Novel Intracellular Mechanisms of NMDA receptor-dependent Spinal Nociceptive Plasticity

by

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I, Richard D'Mello, 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.

Abstract

Prolonged activation of spinal NMDA receptors, after peripheral inflammation or nerve damage, can activate intracellular signalling cascades leading to plastic changes in synaptic transmission. This results in central sensitization of dorsal horn sensory neurones and manifests in patients as increased sensitivity to painful stimuli (hyperalgesia), and pain resulting from normally non-painful tactile stimuli (allodynia). Therefore, targeting NMDA-mediated intracellular signalling pathways could be a successful analgesic strategy, potentially devoid of side-effects associated with receptor blockade. NMDA receptors bind to the intracellular scaffold protein PSD-95, which couples the receptor to cytoplasmic effector pathways. The role of this coupling in spinal sensory transmission and nociceptive plasticity was investigated using biochemical, electrophysiological and behavioural methods. Disruption of binding between PSD-95 and NR2B subunits of NMDA receptors was achieved through the use of a decoy mimetic peptide, Tat-NR2B9c. I show that Tat-NR2B9c selectively reduces wind-up of dorsal horn wide dynamic range neurones and prevents both neuronal and behavioural measures of formalin-induced central sensitization. In the spinal nerve ligation model of chronic pain, Tat-NR2B9c reduced neuronal responses to mechanical and thermal stimulation and was able to reverse behavioural mechanical and cold hypersensitivity, clinical signs of neuropathic pain. In addition, the roles of two kinases, atypical PKCζ/PKMζ and PI3K, known to be involved in hippocampal LTP, were investigated using biochemical, immunohistochemical, electrophysiological and behavioural measures. I found that activation of spinal PKCζ/PKMζ is dependent on coupling between NR2B-subtype receptors and PSD-95, and contributes to central sensitization of dorsal horn neurones. PI3K was also found to be active in the NMDA-dependent formalin model and regulates various intracellular mechanisms in central sensitization. Finally, I investigated the role of DDAH-1, an enzyme which is involved in the regulation of nNOS, in spinal nociceptive plasticity. DDAH-1 inhibition reduced neuronal wind-up and both neuronal and behavioural measures of formalin-induced central sensitization. These findings further our understanding of NMDA-dependent spinal nociceptive plasticity. Disrupting the interaction between NR2B-containing NMDA receptors and PSD-95 or inhibition of downstream intracellular signalling pathways may be successful analgesic strategies for the treatment of chronic pain.

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Declaration of published work

Some of the work presented in this thesis has been published previously:

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Pezet S, Marchand F, D'Mello R, Grist J, Clark AK, Malcangio M, Dickenson AH, Williams RJ, McMahon SB (2008) Phosphatidylinositol 3-kinase is a key mediator of central sensitization in painful inflammatory conditions. *J Neurosci* 28:4261-4270.

Abbreviations

5-HT	Serotonin/5-hydroxytryptamine
7-NI	7-nitroindazole
ADMA	Asymmetric dimethylarginine
AEA	Anandamide
Akt	Protein kinase B
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid
ANOVA	Analysis of variance
AP	Action potential
ASIC	Acid-sensing ion channel
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
BH4	Tetrahydrobiopterin
BK	Bradykinin receptor
CaMKII	Ca^{2+}/Calmodulin-dependent kinase II
cAMP	Cyclic adenosine monophosphate
CB	Cannabinoid receptor
CCI	Chronic constriction injury
CCK	Cholecystokinin
CFA	Complete Freund's adjuvant
cGMP	Cyclic guanosine monophosphate
CGRP	Calcitonin gene-related peptide

CK2	Casein kinase 2
CLB	Complete lysis buffer
CLR	Calcitonin-like receptor
CNS	Central nervous system
CONTRA	Contralateral
CP	Central pain
CPP	3-[2-carboxypi-perazin4-yl]-propyl-I-phosphonic acid
CREB	cAMP response element binding protein
CSF	Cerebrospinal fluid
CVLM	Caudal ventrolateral medulla
DAG	Diacylglycerol
DDAH	Dimethylarginine dimethylaminohydrolase
DFNS	German Research Network on Neuropathic Pain
DMA	Dynamic mechanical allodynia
DMSO	Dimethyl sulfoxide
DOR	Delta (δ) opioid receptor
DRG	Doral root ganglion
EFNS	European Federation of Neurological Societies
eNOS	Endothelial nitric oxide synthase
EPSP	Excitatory post-synaptic potential
ERK	Extracellular signal-regulated kinase
FITC	Fluorescein isothiocyanate
GABA	γ-amino butyric acid
GCH1	GTP cyclohydrolase 1

GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GluR	AMPA receptor subunit
GPCR	G-protein coupled receptor
GSK	Glycogen synthase kinase
GTP	Guanosine triphosphate
HFS	High frequency stimulation
HIV	Human immunodeficiency virus
HRP	Horse radish peroxidase
IASP	International Association for the Study of Pain
IB4	Isolectin B4
Iba1	Ionized calcium binding adaptor molecule 1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP	Immunoprecipitation
IP_3	Inositol 1,4,5-trisphosphate
IPSI	Ipsilateral
KOR	Kappa (κ) opioid receptor
L-291	N^G-[2-methoxyethyl] arginine methyl ester
L-NAME	N omega-nitro-L-arginine methyl ester
L-NMMA	L-N^G-monomethylarginine
L(n)	Lumbar segment (n)
LC	Locus coeruleus
LFS	Low frequency stimulation

LTD	Long-term depression
LTP	Long-term potentiation
MAGUK	Membrane-associated guanylate kinase
MAPK	Mitogen-activated protein kinase
mGluR	Metabotropic glutamate receptor
MOR	Mu (μ) opioid receptor
mPFC	Medial prefrontal cortex
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NA	Noradrenaline
Na_v	Voltage-gated Na^+ channel subtype
NeuN	Neuronal nuclei
NeuPSIG	Neuropathic Pain Special Interest Group
NF200	Neurofilament 200
NGF	Nerve growth factor
NK	Neurokinin receptor
NMDA	N-methyl-D-aspartate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOP1	Nociceptin 1 receptor
NOS	Nitric oxide synthase
NR	NMDA receptor subunit
NS	Nociceptive-specific
OCT	Optimal cutting temperature compound

OFQ/N	Orphanin FQ/nociceptin
OX-42	CD11b
P2	Purinergic receptor
PAG	Periaqueductal grey
PB	Parabrachial area
PBS	Phosphate buffered saline
PBS-T	PBS+Triton X-100
PBS-T+Az	PBS-T+Azide
PD	Post-discharge
PDK-1	3'-PI-dependent protein kinase
PDN	Painful diabetic neuropathy
PDZ	Postsynaptic density 95, discs large, and zonula occludens-1
PFA	Paraformaldehyde
PH	Pleckstrin-homology domain
PHN	Post-herpetic neuralgia
PI	Phophoinositide
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKCγ	PKC gamma
PKCε	PKC epsilon
PKCζ/PKMζ	Atypical PKC zeta

PKG	Protein kinase G
PLC	Phospholipase C
PP1	Protein phosphatase 1
PPN	Peripheral polyneuropathy
PSD-93	Post-synaptic density protein-93
PSD-95	Post-synaptic density protein-95
PSNL	Partial sciatic nerve ligation
PSTH	Post-stimulus histogram
PVDF	Polyvinylidene difluoride
PWT	Paw withdrawal threshold
QST	Quantitative sensory testing
RAMP-1	Receptor activity-modifying protein 1
RCP	Receptor component protein
RIPA	Radioimmunoprecipitation assay
RM	Repeated measures
RSK2	p90 Ribosomal S6 protein kinase 2
RVM	Rostroventromedial medulla
S(n)	Sacral segment (n)
SAP	Saporin
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ser	Serine
SH	Src-homology domain
SLP3	Stomatin-like protein 3
SMA	Static mechanical allodynia

SNL	Spinal nerve ligation
SNP	Sodium nitroprusside
SP	Substance P
SSNRI	Selective serotonin and noradrenaline reuptake inhibitor
SynGAP	Synaptic GTPase-activating protein
TBS-T	Tris-buffered saline+Tween-20
TCA	Tricyclic antidepressant
Thr	Threonine
TNF	Tumour necrosis factor
TREK1	Two-pore domain potassium channel
Trk	Tyrosine receptor kinase
TRP	Transient receptor potential
TRPA1	TRP ankyrin receptor-1
TRPM8	TRP melastatin receptor-8
TRPV1	TRP vanniloid receptor-1
TTX-R	Tetrodotoxin-resistant
TTX-S	Tetrodotoxin-sensitive
Tyr	Tyrosine
WDR	Wide dynamic range
y^+CAT	y^+ system cationic amino acid transporter

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1 Introduction

"Fast moving particles of fire…the disturbance passes along the nerve filament until it reaches the brain…" Descartes (1664)

René Descartes envisaged that pain was transmitted directly from the periphery to the brain by a single, direct, dedicated pain pathway (Fig. 1.1). Today we now recognise this pathway as being the nociceptive system, part of the wider somatosensory system. However, the transmission of pain signals from the periphery, through the spinal cord and up to the cortex of the brain undergoes extensive processing through the nervous system at various anatomical locations. The dorsal horn of the spinal cord, in particular, is a key site for modulation of noxious information. Thus, the relationship between detection of noxious stimuli at the periphery and pain perception in the brain is far from linear.

Fig 1.1: L'Homme, René Descartes (1664)

1.1 Acute Pain

According to the International Association for the Study of Pain (IASP), pain is defined as 'an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage'. This definition reminds us that pain involves both physical and psychological components which contribute to its perception.

Acute or nociceptive pain is a defence mechanism which protects the body from tissue injury by potentially harmful and damaging stimuli. Nociceptors, specialized sensory neurones, detect such threatening stimuli and notify the central nervous system so that an appropriate behavioural response can be made, for example withdrawal from a hot surface. Without such a defence mechanism, injuries would be common and of such severity that survival may be compromised, as highlighted by those individuals born without nociceptors and who suffer from various frequent painless injuries, including burns, fractures and even biting of their own lips and tongues due to their congenital insensitivity to pain (Indo et al., 1996; Woolf and Ma, 2007). Alternatively, pain may be associated with a specific underlying disease or injury and can be a sign of illness that provokes appropriate measures such as rest or medication to be taken. Pain due to a muscular injury, for example, will promote rest of the injured limb and facilitate healing.

1.2 Chronic Pain

It is when any previous underlying injury or disease has not resolved and so the origins of the sensory event continues beyond the acute phase, that pain no longer serves a purpose and loses its usefulness, becoming a primary clinical problem itself. In this scenario, pain is a hindrance to daily life, the quality of which is greatly diminished. Here, pain is caused by dysfunction of the somatosensory system, brought about by inflammation or neuropathy, and should be regarded as a disease in its own right, particularly when it is significantly prolonged and becomes chronic. A recent large-scale European study was conducted to understand the prevalence and impact of chronic pain (Breivik et al., 2006). Patients were defined as having chronic

pain if they had suffered pain for more than six months, had experienced pain within the last month, experienced pain at least two times per week and rated the intensity of their latest pain experience to be at least 5 out of 10 on a numerical rating scale, where 1 represents no pain at all and 10 represents the worst pain imaginable. With these criteria in place, it was found that 19% of European adults were suffering from chronic pain, half of whom suffered pain constantly and a third of whom had pain of severe intensity (Breivik et al., 2006). While the economic cost of chronic pain is estimated to be €200 billion per annum in Europe (Tracey and Mantyh, 2007), significant emotional and social costs are also suffered by patients. Comorbidites such as depression, anxiety and sleep disorders are common. Chronic pain also reduces the ability of patients to socialize and maintain relationships and can have a significant negative impact on employment, further exacerbating the economic burden. In contrast to the adequate management of acute pain, the needs of chronic pain patients are largely unmet, with 40% of chronic pain sufferers feeling that their pain is not managed well, citing a lack of good drug treatments as well as a lack of recognition by their doctor for their pain as being more than just a symptom for this dissatisfaction. Thus, a greater appreciation for chronic pain as a disease entity, improved diagnostic procedures and the development of novel pharmacological treatments are desperately required to combat the clinical problem of chronic pain. Development of novel treatments will be aided by a greater understanding of the sensory system and the pathological mechanisms which convert acute pain to chronic pain.

1.3 The Pain Pathway

1.3.1 Peripheral sensory afferent fibres

The sensory experience begins in the periphery, where the peripheral terminals of primary afferent fibres respond to a myriad of stimuli and translate this information through electrical signals into the dorsal horn of the spinal cord, where the central ends of these fibres terminate. There are three main types of sensory neurone fibres in the peripheral nervous system, Aβ-fibres, Aδ-fibres and C-fibres, with each possessing different properties allowing them to respond to and transmit different types of sensory information. Aβ-fibres are large in diameter and highly myelinated, thus allowing them to quickly conduct action potentials from their peripheral to central terminals, at approximately 30-80 m/s. These fibres have low activation thresholds and normally respond to light touch and joint position and are responsible for conveying tactile information. Aδ-fibres are smaller in diameter and thinly myelinated, making them slower conducting than Aβ-fibres at 6-30 m/s, and they also possess higher activation thresholds. They respond to both thermal and mechanical stimuli. Aδ-fibres can be further subdivided into two main categories, based on their thermal sensitivity. Type I Aδ-fibres respond to very high heat (≈ 50°C) while type II Aδ-fibres are activated at lower noxious heat temperatures (≈ 42°C). C-fibres are the smallest type of primary afferents and are unmyelinated, thus making them the slowest conducting, at 0.5-2 m/s. They have the highest thresholds for activation and therefore detect selectively nociceptive or 'painful' stimuli. There are two main classes of C-fibres, peptidergic and non-peptidergic (see 1.3.1.1 below). Collectively both Aδ-fibres and C-fibres can be termed as nociceptors or 'pain fibres', responding to noxious mechanical, thermal or chemical stimuli. It is assumed that Aδ-fibre nociceptors mediate 'fast' pain which can be described as well-localized, acute, sharp pain, while C-fibre nociceptors mediate 'second pain' which is delayed, more diffuse and dull (Julius and Basbaum, 2001).

1.3.1.1 Development of two classes of C-fibre nociceptors

Approximately 70-80% of all rodent dorsal root ganglion (DRG) neurones require nerve growth factor (NGF) for survival during embryonic life and it is widely accepted that these neurones are small to medium-diameter nociceptors, particularly of the unmyelinated C-fibre class. These nociceptors express TrkA, the tyrosine kinase receptor for NGF, during development and early post-natal life. The fundamental importance of NGF-TrkA signalling in development of the nociceptive system is highlighted by patients who suffer from congenital insensitivity to pain due to mutations in the TrkA gene (Indo et al., 1996). However, three weeks into rodent post-natal life a large proportion of TrkA expression in the DRG is lost, such that in the adult animal, only 40-50% of all DRG neurones express TrkA. Thus TrkA is developmentally regulated (Silos-Santiago et al., 1995; Molliver and Snider, 1997). These TrkA-expressing nociceptors in the adult co-express the neuropeptides calcitonin gene-related peptide (CGRP) and substance P (SP) (Bennett et al., 1996a), and thus are peptidergic C-fibres. In contrast, nociceptors which lack TrkA in adult life are of the non-peptidergic C-fibre class and bind the isolectin IB4 (Silos-Santiago et al., 1995; Bennett et al., 1996b). The downregulation of TrkA in these non-peptidergic C-fibres coincides with an upregulation of Ret, an alternative receptor tyrosine kinase, which is responsive to glial cell line-derived neurotrophic factor (GDNF) (Bennett et al., 1998). The peptidergic, TrkA-expressing C-fibres represent roughly 40% of all DRG sensory neurones, while the non-peptidergic Ret-expressing C-fibres represent approximately 30% (Snider and McMahon, 1998). Regardless of their peptide content, all nociceptors contain and release glutamate as their predominant excitatory neurotransmitter.

1.3.1.2 Peripheral terminals of sensory fibres

The peripheral terminals of polymodal nociceptors are where noxious stimuli are detected and transduced to generate currents which in turn drive action potentials along axons towards the spinal cord and onto higher centres for the conscious perception of pain (Woolf and Ma, 2007). A number of receptors and ion channels on nociceptors can be selectively activated by noxious thermal, mechanical or chemical stimuli and thus confer the sensory specificity of the nociceptor (Fig. 1.2). The expression of such high threshold transduction molecules, including many channels of the transient receptor potential (TRP) family, is in contrast to low threshold transducers found on large diameter sensory neurones which respond exclusively to innocuous stimuli.

In the case of noxious heat, it is widely believed that the TRPV1 (transient receptor potential vanilloid receptor-1) receptor channel, which responds to capsaicin, the extract of chilli peppers, is also responsible for the generation of action potentials following application of noxious heat at or above 42°C (Caterina et al., 1997). Both $IB4^+$ and $CGRP^+$ C-fibre nociceptors as well as type II Aδ-fibre nociceptors respond to capsaicin and thus are believed to express TRPV1 (Snider and McMahon, 1998). The endogenous ligand for this receptor is unclear, however, although the cannabinoid anandamide is one potential candidate (Melck et al., 1999; Zygmunt et al., 1999). More recent work has identified multiple TRP channels, including TRPV2, TRPV3 and TRPV4, which, along with TRPV1, mediate responsiveness of nociceptors in the warm to hot range (Dhaka et al., 2006). In addition, activity of a heat-activated, two-pore domain potassium channel (TREK1) rises in response to increased heat and thus may also contribute to heat-evoked activity of nociceptors (Alloui et al., 2006). Type I Aδ-fibre nociceptors respond to higher intensity noxious heat and this may be mediated by a capsaicin-insensitive, TRPV1-like channel with an activation threshold of approximately 50°C and which is expressed exclusively in medium to large diameter-sized sensory neurones (Caterina et al., 1999).

Sensitivity to cooling and noxious cold is widely accepted to be mediated by another member of the TRP family, the TRPM8 (TRP melastatin 8) channel which is the receptor for menthol. Genetic knockout studies have shown that TRPM8-deficient

sensory fibres have diminished responses to cooling stimuli and mice lacking TRPM8 have reduced behavioural responses to both cooling and noxious cold, and interestingly, injury-induced cold hypersensitivity (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007). However, some sensitivity to particularly intense noxious cold was retained in TRPM8$^{-/-}$ mice, indicating that an as yet unidentified transduction molecule contributes to the sensation of extreme cold pain.

Specific transduction molecules on the peripheral terminals of nociceptors also exist for chemical sensitivity. In addition to capsaicin, TRPV1 can also be activated by protons and spider toxins (Dhaka et al., 2006). The TRPA1 (TRP ankyrin 1) channel is expressed by C-fibre nociceptors and is a major integrator of various noxious chemical stimuli, including allyl isothiocyanate (AITC) and allicin, the pungent ingredients in mustard and garlic extracts, respectively, as well as unsaturated aldehydes, such as acrolein, which mediate the irritant actions of air pollutants (Woolf & Ma, 2007). Furthermore, TRPA1 has been found to be specifically activated by formalin and to mediate formalin-induced pain, a widely used model (McNamara et al., 2007). C-fibres also express a large number of purinergic receptors that respond to ATP (adenosine triphosphate). In particular, the ATP-gated P_2X_3 ion channel is almost exclusively expressed in small-sized sensory neurones and approximately 90% of these are non-peptidergic C-fibre nociceptors (Vulchanova et al., 1997).

High threshold C-fibre and Aδ-fibre nociceptors terminate in the skin as free nerve endings, though some low threshold Aδ-fibres terminate on down hairs in the skin for the detection of light touch. In contrast, Aβ-fibres innervate Merkel cells, Pacinian corpuscles and hair follicles for the detection of texture, vibration and light pressure (Basbaum et al., 2009). At present, it remains unclear precisely how noxious mechanical stimulation is detected and transduced by sensory neurones, though several candidate transducers have been proposed, including acid-sensing ion channels (ASICs). Various mutations in different ASICs can alter mechanoreceptor sensitivity *in vivo* (Price et al., 2000; Price et al., 2001). More recently, Wetzel and colleagues have shown that genetic knockout of an integral membrane protein, called stomatin-like protein 3 (SLP3), results in a loss of mechanosensitivity in both low threshold Aβ-fibres and higher threshold Aδ-fibres. SLP3 was found to immunoprecipitate along with various ASICs, and these ASICs were no longer

functional in SLP3$^{-/-}$ mice. Nerve injury-induced mechanical hypersensitivity was also impaired in SLP3$^{-/-}$ mice (Wetzel et al., 2007). Thus, SLP3 seems to be an essential subunit associated with some unidentified mechanotransduction molecules in A-fibres, possibly ASICs. In contrast, a mechanism for mechanotransduction in high threshold C-fibres has yet to be elucidated.

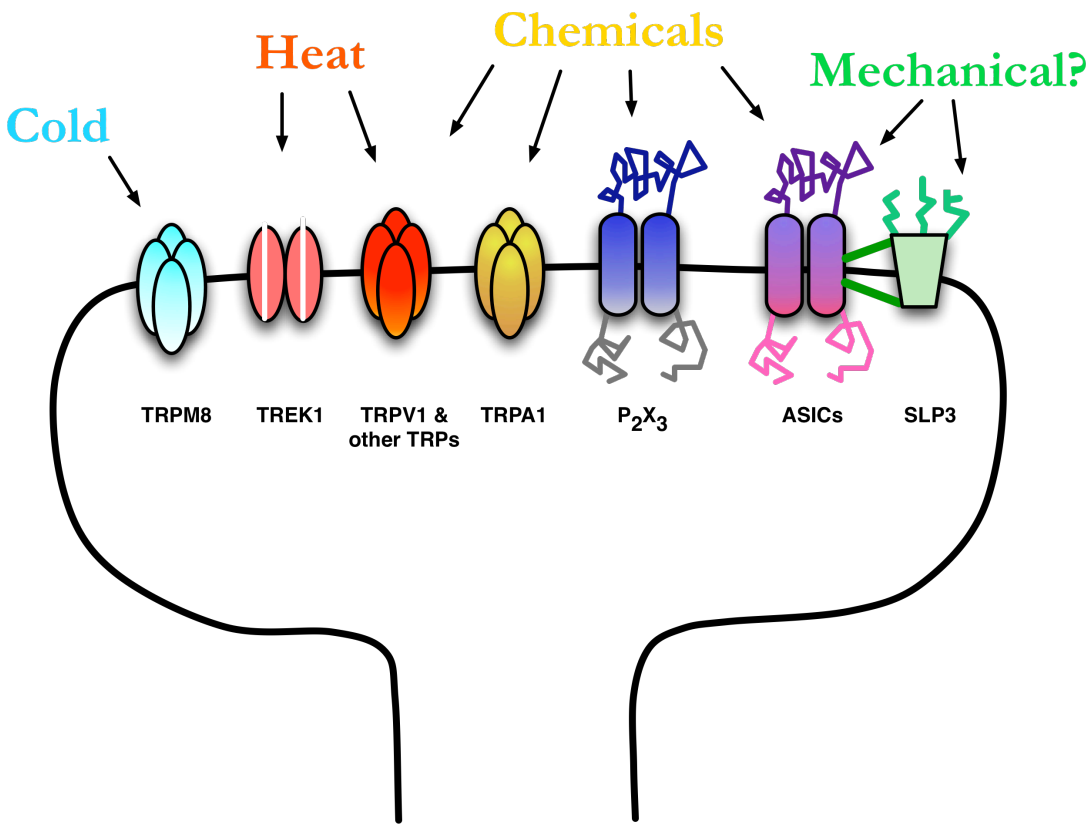


Fig. 1.2: The peripheral terminal of nociceptors. A myriad of transduction molecules are expressed at the peripheral terminals of nociceptors. These receptors and channels respond to different stimuli including heat (TRPV1, other TRPs & TREK1), cold (TRPM8), chemicals (TRPV1, TRPA1, P2X3 & ASICs) and mechanical stimulation (SLP3 & ASICs).

1.3.1.3 Peripheral sensitization

The peripheral terminals of nociceptors can be excited by a number of endogenous chemical mediators, especially in conditions of tissue damage or inflammation, produced and released from local non-neuronal cells, the afferent fibres themselves, the vasculature and from products triggered by activation of the body's defense mechanisms. These chemical mediators, commonly referred to as an 'inflammatory soup' consisting of NGF, ATP, protons, bradykinin, prostaglandins (PGs) such as PGE_2, substance P, CGRP and pro-inflammatory cytokines, act on specific receptors, sensitizing the peripheral terminals of nociceptors so that afferent activity to a given stimulus is increased, heightening pain (Fig. 1.3). This is known as primary hyperalgesia and is characterized by thermal and mechanical hypersensitivity within the territory of the injury.

TRPV1 seems to be a key molecular target activated following tissue damage, resulting in a profound decrease in the thermal activation threshold of the channel as well as increased responsiveness at higher temperatures. The contribution of NGF to peripheral sensitization, in addition to its known developmental effects, has been well demonstrated, not least by the observation that peripheral administration of NGF is proalgesic (Lewin et al., 1994; Petty et al., 1994; McMahon et al., 1995). NGF acting at TrkA receptors on peptidergic C-fibres activates downstream intracellular signalling pathways including phospholipase C (PLC), mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K), the consequence of which seems to be potentiation of TRPV1 responsiveness (Julius and Basbaum, 2001). NGF is also thought to promote increased expression of TRPV1 (Ji et al., 2002b). In addition to NGF, bradykinin, acting via BK_2 receptors, has also been shown to promote TRPV1 sensitization, by stimulating downstream PLC which subsequently leads to activation of protein kinase C (PKC). Accordingly, intraplantar administration of PKC inhibitors has been shown to inhibit bradykinin-induced mechanical hypersensitivity (Souza et al., 2002). PKC can phosphorylate TRPV1 and thus increase capsaicin-induced currents, and can also promote exocytosis and depolarization of TRPV1. In particular, the PKCε isozyme in primary afferents is translocated to the cell membrane in response to bradykinin (Cesare et al., 1999) and $PKC\varepsilon^{-/-}$ mice have reduced primary hyperalgesia in response to chemical

mediators (Khasar et al., 1999). Bradykinin may also increase TRPV1 sensitivity via a PKC-independent mechanism, involving PLC-mediated hydrolysis of phosphatidylinositol 4,5-bisophosphate (PIP_2), thus relieving PIP_2-mediated inhibition of TRPV1 (Chuang et al., 2001). Other chemical mediators, such as protons and lipids, act as allosteric modulators of TRPV1. In addition to TRPV1, TRPA1 and ASICs may also mediate some of the sensitizing effects of peripherally released chemical mediators following tissue damage or inflammation (Basbaum et al., 2009). In relation to this thesis, TRPA1 is activated by formalin, driving central sensitization of dorsal horn neurones (McNamara et al., 2007).

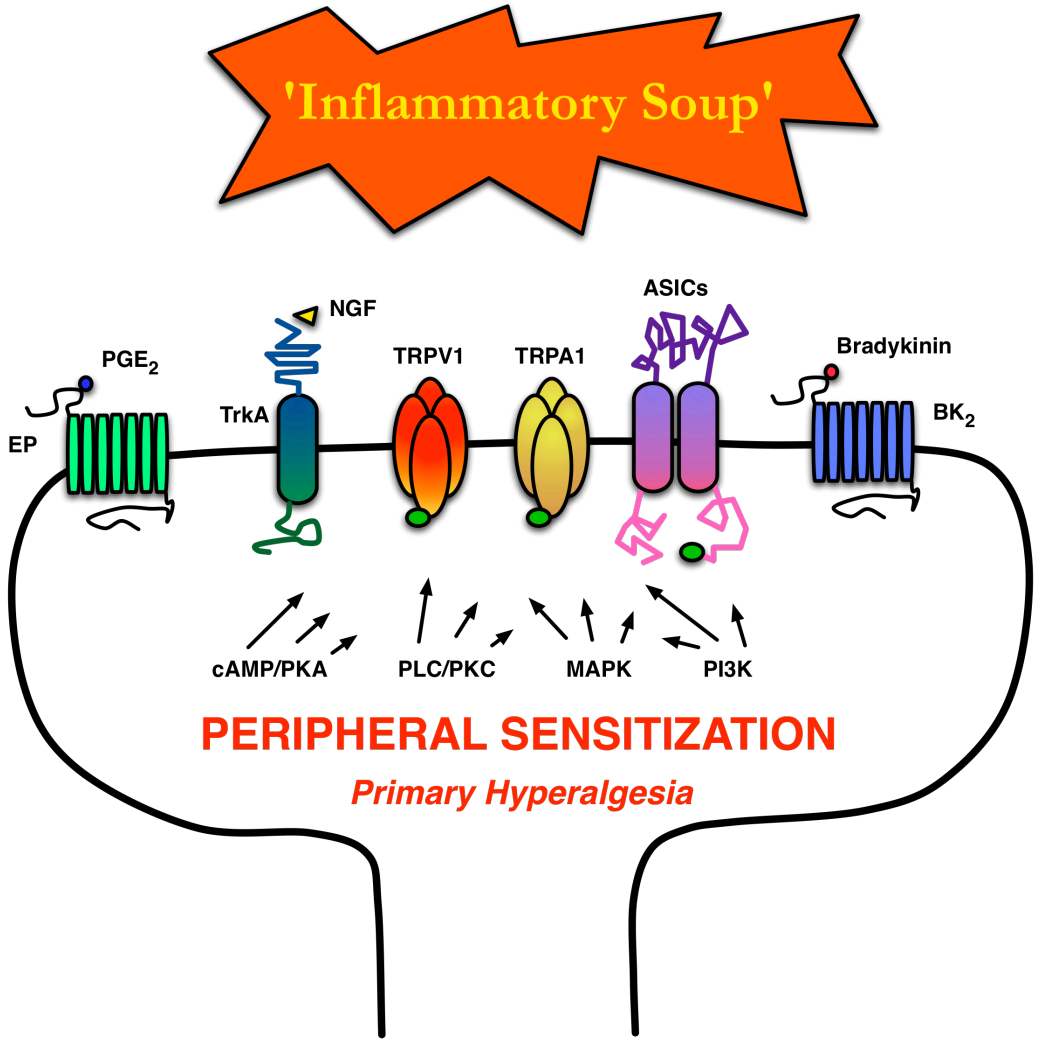


Fig. 1.3: Peripheral sensitization of nociceptors produces primary hyperalgesia. Tissue damage or inflammation causes the release of an 'inflammatory soup', including prostaglandins, NGF and bradykinin. Such mediators activate their receptors and initiate intracellular signalling cascades, such as protein kinase A (PKA), PKC, MAPK and PI3K, which then phosphorylate and sensitize transduction molecules, resulting in heightened nociceptor activity in response to subsequent stimuli, producing mechanical and thermal hypersensitivity within the territory of the injury.

1.3.1.4 Plasticity of sensory fibre Na^+ channels in pain

Voltage-gated sodium (Na^+) channels have a primary role in nervous system signalling. Opening of channels causes influx of Na^+ ions into the cell, resulting in membrane depolarization and action potential generation and propagation from the peripheral to central terminals of primary sensory neurones. Na^+ channels may also regulate cellular excitability to incoming signals. To date, nine different Na^+ channel isoforms, $Na_v1.1$–$Na_v1.9$, have been cloned, and these are expressed in various tissues throughout the body (Chahine et al., 2005). In the adult rodent, three channels, $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$, are normally expressed in primary sensory neurones. $Na_v1.3$ is expressed in embryonic neurones but absent in the adult, except when upregulated following nerve injury (Dib-Hajj et al., 2010). All Na^+ channels can be classified on the basis of their sensitivity to blockade by tetrodotoxin (TTX) and thus a particular channel may be described as being either TTX-sensitive (TTX-S) or TTX-resistant (TTX-R).

Chronic pain states involve a number of peripheral changes in nerves in terms of electrogenesis and activity, in which Na^+ channels are causally implicated. Damaged or inflamed nerves may start to generate ongoing ectopic activity due to the accumulation and clustering of Na^+ channels around the damaged axons. This aberrant activity can then start to spread rapidly to the cell bodies in the dorsal root ganglia. Nerve fibres can start to cross-excite each other (ephaptic transmission) and the same occurs in the cell bodies. These peripheral ectopic impulses can cause spontaneous pain and prime the spinal cord to exhibit enhanced evoked responses to stimuli, which themselves have greater effects due to increased sensitivity of the peripheral nerves. Animal studies have implicated four channels, $Na_v1.3$, $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$, in pathological neuropathic and inflammatory pain states.

$Na_v1.3$ is a TTX-S channel specifically associated with neuropathy, since it is not normally expressed in adult sensory neurones but is greatly upregulated after peripheral nerve injury (Wood et al., 2004). In particular, $Na_v1.3$ is clustered in both rat and human neuromas, implicating the channel in aberrant ectopic action potential generation (Black et al., 1999; Black et al., 2008). However, behavioural data

regarding the involvement of $Na_v1.3$ in neuropathic pain is conflicting since genetic knockout of $Na_v1.3$ does not alter the pain phenotype of mice following nerve injury (Nassar et al., 2006). $Na_v1.7$ is a TTX-R channel preferentially expressed in small-sized primary sensory neurones but also present in some large neurones. Conditional genetic knockout of $Na_v1.7$ in nociceptors abolishes inflammatory pain, while neuropathic pain is normal (Nassar et al., 2004; Nassar et al., 2005). Expression of the TTX-R channel $Na_v1.8$ is specific to small and medium-sized sensory neurones and thus has an exclusive role in nociception. The role of this channel in pain has been extensively investigated in $Na_v1.8$ knockout mice. These mice exhibit deficits in somatic and visceral inflammatory pain (Akopian et al., 1999; Laird et al., 2002). In contrast, any role for this channel in neuropathic pain is not so clear. $Na_v1.8$ knockout mice as well as double knockouts of both $Na_v1.7$ and $Na_v1.8$ develop normal levels of neuropathic pain (Akopian et al., 1999; Nassar et al., 2005). However, damaged sensory axons in $Na_v1.8$ knockout mice show significantly reduced spontaneous activity (Roza et al., 2003), suggesting that $Na_v1.8$ may contribute to nerve injury-induced ectopic activity and spontaneous pain, perhaps not adequately measured in behavioural studies. Indeed, $Na_v1.8$ levels have been shown to increase in non-injured axons, thus possibly contributing to spontaneous action potential generation (Gold et al., 2003). In addition, electrophysiological and behavioural studies in $Na_v1.8$ knockout mice suggest a role for this channel in mechanosensation (Akopian et al., 1999; Matthews et al., 2006). Like $Na_v1.8$, $Na_v1.9$ is a TTX-R channel and is expressed exclusively in non-peptidergic C-fibres. A role for $Na_v1.9$ in contributing to nerve injury-induced pain is unclear since the channel is downregulated after axotomy (Dib-Hajj et al., 1998) and $Na_v1.9$ knockout mice develop neuropathic pain normally. However, inflammatory pain is clearly impaired in $Na_v1.9$ knockout mice (Amaya et al., 2006).

1.3.1.5 Na^+ channels in human genetic pain syndromes

In recent years, $Na_v1.7$ has drawn much attention since various mutations in this channel have been found to cause human pain disorders. Dominant gain-of-function mutations in the gene encoding for $Na_v1.7$ lead to hyperexcitability of the channel and have been linked to inherited erythermalgia, a chronic inflammatory human disease, and to paroxysmal extreme pain disorder (Estacion et al., 2008; Dib-Hajj et al., 2010). Symptoms of both diseases include intense burning sensations (Basbaum et al., 2009). In contrast, recessive loss-of-function mutations in $Na_v1.7$ lead to congenital insensitivity to pain (Cox et al., 2006). Additionally, painful human neuromas have been shown to have increased expression of $Na_v1.3$ and $Na_v1.8$ (Black et al., 2008).

1.3.1.6 Central terminals of sensory fibres

The central terminals of primary afferent fibres enter the spinal cord via the dorsal root entry zones and terminate in specific regions of the dorsal horn (see section 1.3.2), synapsing with second order dorsal horn sensory neurones. All sensory fibres release glutamate (De Biasi and Rustioni, 1988; Broman et al., 1993), the predominant excitatory neurotransmitter in the central nervous system (CNS). Peptidergic C-fibres as well as some Aδ-fibres release CGRP. In addition, peptidergic C-fibres may also release substance P, brain-derived neurotrophic factor (BDNF), somatostatin and galanin (Ju et al., 1987). Action potentials entering the central terminal activate the vesicle-release machinery to induce neurotransmitter release, a process dependent on Ca^{2+} influx into the afferent terminal. Accordingly, nociceptors express various N-, P/Q- and T-type voltage-gated Ca^{2+} channels, of which $Ca_v2.2$ seems to be most important (Woolf and Ma, 2007). Ziconitide, a blocker of $Ca_v2.2$ channels, is analgesic, acting by inhibition of neurotransmitter release. Interestingly, in addition to the main pore-forming α subunit of Ca^{2+} channels, accessory proteins, such as the $\alpha_2\delta$ subunit, are known to have fundamental roles in the control of neurotransmitter release during nociceptive signalling, particularly since C-fibres upregulate $\alpha_2\delta$ in nerve injury-induced pain (Luo et al., 2001). The $\alpha_2\delta$ subunit regulates activation and inactivation kinetics and perhaps also membrane targeting of Ca^{2+} channels (Davies et al., 2007). The anticonvulsant drugs gabapentin and pregabalin have analgesic efficacy, thought to be mediated by binding to $\alpha_2\delta$ subunits, likely reducing trafficking and thus modulating neurotransmitter release (Gee et al., 1996; Field et al., 2006; Hendrich et al., 2008; Bauer et al., 2009). Intriguingly, despite not being exposed to the environment, the central terminals of primary afferent fibres closely resemble their peripheral terminals in that they express similar receptors and channels such as TRPV1, TRPA1, P_2X_3 and BK_2 receptors (Snider and McMahon, 1998; Woolf and Ma, 2007). It is believed that activation of these pre-synaptic receptors in the dorsal horn acts to increase neurotransmitter release, particularly glutamate, from nociceptors. In contrast, inhibition mediated by opioid receptors, γ-amino butyric acid (GABA) receptors and CB_1 receptors for cannabinoids, located on primary afferent terminals, acts to counter neurotransmitter release (Woolf and Ma, 2007).

1.3.2 Anatomical arrangement of the dorsal horn of the spinal cord

Within the grey matter of the dorsal horn, intrinsic spinal neurones can be anatomically divided into two main classes with regard to their terminations. Those cells with axons remaining within the spinal cord are classified as interneurones, and these may be either excitatory, through the release of glutamate, or inhibitory, through the release of GABA and/or glycine. On the other hand, projection neurones have axons which ascend through the white matter and terminate in the brain, and are mainly glutamatergic. Furthermore, descending axons from various brain structures also enter the dorsal horn of the spinal cord and may exert either inhibitory or excitatory effects on intrinsic neurones or afferent fibres. Thus, intrinsic dorsal horn excitatory and inhibitory interneurones, together with descending influences from the brain, can modulate the transmission of sensory information through the dorsal horn, regulating the final output of the spinal cord to higher centres (D'Mello and Dickenson, 2008).

The dorsal horn of the spinal cord is organized into different, anatomically diverse laminae, extending from the superficial to the deep dorsal horn (Fig. 1.4). In the early 1950s, Rexed first divided the spinal cord grey matter of the cat into a series of parallel laminae, numbered I to X, providing a uniform description of the arrangement of the spinal cord regardless of the particular spinal segment under investigation (Rexed, 1952, 1954). This scheme has since been applied to other species, including rodents. Together, laminae I and II are often described as the superficial dorsal horn and this is the main region for terminating nociceptive primary afferents. The remainder of the dorsal horn comprises four further laminae (III-VI), with laminae V-VI often collectively referred to as the deep dorsal horn. Laminae VII-IX form the ventral horn of the spinal cord, while lamina X, or substantia grisea centralis, is the region around the central canal.

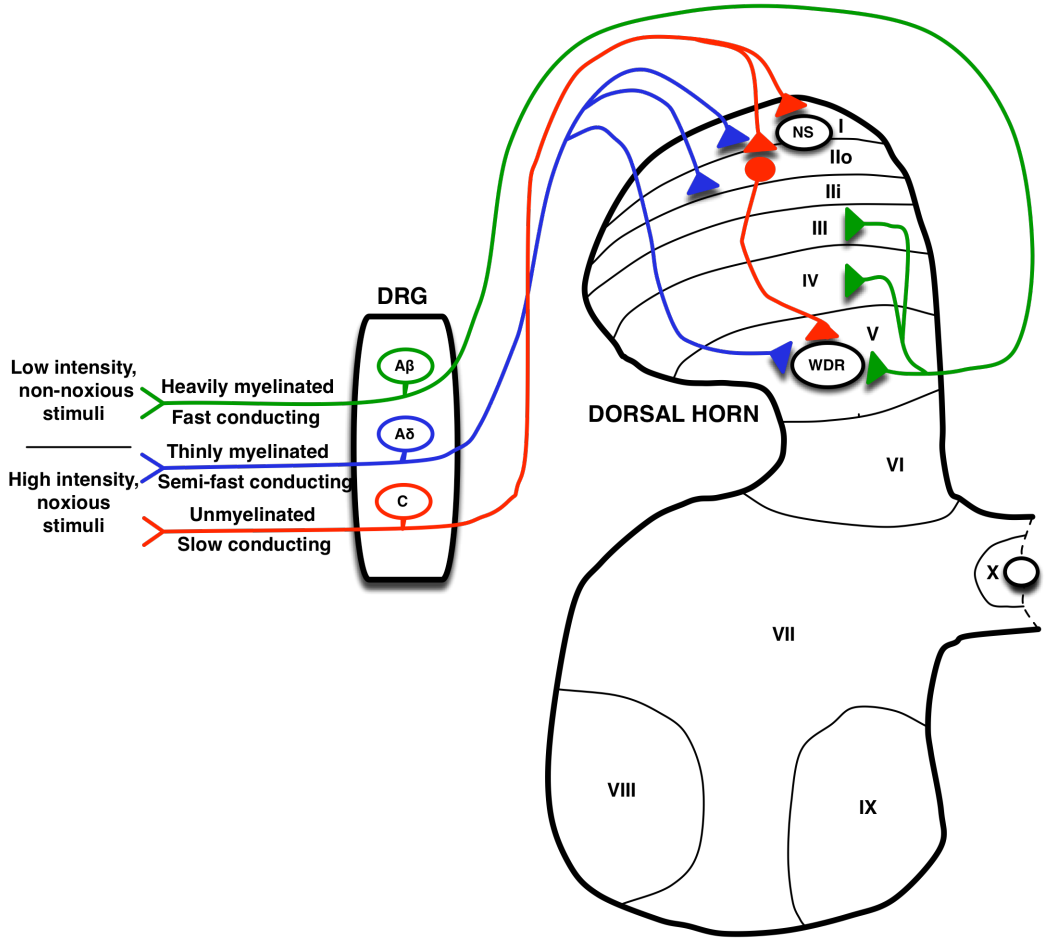


Fig. 1.4: Laminar arrangement of the dorsal horn. Sensory fibres extend from the periphery, via the DRG where their cell bodies are located, into the dorsal horn of the spinal cord, terminating in specific regions. Nociceptive Aδ and C-fibres terminate mainly within the superficial laminae (I-II), synapsing with NS spinal neurones, though some may reach deeper laminae via their deeply penetrating processes. In contrast, Aβ-fibres terminate in deep laminae III-V. Spinal cells in laminae V-VI are mainly WDR neurones. A large population of excitatory interneurones within lamina II connect superficial nociceptor terminals to deep WDR neurones. Alternatively, some WDR neurones may have dorsally extending dendrites which synapse with nociceptors. Adapted from D'Mello and Dickenson (2008).

1.3.2.1 Lamina I

Lamina I is a thin, marginal layer covering the outermost region of the dorsal horn grey matter. Many Aδ-fibre and C-fibre nociceptors terminate in this layer, though some penetrate deeper into the dorsal horn. Specifically, it is the peptidergic CGRP/TrkA-expressing C-fibres which terminate in lamina I, with some also reaching the most outer part of lamina II (IIo). Since mostly nociceptors terminate in this region, approximately 75% of second order spinal cord neurones located here are nociceptive-specific (NS) cells (Bester et al., 2000; Seagrove et al., 2004), which receive synaptic inputs from Aδ- and C-fibres only. These cells fire action potentials when a painful stimulus is detected at the periphery, and have connectivity with other neurones with cell bodies within the same or deeper laminae. In addition, many of the cells resident in lamina I are projection neurones, their axons decussating at the level of the spinal cord and travelling up through the white matter to reach contralateral brain structures such as the parabrachial area (PB) and thalamus (Todd, 2002).

1.3.2.2 Lamina II

Lamina II is also known as the substantia gelatinosa and contains mainly interneurones which make connections with intrinsic spinal neurones but do not project to higher centres. The mid-region of lamina II is where non-peptidergic $IB4^+$ C-fibres terminate, as well as some Aδ-fibres, and thus the majority of cells here are NS neurones. The most ventral portion, lamina II inner (IIi), contains a small subset of dorsal horn neurones which express the γ isoform of PKC (PKCγ). The vast majority of these interneurones (92%) do not contain GABA and thus are deemed to be excitatory (Polgar et al., 1999). Mice lacking the PKCγ isozyme have normal acute pain sensitivity but fail to develop neuropathic pain following nerve injury (Malmberg et al., 1997) and evidence suggests that PKCγ-expressing interneurones may convey tactile inputs from terminating Aβ-fibres in laminae III-IV to superficial NS projection neurones, a pathway which is under inhibitory control in the normal animal (Martin et al., 2001; Miraucourt et al., 2007).

1.3.2.3 Laminae III – IV

Laminae III and IV are primarily responsive to innocuous mechanical stimulation conveyed by terminating Aβ-fibres. Dorsal horn cells in this region are thus tactile or proprioceptive. In addition, many neurones in laminae III and IV have dorsally extending dendrites and project to brain structures, thus contributing to the spinothalamic tract (Todd, 2002; D'Mello and Dickenson, 2008).

1.3.2.4 Lamina V

Dorsal horn neurones in lamina V receive monosynaptic inputs from terminating Aβ-fibres and some deeply penetrating Aδ-fibres. In contrast, polysynaptic inputs from superficially terminating C-fibres are mediated via interneurones (Braz et al., 2005). Alternatively, dendrites of lamina V neurones may extend dorsally to make synaptic contacts with C-fibres (Woolf and King, 1987). Therefore, second order lamina V sensory neurones receive inputs from all three types of sensory fibre and respond to the full range of stimulation, from light touch to noxious pinch, heat and chemicals. These cells are thus appropriately termed wide dynamic range (WDR) neurones. WDRs also reside in lamina VI. Many lamina V WDR neurones are believed to project to higher centres and thus constitute a major output of the dorsal horn, in addition to lamina I projection neurones. Lamina V is of great relevance to the studies presented in this thesis, since all electrophysiological recordings of WDR neurones were conducted in this region of the dorsal horn.

1.3.2.5 Electrophysiological response properties of intrinsic dorsal horn sensory neurones

NS cells fire action potentials only when a noxious stimulus is applied at the peripheral receptive field, since they are normally innervated exclusively by nociceptors. For example, NS cells will not fire action potentials below the heat pain threshold in rats of 42°C, although there can be a paradoxical response to brush (Bester at al., 2000; Seagrove et al., 2004). Transcutaneous electrical stimulation of the receptive field produces little, if any, post-discharge or wind-up of NS cells (Seagrove et al., 2004), two measures of neuronal hyperexcitability and short-term synaptic plasticity (see section 1.5.1). In contrast, WDR neurones fire action potentials in response to both innocuous and noxious thermal and mechanical stimuli, conveyed by the three types of sensory fibres, in a graded fashion (Suzuki et al., 2002b). Responses to heat, for example, begin at approximately 32°C and increase with rising temperatures. WDR neurones also respond to noxious cold as well as innocuous brushing of the receptive field, which is larger than that of NS cells. In addition, WDRs display greater responses, including a large amount of post-discharge and wind-up, following transcutaneous electrical stimulation of the receptive field (Suzuki et al., 2002b). Finally, proprioceptive cells in laminae III-IV, which receive inputs exclusively from Aβ-fibres, are easily identifiable since they respond to light touch and brushing of the receptive field only. This response subsides when stimulus intensity increases and enters the noxious range. Thus these cells do not respond to pinch, for example (personal observations).

1.3.2.6 Contribution of non-neuronal glial cells to dorsal horn nociceptive processing

Traditionally, the role of non-neuronal glial cells was thought to be a supportive one, providing metabolic homeostasis for neurones and immune surveillance within the central nervous system (McMahon and Malcangio, 2009). However, growing evidence from the pain field now suggests that glial cells within the spinal cord, namely microglia and astrocytes, are also able to influence pain transmission through the dorsal horn by regulating neuronal function, particularly under pathological conditions. Injection of inflammatory substances into the hindpaw or peripheral nerve injury has been shown to coincide with induction of a pain-related enhanced response state of glia (McMahon and Malcangio, 2009), as evidenced by immunohistochemical analysis of glial markers such as ionized calcium binding adaptor molecule 1 (Iba1; microglia), CD11b (OX-42; microglia) and glial fibrillary acidic protein (GFAP; astrocytes), as well as alterations of glial cell morphology. Furthermore, pharmacological inhibition of glial activity is analgesic in various pain models (Meller et al., 1994b; Watkins et al., 1997; Coyle, 1998; Jin et al., 2003; Raghavendra et al., 2003; Clark et al., 2007). Thus, although the principal focus of the studies presented in this thesis is on neuronal mechanisms of spinal nociceptive plasticity, the contribution of non-neuronal glial cells should not be underestimated. As this field grows, accumulating evidence suggests that targeting non-neuronal cells may be a potentially beneficial strategy for the treatment of chronic pain (Scholz and Woolf, 2007; McMahon and Malcangio, 2009).

1.3.3 Anatomy of ascending projections from the dorsal horn to the brain

The output from the dorsal horn to higher centres in the brain is carried by spinal projection neurones along ascending pathways (Fig. 1.5). A large population of projection neurones are found superficially in lamina I, estimated to number 400 on each side of a particular spinal segment. The majority of these cells have axons which cross the midline and ascend in the contralateral white matter (Todd, 2002). It is estimated that 80% of these cells express the neurokinin 1 (NK_1) receptor for substance P (Todd, 2002), a neuropeptide which is released by nociceptive afferents, meaning that these cells respond to noxious stimulation (Mantyh et al., 1997; Mantyh and Hunt, 2004). NK_1^+ cells in lamina I have been shown to project to areas in the brain such as the thalamus, the periaqueductal grey (PAG) and brainstem structures such as the caudal ventrolateral medulla (CVLM) and, in particular, the PB. In fact, projections from lamina I to the PB far outnumber those to the thalamus, subsequently reaching limbic structures such as the amygdala and hypothalamus, thus contributing largely to the affective component of pain (Todd, 2002). From the PB, signals are also transmitted to other brainstem areas such as the PAG and the rostroventromedial medulla (RVM), a region which has descending projections back to the dorsal horn. Therefore, lamina I NK_1-expressing cells can modulate spinal processing by activation of descending pathways from the brainstem, thus forming a spino-bulbo-spinal loop (Suzuki et al., 2002b). These descending pathways can be influenced by limbic regions in the brain and so can be altered by mood and attention. A large number of projection neurones, many of them also expressing the NK_1 receptor, are also found deeper in the dorsal horn, scattered throughout lamina III to VI, and these project predominantly to the thalamus, thereby making up a significant proportion of the spinothalamic tract. This ascending pathway carries primarily sensory information and so provides the sensory discriminative component of the pain experience. Many of these deep lying projection neurones have dendrites which extend upwards to the superficial dorsal horn, thus allowing them to make synaptic contacts with terminating C-fibres and receive noxious inputs (Woolf and King, 1987; Todd, 2002).

From the thalamus, nociceptive information is transmitted to cortical regions. There does not exist a single pain centre within the cortex, but rather there are multiple

cortical regions which may or may not be activated during a particular painful experience. These regions make up what is commonly referred to as a 'pain matrix' and include primary and secondary somatosensory, insular, anterior cingulate and prefrontal cortices (Tracey and Mantyh, 2007).

1.3.4 Anatomy of descending projections from the brain to the dorsal horn

Descending pathways from supraspinal structures are able to influence nociceptive transmission in the dorsal horn of the spinal cord. Such descending influences may be either excitatory (descending facilitation) or inhibitory (descending inhibition) in nature (Fig. 1.5) and may equally engage primary afferent terminals or intrinsic dorsal horn cells, both interneurones and projection neurones. In this way, the brain influences the final output from the dorsal horn to cortical regions of the brain where pain is perceived. Several key supraspinal structures have been indentified which can directly or indirectly modulate the dorsal horn, including the PAG in the midbrain and brainstem nuclei such as the PB, RVM and locus coeruleus (LC). Such descending modulatory pathways access the spinal cord via the dorsolateral and ventrolateral funiculi. In addition, many of the structures implicated in descending modulation can exert both facilitatory and inhibitory influences in the dorsal horn, dependent on the context of the situation.

Early studies demonstrated that electrical stimulation of the PAG could produce analgesia in the dorsal horn (Reynolds, 1969; Basbaum and Fields, 1984). However, direct projections from the PAG to the cord are few in number. Rather, the PAG projects to and modulates the output of brainstem nuclei and in this indirect way alters nociceptive processing in the dorsal horn. Similar connectivity exists from the PB to the dorsal horn and these projections predominantly suppress responses of superficial dorsal horn neurones to both innocuous and noxious inputs (Millan, 2002). Numerous transmitters may be released by PAG and PB projection neurones including substance P, cholecystokinin (CCK), GABA and endogenous opioids (Millan, 2002). Recent work has identified a small population of dopaminergic neurones in the PAG which mediate antinociception (Flores et al., 2004).

The RVM in the brainstem receives the majority of its sensory inputs relayed through the PAG and PB, and in turn, sends axons directly to both superficial and deep dorsal horn laminae (Millan, 2002). Initial work suggested that the descending influence from the RVM is inhibitory since electrical stimulation in this region produces analgesia via profound inhibition of noxious inputs to superficial and deep dorsal horn neurones (Basbaum et al., 1976; Fields et al., 1976; Fields et al., 1977). More recent work, however has demonstrated that descending facilitatory pathways also originate from the RVM, particularly in chronic pain states (Burgess et al., 2002; Bee and Dickenson, 2007). This bidirectional control of spinal processing is mediated by two distinct populations of RVM cells. ON cells produce a burst of firing in response to peripheral noxious stimulation and are inhibited by endogenous opioids, thus they are believed to trigger descending facilitation. In contrast, OFF cells are inhibited by noxious inputs since they display a transient disruption in their firing immediately prior to a nociceptive reflex and they are disinhibited by endogenous opioids, thus they are implicated in descending inhibition (Fields et al., 1983; Heinricher et al., 1992; Bannister et al., 2009). Though the precise neurochemistry of these cells remains to be elucidated, evidence implicating serotonin (5-HT) release from the RVM in the dorsal horn in mediating descending facilitation supports the notion that ON cells may be serotonergic. Whatever the neurochemical signature of ON and OFF cells, it is clear that a range of neurotransmitters are released by descending axons from the RVM, including 5-HT, GABA and endogenous opioids, acting at multiple receptor subtypes coupled to different intracellular signalling molecules within the dorsal horn (Millan, 2002).

Noradrenaline (NA), acting predominantly at the α_2-adrenoceptor subclass within the dorsal horn, is believed to contribute significantly to descending inhibition by inhibiting transmitter release from primary afferent terminals and suppressing the firing of projection neurones in the dorsal horn (Millan, 2002; D'Mello and Dickenson, 2008). Brainstem sources of NA include the A1-A7 nuclei, including the locus coeruleus (Kwiat and Basbaum, 1992; Bannister et al., 2009).

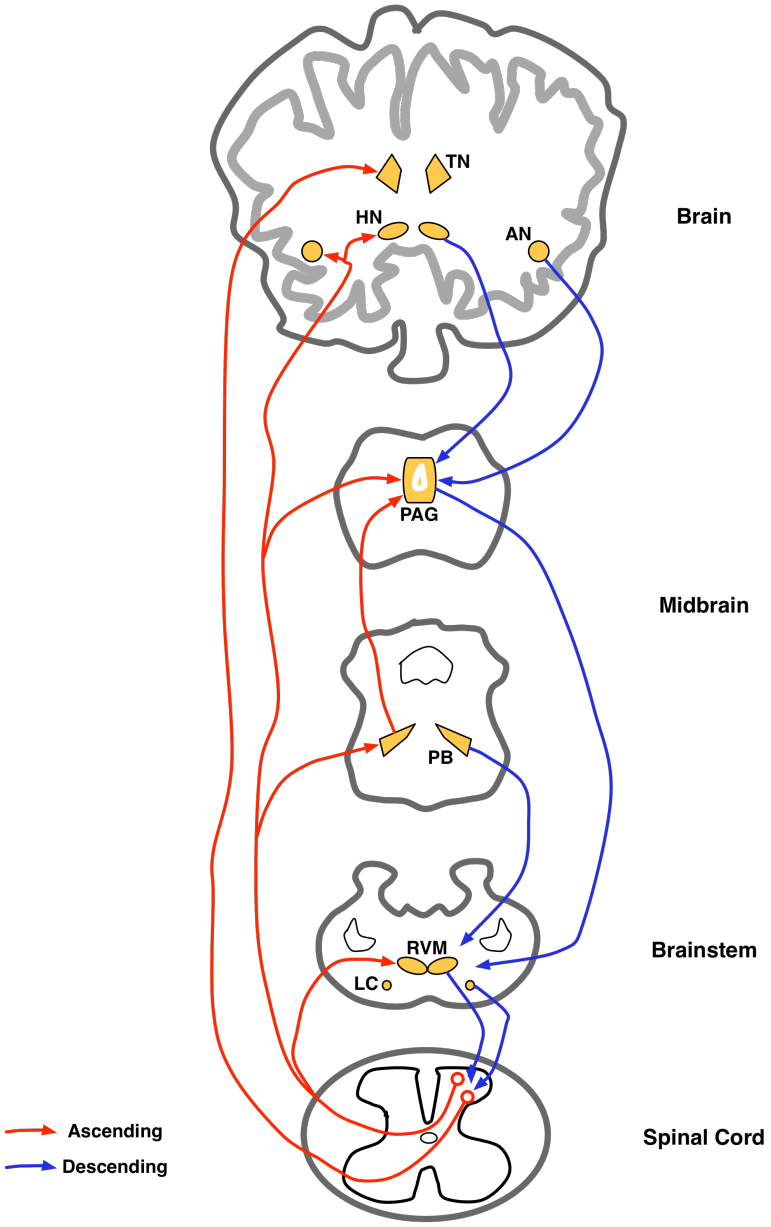


Fig. 1.5: Anatomy of ascending and descending projections between the dorsal horn and the brain. Spinal projection neurones may reach thalamic nuclei (TN) or may engage structures such as the parabrachial area (PB) and periaqueductal grey (PAG). From these sites, projections may reach limbic structures such as the hypothalamic (HN) and amygdala nuclei (AN). Descending modulation is mainly mediated via the locus coeruleus (LC) and rostroventromedial medulla (RVM) in the brainstem. Adapted from Hunt and Mantyh (2001) and Tracey and Mantyh (2007).

1.4 Neurotransmitters and neuromodulators acting in the dorsal horn

A myriad of neurotransmitters and neuromodulators are released within the dorsal horn of the spinal cord and can alter processing of sensory information. Sources of such transmitters include terminating primary afferents, intrinsic dorsal horn neurones, descending axons from supraspinal sites or even dorsal horn microglia and astrocytes. Some key excitatory and inhibitory neurotransmitters and neuromodulators are now described in further detail. (Additional neurotransmitters and neuromodulators are provided in Table 1.1).

1.4.1 Glutamate

Glutamate is an excitatory amino acid and is the major excitatory neurotransmitter found throughout the whole of the nervous system. Since it is released by primary afferent fibres, by dorsal horn neurones, and by neurones in the brain, glutamate is essential for nociceptive signalling at every anatomical level. Descending axons from the brain also release glutamate (Wiertelak et al., 1994; Millan, 2002), as may dorsal horn glia. In particular, astrocytes are not only important for regulating extracellular glutamate concentrations but may also release glutamate (McMahon and Malcangio, 2009). In addition to sensory perception and nociception, glutamate is involved in numerous CNS functions, including movement and learning and memory.

There are two classes of glutamate receptor, metabotropic and ionotropic, present in the dorsal horn (Furuyama et al., 1993; Tolle et al., 1993). The metabotropic class (mGluRs) are G protein-coupled receptors (GPCRs) and are made up of combinations of eight different subunits, mGluR1-8, and several of these have been detected in the dorsal horn. The subunits mGluR3-5 and mGluR7 are located mainly superficially in both primary afferent terminals and local dorsal horn neurones, while mGluR1 is found mainly in deeper dorsal horn neurones (Vidnyanszky et al., 1994; Ohishi et al., 1995; Jia et al., 1999; Azkue et al., 2001; Walker et al., 2001). The ionotropic class comprises three separate families, kainate, α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors. Kainate receptors are tetrameric in structure and generally have an

excitatory role, being expressed both in pre-synaptic afferent terminals and on the post-synaptic membranes of dorsal horn neurones (Bleakman et al., 2006). AMPA receptors are formed from four subunits, GluR1-4, and are also tetrameric. They are found at most, if not all, glutamatergic synapses throughout the spinal grey matter, as well as pre-synaptically in primary afferent terminals (Lee et al., 2002; Nagy et al., 2004b). The third family in this class are the NMDA receptors, which again form tetramers from combinations of seven different subunits, NR1, NR2A-D and NR3A-B. In the dorsal horn, a combination of the NR1 subunit and one NR2 subunit come together to form functional receptors. The NR1 subunit is present throughout the grey matter (Tolle et al., 1993; Nagy et al., 2004a). Both NR2A and NR2B subunits can also be found in most dorsal horn laminae, though the NR2B subunit is densely concentrated in laminae I-II and the NR2A subunit in laminae III-IV (Nagy et al., 2004a). Expression of NR2B has also been detected in primary afferent terminals (Yung, 1998; Boyce et al., 1999) of both A-fibres and C-fibres (Ma and Hargreaves, 2000; Marvizon et al., 2002). Dorsal horn NMDA receptors are the main focus of this thesis and their role in nociceptive processing and plasticity is discussed further below (see sections 1.6 & 1.7).

1.4.2 Substance P

Substance P is part of the tachykinin family of neuropeptides. It is synthesized predominantly in small and some medium-sized neurones in the DRG, and is released from both peripheral and central afferent terminals. In the dorsal horn, substance P-containing afferents terminate mainly in superficial laminae (I and IIo) with a less dense distribution in laminae III-IV (Todd, 2002). In addition, a limited pool of substance P may also be released from descending axons from the RVM or from superficial spinal interneurones which co-express the opioid enkephalin (Hokfelt et al., 2000; Ribeiro-da-Silva and Hokfelt, 2000). Three neurokinin receptors are expressed in the dorsal horn, NK_1, NK_2 and NK_3. Substance P has the greatest binding affinity for the NK_1 receptor, and this receptor is the most abundant of the three in the dorsal horn (Ohkubo and Nakanishi, 1991; Todd, 2002). Spinal NK_1 receptors are most densely located on lamina I neurones (Liu et al., 1994), many of which are projection neurones, while dorsally-extended dendrites of deep projection

neurones found in lamina III and IV also express the NK_1 receptor (Naim et al., 1997; Todd, 2002). NK_2 receptors are present in a population of astrocytes in the superficial dorsal horn, while NK_3 receptors are present in interneurones in laminae I-III (McCarson and Krause, 1994; Seybold et al., 1997; Zerari et al., 1998). Finally, both NK_1 and NK_3 receptors may be present on terminals of peptidergic C-fibres in order to regulate substance P release (Malcangio and Bowery, 1999).

1.4.3 Calcitonin gene-related peptide (CGRP)

Primary afferent terminals are believed to be the major, if not exclusive, source of CGRP in the dorsal horn. These terminals belong mostly to peptidergic C-fibres as well as a few medium-to-large-sized A-fibres. Removal of primary afferent input, by dorsal rhizotomy, results in an almost complete loss of CGRP immunoreactivity in the dorsal horn, evident of the fact that intrinsic spinal neurones and descending axons do not express this neuropeptide (Chung et al., 1988). CGRP-containing fibres terminate in laminae I, II and V and form synapses with spinothalamic projection neurones and interneurones and also have connections with pre-synaptic terminals of non-peptidergic C-fibres. Thus, CGRP receptors not only exist on post-synaptic neurones, but also on pre-synaptic terminals, acting as auto- or hetero-receptors to regulate neurotransmitter release. The densest staining for CGRP receptors is in the superficial dorsal horn (Ye et al., 1999). Such CGRP receptors are trimeric complexes consisting of the calcitonin-like receptor (CLR) (Njuki et al., 1993), the receptor activity-modifying protein 1 (RAMP1) (McLatchie et al., 1998) and a cytoplasmic receptor component protein (RCP) (Luebke et al., 1996). These receptor complexes form GPCRs which activate downstream protein kinase A (PKA).

1.4.4 Brain-derived neurotrophic factor (BDNF)

As well as functioning as a trophic factor during development, BDNF has a neuromodulatory role in the adult. In the dorsal horn, BDNF is mainly released from peptidergic C-fibre terminals in laminae I-II (Zhou et al., 2004). More recent studies have indicated that microglia may also release BDNF in the dorsal horn in response

to peripheral nerve injury (Coull et al., 2005). BDNF-containing afferents terminate in lamina II of the dorsal horn and their terminals also express the tyrosine kinase B receptor (TrkB) for BDNF (Salio et al., 2005) which may function to modulate neurotransmitter release, while lamina II spinothalamic projection neurones also have TrkB (Slack et al., 2005). Evidence suggests that BDNF may facilitate both glutamatergic and GABAergic transmission in the dorsal horn (Pezet et al., 2002; Slack and Thompson, 2002).

1.4.5 Nitric oxide (NO)

Synthesis of NO in neurones is by neuronal nitric oxide synthase (nNOS), which is predominantly located in the superficial dorsal horn (Valtschanoff et al., 1992; Spike et al., 1993; Morris et al., 1994). Intriguingly, various studies have demonstrated that nNOS is almost exclusively expressed in superficial GABAergic interneurones (Valtschanoff et al., 1992; Spike et al., 1993; Laing et al., 1994; Bernadi et al., 1995). The exact role of nNOS in these GABAergic interneurones remains unknown. However, some of the nNOS expression in these cells is found in their dorsally-extending dendrites where GABA is not expressed, and NO may be released from such sites (Bernadi et al., 1995). Since nitric oxide (NO) is a diffusible messenger, its effects may not necessarily be spatially restricted and it may spread up to a radius of 100 μm (Wood and Garthwaite, 1994), thus engaging various neurones within the superficial dorsal horn which may be in close synaptic contact (Ruscheweyh et al., 2006; Schmidtko et al., 2008; Garthwaite, 2008). Cells in the dorsal horn which express the NO receptor, guanylate cyclase, include projections neurones and local excitatory and inhibitory interneurones. Immunohistochemical evidence has shown these cells to lie very close to those cells which contain nNOS, often making contact. Co-localization between nNOS and guanylate cyclase is infrequent, suggesting that NO diffuses and acts in a paracrine manner (Ding and Weinberg, 2006). In the DRG, nNOS expression is low and is restricted to peptidergic C-fibres (Aimi et al., 1991; Zhang et al., 1993). Recently, it has been shown that the NO receptor, guanylate cyclase, is not present in DRG neurones but only in intrinsic dorsal horn cells, including NK_1-expressing projection neurones (Schmidtko et al., 2008). This would suggest that the contribution of NO signalling in pre-synaptic primary afferent

neurones is minimal, and that NO acts predominantly on intrinsic post-synaptic spinal sites. Therefore, these findings challenge the hypothesis that NO acts as a retrograde neurotransmitter, released from spinal sites and acting on peripheral nerve terminals to increase neurotransmitter release. It is more likely that NO is released from afferents and spinal neurones and acts exclusively at intrinsic spinal sites to produce sensitization of nociceptive signalling (Schmidtko et al., 2009).

1.4.6 GABA & Glycine

Inhibitory interneurones are present throughout the grey matter of the dorsal horn. Many of these co-express both GABA and glycine, particularly in superficial laminae, while some contain only GABA and a few in deeper laminae contain only glycine (Todd and Sullivan, 1990). Many of these interneurones are directly stimulated by low threshold mechanoreceptors (Yoshimura and Nishi, 1995), conforming to the Gate Control Theory of pain (Melzack and Wall, 1965), or even by descending axons (Kato et al., 2006). $GABA_A$ and glycine receptors are ionotropic channels permeable to chloride ions (Cl^-) whereas $GABA_B$ receptors are GPCRs. Despite both GABA and glycine receptors being expressed in lamina I, inhibitory post-synaptic currents here are mainly glycinergic, suggesting that only glycine receptors are located at synapses while $GABA_A$ receptors may be extra-synaptic (Chery and de Koninck, 1999). In lamina II, both glycine and GABA exert equal inhibitory influences (Keller et al., 2001). $GABA_A$ receptors on primary afferent terminals also modulate neurotransmitter release (Rudomin and Schmidt, 1999).

1.4.7 Endogenous opioids

Two types of endogenous opioid peptides exist in the dorsal horn, enkephalins and dynorphins. Enkephalins are most abundant and are contained in spinal neurones in laminae I and II (Todd and Spike, 1993), as well as in descending axons from supraspinal sites (Song and Marvizon, 2003), but are not present in primary afferent terminals (Pohl et al., 1994). Additional spinal neurones contain dynorphins and these are mainly located in laminae I, II and V (Cruz and Basbaum, 1985; Todd and

Spike, 1993). Dynorphins may also be present in primary afferent terminals (Botticelli et al., 1981). Both enkephalins and dynorphins are agonists at the three classical opioid receptors, μ (MOR), δ (DOR) and κ (KOR), all of which couple to inhibitory Gi/o proteins that subsequently inhibit cAMP (cyclic adenosine monophosphate) formation, open K^+ channels and inactivate Ca^{2+} channels to mediate analgesia (Watkins et al., 1992; Takemori and Portoghese, 1993; Budai and Fields, 1998). MORs in the dorsal horn are located on superficial neurones which have axons projecting ventrally to laminae III-V (Eckert et al., 2003). KORs are mostly expressed by lamina II neurones whereas DORs are scattered throughout the dorsal horn (Morris and Herz, 1987; Minami and Satoh, 1995). All three types of receptor are expressed on terminals of C-fibres and thus modulate neurotransmitter release (Fields et al., 1980). Finally, pronociceptive effects of dynorphins have been reported, particularly in chronic pain states in which spinal dynorphin content increases. Such effects of dynorphins in pathological conditions may be mediated by actions on spinal bradykinin BK_2 receptors located on primary afferent terminals, thus promoting neurotransmitter release (Lai et al., 2006). Atypical opioid receptors in the dorsal horn include the nociceptin 1 (NOP_1) receptor, also known as the opioid receptor-like 1 (ORL-1) receptor due to its 50% homology to the classical receptors (Mogil and Pasternak, 2001). NOP_1 also couples to an inhibitory Gi/o protein and the endogenous ligand seems to be orphanin FQ/nociceptin (OFQ/N) rather than the known endogenous opioids (Meunier et al., 1995; Reinscheid et al., 1995). At present, the exact effects of NOP_1 activation at the spinal level are unclear and may be both hyperalgesic and analgesic.

1.4.8 Cannabinoids

The cannabinoid system can contribute to the processing of nociceptive transmission (Hohmann, 2002). Cannabinoid receptors CB_1 and CB_2 are associated with sites involved in pain signalling and may be activated by endocannabinoids such as anandamide (AEA). The CB_1 receptor is known to function as an inhibitory receptor, via coupling to an inhibitory Gi/o protein. In nociception, therefore, CB_1 receptors exert an analgesic effect which may be mediated at the peripheral or central terminals of nociceptive afferents or on excitatory spinal dorsal horn neurones (Drew

et al., 2000; Kelly and Chapman, 2001; Kelly et al., 2003; Agarwal et al., 2007). For example, activation of CB_1 receptors on peptidergic C-fibre terminals has been shown to inhibit release of CGRP (Ahluwalia et al., 2003). However, a pronociceptive role for CB_1 receptors has recently been suggested to occur via expression on inhibitory spinal interneurones, thus mediating spinal disinhibition (Salio et al., 2002; Pernia-Andrade et al., 2009). CB_2 receptors are not present in the CNS of normal animals, though peripheral CB_2 receptors do exist and also mediate antinociception (Malan et al., 2001). However, following peripheral nerve injury, CB_2 receptors are functionally upregulated in the spinal dorsal horn (Zhang et al., 2003) and mediate inhibitory effects (Sagar et al., 2005; Yamamoto et al., 2008).

