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Investigation of the central molecular events that maintain persistent inflammatory pain states

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DECLARATION

I, Yuk Ming Wong, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis

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ABSTRACT

Noxious stimulation of the periphery leads to long-term changes in the excitability of dorsal horn neurons of the spinal cord. In the spinal cord the increased excitability of dorsal horn neurons requires the activation of a subset of superficial dorsal horn projection neurons that express the NK1 receptor. These lamina I neurons are crucial for the initiation and maintenance of persistent pain states and are the origin of a spino-bulbo-spinal loop that drives descending spinal facilitation, in part, via serotonergic axons.

In many areas of the central nervous system some aspects of synaptic plasticity are thought to be controlled by the immediate early gene and transcription factor zif268 (also known as Egr-1, Krox- 24 and NGF1-A). In the hippocampus, for example, zif268 is necessary for both late-LTP (long term potentiation) and learning and memory. In the spinal cord, the expression of zif268 is activity dependant and associated with the induction of LTP in the dorsal horn. Here, I have shown that peripheral inflammation of the hindpaw with Complete Freund's Adjuvant (CFA) increased zif268 expression in dorsal horn neurons and was necessary for the development and maintenance of the inflammatory pain state. In addition, descending serotonergic pathways and lamina I projection neurons modulate spinal c-fos and zif268 expression following peripheral inflammation. Depletion of spinal serotonin increased c-fos expression without affecting zif268 expression in the dorsal horn following peripheral inflammation. However, in contrast, the ablation of lamina I NK1 expressing neurons in the dorsal horn that project to the brainstem significantly decreased spinal zif268 expression.

The glucocorticoid receptor (GR) and serum- and glucocorticoid inducible kinase 1 (SGK1) are involved in synaptic plasticity and are potential downstream targets of zif268. I investigated the behavioural role of spinal GR following peripheral inflammation and the consequence of altering zif268 levels on GR and SGK1 expression in the dorsal horn. Using an antisense approach I show that both SGK1 and GR expression are regulated by zif268. I also demonstrated that GR expression in the dorsal horn was crucial for the maintenance of inflammatory pain states. While GR expression was positively regulated, SGK1 was negatively regulated by zif268 during the early stages of peripheral inflammation. In conclusion, these results indicate that zif268 dependent gene regulation in the dorsal horn is pivotal to the maintenance of inflammatory pain states and offer potential new targets for the development of future analgesic drugs.

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LIST OF KEY ABBREVIATIONS

5, 7 DHT	5, 7 -dihydroxytryptamine
5-HT	serotonin
AMPA	aminomethylphosphonic acid
ANOVA	analysis of variance
AS	antisense
CFA	complete Freund's adjuvant
d	day(s)
DAB	diaminobenzidine
Dex-mes	dexamethasone 21-mesylate
DLF	dorsolateral funiculus
DRG	dorsal root ganglia
ERK1/2	extracellular signal-regulated kinase 1/2
FG	fluorogold
g	gram(s)
GABA	gamma-aminobutyric acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GR	glucocorticoid receptor
h	hour(s)
IEG	immediate early gene
i.t.	intrathecal
LTP	long term potentiation
MAPK	mitogen activated protein kinase
min	minute(s)
mGluR	metabotropic glutamate receptor
mRNA	messenger ribonucleic acid
MS	missense
NK1	neurokinin 1
NMDA	N-methyl-D-aspartic acid
Pb	parabrachial nucleus
PKA	protein kinase A
PKC	protein kinase C
RVM	rostral ventromedial medulla
PAG	periaqueductal grey
SGK1	serum and glucocorticoid inducible kinase 1

SP-SAP	substance P-saporin
TTBS	triton x-100 (0.3%) in 0.05M tris-buffered saline pH 7.4
VLM	ventrolateral medulla
Zif268	zinc finger protein 268

CHAPTER 1

General Introduction

1. INTRODUCTION

1.1 Peripheral inflammation and nociceptor sensitisation

Tissue damage leads to local inflammation and the rapid release of neuroactive substances from surrounding tissue and locally from the bloodstream. These chemicals directly activate discrete peripheral sensory organs (nociceptors) that are important for detecting acute noxious stimuli and are partly responsible for the development of persistent inflammatory pain states. Nociceptors are classified as C-fibres (non-myelinated) or A-fibres (myelinated). Each fibre type can innervate a large area of tissue and can respond to different modalities including mechanical, electrical, thermal and chemical. However, in order to become active they require high intensity stimuli that threaten tissue damage. Adenosine triphosphate (ATP), bradykinin (BK), H^+ and K^+ ions released by damaged cells are the only endogenous substances that can directly excite the nociceptor; other substances act by lowering the threshold of the nociceptor, so that nerve ending become more sensitive to less intense stimuli. In addition, during inflammation, the perineurium (which protects and isolates the nerve ending) is disrupted, enabling previous impassable large molecules and hydrophobic peptides to cross and interact with potential targets on the primary afferent fibre.

Receptor phosphorylation and gene expression are responsible for nociceptor sensitisation following inflammation (Fig 1.1). Triggering intracellular pathways by activating protein kinase C (PKC), protein kinase A (PKA) and adenyly cyclase (AC), can for example, lead to the increase phosphorylation and expression of SNS type sodium channels and vanilloid receptor 1 (TRPV1) in the primary afferent and in the DRG. In addition, prostaglandin (PG) and bradykinin receptor interactions at the nerve terminal leads to the sensitisation of the primary afferent to H^+, serotonin and prostaglandins and enhance excitability to both thermal and mechanical stimuli during inflammation (Kumazawa et al, 1991; Khan et al, 1992; Lang et al, 1990; Manning et al, 1991; Neugebauer et al, 1989). Peripheral inflammation can engage axon reflexes. This describes a process whereby the noxious activation of one peripheral branch of a nociceptor results in the antidromic invasion of action potentials into adjacent branches of the nociceptor. This results in the release of vasoactive substances from the terminals of the nociceptor and is thought to underlie flare and the sensitisation of adjacent nociceptors (Schmidt et al, 1997; Klede et al, 2003). Furthermore, about 10–20% of C-fibres are silent nociceptors that do not respond to acute nociceptive stimuli (Schmidt et al, 1995). However, during inflammation silent C-fibres are gradually incorporated into transmitting noxious information to central synapses and therefore fully capable of

participating in the development of hyperalgesia by increasing the noxious input onto second order neurons in the dorsal horn. Hyperalgesia is defined as an increased response to a stimulus which is normally painful (IASP).

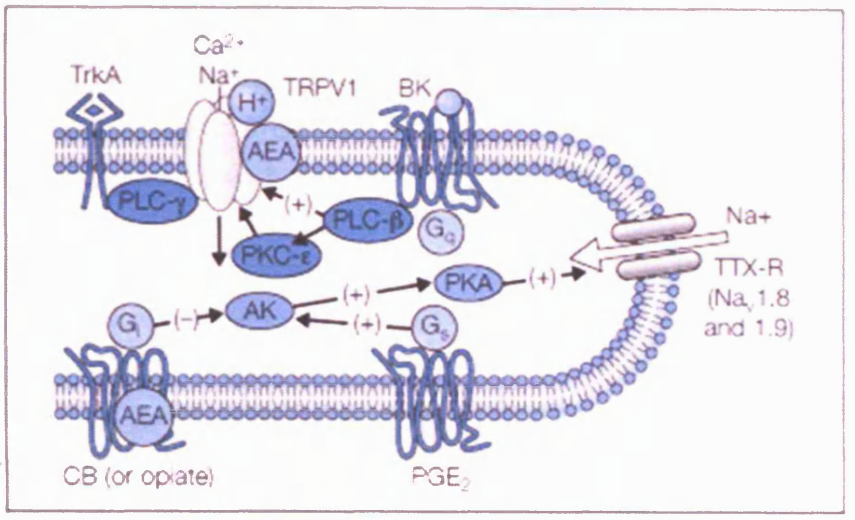


Figure 1.1 Inflammation leads to the activation of multiple intracellular signaling pathways and increases in nociceptor sensitivity. Potentiation of the vanilloid receptor (TRPV1) and the tetrodotoxin-resistant (TTX-R) voltage-gated sodium channels ($Na_v1.8$ and 1.9) can be achieved with direct interactions of the channel with protons (H^+) or lipid metabolites, i.e. anandamide (AEA). The binding of NGF and bradykinin to their putative receptor (TrkA and BK, respectively) stimulates the phospholipase C (PLC-γ or PLC-β) signaling pathway, the hydrolysis of plasma membrane lipids and the subsequent stimulation of protein kinase C isoforms, (i.e. PKC-ε). This leads to the potentiation of TRPV1. Inflammatory products such as prostaglandins (PGE_2) can enhance nociceptor excitability by activating adenylyl cyclase (AC) and Gs-coupled receptors. This occurs, in part, by a cyclic AMP-dependent protein kinase (PKA)-dependent phosphorylation of $Na_v1.8$ and/or $Na_v1.9$. Opioids and cannabinoids activating Gi-coupled receptors can counteract the excitability of the nociceptors; producing peripheral analgesia. (Adapted from The Textbook of Pain, 2005).

1.2 Classification of primary afferents

Cutaneous sensory fibres can be categorized into 3 groups based upon the extent of myelination, conduction velocities and axonal diameter. These are the Aβ-, Aδ- and C-fibres Aβ are large diameter fibres that are heavily myelinated and have the fastest conduction velocity (70-120m/s). In comparison to Aβ fibres, Aδ fibres have a smaller diameter, are less densely myelinated and have a slower conduction velocity (5-30m/s) and respond to high intensity stimuli. C-fibres are small, non-myelinated fibres, have slow conduction velocities (1m/s) and encode and transmit nociceptive stimuli (McMahon and Koltzenburg, 2005). Apart from providing the vast majority of afferent fibres that innervate the viscera, C-fibres also contribute 60–90% of all afferent fibres innervating the skin. The most important C-fibre type in the rat is the polymodal nociceptor, which is able to respond to thermal, mechanical and chemical noxious stimuli. The C-fibre response is dependent on the composition of membrane receptors and the distinctive neurochemical profile that is used to differentiate between the two variants of C-fibres.

C-fibre nociceptors can be divided into peptidergic and non-peptidergic neurons based upon the expression of distinct molecular markers (Fig 1.3). Peptidergic neurons contain substance P, calcitonin gene related peptide (CGRP) and somatostatin. In addition, peptidergic neurons in rat sensory ganglia also express the TrkA receptor, and is dependent on nerve growth factor (NGF) for normal function. Peptidergic neurons make up 50% of all C-fibres and 20% of Aδ fibres (which together make up 40% of the total neuronal DRG population). Non-peptidergic neurons contain fluoride resistant acid phosphatase (FRAP) and are characterized by binding the lectin IB4 from *Griffonia simplicifolia*. IB4 positive dorsal root ganglion cells also express glial cell line-derived neurotrophic factor (GDNF) receptor alpha subunits and receptor tyrosine kinase Ret and respond to the neurotrophin GDNF in adulthood. Classifying non-peptidergic and peptidergic neurons based upon their ability to bind IB4 could be misleading. Several studies have shown colocalisation of IB4 or FRAP and substance P or CGRP in a small number of primary afferent DRG neurons (Fig 1.3) (Carr et al, 1990; Wang et al, 1994; Bergman et al, 1999). The vast majority of visceral afferents are peptidergic (immunoreactive for substance P or CGRP), afferents projecting to the skin are half peptidergic, while a small number of afferents that project to muscle are able to bind IB4 (Ambalavanar et al 2003; Plenderleith & Snow 1993). Furthermore, and in general, the termination pattern of IB4 positive and IB4 negative nociceptors are different. The central projections of IB4 containing afferents project primarily to lamina II (inner). When stained and viewed from the transverse plane, IB4 appears as a distinct band across the dorsal horn. In comparison peptidergic neurons that do not bind IB4, project to lamina I and lamina II (outer) of the dorsal horn (Fig 1.2) (Silverman and Kruger, 1988) and terminate on projection neurons (Torsney et al, 2006).

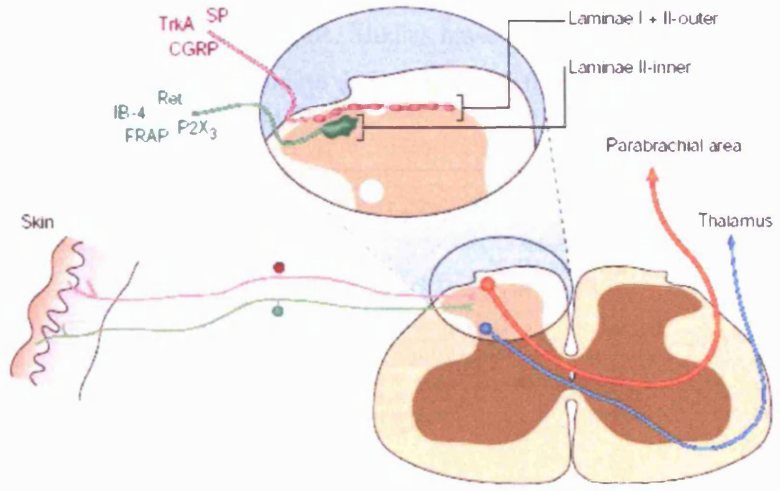


Figure 1.2. The termination pattern of the two main nociceptive C-fibre types in the dorsal horn. The peptide and neurotransmitter content of these C-fibre types differ from each other. The termination site in the dorsal horn also differs, although they innervate similar peripheral tissues. (FRAP, fluoride-resistant acid phosphatase; TrkA, tyrosine kinase receptor A, SP, substance P; CGRP, calcitonin-gene-related peptide). Adapted from Hunt and Mantyh (2001).

The role of Aδ fibres during inflammation is less well understood. In general, Aδ fibres are activated by high threshold stimuli that are higher than those for C fibre activation. Aδ fibres can be classified primarily into two general types. Type I are high threshold, rapidly-conducting mechanoreceptors and respond weakly to high intensity heat or cold (and chemical) stimuli (Handwerker and Kobal, 1993; Simone and Kajander, 1997). Type I fibres can become more heat-responsive if sensitised by rapid repetitive thermal stimulation or following tissue damage. In doing so, they show sustained responses to thermal stimuli of long duration and slow latencies. The second class of Aδ-fibre is type II. This class has been extensively examined in primate skin and respond more rapidly to noxious heat owing to a lower response threshold (in comparison to type I) (Beydoun et al, 1996; Treede et al, 1990). In addition, type II Aδ-fibres are more responsive to cooling than mechanical stimuli, and are capable of encoding the intensity of the stimuli (Craig et al., 1996).

Some Aβ fibres also respond to noxious stimulation. They are believed to contribute to chronic pain by lowering their activation thresholds (Djouhri and Lawson, 2004). Both the superficial layer of the dorsal horn (lamina I and II (outer)) and lamina V are innervated by high threshold A-fibres. In comparison low threshold A-fibres terminate in deeper layers (III-V). A-fibres signal precise localisation and discrimination of the quality of noxious stimuli and are important for triggering precise reflex withdrawal responses. A-fibres also respond to noxious mechanical stimuli with a higher frequency in comparison with C-fibre nociceptors and as such A-fibres are believed to provide information concerning the intensity of noxious mechanical stimuli to a greater extent than C-fibres (Garell et al, 1996; Slugg et al, 2000). Although information coding chemogenic pain is considered to be carried by C-fibres, A-fibre nociceptors may also play a functional role. Studies have shown that several chemical stimuli including bradykinin, capsaicin, histamine and serotonin can activate the A-fibre nociceptors (Davis et al, 1993; Ringkamp et al, 2001).

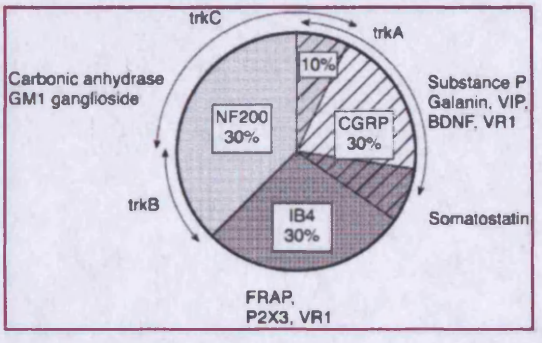


Figure 1.3. Pie chart describing the three main populations of DRG cells in rodents. Large diameter cells are myelinated and express high levels of neurofilament (NF200). Small cells are mainly unmyelinated, generally nociceptive and divided into peptidergic (substance P and calcitonin gene-related peptide, (CGRP) and non-peptidergic (IB4-positive) neurons. The arrows represent different growth factor receptors present on a given neuronal population. In general neuronal populations overlap. BDNF, brain-derived neurotrophic factor; FRAP, fluoride-resistant acid phosphatase; GFRα, Glial cell line-derived factor receptor α; TrkA, tyrosine kinase receptor A; TrkC, tyrosine kinase receptor C; TRPV1, vanilloid receptor 1; VIP, vasoactive intestinal polypeptide (Kidd and Urban, 2001 adapted from J.V.Priestley).

1.3 Peripheral innervation of the dorsal horn

The spinal dorsal horn receives input from the peripheral fibres signalling both non-noxious and noxious stimulation. In 1952, Rexed divided the dorsal horn into six laminae based upon cell shape, size and density. Lamina I, also known as the marginal layer is the most dorsal of all the laminae in the dorsal horn and contain both projection neurons and interneurons. Even though this layer contains the highest density of projection neurons in the dorsal horn, projection neurons only make up 5% of all cells in lamina I with the rest being made up of interneurons. Lamina II, also known as the substantia gelatinosa is divided into two layers (II $_{inner}$ and II $_{outer}$) and receives substantial C fibre input and as a such appears under bright-field microscopy as a translucent band. Lamina III also contains densely packed interneurons and contains many myelinated fibres which make it possible to distinguish the transition between lamina II and lamina III. Lamina IV-V1 contains a range of different size neurons with some being projection neurons. The borders between these layers are more difficult to distinguish.

Innervation of the superficial dorsal horn from the periphery is predominantly high threshold A- and C-fibre (Fig 1.4). The spinal projections of Aδ fibres in the cat was first described in 1979 (Light and Perl, 1979) and were shown to terminate primarily in lamina I and lamina II (outer) with some fibres bifurcating and innervating deeper regions including lamina IV and V. This arrangement was later confirmed in rodents (Woodbury et al, 2004) where high threshold myelinated fibres were also seen to terminate in lamina I and II (outer). In support of this, a recent study (Torsney et al, 2006) revealed that NK1 receptor expressing neurons in the superficial dorsal horn received high threshold Aδ and C-fibre monosynaptic input. In general neurons that lacked the NK1 receptor in lamina I received polysynaptic inputs from the periphery. In addition, Torsney et al (2006) using spinal cord slice preparations showed that a large number of NK1 expressing lamina I projection neurons also received polysynaptic inputs that were capable of being activated by low threshold Aß fibre stimulation. However, this was only possible following the application of bicuculline and strychnine, suggesting that under non-pathological conditions this pathway is under GABAergic inhibition. Furthermore, disinhibition also increased the EPSC response in NK1 expressing projection neurons following stimulation of the dorsal root with Aδ and Aß fibre strengths. This led the authors to suggest that under pathological conditions low-threshold A-beta fibres could activate "nociceptive-specific" neurons. These authors therefore suggested that hyperalgesia and allodynia could in part be explained by the activation of nocispecific pain pathways by non-nociceptive stimuli. Allodynia is defined as a pain caused by a stimulus which does not normally provoke pain (IASP).

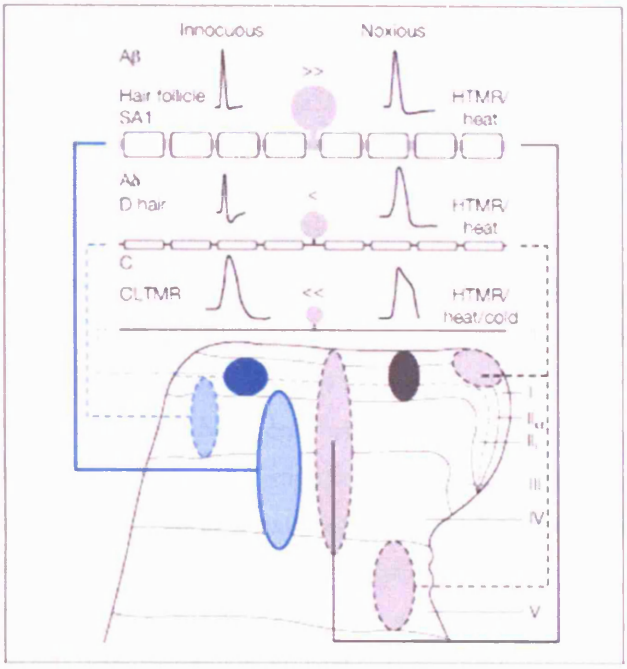


Figure 1.4. Schematic diagram showing innervations to the dorsal horn from the periphery. The predominant inputs into the superficial layer of the dorsal horn are nociceptive and include both C- and Aδ -fibres. Aδ -fibres also project to lamina V, these neurons in this layer are wide-dynamic in nature and can code for stimuli strength. HTMR; high threshold mechanoreceptor. CLTMR; C-fibre low threshold mechanoreceptor. (Adapted from Todd and Koerber. The Textbook of Pain, 2005).

1.4 Morphology of lamina I neurons

Neurons in lamina I generally fall into three morphologies; fusiform, multipolar and pyramidal. At present, it is not clear if direct correlations exists between cell morphology and cell function. Studies on feline lamina I neurons (Han et al, 1998) have indicated that all fusiform cells are nociceptive specific (NS; responsive to heat and/or pinch), while all pyramidal cells are cooling specific and the multipolar cells are a mixture of cells that are heat /pinch/cold responsive (60%) with the remaining (40%) being NS cells. In support of this notion, a study on primate lamina I cells have revealed that the majority of fusiform and multipolar cells are NK1 positive, while pyramidal cells are in general, devoid of NK1 expression (Yu et al, 1999). Little correlation was seen between cell morphology and function in NK1 positive cells in the rat. Using c-fos as a marker of noxious neuronal activity (Hunt et al, 1987), Todd et al (2002) discovered that formalin injection caused an up-regulation of c-fos in all morphological classes. Noxious heat stimulation to activate lamina I projection neurons to the parabrachial nucleus induced c-fos expression in all cell morphologies to the same proportion. In contrast to noxious heat, noxious cold induced c-fos expression in the majority of pyramidal cells, but only in the minority of fusiform and pyramidal cells. This

result led Todd et al (2005) to suggest that in some cases correlations do exist between cell morphology and function in the rat.

1.5 Lamina I neurokinin 1 (NK1) receptor positive projection neurons

The importance of the NK1 receptor in nociceptive processing in the spinal cord has been shown by a number of studies (including King et al, 2000, Martinez-Caro and Laird, 2001, Laird et al, 2000, Bester et al, 2001, Laird et al, 2001, and Suzuki et al, 2002). The cells in lamina I that express the NK1 receptor can intensity code for a number of different noxious stimuli (Doyle and Hunt, 1999) and have been shown to play a fundamental role in maintaining chronic pain states. After noxious stimulation, substance P (SP) is released from a number of nerve terminals including primary afferents (Duggan et al, 1988). SP is then free to bind onto the NK1 receptor located on the post-synaptic membrane. The binding of SP causes the NK1 receptor to internalise. Substance P can be artificially conjugated to saporin (a cytotoxin) forming SP-SAP. This compound is lethal to any cells that express the NK1 receptor. By using intrathecal SP-SAP, Mantyh et al (1997) found that, whilst retaining the ability to code acute pain, the ablation of NK1 cells in lamina I reduced capsaicin induced thermal and mechanical hyperalgesia (by 60-85%). This result was remarkable considering that fact that NK1 cells only make up ~5% of neuronal cells in lamina I. In addition, mechanical allodynia associated with CFA induced peripheral inflammation was attenuated, as well as mechanical and thermal hyperalgesia associated with spared nerve ligation (SNL) (a model of neuropathic pain). The effectiveness of the NK1 neurons at maintaining persistent pain states can be seen by the fact that intrathecal injections of SP-SAP thirty days before or seven days after SNL can both reduce mechanical allodynia (Nichols, et al 1999).

1.6 Ascending projections from the spinal cord

1.6.1 Spinal-projections to the forebrain

Early retrograde labelling studies that injected neuronal tracers into distinct regions of the brain using horseradish peroxidase, wheat germ agglutinin-HRP or fluorogold revealed that both the superficial and the deep layers of the spinal dorsal horn send ascending projections to the brain. However these studies often used large amounts of neuronal tracers that made it difficult to precisely identify individual thalamic targets from individual laminae of the dorsal horn. More recently, a detailed anterograde study (Gauriau and Bernard, 2003) using high resolution neuronal tracers injected into discreet spinal cord laminae revealed that the

thalamic termination pattern of the superficial laminae were uniquely different from deep dorsal horn neurons. Examination of the forebrain revealed clear labelling in a number of contralateral caudal thalamic nuclei following neuronal labelling of the superficial laminae of the dorsal horn. In particular the posterior group triangular part (PoT), the posterior group (Po) and the ventral posterior lateral thalamic nuclei (VPL) were densely innervated. To a lesser extent the mediodorsal thalamic nuclei (MD) was also labelled. In addition, the MD relays lamina I inputs to the rostral cingulate and the frontal cortex. The exact role of the lamina I projections to the VPL/VPM in nociception is unclear. However, the VPL/VPM system belongs to the primary thalamus that is generally considered to relay tactile information from the gracile and the cuneate nuclei towards the somatotopically organised somatosensory cortex SI. Since tactile input to the dorsal column nuclei is conveyed from the periphery by Aß fibres, the authors speculated that the overlay of nociceptive and non-nociceptive inputs in SI may be concerned with the spatial localisation of pain during peripheral noxious stimulation.

As well as receiving innervations from the parabrachial nucleus, the ventral posterior nucleus of the thalamus, parvicellular part (VPPC) also receives direct input from lamina I projection neurons. The VPPC innervates the insular cortex and is believed to be involved with assessment and environmental salience of noxious stimuli. The PoT projects to the amygdala, (LeDoux et al, 1990), insula and SII and is thought to be involved with the emotional and discriminative component of pain (Gauriau and Bernard, 2003).

Neurons belonging to deep laminae of the spinal dorsal horn contribute to nociceptive processing and have the ability to encode peripheral stimuli from low to high intensities (i.e. wide-dynamic range). Projection neurons from this region have been seen to innervate the contralateral centrolateral thalamic nucleus (CL). The CL in turn project to the motor cortex. In addition, and unlike the superficial laminae, the VPL and the Po were found to be sparsely innervated by deep laminae of the dorsal horn (Gauriau and Bernard, 2003). Away from the thalamus, deeper laminae of the dorsal horn also innervate the hypothalamus and striatum. Hypothalamic nuclei with dense labelling include the posterior and lateral hypothalamic nuclei and the paraventricular hypothalamic nucleus (PVN). The central amygdaloid nucleus and the substantia innominata dorsalis (SID) are also innervated by deep dorsal horn neurons. Electrophysiological recordings have shown that noxious stimulation can activate SID neurons (Bernard et al, 1992). Moreover, the SID targets the central amygdaloid structure (Bourgeais et al, 2001) (which is also the termination point of the spino-parabrachial-amygdaloid pathway), and is believed to be involved with the generation of fear, anxiety and alertness during painful conditions.

1.6.2 Spinal projections to the brainstem

The parabrachial nucleus (Pb) receives extensive projections from spinal lamina I neurons (Fig 1.5) (Bernard et al, 1995; Craig, 1995) and are specifically excited by noxious stimuli (Bester et al., 2000). A number of Pb neurons are capable of encoding noxious thermal and mechanical stimuli and in a restricted subgroup cooling as well (Menendez et al, 1996). The activation of Pb neurons can be generated by the electrical stimulation of either Aδ fibres or C-fibres (Bernard and Besson, 1990; Bester et al, 1995; Bernard et al, 1996) as well as visceral stimulation with algogenic substances. Moreover, some Pb neurons can respond to both visceral *and* cutaneous stimuli, a response that may reflect the large receptive fields of the Pb (Bernard et al. 1994). The lateral parabrachial nuclei are densely innervated by projections from the nucleus of the solitary tract (NTS). The NTS receives inputs from lamina I and is important for vital physiological functions including cardiovascular reflexes and respiration and may function to integrate nociceptive and cardio-respiratory afferents (Boscan et al, 2002). A large proportion (~60%) of Pb neurons project to the amygdala and the hypothalamus and are specifically activated by noxious stimuli and may make contributions to the autonomic and emotional homeostatic aspects of pain. Lamina I neurons also project to other brainstem sites including the PAG.

Raboisson et al (1996) have shown that the caudal reticular nuclei – lateral reticular nucleus (LRN), the subnucleus reticularis dorsalis (SRD), the gigantocellular/lateral paragigantocellular reticular nuclei (NGc) are densely innervated by deep dorsal horn neurons. Since the LRN is part of the motor reticular area. Projections to these areas suggest that deep dorsal horn layers are involved with generating the motor reactions that are required during noxious evcnts. In agreement the SRD is also part of the reticular area that can encode the intensity of noxious stimulation and send projections back to the deep dorsal horn, the ventral horn and to motor areas of the brainstem (Bernard et al, 1990; Villanueva et al, 1995).

Broadly speaking projections from lamina I innervate areas of the brain concerned with homeostasis, discrimination, affect and cognition. In contrast, projections from deeper layers of the dorsal horn directly innervate brain areas concerned primarily with motor actions and are likely to contribute to arousal and the generation of motor responses following noxious stimulation. There is, however, some overlap between deep and superficial spinal projection pathways

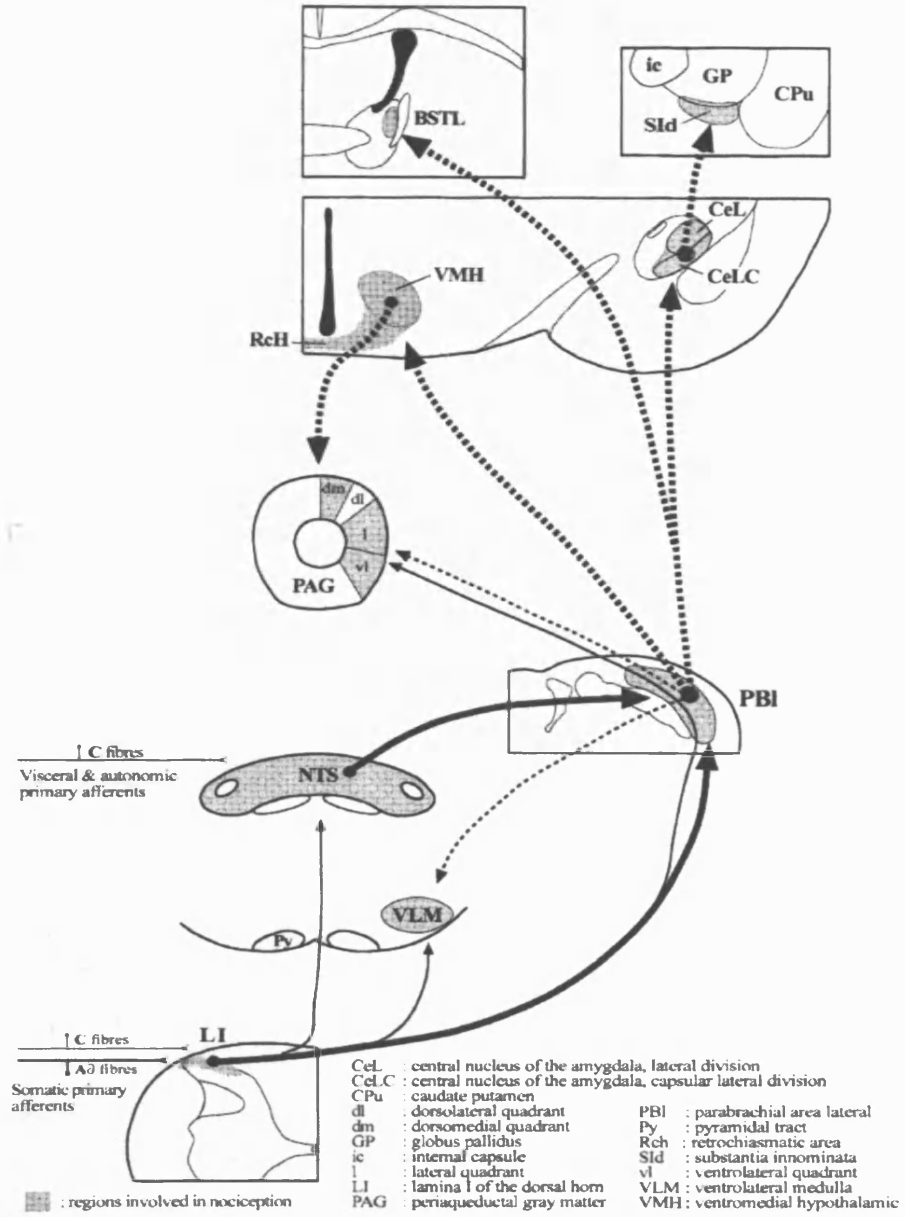


Figure 1.5. The spino-parabrachial nucleus pathway. The parabrachial nucleus (PBN) is regarded as an important locus for integrating nociceptive information from visceral and cutaneous origin, as well as playing an important role in physiological functions such as blood pressure regulation. The strong projections to the amygdala, hypothalamus and the periaquectual gray suggest that the spino-parabrachial pathway from lamina I could modulate autonomic, homeostatic and emotional component of pain. Thick and thin continuous lines with arrowheads, dense and medium 'second order projection' respectively (from lamina I (LI) and NTS); thick and thin dotted lines with arrowheads, dense and medium 'third/fourth order projections', respectively (from PBl, VMH and CeLC) (BSTL; lateral bed nucleus of the stria terminalis). (Taken from Gauriau and Bernard, 2001).

1.7 Spino-PAG projection neurons

1.7.1 The PAG

The PAG, along with the amygdala, are involved with the emotional aspect of nociception. Spino-parabrachial projection neurons can support long term potentiation (LTP) (Ikeda et al,

1999) and changes in the second order neuron could enhance nociceptive transmission to the parabrachial nucleus. The parabrachial nucleus is known to act as a nociceptive and autonomic relay station, both receiving and sending nociceptive information to other key areas of the brain. Pain can elicit a number of effects including affective and emotional behaviours, as well as influencing autonomic homeostatic adaptations to noxious conditions.

Projections from the PB terminate in the lateral, dorsomedial and ventrolateral columns of the PAG. Recent studies have revealed that different nuclei of the PAG are activated during different environmental circumstances. The lateral column is responsible for processing the active emotional coping behaviours that include the 'fight (engagement) or flight' response (Bandler & Shipley, 1994). The dorsomedial column is involved with strong aversive behaviours. In comparison, the ventrolateral column is activated during inescapable persistent pain and is vital for passive emotional coping behaviours. These include disengagement, withdrawal from the external environment and the co-ordination of autonomic and motor support that produces behaviours associated with recuperation and quiescence. Physiological symptoms include hypotension and bradycardia (Keay and Bandler, 2001, 2002).

The dorsolateral and the ventrolateral column of the PAG can be selectively activated by different peripheral fibres (Parry et al, 2001). Selective activation of C-fibre or Aδ-fibre units using different rates of heating produced differential c-fos expression in the PAG. C-fibre activation increased c-fos expression predominantly in the ventrolateral column of the caudal PAG (Mangan et al. 1999; Lumb, 2002) while a greater response was seen in the lateral region following Aδ-fibre activation (in comparison with C-fibre activation). This would suggest that first pain and second pain have different representations in the PAG and that these regions can be selectively activated by different peripheral fibre types.

1.7.2 The hypothalamus

The hypothalamus receives a number of projections from the parabrachial nucleus. Areas that are heavily innervated include the ventromedial nucleus, the retrochiasmatic area, the periventricular and the median preoptic area. To a lesser extent, projections are also sent to the paraventricular nucleus (PVN) (Bester et al, 1997). The ventromedial nucleus projects to the PAG and has been shown to be important for aggressive behaviours. Lesioning of this area reduced aggressive behaviours and territorial behaviours in rodents (Albert et al, 1985; Adams, 1971). As such, the PB-Hypothalamic tract could play an important role in the defensive or aggressive behavioural response during noxious stimulation. Projections to the PVN indicate a potential role for the neuroendocrine system during noxious stimulation.

Activation of the PVN could enhance glucocorticoid (corticosterones) release that aids in the breakdown of substances vital for metabolic reactions. In doing so, this provides the energy needed for the flight or fight response. Projections from the PB also extend to large portions of the ventrolateral medulla (VLM) of the brainstem. The VLM is important for modulating the cardiovascular system (Chamberlin and Saper, 1992, 1994). Projections from the PB to the VLM may provide a supporting role to direct projections from lamina I of the dorsal horn to the VLM. Studies have shown that during noxious stimulation the expression of c-fos increases in the VLM (Pinto et al, 2006) and that, in an indirect manner, the VLM can inhibit nociceptive activity in the dorsal horn by activating spinal alpha 2-adrenoreceptors (Tavares et al, 1996).

1.8 Descending projections from the rostral ventromedial medulla (RVM)

1.8.1 RVM cell types

The PAG has been shown to send projections to the RVM, a structure which sends direct projections to the dorsal horn and participates in the facilitation and inhibition of nociceptive traffic through the dorsal horn. Cells in the RVM can be classified into three classes based upon their electrophysiological response to noxious stimuli. These are 'on' 'off' and 'neutral' cells. 'Off' cells are thought to exert descending inhibition since the activity levels of this cell type is high when nociceptive information is inhibited and because activity decreases during nociceptive transmission before a tail flick response. The firing rate of 'off' cells can be enhanced by blocking GABA mediated synaptic transmission in the RVM and has led to the suggestion that release of GABA from local inhibitory neurons modulate the activity of 'off' cells in the RVM (Heinricher and Tortorici, 1994; Reichling and Basbaum, 1990). Conversely, 'on' cells can be activated just before tail flick using noxious stimuli but not with non-noxious stimuli (Fields, 2000; Ossipov and Porreca 2005) and are thought to facilitate chronic pain states

Anatomical studies have shown that a number of spinal cord laminae receive descending input from the RVM. These include lamina I, II, and V of the dorsal horn and VII, VIII, and X of the ventral horn with 'on' and 'off' cells project to most of these layers (Fields, et al, 1991, 1995). The main pathway in which the RVM can communicate with the dorsal horn is through the dorsolateral funiculus (DLF) (Jones and Gebhart, 1997; Gebhart and Randic, 1990). Disruption of the DLF can negate descending inhibition as well as descending facilitation. During non-pathological conditions the spinal cord is under a constant tonic net inhibition with both the inhibitory and facilitatory components being in fine balance.

However, with the development of persistent inflammatory conditions from an acute pain state, this balance is perturbed gradually allowing for net facilitation to occur. In support of this idea, studies have shown that a stage dependent switch occurs in the dorsal horn. Studies comparing acute monoarthritis (24h-48h post CFA ankle injection) with chronic monoathritis (3-4wks) have shown that descending inhibition is enhanced during the first few hours (following induction) of the pain state (Cervero et al, 1991; Schaible et al, 1991; Ren and Dubner, 1996). However, as the pain state develops this inhibition is gradually lost and facilitation begins to increase.

A number of studies have demonstrated that stimulation of RVM nuclei including the nucleus raphe magnus (NRM), the nucleus raphe pallidus, the nucleus raphe obscurus and adjacent nuclei of the reticular formation, such as the NGc and the NGc-alpha can activate the descending inhibitory pathways to the spinal cord in rodents (Azami et al, 1982; Brodie and Proudfit, 1984; Light et al, 1986). The descending RVM system was classically considered to consist mainly of serotonergic neurons (Bowker et al, 1981), however, neuronal populations in the raphe nuclei are not homogeneous populations of serotonergic neurons. A number (~60%) of RVM neurons that project to the spinal cord are GABAergic (Hama et al, 1997) and are capable of modulating spinal sensory systems (Millan, 2002). In addition, neurotransmitters such as GABA, somatostatin and enkephalin co-exist in RVM-spinal serotonergic neurons (Millan, 2002). Descending inhibition from the RVM is thought to be via GABAergic and/or glycinergic neurons and does not require some 5-HT receptor subtypes. For example, studies (Kato et al, 2006) that activated RVM projection neurons to the substantia gelatinosa of the dorsal horn revealed that 1) descending inhibition is via monosynaptic thinly myelinated fibres that are GABAergic or/and glycinergic. 2) The application of GABA receptor antagonist, bicuculline, and the glycine receptor antagonist, strychnine, completely suppressed RVM induced IPSCs in SG neurons. 3) Ondansetron (5-HT3 receptor antagonist) and ketanserine (5-HT2A receptor antagonist) had no effect in SG IPSCs and 4) depolarisation of SG neurons using paw pinch were depressed during RVM electrical stimulation.

