sparing (Fig. 6.5).

Due to time constraints, analyses on further histological markers were carried out only in the control diet and IV saline, and IV saline and EPA diet groups.

**Table 6.3 Fatty acid composition of red blood cells**. Data expressed as % total fatty acids in animals that received control or EPA enriched diet after SCI.

| Fatty acid | Control diet | EPA diet |
|---|---|---|
| **Saturated** | | |
| 14:00 | 0.26 ± 0.02 | 0.24 ± 0.02 |
| 15:00 | 0.28 ± 0.01 | 0.31 ± 0.02 |
| 16:00 | 23.41 ± 0.26 | 24.01 ± 0.44 |
| 18:00 | 21.51 ± 0.48 | 20.54 ± 0.39 |
| 20:00 | 0.15 ± 0.01 | 0.15 ± 0.01 |
| 22:00 | 0.49 ± 0.03 | 0.50 ± 0.02 |
| 24:00:00 | 1.27 ± 0.07 | 1.35 ± 0.05 |
| **Total** | **47.35 ± 0.74** | **47.13 ± 0.42** |
| **Monounsaturated** | | |
| 16:1n-9 | 0.31 ± 0.02 | 0.34 ± 0.05 |
| 16:1n-7 | 0.26 ± 0.02 | 0.29 ± 0.03 |
| 18:1n-9 | 4.53 ± 0.19 | 4.24 ± 0.21 |
| 18:1n-7 | 2.27 ± 0.07 | 2.18 ± 0.05 |
| 20:1n-9 | 0.08 ± 0.00 | 0.08 ± 0.00 |
| 24:1n-9 | 0.62 ± 0.04 | 0.62 ± 0.03 |
| **Total** | **8.06 ± 0.24** | **7.74 ± 0.22** |
| **Omega-6 PUFA** | | |
| 18:2n-6 | 8.45 ± 0.23 | 8.90 ± 0.35 |
| 20:2n-6 | 0.30 ± 0.01 | 0.38 ± 0.06 |
| 20:3n-6 | 0.33 ± 0.02 | 0.33 ± 0.02 |
| 20:4n-6 **(AA)** | 22.88 ± 0.45 | 20.50 ± 0.65* |
| 22:4n-6 | 1.79 ± 0.05 | 1.18 ± 0.06* |
| 22:5n-6 | 0.64 ± 0.06 | 0.37 ± 0.01* |
| **Total** | **34.39 ± 0.73** | **31.64 ± 0.56*** |
| **Omega-3 PUFA** | | |
| 20:5n-3 **(EPA)** | 0.15 ± 0.04 | 1.22 ± 0.05* |
| 22:5n-3 | 0.91 ± 0.06 | 1.93 ± 0.07* |
| 22:6n-3 **(DHA)** | 1.83 ± 0.09 | 2.39 ± 0.12* |
| **Total** | **2.90 ± 0.14** | **5.53 ± 0.19*** |
| **Dimethylacetal (DMA)** | | |
| 16:0DMA | 2.51 ± 0.08 | 2.67 ± 0.08 |
| 18:0DMA | 3.33 ± 0.08 | 3.39 ± 0.08 |
| 18:1DMAs | 1.48 ± 0.08 | 1.90 ± 0.10* |
| **Total** | **7.31 ± 0.16** | **7.96 ± 0.17** |
| **TOTAL** | **100.00 ± 0.00** | **100.00 ± 0.00** |

Data represents mean ± SEM, n=6 per diet group. *p<0.05 compared to the control group

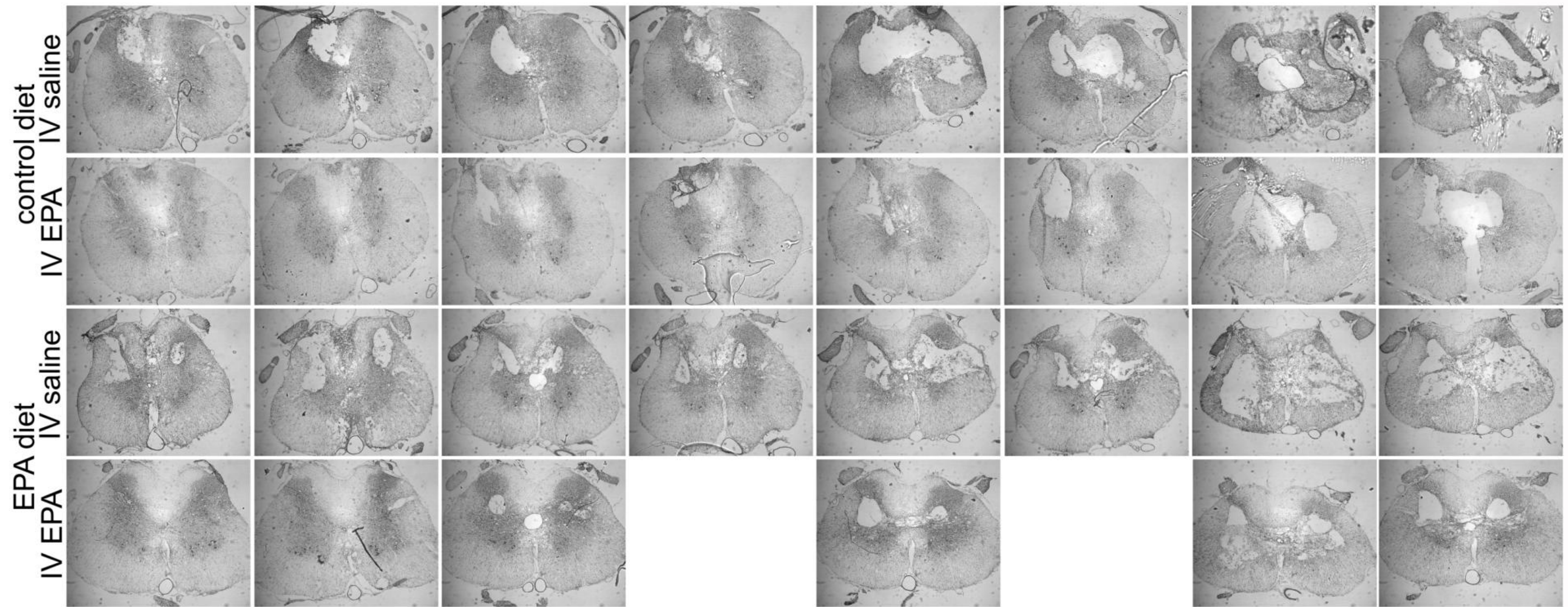



**Figure 6.5 The effect of treatment with dietary EPA on cavity formation after compression SCI.** The animals received saline or EPA (250 nmol/kg), 30 min after SCI and were then maintained on a control or EPA-enriched diet (150-170 mg/kg) for 28 days. Toluidine blue staining on transverse sections throughout the epicentre at 28 days revealed extensive damage and cavitation in all groups. Each row represents one animal from each group and each column is aligned at an equivalent thoracic level.

*Macrophages/microglia and cavitation*

ED1 staining showed that macrophages were present inside the cystic cavities and in the surrounding spared tissue (Fig. 6.6). Quantification revealed that macrophages consisted approximately 8% of the whole section area in the control group (Fig. 6.7A). There was no significant difference in the amount of macrophages between the control and IV saline, and IV saline and EPA diet groups ($p>0.05$; Fig. 6.7A). There appeared to be more extensive cavitation in the IV saline, EPA diet group, which had the worst BBB score. Following quantification there was a slight increase in cavity size in the IV saline, EPA diet group compared to the control group, but this was not significant (Fig. 6.7B). The damage was possibly too severe for accurate analysis in the epicentre; therefore further analyses were carried out on tissue approximately 5 mm rostral to the injury site.

### 6.2.5.2 Rostral to the injury epicentre

*Macrophages/microglia*

There was little or no cavitation 5 mm rostral to the injury site. However, macrophages were found scattered throughout the white matter, as well as confined to the dorsal columns and sometimes clustered in distinct areas (Fig. 6.8A-B). Quantification confirmed a smaller amount of macrophages at this distance compared to the epicentre (approximately 3.5 % compared to 8 % in the control diet and IV saline group in the epicentre; Fig. 6.8C). However, there was no significant difference in the amount of macrophages between the control diet and IV saline and the IV saline, EPA diet groups ($p>0.05$; Fig 6.8C).

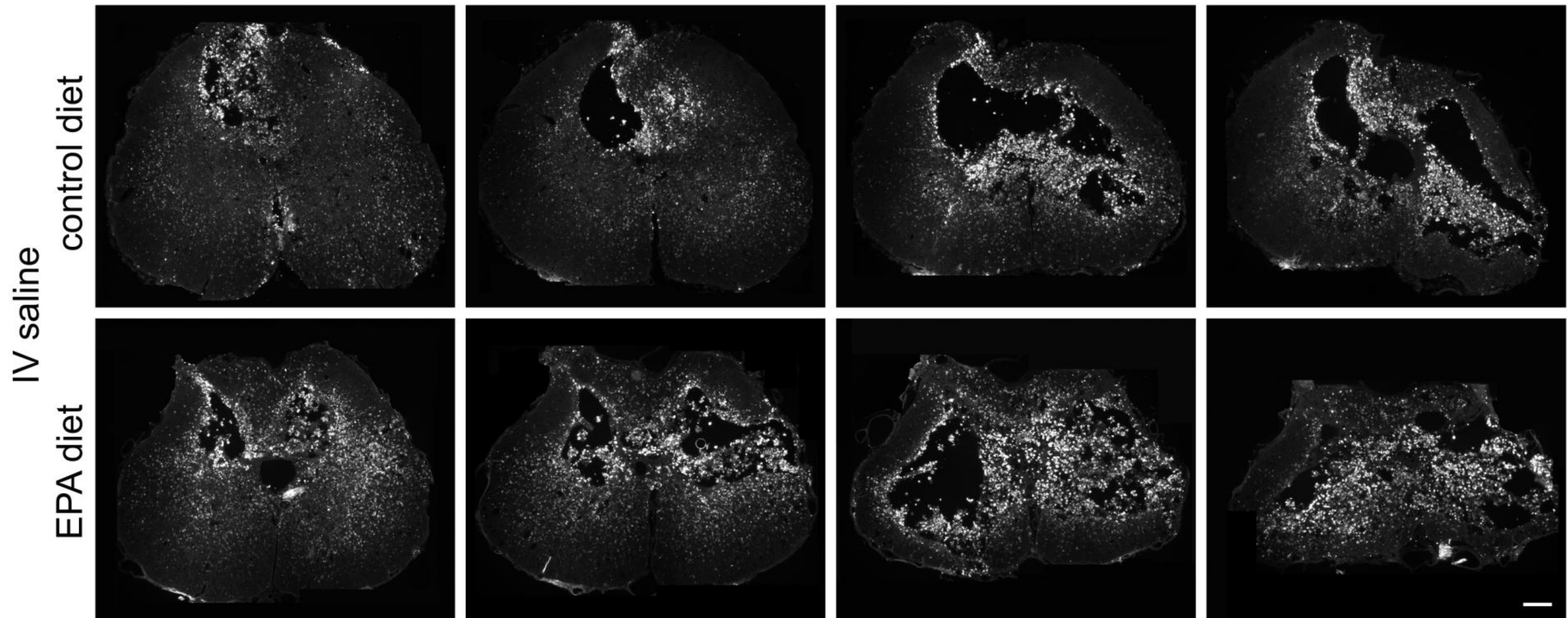
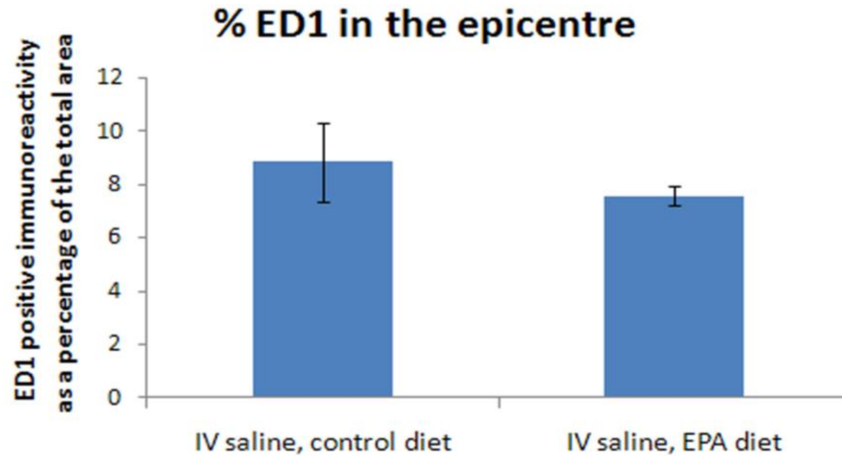



**Figure 6.6 The effect of treatment with dietary EPA on ED1 staining in the injury epicentre after compression SCI.** The animals received saline 30 min after SCI and were then maintained on a control diet or EPA-enriched diet (150-170 mg/kg) for 28 days. Each row of panels represents one animal from each group and each column is aligned at an equivalent thoracic level. There was extensive ED1 labelled cells throughout the epicentre and inside areas of cavitation. Scale bar = 100 μm.

A




B

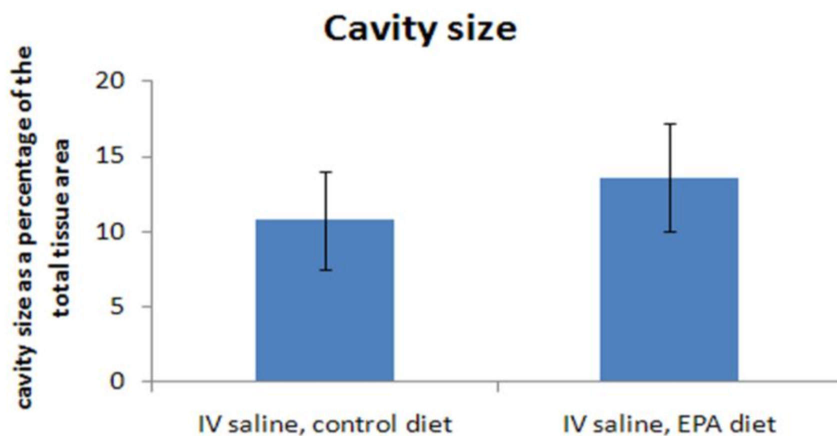



**Figure 6.7. The effect of treatment with dietary EPA on ED1 staining and cavity size after compression SCI.** The animals received saline 30 min after SCI and were then maintained on a control diet or EPA-enriched diet (150-170 mg/kg) for 28 days. **(A)** Quantification of ED1 macrophages in the epicentre at 28 days revealed no significant differences between the two groups ($p>0.05$). **(B)** Measurements were taken to calculate the cavity size in the transverse sections but there was no significant difference between the two groups ($p>0.05$). Results represent mean ± SEM; n=6 per group.