1.4.9 Serotonin (5-HT)

The release of 5-HT within the dorsal horn is almost exclusively from axons descending from supraspinal sites, in particular the RVM in the brainstem, which terminate in both superficial and deep laminae (Millan, 2002). Once released, 5-HT can exert both anti- and pro-nociceptive roles dependent on the receptors activated and the cell types expressing them. Numerous 5-HT receptors, $5\text{-HT}_{1\text{-}7}$, have been detected in the dorsal horn and can influence terminating primary afferents, spinal projection neurones or inhibitory interneurones (Millan, 2002). For example, $5\text{-HT}_{1A/B}$ receptors are inhibitory GPCRs and are believed to directly inhibit dorsal horn neurones (el-Yassir et al., 1988). In addition, 5-HT_{1B} receptors on peptidergic C-fibres can inhibit release of SP and CGRP (Arvieu et al., 1996; Ma et al., 2001). In contrast, the 5-HT_3 receptor is an excitatory ligand-gated cation channel and thus promotes neuronal excitability. 5-HT_3 receptors are abundant in the superficial dorsal horn and a significant proportion of these receptors are found on nociceptor terminals, thus promoting neurotransmitter release (Saria et al., 1990; Maricq et al., 1991; Morales et al., 1998; Zeitz et al., 2002).

1.4.10 Noradrenaline (NA)

NA is released in the dorsal horn by descending axons from noradrenergic brainstem nuclei, such as the locus coeruleus and subcoeruleus (Kwiat and Basbaum, 1992; Millan, 2002). Spinal adrenergic receptors include the α_1 and α_2 subtypes which can account for both inhibitory and excitatory effects of dorsal horn NA release. Inhibitory effects of NA are largely mediated by spinal α_{2A} receptors which may reduce primary afferent release of neurotransmitters and activity of spinal neurones (Yaksh et al., 1995; Suzuki et al., 2002a). Conversely, α_{2C} receptors may be located on inhibitory interneurones and thus may disinhibit spinal projection neurones, producing a facilitatory effect (Olave and Maxwell, 2004). Additionally, excitatory α_1 receptors can exert either facilitatory or inhibitory effects on spinal nociception via their expression on projection neurones and inhibitory interneurones, respectively (Gassner et al., 2009).

Transmitter	Sources in dorsal horn	Receptor	Excitatory or Inhibitory	Location
Somatostatin	C-fibres, Interneurones, Descending axons	sst2A	Inhibitory	Dorsal horn neurones
Adenosine triphosphate (ATP)	GABAergic interneurones?	P_2X_3 P_2X_4 P_2X_7	Excitatory Excitatory Excitatory	Nociceptors Microglia Microglia
Cholecystokinin (CCK)	Interneurones, Descending axons	CCK_B	Excitatory	Afferents
Galanin	Primary afferents, Interneurones	GalR1 GalR2	Inhibitory Excitatory	Interneurones Afferents
Neuropeptide Tyrosine (NPY)	Interneurones	Y1 Y2	Inhibitory Inhibitory	Afferents & Interneurones Afferents

Table 1.1: Additional neurotransmitters acting with the spinal dorsal horn.
(Liu and Hokfelt, 2002; Millan, 2002; Hokfelt et al., 2007; Tsuda et al., 2010)

1.5 Spinal dorsal horn nociceptive plasticity

The spinal cord is an important relay site at which various incoming sensory and nociceptive signals undergo convergence and modulation. Spinal neurones are under ongoing control by peripheral inputs, interneurones and descending controls. One consequence of this modulation is that the relationship between stimulus and response to pain is not always straightforward. The response of output cells can be greatly altered following a peripheral afferent barrage, as produced by electrical stimulation, inflammation or nerve damage, via the interaction of various neurotransmitter systems in the spinal cord, all of which are subject to plasticity, particularly during pathological conditions. This prolonged pre-synaptic input can lead to spinal nociceptive plasticity such as wind-up, long-term potentiation (LTP) and central sensitization of dorsal horn neurones. Central to these events is stimulation of dorsal horn glutamatergic NMDA receptors which can promote activation of intracellular signalling pathways that contribute to such spinal plasticity. As a consequence, electrophysiological characteristics of spinal neurones are altered. NS cells can be activated by low threshold inputs and are effectively converted to WDR neurones. Responses of neurones to afferent inputs increase, as does receptive field size, while spontaneous action potential firing is induced or enhanced. Together, these mechanisms of plasticity contribute to the establishment of chronic pain states which manifest clinically in patients as a heightened response to painful stimuli (hyperalgesia) and pain resulting from normally non-painful stimuli (allodynia), as well as prolonged pain once a stimulus has ended (aftersensations) and spontaneous, ongoing pain in the absence of an evoking stimulus.

1.5.1 Spinal wind-up & temporal summation of pain

Glutamate is released from sensory afferents in response to acute as well as more persistent noxious stimuli, and it is the fast AMPA receptor activation that is responsible for setting the initial baseline response of spinal dorsal horn neurones to both noxious and tactile stimuli. However, if a repetitive and high frequency stimulation of nociceptive C-fibres occurs, at constant intensity, there is then an amplification and prolongation of the response (post-discharge) of spinal dorsal horn neurones to subsequent inputs, so-called wind-up (Fig. 1.6a) (Mendell and Wall, 1965; Mendell, 1966). Stimulation of C-fibres is usually via a train of 16 transcutaneous electrical stimuli at 0.5-2 Hz delivered at the receptive field of the neurone being recorded. It has been shown that 16 stimuli are required to reach maximal discharge and any further stimuli may even lead to a decline of the neuronal response. Furthermore, a neurone resets to its baseline responsiveness by 5 min after electrical stimulation (Schouenborg and Sjolund, 1983). High stimulation frequencies, for example 10 Hz or above, do not induce wind-up, likely due to the failure of nociceptive C-fibres to conduct impulses at such frequencies (Raymond et al., 1990). In addition, electrical stimulation of C-fibres, similar to that used to evoke wind-up, has been shown to produce a short-lasting increase in receptive field size (Cook et al., 1987; Li et al., 1999), a feature of central sensitization.

The enhanced activity during wind-up results from the activation of the NMDA receptor (Dickenson and Sullivan, 1987b). If there are only low level acute or low frequency noxious or tactile inputs to the spinal cord, then activation of the NMDA receptor is not possible, since under normal conditions the ion channel of this receptor is blocked by normal physiological levels of magnesium ions (Mg^{2+}) found in neuronal tissues, including the spinal cord (Mayer et al., 1984; Mayer and Westbrook, 1987). This unique Mg^{2+} plug of the channel requires a sustained depolarization of the membrane in order to be removed and allow the NMDA receptor channel to be activated and opened. It is likely that the co-release of peptidergic transmitters, such as substance P and CGRP, that are found in C-fibres along with glutamate, is responsible for a prolonged slow depolarization of the neurone and subsequent removal of the NMDA block, thus permitting wind-up to occur (Budai and Larson, 1996; Khasabov et al., 2002; Suzuki et al., 2003). AMPA receptor

antagonists have little effect on wind-up (Stanfa and Dickenson, 1999), and the brief depolarization produced by this receptor would not be expected to produce any prolonged removal of the NMDA block, unlike the long-lasting, slow (several seconds) activations caused by peptides. The absence of peptides in large Aβ-fibres explains the lack of wind-up after low threshold stimuli. Once NMDA receptors are activated, Ca^{2+} influx takes place and wind-up occurs. In addition, NMDA receptors may contribute to activation of L-type Ca^{2+} channels which further contribute to the rise in intracellular Ca^{2+}, leading to wind-up (Morisset and Nagy, 2000; Fossat et al., 2007). Blockade of NMDA receptors selectively reduces wind-up without altering the pre-synaptic input to the dorsal horn, indicated by the initial response to electrical stimulation (Davies and Lodge, 1987; Dickenson and Sullivan, 1987b).

Since wind-up occurs only in WDR neurones but not in NS neurones (Schouenborg and Sjolund, 1983), and given that many WDR neurones project to the brain from lamina V, this plasticity is most likely to potentiate the sensory discriminative aspect of pain conveyed by the spinothalamic tract from lamina V, as opposed to the more affective component of pain which largely originates from lamina I NS projection neurones. Furthermore, wind-up is unaffected by spinal administration of ondansetron, a 5-HT_3 receptor antagonist, which blocks 5-HT-mediated descending facilitation from the RVM, thus suggesting that wind-up is predominantly an intrinsic spinally generated event (Suzuki et al., 2002b).

A perceptual correlate of wind-up has been demonstrated in humans, in both healthy volunteers as well as chronic pain patients, whereby repetitive noxious stimulation of C-fibre afferents produces temporal summation of pain ratings (Fig. 1.6b). This increase in pain can be evoked by electrical stimulation of the skin (Price, 1972) or peripheral nerve (Lundberg et al., 1992), as well as by noxious natural stimuli such as pinprick (Magerl et al., 1998) or noxious heat (Price et al., 1992). Price and colleagues showed that this temporal summation of pain was NMDA-dependent, analogous to that already demonstrated in animals (Price et al., 1994). Dextromethorphan, an NMDA receptor antagonist, was administered to healthy subjects and pain was assessed following electrical or noxious heat stimulation of the skin of the hand. First pain, which was described as sharp and deemed to be mediated by Aδ-fibres, was unaltered by dextromethorphan. Second pain, which

followed first pain by about one second and was described as burning, dull or diffuse, is mediated by C-fibres. The first stimulus in each train of stimuli was also unaffected by dextromethorphan. In contrast, dextromethorphan did reliably reduce temporal summation of both electrically and heat evoked second pain. Ketamine, an alternative NMDA antagonist, was shown to inhibit temporal summation of both withdrawal reflexes and pain ratings by human volunteers following repetitive electrical stimulation without any effect on reflexes and pain ratings produced by a single stimulus (Arendt-Nielsen et al., 1995). Kristensen and colleagues reported the case study of a neuropathic pain patient who displayed symptoms of hyperalgesia and allodynia, as well as post-discharge and wind-up, whereby an increase in pain rating was observed from minutes to hours after termination of stimulation, indicating aftersensations and temporal stimulation (Kristensen et al., 1992). Spinal administration of a competitive NMDA receptor antagonist, CPP (3-[2-carboxypi-perazin4-yl]-propyl-I-phosphonic acid) completely abolished aftersensations and temporal summation of pain, as well as increased receptive field size. Thus, NMDA receptors do not contribute to acute first pain, nor do they alter the pre-synaptic input to second order neurones, which is indicated by second pain evoked by the first stimulus in a stimulus train or pain evoked by a single isolated stimulus, but rather contribute exclusively to wind-up and temporal summation of second pain via a post-synaptic spinal mechanism.

Quantitative sensory testing (QST) is the most important approach to characterize the somatosensory phenotype of chronic pain patients in order to define mechanisms and thus aid treatment (Rolke et al., 2006a). A standardized protocol for QST has been developed by the German Research Network on Neuropathic Pain (DFNS) and the battery of tests includes one to assess wind-up pain (Rolke et al., 2006b). Patients are asked to rate the pain produced by both a single pinprick stimulus and also at the end of a train of ten pinprick stimuli, applied at constant intensity and frequency. This procedure is repeated five times and a wind-up ratio is calculated (mean rating of the five series divided by the mean rating of the five single stimuli) to indicate the perceptual correlate of temporal pain summation.

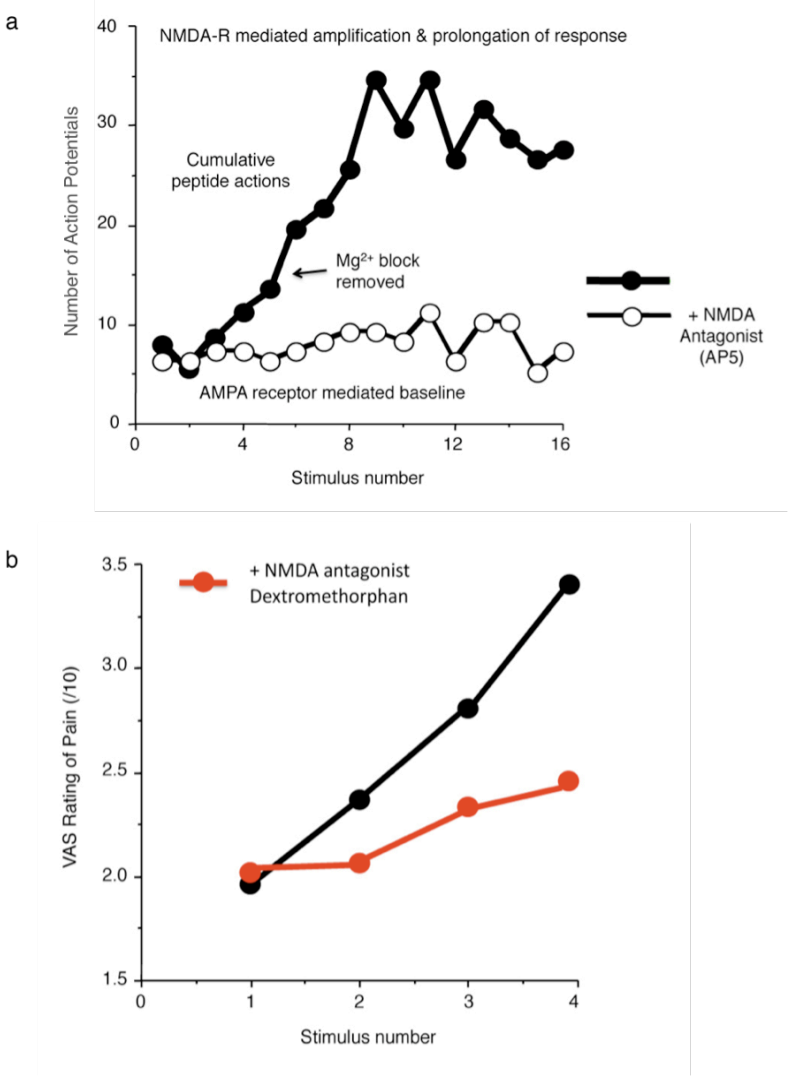


Fig. 1.6: Wind-up of dorsal horn neurones is a likely basis for temporal summation of pain rating in humans. **(a)** Peripheral electrical stimulation at noxious C-fibre intensity produces an amplification and prolongation of the response of spinal neurones, so-called wind-up, recorded in the anaesthetized rat. Wind-up is not mediated by AMPA receptors but is dependent on NMDA receptor activity. The Mg^{2+} block of NMDA receptors is relieved by membrane depolarization due to co-release of peptides by C-fibres. Adapted from Dickenson and Sullivan (1987). **(b)** Repetitive noxious stimulation produces temporal summation of pain ratings in humans on a visual analogue scale (VAS) that is blocked by an NMDA antagonist, dextromethorphan. Adapted from Price et al. (1994).

1.5.2 Spinal long-term potentiation

LTP, first demonstrated in the hippocampus, is a ubiquitous mechanism throughout the central nervous system underlying a long-lasting localized increase in synaptic strength (Bliss and Lomo, 1973). Hippocampal LTP is believed to be the neuronal substrate of learning and memory (Bliss and Collingridge, 1993) and is dependent on activation of NMDA receptors, since NMDA antagonism prevents LTP without effect on normal synaptic transmission (Collingridge et al., 1983). Interestingly, spinal LTP-related phenomena have also been reported in several animal pain models following both inflammation or nerve damage and recent studies have demonstrated that LTP can be induced in pain pathways (Ji et al., 2003; Sandkuhler, 2007). As a result, it has become clear that similarities and probably common intracellular signalling pathways between central sensitization in the spinal cord and LTP in the hippocampus exist. The generation of spinal LTP may be one mechanism whereby acute pain is converted to chronic.

Randic and colleagues first showed that LTP could be induced in the superficial dorsal horn by high frequency (100 Hz) tetanic electrical stimulation at C-fibre intensity (HFS) of dorsal roots using an *in vitro* spinal cord slice preparation (Randic et al., 1993). This LTP was sensitive to pharmacological blockade of NMDA receptors. Subsequently, Liu & Sandkuhler demonstrated superficial dorsal horn LTP *in vivo* by recording superficial C-fibre evoked field potentials (Liu and Sandkuhler, 1995). Here, HFS was applied to the exposed sciatic nerve of anaesthetized rats and produced potentiation for over eight hours. Natural stimuli, such as noxious heat and pinch applied to the skin, formalin injection into the hindpaw and acute physical injury (squeezing with serrated forceps) to peripheral nerve were able to mimic the LTP produced by electrical HFS, though only in spinalized rats, suggesting that in normal animals descending inhibition prevents LTP due to natural noxious stimuli (Sandkuhler and Liu, 1998). Thus, spinal LTP was suggested to be a mechanism underlying afferent-induced hyperalgesia. This LTP in the superficial dorsal horn induced by both electrical HFS and natural noxious stimuli was blocked by NMDA antagonism. Later, it was shown that HFS of the sciatic nerve induces LTP of C-fibre evoked responses specifically in NK_1-expressing neurones in lamina I which project to the parabrachial area (Ikeda et al., 2003). However, the relevance of such studies

to chronic pain has been questioned on the basis that C-fibres do not fire at such high frequencies as 100 Hz, but rather have discharge rates of between 1 and 10 Hz (Puig and Sorkin, 1996). Thus, efforts to induce LTP using more physiological low frequency stimulation (LFS), resembling the afferent barrage during inflammation, were made. Ikeda and colleagues identified *in vitro* a population of lamina I neurones projecting to the PAG in which LTP was induced exclusively by LFS (2 Hz) at C-fibre intensity (Ikeda et al., 2006b). This same protocol could not induce LTP in spino-PB projection neurones, though these cells did still respond positively to HFS. LFS stimulation was subsequently shown to induce LTP in the superficial dorsal horn *in vivo* in anaesthetized rats with intact descending controls (Drdla and Sandkuhler, 2008). Again, this LFS-induced LTP required NMDA receptor activity. The reproducibility of such spinal LTP in the superficial dorsal horn, particularly following HFS, has also been questioned, however. Rygh and colleagues found that HFS stimulation of the sciatic nerve failed to induce LTP of C-fibre evoked action potentials in superficial neurones, including those projecting to the parabrachial area, though, in contrast, LTP was induced in deep dorsal horn WDR neurones (Rygh et al., 2006), suggesting that in the intact animal superficial dorsal horn neurones are under descending inhibitory control which prevents LTP. The discrepancy between this and the aforementioned studies may be explained by examining the stimulus intensities used. Although stimulation frequency was identical (100 Hz), Rygh and colleagues used a stimulation intensity of six times the threshold for activating C-fibre evoked responses, whereas studies from Sandkuhler's lab often employed extreme suprathreshold intensities (e.g. ten times C-fibre threshold; verbal communication from Prof Jurgen Sandkuhler). Thus it is possible that the higher intensity was sufficient to overcome inhibitory influences in the superficial dorsal horn.

Simultaneous to the work demonstrating LTP in superficial laminae, studies also implicated spinal LTP in deep dorsal horn WDR neurones as a potential mechanism for central sensitization (Rygh et al., 2005). Svendsen and colleagues first demonstrated that HFS of the sciatic nerve at C-fibre intensity produced a long-lasting potentiation (> 6 hrs) of not only C-fibre evoked responses, but also Aβ-fibre evoked action potentials of deep WDR neurones, an observation not seen in superficial dorsal horn LTP (Svendsen et al., 1997). The initiation of this deep dorsal horn LTP of WDR firing was mediated by AMPA and NMDA receptors, while

maintenance of potentiation was more NMDA-dependent (Svendsen et al., 1998; Svendsen et al., 1999b). Like in superficial laminae, LTP of deep dorsal horn neurones was found to be influenced by descending inhibitory controls (Svendsen et al., 1999a). Furthermore, Rygh and colleagues demonstrated that descending serotonergic facilitation is also able to modulate manifestation of deep dorsal horn LTP (Rygh et al., 2006). HFS-induced potentiation of WDR neurones was blocked by chemical ablation of superficial NK_1-expressing projection neurones and modulated, though not prevented, by ondansetron, a 5-HT_3 antagonist. It has also been shown that natural, acute, albeit severe noxious peripheral stimulation, achieved by repeated crushing of tissue and bone at the receptive field, can potentiate C-fibre evoked responses of deep WDR neurones (Rygh et al., 1999). More recently, Haugan and colleagues demonstrated that a more physiological LFS (3 Hz) protocol could potentiate responses of deep dorsal horn WDR neurones also (Haugan et al., 2008). However, this electrical LFS-induced potentiation was shorter in duration (< 3 hrs) than that induced by higher frequencies (30 or 100 Hz). This suggests that spinal LTP in the deep dorsal horn, induced by physiological afferent firing frequencies, may contribute only partially to central sensitization and that other plastic mechanisms are required for the manifestation of chronic pain.

Importantly, perceptual correlates of nociceptive LTP have been demonstrated in humans (Klein et al., 2004). Electrical HFS (100 Hz) of noxious intensity was applied to the skin and subjects were asked to rate their pain evoked by mechanical stimulation of the affected area. It was found that HFS alone produced potentiation of pain ratings due to electrical stimuli, while both HFS and extreme high intensity LFS (1 Hz) induced a long-lasting (> 3 hrs) increase in mechanical pain sensitivity at the site of the conditioning stimulus (homotopic hyperalgesia). HFS also caused pain due to light brushing of adjacent skin (secondary dynamic mechanical allodynia). In contrast, sensitivity to thermal modalities was unaltered, as was temporal summation (wind-up) of pain (Lang et al., 2007). A subsequent study showed that potentiation of pain sensitivity to only electrical stimulation at the site of the conditioning stimulus was prevented by ketamine, an NMDA channel blocker (Klein et al., 2007).

1.5.3 Central sensitization of dorsal horn neurones

Central sensitization of dorsal horn neurones was initially demonstrated by Woolf in 1983, providing the first evidence of a central component of nociceptive hypersensitivity in chronic pain (Woolf, 1983). In his study, Woolf recorded responses from α-motoneurone efferents as a measure of the output of dorsal horn processing, while stimulating afferents both at the hindpaw and directly via the sural nerve. Prior to peripheral injury, the majority of efferents exhibited minimal spontaneous activity and responded exclusively to noxious, high threshold mechanical and thermal stimulation at the hindpaw. A thermal injury was then induced at the hindpaw (75°C for 60 s) which caused mild inflammation and produced significant changes in efferent properties. Spontaneous activity increased and mechanical thresholds to von Frey hairs were markedly reduced. The amplitude and duration (post-discharge) of responses evoked by both noxious pinch and electrical stimulation of the sural nerve, at noxious intensities, were also increased. Interestingly, electrical stimulation of Aβ-fibres began to elicit responses in efferents which had been absent prior to peripheral injury indicating that dorsal horn neurones in the pathway had reduced thresholds. Furthermore, the cutaneous receptive fields expanded greatly and even extended to the contralateral hindpaw, providing definitive evidence that this sensitization could not be solely mediated by peripheral sensitization. Expansion of the receptive field is brought about by the lowering of the activation threshold of dorsal horn neurones, since a large proportion of synaptic input to these cells is subthreshold and thus silent under normal conditions whereas following peripheral injury these inputs can evoke action potentials (Cook et al., 1987; Woolf and King, 1990). These changes induced by peripheral thermal injury were mimicked by low frequency electrical stimulation of the sural nerve at C-fibre intensity. Further evidence of a predominant central component of injury-induced changes in muscle efferents was provided by the observations that local anaesthetic block of the hindpaw did not reverse the elevated responses to sural nerve stimulation or receptive field expansion (Woolf, 1983).

Subsequent studies demonstrated that, in addition to peripheral thermal injury and electrical simulation, central sensitization of dorsal horn neurones could also be induced by peripheral injection of chemicals such as formalin (Dickenson and

Sullivan, 1987a), mustard oil (Woolf and King, 1990) and capsaicin (Simone et al., 1991). Furthermore, it was demonstrated that central sensitization is an NMDA-dependent phenomenon and that activity of NMDA receptors is required for both the induction and maintenance of central sensitization of dorsal horn neurones, raising the potential for the use of NMDA antagonists in pre-emptive analgesia and, perhaps more importantly, in the treatment of established pain states (Woolf and Thompson, 1991). For example, NMDA antagonists were able to inhibit both neuronal hyperexcitability and pain-related behaviours induced by the injection of formalin into the hindpaw (Haley et al., 1990; Coderre and Melzack, 1992; Yamamoto and Yaksh, 1992b).

It is important to note that plastic changes induced by peripheral injuries have been demonstrated in neurones at supraspinal sites, representative of the general phenomenon of central sensitization (Lee et al., 2008; Latremoliere and Woolf, 2009). In this thesis, use of the term central sensitization is restricted to the spinal cord and specifically to central sensitization of dorsal horn sensory neurones.

A multitude of studies have now demonstrated that central sensitization within the dorsal horn involves numerous mechanisms which work in concert to increase the excitability of spinal sensory neurones in chronic pain states. These mechanisms include signalling pathways which increase the intrinsic excitability of dorsal horn neurones, diminished spinal inhibition and increased facilitatory influences from supraspinal sites.

1.5.3.1 Intracellular mechanisms of central sensitization of dorsal horn sensory neurones

Downstream of NMDA receptor activation, various intracellular signalling cascades are activated, often via Ca^{2+} influx through the NMDA receptor channel, which promote the increased excitability of dorsal horn neurones, contributing to central sensitization (Fig. 1.7). Such intracellular effectors include the extracellular signal-regulated kinase (ERK), Ca^{2+}/calmodulin-dependent kinase II (CaMKII), PKA, PKC and the tyrosine kinase Src (Ji et al., 1999; Fang et al., 2002; Zou et al., 2002;

Kawasaki et al., 2004; Kohno et al., 2008). These kinases can enhance activity of both AMPA and NMDA receptors during early-onset central sensitization by phosphorylating key regulatory sites, as well as increasing receptor numbers by promoting membrane trafficking and insertion, thus increasing synaptic strength. For example, Ca^{2+}-dependent PKC, inhibition of which prevents central sensitization (Lin et al., 1996), can enhance NMDA receptor activity by increasing the probability of channel opening and decreasing the Mg^{2+} block of the channel (Chen and Huang, 1992). Furthermore, PKC phosphorylates NR1 subunits of NMDA receptors following noxious stimulation and this may lead to the increased trafficking of NMDA receptors to the plasma membrane (Brenner et al., 2004). NR2 subunits are also phosphorylated during central sensitization and this involves PKC and Src (Guo et al., 2002; Guo et al., 2004). AMPA receptor subunits are also phosphorylated by PKA and PKC (Fang et al., 2003) while CaMKII promotes trafficking of AMPA receptors following noxious stimulation (Galan et al., 2004; Pezet et al., 2008), leading to central sensitization. Late-onset central sensitization, like late-phase LTP, requires transcriptional changes driven by transcription factors such as the cAMP response element binding protein (CREB), which is phosphorylated during central sensitization (Ji and Rupp, 1997). This phosphorylation may be regulated by ERK and contributes to expression of genes such as c-Fos, a marker of neuronal activation following noxious stimulation (Hunt et al., 1987).

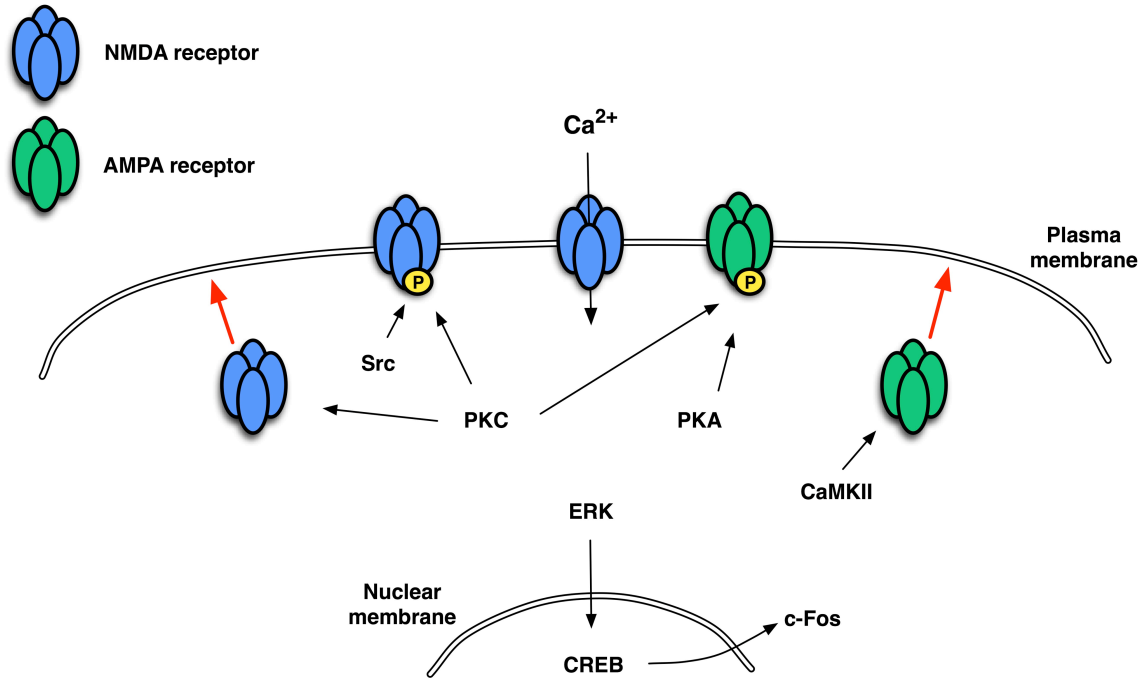


Fig. 1.7: Intracellular mechanisms within dorsal horn neurones contribute to spinal central sensitization. Activation of NMDA receptors following prolonged noxious stimulation leads to a large Ca^{2+} influx into dorsal horn sensory neurones. This activates various Ca^{2+}-dependent intracellular processes that lead to central sensitization. Src kinase, PKA, PKC and CaMKII contribute to early-onset central sensitization via phosphorylation and increased membrane insertion of NMDA and AMPA receptors. Late-onset central sensitization involves ERK-mediated activation of CREB transcription factor and expression of genes such as c-Fos. Adapted from Ji et al. (2003).

1.5.3.2 Loss of spinal inhibition contributes to mechanical hypersensitivity and allodynia

It is believed that low threshold inputs from Aβ-fibres contribute to an inhibitory control of lamina I NS cells, decreasing spinal output of nociceptive information. This is the basis of the Gate Control Theory (Melzack and Wall, 1965). Additionally, it has been suggested that low threshold inputs may activate inhibitory interneurones in the dorsal horn which prevent Aβ-fibre polysynaptic inputs reaching lamina I NS cells from deeper laminae (Torsney and MacDermott, 2006). Yaksh showed that blocking spinal inhibition, through the intrathecal administration of GABA receptor (bicuculline) or glycine receptor (strychnine) antagonists, resulted in pain evoked by light tactile stimulation, equivalent to mechanical allodynia observed in pain patients (Yaksh, 1989). Interestingly, morphine had little effect on this mechanical hypersensitivity, mimicking the clinical situation, whilst NMDA receptor antagonists attenuated strychnine-mediated hypersensitivity. Thus it was suggested that a loss of spinal GABAergic and glycinergic inhibition might underlie the clinical manifestation of mechanical allodynia in chronic pain states. When spinal inhibition is lost, Torsney and MacDermott propose that a novel, polysynaptic, NMDA-dependent input from Aβ-fibres in lamina III is disinhibited and can now activate lamina I NS projection cells, effectively converting them to WDR neurones (Torsney and MacDermott, 2006). In this way, low threshold stimulation may be processed and transmitted to higher centres as being painful, underlying mechanical allodynia. In the clinic, two distinct forms of mechanical allodynia present in patients, static mechanical allodynia (SMA) and dynamic mechanical allodynia (DMA).

Accumulating evidence from preclinical studies suggests that SMA may arise from a loss of GABAergic inhibition, while the loss of glycinergic inhibitory control may produce DMA (Fig. 1.8). Spinal administration of strychnine alone produces behavioural mechanical hypersensitivity (Yaksh, 1989) as well as enhanced neuronal responsiveness to light mechanical stimuli (Sorkin and Puig, 1996). This neuronal hypersensitivity is selectively blocked by NMDA antagonists while opioid agonists have no effect, similar to their behavioural effects. Later, Miraucourt and colleagues showed that spinal strychnine produced mechanical hypersensitivity exclusively to dynamic light tactile stimuli (gentle air puffing). It was found that strychnine

disinhibited a normally silent PKCγ-dependent and NMDA-dependent excitatory spinal circuit which gates low threshold tactile inputs from Aβ-fibres to NS neurones in the superficial dorsal horn (Miraucourt et al., 2007). Furthermore, this circuit did not involve NK_1-expressing projection neurones and the dynamic mechanical hypersensitivity produced was resistant to morphine, again resembling clinical DMA (Miraucourt et al., 2009). In contrast to the selective neuronal effects of strychnine, selective blockade of GABAergic inhibition by spinal bicuculline produces a general enhancement of neuronal responses, including those evoked by light mechanical stimuli, and does not seem to involve NMDA receptors (Yaksh, 1989; Sorkin et al., 1998). This bicuculline-mediated hypersensitivity is not responsive to dynamic stimuli but only to static mechanical stimulation, as produced by von Frey hairs (Miraucourt et al., 2009), thus likely representing clinical SMA.

A loss of inhibitory controls in the dorsal horn may arise from activation of the endocannabinoid system. Noxious stimulation of primary afferents has been shown to lead to the production of spinal endocannabinoids which subsequently activate inhibitory CB_1 receptors located on inhibitory interneurones, thus reducing the synaptic release of GABA and glycine (Pernia-Andrade et al., 2009). The effect of this disinhibition was to promote excitation of normally NS neurones by non-painful stimuli. In addition, it has been suggested that a loss of inhibitory interneurones via neuronal apoptosis may occur in neuropathic pain (Moore et al., 2002; Scholz et al., 2005) though this has since been refuted as neuronal cell death is not required for nerve injury-induced pain to occur (Polgar et al., 2003; Polgar et al., 2004; Polgar and Todd, 2008). Thus, it is more likely that any reduction in spinal inhibition results from an increased inhibitory or reduced excitatory control of inhibitory interneurones. Alternatively, Coull and colleagues proposed that failure of GABAergic inhibitory control of low threshold inputs in the superficial dorsal horn of neuropathic rats might arise from a loss of the Cl^- transporter protein KCC2 (Coull et al., 2003; Coull et al., 2005). This results in accumulation of intracellular Cl^- in lamina I cells which, when activated by GABA released from inhibitory interneurones, are now depolarized rather than hyperpolarized due to Cl^- efflux, thus effectively making GABA excitatory. This mechanism would contribute to the unmasking of a normally silent polysynaptic Aβ-fibre input to lamina I cells, so that these cells now respond to innocuous

mechanical stimulation, leading to behavioural static mechanical hypersensitivity following nerve injury (Keller et al., 2007).

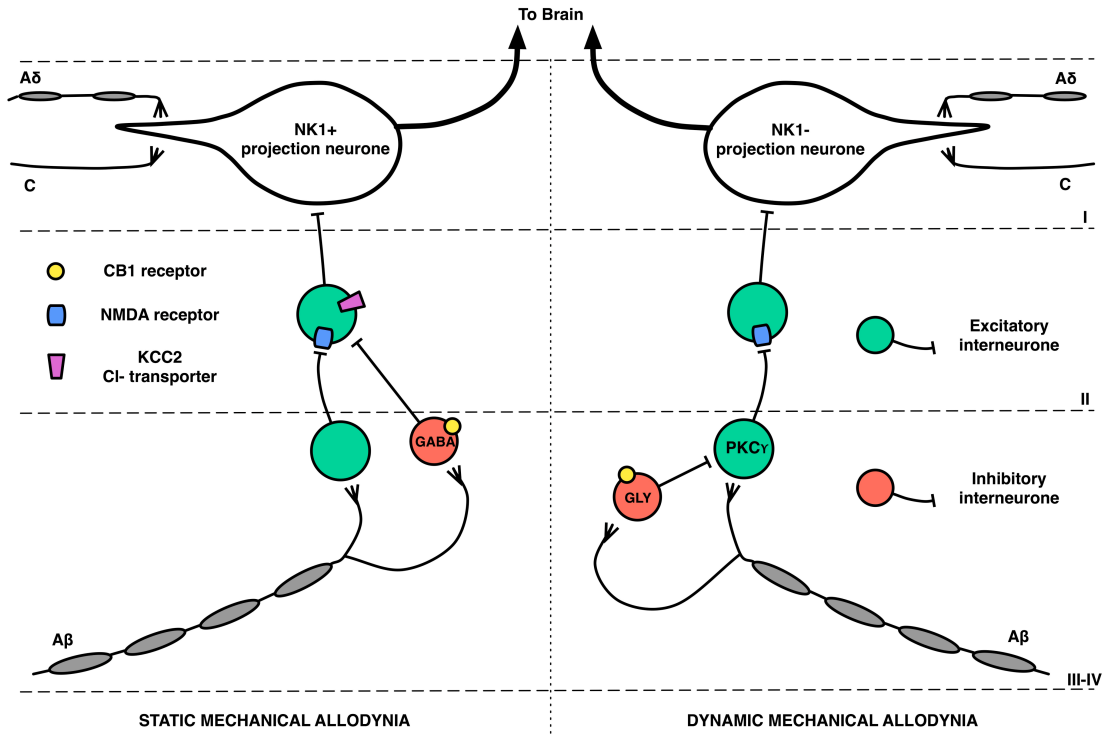


Fig. 1.8: Loss of spinal GABAergic and glycinergic inhibition leads to mechanical allodynia. Under normal conditions low threshold polysynaptic inputs from Aβ-fibres to superficial NS projection neurones are kept silent by inhibitory GABAergic and glycinergic interneurones. Failure of such inhibitory control, perhaps via activation of inhibitory CB_1 receptors on inhibitory interneurones or downregulation of the Cl^- transporter KCC2, may unmask low-threshold inputs to superficial projection neurones. Loss of GABA-mediated inhibition may gate Aβ-fibre inputs to NK_1-expressing projection neurones and may underlie static mechanical allodynia. Loss of glycine-mediated inhibition gates Aβ-fibre inputs to projection neurones lacking NK_1 receptors, via PKCγ-expressing interneurones, leading to dynamic mechanical allodynia. Both pathways involve activation of NMDA receptors.

1.5.3.3 Plasticity of descending modulation in chronic pain states

In addition to the contributions of synaptic plastic mechanisms like wind-up and spinal LTP, plasticity of descending modulation from the brainstem is also involved in central sensitization in abnormal pain states (Fig. 1.9). Descending facilitatory pathways from the RVM in the brainstem have been shown to be involved in the maintenance, rather than the initiation, of nerve injury-induced pain (Kovelowski et al., 2000; Burgess et al., 2002; Vera-Portocarrero et al., 2006). Injection of the local anaesthetic lidocaine, into the RVM reverses established behavioural hypersensitivity in nerve-injured animals, but does not prevent the expression of this hypersensitivity (Burgess et al., 2002). In an electrophysiological study, injection of lidocaine into the RVM reduced dorsal horn neuronal responses to noxious stimuli in normal animals. This effect of lidocaine was greater in nerve-injured animals, and in these animals it was observed that descending facilitation from the RVM now influenced neuronal responses to non-noxious tactile stimulation, thus suggesting a possible mechanism for mechanical allodynia (Bee and Dickenson, 2007). Suzuki and colleagues showed that elimination of the ascending arm of the spino-bulbo-spinal loop, by chemical ablation of lamina I/III NK_1-expressing neurones with a substance P and saporin neurotoxin conjugate (SP-SAP), reduces the excitability of deep dorsal horn WDRs, indicating that descending influences are predominantly facilitatory, acting via spinal 5-HT_3 receptors, since the effect of SP-SAP could be mimicked by spinally-administered ondansetron, a selective 5-HT_3 antagonist (Suzuki et al., 2002b). The contribution of such descending serotonergic facilitation to neuropathic pain was further confirmed by the fact that the efficacy of ondansetron to suppress spinal responses to mechanical punctuate stimuli was significantly enhanced after spinal nerve ligation, suggesting an increase in the descending serotonergic facilitatory drive to the spinal cord (Suzuki et al., 2004). In accordance, depletion of spinal 5-HT attenuates signs of behavioural hypersensitivity following nerve ligation (Rahman et al., 2006). Additionally, it has been shown that spinal SP-SAP treatment attenuates behavioural hypersensitivity and also abnormal neuronal coding exhibited after both nerve ligation (Suzuki et al., 2005) and intraplantar injection of capsaicin (Mantyh et al., 1997; Khasabov et al., 2002). A small double-blinded, placebo-controlled, crossover clinical study has suggested an antinociceptive effect of ondansetron in humans which merits further investigation (McCleane et al., 2003), while SP-SAP,

although not tested in humans, has been found to be without toxic effects when administered in dogs (Allen et al., 2006). Gabapentin is used as a first line treatment for neuropathic pain (Backonja et al., 1998; Rowbotham et al., 1998; Serpell, 2002) and binding studies have revealed interactions with the auxiliary $\alpha_2\delta$ subunit of voltage-gated Ca^{2+} channels (Gee et al., 1996). Interestingly, it has been shown that the efficacy of gabapentin is partly dependent on upregulation of 5-HT_3-mediated descending facilitation following nerve injury. First, spinal SP-SAP was able to block the antinociceptive actions of gabapentin after nerve injury. Second, blocking 5-HT_3 receptors prevented the actions of gabapentin after nerve injury and even more remarkably, their activation allowed the drug to now work in normal animals (Suzuki et al., 2005). Thus, activation of 5-HT_3 receptors enhances pain processing, but at the same time produces a state-dependent or permissive interaction that allows treatment (Suzuki et al., 2005). Together, these studies demonstrate that superficial NK_1-expressing neurones can trigger descending facilitations mediated through parabrachial-RVM connections and regulate the sensitivity of deeper lying neurones to gabapentin through activation of spinal 5-HT_3 receptors. These excitatory influences promote spinal central sensitisation and facilitate nociceptive reflexes, contributing to the pathology of neuropathic pain. It is likely that enhancement of descending supraspinal facilitatory systems represents a central mechanism by which the loss of sensory input resulting from nerve damage is compensated.

Like descending facilitation, inhibitory noradrenergic pathways from the brainstem to the dorsal horn may also undergo plastic changes in chronic pain states. Several studies indicate an increase in descending noradrenergic inhibition following peripheral inflammation (Weil-Fugazza et al., 1986; Stanfa and Dickenson, 1994; Tsuruoka and Willis, 1996a, b; Green et al., 1998; Tsuruoka et al., 2003), coupled with an enhanced efficacy of spinally administered α_2-adrenoceptor agonists (Hylden et al., 1991; Mansikka and Pertovaara, 1995; Suzuki et al., 2002a). This increased inhibitory drive may be a homeostatic mechanism initiated in an attempt to counteract an enhanced facilitatory drive and increased spinal hyperexcitability. It has also been suggested that there is increased noradrenergic innervation to the dorsal horn following nerve injury (Ma and Eisenach, 2003), again analogous to the enhanced facilitatory drive to the dorsal horn mediated by spinal 5-HT_3 receptors. Studies have demonstrated nerve injury-induced changes in noradrenergic pathways, including

upregulation of spinal α_{2A}-adrenoceptors (Cho et al., 1997; Birder and Perl, 1999; Stone et al., 1999) and increased spinal NA content (Satoh and Omote, 1996). While these findings may suggest an enhancement of descending noradrenergic inhibition, it is plausible that the increased noradrenergic receptor density and innervation of the dorsal horn observed after nerve injury is the result of compensatory mechanisms which occur to counteract the actual loss of a tonic descending noradrenergic inhibitory drive. Therefore, it would be expected that α_2-adrenoceptor agonists would increase in potency and efficacy after nerve injury. In support of this, the efficacy of the selective α_2-adrenoceptor antagonist atipamezole is reduced after nerve injury, though only in specific sensory modalities (Rahman et al., 2008), suggesting that there is in fact a loss of descending noradrenergic influences in neuropathic pain. Atipamezole enhanced evoked responses of dorsal horn neurones to low intensity mechanical stimulation. This observation only occurred in control sham-operated animals, and was absent in nerve-injured animals, suggesting a selective control of descending inhibition, via spinal α_2-adrenoceptors, on low-intensity mechanically evoked neuronal responses. No effects of atipamezole were seen following noxious mechanical or both non-noxious and noxious thermal stimulation, in either sham-operated or nerve-injured animals. Further evidence for this differential control of stimulus modalities was shown with the use of a selective and potent α_2-adrenoceptor agonist S18616 (Suzuki et al., 2002a). This compound suppressed dorsal horn neuronal responses to thermal and high intensity mechanical stimulation equally in both sham-operated and nerve-injured rats. However, the suppression of low intensity mechanically evoked responses was greatly enhanced after nerve injury, supporting the loss of descending inhibitory controls of this sensory modality, but also supporting the upregulation of adrenergic receptor density and enhanced sensitivity to α_2-adrenoceptor agonists. Interestingly, atipamezole also enhanced spontaneous activity of dorsal horn neurones, again exclusively in sham-operated rats (Rahman et al., 2008). Overall, these results suggest that there is a loss of tonic descending inhibitory controls of neuronal responses to low intensity mechanical stimulation and also of spontaneous neuronal activity in the dorsal horn following peripheral nerve injury. Coupled with the enhancement of descending serotonergic facilitation, this decrease in descending noradrenergic inhibition would result in an overall enhancement of dorsal horn excitability, manifesting as mechanical hypersensitivity, allodynia and spontaneous pain, common complaints of neuropathic

pain patients. Clonidine, which has been clinically successful in the alleviation of neuropathic pain (Eisenach et al., 1996), and which is licensed for the treatment of cancer pain in the USA (Eisenach et al., 1995), acts by partial agonism at spinal α_2-adrenoceptors.

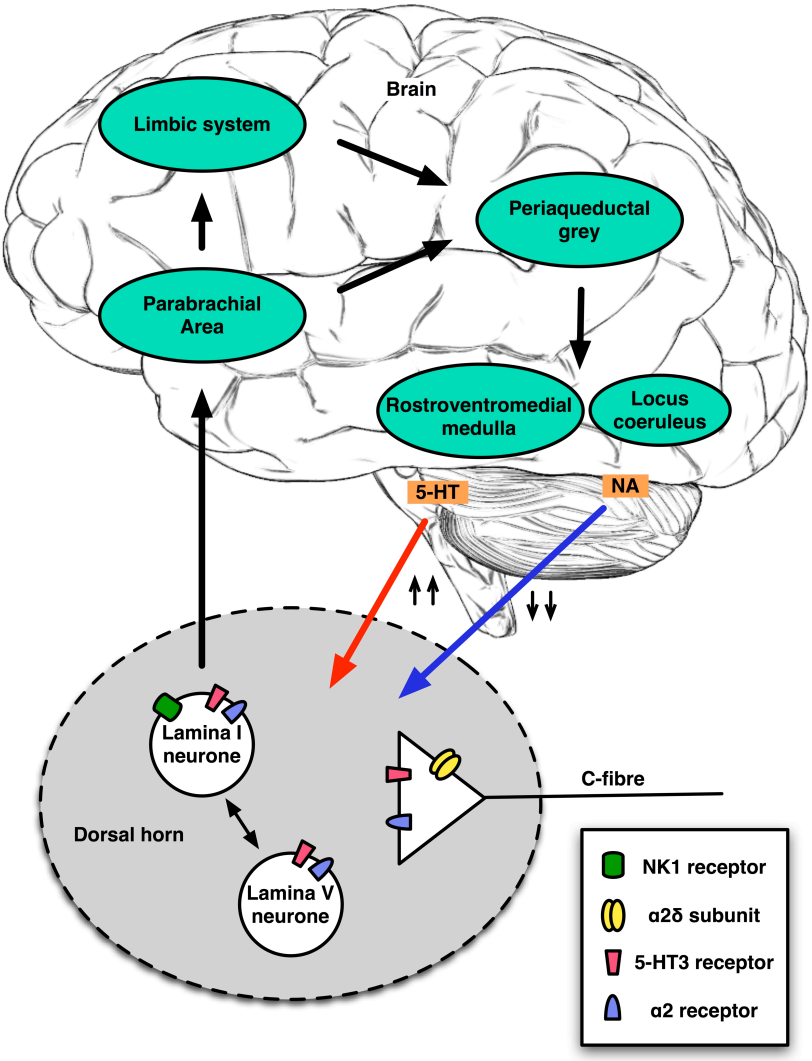


Fig. 1.9: Increased serotonergic and decreased noradrenergic drive from the brainstem contributes to spinal central sensitization. Superficial $NK1^+$ projection neurones activate a spino-bulbo-spinal loop which modulates dorsal horn processing. Activation of the parabrachial area engages the limbic system and the periaqueductal grey, which in turn influence the output from the rostroventromedial medulla (RVM) and locus coeruleus (LC) in the brainstem to the dorsal horn. An increased facilitatory serotonergic drive from the RVM, via activation of spinal 5-HT_3 receptors, coupled with decreased activation of inhibitory α_2 receptors by NA released from the LC, contributes to central sensitization. Activation of 5-HT_3 receptors may contribute to increased function of $\alpha_2\delta$ auxiliary subunits of Ca^{2+} channels, the binding site for gabapentin, leading to increased neurotransmitter release.

1.5.3.4 Human surrogate models of central sensitization

Various models have been developed to study central sensitization in humans and these invariably involve activation of peripheral nociceptors in healthy volunteers to induce dorsal horn plasticity (Schmelz, 2009). Peripheral stimuli which induce central sensitization include chemicals such as capsaicin (Simone et al., 1989; LaMotte et al., 1991), noxious heat (Moiniche et al., 1993; Pedersen and Kehlet, 1998) and electrical stimulation (Koppert et al., 2001). A model employing the combination of heat and capsaicin has also been described (Petersen and Rowbotham, 1999). Outcome measures include neurogenic flare as well as hyperalgesia and allodynia, both within the territory of the injury and beyond, thus representative of both peripheral and central sensitization. Similar to human surrogate models of wind-up and LTP, these models of central sensitization also involve NMDA receptors (Park et al., 1995; Andersen et al., 1996; Warncke et al., 1997; Koppert et al., 2001). For example, ketamine reverses both spatial and temporal aspects of secondary hyperalgesia to punctate and dynamic stimuli, without alteration of thermal detection thresholds (Warncke et al., 1997). While these models further our understanding of peripheral and central mechanisms of pain hypersensitivity in chronic pain, they are incomplete since they do not produce significant spontaneous pain, a highly common complaint of pain patients, and thus spontaneous pain at present can only be investigated in patients. Understanding the mechanisms that cause spontaneous pain will allow this pain to be reproduced in healthy volunteers and improve current surrogate models of central sensitization.

1.6 The NMDA receptor as a therapeutic target for chronic pain

1.6.1 Structure of NMDA receptors

In the dorsal horn, functional glutamatergic NMDA receptors are heterotetramers consisting of a pore-forming NR1 subunit that binds glycine in combination with NR2 subunits which contain the binding site for glutamate (Fig. 1.10). The glycine-binding site may also be bound by endogenous D-serine (Wolosker, 2007). Binding at both co-agonist sites is required for activation of the channel. In addition, opening of the channel pore is voltage-dependent since at normal resting membrane potentials, the channel pore is blocked by extracellular Mg^{2+} (Mayer et al., 1984). This tonic block is only relieved by membrane depolarization, produced by another membrane non-NMDA receptor, thus the NMDA receptor can act as a coincidence detector (see section 1.5.1). Once the channel is open, Na^{+} influx and K^{+} efflux occurs which mediates a slow excitatory post-synaptic potential (EPSP), though this contributes minimally to membrane depolarization and action potential generation, unlike the fast EPSP evoked by AMPA receptors. In contrast to other glutamate receptors, significant Ca^{2+} influx occurs through NMDA receptor channels and subsequently activates numerous downstream intracellular signalling mechanisms (MacDermott et al., 1986). NMDA receptors are also slow to inactivate in comparison to non-NMDA receptors. Thus, the coincidence detection, slow kinetics and Ca^{2+} permeability of NMDA receptors renders them integral components of synaptic plasticity. Various allosteric regulatory sites on the receptor, including a zinc ion (Zn^{2+}) binding site, an inhibitory proton sensor and a polyamine site, can modulate activity (Kalia et al., 2008). Furthermore, the cytoplasmic tail of NR2 subunits can be phosphorylated to further regulate receptor activity and various interactions with intracellular effector proteins are mediated via this cytoplasmic tail (Sheng, 2001).

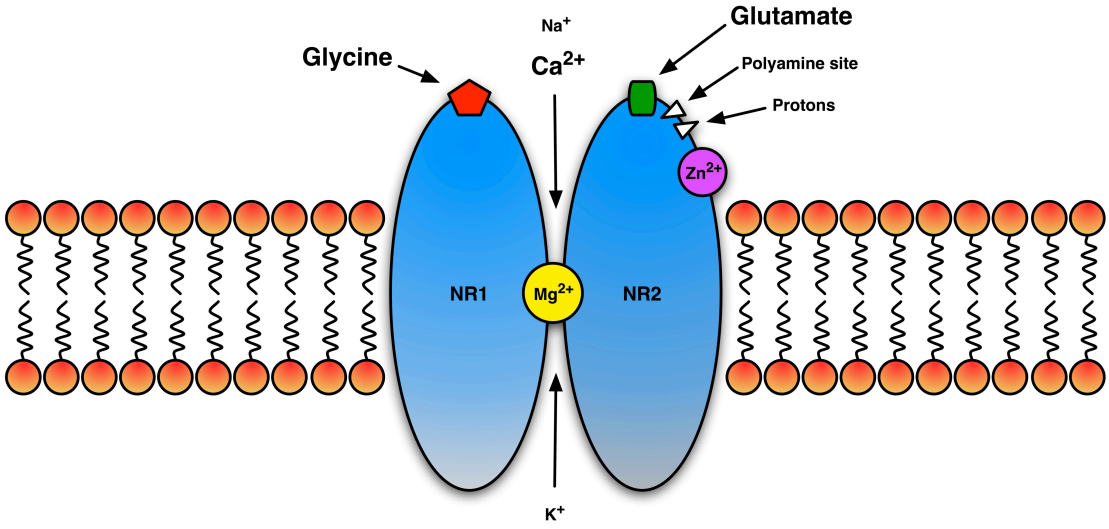


Fig. 1.10: Structure of the NMDA receptor channel. NMDA receptors are tetramers consisting of glycine-binding NR1 subunits and glutamate-binding NR2 subunits. Binding at both co-agonist sites is required for receptor activation. At resting membrane potentials, the channel pore is blocked by Mg^{2+}. This block is removed by membrane depolarization and on subsequent receptor activation and opening of the channel pore, Na^{+} and Ca^{2+} influx and also K^{+} efflux occurs. The large Ca^{2+} influx activates various downstream NMDA-dependent intracellular processes. NR2 subunits also contain various modulatory sites which are bound by polyamines, protons and Zn^{2+} ions. Adapted from Kalia et al. (2008).

1.6.2 Traditional NMDA receptor antagonists and efficacy in chronic pain

Given their expression in the dorsal horn and their essential role in spinal nociceptive plasticity, demonstrated both in animals and humans, NMDA receptors are strong candidates for therapeutic targeting in chronic pain states. Indeed, various drugs targeting NMDA receptor activity have been investigated in animal models of chronic pain following inflammation and nerve damage and there is also evidence from a number of clinical studies to support their use. These drugs act at multiple regulatory sites found on the NMDA receptor and its channel (Bleakman et al., 2006; Childers and Baudy, 2007). Licensed drugs such as ketamine, dextromethorphan and memantine are non-competitive NMDA receptor channel blockers, as is MK-801 (dizocilpine). Competitive antagonists such as CPP bind to the glutamate recognition site on NR2 subunits, while 5,7-dichlorokinurenic acid (5,7-DCK) is an example of a drug which competes for the glycine co-agonist binding site on the NR1 subunit.

Spinal delivery of various NMDA receptor antagonists has been shown to reverse nerve injury-induced hypersensitivity in rodent models of neuropathic pain. These effects were achieved at doses which did not alter the sensitivity of the uninjured paw or the paws of normal rats (Yamamoto and Yaksh, 1992a; Mao et al., 1993; Chaplan et al., 1997). NMDA antagonists also reverse thermal and mechanical hypersensitivity in models of chronic inflammatory pain (Ren and Dubner, 1993; Yamamoto et al., 1993; Eisenberg et al., 1994). Clinical studies have demonstrated analgesic efficacy of NMDA antagonists in various neuropathic pain states (Backonja et al., 1994; McQuay et al., 1994; Felsby et al., 1996; Nelson et al., 1997; Eisenberg et al., 1998; Pud et al., 1998; Jorum et al., 2003). For example, high-dose dextromethorphan reduced pain in patients with painful diabetic neuropathy in a randomized, double-blind, placebo-controlled, crossover trial (Nelson et al., 1997), while amantadine, an alternative NMDA channel blocker, reduced spontaneous pain and wind-up pain in cancer patients with surgical neuropathic pain (Pud et al., 1998). Spontaneous pain, mechanical allodynia and also cold allodynia were reduced by ketamine in patients with post-traumatic neuralgia and post-herpetic neuralgia (Jorum et al., 2003). Overall, these studies indicate that it is likely that aberrant peripheral activity is amplified and enhanced by NMDA receptor-mediated spinal mechanisms following tissue damage and that the receptor is critical for both the induction and the

maintenance of the subsequent pain state. Thus, therapy after the initiating damage can still be effective, in addition to pre-emptive treatment.

However, despite the clear analgesic efficacy of agents acting at the NMDA receptor in both pre-clinical and clinical studies, intolerable cognitive, psychomimetic, motor and memory side-effects are common. This is largely due to the widespread distribution and functionality of NMDA receptors, meaning that the introduction of an antagonist will not only target the underlying pathology, but will also disrupt normal essential NMDA signalling. Patient descriptions of such side-effects include feeling weird, funny in the head, spaced-out, dream-like and having nightmares (Backonja et al., 1994), and all are side-effects that are unlikely to be monitored in animal studies. In addition, sedation, confusion, tremor, ataxia, blurred vision and hypersensitivity to sound have all been reported following use of NMDA receptor antagonists (McQuay et al., 1994; Nelson et al., 1997; Eisenberg et al., 1998; Jorum et al., 2003). Thus, the majority of patients cannot achieve complete pain control since adequate dosing is prevented by the narrow therapeutic window of existing drugs. Nevertheless, the NMDA receptor remains an attractive therapeutic target, requiring novel and perhaps more subtle approaches for modulation.

1.7 Alternative and novel strategies to target spinal NMDA receptors in pain

1.7.1 Specific targeting of NR2B-containing NMDA receptors

Ultimately, the broad use of NMDA antagonists in the treatment of chronic pain will therefore depend on strategies that increase their therapeutic window over existing drugs. These may include drugs with different use-dependent block of the channel, or more practically, the use of low dose NMDA blockers in combination with another agent. Furthermore, drugs acting on specific receptor subtypes, such as antagonists of NR2B-containing NMDA receptors, are analgesic with a better side-effect profile than conventional NMDA antagonists (Taniguchi et al., 1997; Boyce et al., 1999). These NR2B-specific drugs, including ifenprodil, CP-101606 and Ro-256981, bind to a specific allosteric site on NR2B subunits. In addition these compounds may also interact with NR1 subunits and enhance sensitivity to protons which leads to inhibition (Chizh et al., 2001a). In the rat dorsal horn, NR2B-subtype receptors are highly concentrated in superficial layers (Nagy et al., 2004a) and thus are ideally situated to receive and modulate input from terminating nociceptive afferents. Western blot analysis of human post-mortem tissue has demonstrated expression of NR2B subunits in human spinal cord, albeit to a lesser extent than that found in the brain, similar to the expression observed in rodent tissue (Nilsson et al., 2007). Whether the NR2B subunit has a similar pattern of distribution in the human dorsal horn as in the rat remains to be seen.

1.7.2 Targeting NMDA receptor protein interactions and intracellular signalling

The NMDA receptor forms a large, multi-protein complex consisting of scaffolding proteins, enzymes and other membrane-bound receptors, thereby coupling NMDA receptor activation to various cytoplasmic processes. It is recognised that spinal nociceptive plasticity is not brought about solely by NMDA receptor activation, but rather is mediated by intracellular signalling cascades activated downstream of NMDA receptors. Such pathways are activated by the unique and large Ca^{2+} influx through NMDA receptors. Furthermore, this Ca^{2+}-dependent activation of

cytoplasmic effectors may be promoted by physical coupling of such effectors to NMDA receptors via the cytoplasmic tail of NR2 subunits (Tymianski et al., 1993). The cytoplasmic tails of NR2B subunits (residues 862-1481) comprise approximately 42% of the whole subunit size and are capable of numerous binding interactions.

Post-synaptic density protein-95 (PSD-95) is a scaffolding protein which binds to NR2 subunits of NMDA receptors via their cytoplasmic tail (Kornau et al., 1995). In addition, PSD-95 can simultaneously bind to intracellular effector proteins and thus form protein complexes with membrane-associated NMDA receptors which promote downstream NMDA-mediated signalling. For example, by binding to both nNOS and the NMDA receptor, PSD-95 can promote the direct Ca^{2+}-dependent production of NO following NMDA receptor activation (Christopherson et al., 1999; Sattler et al., 1999) and NO has long been known to mediate NMDA-dependent nociceptive hypersensitivity (Kitto et al., 1992; Sorkin, 1993). Thus PSD-95 provides an important link between NMDA receptor activation following the release of glutamate and subsequent stimulation of intracellular signalling cascades which produce spinal nociceptive plasticity, such as central sensitization, contributing to chronic pain states. Therefore, by disrupting such links, it may be possible to prevent pathological NMDA-mediated intracellular signalling, whilst maintaining normal synaptic transmission, thus achieving analgesic efficacy in chronic pain states without the side-effects commonly associated with traditional NMDA antagonists. Furthermore, disruption of coupling specifically between NR2B-subtype receptors and PSD-95 may be an even more successful analgesic strategy.

1.8 Thesis Aims

It is clear that NMDA receptors mediate spinal nociceptive plasticity which contributes to chronic pain states. Indeed NMDA receptor antagonists are efficacious against chronic pain, but their clinical utility is greatly limited by the occurrence of numerous intolerable and unavoidable side-effects. The targeting of NMDA-mediated intracellular signalling is an alternative strategy to receptor blockade and traditional NMDA receptor antagonists, and may improve on current analgesics while potentially avoiding side-effects.

The aims of this thesis are to investigate novel intracellular mechanisms of NMDA receptor-dependent spinal nociceptive plasticity and assess their therapeutic potential as analgesic targets via two main strategies of modulation:

1) Spinal delivery of Tat-NR2B9c, a mimetic peptide believed to perturb binding between PSD-95 and NR2B-containing NMDA receptors, thus disrupting downstream intracellular signalling **(Chapters 3, 4 & 5)**

2) Direct targeting of specific intracellular signalling effector proteins, to reveal potential roles in NMDA-dependent spinal nociceptive plasticity, including:

    - Atypical protein kinase C ζ (PKCζ/PKMζ), known to be important in the late phase of hippocampal LTP **(Chapter 5)**

    - Phosphatidylinositol 3-kinase (PI3K), also known to be involved in hippocampal LTP and pain pathways **(Chapter 6)**

    - Dimethylarginine dimethylaminohydrolase (DDAH), which contributes to endogenous regulation of NOS isoforms implicated in plasticity **(Chapter 7)**

I will employ various techniques such as *in vivo* electrophysiology, behavioural tests, surgery, immunohistochemistry and molecular biology to explore the potential therapeutic benefit of such strategies in pain.

2 Materials and Methods

2.1 *In vivo* Electrophysiology

2.1.1 Set-up

Electrophysiological experiments were conducted using a well-established protocol as previously described (Fig. 2.1) (Urch and Dickenson, 2003b). Anaesthesia of rats was induced using 4-5% isoflurane (66% N_2O & 33% O_2). Once areflexic, a tracheotomy was performed whereby the trachea was exposed and a polyethylene cannula was inserted and fastened with 3-0 silk thread. Isoflurane was now delivered in a closed system through this cannula for the remainder of the experiment. Rats were placed in a stereotaxic frame and secured with ear bars. Core body temperature was maintained at 37°C using a feedback heating blanket connected to a thermal rectal probe. Anaesthesia was reduced to 2.5% isoflurane and a laminectomy was performed. An incision was made into the skin along the length of the vertebra, exposing the underlying muscle. Two incisions were made either side of the vertebral column above the level of the laminectomy and a clamp inserted to stabilize the cord. Muscle and vertebrae were removed at the L1-L3 vertebral level to expose the L4-L5 segments of the spinal cord which receive afferent input from the hindpaw, creating a well for drug administration. Two further incisions were made either side of the vertebral column just below the level of the laminectomy and a clamp inserted to further stabilize and straighten the spinal cord. The dura was carefully removed using a pair of fine watchmaker forceps in order to aid drug penetration. For the remainder of the experiment anaesthesia was maintained at 1.75% isoflurane, whereby animals were deeply anaesthetized and fully areflexic. At the end of each experiment, rats were overdosed on 5% isoflurane and death was ensured by cervical dislocation of the neck.

2.1.2 Recording system

Extracellular recordings from single convergent deep dorsal horn (depth > 600 μm) wide dynamic range neurones (WDRs) were obtained using an AC recording system (NeuroLog system, Digitimer, UK; Fig. 2.2). A parylene coated tungsten electrode (125 μm diameter, 2 MΩ; A-M Systems Inc., Washington USA) was inserted into a head stage attached to a 3-axis manipulator and was manually lowered into the exposed spinal cord (L4-L5 segments). Grounding was achieved via one lead connected to the stereotaxic frame. A second lead was attached to the body of the animal and the signal from the rat was subtracted from the input from the electrode using the NeuroLog differential recording mode to reduce interference. Neuronal activity was amplified, filtered and visualized on an oscilloscope, as well as being heard via a speaker system. Individual neurones were indentified by tapping at the hindpaw receptive field (plantar surface and toes) and looking for series of action potentials of common shape and amplitude with a good signal to noise ratio, such that the isolated neurone could be easily differentiated and counted. WDR neurones respond to both innocuous and noxious stimulation in a graded manner, and can respond to various stimulus modalities including mechanical, thermal, electrical and chemical. Data was captured and analyzed by a CED 1401 interface coupled to a Pentium computer with Spike 2 software (Cambridge Electronic Design, UK; PSTH and rate functions).

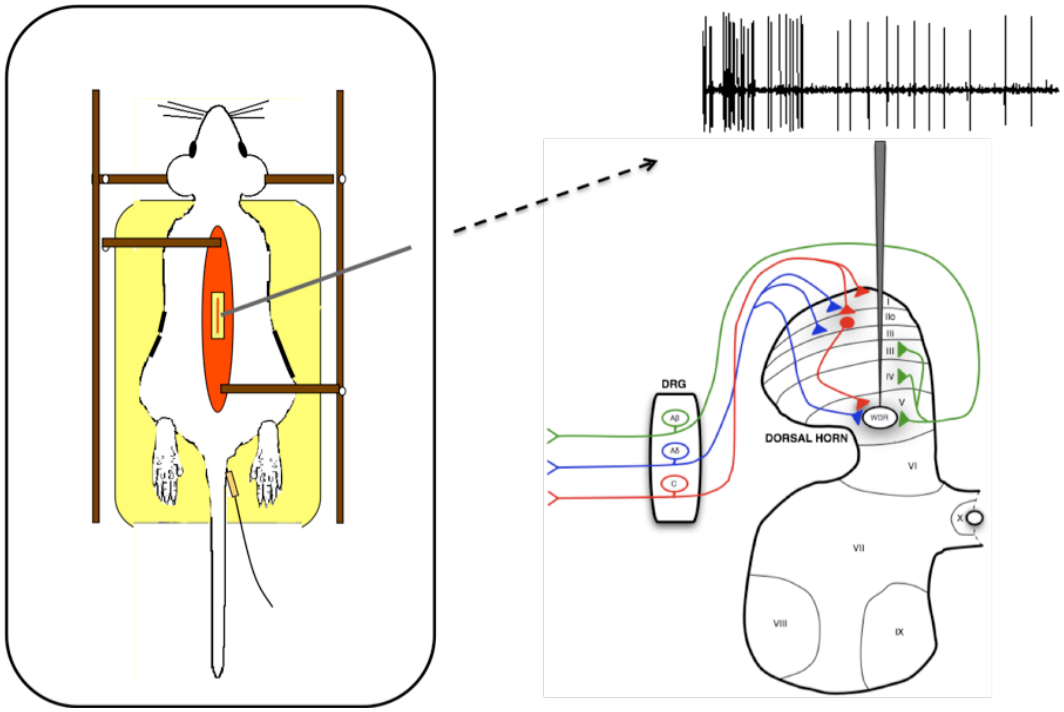


Fig. 2.1: *In vivo* electrophysiology setup. Rats are anaesthetized and fixed to a stereotaxic frame. A laminectomy is performed to expose the lumbar spinal cord and an electrode is driven into the dorsal horn. In all experiments, recordings were conducted from deep (lamina V) dorsal horn WDR neurones. Action potentials were evoked by stimulation at the hindpaw receptive field.

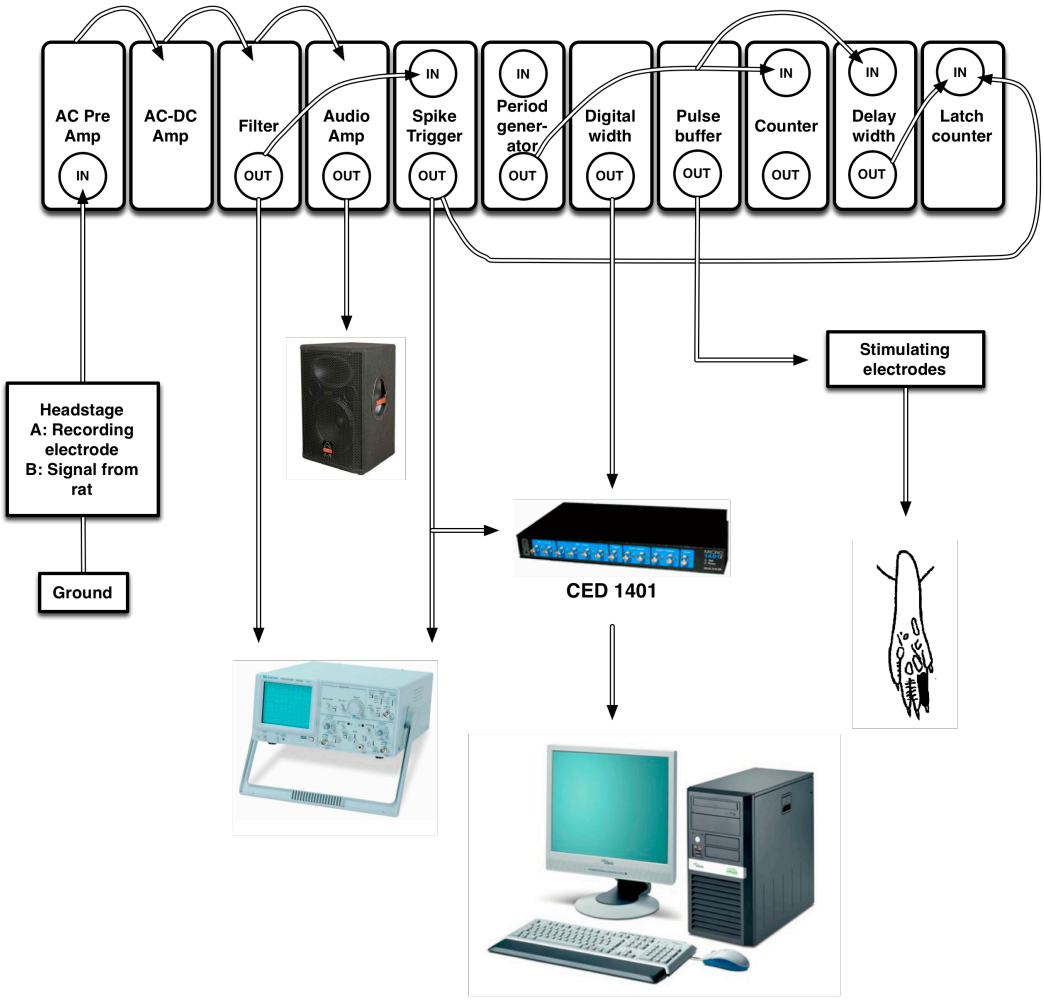


Fig. 2.2: Schematic of the NeuroLog data capture system. A recording electrode (A) is inserted into the spinal cord. The system is grounded via a lead connected to the stereotaxic frame and a second lead connected to the rat (B) allowing differential recording. The signal is then fed into the NeuroLog data capture system, differentiated as A-B, amplified, filtered and then fed to an audio speaker and oscilloscope. Action potentials (APs) above set amplitudes are discriminated and fed into the computer system via the CED 1401 interface. Electrical stimuli are administered via stimulating electrodes inserted in the receptive field. Frequency of stimulation, duration, amplitude of the current and number of pulses are set using the period generator, digital width, pulse buffer and latch counter. The number of APs evoked during a set time frame (90-800 ms) is displayed on the latch counter and the computer. Natural stimuli are also applied to the receptive field and evoked APs are displayed visually as a rate histogram and quantified per 10 s. Adapted from Urch and Dickenson (2003).

