The Contribution of AMPA Receptors to Neuropathic Pain

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DECLARATION

I hereby declare that the composition of this thesis and the work presented are entirely my own. Some of the studies presented have been published as a poster communication. Reprints and abstracts of all published work are included in the appendix.

Dr Mark P Rockett

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ABSTRACT

Neuropathic pain is defined as pain arising as a result of damage to or dysfunction of the nervous system and is often resistant to opiate analgesics, making it difficult to treat using conventional therapies. Underlying the development of this chronic pathological pain state is a process of neuronal plasticity termed central sensitisation, involving changes from the spinal to the cortical levels of the central nervous system. Central sensitisation may result in thermal hyperalgesia and mechanical and cold allodynia which are commonly found in this condition. Neurons in the dorsal horn undergo plastic changes during central sensitisation, markedly altering their response profile to sensory stimulation. Glutamate is the major excitatory neurotransmitter at synapses between primary afferents and spinal dorsal horn neurons and plays a vital role in the generation of neuronal plasticity. Several key receptors are involved in altering the sensitivity of these neurons, including ionotropic and metabotropic glutamate receptors and peptidergic receptors.

This study was designed to investigate the role of AMPA, NMDA, mGlu group I and $VPAC_2$ receptors (Rs) in the generation of neuropathic pain. Firstly, the behavioural reflex responses of naïve rats to intrathecal administration of low doses of these receptor agonists, singly or in combinations were investigated. Combinations of two or more agonists caused increased behavioural responses to thermal and mechanical stimuli. The combination of AMPA with mGluR group I and $VPAC_2$ R agonists, showed a synergistic effect whereas other combinations were less than additive.

Expression of AMPA R GluR1 and GluR2 subunits in the superficial dorsal horn was studied using confocal immunofluorescence. In the chronic constriction injury (CCI) model of neuropathic pain both the number of GluR1-immunopositive cell bodies and the level of GluR1 expression within cells decreased significantly in lamina II of the dorsal horn, ipsilateral to the injury. In contrast, there was no change in GluR2 expression or the degree of colocalisation of GluR1 and GluR2 subtypes within individual cells following CCI.

The subcellular distribution of GluR1 subunits was also noted to change significantly as a result of CCI. GluR1 subunits were found to redistribute distally into neuronal processes in lamina II cells ipsilateral to CCI and also in response to acute

intrathecal AMPA treatment. This redistribution may reflect an increase in the number of GluR1-containing receptors associated with synaptic sites.

The relationship between the presynaptic marker protein, Bassoon and GluR1-immunopositive cells was investigated, showing a significant increase in the number of Bassoon immunopositive puncta associated with each GluR1-immunopositive cell ipsilateral to nerve injury. The subcellular redistribution of GluR1 subunits caused by CCI was reversed upon behavioural recovery and also by intrathecal administration of a combination of antagonists to NMDA R, mGluR1/5 and $VPAC_2$ R, whereas an AMPA R antagonist alone had no effect.

These results suggest that AMPA Rs are important in the central sensitisation underlying neuropathic pain. However, it is clear that several receptors are involved in triggering maximal behavioural responses, and potential therapeutic interventions may be more effective if designed to target more than one receptor type. There is also evidence to suggest that AMPA Rs may have differing roles dependant upon their subtype composition, so subtype-specific antagonists may potentially be useful in treatment of neuropathic pain.

ABBREVIATIONS

ABP	AMPA R binding protein
ACh	acetylcholine
ACPD	trans-1-aminocyclopentane-1,3-dicarboxylic acid
AMH	mechano-/heat-sensitive A fibre units
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
ANOVA	analysis of variance
ASIC	acid sensing ion channel
ATF3	activating transcription factor 3
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
Ca^{2+}	calcium ion
CaMK II	calcium / calmodulin dependent protein kinase II
cAMP	cyclic adenosine 3',5'-monophosphate
CCI	chronic constriction injury
CCK	cholecystokinin
CGRP	calcitonin gene-related peptide
CH	heat-sensitive C fibre units
CMH	mechano-/heat-sensitive C fibre units
CMiHi	heat- and mechano-insensitive C fibre units
CMR1	cold and menthol sensitive receptor
CNS	central nervous system
CREB	cAMP-response-element-binding protein
CTb	cholera toxin B subunit
CTZ	cyclothiazide
CVLM	caudal ventrolateral medulla
DH	dorsal horn
dH_2O	distilled water
DHPG	3,5-dihydroxyphenylglycine
DOR	δ opioid receptor
DRG	dorsal root ganglion
EPSP	excitatory post-synaptic potential
ERK	extracellular signal-regulated kinase
EPSC	excitatory post-synaptic current
G protein	guanyl regulatory protein
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
GAD65 / 67	glutamic acid decarboxylase 65, 67
GluR1-4	ionotropic glutamate receptor type 1 to 4
GRIP	glutamate receptor interacting protein
IB_4	α-D-galactosyl-binding lectin
ICC	immunocytochemistry
IFN-γ	interferon γ
ISHH	in situ hybridisation histochemistry
IT	intrathecal
JNK	c-Jun N-terminal kinase
JsTx	Joro spider toxin

KOR	κ opioid receptor
LAP-3	L-1-amino-3-phosphonopropanoic acid
LC	locus coeruleus
LTP	long term potentiation
MAPK	mitogen-activated protein kinase
MDEG	mammalian degenerin ion channel
Mg^{2+}	magnesium ion
mGluR1-8	metabotropic glutamate receptor type 1 to 6
MOR	μ opioid receptor
MPEP	2-methyl-6-(phenylethynyl)-pyridine
mRNA	messenger ribonucleic acid
Narp	neuronal activity-regulated pentraxin
NBQX	1,2,3,4-Tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide
NGF	nerve growth factor
NK A / B	neurokinin A / B
NK_1 R	neurokinin 1 receptor
NMDA	N-methyl-D-aspartate
NPY	neuropeptide Y
NRM	nucleus raphe magnus
NSF	N-ethylmaleimide-sensitive fusion protein
NT-3/4/5	neurotrophin 3, 4, 5
NTD	N-terminal domain
PACAP	pituitary adenylate cyclase-activating peptide
PAG	periaqueductal grey region
PB	phosphate buffer
PBS	phosphate buffered saline
PGE2	prostaglandin E2
PICK1	protein interacting with C kinase 1
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PPT I/II	preprotachykinin I and II
PSD-95	postsynaptic density protein-95
PSDC	post-synaptic dorsal column
R	receptor
RAIC	rostral agranular insular cortex
Ro 25-1553	[Ac-His^1, Glu^8, Lys^{12}, Nle^{17}, Ala^{19}]VIP (1-24), Asp, Leu, Lys, LysGly, Gly, Thr NH_2 (lactam 21-25)
RVM	rostral ventromedial medulla
SD	standard deviation
SEM	standard error of the mean
SCT	spinocervical tract
SNB	spontaneous nociceptive behaviour
SNI	spared nerve injury
SNL	spinal nerve ligation
SP	substance P
SPET	suspended paw elevation time
STT	spinothalamic tract
t-ADA	trans-azetidine-2,4-dicarboxylic acid

TARP	transmembrane AMPA R regulatory protein
trk A	tropomyosin-related kinase A
TRP	transient receptor potential ion channel
VIP	vasoactive intestinal polypeptide
VR-1	vanilloid receptor 1
WDR	wide dynamic range

CHAPTER 1: INTRODUCTION TO PAIN PROCESSING

Pain is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or expressed in terms of such damage" (Merskey and Bogduk, 1994). Nociception is the term used for a purely sensory event, devoid of the emotional content of pain perception, consisting of the transduction of a noxious stimulus in the periphery and its transmission to the cerebral cortex via the spinal cord.

Physiological pain is an adaptive process, allowing for recovery and healing of an injury by causing immobility of the damaged part and avoidance of further painful stimuli. The sensation of pain ceases once the inflammatory process abates or the noxious stimulus is removed. However, if pain continues once the healing process is complete, or is out of proportion to the degree of tissue injury, this is considered to be a pathological pain state. Nociceptive processing is not a 'hard wired' system; signal modulation and neuronal plasticity may occur at any stage of the pain pathway. Noxious stimuli from injured tissue may lead to long-term changes in nociception and pain perception. In human subjects, these changes result in altered sensitivity to cutaneous stimuli. These plastic changes are termed hyperalgesia and allodynia (Treede et al., 1992), (Willis, 1992).

Hyperalgesia manifests as an increased response to painful stimuli and is described as primary or secondary. Primary hyperalgesia refers to altered nociceptive responses from the area of tissue damage. This is characterised by both thermal and mechanical hyperalgesia. Secondary hyperalgesia refers to the decreased pain threshold experienced in unharmed areas adjacent to damaged tissue. This phenomenon may be triggered experimentally by a number of noxious stimuli including thermal skin injury, mechanical trauma, UV light, freezing or local electrical stimulation. Different modalities of noxious input, such as heat or mechanical stimuli, may be sensitised independently.

Allodynia is the perception of normally innocuous stimuli, such as brushing or fine touch, as noxious. These unpleasant, excessive sensory responses to injury may persist for prolonged periods after tissue healing has occurred, or the noxious stimulus has been removed. Underlying these sensory changes are processes termed peripheral and central sensitisation (Woolf, 1983). Peripheral sensitisation results from release of

inflammatory mediators at the site of injury and causes a reduced firing threshold in peripheral nociceptors. Central sensitisation is an expression of increased excitability of multireceptive or wide dynamic range (WDR) neurons (Mendell, 1966) in the spinal cord (Baranauskas and Nistri, 1998), (Woolf, 1996). Secondary neurons in the dorsal horn show a reduction in threshold so that there is a response to previously sub threshold stimuli (Simone et al., 1989). Spatial and temporal summation of afferent action potentials results in the perception of pain. An increase in receptive field size promotes spatial summation, as demonstrated in vivo in the rat hindpaw (Cook et al., 1987). In human subjects, low threshold Aβ fibre inputs (such as stroking the skin with a brush) take on the characteristics of noxious stimuli and are perceived as painful.

Pathological pain states may be divided into those caused by inflammatory mechanisms and those caused by damage to the peripheral- (PNS) or central nervous systems (CNS). The latter neuropathic form of chronic pain is the subject of the current study. There is good evidence to demonstrate the occurrence of central sensitisation after nerve injury(Bennett and Xie, 1988), (Liu et al., 2000).