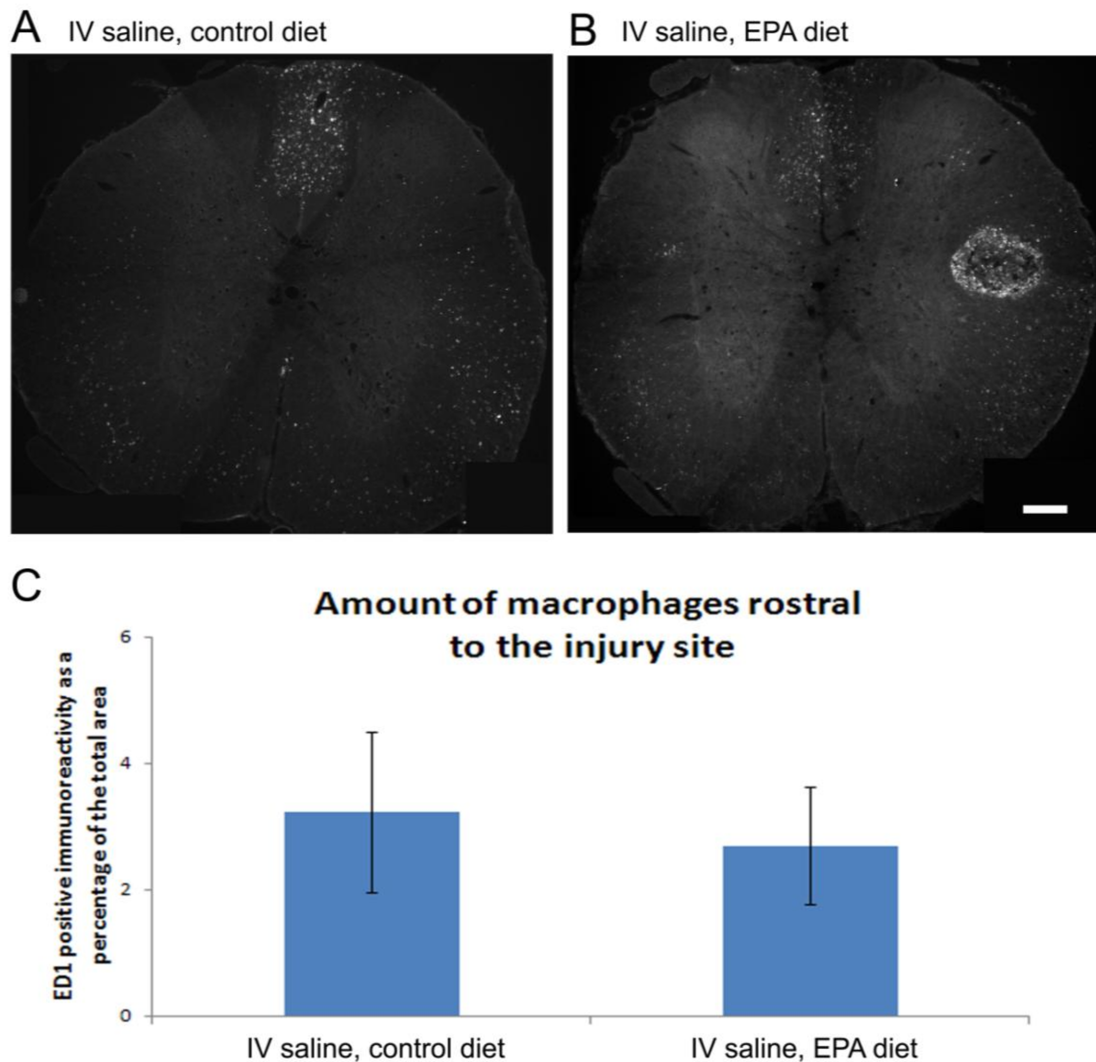



**Figure 6.8 The effect of treatment with dietary EPA on the presence of macrophages in the rostral segment after compression SCI.** The animals received saline 30 min after SCI and were then maintained on a control diet or EPA-enriched diet (150-170 mg/kg) for 28 days. **(A-B)** ED1 labelled macrophages approximately 5 mm rostral to the injury site. **(C)** Quantification revealed that there was no significant difference ($p>0.05$) between the control diet and IV saline group and the IV saline, EPA diet group. Results represent mean ± SEM; n=6 per group. Scale bar = 50 μm.

Phagocytic microglia, identified by large and rounded cells were found in most of the rostral sections, alongside microglia with a 'resting' morphology. Since the phagocytic phenotype was not seen in all areas in all sections and due to the different sizes of phagocytic compared to activated and resting microglia, the quantification was performed in the dorsal and ventral horns only, on activated microglia where no phagocytic microglia/ macrophages were visible (Fig. 6.9A, B). The ED1 analysis above would have already accounted for these since phagocytic microglia/macrophages express both the ED1 and Iba1 markers. There was no significant difference between the two groups in the microglial labelling in the dorsal and ventral horns (p>0.05; Fig. 6.9C, D).

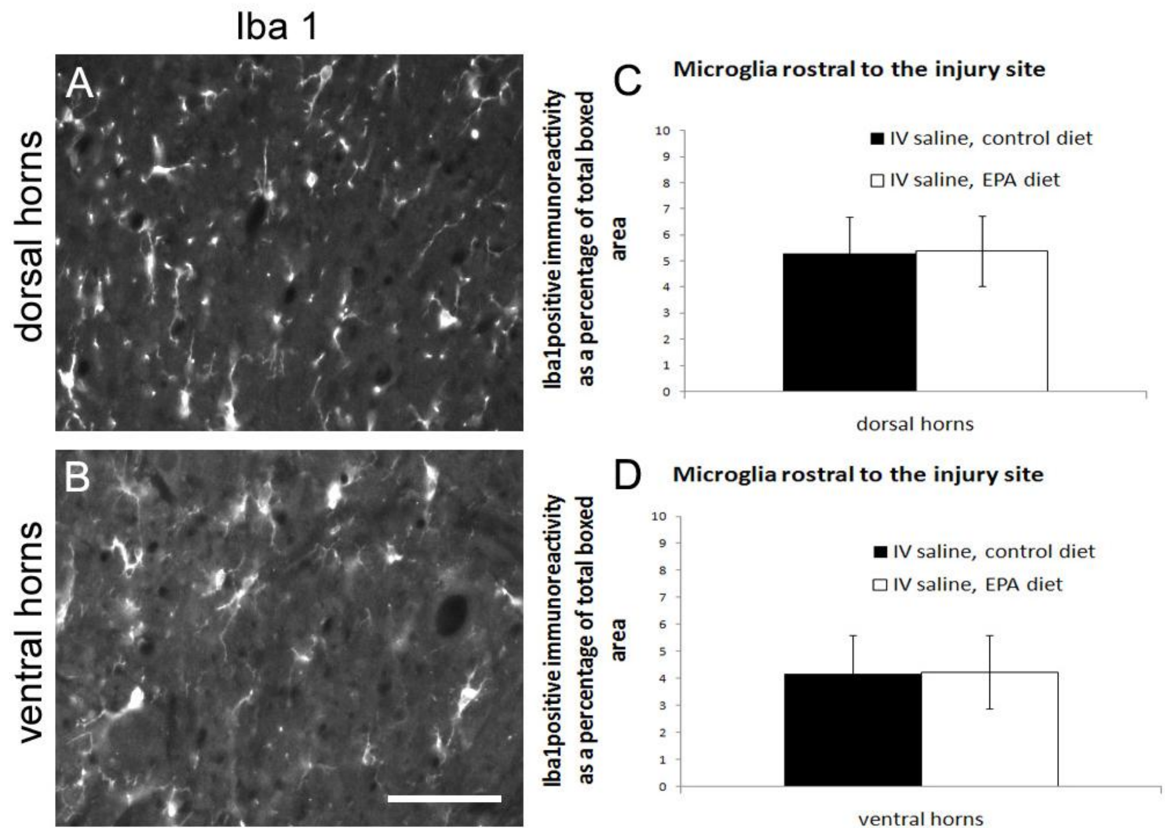



**Figure 6.9 The effect of treatment with dietary EPA on microglia (Iba1) staining after compression SCI.** The animals received saline 30 min after SCI and were then maintained on a control diet or EPA-enriched diet (150-170 mg/kg) for 28 days. **(A, B)** Images showing Iba1 labelling in the dorsal **(A)** and ventral **(B)** horns. **(C, D)** Quantification of microglia (Iba1) in the dorsal **(C)** and ventral **(D)** horns revealed that there was no significant difference between the two groups (p>0.05). Results represent mean ± SEM; n=6 per group. Scale bar = 50 µm.

*Neuronal survival*

Quantification of neurones in the rostral segment was carried out by counting of NeuN labelled cells in specific areas in the dorsal and ventral horns and normalisation to uninjured control tissue at the same spinal level (Fig. 6.10A-F).

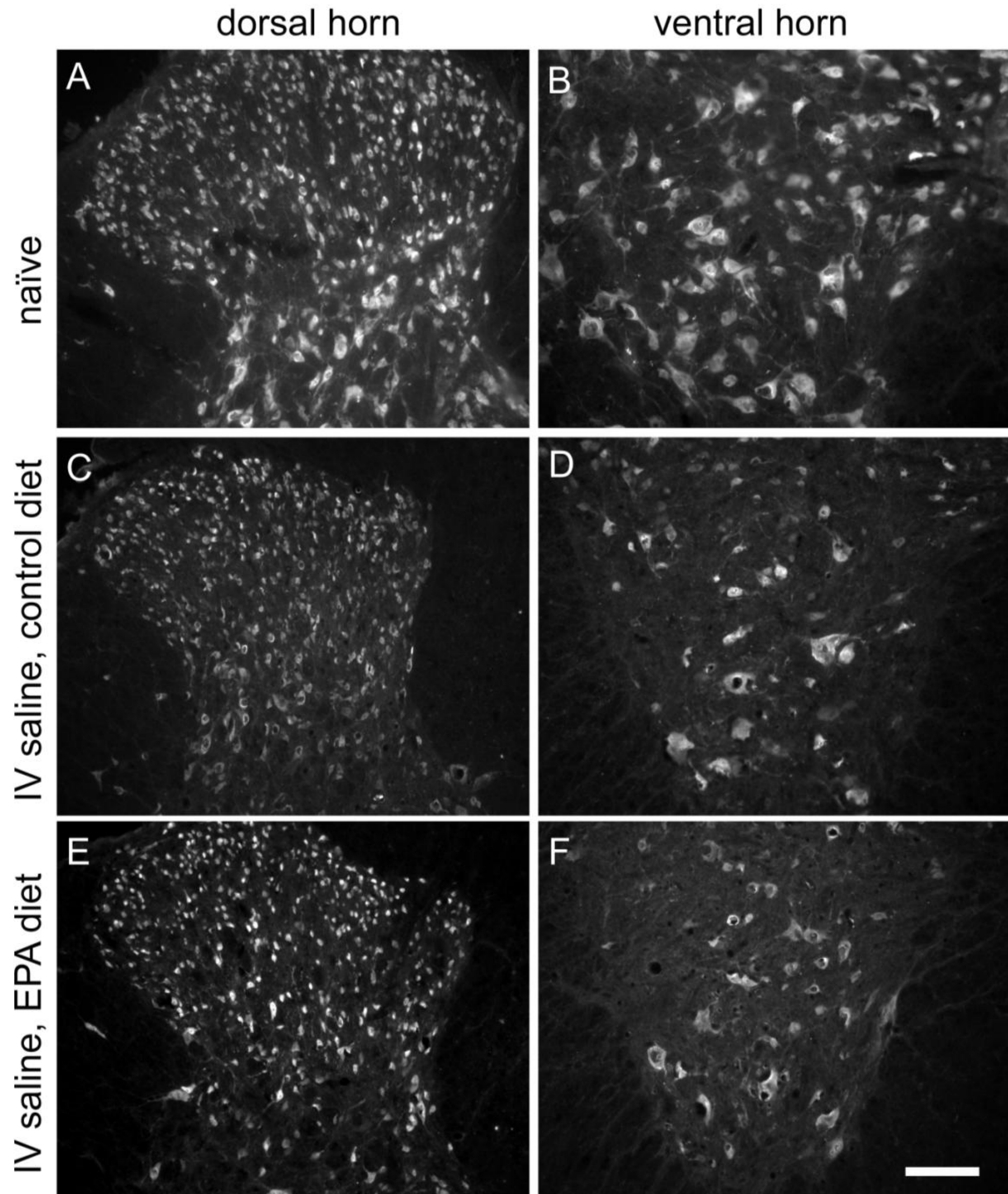



**Figure 6.10 The effect of treatment with dietary EPA on neuronal survival after compression SCI.** Animals received saline 30 min after SCI followed by control diet or EPA-enriched diet (150-170 mg/kg) for 28 days. Representative sections showing NeuN labelled neurones in the dorsal **(A, C, E)** and ventral **(B, D, E)** horns of naive **(A, B)**, IV saline and control diet **(C, D)**, or IV saline and EPA diet **(E, F)**. Scale bar = 100 μm.

In naïve control animals, NeuN-immunoreactive neurones were present throughout the grey matter of the spinal cord (Fig. 6.10A, B) There was a reduction of approximately 20% in the dorsal horn and 40% in the ventral horn at this rostral level after SCI (Figs. 6.10 C, D and 6.11). However, there was no significant difference in neuronal survival at this level between the control diet and IV saline and the IV saline, EPA diet groups ($p>0.05$; Fig. 6.10E, F and 6.11), although there was a tendency to an increased neuronal loss in the dorsal horn after PUFA supplementation.

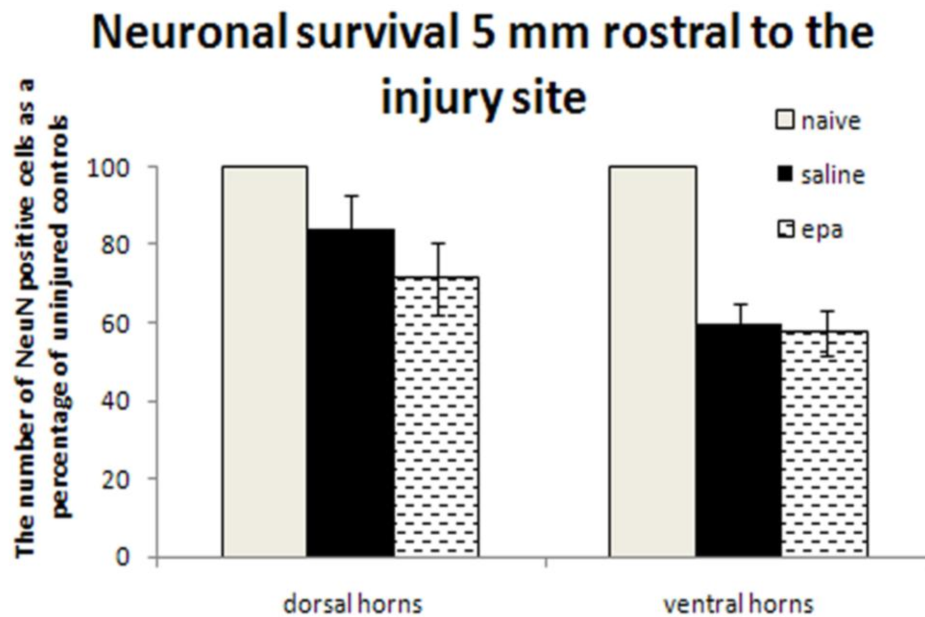



**Figure 6.11 Quantification of neuronal survival in the grey matter.** Quantification revealed a loss of approximately 20% in the dorsal horn and 40% in the ventral horns compared to uninjured control tissue. There was no significant difference in the amount of neuronal survival at this level between the two SCI groups ($p>0.05$). Results represent mean ± SEM; n=6 per group.

*Oligodendrocyte survival*

Images of oligodendrocytes in the rostral segment were captured from the areas shown in Fig. 6.12. Detailed quantification, by counting, was carried out on the control diet and IV saline group and the IV saline, EPA diet group, and this was normalised to uninjured control tissue at the same level (Fig. 6.13). There was a loss of approximately 60±12.4 % in the corticospinal tracts as well as a 20-40 % loss in other areas of the white matter. There was no significant difference in oligodendrocyte survival between the two groups

although there was a clear trend towards a greater loss in the dorsal columns in the IV saline, EPA group compared to control diet and IV saline group (61.4 ± 15.9 % vs 31.6 ± 20.3 %; p>0.05; Fig. 6.13).