2.1.3 Electrical stimulation of hindpaw receptive field to induce wind-up

Two stimulating needles were inserted under the skin of the hindpaw through which electrical pulses could be delivered to the receptive field of the neurone being recorded. The threshold for C-fibre activation was first determined by giving single, incremental, electrical pulses until evoked action potentials in the C-fibre latency range (90-300 ms) were observed. Each test then consisted of a train of 16 transcutaneous electrical stimuli (2 ms wide pulses, 0.5 Hz) applied at three times the threshold current for C-fibres via the stimulating needles. A post-stimulus histogram (Fig. 2.3) was constructed and Aβ- (0-20 ms), Aδ- (20-90 ms) and C-fibre (90-300 ms) evoked responses were separated and quantified on a basis of latency. Responses occurring after the C-fibre latency band were taken to be the post-discharge (PD) of the cell (300-800 ms). Input (non-potentiated response) was quantified as the response after the first stimulus × 16, based on the initial response of the neurone being maintained without further enhancement during the 16 stimuli. Wind-up was calculated as: (total number of action potentials [APs] after 16 stimuli) – (number of APs after the first stimulus × 16 [i.e. input]) so that the additional responses produced by wind-up could be quantified. Stable control responses (no more than 10% variation in the C-fibre response) to electrical stimuli were established by tests at 10 min intervals prior to drug administration. Control values were obtained by averaging responses from the last three tests. Following application of the drug, tests were continued at 10 min intervals for the remainder of the experiment.

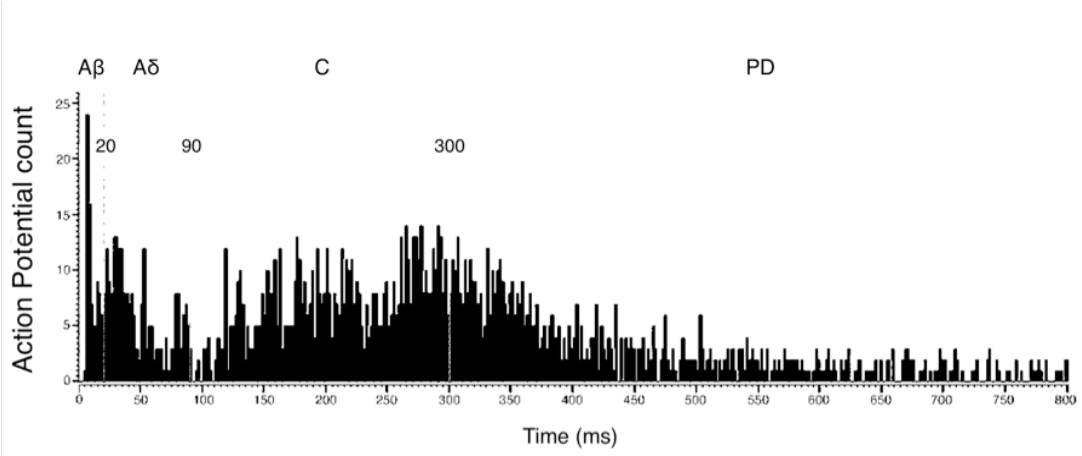


Fig. 2.3: Representative post-stimulus histogram (PSTH) of electrically evoked responses of a WDR neurone to electrical stimulation of the hindpaw. A train of 16 transcutaneous electrical stimuli (2 ms wide pulses, 0.5 Hz, 3 x C-fibre threshold) is applied to the receptive field via two stimulating needles inserted under the skin of the hindpaw. Aβ- (0-20 ms), Aδ- (20-90 ms) and C-fibre (90-300 ms) evoked responses are separated and quantified on a basis of latency. Responses occurring after the C-fibre latency band are taken to be the post-discharge (PD) of the cell (300-800 ms), an indicator of neuronal hyperexcitability. For the first few stimuli, prior to wind-up, neurones fire in the above defined latency bands. Once wind-up is induced neurones continue to fire at latencies beyond the C-fibre range as a consequence of their increased responsivity to afferent input.

2.2 Formalin Model of Central Sensitization

2.2.1 *In vivo* Electrophysiology – Formalin Test

Electrophysiological recordings of deep dorsal horn WDRs (depth > 600 μm) were carried out as described above (see section 2.1). Cells were characterized as being WDR prior to formalin administration. Transcutaneous stimulating needles were used to electrically stimulate the receptive field and induce wind-up (as 2.1.3) and responses were monitored to thermal stimulation using a constant jet of water onto the centre of the receptive field to indicate a strong C-fibre innervation of the WDR neurone, which is required for the response to subcutaneous formalin (Dickenson and Sullivan, 1987a).

Following cell characterization, rats were pre-treated with drug prior to the injection of formalin (for details of individual drug treatments refer to specific results chapter). Formalin (5%, 50 μl) was prepared from a 37% formaldehyde solution and then injected subcutaneously into the hindpaw receptive field. The firing response of the WDR neurone was recorded (Fig. 2.4) for the subsequent 70 min after formalin injection. Activity was displayed as a rate recording and quantified in 10 min time bins.

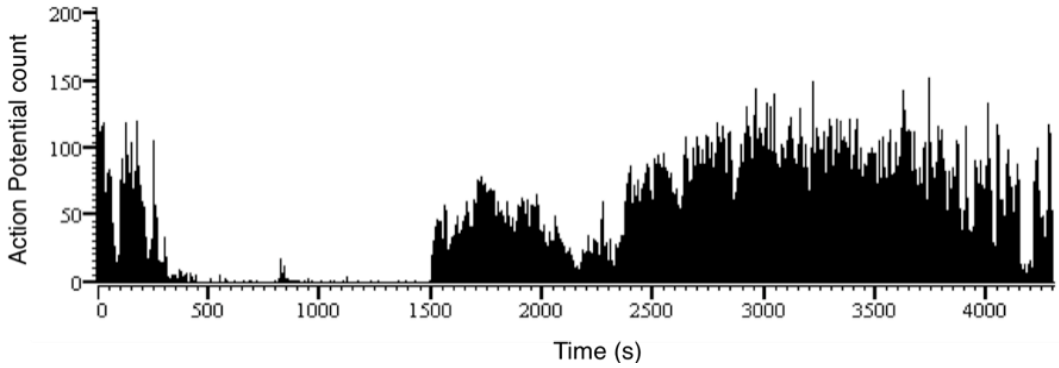


Fig. 2.4: Example of a rate recording of the characteristic biphasic response of a WDR neurone to formalin injection at the hindpaw receptive field. Recordings are conducted for 70 min following formalin injection in continuous 1 s time bins and quantified in 10 min time bins. An initial first phase response to the injection lasts for up to 10 min, followed by a quiescent interphase lasting a further 10-15 min. A prolonged second phase begins at approximately 20-25 min after formalin injection and lasts for the remainder of the recording period.

2.2.2 Behavioural Formalin Test

Rats were placed in a clear Perspex box and were allowed to acclimatize for 30 min. For drug pre-treatment, rats were lightly anaesthetized with isoflurane and then injected intrathecally (lumbar puncture method, see 2.6 below) with drug (for details of individual drug treatments refer to specific results chapter). The experimenter was blind to treatment for the whole experiment. Twenty minutes following drug administration, rats received a subcutaneous injection of 50 μl of formalin (5%) into the plantar surface of the right hindpaw. Lifting, flinching, licking and biting of the injected paw were monitored by measuring the total duration of the response in seconds during the 60 min period following formalin administration. Data was captured in 5 min time bins. At the end of the experiment rats were either culled and disposed of or sacrificed for immunohistochemistry (see 2.7 below).

2.3 Spinal Nerve Ligation (SNL) Model of Neuropathic Pain

2.3.1 Surgery

Male Sprague-Dawley rats (130-150 g, Central Biological Services, University College London, UK) underwent surgery for ligation of L5 and L6 spinal nerves, or sham operation (Kim and Chung, 1992). Under isoflurane anaesthesia (1:1 O_2:N_2O, 5% isoflurane for induction, 2% for maintenance), rats were placed in a prone position and the left paraspinal muscles were separated from the spinal processes at the L4-S2 vertebral level. Part of the L6 transverse process was carefully removed and the left L4-L6 spinal nerves were identified. The L5 and L6 spinal nerves were isolated and tightly ligated with 6-0 silk thread (i.e. thread was looped around the nerve and pulled as tightly as possible), distal to the dorsal root ganglion (DRG) and proximal to the formation of the sciatic nerve (Fig. 2.5). The procedure for sham operation was identical, except for the ligation of spinal nerves which was omitted. Animals were allowed to recover in a warm chamber and were checked for signs of motor impairment, such as dragging or non-weight bearing of the affected hindpaw.

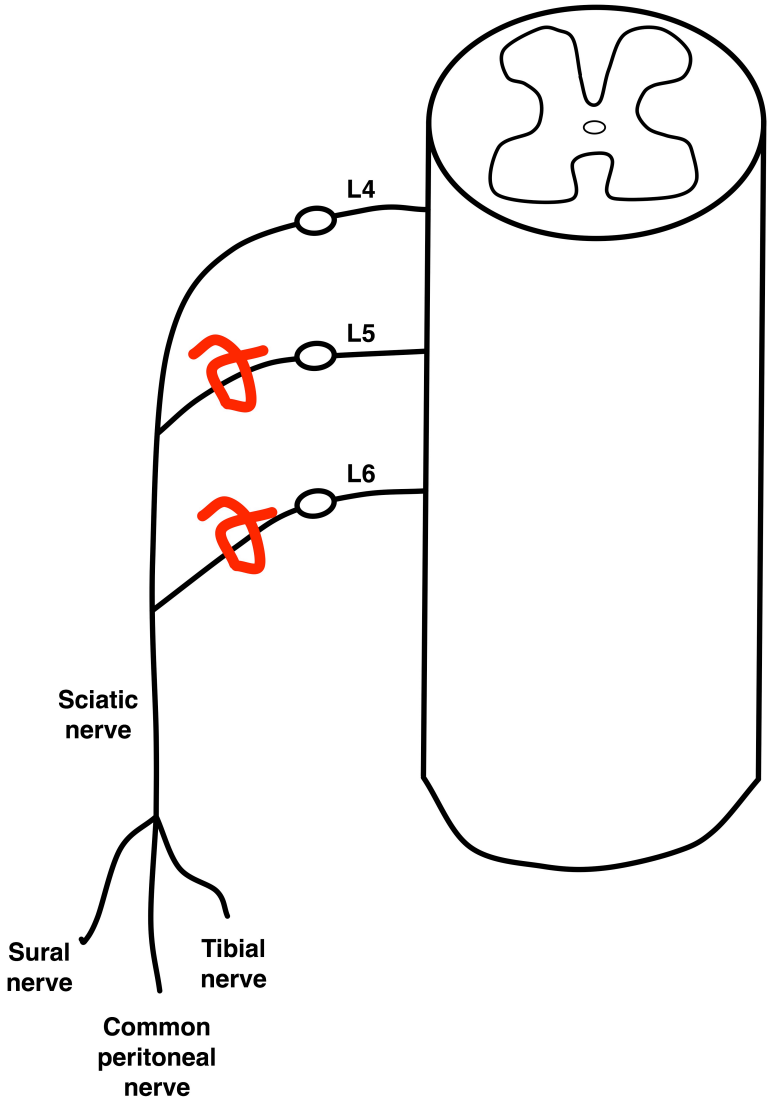


Fig. 2.5: Spinal nerve ligation (SNL) surgery. The L5 and L6 spinal nerves are exposed and tightly ligated with 6-0 silk thread (red). Ligation is made distal to the DRG and proximal to formation of the sciatic nerve.

2.3.2 Behavioural confirmation of mechanical and cold hypersensitivity prior to electrophysiology

Behavioural testing of SNL and sham-operated rats was carried out for up to 14 days post-operatively to assess sensitivity to mechanical and cooling stimuli. Initially rats were allowed to acclimatize to the behavioural testing room for 15 min in their own cages. Rats were then placed in transparent Perspex cubicles with a wire mesh floor, and were allowed to acclimatize to the testing apparatus for a further 15 min. Once rats had settled on all four paws and were fully awake, testing began.

2.3.2.1 Paw Withdrawal Frequency

Mechanical sensitivity of the ipsilateral and contralateral hindpaws in both SNL and sham animals was assessed by recording the frequency of foot withdrawal to normally innocuous mechanical punctuate stimuli. Calibrated von Frey filaments (Touch-Test^{TM}, North Coast Medical Inc., San Jose, USA) of 1, 6 and 8 g (giving bending forces of 9.8, 58.9 and 78.5 mN respectively) were applied 10 times for 2-3 seconds per stimulus to the plantar surface of each hindpaw from below (Fig. 2.6a). Filaments were applied in ascending order to the centre of the paw, to each toe and to the footpads (Fig. 2.6c) and a minimum of 5 min were allowed between application of each von Frey hair to the same paw. A positive response was withdrawal as well as licking and/or biting of the stimulated hindpaw, indicative of nocifensive behaviour. The responses of both the ipsilateral and contralateral hindpaws were scored as a withdrawal frequency (out of 10) and from these results a difference score was calculated for each von Frey hair per animal. The difference score is calculated as:

[the number of paw withdrawals of the ipsilateral paw –
the number of paw withdrawals of the contralateral paw].

Thus, a positive difference score would indicate hypersensitivity of the ipsilateral hindpaw.

2.3.2.2 Acetone Drop Test

Sensitivity to a cooling stimulus was measured by the application of a single drop of acetone, 5 times, to the plantar surface of the ipsilateral and contralateral hindpaws. Acetone was delivered from below through a 10 cm piece of tubing connected to a 0.5 ml syringe (Fig. 2.6b). A positive response was the same as with the application of von Frey hairs except that a short delay would often be noted between application of the acetone drop and foot withdrawal. This is due to the fact that the cooling stimulus is brought about by evaporation of the acetone drop. A difference score (as above) was also calculated for the acetone drop test for each animal. The acetone drop test was performed before, between and after application of von Frey hairs, with a minimum of 5 min between each application to the same paw.

The testing sequence for each session was as follows:

1. Acetone drop test 1, 1 application per hindpaw
2. von Frey hair 1 g, 10 applications per hindpaw
3. Acetone drop test 2, 1 application per hindpaw
4. von Frey hair 6 g, 10 applications per hindpaw
5. Acetone drop test 3, 1 application per hindpaw
6. von Frey hair 8 g, 10 applications per hindpaw
7. Acetone drop test 4, 1 application per hindpaw
8. Acetone drop test 5. 1 application per hindpaw

Rats were monitored for normal grooming behaviour and weight gain throughout the post-operative period and were subsequently used for *in vivo* electrophysiology on days 15-18 after surgery.

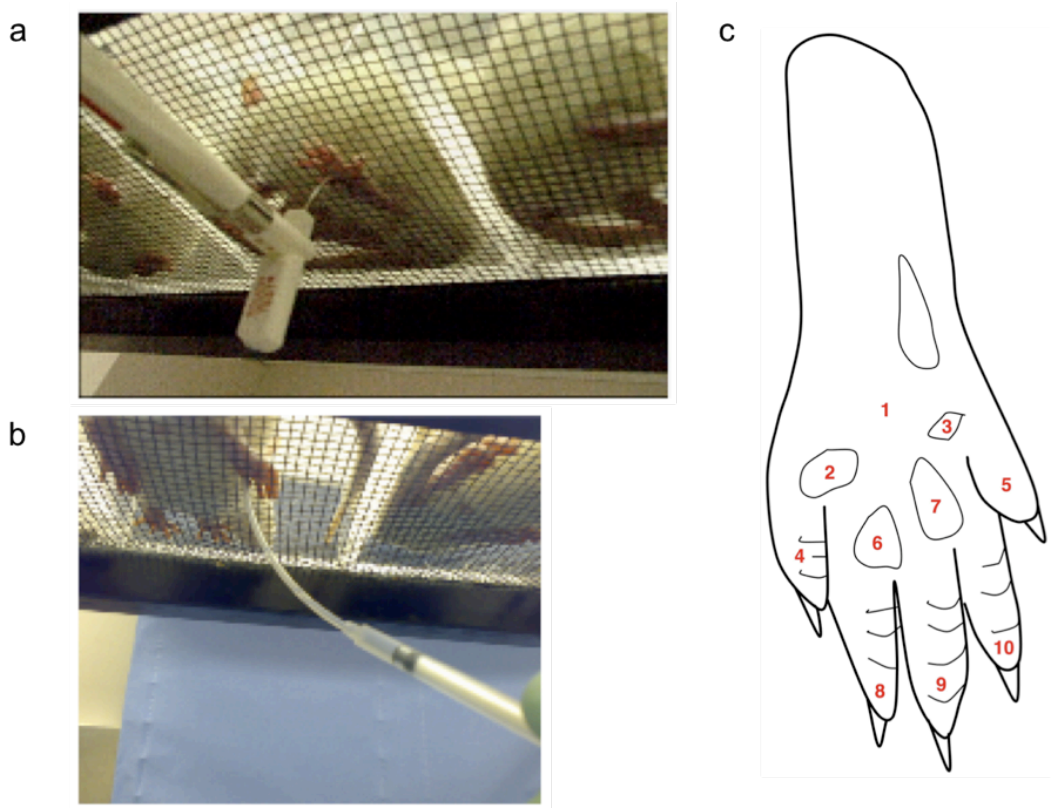


Fig. 2.6: Behavioural testing for neuropathic pain-related behaviour following SNL surgery. **(a,b)** Animals are placed in clear Perspex boxes with wire mesh floors to allow for stimulation of hindpaws from below. Mechanical hypersensitivity is detected using **(a)** calibrated von Frey hairs and cold hypersensitivity is assessed by application of **(b)** acetone delivered to the hindpaw using a syringe. **(c)** Von Frey hairs are applied 10 times to the hindpaw at various positions, including the centre of the paw, footpads and toes.

2.3.3 *In vivo* Electrophysiology – Spinal Nerve Ligation Model

Electrophysiological recordings of WDR neurones (as section 2.1) were conducted in the left side of the dorsal horn, ipsilateral to the site of nerve injury, in both SNL and sham-operated animals on post-operative days 15-18. Rats weighed between 250 and 300 g at this point. Responses to both electrical and natural stimulation of the hindpaw were assessed in separate experiments.

A train of 16 transcutaneous electrical stimuli (2 ms wide pulses, 0.5 Hz) was applied at three times the threshold current for C-fibres via two stimulating needles inserted under the skin of the hindpaw. A post stimulus histogram was constructed and Aβ- (0-20 ms), Aδ- (20-90 ms) and C-fibre (90-350 ms) evoked responses were separated and quantified on a basis of latency. (The latency band for C-fibres was increased up to 350 ms to allow for the larger size of the animals.) Responses occurring after the C-fibre latency band were taken to be the post-discharge (PD) of the cell (350-800 ms). Input and wind-up were calculated as before (see 2.1.3).

In a separate set of SNL and sham-operated animals, neuronal responses to natural stimuli were assessed. The hindpaw peripheral receptive field was stimulated using natural stimuli which extended from the non-noxious to noxious range. Mechanical stimulation was achieved by application of von Frey filaments 1, 8, 15 and 26 g (9.8, 78.5, 147.1 and 255 mN respectively). Filaments 1 and 8 g are considered innocuous while filaments 15 and 26 g are noxious. Each hair was applied in ascending order for 10 s. Next the receptive field was stimulated thermally with the following temperatures: 35, 40, 45 & 48°C. Temperatures including 45°C and above are considered noxious. Heat was applied with a constant water jet onto the centre of the receptive field, again in ascending order for a period of 10 s per stimulus. WDR neurones respond to the whole range of natural stimuli in a graded fashion, from innocuous through to noxious (Fig. 2.7). Data was captured and analyzed by a CED 1401 interface coupled to a Pentium computer with Spike 2 software (Cambridge Electronic Design, UK; PSTH and rate functions).

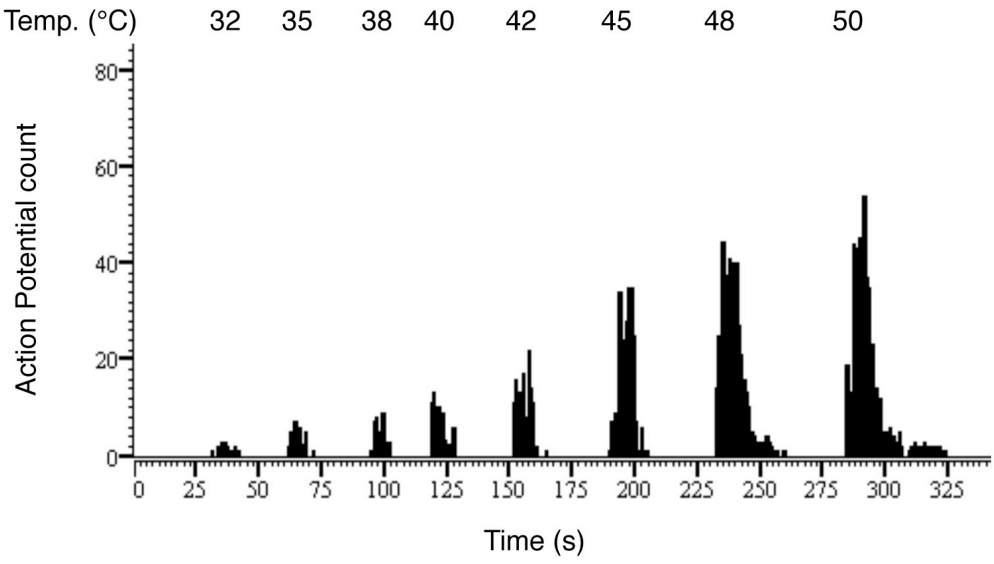


Fig. 2.7: Example of a rate histogram displaying action potentials evoked from a WDR neurone by thermal stimulation of the hindpaw receptive field. Increasing temperatures are applied to the receptive field using a jet of water from a syringe for 10 s. WDR neurones respond to natural stimuli in a graded fashion from the non-noxious to noxious range. At noxious temperatures, WDR neurones produce a prolonged response beyond the duration of the stimulus (post-discharge). Only the first 10 s of each response is quantified and recorded for data analysis.

2.3.4 Behavioural assessment of mechanical and cold hypersensitivity to observe effects of pharmacological interventions

Four days prior to SNL surgery, paw withdrawal thresholds (PWT) and cold sensitivity were assessed for both hindpaws of rats. Following SNL surgery, testing was repeated up to day 14/16. Drug effects were assessed on days 14/16.

2.3.4.1 Paw Withdrawal Threshold (PWT)

As an alternative to measuring paw withdrawal frequencies to selected von Frey filaments, PWTs were measured to assess mechanical sensitivity. Von Frey filaments were applied 10 times to both ipsilateral and contralateral hindpaws (as Fig. 2.6c) in ascending order. The PWT was determined by recording the first von Frey filament to produce 5 positive responses out of 10. A cut-off was set at 15 g (147.1 mN) to avoid tissue damage.

2.3.4.2 Acetone Drop Test

Cold hypersensitivity was assessed by the acetone drop test (as 2.3.2.2) and each rat was given a score out of 5 for the number of positive responses for each hindpaw.

Four rats were assessed during each testing session as follows:

1. Acetone drop test 1, 1 application per hindpaw
2. PWT determined in each hindpaw of rat 1
3. Acetone drop test 2, 1 application per hindpaw
4. PWT determined in each hindpaw of rat 2
5. Acetone drop test 3, 1 application per hindpaw
6. PWT determined in each hindpaw of rat 3
7. Acetone drop test 4, 1 application per hindpaw
8. PWT determined in each hindpaw of rat 4
9. Acetone drop test 5, 1 application per hindpaw

2.4 Assessment of locomotor function – Rotarod

Locomotor function and motor effects of drugs were assessed using an accelerating rotarod device (Fig. 2.8; Rotarod 7750, Ugo Basile, Italy). The apparatus was set to accelerate from 0 to 20 revolutions per minute (rpm) over 60 s and the latency to fall was monitored with a maximum cut-off of 90 s. Rats underwent a training period on days -3 and -1 prior to testing of drug effects in order to ensure that they could remain on the rotarod for a minimum of 60 s.

Fig. 2.8: An accelerating rotarod device to assess locomotor performance. Rats are positioned on the rotarod facing away from the experimenter (i.e. towards the wall). The time spent on the device until the animal falls is recorded.

2.5 Drug administration – *In vivo* Electrophysiology

In all *in vivo* electrophysiological experiments, drugs were applied directly to the surface of the spinal cord of rats, after removal of any residual cerebrospinal fluid (CSF), in a volume of 50 μl, using a Hamilton syringe.

2.6 Intrathecal drug administration for behaviour – Lumbar Puncture Method

For all behavioural pharmacological studies drugs were delivered by intrathecal injections performed by direct lumbar puncture. Briefly, rats were lightly anaesthetized with isoflurane. Their backs were shaved and the skin was cleansed with gauze soaked in an anti-microbial solution. A 25-gauge needle connected to a syringe was then inserted into the subarachnoidal space between the spinal processes of the L5 and L6 lumbar vertebrae. Drug was injected, eliciting a tail-flick. The syringe was held in position for a few seconds after the injection. Drugs were blinded prior to use in all behavioural experiments in the following way. Drugs were made up in identical unlabelled eppendorf tubes in equal volumes. The tubes were then passed on to a colleague who would label the tubes A or B and note down on a separate piece of paper the identity of each drug. This information was retained until the end of the experiment for unblinding.

2.7 Immunohistochemistry

2.7.1 Perfusion

Animals were terminally anaesthetized using pentobarbitone and perfused transcardially with 100 ml saline followed by 400 ml of 4% paraformaldehyde (PFA) with 15% of a saturated solution of picric acid. After perfusion, the lumbar spinal cord (levels L4-L6, identified by tracing of dorsal roots) was excised, post-fixed for 4 hrs in the perfusion fixative and then cryoprotected in 20% sucrose in 0.1 M phosphate buffer overnight at 4°C.

2.7.2 Processing of tissue and staining

Fixed spinal cord samples were embedded in optimal cutting temperature compound (OCT) and frozen using liquid nitrogen. Transverse spinal cord sections (30 μm thickness) were cut using a cryostat and every section was collected and placed in phosphate buffered saline (PBS) solution for free-floating immunohistochemistry. Alternatively, sections were thaw-mounted onto glass slides.

Sections were washed with PBS and then blocked with normal serum (10%) for 30 min. Next sections were incubated with primary antibody made up in PBS+Triton X-100+Azide (PBS-T+Az; see Table 2.1 below and individual results chapters for details of specific primary antibodies). After several washes with PBS, sections were then incubated at room temperature with secondary antibody (in PBS-T+Az). After further washing in PBS, sections were mounted onto slides (if free-floating) and cover-slipped with Vectashield mounting medium (Vector Laboratories, CA, USA). Slides were visualized under a Zeiss Axioplan 2 Microscope (Zeiss, Hertfordshire, UK).

2.8 Western Immunoblotting

2.8.1 Dissection and tissue homogenization

Animals were anaesthetized with urethane and then sacrificed by decapitation with a guillotine. Fresh tissue (spinal cord, DRG or hippocampus) was dissected and placed into 1.5 ml eppendorf tubes and snap frozen in liquid nitrogen. Tissue was subsequently homogenized in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS + 1% DOC [Deoxycholic acid] + Complete protease inhibitor cocktail) using a glass homogenizer. Homogenates were transferred to fresh 1.5 ml eppendorf tubes and incubated on ice for 2 hrs (hippocampus) or at 37°C for 30 min (spinal cord and DRG), and were then centrifuged at 14,000 rpm for 10 min at 4°C. Supernatant containing tissue lysate was collected and an equivalent volume of RIPA buffer was added to bring the final concentration of DOC to 0.5%. The pellet containing insoluble debris was discarded. Spinal, DRG and hippocampal whole cell lysates were next titrated to determine their protein concentration using a BCA (Bicinchoninic acid) Protein Assay kit (Pierce, UK).

2.8.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Lamaelli loading buffer was added to protein lysates and samples were incubated at 70°C for 30 min in order to denature proteins and break disulphide bonds and protein-protein interactions. Samples were then loaded onto 8% gels and separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Gels were initially run at 100 V for 30 min and then at 120 V for a further 60 min. Prior to transfer, gels were equilibrated in transfer buffer for 30 min at 4°C. Proteins were then transferred to either polyvinylidene difluoride (PVDF, pre-soaked in methanol) or nitrocellulose membranes at 100 V for 60 min and subsequently blocked in 5% milk in TBS-T (Tris-buffered saline: Tris 20 mM, pH 7.5, NaCl 500 mM + 0.1% Tween-20) at room temperature for 1 hr with gentle shaking.

2.8.3 Detection and revelation

Next, membranes were incubated overnight at 4°C with primary antibody (see Table 2.2 below and individual results chapters for details of specific primary antibodies), diluted in 5% milk in TBS-T. Membranes were then washed six times (5 min each) at room temperature with TBS-T. Horseradish peroxidase (HRP)-linked secondary antibody was then put on for 1 hr at room temperature, and finally membranes were placed in enhanced chemilluminescence (ECL)-plus™ reagent, for 5 min, for detection of proteins by autoradiography (Fig. 2.9a). Alternatively, following incubation with primary antibody and subsequent washing with TBS-T, membranes were incubated with IRDye-linked secondary antibody for 1 hour at room temperature for detection of fluorescence (Fig. 2.9b) by use of the Odyssey Detection System (Licor, UK). See individual results chapters for details of primary and secondary antibodies used.

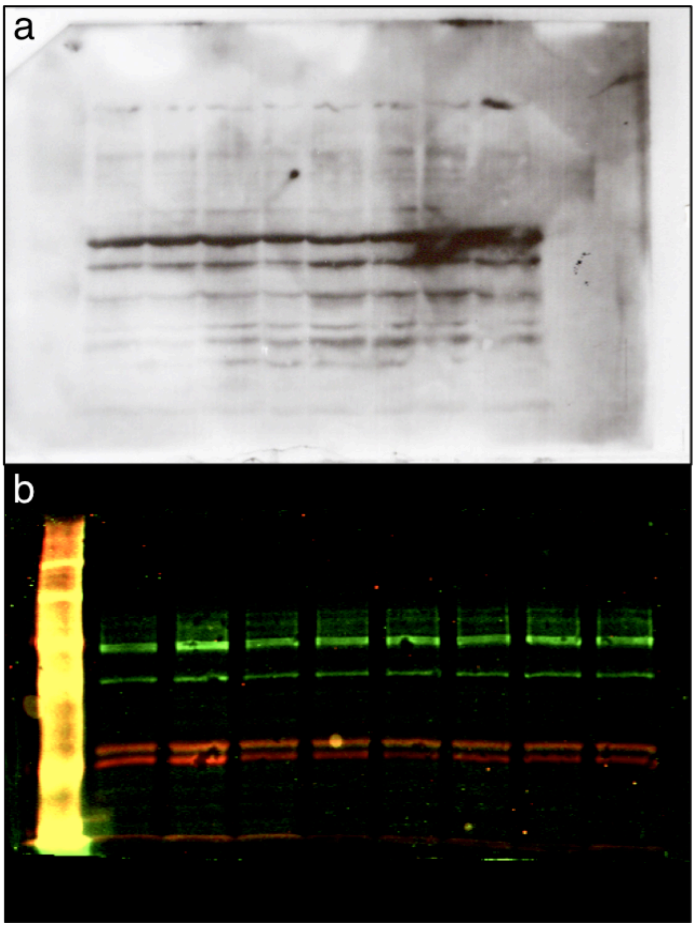


Fig. 2.9: Example western immunoblots obtained using (a) autoradiography or (b) direct fluorescence. **(a)** Following incubation with HRP-linked secondary antibodies, membranes are exposed to ECL-plus™ reagent and then placed with photographic film for detection of bands by autoradiography. **(b)** Alternatively, membranes are incubated with IRDye-linked secondary antibodies for direct detection of fluorescence using the Odyssey detection system (Licor, UK). In both cases, quantification of bands is achieved by densitometric analysis.

2.9 Immunoprecipitation Protocol

2.9.1 Washing/preparation of Dynabeads® Protein G

Magnetic Dynabeads® Protein G (Invitrogen Ltd, UK) were used for immunoprecipitation. Beads (25 µl per sample) were transferred to a 1.5 ml eppendorf tube which was then placed on a magnet (Fig. 2.10a) for 1 min and the supernatant was removed. The tube was removed from the magnet and the beads were resuspended in 100 µl of washing buffer (Citrate-phosphate buffer, pH 5.0). The tube was again placed on the magnet for 1 min and the supernatant was removed. This washing procedure was repeated once more.

2.9.2 Antibody coupling to Dynabeads® Protein G

Antibody (5 µg) against the target antigen was diluted in washing buffer to a final volume of 50 µl. Dynabeads were removed from the magnet and were resuspended using the diluted antibody. The dynabead/antibody mix was incubated at room temperature for 40 min with gentle shaking to achieve coupling of the antibody to beads (Fig. 2.10b). After 40 min, the tube was placed on the magnet for 2 min and the supernatant was removed. The beads were washed three times in washing buffer using the magnet. The final wash was made with citrate-phosphate buffer + 0.1% Tween-20 to prevent aggregation of beads prior to downstream immunoprecipitation.

2.9.3 Target antigen capture

The dynabeads/antibody complex was resuspended in sample containing 500 µg of spinal or hippocampal lysate and the mix was incubated overnight at 4°C with gentle rotation (Fig. 2.10b). Following incubation, beads were washed three times in 200 µl of PBS + 0.1% Tween-20. The beads were resuspended in 100 µl of PBS and

transferred to a clean tube prior to elution (to avoid coelution of non-specific proteins which can bind to the tube wall).

2.9.4 Denaturing elution of target protein

The tube was placed on the magnet for 1 min and the supernatant removed. The dynabeads/antibody/antigen complex was then resuspended in 40 μl of Lamaelli loading buffer and incubated at 70°C for 30 min (Fig. 2.10b). The tube was then replaced on the magnet to separate the dynabeads from the antibody-antigen complex and samples were loaded directly onto 8% gels for SDS-PAGE (as 2.8.2). An input lane was always loaded containing total lysate (no immunoprecipitation) as a positive control.

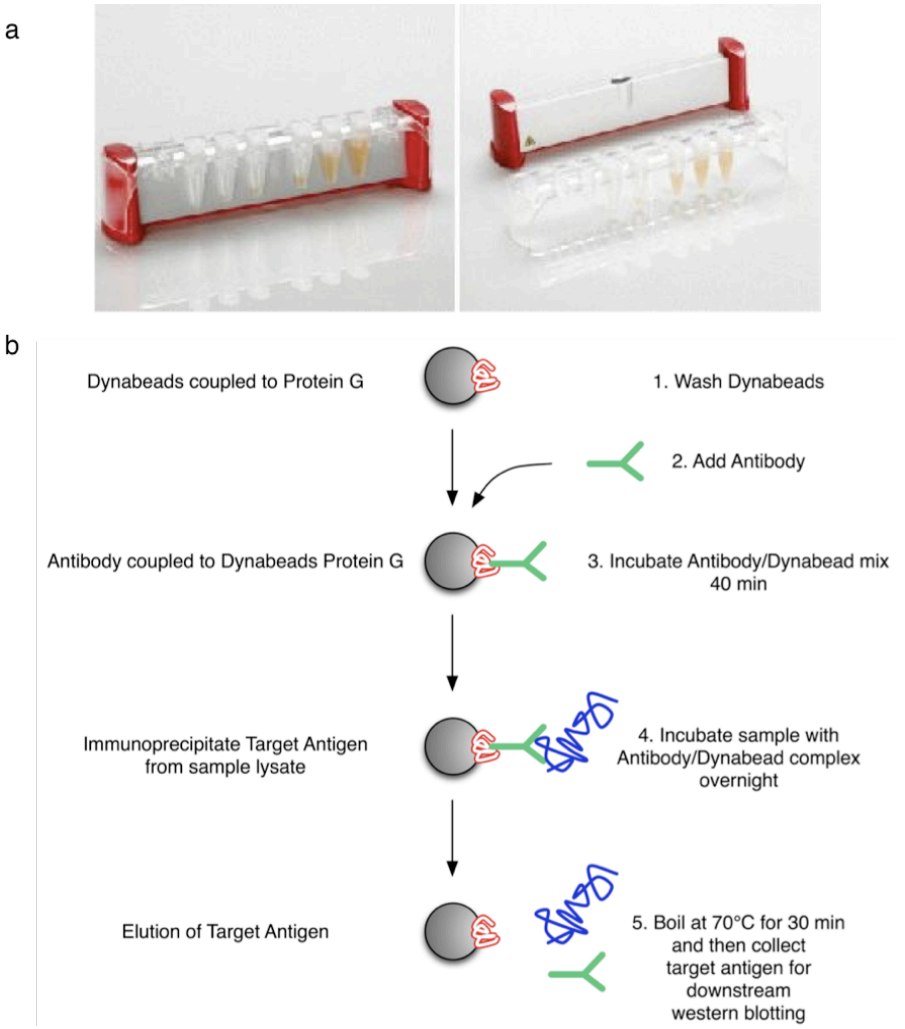


Fig. 2.10: Immunoprecipitation protocol using magnetic Dynabeads® Protein G. **(a)** A magnet is used to capture dynabeads in eppendorf tubes for easy removal of fluid, thus removing the need for centrifugation. **(b)** Dynabeads are washed in washing buffer. Antibody against the target antigen is then incubated with the beads for coupling to dynabeads protein G. The antibody/dynabead complex is then incubated with sample lysate for immunoprecipitation. Target antigen is then eluted and collected for downstream western immunoblotting.

Antibody	Host	Application	Concentration	Cat. Code	Company	Secondary AB	Company	Results Chapter
Anti-phospho-PKCζ Thr410	Rabbit	IHC	1:100	sc-101778	Santa Cruz	Goat anti-Rb IgG-conjugated Alexa Fluor 488	Molecular Probes	5
Anti-c-Fos	Rabbit	IHC	1:2500	AB5	Oncogene Science	Goat anti-Rb IgG-conjugated Alexa Fluor 488	Molecular Probes	5
Anti-phospho Akt1 Ser473	Rabbit	IHC	1:300	unknown	New England Biolabs	Goat anti-Rb IgG-conjugated Alexa Fluor 488	Molecular Probes	6
Anti-phospho ERK	Rabbit	IHC	1:300	unknown	New England Biolabs	Goat anti-Rb IgG-conjugated Alexa Fluor 488	Molecular Probes	6
Anti-phospho CaMKII Thr286	Rabbit	IHC	1:300	ab5683	Abcam	Goat anti-Rb IgG-conjugated Alexa Fluor 488	Molecular Probes	6

Table 2.1: List of antibodies used in immunohistochemistry (IHC) experiments

Antibody	Host	Application	Concentration	Cat. Code	Company	Secondary AB	Company	Results Chapter
Anti-NR2B	Rabbit	IP	μg/100 μg lysat	06-600	Upstate	N/A	N/A	3, 5
Anti-NR2B	Mouse	WB	1:500	75-101	Neuromab	Goat anti-Ms IgG IRDye 680CW	Licor	3, 5
Anti-phospho-NR2B Tyr1472	Rabbit	WB	1:1000	ab5403	Chemicon	Donkey anti-Rb IgG HRP-linked	Amersham	6
Anti-phospho-NR2A Tyr1387	Rabbit	WB	1:500	ab16647	Abcam	Donkey anti-Rb IgG HRP-linked	Amersham	6
Anti-PSD-95	Rabbit	WB	1:1000	ab18258	Abcam	Donkey anti-Rb IgG IRDye 800CW	Licor	3, 5
Anti-nNOS	Rabbit	WB	1:3000	07-571	Millipore	Donkey anti-Rb IgG IRDye 800CW	Licor	3, 5
Anti-PKCζ	Rabbit	WB	1:500	ab59364	Abcam	Donkey anti-Rb IgG IRDye 800CW	Licor	5
Anti-CREB	Rabbit	WB	1:500	ab5803	Abcam	Donkey anti-Rb IgG IRDye 800CW	Licor	5
Anti-P2X3	Rabbit	WB	1:500	ab10269	Abcam	Donkey anti-Rb IgG IRDye 800CW	Licor	5
Anti-GluR1	Rabbit	WB	1:1000	ab1504	Chemicon	Donkey anti-Rb IgG HRP-linked	Amersham	6
Anti-DDAH-1	Goat	WB	1:500	ab2231	Abcam	Donkey anti-Gt IgG IRDye 680	Licor	7
Anti-DDAH-2	Goat	WB	1:500	ab1383	Abcam	Donkey anti-Gt IgG IRDye 680	Licor	7
Anti-neuronal β-III Tubulin	Rabbit	WB	1:3000	ab18207	Abcam	Donkey anti-Rb IgG IRDye 800CW	Licor	3, 7

Table 2.2: List of antibodies used in immunoprecipitation (IP) and western blotting (WB) experiments

2.10 Statistical Analyses

Details of statistical tests used can be found in individual results chapters. All analyses were conducted using GraphPad Prism v.4 software (GraphPad Software Inc., San Diego, CA, USA).

3 Interactions between spinal NR2B-containing NMDA receptors and PSD-95 contribute to spinal nociceptive plasticity

3.1 Introduction

3.1.1 Expression of NR2B-containing NMDA receptors in the dorsal horn

The NR2B subunit of the NMDA receptor is ideally located to play a role in modulation of pain messages from the periphery to the spinal cord, since in the dorsal horn, it is highly concentrated at synapses in superficial laminae where most nociceptive afferents terminate. Studies by Yung and also Boyce and colleagues demonstrated that protein expression of NR2B subunits is highly restricted to the superficial dorsal horn, although some expression is also observed deeper and in the ventral horn also. Initially, the superficial expression was thought to be pre-synaptic in primary afferent terminals (Yung, 1998; Boyce et al., 1999). Accordingly, NR2B immunoreactivity has been shown to co-localize with that of IB4 and CGRP, respective markers of non-peptidergic and peptidergic C-fibers, in DRG neurones, as well as with neurofilament 200 (NF200), a marker of A-fibres (Ma and Hargreaves, 2000; Marvizon et al., 2002). Nagy and colleagues later provided definitive evidence for the presence of NR2B subunits in intrinsic spinal dorsal horn neurones using an antigen-unmasking technique coupled with immunohistochemistry. Fixed spinal sections were briefly treated with pepsin which is believed to digest proteins and thus increase access for antibodies to antigens. The improved immunostaining of NR2B protein showed a punctate expression throughout the dorsal horn with a few weakly stained puncta in the ventral horn. The expression of NR2B subunits was mostly concentrated in the superficial (laminae I-II) dorsal horn (Nagy et al., 2004a). In contrast, NR2A expression was found throughout the spinal cord but was greatest in laminae III-IV of the dorsal horn. Therefore, targeting NR2B subunits, for example through the use of non-competitive NR2B subtype-specific antagonists such as ifenprodil, CP-101606 and Ro-256981, may selectively target nociceptive transmission without interfering with normal sensory function and may have a better side-effect profile compared to non-selective drugs such as ketamine or MK-801.

Both the pre-synaptic and post-synaptic distribution of NR2B subunits in the dorsal horn may contribute to the analgesic efficacy of such drugs.

3.1.2 Involvement of NR2B-containing NMDA receptors in spinal wind-up and inflammatory pain

Very few studies have used electrophysiological techniques to study the role of NR2B-subtype receptors at the spinal level. The limited data has, however, demonstrated that NR2B-subtype receptors contribute to NMDA-dependent wind-up of spinal sensory neurones. Both Boyce and colleagues and also Chizh and colleagues recorded from single motor units as a surrogate measure of dorsal horn neuronal activity, albeit in rabbits and rats respectively. Wind-up in this model was attenuated by systemic administration of NR2B-subtype antagonists. However, whereas Boyce and colleagues observed inhibitions of wind-up in spinalized rats, in which descending controls had been eliminated, thus suggesting a spinal site of action for NR2B-subtype antagonists (Boyce et al., 1999), Chizh and colleagues only saw inhibition of wind-up in sham-spinalized rats, indicating a supraspinal site of action (Chizh et al., 2001b). Therefore, although it was clear that NR2B-subtype receptors were involved in spinal wind-up, a question had arisen regarding the precise location of these receptors. Later, Kovacs and colleagues addressed this question by recording directly from spinal dorsal horn neurones in spinalized rats and found that NR2B-subtype antagonists robustly inhibited spinal wind-up (Kovacs et al., 2004). Minimal inhibitory effects were seen on afferent evoked activity, indicating a selective role for NR2B-subtype receptors in spinal hyperexcitability. More recently, intrathecal administration of the NR2B-antagonist Ro-256981 was shown to reduce C-fibre evoked responses of dorsal horn neurones (Qu et al., 2009). Therefore, it is highly likely that NR2B-containing NMDA receptors in the spinal dorsal horn do in fact contribute directly to nociceptive hyperexcitability of dorsal horn neurones.

In addition to spinal wind-up, plasticity within the dorsal horn can be investigated using the formalin model of inflammatory pain (Dubuisson and Dennis, 1977). Peripheral intraplantar injection of formalin results in central sensitization of dorsal horn sensory neurones and is dependent on activity of spinal NMDA receptors (Haley

et al., 1990; Chaplan et al., 1997). Awake animals injected with formalin respond with pain-related behaviours which characteristically show a biphasic time course, and this biphasic response is replicated at the single neuronal level in electrophysiological recordings. The initial phase of activity is caused by the injection of formalin, which is a highly noxious substance. Recent evidence indicates that formalin activates TRPA1 channels on nociceptive C-fibres (McNamara et al., 2007). This resultant prolonged primary afferent drive leads to central sensitization of dorsal horn neurones and this central component underlies the second phase of the response to formalin. Malmberg and colleagues investigated the specific role of NR2B-subtype receptors in formalin-induced pain. Conantokin peptides derived from cone snail venom, which are selective inhibitors of NR2B-subtype receptors, potently inhibited second phase behaviours induced by formalin at doses which did not affect the first phase response or alter motor performance (Malmberg et al., 2003). Similarly, genetic knockdown of NR2B protein in the dorsal horn with siRNA resulted in the selective reduction of the second phase of the formalin response (Tan et al., 2005). Together, these studies suggest a lack of involvement of spinal NR2B-subtype receptors in acute nociception. Instead, spinal NR2B-subtype receptors contribute to the activity-dependent plasticity of dorsal horn neurones which may underlie chronic pain states.

Involvement of NR2B-containing NMDA receptors has also been demonstrated in more chronic models of inflammatory pain. Taniguchi and colleagues first tested the efficacy of CP-101606 on pain-related behaviours following peripheral carrageenan injection. Systemic administration of CP-101606 was found to inhibit mechanical hypersensitivity whilst having no effect on motor performance in the rotarod test (Taniguchi et al., 1997). Subsequent studies replicated this effect of CP-101606 in the carrageenan model and also showed efficacy of systemic Ro-256981, with both compounds showing no alteration of the sensitivity of the non-inflamed paw or of motor performance (Boyce et al., 1999; Chizh et al., 2001). In an alternative model of chronic inflammatory pain, induced by peripheral injection of complete Freund's adjuvant (CFA), NR2B-selective conantokin peptide inhibitors reversed both thermal and mechanical hypersensitivity when administered intrathecally (Malmberg et al., 2003). Interestingly, no reduction was seen of CFA-induced paw oedema, suggesting that the inhibitory effect of NR2B-subtype blockers was mediated by a

selective spinal action with little peripheral contribution. In addition, CFA has been shown to cause tyrosine phosphorylation of NR2B, but not NR2A, subunits in the spinal cord, further implicating NR2B-subtype receptors in chronic inflammatory pain (Guo et al., 2002; Guo et al., 2004).

3.1.3 Structure of Post-synaptic density protein-95 (PSD-95)

PSD-95, also known as SAP-90, is a 95 KDa protein and a member of the membrane-associated guanylate kinase (MAGUK) protein family found within the post-synaptic density and able to bind and cluster NMDA receptors at synapses (Tao and Raja, 2004). These proteins are able to bind various intracellular signalling proteins and therefore associate them with NMDA receptors in the membrane, thus orchestrating downstream NMDA-mediated signalling pathways. PSD-95 contains three postsynaptic density 95-discs large-zonula occludens-1 (PDZ) domains, a Src homology 3 (SH3) domain and a guanylate kinase (GUK) domain by which it can attach to different proteins with the correct binding motif in their structure (Fig. 3.1). The first and second PDZ domains of PSD-95 are able to bind NR2 subunits of the NMDA receptor, which contain a PDZ-binding motif (TSXV) at the C-terminus (Kornau et al., 1995). In addition, nNOS also contains a PDZ domain which is able to bind the second PDZ domain of PSD-95, thus forming a heterodimeric PDZ-PDZ interaction (Brenman et al., 1996).

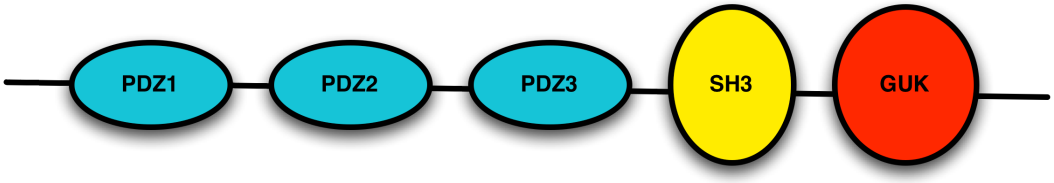


Fig. 3.1: Structure of Post-synaptic density protein-95. PSD-95 is a 95 kDa protein and includes various domains which mediate protein-protein interactions, including three postsynaptic density 95-discs large-zonula occludens-1 (PDZ) domains, a Src homology 3 (SH3) domain and a guanylate kinase (GUK) domain.

3.1.4 Expression of PSD-95 in the dorsal horn of the spinal cord

Immunohistochemical studies have demonstrated that PSD-95 is located in regions of the spinal dorsal horn which are important for nociception. Tao and colleagues first showed that expression of PSD-95 is densest in the superficial dorsal horn, restricted to laminae I and IIo (Tao et al., 2000), thus perfectly situated to receive signals from terminating nociceptive afferents, similar to the expression of NR2B subunits. PSD-95 protein was absent from DRG tissue and deafferentation, produced by cutting spinal nerves, had no effect on PSD-95 immunoreactivity in the spinal cord. Therefore, PSD-95 is exclusively expressed post-synaptically within intrinsic dorsal horn neurones in the spinal cord. In addition, PSD-95 protein was found to bind to dorsal horn NMDA receptors. Garry and colleagues later confirmed the lack of PSD-95 expression in primary afferent fibres, since they did not observe the presence of PSD-95 in dorsal root entry zones, DRG, dorsal roots or sciatic nerve. They did see PSD-95 expression in the dorsal horn restricted to lamina II (Garry et al., 2003). Later, however, Polgar and colleagues used an antigen retrieval technique to unmask epitopes, prior to immunohistochemistry, for better detection of PSD-95 protein. Their results showed that PSD-95 has a punctate expression throughout the grey matter of the dorsal horn, though this was densest in superficial laminae I-II (Polgar et al., 2008). Since both Tao and colleagues and also Garry and colleagues did not use antigen retrieval, they presumably only detected PSD-95 in the region where it is most densely expressed.

3.1.5 Involvement of PSD-95 in acute and inflammatory pain models

The first evidence for a role of PSD-95 in pain was provided by Tao and colleagues, who employed antisense to knockdown the expression of PSD-95 in the spinal cord. The tail flick response of rats to thermal stimulation is potentiated by intrathecal NMDA administration. This effect was blocked by PSD-95 antisense (Tao et al., 2000). Mechanical and thermal hypersensitivity induced by intrathecal NMDA was also abolished in PSD-95 mutant mice, which express a shortened form of the protein truncated at the third PDZ domain (Garry et al., 2003). Surprisingly, these mice did not display any deficits in the response to intraplantar formalin compared to wild-type

mice, suggesting that PSD-95 is not involved in inflammatory pain. However, a later study by Tao and colleagues provided evidence that PSD-95 is in fact important for the manifestation of pain following peripheral inflammation caused by CFA. A decoy peptide was synthesized to mimic the second PDZ domain of PSD-95 which could then be used to prevent protein interactions with PSD-95. It was found that this peptide, Tat-PSD-95 PDZ2, blocked both the development and maintenance of CFA-induced chronic inflammatory pain-related behaviours in mice when administered by either intraperitoneal or intrathecal injections (Tao et al., 2008). No effects were seen in the non-inflamed contralateral paw and no changes were observed in locomotor function as measured by placing, righting and grasping reflexes following peptide treatment. A critical role for PSD-95 has also been established in chronic neuropathic pain and is further investigated in Chapter 4 of this thesis.

3.1.6 Coupling between NMDA receptors and PSD-95 and the source-specificity hypothesis

It is well documented that Ca^{2+} overload of neurones causes excitotoxicity and cell death. This Ca^{2+} overload can be the result of synaptic overactivity, such as during ischaemia, for example. The influx of Ca^{2+} may then trigger intracellular signalling cascades which ultimately contribute to death of the neurone. However, the source of this Ca^{2+} is important in that Ca^{2+} influx through certain channels will cause more excitotoxicity than influx through others. This is the basis of the 'source specificity' hypothesis which was first put forward by Tymianski and colleagues. They showed that when cells were stimulated with either potassium ions, which cause membrane depolarization and subsequent Ca^{2+} influx through non-specific voltage-gated Ca^{2+} channels, or with glutamate, both causing an equivalent amount of Ca^{2+} influx, the level of excitotoxicity differed between the two groups. Cell death was greater following glutamate stimulation and this effect was mediated via NMDA receptors (Tymianski et al., 1993; Sattler et al., 1998). This indicated that Ca^{2+}-dependent processes that trigger neurotoxicity are perhaps physically linked to NMDA receptors and are present in close proximity to the channel pore. In this way, Ca^{2+} influx pathways and downstream intracellular signalling cascades may be compartmentalized within neurones (Tymianski et al., 1993). One effector enzyme

which is involved in this NMDA-induced neurotoxicity is nNOS. Garthwaite and colleagues had previously established that NMDA receptor activation in the brain resulted in the formation of NO from L-arginine, a reaction catalyzed by nNOS (Garthwaite et al., 1988; Garthwaite et al., 1989). Thus, NO is produced in an NMDA-dependent manner. NO then acts as a diffusible messenger, either acting within the same neurone where it is produced or in neighbouring cells, to stimulate the production of cyclic guanosine monophosphate (cGMP) by guanylate cyclase (Bredt and Snyder, 1989). Together, these findings gave rise to the idea that nNOS was in some way physically coupled to the NMDA receptor and contributed to NMDA-mediated neurotoxicity. A likely candidate for mediating this coupling was PSD-95, which was known to bind both NR2 subunits of NMDA receptors as well as nNOS (Kornau et al, 1995; Brenman et al., 1996). Subsequently, Sattler and colleagues showed that knockdown of PSD-95 in cultured cortical neurones, through the use of antisense, blocked NMDA-dependent production of NO, without any alteration of nNOS expression or function, and also suppressed NMDA-dependent excitotoxicity (Sattler et al., 1999). This implicated PSD-95 in the coupling of NO production to Ca^{2+} influx specifically through activated NMDA receptors. This was further confirmed in a study by Christopherson and colleagues who showed that a physical ternary complex exists between NMDA receptors, PSD-95 and nNOS within neurones (Christopherson et al., 1999). PSD-95 is able to bind the cytoplasmic tail of NMDA receptor subunits and to nNOS simultaneously. Thus, nNOS is physically coupled to NMDA receptor activation and is held next to the channel pore where it can be directly activated by incoming Ca^{2+} to generate NO. This complex is not only important in terms of neuronal cell death, but also to plasticity and behaviour (Sattler et al., 1999). As well as nNOS, it is possible that many other proteins may be physically linked to NMDA receptors via PSD-95. Synaptic GTPase-activating protein (SynGAP), for example, is known to bind to PSD-95 in a complex with NMDA receptors and regulates ERK/MAPK signalling in synaptic plasticity, downstream of NMDA receptor activation (Kim et al., 1998; Komiyama et al., 2002). Thus, an extensive network of protein-protein interactions within the post-synaptic density links NMDA receptors to specific intracellular signalling pathways (Fig. 3.2) (Sheng, 2001). By disrupting such links, it may be possible to inhibit pathological NMDA intracellular signalling, whilst maintaining normal synaptic transmission.

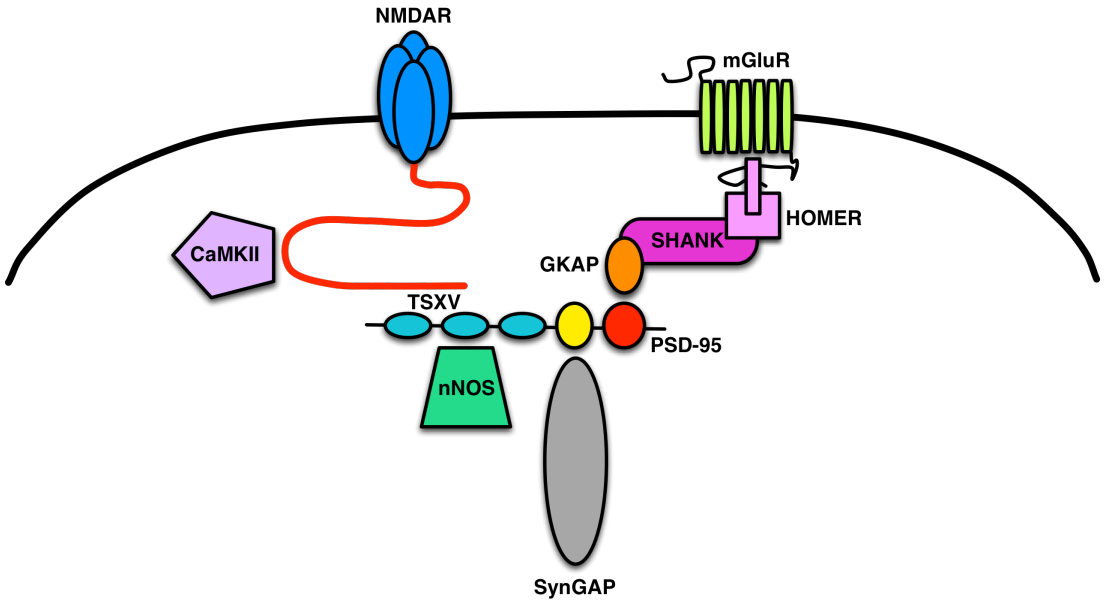


Fig. 3.2: The synaptic NMDA receptor complex. PSD-95 binds to a C-terminal threonine/serine X valine (TSXV) motif in NR2 subunits of the NMDA receptor via its 2nd PDZ domain, as well as with the PDZ domain of nNOS. PSD-95 also binds to other effectors, such as SynGAP, thus coupling NMDA activity to downstream intracellular signalling pathways. Furthermore, NMDA receptors may interact with other membrane-associated receptors such as metabotropic glutamate receptors (mGluRs) via the intracellular scaffold proteins GKAP (guanylate kinase-associated protein), Shank and Homer. CaMKII also binds directly to the cytoplasmic tails of NR2 subunits and participates in NMDA-mediated signalling. Adapted from Tao and Raja (2004).

3.1.7 Disrupting protein-protein interactions specifically between NMDA receptors and PSD-95 through the use of Tat-NR2B9c as a potential treatment strategy

Targeting of the physical link between the NMDA receptor and PSD-95 has previously been investigated in the field of stroke therapy by a group of Canadian researchers (Aarts et al., 2002). They designed a small peptide comprising the final nine amino acids of the NR2B cytoplasmic tail, incorporating the TSXV PSD-95 binding motif, that was conjugated to a human immunodeficiency virus (HIV) cell membrane transduction domain, Tat, for intracellular delivery (see 3.2.2 below). This peptide, termed Tat-NR2B9c, was able to transduce cortical neurones in culture within ten minutes of application. By twenty minutes, intraneuronal accumulation of Tat-NR2B9c had peaked and was detected for a further five hours. In co-immunoprecipitation experiments, Tat-NR2B9c was shown to selectively perturb interactions between PSD-95 and NMDA receptor NR2B but not NR2A subunits. Despite both these NR2 subunits containing the TSXV binding site for PSD-95, it is believed that the preceding five amino acids of Tat-NR2B9c, which are specific to NR2B subunits, confer this selectivity of the peptide for NR2B over NR2A subunits (Aarts et al., 2002). In addition, Tat-NR2B9c was found to have no effect on basal synaptic transmission since it did not alter NMDA receptor-mediated currents or Ca^{2+} uptake in hippocampal neurons. However, Tat-NR2B9c did reduce NMDA-induced generation of cGMP, a downstream measure of NO production by nNOS in neurones. Together, this data suggests that perturbing interactions between PSD-95 and NR2B subunits, through the use of Tat-NR2B9c, does not affect the integrity of NMDA receptors at the cell membrane, but selectively interferes with downstream intracellular signalling. This strategy may improve on the efficacy of conventional NMDA receptor blockers in the treatment of nervous system disorders whilst reducing the side-effects associated with NMDA receptor blockade. Indeed, systemic delivery of Tat-NR2B9c, which can cross the blood-brain barrier, was found to be highly neuroprotective in rats which had experimentally been given stroke by occlusion of a cerebral artery, thus producing cerebral ischaemia. Even when administered one hour after the onset of stroke, thus mimicking a clinical scenario, Tat-NR2B9c reduced cerebral brain damage and significantly improved neurological scores, as measured by postural reflex and forelimb placement tests (Aarts et al.,

2002). In a follow-up study, it was shown that when administered three hours after the onset of stroke, Tat-NR2B9c could permanently reduce ischaemic infarct size and improve neurological function, measured 62 days post-stroke (Sun et al., 2008). As a result of this successful performance in animal models and the evident wide therapeutic window, Tat-NR2B9c has now entered into phase II clinical trials for the treatment of stroke (verbal communication from Prof Mike Salter, Univ. of Toronto).

Since NMDA receptors have pathological roles in various nervous system disorders, including chronic pain, it is plausible that Tat-NR2B9c may be employed successfully for the treatment of many human diseases. So far, it has been demonstrated that Tat-NR2B9c may be useful for the clinical management of status epilepticus and Huntington's disease. Status epilepticus is a life-threatening condition where the brain is in a state of permanent seizure, which causes substantial neuronal damage. In a rat model of status epilepticus, Tat-NR2B9c significantly attenuated neuronal cell loss (Dykstra et al., 2009). Huntington's disease is a neurodegenerative genetic disorder which causes impaired muscle coordination and cognitive function, resulting from NMDA-dependent cell death in the striatum. In a mouse model of Huntington's, association of PSD-95 with NR2B was enhanced in striatal tissue, while Tat-NR2B9c rescued cells from NMDA-mediated toxicity (Fan et al., 2009). Therefore, it would appear that Tat-NR2B9c has the potential to be applied to multiple clinical disorders. It has yet to be demonstrated whether these include abnormal or chronic pain states.

As well as being implicated in excitotoxicity and neurodegenerative disease, NR2B interactions with PSD-95 have also been shown to contribute to the essential role of NMDA receptors in synaptic plasticity in the brain. Gardoni and colleagues demonstrated that Tat-NR2B9c reduced the induction of LTP, but not long-term depression (LTD), at hippocampal synapses. LTP was induced by high frequency stimulation (100 Hz) of hippocampal neurones and was associated with synaptic localization of NR2B-containing NMDA receptors and their association with PSD-95 (Gardoni et al., 2009). This data supported the established role for PSD-95 in synaptic plasticity (Migaud et al., 1998; Stein et al., 2003; Ehrlich and Malinow, 2004).

It is known that overactivity of NMDA receptors in the spinal cord leads to central sensitization of dorsal horn sensory neurones and chronic pain. It is plausible that disruption of NMDA-mediated intracellular signalling via PSD-95 in the spinal cord, through the use of Tat-NR2B9c, may be a highly successful analgesic strategy which lacks the side-effects associated with non-selective NMDA antagonists. By targeting signalling pathways which are known to contribute to neuronal plasticity, we may be able to prevent, and ultimately reverse, pathological pain states. In this chapter, the effects of disrupting the specific interaction between PSD-95 and NR2B-containing NMDA receptors, on dorsal horn sensory transmission and wind-up, as well as formalin-induced central sensitization, have been investigated using electrophysiological and behavioural measures.

3.2 Materials and Methods

3.2.1 Animals

Experiments were conducted in adult male Sprague-Dawley rats (220-250 g, Central Biological Services, University College London, or Harlan, UK), housed in standard laboratory conditions with free access to food and water. Experimental procedures were approved by the UK Home Office and followed guidelines under the International Association for the Study of Pain (Zimmermann, 1983).

3.2.2 Synthesis of Tat-NR2B9c and Tat-NR2BAA peptides

Tat-NR2B9c peptide comprises the final nine amino acids of the NR2B subunit cytoplasmic tail (KLSSIESDV), including the PSD-95 PDZ domain-binding motif (ESDV). This sequence is conjugated to a HIV-1 virus coat protein, Tat, transduction domain (YGRKKRRQRRR), for intracellular delivery (Schwarze et al., 1999). The full peptide is 20 amino acids (YGRKKRRQRRRKLSSIESDV) in length. A control peptide, Tat-NR2BAA, was synthesized with a double substitution in the PDZ domain-binding motif (EADA), thereby rendering it unable to bind to its target and being inactive (YGRKKRRQRRRKLSSIEADA).

Peptides were synthesized using an ACT 396 peptide synthesizer to > 95% purity with high performance liquid chromatography and mass spectrometry analysis (Wolfson Institute for Biomedical Research, University College London). Peptides were dissolved in PBS.

3.2.3 Immunoprecipitation of NR2B subunits and Western Immunoblotting

Rats anaesthetized with urethane were sacrificed by decapitation and fresh spinal cord (dorsal horn only), hippocampus and DRG tissue was dissected out and homogenized for immunoprecipitation (IP) and/or western immunoblotting (see Materials and Methods chapter).

Immunoprecipitation Dynabeads® Protein G (Invitrogen Ltd, UK) were washed and coupled with 5 μg of rabbit anti-NR2B antibody (06-600, Upstate, USA). Next 500 μg of spinal dorsal horn or hippocampus lysate was added to the antibody/dynabeads complex and allowed to incubate overnight for capture of target antigen. Following incubation, the captured protein was eluted from the dynabeads and loaded onto gels for downstream SDS-PAGE. Normal spinal and hippocampal lysate samples were run alongside IP samples as positive controls. Once proteins were separated they were transferred to nitrocellulose membranes. For detection of proteins of interest, membranes were incubated with primary antibody overnight at 4°C. Antibodies used were mouse anti-NR2B (1:500, 75-101, Neuromab, USA), rabbit anti-PSD-95 (1:1000, ab18258, Abcam, UK) and rabbit anti-nNOS (1:3000, 07-571, Millipore, UK). Following incubation with IRDye-linked donkey anti-rabbit 800CW or goat anti-mouse 680 secondary antibodies, proteins were revealed using the Odyssey fluorescence detection system (Licor, UK).

Western blotting of PSD-95 was conducted in hippocampus, spinal cord and DRG lysate samples (40 μg) as above. Membranes were probed with rabbit anti-PSD-95 (1:1000, ab18258, Abcam, UK) and rabbit anti-neuronal βIII-tubulin (1:3000, ab18207, Abcam, UK), which served as a loading control, followed by IRDye-linked donkey anti-rabbit 800CW secondary antibody.

3.2.4 *In vivo* Electrophysiology

Electrophysiological recordings from deep dorsal horn WDR neurones (> 600 μm) were conducted as previously described (see Materials and Methods chapter).

3.2.4.1 Electrical stimulation of the hindpaw

Following the establishment of stable control responses, 12.5 ng of Tat-NR2B9c in a volume of 50 μl was injected onto the surface of the spinal cord (after removal of any residual cerebrospinal fluid) using a Hamilton syringe. Electrical tests were continued at 10 min intervals for 2 hrs. A separate group of animals received 1.25 μg of the control peptide Tat-NR2BAA, in a volume of 50 μl, for comparison.

3.2.4.2 Neuronal Formalin Test

Following cell characterization, rats were pre-treated by topical spinal administration of either 12.5 ng of Tat-NR2B9c or 1.25 μg of Tat-NR2BAA, 20 min prior to the injection of formalin. Both drugs were delivered in a volume of 50 μl using a Hamilton syringe. A separate group of control animals received no drug. Formalin (5%, 50 μl) was prepared from a 37% formaldehyde solution and then injected subcutaneously into the hindpaw receptive field. The firing response of the WDR neurone was recorded for the subsequent 70 min after formalin injection. Activity was displayed as a rate recording and quantified in 10 min time bins.

3.2.5 Behavioural Formalin Test

Rats were lightly anaesthetized with isoflurane and then injected intrathecally (via lumbar puncture method) with either 10 μl of Tat-NR2B9c (12.5 ng or 125 ng) or 10 μl of Tat-NR2BAA (1.25 μg). Experimenters were blind to treatment for the whole experiment. Twenty minutes later, rats received a subcutaneous injection of 50 μl of formalin (5%) into the plantar surface of the right hindpaw. Lifting, flinching, licking and biting of the injected paw were monitored by measuring the total duration of each response in seconds (s) during the 60 min period following formalin administration. Data was captured in 5 min time bins.

3.2.6 Statistical Analysis

Data are presented as mean ± s.e.m. Effects of Tat-NR2B9c and Tat-NR2BAA following electrical stimulation of the hindpaw were assessed by one-way repeated measures analysis of variance (one-way RM ANOVA), followed by Bonferroni multiple comparison post-tests. Cell characteristics from Tat-NR2B9c, Tat-NR2BAA and control (no drug) groups were compared by Student's unpaired t-tests (wind-up study) or one-way ANOVA, followed by Bonferroni post-tests (formalin study. Formalin neuronal and behavioural response time course data was compared between treatment groups and analyzed by two-way RM ANOVA, followed by Bonferroni post-tests. Total activity in the first and second phases was compared between treatment groups by quantifying the area under each curve, and analyzed by one-way ANOVA, followed by Bonferroni post-tests. Statistical analyses were carried out using GraphPad Prism v.4 software (GraphPad Software Inc., San Diego, CA, USA).

3.3 Results

3.3.1 A protein complex between NR2B subunits, PSD-95 and nNOS exists in intrinsic spinal dorsal horn neurones

Protein expression of PSD-95 was confirmed by western immunoblotting in hippocampus and spinal cord dorsal horn tissue. As expected, PSD-95 protein was not present in DRG (Fig. 3.3a). Thus, in the spinal cord, PSD-95 is exclusively found in intrinsic dorsal horn sensory neurones and not in terminating primary afferent fibres.