1.1 Neuronal plasticity, long term potentiation (LTP) and wind-up

During the development of the prolonged sensitised states associated with neuropathic pain, neurons may display plasticity, altering their function, chemical profile or structure (Woolf and Salter, 2000). In addition to changes within neurons, synaptic connections between them also demonstrate plastic changes (Julius and Basbaum, 2001).

Long-term changes in neuronal activity have been studied for some years in the hippocampal region of the brain, where they are thought to mediate learning and memory. In the early 1970s, it was noted that brief trains of high-frequency electrical stimulation (50-100 stimuli at 100Hz or more) to monosynaptic excitatory pathways in the hippocampus caused a rapid increase in synaptic strength lasting hours or days, which was termed LTP (Bliss and Lomo, 1973).

In addition to induction of plasticity by high frequency stimuli in the hippocampus, low frequency stimulation (0.3-20Hz) electrical C fibre stimulation, or cutaneous stimulation of noxious intensity, resulted in an increased number of action potential spikes per stimulus in a subset of dorsal horn interneurons. This effect was

first noted and termed wind-up by Mendell and Wall in 1965 (Mendell and Wall, 1965). Wind-up is an artificially generated process occurring only whilst the train of low frequency stimulation continues. This phenomenon has been investigated using a number of techniques, including recording from extracellular and intracellular electrodes, whole cell patch techniques, and by recording the action potential response from ventral roots (Herrero et al., 2000). Although wind-up is not a physiological process, and LTP occurs only under conditions of very high frequency electrical stimulation, it has been suggested that these processes share some similarities with mechanisms underlying the development of central sensitisation in the spinal cord, leading to hyperalgesia and allodynia (Baranauskas and Nistri, 1998), (Ji et al., 2003).

1.2 Peripheral transduction of sensory information

Sensory information from the external environment is received by the skin and transduced into electro-chemical activity (action potentials) in primary afferent nerve fibres by a wide variety of cutaneous receptors. Subgroups of receptors are activated by specific types of stimulus including heat, cold, fine touch, pressure, vibration, stretch and tissue pH. Primary afferent fibres enter the central nervous system and terminate at the first synapse in the dorsal horn (DH) of the spinal cord forming a highly organised somatotopic map of peripheral structures (Swett and Woolf, 1985).

1.2.1 Peripheral nerve fibres

Cutaneous afferent fibres are subdivided into three main groups depending upon their size and conduction velocity. The largest, most rapidly conducting fibres measure greater than 10μm in diameter, conduct impulses at 30-100ms^{-1} and are termed Aβ fibres. These myelinated fibres are thought to transmit fine touch and proprioceptive information. Smaller myelinated fibres measuring 2-6μm in diameter are termed Aδ. These fibres have conduction velocities varying between 12 and 30ms^{-1} and transmit innocuous and noxious sensory information. The nociceptive information carried by Aδ fibres results in a sensation which has been termed 'first pain' as it usually occurs rapidly after stimulation and is sharp in character. The smallest fibres, termed C fibres, are unmyelinated and measure 2-6μm in diameter, with conduction velocities in the range of 0.5-2.0ms^{-1}. These slow transmission fibres largely carry nociceptive information leading to a sensation, which has been termed 'second pain', as it has a relatively slow onset and burning character (Yaksh, 1986).

Histochemistry of dorsal root ganglion (DRG) cells has revealed two main types of C fibre neurons. One group comprising 40 - 45% of all DRG neurons in the adult mouse express tropomyosin-related kinase A (trk A) receptors for nerve growth factor (NGF). The majority of these cells also coexpress calcitonin gene-related peptide (CGRP) and substance P (SP) (Snider and McMahon, 1998). The other group express α-D-galactosyl-binding lectin (IB_4) and the $P2X_3$ ATP-gated ion channel (references in (Julius and Basbaum, 2001)).

1.2.2 Cutaneous receptors

There are a number of different peripheral receptors involved in the transduction of cutaneous stimuli. In general, receptors triggered by low threshold innocuous stimuli are associated with specific sensory modalities, whereas a stimulus of sufficient strength may trigger a response in a nociceptor no matter what its modality.

Non-nociceptive cutaneous receptors respond specifically to innocuous mechanical, thermal and chemical stimuli. Cutaneous mechanoreceptors are usually associated with myelinated primary afferent fibres. Rapidly adapting receptors are known as Meissner or Pacinian corpuscles and respond to light touch, vibration and pressure. Slow adapting receptors are of two types, Merkel cells and Ruffini corpuscles, which respond to stretch (Willis and Coggeshall, 1991). Hairy skin also has specific receptors associated with hair follicles and responsive to hair bending and movement (Brown and Iggo, 1967), (Lynn and Carpenter, 1982). Skin thermo- and mechanoreceptors have also been identified which are associated with C fibres and respond to cooling and innocuous mechanical stimulation (Bessou et al., 1971).

Cold and warm stimuli activate different receptors depending on the intensity of the stimulus. Innocuous cool sensation is mediated by receptors associated with a population of C and Aδ fibres responding to stimuli in the 15-30°C range (Bessou and Perl, 1969). Mild warmth (30-37°C) would seem to trigger a subset of receptors associated with C fibres (Iggo, 1959).

1.2.3 Nociceptors

Nociceptors respond to stimuli of sufficient intensity to potentially result in tissue damage. The concept of such a receptor is not new, and was first suggested

almost 100 years ago (Sherrington, 1906). These high threshold receptors are less well differentiated in terms of structure and specificity of response than their non-nociceptive counterparts. Peripheral nociceptors consist of unencapsulated nerve endings attached to unmyelinated C fibres or small diameter myelinated Aδ fibres. Cell bodies of these nociceptive afferents lie within the dorsal root ganglia (DRG). Studies based on anaesthetised monkeys, using heat and mechanical noxious stimuli, revealed two basic types of nociceptive afferents: C fibres responsive to heat and mechanical stimuli and Aδ fibres responsive to heat and mechanical stimuli (mechano- / heat-sensitive C fibre units, CMH, mechano- / heat-sensitive A fibre units, AMH) (references in Chapter 3 (Yaksh, 1986)). These afferent fibres show a monotonic response to increasing stimuli of noxious intensity. C fibre polymodal nociceptors have been shown to respond to various high threshold stimuli in a non-specific manner including heat, high threshold mechanical and chemical stimulation (Bessou and Perl, 1969) and in some cases intense cold (Cervero and Iggo, 1980), (Willis and Coggeshall, 1991), (Henley, 1993), (Simone and Kajander, 1996), (Shi, 2001). In addition to the populations of nociceptors described, there are also 'silent' nociceptors which do not respond to any form of stimulus until sensitised by tissue injury (Bessou and Perl, 1969), (Schmidt et al., 1995).

Recordings of action potentials from unmyelinated C fibres have been made from the peroneal nerve of healthy human subjects using microneurography (Weidner et al., 1999). This study found a number of C fibre types, including sympathetic efferents, CMH units, mechano-insensitive C units most of which responded to heat stimuli (heat-responsive C units, CH), and a few that responded to capsaicin alone (mechano- and heat-insensitive C units, CMiHi). Mechano-insensitive units were found to have significantly slower conduction velocity.

Noxious heat transduction

It has been demonstrated that 45% of small and medium nociceptor fibres grown in culture from rat dorsal root ganglia (C and type II Aδ fibres) responded to moderate heat stimuli (45°C), whereas only 5-10% responded to intense heat (52°C) (type I Aδ fibres) (Nagy and Rang, 1999). It is now clear that C and type II Aδ neurons express the vanilloid receptor (VR-1), which transduces heat sensation (Caterina et al., 1997). The vanilloid receptors VR-1 and VRL-1 are members of the

transient receptor potential (TRP) family of ion channels, consisting of six trans-membrane domains with a hydrophobic loop between the fifth and sixth domains. VR-1 is ten times more permeable to calcium than monovalent cations and is activated by capsaicin or its analogue resiniferatoxin and blocked by the antagonists capsazepine and ruthenium red. This receptor responds to noxious heat and its responses are modulated by hydrogen ion concentration (references in (McCleskey and Gold, 1999)), (Caterina et al., 1999). Mutant mice lacking the VR-1 receptor are largely insensitive to moderate heat stimuli but respond normally to intense heat stimuli, which may be transduced by the VRL-1 receptor.

Cold transduction

The molecular mechanisms that transduce cold stimuli have been less clearly elucidated and it has proved difficult to define what degree of cold may be considered noxious. It has been demonstrated that cooling DRG cells in culture to 20°C permits calcium entry into the cells (Reid and Flonta, 2001). However, it was not clear whether cold was having a non-specific effect on many receptors, or acting via a single 'cold-receptor'. Insights into the mechanism of cold transduction originate from the use of pharmacological agents, such as menthol, that elicit a cooling sensation. Using this cooling substance, a menthol receptor has been cloned from trigeminal sensory neurons that is also activated by thermal stimuli in the cool to cold range. This cold- and menthol-sensitive receptor (CMR1) is a member of the TRP family of excitatory ion channels and responds to cold in the region of 8°C to 28°C (McKemy et al., 2002). A further related channel, TRPA1 has also been identified that responds to lower temperatures (<18°C) (Story et al., 2003). The CMR1 receptor was found expressed on small diameter neurons in the trigeminal and dorsal root ganglia. So it would appear that TRP channels detect temperatures over a wide range, and are the principal sensors of thermal stimuli in the mammalian peripheral nervous system.

Noxious chemical transduction

In addition to physical stimuli such as heat, cold and pressure, the local tissue environment may also trigger nociceptive responses. After tissue damage, many chemical products are released. This inflammatory soup includes extracellular protons and adenosine triphosphate (ATP), arachidonic acid and its metabolites, serotonin, bradykinin and nerve growth factor (NGF). Nociceptive neurons express receptors

paired with most of these mediators such as the trk A receptor for NGF, the bradykinin BK_2 receptor, the $5HT_3$ receptor for serotonin and the $P2X_3$ receptor for ATP (McCleskey and Gold, 1999). In addition to this dramatic change in the local environment, nociceptors themselves may release SP when activated by noxious stimuli or via antidromic activation of the peripheral nerve, which may also modulate the release of inflammatory mediators from surrounding tissues (Moskowitz et al., 1983), (Levine et al., 1993).