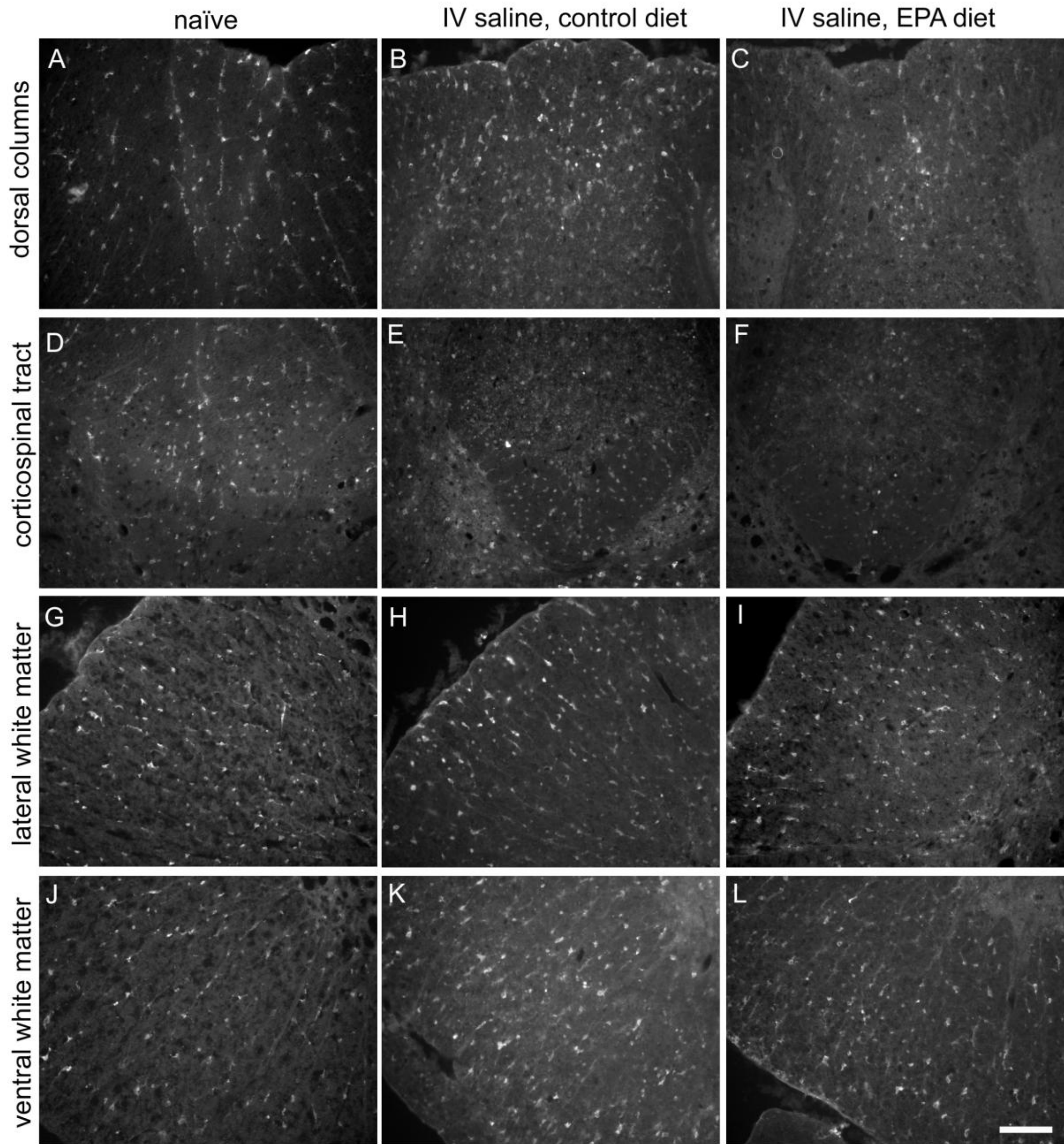



**Figure 6.12 The effect of treatment with dietary EPA on APC staining after compression SCI.** Animals received saline 30 min after SCI followed by control or EPA-enriched diet (150-170 mg/kg) for 28 days. Images show representative APC labelled oligodendrocytes in the white matter of naïve **(A, D, G, J)** IV saline and control diet tissue **(B, E, H, K)**, and IV saline and EPA diet tissue **(C, F, I, L)**. Scale bar = 100 μm.

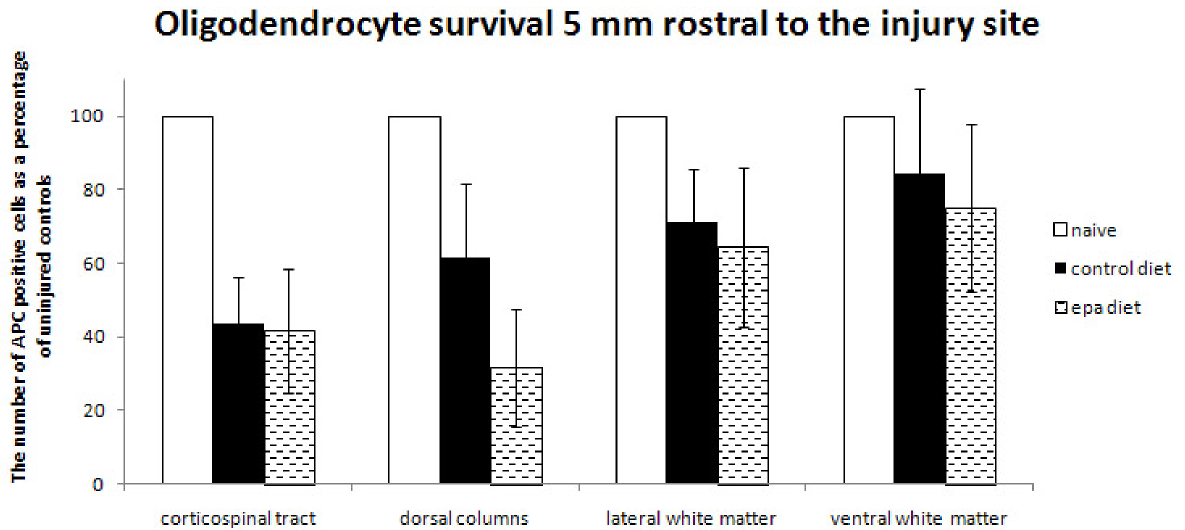



**Figure 6.13 Quantification of treatment with dietary EPA on APC staining after compression SCI.** There was a greater loss in the corticospinal tracts than in other areas of the white matter. There was no significant difference (p>0.05) in oligodendrocyte survival between the two SCI groups although there was a trend towards a greater loss in the IV saline and EPA diet group in the dorsal columns.

*Axonal damage*

Using the non-phosphorylated neurofilament marker SMI32, an analysis was carried out of the number of SMI32 immunoreactive particles in specific areas of the white matter (Fig. 6.14). In normal, uninjured spinal cord at the same level, SMI32 labelling was seen as numerous, regular-sized punctate particles and in an even distribution (Fig. 6.14A, D, G, J). Following SCI, swollen particles of various sizes were seen in a haphazard distribution, indicating severe axonal disruption and cytoskeletal damage, and there was an overall loss in number (Fig. 6.14 B, E, H, K). The greatest loss was in the corticospinal tracts and dorsal columns (95.3 ± 1.4 % and 77.4 ± 6.5 %). There was no significant difference in number between the IV saline and control diet, and IV saline and EPA diet groups (p>0.05; Fig. 6.15).

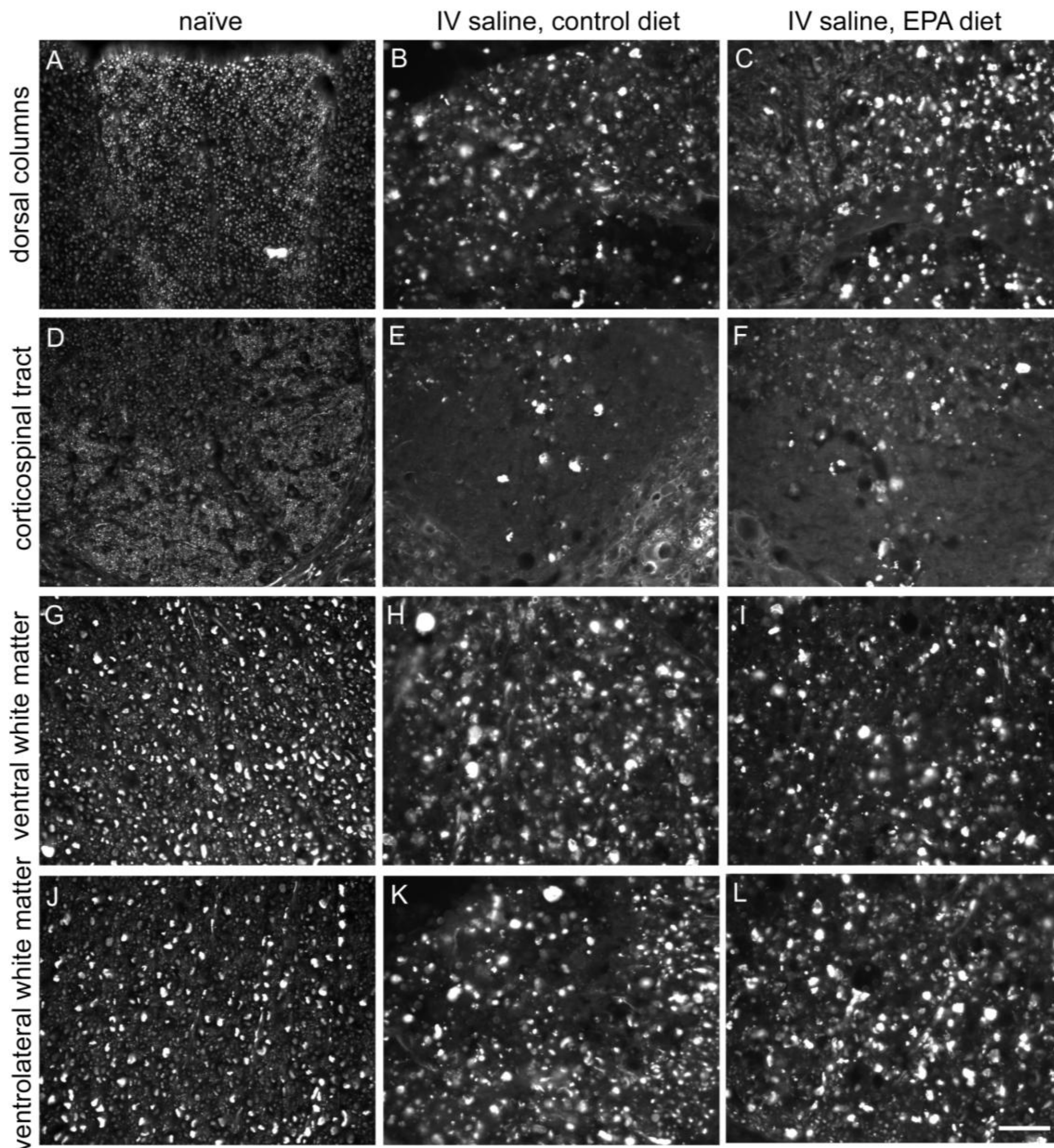



**Figure 6.14 The effect of treatment with dietary EPA on SMI32 staining after compression SCI.** Animals received saline 30 min after SCI followed by control or EPA-enriched diet (150-170 mg/kg) for 28 days. Representative images of SMI32 labelling in areas of uninjured tissue **(A, D, G, J),** IV saline and control diet **(B, E, H, K),** and IV saline and EPA diet tissue **(C, F, I, L)** 5 mm rostral to the injury site. Scale bar = 100 µm.

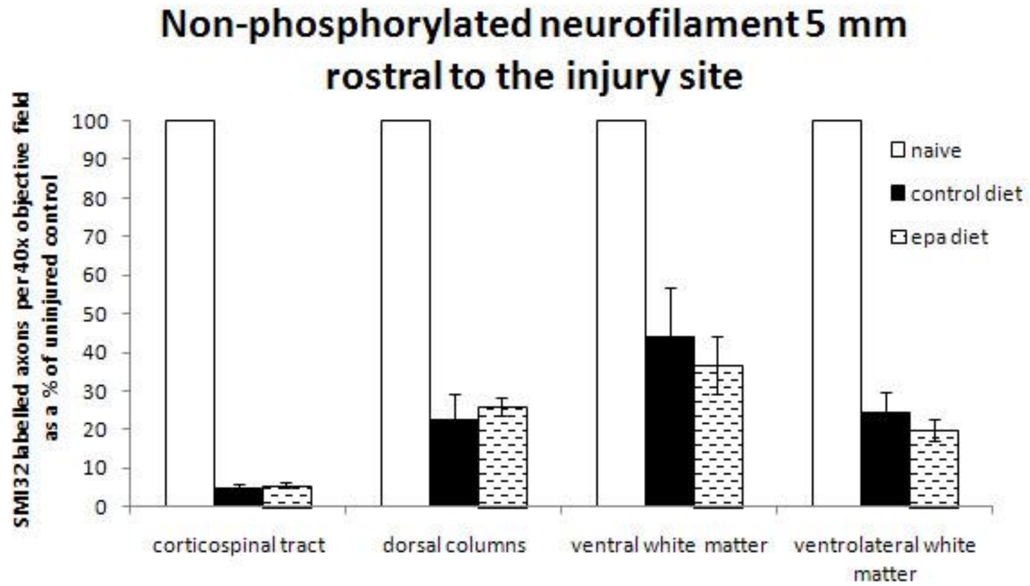



**Figure 6.15 Quantification of treatment with dietary EPA on SMI32 staining after compression SCI.** Animals received saline 30 min after SCI followed by control or EPA-enriched diet (150-170 mg/kg) for 28 days. There was an overall loss after SCI in the dorsal columns, corticospinal tract, ventral white matter and ventrolateral white matter, but no significant difference between the two treatment groups (p>0.05).

### 6.2.6 II: The effect of DHA dietary supplementation pre- and post-SCI

For the DHA pre-treatment study, there were four groups, with 6-7 rats per group. The level of supplementation was approximately 270-320 mg/kg DHA daily.

Groups:

1. **control + control**: 4 weeks control diet, SCI, then 4 weeks control diet
2. **control + DHA**: 4 weeks control diet, SCI, then 4 weeks DHA enriched diet
3. **DHA + control**: 4 weeks DHA enriched diet, SCI, then 4 weeks control diet
4. **DHA + DHA**: 4 weeks DHA enriched diet, SCI, then 4 weeks DHA enriched diet

Unexpectedly, there was an uncharacteristic loss of animals in this first 4 group experiment. In the first week, three animals died in the control group, none in the DHA + DHA group, and one in each of the other two groups. In addition, five were excluded after surgical problems, or because movement of the hindlimbs occurred within four hours of the SCI. This meant that the initial numbers were reduced by approximately 1/3 and the remaining size of the groups decreased markedly the power of the statistical analysis. After a first analysis of the data from this first experiment, it was decided to repeat the experiment, but due to the lack of time, we focused on just two groups. Therefore, the experiment was repeated with 2 of the 4 groups (DHA + control and control + control) but it was decided not to pool the results. Therefore, a summary of the results from the two separate experiments is presented below.

#### 6.2.6.1 Experiment 1

*Locomotor outcome*

Based on the BBB score, recovery in the control group reached a plateau at 28 days of 7.5 ± 1.7; i.e. movement of all joints of the hindlimb/plantar placement but no stepping (Fig. 6.16). Some rats in the other groups regained stepping function (Table 6.4). Two-way RM ANOVA revealed a significant effect for the time factor ($p<0.05$; $F=63.08$), but no significant effect of the treatment factor ($p>0.05$; $F=0.934$)

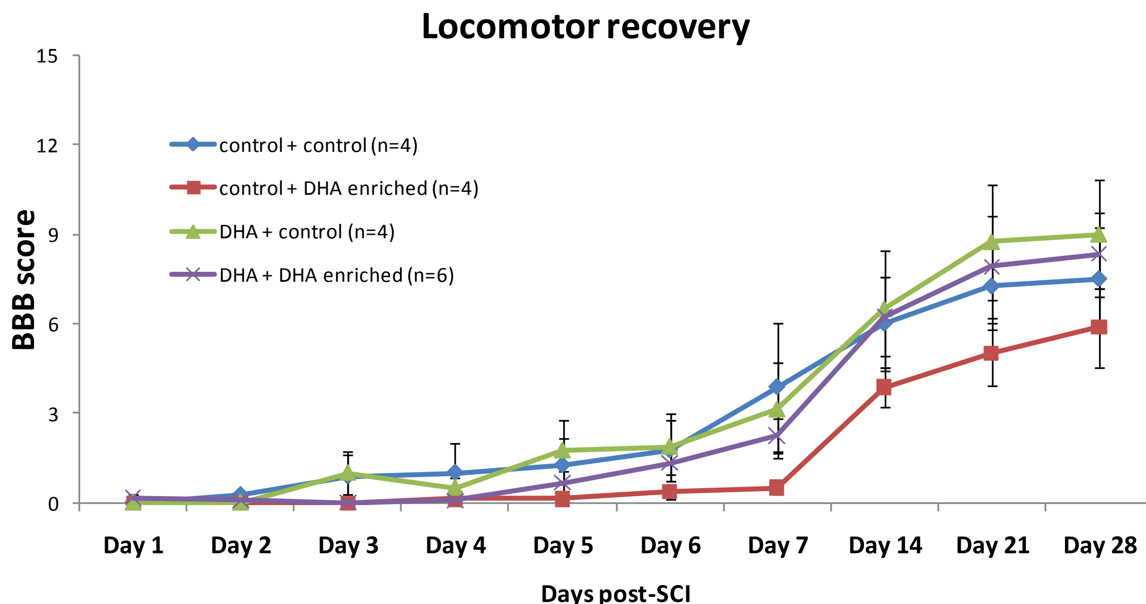



**Figure 6.16 I. Effect of treatment with dietary DHA on locomotor recovery after SCI.** Animals consumed control or DHA (330 mg/kg) for 28 days before and control or DHA (270-320 mg/kg) after SCI. There was no significant difference in the BBB score between treatment groups (p>0.05). Error bars represent SEM; no. of animals per group in brackets.