Immunoprecipitation was used to reveal physical coupling between spinal dorsal horn or hippocampal NR2B subunits and associated proteins using a specific antibody against this subunit. Both PSD-95 and nNOS protein was co-immunoprecipitated by the anti-NR2B antibody, as expected, confirming the presence of a heterotrimeric complex composed of NR2B-containing NMDA receptors, PSD-95 and nNOS in intrinsic spinal dorsal horn neurones and hippocampus (Fig. 3.3b). Normal spinal and hippocampal lysate was run alongside immunoprecipitation samples to act as positive controls. Since PSD-95 is only expressed within intrinsic dorsal horn neurones, the presence of PSD-95 protein in the immunoprecipitated sample indicates that the NR2B-associated protein complex is present within the same dorsal horn neurones.

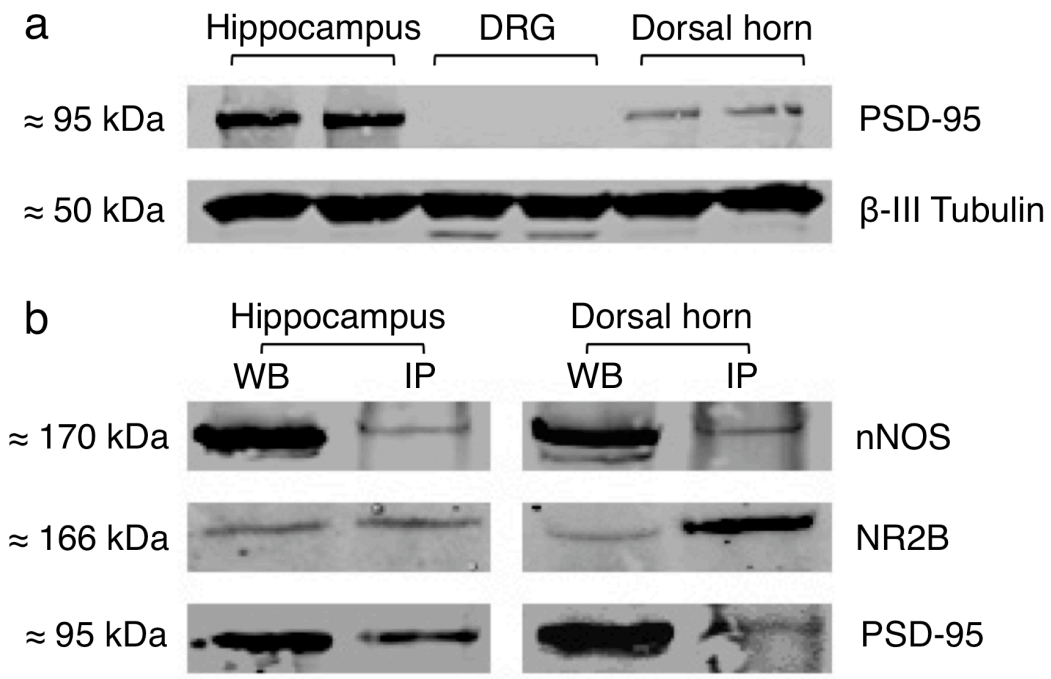


Fig. 3.3: PSD-95 is absent in DRG but present in the dorsal horn and forms a ternary complex with NR2B-subtype NMDA receptors and nNOS within intrinsic dorsal horn neurones. **(a)** Western immunoblot showing expression of PSD-95 in hippocampus (two left lanes), DRG (two centre lanes) and spinal dorsal horn (two right lanes). β-III tubulin served as a loading control. **(b)** Western immunoblots showing immunoprecipitates (IP, right lanes) from hippocampus and dorsal horn obtained using an antibody against NR2B. Left lanes show normal tissue lysates with no immunoprecipitation (WB), acting as positive controls.

3.3.2 Spinal application of Tat-NR2B9c does not alter afferent-evoked responses but inhibits post-discharge of deep dorsal horn WDR neurones

Electrically evoked responses of deep dorsal horn WDR neurones due to primary afferent activity were unaltered following spinal Tat-NR2B9c (12.5 ng, n = 6). No changes were seen in Aβ-fibre, Aδ-fibre or C-fibre evoked responses (Fig. 3.4). The control peptide Tat-NR2BAA (1.25 μg, n = 6) also had no effect on these evoked responses, at a dose 100x greater than the active peptide. WDR neurones continue to fire action potentials (APs) once the afferent barrage has ceased, a measure known as post-discharge, indicating the hyperexcitability of the cell. Tat-NR2B9c produced a significant and robust reduction of post-discharge (Fig. 3.4; % of pre-drug baseline = 45 ± 4%, $p < 0.01$), while Tat-NR2BAA had no effect. Inhibitory effects of Tat-NR2B9c were evident by 20 min following spinal administration, peaked at approximately 40 min and persisted for the duration of the experiment. Pre-drug control responses did not differ between treatment groups (Table 3.1).

	Tat-NR2B9c (*n* = 6)	Tat-NR2BAA (*n* = 6)
C-fibre threshold (mA)	0.68 ± 0.29	0.82 ± 0.24
Depth (μM)	968 ± 70	997 ± 47
Aβ-fibre evoked response (No. of APs)	199 ± 21	102 ± 25
Aδ-fibre evoked response (No. of APs)	266 ± 39	180 ± 14
C-fibre evoked response (No. of APs)	592 ± 50	593 ± 92
Post-discharge (No. of APs)	666 ± 42	460 ± 23
Input (No. of APs)	444 ± 61	442 ± 98
Wind-up (No. of APs)	893 ± 98	682 ± 81

Table 3.1: Pre-drug control responses induced by transcutaneous electrical stimulation of the hindpaw receptive field of WDR neurones in rats treated with Tat-NR2B9c or Tat-NR2BAA. C-fibre threshold, cell depth, afferent-evoked responses, input and wind-up are expressed as mean ± s.e.m. There were no differences between cells recorded in each treatment group for any measure prior to drug delivery; Student's unpaired t-test.

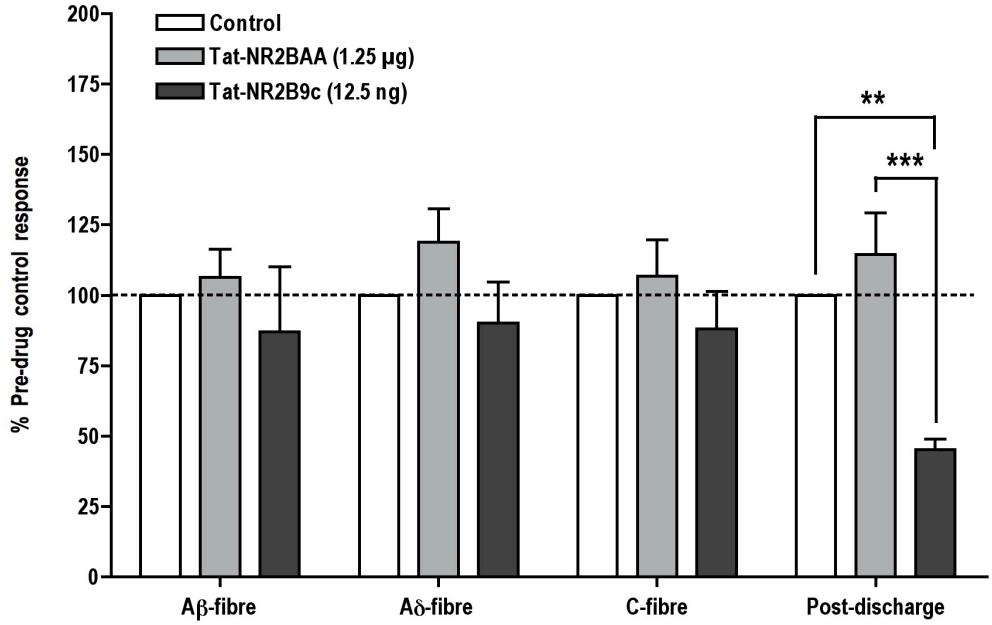


Fig. 3.4: Spinal Tat-NR2B9c decreases hyperexcitability of WDR neurones. Afferent-evoked responses and post-discharge of WDR neurones following transcutaneous electrical stimulation of the hindpaw receptive field, with spinal application of control Tat-NR2BAA (1.25 μg) or Tat-NR2B9c (12.5 ng). White bars represent pre-drug control. All data presented as mean ± s.e.m. of pre-drug control responses; ** $p < 0.01$, *** $p < 0.001$ versus pre-drug baseline or control peptide; one-way RM ANOVA followed by Bonferroni post-tests on raw data; $n = 6$ in each group.

3.3.3 Wind-up of deep dorsal horn WDR neurones is reduced by spinal Tat-NR2B9c

The pre-synaptic input (non-potentiated response) as well as the wind-up of WDR neurones were both assessed (Fig. 3.5a). Wind-up is the NMDA-dependent increasing response of WDR neurones to repetitive noxious peripheral stimulation at constant intensity and frequency (Fig. 3.5b,c). Pre-drug input and wind-up values were similar in both treatment groups (Table 3.1). Both Tat-NR2B9c and Tat-NR2AA had no effect on input (Fig. 3.5a). In contrast, Tat-NR2B9c significantly decreased wind-up of WDR neurones (Fig. 3.5a,c; % of pre-drug baseline = 52 ± 4%, $p < 0.05$), while Tat-NR2BAA had no effect (Fig. 3.5a,b).

Therefore, Tat-NR2B9c, believed to exert its effects by disruption of binding between PSD-95 and NR2B subunits, selectively reduces NMDA receptor-mediated post-discharge and wind-up of deep dorsal horn WDR neurones, while having no effect on afferent-evoked responses and input, indicating a post-synaptic mechanism of action.

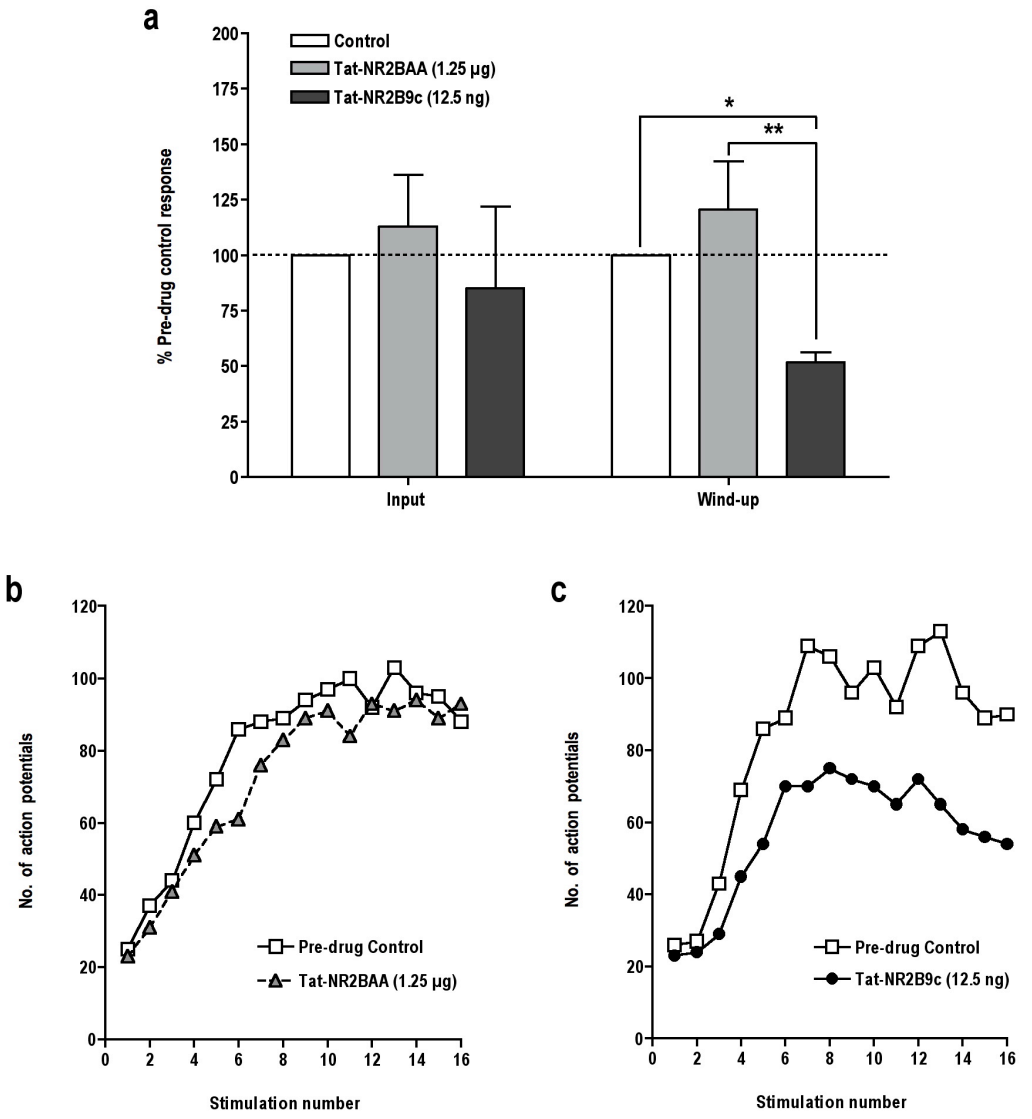


Fig. 3.5: Spinal Tat-NR2B9c selectively reduces wind-up of WDR neurones.

(a) Effect of spinal application of Tat-NR2BAA (1.25 μg) or Tat-NR2B9c (12.5 ng) on input and wind-up of WDR neurones. White bars represent pre-drug control. Data presented as mean ± s.e.m. of pre-drug control responses; $*$ $p < 0.05$, $**$ $p < 0.01$ versus pre-drug baseline or control peptide; one-way RM ANOVA followed by Bonferroni post-tests on raw data; $n = 6$ in each group. (**b,c**) Examples of the wind-up of single WDR neurones following repetitive electrical stimulation in the presence of spinal (**b**) Tat-NR2BAA (1.25 μg) or (**c**) Tat-NR2B9c (12.5 ng).

3.3.4 Formalin-induced central sensitization of deep dorsal horn WDR neurones is reduced by spinal pre-treatment with Tat-NR2B9c

Formalin was injected into the hindpaw receptive field and the firing response of single WDR neurones was recorded (Fig. 3.6c). This stimulus produces an afferent drive with a delayed central NMDA component to the response. In control recordings, where no drug was applied, formalin induced a characteristic biphasic neuronal firing response (Fig. 3.6a-c; 1st phase: 0-10 min, total APs = 9632 ± 1678; 2nd phase: 10-70 min, total APs = 64071 ± 16220; n = 11). Control pre-treatment with spinal Tat-NR2BAA (1.25 μg, n = 11), twenty minutes prior to formalin injection did not alter this response (Fig. 3.6a-c; 1st phase total APs = 13970 ± 2228, $p > 0.05$; 2nd phase total APs = 54483 ± 13673, $p > 0.05$). Spinal pre-treatment with Tat-NR2B9c (12.5 ng, n = 10) did, however, significantly and selectively reduce second phase neuronal firing (Fig. 3.6a-c; total APs = 9695 ± 5386, $p < 0.001$). This second phase activity is attributed to central sensitization of spinal dorsal horn neurones (Dickenson and Sullivan, 1987a; Coderre et al., 1990). Only a small, non-significant reduction of neuronal firing during the acute first phase was observed following Tat-NR2B9c pre-treatment, likely reflecting the selective inhibitory effects seen previously on post-discharge and wind-up. All cells recorded were characterized prior to injection of drug and formalin to ensure that cells were comparable between treatment groups (Table 3.2).

Thus, spinal application of Tat-NR2B9c reduces NMDA-mediated components of formalin-induced central sensitization of deep dorsal horn WDR neurones.

	Control - no drug (*n* = 11)	Tat-NR2BAA (*n* = 11)	Tat-NR2B9c (*n* = 10)
C-fibre threshold (mA)	0.65 ± 0.17	0.95 ± 0.24	0.93 ± 0.12
Depth (*μ*M)	896 ± 42	886 ± 52	905 ± 37
Aβ-fibre evoked response (No. of APs)	208 ± 23	217 ± 13	193 ± 26
Aδ-fibre evoked response (No. of APs)	234 ± 25	256 ± 14	229 ± 33
C-fibre evoked response (No. of APs)	577 ± 73	541 ± 56	546 ± 83
Post-discharge (No. of APs)	641 ± 117	643 ± 70	654 ± 162
Input (No. of APs)	689 ± 215	477 ± 91	525 ± 167
Wind-up (No. of APs)	634 ± 112	814 ± 129	762 ± 207
48°C (No. of APs)	2037 ± 229	1901 ± 200	1834 ± 190

Table 3.2: WDR cell characteristics from Tat-NR2B9c neuronal formalin test. Cells were characterized prior to drug administration and formalin injection. C-fibre threshold, cell depth, afferent-evoked responses, input, wind-up and response to 48°C are expressed as mean ± s.e.m. There were no differences between cells recorded in each treatment group; one-way ANOVA followed by Bonferroni post-tests.

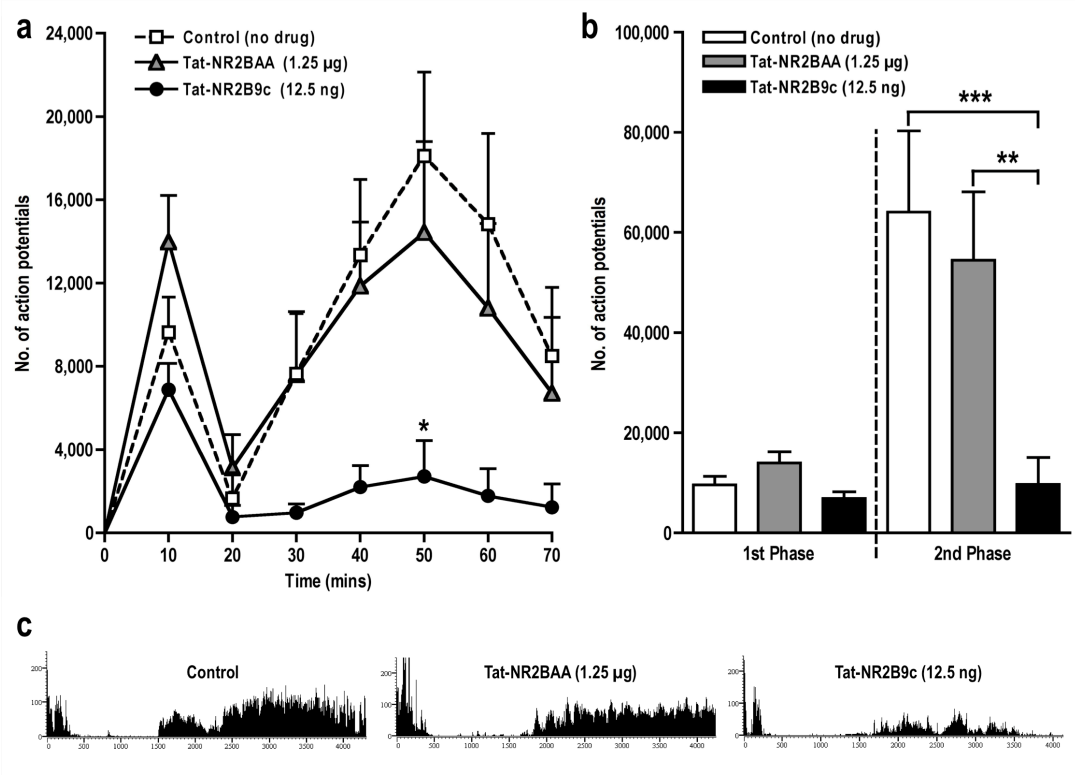


Fig. 3.6: Spinal Tat-NR2B9c reduces formalin-induced central sensitization of WDR neurones. **(a)** Time course of WDR firing response to subcutaneous formalin (5%, 50 μl) injection into the hindpaw receptive field with no drug (control, n = 11) or following spinal pre-treatment with Tat-NR2BAA (1.25 μg, n = 11) or Tat-NR2B9c (12.5 ng, n = 10, $*$ $p < 0.05$ at 50 min versus Tat-NR2BAA, two-way RM ANOVA, followed by Bonferroni post-tests). **(b)** Total neuronal activity during the 1st phase (0-10 min) and 2nd phase (10-70 min) of the formalin response with no drug (control), Tat-NR2AA or Tat-NR2B9c (2nd phase: $**$ $p < 0.01$, $***$ $p < 0.001$ versus Tat-NR2BAA and no drug, respectively, one-way ANOVA, followed by Bonferroni post-tests). **(c)** Representative rate recordings of firing responses of WDR neurones to formalin with no drug (control) or following spinal pre-treatment with Tat-NR2BAA (1.25 μg) or Tat-NR2B9c (12.5 ng). All data presented as mean ± s.e.m.

3.3.5 Spinal Tat-NR2B9c inhibits pain-related behaviours due to formalin-induced central sensitization

Following the inhibitory effects of Tat-NR2B9c observed on neuronal activity related to spinal plasticity, effects of spinal application of Tat-NR2B9c were examined in a behavioural assay, using formalin injected into the hindpaw of freely moving, awake rats. As with neuronal activity, formalin induced a biphasic response of pain-related behaviours in rats pre-treated intrathecally with the control peptide, Tat-NR2BAA (1.25 μg, n = 11), twenty minutes prior to formalin injection (Fig. 3.7a; 1st phase: 0-10 min, total time of pain-related behaviour = 94 ± 9 s; 2nd phase: 10-60 min, total time of pain-related behaviour = 360 ± 26 s). Pre-treatment with Tat-NR2Bc, however, significantly decreased pain-related behaviour during the second phase of the response in a dose-dependent manner (Fig. 3.7a,b; 12.5 ng, n = 10, 2nd phase total time of pain-related behaviour = 255 ± 13 s, $p < 0.01$; 125 ng, n = 6, 2nd phase total time of pain-related behaviour = 174 ± 16 s, $p < 0.001$). No difference was seen during the first phase between either dose of Tat-NR2B9c and the control peptide (Fig. 3.7a,b; 12.5 ng, 1st phase total time of pain-related behaviour = 68 ± 8 s, $p > 0.05$; 125 ng, 1st phase total time of pain-related behaviour = 84 ± 8 s, $p > 0.05$).

Thus, Tat-NR2B9c, perhaps via disruption of NR2B subunits coupling to PSD-95, reduces formalin-induced pain-related behaviours produced by spinal central sensitization.

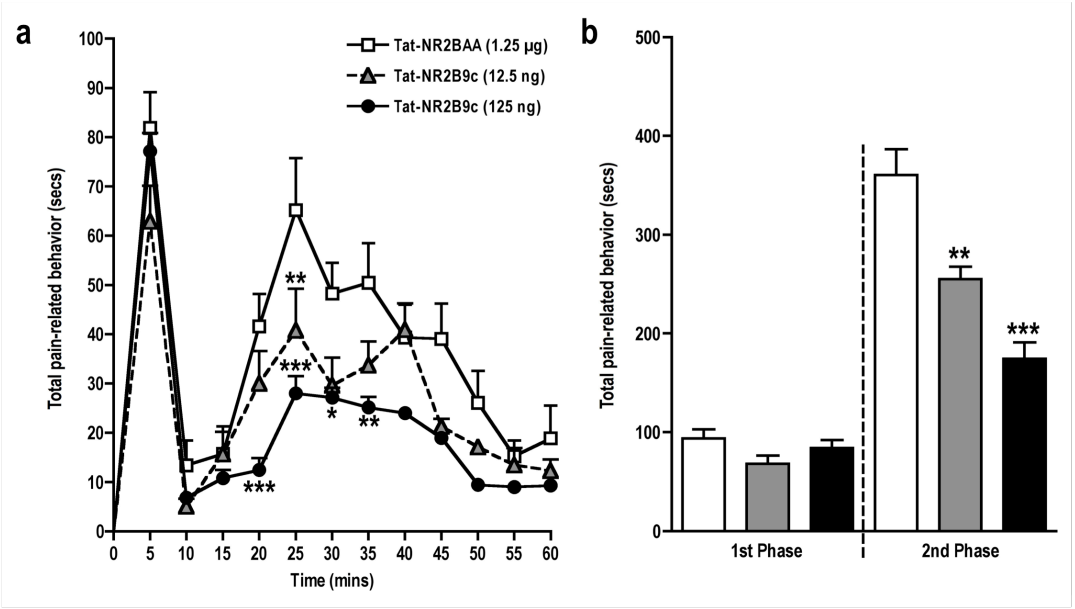


Fig. 3.7: Intrathecal Tat-NR2B9c reduces pain-related behaviours due to formalin-induced central sensitization. (a) Time course of pain-related behaviours induced by intraplantar injection of formalin (5%, 50 μl) following intrathecal pre-treatment with Tat-NR2BAA (1.25 μg, $n = 11$) or Tat-NR2B9c (12.5 ng, $n = 10$, ** $p < 0.01$ at 25 min versus Tat-NR2BAA; 125 ng, $n = 6$, *** $p < 0.001$ at 20, 25 min, * $p < 0.05$ at 30 min, ** $p < 0.01$ at 35 min versus Tat-NR2BAA, two-way RM ANOVA, followed by Bonferroni post-tests). **(b)** Total pain-related behaviour during the 1st phase (0-10 min) and 2nd phase (10-60 min) of the response to formalin following intrathecal pre-treatment with Tat-NR2AA or Tat-NR2B9c (2nd phase: 12.5 ng, ** $p < 0.01$, 125 ng, *** $p < 0.001$ versus Tat-NR2BAA, one-way ANOVA, followed by Bonferroni post-tests). All data presented as mean ± s.e.m.

3.3.6 Spinal Tat-NR2B9c reduces formalin-induced active pain-related licking and biting behaviours

Further analysis of the different pain behaviours showed that rats injected with formalin spent most time licking and biting the injured paw, rather than lifting and flinching (Fig. 3.8). Licking and biting behaviour alone also shows a strong biphasic response following pre-treatment with Tat-NR2BAA (Fig. 3.8a; 1st phase: 0-10 min, total time of licking and biting behaviour = 83 ± 10 s; 2nd phase: 10-60 min, total time of licking and biting behaviour = 285 ± 23 s), which correlates exactly with the biphasic neuronal activity (Fig. 3.6). Pre-treatment with Tat-NR2B9c produced a significant and dose-dependent reduction of this licking and biting behaviour during the second phase of the response (Fig. 3.8b; 12.5 ng, 2nd phase total time of licking and biting behaviour = 200 ± 18 s, $p < 0.05$; 125 ng, 2nd phase total time of licking and biting behaviour = 124 ± 19 s, $p < 0.001$). In contrast, rats spent little time lifting and flinching and this behaviour did not follow such an obvious biphasic time course (Fig. 3.8c). There was no difference in total lifting and flinching behaviour in either the first or second phase between Tat-NR2BAA and Tat-NR2B9c groups (Fig. 3.8d).

Therefore, it is clear that licking and biting behaviour is the predominant pain-related behaviour in rats following intraplantar formalin injection. The reduction in total pain-related behaviour by spinal administration of Tat-NR2B9c was due to a selective action on the active licking and biting responses rather than the more reflexive lifting and flinching.

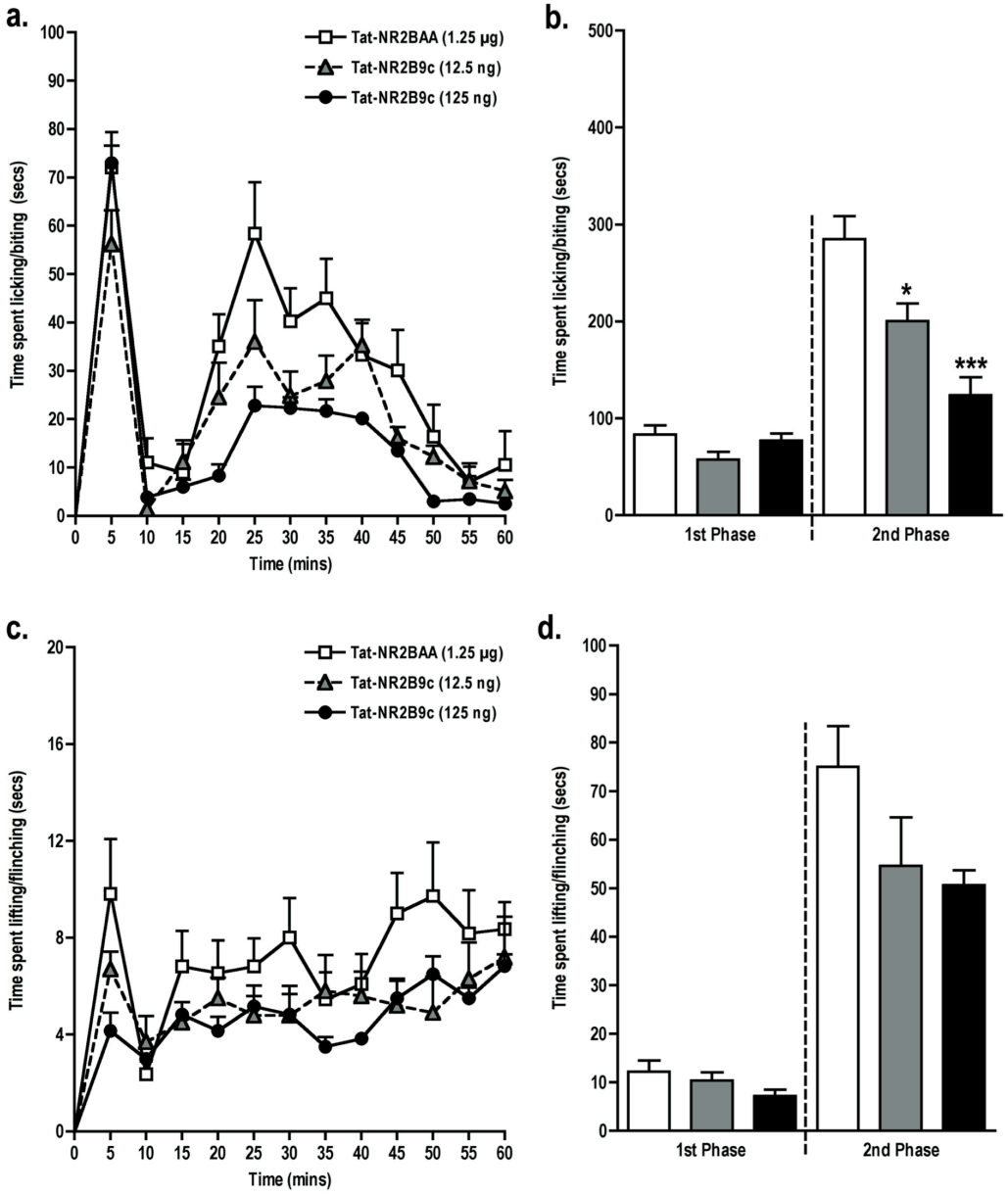


Fig. 3.8: Intrathecal Tat-NR2B9c selectively reduces active pain-related licking and biting behaviours following intraplantar formalin. **(a)** Time course of formalin-induced (5%, 50 μl) licking and biting behaviour following intrathecal pre-treatment with Tat-NR2BAA (1.25 μg, n = 11) or Tat-NR2B9c (12.5 ng, n = 10). **(b)** Total licking and biting behaviour during the 1st phase (0-10 min) and 2nd phase (10-60 min) of the formalin response following intrathecal Tat-NR2AA or Tat-NR2B9c (2nd phase, 12.5 ng, $*$ $p < 0.05$, 125 ng, $***$ $p < 0.001$ versus Tat-NR2BAA, one-way

ANOVA, followed by Bonferroni post-tests). **(c)** Time course of formalin-induced lifting and flinching behaviour following intrathecal pre-treatment with Tat-NR2BAA or Tat-NR2B9c. **(d)** Total licking and biting behaviour during the 1st phase and 2nd phase of the formalin response following intrathecal Tat-NR2AA or Tat-NR2B9c. Note that due to the lower incidence of lifting and flinching behaviours the vertical axes are scaled differently. All data presented as mean ± s.e.m.

3.4 Discussion

Repetitive and prolonged primary afferent stimulation by noxious stimuli, elicited by transcutaneous electrical stimulation and formalin-induced inflammation, leads to NMDA receptor-mediated plastic changes in neuronal activity, producing wind-up and central sensitization of dorsal horn sensory neurones, which may lead to the manifestation of chronic pain states. The novel aim of his study was to specifically target NR2B interactions with PSD-95 in pain using both neuronal and behavioural measures. A mimetic peptide, Tat-NR2B9c, which has been shown to disrupt coupling between NR2B subunits and PSD-95 (Aarts et al., 2002), was employed. The results suggest that binding of PSD-95 to spinal NMDA receptors, composed specifically of NR2B subunits, is important for the sensitizing effects of NMDA receptor activation. Disrupting such interactions by using Tat-NR2B9c reduces neuronal hyperexcitability and plasticity and also abnormal pain-related behaviours.

Despite being a fast-onset and short-lasting phenomenon, wind-up may contribute to long-term changes in the spinal cord which occur during a sustained afferent barrage to the dorsal horn from the periphery, thus leading to central sensitization, such as is produced by peripheral formalin injection (Haley et al., 1990). Use of the disrupting peptide, Tat-NR2B9c, inhibited post-discharge and wind-up, two measures of hyperexcitability and plasticity of dorsal horn WDR neurones. Both of these phenomena are reported clinically in chronic pain patients, and a wind-up ratio, representing the perceptual correlate of temporal pain summation, is routinely assessed during quantitative sensory testing (Kristensen et al., 1992; Jensen et al., 2001; Rolke et al., 2006a). In contrast, responses evoked by primary afferent firing and pre-synaptic input were unaltered by Tat-NR2B9c. These electrophysiological results are similar to those obtained by blocking NR2B-subtype receptors (Boyce et al, 1999; Chizh et al., 2001; Kovacs et al., 2004). Studies by Boyce and colleagues and also Chizh and colleagues recorded wind-up in muscle units, rather than in spinal neurones, to show an antinociceptive effect of systemic NR2B antagonists. However, conclusions in these studies differed with regard to the site of action of these drugs, with Chizh and colleagues concluding that the NR2B antagonist ifenprodil has a supraspinal site of action with respect to its antinociceptive efficacy. Later, however, Kovacs and colleagues recorded directly from spinal WDR neurones,

as in the present study, in spinalized rats, thus eliminating supraspinal influences of descending controls. It was shown that both non-selective NMDA antagonism and selective NR2B antagonism reduced neuronal wind-up while A-fibre and C-fibre evoked responses were unaltered. These results clearly established a role for spinal cord NR2B receptors in hyperexcitability of dorsal horn sensory neurones, and are in accordance with results of the present study.

The importance of NR2B-subtype receptors in pain following peripheral inflammation was first established by Taniguchi and colleagues and later confirmed by Boyce and colleagues in demonstrating anti-hyperalgesic efficacy of NR2B antagonism in rats with carrageenan-induced inflammation of the hindpaw (Taniguchi et al., 1997; Boyce et al., 1999). The formalin model of pain (Dubuisson and Dennis, 1977) produces a biphasic response, both electrophysiologically in single neurones, as well as behaviourally in awake animals. Tat-NR2B9c selectively reduced the second phase of the formalin response, thought to represent central sensitization (Dickenson and Sullivan, 1987a; Coderre et al., 1990) in both neuronal and behavioural tests. The electrophysiological data provides a neuronal substrate for the behavioural effects of disrupting interactions between NMDA receptors and PSD-95. The selective effects of Tat-NR2B9c on formalin-induced central sensitization are mimicked by NR2B-subtype receptor antagonism and genetic knockdown. Malmberg and colleagues used conantokins derived from cone snail venom, which selectively inhibit NR2B-subtype receptors, and found that these could selectively block pain-related behaviours during the second phase of the response to formalin. Interestingly, this effect was only observed when conantokins were delivered intrathecally, whilst systemic administration had no effect, at doses which did not affect motor performance (Malmberg et al., 2003). Genetic knockdown of spinal NR2B subunits by intrathecal delivery of small interfering RNA (siRNA) was employed by Tan and colleagues. This method had no effect on motor performance or on baseline thermal sensitivity, but markedly reduced formalin-induced second phase pain-related behaviours (Tan et al., 2005). Together, these studies further support a role for spinal NR2B-containing NMDA receptors in central sensitization. In contrast, a previous study failed to observe changes in formalin-induced pain-related behaviour in PSD-95 mutant mice with a truncation of PSD-95 at the third PDZ domain (Garry et al., 2003). However, the truncated PSD-95 protein in these mice,

with the first two PDZ domains intact, may still be present at synapses since it is specifically these two domains of PSD-95 which are required for synaptic targeting, while PDZ domain three is dispensable (Craven et al., 1999). In addition, the binding capacity at each individual PDZ domain independently contributes to synaptic clustering of the protein (Nonaka et al., 2006) so that any loss of the third PDZ domain of PSD-95 may not affect its binding to NMDA receptors. Alternatively, if a truncated PSD-95 protein can no longer bind NR2 subunits or be clustered in synaptic regions, other related proteins may compensate for this loss. For example, post-synaptic density protein-93 (PSD-93) has been shown to be important for inflammatory pain (Tao et al., 2003c). It is also notable that Garry and colleagues used a different behavioural endpoint in the formalin test to that in the present study. They measured only lifting and flinching behaviour, which is a spinally-mediated reflex. In the present study, it was found that this behaviour was infrequent and was unaltered by Tat-NR2B9c. In contrast, licking and biting behaviour, which is more of an active pain-related behaviour requiring higher processing, predominated during the formalin response and was sensitive to the inhibitory effects of Tat-NR2B9c, and also exactly paralleled the neuronal firing. It was not reported whether these active behaviours were altered or not in PSD-95 mutant mice. Interestingly, differences were seen with these PSD-95 mutant mice in pain induced by intrathecal NMDA or nerve injury (Garry et al., 2003), suggesting that neuropathic pain may arise from protein interactions involving the third PDZ domain of PSD-95, while inflammatory pain may involve the first and second PDZ domains.

A recent behavioural study using a different disrupting peptide, Tat-PSD-95 PDZ 2, which mimics the second PDZ domain of PSD-95, indicated the importance of protein interactions with PSD-95 in pain following peripheral inflammation (Tao et al., 2008). However, the analgesic effect of this peptide could be attributed to a disruption of binding of PSD-95 to, not only NMDA receptors, but also other signalling proteins, such as Shaker-type K^+ channels (Kim et al., 1995) and nNOS (Brenman et al., 1996), which also interact with PSD-95 via its second PDZ domain. A cyclic peptide, CN2097, which disrupts all PSD-95 interactions mediated via its three PDZ domains, has also been shown to reverse neuropathic pain and spinal LTP, though again, the particular binding partners of PSD-95 involved in this inhibitory effect were not identified (Leblanc et al., 2010). The results presented in this chapter are expected

to be due to a more specific disruption of NR2B and PSD-95 interactions, since the Tat-NR2B9c peptide employed here not only exerted selective neuronal effects but also mimics the specific C-tail of NR2B subunits, so having no effect on the binding of PSD-95 to even NR2A subunits (Aarts et al., 2002). It is therefore expected that other PSD-95 interactions would also remain unaltered. In addition, Tat-NR2B9c was also shown not to alter NMDA-induced currents or Ca^{2+} influx but to reduce the NMDA-induced formation of cGMP, suggesting a lack of effect of Tat-NR2B9c on the receptor and trafficking but supporting an action on signalling downstream of NR2B-subtype receptors via coupling to PSD-95 (Aarts et al., 2002). Furthermore, an alternative peptide, Tat-2ASCV, which disrupts interactions specifically between 5-HT_{2A} receptors and PSD-95, does not alter interactions between NMDA receptors and PSD-95 (Pichon et al., 2010). Thus, through the use of Tat-NR2B9c, it may be concluded that the specific interaction between PSD-95 and NR2B subunits is important for dorsal horn neuronal hyperexcitability and central sensitization.

This study further supports the key role of NR2B-containing NMDA receptors in spinal cord nociceptive plasticity. The NR2B subunit has a concentrated distribution in the superficial laminae (I-II) of the dorsal horn (Nagy et al., 2004a), where it is ideally placed to receive nociceptive inputs from terminating C-fibres. It is likely that the deep dorsal horn neurones recorded in electrophysiological experiments receive nociceptive inputs via interneurones and/or their dorsally extending dendrites (Woolf and King, 1987; Martin et al., 2001; Braz et al., 2005; Kato et al., 2009) and this would account for the effects of Tat-NR2B9c on activity of deep WDR neurones. Physical coupling of PSD-95 and NR2B subunits was demonstrated in spinal neurones by co-immunoprecipitation, in accordance with previous work (Tao et al., 2000). Since PSD-95 was not found to be present in DRG, but only in spinal cord tissue, it can be concluded that a complex between PSD-95 and NR2B-subtype receptors exists in intrinsic spinal neurones and not in primary afferent fibres, and so the target of Tat-NR2B9c is exclusively post-synaptic in the dorsal horn of the spinal cord. This would concur with the electrophysiological results which indicate a lack of effect of Tat-NR2B9c on primary afferent input to WDR neurones.

Further evidence for a role of spinal NR2B in neuronal nociceptive plasticity was provided by studies into spinal LTP. High-frequency stimulation of the rat sciatic

nerve induces a long-lasting potentiation of C-fibre evoked responses of dorsal horn WDR neurones which is sensitive to blockade of spinal NMDA receptors (Svendsen et al., 1998) and is also significantly inhibited by spinal application of the NR2B antagonist Ro-256981 (Pedersen and Gjerstad, 2007; Qu et al., 2009). More recently, LeBlanc and colleagues established that PSD-95 interactions are required for spinal LTP. The potentiation of C-fibre evoked responses of dorsal horn WDR neurones following high frequency stimulation of the sciatic nerve was blocked by a peptide which interferes with interactions via the three PDZ domains of PSD-95 (LeBlanc et al., 2010). However, which specific interactions with PSD-95 involved in this spinal LTP were again not identified. It is likely that the interaction between NR2B subunits and PSD-95 would be involved and this could be established in future studies by assessing the effects of Tat-NR2B9c in spinal LTP. It has, however, been established that coupling between NR2B subunits and PSD-95 is important in hippocampal LTP. Gardoni and colleagues employed Tat-NR2B9c and showed that disruption of binding between PSD-95 and NR2B-subtype receptors reduced induction of LTP but not LTD. This effect was associated with a reduction of NR2B-subtype but not NR2A-subtype receptors at hippocampal synapses (Gardoni et al., 2009). This again demonstrates selectivity of Tat-NR2B9c for NR2B rather than NR2A NMDA receptor subunits. The importance of NR2B subunits in hippocampal LTP has also been shown by studies in both mice and rats which were genetically engineered to overexpress NR2B in their forebrains. These rodents have enhanced LTP, but not LTD, underlying enhanced learning and memory which is maintained in aged animals (Tang et al., 1999; Tang et al., 2001; Cao et al., 2007; Wang et al., 2009). A recent study highlighted the importance of the C-tail of NR2B subunits in LTP. Firstly, NR2B antagonism by Ro-256981 and NR2B siRNA blocked induction of LTP, whereas NR2A siRNA did not. This effect was reversed by overexpression of NR2B but not NR2A. Most interesting was the finding that a chimera of the NR2A subunit fused with the C-tail of the NR2B subunit was able to rescue LTP induction, while the converse chimera could not (Foster et al., 2010). This implicates interactions with the C-tail of the NR2B subunit as being essential for LTP induction, with PSD-95 likely to be involved. CaMKII is known to bind to NR2B subunits via the C-tail and this interaction is essential for LTP (Barria and Malinow, 2005). In a model of neuropathic pain, it was shown that association between CaMKII and NMDA

receptors increases after neuropathy, and this mechanism requires PSD-95 (Garry et al., 2003).

At present, the downstream mechanisms involved in the inhibition by Tat-NR2B9c remain to be elucidated. However, we may speculate as to which effector pathways and intracellular signalling molecules may be involved based on previous studies employing Tat-NR2B9c. This peptide was neuroprotective in a rat model of cerebral ischaemia (Aarts et al., 2002) and reduced the production of NO following NMDA receptor stimulation. Another study showed that Tat-NR2B9c reduced the phosphorylation of ERK, which occurred after co-stimulation of NMDA and mGluR5 receptors (Yang et al., 2004b). In both studies, the mechanism of action of Tat-NR2B9c was a disruption of coupling between NR2B subunits and PSD-95. As mentioned previously, PSD-95 mutant mice fail to exhibit an increase in CaMKII activity following peripheral nerve injury unlike wild-type mice, suggesting that PSD-95 can bind CaMKII for direct activation by Ca^{2+} influx via NMDA receptors (Garry et al., 2003). Both ERK and CaMKII have previously been implicated in central sensitization (Ji et al., 1999; Fang et al., 2002; Pezet et al., 2008). It is also known that binding of PSD-95 to NMDA subunits stabilizes the receptor in the cell membrane (Roche et al., 2001). Thus Tat-NR2B9c may decrease neuronal hyperexcitability by interfering with membrane localization of NMDA receptors. However, the fact that Tat-NR2B9c has no effects on NMDA-mediated excitatory post-synaptic currents (EPSCs) and Ca^{2+} influx in the hippocampus may suggest otherwise (Aarts et al., 2002).

These results demonstrate that NMDA receptor-dependent phenomena, such as wind-up, can be prevented without the requirement for receptor blockade, a likely cause of the numerous neurological side-effects associated with NMDA antagonists. Tat-NR2B9c is suitable for systemic administration since it has been shown to cross the blood-brain barrier (Aarts et al., 2002), raising the potential for its use as a treatment for chronic pain. The next chapter will explore this exciting therapeutic potential in a model of neuropathic pain.

4 Interactions between spinal NR2B-containing NMDA receptors and PSD-95 contribute to maintenance of nerve injury-induced pain

4.1 Introduction

4.1.1 Neuropathic Pain

Neuropathic pain is currently defined by IASP as 'pain initiated or caused by a primary lesion or dysfunction in the nervous system' (Merskey and Boduk, 1994). Recently, however, a group of leading pain clinicians and scientists have come together and put forward their suggestions for improvement of this definition (Treede et al., 2008). One of the issues raised is regarding use of the word 'dysfunction' within the nervous system since it is difficult to distinguish from normal physiological or indeed pathological neuroplasticity that does not involve nerve damage. Therefore 'dysfunction' should be replaced by the word 'disease' which specifies identifiable disease processes such as diabetes, autoimmune conditions or channelopathies. In addition, the current definition does not distinguish between neuropathic pain and pain which may arise from other neurological disorders, for example, musculoskeletal pain which can be associated with spasticity mediated by nociceptive muscle afferents. Therefore, 'nervous system' is replaced in the definition by the more restrictive term 'somatosensory system'. Thus, a new definition for neuropathic pain would read as 'pain arising as a direct consequence of a lesion or disease of the somatosensory system' (Treede et al., 2008).

4.1.2 Clinical presentation, epidemiology & treatment of neuropathic pain

Neuropathic pain constitutes a heterogeneous group of diseases and lesions that produce a common syndrome characterized by pain (Jensen et al., 2001). Such conditions include nerve compression due to cancers, neuropathies in metabolic diseases such as diabetes and CNS disorders such as stroke and multiple sclerosis (Table 4.1 provides an incomplete list of some of these disorders). A subset of

patients suffering from such conditions may develop pain as a direct consequence. These pains also differ by anatomical location, associated with territories which have lost their normal afferent input to the CNS (Jensen et al., 2009). In the clinic neuropathic pain is characterized by the presence of specific symptoms: 1) Pain in a neuroanatomical area with partial or complete sensory loss; 2) The presence of stimulus-independent (spontaneous) ongoing pains; 3) The presence of stimulus-dependent evoked pains (hyperalgesia, allodynia, movement-induced pain); 4) Aftersensations (pain outlasting the stimulus); 5) Abnormal summation of pain (increased pain following repetitive stimulation) i.e. wind-up (Jensen et al., 2009). Thus there are both negative and positive signs and symptoms.

In 2006, Hall and colleagues conducted a large UK study to gain an insight into the incidence of neuropathic pain. In their epidemiological analysis of 6.8 million patients, 39,731 were found to suffer from one of four neuropathic pain conditions, of which 25,200 cases were incident during the period of observation. Post-herpetic neuralgia (PHN) accounted for 12,386 (31%) of these cases, trigeminal neuralgia represented 8,268 (21%) cases, incidence of phantom limb pain was 451 (5%) cases and painful diabetic neuropathy (PDN) accounted for 4,719 (12%) of cases. In terms of the general population, these incidences per 100,000 person years correspond to 40.2 for PHN, 26.8 for trigeminal neuralgia, 1.5 for phantom limb pain and 15.3 for PDN (Hall et al., 2006).

Current management of neuropathic pain is unsatisfactory and often patients continue to suffer pain of moderate severity despite taking prescribed medications. Results from randomized control trials typically report less than half of patients obtaining satisfactory pain relief and side-effects are common (O'Connor and Dworkin, 2009). Pharmacological control of neuropathic pain often involves a trial and error process to find a satisfactory therapy and patients are regularly given combinations of medicines which have not necessarily been validated in clinical trials (Jensen et al., 2009). Numerous agents are prescribed clinically for the treatment of neuropathic pain including anticonvulsants, antidepressants, opioids, topical lidocaine and capsaicin, and NMDA antagonists (Jensen et al., 2009). Recommended first-line treatments, agreed upon by both the IASP Neuropathic Pain Special Interest Group (NeuPSIG) (O'Connor and Dworkin, 2009) and the European

Federation of Neurological Societies (EFNS) (Attal et al., 2006), are the tricyclic antidepressants (TCAs), such as amitriptyline, and the anticonvulsants, gabapentin and pregabalin, for PHN, peripheral polyneuropathy (PPN) and central pain (CP). Selective serotonin and noradrenaline reuptake inhibitors (SSNRIs), such as venlafaxine and duloxetine, are considered first-second line treatments for PPN. Topical lidocaine is used as a first-line treatment for localized peripheral neuropathic pain. Opioid analgesics and tramadol are generally considered second-third line treatments for PHN, PPN and CP, though can be used as first-line treatments for acute and severe neuropathic pain as well as cancer pain. Topical capsaicin and drugs such as mexiletine and the NMDA antagonists, dextromethorphan and memantine, have shown inconsistent results in randomized clinical trials and may be used as third-line treatments based on individual circumstances (Attal et al., 2006; O'Connor and Dworkin, 2009).

Peripheral	Spinal	Brain
Neuropathies (Diabetic, HIV-induced etc)	Multiple Sclerosis	Stroke
Post-herpetic Neuralgia	Spinal Cord Injury	Multiple Sclerosis
Trigeminal Neuralgia	Arachnoiditis	Cancer
Nerve Injuries	Cancer	Syringomyelia
Amputations	Syringomyelia	
Cancer	Spinal stroke (Ischaemia)	
Plexopathies		
Radiculopathies		
Avulsions		

Table 4.1: Some common Neuropathic Pain diseases and conditions.
(adapted from Jensen et al., 2001)

4.1.3 Rodent models of nerve injury-induced neuropathic pain

During the past 25 years or so, animal models of various neuropathies have been used to try to understand the mechanisms contributing to neuropathic pain. The majority of research in this area has utilized rodent models involving some kind of physical trauma of a peripheral nerve, usually the sciatic nerve, to mimic peripheral nerve injury (Zimmermann, 2001). To date, the three most commonly used peripheral nerve injury models are the Chronic Constriction Injury model (CCI), the Partial Sciatic Nerve Ligation model (PSNL) and the Spinal Nerve Ligation model (SNL).

The CCI model is induced by exposing the sciatic nerve at the mid-thigh level and placing four loosely-tied ligatures around the nerve, with 1 mm spacing between each (Bennett and Xie, 1988). The PSNL model involves exposure of the sciatic nerve at the high-thigh level and placing a ligature through the nerve, so that one-third to a half of the nerve thickness is trapped within the ligature (Seltzer et al., 1990). The last of these models to be developed was the SNL model which is induced by exposing and then tightly ligating the L5 and L6 spinal nerves, between the DRG and formation of the full sciatic nerve (Kim and Chung, 1992). This SNL model is sometimes modified to ligation of the L5 spinal nerve only.

A later study by Kim and colleagues compared the three models and showed that rats with all three types of nerve injury developed signs of mechanical and cold hypersensitivity, as well as ongoing (spontaneous) pain (Kim et al., 1997). The time course for evoked pain-related behaviours was similar in all models, developing by day 1 and lasting for a number of weeks. The magnitude of responses to mechanical stimulation was, however, varied between models. The greatest degree of mechanical hypersensitivity was seen in the SNL model and least in the CCI model. Inter-animal variability was greatest in the CCI model and least in the SNL model, suggesting better reproducibility in the SNL model. The contralateral paw was mainly unresponsive in each model. Responses to acetone of the injured paw were similar between models. Ongoing pain was found to be greatest in the CCI model, though with the most variability between animals, while the SNL model displayed the least spontaneous pain. However, frequency of spontaneous behaviours in preclinical

research is rarely monitored due to the difficulty in measurement and interpretation (Mao, 2009). This is arguably a major limitation of these models since spontaneous pain is one of the most common clinical signs observed in neuropathic pain patients.

With these comparisons in mind, our lab routinely uses the SNL model both *in vivo* electrophysiological and behavioural experiments, since this model produces the greatest magnitude of hypersensitivity and is the most reproducible in terms of inter-animal variability. This high reproducibility could be due to the tight ligatures placed around whole spinal nerves rather than loose ones used in CCI and only partial ligation in the PSNL model which is not always consistent in terms of the thickness of the nerve that is ligated. Studies from our lab have validated the use of this model in both electrophysiological and behavioural experiments. Neuronal plasticity has been demonstrated in this model, in particular, an increase in spontaneous neuronal activity in SNL rats (Chapman et al., 1998a). In addition, drugs used in the clinical management of neuropathic pain and which show plasticity in terms of their efficacy following nerve injury, such as gabapentin and morphine, as well as NMDA antagonists such as ketamine and memantine, have been validated in this model using both electrophysiological and behavioural measures (Yaksh et al., 1995; Chaplan et al., 1997; Hunter et al., 1997; Chapman et al., 1998b; Suzuki et al., 1999; Suzuki et al., 2001; Matthews and Dickenson, 2002).

4.1.4 Contribution of NR2B-containing NMDA receptors to neuropathic pain

Numerous studies have shown that activity of NMDA receptors is crucial to the establishment of various chronic pain states, including pain induced by nerve injury. Blockade of NMDA receptors, with antagonists such as MK-801 and ketamine, can inhibit mechanical and thermal hypersensitivity in animal models of neuropathic pain. Accordingly, human studies of various NMDA antagonists have shown analgesic efficacy in various neuropathic pain states, though intolerable cognitive and memory side-effects were common (see Chapter 1).

Targeting the NR2B subunit has been shown to be an improvement on non-selective NMDA antagonists by combining good analgesic efficacy in models of pain with fewer side-effects (Chizh et al., 2001a; Gogas, 2006; Wu and Zhuo, 2009). As already highlighted in Chapter 3, the NR2B subunit is ideally located to play a role in pain processing from the periphery to the spinal cord. This subunit is found in both A and C-fibres (Boyce et al., 1999; Ma and Hargreaves, 2001; Marvizon et al., 2002) and its expression in the spinal cord seems to be highly concentrated in the superficial dorsal horn where nociceptive afferent fibres terminate. In contrast, the NR2A subunit has been found throughout the spinal cord (Nagy et al., 2004a). Therefore, targeting NR2B subunits, for example through the use of non-competitive NR2B subtype-specific antagonists such as ifenprodil, CP-101606 and Ro-256981, may selectively target pain whilst leaving normal sensory function intact. Indeed several studies have shown great efficacy of NR2B-selective antagonists in neuropathic pain states. Boyce and colleagues first demonstrated that systemic administration of ifenprodil, CP-101606 and Ro-256981 could reverse signs of neuropathic pain whilst having no effect on motor performance, as measured by the rotarod, at antinociceptive doses. This was in contrast to the non-selective NMDA antagonist MK-801 and also gabapentin which affected both hypersensitivity and motor performance at the same doses (Boyce et al., 1999). Later, Abe and colleagues showed that CP-101606 reversed mechanical hypersensitivity produced by L5 spinal nerve transection in mice, while not producing motor dysfunction or affecting the non-injured paw. Tyrosine phosphorylation of the NR2B subunit (at Tyr1472) in the spinal dorsal horn correlated with signs of neuropathic pain and was also reversed by CP-101606. Interestingly, both NR2A and NR2D knockout mice

developed neuropathic pain normally in this model, again implicating NR2B as the major player in chronic pain (Abe et al., 2005).

It has been suggested that not spinal but in fact brain NR2B-subtype receptors are responsible for the antinociceptive effects of NR2B-selective antagonists in models of chronic pain (Chizh et al., 2001b; Nakazato et al., 2005). However, more recent studies using spinal administration of these antagonists have clearly demonstrated a role for spinal NR2B-containing NMDA receptors in neuropathic pain. Malmberg and colleagues showed than cone snail venom-derived conantokins, which block NR2B-subtype receptors, were able to suppress both mechanical and thermal hypersensitivity when delivered via intrathecal lumbar puncture at doses which did not impair motor function (Malmberg et al., 2003). Qu and colleagues administered ifenprodil and Ro-256981 directly to the spinal cord via intrathecal injections and were able to inhibit mechanical hypersensitivity, without altering motor function, when given both before L5 SNL surgery and following establishment of the neuropathic pain state (Qu et al., 2009). In addition, intrathecal administration of NR2B-selective antagonists blocked spinal LTP, suggesting that NR2B-subtype receptors contribute to spinal synaptic plasticity in establishing a chronic pain state. Nerve injury is also associated with increased expression of NR2B protein in the spinal dorsal horn, particularly during the maintenance phase of neuropathic pain (Wilson et al., 2005; Geng et al., 2010). Bone cancer pain, a different type of chronic pain state, has recently been shown to involve spinal NR2B-subtype receptors (Gu et al., 2009). The role of NR2B subunit-containing NMDA receptors in the brain cannot be ruled out from being involved in chronic pain. Mice overexpressing the NR2B subunit in the forebrain have enhanced pain following tissue injury (Wei et al., 2001) and peripheral inflammation increases expression of NR2B in the anterior cingulate cortex (Wu et al., 2005). Regardless of the site of action, it is clear that NR2B-subtype selective antagonists demonstrate good analgesic performance in animal models of chronic pain, particularly after nerve injury, without the incidence of psychotomimetic and motor side-effects associated with non-specific NMDA antagonists. Clinical data on the effects of NR2B-subtype antagonists in chronic pain is limited at present. One compound, CP-101606 (traxoprodil) has progressed to phase II clinical trials, and preliminary findings show effective pain relief in patients suffering from central pain, such as following spinal cord injury, without typical psychotomimetic side-effects

(Gogas, 2006; Childers and Baudy, 2007). Aside from their analgesic potential, clinical tolerability of these compounds has been demonstrated with a lack of psychotomimetic side-effects, cognitive disruption and euphoria (Patat et al., 1994; Marquis et al., 1998; Merchant et al., 1999; Chizh et al., 2001a). Therefore, the NR2B-subtype receptor is a viable molecular target for analgesia which should be investigated further in chronic pain states, particularly nerve injury-induced pain.

4.1.5 Involvement of PSD-95 in neuropathic pain

Four previous studies have demonstrated a role for PSD-95 in neuropathic pain. Using antisense oligodeoxynucleotides to specifically knockdown spinal expression of PSD-95, Tao and colleagues investigated the role of PSD-95 in both the development and maintenance of neuropathic pain (Tao et al., 2001; Tao et al., 2003a). The model employed was unilateral ligation and transection of the L5 spinal nerve which resulted in mechanical and thermal hypersensitivity from day 3 onwards. In the first study, rats were treated for four continuous days with antisense against PSD-95 mRNA (messenger ribonucleic acid), beginning on the day before surgery, via an intrathecal catheter. It was found that knockdown of PSD-95 delayed the development of both mechanical and thermal hypersensitivity until day 7 post-surgery, and by day 9 antisense-treated animals showed similar hypersensitivity as control rats treated with saline. Perhaps the effects of the antisense treatment would have diminished by day 9 and expression of PSD-95 may have returned to untreated control levels. These results established that PSD-95 has a role in the development of central sensitization in neuropathic pain. A second study by the same group showed that PSD-95 is involved in the maintenance of nerve injury-induced pain. This time rats were treated with antisense against PSD-95, via an intrathecal catheter, on days 7-10 post-surgery. This resulted in a cessation of both mechanical and thermal hypersensitivity on day 11. On day 13 responses had retuned to pre-antisense levels. Importantly, antisense treatment in normal animals had no effect on baseline mechanical and thermal sensitivity, or on locomotor function, suggesting that PSD-95 is involved specifically in the maintenance of sensory hypersensitivity. Together, both theses studies established PSD-95 as a relevant target for treatment of neuropathic pain. A third study by Garry and colleagues utilized PSD-95 mutant

mice to show the involvement of PSD-95 in neuropathic pain (Garry et al., 2003). These mice express a short form of PSD-95 which is truncated at the third PDZ domain. Both PSD-95 mutant mice and wild-type mice underwent CCI surgery to induce neuropathic pain. Wild-type mice showed clear thermal, mechanical and cold hypersensitivity but this was either absent in PSD-95 mutant mice, or at least markedly reduced, as in the case of cold hypersensitivity. In addition, it was deduced that PSD-95 contributes to neuropathic sensitization via a mechanism involving phosphorylation of NMDA receptors by CaMKII. These results in PSD-95 mutant mice, therefore, reinforced those obtained from studies using antisense knockdown in demonstrating a role for PSD-95 in neuropathic pain. Finally, LeBlanc and colleagues recently showed that PSD-95 mediated protein-protein interactions are essential for nerve injury-induced pain (LeBlanc et al., 2010). A cyclic peptide which binds to the three PDZ domains of PSD-95, thus preventing binding to other proteins via these domains, was found to reduce mechanically evoked neuronal responses in CCI rats as well thermal hypersensitivity in behavioural experiments.

Clearly, both NMDA receptors, particularly those of the NR2B-subtype, and PSD-95 have separately been shown to be important components of central sensitization in neuropathic pain states. The experiments in this chapter now aim to investigate the role of the specific interaction between spinal NR2B-containing NMDA receptors and PSD-95 in contributing to nerve injury-induced pain by employing the mimetic peptide Tat-NR2B9c, believed to disrupt binding between NR2B subunits and PSD-95.

4.2 Materials and Methods

4.2.1 Spinal Nerve Ligation (SNL) Model

SNL surgery was conducted in rats to induce a neuropathic pain state (as described in Materials and Methods chapter). Sham surgery rats were used as controls in electrophysiological experiments.

Establishment of behavioural hypersensitivity to low intensity mechanical (von Frey filaments 1, 6 and 8 g) and cooling stimuli (acetone) was assessed on post-operative days 2, 4, 7, 9, 11 and 14. Responses of both ipsilateral (IPSI) and contralateral (CONTRA) paws were recorded and difference scores were then calculated (see Material and Methods for protocol). Rats were then used for *in vivo* electrophysiological studies on days 15-18. Both behavioural testing and electrophysiological experiments were conducted blind so that it was not known whether each animal had undergone SNL or sham operation.

4.2.2 *In vivo* Electrophysiology

Electrophysiological recordings from deep dorsal horn WDR neurones (> 600 μm) were conducted in both SNL and sham-operated rats, as previously described (see Materials and Methods chapter).

4.2.2.1 Electrical and natural stimulation of the hindpaw

Electrical and natural tests were conduced in separate animals to avoid overstimulation and sensitization of the sensory system. Following the establishment of stable control responses, 12.5 ng of Tat-NR2B9c in a volume of 50 μl was injected topically onto the surface of the spinal cord (after removal of any residual cerebrospinal fluid) using a Hamilton syringe. Electrical tests were continued at 10 min intervals for 1 hr. Natural tests (brush followed by von Frey filaments 1, 8, 15

and 26 g, followed by temperatures 35, 40, 45 and 48°C) were conducted at 20 min intervals for 1 hr.

4.2.3 Behavioural assessment of effects of Tat-NR2B9c on maintenance of mechanical and cold hypersensitivity induced by spinal nerve ligation

Baseline mechanical PWTs and responses to a cooling stimulus (acetone) were detected 4 days prior to SNL surgery (see Materials and Methods for protocol). Testing was then repeated in the post-operative period on days 2, 7, 10 and 14/16 to observe the development of mechanical and cold hypersensitivity in the ipsilateral injured paw versus the contralateral paw. On day 14/16, following initial sensory testing, animals were treated with either Tat-NR2B9c or Tat-NR2BAA (both 125 ng in a volume of 10 μl, n = 8 in each group) via intrathecal lumbar puncture. Following drug treatment, sensory testing recommenced and PWTs and responses to acetone were assessed at 30 min, 60 min, 90 min, 2 hr, 3hr, 4 hr and 5 hr after intrathecal injection. Experimenters were blind to treatment.

4.2.4 Assessment of effects of Tat-NR2B9c on locomotor function

Rats were trained to remain on the rotarod for a minimum of 60 s (see Materials and Methods chapter). Locomotor function was assessed following intrathecal administration of Tat-NR2B9c (125 ng, n = 4) via lumbar puncture. Tests were carried out before injection (baseline) and at 20, 40, 60 and 120 min post-injection.

4.2.5 Statistical Analysis

Data are presented as mean ± s.e.m. Difference scores were compared between SNL and sham-operated rats by the Mann-Whitney rank sum test. The effects of Tat-NR2B9c following electrical stimulation of the hindpaw were assessed by one-way RM ANOVA, followed by Bonferroni multiple comparison post-tests. Cell characteristics from SNL and sham-operated rats were compared by Student's unpaired t-tests, except for responses to graded mechanical and thermal stimulation which were compared by two-way RM ANOVA with Bonferroni post-tests. Effects of Tat-NR2B9c following natural stimulation (von Frey and heat) of the hindpaw were assessed by two-way RM ANOVA, followed by Bonferroni post-tests. Brush-evoked responses were compared using one-way RM ANOVA followed by Bonferroni post-tests. Two-way RM ANOVA followed by Bonferroni post-tests was used to compare PWTs and responses to acetone between ipsilateral and contralateral paws in SNL rats as well as effects of Tat-NR2B9c and Tat-NR2BAA. One-way RM ANOVA, followed by Dunnett's post-test, was used to assess effects of Tat-NR2B9c in the rotarod motor function test. Statistical analyses were carried out using GraphPad Prism v.4 software (GraphPad Software Inc., San Diego, CA, USA).

4.3 Results

4.3.1 SNL induces behavioural mechanical and cold hypersensitivity of the injured paw indicating establishment of a neuropathic pain state

Difference scores were calculated for SNL (n = 10) and sham-operated (n = 8) rats to assess behavioural sensitivity to mechanical and cold stimuli (Fig. 4.1). Von Frey filaments 1, 6 and 8 g each produced a greater number of paw withdrawal responses when applied to the injured ipsilateral paw compared to the non-injured contralateral paw in SNL rats, as indicated by positive difference scores. This mechanical hypersensitivity began on day 2 post-surgery and was maintained throughout the testing period until day 14 (Fig. 4.1a-c). Significantly higher difference scores were seen with von Frey 1 g on post-surgical day 2 (SNL difference score: 2.7 ± 0.8, sham difference score: 0.5 ± 0.6, $p < 0.05$), day 4 (SNL: 2.1 ± 0.6, sham: -1.1 ± 0.5, $p < 0.001$) and day 9 (SNL: 3.3 ± 0.9, sham: -1.0 ± 0.5, $p < 0.05$), with von Frey 6 on post-surgical day 2 (SNL: 4.8 ± 0.6, sham: -0.4 ± 1.2, $p < 0.001$), day 4 (SNL: 3.7 ± 0.7, sham: -0.3 ± 0.5, $p < 0.01$), day 7 (SNL: 3.3 ± 0.8, sham: 0.0 ± 0.8, $p < 0.05$) and day 14 (SNL: 3.8 ± 0.9, sham: -1.3 ± 0.6, $p < 0.05$) and with von Frey 8 on post-surgical day 2 (SNL: 4.3 ± 0.8, sham: 0.0 ± 0.7, $p < 0.01$), day 4 (SNL: 4.1 ± 0.9, sham: -0.4 ± 0.8, $p < 0.01$), day 9 (SNL: 2.8 ± 1.4, sham: -0.5 ± 0.8, $p < 0.05$) and day 11 (SNL: 2.4 ± 1.3, sham: -2.0 ± 0.6, $p < 0.05$) in SNL rats compared to sham rats. Acetone induced greater paw withdrawals of the ipsilateral paw compared to the contralateral paw in SNL rats rather than sham rats. Again this cold hypersensitivity began on day 2 post-surgery and persisted until day 14 (Fig. 4.1d). Significantly higher difference scores in SNL rats compared to sham rats were seen on post-surgical day 2 (SNL: 2.1 ± 0.5, sham: -0.1 ± 0.2, $p < 0.001$), day 4 (SNL: 2.4 ± 0.5, sham: -0.6 ± 0.5, $p < 0.001$), day 7 (SNL: 2.2 ± 0.3, sham: -0.7 ± 0.7, $p < 0.01$) and day 11 (SNL: 2.2 ± 0.4, sham: -0.7 ± 0.7, $p < 0.05$).

Therefore, it is clear that spinal nerve ligation (but not sham) surgery in rats results in mechanical and cold hypersensitivity of the injured ipsilateral hindpaw, beginning on day 2 after surgery and persisting for at least 2 weeks.

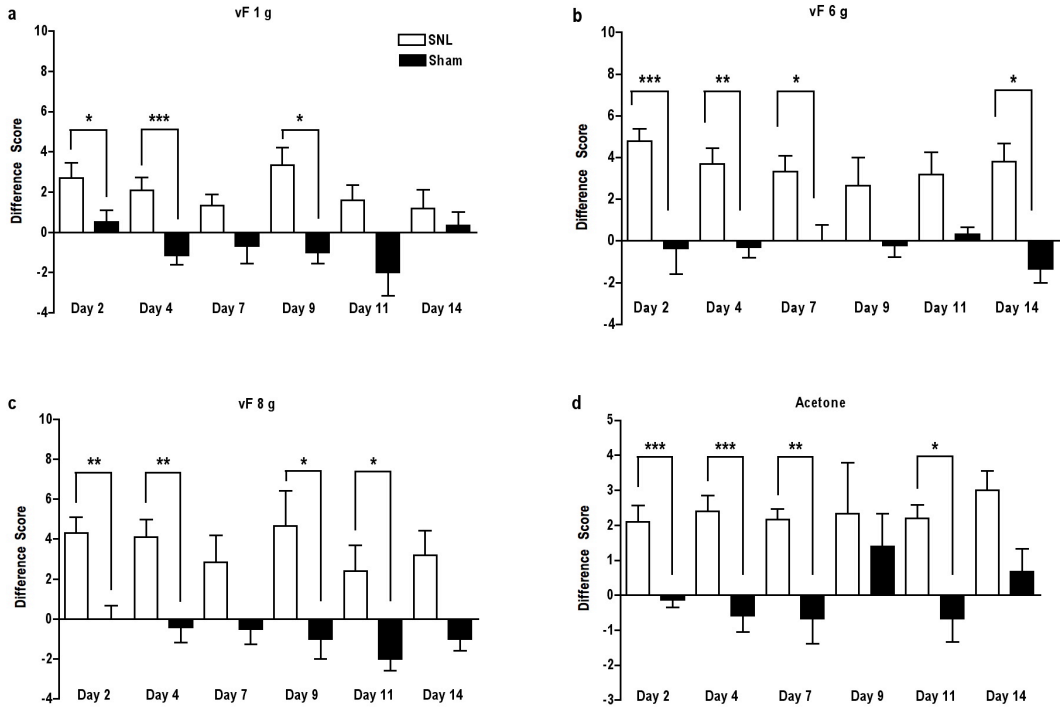


Fig. 4.1: SNL surgery induces robust and long-lasting behavioural mechanical and cold hypersensitivity of the injured paw. Difference scores (the number of paw withdrawals of the ipsilateral paw – the number of paw withdrawals of the contralateral paw) were calculated for responses to von Frey hairs 1, 6 and 8 g and acetone in both sham (n = 8) and SNL (n = 10) rats. Assessment of difference scores was conducted on days 2, 4, 7, 9, 11 and 14 post-surgery to confirm development of nerve injury-induced pain-related behaviour. Data presented as mean ± s.e.m., $*$ $p < 0.05$, $**$ $p < 0.01$, $***$ $p < 0.001$ between surgical groups, Mann-Whitney rank sum test.