It has been suggested that extracellular ATP may play a role in nociception in the periphery. ATP would appear in the extracellular space only after cell lysis in injured tissue. Interest in ATP as a mediator of acute nociception developed when it was found that injection of ATP into inflamed skin causes pain in humans (Bleehen and Keele, 1977). Several subtypes of ATP receptor have now been cloned and some appear to be localised on nociceptive afferents. These nucleotide receptors can be divided into ionotropic forms ($P2X_{1-7}$) and metabotropic forms (P2Y) which are linked to a number of signalling pathways including phospholipase C (PLC) activation (Krishtal et al., 1988), (North and Barnard, 1997).

Local tissue acidity plays a key role in peripheral nociception, (McCleskey and Gold, 1999), (Mamet and Voilley, 2002). Ischaemia, infection and inflammation all lead to a decrease in local tissue pH, which may be as low as 5.4. Steen et al have demonstrated that a subset of C fibres sensitive to mechanical and heat stimuli respond to pH by firing in a dose-dependant manner to increasing hydrogen ion concentration. Threshold levels were found to range from pH 6.9 to 6.1 and mean maximum discharge was at pH 5.2 (Steen et al., 1992).

Hydrogen ions may modulate the response of some receptors (VR-1) and may directly activate others. The acid-sensing ion channel (ASIC) family of cation channels have been shown to respond to pH (Krishtal and Pidoplichko, 1981). These receptors have various roles in different species and tissues, but all are specifically permeable to sodium and may be blocked by the loop diuretic drug, amiloride. It is not entirely clear which receptors are involved in the response to acidity, but the ASIC1 channel is closed at a physiological pH of 7.4, shows a transient response if the local pH drops below 7.0 and a prolonged response below pH 6.0 (Bevan and Yeats, 1991), (McCleskey and Gold, 1999).

Noxious mechanical transduction

No transducer protein for mechanical stimuli has yet been isolated, but some studies suggest it may be a member of the mammalian degenerin (MDEG) ion channel family (Waldman and Lazdunski, 1998), (Chelur et al., 2002). These receptors are a subset of the ASIC family. Nematode studies have identified several degenerins, including MEC-4 and MEC-10. These receptors are expressed on mechanoreceptor neurons of the nematode and are both required for touch sensation. MEC-6 has been more recently identified and colocalises with MEC-4, augmenting amiloride-sensitive sodium currents produced by MEC-4/-10 by a factor of 200 (Chelur et al., 2002). It has been suggested that the MEC-4/-6/-10 complex constitutes a stretch-activated channel. The mammalian equivalent may play a role in mechanical nociception.

1.3 Central transmission of sensory information

The first synapse in the nociceptive pathway occurs in the dorsal horn of the spinal cord. This region acts as a gateway to the CNS for nociceptive information and has been the subject of the present study. Second order neurons vary in their input specificity and response to stimuli. Their response profile may be altered dramatically by the development of central sensitisation, and they are influenced by complex intrinsic and descending modulatory mechanisms, which will be discussed below. The dorsal horn neurons may project to the brainstem via a number of distinct ascending tracts described below (Treede et al., 1992).

Pain information processing also occurs at a cortical level, and the advent of functional MRI scanning has increased our knowledge of these higher brain functions in human subjects. In healthy human volunteers, pain intensity- related haemodynamic changes have been identified in a widespread, bilateral brain system including the primary (SI) and secondary (SII) somatosensory cortices, insular, cingulate, and frontal cortical areas, as well as thalamus, amygdala, and midbrain (Porro, 2003). Data from anatomical and electrophysiological studies show that these cortical regions receive direct nociceptive thalamic input. The rostral agranular insular cortex (RAIC) has recently been identified as a site where neurochemical changes, can alter nociceptive thresholds in awake animals. The connectivity of the RAIC suggests it is involved in multiple aspects of pain behaviour including motivational /

affective components and sensorimotor integration of nociceptive processing (Jasmin et al., 2004).

1.3.1 Anatomy of the spinal cord dorsal horn

The spinal cord consists of a 'butterfly-shaped' region of grey matter arranged around a central canal, surrounded by white matter. Rexed suggested an anatomical division of the spinal cord grey matter into nine laminae based on their cytoarchitechtonic characteristics, comprising the dorsal and ventral horns, and a tenth lamina surrounding the central canal (Rexed, 1952). All afferent sensory information from the skin surface passes into the dorsal horn, which comprises Rexed's laminae I to V. This laminar arrangement of the dorsal horn remains remarkably consistent between mammalian species (Molander et al., 1984).

Peripheral receptors of differing sensitivities tend to terminate in specific regions, with low-threshold mechanoreceptors terminating in laminae III and IV and high- threshold nociceptors in laminae I, II and V. Unmyelinated nociceptor C fibres terminate predominantly in lamina II (Willis and Coggeshall, 1991). Fine calibre myelinated Aδ fibres from the skin may terminate within superficial laminae, or deeper in lamina V, whereas afferents from visceral organs or muscle project to mainly lamina V. Large myelinated Aβ fibres terminate deeper in laminae III and IV of the dorsal horn (Willis and Coggeshall, 1991). It was suggested that this highly specific division of afferent inputs into different regions of the dorsal horn reflected the relationship between a low-intensity stimulus and an innocuous sensation and a noxious stimulus and pain.

In addition to this laminar arrangement, the central terminals of primary afferent neurons also form an ordered topographic map of the skin surface in the dorsal horn of the spinal cord (Swett and Woolf, 1985), (Doubell and Mannion, 1999).

1.3.2 Cytoarchitecture

The three superficial laminae of the dorsal horn contain many small cells and a few larger ones in laminae I and III. Whilst neurons in laminae I and III may project to the brain, most lamina II neurons arborize locally (Willis and Coggeshall, 1991). Unlike neurons in several other areas of the CNS, it would appear that there is little

relationship between cell morphology and function in the dorsal horn (references in (Todd and Spike, 1993)).

Lamina I is the thin outermost zone of the dorsal horn containing relatively few small (5x5μm) and large (15x15μm) cells. Many of these cells are projection neurons joining the spinothalamic tract (STT) and spinomesencephalic tract (SMT), (Menterey et al., 1982), (Todd et al., 2000), (Todd, 2002). There are four main morphological cell types in this lamina, although many are atypical in appearance. The dendrites of fusiform, flattened and pyramidal cells remain within lamina I, whereas those of multipolar cells may enter lamina II or III (Lima and Coimbra, 1986).

Lamina II is known as the 'substantia gelatinosa' due to its appearance under a light microscope. This layer lies ventral to lamina I and consists of an outer zone (lamina II_o) containing densely packed small cells (5x5μm) and a less compact inner zone (lamina II_i). Neurons within this lamina conform to two structural types, islet cells and stalked cells. Islet cells have dendrites that spread rostrocaudally within lamina II with axons that remain close to the cell body. Stalked cells are found in outer lamina II and have dendrites which fan out ventrally and axons which pass dorsally into lamina I (Todd and Lewis, 1986), (Todd, 1988). Very few (~1%) lamina II neurons project to the brainstem, whilst some project into lamina I or have extensive local connections (Willis et al., 1979).

Lamina III contains larger and less tightly packed cells. There are two structural types: those which resemble lamina II islet cells, and others resembling inverted stalked cells with dorsally directed dendrites (Powell and Todd, 1992). Primary afferents of large hair follicles, Pacinian corpuscles and rapidly adapting mechanoreceptors terminate in lamina III. Second order neurons send dendrites from lamina III to laminae I and IV. Lamina III also contains cells of origin of the spinocervical tract (SCT) and the postsynaptic dorsal column (PSDC) tract (Brown et al., 1981).

Lamina IV is a thick layer containing large scattered cells measuring 10x15μm, often with dendrites projecting into lamina I, II and III. This dendritic distribution allows lamina IV cells to receive a direct primary afferent input from fibres that enter the superficial layers as well as indirectly mediated inputs. Significant numbers of larger cells also project to the SCT and PSDC. Lamina V forms the

narrowest part of the dorsal horn and contains the largest cells (15x20μm), many of which join the ascending STT.

1.3.3 Neurotransmitters and peptides within the superficial dorsal horn

The neurotransmitter systems of the dorsal horn have been widely studied, as their pharmacological manipulation may lead to the development of novel analgesic drugs. As the present study involves the superficial laminae of the dorsal horn (I – III), deeper structures will not be discussed. Large numbers of inhibitory and excitatory transmitters and peptides have been isolated within the outer three laminae of the dorsal horn, some of which are considered below.

The inhibitory neurotransmitter γ-aminobutyric acid (GABA) is abundant in the superficial dorsal horn. Early immunohistochemical studies used antibodies directed against glutamic acid decarboxylase (GAD), an essential enzyme in the synthetic pathway of GABA. Samples were also treated with colchicine to inhibit axoplasmic transport of the enzyme and allow labelling of cell bodies. These studies revealed GAD within larger cells in outer and inner lamina II. Utilising antibodies to GABA, it was found that 28% of cells in lamina I contained this neurotransmitter, 31% in lamina II and 46% in lamina III (Todd and Sullivan, 1990). Within lamina II, large islet cells contained GABA whereas small cells did not (Spike and Todd, 1992). GABA also coexists with the calcium-binding protein parvalbumin in 60% of cells and this has been taken as a surrogate marker of GABAergic cells in several studies (Antal et al., 1991). In addition, a proportion of GABAergic cells contain the co transmitter glycine. Of these cells, 33% contained glycine in lamina I, 43% in lamina II and 64% in lamina III (Todd and Sullivan, 1990). There is evidence for the existence of descending GABAergic cells from Lissaur's Tract and the medulla, but the majority are intrinsic to the DH, as spinal cord transection did not decrease the level of GABA within the DH significantly (Rizzoli, 1968). Many GABAergic cells make presynaptic, axo-axonic connections with primary afferents, acting to inhibit sensory inputs (Levy, 1977).

There are also dorsal horn cells containing acetylcholine (ACh), which has an inhibitory action via its muscarinic receptors (Urban et al., 1989). This inhibitory neurotransmitter will not be considered further.

L-glutamate is the major excitatory neurotransmitter within the dorsal horn and is found in the majority of neurons in laminae I and II (Miller et al., 1988). Several studies have substantiated a role for glutamate as a neurotransmitter in primary afferent neurons and in ascending somatosensory pathways (references in (Broman, 1994)). Glutamate and its receptors will be discussed in greater depth below.

Other substances likely to be involved in somatosensory neurotransmission include the neuropeptides and nucleotides such as ATP. Many different peptides have been detected in primary afferent neurons with unmyelinated or thinly myelinated axons, and are thus likely to be directly involved in primary afferent neurotransmission. Some neurons giving rise to ascending somatosensory pathways, primarily those with cell bodies in the dorsal horn, are also immunoreactive for peptides. These peptides are briefly reviewed below.