*Stepping*:

Some rats regained occasional stepping function: 1 out of 4 in the control + control diet group (Table 6.4). The Fischer exact statistical test revealed no significant difference between groups (p>0.05).

**Table 6.4 Effect of treatment with dietary DHA on stepping following SCI**

| Group | Number of stepping animals |
|---|---|
| control + control diet | 1 / 4 |
| control + DHA diet | 0 / 4 |
| DHA + control diet | 3 / 4 |
| DHA + DHA diet | 4 / 6 |

Animals received control or DHA (270-320 mg/kg) for 28 days before and after SCI. There was no significant difference between groups (p>0.05).

Due to the unexpected loss of animals in the preceding experiment, a repeat was performed with two groups, 8-9 rats per group:

1. **Control:** 4 weeks control diet, SCI, then 4 weeks control diet
2. **DHA:** 4 weeks DHA enriched diet, SCI, then 4 weeks control diet

### 6.2.6.2 Experiment 2

*Locomotor outcome*

Based on the BBB score, recovery in the control group reached a plateau at 28 days of 9.19±1.7; i.e. occasional uncoordinated stepping (Fig. 6.17). Two-way RM ANOVA revealed a significant interaction ($p<0.05$; $F=1.981$) and significant difference over time ($p<0.01$; $F=26.96$) but no significant difference between the two treatment groups, despite a trend towards worse recovery in the DHA enriched group ($p>0.05$; $F=1.844$).

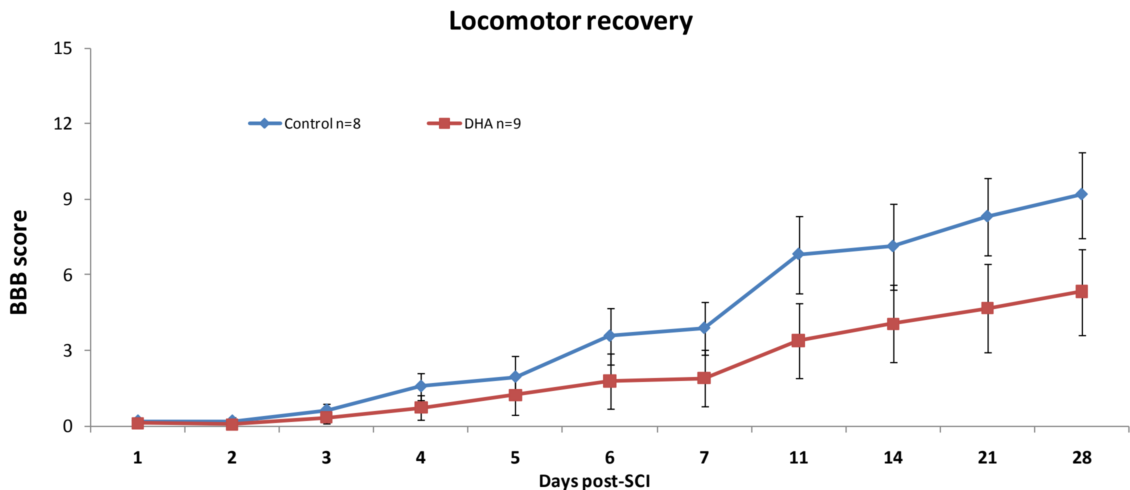



**Figure 6.17 II. Effect of treatment with dietary DHA on locomotor recovery after SCI.** Animals consumed control or DHA (330 mg/kg) for 28 days before SCI and control for 28 days after SCI. There was no significant difference in the BBB score between treatment groups ($p>0.05$). Error bars represent SEM; no. of animals per group in brackets.

*Stepping*:

Some rats regained occasional stepping function: 4 out of 8 in the control diet group; and 3 out of 9 in the DHA pre-treated group. The Fischer exact statistical test revealed no significant difference between the two groups ($p>0.05$; data not shown).

## 6.3 DISCUSSION

In the current chapter the impact of dietary manipulation with EPA or DHA on the response to compression SCI has been assessed. The effects of acute i.v. EPA injection, with or without EPA dietary enrichment were explored and also the potential prophylactic effects of dietary DHA. The EPA study highlighted unexpectedly a possible risk associated with high dietary EPA after SCI. The DHA study remained inconclusive and did not reveal a clear beneficial prophylactic effect of dietary DHA in SCI.

### 6.3.1 EPA study

In the EPA diet study, the animals receiving the EPA bolus injection alone had the best locomotor outcome, but this was not statistically significant. We found that animals treated with an EPA-enriched diet following an i.v. saline injection had a significantly worse functional outcome. EPA injection restored this effect to control values. The poor recovery in the EPA diet group was apparently not correlated with increased neuronal, neurofilament, or oligodendrocyte loss, although there was a trend towards fewer oligodendrocytes and neurones in some areas of the cord; nor was it correlated with a change in the amount of microglia/macrophages at the injury epicentre or 5 mm rostral to the injury site. There was no significant difference in weight between animals before SCI or the amount of food consumed. All groups lost body weight progressively following SCI (average weight loss of 6 % over the first week, data not shown) but regained more than their pre-SCI weight by 28 days, except for the IV saline, EPA diet group which continued to weigh significantly less (10 % less than pre-SCI weight, data not shown). Interestingly, in a parallel assessment of the changes in hindlimb muscle tissue after SCI (i.e. muscle samples collected after 28 days), despite the IV saline and EPA diet group faring worse in locomotor outcome, there was significant prevention of the atrophy of the soleus muscle and prevention of the loss of regenerative satellite/stem cells in the hindlimbs of the same animals compared to the control group (Begum and Robson, unpublished).

*6.3.1.1 Locomotor recovery*

Animals receiving an enriched EPA diet following SCI had a worse neurological recovery, and this was apparent in both the BBB score and BBB sub score analysis. Data from the BBB sub score can provide additional information in a less severe SCI, such as rotation of the paws, but this is once plantar stepping is regained, to distinguish between finer aspects of coordination (Lankhorst et al, 1999). It was interesting to note that considering the recovery of stepping, no animal regained stepping in the IV saline, EPA-enriched diet group, compared to 4 out of 6 in the control group (IV saline, control diet), 5 out of 6 in the IV EPA, control diet group and 3 out of 6 in the IV EPA, EPA diet group. However, likely due to the small group size, this was not statistically significant.

*6.3.1.2 Bladder function*

Bladder volume was calculated using ultrasound measurements. There are alternative methods of measuring urinary tract dysfunction. The most common method is cystometric urodynamic analysis, which is essentially similar to clinical cystometry, where a urethral catheter is implanted into the bladder dome and then filled with saline while recording intravesical pressure to examine the relationship between bladder volume and pressure during filling and micturition. Ultrasound offers a quick, minimally invasive and effective alternative and in rats, increases in bladder volume are proportional to the severity of SCI (Pikov et al., 2001; Wrathall et al., 2006).

The pattern of urinary tract dysfunction observed here is in agreement with other laboratories in thoracic contusion models (Pikov, et al., 2001; Mitsui et al, 2005; de Groat and Yoshimura, 2006). Care was taken to assess the bladder at the same time of day, at each time point: 1-2 h following manual expression of the bladder and performance of the BBB tests. A restriction was not made on the amount of water that was consumed before the measurements, but this was minimal since most eating/drinking takes place during the night in the active phase of the day/night cycle of the animals. Following compression SCI, there was an initial period of flaccid paralysis of the urethral sphincter (noticed by wet abdomens) lasting approximately 3 days. This was followed by areflexia - an

increase in urinary retention (bladder volume) at day 3, which is a result of the development of a dyssynergia between the bladder and urethral sphincter (Blaivas et al., 1981; Kaplan et al., 1991). The IV saline, EPA-enriched diet group had the largest significant increase in bladder volume compared to the control group. Urinary retention (or storage) is sympathetically mediated, whereas micturition is elicited by parasympathetic activation. Normal lower urinary tract function requires the coordinated activity of the sympathetic, parasympathetic and somatic nervous systems. SCI induces profound changes in bladder innervation, particularly afferent, circuitry, morphology and structure (Beattie et al., 1993; for review, Inskip et al., 2009). In the first study to document using this technique, bladder volume was 0.089 ± 0.04 ml in uninjured rats, which was similar to our observations (0.1 ± 0.03 ml); 3.51 ± 0.47 ml on day 4 post-injury and decreased to 1.83 ± 0.50 ml by day 8 (Kierstead et al., 2005). The contusion injury resulted in a more severe paralysis (BBB score of approximately 6 at day 28) compared to our model (BBB 10.8 at day 28 in the control group). If injury severity is correlated with bladder function (Pikov et al., 2001; Wrathall et al., 2006), this explains a less severe bladder impairment in our model based on volume size at day 3. Increased bladder size did not persist in our model, but returned to baseline values at day 8. We did find a permanent change in the width of the bladder at 28 days post-mortem. A similar observation, i.e. an increase in bladder weight, has been reported elsewhere (Mitsui et al., 2005). The increase in bladder capacity is caused by bladder overdistension during the areflexic period. The increased pressure, due to bladder outlet resistance, induces the bladder hypertrophy that is reflected in the increased bladder width (Mitsui et al., 2005). Since there was recovery of bladder function in all groups by approximately day 8, these results imply that there was a partial recovery of coordination by parasympathetic pathways innervating bladder smooth muscle and somatic motor neurons that supply the external urethral sphincter.

Histological studies could be extended to study the sprouting or sparing of pathways implicated in the CNS control of micturition that are located in the lumbosacral cord. These include the bladder afferents projecting to the dorsal horn, and the somatic dorsal lateral motor and spinal parasympathetic nuclei that coordinate the activity of the urinary bladder, urethra, and urethral sphincter. These regions of lumbosacral cord are innervated

by descending pathways from the brainstem, among which are axons containing corticotrophin releasing factor (CRF) and serotonergic (using 5-HT) and noradrenergic (using DβH) axons. Studies elsewhere have correlated a reduced loss of these markers after SCI with an improvement in bladder function (Mitsui et al., 2005).

*6.3.1.3 Sensory outcome*

Hypersensitivity is the phenomenon of enhanced sensitivity to pain, whereas allodynia is a perception of pain in response to an innocuous stimulus. The dynamic plantar aesthesiometer, or automated mechanical plantar withdrawal test has been used to measure hypersensitivity or the development of neuropathic pain following SCI (Anderson et al., 2005), spinal nerve ligation (Ozsoylar et al., 2008) and spinal root avulsion (Chew et al., oral communication, ISRT annual meeting, London, 2009). Before this, manual von Frey filaments have been used to demonstrate at-level and below-level mechanical hypersensitivity or allodynia after SCI (Christensen et al., 1996; Hulsebosch et al., 2000). The fundamental difference is that the automated test can set a fixed 'latency' of withdrawal with more quantitative measurements of both weight and time, removing human error due to variable force applied by the investigator using manual von Frey hairs. It also does not rely on experimenter judgement of positive paw withdrawal. In this way it can be used reliably and robustly between experimenters and laboratories. Here, measurements were taken upon establishment of hind paw plantar placement and weight support, which was on average between 7-14 days after injury; therefore, not all rats were measured at each time point.  However, we noted a significantly decreased withdrawal threshold in all treatment groups, which is indicative of the development of below-level hypersensitivity and central sensitization, documented in previous studies after thoracic hemisection or contusion (Christensen et al., 1996; Christensen and Hulsebosch, 1997; Hulsebosch et al., 2000). Hypersensitivity can be the result of neurones which are part of the ascending somatosensory pathways, such as the neurones in the dorsal horn, becoming pathophysiologically hyperexcitable, leading to the phenomenon of central sensitisation (Woolf, 1983; Christensen and Hulsebosch, 1997; Drew et al., 2001; Hains et al., 2003; Lampert et al., 2006). Central sensitization is the state of heightened excitability of central dorsal horn neurones in that they respond

quicker and their responses are greater and longer lasting: they do not equate to the extent of the stimulus. The central sensitization phenomenon is believed to be the cause of why painful symptoms can last far longer than the initial stimulus, and when put into the context of patients, is commonly termed ‘chronic pain’. Chronic neuropathic pain behaviour in SCI models has been directly related to increases in CGRP immunoreactivity (Christensen and Hulsebosch, 1997), metabotropic glutamate receptors (Mills and Hulsebosch, 2002), and NGF (Gwak et al., 2003). Mechanical allodynia or hypersensitivity in the hindlimbs following clip compression SCI has also been shown to coincide with a reduction in serotoninergic immunoreactivity below the level of the lesion (Bruce et al., 2002), suggesting that in this model, below level neuropathic pain behaviour was due to loss of descending antinociceptive supraspinal control from the brain stem onto dorsal horn neurones. Differences were noted in our study between left/right hindpaws. Based on the criteria implemented, in some animals there was development of hypersensitivity in one hind paw but not the other. These were likely due to asymmetric lesions and were also apparent in the locomotor recovery. In some cases before stepping function was regained, one side showed more extensive movement than the other.

The inflammatory response may also have an important role in pro-nociception and central sensitization after SCI, through release of cytokines and chemokines such as IFN-γ, IL-1β, TNF-α from macrophages, neutrophils and T-lymphocytes, which sensitise dorsal horn neurones (Trivedi et al., 2006; Thacker et al., 2007). Microglia have recently been recognized as powerful modulators of pain in SCI, by producing factors, such as ATP and fractalkine (Milligan and Watkins, 2009), that in turn activate and sensitise dorsal horn neurones (Nesic et al., 2005; Hains and Waxman, 2006; Gwok et al., 2008; Clark et al., 2008; Detloff et al., 2008; Hulsebosch et al., 2009; Marchand et al., 2009). Inhibition of the microglial p38 MAP kinase phosphorylation pathway attenuates the hypersensitivity that develops post SCI (Crown et al., 2008).

Various approaches have been used to model neuropathic pain following SCI, including contusion and hemisection injuries at the thoracic level (Christensen and Hulsebosch, 1997a; Bennett et al., 2000); anterolateral lesions of the spinothalamic tract (Ovelmen-

Levitt et al., 1995;Vierck, Jr. and Light, 2000) and ischaemia (Hao et al., 1991). Not all rats developed hypersensitivity in our model, but this is not unusual. Clinically >65 % of people with SCI develop neuropathic pain (de Miguel and Kraychete, 2009; Miladinovic, 2009) and in the majority of cases, chronic central pain develops within several months to years following injury (Christensen et al., 1996; Christensen MD and Hulsebosch, 1997; Richards et al., 1980). Therefore, it appears that compression to the spinal cord could be a useful and representative model for testing the development of neuropathic pain after SCI. In order to validate the apparent development of hypersensitivity, it would be necessary in future studies to take more than one baseline reading. Other researchers in our laboratories have shown that there is a slight decrease in the latency of withdrawal following the first baseline recording (Chew et al., personal communication). Gender differences have also been documented to play a role in the development of at-level allodynia during different stages of their oestrogen cycle (Hubscher et al., 2010). Females were chosen for our study and are preferred for SCI contusion or compression experiments, since it is easier to manage bladder expression and to avoid urinary tract infections. The best time of day to take readings has also been debated, to the extent that some laboratories reverse the light-dark cycle of their animals, in order to take readings during their dark (active) cycle (Hulsebosch et al., 1996).

Management of chronic pain after SCI is poor and remains a clinical challenge. Downstream metabolites of omega-3 PUFA, such as resolvin E1 and resolvin D1 have recently been shown to have more impact than either morphine or COX-2 inhibitors in central inflammatory evoked pain, and can attenuate inflammatory pain by regulating the synaptic plasticity in spinal cord dorsal horn neurones, which have been strongly implicated in the generation of persistent pain (Woolf and Salter, 2000; Xu et al., 2010). Although no effect was seen in this study, it would certainly be worth pursuing research in this area, in order to explore in more depth the effect of omega-3 PUFA on the development of neuropathic pain after SCI.

*6.3.1.4 Cavitation and the presence of macrophages/microglia*

Histological analysis showed extensive damage in the epicentre of all groups, with widespread cavitation but there was no significant difference in cavity size between the two groups analysed, in the case of the EPA study. Macrophages were present inside the cystic cavities of the epicentre and in the surrounding spared tissue. Due to the extensive cavitation and very little spared tissue, further analysis was carried out in rostral segments, 5 mm from the epicentre.