4.3.2 Spinal Tat-NR2B9c does not alter afferent-evoked responses but inhibits post-discharge of deep dorsal horn WDR neurones in SNL and sham-operated rats

The effects of spinal Tat-NR2B9c on electrically evoked responses of deep dorsal horn WDR neurones were compared in SNL and sham-operated rats (n = 8 in each group). Responses of WDR neurones due to primary afferent activity were unaltered in both groups following spinal Tat-NR2B9c (12.5 ng). No changes were seen in Aβ-fibre, Aδ-fibre or C-fibre evoked responses (Fig. 4.2). Post-discharge, however, was reduced in both SNL and sham rats to a similar extent (Fig. 4.2; SNL: % of pre-drug baseline = 60 ± 11%, $p < 0.01$; Sham: % of pre-drug baseline = 52 ± 13.0%, $p < 0.01$). This inhibitory effect of Tat-NR2B9c on the post-discharge of WDR neurones was similar to that seen in naïve rats (Chapter 3, Fig. 3.4). Pre-drug control responses did not differ between surgical groups (Table 4.2).

	Sham (*n* = 8)	SNL (*n* = 8)
C-fibre threshold (mA)	0.67 ± 0.18	0.88 ± 0.14
Depth (*μ*M)	965 ± 42	935 ± 60
Aβ-fibre evoked response (No. of APs)	111 ± 12	130 ± 21
Aδ-fibre evoked response (No. of APs)	223 ± 29	236 ± 35
C-fibre evoked response (No. of APs)	715 ± 113	726 ± 89
Post-discharge (No. of APs)	603 ± 178	475 ± 85
Input (No. of APs)	373 ± 50	417 ± 47
Wind-up (No. of APs)	1044 ± 272	864 ± 143

Table 4.2: Pre-drug control responses induced by transcutaneous electrical stimulation of the hindpaw receptive field of WDR neurones in sham and SNL rats. C-fibre threshold, cell depth, afferent-evoked responses, input and wind-up are expressed as mean ± s.e.m. There were no differences between cells recorded in each surgical group for any measure prior to treatment; Student's unpaired t-test.

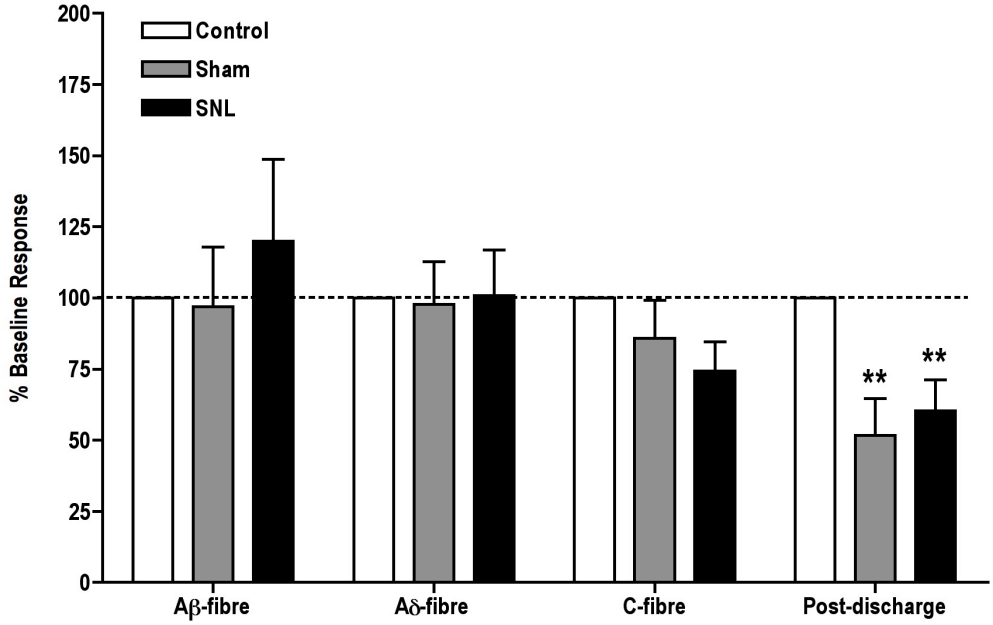


Fig. 4.2: Spinal Tat-NR2B9c decreases hyperexcitability of WDR neurones in both sham and SNL rats. Afferent-evoked responses and post-discharge of WDR neurones induced by transcutaneous electrical stimulation of the hindpaw receptive field in sham and SNL rats and following spinal application of Tat-NR2B9c (12.5 ng). White bars represent pre-drug control. All data presented as mean ± s.e.m. of pre-drug control responses; ** $p < 0.01$ versus pre-drug baseline; one-way RM ANOVA followed by Bonferroni post-tests on raw data; $n = 8$ in each group.

4.3.3 Wind-up of deep dorsal horn WDR neurones is reduced by spinal Tat-NR2B9c in both SNL and sham-operated rats

As in naïve rats, no effect was seen with Tat-NR2B9c on input (non-potentiated response) in either SNL or sham-operated rats (Fig. 4.3a). In contrast, Tat-NR2B9c produced a significant inhibition of wind-up in both surgery groups (Fig. 4.3a-c; SNL: 12.5 ng, % of pre-drug baseline = 56 ± 10%, $p < 0.01$, $n = 8$; Sham: 12.5 ng, % of pre-drug baseline = 54 ± 12%, $p < 0.01$, $n = 8$). Inhibition of wind-up was comparable between groups and to that observed in naïve rats (Chapter 3, Fig. 3.5). Pre-drug input and wind-up values were similar in SNL and sham rats (Table 4.2).

Therefore, Tat-NR2B9c selectively reduces NMDA receptor-mediated post-discharge and wind-up of deep dorsal horn WDR neurones, while having no effect on afferent-evoked responses and input, in both SNL and sham-operated rats. The inhibitory effect of Tat-NR2B9c on post-discharge and wind-up is not altered in neuropathy.

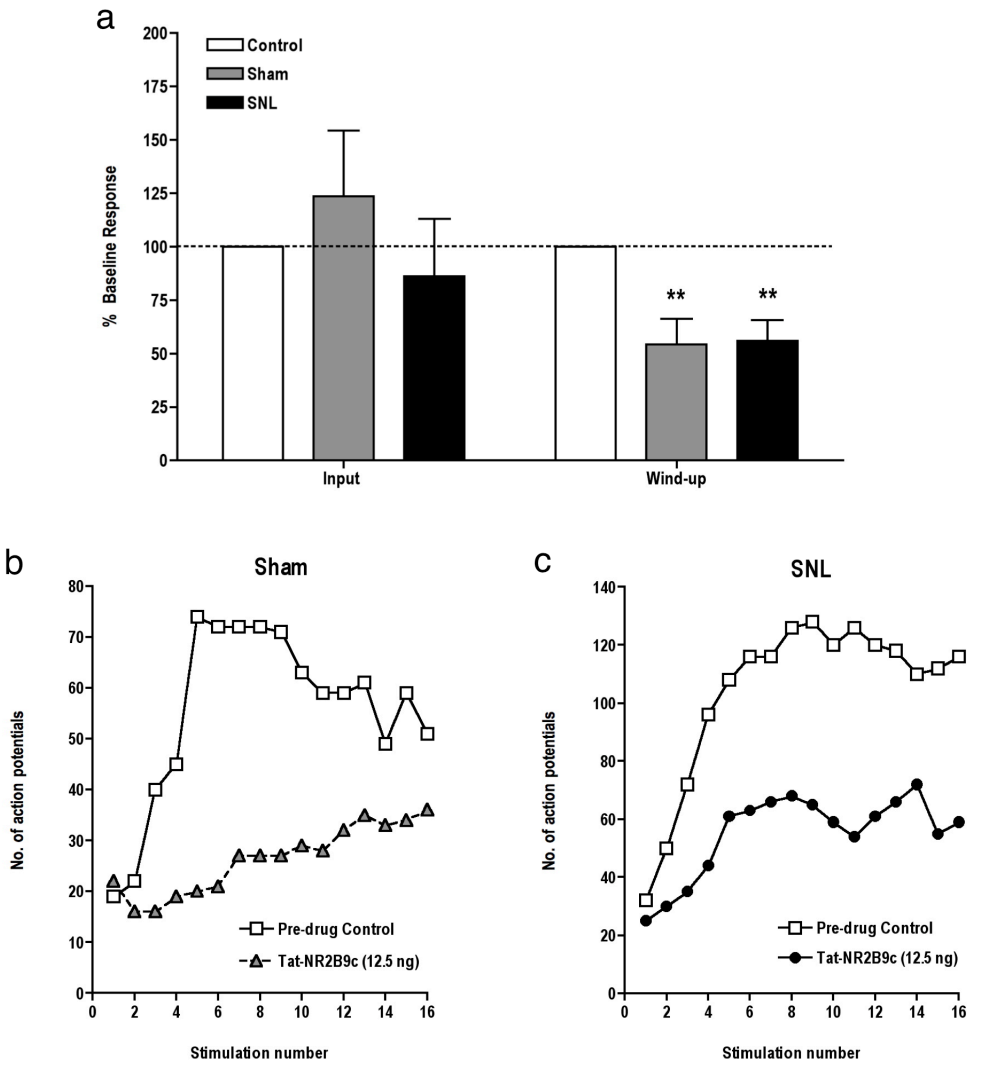


Fig. 4.3: Spinal Tat-NR2B9c selectively reduces wind-up of WDR neurones in both sham and SNL rats. (a) Effect of spinal application of Tat-NR2B9c (12.5 ng) on input and wind-up of WDR neurones in sham and SNL rats. White bars represent pre-drug control. All data presented as mean ± s.e.m. of pre-drug control responses; ** $p < 0.01$ versus pre-drug baseline; one-way RM ANOVA followed by Bonferroni post-tests on raw data; $n = 8$ in each group. **(b,c)** Examples of the wind-up of single WDR neurones following repetitive electrical stimulation in the presence of spinal Tat-NR2B9c (12.5 ng) in **(b)** sham and **(c)** SNL rats.

4.3.4 Responses of deep dorsal horn WDR neurones evoked by natural stimuli are reduced by spinal application of Tat-NR2B9c in SNL and sham-operated rats

Next, the responses of deep dorsal horn WDR neurones to natural stimuli applied to the hindpaw receptive field were assessed. The pre-drug brush evoked response was higher in sham rats compared to SNL rats, though this difference was not statistically significant (Table 4.3; Sham: mean number of action potentials = 473 ± 92, $n = 6$, SNL: mean number of action potentials = 367 ± 73, $n = 6$; $p > 0.05$). Spinal application of Tat-NR2B9c minimally reduced the response to brush in both surgical groups to a similar level, but this effect did not reach statistical significance (Fig. 4.4; 12.5 ng, $n = 6$ in each group, Sham: APs = 298 ± 88, $p > 0.05$; SNL: APs = 270 ± 137; $p > 0.05$).

Mechanical punctate stimulation of the hindpaw was delivered by different von Frey filaments which produced graded responses of WDR neurones that did not differ significantly between SNL and sham-operated rats (Table 4.3; $n = 6$ in each group; 1 g, Sham: mean number of APs = 0.7 ± 0.5, SNL: APs = 0.3 ± 0.3; 8 g, Sham: APs = 225 ± 68, SNL: APs = 227 ± 132; 15 g, Sham: APs = 606 ± 165, SNL: APs = 539 ± 140; 26 g, Sham: APs = 821 ± 140, SNL: APs = 1017 ± 207). Spinal application of Tat-NR2B9c (12.5 ng, $n = 6$ in each group) reduced graded responses to mechanical stimulation in both SNL and sham rats (Fig. 4.5; 1 g, Sham: APs = 0.0 ± 0.0, $p > 0.05$, SNL: APs = 0.2 ± 0.2, $p > 0.05$; 8 g, Sham: APs = 32 ± 13, $p > 0.05$, SNL: APs = 28 ± 12, $p > 0.05$; 15 g, Sham: APs = 214 ± 147, $p < 0.001$, SNL: APs = 161 ± 52, $p < 0.01$; 26 g, Sham: APs = 413 ± 127, $p < 0.001$, SNL: APs = 693 ± 224, $p < 0.01$). The effects of Tat-NR2B9c were similar in both surgical groups.

Graded responses of WDR neurones to thermal stimulation, via a water jet of different temperatures, were similar in SNL and sham-operated rats (Table 4.3; $n = 6$ in each group; 35°C, Sham: APs = 284 ± 97, SNL: APs = 73 ± 30; 40°C, Sham: APs = 346 ± 98, SNL: APs = 138 ± 60; 45°C, Sham: APs = 672 ± 106, SNL: APs = 631 ± 205; 48°C, Sham: APs = 1282 ± 102, SNL: APs = 1254 ± 131). Responses in both surgical groups were reduced by spinal application of Tat-NR2B9c (12.5 ng, $n = 6$ in

each group) to a similar degree (Fig. 4.6; 35°C, Sham: APs = 89 ± 32, $p > 0.05$, SNL: APs = 67 ± 22, $p > 0.05$; 40°C, Sham: APs = 74 ± 27, $p > 0.05$, SNL: APs = 31 ± 11, $p > 0.05$; 45°C, Sham: APs = 161 ± 93, $p < 0.05$, SNL: APs = 99 ± 43, $p < 0.001$; 48°C, Sham: APs = 1119 ± 340, $p > 0.05$, SNL: APs = 857 ± 274, $p < 0.01$). Inhibitory effects of Tat-NR2B9c were evident by 20 min following spinal administration, peaked at approximately 40 min and persisted for the duration of the experiment.

Therefore, spinal administration of Tat-NR2B9c reduces graded responses of deep dorsal horn WDR neurones to mechanical and thermal stimulation in both SNL and sham-operated rats. Brush evoked responses are minimally reduced. It would appear that the effects of Tat-NR2B9c on all evoked responses are unaltered after neuropathy.

	Sham (*n* = 6)	SNL (*n* = 6)
Brush response (No. of APs)	473 ± 92	367 ± 73
Von Frey 1 g (No. of APs)	0.7 ± 0.5	0.3 ± 0.3
Von Frey 8 g (No. of APs)	225 ± 68	227 ± 132
Von Frey 15 g (No. of APs)	606 ± 165	539 ± 140
Von Frey 26 g (No. of APs)	821 ± 140	1017 ± 207
35°C (No. of APs)	284 ± 97	73 ± 30
40°C (No. of APs)	346 ± 98	138 ± 60
45°C (No. of APs)	672 ± 106	631 ± 206
48°C (No. of APs)	1282 ± 102	1254 ± 131

Table 4.3: Pre-drug control responses induced by natural stimulation of the hindpaw receptive field of WDR neurones in sham and SNL rats. Responses evoked by brush, mechanical and thermal stimulation are expressed as mean ± s.e.m. There were no differences between cells recorded in each surgical group for any measure prior to drug treatment; Student's unpaired t-test (brush) or two-way RM ANOVA followed by Bonferroni post-tests (Von Frey and heat).

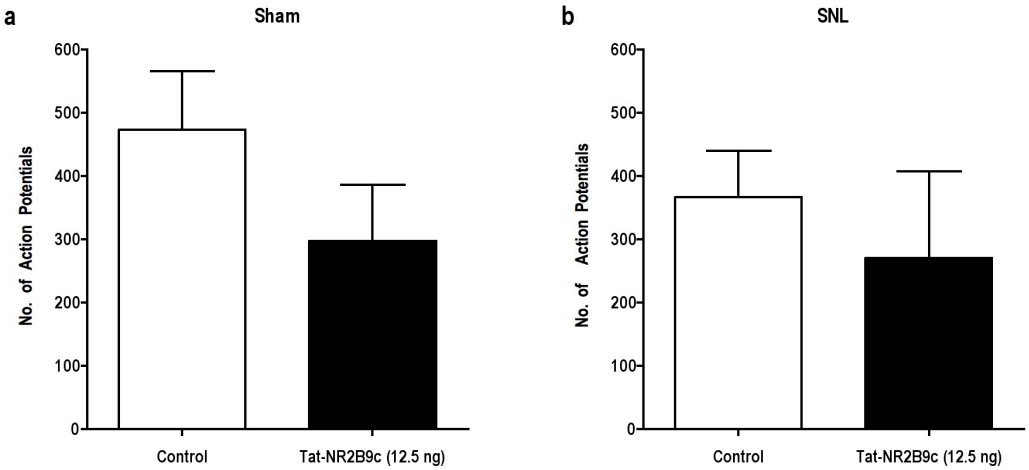


Fig. 4.4: Brush evoked responses of WDR neurones are not significantly altered in either sham or SNL rats by spinal Tat-NR2B9c. (a,b) Responses of WDR neurones to brush stimulation of the hindpaw receptive field in **(a)** sham (n = 6) and **(b)** SNL (n = 6) rats and following spinal application of Tat-NR2B9c (12.5 ng). All data presented as mean ± s.e.m., one-way RM ANOVA followed by Bonferroni post-tests.

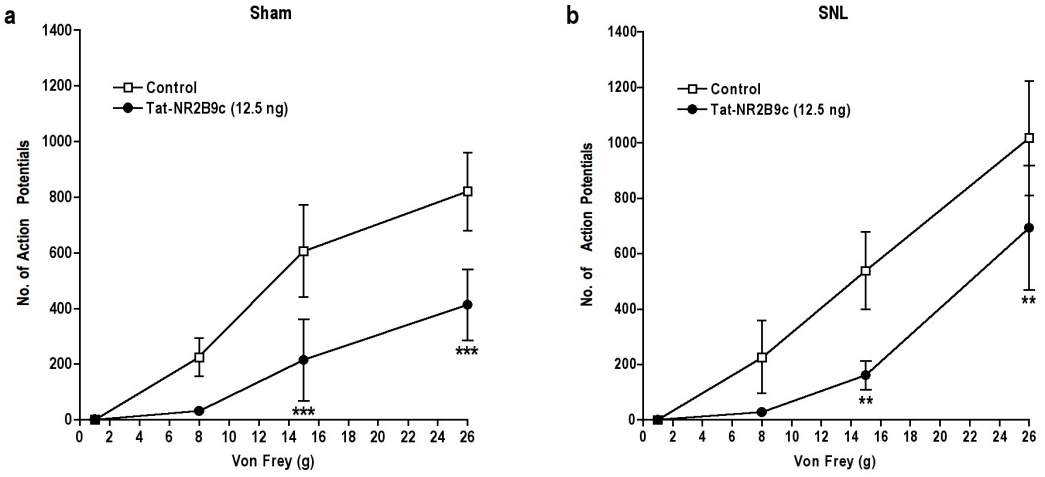


Fig. 4.5: Mechanically evoked responses of WDR neurones are reduced in both sham and SNL rats by spinal Tat-NR2B9c. **(a,b)** Responses of WDR neurones to mechanical stimulation of the hindpaw receptive field with von Frey hairs (1, 8, 15 and 26 g) in **(a)** sham ($n = 6$) and **(b)** SNL ($n = 6$) rats and following spinal application of Tat-NR2B9c (12.5 ng). All data presented as mean ± s.e.m., ** $p < 0.01$, *** $p < 0.001$, two-way RM ANOVA followed by Bonferroni post-tests.

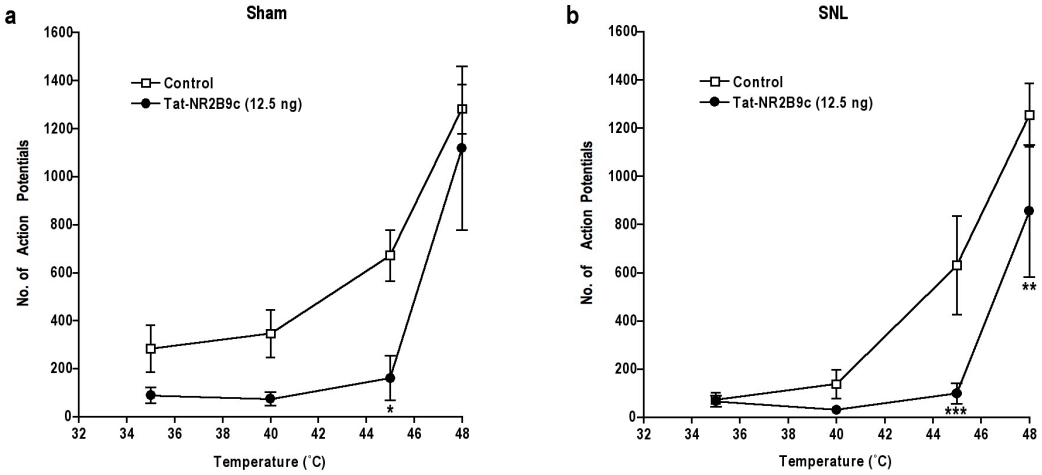


Fig. 4.6: Thermally evoked responses of WDR neurones are reduced in both sham and SNL rats by spinal Tat-NR2B9c. **(a,b)** Responses of WDR neurones to thermal stimulation of the hindpaw receptive field with different temperatures of water (35, 40, 45 and 48°C) in **(a)** sham (n = 6) and **(b)** SNL (n = 6) rats and following spinal application of Tat-NR2B9c (12.5 ng). All data presented as mean ± s.e.m., ∗ $p < 0.05$, ∗∗ $p < 0.01$, ∗∗∗ $p < 0.001$, two-way RM ANOVA followed by Bonferroni post-tests.

4.3.5 Spinal Tat-NR2B9c reverses behavioural mechanical and cold hypersensitivity in SNL rats

As reported above, spinal nerve ligation induces mechanical and cold hypersensitivity of the injured paw (Fig. 4.1). In a separate behavioural study, paw withdrawal thresholds (PWTs), a more sensitive measure of mechanical hypersensitivity, and number of responses to acetone of both hindpaws were measured in rats, 4 days prior to SNL surgery. Rats were assigned to Tat-NR2B9c (n = 8) or Tat-NR2BAA (n = 8) treatment groups in a randomized and blinded fashion. Baseline thresholds of ipsilateral and contralateral paws of rats in each group were identical (Fig. 4.7; Tat-NR2BAA group: IPSI PWT = 10.9 ± 1.3, CONTRA PWT = 10.6 ± 1.4; Tat-NR2B9c group: IPSI PWT = 10.6 ± 1.4, CONTRA PWT = 10.0 ± 1.2). Number of responses to acetone (out of 5) was also comparable between hindpaws of rats in each group (Fig. 4.8; Tat-NR2BAA group: IPSI Acetone responses = 0.8 ± 0.3, CONTRA Acetone responses = 0.4 ± 0.3; Tat-NR2B9c group: IPSI Acetone responses = 0.3 ± 0.2, CONTRA Acetone responses = 0.5 ± 0.3).

Assessment of PWTs and responses to acetone continued throughout the post-operative period. On day 2, PWTs of the ipsilateral paw of rats in each treatment group had significantly decreased indicating mechanical hypersensitivity, while PWTs of the contralateral paws remained at baseline levels (Fig. 4.7; Tat-NR2BAA group: IPSI PWT = 1.5 ± 0.4, CONTRA PWT = 9.8 ± 1.3, $p < 0.001$; Tat-NR2B9c group: IPSI PWT = 2.0 ± 0.9, CONTRA PWT = 10.6 ± 1.5, $p < 0.001$). Similarly, rats had developed cold hypersensitivity of the ipsilateral paw by day 2 as indicated by a greater number of responses to acetone compared to the contralateral paw (Fig. 4.8; Tat-NR2BAA group: IPSI Acetone responses = 3.1 ± 0.5, CONTRA Acetone responses = 0.4 ± 0.3, $p < 0.001$; Tat-NR2B9c group: IPSI Acetone responses = 3.8 ± 0.5, CONTRA Acetone responses = 0.8 ± 0.3, $p < 0.001$). These differences in PWTs and responses to acetone between ipsilateral and contralateral paws were maintained throughout the post-operative period, measured up to day 14 or 16, in both treatment groups (Fig. 4.7 and Fig. 4.8).

Following measurement of PWTs and acetone responses on day 14 or 16, either Tat-NR2BAA (125 ng) or Tat-NR2B9c (125 ng) was delivered spinally to rats via intrathecal lumbar puncture. PWTs and responses to acetone were then re-measured at various time points after treatment (Fig. 4.7 and Fig. 4.8). In the Tat-NR2BAA treatment group there were no changes in PWT or responses to acetone such that mechanical and cold hypersensitivity persisted throughout the testing period. In contrast, treatment with Tat-NR2B9c produced a reversal in both mechanical PWTs and responses to acetone of the ipsilateral hindpaw towards pre-surgery baseline levels and those of the contralateral paw. The ipsilateral PWTs of rats treated with Tat-NR2B9c were higher than those of rats treated with Tat-NR2BAA throughout the 5 hr testing period, though statistically significant differences were only seen from 60 min until 2 hrs after treatment (Fig. 4.7; 60 min: Tat-NR2B9c group IPSI PWT = 8.1 ± 1.5, Tat-NR2BAA group IPSI PWT = 1.6 ± 0.2, $p < 0.01$; 90 min: Tat-NR2B9c group IPSI PWT = 6.6 ± 1.5, Tat-NR2BAA group IPSI PWT = 1.1 ± 0.2, $p < 0.05$; 2 hrs: Tat-NR2B9c group IPSI PWT = 8.7 ± 1.7, Tat-NR2BAA group IPSI PWT = 1.9 ± 0.9, $p < 0.01$). Acetone responses of the ipsilateral hindpaw were lower in the Tat-NR2B9c group than the Tat-NR2BAA following treatment throughout the 5 hr testing period, with statistically significant differences at 30 min, 90 min, 3 hrs, 4 hrs and 5 hrs after treatment (Fig. 4.8; 30 min: Tat-NR2B9c group IPSI acetone response = 2.3 ± 0.3, Tat-NR2BAA group IPSI acetone response = 4.1 ± 0.2, $p < 0.05$; 90 min: Tat-NR2B9c group IPSI acetone response = 2.6 ± 0.6, Tat-NR2BAA group IPSI acetone response = 4.4 ± 0.4, $p < 0.05$; 3 hrs: Tat-NR2B9c group IPSI acetone response = 2.3 ± 0.4, Tat-NR2BAA group IPSI acetone response = 4.5 ± 0.3, $p < 0.01$; 4 hrs: Tat-NR2B9c group IPSI acetone response = 2.3 ± 0.5, Tat-NR2BAA group IPSI acetone response = 4.3 ± 0.4, $p < 0.01$; 5 hrs: Tat-NR2B9c group IPSI acetone response = 2.6 ± 0.6, Tat-NR2BAA group IPSI acetone response = 4.6 ± 0.2, $p < 0.01$).

Thus, Tat-NR2B9c, believed to disrupt coupling between spinal NR2B subunits and PSD-95, reverses SNL-induced mechanical and cold behavioural hypersensitivity. This suggests that the interaction between NR2B-containing NMDA receptors and PSD-95 is involved in the maintenance of nerve injury-induced neuropathic pain.

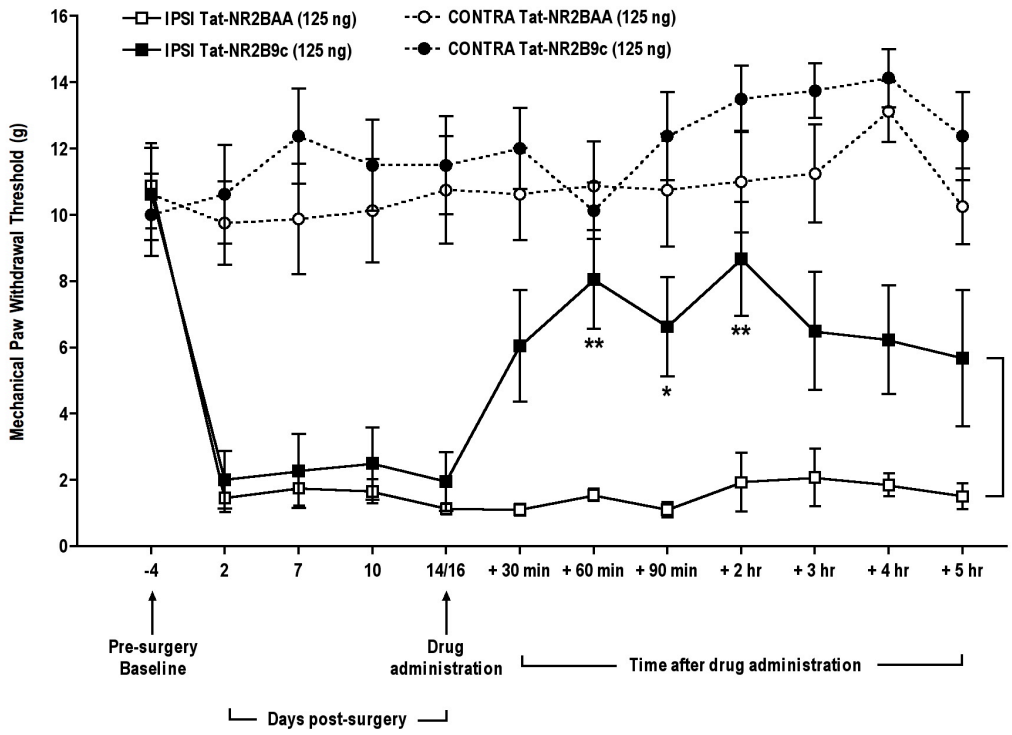


Fig. 4.7: Intrathecal Tat-NR2B9c reverses nerve injury-induced behavioural mechanical hypersensitivity. Mechanical paw withdrawal thresholds (PWTs) of SNL rats at both the ipsilateral (IPSI) and contralateral (CONTRA) hindpaws measured 4 days prior to SNL surgery and on post-surgical days 2, 7, 10 and 14 or 16. Rats received intrathecal Tat-NR2B9c (125 ng, $n = 8$) or Tat-NR2BAA (125 ng, $n = 8$) on day 14 or 16 and PWTs were re-assessed at 30 min, 60 min, 90 min, 2hrs, 3hrs, 4hrs and 5 hrs following drug administration. Data are presented as mean ± s.e.m., $* p < 0.05$, $** p < 0.01$ versus IPSI Tat-NR2BAA group, two-way RM ANOVA followed by Bonferroni post-tests.

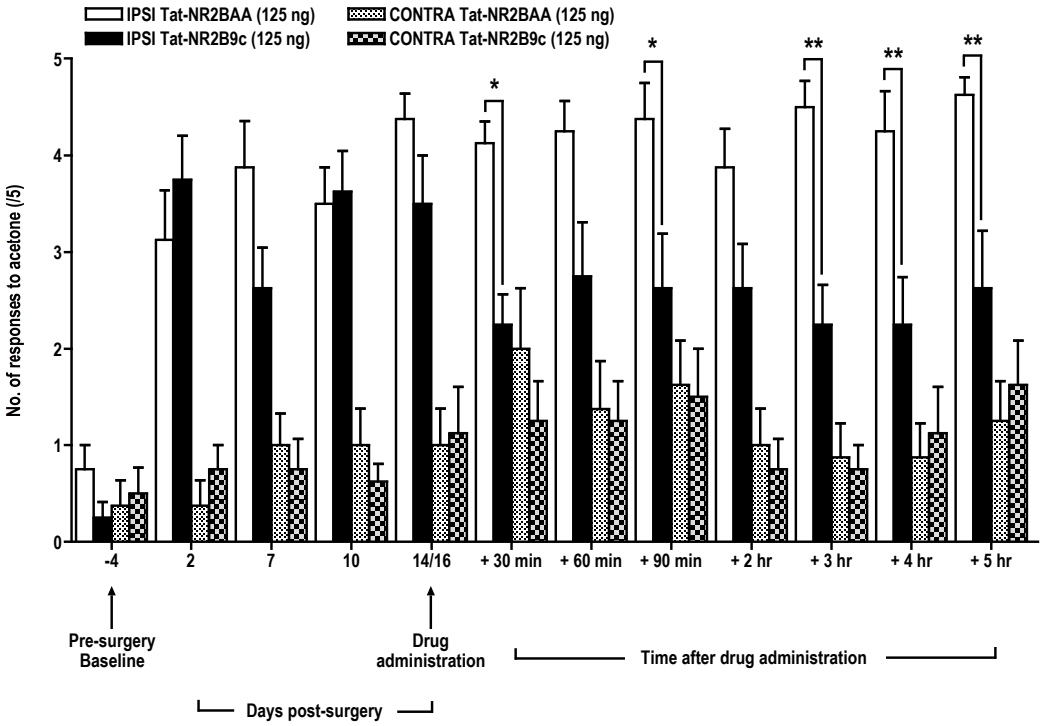


Fig. 4.8: Intrathecal Tat-NR2B9c reverses nerve injury-induced behavioural cold hypersensitivity. Responses to acetone stimulation of SNL rats at both the ipsilateral (IPSI) and contralateral (CONTRA) hindpaws measured 4 days prior to SNL surgery and on post-surgical days 2, 7, 10 and 14 or 16. Rats received intrathecal Tat-NR2B9c (125 ng, $n = 8$) or Tat-NR2BAA (125 ng, $n = 8$) on day 14 or 16 and responses to acetone were re-assessed at 30 min, 60 min, 90 min, 2hrs, 3hrs, 4hrs and 5 hrs following drug administration. Data are presented as mean ± s.e.m., $* p < 0.05$, $** p < 0.01$ versus IPSI Tat-NR2BAA group, two-way RM ANOVA followed by Bonferroni post-tests.

4.3.6 Spinal Tat-NR2B9c does not alter locomotor performance in the rotarod test

Performance on the rotarod was used to assess any effects of spinal administration of Tat-NR2B9c on locomotor function of rats (Fig. 4.9). Rats were initially trained to remain on the rotarod for a minimum of 60 s and up to 90 s. Prior to administration of drug, rats spent an average of 85.3 ± 2.1 s on the rotarod. Intrathecal lumbar puncture treatment with Tat-NR2B9c (125 ng, n = 4) had no effect on rotarod performance during the 2 hr testing period following treatment (mean time spent on rotarod: 20 min = 87.5 ± 2.5 s, 40 min = 86.3 ± 3.8, 60 min = 90.0 ± 0.0, 2 hrs = 88.0 ± 2.0).

Therefore, spinal delivery of Tat-NR2B9c does not affect locomotor performance in rats at a dose which reversed mechanical and behavioural hypersensitivity following spinal nerve ligation and also pain-related behaviours due to formalin-induced central sensitization (Chapter 3). The effects of Tat-NR2B9c can thus be attributed to alterations specifically in sensory processing.

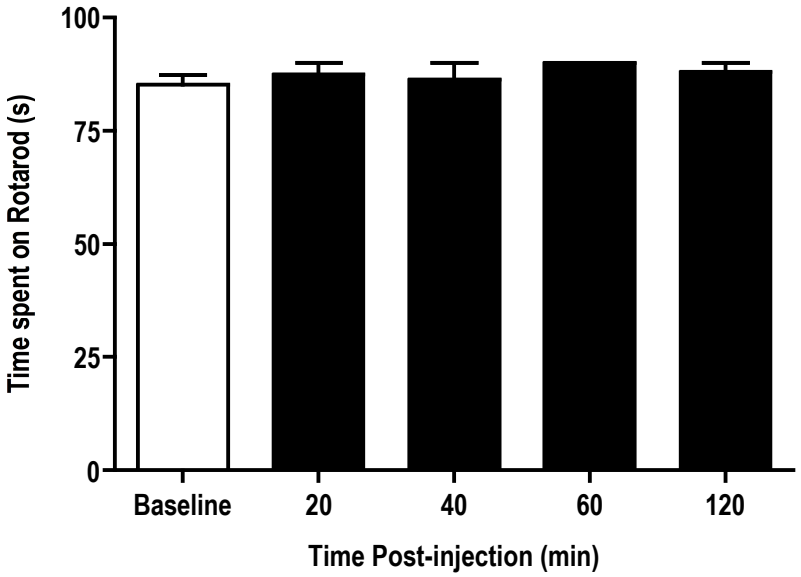


Fig. 4.9: Intrathecal Tat-NR2B9c does not alter locomotor function. Time spent on the accelerating rotarod (i.e. latency to fall) before (baseline, white bar) and after intrathecal injection of Tat-NR2B9c (125 ng, $n = 4$, black bars). A cut-off time of 90 s was used. Data presented as mean ± s.e.m., one-way RM ANOVA followed by Dunnett's post-tests.

4.4 Discussion

The findings reported in this chapter suggest that the physical interaction between spinal NR2B-containing NMDA receptors and PSD-95 is crucial to the maintenance of nerve injury-induced neuropathic pain. The mimetic peptide Tat-NR2B9c, believed to disrupt this physical interaction, reversed mechanical and cold behavioural hypersensitivity in SNL rats, whilst having no effects on locomotor function. Electrophysiological recordings showed inhibition of post-discharge and wind-up as well as of mechanically and thermally-evoked responses of deep dorsal horn WDR neurones following spinal administration of Tat-NR2B9c, suggesting that NMDA-dependent neuronal plasticity contributes to behavioural hypersensitivity in this nerve injury model.

In the previous chapter, it was reported that Tat-NR2B9c produced significant inhibitions of post-discharge and wind-up of WDR neurones in naïve rats. This effect was selective since no changes were seen in primary afferent-evoked responses of WDR neurones or input, suggesting a post-synaptic mechanism of action. This experiment was repeated in animals which had undergone SNL or sham surgery. It was found that Tat-NR2B9c produced identical effects in both surgical groups to those seen in naïve animals. Post-discharge and wind-up were equally reduced in both groups and no effect was seen on afferent-evoked responses or input. This would indicate that NR2B-subtype receptors and PSD-95 do not exert effects on primary afferent activity in nerve-injured rats, but rather specifically regulate the excitability of intrinsic spinal dorsal horn neurones. Naturally evoked responses of WDR neurones, by mechanical and thermal stimulation of the hindpaw, were also inhibited by Tat-NR2B9c to the same extent in both SNL and sham-operated rats. These results are in accordance with previous studies which have assessed effects of NMDA antagonists on spinal neuronal responses. MK-801 inhibited post-discharge and wind-up and also mechanically and thermally evoked responses of WDR neurones and these effects were comparable between SNL and sham groups (Suzuki et al., 2001). A recent study showed that the NR2B-subtype specific antagonist, Ro-256981, reduced C-fibre-evoked responses and blocked spinal LTP equally in normal and SNL rats (Qu et al., 2009). Although trends towards inhibition were seen of brush-evoked responses in both surgical groups in this study, these did

not reach statistical significance, suggesting that coupling between NR2B subunits and PSD-95 may contribute more to static rather than dynamic mechanical allodynia.

Since the neuronal effects of Tat-NR2B9c do not change following neuropathy, it may seem that the role of the interaction between NR2B subunits and PSD-95 is not altered under pathological conditions. However, it is important to remember that major changes in connectivity between primary afferents and spinal neurones have occurred following neuropathy. Two thirds of the input from the sciatic nerve has effectively been eliminated in this SNL model. Within the L4 spinal segment, although afferent inputs are maintained, these are reduced since the ligated L5 and L6 spinal nerves cross-innervate this segment (Pinto et al., 2008; Shehab et al., 2008). Despite this, baseline responses of WDR neurones evoked by electrical, mechanical and thermal stimulation of the hindpaw and conduction through the sciatic nerve were similar in SNL and sham-operated rats. This would suggest that some form of compensation in spinal excitability has occurred in nerve-injured rats to account for the loss of peripheral input and this may involve activity of spinal NMDA receptors. Therefore, it is difficult to conclude as to whether the interaction between NR2B subunits and PSD-95 has changed in this model using pharmacology in electrophysiological experiments alone.

Prior to electrophysiological testing and assessment of the effects of Tat-NR2B9c on pain-related behaviours, neuropathic pain was allowed to develop for two weeks after surgery. Two different ways of behaviourally measuring mechanical hypersensitivity were used in this study. Difference scores were calculated by measurement of withdrawal frequency to specific von Frey hairs. This method is quick and reliably shows the development of mechanical hypersensitivity in SNL rats only over sham rats. However, this method has limitations, since in some rats von Frey filaments 6 and 8 g can induce withdrawal of non-injured paws, giving large withdrawal frequencies, meaning that difference scores, which specifically indicate hypersensitivity of the injured paw, may be lower than expected. For this reason, it was decided that measurement of paw withdrawal thresholds (PWTs) would be a more sensitive approach to assess mechanical hypersensitivity when testing the effects of Tat-NR2B9c on neuropathic pain behaviour. This method allows for the PWT of both paws to be assessed, giving individual values for each paw, as opposed

to a difference score which incorporates measurements at both paws. Importantly, this allows for detection of drug effects of the non-injured paw as well as the injured paw, whereas a difference score does not. Cold hypersensitivity was always assessed by frequency of withdrawal to a single drop of acetone. Behavioural analysis confirmed that unilateral mechanical and cold hypersensitivity developed by day 2 following surgery in SNL rats only and was maintained for the two weeks of testing. Mechanical hypersensitivity has been reported to last for up to 10 weeks post-surgery (Kim and Chung, 1992). This hypersensitivity is confined to the ipsilateral paw, while sensitivity of the contralateral paw of SNL rats remains at pre-surgery baseline levels. Spinal administration of Tat-NR2B9c two weeks after surgery via a single intrathecal injection reversed both mechanical and cold hypersensitivity of the ipsilateral paw, while the control peptide, Tat-NR2BAA, had no effect at all. PWTs were increased and responses to acetone were reduced towards baseline levels and those of the contralateral paw. These effects began 30 min after drug administration and lasted for the full 5 hr testing period. NMDA antagonists, dextromethorphan and ketamine, also reduce pain-related behaviours in rats following neuropathy (Mao et al., 1993; Christoph et al., 2006). More interestingly, spinal inhibition of NR2B-subtype receptors reverses mechanical and thermal hypersensitivity, with this effect also beginning approximately 30 min after administration of an NR2B-selective inhibitor (Malmberg et al., 2003; Qu et al., 2009). Together, all of these results confirm that spinal NR2B-subtype receptors do indeed have a role in chronic pain. In the present study, no effects of Tat-NR2B9c were seen in the contralateral paw. However, because a cut-off of 15 g was applied in mechanical testing (to avoid tissue damage), it can be argued that it was not possible to assess whether or not PWTs were raised above this cut-off following Tat-NR2B9c administration. Nevertheless, it is clear that signs of neuropathic pain can be reversed by Tat-NR2B9c in nerve-injured rats.

The behavioural effects of Tat-NR2B9c were achieved at a dose which was 10 times higher than that used in electrophysiological experiments. This is to account for the fact that a smaller volume is injected intrathecally meaning that the drug may cover a smaller area of spinal cord. Additionally, administration of drug during electrophysiology is into a static system, i.e. a laminectomy exposing the surface of the cord where minimal movement of fluid occurs for the whole duration of the

recording and thus minimal removal of drug, whereas following intrathecal injections, rats quickly awake from brief anaesthesia and move around, limiting the time for the drug to access the target area of cord. Even at this higher dose, though, no effects were seen in the performance of rats on the rotarod, indicating no off-target locomotor effects.

This work has confirmed the findings of previous studies which have demonstrated involvement of spinal PSD-95 in neuropathic pain. Knockdown of PSD-95 in the spinal cord by intrathecal antisense treatment can delay the development of hypersensitivity when administered at the time of L5 spinal nerve injury (Tao et al., 2001). More interestingly, in terms of clinical treatment, PSD-95 antisense treatment was able to reverse hypersensitivity once it had already been fully established, without locomotor effects (Tao et al., 2003a). This finding is in accordance with those of the present study. It has also been shown that PSD-95 mutant mice fail to develop behavioural hypersensitivity following CCI (Garry et al., 2003) again implicating PSD-95 in nerve injury-induced pain. Most recently, LeBlanc and colleagues synthesized a peptide, termed CN2097, which binds to the three PDZ domains of PSD-95, thus non-selectively disrupting PSD-95 interactions with other proteins. It was found that this peptide reduced mechanically evoked responses of dorsal horn WDR neurones as well thermal behavioural hypersensitivity (LeBlanc et al., 2010).

At present, the precise mechanisms by which coupling between NR2B-containing NMDA receptors and PSD-95 contribute to nerve injury-induced pain are unknown. It is likely that specific intracellular signalling events are activated downstream of this interaction which lead to central sensitization of dorsal horn neurones and which ultimately lead to manifestation of pain-related behaviours following peripheral nerve injury as well as intraplantar formalin injection (Chapter 3). Such signalling events will be investigated in the remainder of this thesis.

These results demonstrate that Tat-NR2B9c, a mimetic peptide believed to disrupt protein interactions specifically between NR2B-subtype receptors and PSD-95, can produce similar effects to receptor blockade in a model of neuropathic pain following nerve injury. Since intolerable side-effects are particularly associated with NMDA antagonists, using mimetic peptides which disrupt protein interactions may be a

better analgesic strategy. This is supported by the lack of effect of Tat-NR2B9c on locomotor function. Another mimetic peptide, Tat-PSD-95 PDZ2, which, among other interactions, prevents binding of PSD-95 to NMDA receptors, has been shown to reverse behavioural hypersensitivity following inflammation induced by CFA (Tao et al., 2008). Despite the different pain model used, these results along with those presented here show that binding between NMDA receptors and PSD-95 is involved in regulation of selective sensory modalities in chronic pain, specifically static mechanical hypersensitivity. Targeting of interactions between NMDA receptors and the kinase Src using a peptide mimetic strategy has also shown anti-hyperalgesic efficacy in neuropathic and inflammatory chronic pain models (Liu et al., 2008). Therefore, mimetic peptides, particularly those targeting interactions within the NMDA receptor complex, should be considered further as novel analgesics for the treatment of chronic pain.

5 Involvement of atypical protein kinase C zeta (PKCζ/PKMζ) in NMDA-dependent spinal central sensitization

5.1 Introduction

5.1.1 The Protein Kinase C family

The PKC family of enzymes comprises numerous serine/threonine kinases with varying dependence on Ca^{2+} and diacylglycerol (DAG), and which act as key effectors of intracellular signalling within neurones. The conventional α, βI, βII and γ isozymes are both Ca^{2+} and DAG-dependent, while the δ, ε, η and θ isozymes are Ca^{2+}-independent but DAG-dependent and are termed the novel PKCs. A third group, the atypical PKCs, are independent of both Ca^{2+} and DAG signalling and include the ζ and λ/ι isozymes (Velazquez et al., 2007). Activity of PKCs has been shown to be crucial at all levels of nociceptive processing, from the peripheral primary afferent fibres, through the spinal cord and up to the brain.

5.1.2 PKC in primary afferent fibres

Peripheral sensitization, characterized by thermal hyperalgesia and mechanical allodynia, can occur in response to tissue damage. The release of an 'inflammatory soup' containing various chemical mediators, including ATP, protons, bradykinin, prostaglandins, substance P, CGRP and pro-inflammatory cytokines, to name a few, can activate and sensitize the peripheral terminals of nociceptors, leading to heightened pain. Numerous studies have provided evidence that PKCs play a key role in this sensitization. Phorbol esters, which are activators of PKC, can themselves activate primary afferent fibres and induce nociception (Cesare and McNaughton, 1996; Ferreira et al., 2005), whilst the peripheral administration of PKC inhibitors in models of pain is anti-hyperalgesic. For example, intraplantar administration of PKC inhibitors has been shown to inhibit both bradykinin-induced

mechanical allodynia and also pain-related behaviours during the second phase of the response to intraplantar formalin injection (Souza et al., 2002).

In particular, the PKCε isozyme in primary afferents is thought to have a substantial role in nociception. It is the only PKC isozyme translocated to the cell membrane in response to bradykinin (Cesare et al., 1999). Transgenic mice lacking PKCε have attenuated mechanical allodynia and thermal hyperalgesia in response to chemical mediators, while baseline nociceptive thresholds remain intact (Khasar et al., 1999). Inhibition of PKCε using a selective PKCε inhibitor peptide also blocks peripheral sensitization and hyperalgesia (Cesare et al., 1999; Khasar et al., 1999). It is believed that TRPV1 is a key molecular target for activated PKCε following tissue damage. PKC can promote exocytosis and also depolarization of TRPV1, which means that the channel can be activated at lower temperatures (Zhou et al., 2001; Crandall et al., 2002; Morenilla-Palao et al., 2004). Capsaicin, a TRPV1 agonist, increases immunoreactivity of PKCε in primary afferent fibres (Velazquez et al., 2007) and PKCε is thought to phosphorylate TRPV1, enhancing capsaicin-evoked currents (Numazaki et al., 2002; Mandadi et al., 2006). Thus, it may be that PKCε may act as a switch which allows TRPV1 to integrate heat and tissue damage to produce thermal hyperalgesia (Velázquez et al., 2007). Transmission of such nociceptive signals from the peripheral to the central terminals of primary afferents may also be promoted by PKC activation by enhancement of Na^+ channel currents (Baker, 2005).

PKC also acts at the central terminals of primary afferents by enhancing the release of neurotransmitters such as substance P, CGRP and glutamate (Barber and Vasko, 1996; Malcangio et al., 1998; Frayer et al., 1999) perhaps via phosphorylation of Ca^{2+} channels responsible for neurotransmitter release (Yang et al., 2005). Activation of opioid and GABA receptors on the central terminals of primary afferent fibres can inhibit the release of neurotransmitters and thus produce analgesia. It has been shown that phosphorylation of both types of receptor by PKC can block their inhibitory functions (Yamada and Akasu, 1996; Zhang et al., 1996). Therefore, PKC can both enhance excitatory neurotransmission and reduce inhibitory tone at the central terminals of primary afferents, thus increasing pain.

5.1.3 PKC in the spinal cord

PKCs in the spinal cord can contribute to the excitability of dorsal horn neurones in response to incoming nociceptive signals, and spinal treatment with PKC inhibitors results in analgesia in various inflammatory and neuropathic pain models (Lin et al., 1996; Hua et al., 1999). Most current evidence points to PKCγ as being the main isozyme involved. PKCγ expression is within a small subset of dorsal horn neurones, restricted to lamina II and a few in lamina III (Malmberg et al., 1997). These neurones do not contain GABA and thus are deemed to be excitatory interneurones (Polgar et al., 1999) which are capable of modulating lamina V neurones that belong to an NMDA-dependent circuit (Martin et al., 2001). In addition, Miraucourt and colleagues have proposed a mechanism of dynamic mechanical allodynia in which blockade of spinal glycinergic inhibition gates tactile inputs to superficial nociceptive specific neurones via PKCγ interneurones. Strychnine, a glycine receptor antagonist, produces dynamic mechanical allodynia and induces responses of superficial dorsal horn NS cells to Aβ-fibre inputs. This tactile input was found to be polysynaptic and required activity of PKCγ interneurones. This was demonstrated in two ways. First, a brush stimulus, under glycinergic disinhibition, induced expression of c-Fos in cells of the superficial dorsal horn and a significant proportion of these cells were also immunopositive for PKCγ. Secondly, pharmacological inhibition of PKCγ reduced strychnine-induced dynamic mechanical allodynia (Miraucourt et al., 2007). Mice lacking the PKCγ isozyme have normal acute pain sensitivity but fail to develop neuropathic pain following nerve injury, thus implicating PKCγ as a mediator of central sensitization (Malmberg et al., 1997).

It has been shown that PKC reduces the Mg^{2+} block of the NMDA receptor thus increasing the open probability of the channel and contributing to central sensitization (Chen and Huang, 1992). Here, Chen and Huang injected PKC intracellularly in trigeminal neurones and found that this potentiated NMDA-mediated currents. PKC also increased the dissociation of Mg^{2+} from the NMDA receptor and allowed for receptor activation at more hyperpolarized membrane potentials. GPCRs, such as mGluRs and NK_1 receptors, can activate PKC in the dorsal horn and this can lead to activation of spinal ERK, a marker of central sensitization (Kawasaki et al., 2004).

Group I mGluRs activate PKC in the dorsal horn which in turn can activate the tyrosine kinase Src which then phosphorylates the NR2B subunit of NMDA receptors, increasing channel activity (Guo et al., 2002; Guo et al., 2004). Thus, spinal PKC can directly modulate the excitability of dorsal horn neurones and contribute to central sensitization.

5.1.4 Atypical PKCζ/PKMζ and role in hippocampal LTP

PKCζ belongs to the atypical class of the PKC family. This enzyme consists of four functional domains, including a regulatory pseudosubstrate sequence and a kinase domain in the C-terminus (Fig. 5.1). The pseudosubstrate domain is a short sequence of amino acids which closely resembles a substrate sequence except for a substitution of a single amino acid (alanine for serine/threonine). This sequence is therefore thought to block the substrate-binding cavity of the kinase domain, thus acting as an autoinhibitory mechanism. The kinase domain of PKCζ contains an activation loop which is phosphorylated at Thr410 by 3'-PI-dependent protein kinase (PDK-1) for activation. As well as phosphorylation of the kinase domain, liberation from pseudosubstrate autoinhibition is required for PKCζ activation and this is achieved by interaction with phosphatidylinositol 3,4,5-triphosphate (PIP_3) (Hernandez et al., 2003; Hirai and Chida, 2003).

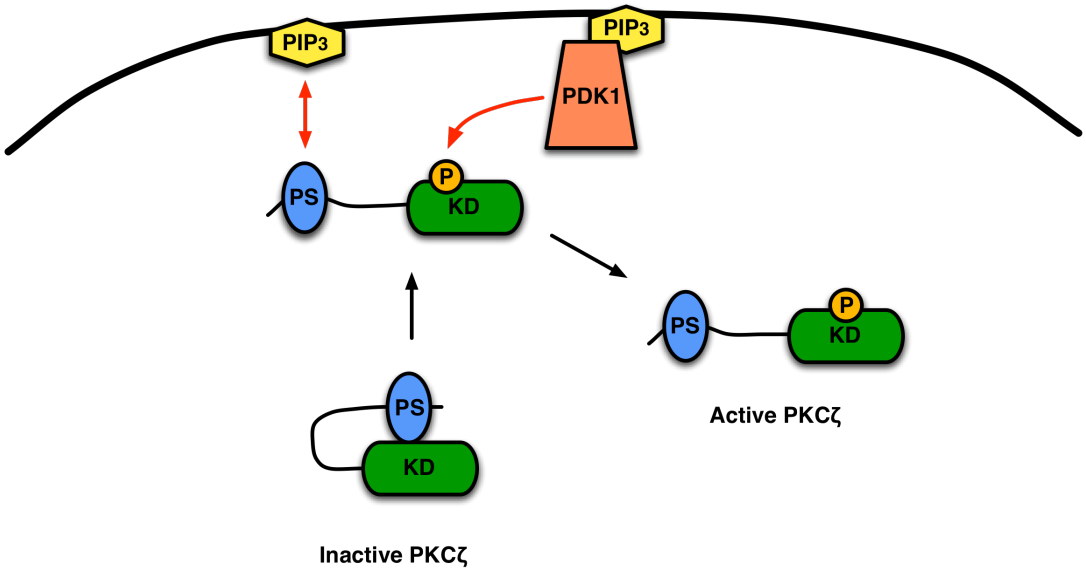


Fig. 5.1: Activation of PKCζ/PKMζ. PKCζ contains a kinase domain (KD) which at rest is blocked by a regulatory pseudosubstrate domain (PS). Two steps are required for full activation of PKCζ. First, interaction with PIP_3 liberates the KD from the PS. Second, PIP_3 activates PDK-1 which phosphorylates the KD of PKCζ. PKMζ is a shorter form of PKCζ and has no PS domain. However, phosphorylation of the KD of PKMζ is still required for full activity. Adapted from Hirai and Chida (2003).

PKCζ has been reported to be involved in several cellular functions, from receptor signalling complexes (i.e. TNF-α and IL1-β receptors) to activation of NFkB transcription factor during immune reactions, but more importantly also in LTP in the hippocampus (Hirai and Chida, 2003). Two forms of the enzyme are expressed in the brain. These are the native PKCζ (75 kDa) and also a shorter protein (51 kDa), termed PKMζ, which consists solely of the independent catalytic domain of PKCζ, and is therefore persistently active. It had been thought that PKMζ was simply a proteolytic fragment of PKCζ. A recent study, however, found that PKMζ is in fact an independent protein and is the product of a novel mRNA which encodes a PKCζ catalytic domain but without an autoinhibitory domain. This independence is shown by the mRNA encoding PKMζ being still expressed in a PKCζ knockout mouse (Hernandez et al., 2003). LTP has been shown to increase *de novo* synthesis of PKMζ which enhances synaptic transmission by doubling the number of post-synaptic AMPA receptors (Ling et al., 2006). Several studies have demonstrated that PKMζ is the only kinase involved in the late phase of LTP as shown by inhibition of LTP maintenance using a myristoylated pseudosubstrate inhibitor (see 5.2.2), which presumably mimics the autoinhibitory domain of the full-length PKCζ isoform (Sacktor et al., 1993; Ling et al., 2002; Pastalkova et al., 2006). Most interestingly, inhibition of PKMζ was found to erase established memories (Pastalkova et al., 2006).

5.1.5 Atypical PKCζ/PKMζ in pain

In stark contrast to the roles of other PKCs in pain, very little is known regarding the involvement of PKCζ/PKMζ. One previous study attempted to examine the role of PKCζ in neuropathic pain (Narita et al., 2004). In this study, the authors observed increased phosphorylation of PKCζ in the ipsilateral dorsal horn after nerve injury. Thermal hyperalgesia and morphine-induced place preference in this neuropathic pain state were attenuated but through the use of a general inhibitor of PKC, thus limiting conclusions on the role of PKCζ in particular in pain. Therefore, the aim of this study was to investigate the expression and specific involvement of atypical PKCζ/PKMζ in pain signalling in the spinal dorsal horn using the formalin model of central sensitization.

5.2 Materials and Methods

5.2.1 Animals

All experiments were carried out using adult male Wistar rats (220-250g, Harlan, UK or Central Biological Services, University College London, UK) housed in standard laboratory conditions with free access to food and water, and were approved by the UK Home Office and followed guidelines set by the International Association for the Study for Pain (Zimmermann, 1983).

5.2.2 PKCζ/PKMζ inhibitor

Inhibition of PKCζ/PKMζ activity was achieved through the use of a myristoylated pseudosubstrate peptide inhibitor (Myr-SIYRRGARRWRKL-OH, Biosource International Inc., USA) which mimics the autoinhibitory domain of the full-length PKCζ isoform and thus prevents catalytic activity. A control scrambled pseudosubstrate peptide (Myr-RLYRKRIWRSAGR-OH) was synthesized (Genscript, USA). Both peptides were dissolved in 0.9% saline. Doses for experiments were chosen with regard to previous *in vitro* studies using the inhibitor (Ling et al., 2002; Serrano et al., 2005; Kelly et al., 2007).

5.2.3 Behavioural Formalin Test

Rats were lightly anaesthetized using isoflurane and then injected intrathecally (lumbar puncture method) with either 5 or 10 μg of the myristoylated PKCζ/PKMζ pseudosubstrate inhibitor, in a volume of 10 μl. Two control groups of rats received either the scrambled version of the pseudosubstrate (10 μg) or vehicle (0.9% saline). Experimenters were blind to treatment for the whole experiment. Thirty minutes later, rats received a subcutaneous injection of 50 μl of formalin (5%) into the plantar surface of the right hindpaw. Lifting, flinching, licking and biting of the injected paw were monitored by measuring the total duration of the response in seconds (s) during the 60 min period following formalin administration. Data was captured in 5 min time bins. An additional group of rats were pre-treated with 125 ng of Tat-NR2B9c in 10 μl and subsequently received intraplantar formalin thirty minutes later (as Chapter 3). These rats were used to assess the effects of Tat-NR2B9c on activation of PKCζ/PKMζ. At the end of the test, all animals were terminally anaesthetized and perfused for immunohistochemistry.

5.2.4 *In vivo* Electrophysiology

Experiments were conducted in adult male Wistar rats (220-250 g, Harlan, UK or Central Biological Services, University College London, UK). Electrophysiological recordings from deep dorsal horn WDR neurones (> 600 μm) were conducted as previously described (see Materials and Methods chapter).

5.2.4.1 Neuronal Formalin Test

Following cell characterization, rats were pre-treated 30 min prior to the injection of formalin by topical spinal administration of 10 μg of the PKCζ/PKMζ pseudosubstrate inhibitor, in a volume of 50 μl using a Hamilton syringe. A separate group of control animals received 10 μg of the scrambled pseudosubstrate. Formalin (5%, 50 μl) was prepared from a 37% formaldehyde solution and then injected subcutaneously into the hindpaw receptive field. The firing response of the WDR neurone was recorded for the subsequent 70 min after formalin injection. Activity was displayed as a rate recording and quantified in 10 min time bins.

5.2.5 Statistical Analysis for Formalin Test

Data are presented as mean ± s.e.m. Cell characteristics from scrambled pseudosubstrate and PKCζ/PKMζ pseudosubstrate inhibitor groups were compared by Student's unpaired t-tests. Formalin neuronal and behavioural response time course data was compared between treatment groups and analyzed by two-way RM ANOVA, followed by Bonferroni post-tests. Total activity in the first and second phases was compared between treatment groups by quantifying the area under each curve, and analyzed by one-way ANOVA, followed by Bonferroni post-tests. Statistical analyses were carried out using GraphPad Prism v.4 software (GraphPad Software Inc., San Diego, CA, USA).

5.2.6 Immunohistochemistry for phospho-PKCζ and c-Fos

At the end of the formalin tests, animals were terminally anaesthetized using pentobarbitone and perfused, after which the lumbar spinal cord was excised (see Material and Methods chapter for details). Transverse sections (30 μm) were cut on a cryostat and thaw-mounted onto glass slides.

Sections were stained for p-PKCζ immunohistochemistry as follows: after 3 washes in PBS, sections were incubated for 48 hrs at 4°C with a rabbit primary antibody for anti-phospho-atypical protein kinase C-ζ (anti-p-PKCζ, Thr410; 1:100; sc-101778, Santa Cruz Biotechnology, USA) followed by a secondary antibody solution for 4 hrs (goat anti-rabbit IgG-conjugated Alexa Fluor 488^{TM}; 1:1000; Molecular Probes, USA), as previously described (Narita et al., 2004). Slides were washed in PBS and cover-slipped with Vectashield mounting medium (Vector Laboratories, CA, USA). Quantitative assessment of p-PKCζ staining was carried out by Dr Fabien Marchand, by determining the immunofluorescence intensity (densitometric analysis) using grey scale within a fixed area of the dorsal horn of the spinal cord (ipsilateral and contralateral to injury). A box measuring 10^4 μm^2 (100x100) was placed over areas of the medial dorsal horn and the mean intensity of each area recorded. The measurement protocol was carried out on L4-L6 spinal sections from each animal (at least 5 sections from each animal). The background fluorescence intensity of each tissue section was also determined and subtracted from recorded values. Specificity of p-PKCζ staining was confirmed by omission of the primary antibody.

To determine the cellular distribution of p-PKCζ, sections were counterstained with primary antibodies against markers for neurones [mouse anti-neuronal nuclei (anti-NeuN); 1:500; Chemicon, Hampshire, UK], astrocytes [mouse anti-glial fibrillary acidic protein (anti-GFAP); 1:1000; Abcam, Cambridge, UK] and microglial cells [rabbit anti-ionized calcium binding adaptor molecule 1 (anti-Iba1); 1:100; Wako Pure Chemical Industries Ltd, Japan], followed by the appropriate secondary antibody solution: goat anti-mouse or goat anti-rabbit IgG-conjugated Alexa Fluor 546^{TM} (1:1000; Molecular Probes, OR).

Sections were processed for c-Fos immunohistochemistry by Dr Fabien Marchand as follows: following 3 washes in PBS, sections were incubated overnight, at room temperature, with the primary rabbit anti-c-Fos antiserum (1:2500; AB5, Oncogene Science, USA). Sections were then incubated with AlexaFluor^{TM} 488 goat anti-rabbit antibody (1:1000; Molecular Probes, USA) for 4 hrs and coverslipped. Sections were visualized using a Zeiss Axioplan 2 fluorescent microscope running Axiovision 3.1 image analysis software and classified according to spinal level (L4, L5 and L6). From each animal 4-6 sections (L4-L6) were randomly selected for counting c-Fos positive cells by a blinded investigator and an average of these counts was taken. The contralateral dorsal horn served as an internal control for the effects of formalin injection.

5.2.7 Statistical Analysis for Immunohistochemistry

Immunostaining of p-PKCζ and c-Fos was analyzed by one-way and two-way ANOVA, respectively, followed by Bonferroni post-tests, using GraphPad Prism v.4 software (GraphPad Software Inc., San Diego, CA, USA).

5.2.8 Immunoprecipitation of NR2B subunits and downstream Western Immunoblotting

Rats anaesthetized with urethane were sacrificed by decapitation and fresh spinal cord tissue (dorsal horn only) was dissected out and homogenized for immunoprecipitation (IP) and western immunoblotting (see Materials and Methods chapter).

Immunoprecipitation Dynabeads® Protein G (Invitrogen Ltd, UK) were washed and coupled with 5 μg of rabbit anti-NR2B antibody (06-600, Upstate, USA). Next 500 μg of spinal cord dorsal horn lysate was added to the antibody/dynabeads complex and allowed to incubate overnight for capture of target antigen. Following incubation, the captured protein was eluted from the dynabeads and loaded onto gels for downstream SDS-PAGE. Normal spinal lysate samples were run alongside IP samples as positive controls. Once proteins were separated they were transferred to nitrocellulose membranes. For detection of proteins of interest, membranes were incubated with primary antibody overnight at 4°C. Antibodies used were mouse anti-NR2B (1:500, 75-101, Neuromab, USA), rabbit anti-PSD-95 (1:1000, ab18258, Abcam, UK), rabbit anti-PKCζ (1:500, ab59364, Abcam, UK) and rabbit anti-nNOS (1:3000, 07-571, Millipore, UK). Membranes were also probed with rabbit anti-CREB (1:500, ab5803, Abcam, UK) and rabbit anti-P_2X_3 (1:500, ab10269, Abcam, UK) antibodies which served as negative controls. Following incubation with IRDye-linked donkey anti-rabbit 800CW or goat anti-mouse 600 secondary antibodies, proteins were revealed using the Odyssey fluorescence detection system (Licor, UK).

5.3 Results

5.3.1 Spinal pre-treatment with an inhibitor of PKCζ/PKMζ reduces pain-related behaviours due to formalin-induced central sensitization

In control rats pre-treated intrathecally with saline (0.9%, $n = 8$), formalin injected into the hindpaw induced a biphasic response of pain-related behaviours (Fig. 5.2a,b; 1st phase: 0-10 min, total time of pain-related behaviour = 97 ± 8 s; 2nd phase: 10-60 min, total time of pain-related behaviour = 328 ± 28 s). A second group of control rats received a scrambled version of the PKCζ/PKMζ pseudosubstrate (10 μg, $n = 6$), via intrathecal lumbar puncture, 30 min prior to formalin. In these rats, formalin induced a biphasic behavioural response which was no different to that in saline-treated rats (Fig. 5.2a,b; 1st phase: 0-10 min, total time of pain-related behaviour = 87 ± 6 s; 2nd phase: 10-60 min, total time of pain-related behaviour = 317 ± 13 s). In contrast, pre-treatment with the myristoylated PKCζ/PKMζ pseudosubstrate inhibitor significantly decreased pain-related behaviours during both the first and second phases of the response to formalin in a dose-dependent manner (Fig. 5.2a,b; 5 μg, $n = 6$, 1st phase: 0-10 min, total time of pain-related behaviour = 59 ± 9 s, $p > 0.05$; 2nd phase total time of pain-related behaviour = 207 ± 13 s, $p < 0.01$; 10 μg, $n = 9$, 1st phase: 0-10 min, total time of pain-related behaviour = 46 ± 8 s, $p < 0.05$; 2nd phase total time of pain-related behaviour = 145 ± 14 s, $p < 0.001$) compared to the scrambled pseudosubstrate group.

Thus, inhibition of the PKCζ/PKMζ pathway reduces formalin-induced pain-related behaviours associated with spinal central sensitization.

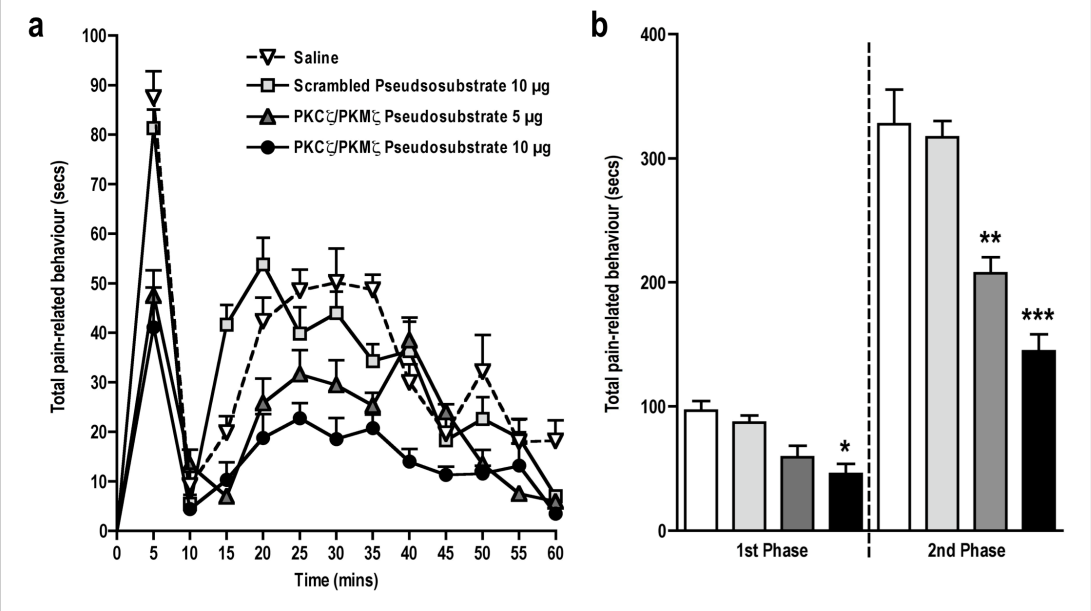


Fig. 5.2: Intrathecal PKCζ/PKMζ pseudosubstrate inhibitor reduces pain-related behaviours due to formalin-induced central sensitization. **(a)** Time course of pain-related behaviours induced by intraplantar formalin (5%, 50 μl) following intrathecal pre-treatment with saline (n = 8), scrambled pseudosubstrate (10 μg, n = 6) or PKCζ/PKMζ pseudosubstrate (5 μg, n = 6, $***$ $p < 0.001$ at 5, 15 and 20 min versus scrambled pseudosubstrate; 10 μg, n = 9, $*$ $p < 0.05$ at 25 min, $**$ $p <$ 0.01 at 40 min, $***$ $p < 0.001$ at 5, 15, 20 and 30 min versus scrambled pseudosubstrate; two-way RM ANOVA followed by Bonferroni post-tests). **(b)** Total pain-related behaviour during the 1st (0-10 min) and 2nd (10-60 min) phases of the formalin response with saline, scrambled pseudosubstrate or PKCζ/PKMζ pseudosubstrate (1st phase: 10 μg, $*$ $p < 0.05$ versus scrambled pseudosubstrate; 2nd phase: 5 μg, $**$ $p < 0.01$, 10 μg, $***$ $p < 0.001$ versus scrambled pseudosubstrate; one-way RM ANOVA followed by Bonferroni post-tests). All data presented as mean ± s.e.m.