Tachykinins are a group of related neuropeptides derived from the precursors preprotachykinin I and II (PPT I and PPT II). SP, neurokinin A (NK A), and neuropeptides K and γ are derived from PPT I, whereas neurokinin B (NK B) is derived from PPT II. Immunohistochemical analysis has provided an insight into the distribution of these peptides within the dorsal horn, but antisera tend to cross react. Tachykinin immunopositive fibres have been demonstrated in laminae I – III, generally decreasing in number with increasing depth, many of which would appear to be primary afferents (Rusa et al., 1986). Many cell bodies have also been shown to contain tachykinins and colchicine treatment revealed large cells in lamina I and smaller cells in laminae II and III (RibieroDaSilva et al., 1991). In situ hybridisation histochemistry (ISHH) revealed neurons expressing PPT I in laminae I and II and neurons expressing PPT II in a band within lamina III (Warden and Young, 1988). More recently, application of specific antisera to NK B and SP has revealed that these tachykinins can coexist within individual axons, suggesting that a subset of cells may express both PPT I and II (Too and Maggio, 1991). Tachykinins have also been shown to colocalise with calcitonin gene-related peptide (CGRP), galanin and cholecystokinin (CCK) within primary afferents (Tuchscherer and Seybold, 1989), (Battaglia and Rustioni, 1992). Some STT projection neurons in lamina I also contain tachykinins.

The endogenous opioids are also present within nerve fibres in the superficial dorsal horn, largely within laminae I and II. The cell bodies of these fibres mostly lie within lamina II (Todd and Spike, 1992), (Todd et al., 1992b). There are several types of endogenous opioids derived from two precursor peptides. Endorphin and dynorphin are derived from preprodynorphin (PPD). The enkephalins may be subdivided further into Met- and Leu-enkephalin; the Met- form is derived solely from preproenkephalin (PPE), whereas the Leu- form may derive from PPE or PPD (Standaert et al., 1986).

Dynorphin- and enkephalin- immunoreactive perikarya in lamina I of the dorsal horn represent neurons with extensive local collateral connections, which are the main source of a long ascending projection to the parabrachial nucleus in the rat. Furthermore, lamina I opioid-containing neurons may receive SP-immunoreactive primary sensory afferents. (Standaert et al., 1986). This finding underlies the importance of these cells in sensory processing, as the pontine parabrachial nucleus, which has been implicated in nociceptive as well as antinociceptive processes, expresses high levels of opioid receptors and is reciprocally connected with the spinal cord dorsal horn.

Endogenous opioids are often coexpressed with other peptides in the dorsal horn. Within lamina II, 50% of cells containing enkephalin were also tachykinin immunopositive (this represents 95% of cells containing SP) (Senba et al., 1988). Metenkephalin and somatostatin have also been shown to coexist in cell bodies and nerve terminals in the dorsal horn (Todd and Spike, 1992). Enkephalin and GABA may also coexist in cells in laminae II and III (Todd et al., 1992b).

Dynorphin mRNA has been demonstrated in laminae I and II of the dorsal horn in rat, cat and monkey spinal cord. In rats the immunoreactive fibres would seem to be distributed within inner lamina I and outer and inner lamina II and, after colchicine treatment, the cell body staining matches this pattern (Cho and Basbaum, 1989). In keeping with its likely role in nociceptive processing, the axons of some dynorphin containing cells within lamina I extend to the parabrachial midbrain areas and some to the nucleus of the solitary tract. In addition, levels of this opioid have been shown to increase within the dorsal horn in response to peripheral inflammation (Ruda et al., 1988). Preprodynorphin mRNA and dynorphin peptide are increased after various chronic noxious stimuli such as CCI and peripheral inflammation (Cho and Basbaum, 1988). In these conditions, there is an increase in opioid levels in local lamina I neurons and spinal mesencephalic neurons. It has been suggested this is

significant in bringing about the increase in spinal cord excitability seen in these conditions (Dubner and Ruda, 1992). In addition to enkephalin, dynorphin is also coexpressed with SP in some cells in the dorsal horn or in descending neurons (Tuchscherer and Seybold, 1989).

Neurotensin is a tridecapeptide which acts to excite dorsal horn neurons when applied iontophoretically, but produces antinociception when administered intrathecally (references in (Furst, 1999)). Fibres containing this neuropeptide are found in lamina I and II in two bands. Cell bodies are found in ventral lamina II and dorsal lamina III (references in (Todd and Spike, 1993)). The majority of these axons contain the glutamate transporter molecule VGLUT2 and are probably glutamatergic (Todd et al., 1994), (Todd et al., 2003). Cells containing neurotensin do not seem to contain GABA and 75% contain calbindin (Todd et al., 1992a).

Cells containing somatostatin do not express GABA and would seem to represent a different population from those containing neurotensin. These cells also express VGLUT2 and arborize locally within dorsal lamina II and lamina I (Todd et al., 2003). These small cells have cell bodies within ventral lamina II and lamina III and may represent excitatory interneurons (Todd et al., 1994).

Fibres containing vasoactive intestinal polypeptide (VIP) are found within laminae I and II. Most of these fibres regress with primary afferent sectioning, suggesting that they arise peripherally. After colchicine treatment, some cell bodies containing VIP are found in laminae I and II. Some of the lamina I cells have been shown to have processes extending to the midbrain (Leah et al., 1988).

Finally, while some fibres within laminae I and II have been shown to contain galanin, most degenerate following spinal cord section suggesting that they arise from descending neurons (Melander et al., 1986). It has been demonstrated that these peptide-containing neurons originate at least in part in the locus coeruleus (Holets et al., 1988).

1.3.4 The electrophysiological responses of dorsal horn neurons to stimuli

It is possible to classify dorsal horn cells according to their electrophysiological response to peripheral stimuli. Three major groups exist, depending upon their response to noxious stimulation. Class I neurons do not respond to noxious stimulation and are involved in the transmission of innocuous information via low-threshold A-fibres (Dubner and Bennett, 1983). Class II neurons respond to

both innocuous and noxious cutaneous stimuli and are termed multireceptive or wide dynamic range (WDR) neurons (Mendell, 1966). These cells may respond to stimulation of all types of primary afferent from C fibres to Aβ fibres (reviewed in (Besson and Chaouch, 1987)) and are most commonly found in laminae IV-VI. Neurons of the WDR type may show tonic activity without stimulation and have a graded response across their receptive fields. Some are inhibited by Aβ fibre input at the periphery, whereas others respond positively to all stimuli across their receptive fields (Cervero and Iggo, 1980), (Iggo et al., 1985). Class III, nocispecific neurons respond to input from myelinated and unmyelinated nociceptive afferents only (Cervero et al., 1976). These neurons have relatively small receptive fields and display little spontaneous activity. Nocispecific neurons tend to occur in lamina I and include projection neurons. A fourth group, consisting of proprioceptive neurons will not be considered here.

It has been demonstrated that 74% of lamina I neurons are nociceptive specific. The remaining 26% were shown to be wide dynamic range class II neurons. It was demonstrated that application of an AMPA R antagonist resulted in an inhibition of electrically evoked responses, whereas application of an NMDA antagonist had no effect. This suggests that transmission of nociceptive information is mediated in part by AMPA Rs rather than NMDA Rs (Seagrove et al., 2004).

1.3.5 Projection of dorsal horn neurons

In addition to this functional classification, dorsal horn neurons may also be grouped according to their axon projections. The vast majority of dorsal horn neurons are interneurons, which may be excitatory, or inhibitory. Projection neurons enter ascending spinal tracts and convey sensory information to higher centres. Inhibitory interneurons may synapse with primary afferents or post-synaptically with dorsal horn neurons. This inhibitory influence can be seen on lamina III / IV neurons, which have been shown to receive synapses from local GABAergic interneurons (Todd, 2002) and on a subset of lamina I projection neurons which also receive a dense inhibitory input (Puskar et al., 2001).

Projection neurons from lamina I send axons to a number of brain regions which are known to be involved in nociception, including the caudal ventrolateral medulla (CVLM), the parabrachial area, periaqueductal grey area (PAG) and thalamus (Willis and Coggeshall, 1991), (Craig, 1995), (Gauriau and Bernard, 2004).

These neurons have been shown to be highly excitable and to display unique firing patterns (Ruscheweyh et al., 2004).

Up to 80% of lamina I projection neurons express the SP receptor, NK_1 (Todd et al., 2002). These cells are likely to have a pro-nociceptive function, as their ablation in chronic inflammatory and neuropathic pain states led to a marked reduction in hyperalgesia but no change in innocuous sensory transmission (Mantyh et al., 1997), (Nichols et al., 1999). In addition to cells expressing NK_1, lamina I contains a number of large cells lacking this receptor, which may have an inhibitory function. These cells contain high levels of the glycine receptor-associated protein gephyrin and are found close to GABA containing terminals (Puskar et al., 2001). There is evidence that a population of cells within laminae III and IV expressing the NK_1 receptor (Naim et al., 1997), project to the CVLM and parabrachial area (Todd, 2002), and may also target lamina I projection neurons (Sakamoto et al., 1999).

In addition to this complex local circuitry within the dorsal horn, there are also descending fibre tracts, which have a significant modulatory affect on the dorsal horn output. For example, the descending serotonergic axons from the medullary raphe nuclei have been found to exhibit inhibitory influence over lamina I and lamina III/IV projection neurons (Todd, 2002). It can be seen that signal modulation by local interneurons may affect both the input to and output from the dorsal horn, and these cells have been implicated in models of pain and analgesia.

1.3.6 Ascending tracts

The anatomy of the fibre tracts from the dorsal horn to the brainstem is considered below. These tracts are described with reference to the rat; other mammalian species may show variations in their relative importance.

Spinothalamic tract (STT)

The spinothalamic tract conveys sensory information from the contralateral dorsal horn to thalamic nuclei. Although mostly concerned with transmission of noxious mechanical sensory information, temperature and innocuous mechanical stimuli may also be conveyed (Giesler et al., 1976). Cells of origin of this tract can be found in laminae I, II, IV and V (Todd et al., 2000), (Todd, 2002). These excitatory pro-nociceptive neurons within lamina I also receive synapses from local inhibitory

interneurons, as well as descending input from inhibitory fibre tracts (Polgar et al., 2002).

Spinomesencephalic tract (SMT)

The SMT projects from cells in laminae I, V and VI to the mesencephalic tegmentum region including the periaqueductal grey area. It is likely that the contribution from lamina I cells is nocispecific (Menetrey et al., 1982).