In rostral segments, there was no difference between the amounts of ED1- or Iba1-labelled macrophages/microglia in the two groups. As seen by Popovich at al. (1997), there were macrophages predominantly confined to the dorsal columns but also scattered throughout the tissue, as well as in clusters, the location of which was variable from animal to animal. These walled off clusters have previously been documented to be surrounded by reactive astrocytes, demarcating damaged tissue and preventing the spread of leukocytes through the CNS parenchyma (Sofroniew et al., 2009, Fig. 3.12 in chapter 3). However, this also presents a barrier to regeneration of axons through the injury site (Silver and Miller, 2004). Most research is aimed at disrupting this barrier, or preventing its formation, for example by digesting CSPGs, to enhance axon regeneration or sprouting (Moon et al., 2001; Bradbury et al., 2002). It would be interesting to explore the spread of inflammation above and below the injury site, and the integrity of the astroglial barrier, and generally, the astrocytic response, after PUFA treatment.

The analysis was carried out differently for ED1 and Iba1. In the rostral region, an overall measurement of the entire transverse section appeared to best represent the amount of ED1 macrophage/microglia in the tissue compared to high power images in specific locations, which might have missed some of the accumulations of cells. However, for Iba1, high power images were preferred, since using low power images meant it was not possible to detect the fine processes of the resting microglia phenotype. In future, it would be useful to quantify the ratio of ED1:Iba1 double-labelled macrophages/microglia. An attempt to do this was made using MetaMorph® software. However, there were difficulties in setting the analysis criteria to get an accurate output,

due to the variability in background staining in each animal, combined with the variable morphologies of the microglia.

*6.3.1.5 Neuronal loss*

There was no difference in neuronal number in the dorsal and ventral horns between the EPA-enriched diet and control diet groups. The extent of neuronal loss at this time point correlates with the protracted neuronal loss seen by Huang et al. (2007b) in the establishment of the compression model of SCI in our laboratory (approximately 20% at 28 days in the rostral region), although the analysis carried out here was slightly different. Huang et al. (2007a, b), analysed images taken at 40x magnification and the entire image was counted at 7 days and 6 weeks, rather than images taken at 20x magnification here, with a count made on a box placed over the image. Despite this, consistent with their observations, a greater relative loss was found in the ventral, compared to the dorsal horn areas (40 % vs. 20 %). The reason for this dorsolateral sparing is not known, but may reflect differences in the intrinsic vulnerability of the cells, or mechanical effects such as transmission of compressive stress into the intermediate and ventral grey matter by the overlying dorsal columns (Huang et al., 2007b).

Most neuronal loss is considered to be the result of necrosis caused by the original injury (Faden et al., 1988; Panter et al., 1990; Wrathall et al., 1992) and it has been documented that in contrast to compression SCI, after contusion SCI there is no further neuronal loss after 3 days (Grossman, et al., 2001). However, a number of other studies have shown the occurrence of neuronal apoptosis after contusion SCI (Crowe et al., 1997; Liu et al., 1997; Lou et al., 1998). Secondary pathogenesis such as ischaemia, glutamate-induced toxicity (Liu et al., 1999), inflammation and free radical production could contribute to neuronal loss. Since there is a prolonged inflammatory response and microglia are known to produce toxic substances, such as nitric oxide and TNF-α (Chao et al., 1992; Si et al., 1998; Liu et al., 2002), these glial cells have been implicated in neuronal loss after SCI.

*6.3.1.6 White matter pathology*

Ward et al. (2010) showed a decrease in the non-phosphorylated component of neurofilament (SMI32) following compression SCI. The method of analysis was slightly different, with the use of multiple small sample boxes, rather than using the whole image taken with the 40x objective, and the results expressed counts per area. After converting these values to a percentage of loss compared to the uninjured control tissue, they equated to an approximate 60 % loss in the DC; and 50 % in the VWM and 42 % in the VLWM at 7 days (Ward et al., PhD thesis). In our study, there was a more dramatic loss of non-phosphorylated neurofilament rostral to the injury site at 4 weeks following SCI - 95 % in the CST and 77 % in the DC. This might be due to the later time point of analysis, but also the injury severity was worse in this study (BBB score of 11 vs.12 in the control group at 4 weeks here).There was no significant difference in the number of SMI32-labelled axons between the control group and the group exposed to a higher level of dietary EPA. The loss of axons in the white matter following human SCI is almost always incomplete (Bunge et al., 1993; Norenberg et al., 2004; Guest et al., 2005). Studies of these surviving axons demonstrate that they are abnormally myelinated, with thinner myelin sheaths than uninjured axons (Gledhill et al., 1973; Griffiths, 1983; Totoiu and Keirstead, 2005). Spinal cord axons can remyelinate following injury (Bunge et al., 1961). However, abnormally myelinated axons are still observed at chronic time points following injury. It would be interesting to look in the tissue from this study, at the effects of the dietary treatment on myelin, such as proteolipid protein (PLP) and myelin basic protein (MBP), two major structural proteins that make up CNS myelin (Norton and Cammer, 1984).

Myelin is essential for normal nervous system function and a loss of myelin after SCI has critical effects on nerve physiology. Demyelination results in a larger membrane capacitance and a decrease in conduction velocity which, in turn, contributes to an increased refractory period and a higher frequency of overt conduction failure. These alterations have a critical effect on neurological function following experimental (Bunge et al., 1961; Blight, 1983; Nashmi and Fehlings, 2001b) and human (Norenberg et al., 2004; Guest et al., 2005; Smith and Jeffery, 2006) SCI. Strategies to prevent

demyelination or to promote remyelination are now extensively investigated in the field of SCI (for review see McDonald and Belegu, 2006).

Oligodendrocytes are the cells responsible for the myelination of axons in the CNS, and remyelination of injured axons. There was a loss in the number of oligodendrocytes after compression SCI compared to naïve controls and this was greatest in the CST, consistent with other laboratories (Crowe et al., 1997; Li et al., 1999; McTigue et al., 2001; McEwen and Springer, 2005) and with what was found at 7 days in the epicentre in one of our previous studies (Huang et al., 2007a). There was no significant difference between treatment groups, although there was a trend towards a greater loss in the dorsal columns of the segment rostral to the injury epicentre in the IV saline, EPA diet group, compared to the control group. It is generally recognized that white matter damage is more critical in SCI than grey matter damage (Eidelberg et al., 1981).

In summary, there was no correlation between the marked worsening of the motor outcome after dietary intake of EPA, and tissue markers of injury. It is not clear why there was no correlation of the usual injury markers with behavioural outcome in this study. Studies where there is an improvement in locomotor outcome tend to show, overall, a correlation with neuronal and/or oligodendrocyte loss (Huang et al., 2007a; Lim et al., *in press*). However, there are exceptions – Kostyk et al. (2008) reported worse locomotor recovery in a transgenic mouse compared to wildtype controls after spinal contusion, whilst paradoxically, the tissue exhibited robust axon growth within the lesion.

*6.3.1.7 The EPA diet and fatty acid composition of red blood cells*

Analysis of serum or RBCs is a widely used method of indicating uptake of dietary fatty acids. In the EPA study, animals consumed approximately 150-170 mg/kg, and this was enough to enrich the EPA content in RBCs by eight fold compared to the control group. DHA content also increased by a relatively smaller but significant amount and this is likely to come from the small percentage present in the oil (5-10 %), or conversion of EPA to DHA, which is also possible (Rapopport et al., 2007). In our study, the omega-6 content was significantly lowered in parallel, particularly AA, docosapentaenoic (22:5)

and docosatetraenoic (22:4). Supplementation with omega-3 PUFA can displace AA structurally from phospholipids, causing a subsequent decrease in AA membrane content, and this has been demonstrated in several other dietary studies (Carlson et al., 1992; Watanabe et al., 2003; Valenzuela et al., 2005; Calder 2007; Dyall et al., 2007; this list is not exhaustive). In future studies, baseline levels should be obtained prior to SCI, to confirm the fatty acid level content was similar for all animals preceding the injury.

The control diet used here contained a small percentage of fatty acids (1.38 % linoleic acid, 0.11 % linolenic acid, 0.11 % omega-3 and 0.52% saturated fatty acids, see Appendix 2). A control diet should not lack omega-3 PUFA completely, since omega-3 PUFA confer neuroprotection intrinsically, as demonstrated in an *ex vivo* study after omega-3 PUFA dietary deficiency in a model of ischaemia on hippocampal slices exposed to oxygen and glucose deprivation (Moreira et al., 2009). For the EPA oil, the composition was 70 % EPA and the rest made up of a combination of DHA (5-10 %), AA and other fatty acids (see materials and methods, Chapter 2). The EPA delivered in the diet was in ethyl ester form.

Based on another study carried out in our laboratory (Dyall et al., 2007), we have established that after this type of supplementation, omega-3 PUFAs are taken up into all major classes of nervous tissue phospholipids. The brain is highly enriched in DHA, with phosphatidylethanolamine (PE) and phosphatidylserine (PS) containing the highest levels (Salem Jr et al., 2001). Many previous studies have shown that dietary intake of omega-3 PUFAs affects brain levels of fatty acids in all the major classes of phospholipids (Chernenko et al., 1989; Saito et al., 2003; Dyall et al., 2007). The change in these levels will likely have a significant impact on membrane fluidity, cell signaling and competition of PUFA for enzymatic reactions. Evidence for this could be obtained in a repeat experiment, using freshly harvested tissue samples and Western blot analysis, to measure activation of the proteins involved in downstream signaling pathways of PUFA, such as Akt and ERK (Lu et al., 2010; Moereira et al., 2010) or COX2 expression (Huang et al., 2007a).

*6.3.1.8 The EPA bolus and EPA diet target different SCI processes*

It is not clear whether i.v. EPA alone is significantly neuroprotective, as although our results showed that the BBB outcome was better after the i.v. EPA injection alone in the present study compared to control, this was not statistically significant. In another study in our laboratory, i.v. EPA administered 30 min after compression SCI demonstrated significant neuronal and oligodendrocyte protective effects at 7 days, although the effect for locomotor outcome was not significant at 7 days, but was significantly better in the EPA group when all time points were considered during the first recovery week (Lim et al, *in press*). However, overall it was not as effective as an equivalent dose of DHA administered as a bolus. In our laboratory, we do experience some differences from surgeon to surgeon in the severity of the injury, which makes it difficult to directly compare results between experimenters. It is possible that the severity of SCI could affect the efficacy of a treatment. However, the BBB score of control groups in both this study and that of Lim et al. (*in press*) were approximately 3 at day 7. It is also not clear yet why the acute and chronic effects of EPA intervention are so different, and in particular why EPA in the diet immediately after SCI has such a significant negative effect. The combination of an acute i.v. bolus of EPA with dietary enrichment is likely to affect a multitude of pathogenetic mechanisms which are involved in trauma-induced damage and both are likely to affect different SCI processes. The acute bolus will likely be targeting ischemic, apoptotic and excitotoxic events, whereas the dietary enrichment will only start to increase levels of EPA in the blood and tissue after a delay of several hours. This is at a time when secondary injury events will be occurring, such as interaction with the immune system, damage to the blood-brain-barrier, degeneration of surviving axons and further loss of neuronal and non-neuronal cells. We need to characterize in detail the exact mechanism and targets of the omega-3 PUFAs, in order to confirm whether one or a combination of these processes are being targeted. Further studies could also address the issue of whether the negative effects are dose-related.

The clearance of omega-3 in plasma after intravenous delivery is within seconds-minutes. Large proportions of omega-3 taken up from blood as free fatty acids are bound to bovine serum albumin or acylated into phospholipids and retained in the liver, heart, lung,

kidney, bone marrow, spleen, gastrointestinal tract, brown adipose tissue and skeletal muscle (Nilsson and Becker, 1995), whereas the remainder is either transported by a fatty acid binding protein to the endoplasmic reticulum, or lost by β-oxidation in mitochondria or peroxisomes (Rapopport 2007). Similar to DHA, a fraction of the EPA bolus could be targeting TREK channels, while another portion is likely to be converted into its metabolites by COX and lipoxygenase activities and affect inflammatory cell trafficking. Results from this thesis suggest that the acute EPA injection would have a modest influence on neutrophils after SCI. Retinoid X receptors and the peroxisome proliferator-activated receptors are also targets for omega-3 fatty acids, and they are upregulated after SCI (Genovese et al., 2005; Schrage et al., 2006; Mucida et al., 2007). Our laboratory is currently exploring the importance of TREK channels in the effect of the i.v. omega-3 bolus, using a specific TREK-1 antagonist called spadin, a secreted peptide derived from the neurotensin receptor 3 (Mazella et al., 2010).

*6.3.1.9 Possible explanation of the apparent detrimental effects of the EPA diet*

The results from the study using dietary EPA-enrichment are somewhat unexpected. On the one hand, the EPA diet appeared to have a detrimental effect on locomotor outcome, whilst the acute injection of EPA did not. There was no change in global markers of macrophages/microglia after i.v. (Chapter 4) or dietary EPA treatment, but this does not rule out the possibility that certain subtypes of these cells - such as the less neurotoxic, growth-promoting “M2” macrophage phenotype Kigerl et al. (2009), or the blood-derived population, could have been reduced by the actions of EPA or its metabolites at earlier time points. It was not possible to assess this with the markers used. Resolvin E1 is known to be active at nanomolar concentrations. Therefore, even after the ingestion of moderate amounts of the EPA-enriched diet, active levels of E-series resolvins could have been reached in the first few days after SCI. As discussed in Chapter 3, resolvins have potent effects as immune regulators and they interact with receptors expressed on phagocytic leukocytes (Arita et al., 2007; Spite et al., 2009; Krishnamoorthy et al., 2010; Ohira et al., 2010). The use of intra-vital microscopy has demonstrated that i.v. administration of RvE1 (100 ng) rapidly reduced leukocyte rolling by approximately 40% in mouse cremaster muscle venules (Spite et al., 2009). Therefore, it could be speculated

that the detrimental effects of an enriched EPA diet may be due to the early reduction of the M2 macrophage response, or the blood-derived macrophages, leaving the neurotoxic “M1” phenotype to dominate the lesion and persist there indefinitely.

Schechter et al., (2009) demonstrated with the use of adoptive transfer experiments and bone marrow chimeras, which were able to functionally distinguish between the resident microglia and the infiltrating monocyte-derived macrophages, that selective ablation of infiltrating monocyte-derived macrophages following SCI using either antibody-mediated depletion or by diphtheria toxin, impaired recovery. Most studies have shown that depleting this subset of macrophages is beneficial to recovery from SCI (Blight, 1994; Taoka et al., 1997; Popovich et al., 1999; Eng and Lee, 2003; Gris et al., 2004). Schechter et al. (2009) alluded to a novel unique role associated with this subset of infiltrating blood-derived macrophages in SCI recovery, which cannot be provided by the activated resident microglia. The authors go on to propose that limited recovery following SCI can be attributed in part to the inadequate, untimely, spontaneous recruitment of monocytes (Schechter et al., 2009). They showed that boosting this pool of monocytes by adoptive transfer resulted in an improved outcome.

Since the detrimental effects to locomotor recovery were already apparent in the first few days after starting the diet, it is also possible that EPA could be affecting repair of the blood-brain barrier or prolonging bleeding time (Mortensen et al., 1983; Nelson et al., 1991) by decreasing aggregation of platelets (Goodnight et al., 1981; Nelson et al., 1991; Wander et al., 1991). It would be possible to testblood-brain barrier integrity by using permeability tests, e.g. with HRP injection (Schnell et al., 1999), or Evans blue (Uyama et al., 1988). Both DHA and EPA, but EPA in particular is known to reduce platelet aggregation (Hirai et al., 1982; Park and Harris, 2002). PUFA are known to have reversible anti-coagulant/platelet aggregation and clotting properties, and are given with warfarin or aspirin in the prevention of stroke, and this has been without clinical concerns (Bays et al, 2007). However, the anti-aggregating impact of omega-3 PUFAs can vary between species. In particular, intake of EPA and DHA has been reported to increase PUFA levels in platelets (Zhu et al., 1994), lead to increased mesenteric bleeding time (Mark et al., 1994), and be effective in limiting platelet reactivity in rats and cats

(Wojenski et al., 1991; Bright et al., 1994). It would therefore be worth considering whether the EPA enriched diet prolongs bleeding duration after SCI, by analysing the spread of haemorrhage by staining tissue with the diaminobenzidine (DAB) method, such as shown in the image below (Fig. 6.18), generated in tissue from our SCI animals, testing platelet reactivity as in the above studies; or by measuring clotting time from fresh blood samples.