5.3.2 Formalin-induced central sensitization of deep dorsal horn WDR neurones is reduced by spinal inhibition of PKCζ/PKMζ

In rats pre-treated with the control scrambled pseudosubstrate peptide (10 μg; n = 8) formalin injected into the hindpaw receptive field produced the characteristic biphasic neuronal firing response of spinal WDR neurones (Fig. 5.3a,b; 1st phase: 0-10 min, total APs = 8743 ± 1778; 2nd phase: 10-70 min, total APs = 83358 ± 11531). Spinal pre-treatment with the myristoylated PKCζ/PKMζ pseudosubstrate inhibitor (10 μg, n = 8) significantly reduced the second phase neuronal firing, while the first phase was only minimally reduced (Fig. 5.3a,b; 1st phase: 0-10 min, total APs = 4847 ± 979, $p > 0.05$; 2nd phase: 10-70 min, total APs = 29643 ± 11132, $p < 0.001$). All cells recorded were characterized prior to injection of drug and formalin to ensure that cells were comparable between treatment groups (Table 5.1).

Thus, inhibition of PKCζ/PKMζ reduces formalin-induced central sensitization of deep dorsal horn WDR neurones.

	Scrambled pseudosubstrate (*n* = 8)	PKCζ/PKMζ pseudosubstrate (*n* = 8)
C-fibre threshold (mA)	0.41 ± 0.08	0.64 ± 0.07
Depth (*μ*M)	1033 ± 55	1029 ± 56
Aβ-fibre evoked response (No. of APs)	147 ± 9	148 ± 9
Aδ-fibre evoked response (No. of APs)	165 ± 16	133 ± 17
C-fibre evoked response (No. of APs)	496 ± 54	507 ± 65
Post-discharge (No. of APs)	676 ± 122	569 ± 93
Input (No. of APs)	220 ± 29	266 ± 46
Wind-up (No. of APs)	1050 ± 185	897 ± 138
48°C (No. of APs)	1363 ± 171	1213 ± 129

Table 5.1: WDR cell characteristics from PKCζ/PKMζ pseudosubstrate inhibitor neuronal formalin test. Cells were characterized prior to drug administration and formalin injection. C-fibre threshold, cell depth, afferent-evoked responses, input, wind-up and response to 48°C are expressed as mean ± s.e.m. There were no differences between cells recorded in each treatment group; Student's unpaired t-test.

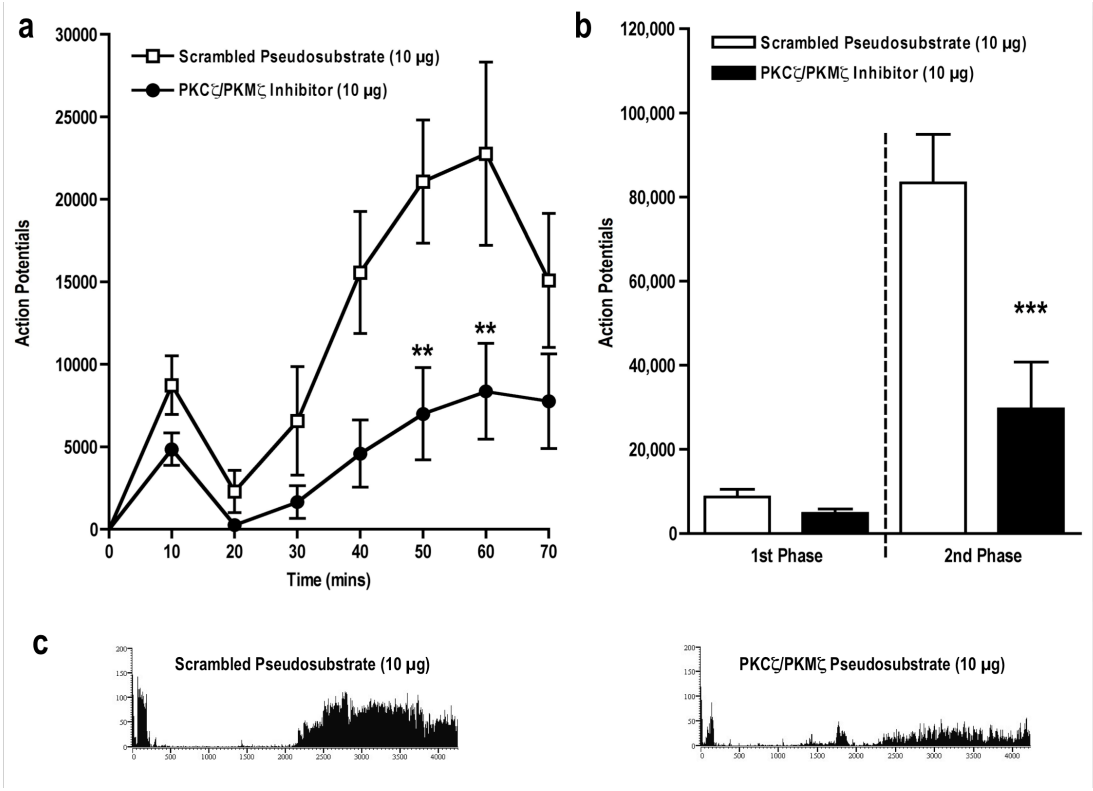


Fig. 5.3: Spinal PKCζ/PKMζ pseudosubstrate inhibitor reduces formalin-induced central sensitization of WDR neurones. **(a)** Time course of WDR firing response to formalin (5%, 50 μl) injected into the hindpaw receptive field following spinal pre-treatment with the scrambled pseudosubstrate (10 μg, n = 8) or PKCζ/PKMζ pseudosubstrate (10 μg, n = 8, ** $p < 0.01$ at 50 and 60 min versus scrambled pseudosubstrate; two-way RM ANOVA followed by Bonferroni post-tests). **(b)** Total neuronal activity during the 1st (0-10 min) and 2nd (10-70 min) phases of the formalin response with the scrambled pseudosubstrate or PKCζ/PKMζ pseudosubstrate (2nd phase: *** $p < 0.001$ versus scrambled pseudosubstrate; one-way RM ANOVA followed by Bonferroni post-tests). **(c)** Representative rate recordings of firing responses of WDR neurones to formalin following spinal pre-treatment with scrambled pseudosubstrate or PKCζ/PKMζ pseudosubstrate. All data presented as mean ± s.e.m.

5.3.3 PKCζ/PKMζ is involved in the upregulation of c-Fos after formalin injection

Noxious peripheral stimulation induces expression of the immediate early-onset gene c-Fos in dorsal horn neurones (Hunt et al., 1987). The involvement of the PKCζ/PKMζ pathway on induction of spinal c-Fos expression following formalin injection in the hindpaw was assessed using immunohistochemistry. Intraplantar formalin injection induced an increase of c-Fos in superficial (laminae I-II) and deep laminae (V-VI) of the dorsal horn in the lumbar cord (L4, L5 and L6) 60 min after formalin injection in rats pre-treated with the scrambled pseudosubstrate (n = 5; Fig. 5.4a,c), as previously described (Presley et al., 1990). Intrathecal pre-treatment with the myristoylated PKCζ/PKMζ pseudosubstrate inhibitor (10 μg, n = 5) resulted in a significant reduction of c-Fos expression in the superficial dorsal horn (L4: 45.5% ± 5.8 reduction; L5: 51.3 % ± 5.9 reduction; L6: 59.3% ± 1.1 reduction, all $p < 0.001$) and deeper dorsal horn (L4: 53.2% ± 8.4 reduction; L5: 51.6% ± 9.2 reduction; L6: 52.9 ± 11.4 reduction, all $p < 0.001$) in comparison to scrambled pseudosubstrate-treated rats (Fig. 5.4b,c).

Thus, the formalin-induced upregulation of c-Fos in spinal dorsal horn neurones involves activity of the PKCζ/PKMζ pathway.

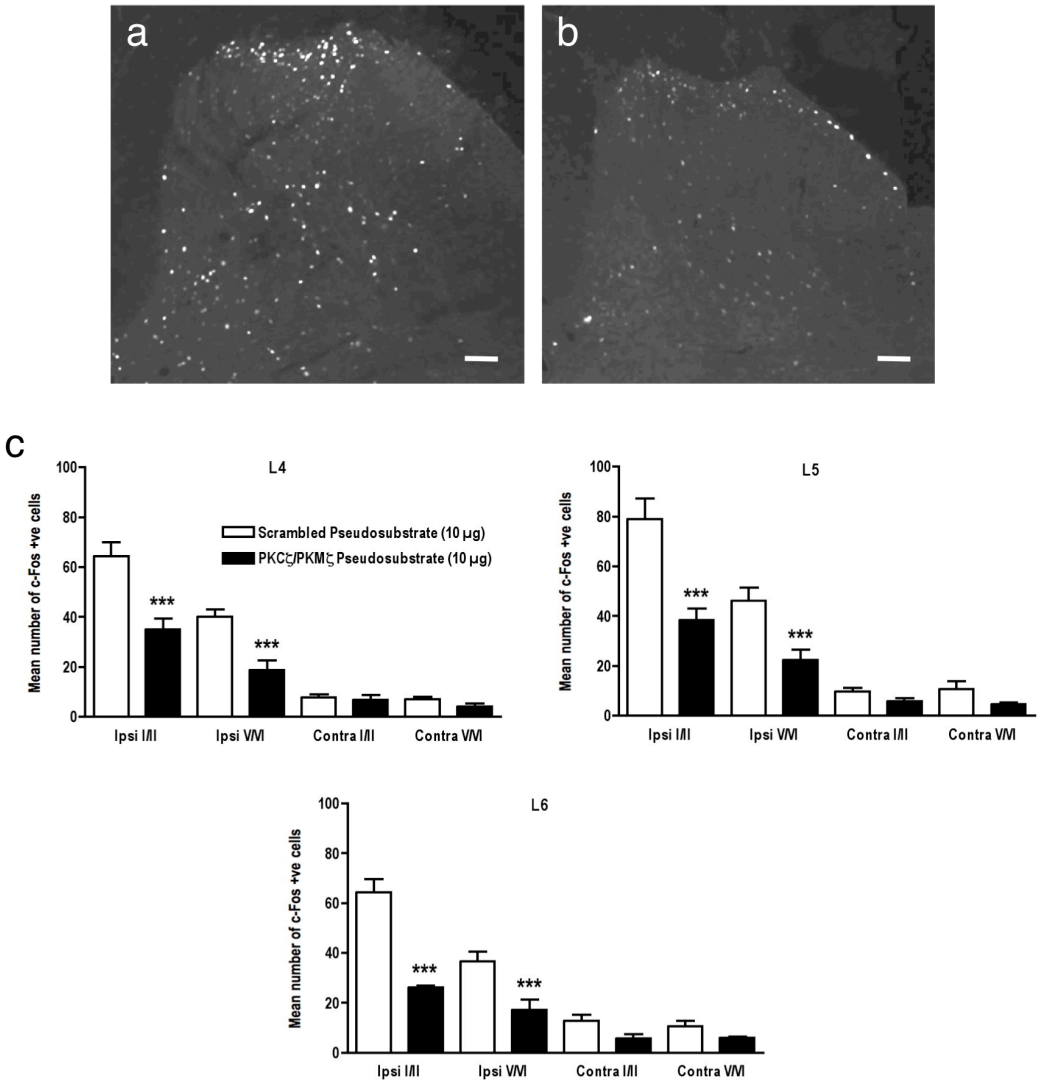


Fig. 5.4: Inhibition of spinal PKCζ/PKMζ reduces formalin-induced upregulation of c-Fos. **(a,b)** Representative photomicrographs of formalin-induced upregulation of c-Fos expression in superficial (I-II) and deep laminae (V-VI) of the ipsilateral lumbar (L5) dorsal horn in rats pre-treated with intrathecal **(a)** scrambled pseudosubstrate (10 μg, n = 5) or **(b)** PKCζ/PKMζ pseudosubstrate (10 μg, n = 5). **(c)** Quantification of number of c-Fos-positive cells per section in superficial (I-II) and deep laminae (V-VI) of the lumbar (L4, L5 and L6) dorsal horn. All data presented as mean ± s.e.m., *** $p < 0.001$, two-way ANOVA followed by Bonferroni post-tests. Scale bars = 100 μm.

5.3.4 Phosphorylation of dorsal horn PKCζ/PKMζ is induced by intraplantar formalin and is prevented by spinal administration of Tat-NR2B9c

Activation of the PKCζ pathway was assessed by immunohistochemistry using a phospho-specific antibody which recognizes the Thr410 phosphorylation site of both PKCζ and PKMζ isoforms, 60 min after intraplantar formalin (Fig. 5.5). In control rats pre-treated intrathecally with saline (0.9%, n = 4) or scrambled pseudosubstrate peptide (10 μg, n = 5), and in rats which received the myristoylated PKCζ/PKMζ pseudosubstrate inhibitor (10 μg, n = 5), intraplantar formalin injection into the hindpaw produced a significant increase of p-PKCζ in the superficial (I-II) layers of the ipsilateral dorsal horn (levels L4-L5) compared to the contralateral side (saline: mean percentage increase = 26 ± 3 %, $p < 0.05$; scrambled peptide: mean percentage increase = 22 ± 2 %, $p < 0.05$; pseudosubstrate inhibitor: mean percentage increase = 25 ± 2 %, $p < 0.05$; Fig. 5.5a-c). No difference was seen between saline, scrambled peptide and PKCζ/PKMζ pseudosubstrate inhibitor groups (Fig. 5.5e). In sections where the primary anti-p-PKCζ antibody had been omitted from the staining protocol, a complete loss of immunoreactivity was observed, indicating specificity of the antibody for phosphorylated PKCζ (not shown).

An additional group of rats were pre-treated with Tat-NR2B9c (125 ng, n = 5). The formalin-induced upregulation of p-PKCζ in the ipsilateral dorsal horn was prevented by intrathecal pre-treatment with Tat-NR2B9c (mean percentage increase = 4 ± 2 %, $p < 0.001$; Fig. 5.5d,e).

Therefore, PKCζ/PKMζ is activated in the spinal dorsal horn after peripheral injection of formalin into the hindpaw. The effect of the myristoylated PKCζ/PKMζ pseudosubstrate inhibitor seems to be inhibition of activity of this pathway, rather than prevention of its activation. Furthermore, upregulation of p-PKCζ was prevented by Tat-NR2B9c, suggesting that phosphorylation of PKCζ/PKMζ is mediated by coupling between NR2B-containing NMDA receptors and PSD-95 in spinal dorsal horn neurones.

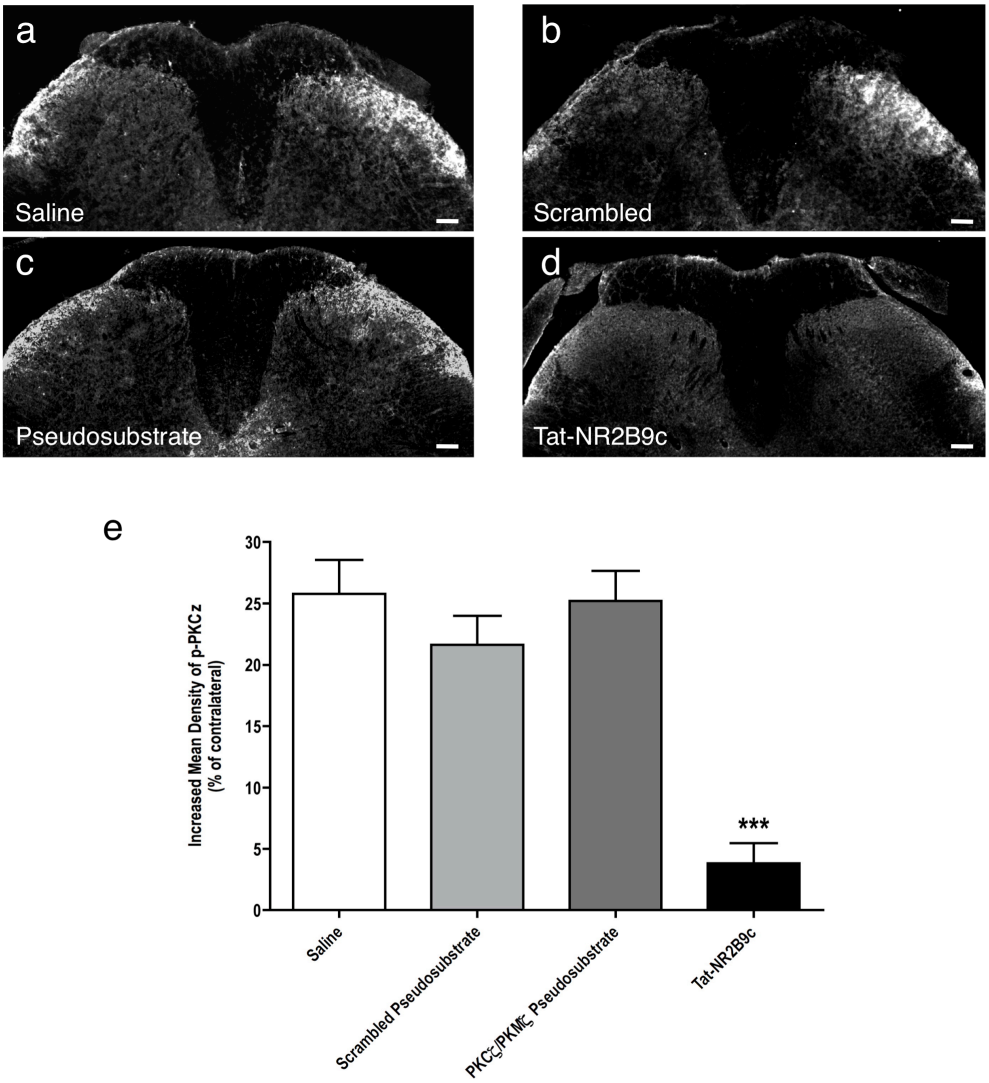


Fig. 5.5: Intrathecal Tat-NR2B9c reduces formalin-induced phosphorylation of PKCζ/PKMζ. **(a-d)** Representative photomicrographs of expression of p-PKCζ in superficial laminae of the ipsilateral (right) lumbar dorsal horn following intraplantar formalin in rats pre-treated with intrathecal **(a)** saline (0.9% *n* = 4), **(b)** scrambled pseudosubstrate (10 *μ*g, *n* = 5), **(c)** PKCζ/PKMζ pseudosubstrate (10 *μ*g, *n* = 5) or **(d)** Tat-NR2B9c (125 ng, *n* = 5). **(e)** Quantification of increased expression of p-PKCζ in the ipsilateral dorsal horn presented as a percentage of contralateral dorsal horn expression, mean ± s.e.m., *** $p < 0.001$, one-way ANOVA followed by Bonferroni post-tests. Scale bars = 100 *μ*m.

5.3.5 Formalin-induced upregulation of p-PKCζ is within intrinsic dorsal horn neurones

Phospho-PKCζ immunoreactivity was observed in NeuN-positive cells, marking neuronal nuclei (Fig. 5.6c), and seemed to be located in the membrane of neurones, since there was no overlap with NeuN staining. No obvious co-localization was observed with GFAP-positive astrocytes or Iba1-positive microglial cells (Fig. 5.6a-b). Thus, phosphorylated PKCζ/PKMζ, following intraplantar formalin, seems to be exclusively present in neurones within the spinal dorsal horn.

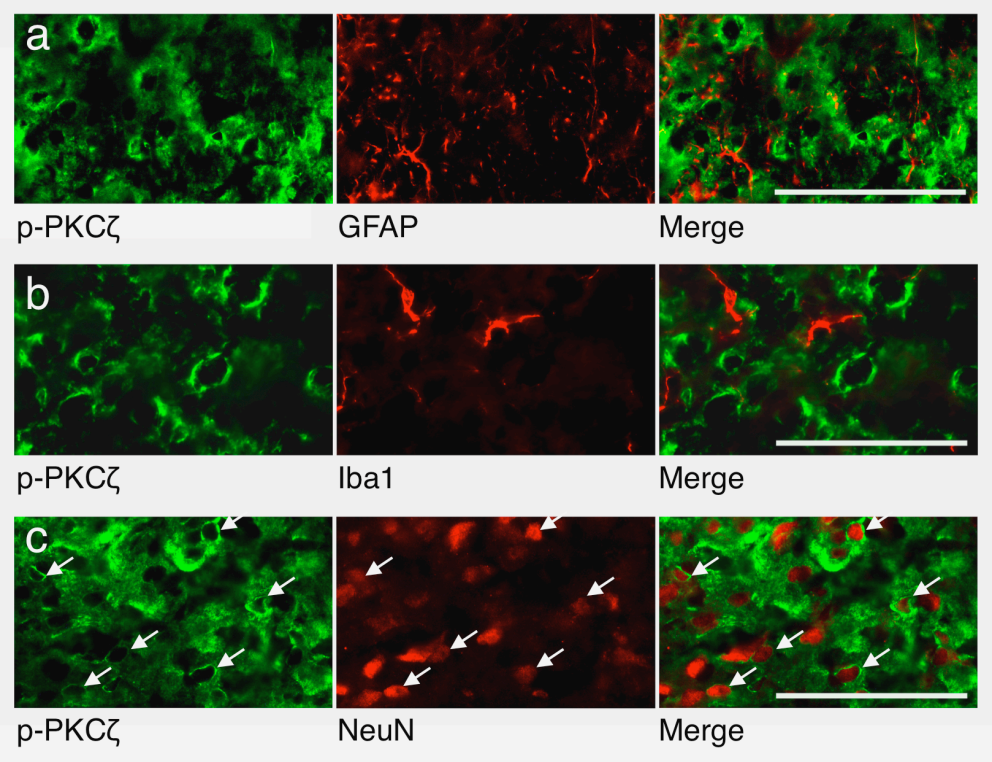


Fig. 5.6: Identification of spinal cell types expressing phospho-PKCζ following intraplantar formalin. **(a-c)** Double staining of p-PKCζ with **(a)** GFAP (astrocyte marker) and **(b)** Iba1 (microglia marker) did not show any co-expression. However, p-PKCζ was co-expressed with **(c)** NeuN (neuronal marker). Scale bars = 50 μm.

5.3.6 Co-immunoprecipitation of NMDA receptor NR2B subunits and PSD-95 reveals a novel interaction with PKCζ in spinal dorsal horn neurones

Immunoprecipitation can be used to reveal physical coupling between proteins *in vivo.* An antibody against NR2B subunits was used to immunoprecipitate NR2B protein from spinal dorsal horn tissue lysate. The immunoprecipitated sample was then separated by SDS-PAGE and associated proteins were revealed by western blotting (Fig. 5.7). As expected, PSD-95 protein, known to bind to the cytoplasmic tail of NR2B subunits, was co-immunoprecipitated by the anti-NR2B antibody, as was nNOS. In addition, PKCζ was also detected in the immunoprecipitated sample, revealing a novel protein-protein interaction of this kinase with spinal NMDA receptors and specifically with NR2B subunits. Normal spinal lysate was run alongside immunoprecipitation samples to act as positive controls. To ensure that the immunoprecipitation step had worked, and that proteins were not being detected non-specifically, two negative control proteins, CREB and P_2X_3, were probed for. CREB is a nuclear protein which should therefore not be physically linked to membrane-bound NMDA receptors. The P_2X_3 receptor is located on the central terminals of primary afferents rather than on intrinsic dorsal horn neurones, and thus should not be found in a complex including NR2B subunits and PSD-95. Accordingly, both CREB and P_2X_3 were detected in the normal spinal tissue lysates, but were not found in the immunoprecipitation samples, as expected, indicating that the immunoprecipitation had worked and that proteins found in this sample are physically coupled to NR2B subunits of NMDA receptors. Since PSD-95 is not expressed pre-synaptically in the spinal dorsal horn (i.e. in primary afferent terminals), but only within intrinsic dorsal horn neurones, the presence of PSD-95 protein in the immunoprecipitated sample would suggest that the NR2B-associated complex is present within the same intrinsic dorsal horn sensory neurones.

These data confirmed the physical interaction between NR2B subunits of spinal NMDA receptors and PSD-95, an association which modulates receptor activity and couples NMDA receptor activity to downstream intracellular signalling. Western blotting of PKCζ confirmed its expression in the dorsal horn of the spinal cord and co-immunoprecipitation with NR2B subunits revealed a possible novel physical interaction between these proteins within intrinsic spinal dorsal horn neurones,

perhaps mediated via the scaffolding protein PSD-95. Thus, these findings suggest that activation of NR2B-containing NMDA receptors and coupling with PSD-95 may lead to the direct activation of signalling via the PKCζ/PKMζ pathway, contributing to spinal central sensitization.

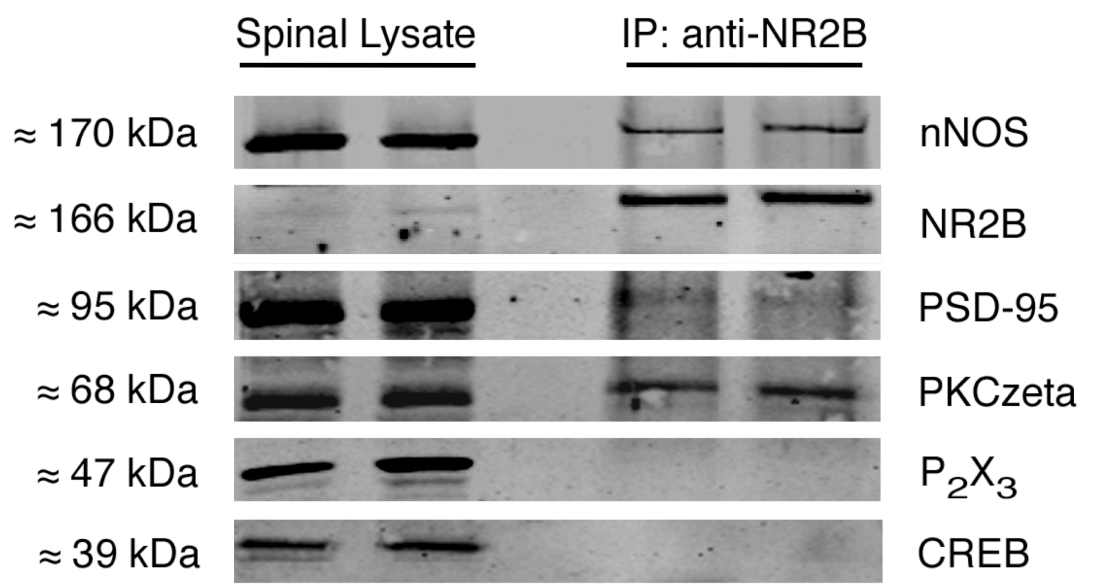


Fig. 5.7: Identification of a novel NR2B-associated complex with PSD-95 and PKCζ in spinal dorsal horn neurones. Western immunoblot of normal lumbar spinal dorsal horn lysates (left two lanes) and co-immunoprecipitates from lumbar spinal dorsal horn lysate obtained using an antibody against NR2B (right two lanes), probed with antibodies against NR2B, nNOS, PSD-95, PKCζ, CREB and P_2X_3.

5.4 Discussion

This study demonstrates that atypical PKCζ/PKMζ is involved in central sensitization of spinal dorsal horn neurones following peripheral formalin injection. A specific pseudosubstrate inhibitor of PKCζ/PKMζ, injected intrathecally, reduced pain-related behaviours and neuronal expression of c-Fos elicited by intraplantar injection of formalin. In addition, direct spinal application of this inhibitor reduced the firing response of WDR neurones to formalin administration into the hindpaw receptive field. Pain-related behaviours were associated with increased expression of phospho-PKCζ in the ipsilateral dorsal horn, specifically within neurones, which was abolished by spinal administration of the mimetic peptide Tat-NR2B9c, believed to disrupt binding between NR2B-subtype receptors and PSD-95. Immunoprecipitation revealed the presence of an NR2B-associated complex with PSD-95 and PKCζ within dorsal horn neurones, implicating atypical PKCζ/PKMζ as a novel downstream effector of NMDA-dependent nociceptive plasticity.

Both neuronal and behavioural formalin tests showed a significant reduction in second phase activity with the use of the PKCζ/PKMζ inhibitor. In contrast, a significant reduction of first phase activity was observed in the behavioural response only with the high dose (10 μg) of the inhibitor. The lower dose (5 μg) did not affect the first phase yet selectively and significantly reduced second phase pain-related behaviours. Neuronal firing during the first phase was reduced only slightly. This discrepancy between neuronal and behavioural first phase effects of the PKCζ/PKMζ inhibitor could be explained by the fact that electrophysiological recordings were conducted exclusively from deep dorsal horn WDR neurones. It is possible that other spinal sensory neurones, such as NS cells, which are mainly populated in the superficial dorsal horn, also contribute to the behavioural response to formalin. Inhibition of PKCζ/PKMζ within NS neurones or other cells in the superficial dorsal horn may reduce the responsiveness of these cells to incoming signals from nociceptive C-fibres, which is a major component of the first phase. It must also be remembered that there is a fundamental difference between recording neurones and behavioural activity in terms of what that activity means. In behavioural studies, a rat will produce a pain-related behaviour once the threshold for that response has been

reached. An increase in stimulus intensity cannot increase this behaviour in any detectable way and rats cannot communicate heightened pain to us. Therefore, in behavioural experiments we are dealing simply with thresholds: the rat will either respond or it will not, depending on whether the threshold for that particular behaviour is reached. In contrast, neurones can give us much more information. They will respond to sub-threshold, threshold and supra-threshold stimulation, particularly WDR neurones which produce graded responses as stimulus intensity increases. This gives us a quantifiable output, which correlates with heightened pain, that behavioural measures cannot. Therefore, the neuronal responses to formalin will consist of activity induced by sub-threshold, threshold and supra-threshold stimulation of the hindpaw receptive field. What is not clear, in this model, is how much neuronal activity is induced by each category of stimulus and therefore, we cannot say how much neuronal activity is required to reach the threshold of formalin-induced pain-related behaviour. Subsequently, we cannot say how much inhibition of neuronal activity is required to reduce behaviour. It may be that a small reduction on neuronal firing will greatly reduce behaviour. Additionally, it cannot be ruled out that PKCζ/PKMζ may function within primary afferent fibres, thus contributing to the first phase response. However, the immunohistochemical and biochemical data, which indicates that phosphorylation of PKCζ/PKMζ is predominantly within intrinsic dorsal horn neurones in this formalin model, would suggest otherwise. Lumbar rhizotomy, which causes a loss of primary afferent terminals within the dorsal horn, did not alter spinal expression of PKCζ/PKMζ (verbal communication from Dr. Fabien Marchand). Ultimately, the electrophysiological and behavioural data together clearly implicate PKCζ/PKMζ in spinal mechanisms of central sensitization.

Others studies have demonstrated reduction of pain-related behaviours in inflammatory pain models using non-selective blockers of PKC (Coderre, 1992; Coderre and Yashpal, 1994; Sweitzer et al., 2004). Interestingly, PKCγ knockout mice exhibit a reduction of the second phase of the formalin test (Malmberg et al., 1997). However, PKCγ only contributes to increased hyperexcitability of a subset of lamina V NMDA-dependent neurones following an inflammatory stimulus, suggesting only a partial contribution of this particular kinase in central sensitization (Martin et al., 2001). Therefore, other PKCs must be involved, with PKCζ/ PKMζ a likely

contributor. Following intraplantar injection of formalin, upregulation of c-Fos was observed in superficial and deep laminae of the dorsal horn, which was reduced following PKCζ/PKMζ inhibition, reinforcing the hypothesis of a profound effect on neuronal activity. Another kinase, PKCε, also influences c-Fos expression in the lumbar dorsal horn following formalin, though its effects are mediated by inhibition of neurotransmitter release from primary afferent fibre terminals (Velazquez et al., 2007). Furthermore, decreased expression of c-Fos following formalin injection in PKCγ knockout mice only occurs in laminae I-II (Malmberg et al., 1997).

The Tat-NR2B9c mimetic peptide, believed to disrupt a protein complex located specifically within intrinsic dorsal horn neurones, almost completely abolished phosphorylation of PKCζ. This result further corroborates that the formalin-induced upregulation of p-PKCζ is restricted to intrinsic dorsal horn neurones rather than terminating primary afferent fibres. The pseudosubstrate PKCζ/PKMζ inhibitor did not affect the phosphorylation of PKCζ following formalin, as previously described, at least for PKMζ in hippocampal slices following electrically-induced LTP (Kelly et al., 2007). Therefore, the antinociceptive effect of this inhibitor may be due to a reduction of the phosphorylating capacity of PKCζ via specific blockade of its enzymatic site, rather than its ability to be phosphorylated and further activated. The phospho-specific antibody used in this study recognizes the Thr410 phosphorylation site of both PKCζ and PKMζ isoforms, so it is likely that both isoforms are activated following intraplantar formalin. In addition, the pseudosubstrate inhibitor blocks both isoforms and therefore, the behavioural and electrophysiological results are likely due to blockade of activity of both PKCζ and PKMζ proteins.

Phosphorylation of PKCζ/PKMζ was observed in the superficial dorsal horn where expression of NR2B subunits is also thought to be concentrated (Nagy et al., 2004a). As mentioned previously (Chapter 3), the deep dorsal horn neurones recorded here likely receive nociceptive inputs from the superficial dorsal horn via interneurones and/or their dorsally extending dendrites (Woolf and King, 1987; Braz et al., 2005). This would account for the effects of the PKCζ/PKMζ inhibitor on activity of deep WDR neurones in electrophysiological experiments. Also, the PKCζ/PKMζ inhibitor was able to reduce c-Fos expression in both superficial and deep laminae of the

dorsal horn, indicating some connectivity and crosstalk between superficial neurones expressing PKCζ/PKMζ and deeper cells.

One of the major findings of this study is that PKCζ/PKMζ is not simply activated downstream of the NMDA receptor, but is physically linked to the receptor in a complex which also involves PSD-95. Spinal treatment with Tat-NR2B9c, believed to disrupt the interaction between NR2B subunits and PSD-95, not only inhibits nociceptive plasticity and pain-related behaviour (Chapters 3 & 4), but also prevents activation of PKCζ/PKMζ. Along with the data from the co-immunoprecipitation experiment, this result suggests the presence of a signalling complex between NR2B-containing NMDA receptors, PSD-95 and PKCζ/PKMζ, with activation of PKCζ/PKMζ being downstream of coupling between NR2B subunits and PSD-95. Further evidence, such as the use of immunohistochemistry and confocal microscopy to assess co-localization of these proteins, is required to strengthen this hypothesis. Cells expressing p-PKCζ are neuronal, as suggested by association with cells expressing NeuN. Specifically, p-PKCζ staining was observed around the NeuN staining, thus confirming that p-PKCζ is non-nuclear, perhaps located in the membrane of neurones, consistent with the finding that PKCζ was found to be in a complex with NR2B-subtype receptors and PSD-95, two membrane-associated proteins. Again, confocal microscopy may be used to shed further light on the precise subcellular location of PKCζ. The presence of PKCζ in such a complex may allow it to be specifically regulated by other kinases such as PI3K, CaMKII, ERK and mTOR (mammalian target of rapamycin), all of which have been shown to regulate PKMζ in hippocampal LTP as well as regulate spinal central sensitization (Ji et al., 1999; Fang et al., 2002; Choi et al., 2006; Kelly et al., 2007; Pezet et al., 2008; Asante et al., 2009). CaMKII is known to interact with PSD-95 in contributing to neuropathic pain (Garry et al., 2003), while disrupting NR2B interactions with PSD-95 in the brain using Tat-NR2B9c has been shown to regulate ERK (Yang et al., 2004). The mTOR pathway has been shown to be functionally linked to NMDA receptor activity in neuronal protein synthesis (Gong et al., 2006; Huang et al., 2007). PI3K has been shown to be activated by NMDA receptor stimulation (Sutton and Chandler, 2002; Man et al., 2003) and can influence synaptic trafficking of PSD-95 (Yoshii and Constantine-Paton, 2007), while Akt (PKB), a downstream target of PI3K, is

complexed with NR2B subunits in the dorsal horn (Peng et al., 2009). In fact, PI3K itself has been shown to bind NR2B subunits of NMDA receptors (Hisatsune et al., 1999). Phosphorylation of PKCζ at Thr410 of its activation loop is by PDK-1 (Chou et al., 1998; Le Good et al., 1998) and activation of PDK-1 requires binding to PIP_3, which is produced by PI3K. PIP_3 is also required to relieve PKCζ from autoinhibition (Hirai and Chida, 2003). Thus, PI3K is significantly involved in regulation of PKCζ. The role of PI3K in formalin-induced central sensitization is investigated in Chapter 6 of this thesis.

This study highlights a new spinal mechanism contributing to abnormal pain states with potential therapeutic applications. PKCζ/PKMζ, a kinase expressed in spinal sensory neurones, is involved in central sensitization and inhibition of this pathway may be a novel analgesic target. A physical interaction exists between PKCζ and NR2B-containing NMDA receptors in a complex also including PSD-95, thus implicating PKCζ/PKMζ as an effector of NMDA-dependent nociceptive plasticity in the dorsal horn of the spinal cord.

6 The role of phosphatidylinositol 3-kinase (PI3K) in NMDA-dependent spinal nociceptive plasticity

6.1 Introduction

6.1.2 Structure & function of Phosphatidylinositol-3-kinase

PI3K is a heterodimer enzyme associated directly with growth factor tyrosine kinase receptors and adaptor proteins (Cantley, 2002). The 85 kDa regulatory subunit (p85) maintains the 110 kDa catalytic subunit (p110) in a low activity state. Upon receptor activation, the regulatory subunit binds to phosphotyrosine residues of receptors which promotes the endogenous enzymatic activity of the catalytic subunit (Kapeller and Cantley, 1994). The Src-homology domains (SH2 and SH3) of the regulatory subunit (Fig. 6.1a) bind directly to specific phosphotyrosine motifs and proline-rich short amino acid sequences and thus mediate the specificity of association between PI3K and other proteins. The p110 catalytic subunit binds to the p85 regulatory subunit via its N-terminal region (Kapeller and Cantley, 1994).

PI3K is part of a larger family of phosphatidylinositol kinases (PIKs) which phosphorylate and synthesize phosphoinositides (PIs). Sequential phosphorylation of PI to produce phosphatidylinositol 4-phosphate (PI4-P) and phosphatidylinositol 4,5-bisphosphate (PI4,5-P_2) had been known to involve PI4K and PI5K respectively. The resultant PI4,5-P2 acts as a substrate for PLC and is hydrolysed to inositol 1,4,5-trisphosphate (IP_3), which promotes release from intracellular Ca^{2+} stores, and DAG, which activates PKC (Kapeller and Cantley, 1994). It was later discovered that a distinct PIK lead to the production of a novel PI, termed phosphatidylinositol 3-phosphate (PI3-P). Phosphorylation of PI3-P had occurred at the D-3 position of the inositol ring (Whitman et al., 1988), rather than at the D-4 position which produces PI4-P via PI4K. Thus, PI3K selectively phosphorylates the D-3 position of the inositol ring of PIs, generating membrane-associated second messengers. In addition to producing PI3-P, PI3K can phosphorylate PI4-P and PI4,5-P_2 to generate PI3,4-P_2

and PI3,4,5-P_3 (from this point onwards referred to as PIP_2 and PIP_3) respectively (Fig. 6.1b).

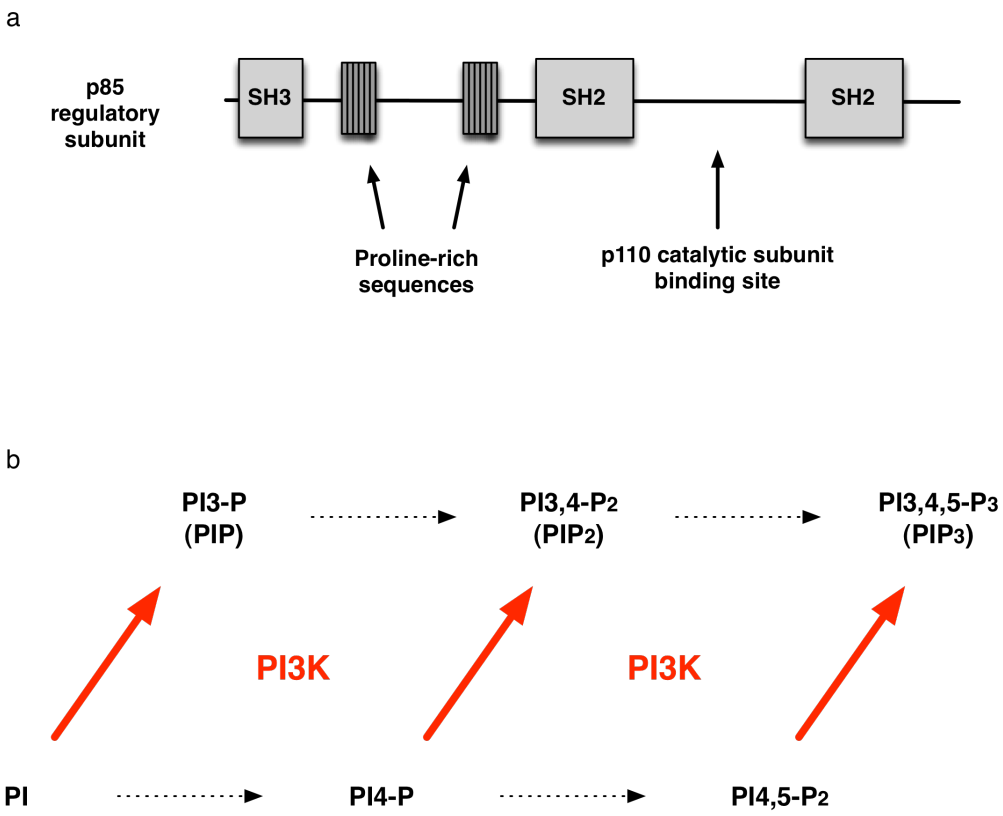


Fig. 6.1: Structure of the PI3K p85 regulatory subunit and phosphoinositide pathway. **(a)** The p85 regulatory subunit of PI3K consists of three Src-homology (SH) domains and proline-rich sequences which mediate interactions with other proteins. The p110 catalytic subunit of PI3K binds to the p85 subunit via its N-terminal region. **(b)** PI3K phosphorylates the D-3 position of the inositol ring of phosphoinositides to generate the second messengers PIP, PIP_2 and PIP_3. Adapted from Kapeller and Cantley (1994).

6.1.3 Protein kinase mediators of PI3K signalling

PI3K has been implicated in diverse cellular functions, including mitogenesis, inhibition of apoptosis, intracellular vesicle trafficking and regulation of the cytoskeleton (Carpenter and Cantley, 1996). More recently, biochemical targets of PIs generated by PI3K have been identified, including various protein kinases, for example Akt, PDK-1, PKCζ and MAPKs. PIP_2 and PIP_3 are not, however, involved in the release of Ca^{2+} from intracellular stores.

Akt is a major downstream effector of PI3K signalling and contains a pleckstrin-homology (PH) domain to which PIP_2 binds, with greater affinity than PIP_3, contributing to activation of the enzyme (Franke et al., 1997). PIP_2 recruits Akt to the plasma membrane and facilitates dimerization and partial activation of the enzyme. This produces a conformational change in Akt which exposes it to upstream kinases, such as PDK-1, which itself is regulated by PI3K-generated PIs, to be phosphorylated and fully activated (Toker and Cantley, 1997). For full activation of Akt, phosphorylation is required at Thr308 in the activation loop by PDK-1 and at Ser473 in the C-terminal region by an, at present, unidentified kinase, though phosphorylation at Thr308 may trigger autophosphorylation at Ser473 without the need for an additional kinase (Toker, 2000). Once activated, Akt leaves the plasma membrane to phosphorylate various intracellular targets, including the nuclear transcription factor CREB. Akt also phosphorylates and suppresses Bcl-2 family pro-apoptotic factors such as BAD. A third target of Akt is glycogen synthase kinase 3 (GSK3) which, when phosphorylated, can no longer inhibit the synthesis of glycogen (Cantley, 2002).

PDK-1 also contains a PH domain allowing it to be translocated to the plasma membrane by PIP_3, which also promotes intrinsic catalytic activity of the kinase (Toker and Newton, 2000). Several kinases are phosphorylated by PDK-1 including conventional PKCs and atypical PKCζ, and also p70S6K, involved in cell growth, and p90RSK, both of which are involved in signalling in the MAPK pathway (Toker, 2000). PKCζ, known to be involved in LTP, is itself thought to be regulated directly by PIP_3, despite the fact that a PIP_3-binding site has not been located in its structure. It is believed that PIP_3 directly modulates the pseudosubstrate domain of PKCζ, thus

relieving autoinhibition and promoting catalytic activity (Hirai and Chida, 2002). Additionally, PKCζ has been shown to be activated downstream of the mTOR (Kelly et al., 2007) which itself is downstream of both PDK-1 and Akt in a PI3K-dependent pathway.

All of the kinases mentioned thus far are able to contribute to PI3K-mediated regulation of the MAPK pathway. Aside from its function as a lipid kinase, PI3K may also possess the ability to directly phosphorylate proteins. This protein kinase activity was retained in a mutant of PI3K which was deficient of lipid kinase function but was still able to activate MAPK (Bondeva et al., 1998). This shows that PIs are not always necessary for regulation of MAPK by PI3K. Therefore, PI3K can activate the MAPK pathway at various points in the cascade involving activation of protein kinases by PIP_3, as well as via its own intrinsic protein kinase function (Toker, 2000).

6.1.4 PI3K in brain LTP

Several reports have demonstrated a requirement of PI3K activity in brain synaptic plasticity, including LTP. An electrophysiological study in rat hippocampal slices showed that PI3K activity was induced during LTP. Conversely, established LTP was reduced by two widely-used inhibitors of PI3K, LY294002 and wortmannin. However, following drug washout, LTP recovered, suggesting a predominant role of PI3K in the expression of LTP (Sanna et al., 2002). A subsequent study confirmed a role for PI3K in hippocampal LTP, when PI3K inhibitors, applied at the time of the conditioning stimulus, prevented LTP. However, inhibition of PI3K had no effect on LTP once it had already been established, indicating that PI3K is more involved in the induction rather than maintenance or expression of LTP (Opazo et al., 2003). Despite the obvious discrepancies regarding the timing of activation of PI3K during LTP, it is clear that PI3K plays a crucial role in this form of neuronal synaptic plasticity. One potential mechanism by which PI3K might contribute to synaptic strengthening could be by promoting the membrane insertion of AMPA receptors. The NMDA-dependent increase in AMPA receptor surface expression during hippocampal LTP requires PI3K activity (Man et al., 2003) and further studies showed that PI3K activity in LTP is downstream of NMDA receptor activation (van

der Heide et al., 2005; Peineau et al., 2007). In addition, PI3K is involved in LTP via both ERK-dependent and ERK-independent mechanisms since not all forms of PI3K-dependent LTP, such as, for example, that induced by LFS paired with post-synaptic depolarization, can be blocked by ERK inhibitors (Opazo et al., 2003; Tsokas et al., 2007). Interestingly, stress-induced alterations of LTP involve PI3K signalling, perhaps via upregulation of PSD-95, since stress increased dendritic expression of PSD-95 in hippocampal neurones (Yang et al., 2008). Finally, mTOR has been shown to be an important effector of PI3K signalling in LTP (Tsokas et al., 2007). High frequency stimulation in the medial prefrontal cortex (mPFC) induced LTP and caused phosphorylation of the PI3K/Akt-mTOR pathway, both of which were suppressed by pharmacological inhibition of either PI3K or mTOR (Sui et al., 2008).

6.1.5 PI3K in pain pathways

A role for PI3K in nociceptive pathways was first established in terms of sensitization of peripheral nociceptors. Bonnington and McNaughton used Ca^{2+} imaging to show that NGF potentiated the capsaicin-induced rise in intracellular Ca^{2+} in a subset of nociceptive sensory neurones. This effect of NGF was abolished by application of the PI3K inhibitor wortmannin (Davies et al., 2000) indicating that activation of the NGF tyrosine kinase receptor, TrkA, leads to signalling via PI3K in nociceptors (Bonnington and McNaughton, 2003). This work was expanded on by Zhuang and colleagues who showed that intradermal injection of capsaicin and NGF in rats caused activation of PI3K and ERK in C-fibres. Inhibition of PI3K prevented both ERK activation and heat hyperalgesia, without effect on basal heat sensitivity (Zhuang et al., 2004). Subsequently it was shown that PI3K activates Src kinase which phosphorylates TRPV1, inducing insertion of TRPV1 into the plasma membrane following NGF (Zhang et al., 2005). An alternative mechanism for this potentiation of TRPV1 by PI3K was suggested by Stein and colleagues who demonstrated that PI3K is physically linked to TRPV1 via its p85 subunit and that NGF increased the number of channels in the membrane. Thus, it was proposed that NGF-mediated heat hyperalgesia is brought about by a physical coupling between TRPV1 and PI3K which facilitates trafficking of TRPV1 to the membrane of primary sensory neurones (Stein et al., 2006), perhaps via activation of the MAPK

pathway (Zhu and Oxford, 2007). Accordingly, phosphorylation of Akt has been detected in small and medium-sized nociceptive DRG neurones, following intradermal capsaicin, confirming activation of PI3K signalling. Neurones expressing phospho-Akt (p-Akt) were also positive for TrkA and TRPV1 and were both peptidergic ($CGRP^+$) and non-peptidergic ($IB4^+$) (Pezet et al., 2005; Sun et al., 2007). In contrast to NGF-induced hyperalgesia, acute peripheral administration of GDNF induces mechanical hyperalgesia involving non-peptidergic nociceptors which can also be attenuated by pharmacological inhibition of PI3K (Bogen et al., 2008).

Prior to publication of the work presented in this chapter (Pezet et al., 2008), little was known regarding involvement of PI3K in spinal cord pain transmission. One study had shown increased expression of p-Akt in the dorsal horn following intradermal capsaicin, measured using both immunohistochemistry and western blotting (Sun et al., 2006), while another study had demonstrated a role for PI3K in neuropathic pain following L5 SNL (Xu et al., 2007). It was found that p-Akt increased in both injured (L5) and non-injured (L4) DRG neurones, particularly non-peptidergic C-fibres. Interestingly, phosphorylation of Akt was also detected in the L5 spinal cord. Inhibition of PI3K signalling reduced hypersensitivity in both pain models.

The work in this chapter explores the specific contribution of spinal dorsal horn PI3K to NMDA-dependent nociceptive plasticity in wind-up and the formalin model of central sensitization.

6.2 Materials and Methods

6.2.1 *In vivo* Electrophysiology

Experiments were conducted in adult male Sprague Dawley rats (220-250 g, Central Biological Services, University College London, UK). Electrophysiological recordings from deep dorsal horn WDR neurones (> 600 μm) were conducted as previously described (see Materials and Methods chapter). Halothane anaesthesia was used in this study prior to the changeover to the safer isoflurane.

6.2.1.1 Electrical stimulation of the hindpaw

Stable control responses to electrical stimuli were established at 10 min intervals before drug administration. LY294002 (Tocris Biosciences, Bristol, UK), a potent inhibitor of PI3K, at a dose of 0.8 μg in 50 μl, was applied directly to the exposed surface of the spinal cord (after removal of any residual cerebrospinal fluid) using a Hamilton syringe. Electrical tests were continued for 1 hr at 10 min intervals. After 1 hr any remaining solution was removed from the surface of the spinal cord and replaced by 1.6 μg of LY294002 in 50 μl and tests were continued for a further 1 hr. A separate group of control animals received vehicle, 10% dimethyl sulfoxide (DMSO).

6.2.1.2 Statistical Analysis

Data are presented as mean ± s.e.m. The effects of vehicle or LY294002 versus pre-drug control values were assessed by one-way RM ANOVA, followed by Bonferroni post-tests, using GraphPad Prism v.4 (GraphPad Inc., San Diego, CA, USA).

6.2.2 Behavioural Formalin Test

Adult male Wistar rats (Harlan, UK) were injected intrathecally (lumbar puncture method) with LY294002 (50 or 100 μg in a volume of 10 μl) or vehicle (10% DMSO). Fifteen minutes later rats received 50 μl of 5% formalin injected subcutaneously into the plantar surface of the right hindpaw. Lifting, shaking, biting and licking of the injected paw were monitored by measuring the total duration of the response in seconds during 60 minutes following formalin administration. Data are presented in 5 min bins.

6.2.2.1 Statistical Analysis

All data is presented as mean ± s.e.m. Formalin behavioural response time course data was compared between treatment groups and analyzed by two-way RM ANOVA, followed by Bonferroni post-tests. Total activity in the first and second phases was compared between treatment groups by quantifying the area under each curve, and analyzed by one-way ANOVA, followed by Bonferroni post-tests. Statistical analyses were carried out using GraphPad Prism v.4 software (GraphPad Software Inc., San Diego, CA, USA).

6.2.3 Preparation of tissues for Immunohistochemistry and Western Immunoblotting

6.2.3.1 For phospho-Akt, phospho-ERK & phospho-CAMKII Immunohistochemistry

Adult male Wistar rats (Harlan, UK) were first anaesthetized with urethane (1 mg/kg), then pre-treated intrathecally via lumbar puncture method with LY294002 (100 μg in 10 μl) or vehicle (10 μl of 10% DMSO). Fifteen minutes later, rats were injected with 50 μl of 5% formalin into the plantar surface of the right hindpaw. A further five minutes after formalin administration, rats were perfused transcardially with 100 ml of heparinized saline followed by 400 ml of 4% PFA containing 15% of a saturated solution of picric acid. Rats were dissected and lumbar cord (levels L3-L6) was collected for immunohistochemistry.

6.2.3.2 For Western Immunoblotting of NMDA & AMPA receptor subunits

Rats anaesthetized with urethane were pre-treated intrathecally via lumbar puncture with LY294002 (100 μg in 10 μl) or vehicle (10 μl of 10% DMSO) and then injected with 5% formalin into the right hindpaw fifteen minutes later. After a further five minutes, rats were sacrificed by decapitation and fresh tissues were dissected out for western immunoblotting of NR2A and NR2B NMDA receptor subunits. Alternatively, for the detection of GluR1 AMPA receptor subunits, animals were sacrificed by decapitation 10, 45 and 90 min after the injection of formalin into the hindpaw. A group of control rats were also sacrificed without the injection of formalin (0 min) for comparison. Dorsal spinal cord, both ipsi- and contralateral, from the L4 and L5 levels, was collected and immediately frozen in liquid nitrogen.

6.2.4 Immunohistochemistry for phospho-Akt, phospho-ERK & phospho-CaMKII

Transverse spinal cord sections (30 μm thickness) were cut using a cryostat and every section was collected and placed in PBS solution for free-floating immunohistochemistry. Sections were washed with PBS and then incubated with primary antibody, either rabbit anti-phospho-Akt1 Ser473 (1:300, New England Biolabs, Beverly, MA, USA), rabbit anti-phospho-ERK (1:300, New England Biolabs, Beverly, MA, USA) or rabbit anti-phospho-CaMKII Tyr286 (1:300, ref 5683, Abcam, UK) overnight at room temperature.

Immunostaining against p-ERK was performed using direct fluorescence. After several washes with PBS, sections were incubated for 2 hrs at room temperature with secondary antibody (goat anti-rabbit IgG-conjugated Alexa Fluor^{TM} 488, 1:1000, Molecular Probes, Oregon, USA). Immunostaining against phospho-Akt Ser473 and phospho-CaMKII Tyr286 required tyramide amplification. Sections were incubated with the secondary antibody, goat anti-rabbit biotin (1:300 in PBS-T, Vector Laboratories, CA, USA) for 1 hr at room temperature. Following several washes, sections were incubated in avidin biotinylated enzyme complex (1:500 in PBS, Vector Laboratories, CA, USA) for 1 hr, followed by several washes in PBS. The sections were then incubated in biotinyl tyramide (1:75 in amplification buffer, Perkin Elmer, UK) for 10 min. After several washes in PBS, sections were finally incubated in extravidin fluorescein isothiocyanate (FITC, 1:500, diluted in PBS, Sigma).

After staining, sections were mounted onto slides and cover-slipped with Vectashield mounting medium (Vector Laboratories, CA, USA). Slides were visualized under a Zeiss Axioplan 2 fluorescent microscope and L4 and L5 sections were identified for quantification (5 sections per level per animal, chosen randomly). For p-ERK, labelled spinal cord neurones were counted in laminae I-II and V-VI, for both ipsilateral and contralateral (control) sides by one blinded investigator. For assessment of p-Akt and p-CaMKII upregulation, images of dorsal horn (ipsilateral and contralateral at the level of lamina II, where changes are mostly observed) from 5 sections per animal, chosen randomly, were taken at magnification x20, using the same set-up of acquisition for all animals. Densitometric analysis of medial p-Akt

and p-CaMKII staining (since staining was too abundant to count individual neurones) was carried out using Scion Image software. Results for p-Akt and p-CaMKII expression are given as a percentage increase in the ipsilateral versus contralateral (control) dorsal horn. Statistical significance was tested by the Kruskal-Wallis test followed by Dunn's multiple comparison test or the Mann-Whitney Rank Sum test using GraphPad Prism v.4 software (GraphPad Software Inc., San Diego, CA, USA). All imaging and quantification was conducted by Dr Sophie Pezet.

6.2.5 Western Immunoblotting of phospho-NR2A & phospho-NR2B

Proteins (30 μg/sample) were separated using 8% SDS-PAGE, and transferred to PVDF membranes. Membranes were then incubated with primary antibody, either rabbit phospho-NR2A Y1387 (1:500, ab16647, Abcam, UK) or rabbit phospho-NR2B Y1472 (1:1000, ab5403, Chemicon, USA), overnight at 4°C. After several washes in TBST, membranes were incubated with donkey anti-rabbit HRP-linked secondary antibody (1:5000, Amersham, UK) for 1 hr at room temperature, and revealed using ECL-plus^{TM} reagent (5 min) for detection of phosphorylation by autoradiography. Gels were scanned and bands were quantified by densitometric analysis using Scion Image software.

Results are expressed as percentage phosphorylation in the ipsilateral dorsal horn compared to the contralateral (control) dorsal horn for each animal (both ipsilateral and contralateral samples were run on the same gel). Mann-Whitney Rank Sum tests were carried out using GraphPad Prism v.4 software (GraphPad Software Inc., San Diego, CA, USA) to test the statistical significance of the results. Reprobing of the membranes with Ponceau red showed a lack of significant differences between the protein content of samples loaded on the same gel. Analysis of gels and quantification was conducted by Dr Sophie Pezet.

In all biochemical experiments, the contralateral dorsal horn served as an internal control for the effects of formalin injection. Urethane anaesthesia was used to allow for quick perfusion at multiple timepoints following formalin injection.

6.2.6 Translocation of AMPA receptor subunit GluR1

Subcellular fractioning (isolation of cytoplasm and plasma membranes) was carried out using a protocol previously described by Galan et al. (2004). In brief, spinal cord samples were homogenized in complete lysis buffer (CLB: 10 mM Tris pH 7.5, 300 mM sucrose, 1 mM EDTA, 1 mM sodium orthovanadate and protease inhibitor cocktail [Complete, Roche, UK]), and then centrifuged at 7000 g for 5 min. Supernatant containing cytoplasm (S1) was separated from the pellet containing nuclei and debris (P1), which was discarded. Supernatant (S1) was then centrifuged at 40,000 g for 30 min to obtain pure cytoplasmic extract in the supernatant (S2) and crude membrane in the pellet (P2). This pellet (P2), which includes plasma and cellular organelle membranes, was resuspended in PBS with protease inhibitor cocktail and sodium orthovanadate. Protein titration was conducted using a BCA Protein Assay Kit (Pierce, UK) to determine protein concentrations in the cytoplasmic and membrane samples and 7.7 μg and 6.0 μg of proteins were loaded onto gels for each cytoplasmic (S2) and membrane (P2) sample, respectively. Proteins were separated using 8% SDS-PAGE, and transferred to PVDF membranes. Membranes were incubated with rabbit anti-GluR1 primary antibody (1:1000, Chemicon, ab1504, Temecula, CA, USA) overnight at 4°C. After several washes in TBS-T, membranes were incubated with donkey anti-rabbit HRP-linked secondary antibody (1:5000, Amersham, UK) for 1 hr at room temperature and revealed using ECL-plus™ reagent (5 min) for detection by autoradiography. Gels were scanned and bands were quantified by densitometric analysis using Scion Image software. Analysis of gels and quantification was conducted by Dr Sophie Pezet.

Results are expressed as percentage of GluR1 expression in naïve rats (0 min, 100%) at each time point. Mann-Whitney Rank Sum tests were carried out using GraphPad Prism v.4 software (GraphPad Software Inc., San Diego, CA, USA) to test the statistical significance of the results.

6.3 Results

6.3.1 Spinal application of LY294002 selectively reduces C-fibre evoked responses and post-discharge of deep dorsal WDR neurones

Electrically evoked responses of deep dorsal horn WDR neurones were reduced by spinal application of LY294002 (0.8 μg, n = 6; 1.6 μg, n = 6) in a dose-dependent manner (Fig. 6.2) in comparison to vehicle (10% DMSO, n = 6). Statistically significant reductions were seen of C-fibre evoked responses (0.8 μg, % of pre-drug control response = 78 ± 4%, $p < 0.05$; 1.6 μg, % of pre-drug control response = 73 ± 4%, $p < 0.01$). These effects were selective as reductions of Aδ-fibre evoked responses did not reach statistical significance, while no changes were seen in Aβ-fibre evoked responses. The post-discharge of WDR neurones was also reduced by LY294002 (0.8 μg, % of pre-drug control response = 61 ± 14%, $p < 0.05$; 1.6 μg, % of pre-drug control response = 49 ± 5%, $p < 0.05$; Fig. 2) compared to vehicle. Inhibitory effects of LY294002 were evident by 10 min following spinal administration and persisted for the duration of the experiment. Administration of vehicle (10% DMSO) alone had no effect on any evoked response. Pre-drug control responses did not differ between treatment groups.

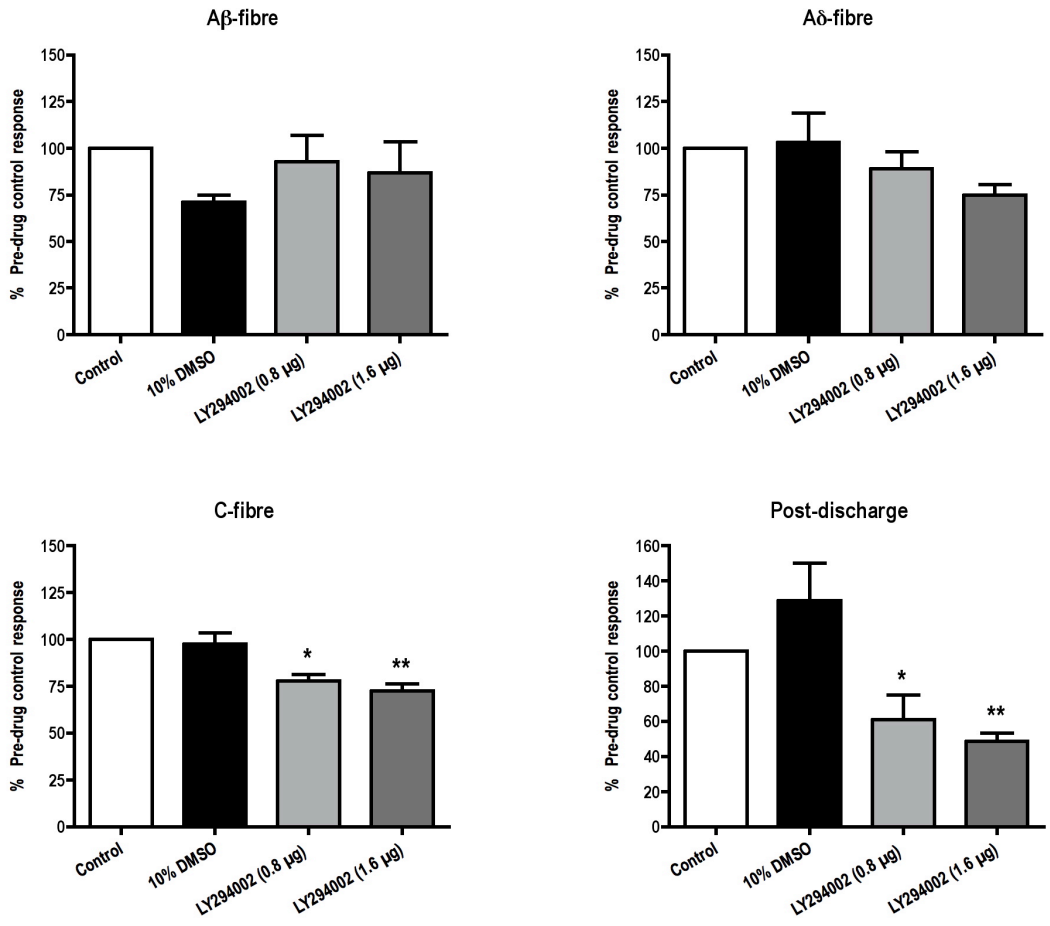


Fig. 6.2: Spinal LY294002 decreases C-fibre evoked responses and post-discharge of WDR neurones. Afferent-evoked responses and post-discharge of WDR neurones induced by transcutaneous electrical stimulation of the hindpaw receptive field, following spinal application of vehicle (10% DMSO) or LY294002 (0.8 μg or 1.6 μg). White bars represent pre-drug control. All data presented as mean ± s.e.m. of pre-drug control responses; $* \; p < 0.05$, $** \; p < 0.01$ versus vehicle; one-way RM ANOVA followed by Bonferroni post-tests on raw data; $n = 6$ in each group.

6.3.2 Wind-up of deep dorsal horn WDR neurones is reduced by spinal LY294002

Inhibition of PI3K by application of LY294002 directly onto the spinal cord produced a significant reduction of wind-up of WDR neurones (Fig. 6.3b,c; 0.8 μg, % of pre-drug control response = 43 ± 8%, $p < 0.01$; 1.6 μg, % of pre-drug control response = 50 ± 6%, $p < 0.01$) compared to vehicle (10% DMSO). No change in wind-up was seen with vehicle (10% DMSO) alone (Fig. 6.3b,d). Importantly, neither LY294002 nor vehicle had any effect on input (Fig. 6.3a), a measure of the non-potentiated neuronal response, suggesting that effects of LY294002 were more likely due to a direct effect on dorsal horn neuronal excitability rather than via pre-synaptic mechanisms. Pre-drug input and wind-up values were similar in both treatment groups.

Therefore, inhibition of PI3K reduces responses of deep dorsal horn WDR neurones evoked by electrical stimulation of nociceptive C-fibres. Favourably, A-fibre evoked responses remained unaltered. The post-discharge and wind-up of WDR neurones were also reduced by inhibition of PI3K, while there was no effect on input, indicating a post-synaptic mechanism of action.

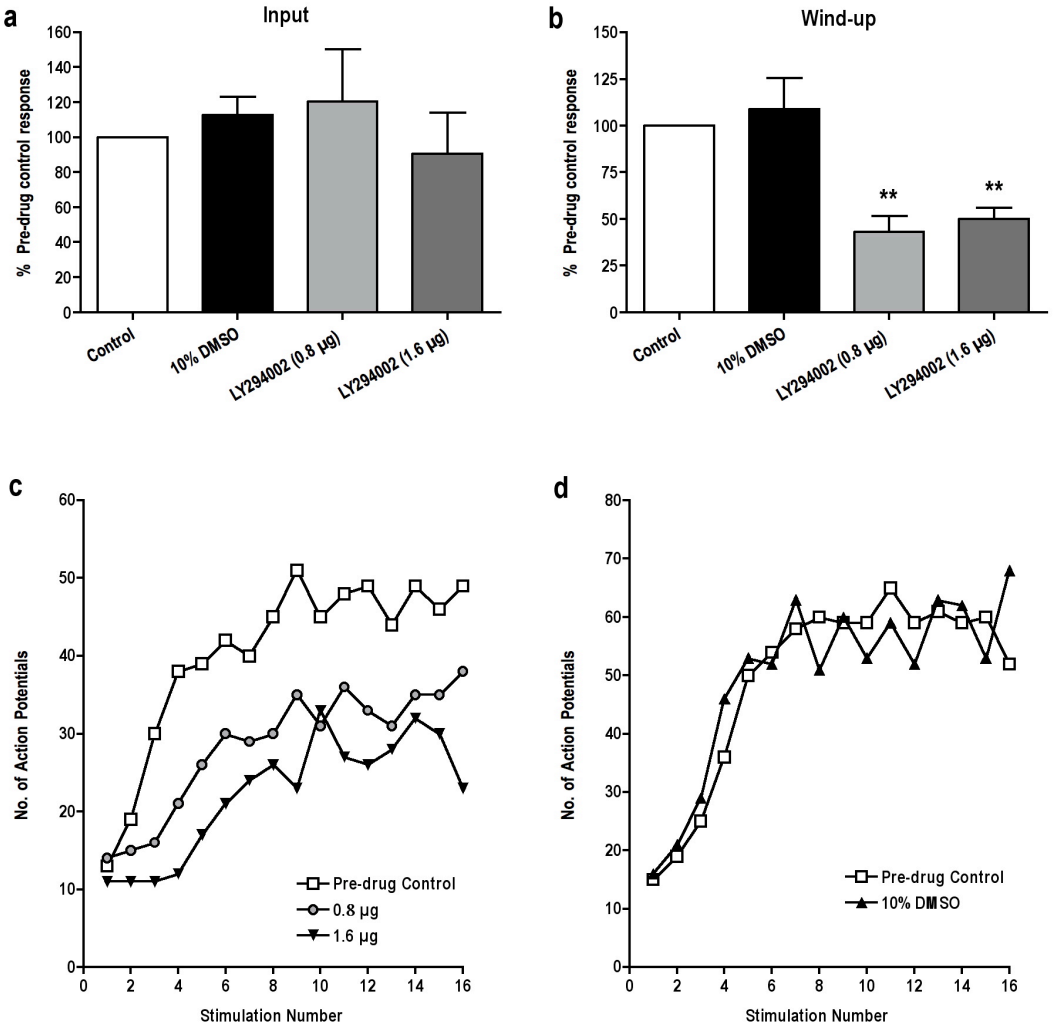


Fig. 6.3: Spinal LY294002 selectively reduces wind-up of WDR neurones.

(**a,b**) Effect of spinal application of vehicle (10% DMSO) or LY294002 (0.8 μg and 1.6 μg) on (**a**) input and (**b**) wind-up of WDR neurones. White bars represent pre-drug control. All data presented as mean ± s.e.m. of pre-drug control responses; ** $p <$ 0.01 versus vehicle; one-way RM ANOVA followed by Bonferroni post-tests on raw data; n = 6 in each group. (**c,d**) Examples of the wind-up of single WDR neurones following repetitive electrical stimulation in the presence of spinal (**c**) LY294002 (0.8 μg and 1.6 μg) or (**d**) vehicle (10% DMSO).

6.3.3 Inhibition of PI3K reduces pain-related behaviours due to central sensitization induced by intraplantar formalin

The intraplantar injection of formalin into the hindpaw of awake and freely-moving rats induced the characteristic biphasic response of pain-related behaviours in those rats pre-treated intrathecally with vehicle, 10% DMSO (n = 8, Fig. 6.4; 1st phase: 0-10 min, total time of pain-related behaviour = 131 ± 11 s; 2nd phase: 10-60 min, total time of pain-related behaviour = 368 ± 29 s). Pre-treatment with the PI3K inhibitor LY294002, however, 15 min prior to the injection of formalin, via intrathecal lumbar puncture, significantly decreased pain-related behaviours in a dose-dependent manner. Both the first phase response (Fig. 6.4; 50 μg, n = 8, 1st phase total time of pain-related behaviour = 96 ± 10 s; 100 μg, n = 6, 1st phase total time of pain-related behaviour = 82 ± 13 s, $p < 0.05$) and the second phase response (Fig. 6.4; 50 μg, n = 8, 2nd phase total time of pain-related behaviour = 254 ± 47 s; 100 μg, n = 6, 2nd phase total time of pain-related behaviour = 193 ± 36 s, $p < 0.05$) were reduced by LY294002 in comparison to vehicle treatment.

Thus, inhibition of PI3K reduces formalin-induced pain-related behaviours, suggesting that PI3K is involved in the transmission of nociceptive signalling within the spinal cord and development of spinal central sensitization.