Dorsal column pathways

This ascending tract can be divided into the postsynaptic dorsal column system (PSDC) and the spinocervical tract (SCT). The PSDC originates in laminae III and IV and projects ipsilaterally to the gracile and cuneate nuclei of the medulla via the dorsal funiculus. This tract conveys a multitude of sensory modalities including pressure, cooling, pinch and hair movement as well as nociceptive information (Angaut-Petit, 1975). The SCT originates from cells in laminae IV to VII and projects ipsilaterally to the lateral funiculus. Some fibres of this tract pass into the PSDC or cross the midline to run contralaterally in the ventral funiculus (Cao et al., 1993). Most neurons of the SCT respond to innocuous hair movement, however, many are multireceptive and can also respond to nociceptive stimulation (Fleetwood-Walker et al., 1988).

1.3.7 Endogenous inhibitory modulation

As suggested above, there are local and descending mechanisms within the CNS designed to modulate nociceptive transmission. Melzack and Wall first designed a model to describe the functioning of such an intrinsic inhibitory mechanism in their gate control theory of pain (Melzack and Wall, 1965). They proposed that spinal nociceptive transmission could be inhibited by non-nociceptive large diameter myelinated Aβ fibre inputs acting on second order neurons via spinal inhibitory interneurons. The inhibitory influence of cutaneous stimulation of large A fibres on C fibre- and noxious stimulation-evoked excitation of dorsal horn neurons has been well demonstrated (Woolf and Wall, 1982), (Besson and Chaouch, 1987). Although many additional receptors and transmitters have since been discovered, the gate theory remains relevant to our current understanding of nociception (Dickenson, 2002). Local inhibitory mechanisms may be augmented by descending inhibitory pathways

from the brain, damping down nociceptive transmission (Besson and Chaouch, 1987), (Polgar et al., 2002).

Tonic descending modulation

Several brain-stem regions are known to be involved in the descending control of nociceptive transmission. Two key regions would appear to be the medullary nucleus raphe magnus (NRM) and adjacent structures of the rostral ventromedial medulla (RVM). The NRM provides a major descending serotonergic input to the dorsal horn laminae I, II and V (Basbaum and Fields, 1984), (Polgar et al., 2002). In addition, electrical stimulation of this region has been shown to produce analgesia.

It has been suggested that RVM cells are tonically active during mild sensory stimulation, inhibiting nociceptive transmission from the periphery. However, intense noxious stimulation may cause the release of GABA onto RVM cells, which in turn suppresses activity in this pathway and removes descending inhibition on dorsal horn cells, allowing transmission of nociceptive input via the dorsal horn (Gilbert and Franklin, 2001). The RVM may also be activated via opioid-mediated mechanisms involving higher brain centres such as the amygdala (McGaraughty and Heinricher, 2002).

The locus coeruleus (LC) also plays a role in modulating nociceptive transmission, via its actions on the parafascicular neurons (PF) of the thalamus (Zhang et al., 1997). The LC exerts two opposing effects on nociceptive transmission. Descending noradrenergic fibres from the LC to spinal cord play a predominantly inhibitory role, and ascending fibres may facilitate the responsiveness of the PF to noxious input.

Noradrenaline has been shown to cause hyperpolarization of lamina II cells when applied to the spinal cord, and to induce an outward current by G- protein-mediated activation of potassium channels through α_2 adrenoceptors, thereby producing an antinociceptive effect (Sonohata et al., 2004). From this brief summary, it can be seen that descending modulation of nociceptive transmission involves complex interactions between several brain and spinal cord regions.

The endogenous opioid system

Exogenous opioids have been used as effective analgesics in man for over one thousand years. These drugs have been shown to be effective when administered by

virtually every route, but intrathecal opioids have been shown to be particularly effective at very low concentrations, suggesting that receptors may be concentrated in the CNS, including the spinal cord. Three members of the opioid receptor family have been cloned: the delta-opioid receptor (DOR) (Evans et al., 1992), the mu-opioid receptor (MOR) (Chen et al., 1993) and the kappa-opioid receptor (KOR) (Meng et al., 1993).

Opioid receptors have been found in the superficial dorsal horn, both on primary afferents and secondary interneurons (Atweh and Kuhar, 1977). The highest concentrations of receptors in the spinal cord are around the C fibre termination zone in lamina I and in the substantia gelatinosa. In the rat spinal cord, 70% of opioid receptors are of the mu type, 24% delta and 6% kappa (Besse et al., 1990). Endogenous ligands for these receptors have been identified, and opioid peptides such as dynorphin and enkephalin are present at synapses within the dorsal horn (Willis and Coggeshall, 1991). These findings provide evidence for the role of the endogenous opioids in nociceptive processing. The antinociceptive action of these compounds has been confirmed in electrophysiological studies, where iontophoretic application of opioids to lamina II of the dorsal horn produced inhibition of neuronal responses to noxious peripheral stimulation (Fleetwood-Walker et al., 1988).

The endogenous cannabinoid system

This recently discovered endogenous inhibitory system is currently under intense investigation due to the relative efficacy of natural and synthetic cannabinoid compounds in providing analgesia in conditions such as multiple sclerosis and neuropathic pain (Robson, 2001). The two identified cannabinoid receptors, CB_1 and CB_2 (Pertwee, 1997), (Pertwee, 1998) have been shown to be present in the peripheral and central nervous system, particularly in areas associated with nociceptive processing such as the superficial laminae of the spinal dorsal horn. CB_1 receptors in particular, have been reported to be located on spinal interneurons in the outer part of lamina II (Farquhar-Smith et al., 2000). The antinociceptive role of endogenous cannabinoids has been demonstrated in behavioural (Lichtman and Martin, 1997), (Smith and Martin, 1992) and electrophysiological studies of acute pain (Drew et al., 2000). In addition to the antinociceptive role of cannabinoids alone, CB_1 and MORs have been shown to colocalise in lamina II interneurons, and could potentially act in a synergistic manner (Salio et al., 2001).

1.4 ANIMAL MODELS OF CHRONIC PAIN LEADING TO SPINAL SENSITISATION

Progress in pain research has been aided by the use of animal models of human pain conditions. Simple tests of acute nociception such as the tail flick, hot plate test were used to develop analgesic drugs. More recently, complex models of persistent inflammatory or neuropathic pain have been developed. The majority of these models reflect relevant clinical conditions in man and leave the animal in relatively good health with the minimum of distress.

Measuring the behavioural responses of animals to painful stimuli also presents difficulties. It is clearly not possible to assess the character of the pain experienced by animal subjects (eg: stabbing, burning, shooting pain and so on). Aversive behaviour such as vocalising, biting or scratching can be measured in certain circumstances, but nociceptive responses are often too subtle to result in such clear behavioural changes. Generally, spinal reflexes have been utilised in studies of pain mechanisms in animals and man. The magnitude of the nocifensive hindpaw flexor reflex to electrical stimulation or to physiological stimuli, has been positively correlated to the activity of spinal dorsal horn neurons (Schouenborg and Sjolund, 1983). This reflex is subject to local and descending modulation and can be used to investigate responses to a number of experimental interventions. Changes in the latency and magnitude of the nocifensive response can be measured relatively easily, providing a graded behavioural response to stimuli perceived as increasingly noxious. A number of behavioural tests have been developed to assess different sensory modalities. Heat hyperalgesia has been assessed using an apparatus developed by Hargreaves to measure the latency of hindpaw withdrawal from a calibrated heat stimulus (Hargreaves et al., 1988) (see Figure 3.1). Mechanical allodynia can be assessed using von Frey filaments (or pressure analgesymeter). These calibrated fibres flex at a given force and produced a graded, non-noxious pressure stimulus. In the presence of mechanical allodynia (characteristic of some neuropathic pain states), this usually innocuous stimulus will cause paw withdrawal. Cold allodynia is assessed using a cold water bath and noting the presence and duration of limb withdrawal from the cold stimulus.

Both inflammatory and neuropathic chronic pain models have been developed. Briefly, the rat model of inflammatory chronic pain utilises peripheral injection of

irritants such as formalin or Freund's adjuvant (Walker et al., 1999). These models result in mechanical and thermal hyperalgesia with a large peripheral component.

Neuropathic pain would seem to have a somewhat different mechanism to chronic pain caused by inflammatory processes and leads to mechanical allodynia and, in some models, cold allodynia and thermal hyperalgesia (see section 1.9).

The model adopted for the current study was developed by Bennett (Bennett and Xie, 1988). It is known as the sciatic chronic constriction injury (CCI) model. Four loose chromic ligatures are tied around the sciatic nerve (see section 4.2.1). Spontaneous pain, allodynia and protective posture are all demonstrated to a variable degree after surgery. The de novo development of cold allodynia is also a marked feature of this model. These behavioural changes manifest within 36 hours of the nerve constriction, are most pronounced 10-14 days after induction of the injury and can last for up to three months.

1.5 Receptors and neurotransmitters involved in nociception

Peripheral stimuli of sufficient intensity to trigger action potentials in primary afferent fibres result in the release of a variety of neurotransmitters and neuropeptides from the presynaptic nerve terminals in the spinal dorsal horn. After crossing the synaptic cleft, these neurotransmitters bind to the postsynaptic receptors on dorsal horn cells where they result in changes in the conductance of the postsynaptic membrane.

The major excitatory amino acid neurotransmitter released from primary afferents is thought to be L-glutamate (Watkins and Evans, 1981). Electrical nerve stimulation causes glutamate release by terminals of both myelinated and unmyelinated axons, suggesting that this neurotransmitter has an important role to play in both nociception and transmission of non-noxious information (Davies et al., 1979). L-glutamate has subsequently been identified as an important excitatory amino acid neurotransmitter for fibres transmitting mechanical, chemical and thermal stimuli from the periphery (Gerber and Randic, 1989), (King and Lopez-Garcia, 1993). Several subtypes of glutamate receptor have been identified in both the superficial and deep dorsal horn. These can be broadly subdivided into ionotropic ligand-gated ion channels and metabotropic receptors, which are coupled to various second messenger systems through GTP-binding proteins (Willis and Coggeshall, 1991).

In addition to the glutamatergic systems discussed above, peptides also play an important role in neurotransmission and neuromodulation. The current study was designed to investigate the interaction between key neurotransmitter systems in the development of neuropathic pain. These systems will be discussed in detail below, with emphasis on the role of the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor for glutamate (see section 1.9).