1.8.2 Descending serotonergic neurons

Serotonergic neurons make up one fifth of the total number of neurons in the RVM with a large proportion projecting to the dorsal horn. Owing to the large array of serotonin receptor (5-HTR) subtypes expressed in the dorsal horn, serotonin (5-HT) released into the dorsal horn can be both pro- and anti-nociceptive. Electrical stimulation of the RVM promotes the release of serotonin into the dorsal horn and causes analgesia which is reversible by serotonin

antagonists (Le Bars, 1988). The direct application of serotonin inhibits nociceptive dorsal horn neurons (Hylden and Wilcox, 1983). This effect is largely mediated by the activation of the 5-HT1 and 5-HT2 receptors in the dorsal horn, but also by the activation of spinal GABAergic inhibitory neurons (that express the 5-HT3 receptor) (Alhaider et al, 1991). Furthermore, spinal 5-HT3 receptors have been implicated as having a role in facilitating pain states. The intrathecal injection of ondansetron (a 5-HT3 receptor antagonist) was seen to reduce pain behaviour associated with the formalin model (Oyama et al, 1996).

1.9 Serotonin receptors

The serotonin receptor consists of 14 different subtype and are, in general, G-protein coupled receptors. Depending on the subtype, serotonin receptors can be facilitatory or inhibitory. For instance, the 5-HT1 receptors are a large family that are negatively coupled with adenylyl cyclase through the G_i family of G proteins. This is also the case for the 5-HT4 receptor subtype. Dual coupling with potassium channels and adenylyl cyclase characterises the G_i linked receptors, and occurs in other members of the 5-HT1 receptor family. The activation of potassium channels and the inhibition of calcium channels leads to the hyperpolarisation of the neuron and reduces neuronal activity

The 5-HT2 receptor family are coupled to phospholipase C (PLC) and is in general excitatory owing to the closure of potassium channels. PLC is a membrane-bound enzyme and responsible for the degradation of inositol lipid, phosphatidylinositol 4, 5-bisphosphate (PIP_2), with the production of inositol 1, 4, 5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 causes the release of calcium from intracellular storage site by interacting with specific receptors. The increase of cellular calcium induces multiple effects in the cell, including activation of important enzymes which are responsible for phosphorylating protein substrates such as membrane receptors in the cell. DAG activates another kinase family, protein kinase C (PKC). PKC regulates numerous processes of cell function.

The 5-HT3 receptor is different from other 5-HT receptors by being ligand gated. The receptor forms an ion channel that regulates ion flux in a G-protein-independent manner. The 5-HT3 receptor is a member of the ligand-gated ion channel and was first found on peripheral sensory neurons including C-fibre neurons that innervate the dorsal horn where they mediate excitation and regulate neurotransmitter release.

The release of 5-HT from descending pathways can act on a number of 5-HTRs Immunohistochemical studies have revealed positive staining for 5-HT1A, 5-HT1B, 5-HT1D

and 5-HT3 receptors in lamina I and II of the spinal dorsal horn (Tecott et al, 1993; Morales et al, 1998; Maricq et al, 1991; Maxwell et al, 2003). The 5-HT3A receptor subtype is found on axon terminals of both IB4 and CGRP expressing primary afferents and also terminals of non-primary afferent origin. Moreover, it appears that the receptor is not located on GABAergic or serotonergic neurons in the dorsal horn, but on primary afferent fibres and interneurons (Maxwell et al, 2003) Although the relative percentages are small in comparison with the overall number of primary afferents, the expression of the receptor on primary afferents could provide a possible mechanism by which this receptor could be pro-nociceptive. Electrophysiological recordings from the dorsal horn suggest that 5-HT3 receptor does not play a functional role in normal acute nociceptive processing but is activated under peripheral inflammatory conditions (formalin) (Green et al, 2000). The 5-HT3 receptor antagonist ondansetron significantly reduced second phase formalin response but did not affect carrageenan hyperalgesia. An explanation for this is that carregeenan, unlike formalin is a much weaker stimulus and might not be adequate to provoke a descending serotonergic response.

Recently, there is evidence to suggest that some lamina I NK1 positive projection neurons form direct contacts with 5-HT3A receptor positive afferents (Conte et al, 2005). This anatomical arrangement would allow descending serotonergic neurons to facilitate pain states by increasing neurotransmitter release from 5-HT3A positive afferents and enhance activity in lamina I projection neurons. Lamina I NK1 projection neurons form part of a spino-bulbo-spinal loop and provides a mechanism whereby descending pathways can modulate the levels of excitation in the dorsal horn neurons. The serotonergic pathway only makes up part of the descending component but has been shown to be both anti- and pro-nociceptive. By selectively antagonising the 5-HT3 receptor with intrathecal ondansetron it was found that it was possible to facilitate both thermal and mechanical stimuli. Furthermore the receptive fields of deep dorsal horn neurons were also altered. A similar result can also be ascertained by ablating NK1 lamina I projection neurons suggesting that 5-HT3 receptors on these neurons could play a role in pain facilitation (Suzuki et al, 2002; Suzuki et al, 2005). The 5-HT3 receptor (mRNA) have been found in (non-capsacin sensitive, non-IB4) small cell bodied neurons and in myelinated primary afferents of the dorsal root ganglion. 5-HT3 receptor protein are expressed in; peptidergic unmyelinated primary afferent fibres, excitatory interneurons and lamina I projection neurons of the dorsal horn. Electrophysiological studies using 5-HT3 agonists have suggested that 5-HT3 receptors are present in neurons that can be activated by noxious chemical and natural stimuli (Zeitz et al, 2002; Conte et al, 2005). As such it has been suggested that spinally expressed 5-HT3 receptors in the dorsal horn

including lamina I projection neurons may play a pivotal role in facilitating persistent pain states during pathological conditions.

1.10 Dorsal horn: synaptic plasticity and long term potentiation (LTP)

Synaptic efficacy in the dorsal horn can be modulated in a use dependent manner. Changes in synaptic efficacy can have profound effects on nociceptive processing in the dorsal horn. Many dorsal horn neurons including neurons that project to the parabrachial nucleus and the periaqueductal grey (PAG) can be sensitised following high intensity peripheral stimulation leading to a prolonged enhancement in synaptic efficacy and neuronal excitability. This is known as central sensitisation. Central sensitisation can take various forms, including wind-up, classic heterosynaptic sensitisation, classic homosynaptic sensitisation, long term potentiation (LTP) and transcription-dependent central sensitisation.

1.10.1 Wind-up

Repetitive noxious C-fibre stimulation at the same strength can result in prolonged discharge in dorsal horn neurons. This phenomenon is termed wind-up and results in the progressive increase in the number of action potentials generated in second order neurons in the dorsal horn per unit stimulus. NMDA receptors and NK1 receptors are required for the generation of wind-up, although other receptors such as calcium channels and potassium channels have been implicated as well. Wind-up is a form of activity dependent plasticity that is extremely short lived. Wind-up manifests itself over the course of the stimulus and does not last beyond this point. The behavioural correlate of wind-up has been seen in human studies (Price et al, 1977) with both thermal and mechanical stimuli. Although the stimulus strength remained the same, repetitive stimulation increased the pain experienced by human volunteers. This occurrence has led some to believe that wind-up is a phenomenon that is part of normal acute nociceptive processing (a defensive behaviour to potential tissue damage).

1.10.2 Heterosynaptic central sensitisation

This form of central sensitisation can be induced by intense noxious stimulation with capsaicin, C-fibre electrical stimulation or tissue damage. Following the induction, the sensitisation of dorsal horn neurons outlasts the duration of the stimulus. The reduction in the threshold of second order neurons in the dorsal horn following sensitisation enables innocuous stimuli (via low threshold sensory fibres) to activate the sensitised cell. This

therefore produces behavioural allodynia, and increases the receptive field. The unmasking of sub-threshold inputs maybe responsible for the generation of secondary hyperalgesia.

The maintenance of heterosynaptic central sensitisation is consequence of two general mechanisms 1) post-translational modifications on ion channels, receptors and regulatory proteins 2) trafficking of receptors to the membrane of the post-synaptic cell in the dorsal horn. Post-translational modifications to receptors and ion channels can dramatically alter gating kinetics including increasing opening probability and open time durations. In addition, the permeability of the membrane is further increased with the increased trafficking and insertion of receptors into the membrane. For instance following inflammation dorsal horn NR1 and NR2 subunits of the NMDA receptor are rapidly phosphorylated (Guo et al, 2002; Caudle et al, 2005) resulting in altered channel kinetics (Yu et al, 1997; Suen, et al, 1998). Changes include an increase in the opening probability and a reduction in voltage-dependent magnesium block. The phosphorylation of the NMDA receptor can be achieved by activating a series of protein kinases including Src (activated by TrkB and EphB) (Simone et al, 1989), protein kinase A and protein kinase C (Chen and Huang, 1992). Moreover, recent studies have shown that dorsal horn AMPA receptors (GluR1) are readily trafficked and inserted into the membrane following noxious stimulation (Galan et al, 2002; Tao et al, 2006) in order to strengthen synaptic efficacy.

1.10.3 Transcriptional-dependent central sensitisation

Peripheral inflammation increases afferent input into the dorsal horn and can drive transcriptional events both peripherally and centrally. Transcription of key proteins in the dorsal horn can sustain altered nociceptive functions for long periods of time and maintain central sensitisation (Woolf and Costigan, 1999). The release of neurotransmitters as a consequence of intense nociceptive activation can result in the activation of NMDA, AMPA, NK1 and TrkB receptors in dorsal horn neurons and results in protein kinase A, protein kinase C and ERK phosphorylation (activation). ERK activation can alter gene transcription by regulating the activity of CREB and other IEGs (Adwanikar et al, 2004). Indeed, to support this idea, the expression of the immediate early genes (IEGs) such as c-fos and zif268 occurs rapidly (2h) and transiently in dorsal horn neurons following peripheral stimulation (Hunt et al, 1987; Rygh et al, 2005; Ji et al, 2002) and are known to be important for gene transcription. Microarray studies have shown that peripheral inflammation can increase mRNA expression in the dorsal horn for a number of genes (Rodriguez-Parkitna et al, 2006), while other studies have shown that TrkB (mRNA) expression increases in the dorsal horn following electrical C-fibre stimulation (Woolf and Costigan, 1999).

1.10.4 Long term potentiation

LTP has often been used as a model for central sensitisation. However it remains unclear if the two events describe the same phenomenon. LTP is one form of synaptic plasticity and is defined as an increase in synaptic strength and excitability (that outlives the stimulus that induced the event) within a neuron. LTP-like events can be artificially induced in dorsal horn neurons with high intensity stimulation (including lamina I projection neurons) (Liu and Sandkuhler, 1995, 1997; Svendsen et al, 1999; Ikeda et al, 2006).

Hippocampal LTP can be divided into two time dependent components; early LTP (E-LTP) and late LTP (L-LTP). Although the temporal distinctions are not always clear as to when the onset of L-LTP actually occurs following E-LTP (reviewed in Kelleher et al 2004), a distinction which most authors agree upon is that L-LTP requires the expression of new mRNA and *de novo* protein synthesis, which E-LTP does not. LTP and central sensitisation share a number of characteristics. Importantly, both require the synthesis of the same proteins including mediators of LTP such as the NMDA receptor, CaMKII, and group 1 mGluRs (Ji et al, 2003; Azkue et al, 2003), as well as a wide range of modulatory proteins. Noxious stimuli such as electrical C-fibre stimulation in the noxious range, noxious skin heating or subcutaneous CFA injection leads to the induction of the same IEGs seen in hippocampal LTP such as c-fos, c-jun. Non-noxious stimuli usually fail to induce the expression of these IEGs. Furthermore, the intrathecal administration of protein synthesis inhibitors such as anisomycin or cycloheximide attenuates L-LTP in the dorsal horn without effecting the induction or the baseline response (Hu et al, 2003). Importantly, this also blocked proteins that are important for the expression of allodynia and hyperalgesia in both the primary afferent (Tohda et al, 2001) and in the dorsal horn (Hou et al, 1997). By blocking de-novo synthesis in the dorsal horn, cycloheximide dose dependently reduced formalin dependent spinal c-fos expression and reduced nociceptive responses in a similar manner to c-fos antisense treatment (Hou et al, 1997) suggesting a behavioural role for c-fos in nociception.

Another signalling cascade that is important for hippocampal LTP is the MAPK pathway. The inhibition of this pathway at time points that are too early for gene expression can prevent the induction of hippocampal LTP and also prevent second phase formalin, these results suggest that MAPK in the dorsal horn could play a role in amplifying nociceptive processing (Ji et al, 2003; Karim et al, 2006). In addition, hippocampal LTP can also be affected by the AMPA receptor subunit composition. Calcium influx into hippocampal neurons was found to be significantly larger in mutant mice that lacked the GluR2 subunit. This resulted in hippocampal neurons that were more excitable and enhanced LTP expression (Gerlai et al,

1998). In a similar manner to the hippocampal neurons, GluR2 subunits can also affect the excitability of nociceptive neurons in the dorsal horn. It has been suggested that the excitable nature of the neurons located in the superficial layers of the dorsal horn can be enhanced with AMPA receptors that lack the GluR2 subunit (Hartmann et al, 2004). AMPA receptors that lack the GluR2 subunit are more calcium permeable in comparison with other AMPA receptors and are therefore likely to mediate synaptic changes that amplify noxious input. Altering the neuronal composition of calcium impermeable to calcium permeable subtype during induction would increase calcium permeability of the cell and enhance gene transcription.

1.11 Zif268: an immediate early gene important for synaptic plasticity

Zif268 is an immediately early gene associated with learning and memory in the hippocampus (Jones et al, 2001). Zif268 is also expressed spinally. The dorsal horn of naïve (unstimulated) animals contains very few zif268 positive cells. Zif268 expression in the dorsal horn is stimulus dependent and can be induced with C-fibre HFS (Wisden et al, 1990), formalin, carrageenan and CFA (Koganemaru et al, 2000; Rahman et al 2002; Otahara, 2003). The expression of zif268 using these agents all produced a similar time course, significant increases were seen at 45min with peak expression occurring at approximately 2h post stimulation. The levels returned to baseline values at the 6h time point. Concomitantly pCREB in the dorsal horn (including lamina I) are also upregulated with the same stimuli (Ji and Rupp, 1997; Impey et al, 1999; Ji et al, 2002) following the same time course. Since zif268 contains the CRE binding site in its promoter region (Sakamoto et al, 1991), it is possible that the increase in pCREB levels in the dorsal horn can contribute to the activation of zif268. To support this idea, evidence from learning and memory studies show that pCREB often binds to the zif268 promoter with both molecules being essential for long term stability of memory traces (Bourtchuladze et al, 1994, also reviewed by Josselyn and Nguyen 2005). However it must also be noted that zif268 also contains several SRE sites within its promoter region, which may also provide a potential target for Elk-1. Elk-1 is an important molecule for gene transcription that lies downstream of ERK. Elk-1 is known to be phosphorylated in the dorsal horn following nerve injury (Yu and Yezierski, 2005) as such it may also be an important activator of zif268 providing a CREB independent manner of transcription (Widmann et al, 1999). It might also be the case that zif268 requires the stimulation from both the CREB pathway as well as the Elk-1 pathway for its full activation during the induction of chronic pain states, although this has yet to be proven.

The behavioural contributions of zif268 to nociceptive processing were nicely demonstrated using zif268 knockout animals (Ko et al, 2005). It was noted that the response to acute pain was normal in the zif68 knockout animal, indicating that zif268 was unnecessary for normal pain transmission. This is not surprising since zif268 is not expressed following acute noxious stimulation. Peripheral injection of either formalin or CFA leads to hyperalgesia and allodynia to both thermal and tactile stimulation. Formalin injection is accompanied by gross nocifensive behaviours such as paw flinching and licking. These behaviours were also significantly reduced with mice that lacked the zif268 gene. Since zif268 has been found to be expressed in other parts of the nervous system involved with pain processing (the anterior cingulate cortex, the amygdala, the parabrachial nucleus, the periaqueductal grey and the hypothalamus (Herdegen et al, 1991; Herdegen and Leah, 1998)) it remains unclear if loci other then the dorsal horn contributed to the phenotype.

1.12 Thesis outline

In this thesis I have examined a number of molecular components that have been associated with the setting up of persistent pain states. Initially, I looked at the localization of zif268 and found it to be expressed by lamina I projection neurons. I then examined the influence of descending serotonergic pathways on gene expression in the dorsal horn and finally examined the expression of the glucocorticoid receptor (GR) in inflammatory pain states. Both GR and zif268 expression in dorsal horn neurons and the descending serotonergic pathways play an important role in maintaining inflammatory pain states.

CHAPTER 2

Materials and Methods

2. MATERIAL AND METHODS

2.1 Animals

Rats were housed 4 per cage. Adult male Sprague-Dawley rats weighing between 180-300g were used. The animals were housed in artificial lighting on a 12:12 h light cycle and the temperature was kept at a constant temperature of 21°C. Food and water were given *ad libitum* and all procedures were carried out in accordance with the United Kingdom Animal Procedure Act 1986.

2.2 Behaviour assessment – mechanical allodynia

The 37400 Dynamic Plantar Aesthesiometer (or mechanical plantar test apparatus) (Ugo Basile, Varese, Italy) was used to test for mechanical allodynia. The apparatus consisted of a movable force-actuator situated below a perforated platform upon which the rats are situated. A modular Perspex enclosure, with 8 spaces, leaves the animals unrestrained for the duration of the experiment.

Animals were placed in the testing cage and were habituated for 1h every day for 7d to acclimatize the rats to the test equipment and test environment. Using the mechanical plantar test apparatus, animals were tested for their baseline paw withdrawal threshold to mechanical stimuli on days 6 and 7 of the habituation period. An increasing upwards linear force was exerted onto the middle of the plantar region on both the right and left hindpaw with the actuator. The apparatus was calibrated as follows: max force 50g with a linear ramp speed of 20s (i.e. 0-50g in 20s). Paw withdrawal thresholds were tested four times per paw with each ramp separated by a 5 min interval and the mean score taken. Since the maximum force generated by the test apparatus was 50g, any animals with a threshold above 50g were excluded from the study, as it was impossible to ascertain the true baseline score. Following determining the baseline score, animals were taken to surgery (as described in section 2.5). The animals were left to recover for 1d and inspected for behavioural abnormalities. The post-surgical baseline was then established over the next three days using the same technique as described above. Three days following mini-pump insertion, or 4d following a bolus injection, rats received an intraplantar injection of CFA (100µl, 50%, Sigma, Poole, UK) into the left hindpaw. Changes in paw withdrawal thresholds were assessed a day later for the next 4d for osmotic pump (zif268 and GR antisense) studies and a week for bolus injection (5, 7 DHT) studies. Behavioural testing post-CFA used the same technique as that used for

determining baseline values. Changes in paw withdrawal thresholds were assessed by comparing post-surgical baseline scores with post-CFA scores for each day Behavioural results were presented as either net change from baseline or percentage change from baseline. Net change from baseline was determined by subtracting the mean post-surgical baseline from the mean score for each animal on each day after CFA. Percentage change from baseline was determined by taking the mean score for each animal (for each day) and dividing the score by the appropriate post-surgical baseline, before being multiplied by 100. Results were statistically analyzed using a two-way ANOVA repeated measures test, Tukey's post-hoc test and paired t-tests.

2.3 Anaesthesia

In order to induce anaesthesia, rats were placed into a halothane chamber for about 3min. When unconscious the animals were then gently transferred onto a heat mat to prevent hypothermia. Anaesthesia was maintained by a continuous flow of 2% halothane through a specialised mask (flow rate of 1.5L min^{-1}). To ensure the animal was adequately anaesthetised paw pinch and corneal reflexes were tested. Further procedures were only carried out if these responses were negative.

2.4 Retrograde labelling with fluorogold (FG)

2.4.1 Fluorogold as a retrograde tracer (FG)

Fluorogold (Hydroxystilbamidine) (FG) is a widely used hydrophilic (Fig 2.1) retrograde label used in neuronal tracing. Under physiological conditions FG has a peak excitation wavelength of 325nm and emits a maximum wavelength at 440nm (Kobbert et al, 2000). Fluorogold is a weak base that enables it to diffuse across membranes and into intracellular vesicles (lysosomes and endosomes) using the favourable pH gradient. Within vesicles FG is protonised due to the acidic environment. In this modified form, FG cannot cross back over the lipophilic membrane and is trapped and accumulated. The FG is then retrogradely transported to the cell body (Wessendorf, 1991).

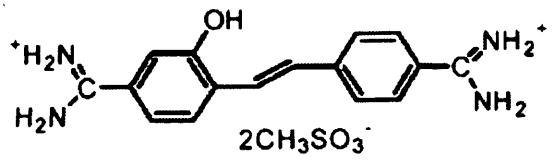


Figure 2.1. Chemical structure of FG

2.4.2 Stereotaxis and FG injection into the parabrachial nucleus

Anaesthetised rats were gently transferred onto a stereotaxic frame (Kopf), held in position and maintained under anaesthesia. Once stable, a 2cm epidermal incision was made running rostro-caudally into the scalp. A Goldstein eye retractor gently held the skin open. Sub-dermal tissue was pushed to the side to expose the skull. Target co-ordinates were identified using the brain atlas of Paxinos and Watson (1998). The skull was briefly cleaned with sterile water and a Hamilton syringe (Hamilton, Microliter No 75 with a 26s/25/pst4 needle) filled with 800nl of 4% fluorogold (Fluorochrome Ltd) was attached to the stereotaxic frame. Bregma (the point of intersection of 3 skull plates) was then localised under a freestanding surgical microscope. After successfully locating bregma, the needle, now located over bregma was moved 9.3mm caudally and +/- 1.7mm medio-laterally. The co-ordinates correspond to points directly dorsal to each parabrachial nucleus. In order to expose the dura, openings in the skull (2mm in diameter) were made at both these co-ordinates using a dental drill (RS model 547 616). The Hamilton syringe was then lowered until the needlepoint was in touch with the dura. The syringe was then advanced 6.7mm ventrally into the parabrachial nucleus and an injection of 400nl of FG was given (at a rate of approximately 100nl min^{-1}). To ensure the injection were adequate and to minimise FG drag back, the needle was left in the parabrachial nucleus for a further 6 minutes. The same injection technique was used for both sides. Bone wax was used to fill the skull openings after the injection and the sub-dermal and the epidermal layers were pulled back into position and the skin surgically clipped. The animals were left for 3-4d to allow time for the FG to be transported down to the lumbar spinal cord before being perfused.

2.5 Intrathecal bolus injections and implantation of osmotic mini-pumps

2.5.1 The cannula

To deliver compounds intrathecally using bolus injections or osmotic pumps (to the lumbar spinal cord) it was necessary to cannulate the animals. This method is relatively non-invasive compared to other approaches. The cannula used was a 15cm PE-10 polythene tubing (Portex) with an internal diameter of 0.28mm and an external diameter of 0.61mm. To reduce the likelihood of damaging the spinal cord during cannula insertion, the tubing was placed into hot water (~80-90°C) and stretched to reduce the diameter of the tubing by 40%. The tubing was then rapidly submerged into cold water and held for 1min to "fix" the length of tubing. For osmotic pumps the cannula diameter was only reduced (by 40%) at distal end. This

technique not only reduced the damage to the spinal cord but also allowed the cannula (at its proximal end) to be tightly fitted to the bridging tubing that separates the cannula from the osmotic pump.

To ensure the cannula was inserted the correct distant (3cm) into the intrathecal space the cannula was pre-inserted into the catheter so that the distal tip of the catheter was flush with the distal tip of the cannula. A reference point was then created on the cannula using a non-toxic pen 3 cm from the proximal end of the catheter to aid surgical accuracy (Fig 2.5).

2.5.2 Cannulation

The cannulation technique used was similar to that developed by Pogatzki et al (2000). Adult rats weighing between 180-200g were anaesthetised as described above (section 2.3). A small rostro-ventral incision (~3cm) was made to the skin along the midline above the lumbo-sacral spinal column (Fig 2.5). The incision was made with a scalpel blade (size 15) with the start point 2cm above the level of the iliac spine. The incision exposed the underlying muscle but did not damage it. To ensure sterility during the surgery, the skin was shaved and swabbed with povidone-iodine solution (Betadine, Seton Healthcare Group Plc, UK) before the incision was made. Once the incision was complete, a guide catheter (19g needle) was inserted and directed rostrally (i.e. cranially) into the muscle overlying the spine. The point of entry into the muscle was made ~2mm lateral to the midline at the level of the iliac crest. The catheter was then advanced approximately 4mm so that the tip of the needle was residing between the gap of the L5 and L6 vertebrae. The cannula was then gently slid through the catheter so that the reference point on the cannula was flush with the proximal end of the catheter. This ensured that the cannula was 3cm inside the intrathecal space and lying dorsal of the lumbar 5 (L5) region of the spinal cord. The catheter was then carefully removed from the animal, ensuring the cannula remained in place. Correct placement into the intrathecal space was accompanied by minor tail and/or hindpaw flick during insertion and the back-flow of spinal fluids from the intrathecal space after insertion. However, back-flow of spinal fluids is not guaranteed even with correct cannula positioning.

2.5.3 Intrathecal bolus injections via cannulation

For bolus injections, the cannula was pre-filled with the same solution as the injection medium and inserted as described above (section 2.5.2). Injections were made using Hamilton syringes (50µl, 705N). The injections consisted of 10µl of drug followed by a 10µl flush of saline. Both flush and drug were delivered at a rate of ~10µl min^{-1}. The cannula was then

slowly and carefully removed from the rat and Aureomycin (chlortetracycline hydrochloride 0.5g, 2%) powder was applied onto the dorsal layers of the cutaneous maximus muscle to prevent infection. The incision was then closed using 4 equally spaced surgical Michel clips (size 2x5mm). After closing the animals were left to recover from the effects of anaesthesia for approximately 2h before returning to their home cages. Any animals with any motor deficits or in discomfort were immediately sacrificed.

2.5.3.1 Bolus intrathecal injections of 5, 7 dihydroxytryptamine (5, 7 DHT)

Bolus injections of 5, 7 DHT were given to ablate local serotonergic neurons in the lumbar region of the spinal cord. Firstly, animals were anaesthetised and injected i.p. with 1ml desipramine hydrochloride (in saline, 25mg kg^{-1}, Sigma, D3900-SG) in order to protect noradrenergic axons. The animals were then left to recover for 45min before being anaesthetised again and cannulated. The animals were injected with either 5, 7 DHT (dissolved in saline, 10µl, 6µg μl^{-1}, Fluka) or saline (10µl, vehicle control) before receiving an intrathecal saline flush (10µl). The animals were left for 3-4d to allow time for the depletion (~90%) of serotonin from local axons and separated into two groups. The first group was assessed for behavioural changes (section 2.2) and the second group was immunostained (Fig 2.6) for 5-HT, c-fos and zif268 following a 2h CFA injection into the intraplantar region of the hindpaw (section 2.9 and table 2.2). If applicable, neuronal counts were performed using a Nikon E-800 Eclipse microscope and MCID software (section 2.11.2).

2.5.3.2 Bolus intrathecal injections of SP-SAP and SAP

Substance P-Saporin (SP-SAP) specifically targets and ablates cells expressing the NK1 receptor. The active component leading to cell death is saporin (SAP) (see Fig 2.2). Animals were cannulated as above (section 2.5.2) and injected with either SAP (vehicle control) (1.0µm, 10µl, Advanced Targeting Systems) or SP-SAP (1µm, 10µl, Advanced Targeting Systems) followed by a saline flush (10µl). The animals were then left for 28d (Mantyh et al, 1997; Nichols et al, 1999) before being briefly anaesthetised and injected with CFA (50% 100µl, Sigma) into the intraplantar region of the hindpaw. 2h later animals were perfused with 4% PFA (section 2.6). Lumbar spinal cord sections (40µm) were then immunostained for the NK1 receptor and zif268 using the three step indirect fluorescence protocol (Fig 2.6). The primary antibodies concentrations were used as detailed in table 2.2. Following immunostaining, zif268 and NK1 positive neurons were counted as described below (section 2.11.2).

Saporin and SP-SAP

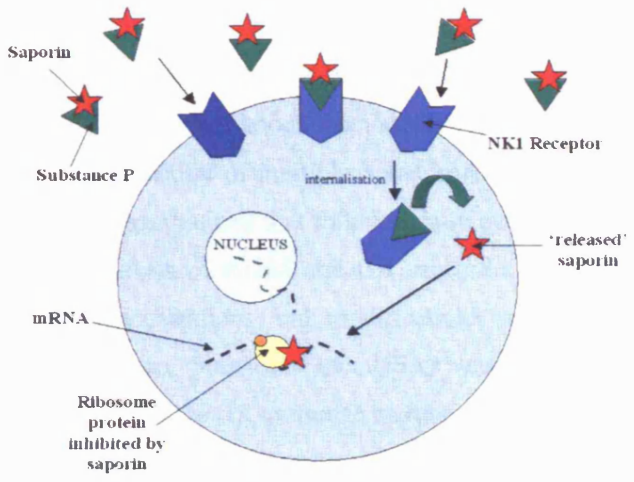


Figure 2.2. Substance P and saporin mediates NK1 receptor positive cell ablation. Since saporin is a type 1 RIP it has more difficulty entering cells (however, it can still be internalised by binding to either alpha-microglobulin or the megalin receptor). Saporin is stable from protease activity (compared with other RIPs) and this property has made it useful. By conjugating saporin to a specific substrate it is possible to selectively ablate cells that contain its receptor. Here saporin has been conjugated to substance P (SP-SAP) the putative substrate for the NK1 receptor. As such when SP-SAP binds onto the NK1 receptor the receptor is internalised and the saporin is then detached and free to inhibit ribosomal activity leading to the cell death

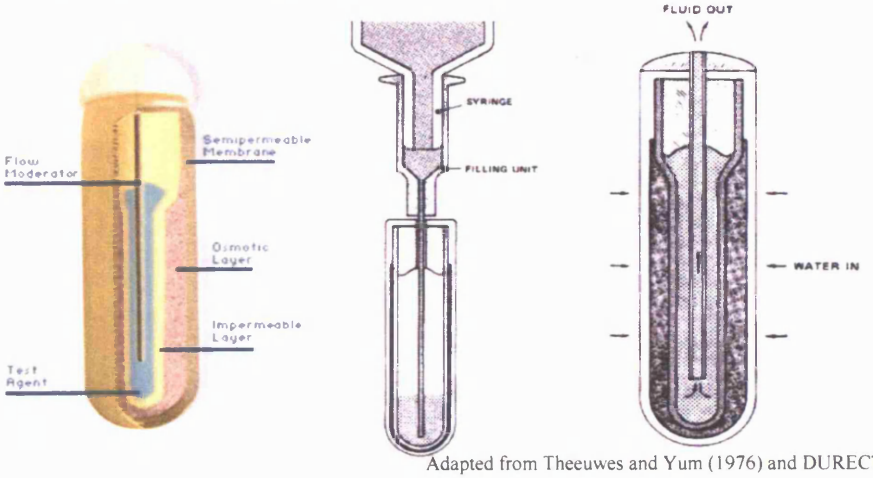
The 30KDa protein Saporin was first discovered in the seeds of *Saponaria officinalis* (Stirpe et al, 1983) and is a ribosome inactivating protein (RIP). RIPs are believed to have evolved in part to protect plants from viral infections and insect parasites. Located in rRNA is an evolutionary conserved 14 nucleotide region (Endo et al 1987) (the alpha-sarcin loop). RIPs are capable of modifying the first adenosine in a GAGA sequence. This irreversible event blocks elongation factor (EF) 1- and EF 2-dependant GTPase activity. This action inhibits translation and leads to cell death since the ribosome is unable to bind EF 2. RIPs have been classified into 3 main groups based on physical characteristics these are Type 1, 2 and 3. Type 1 includes saporin, PAP, and barley. Type 2 RIPs are more toxic compared to type 1 and 3 and include ricin and abrin. The toxicity is due to the ease in which they enter cells. These RIPs contain two subunits that are linked to each other by disulphide bonds. One subunit contains the RIP activity (A Chain) and the other (B Chain) has enzymatic and lectin binding properties. The B chain can bind to galactosyl moieties of glycoproteins or glycolipids found on eukaryotic cell membranes. These are internalised with the RIP and retrogradely transported to the nucleus via endosomes. Type 3 RIPs are less common, and are made from inactive precursors and are found in maize and barley.

2.5.4 Continuous infusion with mini-osmotic pumps

In order to look at the behavioural consequences of knocking down protein levels of zif268 and the glucocorticoid receptor (GR), as well as their potential regulatory relationship with each other and with SGK1, this study used mini-osmotic pumps to apply a continuous supply of antisense, missense and vehicle for chronic knockdown studies (Fig 2.3). Osmotic pumps eliminated the repetitive and stressful nature of multiple bolus injections. Furthermore, they remove large fluctuations in drug concentrations that follow a daily cycle of bolus injections. This study looked at reducing the levels of zif268 and GR using the appropriate modified oligonucleotide (for sequences, concentrations and modifications see table 2.1). A GR antagonist (dexamethasone 21-mesylate, Steraloids Inc. USA) was also delivered in this manner in order to confirm the actions of the GR antisense treatment.

Gene	Antisense sequence	Missense Sequence	Modification
Zif268 (42µg)	5'-GGT-AGT-TGT-CCA-TGG-TG*G-3`; (Lee et al, 2004)	5`-GTG-TTC-GGT AGG-GTG-TC*A-3`. (Lee et al, 2004)	Phosphorothioate linkages at *
Glucocorticoid Receptor (200µg)	5'-TGG-AGT-CCA-TTG-GCA-AA*T-3' (Wang et al, 2005)	5'-TGA-AGT-TCA-GTG-TCA-AC*T-3' (Wang et al, 2005)	Phosphorothioate linkages at *

Table 2.1 Oligonucleotide probes used for knockdown. The oligonucleotides were made to order (Eurogentec, Belgium) and were delivered using mini-osmotic pumps. Sequences were ascertained from previous studies and were modified at point * in order to stabilise the oligonucleotide from enzymatic degradation. GenBank database searches of the probes did not match any other coding region of the rat.



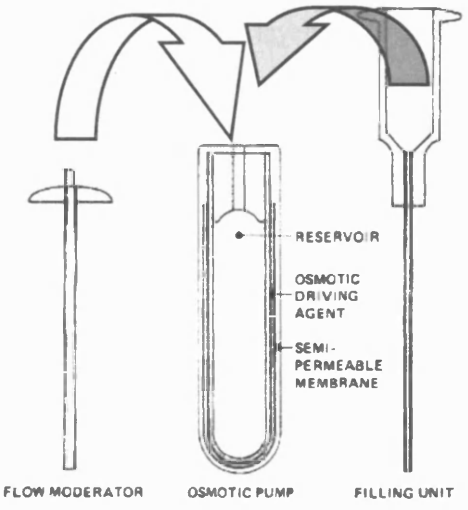
Adapted from Theeuwes and Yum (1976) and DURECT Corp

Figure 2.3 Principles of operation. The mini-osmotic pump utilises the principle forces of osmosis as its driving force to deliver drug solutions to the target site. The drug is placed into a sealed, but fully collapsible bladder. Surrounding the bladder is a layer called the osmotic layer that acts as the driving agent to compress the bladder. In order to secure the driving agent from the external environment and to allow water to enter the pump, a rigid semi-permeable membrane encloses the pump. Since the semi- permeable membrane is rigid and water is highly incompressible, the pump, when placed into an aqueous environment creates a net flux of water that crosses the membrane and into the pump. This entry into the pump causes an equal amount of drug to be expelled out of the pump orifice. After 5-6h (37°C, 0.9% saline) the amount of drug being expelled is fixed at a constant rate until the bladder is empty (Theeuwes and Yum, 1976), therefore allowing for a continuous infusion of drug towards its target site for extended periods of time.

2.5.4.1 Filling the pump

Mini-osmotic pumps (Model 2001, Durect, CA) were filled in a sterile environment (using a vertical laminar flow hood (VLF, Microflow 4) one day prior to inserting the pump into the animal. Each pump was overfilled beyond its capacity (250µl) using a specialised filling needle (Durect Corp, USA) (Fig 2.3 and Fig 2.4) attached to a 1ml syringe (BDH) containing the drug in question (vehicle, missense or antisense). The procedure of overfilling reduces the chances of air being trapped inside the bladder (or reservoir) of the pump, which could impede pumping. The VLF hood prior to use had been thoroughly wiped down with 70% ethanol and any equipment entering the hood had been sterilised in the autoclave. The procedure itself only required the partial insertion of the filling unit; if the filling unit was totally inserted no air could escape and filling was hampered. Once inserted, the bladder will begin to fill with the chosen solution as the syringe is compressed. Filling was stopped when fluid began to exit out of the pump orifice. The flow moderator was then inserted into the pump and a piece of silicon ('bridging') tubing (Sani-tech, STHT-C-025-0F) was placed over the exposed end and secured with cyanoacrylate gel (RS). The filled pump was then placed into saline and placed into an incubator set at 38.5°C until surgery.

On the day of the surgery each pump was observed for its pumping efficiency. If the pump was working a meniscus was seen in the silicon tubing, however if the meniscus was not present then the pump was not surgically implanted but discarded.



Adapted from Theeuwes and Yum (1976)

Figure 2.4. Filling of osmotic pump. Pumps were filled with chosen solution. The filling unit was attached to a syringe and filled with solution of choice. The filling unit was then inserted into the pump (shaded arrow). After the reservoir was overfilled the flow moderator was inserted (open arrow) and a piece of silicon tubing (~3cm) was attached and glued onto the end.

2.5.4.2 Implantation of the mini-osmotic pump

The surgical procedure of pump implantation was similar to that used for intrathecal bolus injections. However, modifications were made to ensure the implantation of the pump was successful and as comfortable for the animal as possible.

The animals were shaved and the incision site was swabbed with Betadine. An incision into the skin was made (Fig 2.5) similar to that used for i.t. bolus injections. However unlike the bolus injection, a subdermal pocket was created. The dimensions of the pocket were such that it was just larger than the osmotic pump and allowed the pump to slip gently into the pocket The size of the pocket minimised lateral movements that could displace the cannula. The pocket was located immediately dorsal to the dorsal most point of the incision and was made using a pair of blunt-ended Mayo scissors. Following formation of the pocket, a 19-gauge needle (the catheter) was then inserted (as described in section 2.5.3). After successfully implanting the cannula into the correct position (L5), the catheter was gently removed. Since the cannula is left 'in-dwelling' in awake animals, the cannula needed to be secured to avoid displacement. The cannula was held onto the underlying muscle with a suture (Ethicon Mersilk 3-0) at the entry point of the cannula and a loop was formed in the cannula. The loop was held in position by loose sutures with the underlying tissue acting as anchor points. If the cannula was pulled at the proximal end, the loop would absorb the force by tightening itself rather than displacing the cannula. The proximal end of the cannula was then inserted (5mm) into the bridging tubing (attached to the mini-osmotic pump). For added security, the two tubing components were then coated with 2 layers of cyanoacrylate gel (159-3935, RS components) at the connection point and left to dry. Ensuring no kinks occurred in the tubing, the pump was then carefully inserted into the subcutaneous pocket. The wound was then dusted with antibiotic powder (Aureomycin) and closed with 3-4 Michel clips, ensuring the clips did not compress the tubing.

2.5.5 Specificity of antisense treatment

In order to be certain that knocking down GR and its behavioural effects were specific we also used the GR antagonist dexamethasone 21-mesylate. The drug is stable at 37°C for extended periods of time and dissolvable in saline which allowed us to simply substitute the antisense for the drug while maintaining the same surgical and behavioural paradigm as the antisense study. Each animal received a total of 28nmol over a 7d period.