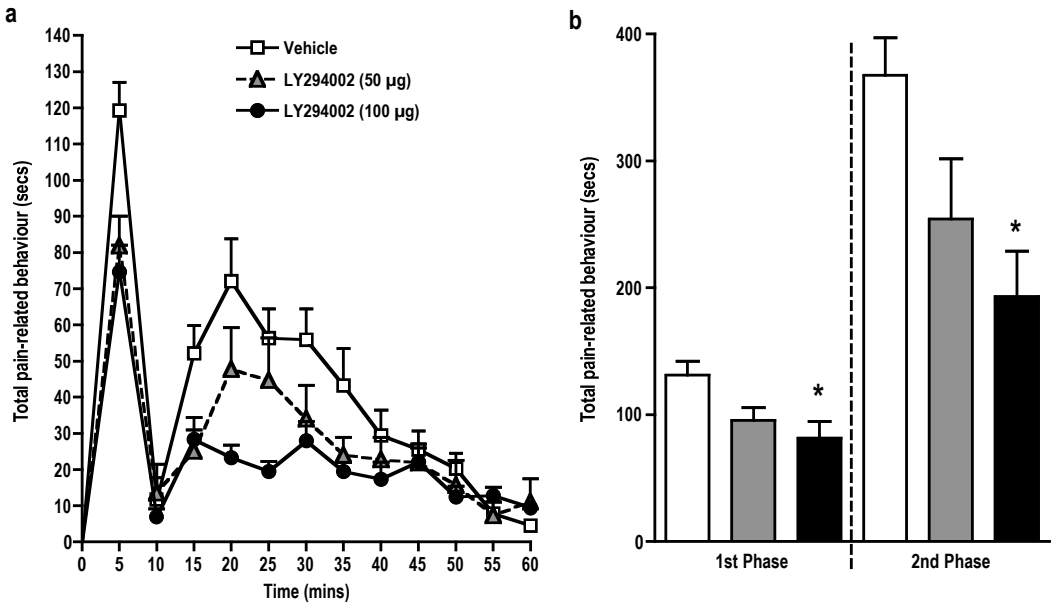


Fig. 6.4: Intrathecal LY294002 reduces pain-related behaviours due to formalin-induced central sensitization. **(a)** Time course of pain-related behaviours induced by intraplantar formalin (5%, 50 μl) following intrathecal pre-treatment with vehicle (10% DMSO, $n = 8$) or LY294002 (50 μg, $n = 8$; 100 μg, $n = 6$). **(b)** Total pain-related behaviour during the 1st (0-10 min) and 2nd (10-60 min) phases of the formalin response with vehicle (10% DMSO) or LY294002 (1st phase: 100 μg, $* p < 0.05$ versus vehicle; 2nd phase: 100 μg, $* p < 0.05$ versus vehicle; one-way RM ANOVA followed by Bonferroni post-tests). All data presented as mean ± s.e.m.

6.3.4 Phosphorylation of Akt in spinal neurones following intraplantar formalin

The phosphorylation of downstream kinase Akt (Ser473) can be measured using immunohistochemistry to indicate activation of the PI3K pathway. Intraplantar formalin injection into the hindpaw of rats produced a large increase of p-Akt in the medial part of the superficial layers (I-II) of the ipsilateral dorsal horn (levels L4-L5) compared to the contralateral side in vehicle-treated rats (10% DMSO, $n = 5$) within five minutes (mean percentage increase = 82 ± 22%, $p < 0.05$; Fig. 6.5). Inhibition of PI3K by intrathecal injection of LY294002 (100 μg, $n = 5$), fifteen minutes prior to formalin injection, prevented the increase in Akt phosphorylation in the ipsilateral dorsal horn such that the level of p-Akt staining did not differ significantly from that of the contralateral side (mean percentage increase = 18 ± 21%, Fig. 6.5c).

This result confirms that Akt, a downstream marker of the PI3K pathway, is activated in the spinal dorsal horn after peripheral administration of formalin in the hindpaw and this effect is dependent on upstream PI3K activity.

Taken together, these results show that activity of the PI3K pathway is involved in neuronal plasticity which contributes to formalin-induced spinal central sensitization and the resultant pain-related behaviours. In order to define the molecular mechanisms by which PI3K is involved in the development of central sensitization, the effect of PI3K inhibition on the activation of various intracellular signalling pathways and glutamate receptors, known to be involved in synaptic plasticity in the hippocampus and activated in the spinal cord following an inflammatory stimulus, was studied.

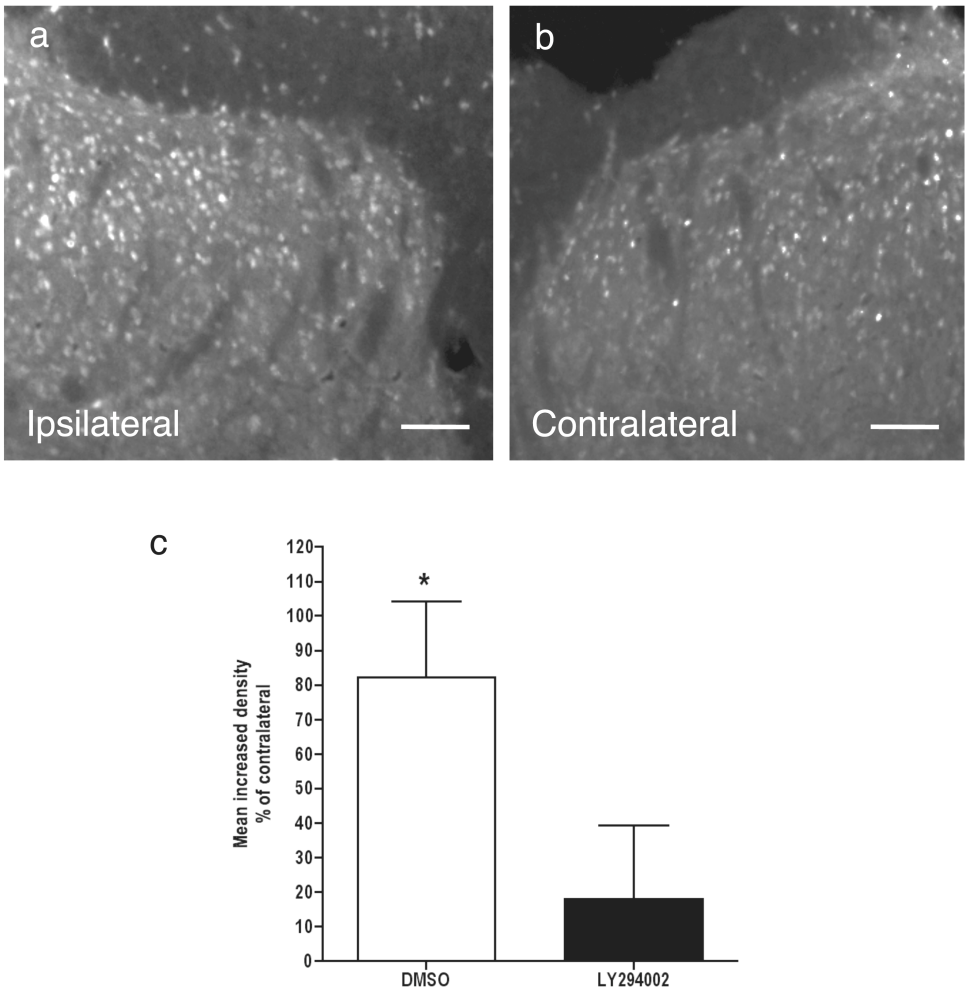


Fig. 6.5: Intraplantar formalin-induced phosphorylation of spinal Akt is reduced by LY294002. Representative photomicrographs of medial expression of p-Akt in superficial laminae of the **(a)** ipsilateral and **(b)** contralateral lumbar dorsal horn following intraplantar formalin in rats pre-treated with vehicle (10% DMSO). **(c)** Quantification of formalin-induced upregulation of p-Akt in the medial part of the ipsilateral dorsal horn following intrathecal pre-treatment with vehicle (10% DMSO, n = 5) or LY294002 (100 μg, n = 5). Data presented as a percentage of contralateral dorsal horn expression, mean ± s.e.m., $*$ $p < 0.05$ versus contralateral, Kruskal-Wallis test followed by Dunn's Multiple Comparison post-test. Scale bars = 80 μm.

6.3.5 Spinal LY294002 reduces the increase in ERK phosphorylation in the dorsal horn after formalin injection in the hindpaw

Using immunohistochemical methods, it was shown that p-ERK expression is increased in the superficial layers (I-II) of the ipsilateral dorsal horn (levels L4-L5) five minutes after formalin injection compared to the contralateral side (Fig. 6.6a-c; mean number of positively-labelled cells per section: ipsilateral = 18.3 ± 0.5, contralateral = 2.9 ± 1.9, $p < 0.001$), in vehicle-treated rats (10% DMSO, $n = 5$). Some p-ERK expression was also induced in the ipsilateral dorsal horn in deeper laminae (V-VI) compared to the contralateral side, though this was much less than in superficial laminae (mean number of positively-labelled cells per section: ipsilateral = 5.1 ± 1.0, contralateral = 0.1 ± 0.1, $p < 0.001$). Intrathecal pre-treatment with LY294002 (100 μg, $n = 5$) significantly reduced p-ERK expression in both superficial (Fig. 6.6d) and deep laminae of the ipsilateral dorsal horn compared to vehicle-treated rats (mean number of positively-labelled cells in ipsilateral dorsal horn per section: superficial = 12.6 ± 1.2, $p < 0.01$; deep = 1.8 ± 0.7, $p < 0.05$). However, levels of p-ERK expression in LY294002-treated rats were still significantly greater in the ipsilateral dorsal horn than the contralateral side (mean number of positively-labelled cells in contralateral dorsal horn per section: superficial = 2.7 ± 1.0, $p < 0.001$; deep = 0.1 ± 0.1, $p < 0.05$). No changes were seen in the contralateral side with LY294002 treatment.

Thus, formalin injection in the hindpaw induces phosphorylation of ERK in the spinal dorsal horn, indicating activation of the ERK pathway. Inhibition of PI3K by spinal administration of LY294002 reduces ERK activation but does not completely reverse it, suggesting that activation of ERK is in part mediated by the PI3K pathway, contributing to spinal central sensitization.

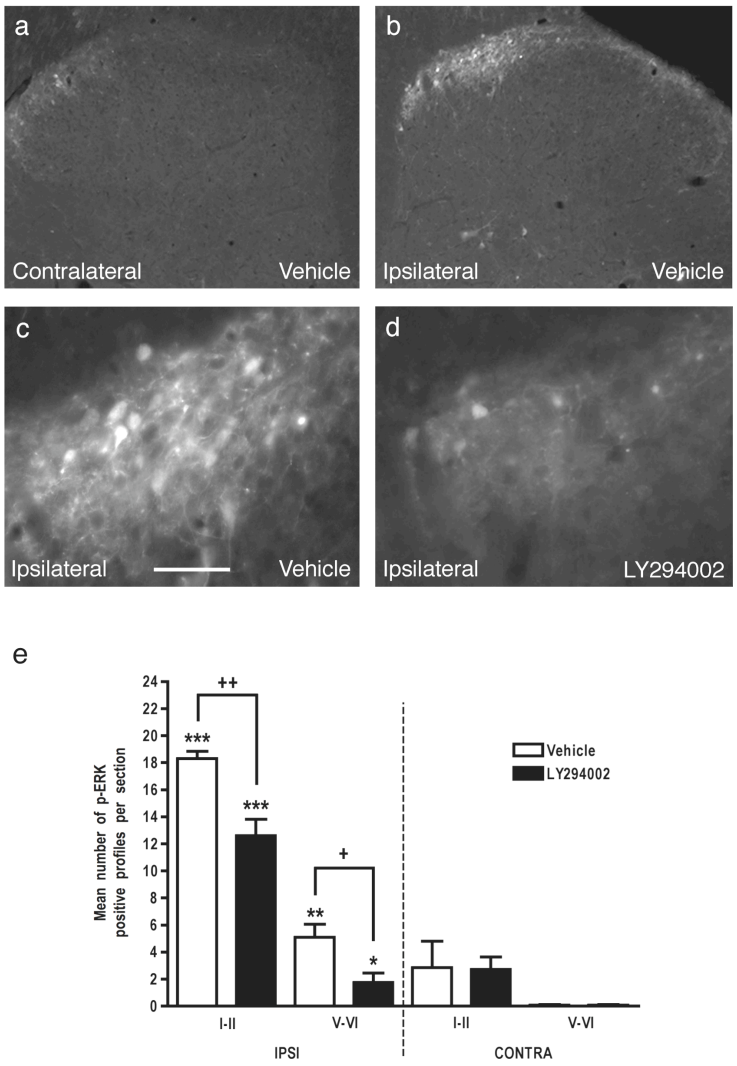


Fig. 6.6: LY294002 reduces formalin-induced phosphorylation of spinal ERK. **(a,b)** Representative photomicrographs of formalin-induced p-ERK expression in the **(a)** contralateral and **(b)** ipsilateral lumbar dorsal horn of rats pre-treated with intrathecal vehicle (10% DMSO). **(c,d)** High power photomicrographs of formalin-induced ipsilateral p-ERK expression following intrathecal (c) vehicle (10% DMSO) or **(d)** LY294002 (100 μg). **(e)** Quantification of the number of formalin-induced p-ERK positive profiles per section in both superficial (I-II) and deep (V-VI) dorsal horn laminae following intrathecal pre-treatment with vehicle (10% DMSO, n = 5,) or LY294002 (100 μg, n = 5). Data presented as mean ± s.e.m., $*$ $p < 0.05$, $**$ $p < 0.01$, $***$ $p < 0.001$ versus contralateral side; + $p < 0.05$, ++ $p < 0.01$ between treatment groups; Mann-Whitney Rank Sum tests. Scale bar = (a,b) 125 μm, (c) 32 μm.

6.3.6 Spinal LY294002 prevents the increase in CaMKII phosphorylation in the dorsal horn after formalin injection in the hindpaw

Expression of p-CaMKII was quantified in the medial part of the superficial (I-II) layers of the dorsal horn (levels L4-L5). Formalin injection produced a significant increase in the expression of p-CaMKII in the ipsilateral dorsal horn of vehicle-treated rats (10% DMSO, $n = 5$) within five minutes, when compared to the contralateral side (Fig. 6.7a-c; mean density per section: ipsilateral = 108.7 ± 4.4, contralateral = 93.2 ± 2.9, $p < 0.05$; Fig. 6.7e, main). There was no difference observed in p-CaMKII expression in animals pre-treated via intrathecal lumbar puncture with LY294002 (100 μg, $n = 5$) between the ipsilateral and contralateral sides of the dorsal horn (Fig. 6.7e; mean density per section: ipsilateral = 96.3 ± 3.6, contralateral = 93.7 ± 4.4, $p > 0.05$). Thus, LY294002 attenuated the formalin-induced increase in p-CaMKII expression in the ipsilateral dorsal horn compared to vehicle-treated rats (Fig. 6.7d; mean % of contralateral: vehicle group = 12.9 ± 2.9%, LY294002 group = 3.9 ± 1.6%, $p < 0.05$; Fig. 6.7e, inset). Constitutive expression of p-CaMKII was found in the lateral regions of the superficial dorsal horn (Fig. 6.7a,b) and this was not altered following intraplantar formalin or drug treatment.

Thus, formalin injection in the hindpaw induces phosphorylation of CaMKII in the spinal dorsal horn, indicating activation of the CaMKII pathway. Inhibition of PI3K by LY294002 inhibits CaMKII phosphorylation suggesting that activation of the CaMKII pathway is downstream of PI3K activity, contributing to spinal central sensitization.

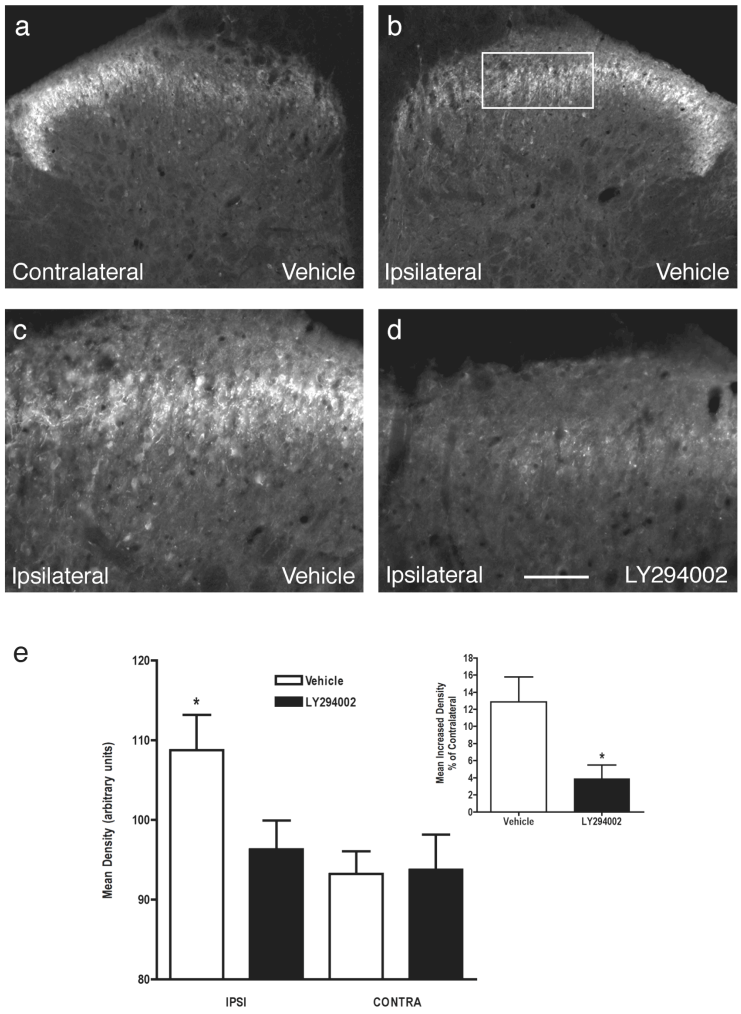


Fig. 6.7: LY294002 reduces formalin-induced phosphorylation of spinal CaMKII. **(a,b)** Representative photomicrographs of formalin-induced p-CaMKII expression in the **(a)** contralateral and **(b)** ipsilateral lumbar dorsal horns of rats pre-treated with intrathecal vehicle (10% DMSO). Box shows p-CaMKII upregulation in the medial part of the superficial dorsal horn. **(c,d)** High power photomicrographs of formalin-induced p-CaMKII expression in medial superficial dorsal horn following intrathecal **(c)** vehicle (10% DMSO) or **(d)** LY294002 (100 μg). **(e)** Quantification of mean density (main: arbitrary units; inset: percentage of contralateral) of formalin-induced p-CaMKII expression in medial part of the superficial dorsal horn following intrathecal vehicle (10% DMSO, n = 5,) or LY294002 (100 μg, n = 5). All data presented as mean ± s.e.m; $*$ $p < 0.05$ versus contralateral side (main) or between treatment groups (inset); Mann-Whitney Rank Sum tests. Scale bar = (a,b) 130 μm, (c) 32 μm.

6.3.7 Spinal LY294002 prevents phosphorylation of NMDA receptor NR2B subunits in the dorsal horn after formalin injection in the hindpaw

Western blot analysis was used to test the role of PI3K in phosphorylation of NMDA receptor subunits NR2A and NR2B in the dorsal horn (levels L4-L5) five minutes after formalin injection. Vehicle-treated rats (10% DMSO, n = 4) showed a marked increase in both ipsilateral NR2A and NR2B phosphorylation after formalin in comparison to the contralateral dorsal horn, though this effect was only significant for p-NR2B (Fig. 6.8; % of contralateral side: p-NR2A = 180.5 ± 48.1%; p-NR2B = 154.4 ± 18.4%, $p < 0.05$). Intrathecal LY294002 (100 μg, n = 4) attenuated both p-NR2A and p-NR2B upregulation in the ipsilateral dorsal horn (Fig. 6.8; % of contralateral side: p-NR2A = 103.0 ± 12.3%; p-NR2B = 77.0 ± 7.0%), such that levels of phosphorylation of both subunits no longer differed from the contralateral side. The expression of p-NR2A and p-NR2B in the ipsilateral dorsal horn differed between vehicle and LY294002-treated groups, however this difference was only statistically significant for p-NR2B expression ($p < 0.05$).

Therefore, intraplantar formalin injection in the hindpaw induces a significant increased phosphorylation of NR2B NMDA receptor subunits in the ipsilateral dorsal horn. This phosphorylation is attenuated by spinal inhibition of PI3K indicating that activity of the PI3K pathway leads to heightened activity of spinal NMDA receptors, particularly of the NR2B-subtype, contributing to spinal central sensitization.

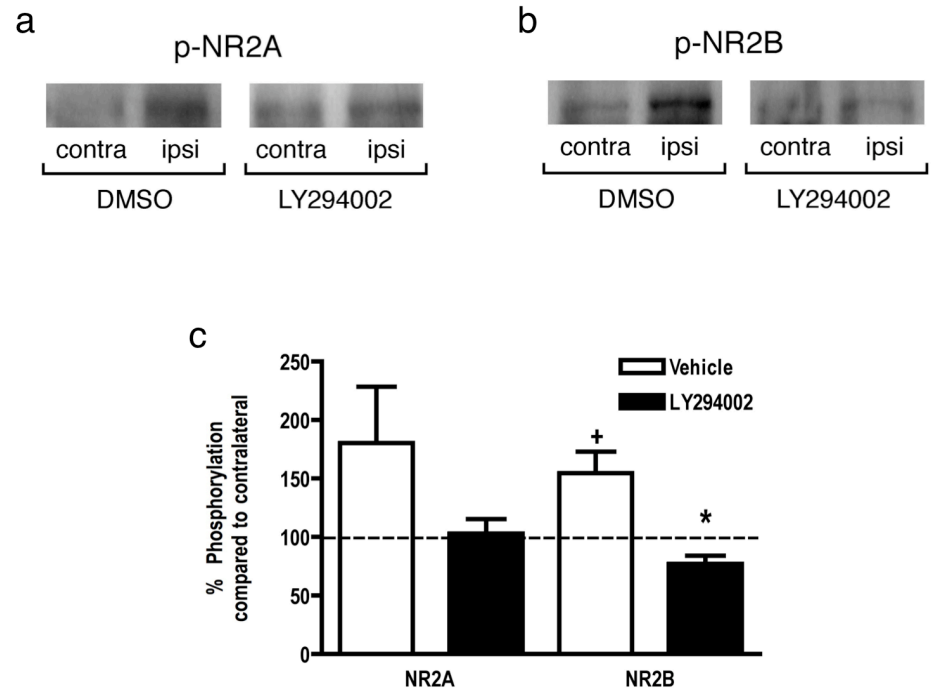


Fig. 6.8: LY294002 prevents formalin-induced phosphorylation of spinal NMDA receptor NR2B subunits. **(a,b)** Western immunoblots showing **(a)** p-NR2A and **(b)** p-NR2B expression in the dorsal horn induced by intraplantar formalin in rats pre-treated with intrathecal vehicle (10% DMSO) or LY294002 (100μg). **(c)** Quantification of formalin-induced expression of p-NR2A and p-NR2B. Data expressed as mean ± s.e.m., + $p < 0.05$ versus contralateral side, $*$ $p < 0.05$ between treatment groups, Mann-Whitney Rank Sum test.

6.3.8 PI3K regulates the trafficking of dorsal horn AMPA receptor GluR1 subunits after formalin injection in the hindpaw

Using a combination of subcellular fractioning and western blot analysis, the effect of inhibition of PI3K on the trafficking of AMPA receptor GluR1 subunits from the cytoplasm to the plasma membrane of dorsal horn neurones after intraplantar formalin injection was tested. As previously demonstrated following visceral inflammatory stimulation (Galan et al., 2004), intraplantar injection of formalin induced an initial significant reduction of the relative amount of GluR1 receptor in the cytoplasm in vehicle-treated rats (10% DMSO, $n = 3$), 10 min after formalin injection, in comparison to naïve rats (34% decrease, $p < 0.05$; Fig. 6.9a,c). This reduction was prevented by spinal pre-treatment with LY294002 (100 μg, $n = 3$). This was followed by an increase at 45 min and a significant increase at 90 min after formalin injection in the amount of cytoplasmic GluR1 (90 min: 111% increase, $p < 0.05$, Fig. 6.9a,c) which, again, was blocked by spinal LY294002. The changes in the cytoplasm following formalin were paralleled by a trend towards increased GluR1 in the membrane fraction at 45 and 90 min in the vehicle-treated group only (Fig. 6.9b,d), though these increases did not reach statistical significance.

Therefore, injection of formalin into the hindpaw produces changes in cytosolic and membrane GluR1 content, perhaps suggesting an alteration of trafficking of GluR1 AMPA receptor subunits from the cytoplasm to the plasma membrane of spinal dorsal horn neurones, a mechanism contributing to synaptic plasticity. Inhibition of PI3K, by administration of LY294002, blocks such changes, suggesting that the PI3K pathway may regulate trafficking of GluR1 in response to noxious peripheral stimulation, contributing to spinal central sensitization.

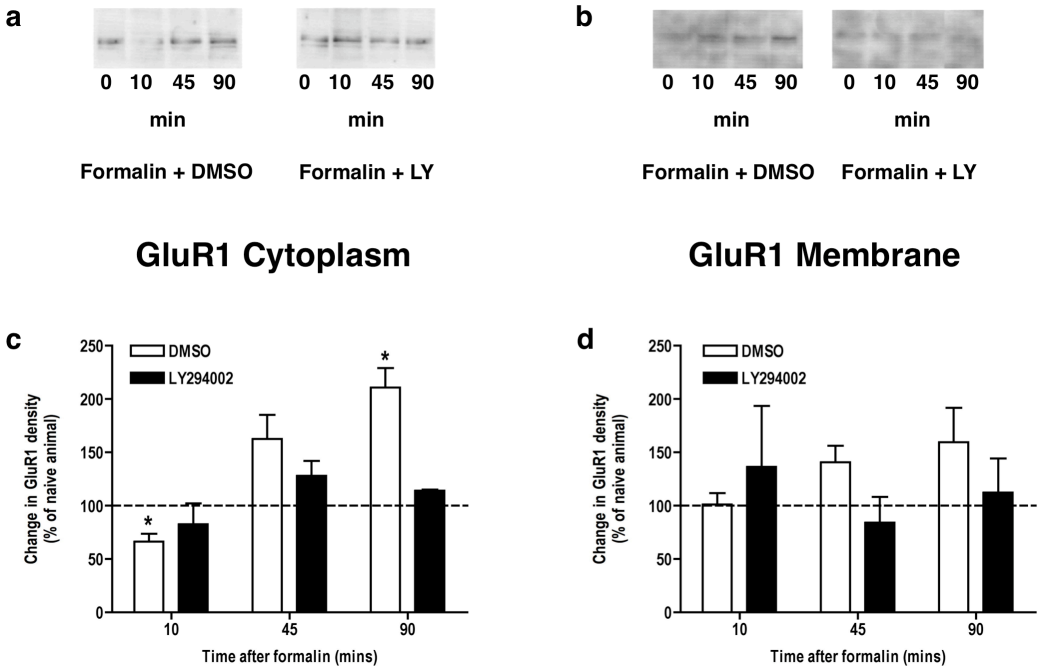


Fig. 6.9: LY294002 alters trafficking of AMPA receptor GluR1 subunits following intraplantar formalin. **(a,b)** Western immunoblots of GluR1 expression in **(a)** cytoplasmic and **(b)** membrane fractions of the ipsilateral dorsal horn at 0 (naïve rats), 10, 45 and 90 min following intraplantar formalin (5%, 50 μl) injection in rats pre-treated with intrathecal vehicle (10% DMSO) or LY294002 (100 μg). **(c,d)** Quantification of formalin-induced changes in GluR1 expression in **(c)** cytoplasmic and **(d)** membrane fractions of the ipsilateral dorsal horn following intrathecal vehicle (10% DMSO, n = 3) or LY294002 (100 μg, n = 3). Data are presented as a percentage of GluR1 expression in naïve rats (0 min) mean ± s.e.m., $*$ $p < 0.05$ versus naïve animals (0 min, 100%), Mann-Whitney Rank Sum test.

6.4 Discussion

The results presented in this chapter suggest that PI3K is involved in the expression of spinal neuronal nociceptive plasticity following electrical stimulation of the hindpaw and intraplantar injection of formalin. Spinal treatment with a PI3K inhibitor, LY294002, robustly inhibited C-fibre evoked responses, post-discharge and wind-up of spinal WDR neurones. The selectivity of these effects indicates a post-synaptic site of action. Intraplantar formalin activated Akt, a downstream marker of the PI3K pathway, and produced pain-related behaviours, both of which were reduced by intrathecal LY294002. A reduction in formalin-induced central sensitization by LY294002 coincided with reduced formalin-induced phosphorylation of NMDA receptor subunits, ERK and CaMKII, as well as abolishment of the formalin-induced changes of GluR1 AMPA receptor subunit cytosolic content. Each of these intracellular mechanisms is considered to have essential roles in the manifestation of both hippocampal LTP in learning and memory and spinal central sensitization (Ji et al., 2003).

PI3K has been shown to have a key role in the various stages of LTP in the mammalian hippocampus (Sanna et al., 2002; Opazo et al., 2003), amygdala (Lin et al., 2001) and medial prefrontal cortex (Sui et al., 2008). Based on the hypothesis that central sensitization represents a spinal form of LTP (Ji et al., 2003), the aim of this study was investigate whether PI3K plays a similar role in spinal plasticity of pain pathways to that in the brain. Firstly, it was demonstrated that PI3K is required for the full expression of wind-up, as LY294002 was able to reduce wind-up by approximately 50%. This form of spinal plasticity is NMDA-dependent, as is LTP, and seems to be the neuronal correlate of pain hypersensitivity. Reductions were seen of the post-discharge of WDRs, which is considered to be a measure of hyperexcitability of these cells. In addition, spinal inhibition of PI3K produced significant reductions of C-fibre evoked responses, as well as a small inhibitory effect on Aδ-evoked responses, suggesting that PI3K blockade mainly interferes with nociceptive signalling. Favourably, no effect was seen on Aβ-fibre evoked responses. Importantly, input was also unaltered by LY294002, indicating that the reductions observed in evoked neuronal responses and wind-up were most likely not due to an inhibition of primary afferent signalling, but rather due to a direct effect on

post-synaptic dorsal horn neurones themselves. In support of this, inhibition of PI3K does not affect pre-synaptic afferent release of substance P following electrical stimulation of dorsal roots attached to isolated dorsal horn slices (Pezet et al., 2008), though an effect on glutamate release from primary afferent fibres cannot be ruled out. Thus, it is likely that PI3K is predominantly involved at the post-synaptic level in the dorsal horn in contributing to the expression of neuronal wind-up.

Activation of the PI3K pathway five minutes after formalin administration was demonstrated by an increase in phosphorylation of downstream Akt, similar to that observed following intradermal capsaicin (Sun et al., 2006). This indicates an important involvement of PI3K signalling in early central sensitization following noxious peripheral stimulation. Accordingly, a dose-dependent inhibitory effect of PI3K blockade on pain-related behaviours during both the first and second phase of the response to intraplantar formalin was observed. The first phase of the formalin test is thought to be mediated mainly by direct stimulation of second order neurones in the superficial laminae of the dorsal horn by C-fibres. Reduction of this acute phase by LY294002 could reflect the decreases in C-fibre evoked responses, as well as post-discharge and wind-up of WDR neurones, observed electrophysiologically following electrical stimulation of the hindpaw. A peripheral effect of LY294002 on primary afferent terminals cannot be entirely ruled out, particularly since peripheral noxious stimulation has extensively been shown to activate PI3K in nociceptive primary afferent fibres (Zhuang et al., 2004; Pezet et al., 2005; Sun et al., 2007). However, the lack of effect on peripheral input with LY294002 coupled with the evidence from release studies following electrical stimulation would point to an effect on intrinsic spinal excitability. Thus, inhibition of PI3K may reduce the responsiveness of spinal neurones to incoming nociceptive signals induced by formalin and consequently reduce pain perception and resultant behaviours during the first phase. Since LY294002 significantly reduced pain-related behaviours during the second phase of the response to formalin, it is evident that spinal PI3K activity is required for the manifestation of central sensitization. Formalin-induced expression of c-Fos in dorsal horn neurones is also markedly reduced by spinal administration of LY294002 (Pezet et al., 2008) reinforcing the hypothesis of a role of PI3K in central sensitization of spinal sensory neurones.

Formalin-induced central sensitization is dependent on activation of spinal NMDA receptors (Haley et al., 1990; Chaplan et al., 1997) and accordingly, increased phosphorylation of NR2A and NR2B subunits was observed in this study five minutes after intraplantar formalin injection. PI3K inhibition significantly reduced phosphorylation of NR2B subunits, and to a lesser extent that of NR2A subunits, suggesting that increased activity and/or responsiveness of NMDA receptors is regulated by PI3K signalling. NMDA receptor subunit phosphorylation, particularly of the NR2B subunit, has been correlated with pain hypersensitivity in various pain models (Guo et al., 2002; Abe et al., 2005) and inhibition of NR2B-subtype receptors produces a potent antinociceptive effect (Taniguchi et al., 1997; Boyce et al., 1999; Malmberg et al., 2003; Tan et al., 2005). In addition, NR2B-subtype receptors play a key role in LTP in the hippocampus (Tang et al., 1999; Gardoni et al., 2009; Foster et al., 2010) as well as in the spinal cord (Pedersen and Gjerstad, 2007; Qu et al., 2009).

Inhibition of PI3K by intrathecal administration of LY294002 produced a significant reduction in formalin-induced p-ERK expression, implicating PI3K as an upstream activator of ERK in the dorsal horn. LTP has also been shown to involve activation of ERK via PI3K signalling (Opazo et al., 2003) and PI3K inhibitors suppress NMDA receptor-mediated ERK activation in hippocampal neurones (Chandler et al., 2001; Perkinton et al., 2002). Although the exact mechanisms for this role of ERK in LTP have not been identified, it is postulated that ERK activation facilitates synaptic NMDA function and may also contribute to activity-dependent membrane insertion of AMPA receptors (Opazo et al., 2003). PI3K signalling may thus promote ERK to act through similar mechanisms in the spinal cord to produce central sensitization. The phosphorylation of ERK has been previously shown in inflammatory pain models (Ji et al., 2002a; Obata and Noguchi, 2004). For example, ERK activation occurs rapidly in dorsal horn neurones after formalin injection and ERK inhibition blocks the resulting pain-related behaviour (Ji et al., 1999). Zhuang and colleagues demonstrated that PI3K and ERK signalling pathways were intricately associated in peripheral primary sensory neurones (Zhuang et al., 2004).

CAMKII has a crucial role in LTP, both in the hippocampus (Malinow et al., 1988; Malinow et al., 1989) as well as in the spinal cord (Yang et al., 2004a; Pedersen et

al., 2005). In addition, influx of Ca^{2+} via NMDA receptors leads to the phosphorylation and activation of CaMKII, and its inhibition prevents central sensitization in the spinal cord in inflammatory and neuropathic pain models (Fang et al., 2002; Dai et al., 2005; Choi et al., 2006). The results of this study show that PI3K regulates activity of CaMKII in the dorsal horn, a particularly intriguing finding since a dependence of CaMKII on upstream PI3K has not been shown in LTP. Intraplantar formalin increased expression of p-CaMKII in the ipsilateral dorsal horn, an effect which was significantly reduced by intrathecal LY294002. Similarly, Fang and colleagues saw increased expression of p-CaMKII after a noxious stimulus, in this case capsaicin. Inhibition of CaMKII during behavioural testing prevented the hypersensitivity associated with central sensitization (Fang et al., 2002). More recently, Dai and colleagues have shown upregulation of p-CaMKII after peripheral nerve injury. Again, inhibition of CaMKII activity prevented behavioural hypersensitivity (Dai et al., 2005).

Finally, trafficking of AMPA receptor GluR1 subunits to the plasma membrane of dorsal horn neurones may occur after formalin injection. Increased numbers of AMPA receptors on the membrane of spinal sensory neurones would increase the responsiveness of these cells to glutamate release from primary afferent fibres and thus amplify responses to and consequently perception of peripheral stimulation. Prior to this study, this mechanism had never been demonstrated before at the spinal cord level in a model of somatic inflammatory pain. However, it had been shown that AMPA GluR1 subunits are recruited to the plasma membrane of spinal cord neurones after noxious visceral stimulation (Galan et al., 2004). Therefore, this is a mechanism which could contribute to the expression of central sensitization following noxious inputs from varying locations and via different pathways. In the hippocampus, it has been shown that exocytosis of GluR1 is PI3K-dependent, whereas that of GluR2 is not (Passafaro et al., 2001). Subsequently, Man and colleagues showed that PI3K is required for AMPA receptor insertion during LTP (Man et al., 2003). The results here suggest that PI3K may regulate GluR1 trafficking in the dorsal horn after intraplantar formalin. Formalin-induced changes in both cytosolic and membrane AMPA receptor content were prevented by spinal LY294002. An initial decrease in cytosolic GluR1 content may be due to increased trafficking to the plasma membrane. This is supported by the increasing trend in

membrane AMPA receptor content observed up to 90 min after formalin injection, even after pain-related behaviour has subsided, suggesting that central sensitization has been persistently established. The later increase in cytosolic GluR1 content may serve to replenish and increase the overall cellular content of GluR1 and contribute to neuronal plasticity. Furthermore, the effect of CaMKII on pain transmission in this model may be due to an alteration of the trafficking and/or activation of GluR1 subunits as previously observed following visceral inflammatory stimulation (Galan et al., 2004). Phosphorylation of GluR1 and membrane insertion are also essential components of LTP and both involve CaMKII (Fukunaga et al., 1996; Barria et al., 1997; Derkach et al., 1999; Hayashi et al., 2000).

It is also important to consider that in these experiment rats were sacrificed five minutes after intraplantar formalin injection and so central sensitization may not yet have been fully established. Changes in the spinal expression of p-ERK, p-CaMKII and p-NR2B may be components which lead to central sensitization, suggesting that PI3K is an initiatory upstream molecule which triggers multiple changes in spinal sensory neurones, resulting in central sensitization. It therefore follows that inhibition of PI3K activity soon after a noxious stimulus may be a highly effective way of preventing central sensitization by blocking initiation of various signalling pathways. Further studies are required to establish whether PI3K is involved in the maintenance of central sensitization.

Of note in these studies is the fact that doses of LY294002 used in electrophysiological experiments are much lower than those used in the behavioural formalin test. The ability to apply the drug locally to a restricted cord zone in a static system during electrophysiological recordings explains the low effective doses as compared to the behavioural study where CSF flow and dilution, and the smaller volume make higher doses necessary. It is possible that at the higher behavioural doses LY294002 may exert non-specific effects, inhibiting other kinases such as glycogen synthase kinase 3 (GSK3), casein kinase 2 (CK2) and p90 ribosomal S6 protein kinase 2 (RSK2) (Davies et al., 2000), though this is made less likely since the actual effective dose should be much lower than the administration dose due to dilution by CSF and minimal access to the cord.

In conclusion, this study reinforces that similarities exist between LTP in the brain and central sensitization in the spinal cord. PI3K regulates various intracellular mechanisms which collectively contribute to the induction of central sensitization of dorsal horn sensory neurones.

7 Involvement of dimethylarginine dimethylaminohydrolase-1 (DDAH-1) in NMDA-dependent spinal nociceptive plasticity

7.1 Introduction

7.1.1 NMDA-NO signalling in the brain

Activation of nNOS and subsequent generation of NO is coupled to upstream stimulation of NMDA receptor activity. This was first demonstrated by Garthwaite and colleagues who showed that stimulation of NMDA receptors in cerebellar cells by glutamate induced release of a diffusible messenger, NO, in a Ca^{2+}-dependent manner, and lead to a rise in cGMP levels. In addition, they showed that this NMDA-mediated rise in cGMP could be blocked by L-N^G-monomethylarginine (L-NMMA), an inhibitor of NO production from L-arginine by NOS. This block was reversed by subsequent application of L-arginine (Garthwaite et al., 1988; Garthwaite et al., 1989). In addition, the relationship between NMDA receptors and NO was shown to exist in the hippocampus (East and Garthwaite, 1991). Later it was shown that nNOS is in fact physically coupled to NMDA receptors via PSD-95 in a ternary complex (Brenman et al., 1996; Christopherson et al., 1999) such that $Ca2^+$ influx through the NMDA channel pore can directly drive the production of NO in an NMDA-dependent manner. This complex was found to contribute to NMDA-mediated excitotoxicity and is believed to be involved in plasticity and behaviour (Sattler et al., 1999).

Various studies have demonstrated that NO is required for hippocampal LTP (Bohme et al., 1991; Haley et al., 1992b; O'Dell et al., 1994; Doyle et al., 1996; Kantor et al., 1996; Son et al., 1996). Together, this work suggested that both eNOS (endothelial nitric oxide synthase) and nNOS are required for LTP, and that loss of one enzyme may be compensated for by the presence of the other so that LTP may be normal. Hopper and Garthwaite confirmed the requirement for both eNOS and nNOS in hippocampal LTP and concluded that eNOS produces tonic NO while nNOS

generates phasic NO signals which are particularly important for late LTP (Hopper and Garthwaite, 2006). Thus, NO is a key messenger in brain synaptic plasticity.

7.1.2 A role for nitric oxide and nitric oxide synthases in pain at the spinal level

In the dorsal horn of the spinal cord, it has clearly been established that nitric oxide and downstream cGMP, which is produced by the NO receptor guanylate cyclase, contribute to sensitization of nociceptive signalling in both inflammatory and neuropathic pain states, though apparently not to basal pain perception (Schmidtko et al., 2009). Two very similar studies showed that the facilitation of the nociceptive tail-flick reflex in response to thermal stimulation that occurs after intrathecal administration of NMDA is abolished by spinal treatment with the non-selective NOS inhibitor N omega-nitro-L-arginine methyl ester (L-NAME), while L-arginine, which is converted to NO, and NO donors, such as sodium nitroprusside (SNP), produced hypersensitivity (Kitto et al., 1992; Meller et al., 1992a). Thus it was shown that downstream NO signalling contributes to acute NMDA-dependent hypersensitivity. Sorkin subsequently demonstrated that intrathecal NMDA caused extracellular release of glutamate and citrulline, a metabolite of NO synthesis, in the spinal cord. This NMDA-induced release of glutamate was blocked by L-NAME (Sorkin, 1993), thus suggesting that activation of NMDA receptors and subsequent generation of NO leads to further release of glutamate as part of a feed-forward control of spinal nociceptive processing. However, where this glutamate originates from could not be determined and thus could be of either pre- or post-synaptic origin. Citrulline was measured rather than NO in this study since the short half-life of NO makes it particularly difficult to measure in the extracellular space. In addition, Malmberg and Yaksh showed that L-NAME could block the second phase of the behavioural formalin test with no effect on baseline nociceptive thresholds (Malmberg and Yaksh, 1993), while a similar antinociceptive effect on the neuronal response to formalin was shown in an electrophysiological study using L-NAME (Haley et al., 1992a).

A role for NOS and NO was shown in more chronic inflammatory pain, as induced by hindpaw injection of carrageenan. L-NAME produced a dose-dependent block of

thermal hypersensitivity in the injected paw, while there was no change in the non-injected paw or of baseline nociceptive thresholds prior to inflammation (Meller et al., 1994a). In particular, it would appear that nNOS is most important in producing inflammatory hyperalgesia, with eNOS and iNOS (inducible nitric oxide synthase) having little, if any, involvement. The selective nNOS inhibitor 7-nitroindazole (7-NI) reduced both early (development) and late (maintenance) phases of carrageenan-induced thermal hypersensitivity. Interestingly, however, nNOS knockout mice had reduced hypersensitivity during the late phase only. The intact early phase hypersensitivity in these mice was explained by an upregulation of eNOS which compensated for the loss of nNOS (Tao et al., 2004). Mice lacking iNOS had normal carrageenan-induced thermal hyperalgesia, though this may have been due to a compensatory upregulation of nNOS (Tao et al., 2003b). Similarly, genetic knockout of nNOS selectively reduced late phase hypersensitivity following CFA-induced inflammation of the hindpaw, while pharmacological inhibition with 7-NI reduced both early and late phases (Chu et al., 2005). Together these studies would suggest that nNOS plays differing but critical roles in the development and maintenance phases of chronic inflammatory hyperalgesia.

It has also been demonstrated that NO signalling is required for the expression of nerve injury-induced pain. Meller and colleagues found that acute administration of L-NAME could block thermal hypersensitivity in rats with CCI-induced neuropathic pain for two hours (Meller et al., 1992b). This finding was later confirmed by Salter and colleagues, who additionally found that L-NAME administered intracerebroventricularly could reverse thermal hypersensitivity, though this effect was not as prolonged as that achieved by intrathecal administration of the drug (Salter et al., 1996). Later, Guan and colleagues combined genetic and pharmacological approaches to elucidate a role specifically for nNOS in nerve injury-induced pain. Mice lacking nNOS failed to develop mechanical hypersensitivity following L5 SNL and intrathecal administration of L-NAME or 7-NI robustly inhibited mechanical hypersensitivity in nerve-injured wild-type mice. No effect of either drug was seen on mechanical sensitivity of the contralateral paw (Guan et al., 2007). Most interestingly, especially in the context of this thesis, Florio and colleagues have recently demonstrated the importance of interactions between nNOS and PSD-95 in neuropathic pain. Small molecule inhibitors which can disrupt association of nNOS

with PSD-95 were found to attenuate the NMDA-induced facilitation of the tail-flick reflex and also reverse mechanical hypersensitivity following CCI, when administered intrathecally at doses which did not alter baseline nociceptive thresholds or motor performance on the rotarod (Florio et al., 2009). Thus, nNOS activity is implicated in the nociceptive sensitization that contributes to neuropathic pain states.

Additional evidence of a role for NO in pain sensitization was provided by Tegeder and colleagues who showed that inhibition of tetrahydrobiopterin (BH4), an essential cofactor for NO production by NOS, could inhibit inflammatory and neuropathic pain in rodents. Furthermore, in humans, a polymorphism in the gene encoding GTP cyclohydrolase 1 (GCH1), the rate-limiting enzyme for BH4 synthesis, was found to be protective against neuropathic pain and was associated with reduced experimental inflammatory hyperalgesia in healthy individuals (Tegeder et al., 2006). GCH1 was one of the first susceptibility genes identified for chronic pain.

7.1.3 Endogenous regulation of NOS by ADMA and DDAH

The majority of studies described above investigating the role of NO have utilized exogenously applied direct inhibitors of NOS as a means for targeting this signalling pathway. However, regulation of NOS, and thus regulation of NO generation, involves multiple enzymes and substrates as part of a complex regulatory pathway which may equally be targeted. A key endogenous inhibitor of NOS activity is asymmetric dimethylarginine (ADMA), which is formed as a by-product of continuous protein turnover in all cells of the body. ADMA is structurally similar to L-arginine, which is the substrate converted to NO by NOS. Therefore, ADMA can compete with L-arginine and inhibit NOS activity and in this way regulate the formation of NO. For example, Vallance and colleagues showed that impaired renal function can lead to accumulation of ADMA in the body, which may in turn impair NO synthesis and cause hypertension, since NO is a vasodilator in blood vessels (Vallance et al., 1992). Later, Cardounel and Zweier measured the levels of ADMA in the brain and in neurones and found that physiological concentrations were sufficient to modulate nNOS function and suppress NO-mediated excitotoxic injury (Cardounel and Zweier, 2002). ADMA is itself actively regulated by an enzyme called dimethylarginine

dimethylaminohydrolase (DDAH), of which there are two isoforms, DDAH-1 and DDAH-2, strengthening the suggestion that ADMA is more than simply a by-product of protein turnover, but rather has an important physiological function (Leiper et al., 1999). DDAH metabolizes ADMA and converts it to citrulline and dimethylamine (Rossiter et al., 2005). Inhibition of DDAH has been shown to sufficiently increase levels of ADMA to inhibit synthesis of NO (MacAllister et al., 1996). Therefore, activity of DDAH can indirectly influence NOS activity, by relieving the inhibitory influence of ADMA and thus increasing the generation of NO (Fig. 7.1).

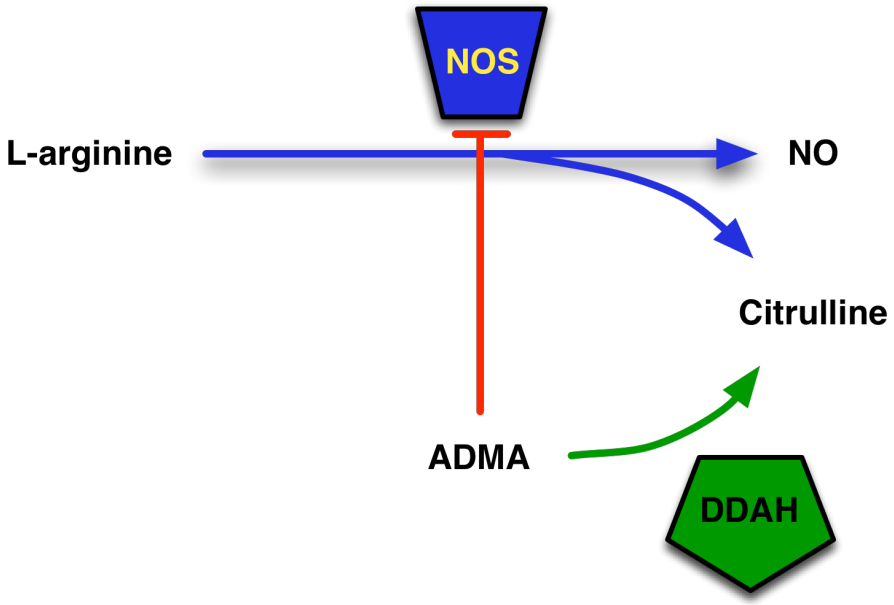


Fig. 7.1: DDAH/ADMA regulation of NOS. NOS converts L-arginine to NO and citrulline. ADMA is produced endogenously by protein turnover and is structurally similar to L-arginine. Thus, ADMA can act as an endogenous competitive inhibitor of NOS and decreases generation of NO. ADMA itself is regulated and metabolized by DDAH. Thus, activity of DDAH indirectly modulates NOS activity by relieving endogenous inhibition by ADMA and so increasing generation of NO.

7.1.4 Expression of DDAH-1 and DDAH-2

The distribution of DDAH mRNA correlates well with NOS in various tissues, with DDAH-1 found predominantly in tissues expressing nNOS and DDAH-2 associated more with eNOS (Leiper et al., 1999). DDAH-1 mRNA was strongly detected in brain, liver, kidney and pancreas, while the greatest expression of DDAH-2 was in heart, kidney, placenta, pancreas and lung. DDAH-1 was completely absent from heart, placenta and lung, while DDAH-2 was barely detectable in brain (Leiper et al., 1999). Perhaps surprisingly, DDAH-2 mRNA is abundant in the spinal cord and this expression was found to be greater than that of DDAH-1 mRNA (Tran et al., 2000).

7.1.5 Novel targeting of DDAH isoforms in disease

Interest in the DDAH/ADMA pathway has increased with the accumulating evidence that ADMA is involved in numerous pathological processes and diseases, while a role for NOS in disease has been established for some time. Therefore, recent attempts have been made to design novel inhibitors of DDAH isoforms in an effort to elucidate precise roles for DDAH in both physiological and pathophysiological processes. To that end, a selective inhibitor of DDAH-1, L-291 (N^G-[2-methoxyethyl] arginine methyl ester), has recently been synthesized. This compound is based on the structure of ADMA and acts as a competitive inhibitor of DDAH-1 (IC_{50} 20 μM), with no direct effect on NOS activity, but rather reduces NO signalling through accumulation of ADMA (Rossiter et al., 2005; Leiper et al., 2007). L-291 is specific for DDAH-1 based on recombinant protein activity assays and on functional assays in culture (unpublished observations, verbal communication from Dr Manasi Nandi, UCL). In addition, transgenic mice globally lacking DDAH-1 have been genetically engineered. Although complete deletion of DDAH-1 is lethal *in utero* (indicating the importance of DDAH-1 in embryonic development), heterozygous mice (DDAH-$1^{+/-}$) are viable. These mice have been fully characterized biochemically and show reduced DDAH-1 mRNA, protein and activity, with no compensatory change in DDAH-2, as well as increased circulating and tissue levels of ADMA (Leiper et al., 2007). From a therapeutic point of view, pharmacological and genetic inhibition of DDAH-1 was found to be protective in a model of endotoxic shock, in which NO

production is known to reach excessive and pathological levels (Leiper et al., 2007). Thus, targeting of DDAH isoforms may be beneficial in other diseases where increased NO signalling is implicated, particularly in the nervous system.

7.1.6 DDAH in the nervous system and possible role in pain

As outlined above, mRNA for DDAH isoforms has been detected in various neuronal tissues. To date, however, nothing is known regarding the functional role of DDAH in the nervous system. A single study has demonstrated that mRNA for DDAH and nNOS are both upregulated in injured motoneurones following axotomy (Nakagomi et al., 1999), though the functional significance of this finding remains unknown. Since mRNA for DDAH isoforms has been detected in the spinal cord, and that nNOS is known to be important for sensitization of spinal pain processing, it was hypothesized that DDAH may too influence spinal pain processing, perhaps via indirect regulation of nNOS. Thus, the aim of this study was to first detect protein expression of DDAH isoforms in the nervous system, including the spinal cord, and to subsequently assess the functional contribution of DDAH-1 to spinal pain processing using both pharmacological and genetic strategies.

7.2 Materials and Methods

7.2.1 Animals

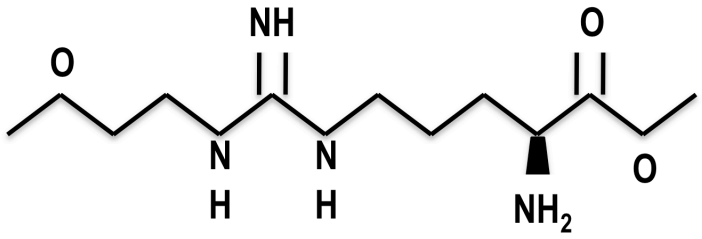
Experiments were conducted in adult male Sprague-Dawley rats (220-250 g, Central Biological Services, University College London, or Harlan, UK) and C57BL/6 DDAH$^{+/-}$ or wild-type mice (25-30 g, donated by Dr Manasi Nandi (UCL) and Dr James Leiper (UCL), housed in standard laboratory conditions with free access to food and water. Experimental procedures were approved by the UK Home Office and followed guidelines under the International Association for the Study of Pain (Zimmermann, 1983).

7.2.2 Western Immunoblotting of DDAH-1 and DDAH-2 in neuronal tissues

Rat dorsal horn, DRG and hippocampus tissue was homogenized and 40 μg of each lysate was run on an 8% gel for SDS-PAGE (see Material and Methods). Proteins were transferred to nitrocellulose membranes which were then probed overnight at 4°C with goat anti-DDAH-1 (1:500, ab2231, Abcam) or goat anti-DDAH-2 (1:500, ab1383, Abcam, UK) primary antibodies. Rabbit anti-neuronal β-III tubulin served as a loading control (1:3000, ab18207, Abcam, UK). Next membranes were incubated with IRDye-linked donkey anti-goat 680 or donkey anti-rabbit 800CW secondary antibody for 1 hr at room temperature, and proteins were subsequently revealed using the Odyssey fluorescence detection system (Licor, UK).

7.2.3 DDAH-1 specific inhibitor, L-291 and control inactive enantiomer, L-456

L-291 (N^G-[2-methoxyethyl] arginine methyl ester; Fig. 7.2) is a selective small molecule inhibitor of DDAH-1 while L-456 is its mirror image inactive D-enantiomer and therefore serves as a control. Both compounds were donated by Dr Manasi Nandi (UCL) and Dr James Leiper (UCL) and subsequently dissolved in 0.9% saline.



L-291

Fig. 7.2: Chemical structure of DDAH-1 inhibitor L-291 (IC_{50} 20 μM)

7.2.4 *In vivo* Electrophysiology

Electrophysiological recordings from deep dorsal horn WDR neurones (> 600 μm) were conducted as previously described (see Materials and Methods chapter).

7.2.4.1 Electrical stimulation of the hindpaw

Following the establishment of stable control responses, L-291 was injected directly onto the surface of the spinal cord (after removal of any residual cerebrospinal fluid) in cumulative doses of 1.2, 12 and 120 μg in a volume of 50 μl using a Hamilton syringe. Electrical tests were continued at 10 min intervals with the effect of each dose followed for 1 hr. A separate group of animals received 120 μg of the control peptide L-456, in a volume of 50 μl, for comparison.

7.2.4.2 Neuronal Formalin Test

Following cell characterization, rats were pre-treated by topical spinal administration of either 12 μg of L-291 or 120 μg of L-456, 20 min prior to the injection of formalin. Both drugs were delivered in a volume of 50 μl using a Hamilton syringe. Formalin (5%, 50 μl) was prepared from a 37% formaldehyde solution and then injected subcutaneously into the hindpaw receptive field. The firing response of the WDR neurone was recorded for the subsequent 70 min after formalin injection. Activity was displayed as a rate recording and quantified in 10 min time bins.

7.2.5 Behavioural Formalin Test

Rats were lightly anaesthetized with isoflurane and then injected intrathecally (lumbar puncture method) with either 10 μl of L-291 (12 μg or 24 μg) or 10 μl of L-456 (24 μg). Experimenters were blind to treatment for the whole experiment. Twenty minutes later, rats received a subcutaneous injection of 50 μl of formalin (5%) into the plantar surface of the right hindpaw. Lifting, flinching, licking and biting of the injected paw were monitored by measuring the total duration of the response in seconds (s) during the 60 min period following formalin administration. Data was captured in 5 min time bins.

7.2.6 DDAH-1$^{+/-}$ mice Behavioural Formalin Test

Heterologous null DDAH-1$^{+/-}$ and wild-type littermates received an intraplantar injection of formalin (2%, 20 μl). As with rats, lifting, flinching, licking and biting of the injected paw were monitored by measuring the total duration of the response in seconds (s) during the 60 min period following formalin administration. Data was captured in 5 min time bins. No obvious phenotypic differences were observed between DDAH-1$^{+/-}$ and wild-type mice, and experimenters were blind to genotype throughout the testing period. Global homologous deletion of DDAH-1 is lethal *in utero*.

7.2.7 Statistical Analysis

All data is presented as mean ± s.e.m. Effects of L-291 and L-456 following electrical stimulation of the hindpaw were assessed by one-way RM ANOVA, followed by Bonferroni multiple comparison post-tests. Cell characteristics from L-291 and L-456 groups were compared by Student's unpaired t-tests. Formalin neuronal and behavioural response time course data was compared between treatment groups and analyzed by two-way RM ANOVA, followed by Bonferroni post-tests. Total activity in the first and second phases was compared between treatment groups by quantifying the area under each curve, and analyzed by one-way ANOVA, followed by Bonferroni post-tests. Statistical analyses were carried out using GraphPad Prism v.4 software (GraphPad Software Inc., San Diego, CA, USA).

7.3 Results

7.3.1 DDAH-1 and DDAH-2 protein is expressed in DRG, spinal dorsal horn and hippocampus

Protein expression of DDAH-1 or DDAH-2 has never been shown previously in rat spinal cord dorsal horn and DRG. Using western blotting, both DDAH-1 and DDAH-2 protein was found to be present in the hippocampus, DRG and the dorsal horn of the spinal cord (Fig. 7.3). Protein content of DDAH-1 was greatest in DRG, followed by similar expression in spinal dorsal horn and hippocampus. DDAH-2 expression was also greatest in DRG followed by spinal dorsal horn and then by hippocampus. DDAH-1 expression in all three types of neuronal tissue was greater than DDAH-2. This would agree with previous findings which have suggested that DDAH-1 is mainly found in tissues which express nNOS, while DDAH-2 seems to co-localize more with eNOS (Leiper et al., 1999).

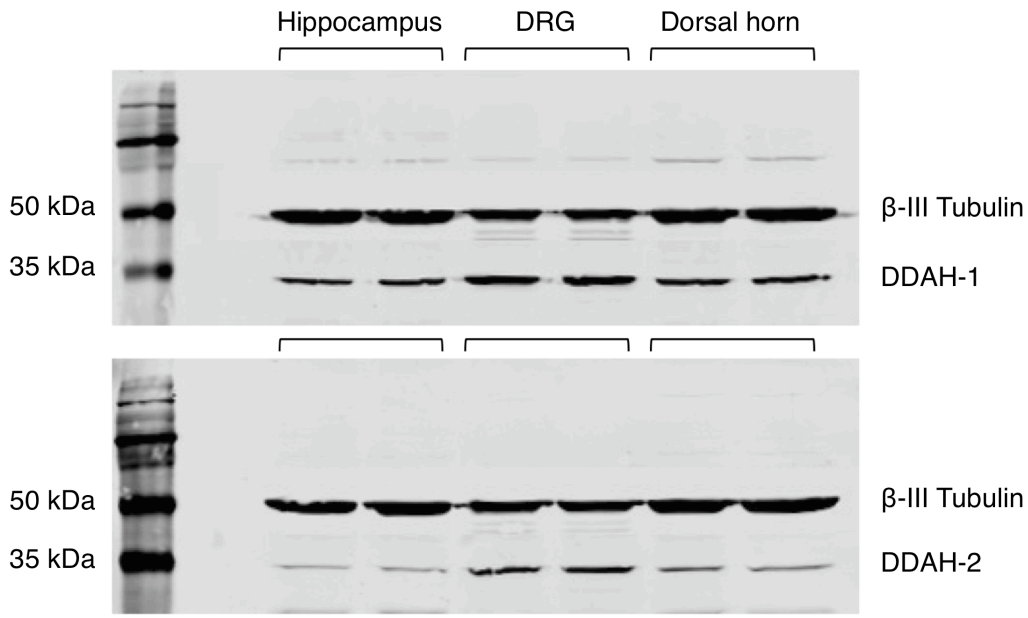


Fig. 7.3: Expression of DDAH-1 and DDAH-2 protein in neuronal tissues. Western immunoblots showing expression of DDAH-1 (top) and DDAH-2 (bottom) in hippocampus (two left lanes), DRG (two centre lanes) and spinal dorsal horn (two right lanes). β-III tubulin served as a loading control.

7.3.2 Spinal L-291 inhibits C-fibre evoked responses and post-discharge of deep dorsal horn WDR neurones

Having observed expression of DDAH protein in DRG and spinal cord dorsal horn, the function of DDAH-1 in sensory and nociceptive signalling was assessed using the specific DDAH-1 inhibitor L-291. Spinal administration of L-291 produced selective and significant dose-dependent inhibitions of C-fibre evoked responses of WDR neurones (Fig. 7.4; 1.2 μg, n = 9, % of pre-drug baseline = 89 ± 8 %, $p > 0.05$; 12 μg, n = 9, % of pre-drug baseline = 78 ± 7 %, $p < 0.05$; 120 μg, n = 6, % of pre-drug baseline = 74 ± 4 %, $p < 0.01$) as well as of post-discharge (Fig. 7.4; 1.2 μg, n = 9, % of pre-drug baseline = 74 ± 13 %, $p > 0.05$; 12 μg, n = 9, % of pre-drug baseline = 62 ± 12 %, $p < 0.05$; 120 μg, n = 6, % of pre-drug baseline = 57 ± 16 %, $p < 0.01$). No changes were seen in Aβ-fibre or Aδ-fibre evoked responses (Fig. 7.4). Peak inhibitory effects of L-291 were evident by 40 min post-administration and persisted for the remainder of the recording period. The control compound L-456 (120 μg, n = 8) did not affect any evoked response or post-discharge. Pre-drug control responses did not differ between treatment groups (Table 7.1).

	L-456 (*n* = 8)	**L-291 (*n* = 9)**
C-fibre threshold (mA)	0.68 ± 0.21	0.63 ± 0.10
Depth (*μ*M)	934 ± 58	922 ± 45
Aβ-fibre evoked response (No. of APs)	153 ± 22	142 ± 13
Aδ-fibre evoked response (No. of APs)	216 ± 31	172 ± 22
C-fibre evoked response (No. of APs)	512 ± 37	495 ± 48
Post-discharge (No. of APs)	668 ± 81	564 ± 61
Input (No. of APs)	327 ± 46	368 ± 64
Wind-up (No. of APs)	940 ± 91	776 ± 62

Table 7.1: Pre-drug control responses induced by transcutaneous electrical stimulation of the hindpaw receptive field of WDR neurones in rats treated with L-456 or L-291. C-fibre threshold, cell depth, afferent-evoked responses, input and wind-up are expressed as mean ± s.e.m. There were no differences between cells recorded in each treatment group for any measure prior to drug delivery; Student's unpaired t-test.

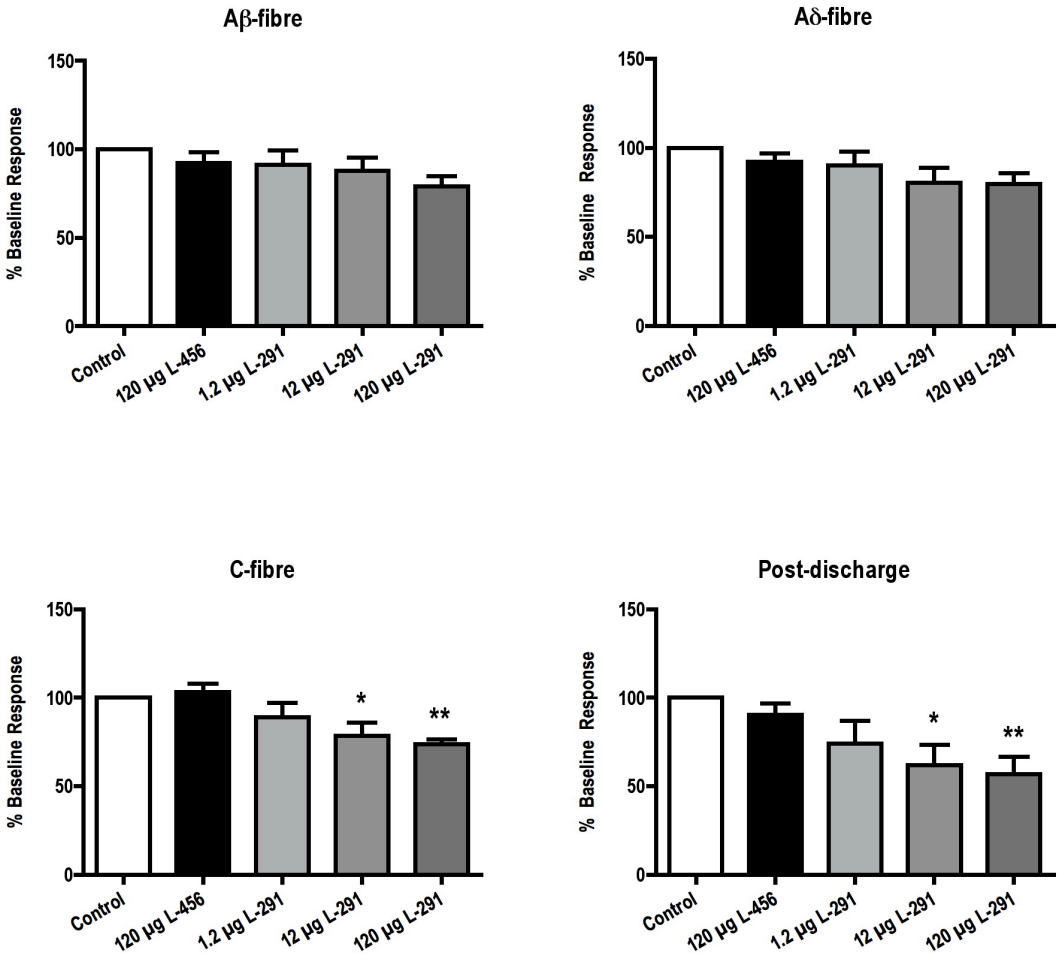


Fig. 7.4: Spinal L-291 decreases C-fibre evoked responses and post-discharge of WDR neurones. Afferent-evoked responses and post-discharge of WDR neurones induced by transcutaneous electrical stimulation of the hindpaw receptive field, following spinal application of L-291 (1.2 μg, $n = 9$; 12 μg, $n = 9$; 120 μg, $n = 6$) or L-456 (120 μg, $n = 8$). White bars represent pre-drug control. Data presented as mean ± s.e.m. of pre-drug control responses; $* p < 0.05$, $** p < 0.01$ versus pre-drug control, one-way RM ANOVA followed by Bonferroni post-tests.

7.3.3 Wind-up of deep dorsal horn WDR neurones is reduced by spinal L-291

No effect was seen on input following spinal application of L-291 or L-456 (Fig. 7.5a). In contrast, L-291 produced a significant and dose-dependent inhibition of wind-up of WDR neurones (Fig. 7.5b,c; 1.2 μg, n = 9, % of pre-drug baseline = 67 ± 8 %, $p <$ 0.05; 12 μg, n = 9, % of pre-drug baseline = 63 ± 11 %, $p < 0.01$; 120 μg, n = 6, % of pre-drug baseline = 58 ± 13 %, $p < 0.01$), while L-456 had no effect (Fig. 7.5d). Pre-drug input and wind-up values were similar in both treatment groups (Table 7.1).

Therefore, inhibition of spinal DDAH-1 by L-291 selectively reduces C-fibre evoked responses, post-discharge and NMDA-dependent wind-up of WDR neurones, indicating a role for DDAH-1 in nociceptive signalling and plasticity within the dorsal horn. No changes were seen in A-fibre evoked responses and input, indicating minimal pre-synaptic effects.

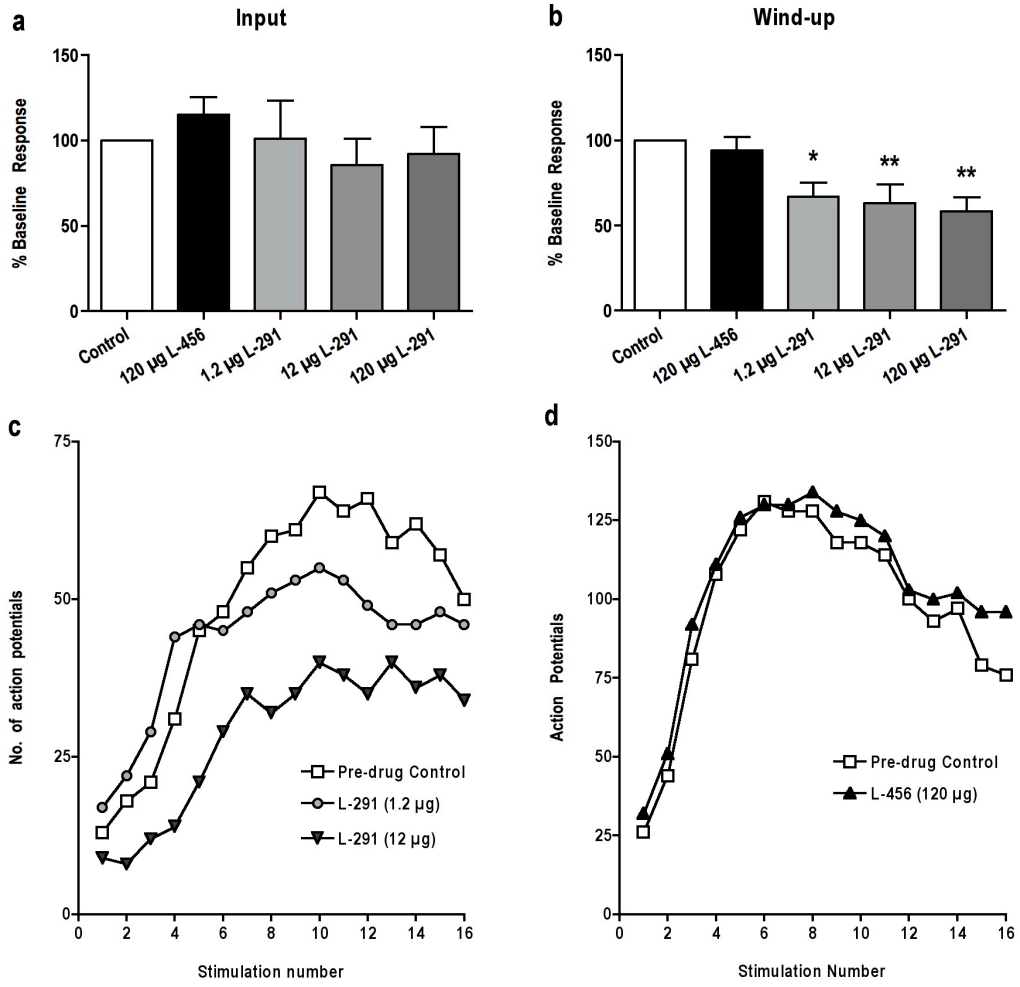


Fig. 7.5: Spinal L-291 selectively reduces wind-up of WDR neurones.