1.5.1 Ionotropic glutamate receptors

The ionotropic glutamate receptors (iGluR) have been divided into two major groups depending upon their pharmacological response characteristics to various agonists (Young and Fagg, 1990). These are the N-methyl-D-aspartate (NMDA), and the non-NMDA iGluRs. Non-NMDA Rs are further subdivided into AMPA and kainate receptors. AMPA R subunits have been demonstrated in neurons of the superficial laminae of the dorsal horn (Furuyama et al., 1993), (Henley, 1993), (Tolle

et al., 1993), (Popratiloff et al., 1996), and NMDA receptors (NMDA Rs) have been shown to be located throughout the brain and spinal cord particularly in the superficial dorsal horn (Cotman and Monaghan, 1985), (Greenamyre et al., 1985), (Monaghan and Cotman, 1985).

There is a great deal of evidence to support the critical role of NMDA and AMPA Rs in nociceptive processing and in the neuronal plasticity underlying processes such as memory, learning and the development of chronic pain states (Coderre et al., 1993). Activation of NMDA Rs may play an important role in neuronal plasticity and specifically in the development of central sensitisation (Woolf and Thompson, 1991). Recent studies have demonstrated a more significant role for AMPA Rs in synaptic plasticity than had been previously appreciated (Luscher et al., 1999), (Luscher and Frerking, 2001), and this receptor is considered in more detail below (see section 1.6).

NMDA receptors

During non-noxious stimulation, A fibres release glutamate from pre-synaptic end plates and excitatory postsynaptic potentials (EPSPs) are generated in the dorsal horn neuron by activation of non-NMDA glutamate receptors. In this situation the NMDA R calcium channels remain blocked by magnesium ions in a voltage-dependant manner. Once the stimulus is removed, action potentials cease and no further signal transduction occurs. During acute pain transmission, NMDA Rs are largely inactive due to channel blockade by magnesium ions (Nishiyama, 2000).

Prolonged noxious stimuli, however, cause a release of glutamate from Aδ and C fibres which may be sufficient to activate NMDA receptors. NMDA receptor activation may occur when the dorsal horn neuron is sufficiently depolarised (by alternative glutamate receptors or alternative mechanisms), or the receptor is phosphorylated via second messenger systems triggered by activation of non-NMDA glutamate and peptide receptors. When NMDA receptors are activated by removal of the magnesium block, there is an increase in calcium flux through the postsynaptic membrane with a prolonged channel opening time. The channel blockade of NMDA Rs by magnesium ions may be removed by progressive depolarisation of the cell membrane or by reducing the receptor affinity for the ion by, for example, conformational change following its phosphorylation. Thus, glutamate produces a fast

excitatory potential when acting at the AMPA R site and a long synaptic potential when acting at the NMDA receptor site.

The NMDA receptor is distributed throughout the dorsal and ventral horns of the spinal cord (Furuyama et al., 1993). It is an ionotropic glutamate receptor, permeable to calcium and sodium ions. The receptor is a tetramer of NR1 and NR2A-D subunits (Dingledine et al., 1999). Following the induction of central sensitisation, binding of L-glutamate to this receptor allows calcium and sodium to enter the cell, triggering further depolarisation and activation of calcium dependent processes (Davies and Lodge, 1987). The activation of NMDA receptors appears to be central to the generation of prolonged states of nociception. The hyperalgesia occurring following the induction of sensitised states in a number of experimental paradigms was attenuated by intrathecal administration of NMDA receptor antagonists. These results suggest that NMDA receptor activation may contribute to the mechanical hyperalgesia that follows peripheral tissue inflammation (Ren and Dubner, 1993) and peripheral mononeuropathy (Tal and Bennett, 1993), (Tal and Bennett, 1994). Human studies have demonstrated that the NMDA antagonist ketamine may be effective in the treatment of various chronic pain conditions, but the evidence for its efficacy is moderate or weak and side effects may preclude its use (Hocking and Cousins, 2003).

The NMDA receptor also plays a central role in other processes involving neuronal plasticity, such as LTP, necessary for spatial learning and memory formation in the hippocampus (Morris et al., 1986). NMDA receptor-dependent LTP is considered to share some similarities with processes occurring in central sensitisation of the spinal cord in hyperalgesic pain states (Dougherty et al., 1992), (Sandkuhler, 2000), (Rueter et al., 2003). However, there are clearly significant mechanistic differences between the two processes. Sensitisation in neuropathic pain is a heterosynaptic process, triggered by relatively low frequency input from more than one source, whereas LTP requires high frequency neuronal stimulation (Ji et al., 2003). An LTP-like event can be triggered in spinal cord slices, using high frequency stimulation, but this is not seen in intact cord in the presence of segmental and descending modulation (Sandkuhler and Liu, 1998).

In addition to the role of the NMDA R in LTP in the hippocampus, application of NMDA R antagonists also attenuated the increased responses seen following wind-up of a subset of dorsal horn interneurons by low frequency C fibre stimulation, or

cutaneous stimulation of noxious intensity (Woolf and Thompson, 1991), (King and Lopez-Garcia, 1993).

The effect of glutamate binding to the NMDA receptor may be modulated by a regulatory glycine binding site (Kleckner and Dingledine, 1988). Glycine appears to be important in regulating a number of NMDA receptor-mediated responses, and may act as a co-agonist. Activation of the Gly_{NMDA} site would also appear to be an essential component of the NK1 receptor facilitation of dorsal horn neuronal responses towards NMDA receptor agonists (Heppenstall and Fleetwood-Walker, 1997b). Gly_{NMDA} site antagonists have been shown to suppress the enhanced responses of spinal neurons resulting from repetitive C fibre stimulation (Dickenson and Aydar, 1991). In vivo, blockade of the Gly_{NMDA} ($glycine_B$ receptor (Kleckner and Dingledine, 1988)) site using the antagonist 7-chlorokynurenate, has been shown to inhibit NMDA-induced thermal hyperalgesia in the rat (Kolhekar et al., 1994).

AMPA receptors

AMPA Rs are considered extensively in section 1.6.

Kainate receptors

Until recently, this family of ionotropic glutamate receptors has been difficult to study due to a lack of specific agonists and antagonists. Available agents tended to cross-react with AMPA iGluRs. Kainate receptors incorporating the GluR5 subunit are present at high levels on small diameter primary afferent neurons thought to mediate nociceptive inputs. Procter et al investigated the effect of GluR5 agonists and antagonists on nociceptive stimuli in single cells, hemisected rat spinal cords and intact mice (Procter et al., 1998). It was found that the GluR5 agonist had a small effect in reducing C fibre-mediated reflexes in a spinal cord preparation, but had no analgesic effect in vivo.

Using spinal cord slices from knockout mice, GluR5 or GluR6 subunits were found to either suppress or facilitate glutamatergic excitatory transmission in the substantia gelatinosa. Kainate treatment had a biphasic effect in the absence of GABA inhibition, with facilitation apparent at a low concentration (30 nM) and depression at a higher concentration (3 μM). This suggests that kainate receptors are involved in bi-directional regulation of excitatory synaptic transmission in the dorsal horn lamina II

region, and that these actions may be of importance for nociception (Youn and Randic, 2004).

1.5.2 Metabotropic glutamate receptors

The metabotropic glutamate receptors (mGluRs) form a family of eight subtypes (Pin and Duvoisin, 1995). They are divided functionally in to three groups: group I metabotropic receptors (mGluR1 and mGluR5) are concentrated in the superficial dorsal horn of the spinal cord (Jia et al., 1999). Some of these receptors can be further sub-divided into splice variants for example (mGluR1a-d and mGluR5a-b), with varying physiological roles (Tanabe et al., 1992), (Joly et al., 1995). Antisera to mGluR5 detected marked immunoreactivity within laminae I and II of the dorsal horn, and autonomic regions in rat spinal cord. Relatively fewer mGluR1a were found in laminae I and II, the majority of these receptors being localised to the deep dorsal horn (laminae III-V), and the ventral horn (laminae VI-IX). Somatic motoneurons expressed mGluR1a. The mGluR1b appeared to be localised to lamina X around the central canal, and in the ventral horn (Alvarez et al., 2000).

The group I mGluRs are coupled to $G_{q/11}$ proteins and activate protein kinase C (PKC) in addition to releasing calcium from intracellular stores and possibly increasing intracellular cyclic adenosine 3',5'-monophosphate (cAMP) (Abe et al., 1992), (Aramori and Nakanishi, 1992). All these functions lead to an increase in cellular excitability and are likely to underlie the role group I metabotropic glutamate receptors are thought to play in various forms of synaptic plasticity including LTP in the hippocampus, wind-up and the development of chronic pain states.

Group II and III mGluRs are negatively coupled to adenylate cyclase via $G_{i/o}$ proteins (De Blasi et al., 2001). Presynaptic group II receptors (mGluR2 and 3) decrease glutamate release. Group III mGluRs are involved in varied processes: mGluR6 is found only in the retina and plays a role in the amplification of visual inputs. The remainder of group III receptors; mGluR4, 7 and 8 are also found at presynaptic sites. The group II and III mGluRs will not be discussed further here.

Group I metabotropic glutamate receptors and nociception

Application of mustard oil peripherally to rats selectively activates C fibres and brings about a state of sustained activation in dorsal horn neurons (Munro et al.,

1993). The importance of mGluR receptor activation in nociception has been demonstrated in vivo by reversal of DH neuron mustard oil evoked sensitisation using iontophoretic application of L-1-amino-3-phosphonopropanoic acid (L-AP3), a selective mGluR antagonist (Young et al., 1995). It was also found that cyclothiazide (CTZ), a selective mGluR1 antagonist, markedly reduced the electrophysiological response of DH neurons to mustard oil but not to brushing (Young et al., 1997). This study provides evidence that mGluR1 plays an important role in nociceptive transmission, particularly after prolonged noxious input.

The effect of removal of mGluR1 by antisense ablation on single cell electrophysiological responses to stimulation has also been studied (Young et al., 1998). Multireceptive neurons in the dorsal horn of mGluR1-ablated animals showed a normal response to innocuous stimuli, but a markedly reduced response to mustard oil and the specific mGluR1/5 agonist 3,5-dihydroxyphenylglycine (DHPG). In parallel to the electrophysiological changes, it was noted that the rats demonstrated a marked analgesia to thermal stimuli on behavioural testing. Once again, this provides evidence for the role of mGluR group I in nociception.