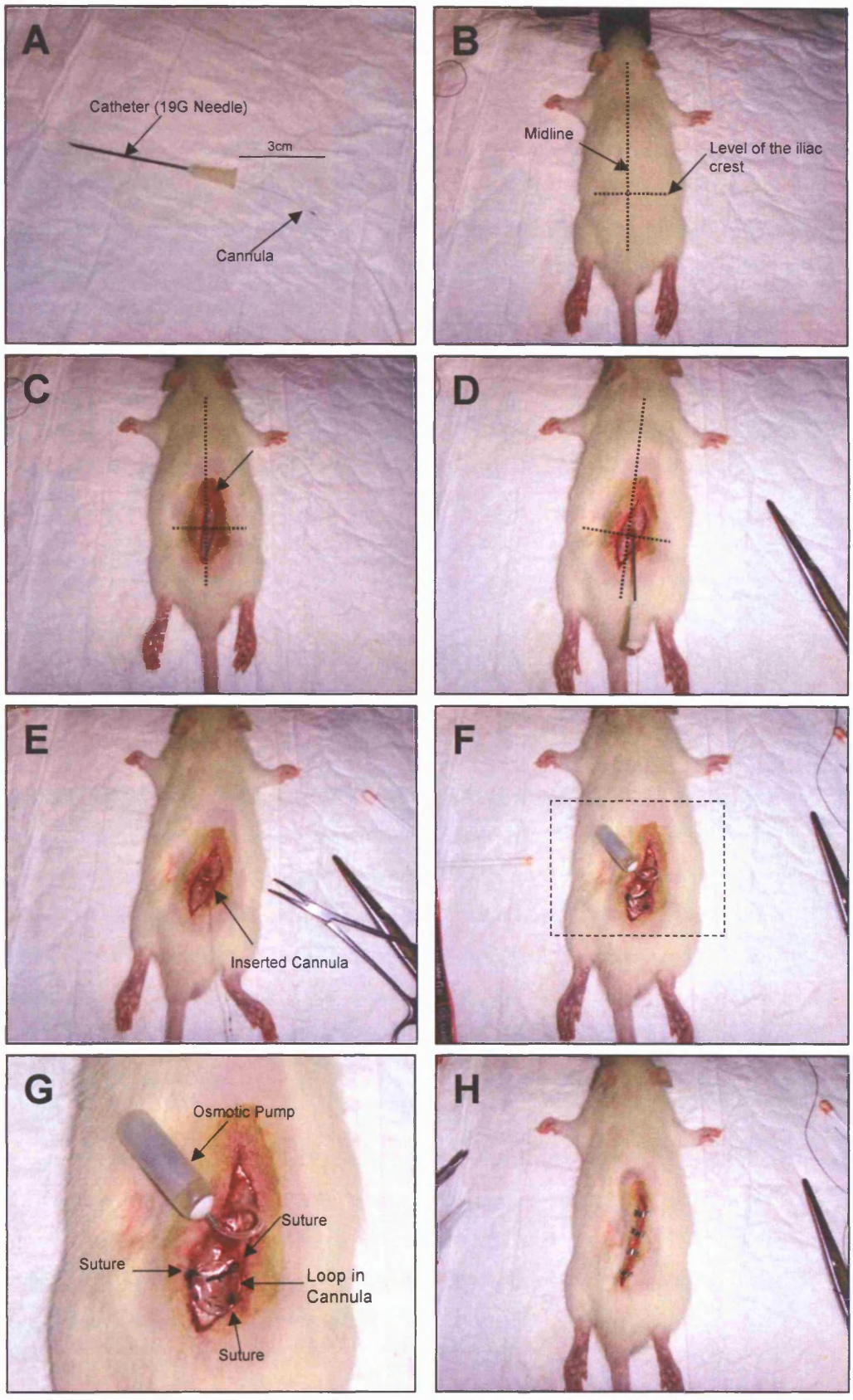


Fig 2.5. Implantation of osmotic pump. Prior to insertion into the intrathecal space, catheters were pulled by 40% to ease passage into the i.t. space and were attached to the cannula (A). The cannula was inserted into the space between L5-L6 following skin incision (C). The point of entry for the cannula was lateral of the midline and at the level of the iliac crest (B,D). The catheter was then pushed forward by 3cm before the catheter was removed (E). A loop was formed in the middle of the exposed end of the cannula and were held in position using 3 sutures into the underlying muscle. The mini osmotic pump was then attached onto the distal end of the cannula and secured with glue (F,G). The pump was then placed into a specially created subdermal pocket Finally the wound was dusted with anti-biotic powder and closed using skin clips (H).

2.5.6 Post experimental pump check

Since such a small amount of substance was expelled from the pump per unit time (1µl h^{-1}) it was essential to check that the placement of the cannula was precisely placed over the L4-L5 region (lumbar enlargement) of the spinal cord. To do this, animals were subsequently perfused (see 2.1.6) and the overlying muscle and spinal vertebrae were carefully removed to ensure that the pump and tubing were not displaced as a consequence of the dissection. Only animals with correctly positioned cannula were included in the results. After positional verification, the pump and cannula were removed from the perfused rat and the pump was then separated from the silicon tubing and checked. Only pumps that were fully empty or had minimal fluid left were included in this study. In addition, the bridging tubing and the cannula were checked for leaks. An injection of water was made using a Hamilton syringe. If the connection was still tight, the animal data was included in the study.

2.6 Perfusion and dissection of animals

Animals were transcardially perfused after administration of a lethal intraperitoneal injection of pentobarbitone at a dose of 3.5 ml kg^{-1}. The ribcage and diaphragm were removed and a catheter inserted into the aortic arch via the left ventricle. The right atrium was then cut and the animal was flushed (40ml min^{-1}) for 5 min with saline containing 0.01% heparin. To fix the tissue, a solution of 4% paraformaldehyde was then pumped into the rat for 15min (30 ml min^{-1}). The spinal cord and brain were removed, post-fixed for 4h and then cryoprotected in 30% sucrose solution overnight at 4°C. Tissues were then made ready for immunohistochemical processing (section 2.9).

2.7 Verification of knock-down

In order to verify protein knock-down efficacy, two separate techniques were run in parallel. The first was immunohistochemistry which had the advantage of looking at protein concentrations per unit cell (section 2.11.3) and second western blotting (section 2.12). This technique is more analytically accurate and quantifiable but requires larger amount of tissue and lacks the spatial resolution available with immunohistochemistry.

2.8 Toxicity from oligonucleotides

On the last day of behaviour, animals were perfused, the spinal cord sections were stained for IB4 (a marker for non-peptidergic C-fibres) (1:500, 24h, RT, Vector Labs) to verify neuronal damage from the surgical procedure if any. Since concentrations of oligonucleotide probes are often higher in more superficial layers of the dorsal horn, sections were also immunostained for the NK1 receptor and NeuN using the 3 step fluorescence protocol (Fig 2.6) and stained using Hoechst (1:100,000, 5min, RT) to look for any significant increases in neuronal cell death arising as a consequence of the oligonucleotide probes. The antibodies were visualised using Alexa 488 under a Nikon E-800 eclipse microscope and counted as described in section 2.11.2.

2.9 Immunohistochemistry (also see Principles of immunohistochemistry below)

2.9.1 Single immunohistochemistry

Lumbar spinal cord tissue (L4-L6) was transversely sectioned (40μm) using a freezing microtome (Leica). Sections were then placed into 5% normal goat serum (Vector labs) (and 2% hydrogen peroxide for serotonin immunostaining) diluted with distilled water containing 0.2% Triton X-100 and 0.05M Tris Saline (TTBS) for 1h before being placed into either rabbit anti-zif268 (1:5000, Santa Cruz, sc-189), rabbit anti-glucocorticoid receptor (1:500, Santa Cruz, sc-8992), rabbit anti-NK1 (1:5000, Eurogentec), mouse anti-NeuN (1:200, Chemicon, MAB377) or rabbit anti-serotonin (1:200, Chemicon, AB938) and incubated overnight at room temperature (RT). The sections were then washed (3x10min PB wash i.e. a standard wash) and placed into biotinylated anti-rabbit or anti-mouse secondary antibody (Vector Labs) (1:500 in TTBS for 2h, RT) before being washed again and incubated with fluoroscein avidin D (1:200, Vector Labs) or Alexa Fluor 488 (1:500, Molecular Probes) in TTBS for 2h at RT. (Sections undergoing serotonin immunostaining were instead placed into Avidin-Biotin Complex (ABC, Vector Labs) solution in TTBS for 1h, washed and placed into DAB solution until positive staining occurred). The sections were washed, mounted onto slides and coverslipped with gel mount (Sigma). In the case of DAB (serotonin staining), sections were dehydrated using a range of ethanol concentrations (70, 95, 100%, 4min in each), dipped into Histoclear I (National Diagnostics) and mounted onto slides and coverslipped with DPX (Fig 2.6).

2.9.2 Double immunohistochemistry

Serial sections (40 μm thick) were cut (with a freezing microtome, Leica) from segments of the lumbar enlargement (L4-L6). The sections were pre-treated for 1h with 5% normal goat serum (Vector labs) diluted in TTBS before being placed in rabbit anti-zif268 (1:80,000 in TTBS, Santa Cruz sc-189). Sections were then left overnight at RT or 3d at 4°C. After the allotted time in the primary antibody, sections were then washed, before being placed into biotinylated anti-rabbit secondary antibody (Vector Labs) (1:400 in TTBS for 2h, RT). After 2h the tissues were given a standard wash and placed into ABC solution (containing 4% A solution and 4% B solution (from Vector Labs ABC kit) in TTBS) for 30min. Tissues were again given a standard wash before being amplified by a tyramide based signal amplification (TSA) kit (NEL 700, Perkin Elmer). TSA was used at a concentration of 1:75 and left for 7 minutes. After TSA amplification, tissues were washed and left in fluoroscein avidin D (1:600) for 2h. Afterwards the tissue were given a standard wash and placed into 50% ethanol for 30min, before being placed into 5% normal chicken serum for 1h. The tissues were then transferred into rabbit anti-glucocorticoid receptor (1:500 in TTBS, 24h at RT, Santa Cruz sc-8992). A standard wash followed and the sections were placed into chicken anti-rabbit Alexa Fluor 594 (1:500 in TTBS, 2h at RT, Molecular Probes). The sections were washed and mounted onto slides before being coverslipped with gel mount (Sigma) (Fig 2.6).

2.9.3 Immunohistochemical controls

Standard immunohistochemical controls were utilised and ran in parallel. Primary antibodies were omitted to check for specificity and in the case of dual immunofluoresence, one of the primary antibodies was removed to check for cross-reactivity since two rabbit primary antibodies were used on the same sections. With TSA, the concentration required to detect the first primary antibody was generally 10 times less than required for standard immunofluorescence. However it was still important to check that cross over between secondary antibodies had not occurred. Firstly, sections were amplified with TSA giving a positive signal for zif268 in the FITC range. Following this, and unlike a normal double staining, no second primary antibody was added: instead only the fluorophore aimed at detecting this second primary antibody was added. If no signal was detected on this channel then cross-reactivity had not occurred. Cross-reactivity was not in evidence in any of the immunostaining achieved in this thesis.

Primary Antibody	Company	Code	Raised in	[DAB]	[Fluoroscence]	[TSA]
Glucorticoid Receptor	Santa Cruz	sc-8992	Rabbit	N/A	1 in 5000	N/A
NeuN	Chemicon	MAB377	Mouse	N/A	1 in 200	N/A
NK1 Receptor	Eurogentec	N/A	Rabbit	N/A	1 in 5000	N/A
Serotonin	Chemicon	AB-938	Rabbit	1 in 200	N/A	N/A
Zif268	Santa Cruz	sc-189	Rabbit	N/A	1 in 5000	1 in 80,000
Secondary Antibody	**Company**	**Code**	**Raised in**	**[DAB]**	**[3x Fluoroscence]**	**[TSA]**
Biotinylated Anti-Mouse	Vector Labs	BA-2000	Horse	1 in 500	1 in 500	1 in 400
Biotinylated Anti-Rabbit	Vector Labs	BA-1000	Goat	1 in 500	1 in 500	1 in 400
Chicken Anti-Rabbit	Chemicon	AP-159	Chicken	N/A	N/A	1 in 500
Flurophores	**Company**	**Code**	**[3x Fluorescence]**	**[TSA]**		
Fluoroscein Avidin D	Vector Labs	A2001	1 in 200	1 in 600		
Strepavidin Alexa 488	Molecular Probes	S-11223	1 in 500	1 in 600		
Strepavidin Alexa 594	Molecular Probes	S-11227	1 in 500	N/A		

Table 2.2. Primary and secondary antibodies used in this thesis. Concentrations for the separate immunohistochemistry protocols are stated here and were diluted with TTBS.

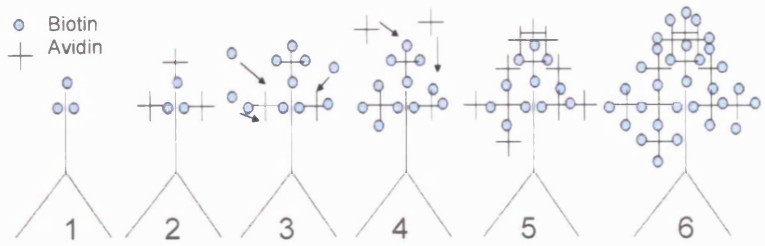
Principles of immunohistochemistry

Immunohistochemistry (IHC) describes a process of localising proteins on biological tissues using specific antibody-antigen interactions. The pivotal reagent in the process is the antibody. Antibodies are immunoglobulins, of which there are 5 main classes. The most popular class used in IHC are the IgG and the IgM classes. Immunoglobulins contain two identical heavy (H) chains and two identical light (L) chains. Constant and variable parts make up the L and H chain, the latter part binds to the antigen.

There are two types of antibodies used in IHC each with different characteristics Polyclonal antibodies can target and bind onto a large array of different number of epitopes located on a specific antigen whereas monoclonal antibodies only target a specific epitope on a specific antigen located in the target tissue.

The primary antibody can be conjugated to a number of substances in order to visualise the antibody and thus when bound to its specific antigen defines areas of tissue where the protein is located. Similarly, the second antibody which targets the primary antibody can also be conjugated to visualisation substances. The second antibody generally targets the antigens of the species in which the primary antibody is made in, thus the same secondary antibody can target any primary antibodies that are made in the same species.

However in some cases the primary antibody alone is not adequate to yield a strong enough signal. This problem can be solved if the signal can be amplified above the background noise. The most commonly used method is the avidin-biotin interaction. Since avidin has a strong affinity for biotin and has 4 binding sites for biotin, the potential to create large complexes exist (see below). 1) Here the secondary antibody is conjugated with biotin molecules. 2) The biotin can bind free avidin. 3) This in-turn allows free biotin molecules to bind onto the avidin molecules. 4) This 'extra' biotin then allows yet more avidin to bind and so on (5) to create a large avidin-biotin complex (6). An enzyme can also be added to the avidin, in the case of DAB it is horseradish peroxidase. As such when hydrogen peroxidase is added with DAB it catalyses a reaction whereby deposits are left around the immediate area and can be visualised. This is why endogenous peroxidase activity needs to be quashed with hydrogen peroxide before the secondary antibody is added to prevent unwanted reactions from occuring



This is similar to TSA where deposits of tyramide are left in the immediate area of the primary antibody due to an enzymatic reaction. An avidin based fluorophore is then added which binds onto both the biotin sites on the avidin-biotin-secondary antibody complex but also the deposited tyramide particles. Thus the tyramide particles amplify the signal. Fluorophores are molecules that are 'excited' by specific wavelengths of light in doing so they emit light at specific wavelengths. Different fluorophores have different excitation and emission properties as such one can use different fluorophores in the same tissue as long as their characteristics do not interfere with one another.

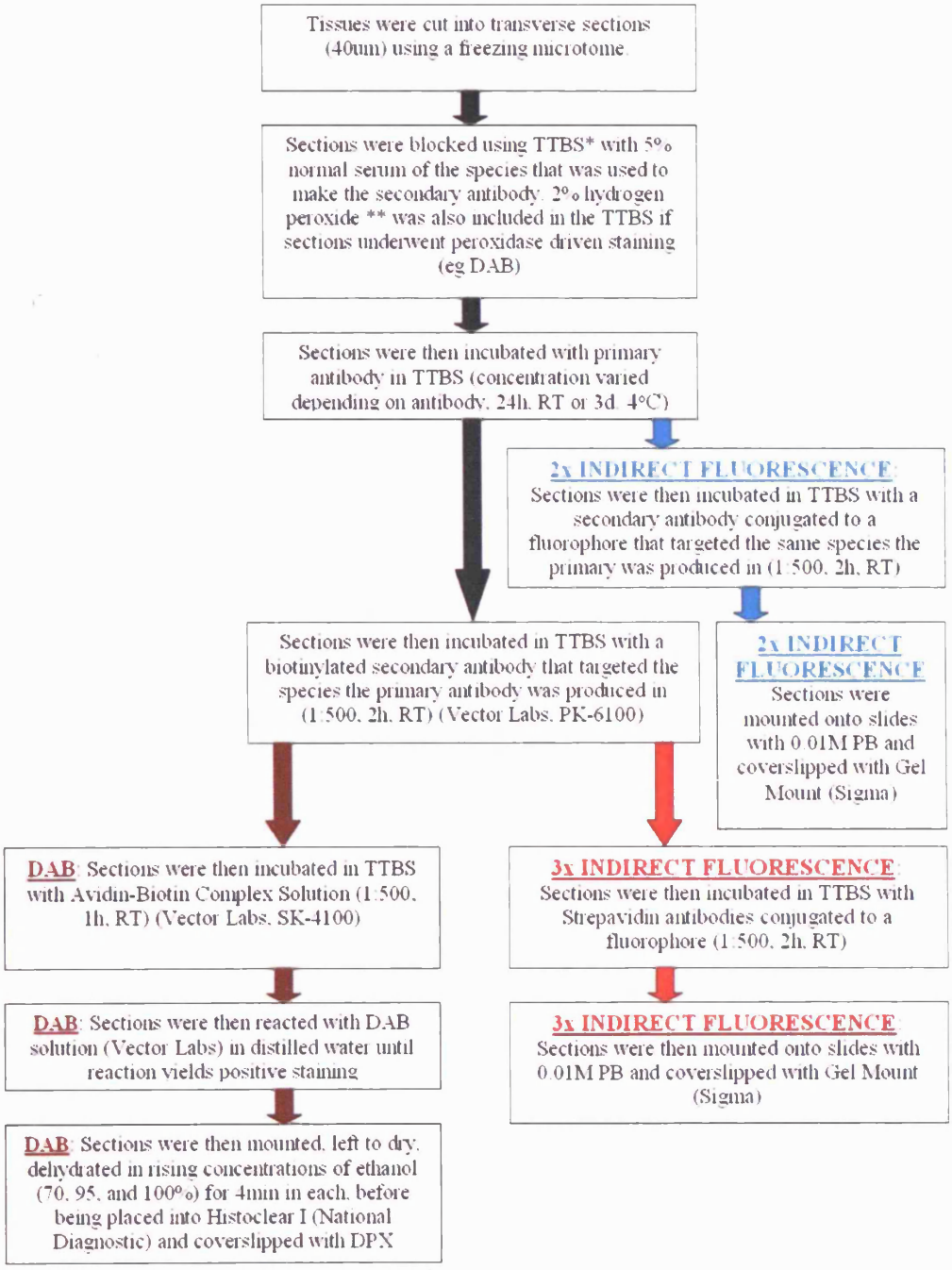


Figure 2.6 Immunohistochemical protocol framework. There were 4 main protocols that were used in this thesis for immunohistochemistry; these were 2-step indirect fluorescence, 3-step indirect fluorescence and DAB (as represented by blue, red, and brown arrows respectively). TSA was also used but not represented in this figure. Each arrow represents a wash (3x10min with 0.1M PB) and a continuation to the next step. *TTBS consisted of 0.3% Triton X-100 (for cell permeation) and 0.05M Tris-HCL in distilled water. ** Hydrogen peroxide was used to remove any endogenous peroxidase activity that could have resulted in a non-specific DAB reaction in the sections.

2.10 Microscopy

Sections were examined under a 40X optical lens using the Leica DMR microscope with appropriate light filter to allow for independent viewing of fluorophores in the UV, FITC and CY3 range. Images were taken via a charge coupled density (CCD) camera (Hamamatsu C5985) attached to the microscope and by using the software Vision Explorer VA1.11 (Graftek Imaging). Images were also taken using a Nikon E-800 Eclipse microscope and MCID software. For colocalisation studies images were merged using Photoshop 7.0 (Adobe).

2.11 Neuronal cell counts and intensity scores

2.11.1 Counts on lamina I projection neurons

Zif268 and GR expression in lamina I projection neurons was quantified by manually counting the total number of zif268 and GR neurons on FG positive neurons in the marginal layer of the dorsal horn. This was then divided by the total number of FG neurons on the same section before being multiplied by 100. Zif268 or glucocorticoid receptor positive neurons and FG neurons were counted as positive only if they were in the same focal plane

2.11.2 Neuron counts using MCID software

The density threshold criteria were set on MCID to allow for target detection following immunohistochemistry. MCID was given an upper and a lower density threshold (a segmentation range). Pixels lying within the segmentation range were regarded as valid targets; pixels lying outside of the range were ignored by MCID. Owing to the nature of MCID, a threshold operation alone does not successfully eliminate all invalid targets. There are pixels that have densities similar to genuine targets. To prevent artefacts from being accepted as valid targets MCID was made to use logical target acceptance criteria. Logical criteria included minimum and maximum area and minimum roundness. As such, in this mode MCID was fully capable of detecting individual neuronal cell bodies by taking adjacent pixels within the segmentation range and then evaluating the minimum and maximum pixels that equates to a single cell.

Positive neurons located within a 100μm boundary from the dorsal edge of lamina I (L4-L5) sections were counted using MCID software and a Nikon E800 Eclipse microscope (with an appropriate filter). The sensitivity of the software in detecting positive (immunostained)

neurons had been calibrated so that the accuracy was within 95% to the counts made manually. Calibrations were made by manually counting positive neurons on 6 randomly selected sections before finely adjusting the software so that automatic counts were accurate. In the actual count, a minimum of 10 sections per animal were counted and the mean taken before statistical analysis.

2.11.3 Neuron intensity scores

Neuron luminosity (or intensity) was measured in the ipsilateral superficial dorsal horn using confocal microscopy (Bio-Rad 600). L4-L5 sections were randomly chosen from each animal. All measurements were taken from one single session; any adjustments (to gain, intensity or black level settings) were not made during the session. Using the integrated software, an analytical marquee was generated to measure intensity scores. The marquee had an area no larger than the cell body being analysed. Using the marquee the mean background intensity for each selected section was taken at four points on the dorsal horn. The intensity score for each positive cell body was then taken using the same marquee and the mean background level for that section was subtracted from the luminosity score. Background luminosity had a value of zero. The mean was then taken from the top 20 of the most highly labelled cell bodies from each selected section and statistically compared with sections from other treatment group(s).

2.12 Western blots

Rats were implanted as above (section 2.5.4.2) with pumps containing vehicle (saline), missense or antisense to zif268 or the glucocorticoid receptor and were left for 4d and then given a unilateral injection of CFA (100µl. 50%). Rats where then killed under CO_2 before being quickly decapitated 2h after the CFA injection (corresponding to the peak expression of zif268 (Liang and Jones, 1996)). The pump and ancillary components were removed from the animal and the L4-L6 region of the spinal cord was dissected out of the animal and placed onto an ice-cold surface. The cord was then further dissected into quadrants and each quadrant separately placed into a pre-chilled Eppendorf tube. The tissue was then individually homogenized in 1ml RIPA buffer (1% NP-40, 20mM Hepes pH7.4, 100mM NaCl, 100mM NaF, 1mM Na_3V0_4, 5mM EDTA) with 10µl of protease inhibitor (P8340, Sigma) and left on ice for 2h. The samples were then centrifuged (12,000rpm, 15min, 4°C) and the supernatant were transferred into new Eppendorf tubes. The samples were then normalised using a BCA kit (Pierce) and a Biotrak II plate reader (Amersham) following manufacturers instructions. Loading buffer (100mM Tris-HCL pH6.8, 200mM dithiothreitol, 4% SDS (electrophoresis

grade), 0.2% bromophenol Blue, 20% glycerol) was then added to each sample in such a manner that each sample contained the same concentration of protein (1μg μl^{-1}) before being boiled for 5min, The samples were then individually loaded into the lanes of the SDS-PAGE ready gel (submerged in running buffer (3.72% glycine, 0.5% trizma base, 0.2% SDS)) with each lane containing 10μg of protein (10% HCl-Tris, Bio-Rad, Hercules, CA), reserving one lane for the molecular marker (Amersham). The protein samples were then separated into different molecular weights using the Bio-Rad Power-Pac 300 (1h, 120V). After separation, protein samples were then transferred from the gel onto polyvinylidene difluoride filter (PVDF) membranes (Bio-Rad, Hercules, CA) in transfer buffer (1.16% Trizma Base, 0.58% glycine, 0.74% SDS, 20% methanol). To prepare the transfer sandwich, the membrane was first primed and submerged with 100% methanol (10min) and then left in transfer buffer for 5min. The gel was removed from its holding shell and submerged in transfer buffer (5min) before the transfer sandwich was assembled as shown in figure 2.7 and ran for 1h at 100V. The membrane was then blocked (1h) in PBS-Tween (1M PBS, 0.1% Tween 20 (Sigma)) containing 5% milk

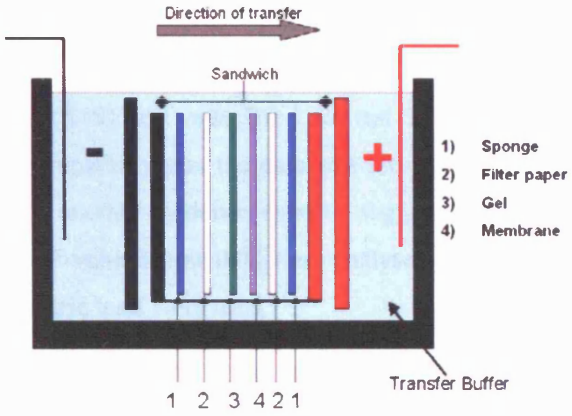


Figure 2.7 Bio-Rad mini transblot system. Following electrophoresis protein samples were transferred onto PVDF membranes using the transfer sandwich submerged with transfer buffer (1h, 100V)

The membrane was then incubated (24h, 4°C) in PBS-Tween with 5% powdered milk with a primary antibody either rabbit anti-zif268 (1:200, Santa Cruz, sc-110), rabbit anti-GR (1:150, Santa-Cruz, sc-8892) or rabbit anti-SGK1 (1:1000, Upstate Biotechnology, 07-315) antibody and then washed 6x5min in PBS-Tween. The membrane was then transferred into PBS-Tween containing anti-rabbit-HRP (1:2000, Santa Cruz, sc-2301)) and incubated for 1h (RT) before being washed with PBS-Tween (6x5min). The blots were visualised using the ECL western blotting detection system (Amersham, RPN-2106) and the Bio-Rad Chemi-Doc XRS system (Bio-Rad, Hercules, CA) for 1-10min. The blots were then incubated (2x10min) in stripping buffer (0.15% glycine, 0.1% SDS, 1% Tween 20) before being reprobed with mouse anti-GAPDH (1:1000, RT, Chemicon, MAB374), washed and then incubated in goat anti-mouse-HRP (Santa Cruz, sc-2302) before being visualised. The westerns were performed in

duplicates. The density of the specific bands were measured using the software package Quantity One v 4.4.1 (Bio-Rad, Hercules, CA) and were normalised against the corresponding band of loading control (GAPDH) to attain a ratio score. By doing this a ratio score for each lane was attained. The ratio scores for each treatment group were then compared with the scores from the other treatment group and statistically analysed using a unpaired t-test.

2.13 Statistical analysis

A number of different statistical tests were used to analyse experimental data presented in this thesis. Fundamental principles underlying the statistics are described below. Statistical analysis was carried out using SPSS 12.0.1 for Windows (SPSS Inc., Chicago). All data sets were tested for homogeneity of variance and normal distribution. If the data did not meet these requirements, they were transformed with the most appropriate method or another statistical test was chosen. $P<0.05$ was the criterion for statistical significance.

2.13.1 Kolmogorov-Smirnov test

The Kolmogorov-Smirnov (K-S) test was used to test if the data followed a normal distribution K-S gives the probability that the data sets are not normally distributed. Values above 0.05 implies that not enough evidence exist to suggest that the data set is normally distributed. Data sets with a P-value below 0.05 were analysed using non-parametric tests and for those above 0.05, parametric tests were used

2.13.2 T-test

The t-test is a parametric test that compares the difference in the means of two data sets to evaluate if the data had come from the same population or not. This test can only be applied if the data are normally distributed and with similar standard deviations. There are two forms of t-tests: paired and unpaired (Student's t-test). Data from paired t-tests are derived from study subjects that have been measured twice at two independent time points. In most cases the measurements are taken before and after treatment. Unpaired t-tests are used for two independent groups and can be used in samples with different sizes. With 2 independent samples of measurements, a non-parametric alternative to the independent-samples t-test is the Mann-Whitney U test. With two related samples, two nonparametric equivalents of the related-samples t-test are the Wilcoxon test and the Sign test. The latter two tests were not required to be used in this thesis.

2.13.3 Mann-Whitney U (MWU) test

The MWU test is a non-parametric statistical test that assesses if two samples are taken from the same distribution. The null hypothesis of the MWU test is that the two samples are taken from a single population which makes the probability that the distributions for the samples are equal to one another. The assumption that the MWU makes is that the two samples are independent to one another and the data are ordinal or continuous in nature.

2.13.4 Analysis of Variance (ANOVA)

ANOVA is a parametric statistical test that is used to test the hypothesis that the means among two or more groups are equal and makes the assumption that the data sets are normally distributed. ANOVA also assumes that within-subject variation is lower than among subject variations. The repeated measure ANOVA is used when all members of a sample are measured under a number of different conditions. As the sample is exposed to each condition in turn, the measurement of the dependent variable is repeated. If the ANOVA test revealed a significant interaction Tukey's post-hoc test was performed.

CHAPTER 3

Zif268 and Inflammatory Pain

3.1 INTRODUCTION

3.1.1 Zif268: an immediate early gene

The increase in synaptic efficacy that underlies persistent inflammatory pain (hyperalgesia and allodynia) requires macromolecular synthesis including changes in gene expression in order to convert extracellular signals from the periphery into long term functional changes. A number of studies have shown that regulation of protein synthesis is a necessary step for the formation of long-lasting changes in pain states, a step which normally requires transcription factors to be activated. Transcription factors are proteins that bind onto specific promoter regions of genes and alter the expression of the gene. Immediate-early genes (IEGs) such as zif268 and c-fos are transcription factors that convert synaptic signals into long-term changes in gene expression and are thought to influence synaptic efficacy. IEGs can be categorised into two general types: regulatory and effector. Regulatory IEGs encode proteins that increase or decrease downstream gene expression. Effector IEGs (e.g. arg3.1, Homer and BDNF) encode for proteins that have a more direct role. The IEG zif268 (also known as EGR-1, Krox-24, NGF1-A and Zenk) is an inducible transcription factor and can exist in two species, each with different molecular weights (82 and 88KDa). Zif268 is pivotal to some forms of synaptic plasticity such as hippocampal LTP and long-term memory (Bozon et al, 2003).

The importance of zif268 in signal transduction is emphasised by numerous regulatory domains presented on its promoter region (Fig 3.1). Zif268 can be regulated by a number of kinases. Studies have shown that, when bound, each of the (six) serum response elements (SREs) (which can bind Elk-1) on the promoter region can increase the expression of zif268. Fibroblast zif268 can be activated with just one SRE site being occupied. In addition, since the zif268 gene also contains an EGR response element (ERE) binding domain it affords the opportunity for zif268 to autoregulate itself and the potential to amplify its own intracellular signal (James et al, 2005; Cao et al, 1993). Zif268 also contains binding domains that negatively regulate the activity of the protein; animals with mutations occurring at the 5' end of the zinc finger R1 displayed an enhanced transcriptional activity (Gashler et al, 1993; Russo et al, 1995). Certain proteins such as NAB1 and NAB2 can negatively regulate zif268 by binding onto the R1 domain. NAB1 operates as an active repressor and directly inhibits zif268 activity. NAB2 unlike NAB1 can be activated by the same stimuli as zif268 which enables NAB2 to control the transcription of zif268 in circumstances where both genes are simultaneously activated (Svaren et al, 1996; Swirnoff et al, 1998). Other motifs on the promoter region of zif268 include two cAMP response element-binding (CREB) response

element (CRE) sites (binds with CREB), a Sp1-like motif, CCAATT sequences, an AP-1 like motif (binds c-jun-fos dimer), and an AP-2-like binding sequence.

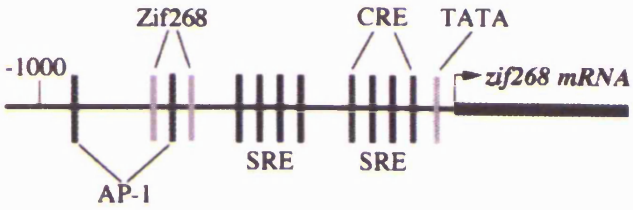


Figure 3.1 Promoter region of zif268. The promoter region of zif268 contains a large number of binding motifs including AP-1, zif268 (ERE) and the putative binding sites for the transcription factors AP-1, serum response element (SRE); cyclic AMP-responsive element (CRE); TATA, TATA box. (Adapted from Hirabayashi and Saffen, 2000).

Zif268 belongs to the early growth response factor (EGR) family and is located to the nucleus when activated. Its residency in the nucleus is due to a bipartite signal in the DNA binding domain of the second and third zinc finger (Gashler et al, 1993). The zinc fingers are also important for physical interactions with target DNA molecules. The binding domain of zif268 consist of three zinc fingers (Fig 3.2) which recognises and bind onto DNA targets that contain a sequence of 9 base pairs (bp). The zif268 binding consensus sequence is GCG(G/T)GGGCG. With each zinc finger recognising a sequence of 3bp (Christy and Nathans, 1989; Cao et al, 1990).

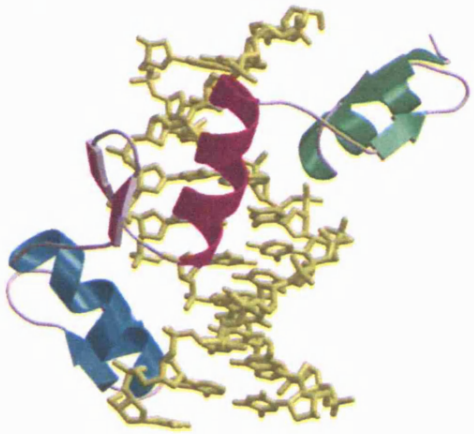


Figure 3.2 Three dimensional representation of zif268. Zif268 is an 82 or 88KDa protein and consists of three zinc fingers (blue, green, purple) which are responsible for binding on specific DNA sequences to cause the transcription of a number genes. (Adapted from http://www.biochem.ucl.ac.uk/bsm/nucplot/1zaa_molscript.html)

3.1.2 Zif268 expression

Zif268 mRNA is constitutively expressed at low levels in a number of brain regions, including the amygdala, cerebellum, entorhinal cortex, neocortex, nucleus accumbens and the striatum, (Christy et al, 1988; Mack et al, 1990; Schlingensiepen et al, 1991; Worley et al,

1991; Herms et al, 1994). Zif268 expression also occurs in the hippocampus (Saffen et al, 1988; Hughes et al, 1992) where it has been implicated as a key protein involved with synaptic plasticity including long term potentiation (LTP) and learning and memory (Jones et al, 2001; Tischmeyer and Grimm, 1999). Hippocampal zif268 was found to be expressed in the CA1 region with less in the CA2 region and minimally expressed in the dentate gyrus. Induction of LTP by stimulation of the perforant path results in massive upregulation of zif268 in the dentate gyrus. Zif268 is also expressed in the spinal cord. Immunohistochemistry has shown that zif268 is lightly expressed, constitutive, and restricted primarily to lamina I at post natal day 6 (P6). However, by P11, the expression of zif268 was found to have extended ventrally into lamina III, before disappearing at P26 (Liang and Jones, 1996). In adult rats, the expression of zif268 in the dorsal horn requires noxious stimulation to the periphery. Innocuous stimulation such as light brushing does not increase the expression of zif268 in the dorsal horn. Induction of spinal zif268 expression (including superficial layers) requires a more severe input into the spinal cord (Liang and Jones, 1996) and expression has been seen after high threshold stimulations (HFS) on C-fibres (Rygh et al, 2006); HFS can also induce LTP in this region.

3.1.3 Zif268 and LTP

The induction of LTP in the dentate gyrus is accompanied by a rapid increase in the expression of zif268. The mRNA increase can occur within 10min (Wisden et al, 1990) and is dependant on the activation of the NMDA receptor. This suggests that calcium entry through the NMDA receptors elicited by LTP inducing stimuli may serve as the initial step in the signalling pathway leading to enhanced expression of the zif268 protein. Furthermore, the expression of zif268 is directly correlated with the persistence of LTP and not with the induction protocol used to induce LTP in awake animals (Richardson et al, 1992). In comparison with other IEGs, zif268 has been shown to be related to LTP. In comparison c-fos is not expressed in the dentate gyrus following LTP (Dragunow et al, 1989) whereas Jun-B and C-Jun are less reliable markers of LTP and are not always expressed during LTP. However, Schreiber et al (1991) reported a reduction in the expression of zif268 following the induction of LTP, while stimulation that failed to generate LTP increased zif268 expression. Suggestions to explain this discrepancy include the use of different anaesthetics and a difference in the LTP induction protocol used (Knapska and Kaczmarek, 2004). In addition, more recent research has demonstrated that the expression of zif268 did not change in certain regions (subfields) of the hippocampus following LTP induction (French et al, 2001). In addition, in-vivo LTP induction in the dentate gyrus was shown to increase the expression of

zif268 mRNA whereas LTP induction with high frequency stimulation on the commissural projection neurons did not change zif268 expression in CA1 pyramidal cells.

The expression of hippocampal zif268 can be increased by a number of behavioural tests including the water maze test, the elevated plus maze and open field tests (Pollak et al, 2005). The requirement of zif268 in synaptic plasticity and for the consolidation of object recognition memory was seen in zif268 knockout mice (Jones et al, 2001). Electrophysiological analysis of the dentate gyrus in the hippocampus in awake mutant mice showed that the induction of LTP was unaffected by the mutation. However synaptic plasticity could not be maintained to the same extent as in wildtype mice. In correlation to electrophysiological data, behavioural tests showed that these mice, although retaining short-term memory, did not perform well in memory tests that required memories that lasted longer than a few hours. Mutant mice were less effective at spatial memory tasks, recognition tasks, olfactory and gustatory memory.

Zif268 antisense oligonucleotides that targeted the zif268 gene in the hippocampus suggested that zif268 is important for reconsolidation of long term fear conditioned contextual memories (Lee et al, 2004). Hall et al (2001) revealed that zif268 may be important for temporal consolidation of contextual fear memories due to an increase expression of zif268 in CA1 neurons of the hippocampus after the retrieval of recently formed contextual fear memories, but not for the retrieval of older memories, suggesting a temporal limit for zif268. In addition, the retrieval of both cued and contextual fear memories were also associated with an increase in the expression of zif268 in the amygdala. This would suggest the amygdala is an important locus for supporting retrieval and acquisition of fear-related memories and suggests a role for zif268 in neuronal plasticity associated with reconsolidation of memories accompanying the retrieval process.

3.1.4 Zif268: pain and synaptic plasticity

Noxious stimulation and injury leads to central sensitisation of dorsal horn neurons and the development of allodynia and hyperalgesia. Wind-up has often been included as a form of central sensitisation and describes spinal cord amplification of nociceptive information from repetitive stimulation of C-fibre nociceptors. The physiological importance of wind-up is unclear; due to its short lasting nature wind-up has been suggested to be either 1) a correlate of central sensitisation or 2) part of normal acute pain processing (reviewed by Herrero et al, 2000).