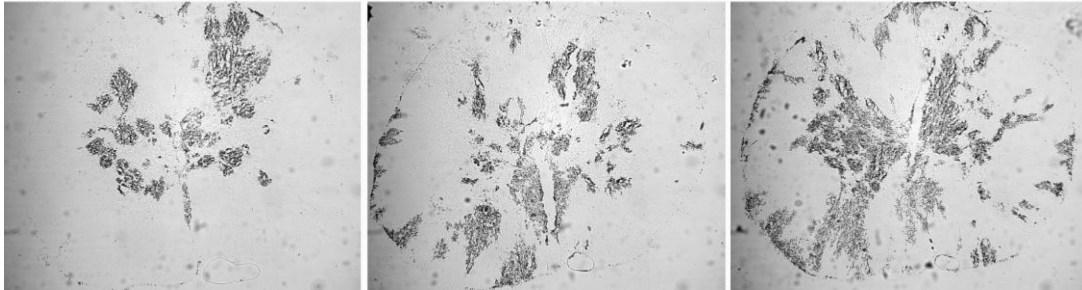


**Figure 6.18 Areas of haemorrhage in transverse sections of the injury epicentre 4 h after SCI.** Transverse sections were labelled with DAB, which revealed the presence of red blood cells. Images are serial sections, approximately 150 μm apart, taken from the same animal.

PUFAs are anti-oxidant and anti-inflammatory compounds (Mori et al, 2003; Sarsilmaz et al., 2003; Richard et al., 2008). The degree of unsaturation makes these compounds more oxidisable and omega-3 fatty acid preparations can undergo extensive oxidation, especially if stored under inadequate temperature and light conditions. This could contribute to potential toxicity. Several studies have suggested that increased PUFA incorporation may exacerbate the susceptibility of tissues to free radical-mediated lipid peroxidation (Lamers et al., 1988, Luostarinen et al., 1997; Véricel et al., 2003). Bays (2007) recently reviewed the safety considerations of omega-3 fatty acid therapy. For example, due to the potential presence of environmental toxins such as mercury, polychlorinated biphenyls, dioxins, and other contaminants, results in swine suggest that EPA may functionally impair the induction/expression of adhesion molecules (Kim et al., 1995). Therefore, PUFA ingestion may reduce cell membrane integrity and tissue viability under circumstances of an increased oxidative stress, such as an ischaemic/reperfusion challenge (McCord et al., 1985), or compression SCI. Results from our laboratory with DHA supplementation so far suggest otherwise, however this cannot be ruled out for EPA.

### 6.3.2 DHA studies

The results from the DHA pre-treatment experiments did not reveal a significant beneficial prophylactic effect of DHA. This was surprising, but may be due to the insufficient power of the studies or masked by the severity of the injury as discussed earlier. Others have shown improved neurological outcome when animals were pre-treated with DHA before in other models involving neuronal loss (Umemura, et al., 1995; Pan et al., 2009), and when i.v. and dietary DHA were given after SCI (Huang, et al., 2007a), as well as pre- and post- injury DHA exposure in *in vitro* models of neuronal injury (Gladman et al., communication FENS meeting Amsterdam July 2010). Moreover, preliminary results obtained in our laboratory using *fat-1* transgenic mice (Kang et al., 2004), which produce a higher level of endogenous omega-3 PUFA from omega-6 PUFAs in the diet, showed better locomotor outcome after SCI than their wild-type littermates (Lim et al., communication FENS meeting Amsterdam July 2010). These results suggest that a potential prophylactic treatment with DHA for SCI cannot be ruled out yet.

Based on the mortality and number of stepping animals in the first study with dietary DHA, there appeared to be a slight indication that there was a beneficial effect from DHA prophylactic treatment. There was no significant difference between the groups in locomotor recovery following pre-treatment with DHA in the second study. There was no significant difference in the intake of the two diets and there was no difference in body weight between the two groups before SCI, or after SCI in the first study. It was estimated that three quarters of the intended dose (400 mg/kg/rat) was ingested during the experiment. This was less than the dose given by Huang et al. (2007a), but the beneficial dietary effect in that study was only seen when intravenous DHA was also administered. It is possible that sufficient levels were not reached in our study. Two brain injury studies showing a beneficial effect of DHA, administered the compounds for 6 weeks intraperitoneally (Pan et al., 2009) and 8 weeks via the diet (300 mg/kg/day Umemura et al., 1995). From data generated in a parallel experiment in our laboratory, where uninjured Sprague-Dawley rats received 400 mg/kg/day for 8 weeks, we know that levels of DHA in liver tissue increase after this exposure up to two-fold and the amount of AA

is reduced (Osman communication, British Pharmacological Society, UK, 2007), therefore there are tissue structural changes. However, the levels reached in the spinal cord by 4 weeks may not have been sufficient to exert effects on recovery after SCI. Or it may be, as found before, that the mechanism of neuroprotection by DHA after SCI requires the fast, intravenous delivery of DHA after SCI (Huang et al., 2007a). It has been shown that DHA may activate positive regulators of cell survival by up-regulating Akt, extracellular-signal-regulated kinase (ERK) and/or the Bcl-2-mediated pro-survival cascade. DHA can also attenuate inflammatory cytokine and free radical production (Akbar et al., 2005; Florent et al., 2006; German et al., 2006). DHA pre-treatment in rats subjected to focal cerebral ischemia/reperfusion injury reduced the size of infarction, decreased blood-brain barrier disruption, brain oedema and inflammatory cell infiltration (Pan et al., 2009; Umemera et al., 1995a).

There has been no evidence in our laboratory so far of a detrimental effect of dietary DHA post-SCI. There has been only one study reported, where post-treatment with DHA and AA by intraperitoneal injection was reported to exacerbate neurological damage following ischemia/reperfusion injury, possibly via increasing damage from reactive oxygen species (ROS) and oxidative stress (Yang., et al, 2007). DHA induced enlargement of areas of cerebral infarction and increased impairment of motor activity, in a dose-dependent manner. The mechanisms explored showed an increase in metalloproteinase (MMP) activity, inflammatory cell infiltration, COX-2 expression and caspase 3 activity. In future, it will be necessary to look at histological markers at the injury site to address the effects of DHA prophylactic treatment for SCI in more detail.

Before SCI there was no difference in weight between the two groups in the second DHA experiment, but 4 weeks after SCI the DHA group weighed significantly less (25 g difference between groups, data not shown) with no significant decrease in food intake. It has been shown that dietary supplementation of n-3 PUFA in fish oil reduces weight gain (Hassanali et al., 2010). Anti-obesity effects of DHA are not well-understood or documented (for review, Li et al., 2008). In one study where weight measurements were recorded, Umemura et al. (1995) reported no difference in weight between the control and DHA groups before the injury (middle cerebral artery thrombosis) but tissue was

harvested at 24 h post-injury. Therefore, it is not possible to ascertain whether weight similarity was maintained between groups after the brain injury, or even if the protective effects of the DHA pre-treatment were sustained.

In conclusion, EPA dietary treatment following SCI revealed a potential detrimental effect in locomotor recovery but this was not correlated with histological markers. DHA prophylaxis appeared to have no significant effect on outcome following SCI. Further work is required in order to explore any detrimental or neuroprotective effect of dietary manipulation with these PUFA in SCI.

# Chapter 7: General Discussion

## 7.1 Summary

The most important findings from the studies carried out in this thesis were as follows: 1) the time course of the neutrophil and macrophage/microglial response in the rat static compression model of SCI is similar to the inflammation time course documented in other models of SCI, although lymphocytes detected in tissue were few in number. A systemic response was also observed in the liver after SCI and laminectomy surgery. 2) The neuroprotective effects of acute DHA or EPA injection following compression SCI are unlikely to be related to a major effect on the acute component of the inflammatory response. An overall modest effect of the two omega-3 PUFA was observed on neutrophil infiltration to the epicentre. Cytokine/chemokines in the epicentre and an acute phase protein (CRP) in the plasma related to leukocyte trafficking and systemic inflammation were all observed to precede the peak of neutrophil infiltration to the injury site. DHA reduced CRP levels, but neither EPA nor DHA reduced levels of the cytokine/chemokines. 3) In an alternative injury model, rat contusion SCI, acute DHA injection was found to improve hind limb function at a higher dose (500 nmol/kg) than in compression SCI (250 nmol/kg), but was not significantly correlated with neuronal and non-neuronal histological markers in the spinal cord. 4) The dietary treatment with EPA and DHA in rat compression SCI revealed a possible detrimental effect of EPA, whereas pre-treatment with DHA had no significant effect on locomotor outcome.

## 7.2 DHA and EPA are pleiotrophic compounds with functional differences

As the field has progressed alongside the course of this doctoral research project, functional differences between DHA and EPA are becoming more apparent and documented in the literature. Parallel *in vitro* studies in our laboratory in neuronal models of mechanical stretch and hypoxia (Gladman et al., 2010), are helping to tease apart the differences between DHA and EPA in their potency, targets and effects in protection against apoptotic cell death. Pre-treatment with DHA and EPA reduced neuronal and non-neuronal cell death in the mechanical stretch and hypoxia models; but EPA did not

have protective effects after hypoxic injury, in clear contrast with DHA (Gladman et al, communication, FENS meeting Amsterdam July 2010).

Historically, both DHA and EPA have been used together in their naturally occurring form, e.g. as components of fish oil, in fish oil supplements or other preparations containing fish oil, and until recently, were thought to share very similar characteristics. In combination, they are well tolerated and have been shown to have significant potential for beneficial effects in various neurological conditions, reinforcing their potential for rapid translation into clinical use. Used separately, after SCI, in acute bolus or in a dietary enrichment, of these two PUFA, DHA appears to be the leading molecule with neuroprotective properties after SCI (King et al., 2006; Huang, et al., 2007a; Lim et al., *in press*). A detailed dose-response study is yet to be performed, with both administration regimes, in order to see if the neuroprotective effects of DHA or EPA can be further enhanced after SCI. Multiple injections might also increase neuroprotection, but only if the processes that the omega-3 PUFAs are targeting are still relevant within the window of treatment considered. This latter aspect is particularly important in the context of neurotrauma, where beneficial mechanisms may only be possible to harness within a very limited and well-defined time period after injury.

Differences between DHA and EPA have been demonstrated in models of inflammation. For example, both DHA and EPA suppress proliferation of T cells, but by different mechanisms: EPA, but not DHA, enhances expression of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), a protein associated with the inhibition of T cell proliferation (Ly et al., 2006). DHA is more effective than EPA in reducing acute inflammatory processes such as infiltration of lymphocytes and expression of cytokines (Tomobe et al., 2000). However, others have found that pure EPA is the more potent inhibitor for upregulation of proinflammatory cytokines from LPS-stimulated human macrophages, compared to EPA-rich media (45% EPA /10% DHA), pure DHA and DHA-rich media (10% EPA/50% DHA) (Mickleborough et al., 2009). DHA demonstrated a much less potent effect on neutrophil chemotaxis after 4 weeks of dietary enrichment with EPA ethyl ester and DHA ethyl ester (Terano et al., 1987). Clearly, there remains a lot to be unravelled regarding the differences between the effects of EPA and DHA and it is very likely that these differences are model/context/species dependent.

One of the biggest recent advances in the field of lipidomics is the characterisation and classification of a large family of bioactive metabolites of DHA and EPA, including the resolvins, maresins and neuroprotectins (Serhan et al., 2000; Hong et al., 2003; Bannenberg et al., 2005; Serhan et al., 2009). These compounds were isolated from inflammatory exudates and appear to be more potent than either of their parent compounds, playing an active role in the switch to resolution of inflammation (For review, see Lawrence, et al., 2002; Serhan et al., 2004). It is very likely therefore, that the different effects of DHA and EPA are related to the action of these metabolites.

## 7.3 The acute PUFA injection targets different SCI processes to those targeted by dietary enrichment

The cascade of events that occur following SCI involve a complex sequence of processes. The acute bolus appears to be targeting pathological events that occur within the first 30 min – 1 h after SCI such as cell death, excitotoxic signalling, and eicosanoid synthesis initiated from the fast release of AA from cell membranes. The effect of the acute bolus is lost if delayed to 3 h post-SCI (Huang et al., 2007a). PUFA delivered in the diet will start to accumulate in tissue hours after SCI, therefore targeting different events to the bolus, such as prolonged secondary damage, causing spread of neuronal and non-neuronal loss from the injury site.

The results presented here on the effect of the omega-3 PUFA dietary treatments in SCI are in contrast to a number of studies in the literature indicating intrinsic beneficial neurological effects of PUFA supplementation, such as in TBI models (Bailes et al., 2010), or stroke (Nguemeni et al., 2010). It will be important to analyse in future, the tissue levels of omega-3 PUFA achieved in the spinal cord in our DHA dietary studies. The results of the dietary DHA post-SCI treatment data here are in agreement with Huang et al. (2007a), where the diet was only effective after combination with the i.v. bolus of DHA. Due to the characteristics of this injury, it cannot be ruled out that neuroprotection in SCI can be achieved exclusively with oral exposure to high levels of DHA.

## 7.4 The inflammatory response after SCI: a double-edged sword?

The modest effect of omega-3 PUFAs on inflammation after SCI is in contrast with several studies demonstrating their anti-inflammatory properties (see Table 6.1, chapter 6). It is likely that the dynamics of the inflammatory response are different after SCI in comparison to those in non-CNS inflammatory models, or the timing of intervention of the omega-3 PUFA bolus or dietary treatments after SCI are not in synchrony with the timing of the same critical targets of the fatty acids that influence leukocyte trafficking or cytokine regulation in non-CNS models. The effects of omega-3 PUFAs on chronic inflammatory markers after SCI are yet to be studied in full detail histologically (see Future Work).

It is an exciting time to be researching the inflammatory response after SCI. A lot has been learned recently about the dual nature of the response, which is a complex conflict between both neurotoxic and neuroprotective processes. We still have a long way to go before we will understand all the factors that could contribute towards a ‘successful’ outcome when manipulating the inflammatory response after SCI and why residual inflammation persists in chronic SCI, despite improvements in neurological outcome after several anti-inflammatory treatment strategies (Bao et al., 2004, 2005; Fleming et al., 2008, 2009). It is considered by some researchers that there is a significant paucity of blood-derived immune infiltration after injuries to the CNS in vertebrates, particularly in the brain compared to the spinal cord (Schnell et al., 1999; Schecter et al., 2009). These discoveries encourage the exploration of the potential for the immune response to play an active beneficial role in recovery of the CNS to injury. It is tempting to consider that perhaps we can manipulate the response after SCI and drive it to a more beneficial resolution of inflammation. There have already been several successful attempts preclinically (Rapalino et al., 1998; Popovich et al., 1999; Hauben et al., 2000a, b; Bao et al., 2004, 2005; Gris et al., 2004; Schechter et al., 2009) but there remains disagreement on whether the enhancement or the prevention of particular processes of inflammation is the way forward. Animal strain differences highlight the fact that within a genetically heterogeneous human population, attempts to manipulate inflammation to promote repair or minimize secondary injury will yield variable results (Kigerl et al., 2006). Since diverse strains and species will continue to be used to model the human condition, future

studies need to continue to define how leukocyte populations vary between strains/species in the context of outcomes that are relevant to CNS repair (Donnelly et al., 2007).

### 7.5 Consideration of the histological evaluation following SCI

The histological markers assessed in our studies showed robust changes related to SCI, but they did not correlate with the behavioural outcome, for example in the study on dietary EPA. The markers used here have been effective for histological assessment of omega-3 PUFA treatment in previous SCI studies in our laboratory (King et al., 2006; Huang et al., 2007a; Lim et al., *in press*; Ward et al., 2010). Neuronal or oligodendrocyte loss at the thoracic level in the injury epicentre is not directly related to locomotor function *per se*, but is an indicator of neuroprotection and the amount of spread of the initial injury. Spared tissue, particularly the white matter, is a more direct correlate of locomotor function measured using the BBB score (Bresnahan, et al., 1987; Stokes et al., 1992; Basso et al., 1995, 1996, 2002; Popovich et al., 1999). The SMI32 labelling after SCI revealed different sizes of axons, which may invalidate the method of making a total count to reflect axonal pathology and protection. A more extensive and detailed analysis of the white matter, for example, with the axonal (SMI32 immunoreactive) counts, would be to separate the large swollen axons from the smaller labelled axons and might better reflect the behavioural outcome. It will also be worth in future to look at preservation of specific tracts related to locomotor function, such as the corticospinal or rubrospinal tracts for example, by labelling spinal cord sections with PKCγ (Mori et al, 1990), or by injecting a tracer such as BDA (biotinylated dextran amine), before harvesting the tissue (Angelucci et al., 1996). This would give an idea of the amount of regeneration or sprouting of axons, which may account more directly for locomotor outcome. The use of MR *in vivo* imaging of the cord to assess lesion size will reduce the need to collect separate tissue for multiple time points. Further development of this technique is needed, regarding the quality of imaging, but some studies have begun to use this technique (Fleming et al., 2009).