DHPG has also been shown to cause spontaneous nociceptive behaviour (SNB) and mechanical hyperalgesia when given via the intrathecal (IT) route to naïve rats, whereas the non-specific mGluR agonist trans-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) caused less SNB and a specific mGluR5 agonist trans-azetidine-2,4-dicarboxylic acid (t-ADA) produced no SNB. This suggests a more significant role for mGluR1 than for mGluR5 in acute nociception (Fisher and Coderre, 1996).

The role of mGluR5 in a number of pain states was investigated using the specific antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) (Walker et al., 2001). MPEP given intrathecally resulted in some reversal of mechanical hyperalgesia in chronic inflammation using a Freund's Adjuvant model at 24hrs post injection. In acute inflammation, MPEP had a moderate effect on mechanical hyperalgesia, but no effect on oedema. In a sciatic nerve ligation model of neuropathic pain, no effect was found on mechanical hyperalgesia, suggesting that the mGluR5 has a more significant role in inflammation than in neuropathic pain.

In contrast to these findings, intrathecal treatment with the specific mGluR5 antagonist SIB 1757 was effective in reversing thermal hyperalgesia in a spinal nerve

ligation (SNL) model of neuropathic pain at a low dose of 0.01μg but only showed a partial dose-dependant reversal of mechanical hyperalgesia (Dogrul et al., 2000).

It has been suggested that the relative efficacy of mGluR5 antagonists in ablating thermal but not mechanical hyperalgesia in nerve injury emphasizes the possibility that differing mechanisms exist for each of these abnormal pain states. In addition, antibody ablation of mGluR1 and mGluR5 had little effect on reducing nociceptive behaviour caused by noxious heat or chemical irritation (with formalin). However, these antibodies were effective in reducing cold allodynia after sciatic nerve constriction induced neuropathic pain (Fundytus et al., 1998). It has recently been suggested that the role of mGluR5 in neuropathic pain is mediated via modulating apoptotic cell death (de Novellis et al., 2004). However, the relative importance of this process in neuropathic pain is not yet clear as reports vary widely as to the degree of cell death occurring, and a recent carefully conducted study revealed no differential loss of GABAergic inhibitory cells (Polgar et al., 2003), (see section 1.11.4).

1.5.3 Neuropeptides

Various neuropeptides are synthesised in DRG neurons and are often co-released with glutamate from peripheral afferent terminals causing a variety of synergistic effects on postsynaptic dorsal horn cells. As they may play an important role in the development of sensitised states within the CNS, they are considered in some detail below.

Levels of peptide receptors and their ligands alter markedly after peripheral nerve injury, both in the dorsal root ganglion and spinal cord. In large-diameter neurons, neuropeptide Y (NPY) and cholecystokinin increase, whereas levels of calcitonin gene-related polypeptide are reduced. In small diameter nociceptive neurons in the DRG, levels of SP, CGRP and somatostatin decrease, while levels of vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase-activating peptide (PACAP) and galanin markedly increase (Hokfelt et al., 1994).

Substance P

Substance P is a member of the tachykinin family and is expressed by a significant proportion of small diameter primary afferent neurons, neurons within the superficial spinal dorsal horn and small diameter nocispecific DRG neurons (Hokfelt

et al., 1975), (Ju et al., 1987). This peptide is an endogenous ligand for the neurokinin 1 (NK_1) receptor.

The NK_1 receptor has been shown to be expressed by approximately 80% of lamina I neurons projecting to the thalamus, periaqueductal grey matter, lateral parabrachial area and caudal ventrolateral medulla (Todd et al., 2000). It would therefore seem likely that NK_1-expressing cells are involved in several aspects of nociceptive processing. The effects of NK_1 expressing projection neurons on brainstem structures may be modulated by descending serotonergic inhibitory pathways, which have been shown to form connections with these cells in the superficial dorsal horn (Polgar et al., 2002).

Evidence for the pro-nociceptive role of SP has been provided using a variety of experimental techniques. When given intrathecally, SP caused a reduction in the latency of the tail-flick response to peripheral stimuli in naïve rats (Yashpal et al., 1982) and triggered biting and scratching behaviours in mice that may be indicative of pain sensation (Hylden and Wilcox, 1981), (Courteix et al., 1993).

The SP tachykinin receptor NK_1 is linked to second messenger systems via G proteins and its activation leads to a prolonged depolarising effect on secondary neurons. It has been suggested that this prolonged activation of second order neurons by SP may lead to central sensitisation, as antagonists to the NK_1 receptor have been shown to block cumulative depolarisation and hence wind-up (Baranauskas and Nistri, 1996), (Baranauskas and Nistri, 1998). However, NK_1 antagonists have not been shown to be generally effective in reducing neuronal responses to brief noxious stimulation in rats (Fleetwood-Walker et al., 1990), (Yamamoto and Yaksh, 1992). These results would suggest that SP and NK_1 receptors might play a more prominent role in sustained noxious states. This suggestion is supported by the finding that noxious mechanical and severe thermal stimuli specifically evoked SP release from primary afferents using microdialysis techniques and antibody probes (Kuraishi et al., 1989), (Duggan and Furmidge, 1994), (Duggan et al., 1995). Noxious stimuli resulting in tissue damage were also shown to result in a significantly greater release of SP than less intense stimuli. In addition, ablation of SP containing neurons in the superficial dorsal horn in vivo resulted in marked attenuation of behavioural responses to highly noxious stimuli, but left responses to mild noxious stimuli intact (Mantyh et al., 1997).

It is known that the effect of glutamate on the dorsal horn neuron may be modulated by co-released SP which coexists with this neurotransmitter in dorsal root ganglion (DRG) neurons and primary afferent terminals (Battaglia and Rustioni, 1988), (Debiasi and Rustioni, 1988). Electrophysiological studies have demonstrated that iontophoretic application of SP may enhance NMDA receptor-induced activity of spinothalamic tract (STT) neurons (Dougherty and Willis, 1990), (Dougherty and Willis, 1991), and rat dorsal horn neurons (Cumberbatch et al., 1995). This NK_1 receptor facilitation of the NMDA receptor can be blocked by Gly_{NMDA} site antagonists (Heppenstall and Fleetwood-Walker, 1997a), (Heppenstall and Fleetwood-Walker, 1997b). These results suggest that there is a clear interaction between the glutamatergic NMDA receptor, glycine and SP in the transmission of nociceptive information.

These studies suggest that SP is involved in nociception in situations where tissue damage occurs and inflammatory mediators are released. In addition to this role, SP has recently been implicated in the generation and maintenance of neuropathic pain, as pre-emptive intrathecal administration of the NK_1 receptor antagonist L-732 138 has been shown to prevent the development of mechanical and cold allodynia following CCI (Cahill and Coderre, 2002). It would therefore appear that an NK_1 antagonist would be an effective analgesic drug in chronic pain states. Unfortunately, such compounds have been shown to be largely ineffective in clinical trials in humans, possibly due to the existence of multiple alternative pathways that may potentially activate iGluRs in the development of chronic pain states.

Vasoactive intestinal polypeptide and pituitary adenylate cyclase-activating polypeptide

These polypeptides are widely expressed within the CNS of a variety of species (Yaksh et al., 1988). VIP and PACAP are present throughout the spinal cord of the cat and rat, with increasing concentration towards the caudal end (Gibson et al., 1981), (Yashpal et al., 1991). Nerve fibres immunopositive for these compounds are concentrated in laminae I and II, an area known to be important in the transmission of nociceptive information (Cervero and Iggo, 1980), (Moller et al., 1993). Recently, PACAP mRNA expression has been demonstrated in neurons in laminae I and II of the DH. Interestingly, increased expression of PACAP was seen in ventral horn neurons, in response to sciatic nerve transection, suggesting a role for PACAP in

repair or regeneration of motor neurons (Pettersson et al., 2004). The wide distribution of VIP and PACAP within the CNS, in particular in primary sensory neurons of the spinal dorsal horn, suggests a possible role for these peptides in the transmission of somatosensory information.

VIP and PACAP are recognised by a family of three receptors. As these two peptides share a relatively high degree of sequence homology, they may combine with the same receptor binding sites (Harmar and Lutz, 1994). The PAC_1 receptor is relatively specific for PACAP, whereas the $VPAC_1$ and $VPAC_2$ Rs have similar binding affinity for both agonists (Hosoya et al., 1992), (Ishihara et al., 1992). These receptors each consist of seven trans-membrane domains and are coupled to stimulatory G_s proteins, increasing cAMP levels on activation (Spengler et al., 1993), (Dickinson and Fleetwood-Walker, 1999).

Intrathecal application of VIP has been shown to facilitate spinal cord reflex excitability in rats (Hokfelt et al., 1987), (WiesenfeldHallin, 1989), (Xu and WiesenfeldHallin, 1991). Despite this demonstration of the role of VIP in acute nociception, this peptide is expressed at very low levels in DRG neurons and in lamina I and lamina II neurons of the spinal dorsal horn in the normal state (Gibson et al., 1981), (Knyiharcsillik et al., 1991), (Moller et al., 1993), (Noguchi et al., 1993). Intrathecal application of the VIP antagonist (Ac-Tyr^1, D-Phe^2)-GRF(1-29)-NH_2 did not prevent C fibre stimulation induced reflex facilitation in normal animals (WiesenfeldHallin et al., 1990), (Xu and WiesenfeldHallin, 1991). These studies suggest that endogenous VIP does not appear to play a significant role in the C fibre-induced spinal sensitisation under normal conditions.

Following nerve injury, however, the situation would appear to be rather different, with characteristic changes occurring in the expression of various neuropeptides in the DRG and DH (Hokfelt et al., 1994). In small diameter, nociceptive neurons in the DRG, levels of SP, CGRP and somatostatin decrease, whereas VIP and PACAP increase (WiesenfeldHallin et al., 1990). These results suggest that there may be a switch in the significance of various neuromodulatory substances following peripheral nerve injury, with the role of major excitatory neuropeptide transferring from SP in the naïve animal to VIP in the sensitised state.

PACAP levels in the DRG increase early following injury and return to resting levels after one to two weeks (Zhang et al., 1995), whereas VIP levels increase late in

the superficial dorsal horn and remain elevated (Nahin et al., 1994), (Zhang et al., 1995b). The marked difference in the time course of these changes suggests that PACAP and VIP may play a role in the induction and maintenance of a sensitised state respectively.