Central sensitisation shares many features with hippocampal LTP (Miyamoto, 2006; Ji et al, 2003) including the phosphorylation of glutamate receptors (including AMPA and NMDA receptors) and the insertion of AMPA receptors into the post-synaptic density (Galan et al, 2004). The activation of the same intracellular pathways and protein kinases are also seen, including mitogen-activated protein kinase (MAPK) (Ji et al, 1999; Ji et al, 2002), protein kinase A (PKA), protein kinase C (PKC) and calcium/calmodulin-dependent kinase II (CaMKII) (Yang et al, 2004). Spinal LTP has also been reported and may be an important process that allows acute pain to shift into a more persistent pain state (Klein et al, 2004). Lamina I neurons that project to the brain from the spinal dorsal horn are critical for maintaining persistent inflammatory pain states and the formation of neuropathic pain states following spinal nerve ligation (SNL) or chronic constriction injury (CCI) (Mantyh et al, 1997; Nichols et al, 1999). These neurons have been shown to support LTP in deep dorsal horn neurons although synapse specificity has not been shown. A recent study by Ikeda et al (2006) has shown that low frequency, high threshold stimulation can induce LTP in spinal-PAG lamina I projection neurons.

Nociceptive neurons can express zif268 following most types of peripheral noxious stimulation. Herdegen et al (1991) showed that electrophysiological stimulations on the sciatic nerve at C-fibre intensity increased the expression of zif268 (reaching peak at 1h post stimulation). While noxious thermal stimulation using hot water (52 °C) induced a NMDA receptor dependent rise in the expression of zif268, little induction was seen with non-noxious warm water (Wisden et al, 1990; Rahman et al 2001). Mechanical stimulation that was capable of inducing the expression of c-fos in the dorsal horn can also induce spinal zif268 (Rahman et al, 2001), with a similar response in the trigeminal nucleus caudalis following the injection of formalin into the rat whisker pad (Otahara et al, 2003).

Although increases of zif268 in post-synaptic neurons are seen following hippocampal LTP and noxious stimulation, the importance of the molecule in pain behaviours (mechanical hyperalgesia and allodynia) is not entirely certain. Studies using mice that lacked the zif268 gene showed that while normal pain processing remained unaffected by the mutation, the generation of chronic pain states and central sensitisation were attenuated. Hindpaw inflammation with subcutaneous formalin in the mutant mice did not alter the first phase but reduced the behavioural response (paw licking and biting) to the second phase. Mechanical allodynia following CFA induced inflammation was also attenuated in comparison with wildtype controls. Furthermore, inflammation of the hindpaw in wildtype mice increased zif268 expression in the anterior cingulate cortex (ACC) (Ko et al, 2005). Previously, tetanic stimulation in the ACC was shown to enhance C-fibre evoked field potentials and nociception

in the spinal cord via a descending modulatory pathway that included the dorsal reticular nucleus (Zhang et al, 2005). Similar to other studies using mutant mice (Jones et al 2001) synaptic plasticity was altered in the study by Ko et al (2005). Theta burst stimulations on layer V neurons of the ACC that usually generates LTP in layers II and III of the ACC failed to do so in animals that lacked the zif268 gene. This led the authors (Ko et al, 2005) to suggest that zif268 in the ACC played a prominent role in long-term inflammatory pain but did not clarify the role of zif268 induced in the dorsal horn neurons.

3.1.5 SGK1: regulation and synaptic plasticity

The IEG serum and glucocorticoid inducible kinase (SGK) consists of three isoforms (SGK1-3) (Kobayashi et al, 1999) and is expressed in a number of tissues including the spinal dorsal horn. Its activation can be modulated by serum and glucocorticoids. Neuronal SGK1 can regulate both AMPA and kainate receptors as well as glutamate transporters, which are important for the removal of excess glutamate from the synaptic extracellular space. The balance of glutamate transporters in the synapse is important, the lack of glutamate transporters can lead to both neurotoxicity and altered synaptic efficacy. Although studies using zif268 knockout animals and microarray studies have shown that SGK1 expression can be negatively regulated by zif268 in the brain (James et al, 2005, 2006), it was unclear if spinal SGK1 had a part in persistent inflammatory pain. SGK1 can be phosphorylated at a number of locations and can itself phosphorylate a large number of other proteins regulating transcription. In order to become fully active, SGK1 needs to be phosphorylated at several residues. Phosphorylation of SGK1 is partly achieved by PDK1 (PI3-dependent kinase 1) and PDK2. PDK2 is responsible for the phosphorylation of serine422 which facilitates the phosphorylation of threonine256 by PDK1. SGK1 can also be activated and phosphorylated by ERK5 at serine78 (Lang and Cohen, 2001) and by ERK1/2 pathway (Lee et al, 2006).

SGK1 in the hippocampus may be involved with spatial memory consolidation and reconsolidation. SGK1 expression in the hippocampus increased during both the consolidation and reconsolidation phase following contextual fear conditioning. Levels of SGK1 also increased after 1h and returned to baseline by 24h. During reactivation of consolidated memories the expression of SGK1 increased to a similar level to those seen during consolidation (von Hertzen and Giese, 2005), although the expression of SGK1 was not specifically regulated after contextual fear conditioning, SGK1 is important for hippocampal memories (Tsai et al, 2002) and requires the activation of the ERK1/2 pathway (Lee et al, 2006). Animals that were initially classified as fast or slow learners were found to express SGK1 (mRNA) at different levels in the dorsal hippocampus. Fast learners are by

definition animals that can find the hidden platform in the water maze test within 30 seconds before the third session. In comparison, slow learners only find the platform during the seventh or eighth session and not before. The levels of SGK1 were found to be higher in fast learners in areas such as the CA1, CA2 and the dentate gyrus. Furthermore, DNA transfection of SGK1 into the hippocampus facilitated water maze spatial learning, while transfection of mutant SGK1 DNA hindered learning; an effect that can be reversed by enrichment training. SGK1 is also critical for novel object recognition and enrichment training and is upregulated in hippocampal neurons during these events (Lee et al, 2003). The transfection of mutant DNA (a dominant-negative mutant of SGK1) that inhibits the actions of SGK1 in the hippocampus blunted the ability of the animal to perform well in these tasks (relative to the control group). Antagonists targeting the AMPA receptor in the hippocampus prevented both spatial learning and SGK1 upregulation, suggesting SGK1 expression is dependent on the activation of AMPA receptors. Using a dominant-negative mutant of SGK1 (DNA) it was found that SGK1 also can impede the expression of LTP, while the constitutively active SGK1, SGKS422D, up-regulated postsynaptic density-95 (PSD-95) expression in the hippocampus. (Ma et al, 2006)

Using oligonucleotides with the same sequence that successfully reduced the levels of zif268 in other studies (Lee et al, 2004; Malkani et al, 2006), I have assessed the importance of spinally expressed zif268 on the induction and maintenance of inflammatory pain states. Furthermore since SGK1 expression is regulated by zif268 in the brain, I also investigated if spinal SGK1 is affected by changes in the expression of zif268 during inflammatory pain states.

3.2 METHODS (see chapter 2 for details)

3.2.1 Temporal expression of zif268 expression after inflammation

To determine the time-line of zif268 expression following peripheral inflammation, adult rats (280g) were either perfused without peripheral stimulation (naïve) or at 1h, 2h, 6h and 24h after CFA injection into the intraplantar region of hindpaw. Animals were perfused and 40μm sections were then taken from the spinal lumbar region L4-L5 and immunostained for zif268. Sections were counted automatically using MCID software specifically calibrated for counting zif268 positive cells. Cell counts were made to the ipsilateral medial half of the superficial layer of the dorsal horn.

In order to make lamina specific cell counts lamina boundaries were defined by using bright-field microscopy and MCID software. Under bright-field microscopy it was possible to distinguish lamina II (owing to the lack of myelination). Two boundary markers were drawn using MCID across the dorsal horn. The first separated lamina I and lamina II and the second was drawn at the point between lamina II and lamina III. Thus, it was possible to distinguish lamina I and lamina II. Deeper lamina were more difficult to differentiate but cells lying within 150μm from the second boundary marker of lamina II (the most ventral marker) were regarded as lamina III-V.

In order to determine if zif268 expression occurred in lamina 1 projection neurons to the parabrachial nucleus, adult rats received bilateral injections of 5% fluorogold (FG) into the parabrachial nucleus under halothane anaesthesia. Animals were then injected intraplantarly into the hindpaw 4d afterwards with complete Freund's adjuvant (CFA, 50%, 100μl) and perfused 2h later with 4% paraformaldehyde. The time point at which rats were perfused coincided with peak expression of zif268 protein in the dorsal horn. 40μm sections were then taken from the spinal lumbar region L4-L5 and immunostained for zif268. Sections were then analysed for colocalisation with FG in the marginal layer of the dorsal horn.

3.2.2 Intrathecal catheterization and drug delivery.

Osmotic pumps were implanted sub-dermally. Pumps were connected to a PE10 catheter that was inserted at the level of the lumbar enlargement under halothane anaesthesia. For experiments using zif268 oligonucleotides, the antisense sequence was 5'-GGT-AGT-TGT-CCA-TGG-TG*G-3' and the missense was 5'-GTG-TTC-GGT-AGG-GTG-TC*A-3'. Each pump administered 0.16μg/μl/h of oligonucleotide dissolved in 0.9% saline for 7d. A vehicle

control (saline) group and a no pump group (naïve) was also included into this study to ensure that saline or the pump itself did not have any untoward effects on pain behaviours. Following behavioural tests, animals were perfused and examined for catheter placement and damage to spinal cord. Animals with damaged cords and displaced cannula were discarded from the study (also see Methods and Materials). Oligonucleotides were thio-capped (*) to reduce oligonucleotide degradation following intrathecal injection. Phosphothioate modification replaces an oxygen atom with a sulphur atom in the internucleotide linkage of DNA. This enables the oligonucleotide to be more resistant to nuclease degradation than natural DNA, while maintaining the ability to bind onto complementary nucleic acid sequences.

3.2.3 Behavioural tests and statistical analysis

Animals belonging to each treatment group (naïve, saline, missense, antisense) were habituated to the test environment before surgery. 3d following surgery, the baseline score to ramped mechanical stimulation was taken (mean of 4 scores taken, each separated by a minimum of 5 min). On the third day, rats received a unilateral intraplantar injection of CFA (100µl, 50%) into the hindpaw. Over the following 4d, animals were tested for mechanical allodynia. The change in paw withdrawal thresholds from the baseline score was used as a measure of mechanical allodynia and hyperalgesia. Comparisons were made between the baseline score for each group with the respective group behaviour scores for each day following CFA treatment. Tests were statistically analyzed using two-way ANOVA (repeated measure) and Tukey's post hoc test.

3.2.4 Immunocytochemical staining for oligonucleotide toxicity

Following oligonucleotide administration and behavioural analysis, rats were deeply anaesthetized with pentobarbital and transcardially fixed with 400-500ml of 4% paraformaldehyde. The lumbar spinal cords were dissected, and postfixed for 4h, left overnight in 30% sucrose and 40µm transverse sections cut on the microtome. Immunocytochemical staining was used to detect zif268, neuronal-specific nuclear protein (NeuN), and NK1 (see table 2.2 for concentrations). Sections were also stained for Hoescht and IB4 to check for toxicity.

3.2.5 Immunocytochemical staining for zif268

Osmotic pumps containing zif268 antisense or missense were implanted into adult rats sub-dermally. 4d later, rats received a unilateral injection of CFA (50%, 100µl) into the intraplantar hindpaw and were perfused 2h later. Luminosity scores were made on an MRC

Bio-Rad 600 confocal microscope. This technique makes the assumption that the greater the luminosity then the more protein is being expressed by the cell. Sections from the L4-L5 region of the spinal dorsal horn were taken from animals treated with either zif268 antisense or missense. Sections were immunostained for zif268 and the luminosity scores were ascertained from 10 sections per animal. Since the background for each section varied slightly, sections were normalised by subtracting the background value taken from adjacent neuropil of that section. The mean from the twenty highest scores from each section were taken and statistical comparisons were made to the other treatment group.

3.2.6 Western blotting for zif268 and SGK1

Following 4d of zif268 antisense and missense treatment (delivered via osmotic pumps), the dorsal horn of animals stimulated by 2h intraplantar CFA were analysed using western blot techniques to examine the effectiveness of the antisense knockdown and to look for any changes to the expression levels of SGK1 protein (also see Methods and Materials). Signal intensity (density) was quantified using the software package Quantity One 4.4.1 (Bio-Rad). Densitometric values were expressed as the ratio of zif268 or SGK1 to GAPDH (loading control). Statistical comparisons between the ratio scores of missense and antisense treatment were then assessed using an unpaired t-test.

3.3 RESULTS

3.3.1 Zif268 expression following CFA

Zif268 positive cells were counted (using MCID software) in the superficial layers and deeper regions of the dorsal horn. Counts were restricted to the medial half of the dorsal horn that represented the region of hindpaw innervation. Unstimulated (naïve) dorsal horn neurons, in general, were void of zif268 expression (10 +/- 1 cells per 40μm section in the marginal layer per section). However, in comparison with naïve animals the intraplantar injection of CFA was associated with a transient rise in zif268 expression in the dorsal horn. Significant (Mann Whitney U test, $P<0.05$) increases were seen at 1h (69 +/- 7 cells per 40μm section in the marginal layer per section) and at 2h where zif268 expression peaked (84 +/- 9 cells per 40μm section in the marginal layer) (Mann Whitney U test, $P<0.05$) before gradually returning to basal levels after 6h (Fig 3.3) where no significant differences were seen compared to naïve animals ($P=0.10$). No zif268 expression was seen in the ventral horn either in the naïve or CFA injected animals at any of the time points analysed above. Zif268 expression was present from L4-L6. Analyses beyond these lumbar segments were not made. Zif268 expression followed a similar time course across L4-L6.

3.3.2 Expression pattern of zif268 in the dorsal horn

Zif268 expression was induced by an injection of CFA into the intraplantar region of the hindpaw and analysed using immunohistochemistry at peak expression (2h). Substantial increases of zif268 expression were seen in the ipsilateral dorsal horn and the majority was in the medial half of the dorsal horn. This is not surprising as this region more accurately represents the region of innervation of the intraplantar region of the hindpaw that was stimulated. In addition, zif268 expression in terms of individual laminae was strongest in lamina I, II (outer) and lamina II (inner). Lamina I expressed 19 +/- 4 cells and lamina II (combined (inner and outer)) expressed 65 +/- 8 cells. In comparison, zif268 expression in deeper layers of the dorsal horn (lamina III, IV and V) were less intense and offered a scattered distribution with no expression in the ventral horn (Fig 3.3). Combined, these deeper layers of the dorsal horn expressed 34 +/- 10 cells following 2h of CFA injection. Statistical analysis (Mann Whitney U test) comparing the expression of zif268 in the superficial layers (lamina I and II) of the dorsal horn with deeper regions of (lamina III-V) showed a significant difference ($P<0.05$).

3.3.3 Zif268 expression in lamina I projection neurons

Bilateral injections of fluorogold into the parabrachial nucleus labelled a subset of neurons in the marginal layer of the spinal dorsal horn (lamina I projection neurons). Immunohistochemistry revealed that 47 +/- 8 % of these neurons expressed zif268 following CFA induced peripheral inflammation (Fig 3.4 and see chapter 5).

3.3.4 Zif268 antisense blunts the maintenance of inflammatory pain states

To examine the importance of spinal zif268 in persistent inflammatory pain states, animals were implanted with mini-osmotic pumps that either contained oligonucleotides that targeted zif268 mRNA, missense or saline. Treatment baseline scores were similar (one way ANOVA) with each other prior to CFA injection ($F_{3, 40} = 0.7$, $P = 0.5$) Animals then received a unilateral injection of CFA into the intraplantar surface of the hindpaw. Statistical analysis (paired t-tests) indicated that on the first day after CFA all treatment groups displayed allodynia to mechanical stimuli ($P<0.005$). Percent change in paw withdrawal thresholds on the first day after CFA were 36 +/- 8% for naïve, 37 +/- 4% for saline, 50 +/- 5% for missense, and 30 +/- 6% for antisense treatment. Further statistical analysis (two way ANOVA repeated measures) revealed that paw withdrawal thresholds remained stable for the duration of the study (days 1-4 after CFA) ($F_{2, 74} = 2.6$, $P = 0.08$) with no interactions occurring between time and treatment ($F_{6, 74} = 1.9$; $P = 0.10$). In addition, an overall effect of treatment was seen ($F_{3, 37} = 13.2$; $P<0.001$) that affected paw withdrawal thresholds. Tukey's post-hoc testing (HSD) revealed that overall zif268 antisense treatment significantly attenuated mechanical allodynia following peripheral inflammation (antisense in comparison to naïve, $P<0.005$; to saline, $P<0.05$; to missense, $P<0.005$). Analyses of individual days, revealed that mechanical sensitivity on day 1 were similar across all treatment groups. Two days following CFA injection, mechanical allodynia and hyperalgesia was significantly reduced in antisense treated animals when compared with saline (21% reduction, $P<0.03$), missense (35% reduction, $P<0.05$) and CFA only treated animals (40% reduction, $P<0.005$) (Fig 3.5). A similar result was seen on day 4 post CFA where mechanical allodynia and hyperalgesia were fully attenuated in the antisense treated group. Importantly, throughout the duration of the behavioural experiment, no difference was seen between the naïve (no pump) group, the saline treated group and the missense treated group indicating that the implantation of the pump did not affect the paw withdrawal thresholds to mechanical stimuli. Paw withdrawal thresholds of the naïve group were not significantly different to missense ($P = 0.98$) or the saline group ($P = 0.28$) and the missense group was not different to saline group ($P = 0.12$).

3.3.5 Zif268 antisense reduces zif268 protein expression in the dorsal horn

The effectiveness of zif268 knockdown was assessed using immunohistochemistry and western blotting. Animals were treated with zif268 oligonucleotides in the same manner as for behaviour and were followed by a unilateral injection of CFA for 2h. Analysis of zif268 inmmunoreactivity in the superficial layers of the dorsal horn revealed that in comparison with missense (70 +/- 14 cells per 40μm section) antisense treatment significantly reduced (unpaired t-test, $P<0.05$) the number of zif268 positive cells (48 +/- 9 cells per 40μm section). In addition, cell luminosity was also significantly reduced (unpaired t-test, $P<0.05$) following zif268 antisense treatment in comparison with missense treatment (relative luminosity, 85 +/- 9 compared with 53 +/- 9) (Fig 3.6). In agreement with cell counts western blot analysis (Fig 3.7) also revealed a significant reduction (38%, unpaired t-tests $P<0.05$) in zif268 expression in animals treated with antisense in comparison with missense

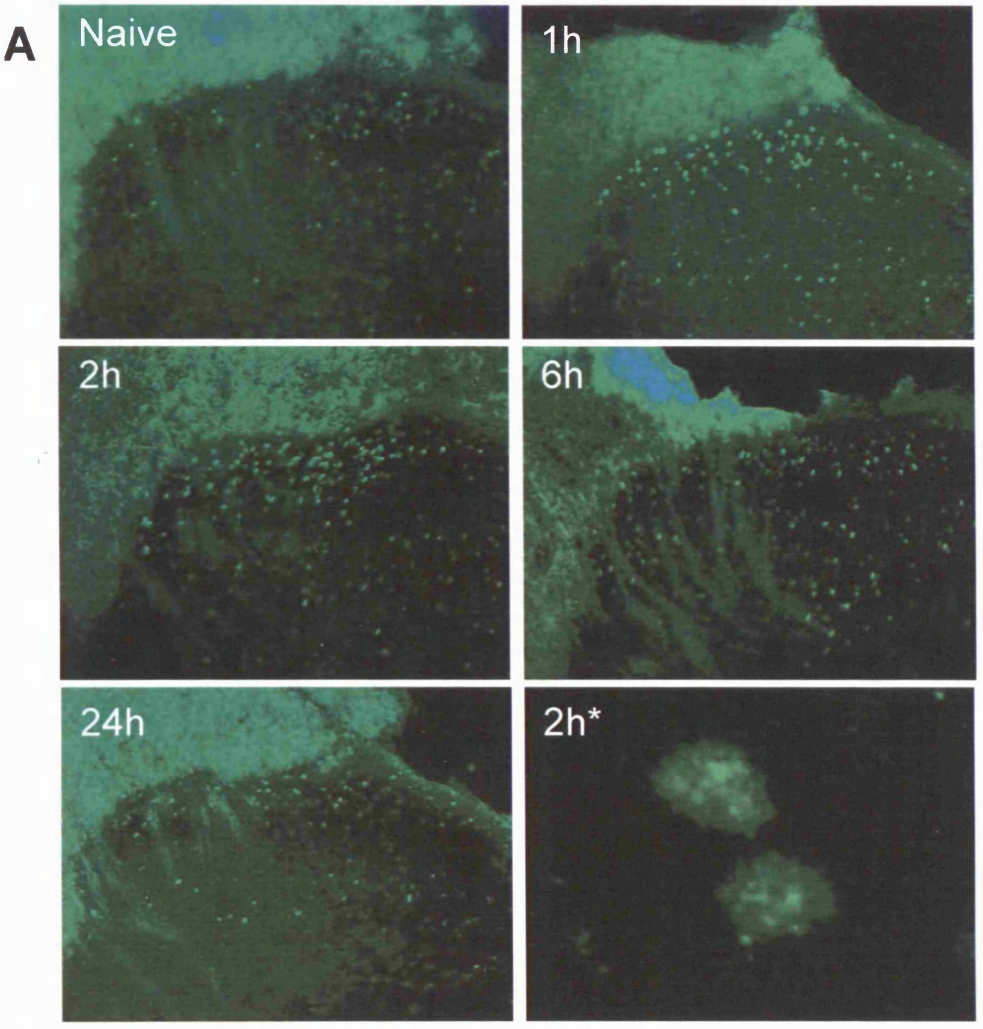
3.3.6 Lack of toxicity and cell death using antisense

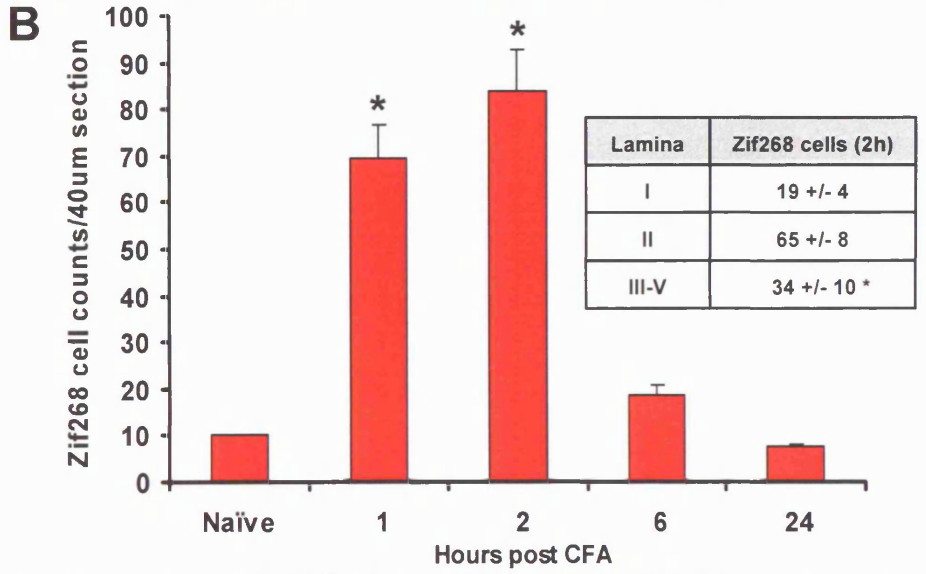
To examine if oligonucleotide treatment resulted in unwanted tissue toxicity or damage, spinal cord tissue was stained for IB4, NeuN and NK1. In the majority of cases, no gaps in the IB4 staining were present. Under normal circumstances IB4 staining is observed as a distinct band that spans across the entire lamina II region of the dorsal horn (Fig 3.5). Any gaps or disruptions in the IB4 staining represented neuronal cell loss caused by primary afferent damage. Animals with damaged spinal cords were removed from the experiment and behavioural data collected discounted ($n = 3$). Cell counts made in the superficial layers of the dorsal horn using NeuN revealed no oligonucleotide toxicity. The number of NeuN cells was similar across all treatment groups (naïve 730 +/- 46; missense 640 +/- 58; antisense 710 +/- 51 per 40μm section). Furthermore, Hoescht labelling did not detect any pyknotic nuclei in any of the groups. NK1 expressing cells are important for persistent inflammatory pain. Owing to their proximity to the catheter, NK1 cells would be most susceptible to any toxic effects. This would have dramatic effects on nociceptive behaviours. We found that NK1 positive cells were healthy in all groups and no toxic effects were in evidence (Fig 3.8).

3.3.7 Zif268 regulates the expression of SGK1

The significant reduction (29%, $P<0.05$) of zif268 expression in the spinal cord following zif268 antisense treatment also had a profound effect on the levels on serum and glucocorticoid inducible kinase 1 (SGK1) in the dorsal horn. SGK1 expression was

significantly ($P<0.05$) increased in the zif268 antisense treated group in comparison with the missense group indicating a negative regulatory role of zif268 on SGK1 expression 2h following injection of CFA into the intraplantar hindpaw (Fig 3.9).





Lamina	Zif268 cells (2h)
I	19 +/- 4
II	65 +/- 8
III-V	34 +/- 10 *

Figure 3.3. Time course of zif268 following peripheral inflammation. Animals were perfused and immunostained for zif268 at different time points following a CFA injection into the intraplantar region of the hindpaw. A) Representative images of animals immunostained at different time points. 2h* shows nuclear staining of zif268 at 2h following CFA treatment. B) Cell counts of zif268 positive neurons in the medial portion of the dorsal horn (superficial layer). Naïve rats expressed little zif268. However following CFA induced inflammation, zif268 expression increased dramatically within 1h, peaked at 2h before dropping to baseline levels at 6h. B, inset table) indicates that 2h following CFA treatment more zif268 positive cells are expressed in the marginal layers (lamina I and II) in comparison to deeper regions of the dorsal horn (lamina III-V) (Confidence levels in comparison with naïve group. Mann Whitney U test * $P<0.05$, 10 sections counted per animal).

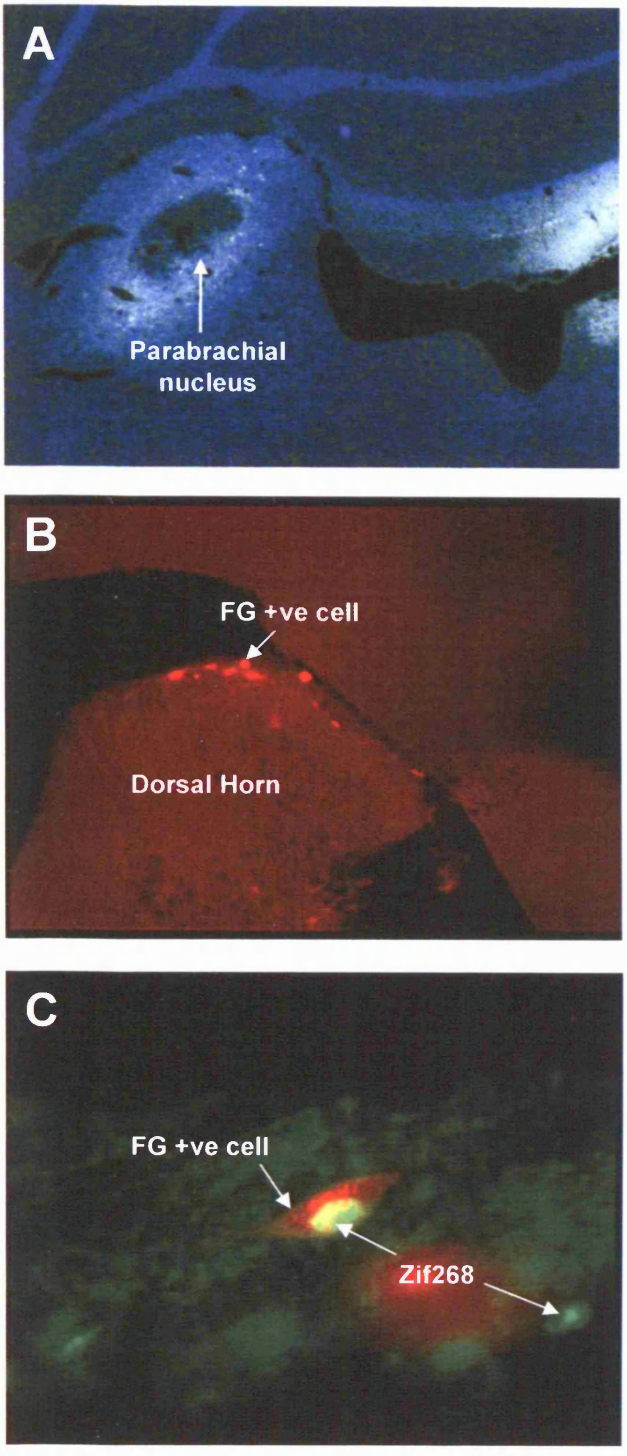


Figure 3.4. Lamina I projection neurons to the parabrachial nucleus (LI-Pb) express zif268 during inflammation. A) Image taken from a rat brain section showing site of Fluorogold (FG) injection into the parabrachial nucleus (Pb). B) FG injection successfully labelled LI-Pb projection neurons in the dorsal horn. C) Image showing zif268 expression (green) in the dorsal horn following intraplantar CFA injection. Zif268 expression was also found to be expressed in LI-Pb projection neurons (red) (with zif268 seen as yellow). Cell counts revealed that 47% of LI-Pb projection neurons expressed zif268 (n = 5).

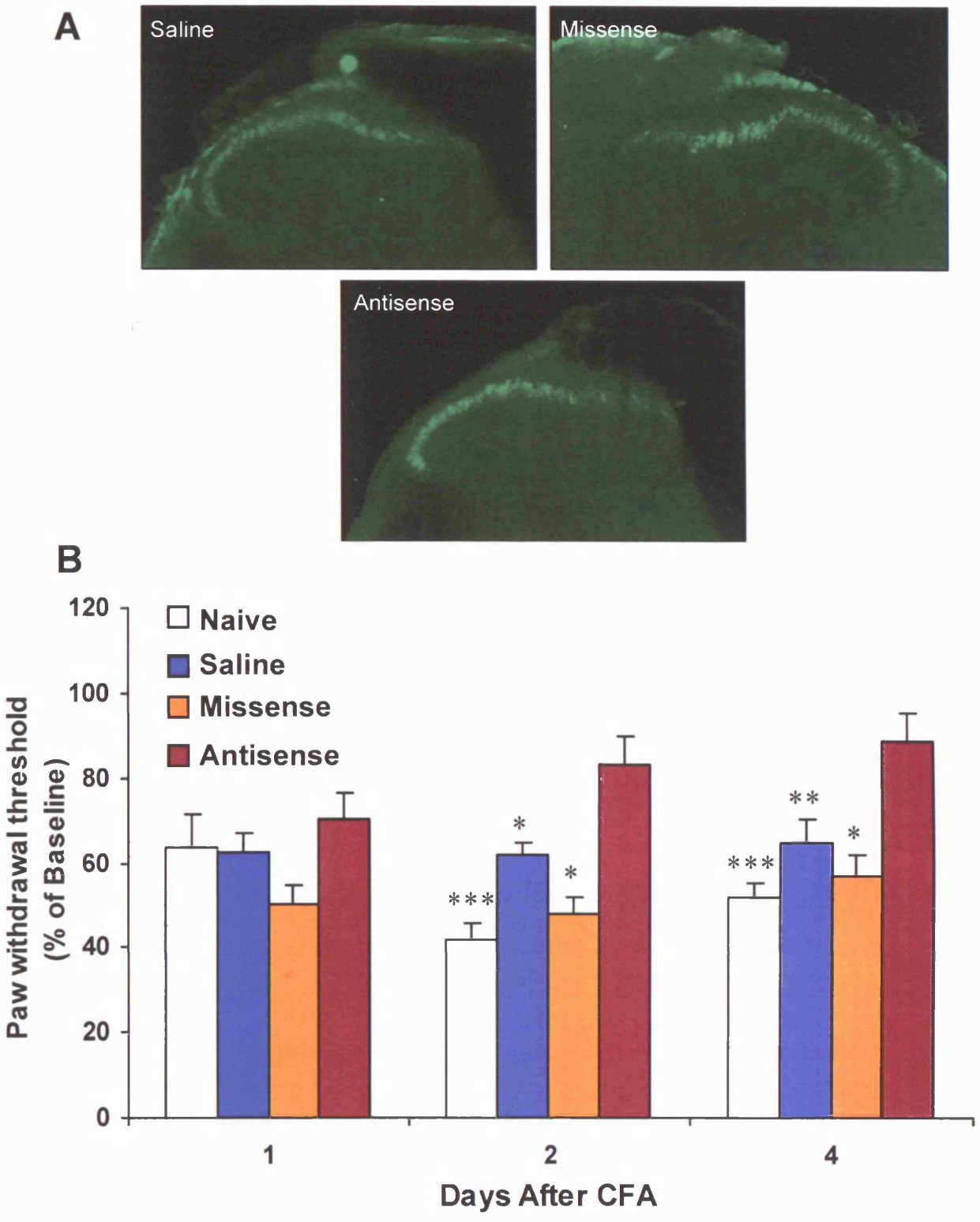


Figure 3.5. Zif268 contributes to the maintenance but not the induction of mechanical allodynia in the CFA model. A) IB4 was chosen to assess primary afferent damage following surgery and the intrathecal implantation of osmotic pumps. Representative images showing IB4 staining in the dorsal horn. Only animals without primary afferent damage were included into the behavioural studies. B) Comparison of the mechanical sensitivity (allodynia) of CFA injected animals receiving pumps containing saline (blue bar, n = 10), mismatch ODN (orange bar, n = 12), antisense ODN (purple bar, n = 12) or no pump (open bar n = 7). Animals receiving zif268 antisense treatment displayed markedly reduced mechanical allodynia and hyperalgesia. Data are plotted as percentage of baseline response compared with antisense treated group (Two way ANOVA repeated measure * $P<0.05$, ** $P<0.01$, *** $P<0.001$).

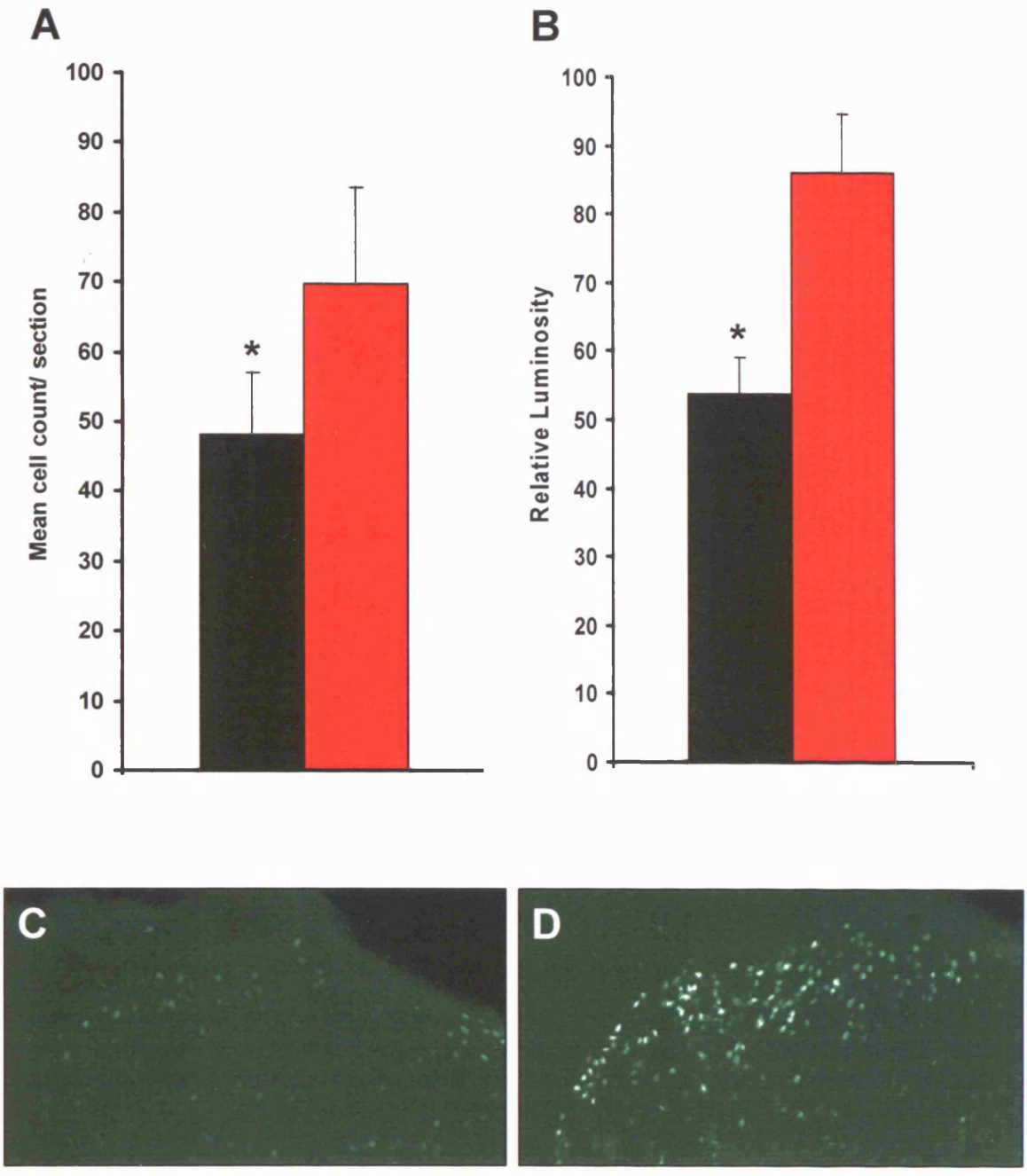


Figure 3.6. Analysis of sections treated with zif268 antisense or missense after intraplantar CFA injection into the hindpaw (2h, 100µl, 50%). (A) Immunohistochemical techniques indicated a significant difference between the mean number of cells positive for zif268 per 40µm section following treatment with either zif268 antisense (black bar) or missense (red bar). (B) The difference in relative luminosity of cells treated with either zif268 antisense (black bar) or missense (red bar) relative to background. (C,D) Representative example sections treated with (C) antisense or with (D) missense (Unpaired t-test * $P<0.05$, $n = 8$).

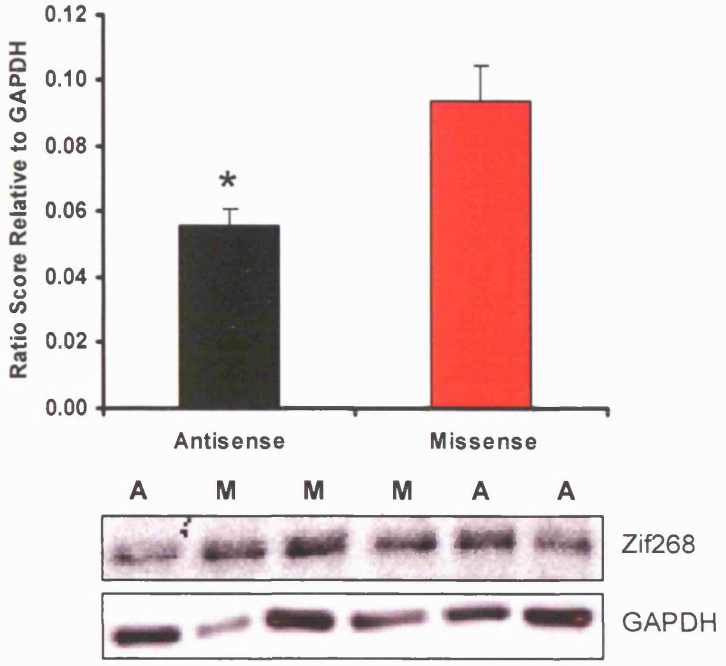


Figure 3.7. Verification of zif268 knockdown. Rats were implanted with mini-osmotic pumps containing oligonucleotides that targeted zif268 mRNA in the spinal dorsal horn. Nominal infusion rate was 0.5µg/µl/h. Animals were infused for 4d before receiving an intraplantar injection of CFA (2h, 100µl, 50%) into hindpaw. Ipsilateral dorsal horn quadrants were freshly dissected and analysed using western blotting techniques. Zif268 protein levels were significantly lower in animals treated with the zif268 antisense in comparison with missense treatment (Confidence levels in comparison with missense. Unpaired t-test * P <0.05, AS: n = 4, MS: n = 4).