Immunohistological analysis is limited methodologically when there are problems with a high noise: signal ratio in damaged tissue, this was particularly a problem when using the

diamonobenzidine (DAB) method at earlier time points after injury (Chapter 3). This makes cell counting tricky, especially of faintly labelled cells. The markers used to assess macrophages in this thesis allowed some insight into the spread of distribution of macrophages. However, ED1 or Iba1 labelling, expressed as a percentage of the area of tissue is only a blunt tool and does not reflect the functional properties of macrophages. Instead, function will be better predicted by quantifying how much a selection of surface or cytoplasmic antigens are up- or down-regulated. These changes may occur without changes in absolute cell numbers. Another quantitative assessment might be made based on real-time PCR (RT-PCR) of the injury site, but this cannot distinguish between the cell types contributing to the release of various factors. If a proinflammatory phenotype of macrophages is defined by increased release of TNF-α or IL-1β for example, RT-PCR can measure this increase but neurones and astrocytes are also known to release these products. FACS analysis offers an alternative method, by giving the opportunity to isolate and label the different cell types in the injury site. However, it is not easy to remove the leukocytes present in blood vessels in this location. A vascular rinse may not remove all blood cells from the blood vessels in the injury epicentre.

**7.6 Future work**

The findings from the studies documented in this thesis have resulted in a series of questions that remain to be answered and some of these are summarised below, with a set of proposed experiments to address them. Some preliminary experimental steps have already been taken to explore these questions.

*Is there a linear dose-response to omega-3 PUFA treatment after SCI?*

In chapter 5, after contusion SCI, i.v. DHA was found to be effective on a specific motor aspect of functional recovery at a higher dose (500 nmol/kg) than used after compression and hemisection SCI (250nmol/kg) (King et al., 2006; Huang et al., 2007a) but the effect was not significant overall in the global BBB score. It could be that a different, higher dose would be more effective in this model. Heurteaux et al. (2006) found a U-shaped response for acute i.v. ALA in a model of cerebral ischemia. The processes of ischaemia and the targets for DHA and EPA could be similar to the targets for ALA, and therefore,

multiple doses and/or multiple injection studies should be considered. Specific antagonists for postulated targets, such as for TREK-1, could be used to confirm the mechanisms underlying the effect of PUFA. Such experiments are in progress in our laboratory.

In addition, it would be useful to directly compare the efficacy of omega-3 PUFA with another potentially translatable treatment, such as minocycline or riluzole. This would enable a direct comparison of our treatment, in our experimental conditions, to other neuroprotection strategies which are considered for clinical translation.

*Could the study of PUFA and the inflammatory response after SCI be expanded?*

Since there is a lot of research demonstrating the anti-inflammatory potential of omega-3 PUFA and their metabolites, and the inflammatory reaction after SCI remains to be understood fully, it would be worth expanding the exploration of the inflammatory response after PUFA in SCI:

- What happens to neutrophil numbers between 1-7 days after SCI? The immunohistochemical analysis could be expanded to include further time points to more accurately measure indices of inflammation such as the precise time point of resolution of the neutrophil response at the injury epicentre. This time point may be altered by treatment with omega-3 PUFAs in a similar way to that demonstrated with resolvin E1 (Serhan et al., 2008; Fig. 7.1).
- Since COX and LOX metabolise EPA and DHA, do levels of COX change in the epicentre after SCI after PUFA treatment? This could be addressed by Western blot analysis as shown by Huang et al. (2007a). An increase in COX-2 expression was reported 3 and 24 h after compression SCI.
- Are resolvins present in the SCI injury site? These can be measured using lipid analysis by mass spectrometry, and will be possible when we obtain the resolvin standards, which are not yet widely available.

- Is there a prolonged systemic inflammation in other vital organs after SCI, and how does it respond to PUFA? Gris et al. (2008) have reported the presence of neutrophils in other tissues after SCI. Alongside the tissue harvested in our studies, lung and kidney tissue has been collected in order to answer this question in future by immunohistochemical analysis of the tissue.

- Does the i.v. injection of DHA or EPA affect the dynamics of cell adhesion or transmigration through the endothelium/blood-brain barrier? Intra-vital microscopy is a tool that has been used to demonstrate the effects of RvE1 on leukocytes (Spite et al., 2009). Even more powerful imaging techniques are available *in vivo* to allow the visualization and quantification of a robust recruitment of neutrophils to spinal cord microvasculature within the lesion site, where they begin to tether and roll onto endothelial cells as early as 15 min after SCI, before their entry into the injured spinal cord (Stirling et al., 2009). Visualization in real-time of leukocyte dynamics in blood vessels around SCI lesion sites should be helpful in future studies that target particular immune subsets in the circulation to modulate the extent of secondary injury after SCI.

- Are there macrophages of the M1 or M2 phenotype in the injury site after rat or mouse compression SCI, and what is the time course of their response? The immunohistochemical markers documented by Kigerl et al. (2009) have so far been characterised in mouse tissue but not yet in the rat, or in our laboratory.

- Does DHA or EPA applied directly to isolated macrophages, affect the activation of the M1/M2 phenotypes? Bone-marrow derived macrophage cultures were established while on a short visit to the laboratory of Dr. Philip Popovich, during this doctoral work (Fig. 7.2). The nitrite assay and real-time PCR analysis of secreted cytokines from the supernatant of unstimulated and stimulated macrophages in culture were used to define the M1 and M2 phenotypes. It would be interesting to see if omega-3 PUFA applied to these macrophage cultures affect the phenotype, based on the release of the same products.

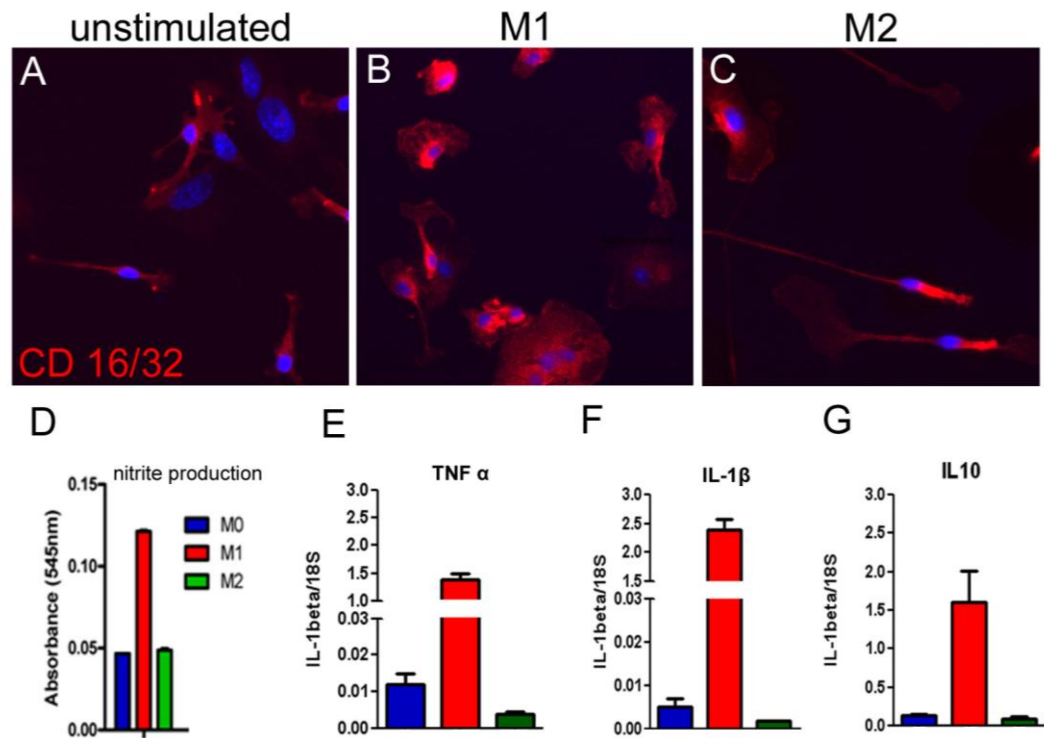



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**Figure 7.1 Creating M1 or M2 macrophages *in vitro*.** **(A-C)** Bone marrow-derived macrophages **(A)** stimulated with LPS + interferon-γ or IL-4 differentiate into M1 **(B)** or M2 **(C)** phenotypes, respectively. CD 16/32 labels both polarized phenotype of M1 (red) or M2 macrophages *in vitro*. Nitrite assay (D) and Q-RT-PCR confirmation of gene expression profiles **(E-G)** specific for M1 or M2 macrophages. MO represents unstimulated macrophages.

*Can the detrimental effects of the EPA diet after SCI be confirmed and what are the mechanisms underlying it?*

- What is the cause of the apparent detrimental effect of dietary EPA after SCI? Tissue could be taken at early time points (1-3 days) to assess coagulation, haemorrhage and blood-brain barrier damage. There is a simple test to measure clotting time in fresh blood samples that has been developed in the trauma research group at Barts and The London (Brohi et al., 2003). DAB labelling on perfused spinal cord could be assessed for spread of haemorrhage from the same injured animals (Chapter 6). Injection of HRP or Evans Blue before perfusion could be used to assess permeability of the blood-brain barrier (Chapter 6).

- The histological assessment of macrophages/microglia could be improved. In SCI tissue labelled with both Iba1 and ED1 (Fig. 7.3), there was colocalisation of most, but not all cells. It would be interesting to measure the degree of colocalisation and whether treatment affects it. This is difficult to assess at present due to the different morphologies of resting and activated/phagocytic microglia.

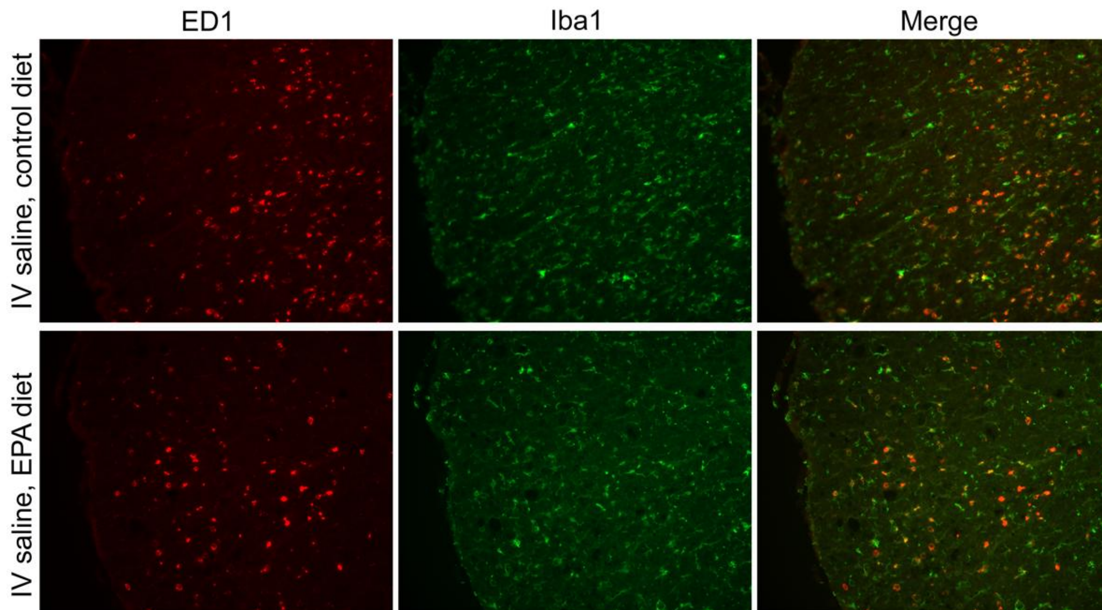


**Figure 7.2 Macrophages/microglia in the ventral white matter 5 mm rostral to the injury site**. Each row of panels represents one section from each group labelled with ED1 (red) or Iba1 (green). Yellow represents co localisation of the two markers.

### 7.7 The challenges in SCI preclinical research/methodological design and translation

Several lessons have been learned from managing the rat SCI studies documented in this thesis. For functional neurological recovery, the BBB test is a good starting point and the best for the early stages of locomotor recovery, before stepping is recovered. When recovery of finer movements and coordination begins, other tests such as the grid, ladder, or beam walk and sensory tests such as tape removal, mechanical or thermal sensitivity will reflect a wider set of functions and continuing recovery that might not be apparent in the BBB scale. However, SCI models are demanding, and multiplying tests in the same animals can prove very challenging.

When working alone, the significant amount of care required in the recovery period post-SCI, besides the behavioural testing, limits the addition of extra tests and/or experimental groups. It takes a considerable amount of time to master the SCI surgery and create reproducible injuries. The severity of the SCI could influence the efficacy of compounds so it is imperative that the severity of the injury or tissue sparing is set to one that offers a wide window of opportunity to assess the efficacy of treatments.

In future studies, an alternative delivery method of the diet could be considered, e.g. by gavage (although more stressful for the animal), since the amount of the powder diet consumed per rat was difficult to monitor accurately. We preferred not to use the gavage method in this study to avoid possible damage to the SCI site from restraining the animal. Rats were housed in groups and there was a different number of animals per cage (n=2-4/cage). The most accurate method would be to house animals individually to monitor the exact dose ingested, so that this data could be correlated with individual outcome measurements. However, this is not ideal and can cause distress. A more concentrated dose could be delivered in a smaller volume or given in a concentrated pellet in addition to normal diet to ensure that the full dose is given and the same per rat. In addition it would be important to establish the fatty acid tissue levels before and after dietary intervention and SCI.

The choice of female rats was preferred in our experiments, since it was less complicated to manually express their bladders after SCI than males and they tend to have a lower rate of bladder infections. It is important to note that some variability in outcome after SCI may be influenced by the timing of the oestrus cycle of female rats, which is every 3 days. Indeed, there are studies that have shown the protective effect of oestrogen (Samantaray et al., 2010; Chaovipoch et al., 2006), so the influence of oestrogen should be considered in the analysis of results of SCI experiments. It would be worth considering the use of male rats in future experiments, or to use females at synchronised oestrus cycles in order to reduce the amount of variability between groups.

Different injury levels were used for the contusion (T8) and compression (T12) SCI experiments in this thesis and similar comparisons between these models and levels have been made in the literature. Although the purpose here was to repeat the effect of DHA

(Huang et al., 2007a) in another SCI model, it was not strictly a direct replication. In addition, although it was attempted to replicate the severity in both models, the severity was higher in the contusion model (BBB=10 at 28 days) compared to the compression model (BBB=12 at 28 days), which may have contributed to the need for a higher dose in the contusion experiment.

The clinical relevance of making an intervention within 30 minutes of SCI is debateable. This timeframe is possible to achieve by some emergency services in largely populated areas with helicopter assistance. However, in more remote places in the world, an SCI patient may only receive medical attention hours after their injury. This means a treatment with a larger window of efficacy would be more likely to reach translation. Pre-treatment studies are useful to gain mechanistic information and indeed, prophylactic treatment by ingesting high levels of omega-3 PUFAs would be possible for the military, before going into areas of conflict. However, it might not be realistic for ordinary civilians to increase their consumption of omega-3 PUFAs in order to prevent the rare chance of a SCI.

When considering the transfer of a regime of treatment with DHA or EPA to humans, the interpretation of experimental data with regard to physiological relevance is complicated by the fact that different forms of omega-3 PUFAs have been used by researchers, and their bioavailability/ bioactivity may differ (Kim et al., 2010). Studies have used processed oil supplements, where DHA and EPA are present as glyceride esters (Christensen, et al., 1994; Sadou, et al., 1995). Ethyl esters of PUFA are another form used, such as in the dietary experiments performed in this research project, as well as the free fatty acid (FFA) form, used here for the i.v. bolus studies. Fatty acid absorption and supply to tissues can vary considerably with the nutritional state and the herbivore–carnivore status of the species examined (Zhou et al., 2001). It is worth taking these factors into account when designing a protocol for clinical trials and deciding on the optimum dietary or parenteral delivery method of fatty acids, when a certain level/tissue and plasma exposure or composition is required.