(**a,b**) Effect of spinal application of L-291 (1.2 μg, n = 9; 12 μg, n = 9; 120 μg, n = 6) or L-456 (120 μg, n = 8) on (**a**) input and (**b**) wind-up of WDR neurones. White bars represent pre-drug control. Data presented as mean ± s.e.m. of pre-drug control responses; $*$ $p < 0.05$, $**$ $p < 0.01$ versus pre-drug control; one-way RM ANOVA followed by Bonferroni post-tests. (**c,d**) Examples of the wind-up of single WDR neurones following repetitive electrical stimulation in the presence of spinal (**c**) L-291 (1.2 μg and 12 μg) or (**d**) L-456 (120 μg).

7.3.4 Formalin-induced central sensitization of deep dorsal horn WDR neurones is reduced by spinal pre-treatment with L-291

In recordings where rats were pre-treated with spinal application of control L-456 (120 μg, n = 14), formalin injection into the hindpaw induced a characteristic biphasic neuronal firing response (Fig. 7.6a,b; 1st phase: 0-10 min, total APs = 9524 ± 1778; 2nd phase: 10-70 min, total APs = 37855 ± 7595). Spinal pre-treatment with L-291 significantly and selectively reduced second phase neuronal firing (12 μg, n = 10; total APs = 8258 ± 5198, $p < 0.001$). No change in neuronal firing during the first phase was observed. All cells recorded were characterized prior to injection of drug and formalin to ensure that cells were comparable between the two treatment groups (Table 7.2).

Thus, spinal inhibition of DDAH-1 reduces NMDA-dependent central sensitization of deep dorsal horn WDR neurones following intraplantar formalin.

	L-456 (*n* = 14)	L-291 (*n* = 10)
C-fibre threshold (mA)	0.60 ± 0.11	0.54 ± 0.09
Depth (*μ*M)	859 ± 26	886 ± 31
Aβ-fibre evoked response (No. of APs)	139 ± 9	162 ± 16
Aδ-fibre evoked response (No. of APs)	168 ± 19	250 ± 33
C-fibre evoked response (No. of APs)	481 ± 74	536 ± 70
Post-discharge (No. of APs)	518 ± 78	488 ±75
Input (No. of APs)	489 ± 122	424 ± 75
Wind-up (No. of APs)	602 ± 69	665 ± 99
48^{o}C (No. of APs)	1295 ± 164	1450 ± 138

Table 7.2: WDR cell characteristics from L-291 neuronal formalin test. Cells were characterized prior to drug administration and formalin injection. C-fibre threshold, cell depth, afferent-evoked responses, input, wind-up and response to 48°C are expressed as mean ± s.e.m. There were no differences between cells recorded in each treatment group; Student's unpaired t-test.

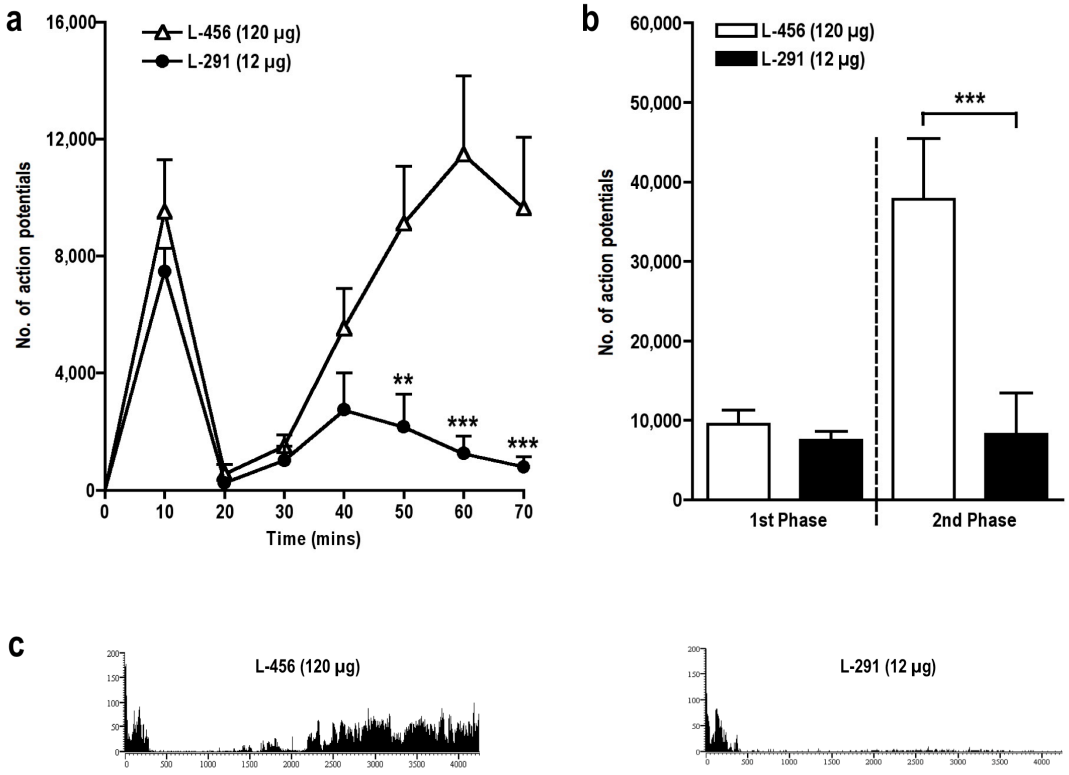


Fig. 7.6: Spinal L-291 reduces formalin-induced central sensitization of WDR neurones. **(a)** Time course of WDR firing response to subcutaneous formalin (5%, 50 μl) injection into the hindpaw receptive field following spinal pre-treatment with control L-456 (120 μg, $n = 14$) or L-291 (12 μg, $n = 10$; ** $p < 0.01$ at 50 min, *** $p < 0.001$ at 60min, *** $p < 0.001$ at 70min versus L-456; two-way RM ANOVA followed by Bonferroni post-tests). **(b)** Total neuronal activity during the 1st (0-10 min) and 2nd phases (10-70 min) of the formalin response with spinal L-456 or L-291 (2nd phase: *** $p < 0.001$ versus L-456, one-way ANOVA followed by Bonferroni post-tests). **(c)** Representative rate recordings of firing responses of WDR neurones to formalin following spinal pre-treatment with L-456 or L-291. All data presented as mean ± s.e.m.

7.3.5 Spinal L-291 inhibits pain-related behaviours in rats due to formalin-induced central sensitization

Effects of spinal inhibition of DDAH-1 were next assessed in the formalin behavioural test. In rats pre-treated intrathecally with control L-456 formalin induced a biphasic response of pain-related behaviours as expected (Fig. 7.7a,b; 24 μg, $n = 6$; 1st phase: 0-10 min, total time of pain-related behaviour = 61 ± 5 s; 2nd phase: 10-60 min, total time of pain-related behaviour = 487 ± 83 s). Pre-treatment with the DDAH-1 inhibitor L-291 significantly and selectively decreased pain-related behaviour during the second phase of the response (Fig. 7.7a,b; 24 μg, $n = 6$, 2nd phase total time of pain-related behaviour = 218 ± 54 s, $p < 0.01$). No difference was seen during the first phase between L-291 and L-456 treatment groups.

Thus, inhibition of spinal DDAH-1 reduces formalin-induced pain-related behaviours produced by spinal central sensitization.

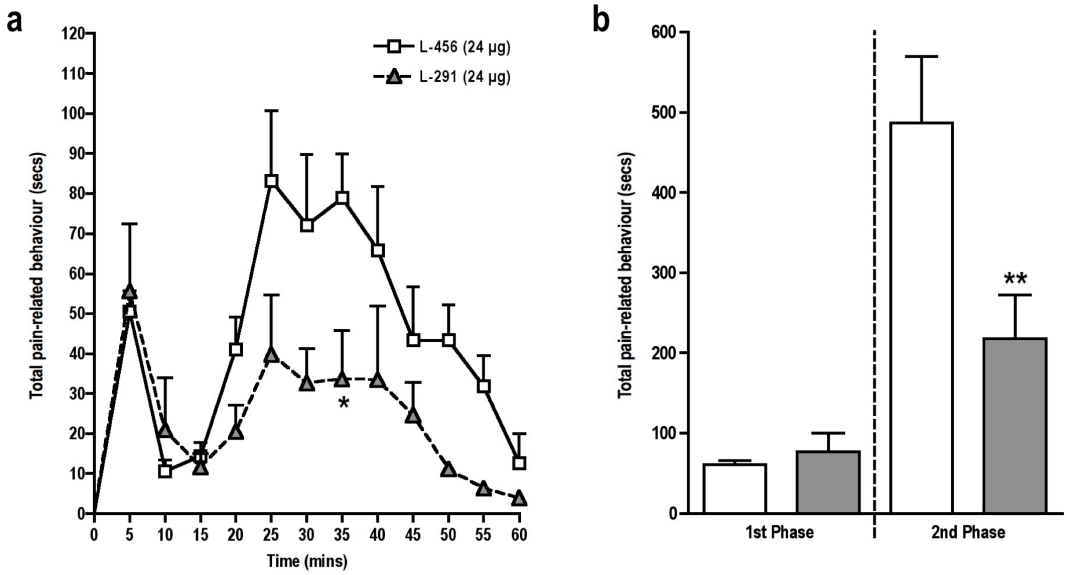


Fig. 7.7: Intrathecal L-291 reduces pain-related behaviours due to formalin-induced central sensitization. **(a)** Time course of pain-related behaviours induced by intraplantar injection of formalin (5%, 50 μl) following intrathecal pre-treatment with L-456 (24 μg) or L-291 (24 μg, $*$ $p < 0.05$ at 35 min versus L-456; two-way RM ANOVA followed by Bonferroni post-tests). **(b)** Total pain-related behaviour during the 1st phase (0-10 min) and 2nd phase (10-60 min) of the response to formalin following intrathecal pre-treatment with L-456 or L-291 (2nd phase: $**$ $p < 0.01$ versus L-456; one-way ANOVA followed by Bonferroni post-tests). All data presented as mean ± s.e.m., $n = 6$ in each group.

7.3.6 Spinal L-291 reduces formalin-induced pain-related active licking and biting and reflexive lifting and flinching behaviours

Pain-related behaviours produced by intraplantar formalin were separated into two categories: the reflexive lifting and flinching behaviours and the active licking and biting of the injured paw. Further analysis showed that L-291 reduced both licking and biting (Fig. 7.8a,b; 2^{nd} phase total time of licking and biting behaviour = 138 ± 48 s, $p < 0.01$) and lifting and flinching (Fig. 7.8c,d; 2^{nd} phase total time of lifting and flinching behaviour = 81 ± 10 s, $p < 0.01$) during the second phase of the formalin response only in comparison to L-456 (Fig. 7.8a,b; 2^{nd} phase total time of licking and biting behaviour = 318 ± 51 s; Fig. 7.8c,d; 2^{nd} phase total time of lifting and flinching behaviour = 169 ± 37 s). No differences were seen during the first phase in any behaviour between L-291 and L-456 treatment groups.

Therefore, the reduction in total pain-related behaviour produced by spinal administration of L-291 was due to action on both the active licking and biting responses and also the more reflexive lifting and flinching.

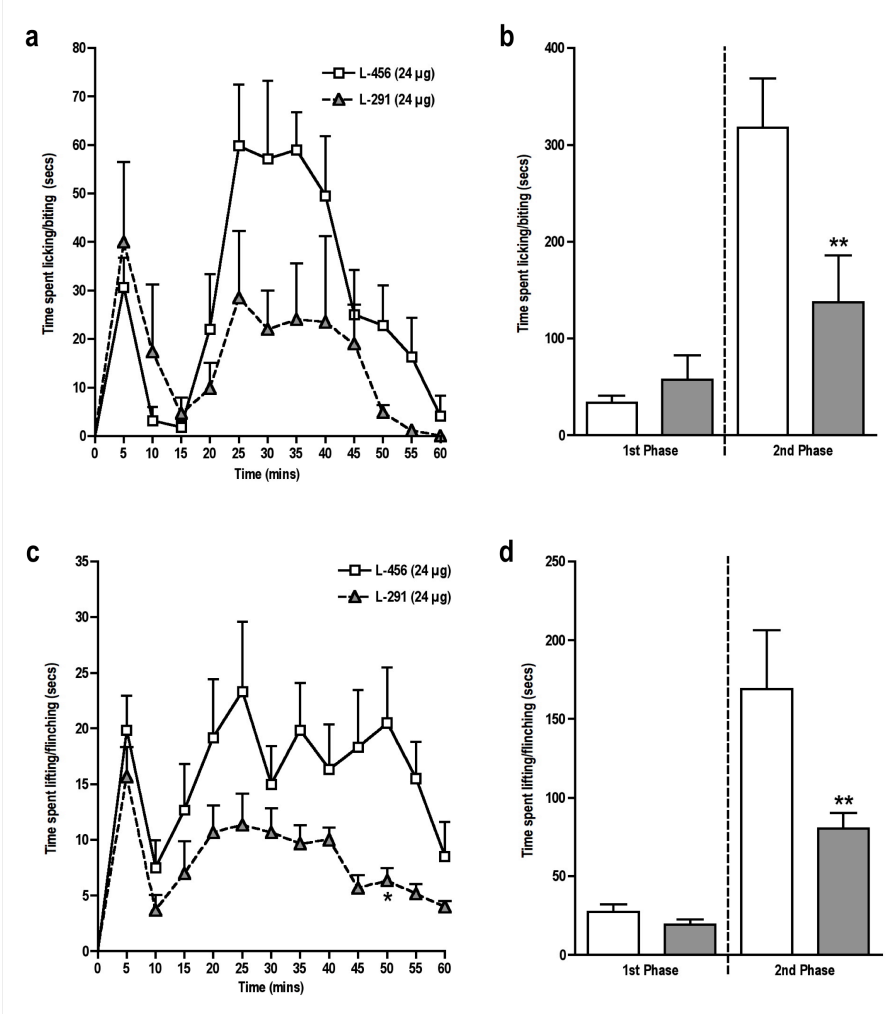


Fig. 7.8: Intrathecal L-291 reduces both active licking and biting and reflexive lifting and flinching pain-related behaviours following intraplantar formalin. (a) Time course of formalin-induced (5%, 50 μl) licking and biting behaviour following intrathecal pre-treatment with L-456 (24 μg, n = 6) or L-291 (24 μg, n = 6). **(b)** Total licking and biting behaviour during the 1st (0-10 min) and 2nd phases (10-60 min) of the formalin response following intrathecal L-456 or L-291 (2nd phase, $* p < 0.01$ versus L-456; one-way ANOVA followed by Bonferroni post-tests). **(c)** Time course of formalin-induced lifting and flinching behaviour following intrathecal pre-treatment with L-456 or L-291. **(d)** Total lifting and flinching behaviour during the 1st and 2nd phases of the formalin response following intrathecal L-456 or L-291 (2nd phase, $* p <$ 0.01 versus L-456; one-way ANOVA followed by Bonferroni post-tests). Note that due to the lower incidence of lifting and flinching behaviours the vertical axes are scaled differently. Data presented as mean ± s.e.m.

7.3.7 No change in formalin-induced pain-related behaviours in DDAH-1$^{+/-}$ mice

The effect of global heterologous deletion of the DDAH-1 gene was assessed in the formalin behavioural test (Fig. 7.9). DDAH$^{+/-}$ mice (n = 5) produced the characteristic biphasic behavioural response to intraplantar formalin which surprisingly did not differ from the response in wild-type DDAH$^{+/+}$ mice (n = 7). No differences were seen at any time point in either phase (Fig. 7.9a,b). Further analysis of the different pain behaviours showed that mice spent most time licking and biting (Fig. 7.9c,d) the injected paw, rather than lifting and flinching (Fig. 7.9e,f). Both behaviours showed a biphasic pattern though this was much reduced and arguably less obvious for lifting and flinching. It would seem clear that active licking and biting behaviours predominate in mice, at least in this experiment, over the more reflexive lifting and flinching behaviour. Again no differences were seen in these behaviours between DDAH$^{+/-}$ and wild-type mice (Fig. 7.9d,f).

Thus, global heterologous deletion of DDAH-1 does not alter formalin-induced behaviours and spinal central sensitization.

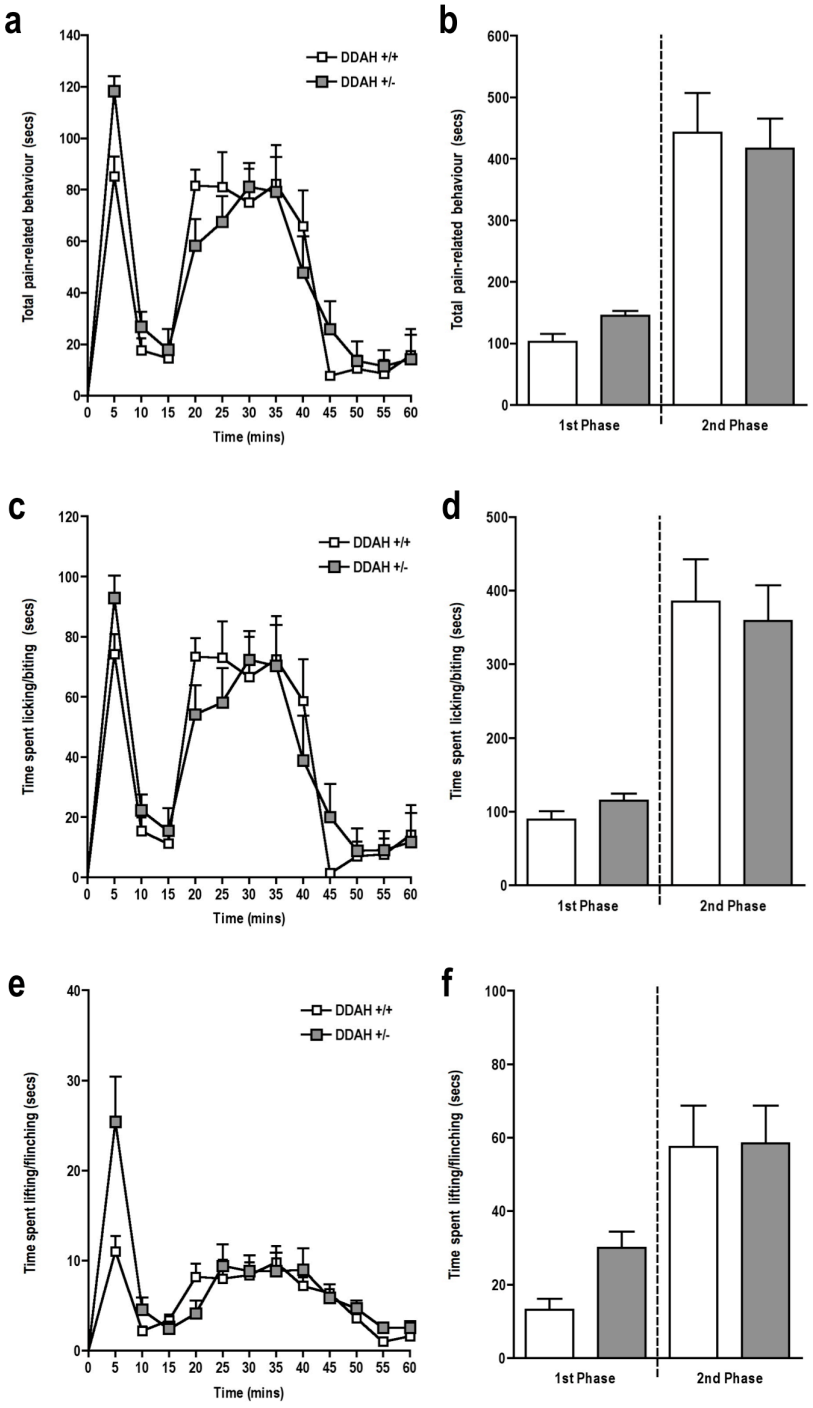


Fig. 7.9: DDAH$^{+/-}$ mice have normal behavioural responses to intraplantar formalin. **(a)** Time course of pain-related behaviours induced by intraplantar formalin (5%, 50 μl) injection in DDAH$^{+/-}$ (n = 5) and wild-type DDAH$^{+/+}$ mice (n = 7). **(b)** Total pain-related behaviour during the 1st phase (0-10 min) and 2nd phase (10-60

min) of the response to formalin in DDAH$^{+/-}$ and wild-type mice. **(c)** Time course of formalin-induced licking and biting behaviour in DDAH$^{+/-}$ and wild-type mice. **(d)** Total licking and biting behaviour during the 1st and 2nd phases of the formalin response in DDAH$^{+/-}$ and wild-type mice. **(e)** Time course of formalin-induced lifting and flinching behaviour in DDAH$^{+/-}$ and wild-type mice. **(f)** Total licking and biting behaviour during the 1st and 2nd phases of the formalin response in DDAH$^{+/-}$ and wild-type mice. Note that due to the lower incidence of lifting and flinching behaviours the vertical axes are scaled differently. All data presented as mean ± s.e.m.

7.4 Discussion

The results of this study suggest that DDAH-1, an enzyme which metabolizes methylarginines, is involved in NMDA-dependent plasticity of spinal dorsal horn sensory neurones following noxious peripheral stimulation. Pharmacological inhibition of DDAH-1, using a novel DDAH-1 specific small molecule inhibitor, L-291, reduces neuronal hyperexcitability as well as pain-related behaviours produced by formalin-induced spinal central sensitization in rats. Surprisingly, however, global heterologous deletion of DDAH-1 had no effect on formalin-induced pain-related behaviours in transgenic mice.

Spinal administration of L-291 produced significant dose-dependent inhibitions of post-discharge and wind-up of WDR neurones induced by transcutaneous electrical stimulation of the hindpaw receptive field. C-fibre evoked responses were also dose-dependently reduced, although to a lesser extent than post-discharge and wind-up. No effect was seen on A-fibre evoked responses or on input at any dose. These selective inhibitory effects of spinal DDAH-1 antagonism on NMDA-mediated neuronal events suggest a post-synaptic site of action and are similar to those produced by spinal inhibition of nNOS (Stanfa et al., 1996; Urch and Dickenson, 2003a) as well as drugs targeting spinal NMDA receptors (Chapter 3) (Dickenson and Sullivan, 1987b). Together, these data confirm the intimate relationship between NMDA receptor activation and downstream NO signalling. Targeting of DDAH-1 may be a novel way of modulating this pathway. The fact that effects of DDAH-1 inhibition were selective would also suggest that there were no vascular effects, as these would be expected to alter all spinal responses in a non-selective manner. Indeed, no obvious changes in the central blood vessel shape or size were observed during electrophysiological recordings following administration of L-291.

In both neuronal and behavioural formalin tests, spinal administration of L-291 reduced central sensitization of dorsal horn neurones, as shown by a reduction of second phase activity. No effects were seen in the first phase of either response, suggesting that L-291 does not alter normal acute pain, but rather alters potentiation of pain transmission. These findings are consistent with those of several studies which investigated the effect of inhibition of NOS in the formalin test. Both systemic

and spinal administration of NOS inhibitors has been shown to selectively reduce second phase pain-related behaviours induced by intraplantar formalin injection, with no effect on the first acute phase (Malmberg and Yaksh, 1993; Moore et al., 1993; Coderre and Yashpal, 1994). Interestingly, Wiertelak and colleagues showed that hindpaw injection of formalin lead to a facilitation of the noxious tail-flick response, and this hypersensitivity was found to be mediated by the NMDA-NO signalling cascade at the level of the spinal cord (Wiertelak et al., 1994). Formalin injection in the hindpaw has also been shown to upregulate nNOS in the dorsal horn of the spinal cord (Herdegen et al., 1994; Lam et al., 1996), and formalin-induced c-Fos is reduced by spinal inhibition of NOS, primarily in the superficial dorsal horn (Roche et al., 1996). Together, these data establish a significant contribution of NO and NOS in central sensitization of spinal sensory neurones, and provide a context for the inhibitory effects of DDAH-1 antagonism, which indirectly suppresses generation of NO by NOS. Interestingly, the DDAH-1 antagonist L-291 reduced both active and reflexive pain-related behaviours induced by formalin, in contrast to disruption of NR2B interactions with PSD-95 which altered only active licking and biting behaviours (Chapter 3, Fig. 3.8). This suggests that activation of nNOS and regulation by DDAH-1 may involve alternative mechanisms in addition to upstream NR2B-subtype receptor activation via PSD-95. For example, the sensitizing effects of substance P are also known to involve downstream nNOS (Coderre and Yashpal, 1994). Nevertheless, disruption of binding between PSD-95 and nNOS has recently been shown to inhibit NMDA-induced thermal hypersensitivity and also neuropathic pain (Florio et al., 2009), confirming the importance of the ternary complex between NMDA receptors, PSD-95 and nNOS in pain hypersensitivity.

Surprisingly, DDAH-$1^{+/-}$ mice showed no differences in their behavioural response to intraplantar formalin compared to wild-type littermates. This may be because only 50% of DDAH-1 was absent in these mice, and the remaining DDAH-1 may have been sufficient for the full expression of the formalin response. A greater loss of DDAH-1 may be required and this hypothesis is supported by the fact that relatively high doses of L-291 were required to inhibit formalin-induced pain-related behaviours in rats, though neuronal effects of L-291 were still selective at these doses. The lowest effective dose in electrophysiological experiments was 12 μg in 50 μl (1 mM), which is 50 times the IC_{50} of the drug, whilst in behavioural studies a dose of 24 μg in

20 μl (5 mM), at 250 times the IC_{50} of L-291 was effective, suggesting that a major blockade of DDAH-1 is required. Lower doses were ineffective (data not shown). Unfortunately, total global knockout of DDAH-1 is lethal *in utero*, and therefore, tissue-specific (conditional) knockouts of DDAH-1 are now being constructed. Complete loss of DDAH-1 in nociceptors and/or spinal cord may reveal a phenotype in the formalin test. Additionally, DDAH-2 may also be involved in the response to formalin and so may functionally compensate for the loss of DDAH-1, even though expression levels of DDAH-2 are unaltered in DDAH-$1^{+/-}$ mice (Leiper et al., 2007). DDAH-2 knockout mice (DDAH-$2^{-/-}$) have been bred successfully and may reveal a role for DDAH-2 in the formalin test in future studies. Furthermore, DDAH-$2^{-/-}$ mice have been successfully crossed with heterozygous DDAH-$1^{+/-}$ mice and so the effects of complete DDAH-2 deletion on a DDAH-1 haploinsufficient background can be investigated (for further information visit www.ddah.org.uk). Functional compensation in these mice by DDAH-2 for the loss of DDAH-1, or vice-versa, will have been eliminated or reduced, respectively. Alternatively it may be argued that L-291 may act via another mechanism which does not involve DDAH-1 inhibition, explaining the lack of behavioural changes in DDAH-$1^{+/-}$ mice. However, inhibition of DDAH-1 is the most likely mechanism of action of L-291 since this compound is based on the structure of ADMA and acts as a competitive inhibitor of DDAH-1 (IC_{50} 20 μM) by binding directly to the active site of the enzyme, as shown by crystallography (Leiper et al., 2007). Furthermore, recombinant protein activity assays and functional assays in culture have demonstrated the specificity of L-291 for DDAH-1 (unpublished observations, verbal communication from Dr Manasi Nandi, UCL).

Western blot analysis confirmed protein expression of both DDAH-1 and DDAH-2 in neuronal tissues, specifically in DRG, spinal dorsal horn and hippocampus, which not only contain neurones but also vascular structures which would be expected to express DDAH isoforms. Immunohistochemical studies are required to elucidate the exact neuronal expression patterns of DDAH isoforms, particularly in the dorsal horn of the spinal cord, though this would be expected to be similar to that of nNOS. Early studies demonstrated that nNOS is predominantly located in the superficial dorsal horn (Valtschanoff et al., 1992; Spike et al., 1993; Morris et al., 1994) and formalin injection in the hindpaw has been shown to increase this expression (Herdegen et al., 1994; Lam et al., 1996). Intriguingly, extensive evidence suggests that nNOS is

present almost exclusively in GABA-positive interneurones in the superficial dorsal horn (Valtschanoff et al., 1992; Spike et al., 1993; Laing et al., 1994; Bernardi et al., 1995). This finding is perhaps confusing considering the abundance of functional evidence implicating nNOS in NMDA-mediated excitatory events as well as the presence of nNOS in NMDA receptor complexes (Chapter 3, Fig. 3.3 & Chapter 5, Fig. 5.7). The exact role of nNOS in these GABAergic interneurones remains unknown. However, some of the nNOS expression in these interneurones is found in their dorsally-extending dendrites where GABA is not expressed, and NO may be released from such sites (Bernardi et al., 1995). NMDA receptors on inhibitory interneurones may cause NO to be generated which may then counteract GABA-mediated inhibitions, permitting NMDA-dependent spinal events such as wind-up, LTP and central sensitization to occur. In addition, since NO is a diffusible messenger, its effects are not restricted to inhibitory interneurones and may easily engage many types of neurones within the superficial dorsal horn, including lamina I projection neurones (Ruscheweyh et al., 2006; Schmidtko et al., 2008), to increase excitations. In the DRG, nNOS expression is much less abundant and is restricted to peptidergic C-fibres (Aimi et al., 1991; Zhang et al., 1993). More recently, it has been shown that NO-sensitive guanylate cyclase is absent in DRG neurones but present in astrocytes (Schmidtko et al., 2008). Together, these studies would suggest that the contribution of NO signalling in pre-synaptic primary afferent neurones is minimal, and that spinally administered drugs targeting nNOS and NO signalling act predominantly on intrinsic post-synaptic spinal sites. These findings also challenge the hypothesis that NO may act as a retrograde transmitter, released from spinal sites and acting on peripheral nerve terminals to increase neurotransmitter release. It is more likely that NO is released from spinal sites and acts exclusively on spinal neurones to produce sensitization of nociceptive signalling (Schmidtko et al., 2009). DDAH-1 expression in DRG may also be minimal in neurones and this would fit with the lack of effect of L-291 on input and afferent-evoked responses in comparison to post-discharge and wind-up, which are neuronal events induced within intrinsic dorsal horn neurones.

It would be interesting to assess the effects of DDAH-1 inhibition on LTP in spinal pain pathways. Ikeda and colleagues demonstrated that LTP in the superficial dorsal horn increases NO production, as indicated by the use of an NO-sensitive dye. The

amount of NO produced correlated well with the level of LTP. In addition, inhibitors of NOS blocked both LTP and NO production (Ikeda et al., 2006a).

A question remains regarding exactly how L-291 gains access to the intracellular compartment of cells to exert its effects on DDAH-1. Since L-291 mimics L-arginine structurally, it is believed that the same mechanisms which transport L-arginine across cell membranes from the extracellular space will also transport L-291. This is in fact the case for direct NOS inhibitors such as L-NMMA and L-NAME which are also based on the structure of L-arginine. The y^+ system cationic amino acid transporters (y^+CATs) are thought to be the primary transmembrane proteins responsible for the transport of L-arginine into cells, and have been characterized in a variety of neuronal populations (Wiesinger, 2001). It is possible that, in addition to binding to and inhibiting DDAH-1, L-291 may limit the availability of L-arginine by competing for transporters, thus further reducing NO signalling. Accordingly, addition of L-arginine can reverse the effects of L-291 on blood vessels (Leiper et al., 2007).

The results presented in this study are the first to demonstrate protein expression of DDAH isoforms in the nervous system as well as suggesting a functional role for DDAH-1 in neuronal signalling. Similar to inhibition of nNOS, inhibition of DDAH-1 by L-291 modulates NMDA-dependent nociceptive plasticity in the dorsal horn of the spinal cord. Thus, DDAH-1 may be a novel analgesic target in chronic pain states.

8 Final Discussion

The main aims of this thesis were to investigate novel ways of modulating NMDA receptors and downstream intracellular signalling cascades in the dorsal horn of the spinal cord as a strategy for analgesia whilst avoiding side-effects associated with receptor blockade by traditional NMDA receptor antagonists. I attempted to modulate NMDA-mediated intracellular signalling in two ways: first, through disruption of binding to the scaffolding protein PSD-95, and second, by directly targeting specific signalling pathways in models of NMDA-dependent spinal nociceptive plasticity. The studies presented here demonstrate that NMDA-dependent spinal mechanisms of plasticity, particularly central sensitization, are important for the manifestation of persistent pain states. I have used behavioural studies, *in vivo* electrophysiology, immunohistochemistry and molecular biology to draw these conclusions. The main findings are now summarized.

8.1 NMDA-dependent spinal nociceptive plasticity can be prevented without NMDA receptor blockade but through modulation of protein interactions

Both spinal dorsal horn wind-up, produced by electrical stimulation at the peripheral receptive field, and formalin-induced central sensitization are robust models for studying spinal nociceptive plasticity. I have confirmed that both of these phenomena require activity of dorsal horn NMDA receptors and interactions with the scaffolding protein PSD-95, which couples the NMDA receptor to downstream intracellular signalling pathways. Furthermore, I have shown that, rather than receptor blockade, disruption of NMDA-mediated signalling, by perturbing the interaction with PSD-95, can prevent such spinal nociceptive plasticity without altering primary afferent input. A lack of effect on acute sensory and noxious processing was evident by unaltered afferent-evoked responses of WDR neurones and 1st phase neuronal and behavioural activity following formalin injection into the hindpaw, which is driven by peripheral C-fibres. This data, together with the fact that PSD-95 is absent from DRG neurones, suggests an exclusively post-synaptic action

of the disrupting peptide Tat-NR2B9c on intrinsic dorsal horn neurones, which is selective for neuronal and behavioural hypersensitivity.

8.2 Disruption of interactions between NR2B-containing NMDA receptors and PSD-95 can reverse neuropathic pain

A single acute injection of Tat-NR2B9c, delivered spinally via intrathecal lumbar puncture, was able to produce a significant, though not complete, reversal of mechanical and cold hypersensitivity in nerve-injured rats. No effects were observed in the sensitivity of the non-injured paw, supporting the idea of selective roles for NR2B-subtype receptors and interactions with PSD-95 in aberrant pathophysiological signalling and not in normal acute transmission. In addition, Tat-NR2B9c robustly reduced mechanically and thermally evoked responses and also wind-up of dorsal horn WDR neurones in nerve-injured rats as well as sham rats, though this inhibitory effect was translated behaviourally only when associated with nerve injury, again suggesting a selective action of Tat-NR2B9c on the hypersensitivity. Here, it is likely that the NMDA receptor is recruited by intense suprathreshold and prolonged stimulation used in electrophysiological studies, whilst playing a minimal role at behavioural thresholds. Spinal Tat-NR2B9c produced no adverse motor impairment in the rotarod test at a therapeutically effective dose, suggesting improved tolerability over conventional NMDA antagonists. In these studies, efficacy of Tat-NR2B9c in both wind-up and formalin tests was a good predictor of efficacy in the nerve injury model of neuropathic pain. Thus, wind-up and the formalin test may be used to predict efficacy of novel pharmacological interventions in chronic pain states, at least for nerve injury-induced neuropathic pain.

8.3 Importance of dorsal horn NR2B-containing NMDA receptors in nociceptive plasticity and neuropathic pain

Although numerous studies have implicated NR2B-subtype receptors in pain, the precise location of these receptors has been controversial, with some studies indicating the importance of supraspinal over spinal NR2B-subtype receptors, though

most support an intrinsic spinal role. By employing exclusively spinal administration of Tat-NR2B9c, I have confirmed that spinal NR2B-subtype receptors do in fact have a crucial role in neuronal and behavioural hypersensitivity. In addition, PKCζ/PKMζ was found to be coupled to spinal NR2B-subtypre receptors and to contribute to central sensitization, while intraplantar formalin also increased phosphorylation of spinal NR2B subunits. Therefore, spinal NR2B-containing NMDA receptors remain an important drug target for the treatment of chronic pain.

8.4 PKCζ/PKMζ and PI3K signalling pathways are implicated downstream of NMDA receptor activation in NMDA-dependent spinal nociceptive plasticity

Activation of PKCζ/PKMζ and PI3K signalling pathways contributes to central sensitization, and blockade of both kinases inhibits the resultant neuronal plasticity and behavioural hypersensitivity, similar to Tat-NR2B9c. Inhibition of PI3K reduced NMDA-dependent wind-up of WDR neurones without affecting primary afferent input, again similar to disruption of binding of PSD-95 to NR2B subunits. In addition, PI3K was found to be a key upstream modulator of ERK and CaMKII activity in central sensitization. PKCζ/PKMζ has an intimate role in NMDA-dependent nociceptive plasticity. I have demonstrated the presence of a novel physical complex between NR2B-containing NMDA receptors, PSD-95 and PKCζ/PKMζ in spinal dorsal horn neurones. Binding between NR2B subunits and PSD-95 mediates activation of PKCζ/PKMζ signalling, contributing to central sensitization. While Tat-NR2B9c itself had no effect on the 1st phase behavioural response to formalin, direct inhibition of PKCζ/PKMζ did produce a small but significant reduction of this acute phase. This may suggest that not all of the PKCζ/PKMζ in the dorsal horn is bound to and regulated by NR2B-containing NMDA receptors and PSD-95.

8.5 DDAH-1 may be an alternative target to nNOS in the treatment of pain

I confirmed that nNOS is coupled to NR2B-containing NMDA receptors via PSD-95 in dorsal horn neurones, implicating the generation of NO as a key downstream

signalling molecule of NMDA-dependent nociceptive plasticity. In addition, I showed that DDAH isoforms, which indirectly modulate NOS isoforms, are present in the DRG and dorsal horn of the spinal cord. DDAH-1, which is believed to indirectly modulate nNOS activity, was shown to be involved in NMDA-dependent wind-up and central sensitization. As with Tat-NR2B9c, inhibition of DDAH-1 was without effect on pre-synaptic input from primary afferents and on acute pain processing as observed in the 1st phase of the neuronal and behavioural responses to intraplantar formalin. These results are similar to those achieved with direct nNOS inhibitors, thus implicating DDAH-1 as an alternative target to nNOS for the treatment of chronic pain.

8.6 Possible interplay between NMDA-dependent intracellular signalling pathways in dorsal horn neurones

The implications of these findings and their relationship to what is already known in the field have been discussed previously in the relevant results chapters. I will now put forward some ideas and evidence which may link these findings together and are summarized in the subsequent schematic (Fig. 8.1).

In addition to PSD-95, nNOS and PKCζ/PKMζ (Chapters 3 & 5), the PI3K signalling pathway has also been shown to couple directly to NR2 subunits of NMDA receptors. The p85 regulatory subunit of PI3K binds directly to a phosphorylated tyrosine (Tyr1336) within the cytoplasmic tail of NR2 subunits (Hisatsune et al., 1999). This interaction between PI3K and NR2 subunits does not require PSD-95. In addition, it has been shown that Akt, a downstream effector of PI3K, forms a complex with NR2B subunits and PSD-95 in the dorsal horn (Peng et al., 2009). It may be that generation of PIP_2 by PI3K causes translocation of Akt to the plasma membrane where it now interacts with NMDA receptors, via PSD-95, and can be phosphorylated and fully activated, as occurs in formalin-induced central sensitization (Chapter 6). Furthermore, PI3K signalling is believed to increase trafficking of PSD-95 to the plasma membrane (Yoshii and Constantine-Paton, 2007) and this may be a mechanism for maintenance and consolidation of NMDA receptor complexes. Together these studies demonstrate that the PI3K signalling cascade is directly

linked to NMDA receptor NR2 subunits and has a key regulatory role within the NMDA receptor complex.

PI3K also phosphorylates PIP_2 to generate PIP_3 which acts as an intracellular second messenger. PIP_3 has a dual role in the activation of PKCζ/PKMζ. First, PIP_3 can directly modulate the pseudosubstrate domain of PKCζ, thus relieving autoinhibition and promoting catalytic activity. Second, PIP_3 is required for activation of PDK-1, which in turn phosphorylates PKCζ/PKMζ on its activation loop (Thr410). This phosphorylation is upregulated in formalin-induced central sensitization (Chapter 5). These PIP_3-regulated steps are required for complete and stable activation of PKCζ/PKMζ (Hirai and Chida, 2002). PDK-1 may also phosphorylate Akt in a PI3K-dependent manner (Toker and Cantley, 1997). Activation of both ERK and CaMKII signalling is also regulated by upstream PI3K in central sensitization (Chapter 6), as is mTOR, which is also implicated in central sensitization (Asante et al., 2009). All of these aforementioned kinases (PI3K, Akt, PDK-1, ERK, CaMKII and mTOR) have been shown to regulate PKCζ/PKMζ in the hippocampus during LTP (Kelly et al., 2007) and this may also be the case in spinal central sensitization.

CaMKII has also been shown to bind directly to NR2B subunits of NMDA receptors at residues 1290-1306 of the cytoplasmic tail (Strack et al., 2000). This binding is facilitated by autophosphorylation of CaMKII at Thr286 (Strack and Colbran, 1998), which activates the enzyme and occurs in formalin-induced central sensitization (Chapter 6).

Binding of nNOS to NR2B-containing NMDA receptors is mediated via PSD-95 (Chapter 3). DDAH-1 indirectly regulates nNOS by metabolizing ADMA, an endogenous inhibitor of nNOS and is involved in central sensitization (Chapter 7). At present, it is not clear if DDAH-1 regulates only the nNOS which is coupled to NMDA receptors or only free nNOS, or both. Therefore, it is unknown if the regulation of nNOS by coupling between NR2B subunits and PSD-95 overlaps with the regulation by DDAH-1. Phosphorylation of nNOS is also required for regulation of activity and involves CaMKII. Two sites on nNOS, Ser741 and Ser847 are phosphorylated by CaMKII (Hayashi et al., 1999; Song et al., 2004), perhaps mediated by PSD-95

(Watanabe et al., 2003), the effect of which is a decrease in activity and synthesis of NO. Interestingly, phosphorylation of nNOS is bidirectionally regulated by NMDA receptors whereby low glutamate concentrations induce phosphorylation of Ser847 by CaMKII but high glutamate concentrations, as occurs during heightened stimulation, induces dephosphorylation of Ser847 by protein phosphatase 1 (PP1) which increases nNOS activity (Rameau et al., 2004). Additional NMDA-induced activation of nNOS is achieved by phosphorylation of Ser1412 by Akt (Rameau et al., 2007), again implicating the PI3K pathway in NMDA-mediated signalling.

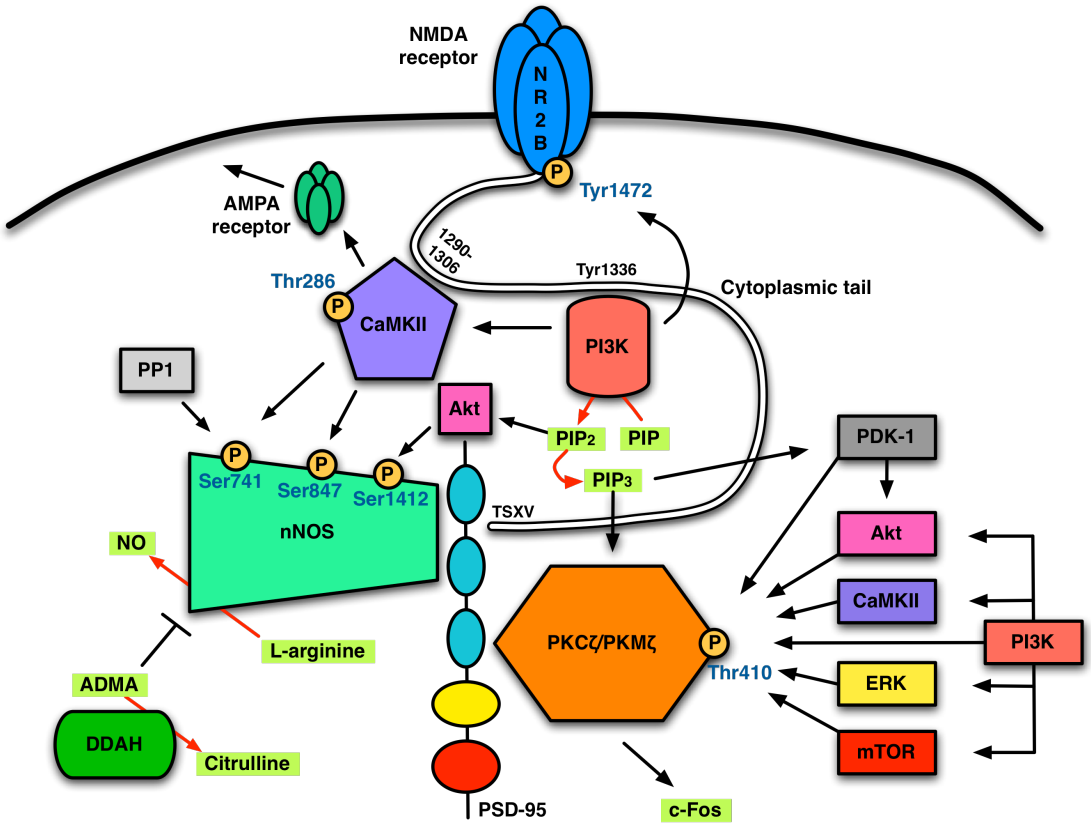


Fig. 8.1: NMDA-dependent intracellular signalling within dorsal horn sensory neurones promotes spinal central sensitization. The cytoplasmic tails of NR2B subunits of NMDA receptors bind to PSD-95, a physical interaction which mediates NMDA-dependent downstream signalling via cytoplasmic effectors such as PKCζ/PKMζ and nNOS. PKCζ/PKMζ is phosphorylated and activated by various kinases including PI3K, PDK-1, Akt, CaMKII, ERK and mTOR, promoting central sensitization and expression of genes such as c-Fos. PI3K is a key regulator of central sensitization and also binds to the cytoplasmic tail of NR2 subunits. PI3K promotes activation of multiple intracellular signalling pathways via generation of PIP_2 and PIP_3 and phosphorylation of Akt, CaMKII and ERK, as well as phosphorylation of NMDA receptor NR2 subunits and increased membrane trafficking of AMPA receptors via CaMKII. NMDA-dependent generation of NO by nNOS is regulated by ADMA which itself is metabolized by DDAH. Activity of nNOS is further regulated by phosphorylation by CaMKII and dephosphorylation by PP1.

8.7 Wind-up initiates central sensitization and is a neuronal correlate of pain hypersensitivity

In many of my studies I have used wind-up as a model of short-term spinal nociceptive plasticity. I found that drugs which reduced wind-up also reduced central sensitization. Wind-up is widely regarded as a neuronal correlate of the pain hypersensitivity which occurs after central sensitization. Indeed, there are many similarities between these two phenomena. Both wind-up and central sensitization are dependent on activation of NMDA receptors (Dickenson and Sullivan, 1987b; Woolf and Thompson, 1991) and both can increase responsiveness and receptive field size of dorsal horn neurones (Woolf, 1983; Cook et al., 1987; Li et al., 1999). However, it is not correct to say that wind-up is equivalent to central sensitization, since there are also differences between the two. The most obvious difference between wind-up and central sensitization is the duration of effects. Wind-up is short-lasting and its effects, both the enhanced responsiveness and expansion of receptive field size of dorsal horn neurones, are gone within a matter of minutes, while central sensitization and the resultant hypersensitivity persist for a long period beyond the conditioning stimulus. Furthermore, unlike in central sensitization where conditioning stimulation of C-fibres can increase A-fibre evoked responses (heterosynaptic facilitation) of dorsal horn neurones (Woolf, 1983), this is not the case in wind-up since only C-fibre evoked responses are enhanced (homosynaptic facilitation), suggesting that wind-up may be a selective reflection of central sensitization to C-fibre inputs only (Li et al., 1999). Therefore, while there are important similarities between wind-up and central sensitization, they are in fact separate phenomena. It would seem that wind-up is in fact an initiator of central sensitization. This notion is compatible with the short duration of wind-up. Noxious stimuli applied immediately after the initial conditioning stimulus can prolong and maintain wind-up (Li et al., 1999) and might lead to long-lasting central sensitization if they are of sufficient intensity, frequency and duration. It is likely that the Ca^{2+} influx which occurs following NMDA receptor activation during wind-up is a key step in initiating central sensitization, which is also known to involve Ca^{2+}-depdendent processes. Thus, drugs that block wind-up, such as NMDA antagonists, also block the initiation of central sensitization (Dickenson and Sullivan, 1987b; Haley et al., 1990; Woolf and Thompson, 1991). Indeed, in my studies I have shown that the

peptide Tat-NR2B9c, which interferes with NMDA receptors, reduces both wind-up and central sensitization of dorsal horn neurones. A trend towards inhibition of the first phase of the neuronal response to formalin, where central sensitization is initiated, was observed following Tat-NR2B9c, likely reflecting the inhibitory effects of this peptide on spinal wind-up. Therefore, in my opinion, wind-up is certainly a useful model for studying the neuronal basis of pain hypersensitivity, particularly central mechanisms since wind-up is a purely central phenomenon (Herrero et al., 2000). Recording wind-up is quick, reproducible and effects of pharmacological manipulations are easily observed. Finally, as described previously (see Chapter 1), several studies have demonstrated that a perceptual correlate of wind-up manifests in healthy human volunteers as an NMDA-dependent temporal summation of pain following repetitive noxious stimulation of C-fibres (Price, 1972; Price et al., 1994). Furthermore, symptoms of wind-up, temporal summation of pain and aftersensations, have been observed in chronic pain patients (Kristensen et al., 1992). Together, these human representations of wind-up support the clinical relevance of this model of nociceptive plasticity.

8.8 The Formalin Model of Pain

In this thesis, I have utilized the formalin model of pain in many studies, and so it is important to discuss both the merits and disadvantages of this test. The behavioural formalin pain test for rodents was first described by Dubuisson and Dennis in 1977, whereby 50 μl of 5% formalin was injected into the hindpaw of rats, as a method for studying pain and assessing analgesic potential of treatments (Dubuisson and Dennis, 1977). At that time, most pain studies employed acute pain tests, such as the tail-flick test, the hot-plate test and the pinch test. Whilst useful, these tests had the disadvantages that they were testing only transient pain without any underlying clinically relevant pathology and would only determine pain thresholds through predominantly reflex responses. Significant restraint of animals was required to conduct some of the tests which may affect results by exacerbating fear and anxiety of the animal. In addition, assessment of pain is perhaps overly subjective to interpretation by the experimenter, particularly in cases of evoked pain. Thus, the formalin test has significant advantages over these other tests. Little or no restraint

is required to conduct the test. The pain produced is prolonged, with pain-related behaviours routinely observed for a one-hour testing period. Inflammation occurs following formalin injection, as indicated by redness and swelling of the injected paw, and drives the pain response, thus giving the test clinical relevance to inflammatory pain conditions. Pain-related behaviours, such as lifting, flinching, licking and biting of the injected paw, as later characterized by Wheeler-Aceto and colleagues (Wheeler-Aceto et al., 1990), are ongoing and can be easily distinguished and recorded. In the case of licking and biting behaviours, it is clear that the animal is addressing the injured paw and hence processing in higher centres must be involved in these active behaviours. Pain-related behaviours were found to be sensitive to morphine and also stimulation of the PAG (Dubuisson and Dennis, 1977). The formalin-induced behavioural response is highly reproducible and is similar in both rats and mice (Chapter 7) (Hunskaar et al., 1985). Dubuisson and Dennis also described the extent of tissue damage following formalin injection. In addition to significant swelling, which lasts for several weeks, a small blister develops. However, despite this damage, approximately two hours after formalin administration the injected paw is used normally again for locomotion, grooming and eating (Dubuisson and Dennis, 1977). Since animals are usually observed for one hour following formalin injection, and not often assessed after this testing period, the tissue damage caused by formalin is not really a disadvantage. Perhaps most significantly, Dubuisson and Dennis reported that formalin injection in humans produced intense, burning and throbbing pain that had a similar time-course to the behavioural response in rodents (Dubuisson and Dennis, 1977).

The main reason why I find the formalin model particularly useful is the unquestionable correlation between pain-related behaviours in awake animals and activity of individual dorsal horn neurones recorded electrophysiologically in anaesthetized animals, both induced by hindpaw administration of formalin. This correlation makes it easier to understand neuronal changes which bring about an abnormal pain state. Neuronal changes are not often observed or particularly obvious in many chronic pain models and are often inferred on the basis of altered efficacy of drugs following a particular injury. In nerve injury models, interpretation of neuronal changes is further complicated by the loss of some afferent input as a result of the peripheral nerve injury. This is not the case for the formalin test where spinal

neuronal activity is directly driven by formalin and can be observed before, during and after formalin administration. Both behavioural and neuronal responses follow the same biphasic time course and there is clear differentiation between peripheral mechanisms which mainly drive the first phase and central mechanisms which mainly contribute to the second phase. For example, I have observed during electrophysiological recordings that fixation of the receptive field occurs following injection of formalin (taking some minutes so not affecting the first phase firing response). Despite this, firing of dorsal horn neurones during the second phase persists, suggesting that this activity is mainly generated centrally following the initial afferent barrage. This was evident by the fact that pinching of the injected toe (i.e. the receptive field) at the end of the experiment no longer evoked firing of the neurone, though spontaneous firing still occurred indicating that the same cell was still being recorded. Most interestingly, pinching of an adjacent toe, which prior to formalin injection did not excite the neurone being recorded, now evoked firing, indicating expansion of the receptive field, a known feature of spinal central sensitization observed clinically in chronic pain patients (Woolf, 1983; Cook et al., 1987; Dubner et al., 1987). The formalin-induced second phase is a well-established model for spinal central sensitization and this plasticity is known to be dependent on NMDA receptor activation (Haley et al., 1990; Chaplan et al., 1997). Drugs such as ketamine reduce this central sensitization without alteration of responses to acute stimuli (see Chapter 3). In addition, gabapentin, which is used clinically in the treatment of chronic pain, also selectively reduces the formalin-induced second phase response with no effect on the tail-flick reflex, a test of acute pain (Singh et al., 1996; Shimoyama et al., 1997; Yoon and Yaksh, 1999). Conversely, morphine is known to alter acute pain and, accordingly, reduces both phases of the formalin response (Wheeler-Aceto and Cowan, 1991). Thus, clinically relevant drugs are effective in the formalin test and clear distinctions can be made between effects on acute pain and on hyperalgesia. This further increases the clinical relevance of the formalin model.

The question of clinical relevance in the formalin model has been put to me several times. One common argument made against the use of the formalin model is that injection of formalin does not specifically mimic any known cause of a human chronic pain syndrome, as is the case of injuring a nerve to mimic neuropathic pain, for

example. However, as I mentioned above, formalin produces a clear inflammatory response which is localized to the injected paw and the late phase is sensitive to non-steroidal anti-inflammatory drugs (Hunskaar and Hole, 1987). Thus it can be argued that the formalin test is a good model of pain induced by inflammation. In my opinion, one of the best arguments for the use of the formalin model, in terms of its clinical relevance, is not to do with the particular injury which produces the pain, but rather the specific behavioural outcome of that pain, that is spontaneous pain-related behaviours. Spontaneous pain is one of the most common symptoms observed in chronic pain patients (Jensen et al., 2001), and yet, despite this fact, many chronic pain models do not reflect this. The incidence of spontaneous pain in nerve injury models is low or highly variable (Kim and Chung, 1997) and not routinely assessed. Wheeler-Aceto and colleagues compared the incidence of spontaneous pain in various models of peripheral inflammation, including the formalin test. Formalin was confirmed to produce significant spontaneous pain-related behaviour in rats. In contrast, little or no spontaneous pain occurred following hindpaw injection of numerous noxious agents including carrageenan, kaolin, serotonin and mustard oil and so these compounds were found to offer no advantage over formalin in assessing spontaneous, ongoing pain (Wheeler-Aceto et al., 1990). Therefore, I would argue that the formalin model should not be seen plainly as a model of inflammatory pain, but can be thought of as a model of spontaneous pain, which is a clinically relevant symptom of chronic pain states. As we try to move towards a mechanism-based approach for the treatment of chronic pain (Woolf et al., 1998; Woolf and Decosterd, 1999), we need to understand the mechanisms for different types of pains and so we require models which exhibit different pain characteristics. Studying the mechanisms which cause spontaneous pain-related behaviours in the formalin model may provide insights which can then be applied to more disease-specific models and ultimately to patients. For example, it is now known that stimulation of peripheral nociceptors by formalin, which in turn drives spinal central sensitization, is not non-specific but rather occurs through activation of TRPA1 channels on C-fibres (McNamara et al., 2007). This may implicate TRPA1 in spontaneous pain. Interestingly, a mutation in human TRPA1 has recently been detected in a Colombian family who have an abnormal genetic pain syndrome characterized by episodic spontaneous pains (unpublished observations from the lab of Prof John Wood, UCL). If a nerve injury model in a rat produces little or no

spontaneous pain, then should this model be described as being clinically relevant to human neuropathic pain? If we consider clinical relevance to be more important with respect to the symptoms produced by the model rather than the cause, then the answer would be no. If we believe that disease specificity is most important in terms of measuring clinical relevance, then the answer is yes. The formalin model may thus be considered to be more clinically relevant than the nerve injury model in terms of symptoms, since it produces symptoms which are common to patients, but less clinically relevant in terms of the underlying disease. Despite the various aetiologies of neuropathic pain in humans, ranging from diabetes to viral infection, trauma and chemotherapy, these diverse causes have no relation to symptoms, since patients with the same disease can present with very different symptoms. Thus, signs and symptoms are arguably more important than the underlying disease.

A second argument used to dispute the clinical relevance of the formalin model is regarding the relatively short time-course of the response. I agree that pain-related behaviours produced over a period of one hour cannot be considered to be chronic. This argument would conclude that a nerve injury model which produces pain-related behaviours over a number of weeks is more clinically relevant in terms of chronic duration. I do not see this is a problem or as a reason not to use the formalin test as I have never considered it as a model of chronic pain. However, it is clear that the injection of formalin is not a simple acute pain test like the tail-flick reflex, for example. Thus the formalin model sits somewhere in between acute and chronic models of pain and may be referred to as tonic. I would argue that the short time course of the formalin test is an advantage and makes this model a good screen to assess analgesic potential of novel compounds and a good predictor of performance in chronic pain models. Studies are relatively short and results are obtained quickly. We should also note that although pain-related behaviours are assessed routinely for only one hour, hypersensitivity of the injected paw is likely to persist for a prolonged period after, perhaps lasting days and even weeks. Many of the neuronal changes that occur during the initial hour following formalin injection are likely to be sustained, thus contributing to a prolonged hypersensitivity of the injured paw. In Chapter 6, for example, changes in AMPA receptor trafficking were observed at 90 minutes after formalin administration (Fig. 6.9). Formalin-induced upregulation of protein kinase G (PKG) has been shown to occur one hour after formalin injection and persist for up to

96 hours (Schmidtko et al., 2003). Finally, formalin injection can induce mechanical hypersensitivity of the hindpaw which lasts for days and is maximal at 48 hrs (Shi et al., 2005). Despite the short duration of the observed response to formalin injection, it is clear (from the studies presented in this thesis) that the formalin model shares many spinal mechanisms with LTP, as well as chronic pain states, making the formalin test an excellent model for central sensitization. Formalin-induced pain-related behaviours during the second phase, representing central sensitization, are dependent on NMDA receptor activation and downstream signalling via PSD-95. Activation of c-Fos and ERK, common markers of neuronal activity in nociceptive pathways, and activation of PI3K and PKCζ/PKMζ, both of which are known to be important for hippocampal LTP, occur in response to formalin. Together these findings confirm that the formalin test is an excellent model for studying neuronal plasticity in pain pathways, particularly in the spinal cord, resulting in central sensitization.

Although the formalin test is not used as a pain model in humans, injection of another chemical irritant, capsaicin, is widely used to study mechanisms of hyperalgesia in humans as well efficacy of analgesics. Intradermal injection of capsaicin in healthy individuals produces intense burning pain and peripheral inflammation, indicated by a flare, similar to that reported for formalin (Dubuisson and Dennis, 1977) and results in secondary hyperalgesia, caused by central sensitization, in the area of skin outside of the territory of the flare (Simone et al., 1989; LaMotte et al., 1991; Hughes et al., 2002). This model of central sensitization has been validated using both psychophysical and imaging measures (LaMotte et al., 1991; Iadarola et al., 1998; Baron et al., 1999; Zambreanu et al., 2005). Interestingly, both formalin and capsaicin activate specific TRP channels, TRPA1 and TRPV1 respectively, located on nociceptive C-fibres (Caterina et al., 1997; McNamara et al., 2007). Similar to formalin-induced central sensitization in rodents, this capsaicin-induced central sensitization in humans is also NMDA-dependent (Park et al., 1995; Andersen et al., 1996). Thus the injection of chemical irritants can be considered to be clinically relevant since the symptoms produced mimic those seen in chronic pain patients. Similar symptoms, such as secondary hyperalgesia and allodynia, are also produced in humans in a surrogate model of LTP, whereby conditioning electrical stimulation of cutaneous afferents of the forearm produces an NMDA-dependent potentiation of

pain ratings (Klein et al., 2004; Klein et al., 2007). Again, understanding the mechanisms producing these symptoms in both preclinical and human surrogate models of central sensitization should aid drug development for chronic pain. Both the capsaicin test and LTP induced by electrical stimulation in humans are good models for testing efficacy of novel analgesics which have been identified by preclinical models, such as the formalin test. Development of newer and better human surrogate models may further our understanding of mechanisms of central sensitization.

In my opinion, the formalin test is an excellent pre-clinical model for central sensitization. Factors such as the high incidence of spontaneous, ongoing pain and evidence of receptive field expansion, as well as excellent correlation between pain-related behaviours and neuronal activity, make the formalin test a highly useful model for developing and assessing novel analgesics for potential use in chronic pain patients.

8.9 Spinal administration of drugs

In all of the studies presented in this thesis, drugs were administered spinally, either directly onto the surface of the spinal cord during electrophysiological experiments or via intrathecal lumbar puncture in behavioural studies. The reason for this selective spinal administration was to allow for investigation of the importance of intrinsic spinal mechanisms of nociceptive plasticity in the various models used. The spinal cord is a key site for modulation of nociceptive processing (D'Mello and Dickenson, 2008).

It may be argued, however, that while drugs are administered to the lumbar spinal cord, spread via CSF or blood flow may occur to other sites, including supraspinal structures, which may then contribute to drug effects. I believe that this is unlikely or, at most, minimal in the studies presented in this thesis for various reasons. In electrophysiological studies, drugs are administered into a ‘well’ on the surface of the lumbar spinal cord, created by a laminectomy. The drug sits within this ‘well’ for the duration of the entire recording in a static system with no CSF flow and so it is

unlikely to be transported elsewhere via this route. In all studies, drug effects on the responses of neurones were selective and, importantly, were regulated by afferent pathophysiology, indicating a lack of non-specific effects. Low drug doses were effective in the majority of cases. Effects were also seen relatively quickly following drug administration, sometimes within ten minutes, as in the case of wind-up studies, which is unlikely to be enough time to allow for significant spread of the drug to other sites. In behavioural studies, drugs were administered in low volumes and effects were again found to be selective at relatively low doses. Of all the compounds tested, only L-291 (DDAH-1 inhibitor) was administered at seemingly high doses, though these corresponded to the IC_{50} of the drug, and these doses were also found to be selective in both electrophysiological and behavioural studies. In addition, a lack of motor impairment following intrathecal administration of the highest dose of Tat-NR2B9c would suggest a lack of off-target effects. Finally, during control intrathecal injections where lidocaine was administered, paralysis was observed of the hindpaws only. This would suggest that rostral spread of drug further along the surface of the spinal cord was minimal and insignificant, and that the majority of drug action was in the lumbar cord. In agreement, a study testing the safety of lumbar intrathecal injection of a drug (SP-SAP) in dogs found that there was no effect of the drug in the cervical cord, with no generalized neurotoxicity as indicated by a lack of significant neuronal loss, astrocytic activation or behavioural side-effects (Allen et al., 2006). Together, all the evidence suggests that, even if spread of drug to non-spinal sites cannot be entirely ruled out, the effect of this spread is minimal and of little consequence. I am confident that drug effects in my studies were mediated via spinal sites of action.

A further point of interest in these studies is the fact that drug doses used in electrophysiological experiments were often lower than those used in behavioural studies. This reflects the ability to apply the drug locally to a restricted cord zone in a static system during electrophysiological recordings as compared to intrathecal injections in behavioural studies where CSF flow and dilution, and the smaller volume make higher doses necessary. Nevertheless, in all studies, drug effects were found to be selective in both electrophysiological and behavioural studies.

8.10 Ideas for future work

Here I will outline some ideas for future experiments which may further the work presented in this thesis. These experiments were not conducted during my PhD mainly due to time constraints.

In my studies I have mainly focused on the activation of downstream intracellular signalling as being a major consequence of binding of NR2B subunits by PSD-95. However, it is also known that PSD-95 may contribute to trafficking and membrane stability of NMDA receptors (Roche et al., 2001). Therefore, it would be interesting to see if Tat-NR2B9c alters membrane localization of NMDA receptors on dorsal horn neurones, as this would be another potential mechanism of action for altering spinal nociceptive plasticity.

I believe I have identified two potential novel analgesic applications for disrupting interactions between NR2B-containing NMDA receptors and PSD-95, through the use of Tat-NR2B9c. The first is in the treatment of pain in osteoarthritis. Ramage and colleagues showed that in human chondrocytes, obtained from osteoarthritis patients, expression of NR2B-subtype receptors is upregulated. In normal chondrocytes this subtype is absent. This upregulation of NR2B-subtype receptors produced altered Ca^{2+} influx and membrane depolarization of chondrocytes, which were both blocked by NMDA antagonists, removal of extracellular Ca^{2+}, inhibition of nNOS and also by Tat-NR2B9c (Ramage et al., 2008). I propose that this altered NMDA receptor activity in chondrocytes drives the abnormal pain state in osteoarthritis patients. Thus, intra-articular administration of Tat-NR2B9c into the knee joint may not only normalize excitability of chondrocytes, but may also reduce nociceptive drive to the spinal cord, producing pain relief. In addition, peripheral NMDA receptors have been suggested to have a role in chronic pain states (Carlton, 2009). Drugs which target NMDA receptors and are restricted peripherally would avoid CNS-mediated intolerable side-effects. Second, I propose that Tat-NR2B9c may have analgesic potential in HIV-induced neuropathic pain. It is not entirely clear how HIV affects neurones, though one proposed mechanism is through the coat glycoprotein gp120, which has neurotoxic effects (Wallace et al., 2007). An alternative mechanism for neurotoxicity is through the full-length HIV transduction

molecule Tat, which can potentiate glutamate-induced excitotoxicity and promote neuronal apoptosis (Eugenin et al., 2007). Eugenin and colleagues demonstrated that the Tat receptor, the lipoprotein receptor-related protein (LRP), forms a macromolecular complex, via PSD-95, with the ternary NMDAR-PSD-95-nNOS complex, triggered by Tat (Eugenin et al., 2007). Thus, it may be that Tat also triggers neuronal changes in pain pathways, for example in the spinal cord, that involve this complex. Disruption of this complex with Tat-NR2B9c and/or other interfering peptides may inhibit or reverse HIV-induced neuropathic pain.

Signalling pathways, in addition to PKCζ/PKMζ, activated downstream of NR2B-containing NMDA receptors via PSD-95 can be identified. It would be of interest to investigate the role of PDK-1 in spinal nociceptive plasticity, since this kinase is involved in both PI3K and PKCζ/PKMζ signalling (Hirai and Chida, 2003) and, as yet, has not been studied in pain.

Spinal LTP is another model of nociceptive plasticity requiring activity of NMDA receptors (Liu and Sandkuhler, 1995; Svendsen et al., 1998) and can provide further insights into chronic pain mechanisms. I would like to examine the effects of Tat-NR2B9c and inhibition of PI3K, PKCζ/PKMζ and DDAH-1 in this model. In addition, the effects of inhibition of PI3K, PKCζ/PKMζ and DDAH-1 should also be investigated in chronic pain models, such as the SNL model.

Although I have demonstrated a role for DDAH-1 in spinal pain processing and plasticity, it remains unclear which types of cells in the spinal cord and DRG express DDAH-1. Thus, immunohistochemical approaches should be employed to identify DDAH-1-expressing cells. It would be expected that DDAH-1 would co-localize with nNOS. Surprisingly, I did not observe an altered phenotype of DDAH-$1^{+/-}$ mice in the formalin test. Conditional knockout mice are currently being generated and these may have altered sensitivity in the formalin test and other pain models.

As discussed above, I utilized spinal administration of drugs to specifically target spinal cord mechanisms of pain processing. It would be of interest to look at other areas of the pain pathway where these mechanisms may also act. For example, brain administration can be achieved through intracerebroventricular injections or

through microinjection into specific brain structures. Additionally, intraplantar hindpaw administration can be used to assess peripheral contributions of relevant targets. Finally, systemic administration of all compounds should be investigated since this is the most likely route of administration to be tested clinically in patients. Systemic administration would also allow for greater assessment of side-effects. Studies have demonstrated that peptides, including Tat-NR2B9c, can cross the blood-brain barrier, such that systemic administration results in spinally-mediated effects (Aarts et al., 2002; Liu et al., 2008; Tao et al., 2008). Although systemic administration of Tat-NR2B9c may raise the potential for brain-mediated side-effects, clinical testing of Tat-NR2B9c in stroke patients has not revealed any adverse psychotropic or neurological effects (verbal communication from Prof Mike Salter, Univ. of Toronto).

8.11 Closing Remarks

NMDA receptors are undeniably implicated in the pathophysiology of many neurological and psychiatric diseases. Therefore, the NMDA receptor remains an important therapeutic target in such diseases, including chronic pain. However, efforts to modulate NMDA receptor activity with non-selective antagonists have failed in the clinic due to the occurrence of intolerable side-effects and this has perhaps led to the NMDA receptor being ignored or disregarded as a viable drug target. These side-effects inform us that we rely on NMDA receptor activity in various neuronal systems for everyday functions, such as memory formation or learning of new movements. Clear distinctions do exist, however, between NMDA receptor-mediated physiological and pathological activity. These distinctions may be made by the varying arrangement of NMDA receptor subunits or by coupling to different intracellular signalling pathways. By identifying which receptor subtypes, such as NR2B-containing NMDA receptors, or which signalling cascades are important in each system, we can design interventions which specifically target one system and leave others intact. Over one hundred different proteins have been found to exist within the NMDA receptor complex (Husi et al., 2000) and each one may thus be a potential candidate for regulation in NMDA-dependent pathophysiology, including pain hypersensitivity. Additionally, we can try different routes of drug delivery, such

as spinal administration, to make use of spatial boundaries which exist between different NMDA-dependent neuronal circuits, therefore allowing for different systems to be targeted individually.

Thus, in my opinion, successful targeting of pathological NMDA receptor activity is possible, not through the use of non-selective NMDA receptor antagonists, which will inhibit NMDA receptor function in all systems and consequently produce side-effects, but rather through more selective approaches which avoid receptor blockade. As I have shown in this thesis, alternative modulation of NMDA receptor-mediated pathological activity may be achieved by disrupting interactions with intracellular signalling proteins, such as PSD-95, which orchestrate pathological signalling cascades downstream of NMDA receptor activation, or through the direct inhibition of such signalling cascades. I have proved these strategies to be successful in reducing spinal nociceptive plasticity and therefore the potential is there for them to be applied to chronic pain patients. It is my hope that the studies presented here will further our understanding of pain processing in the spinal cord, particularly under pathological conditions, and will lead to the development of new and more selective treatments for chronic pain.

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