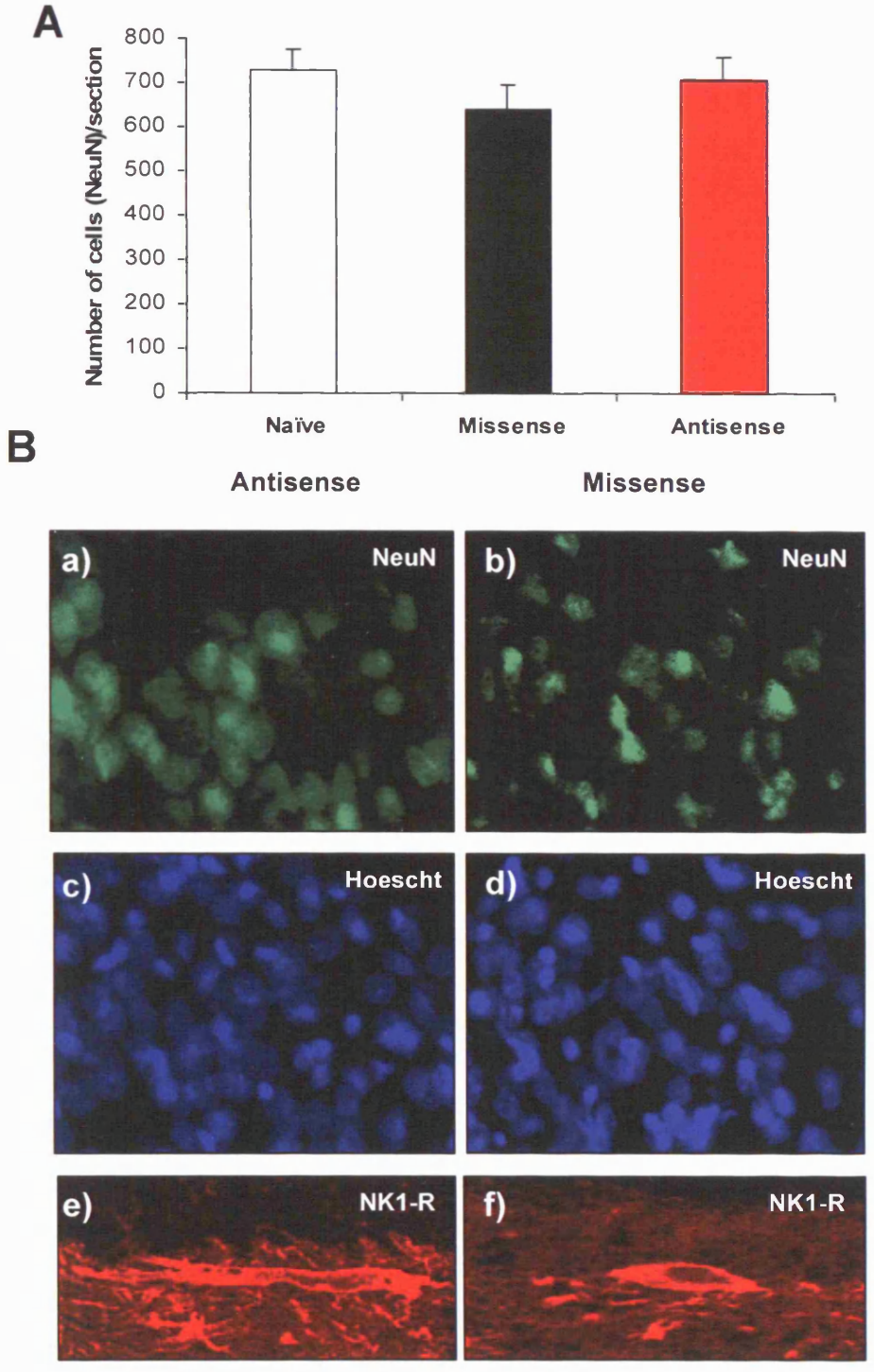


Figure 3.8. No toxic effect of zif268 antisense or missense on dorsal horn neurons. Animals received chronic intrathecal infusion of zif268 antisense or missense oligonucleotides via mini-osmotic pumps followed by an intraplantar injection of CFA for 4d. Sections of spinal cord were immunostained for NeuN, and NK1 and stained for Hoescht. A) NeuN positive cells located in lamina I and II were automatically counted using MCID software. No significant differences were found between naïve, antisense or missense treated animals. B) Representative images showing (a) NeuN staining, (c) Hoescht staining and (e) NK1-R immunostaining for zif268 antisense treated animals and (b) NeuN staining, (d) Hoescht staining and (f) NK1-R immunostaining for missense treated animals. Hoescht staining did not pick up any pyknotic cells in either the antisense or the missense group. NK1 staining was similar in both antisense and missense treated rats. Cells appeared healthy and intact (Mann Whitney U test n = 5, 10 sections per animal).

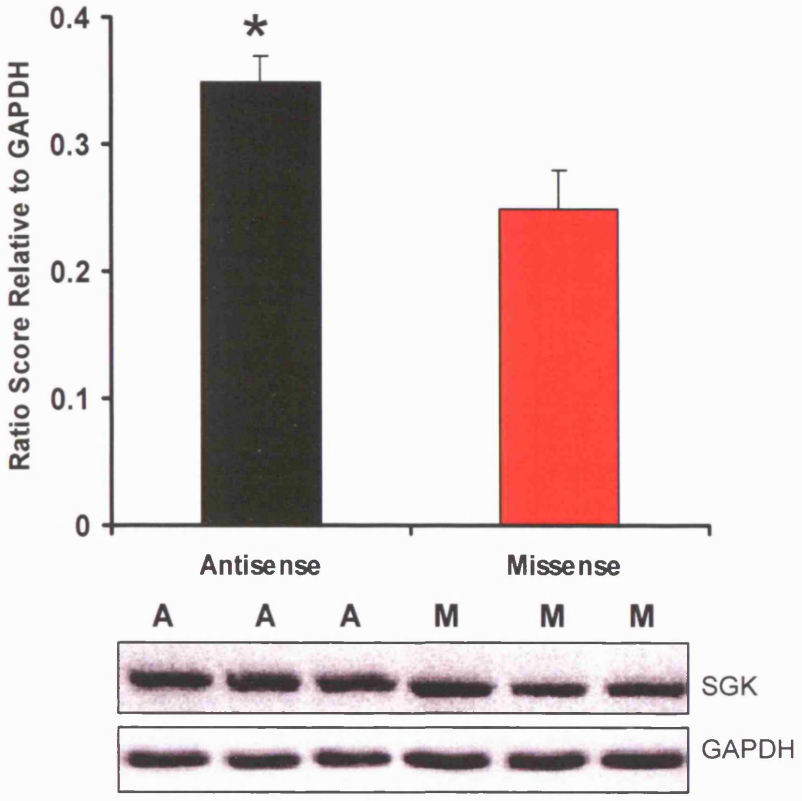


Figure 3.9. Zif268 negatively regulates the expression of SGK1. Rats were implanted intrathecally with mini-osmotic pumps containing oligonucleotides that targeted zif268 mRNA. Nominal infusion rate was 0.5µg/µl/h. Animals were infused for 4d before receiving an intraplantar injection of CFA (2h, 100µl, 50%) into hindpaw. Ipsilateral dorsal horn quadrants were freshly dissected out and analysed using western blotting techniques. SGK protein was significantly higher in animals treated with zif268 antisense in comparison with missense treatment (Confidence levels in comparison with missense. Unpaired t-test * $P<0.05$, AS: n = 6. MS: n = 6).

3.4 DISCUSSION

3.4.1 Zif268 expression: dorsal horn and lamina I projection neurons

This study has shown that an injection of CFA into the hindpaw can transiently increase the expression of zif268 in selective neurons throughout the dorsal horn. Antisense treatment targeting zif268 suggests a functional role for this protein in the maintenance of persistent inflammatory pain states and the repression of SGK1 expression. The pre-treatment of zif268 antisense did not alter the normal acute pain processing.

The expression of the IEGs c-fos and zif268 can be induced in the dorsal horn following noxious peripheral stimuli or HFS on C-fibres. The expression of zif268 is strongly linked to the successful establishment of LTP in both the hippocampus and the dorsal horn. The ablation of lamina I/NK1 projection neurons reduces both spinal LTP and zif268 expression (Rygh et al, 2006). This strongly suggests that zif268 expression is involved with long-term changes in pain sensitivity. In support of this, inflammatory hyperalgesia was attenuated in studies using zif268 knockout mice (Ko et al, 2005). In the hippocampus zif268 knockout mice were unable to maintain LTP (after 24h) and learning and memory type behaviours were severely inhibited in comparison to wildtype mice (Davis et al., 2003). Therefore, it is likely that zif268 drives a chain of molecular events that lead to long-term changes in synaptic efficacy. Since protein expression is vital for maintaining LTP, the data strongly suggest that gene expression leading to physiological changes in neurons belonging to the superficial layers of dorsal horn is crucial for maintaining long-term changes in sensitivity. This is a hypothesis that is taken up in a later chapter.

3.4.2 Antisense treatment reveals a role for zif268 in inflammatory pain

Zif268 is expressed in a number of regions in the nervous system. Although previous work using a global knockout (Ko et al, 2005) have shown the importance of zif268 in persistent inflammatory pain, our work using intrathecal zif268 antisense has helped pin-point the spinal cord as an anatomical loci where zif268 modulates the transition from acute pain to a persistent pain state. Under normal conditions the adult spinal dorsal horn does not express zif268 except following noxious stimulation or HFS. Zif268 expression was also seen in lamina I projection neurons following peripheral inflammation. Previous studies (Mantyh et al, 1997; Nichols et al, 1999; Ikeda et al, 2006) have shown that these neurons are vital for the maintenance of persistent inflammatory pain. The expression of zif268 here could modulate

transcriptional events that are crucial for setting-up long term changes in synaptic efficacy and pain behaviours.

Synaptic plasticity such as LTP in hippocampal neurons requires the expression of zif268. Recent studies have shown that synaptic plasticity may be dependent on the levels of proteosomic activity in the synapse and that proteosome expression itself is dependent on zif268. The inhibition of proteosomes can prevent long term facilitation in *Aplysia* (Hegde et al, 1997) and can have negative effects on long-term memory in the hippocampus (Lopez-Salon et al, 2002). Through protein degradation, proteosomes are responsible for regulating protein composition in the post-synaptic density and therefore vital for the efficacy of synaptic transmission. Microarray studies (James et al, 2005) have shown that the activation of zif268 can modulate the expression of 26S (a proteosome protein). It has been proposed that post-synaptic proteosome activity suppresses some forms of synaptic plasticity (Zhao et al, 2003). The repressive nature of zif268 was further highlighted with the use of the zif268 knockout mice. Proteosomes are heteromeric complexes that are made up from a number of subunits. Using the zif268 knockout mice it has been noted that the expression of some proteosome subunits were increased in the zif268 knockout mouse in comparison with the wildtype (James et al, 2006). This report would indicate that by decreasing proteosomic activity, zif268 could aid in synaptic plasticity and the generation of persistent pain states. In support of this, a study has also shown that protein kinase A (PKA) was subject to rapid degradation by proteosomes (James et al, 2005). PKA may be involved with persistent inflammatory pain since increased phosphorylation of PKA occurs ipsilaterally in the dorsal horn during long term CFA treatment and the use of selective PKA inhibitors (KT-5720) applied intrathecally can attenuate thermal hyperalgesia (Yajima et al, 2003). Therefore zif268 by preventing the degradation of certain key synaptic proteins at specific synapses could help facilitate persistent pain.

3.4.3 Regulation of SGK1 by zif268

Data from this study has shown that SGK1 in the dorsal horn can be negatively regulated by spinal zif268. SGK1 can enhance the expression of EAAT4 in cerebellar climbing fibres by protecting the glutamate transporters from degradation (Bohmer et al, 2004). EAAT4 can offer a neuroprotective role by preventing glutamate neurotoxicity by regulating levels of glutamate in the synaptic cleft. Therefore it is possible that zif268 by decreasing the levels of SGK1 can also reduce the expression of EAAT4. Although this would increase the risk of neurotoxicity it can also enhance synaptic efficacy by activating mGlu receptors lying in the perisynaptic region (Wadiche and Jahr, 2006). SGK1 also positively regulates the EAAC1

(EAAT3) glutamate transporter in retinal tissue (Schniepp et al, 2004). EAAC1 like EAAT4 prevents excess glutamate accumulation from occurring, importantly dorsal horn neurons, glial cells and the DRG express EAAC1 (Shashidharan et al, 1997). Furthermore, EAAC1 expression has been shown to be downregulated in the dorsal horn following rhizotomy (Tao et al, 2004) and CC1 (Wang et al, 2006) and may well play a role in other long lasting pain states.

Another mechanism by which SGK1 could affect synaptic plasticity is by modulating the expression of stargazin. Recently, stargazin has been implicated in the expression of formalin induced inflammatory pain (Tao et al, 2006). Stargazin is an AMPA receptor interacting protein that regulates the targeting and clustering of AMPA receptors in the post-synaptic membrane. Stargazin can also directly alter the biophysical properties of AMPA receptors by reducing the desensitization and deactivation of the receptor (Tomita et al, 2005, Priel et al, 2005). Stargazin contains a consensus sequence that can bind SGK1. However, it would appear that SGK3 at least in xenopus oocytes and in hippocampal neurons is more effective at binding this motif in comparison with SGK1 (Strutz-Seebohm et al, 2005; Strutz-Seebohm et al, 2006).

During CFA induced inflammation, GluR6 (mRNA) is upregulated (2 to 5h post stimulation) in the dorsal horn. The attenuation of thermal hyperalgesia can be seen with the intrathecal use of antagonists aimed at GluR6 (Guo et al, 2002). Since SGK1 can modify the expression of GluR6 (a kainate receptor subunit) in hippocampal neurons (Strutz-Seebohm et al, 2005), spinal SGK1 may also enhance GluR6 expression in the dorsal horn.

The expression levels of hippocampal glucocorticoid receptors (GR) have been shown to be modulated by epigenetic factors. Offspring that receive maternal attention such as licking and grooming have a higher expression of GR in comparison to littermates that do not (Weaver et al, 2004). Since zif268 can bind onto the GR promoter the authors have suggested that GR expression may be modulated by zif268. In a similar manner it would be interesting to investigate if GR modulation in the dorsal horn can be achieved by spinally expressed zif268 and whether GR has a role in persistent inflammatory pain states (see chapter 5).

Although changes in synaptic sensitivity of central neurons are important for the full expression of long term inflammatory pain, continuous peripheral input to these neurons may be needed for the maintenance of this pain. Indeed protein expression in the DRG changes with time. A recent study (Inglis et al, 2005) has revealed that during CFA induced inflammation of the knee, the TNF receptors, TNFR1 and TNRF2, are chronically increased

(mRNA) but at different anatomical locations in the DRG. TNFR1 expression increased bilaterally in DRG neuronal cells for 7d while TNFR2 increased ipsilaterally on macrophages (but not in neuronal cells). Etanercept, a TNF antagonist, administered before and (7 days) after knee inflammation reduced long term mechanical hyperalgesia. Suggesting TNF and the TNF receptors may be involved with both the induction and the maintenance phase of this pain state.

3.5 CONCLUSION

Zif268 is rapidly and transiently expressed in the dorsal horn (inclusive of lamina I projection neurons) during peripheral inflammation. The expression of zif268 in the dorsal horn maintains mechanical allodynia and hyperalgesia and is important for the repression of SGK1 in the dorsal horn

CHAPTER 4

Descending Serotonergic Modulation of Persistent Inflammatory Pain

4.1 INTRODUCTION

4.1.1 Lamina I and the spino-bulbo-spinal loop

Noxious stimulation can engage spino-bulbo-spinal circuits. From lamina I, projections to the brainstem and forebrain are thought to activate a series of pathways that converge onto the rostral ventromedial medulla (RVM). Descending pathways from RVM impinge upon the dorsal horn and regulate nociceptive traffic. Incoming nociceptive information can thus be either facilitated or inhibited depending on environmental contingencies recorded by higher brain centres.

4.1.2 Primary and secondary hyperalgesia

In 1950, Hardy et al investigated two types of experimentally produced cutaneous hyperalgesia; primary and secondary. Primary hyperalgesia occurs at the site of injury, while secondary hyperalgesia remains associated with the injury but occured in "undamaged tissues adjacent to and at some distance from the site of an injury." It is now widely accepted that mechanisms of primary and secondary hyperalgesia are, respectively, peripheral and central. The activation of capsaicin sensitive C-fibres are essential for the development of primary hyperalgesia at the site of damage and secondary hyperalgesia in areas around the site of damage. (Magerl et al, 1998; Magerl et al, 2001). Activation of capsaicin sensitive C-fibres leads to the sensitisation of dorsal horn neurons which leads, heterosynaptically, to the generation of secondary hyperalgesia and allodynia (Zeigler et al, 1999). The activation of mechanical low threshold A-fibre type (such as light touch) corresponds with mechanical allodynia and the activation of nociceptive A-fibres (e.g with pin-prick) is associated with mechanical hyperalgesia. This increase in excitability of spinal neurons (central sensitization) after peripheral injury has been extensively studied (Woolf, 1995; Woolf, 2004). It has been shown that the enhanced reflex excitability after peripheral tissue damage did not necessarily require ongoing peripheral input and that spinal dorsal horn neuron receptive fields expanded, responsiveness to suprathreshold stimuli increased, response thresholds decreased, and sensitivity to novel stimuli was acquired after peripheral injury.

Urban and Gebhart (1999) hypothesized that facilitatory influences from the brainstem significantly contribute to secondary, but not primary hyperalgesia. Intra-RVM injection of lidocaine reverses established secondary hyperalgesia, suggesting a clear role for the RVM in maintenance of secondary hyperalgesia. Other studies revealed that spinal cord transection or

cell specific lesions of the RVM prevented the development of secondary, but not primary, hyperalgesia. They argue that the evidence suggests that the RVM is important to both the development and maintenance of secondary hyperalgesia but perhaps not primary hyperalgesia.

But is RVM input necessary for central sensitization? Schaible et al (2002) examined the effect of acute inflammation of the knee joint on spinal dorsal horn neurons and reported that spontaneous activity and responses to both innocuous and noxious stimulation of the joint increased as inflammation progressed but was increased further by cold block of the spinal cord. They concluded that spinal neuron hyperexcitability associated with a peripheral inflammation was counteracted by an enhancement of descending inhibitory influences. Lidocaine injected into the midline RVM (Ren and Dubner. 1996) increased the spinal neuron response to mechanical and thermal stimulation applied to an inflamed hindpaw (complete Freund's adjuvant induced). This result was interpreted to indicate that peripheral inflammation leads to an enhanced descending inhibition. Both of these studies used models of primary hyperalgesia because stimuli were applied to injured tissue and suggest that activation by peripheral noxious inputs of descending excitation and inhibition that can modulate spinal nociceptive transmission.

4.1.3 The rostral ventromedial medulla (RVM)

Neurons in the RVM have been broadly classified into three cells types based upon their reflexive (tail flick or paw withdrawal) response to noxious heat. Electrophysiological characterisation of neurons in the RVM suggests that two cell classes could account for the inhibitory and the facilitatory drive from the RVM. 'On' cells are believed to facilitate nociception and respond by increasing their firing rate to noxious heat (just before a nociceptive reflex response). 'Off' cells are tonically active and just prior to a nociceptive reflexive response they reduce or cease firing. 'Off' cells are inhibitory and their cessation in firing allows for the successful transmission of nociceptive input into dorsal horn (Fields et al, 1983; Heinricher et al, 1989). The third group respond to mechanical stimulation (Schnell et al, 2002) and are called 'neutral' cells. Neutral cells do not initially respond to peripheral thermal noxious stimulation, but with time, are activated by stimulations made at other areas of the body. However, stimuli such as noxious colonic distension can activate 'neutral' cells but do not activate 'on' cells nor inhibit 'off' cells (Brink and Mason, 2003). During normal circumstances numbers of 'on' and 'off' cells are relatively stable. However during long term persistent pain, there may be a shift in response towards the 'on' cell category.

4.1.4 Descending inhibition from RVM

Descending inhibition from the RVM can be mediated by GABAergic, serotonergic and noradrenergic receptors in the spinal cord (Millan, 2002). Classically, the descending RVM projections (especially from the NRM) were believed to be mainly serotonergic fibres (Bowker et al, 1981). However, recent studies have shown that this is not that case and those projections to the spinal cord from the RVM are heterogeneous in nature and only about 20% of RVM neurons are serotonergic and are generally thought to be 'neutral' cells. Retrograde labelling from the cervical and the lumbar spinal cord using horseradish peroxidase and fluorogold respectively have shown that a large number of RVM projection neurons to the dorsal horn do not co-localise with serotonin (5-HT) (Jones et al, 1991; Wang and Wessendorf, 1999; Marinelli et al, 2002) indicating the presence of a non-serotonergic descending system. Indeed studies have shown that the majority of descending projections from the RVM are not serotonergic but GABA-ergic, indeed the vast majority of 'off' cells are GABA-ergic (Winkler et al, 2006).

5-HT is released in the spinal cord following electrical activation of NRM but an added complexity is that descending neurons do not express a single neurotransmitter. For instance, a subset of serotonergic neurons from the RVM co-exists with a number of other neurotransmitters including substance P, GABA, dynorphin, glycine, galanin and/or CCK. It has been found that 25% of serotonergic neurons innervating the superficial dorsal horn contain GABA, while those projecting to deeper layers (lamina V) do not contain GABA. Therefore, in a subset of descending neurons their activation could co-release both GABA and 5-HT to inhibit the post-synaptic response of dorsal horn neurons (Millhorn et al, 1987, 1988; Bowker and Abbott, 1988; Reddy et al, 1990; Millan, 2002).

4.1.5 Descending facilitation from RVM

Following spinal nerve ligation, Burgess et al (2002) discovered that the RVM cells expressing the mu-opioid receptor and their projections through the dorsolateral funiculus (DLF) are vital for the maintenance of neuropathic pain. The ablation of mu-opioid receptor expressing neurons with dermorphin-saporin conjugates or the transection of the DLF reduced both long term mechanical and thermal hyperalgesia (Burgess et al, 2002).

Numerous studies (reviewed by Urban and Gebhart, 1999) have shown that peripheral inflammation or spinal nerve ligation with either the spinal cord transected or the RVM

lesioned can significantly reduce behavioural hyperalgesia suggesting a facilitatory role for the RVM. Reviewing the extensive literature, Urban and Gebhart (1999) suggest that the principal effect of descending controls is on secondary (and therefore) mechanical hyperalgesia. In addition, studies have also revealed that the descending facilitation can also affect pain behaviour and spinal physiology of deeper layers of the dorsal horn (lamina V). The response is driven, in part, by NK1-positive projection neurons from lamina I (Suzuki et al, 2002; 2003). In fact the selective ablation of lamina I cells with SP-SAP shows strong similarities to that following selective ablation of descending serotonergic axons. The effects on lamina V cells include the reduction of the second phase of the biphasic response to formalin, as well as the reduction in the coding response to both thermal (noxious range) and mechanical (mid-noxious range) stimuli.

Descending serotonergic pathways originating from the RVM are fundamental to the maintenance of persistent pain and are believed to be part of the spinal-bulbo-spinal loop. For example, the removal of serotonergic axons with 5, 7-dihydroxytryptamine (5, 7 DHT) can attenuate cooling and mechanical hyperalgesia following spinal nerve ligation (SNL) (Rahman et al, 2006). Release of 5-HT from serotonergic axons can activate a number of 5-HT receptors. The 5-HT3 receptor is ligand gated and gates calcium poorly, however it can indirectly activate calcium channels (Jones and Yakel, 1998) and facilitates the maintenance of neuropathic pain (Suzuki et al, 2002). The administration of ondansetron (a selective inhibitor of the 5-HT3 receptor) intrathecally can attenuate SNL induced mechanical allodynia.

RVM serotonergic activity is essential for the maintenance of neuropathic mechanical and cold allodynia (Rahman et al, 2004). I have investigated if serotonergic drive is required for persistent inflammatory pain by locally ablating 5-HT axons within the lumbar dorsal horn. In addition, levels of zif268 and c-fos expression (after CFA induced inflammation) were also examined using immunohistochemistry.

4.2 METHODS (see Chapter 2 for details)

4.2.1 Bolus injections of 5, 7 DHT and behaviour

Adult rats were anaesthetised using halothane and injected i.p. with 1ml desipramine hydrochloride (25mg kg^{-1}) before being given an (i.t.) injection of 5, 7 DHT (60μg) or saline (10μl). Following 5, 7 DHT injections the animals were left for 3-4d and separated into two groups; behaviour or immunohistochemistry, before being given a unilateral injection of CFA into either the hindpaw (50%, 100μl, intraplantar) or into the ankle joint (50%, 10μl) under halothane anaesthesia.

The immunohistochemical group were perfused 2h after the CFA injection and processed for immunohistochemistry using antibodies that target 5-HT, c-fos and zif268. Cells located in the superficial layer of the dorsal horn that were positive for c-fos or zif268 were counted using a calibrated computer software programme (MCID). While the behavioural group were tested for mechanical allodynia and hyperalgesia (change in paw withdrawal thresholds) for 7d after the CFA injection

4.2.2 SP-SAP and SAP injections

To ablate superficial NK1 neurons in the lumbar segments L4-L6, adult rats were intrathecally cannulated under halothane anaesthesia and injected (in a bolus manner) with SP-SAP (1μm 10μl) followed by a intrathecal saline flush (10μl) or SAP (control) (1μm, 10μl) followed by a intrathecal saline flush (10μl) using a Hamilton syringe. The animals were left for 28d in their home cage, injected with CFA (50% 100μl, hindpaw, intraplantar) and perfused 2h later (section 2.1.6). Spinal cord sections were then immunostained for NK1 and zif268. Positive cells located within lamina I and II were counted using the MCID software.

4.3 RESULTS

4.3.1 Descending serotonergic axons facilitates persistent inflammatory pain

An overall significant decrease in mechanical withdrawal thresholds was found following the intraplantar injection of CFA into the hindpaw in animals receiving intrathecal 5, 7 DHT animals and intrathecal saline ($F_{2, 27} = 61.49$; $P<0.001$). This effect was persistent in nature and lasted for the remainder of the study (day 1-7 post CFA) ($F_{5, 135} = 0.412$; $P<0.77$) (Fig 4.1B). In addition post hoc analysis (Tukey's HSD) revealed that each treatment group were significantly different from each other ($P<0.005$) with regards to paw withdrawal thresholds. Unsurprisingly, animals receiving only 5, 7 DHT but without paw inflammation did not show any significant ($P=0.50$) reductions in paw withdrawal thresholds, suggesting that intrathecal 5, 7 DHT alone did not affect paw sensitivity in this study. Importantly, although sensitivity in animals receiving 5, 7 DHT and CFA were allodynic the paw withdrawal thresholds were significantly higher ($P<0.005$) than animals receiving intrathecal saline (vehicle) and CFA. This difference became apparent by 6 hours post CFA. Animals that received 5, 7 DHT and CFA had a drop in threshold of 11 +/- 3g, in comparison the vehicle group experienced a drop in threshold of 20 +/- 1g (Fig 4.1A). However, the induction of the inflammatory pain state were unaffected by either treatments with both showing a significant decrease in paw withdrawal thresholds early on (0-6h) after CFA injections ($F_{4, 32} = 13.2$; $P<0.001$).

In a similar manner to peripheral inflammation, CFA injection into the ankle joint (monoarthritic model) resulted in a persistent mechanical allodynia and hyperalgesia (Fig 4.1C). This pain state was partially alleviated with the pre-treatment of intrathecal 5, 7 DHT. On an overall basis, treatment had a significant effect on paw withdrawal thresholds ($F_{1, 15} = 5.06$ $p<0.04$). However further analysis (post hoc testing) that took individual days into account, found that the pre-treatment of 5, 7 DHT only significantly reduced paw withdrawal thresholds on day 1 ($P<0.01$) and day 4 ($P<0.05$) Paw withdrawal thresholds on day 1 were reduced by 19 +/- 1g in the vehicle and CFA group and 8.9 +/- 3g in the 5, 7 DHT and CFA group. On day 4 the thresholds were reduced by 17 +/- 3g in the 5, 7 DHT and CFA group and 11.9+/-2g in the vehicle and CFA group.

4.3.2 Zif268 and c-fos expression are regulated by the spino-bulbo-spinal loop

The expression of immediate early gene zif268 and c-fos in the dorsal horn are markers of CFA induced peripheral inflammation. Cell counts made in the superficial layer of the dorsal horn (using MCID software) revealed that serotonergic axons innervating the dorsal horn are

not required for the expression of spinal zif268 following peripheral inflammation. No significant differences were seen in zif268 expression levels in animals pre-treated with intrathecal 5, 7 DHT or intrathecal saline. Cell counts were 157 +/-7 and 162 +/- 9 per 40μm section respectively (Fig 4.2A and Fig 4.3A, B). In comparison, intrathecal 5, 7 DHT pre-treatment did have an affect upon c-fos expression in the dorsal horn. C-fos expression was significantly higher (unpaired t-test, $P<0.05$) in animals pre-treated with intrathecal 5, 7 DHT in comparison to vehicle control (Fig 4.2B and Fig 4.3C, D). Mean number of c-fos positive cells (per 40μm section) were 77 +/- 8 after intrathecal 5, 7 DHT and 46 +/- 5 for intrathecal saline.

In comparison with the SAP control, the ablation of NK1 expressing cells in lamina I with intrathecal SP-SAP significantly reduced (unpaired t-test, $P<0.05$) zif268 expression in the dorsal horn (Fig 4.2C). Mean cell counts for zif268 per 40μm section were 55 +/- 3 for SP-SAP and 84 +/- 13 for SAP treated animals. However c-fos levels were not analysed after CFA injection into the hindpaw in SP-SAP treated animals. Previously it has been shown that induction of c-fos following high frequency stimulation was the same in sham and SP-SAP treated animals (Rygh et al, 2006).

4.3.3 Successful depletion of local serotonin using i.t. 5, 7 DHT

To verify that 5, 7 DHT successfully depleted 5-HT in the lumbar regions of the spinal cord, immunohistochemistry was carried out on all animals used in the experiment. Following intrathecal 5, 7 DHT treatment a marked reduction in 5-HT immunoreactivity was seen in the entire lumbar enlargement in comparison with vehicle (saline) treated animals (Fig 4.4). Clear 5-HT depletion was seen from L4-L6. It was, however, not clear if the depletion exceeded this point since visual inspection was not made beyond these lumbar regions. 5-HT expression in the whole dorsal horn was extremely low following 5, 7 DHT. Lamina I was visibly reduced following 5, 7 DHT treatment. The level of immunostaining for 5-HT was similar in animals perfused 3-4d to those after 10-11d post 5, 7 DHT, suggesting the re-growth of serotonergic axons does not occur within this time frame. Animals treated with 5, 7 DHT that did not show any serotonergic depletion (especially in the superficial layers of the dorsal horn) were discarded from the experiment. Although 5, 7 DHT can also affect noradrenergic neurons, other studies have shown that the use of desipramine (at similar to concentrations to ours) prevented 5, 7 DHT induced loss of noradrenergic neurons (Sawynok and Reid, 1996; Choi et al, 2004).

4.3.4 SP-SAP successfully ablated NK1 receptor expressing neurons in lamina I

Immunohistochemistry and cell counts revealed that SP-SAP treatment successfully reduced the expression of the NK1 receptor in lamina I of the spinal dorsal horn in comparison with SAP treated animals (Fig 4.5) (unpaired t-test, $P<0.05$). The number of NK1 positive cells after SP-SAP was 15 +/- 3 per 40μm section and 30+/-2 for SAP.

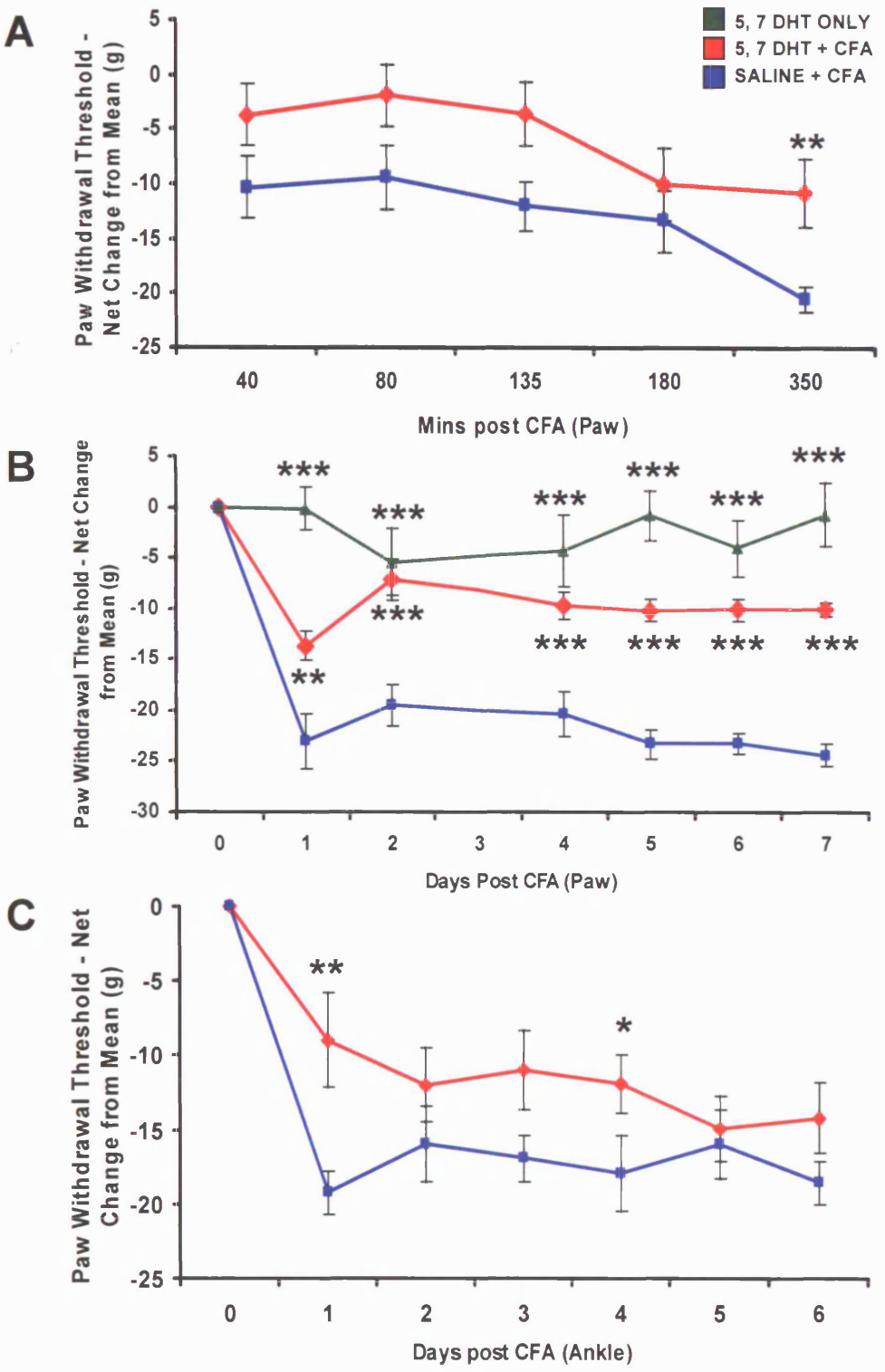


Figure 4.1. Descending serotonergic axons facilitate persistent inflammatory pain. A) The local depletion of serotonin in descending axons with 5,7 DHT did not affect paw withdrawal thresholds (PWTs) in the early stages of peripheral inflammation until 6h after CFA treatment (n = 5 for each group). B) Depletion of local (L4-L6) serotonergic axons alone (without inflammation) did not change PWTs (green line, n = 6). Peripheral inflammation with CFA caused persistent mechanical allodynia and hyperalgesia (blue line, n = 12) that can be attenuated by 5, 7 DHT (red line, n = 12). C) Monoarthritis (injection of CFA into the ankle) caused persistent mechanical allodynia (blue line, n = 8) which was partially reversed by intrathecal 5, 7 DHT (red line, n = 9). Confidence levels are comparisons between 5,7 DHT + CFA or 5, 7 DHT only with Saline + CFA. (Two way ANOVA repeated measure and Tukey's post hoc testing *** P<0.005, ** P<0.01, * P<0.05).

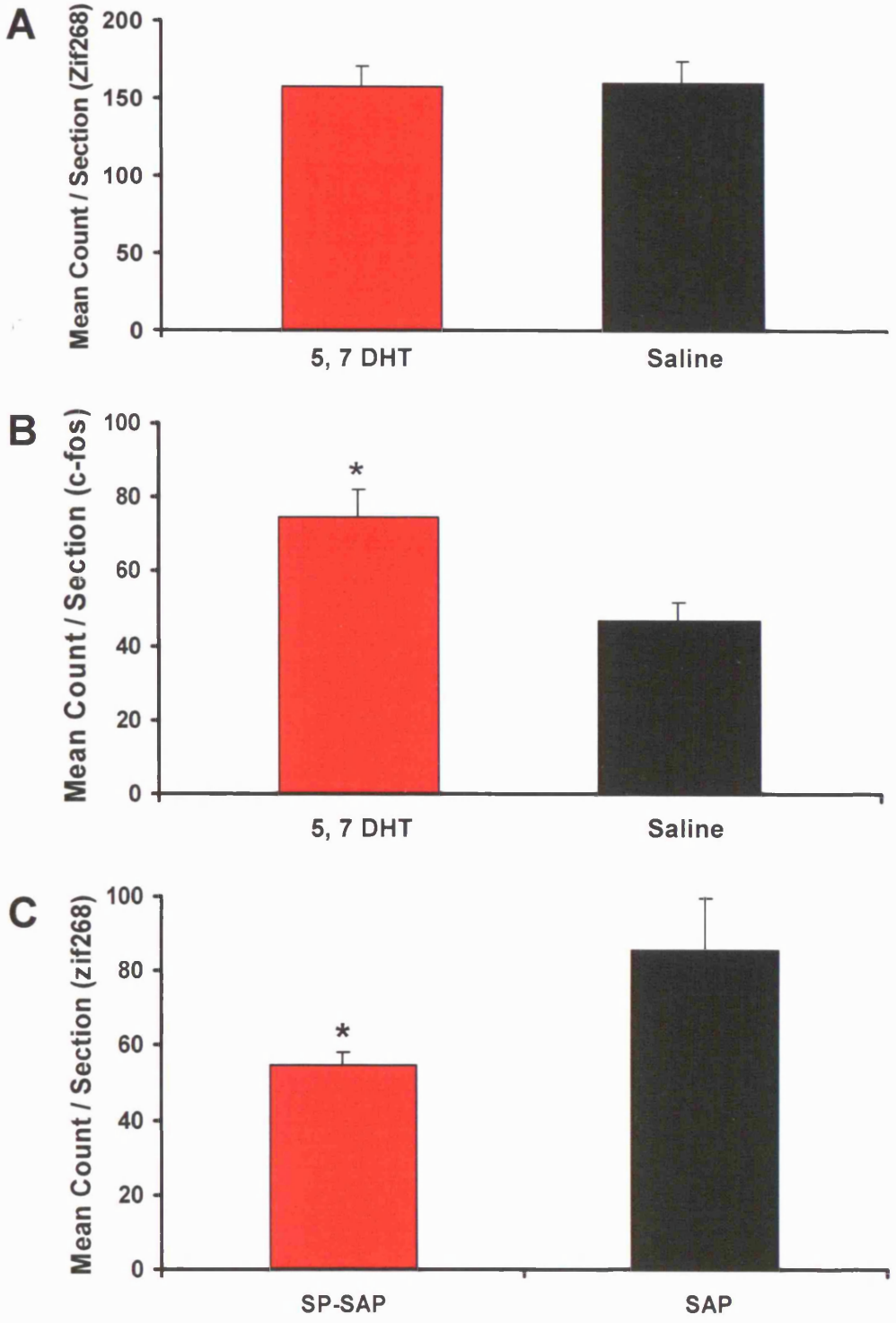


Figure 4.2. Descending serotonergic axons and NK1 positive projection neurons in the dorsal horn differentially modulates the expression of zif268 and c-fos in the spinal dorsal horn. Adult rats were administered with i.t 5, 7 DHT (n = 4), saline (n = 4). SP-SAP (n = 4) or SAP (n=4) before receiving a unilateral intraplantar injection of CFA (50%, 100µl). Animals were then perfused 2h later to allow for the peak expression of both zif268 and c-fos. A) The local depletion of descending serotonergic axons in the dorsal horn did not modulate the expression of zif268 in the dorsal horn during peripheral inflammation in comparison to saline control. B) The local depletion of descending serotonergic axons in the dorsal horn increased the expression of c-fos in the dorsal horn during peripheral inflammation. C) The ablation of lamina I NK1 expressing cells in the dorsal horn reduced the expression of spinal zif268. (Unpaired t-test in comparison with controls * $P<0.05$, 10 sections per animal).