A grading system for translation of systemically administered preclinical treatments has recently been proposed, in order to identify the strongest candidates for translation to the clinic (Kwon et al., 2010; Appendix 5). It represents a systematic approach to developing an objective method of evaluating the extent to which the preclinical literature supports the translation of a particular experimental treatment into human trials. The approach used included an evaluation of a number of factors that are thought to be important in considering the "robustness" of a therapy's efficacy, including the animal species and injury models that have been used to test it, the time window of efficacy, the types of functional improvements affected by it, and whether efficacy has been independently replicated. These factors were then scored and the grading system applied to a series of potential neuroprotective treatments for acute SCI. For example, erythropoeietin would score 34 points out of a potential 100, in part due to the large number of studies. Using the same criteria, based on published studies (including poster presentations), DHA would score 16. EPA would score 13. To put these scores into perspective, the highest score using this model was 48, by minocycline. The authors suggested that one could consider translating a treatment that was found in a single laboratory to be effective in a rodent model of SCI with a score of 13 (Kwon et al., 2010). Assessments such as these will likely play a great role in future decisions for funding SCI research. Meetings such as the most recent Spinal Research Trust meeting (September 2010, Zurich) themed "On the way to translation" will likely recommend the use of objective modelling and scoring of this type to reduce the risk of failure in translation, as the common goal remains: to find realistic therapies for the future of SCI management.

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# Appendices

## Appendix 1. Care sheets for SCI studies

### Compression Daily Checks

PREOP WEIGHT:

**Date:** **Day 1 after SCI** **Animal no:**

Weight: Dehydrated: Yes No (saline given?)

**AM** Bladder: tight average flaccid Urine: cloudy clear blood

large medium small

**PM** Bladder: tight average flaccid Urine: cloudy clear blood

large medium small

| Notes: animal wet, dry, infection? If dry for 3 consecutive days, bladder checks can stop |
|---|

**Date:** **Day 2**

Weight: Dehydrated: Yes No WET: Yes No

**AM** Bladder: tight average flaccid Urine: cloudy clear blood

large medium small

**PM** Bladder: tight average flaccid Urine: cloudy clear blood

large medium small

| Notes: |
|---|

**Date:** **Day 3**

Weight: Dehydrated: Yes No WET: Yes No

**AM** Bladder: tight average flaccid Urine: cloudy clear blood

large medium small

**PM** Bladder: tight average flaccid Urine: cloudy clear blood

large medium small

Continue to check until day 7 and then vdaily until bladder function resumes

## Appendix 2. Composition of the control diet used in chapter 6

# Certified EURodent Maintenance Diet 14%

# 5KB3*

### DESCRIPTION

Certified EURodent Diet is a constant-nutrient formulation recommended for rats, mice and hamsters. The constant formula feature is designed to minimize nutritional variables in long-term studies.

#### *Features and Benefits*

- Formulated to be free from animal by-products´
- Constant Nutrition™ formula helps minimize nutritional variables
- Designed for longterm low protein maintenance of rodents
- Each package is assayed for environmental contaminants prior to shipment
- Preanalysis monitoring assures maximum diet control
- Fulfills GLP requirements

#### *Product Forms Available*

- Meal

### GUARANTEED ANALYSIS

Crude protein not less than ...................14.0%
Crude fat not less than .......................2.5%
Crude fibre not more than ....................6.0%

### INGREDIENTS

Ground corn, ground wheat, dehulled soybean meal, wheat middlings, dried beet pulp, brewers dried yeast, dehydrated alfalfa meal, calcium carbonate, dicalcium phosphate, monocalcium phosphate, salt, soybean oil, L-lysine, DL-methionine, menadione dimethylpyrimidinol bisulfite (source of vitamin K), choline chloride, potassium chloride, pyridoxine hydrochloride, dl-alpha tocopheryl acetate, vitamin A acetate, cholecalciferol, folic acid, biotin, calcium pantothenate, cyanocobalamin, thiamin mononitrate, nicotinic acid, riboflavin, manganous oxide, zinc oxide, ferrous carbonate, copper sulfate, zinc sulfate, calcium iodate, cobalt carbonate, sodium selenite.

### FEEDING DIRECTIONS

Feed ad libitum to rodents. Plenty of fresh, clean water should be available to the animals at all times.

***Rats***- All rats will eat varying amounts of feed depending on their genetic origin. Larger strains will eat between 15-30 grams per day. Smaller strains will eat between 12-15 grams per day.

***Mice***- Adult mice will eat 4 to 5 grams of diet per day. Some of the larger strains may eat as much as 8 grams per day per animal.

***Hamsters***- Adults will eat 10 to 14 grams per day.

### INGREDIENT COMPOSITION

**Cereal Products** (Corn, Wheat, Wheat Middlings) .77.5%
**Vegetable Proteins** (Dehulled Soybean Meal, Dehydrated Alfalfa, Dried Beet Pulp, Dried Brewers Yeast) .....19.0%
**Energy Sources** (Soybean Oil) ...............0.50%
**Supplementation** (Vitamins, Major Minerals, Trace Minerals, Amino Acids) .....................3.00%

11/13/07

### CHEMICAL COMPOSITION[1]

#### *Nutrients*[2]

**Protein, % ..............14.3**
Arginine, % ...............0.70
Cystine, % ...............0.21
Glycine, % ...............0.61
Histidine, % ..............0.34
Isoleucine, % ..............0.63
Leucine, % ...............1.16
Lysine, % ................0.75
Methionine, % ............0.39
Phenylalanine, % ...........0.61
Tyrosine, % ...............0.33
Threonine, % ............0.50
Tryptophan, % ............0.17
Valine, % .................0.66
Serine, % ................0.74
Aspartic Acid, % ...........1.43
Glutamic Acid, % ..........3.65
Alanine, % ...............0.87
Proline, % ................1.27
**Fat (ether extract), % ......2.5**
**Fat (acid hydrolysis), % ....3.3**
Linoleic Acid, % ...........1.38
Linolenic Acid, % ..........0.11
Omega-3 Fatty Acids, % .....0.11
Total Saturated Fatty Acids, % .0.52
Total Monounsaturated Fatty Acids, % .............0.53
**Fibre (Crude), % ..........3.7**
Neutral Detergent Fibre[3], % ...15.4
Acid Detergent Fibre[4], % ......5.5
**Nitrogen-Free Extract (by difference), % ..........65.2**
Starch, % ................50.6
Glucose, % ...............0.26
Fructose, % ...............0.29
Sucrose, % ...............0.94
**Total Digestible Nutrients,% ..76.4**
**Gross Energy, kcal/gm .....3.90**
**Physiological Fuel Value[5], kcal/gm ..................3.41**
**Metabolizable Energy, kcal/gm ..................3.18**

#### *Minerals*

**Ash, % ..................4.1**
Calcium, % ...............0.65
Phosphorus, % ............0.46
Phosphorus (non-phytate), % ..0.24
Potassium, % ..............0.61
Magnesium, % ............0.17
Sulphur, % ...............0.20
Sodium, % ...............0.27
Chlorine, % ..............0.49
Fluorine, ppm ..............5.1
Iron, ppm ................140
Zinc, ppm .................72
Manganese, ppm ............65
Copper, ppm ..............9.9
Cobalt, ppm ..............0.52
Iodine, ppm ..............0.84
Chromium, ppm ...........0.58
Selenium, ppm ............0.28

#### *Vitamins*

Carotene, ppm .............1.4
Vitamin K (as menadione),ppm .3.4
Thiamin Hydrochloride, ppm ..9.4
Riboflavin, ppm ............5.0
Niacin, ppm ...............74
Pantothenic Acid, ppm ........15
Choline Chloride, ppm .....1500
Folic Acid, ppm ............2.9
Pyridoxine, ppm ............8.0
Biotin, ppm ..............0.20
$B_{12}$, mcg/kg ................25
Vitamin A, IU/gm ...........10
Vitamin $D_3$ (added), IU/gm ....1.0
Vitamin E, IU/kg ..........110
Ascorbic Acid, mg/gm ........—

#### *Calories provided by:*

Protein, % ..............16.799
Fat (ether extract), % .......6.608
Carbohydrates, % .........76.593

*Product Code

1. Based on the latest ingredient analysis information. Since nutrient composition of natural ingredients varies, analysis will differ accordingly.
2. Nutrients expressed as percent of ration except where otherwise indicated. Moisture content is assumed to be 10.0% for the purpose of calculations.
3. NDF = approximately cellulose, hemi-cellulose and lignin.
4. ADF = approximately cellulose and lignin.
5. Physiological Fuel Value (kcal/gm) = Sum of decimal fractions of protein, fat and carbo-hydrate (use Nitrogen Free Extract) x 4,9,4 kcal/gm respectively.

## Appendix 3. BBB score sheet

Rat #: ______ Date: ______ DPO: ______ Rater: ____________ Score: L ____ R ____

| Limb Movement | | | | | | Trunk Position | | Abdomen | Paw Placement | | | Stepping | | | | Coordination | Toe Clear | | Predominant Paw Position | | | | Trunk Instability | Tail |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Hip | | Knee | | Ankle | | | | | | Plantar Pl. | | Dorsal | | Plantar | | | | | Initial Contact | | Lift Off | | | |
| L | R | L | R | L | R | Side | Prop | | Sweep | W/O Supp. | W Supp. | L | R | L | R | | L | R | L | R | L | R | | |
| Ø | Ø | Ø | Ø | Ø | Ø | L R | L R | Drag | L R | L R | L R | Ø | Ø | Ø | Ø | Ø | Ø | Ø* | I | I | I | I | | |
| S | S | S | S | S | S | Mid | | Parallel | | | | O | O | O | O | O | O | O | E | E | E | E | | Up |
| E | E | E | E | E | E | | | High | | | | F | F | F | F† | F | F** | F** | P | P | P | P | | Down |
| | | | | | | | | | | | | C | C | C | C | C | C | C | | | | | | |

Comments: ____________________________________________

(Reproduced from material from the Reeve-Irvine Research Techniques course, 2008)

## Appendix 4. Basso, Beattie, and Bresnahan Locomotor Rating Scale

**0** No observable hindlimb (HL) movement
**1** Slight movement of one or two joints, usually the hip and/or knee
**2** Extensive movement of one joint or extensive movement of one joint *and* slight movement of one other joint
**3** Extensive movement of two joints
**4** Slight movement of all three joints of the HL
**5** Slight movement of two joints *and* extensive movement of the third
**6** Extensive movement of two joints *and* slight movement of the third
**7** Extensive movement of all three joints of the HL
**8** Sweeping with no weight support or plantar placement of the paw with no weight support
**9** Plantar placement of the paw with weight support in stance only (i.e., when stationary) or occasional, frequent, or consistent weight-supported dorsal stepping and no plantar stepping
**10** Occasional weight-supported plantar steps; no FL–HL coordination
**11** Frequent to consistent weight-supported plantar steps *and* no FL–HL coordination
**12** Frequent to consistent weight-supported plantar steps *and* occasional FL–HL coordination
**13** Frequent to consistent weight-supported plantar steps *and* frequent FL–HL coordination
**14** Consistent weight-supported plantar steps, consistent FL–HL coordination, *and* predominant paw position during locomotion is rotated (internally or externally) when it makes *initial contact* with the surface as well as just before it is *lifted off* at the end of stance; or frequent plantar stepping, consistent FL–HL coordination, and occasional dorsal stepping
**15** Consistent plantar stepping and consistent FL–HL coordination *and* no toe clearance or occasional toe clearance during forward limb advancement; predominant paw position is parallel to the body at initial contact
**16** Consistent plantar stepping and consistent FL–HL coordination during gait *and* toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off
**17** Consistent plantar stepping and consistent FL–HL coordination during gait *and* toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact *and* lift off
**18** Consistent plantar stepping and consistent FL–HL coordination during gait *and* toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off
**19** Consistent plantar stepping and consistent FL–HL coordination during gait, toe clearance occurs consistently during forward limb advancement, predominant paw position is parallel at initial contact *and* lift off, and tail is down part or all of the time
**20** Consistent plantar stepping and consistent coordinated gait, consistent toe clearance, predominant paw position is parallel at initial contact and lift off, *and* trunk instability; tail consistently up
**21** Consistent plantar stepping and coordinated gait, consistent toe clearance, predominant paw position is parallel throughout stance, and consistent trunk stability; tail consistently up

(Reproduced from Basso et al., 2006)

## **Appendix 5. Preclinical Grading Scale** (Reproduced from Kwon et al., 2010)

| **Animal Species In Which Efficacy* Has Been Demonstrated** | **Points** |
|---|---|
| Primate model of traumatic SCI | 8 |
| Large animal model of traumatic SCI (dog, cat, rabbit, pig, sheep) | 6 |
| Rat model of traumatic SCI | 4 |
| Mouse model of traumatic SCI | 2 |
| **Maximum Score:** | **20** |
| **Injury Paradigms In Which Efficacy* Has Been Demonstrated In** | **Points** |
| Cervical contusion SCI models | 6 |
| Thoracic contusion SCI model | 3 |
| Cervical clip compression SCI model | 6 |
| Thoracic clip compression SCI models | 3 |
| Cervical partial transection sharp SCI model | 1 |
| Thoracic partial transection sharp SCI model | 1 |
| **Maximum Score:** | **20** |
| **Time Window of Efficacy*** | **Points** |
| Efficacy demonstrated with treatment delay of 12 or more hours | 8 |
| Efficacy demonstrated with treatment delay of 4 or more hours, but less than 12 hours | 6 |
| Efficacy demonstrated with treatment delay of 1 hour or more, but less than 4 hours | 3 |
| Efficacy demonstrated when treatment given immediately at time of injury /within/less than 1 h | 2 |
| Efficacy demonstrated when treatment given prior to injury | 1 |
| **Maximum Score:** | **20** |
| **Demonstration of "Clinically Meaningful" Efficacy** | **Points** |
| THORACIC SCI MODEL: Achievement of plantar weight-support (i.e. BBB of 9) versus controls that do not, or the achievement of consistent forelimb-hindlimb coordination (i.e. BBB of 14) versus. controls that do not, in a study with associated improvements in non-behavioral outcomes (eg. Tissue sparing). | 4 |
| THORACIC SCI MODEL: Significant improvement in other locomotor or motor behavioral tests (e.g. quantitative gait analysis, inclined plane, swimming) or other non-motor behavioral tests (eg. Pain, autonomic dysreflexia) in a study that also demonstrates associated improvements in non-behavioral outcomes (e.g. Tissue sparing). | 4 |
| CERVICAL SCI: Significant improvement in some motor function test (e.g. Food pellet reaching, grasping, quantitative "gait" assessment) in a study that also demonstrates associated improvements in non-behavioral outcomes. | 4 |
| CERVICAL SCI: significant improvements in other non-motor behavioral tests (e.g. Pain, autonomic dysreflexia), in a study that also demonstrates associated improvements in non-behavioral outcomes | 4 |
| DOSE RESPONSE demonstrated in a single study using either the thoracic or cervical SCI model. The dose "response" is defined by improvements in either behavioral or non-behavioral outcomes with changing doses of the therapy. | 4 |
| **Maximum Score:** | **20** |
| **Independent Reproducibility / Replication** | **Points** |
| More than 10 independent laboratories report on the beneficial effects of the therapy | 20 |
| 5-10 Independent laboratories report on the beneficial effects of the therapy. | 12 |
| 3-4 Independent laboratories report on the beneficial effects of the therapy. | 7 |
| 2 Independent laboratories report on the beneficial effects of the therapy. | 3 |
| 1 independent laboratory reporting on the beneficial effects of the therapy | 0 |
| 1 independent laboratory reports on the negative results on the therapy | -3 |
| 2-3 independent laboratories report on the negative results of the therapy | -7 |
| 4-9 independent laboratories report on the negative results of the therapy | -12 |
| More than 9 independent laboratories report on the negative results of the therapy | -20 |
| **Maximum Score:** | **20** |

**Appendix 6. Calculation for preperation of PUFA supplemented food**

e.g. DHA 400 mg/kg/day

For rat weight 200 g: 80 mg DHA/rat/day
CRODA oil approx 75 % DHA, therefore: 107 mg/oil/rat/day

s.g. oil = 1.0, therefore 107 mg oil = 107 µl oil
if rat eats 30 g/day, then 107 µl oil in 30 g food, or 3.6 ml oil/kg diet

EPA oil purity: 70 %

Therefore, 114 mg oil (114 µl) in 30 g powder or 3.8 ml oil/kg diet