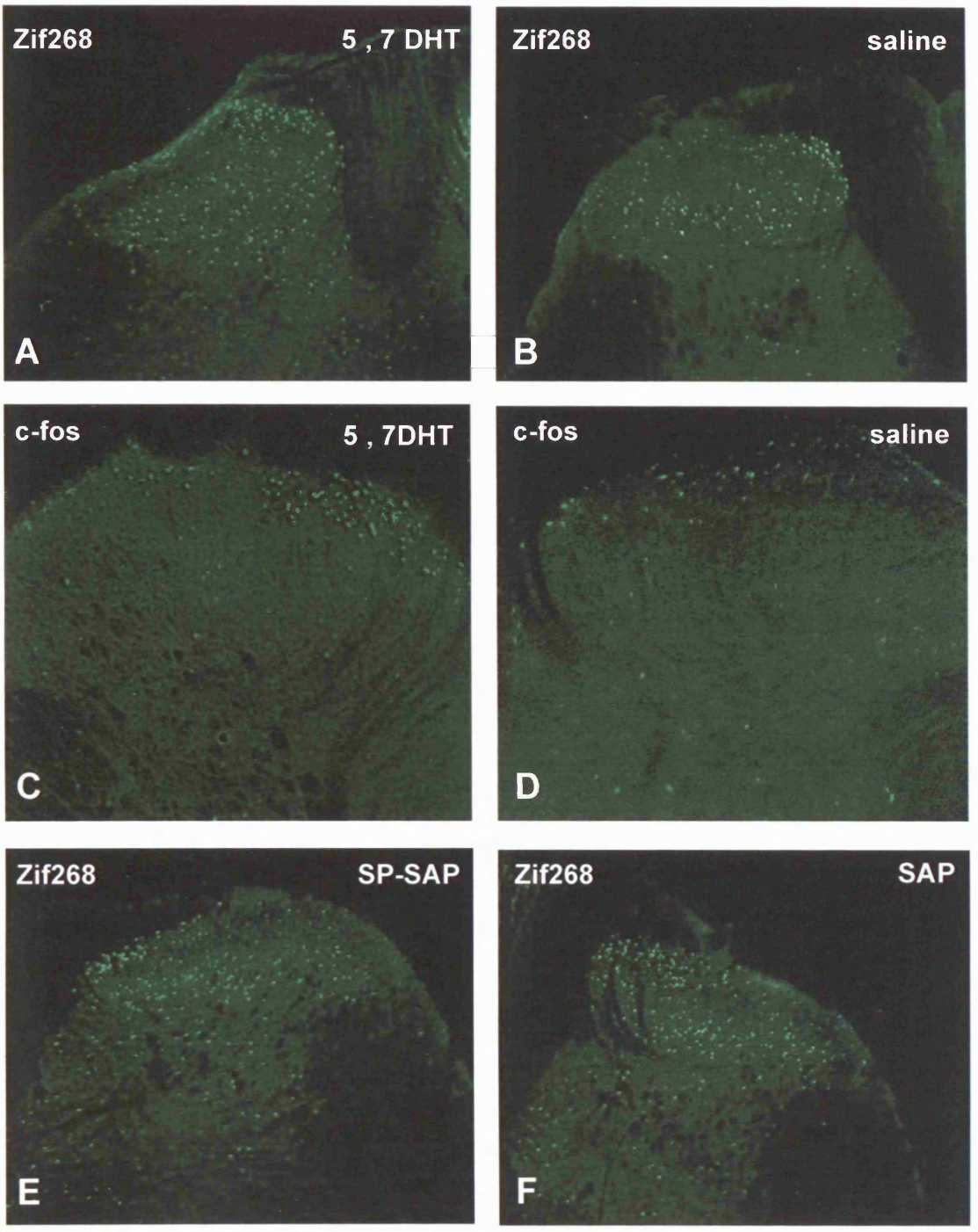


Figure 4.3. Spinal cord sections (lumbar) from animals during peripheral inflammation (50% CFA, 2h, 100µl) Animals were pre-treated with i.t. injections of SP-SAP, SAP, 5, 7 DHT or saline. A) Spinal zif268 expression was unaffected by the ablation of descending serotonergic axons (using 5, 7 DHT). Cell counts revealed that zif268 expression was similar to (B) saline treated animals. C) In comparison, spinal c-fos expression increased following 5, 7 DHT pre-treatment in comparison with (D) animals receiving saline. Spinal zif268 expression can be modulated by spinal NK1-R expressing projection neurons. E) The ablation of these neurons with SP-SAP reduced zif268 expression in the dorsal horn in comparison with F) SAP pre-treated animals.

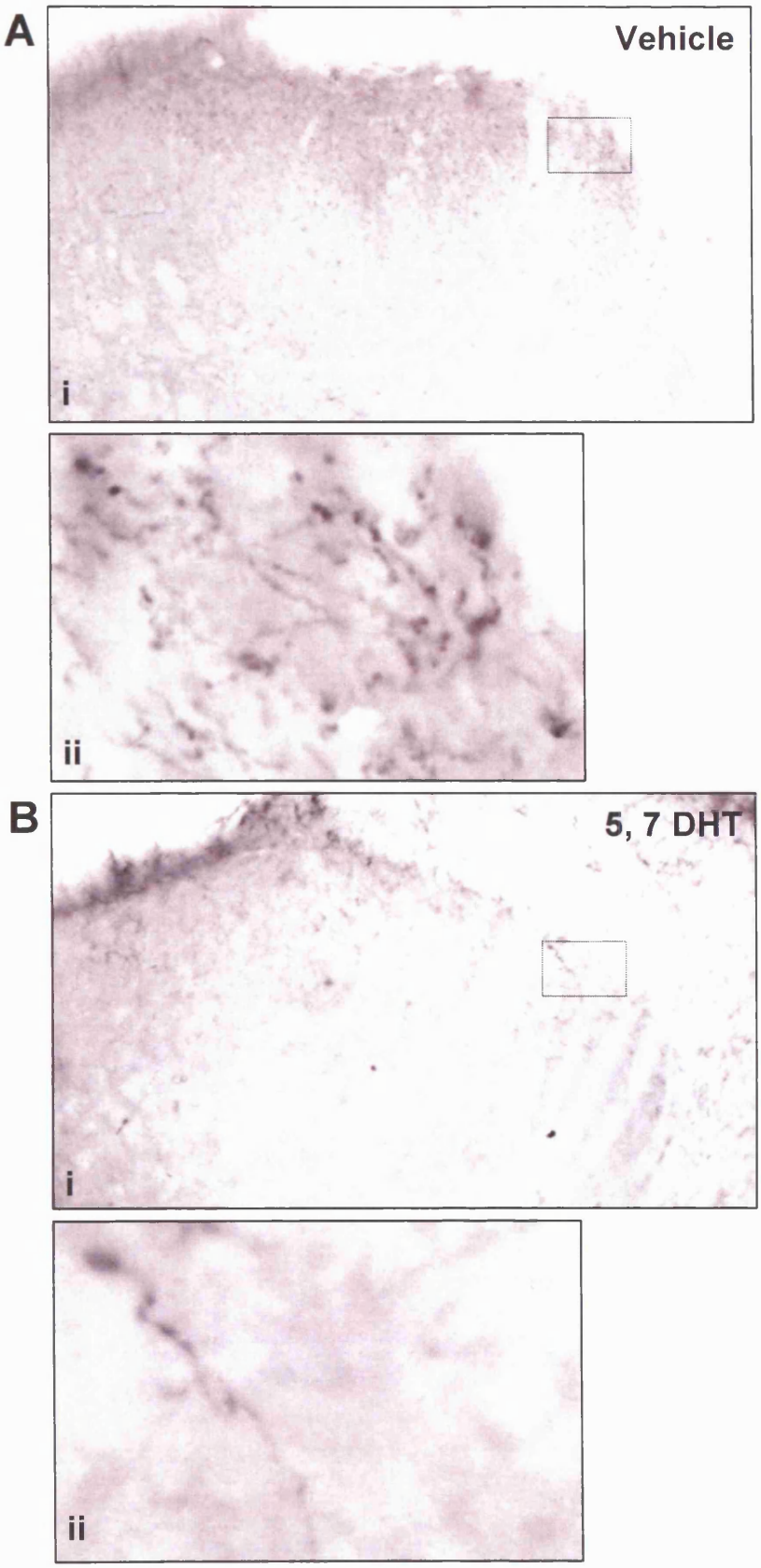


Figure 4.4. Intrathecal administration of 5, 7 DHT depleted serotonin axons in the lumbar region of the dorsal horn. Representative sections of adult rat spinal cord (L5) immunostained for serotonin. Ai) In animals treated with vehicle (saline), serotonergic axons were still present in the superficial layers of the dorsal horn. Aii) Magnification of the marquee area from image Ai. Bi) Treatment with intrathecal 5, 7 DHT depleted serotonin expression in the superficial layers of the dorsal horn. Bii) Magnification of the marquee area from image Bi.

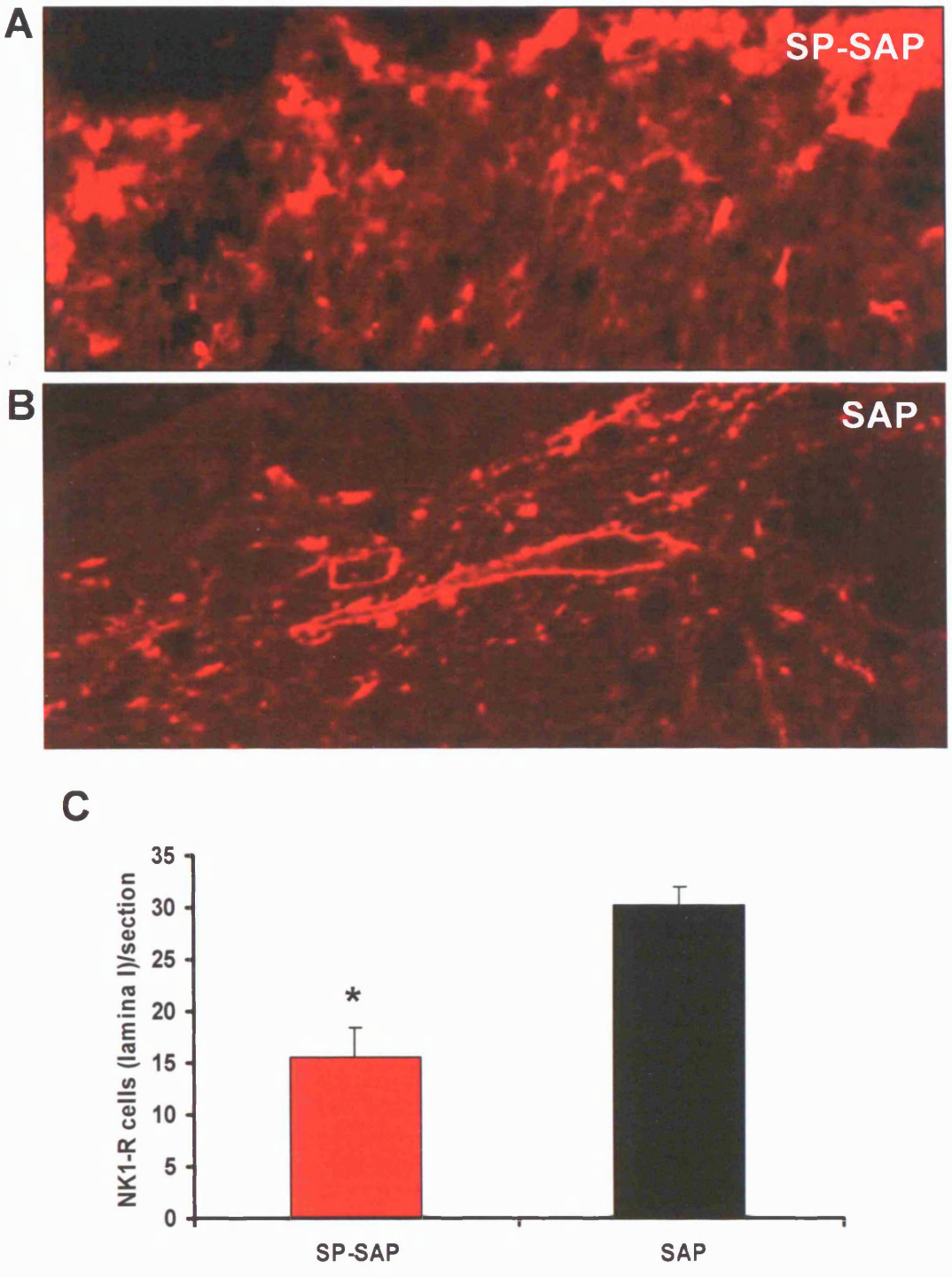


Figure 4.5. Intrathecal SP-SAP successfully ablated neurons that expressed the NK1 receptor in lamina 1. A) A representative image of a lumbar dorsal horn section immunostained for the NK1 receptor following an intrathecal injection of SP-SAP 30 days earlier. B) A representative image of a lumbar dorsal horn section immunostained for the NK1 receptor following an intrathecal injection of SAP 30 days earlier. C) Cell counts for NK1-R expressing neurons in lamina I of the spinal dorsal horn revealed that SP-SAP (red bar) significantly reduced the number of NK1-R expressing neurons in lamina I in comparison with SAP (black bar). (Confidence levels in comparison with SAP. Unpaired t-test * $P<0.05$, n = 4, 10 sections per animal).

4.4 DISCUSSION

4.4.1 Descending serotonergic axons facilitate inflammatory pain states

This study has shown that 1) the early development of inflammatory pain sensitivity does not require local serotonergic activity 2) maintenance of inflammation induced mechanical hyperalgesia can be attenuated by depleting 5-HT in the spinal cord (L4-L6). 3) Depletion of 5-HT axons alone did not influence acute pain response and 4) zif268 and c-fos expression in dorsal horn neurons are differentially modulated by different components of the spino-bulbo-spinal loop. These data supports the hypothesis that descending serotonergic axons are involved in the maintenance of mechanical hyperalgesia but not with its induction.

Our behavioural data has shown a decrease in paw withdrawal thresholds following 5-HT depletion in the lumbar spinal cord. Results from this experiment compliments a number of other studies that show descending projections originating from the RVM facilitate and maintain chronic neuropathic pain states (Pertovaara et al, 1996; Ossipov et al, 2000; Porreca et al, 2001; Burgess et al, 2002; Suzuki et al, 2004 and Rahman et al, 2006). This would suggest that chronic neuropathic pain and persistent inflammatory pain may share a similar mechanism in the maintenance of their respective pain states.

4.4.2 Descending serotonin axons do not regulate spinal zif268 expression

The activation of the ERK1/2 pathway in dorsal horn neurons is vital for the full expression of hyperalgesia and allodynia (Ji et al, 1999). Recently it has been suggested that ERK1/2 expression is under the influence of descending serotonergic axons. The ablation of 5-HT neurons with intrathecal 5, 7 DHT before peripheral inflammation with CFA decreases ERK1/2 expression in the spinal dorsal horn (Svensson et al, 2006). In addition pain behaviours (paw lifting, biting and licking) to formalin was reduced following a similar pre-treatment with 5, 6 DHT (Tjolsen et al, 1991). Given that zif268 lies downstream of ERK1/2 in the hippocampal neurons, the local depletion of 5-HT in the dorsal horn surprisingly did not affect the expression of zif268 in the dorsal horn. However, the ablation of the ascending portion of spino-bulbo-spinal loop with SP-SAP significantly reduced zif268 expression following peripheral inflammation. Previously it has been seen that induction of zif268 after C-fibre high frequency stimuli can also be reduced in animals previous treated with SP-SAP with no change in levels of c-fos expression (Rygh et al, 2005). This result would suggest that, depletion of 5-HT alone is not sufficient to reduce zif268 expression but nevertheless

attenuates the maintenance but not induction of the inflammatory pain state. Given that 5-HT depletion attenuates the maintenance phase of inflammatory pain but only lamina I/NK1 projection neuron ablation attenuates the maintenance and induction phase, it might be concluded that modulation of zif268 expression is more closely related to induction of the pain state. However, the previous experiments (chapter 3) suggest that this may not be the case. Antisense knockdown of zif268 expression attenuates the maintenance, but not the inductive phase of inflammatory pain. An alternative explanation is that NK1 positive lamina I projection neurons may activate a number of different descending pathways in a coordinated fashion. Thus SP-SAP lesions of lamina I neurons would be expected to produce a more profound effect on spinal processing than simple isolation and ablation of one small component of the descending pathways (the serotonergic component). We were recently able to show that LTP in deep dorsal horn neurons was lost following ablation of lamina I projection neurons and this was accompanied by an attenuated rise in zif268 expression in superficial dorsal horn neurons but did not influence c-fos expression (Rygh et al, 2006). Depletion of spinal 5-HT had a much more limited influence on the development of LTP in deep dorsal horn neurons. Increased c-fos expression suggests that inhibitory components of serotonergic control were revealed by 5, 7 DHT treatment which are not seen following SP-SAP treatment. Interestingly, this may mean that the integrated activation of descending pathways can be modulated to produce either inhibition or facilitation as indeed has been suggested experimentally (Urban and Gebhart, 1999).

4.4.3 Spinal c-fos expression is modulated by descending serotonergic axons

Along with a number of other studies, the data has revealed that c-fos expression in the dorsal horn can be modulated by descending serotonergic axons. C-fos expression is widely accepted as a marker of neuronal activity that can lead to long term changes in cellular activity. C-fos is an immediately early gene that is expressed in the dorsal horn following a number of different types of noxious peripheral stimulation (Doyle and Hunt, 1999). The protein itself forms heterodimers with members of the jun family in order to form an active transcription factor. In this state c-fos is able to regulate the expression of a number of second response genes. The expression of spinal c-fos has been correlated with behavioural hyperalgesia following peripheral inflammation (Dubner and Ruda, 1992) with both being simultaneously attenuated with the intrathecal administration of NMDA receptor antagonists (Kehl et al, 1991). More recently, the importance of c-fos in nociception processing has been highlighted with the use of oligonucleotides that targeted the c-fos gene in the spinal cord.

Peripheral injection of formalin into the hindpaw normally produces gross nocifensive behaviours including paw licking and paw lifting. There are two distinct phases to the formalin response that are separated by quiescence in the nocifensive behaviours. Pre-treatment with c-fos antisense significantly reduced the second phase formalin in a dose dependent manner (Hou et al, 1997) but did not affect the first phase. The reduction of c-fos also reduced the transcription of preprodynorphin (Hunter et al, 1995) which has been suggested to be pro-nociceptive (Zhang et al, 2003) stressing the importance of c-fos in the regulating pain-related genes in inflammatory pain states. However the transcriptional role of c-fos in CFA induced behaviours is still unknown.

Interestingly, our data showed that the depletion of 5-HT in the dorsal horn increased local c-fos expression. The results we obtained would suggest, in part, an inhibitory role for descending serotonergic axons. In support, previous experiments using spinally transected animals have yielded similar results. Ren and Ruda (1996) discovered that c-fos expression following peripheral inflammation was higher in animals with transected cords in comparison with whole animals. Since c-fos expression is related to neuronal activity in the spinal cord the authors believed that the descending pathways net role is to dampen down central hyperexcitablity in the spinal cord during peripheral inflammation. A possible solution to account for both the excitatory and inhibitory activity of descending serotonergic pathways is that during the early phases (hours) of persistent inflammatory pain, the dorsal horn is under increased descending inhibition (Cervero et al, 1991; Schaible et al, 1991). This event may be dependent on a serotonergic drive from the RVM onto inhibitory spinal 5-HT receptors. This is then followed by a gradual switch from inhibition to descending facilitation after a few hours (Danziger et al, 2001), possibly because of changing patterns of gene expression in the RVM (Imbe et al, 2005; Imbe et al, 2007) and 5-HT acting at the excitatory 5-HT3 receptor playing a prominent role. This would explain both the behavioural response and the c-fos response seen in this study and that c-fos expression may be modulated by different 5-HT receptors.

4.4.4 5-HT3 receptors mediate descending facilitation

The results gathered from this study have shown that descending serotonergic axons play a facilitatory role in mediating CFA induced hyperalgesia. The removal of 5-HT axons with 5, 7 DHT significantly increased paw withdrawal latencies at later time points. It still remains uncertain as to which particular 5-HT receptor subtype or subtypes are involved with the

maintenance of CFA induced inflammatory pain state, but other inflammatory models have shown the 5-HT receptors are intimately involved with nociception.

While a strong case for the 5-HT3 receptor in mediating descending facilitation has been made, a growing number of studies using electrophysiological and behavioural techniques have shown that other 5-HT receptor subtypes and the release of 5-HT plays a vital role in inhibiting (Eide et al, 1988; Eide et al, 1990; Xu et al, 1994; Gjerstad et al, 1996; Millan et al, 1996; Oyama et al, 1996), or facilitating (Murphy and Zemlan, 1990; Crisp et al, 1991; Ali et al, 1994) the nociceptive response. The inhibitory or excitatory response of the neuron can be partly determined by the 5-HT receptor subtype that is being activated, making it conceivable that other spinally expressed 5-HT receptor subtypes could play a role in the maintenance of CFA induced inflammatory pain (reviewed by Millan, 2002). For instance the activation of 5-HT2 receptor can evoke long-lasting synaptic facilitation (Hori et al, 1996), and produce nocifensive-like behaviours that are reversible by substance P antagonist (Eide and Hole, 1991; Eide and Hole, 1993). Peripheral inflammation with carrageenan increases the expression of 5-HT1A and 5-HT2A receptors in the dorsal horn, including lamina I and II but also in GABAergic neurons (Zhang et al, 2002; Zhang et al, 2001). Activation of 5-HT1A and 5-HT1B enhances the behavioural response to noxious stimulation (Zhang et al, 2001).

However, although a compelling case has been made for a role of the 5-HT3 receptor in the maintenance of persistent inflammatory pain, studies on animals that lacked the 5-HT3 receptor gene (Zeitz et al, 2002) have concluded that this receptor does not alter the noxious behaviours associated with intraplantar CFA injections (measured at day 1 and 2). No difference was seen between mutant and wildtype animals in the response to thermal and mechanical stimulation. However, it still remains unclear if this was specifically due to a lack of 5-HT3 receptor at the level of the spinal cord or due an effect of 5-HT3 receptor loss throughout the CNS. In comparison, other inflammatory models have shown that the activation of the 5-HT3 receptor is vital for facilitating noxious heat (Ali et al, 1996).

Oyama et al (1996) examined the role of the descending serotonergic system in the regulation of nociceptive processing. Intrathecal 5-HT showed a dual effect on second phase formalin response in 5, 7 DHT treated animals. 5-HT at low dose inhibited aversive effects of formalin while high dose 5-HT facilitated the pain response. The effectiveness of both doses was affected by antagonising the 5-HT1A and the 5-HT3 receptor respectively. The results suggest that spinal 5-HT1A receptor suppresses the formalin response and that spinal 5-HT3 receptors facilitates the formalin response.

4.4.5 Spino-bulbo-spinal loops are essential for the induction and maintenance of persistent pain states

Spino-bulbo-spinal loops are crucial for the maintenance of chronic and persistent pain states. The interruption of the loops with either the ablation of ascending projection neurons from lamina I (Nichols et al, 1999; Suzuki et al 2002), the use of NK1 mutant mice (Kidd et al, 2003), transection of the dorsolateral funiculus (DLF) (Ossipov et al, 2000) or disruption of the RVM can alleviate long term pain. Neuronal protein expression in the RVM can be modulated by ascending projections from lamina I. For example, CFA induced peripheral inflammation can increase the expression of proteins associated with plasticity within hours in the RVM, including the NMDA receptor subunits (NR1, NR2A and NR2B) (Miki et al, 2002), GluR1 (Guan et al, 2003) as well as increasing ERK1/2 expression in the majority (60%) of serotonergic neurons in the RVM (Imbe et al, 2005). Moreover, persistent peripheral inflammation can also induce unresponsive cells to start firing in a subpopulation of RVM cells (Miki et al, 2002). Gene transcription in the RVM may enhance the drive onto descending serotonergic axons. Consistent with the idea that descending facilitatory drive originating from the RVM is a component of the spino-bulbo-loop, the depletion of 5-HT from the spinal cord, in this experiment, can attenuate persistent mechanical allodynia in the same manner as SP-SAP treatment on NK1 neurons in lamina I. In addition the attenuation occurred within hours after CFA administration and may reflect the time needed for protein modification and expression to occur in the RVM.

4.5 CONCLUSION

In conclusion, these studies have shown that the induction of inflammatory pain states may be independent of descending serotonergic axons. This behavioural response would seem to be dissociated with dorsal horn c-fos expression since local depletion of 5-HT increased c-fos expression. Moreover, in contrast to the early stages of inflammation, persistent mechanical allodynia caused by peripheral inflammation to the hindpaw and ankle is dependent on descending 5-HT released from descending axons of the RVM. However, it must be noted that since we did not fully examine the effects of 5, 7 DHT on other neuronal populations in the dorsal horn we cannot fully exclude the possibility that our results are also influenced by other non-serotonergic activity.

CHAPTER 5

The Glucocorticoid Receptor: A Role in Persistent Inflammatory Pain

5.1 INTRODUCTION

5.1.1 The HPA axis

Stress can be defined as any stimulus that can perturb normal homeostasis and therefore activate the hypothalamus-pituitary-adrenal (HPA) axis. The HPA axis regulates the production and release of glucocorticoids, including the steroid hormone cortisol (in humans) and corticosterone (in rodents). The responses of the HPA axis to stressful and/or harmful stimuli are controlled by a subset of hypophysiotrophic neurons located in the medial parvocellular division of the paraventricular nucleus (PVN) within the hypothalamus. These neurons produce and secrete the corticotrophin releasing hormone (CRH) and vasopressin that act in a synergistic manner with arginine vasopressin to stimulate corticotrophs in the adrenal gland to release adrenocorticotropic hormone (ACTH) (Aguilera et al, 1994; Romero et al, 1996). ACTH is synthesised from the precursor pro-opiomelanocorticortin (POMC), which when cleaved produces both ACTH and beta-endorphin. In the presence of ACTH, the adrenal gland is responsible for converting cholesterol into androgens, glucocorticoids and mineralcorticoids. The major glucocorticoid produced by the adrenal gland is corticosterone.

Glucocorticoids are steroids that are released in times of stress and require strict regulation to avoid excess production. Glucocorticoids released by the activation of the HPA axis can form part of a negative feedback system to inhibit any further release (Herman and Cullinan, 1997; Sapolsky et al, 2000). The inhibitory feedback effect of glucocorticoids on the hypothalamus can occur rapidly and dissipate within minutes, but also in a much slower manner that can take minutes to hours for an effect to take place (Keller-Wood and Dallman, 1984). While slow glucocorticoid effects are believed to involve gene regulation, the rapid feedback mechanism is too fast for transcriptional activity to occur and involve non-genomic events (Falkenstein et al, 2000). In support, in-vivo electrophysiology studies using ionophoretically applied corticosterone produced a rapid excitatory effect in a number of brainstem loci (Avanzino et al, 1987). The reticular formation, the locus coeruleus and the RVM (Rong et al, 1999) were all found to be affected by corticosterones in a non-genomic manner, with the RVM seeing an increase in firing frequency in 75% of neurons that project to the spinal cord. In addition electrophysiological recordings have shown that PVN neurons can also rapidly respond to corticosteroid application (Kasai et al, 1988; Kasai and. Yamashita, 1988; Saphier and Feldman, 1988). It is believed that both forms of feedback are achieved by using different corticosterone binding receptors, although this has yet to be fully resolved (Tasker et al, 2005). There are two receptors that can bind corticosterones; these are the glucocorticoid

receptor (GR or Type II) and the mineralocorticoid receptor (MR or Type I). The GR has a ten-fold lower affinity for corticosterones in comparison with MR. As a consequence, higher levels of circulating corticosterone are needed for GRs to cause any cellular changes. Glucocorticoids when released can target several anatomical regions of the HPA axis to initiate the negative feedback loop. There is a large population of GR and MR located within the hippocampus, hypothalamus, and pituitary. Targeting any one or all of these regions can reduce the release of the steroid (Fig 5.1).

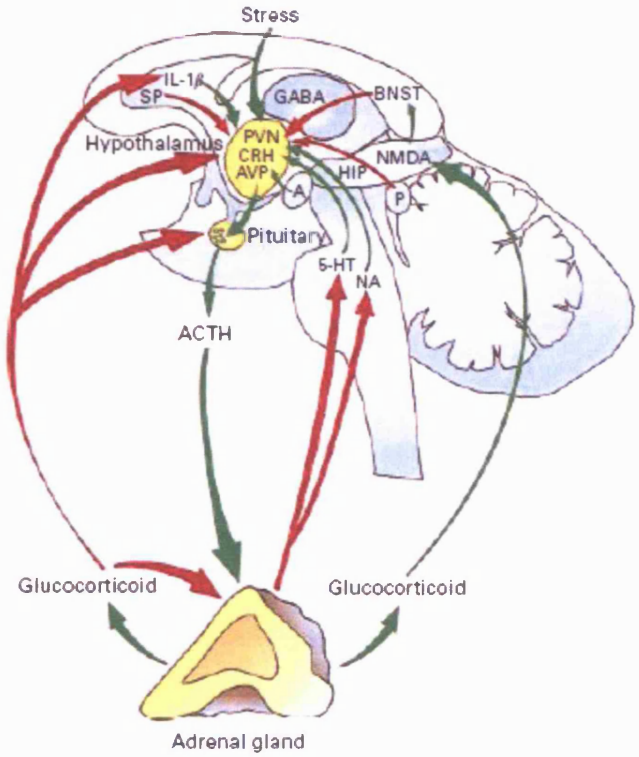


Figure 5.1. Stressors can stimulate the HPA axis by activating the paraventricular nucleus (PVN) (parvocellular division). The PVN contains both CRH and arginine vasopressin (AVP). These are released into the hypophyseal portal system and transported to the anterior pituitary gland. CRH acts at anterior pituitary corticotropes as a secretagogue in synergy with the weaker secretagogue vasopressin to release adrenocorticotrophic hormone (ACTH) into the systemic circulation. ACTH acts upon the adrenal cortex (zona fasciculate) and causes both the production and the release of cortisol or corticosterone (glucocorticoids) into the blood. Excess glucocorticoid release can be prevented using a negative feedback loop where glucocorticoids can prevent the release of ACTH from the pituitary, CRH and vasopressin from the PVN and finally glucocorticoids from the adrenal cortex. In additional glucocorticoids may act on GR and MR in the hippocampus by increasing the activity of NMDA receptors that stimulate the activity of inhibitory neurones (GABA) located in the bed nucleus of the stria terminalis. Noradrenergic and serotonergic input from the brainstem may also negatively modulate the activity of the HPA axis. Further negative feedback may be supplied by the amygdala and by the cytokine IL-1b. (Adapted from Blackburn-Munro and Blackburn-Munro, 2001)

Stimuli from internal or external sources can reach the PVN via a number of pathways from the spinal cord. These pathways include the spino-hypothalamic tract that relays to the lateral hypothalamic neurons as well as the spino-recticular-thalamic tract which includes fibres that

terminate in the ventrolateral and dorsomedial medulla where catecholaminergic neurons of the A1 and A2 cell group project to the PVN. Moreover, spinal lamina I projections terminate on the A1 and A2 group of the medulla, as well as in a number of other regions including the parabrachial nucleus, the periaqueductal gray (PAG), the solitary nucleus, the locus coeruleus, and the subcoerulear region (Westlund and Craig, 1996; Gauriau and Bernard, 2002; Spike et al, 2003). The HPA axis can also be activated indirectly via the amygdala (both the medial and central nuclei). The amygdala is known to receive direct and indirect projections from the spinal cord. The parabrachial nucleus is located in the hindbrain and receives nociceptive information direct from spinal lamina I neurons (Hermanson and Blomqvist, 1996) and has been shown to send collaterals to specific amygdaloid nuclei. Anatomical studies using retrograde tracers injected into the amygdala have identified projections from the parabrachial to the amygdala. Small subsets of these neurons are activated by formalin injected into the hindpaw. Therefore it remains possible that peripheral noxious stimulation may also be able to activate the HPA axis by activating neurons in the amygdala (Richard et al, 2005).

5.1.2 Glucocorticoid release and pain

Glucocorticoids are secreted in situations where homeostasis has been perturbed. Studies have shown that pain can cause the HPA axis to respond. Glucocorticoid release can be initiated by direct PVN activation via nociceptive pathways. Injection of dilute formalin into the hindpaw of rats can lead to the bilateral c-fos expression within catecholominergic cells in the brainstem including the cell groups A1 and A2 of the medulla which send projections to the hypothalamus. Peripheral injection of formalin (after 30min) can increase both noradrenaline release into the PVN (4 fold) and ACTH and glucocorticoid release (Palkovits et al, 1999).

5.1.3 The HPA axis and persistent pain states

Formalin and adjuvant induced arthritis (AA) has been shown to affect the HPA axis. The AA model of polyarthritis is a T cell dependent inflammatory model and is a model of rheumatic diseases. This model generates both persistent hyperalgesia and allodynia and is on-going (Bomholt et al, 2004). AA rats show a number of physiological disturbances including perturbations in their circadian rhythm (increase wakefulness) (Shanks et al, 1998; Harbuz et al, 1998) as well as increased EEG amplitudes (Andersen and Tufik, 2000). These phenomena can be reduced by the administration of analgesic compounds (Calvino et al, 1996). The AA model is also associated with abnormally high levels of plasma corticosterones and ACTH. Increases of propiomelanocortin (POMC, a precursor to ACTH) mRNA was also seen in the

anterior pituitary (Stephanou et al, 1992). The circadian rhythm of corticosterone release was found to be significantly altered. In healthy animals, corticosterone release occurs in a rhythmical manner, but this did not occur in animals that suffered from polyarthritis; the usual peaks and troughs of corticosterone release were flattened. In addition, high levels of corticosterone and ACTH that are usually seen in the active evening period (in nocturnal rodents) were significantly reduced. Instead, high levels of corticosterone were released during daylight hours including the non-active dawn period (Persellin et al, 1972; Sarlis et al, 1992; Selgas 1997).

Under normal circumstances corticosterones are released in a pulsatile manner, however in arthritic animals the interpulse phase is reduced. The outcome is an increased release of corticosterone release per unit time (Windle et al, 2001). Other effects of AA include an alteration in the level of CRH and AVP release. CRH was found to be decreased, while AVP was found to have increased with time. It is believed that the upregulation of AVP arises in order to compensate for the loss of CRH drive in the HPA axis. Using adrenolectomised rats it was noted that formalin induced peripheral inflammation increases the release of corticosterone which does not feedback and modulate the HPA axis (Taylor et al, 1998). There are two possible mechanisms whereby peripheral inflammation can increase the levels of ACTH or corticosterone. Subcutaneous formalin injection can lead to an increase production of interleukin-6 (IL-6) and ACTH in the plasma (Aloisi et al, 1995). Interleukin 6 has been shown to directly activate the PVN portion of the HPA axis (Rivier, 1995). The second possible mechanism is achieved by activating the ascending pathways that directly or indirectly influence the HPA axis. Destruction of C-fibres has been shown to decrease the effectiveness of formalin and CFA to cause the secretion of ACTH from the HPA axis (Amann and Lembeck, 1987). The efficacy of formalin to increase circulating corticosterone in the plasma can be inhibited by lesions to the PVN, although lesioning of this structure does not affect the pain state of the animal. Furthermore, the expression of substance P and CGRP in the DRG are increased in adrenolectomised rats (which have reduced plasma levels of corticosterones) whereas the levels of somatostatin are decreased. The effects of adrenolectomy can be successfully reversed with the GR agonist dexamethasone (Smith et al, 1991).

Several studies using the CCI (chronic constriction injury) model have shown the HPA axis was perturbed, but to a lesser degree than in the AA model. For instance, the glucocorticoid negative feedback loop remained intact. No significant increases were seen in corticosterone or ACTH release (Vissers et al, 2003; Bomholt et al, 2005) and the rhythmical release of corticosterone, which is perturbed in the AA model, remained normal (Bomholt et al, 2005).

The expression of c-fos in the PVN were not significantly altered, indicating that the basal function of the HPA axis remained unaffected. The basal expression of CRF (mRNA) also remained unaltered. However the release of CRF during the restraint test was reduced (in comparison to their controls). The levels of AVP secretion also remained the same (unlike in inflammation). Overall it would seem that the CCI model does not perturb the HPA axis to the same extent as some inflammatory pain models.

5.1.4 The glucocorticoid receptor and its role in pain

The GR is a member of the nuclear receptor superfamily, and like other members of the superfamily the GR functions as a ligand-dependant transcription factor. The GR can bind steroids such as cortisol and corticosterone and can affect the transcriptional activity of the cell. The GR when unbound to corticosterones are unable to dimerise and are restricted to the cytoplasm. However following corticosterone binding, GR changes conformation state and dissociates itself from chaperone molecules allowing the GR to dimerise and translocate to the nucleus. GRs can bind directly to specific DNA sequences that recognise activated GR; these regions are termed glucocorticoid response elements (GRE). There are 2 types of GRE, each with opposing effects. Positive GREs, as the name suggests, are responsible for allowing the process of transcription to occur, conversely, negative GREs direct transcription repression. Furthermore, GRs can interact with other transcription factors such as activator protein-1 (AP-1) and nuclear factor kappa beta (reviewed by Slater et al, 1986; Schoneveld et al, 2004).

Even though the corticosterone levels remained unaltered in neuropathic pain models, in the spinal cord, the GR has been shown to be a key molecule in modifying neuropathic pain states. Several studies by Wang et al (2004, 2005) have helped to elucidate the role of the GR in neuropathic pain states. GR expression in the dorsal horn is predominantly neuronal and increases during neuropathic injuries (CCI), the use of either GR antagonists (dexamethasone 21-mesylate) or adrenolectomy prior to CCI reduced both mechanical and thermal allodynia that normally accompanies CCI (Wang et al, 2004; Takasaki et al, 2005). In addition, Mao's group (2004) further investigated the regulatory role of the GR in the CCI model. The GR was found to regulate a number of proteins and receptors in the dorsal horn. The increase expression in the dorsal horn of the NMDA receptor subunit NR1 and NR2 caused by CCI can be successfully attenuated with the use of a GR antagonist or GR antisense when applied chronically and intrathecally, indicating a regulatory role of spinal GR on NMDA receptors in the dorsal horn (Wang et al, 2005). Furthermore, the contribution to pain does not lie solely with GRs ability to regulate the expression of NMDA receptors. Studies have also shown that

the GR can regulate EAAC1 (a neuron specific high capacity glutamate transporter). Under normal circumstances EAAC1 prevents the over-stimulation of glutamate receptors by regulating the amount of extracellular glutamate that is present in the synaptic cleft (Brustovetsky et al, 2004). However, during neuropathic injuries EAAC1 is down-regulated in the dorsal horn (Sung et al, 2003) a process that can be prevented with the use of GR antagonists and antisense aimed at disrupting GR expression in the dorsal horn (Wang et al, 2006). GR can also modulate the expression of cannabinoid receptor in the dorsal horn (Lim et al, 2005) as well as being a key molecule in the development of morphine tolerance. The mechanism by which the expression of GR is regulated in the CCI model remains unknown. However, owing to the existence of a zif268 binding site on exon 1 of the GR promoter (Fig 5.2) it remains possible that GR expression in the dorsal horn is dependent on the activity of zif268 (Weaver et al, 2004).

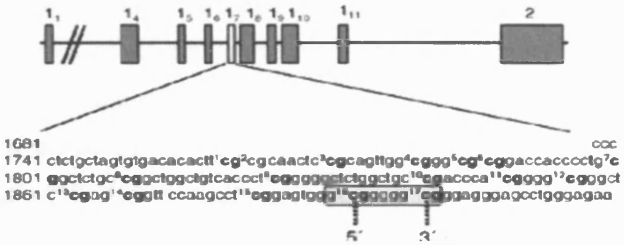


Figure 5.2. The promoter region of GR (exon 1_7) contains a binding site for zif268. This binding site may provide a site where zif268 can regulate the expression of GR. (Adapted from Weaver et al, 2004)

Since CCI does not increase corticosterone plasma levels, it is possible that in the absence of increased corticosterone levels, local changes in the GR signalling in the dorsal horn may be restricted to regions of the CNS that have received substantial activation such as after nerve injury or peripheral inflammation. Thus activation leading to central sensitisation would be accompanied by increased expression of GR in the dorsal horn neurons. The experiments presented here attempted to determine the role of the GR in the expression of mechanical allodynia and hyperalgesia and whether spinal GR expression changes during persistent peripheral inflammation. In addition, focus was also placed on the potential regulatory role of zif268 in determining the expression levels of the GR in the dorsal horn following peripheral inflammation.

5.2 METHODS (see chapter 2 for more details)

5.2.1 General GR immunostaining

Adult Sprague-Dawley rats were rapidly anesthetised using halothane, half the animals were injected with CFA into the intraplantar region of the hindpaw, the remaining animals did not receive CFA and remained non-inflamed. Animals were then perfused 2h later with 4% PFA. Dorsal horn sections (lumbar 4-6) were cut into 40μm sections and immunostained for GR with rabbit anti-GR (1:500, 4°C, overnight). Biotinylated anti-rabbit secondary antibody (1:200, 2h, RT) was used to enhance the signal and visualised using Alexa Fluor 488 (1:500, 2h, RT). Sections were mounted onto glass slides and coverslipped with Gel Mount (Sigma).

5.2.2 Intrathecal catheterization and drug delivery

Mini-osmotic pumps were implanted sub-dermally. Pumps were connected to a PE10 catheter that was inserted at the level of the lumbar enlargement (L4-L5) under halothane anaesthesia. For experiments using GR oligonucleotides, the antisense sequence was 5'-TGG AGT CCA TT GCA AA*T-3' and the missense was 5'-TGA AGT TCA GTG TCA AC*T-3'). The GR antagonist dexamethasone 21-mesylate (dex-mes) was also used in this study. For zif268, the antisense sequence was 5'-GGT-AGT-TGT-CCATGG-TG*G-3 and for the missense, 5'-GTG-TTC-GGT-AGG-GTG-TC*A-3'. Each mini-osmotic pump administered 0.5μg/μl/h of oligonucleotide for the GR study, 0.16μg/μl/h for the zif268 study and 28nmol (in total) of dex-mes (dissolved in 0.9% saline) for 7d. Following behavioural tests, animals were perfused and examined for correct catheter placement and damage to spinal cord. Animals with damaged cords and displaced cannula were discarded from the study (see Methods and Materials 2.2).

5.2.3 Behavioural tests and statistical analysis

Animals were habituated to the testing environment before surgery. 3d following surgery, the baseline score to ramped mechanical stimulation was taken (mean of 4 scores taken, each separated by a minimum of 5min). On the third day, rats received a unilateral intraplantar injection of CFA (100μl, 50%). Over the next 4 days animals were tested for mechanical allodynia and hyperalgesia. Comparisons were made between antisense and missense animals. Results were statistically analyzed using a two-way ANOVA repeated measure.

5.2.4 Retrograde labelling with FG

Rats were anaesthetised continuously with halothane throughout the surgical procedure and mounted onto a stereotaxic frame. Co-ordinates for the parabrachial nucleus were ascertained using an appropriate brain atlas (Paxinos and Watson, 1998). An injection (4%, 300nl) of fluorogold (FG) was made into the parabrachial nucleus (see Methods and Materials). Animals were left to recover for 3-4d before being given a unilateral injection of CFA (100µl, 50%) into the intraplantar surface of the hindpaw. Rats were then perfused and immunostained for zif268 and GR (see below)

5.2.5 Immunocytochemical staining for oligonucleotide toxicity

Following oligonucleotide administration and behavioural analysis Rats were deeply anaesthetized with pentobarbital and transcardially fixed with 400-500 ml of 4% paraformaldehyde. The lumbar spinal cords were dissected, and postfixed for 4h, left overnight in 30% sucrose and 40µm transverse sections cut on the microtome. Immunocytochemical staining was used to detect Neuronal-specific nuclear protein (NeuN), NK1 (see table 2.2 for concentrations). Sections were also stained for Hoescht and IB4 to check for toxicity.

5.2.6 Immunocytochemical staining for GR after oligonucleotide treatment

Osmotic pumps containing zif268 or GR antisense or missense were implanted into adult rats sub-dermally. 4d later, rats received a unilateral injection of CFA (50%, 100µl) into the intraplantar hindpaw and were perfused 2h later. The immunocytochemistry then followed the same protocol as described in the Chapter 2 and Section 5.2.1 (above). Individual GR expressing neurons were then analysed for pixel luminosity using the BioRad 600 confocal microscope. Luminosity scores are a measurement of the brightness emitted from the fluorophore during the excitation. The technique makes the assumption that the greater the luminosity score the more of the detected protein that is present in the cell. Sections from the L4-L5 region of the spinal dorsal horn were taken from animals treated with either zif268 or GR antisense or missense. Sections were immunostained for GR and the luminosity scores were ascertained from 10 sections per animal. Since the background for each section varied slightly, sections were normalised by subtracting the background value from adjacent neuropil. The mean from the twenty highest score from each section were taken and comparisons were made with the other treatment group.

5.2.7 Western blotting

Following zif268 and GR antisense and missense treatment for 4d, and 2h following intraplantar CFA injection to the hindpaw, protein levels of GR residing in the ipsilateral dorsal horn of the spinal cord were analysed using western blotting techniques. This technique was also used to analyse the effectiveness of the antisense knockdown as well as to study the potential role of zif268 in regulating GR in the dorsal horn (see Methods and Materials). Quantification of individual protein bands of GR and GAPDH were analysed using the software Bio-Rad Quantity One 4.4.1. To analyse if the GR protein levels changed following GR or zif268 antisense or missense treatment, the GR quantification value was divided with the appropriate GAPDH (loading control) quantification value for each lane. By doing this a ratio score for each lane was made. The ratio scores for each treatment group were then compared with each other.

5.3 RESULTS

5.3.1 Increased expression of GR after peripheral inflammation

Immunohistochemistry revealed that dorsal horn neurons constitutively express GR. The majority of GR positive cells were present in both the inner and outer regions of lamina II, with some positive cells also being located in lamina I, although the levels were lower than in lamina II (Fig 5.8) (LI 12 +/- 3 and LII 48 +/- 5). In order to examine the role of GRs in persistent inflammatory pain, a unilateral injection of CFA (50%, 100µl) was made to the intraplantar region of the hindpaw of adult rats. CFA injections did not increase the number of cells in either the ipsilateral (90 +/- 7 cells per 40µm section) or the contralateral (98 +/- 3 per 40µm section) dorsal horn in comparison with naïve animals (105 +/- 16 cells per 40µm section) (Fig 5.3). However, confocal microscopy detected a significant increase in GR cell body luminosity following CFA in the ipsilateral dorsal horn (relative luminosity 112 +/- 6) in comparison with naïve animals (relative luminosity 83 +/- 3) ($P<0.05$). However, no significant differences were found in GR cell luminosity between naïve animals and the contralateral side of CFA treated animals (relative luminosity 83 +/- 2).

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5.3.2 GR antisense blunts the maintenance of inflammatory pain states

To examine if GR expression affected the paw withdrawal thresholds to mechanical stimuli during inflammatory pain, adult rats received a continuous intrathecal infusion of missense or antisense targeting GR mRNA. The administration of GR antisense or the missense alone did not cause any significant changes in the respective baseline scores to mechanical stimulation (Fig 5.4). Paw withdrawal thresholds were 26 +/- 5g for antisense and 36 +/- 4g for missense treated animals before surgery and 32 +/- 2g and 33 +/- 6g respectively after surgery (before CFA). Following a unilateral injection of CFA into the intraplantar region of the hindpaw all animals developed mechanical allodynia and hyperalgesia 1d after the injection (Fig 5.5). A significant reduction in paw withdrawal thresholds was seen in both the missense ($P<0.04$) and the antisense group ($P<0.05$) compared with baseline scores one day after CFA injections. Overall, there was a significant affect of oligonucleotide treatment on paw withdrawal thresholds following peripheral inflammation ($F_{1, 15} = 10.6$; $P<0.005$). This response was stable and remained unaffected with time (day 1 to day 4 post CFA) ($F_{3, 45} = 2.12$; $P = 0.14$). Analysis on individual days showed that the two treatment groups were not significantly different from each other on the first day, where the mean paw withdrawal threshold decreased by 18 +/- 2g following antisense treatment and 24 +/- 4g after missense treatment in comparison with their respective baselines. However, on the second day, paw withdrawal

thresholds were significantly higher (P<0.01) in antisense group in comparison with the missense group, so that paw withdrawal thresholds decreased by 20 +/- 2g with antisense and 29 +/- 2g with missense from baseline. The paw withdrawal threshold of the antisense group remained significantly higher than the missense group from day 2 onwards until day 4 post CFA (day 3 P<0.01; day 4 P<0.01).

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5.3.3 Examination of glucocorticoid receptor knockdown

In order to verify GR knockdown was successful, western blots and immunohistochemistry were performed on a further two groups treated with GR antisense or missense at the same concentration as that used in the behavioural study. Relative luminosity scores for antisense (96 +/- 8) were significantly lower (P<0.05) in comparison with missense (126 +/- 3). In addition, cell counts revealed that GR antisense treatment significantly (P<0.05) reduced the number of GR positive cells in the dorsal horn in comparison with the missense group (61 +/- 13 compared with 112 +/- 21 cells per 40μm section). Western blots also showed a tendency towards a reduction in the levels of GR, however, the difference between the antisense and missense was not significant (Fig 5.6). An increase in the number of animals used for this analysis may have helped to reveal a significant drop in protein levels. Signal intensity (density) was quantified using the software package Quantity One 4.4.1 (Bio-Rad). Densitometric values were expressed as the ratio of GR to GAPDH (loading control). Statistical comparisons between the ratio scores of missense and antisense treatment were assessed using an unpaired t-test.

5.3.4 Lack of toxicity and cell death using antisense

To examine if oligonucleotide treatment resulted in unwanted tissue toxicity or damage, spinal cord tissue was stained for IB4, NeuN and NK1. In the majority of cases, no gaps in the IB4 staining were present. Under normal circumstances IB4 staining is observed as a distinct band that spans across the entire lamina II region of the dorsal horn (Fig 5.5). Any gaps or disruptions in the IB4 staining represent primary afferent damage. Animals with damaged spinal cords were removed from the experiment and behavioural data collected discounted (n = 5). Cell counts made in the superficial layers of the dorsal horn using NeuN revealed no oligonucleotide toxicity. The number of NeuN cells was similar across all treatment groups (naïve 703 +/- 16; missense 737 +/- 17; antisense 722 +/- 21 per 40μm section), Furthermore, Hoescht labelling did not detect any pyknotic nuclei in any of the groups. NK1 expressing cells are important for persistent inflammatory pain. Owing to their proximity with the catheter, NK1 cells would be most susceptible to any toxic effects. This would have dramatic

effects on nociceptive behaviours. We found that NK1 positive cells were healthy in all groups and no toxic effects were in evidence (Fig 5.7).

5.3.5 Behavioural effects of glucocorticoid receptor antagonist

In order to provide further evidence of the importance of the GR in persistent inflammatory pain it was decided to use dex-mes (a GR antagonist). The chronic intrathecal administration of dex-mes (28nmol) alone did not affect paw withdrawal thresholds (36 +/- 3g before surgery compared with 38 +/- 2g after surgery) (Fig 5.4). However, the antagonist did significantly inhibit mechanical allodynia and hyperalgesia following peripheral inflammation with CFA ($F_{1, 12} = 11.8$; $P<0.005$). Overall paw withdrawal latencies in dex-mes treated animals were significantly different from those treated with saline. This effect was stable for the entirety of the experiment (day 1-4 post CFA injection) ($F_{3, 36} = 0.54$; $P = 0.70$). In addition, analysis of individual days revealed that unlike the antisense treatment, dex-mes treatment partially attenuated mechanical sensitivity on all days following CFA induced peripheral inflammation. Paw withdrawal thresholds on day 1 after CFA decreased by 10 +/- 3g in animals treated with dex-mes and 17 +/- 2g for saline treated animals.

5.3.6 Zif268 and GR colocalisation in the dorsal horn and projection neurons

One of our hypotheses was that spinal GR may be regulated by zif268 during chronic inflammatory pain. In order for zif268 to regulate GR the two proteins must reside in the same cell. Immunohistochemistry targeting both GR and zif268 was applied to 40μm sections from animals that had previously been injected with CFA (2h) before perfusion. Results indicate that both zif268 and GR colocalise in a subpopulation of cells in the dorsal horn (Fig 5.8). Cell counts on the medial half of the dorsal horn revealed that the population of zif268 positive cells (99 +/- 11 per 40μm section) outnumbered the population of the GR positive cells (62 +/- 6 per 40μm section). 19 +/- 3% of cells containing zif268 colocalised with GR, while 31 +/- 5% of GR cells colocalised with zif268 (Fig 5.9). The level of colocalisation between GR and zif268 in LI-Pb projection neurons was 34 +/- 2 %. Using retrograde labelling it was noted that 61% of projection neurons to the parabrachial nucleus were GR positive of which half colocalised with zif268 (Fig 5.9).

5.3.7 Regulation of GR expression by zif268

Having established that zif268 colocalised with a small number of dorsal horn neurons (including projection neurons to the parabrachial nucleus), it was still unclear as to whether or

not zif268 had any regulatory role over GR during persistent inflammatory pain states. Using osmotic pumps to continuously deliver the oligonucleotides for a week, it was noted that the application of zif268 antisense not only attenuated the mechanical allodynia seen with peripheral CFA inflammation (see chapter 3) but also significantly reduced the expression of GR during peripheral inflammation. Comparisons of luminosity scores between zif268 antisense and missense treated animals revealed that immunostaining of GR positive cells in the missense group (with a relative luminosity score of 109 +/- 8) was 34% more intense in comparison with antisense treated animals (with a relative luminosity score of 69 +/- 5). Baseline values for both groups were normalised to zero. Western blots also showed a reduction of GR protein in the dorsal horn following zif268 antisense treatment. Levels were reduced by ~21% in comparison with the missense group although this did not reach significance (Fig 5.10).

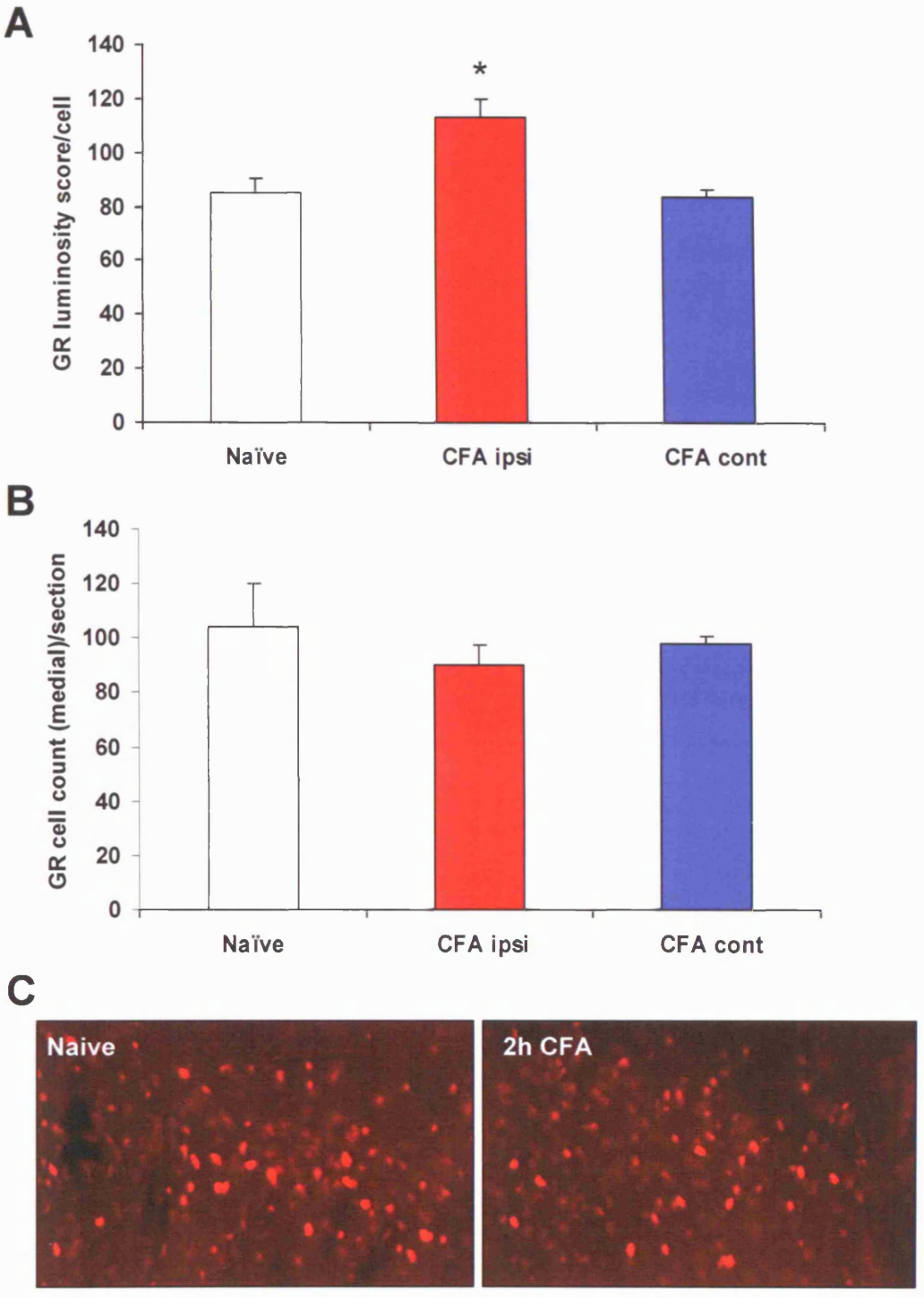

Figure 5.3. Peripheral inflammation with CFA increased GR expression in the superficial dorsal horn. Immunohistochemistry for GR provided evidence that GR levels increased following a unilateral injection of CFA (100µl, 50%, 2h) into the hindpaw. A) Luminosity scores (normalised to background scores) revealed that CFA caused a significant increase in the levels of GR in the dorsal horn. There were no difference between the levels of GR in the contralateral side compared to naïve animals. B) The total number of cells expressing GR in the medial portion of the dorsal horn remained unchanged following peripheral inflammation. C) Representative images of GR expression in the medial portion of the ipsilateral dorsal horn without CFA (naive) or 2h after intraplantar injection of CFA. (Confidence levels in comparison with naïve group. Unpaired t-test * $p<0.05$, n = 5, 10 sections per animal).

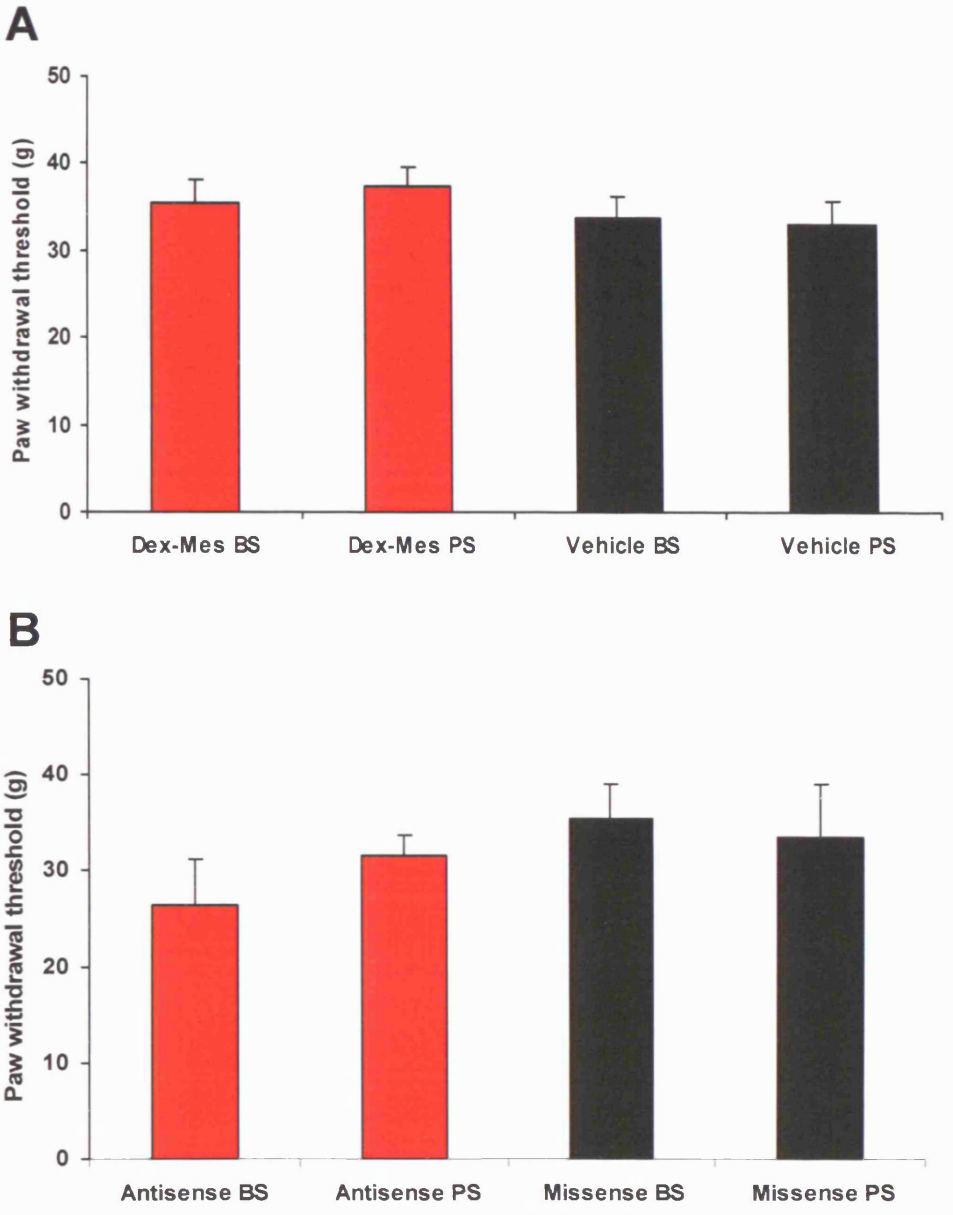

Figure 5.4. GR antisense, missense and dexamethasone 21-mesylate (dex-mes) did not affect paw withdrawal thresholds to mechanical stimuli without inflammation. Paw withdrawal thresholds before surgery (BS) were compared to those 2d after the implantation of the osmotic pumps (PS) containing either GR antisense, missense or dex-mes but before CFA injection. A) Intrathecal administration of dex-mes alone did not significantly affect the baseline paw withdrawal thresholds in non-inflamed animals. B) The intrathecal administration of either GR antisense (red bars) or missense (black bar) did not affect paw withdrawal responses in non-inflamed animals. (Unpaired t-test, AS: n = 9, MS: n = 8, dex-mes: n = 7, vehicle: n = 7).

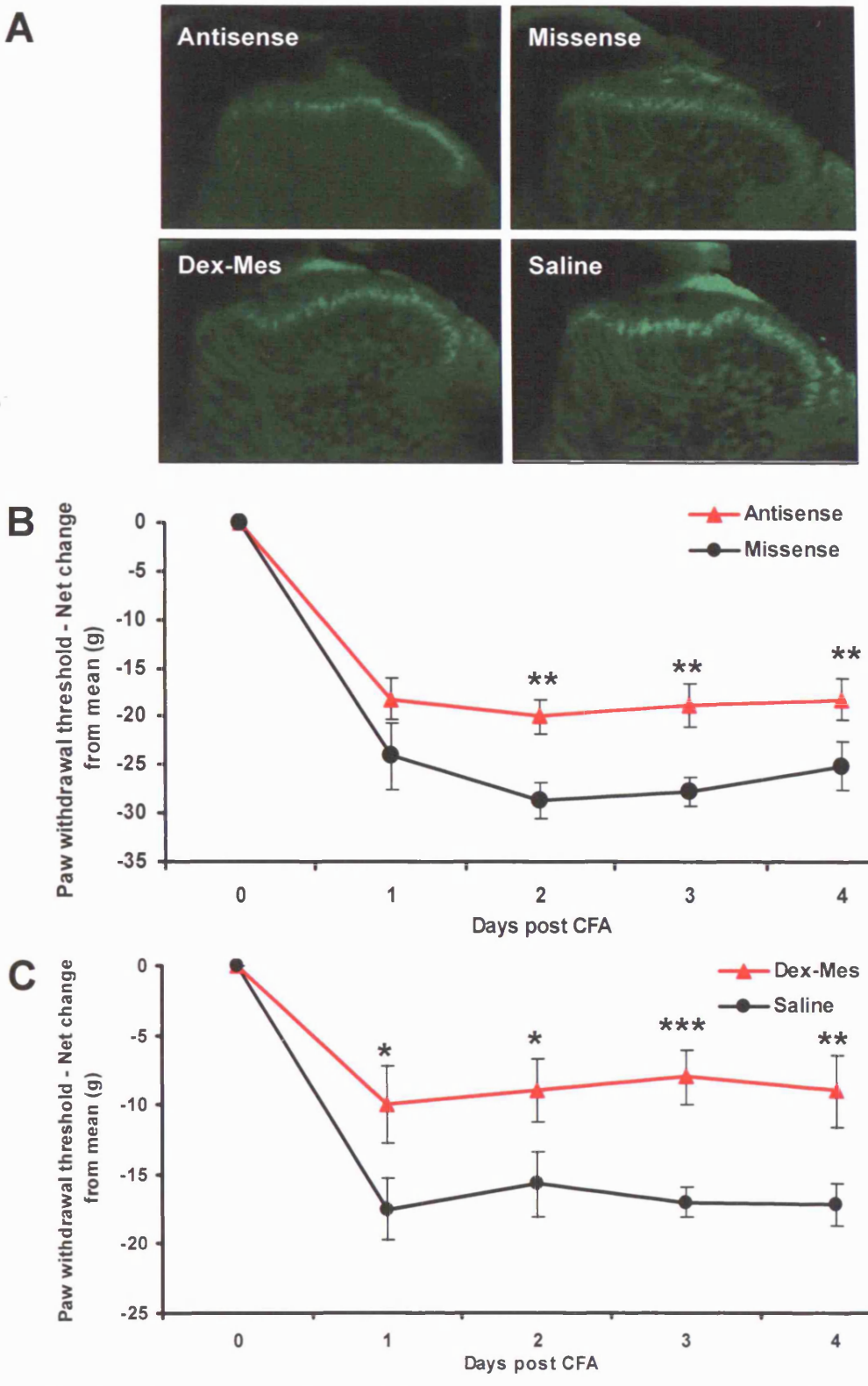

Figure 5.5. Glucocorticoid receptors (GRs) are important for the maintenance of persistent inflammatory pain states. A) IB4 was chosen to assess primary afferent damage following implantation of mini-osmotic pumps. Representative images showing IB4 staining in the dorsal horn. Only animals without primary afferent damage were included into the behavioural studies B) Mechanical allodynia and hyperalgesia associated with peripheral inflammation was significantly reduced with the chronic i.t. infusion of GR antisense using mini-osmotic pumps (red bar, n = 9) in comparison with missense treatment (black bar, n = 8) on days 2 to 4 following intraplantar CFA injection (100µl, 50%). C) Mean paw withdrawals following chronic i.t. administration using mini-osmotic pumps containing dexamethasone 21-mesylate (a GR antagonist, red bar, n= 7) was significantly reduced in comparison with vehicle (saline) control (black bar, n = 7). (Two way ANOVA repeated measure and Tukey's post hoc testing *P < 0.05, **P < 0.01, ***P < 0.001).

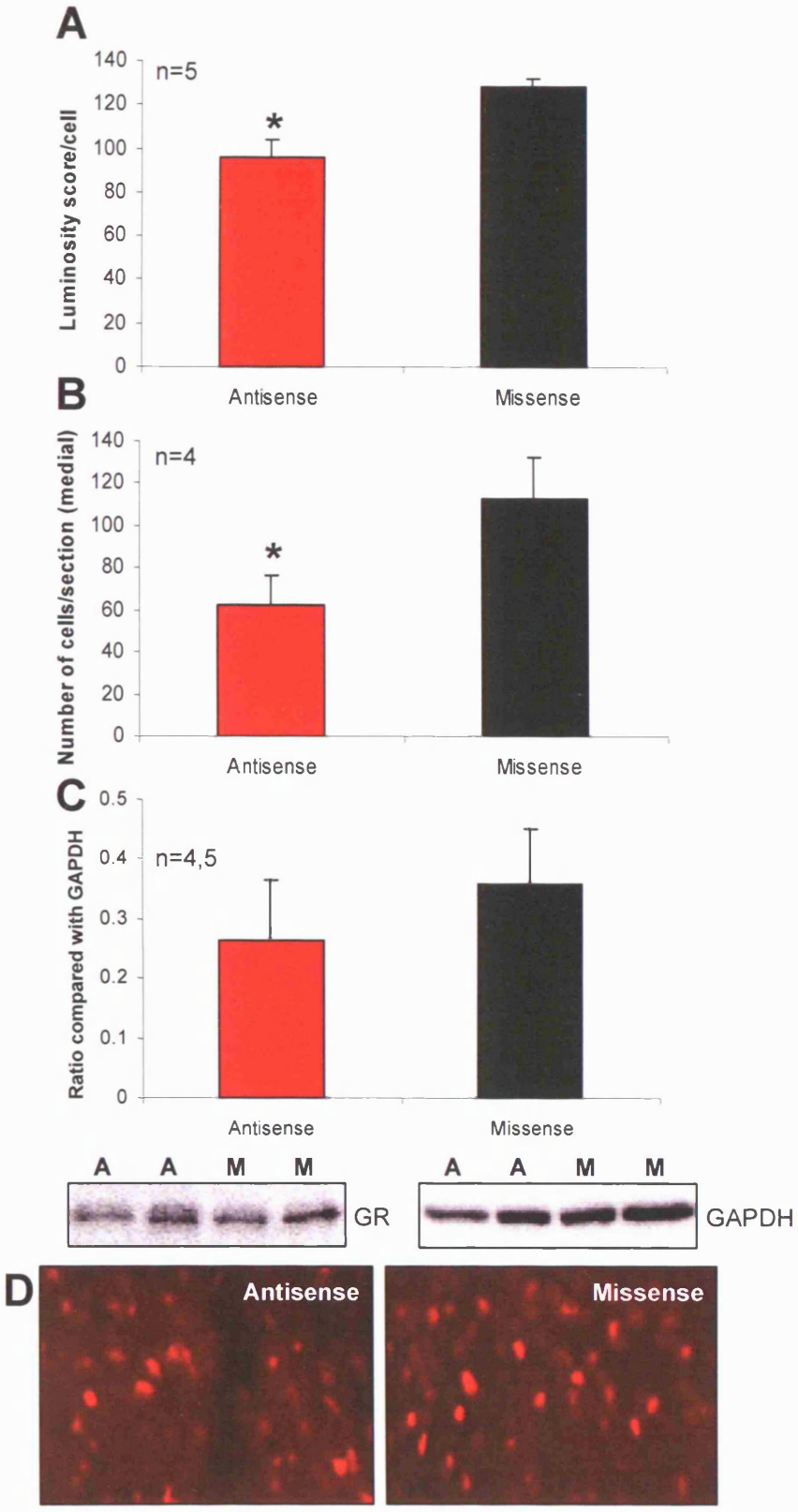

Figure 5.6. Verification of GR knockdown following antisense treatment. Adult rats were implanted with mini-osmotic pumps delivering GR antisense or missense oligonucleotides onto the dorsal horn. Animals were infused for 4d before receiving an intraplantar injection of CFA (2h, 100µl, 50%) into hindpaw. A) Luminosity scores (normalised to background) were analysed following immunostaining. Scores were significantly lower in animals treated with antisense in comparison to the missense group. B) Cell counts for GR positive cells in the dorsal horn revealed that GR antisense treatment significantly reduced the mean number of GR positive cells in comparison with missense group. C) Animals were also analysed for changes in GR protein levels using western blotting techniques. GR protein levels were somewhat lower in animals treated with GR antisense in comparison with the missense. However the reduction did not reach significance D) Representative images taken from the medial ipsilateral dorsal horn in antisense and missense treated animals 2h after CFA injection. (Unpaired t-test, *p <0.05).

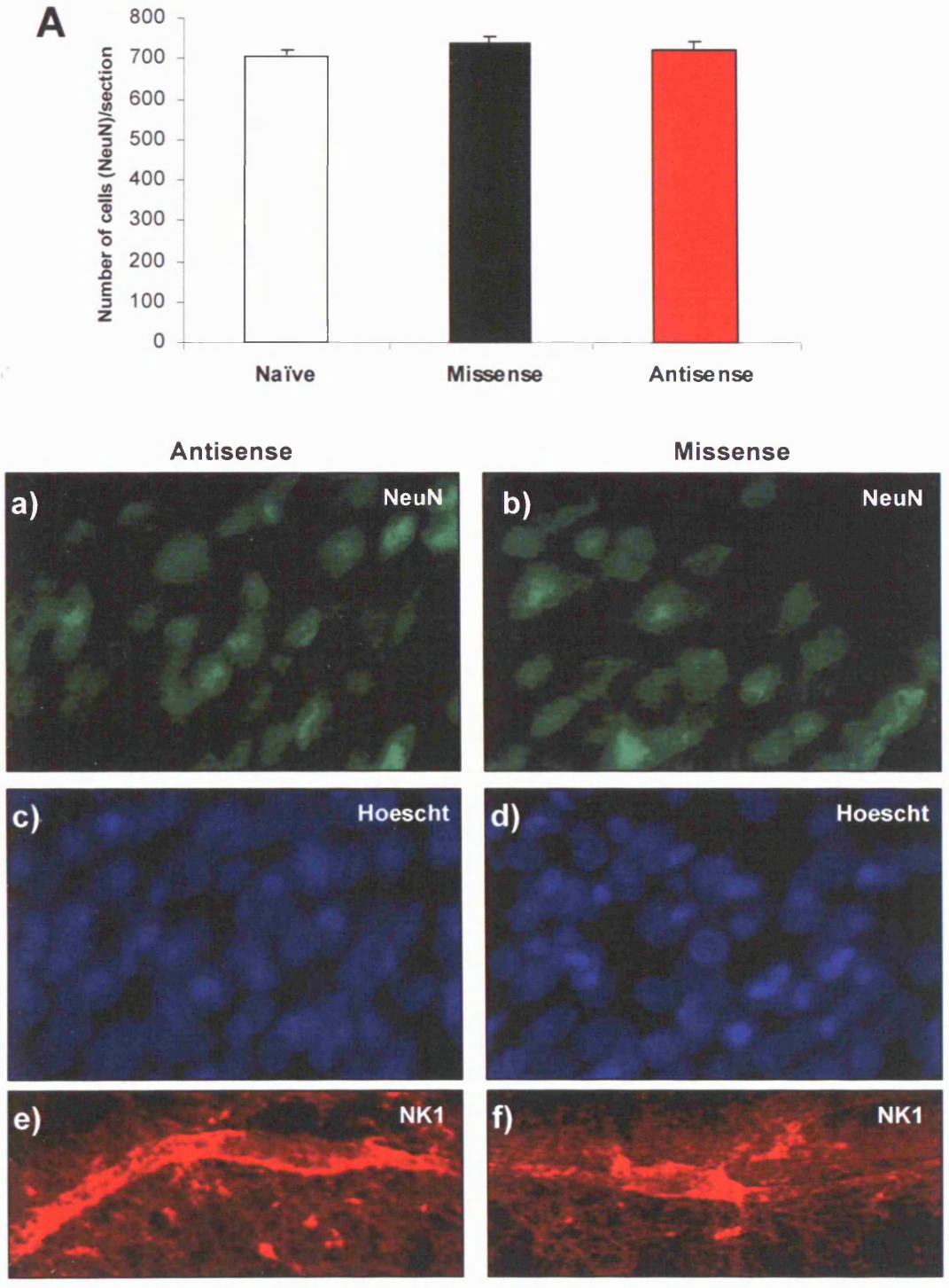

Figure 5.7. No toxic effect of GR antisense or missense on dorsal horn neurons. Animals received chronic intrathecal infusion of GR antisense or missense via mini-osmotic pumps followed by an intraplantar injection of CFA for 4d. Sections of spinal cord were immunostained for NeuN, and NK1 and stained for Hoescht. A) NeuN positive cells located in lamina I and II were automatically counted using MCID software. No significant difference was found between naïve, antisense or missense treated animals in the spinal dorsal horn. B) Representative images showing (a) NeuN staining, (c) Hoescht staining and (e) NK1-R immunostaining for GR antisense treated animals and (b) NeuN staining, (d) Hoescht staining and (f) NK1-R immunostaining for missense treated animals. Hoescht staining did not pick up any pyknotic nuclei in either the antisense or the missense groups. NK1 receptor staining was similar in both antisense and missense treated rats; with cells appearing healthy in both groups (Unpaired t-test, n = 7).

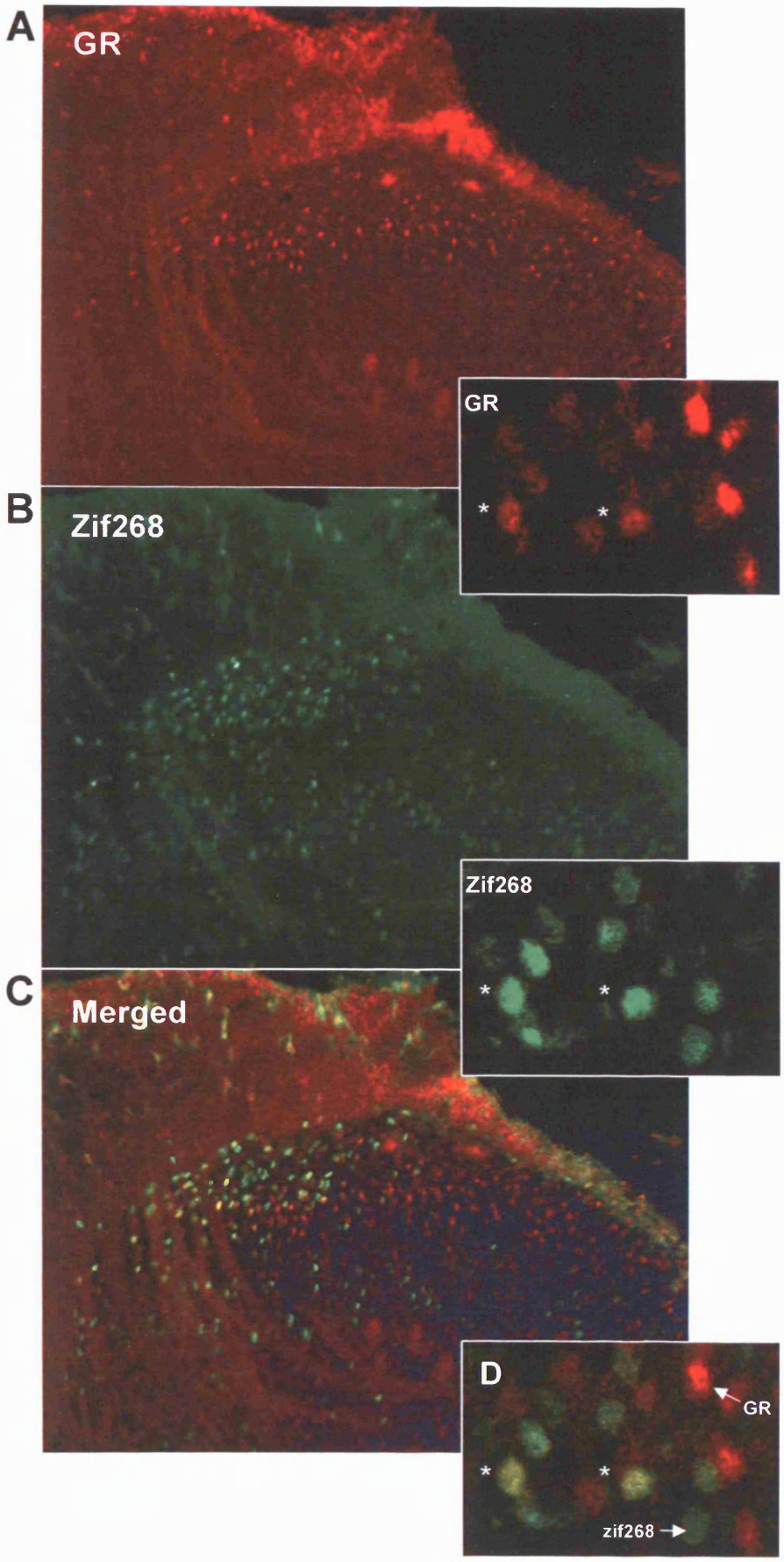

Figure 5.8. Zif268 and GR colocalise in spinal dorsal horn neurons. A) GR expression and B) zif268 expression in the dorsal horn 2h after an intraplantar injection of CFA. C) Merged image (of A and B) showing colocalisation of zif268 and GR. D) High magnification image of zif268 (green) and GR (red) in the medial portion of the dorsal horn. * cells that express both zif268 and GR. Inset images were taken from different animals to the main pictures.

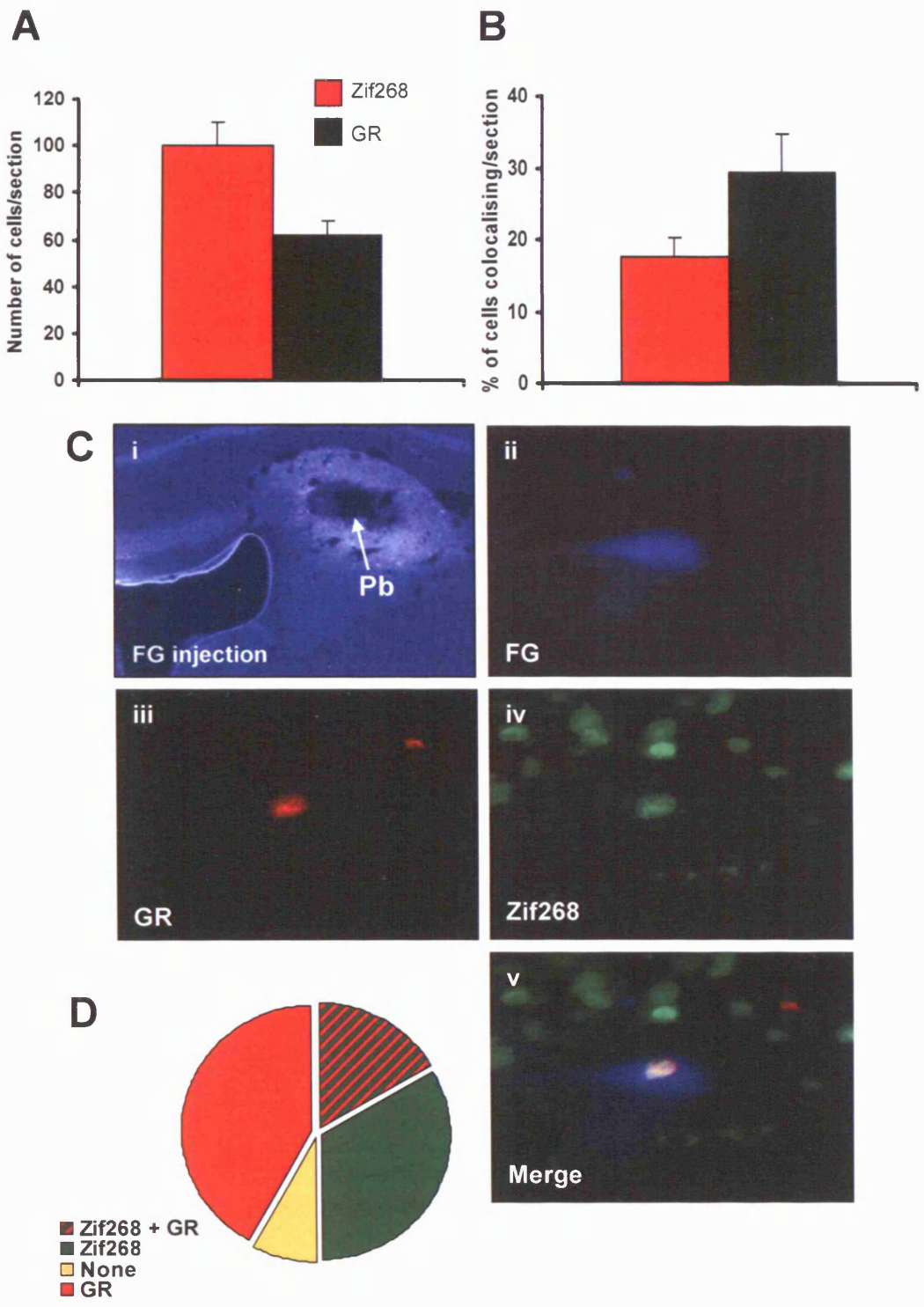

Figure 5.9. Zif268 and GR colocalise in spinal dorsal horn neurons and in lamina I - projection neurons following peripheral inflammation (intraplantar CFA, 2h, 50%, 100µl). A) Mean number of neurons expressing zif268 (red bar) and GR (black bar) in the superficial layers of the lumbar dorsal horn (per 40 µm section, medial portion). B) 19 +/- 3% of zif268 positive cells colocalised with GR positive cells (red bar) and 31 +/- 5% GR positive cells colocalised with zif268 positive cells (black bar) in the superficial layers (medial portion) of the dorsal horn. C) (i) Fluorogold (FG) injection into the parabrachial nucleus successfully labelled lamina I - projection neurons (LI-Pb) (ii). A number of LI-Pb neurons co-expressed (iii) GR with (iv) zif268 and can be seen in figure Cv. D) Pie chart indicating percentage of zif268 and GR expression in LI-Pb projection neurons. Retrograde labelling from the parabrachial nucleus with fluorogold showed that in total 61% of LI-Pb neurons expressed GR (red and hashed slice). A total of 47% of LI-Pb neurons expressed zif268 (green + hashed slice), while 34% of LI-Pb neurons co-expressed zif268 *and* GR (hashed slice) (n=5, ten sections per animal).

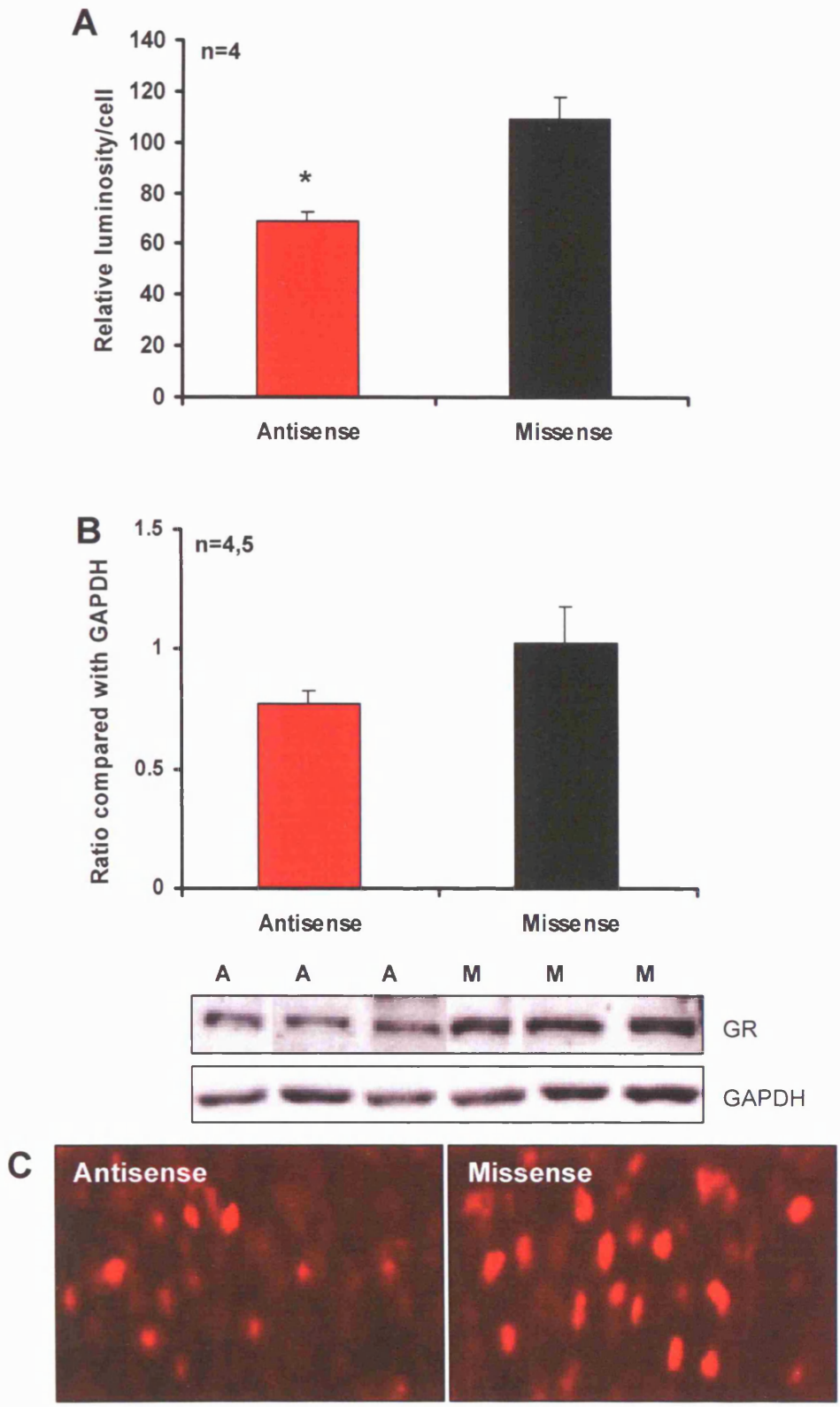

Fig 5.10. Zif268 regulates the expression of GR during peripheral inflammation. A) Chronic intrathecal administration of zif268 antisense using mini-osmotic pumps significantly reduced GR expression in the medial half of the dorsal horn by 34%. in animals with peripheral inflammation (CFA, 100µl, 50%, 2h) in the hindpaw. B) Western blot confirmed a reduction in GR expression in zif268 antisense treated animals. However this did not reach significance ($p<0.12$). C) Representative images of GR expression in the medial dorsal horn following chronic intrathecal infusion of zif268 antisense and missense with 2h CFA treatment. (Confidence levels in comparison with missense) (Unpaired t-test *$p<0.05$).

5.4 DISCUSSION

5.4.1 Peripheral inflammation induces the upregulation of spinal GR

Using immunohistochemical approaches we found that spinal GR expression was predominantly located in lamina I and II of the dorsal horn. Deeper lamina of the dorsal horn expressed a much lower level of GR. The spinal ventral horn (motorneurons) also expressed GR. As such our immunohistochemical analysis is in agreement with other studies that have localised GR to the same spinal cord regions (Clarck et al, 1981; Duncan and Stumpf, 1984; Fuxe et al, 1985; Wang et al, 2004).

This study has shown that peripheral inflammation induced by CFA can increase the expression of GR in a subset of dorsal horn neurons. There were no significant changes to the number of GR positive cells in the dorsal horn indicating that the increase in GR expression was within neurons that already expressed the protein. This increase expression in a restricted population of neurons suggests that these GR positive neurons play a certain role in nociception possibly by increasing signalling to corticosterone. The use of intrathecally applied antisense that targeted GR expression in the dorsal horn during CFA inflammation reduced GR expression and subsequently reduced mechanical hyperalgesia. Using antisense that targeted zif268 mRNA, GR expression was found to decrease in the dorsal horn

During CCI the expression of both NR1 and EAAC1 are regulated by GR activation (Wang et al, 2006). Studies have shown that the NMDA receptor is important in the establishment of inflammatory pain both for visceral pain (Zhou et al, 2006) and peripherally derived inflammatory pain states (Guo et al, 2005). Indeed in the dorsal horn the NR1 subunit undergoes rapid phosphorylation at Ser897 following CFA injection into the hindpaw. Moreover, a rise in the NR1 protein in the dorsal horn was seen in the first day following CFA injection (Guo et al, 2005). A similar response with the NR1 subunit can also be seen in rats with chronic colonic inflammation (Zhou et al 2006). The evidence for the importance of EAAC1 during inflammatory pain is less clear due to the fact that no specific inhibitor exists for this protein. However, studies using a combination of inhibitors aimed at a number of glutamate transporters have given clues that EAAC1 may be important for the full behavioural expression of pain following formalin and CFA (Tao et al, 2005a, Tao et al, 2005b). Therefore, it remains possible that these proteins seen to be regulated by the GR in neuropathic pain, may also be regulated by the GR in models of inflammatory pain.

5.4.2 Spinal GR: a role in inflammatory pain behaviour

The use of antisense that targeted the mRNA of the GR has shown that chronic inflammatory pain requires the activation of the GR in the dorsal horn. This result was further verified with the use of a GR antagonist (dexamethasone 21-mesylate). The effect of dex-mes was more potent in comparison with the antisense treatment being effective at reducing mechanical allodynia and hyperalgesia a day earlier. A possible explanation could reside in the method and rates in which they disrupt the actions of the GR. The antagonist forms permanent covalent bonds with GR (Stoney-Simons and Thompson, 1981; Miller et al, 1984) and inhibits the actions of both constitutive GR and de-novo GR. GR antisense, unlike dex-mes, does not interact with GR at the protein level. Instead, the antisense interferes with GR translation and therefore does not immediately interfere with constitutive GR.

The activation of constitutively expressed GR may lead to non-genomic changes. Non-genomic effects of GR have been seen in other parts of the nervous system: GR have been shown to modulate synaptic plasticity and drive intracellular changes. GR can increase the levels of intracellular calcium in hippocampal neurons by activating voltage dependent calcium channels (VDCC) (Coussens et al, 1997). GR can also facilitate synaptic potentiation by affecting VDCC. Furthermore, GR has also been implicated in the inhibition of the NMDA receptor activation (Krugers et al, 2005). In the dorsal horn, mice with mutated VDCCs respond in a different manner to wildtype mice to the intrathecal application of a GR antagonists, indicating a potential interaction in the dorsal horn between the N-type calcium channel and GR (Takasaki et al 2005). There is also the possibility that constitutively expressed GR can lead to the production of more GR and other proteins by either a direct or indirect signalling pathway (Meaney et al 2000, Revest et al 2005) which may lead to an amplification of GR mediated signalling in the dorsal horn.

The effectiveness of the GR antisense treatment became apparent two days after the injection of CFA. This may reflect the time needed for GR to be transcribed and cause other transcriptional events in the dorsal horn following initial inflammatory stimulation. It is possible that the time point represents a switch from non-genomic effects to a more genomic effect. Therefore GR may have a dual role: constitutively expressed GR is responsible for signalling events that lead to persistent pain states, while transcribed GR maybe important for the maintenance of chronic pain states i.e. increasing the transcription of key proteins involved with maintenance of chronic pain. However this will need to be investigated further.

In contrast to our results, GR activation in the dorsal horn has been suggested to have anti-nociceptive qualities. The GR agonist dexamethasone can reduce spinal cyclo-oxygenase-2 (COX-2) expression in the dorsal horn (Hay and de Belleroche, 1999). COX-2 activation in dorsal horn has been linked to the facilitation of mechanical allodynia and hyperalgesia in inflammatory pain models (Hay et al, 1997; Seybold et al, 2002) and in neuropathic conditions (partial sciatic nerve ligation) (Ma et al, 2002). There are no clear explanations for the discrepancy with our work and that of Hay and de Belleroche (1999). However, they administered dexamethasone subcutaneously, with the first dose beginning 30min before CFA, while we applied oligonucleotides intrathecally, continuously and 3d before CFA. The *chronic* infusion of oligonucleotides itself into the spinal cord or the down-regulation of the GR receptors with chronic dex-mes in the dorsal horn could have led to neuronal and non-neuronal adaptations in the dorsal horn that may have compromised the activation of spinal COX-2 during inflammation. To resolve this issue, changes in COX-2 activity could be analysed following our GR regime.

5.4.3 Zif268 regulates the expression GR

Results from chapter 3 have made suggestions that spinal zif268 expression during peripheral inflammation was vital for the maintenance of the pain state. The knockdown of zif268 attenuated mechanical allodynia and hyperalgesia. Immunohistochemistry revealed that both zif268 and GR colocalised in the dorsal horn neurons including lamina I projection neurons. Lamina I projection neurons to the brain provide important pathways for chronic pain states and undergo long-term changes in their synaptic sensitivity (Ikeda et al, 2003; Ikeda et al, 2006). The vast majority of lamina I projection neurons express the NK1 receptor (Bester et al, 2001; Todd et al, 2002). The response to chronic but not acute pain can be significantly attenuated following the ablation of NK1 positive cells in lamina I (Mantyh et al, 1996; Nicholls et al, 1999). Using high frequency stimulation of C-fibres it was shown that lamina I projection neurons that also expressed the NK1 receptor were capable of supporting long term potentiation (LTP) with an enhancement in the excitatory post synaptic current (EPSC) (Ikeda et al, 2003).

It is currently unclear which signalling pathways are involved with the increase expression of GR in the dorsal horn. This study suggests that the immediate early gene zif268 is involved. During peripheral inflammation and HFS the protein expression of zif268 in the dorsal horn dramatically increases within the first 2 hours. By binding to the GR promoter, zif268 expression could dramatically increase transcription of GR on lamina I projection neurons, and in general, in dorsal horn neurons. Previous studies on the hippocampus have shown that

LTP in the dentate gyrus was dependent on the transient activation of the ERK1/2 pathway (Davis et al, 2000). The inhibition of this pathway with MEK inhibitors not only prevented the induction of LTP but also prevented Elk-1 and CREB phosphorylation and reduced zif268 expression. Zif268 is vital for persistence hippocampal LTP and is essential for the maintenance of persistent inflammatory pain. Peripheral inflammation induces phosphorylation of ERK1/2 within 10min in the dorsal horn (Ji et al, 2002). In a similar manner to hippocampal ERK1/2 activation, spinal ERK1/2 expression is dependent upon the activation of protein kinase C (PKC) and the subsequent phosphorylation on raf-1 which lies upstream of ERK1/2. The activation of PKC requires high intracellular calcium concentrations through the NMDA receptors in the dorsal horn. Noxious stimulation can activate and phosphorylate the NMDA receptor. We propose that peripheral inflammation activates and phosphorylates the NMDA receptor, which facilitates calcium influx and the phosphorylation of ERK1/2 via PKC and Raf. The activation of the ERK1/2 pathway would lead to the phosphorylation of CREB and Elk-1 in the dorsal horn and the subsequent transcription of zif268. Zif268 would then bind onto the GR promoter leading to an increase in GR expression.

At present the temporal profile of corticosterone release following intraplantar injection of CFA is currently unclear, however a number of other inflammatory pain models have suggested that circulating corticosterones can be increased rapidly and possibly for extended periods of time. The intraplantar injection of formalin can lead to the activation of the HPA axis and the rapid secretion of both ACTH and corticosterones. Plasma analysis suggests that ACTH and corticosterones are secreted within 5min and peaks at 30min after formalin stimulation (Taylor et al, 1998). The injection of CFA into the temporo-mandibular joint (TMJ) can also increase the levels of corticosterone for 2 days (Harper et al, 2000). It is therefore possible that, in a similar manner to hippocampal neurons (Revest et al, 2005), the increase concentrations of corticosterones caused by peripheral inflammation with CFA can lead to the activation of the MAPK pathway through constitutively expressed GRs leading to changes in gene transcription.

5.5 CONCLUSION

Long term changes in synaptic sensitivity in the superficial dorsal horn are dependent on gene expression. These essential molecular changes may be crucial in the maintenance of long-term pain states that are mediated by lamina I projection neurons. Dorsal horn neurons increase GR synthesis when stimulated and are vital for the full expression of mechanical allodynia and hyperalgesia following CFA induced peripheral inflammation. Reducing zif268

expression in dorsal horn neurons can also reduce the expression of GR, suggesting that GR expression in the dorsal horn is subject to zif268 regulation. This work has provided a new insight into the molecular complexities surrounding inflammatory pain states.

CHAPTER 6

General Discussion

6. GENERAL DISCUSSION

Molecular changes in the dorsal horn are essential to support the modifications in synaptic efficacy that underlie persistent inflammatory pain states. In this thesis the role of the immediate early gene zif268, has been shown to play an important role in the maintenance of inflammatory pain in part through the regulation of two downstream genes, serum and glucocorticoid inducible kinase 1 (SGK1) and the glucocorticoid receptor (GR).

6.1 Zif268 expression and regulation of pain states

Spinal zif268 expression has been shown to be induced in dorsal horn neurons following a number of different stimuli including formalin and high frequency, high threshold stimulation of primary afferents (Rygh, et al, 2006). In this study, we have shown that CFA injected peripherally into the intraplantar region of the hindpaw can likewise increase the expression of spinal zif268. Time course studies have shown the expression of spinal zif268 occurred by 1h and disappeared by 6 hours following peripheral inflammation with CFA. Expression of zif268 was predominantly found in the superficial layers of the dorsal horn and ipsilateral to the CFA injection, closely matching the innervation pattern from the hindpaw. Retrograde labelling from the parabrachial nucleus indicated that zif268 expression following CFA injection into the periphery occurred in lamina I projection neurons. The expression of zif268 in these neurons would suggest that alterations in gene transcription are obligatory for the development of persistent inflammatory pain regulating the synaptic plasticity of these key neurons (Nichols et al., 1999; Ikeda et al., 2006).

CFA injection into the intraplantar region of the hindpaw resulted in mechanical allodynia. Mechanical pressure that were previous insufficient to generate a withdrawal response did so following inflammation. This affect was attenuated with zif268 antisense treatment; suggesting an intimate relationship between mechanical allodynia and zif268. At present it is not clear if zif268 has a function in mechanical hyperalgesia. To resolve this issue, future work should focus on paw withdrawal thresholds in inflamed animals using von Frey filaments in the noxious range during intrathecal zif268 antisense (and missense) treatment.

Perhaps surprisingly ablation of 5-HT axons in the lumbar cord reduced inflammatory allodynia and hyperalgesia but did not reduce zif268 expression. We have previously shown that CFA induced zif268 expression is however reduced by lesioning lamina I/NK1 positive projection neurons that form the ascending component of a spinal-brainstem-spinal loop (Suzuki et al 2002; Rygh et al., 2006). The reduction in zif268 positive neurons was seen

principally in lamina II neurons and there was an accompanying reduction in mechanical sensitivity. Given that zif268 seems to be essential for the full development of inflammatory pain states the result of the 5-HT ablation study is puzzling given a similar behavioural change. I have suggested above that destruction of 5-HT axons may disrupt both descending inhibition and excitation and changes in zif268 levels may not therefore be evident. This might also explain why c-fos levels are increased in 5-HT ablated rats even though this should have predicted increased sensitivity to mechanical stimulation in sensitized rats. It also suggests that an increase in c-fos expression is not directly correlated to increased mechanical sensitivity despite earlier claims to the contrary (Dubner and Ruda, 1992; Hunter et al, 1995; Hou et al, 1997).

6.2 Zif268 regulation of SGK1

Zif268 differentially regulates the transcription of two other genes in the dorsal horn; SGK1 and the GR following peripheral inflammation. The SGK1 promoter region has been characterised and contains several motifs including GRE (glucocorticoid response element) (Webster et al, 1993) and several ERE (EGR response elements) motifs (James et al, 2006). The presence of these motifs, allow for the direct regulation of SGK1 expression by both glucocorticoids and zif268 respectively. The SGK1 promoter also contains a TATA box and Sp-1 elements as well as putative binding sites for a variety of other transcriptional regulators including the AP-1 complex and NF-kB (Firestone et al, 2003). Analysis from both immunohistochemistry and western blots revealed that SGK1 expression was repressed and GR transcription was induced as a consequence of spinal zif268 expression (Fig 6.1).

However, previous studies have demonstrated that, at later time points, *increased* expression of SGK1 occurred in models of joint inflammation. The expression of SGK1 in the dorsal horn of monoarthritic rats peaked at 6h, corresponding with a decline of zif268 expression in both the monoarthritic model and the peripheral inflammatory model. Moreover, the expression of SGK1 also occurred in approximately half of all projection neurons to the parabrachial nucleus from lamina I. A large proportion of these neurons also expressed zif268. The intrathecal application of antisense targeting SGK1 also delayed the induction of mechanical allodynia and hyperalgesia (Geranton et al, 2007). However, it seems likely that this later increase in SGK1 mRNA and protein follows phosphorylation of the MeCP2 gene repressor complex resulting in de-repression of SGK1 gene expression (Geranton et al, 2007). This would suggest that 1) the expression of zif268 represses constitutive levels of SGK1, 2) that by 6h post inflammation, zif268 expression has returned to baseline levels and 3) at 6h levels of SGK1 rise following de-repression by MeCP2. However, Geranton et al (2007) did

not show any change in SGK1 expression following adjuvant-induced knee inflammation at the 2h time point. This discrepancy may reside in the fact that the models used were different. First, the amount of CFA used was higher in our study. Second, the innervation pattern from the ankle joint to the dorsal horn is different from that of the hindpaw. Several studies using c-fos as a marker of neuronal activity have shown that c-fos expression in the arthritic model (ankle) was predominantly expressed in lamina I and V of the dorsal horn (Honore et al, 1995; Leah et al, 1996). In comparison hindpaw inflammation in our studies resulted in c-fos expression in lamina I, II and V. In addition, while Geranton et al (1997) analysed SGK1 expression at the mRNA level with RT-qPCR, we analysed SGK1 protein using western blot techniques. Moreover, the time course of allodynia and hyperalgesia of the two models are slightly different and may reflect different use-dependent transcription of SGK1.

6.3 Zif268 regulation of GR

In contrast to SGK1, the expression of GR was positively regulated by zif268. The expression of GR during peripheral inflammation has been shown to be integral to the development of the full pain state. The knockdown of this gene with GR antisense or antagonism of the receptor significantly attenuated pain states associated with CFA induced inflammation.

The pro-nociceptive role of GR has also been implicated in other pain states. Studies have shown that the glucocorticoid receptor in the dorsal horn during the chronic constriction model is upregulated and is essential for the full expression of this pain state (Wang et al, 2004). In addition, GR expression in this pain state is dependent on the activities of interleukin-6 (IL-6) and protein kinase C (PKCgamma). Spinal PKCgamma facilitates inflammatory pain (Guo and Huang, 2001; Sweitzer et al, 2004), and I have shown that spinal GR plays a functional role in regulating hyperalgesia and allodynia during inflammation. Our results suggest that neuropathic pain and inflammatory pain may share a common mechanism.

The enhancement of GR expression in a subset of neurons (including lamina I parabrachial projection neurons) in the dorsal horn during peripheral inflammation would allow for specific neuronal populations to become more responsive to circulating corticosteroids (i.e. only neurons that are involved with peripheral inflammation). The increase in GR expression in specific neuronal populations would increase transcriptional activity driven by circulating corticosterones and activate downstream genes that regulate synaptic plasticity. It has been shown for example that expression of the NMDA receptor in the dorsal horn can be modulated by GR in the dorsal horn following CCI (Wang et al 2005) as well as EAAC1 (Wang et al, 2006) and voltage gated calcium channels (Takasaki et al, 2005).

Hippocampal studies have also revealed that GR expression is closely associated with neuronal plasticity. ERK1/2 expression following stress can be prevented with the GR antagonist (RU38486) and the induction of LTP can also be altered (Yang et al, 2004). Furthermore, the activation of GR greatly increased the expression and the enzymatic activity of a number of proteins in the mitogen-activated protein kinase (MAPK) signalling pathway. Kinases that were seen to be affected include Ras and Raf-1, both of which can be directly activated via their GRE motifs. The inhibition of this pathway abolished the increase in contextual fear conditioning induced by glucocorticoids (Revest et al, 2005).

The ERK1/2 pathway has been implicated in the transcriptional activity that is necessary for the maintenance of both neuropathic and inflammatory pain states. As such, this would suggest that the GR can affect transcription by activating the ERK1/2 pathway. However, ERK1/2 expression in the superficial layers of the dorsal horn occurs rapidly (within 10min) in the peripheral inflammatory model and remains above baseline for over 24h (Ji et al, 2002). Since GR expression is in part dependent on zif268 expression in the dorsal horn (which increases at 1h) it seems unlikely that the early ERK1/2 expression is GR dependent. However this does not exclude the possibility that the maintenance of ERK1/2 expression following the 1h-2h time-point is GR dependent.

6.4 Technical limitations

This thesis used a number of techniques in order to analyse the role of zif268 in persistent inflammatory pain. However, these techniques have their limitations and inherent problems. These are briefly discussed below.

6.4.1 Chronic oligonucleotide treatment

The use of phosphorothioate modified oligonucleotides in this thesis successfully reduced the spinal expression of zif268 and GR in the dorsal horn. Oligonucleotides, in general have inherent limitations including cell toxicity and lack of specificity. While our studies checked for toxicity (and found no evidence of this), the observation for oligonucleotide specificity was more problematic. Phosphorothioate oligonucleotides were chosen for their ability to withstand nuclease digestion. However, studies have shown that this type of modified oligonucleotide may have non-specific effects, including binding to membrane proteins, DNA polymerases and the induction of transcription factors. For example, studies using c-*myc* antisense have shown that cell growth could be affected in a number of ways including,

sequence-specific antisense inhibition, sequence-specific non-antisense (aptameric) inhibition, and non-sequence-specific inhibition (Chavany et al, 1995). Moreover, sequence-specific effects of control oligonucleotides were also observed (Sun et al, 1996). As such, non-specific down-regulation of other genes may have occurred in our studies that may have played an influential role on our results. The use of microarray technology may provide a possible solution to help identify oligonucleotide specificity by monitoring other changes in gene expression (Delihas, 2001). However, given the fact that our oligonucleotides are designed to target transcription factors (GR and zif268), it may prove difficult even with microarray technology to determine specificity, as the expression of downstream genes will be modulated. In addition, chronic infusion of oligonucleotide could lead to compensatory effects in the spinal cord, adding to the problem, although vehicle controls were always used.

6.4.2 Immunohistochemistry

Immunohistochemistry was widely used in this thesis. Although we used negative controls (omission of the primary antibody) to test for specificity, ideally the antibodies should have been controlled further. The antibodies could have been used after adsorption of the primary antibody with the purified antigens and/or using dorsal horn sections from GR or zif268 knockout animals. This would have reduced the likelihood of false positives. However, knockout animals were not available but zif268 and GR antibodies run on western blots provided a second method for testing specificity and revealed distinct bands at the correct molecular weight for the proteins.

6.4.3 Cell counts

A potential problem with automatic cell counting is an over-estimation of the number of cells present on the tissue due to non-specific staining. In addition, the possibility of under-estimating the number of positive cells can also occur if the cell is not bisected through the maximum diameter. However, these problems would affect sections from both control and treated animals alike. In addition, the MCID cell count criteria was set at a rigorous enough setting to reduce the likelihood of false negatives. Cells were only counted if they fell within a certain size and fluorescence limit.

6.4.4 Luminosity scores

Luminosity scores was used to analysis changes in protein content of dorsal horn cells following oligonucleotide treatment. The major problem with analysing protein concentrations using this method is the assumption that the greater the signal from the cell, the greater the proportion of protein that is present in the cell. This assumption does have its problems; increases in protein would be rendered undetectable if the signal was saturated. To avoid this complication the protein was never immunohistochemically amplified with TSA. In addition, the settings of the confocal microscope and software had an upper limit of detection that was well above the maximum emission of the immunoreactive cells. Despite the limitations of this method, the approach has helped identify changes in protein content of individual cells in the dorsal horn undetectable by western blots.

6.4.5 Fluorogold tracing

The parabrachial nucleus forms part of the spino-bulbo-spinal loop and is involved with modulating spinal cord activity (through descending projections from the RVM). Fluorogold injections into the parabrachial nucleus successfully labelled lamina I projection neurons. However, necrosis was present at the injection site possibly as a consequence of the physical trauma of the FG injection and/or the FG itself. Damage to parabrachial region would affect descending modulation of the spinal cord and thus may play a role in the changes in gene expression seen in the dorsal horn (Svensson et al, 2006), affecting cell count analysis. In addition, the presence of FG in viable cells may have directly led to unwanted transcriptional changes in labelled lamina I projection neurons, leading to an increase (and over-estimation) of GR and/or zif268 expression in these neurons. However, this is unlikely due to rapid and transient nature of zif268 expression. Nevertheless, since we did not analyse the dorsal horn for gene expression immediately (1-6h) after FG injection we cannot exclude the possibility that zif268 (or indeed any other transcription factors) may have 'primed' the ascending FG positive neurons to react to inflammation (CFA) to a greater extent than normal (i.e. more likely to express GR or zif268). In addition, the injection of FG into the parabrachial nucleus may have labelled other descending fibres that just transverse the injection site. Therefore, some of the labelled lamina I projection neurons may not be LI-Pb neurons.

6.4.6 Ablation of serotonergic axons with 5, 7 DHT

The use of 5, 7 DHT successfully depleted serotonin in the dorsal horn. However, the absence of descending serotonergic axons may have resulted in compensatory events in the dorsal horn leading to long-term adaptations. Studies have shown that 5, 7 DHT can lead to gliosis.

Astroglia increases during inflammation (Frankfurt et al, 1991; Brezun and Daszuta, 2000) and have been suggested to have a role in chronic pain by releasing inflammatory cytokines (DeLeo and Yezierski, 2001; Watkins et al, 2003; Guo et al, 2007). 5, 7 DHT can also affect noradrenergic neurons. Although we did not check the noradrenergic population in the dorsal horn following intrathecal 5, 7 DHT, desipramine, can protect noradrenergic (NA) neurons from the effects of 5, 7 DHT (Sawynok and Reid, 1996; Choi et al, 2004). In addition, as far as we are aware there is little or no evidence to indicate that other descending monoaminergic neurons are affected by 5, 7 DHT. Therefore, we are fairly confident that monoaminergic systems (other than serotonin) remained intact following 5, 7 DHT.

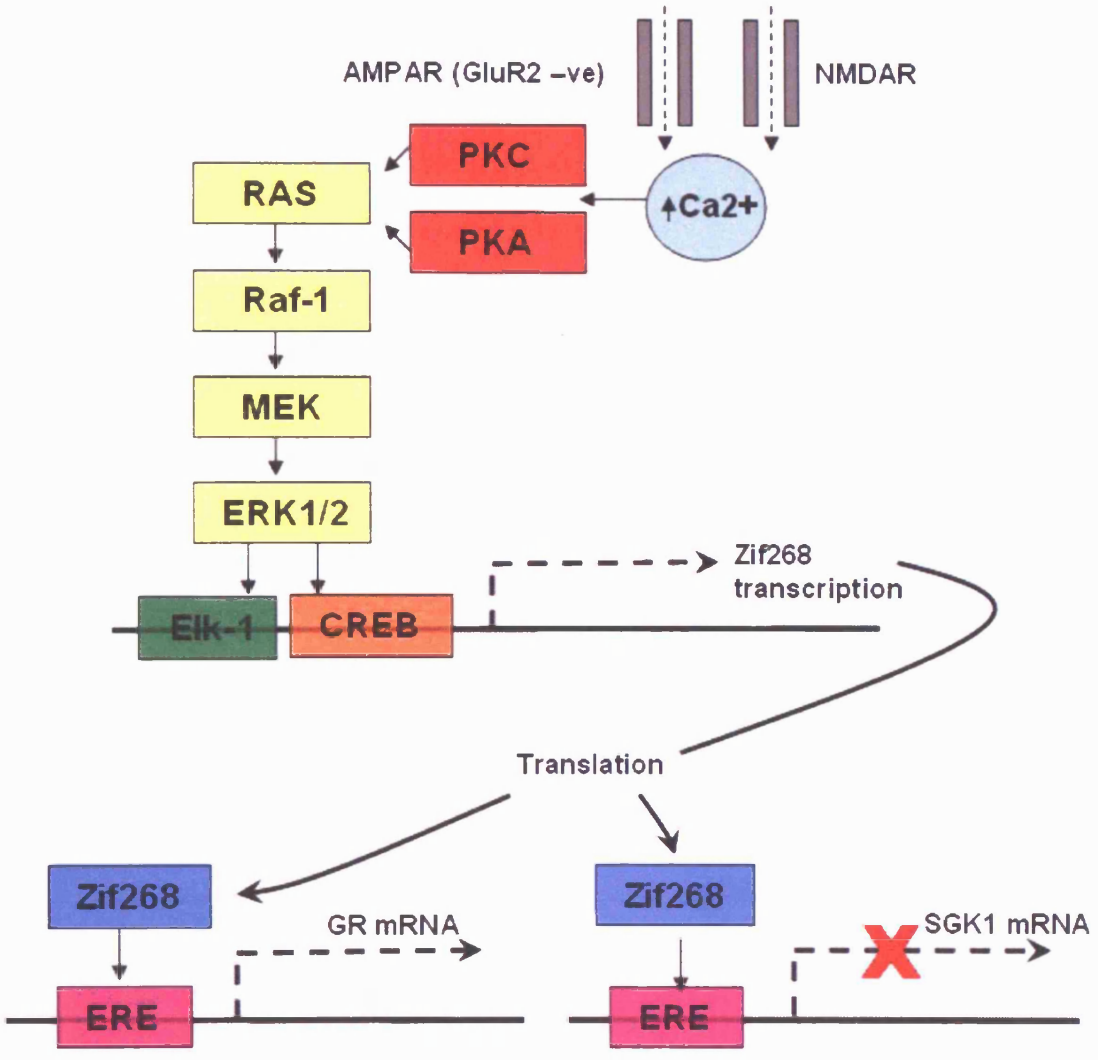

Figure 6.1. Molecular events that occurred following peripheral inflammation in lamina I projection neurons. Noxious stimuli can increase intracellular calcium levels via the AMPA receptors (those lacking the GluR2 subunit), the NMDA receptor and from intracellular stores. A rise in the calcium concentration activates protein kinases that can switch on molecular pathways including the MAPK pathway (yellow boxes). The MAPK pathway is important for gene transcription and can lead to the expression of the immediate early gene zif268 within an hour. Zif268 when translated, positively modulates the expression of the glucocorticoid receptor (GR) and the repression of serum and glucocorticoid inducible kinase 1 (SGK1), possibly by binding onto the ERE binding domain.

CHAPTER 7

General Conclusions

7. GENERAL CONCLUSIONS

Peripheral inflammation leads to an increase in transcriptional events in the dorsal horn. Our results suggest that zif268 plays a central role in gene regulation, in particular in lamina I projection neurons during inflammatory pain. The increase of zif268 can lead initially to the repression of SGK1 and increase expression of GR (Fig 6.1). GR is then capable of causing multiple changes to transcriptional events in the dorsal horn following activation by circulating corticosterone involving increased activation of the NMDA receptor subunits (Wang et al, 2005), EAAC1 (Wang et al, 2006) and voltage gated calcium channels (Takasaki et al, 2005). Once zif268 expression returns to baseline (post 6h) and following the relief of MeCP2 repression, SGK1 expression increases resulting in increased neuronal excitability (Geranton et al, in press). Neuronal changes regulated by changes in SGK1 expression may include regulating glutamate transporter and GluR1 expression in the dorsal horn (Strutz-Seebohm et al, 2006).

This thesis has shown that zif268 in the dorsal horn following CFA injection into the hindpaw is essential for the maintenance of chronic pain. Zif268 regulates the expression of both SGK and GR. As both of these molecules have independently been shown to be pro-nociceptive it would be worthwhile exploring these genes as potential targets for new analgesic drugs.

CHAPTER 8

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8. REFERENCES

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