It has been demonstrated that iontophoretic spinal application of a specific $VPAC_2$ R antagonist inhibited the neuronal response to sustained noxious input from the application of mustard oil, but had no effect on the response to innocuous brushing in the naïve animal. In neuropathic animals, $VPAC_1$, $VPAC_2$ and PAC_1 R antagonists had no effect on innocuous sensory transmission (Dickinson et al., 1999).

Spinal application of agonists to the $VPAC_1$, $VPAC_2$ and PAC_1 Rs resulted in increased firing of DH neurons in naïve animals. After the induction of neuropathy (CCI), the response to $VPAC_1$ and PAC_1 R agonists was not increased, whereas the response to the $VPAC_2$ R agonist Ro 25-1553 was dramatically augmented (Dickinson et al., 1999). These findings suggest that the $VPAC_2$ R may play a specific role in the transmission of noxious stimuli in neuropathic pain.

The role of PACAP has been less well elucidated. Intrathecal delivery of PACAP-38, activating both VPAC and PAC Rs, has been shown to reverse thermal hyperalgesia in mice at low doses and causes biting and scratching pain related behaviour at higher doses, suggesting that this peptide may have a complex role in the spinal cord, dependant upon its concentration (Narita et al., 1996). In contrast to these studies in mice, intrathecal PACAP-27 produced a significant and long-lasting suppression of C fibre-evoked flexion reflex in the rat (Zhang et al., 1993). In addition, intrathecal PACAP also reduced spontaneous pain behaviours in the rat following formalin inflammation of the hind paw (Yamamoto and Tatsuno, 1995). The role of PACAP remains unclear, therefore, and may alter depending upon the background state of the nociceptive system and the drug dosage. Interspecies differences may further complicate the situation.

Blockade of the $VPAC_2$ R would seem to have a specific effect in suppressing neuronal responses to prolonged noxious input in both normal and nerve-injured rats, suggesting that this receptor may mediate noxious sensory transmission, particularly after sensitising nerve injury (Dickinson et al., 1999). This finding highlights a role for specific $VPAC_2$ R antagonists as potential analgesics in intractable neuropathic pain.

Calcitonin gene-related peptide (CGRP)

This peptide is an important mediator of nociceptive transmission. CGRP is found in 30% of all unmyelinated C fibre primary afferents and Aδ fine myelinated fibres, and is down regulated in large dorsal root ganglia (DRG) neurons following axotomy (Levine et al., 1993), (Hokfelt et al., 1994). Noxious thermal and mechanical stimulation of primary afferents has been shown to trigger CGRP release into the superficial dorsal horn (Morton and Hutchison, 1990). The majority of CGRP positive DRG neurons also contain SP (Battaglia and Rustioni, 1988). These two compounds are co-released and CGRP modulates the effect of SP, inhibiting SP endopeptidase and potentiating its effect (Mao et al., 1992c).

The role of CGRP in various pain conditions has been investigated using CGRP(8-37), a truncated form of CGRP that binds to the CGRP receptor with similar affinity to the endogenous ligand but does not activate the receptor and thus acts as a highly selective CGRP receptor antagonist. Mechanical hyperalgesia following intradermal injection of capsaicin in rats has been reversed using CGRP(8-37) applied via a microdialysis catheter into the dorsal horn (Sun et al., 2003). This study suggests that CGRP may play a role in inflammatory hyperalgesia.

This peptide has also been implicated in the maintenance of neuropathic pain. In a rat model of mononeuropathy after left common sciatic nerve constriction, CGRP immunoreactivity was significantly decreased in the DH of the spinal cord and DRG ipsilateral to injury. In the same study, intrathecal CGRP(8-37) induced a significant bilateral increase in hindpaw withdrawal latency to both thermal and mechanical stimulation in both neuropathic and naïve animals (Yu et al., 1996). Interestingly, the analgesic effect of CGRP blockade using CGRP(8-37) was less marked in rats following nerve injury, suggesting that the contribution of CGRP and its receptors to transmission of presumed nociceptive information appears to be reduced in the sciatic nerve constriction model. In addition to peripheral nerve injury, intrathecal CGRP(8-37) was effective in alleviating mechanical and thermal allodynia in a dose-dependent manner in a model of chronic central neuropathic pain after spinal hemisection (Bennett et al., 2000).

1.5.4 Inhibitory and neuromodulatory peptides

Neuropeptide Y (NPY)

In the resting state, NPY is not expressed by DRG neurons (Lundberg et al., 1983). However, following axotomy, (Wakisaka et al., 1991), (Kashiba et al., 1994), (Malmberg et al., 1997) or CCI of the sciatic nerve (Nahin et al., 1994), (Munglani et al., 1995) its expression increases in large diameter neurons. However, there are also many NPY-positive fibres innervating the superficial dorsal horn, which originate both locally and from supraspinal descending tracts (Wakisaka et al., 1991).

In a study of neuropathic pain following spinal nerve injury in rats, administration of anti-NPY antiserum into the nucleus gracilis ipsilateral to nerve injury reversed tactile, but not thermal, hypersensitivity. These data suggest that nerve injury-induced tactile hypersensitivity may be mediated by upregulated NPY via large fibre input into the nucleus gracilis (Ossipov et al., 2002).

In inflammation, intrathecal NPY has been shown to reduce thermal hyperalgesia on the side ipsilateral to carrageenan or CFA injection in a dose dependant manner, with only a small effect on the contralateral side. The effect of NPY may be inhibited by administration of an antagonist at the NPY Y1 receptor, but not by an NPY Y2 receptor antagonist. These data suggest that spinal Y1 receptors contribute to the inhibitory effects of NPY on thermal hypersensitivity in the rat (Taiwo and Taylor, 2002). However, NPY expression does not appear to alter in inflammatory states, in contrast to the marked effects of neuropathy (Wakisaka et al., 1992).

It would seem that the role of NPY in nociception is far from clear: inhibition of this peptide in the nucleus gracilis results in decreased mechanical hyperalgesia in neuropathy, and activation of the Y1 receptor decreases thermal hyperalgesia in inflammation. It is likely that differing NPY receptors have differing modulatory roles within different regions of the CNS.

γ-Aminobutyric acid (GABA) and glycine

An important role for GABA in sensory processing is suggested by both electrophysiological and behavioural studies, which indicate an inhibitory function in the transmission of noxious stimuli. Neurons containing GABA and glycine exert tonic inhibitory effects on excitatory dorsal horn inputs (Curtis et al., 1970),

(Zieglgansberger and Sutor, 1983). Both ionotropic $GABA_A$ receptors and metabotropic $GABA_B$ receptors are expressed on neurons in the superficial DH (Price et al., 1984), (Bowery et al., 1987). Activation of $GABA_B$ receptors leads to inhibition of presynaptic neurotransmitter release and to inhibition of postsynaptic neuronal activity (references in (Bowery et al., 1993) and (Bowery, 1993)).

$GABA_B$ receptors clearly play a role in neuropathic pain, as treatment with agonists to this receptor subtype resulted in reduction in mechanical allodynia following partial sciatic ligation and a dose dependant reduction in thermal hyperalgesia following CCI, with little effect on inflammatory hyperalgesia (Patel et al., 2001), (Franek et al., 2004).

Following unilateral sciatic nerve ligation in the mouse, intraperitoneal injection of muscimol ($GABA_A$ agonist) or baclofen ($GABA_B$ agonist) induced a dose-related thermal antinociception in both intact and ligated animals (Zarrindast and Mahmoudi, 2001). Activation of $GABA_A$ receptors using intrathecal muscimol has also been shown to prevent the long-lasting potentiation of dorsal horn neuron electrophysiological responses usually seen after tetanic stimulation in rats with chronic constriction injury (Miletic et al., 2003).

It has been suggested that loss of GABAergic inhibition within the dorsal horn may be required for the development of spinal central sensitisation and this mechanism is discussed further below (see section 1.11.4), (Ibuki et al., 1997), (Moore et al., 2002).

Galanin

Galanin is a 29-amino-acid neuropeptide normally expressed at very low levels in sensory and sympathetic neurons (Ju et al., 1987), (Baranowski et al., 1994), (Ma and Bisby, 1997). It is widely distributed in the CNS, and is concentrated both in the superficial dorsal horn of the spinal cord and within primary afferent fibres (Kar and Quirion, 1995).

Following peripheral nerve injury, galanin is strongly upregulated in small and medium diameter unmyelinated neurons in the DRG, which commonly co-express SP and CGRP (Hokfelt et al., 1987), (Villar et al., 1989). It has been suggested that galanin up regulation is important in the development of central sensitisation (Kerr et al., 2001).

The effects of intrathecal galanin on behaviour are complex and variable, seemingly dependant on the endogenous levels of galanin, the drug dose, and the type of experimental model (Xu et al., 2000). In general, an inhibitory effect on neuropathic pain was noted (Liu and Hokfelt, 2002). It is possible that this peptide plays a neuromodulatory role, as it antagonises the excitatory effects of CGRP and SP when used as an intrathecal pre-treatment (Xu et al., 1990), (WiesenfeldHallin et al., 1991b). In addition to damping down the excitatory effects of pro-nociceptive peptides (Ferhat et al., 1996), galanin also inhibits the analgesic effect of morphine on noxious thermal and mechanical stimuli in naïve animals (WiesenfeldHallin et al., 1991a).

As might be expected from its up-regulation following nerve injury, galanin would seem to play a more significant role in modulating nociception in rats with spinal nerve ligation. There was a marked inhibition of up to 80% of responses to both physiological and electrical stimuli in SNL rats following intrathecal galanin administration. This suggests that the inhibitory role of galanin may become more significant following nerve injury (Flatters et al., 2002).

Somatostatin (SOM)

Somatostatin is a neuropeptide expressed by small diameter primary afferent neurons, which do not express SP (Nagy and Hunt, 1982) and in lamina II cells of the dorsal horn (Hokfelt et al., 1976). Its role in nociception is not entirely clear. Intrathecal SOM application has been reported to increase the excitability of the spinal cord neurons following mechanical and thermal stimuli (WiesenfeldHallin, 1985). In contrast to this finding, intrathecal and epidural application of somatostatin in humans results in analgesia, however concerns regarding neurotoxicity have limited its use via these routes. Receptors for this peptide are also found peripherally, and their activation reduces both inflammatory pain and the activity of sensitised nociceptors in rats (Carlton et al., 2001). It may be that SOM has a neuromodulatory effect, controlling the release of other neurotransmitters, and so may act in a pronociceptive or analgesic manner depending upon the overall state of activity of the nociceptive pathway.

1.6 AMPA RECEPTORS

AMPA Rs are involved in the fast transmission of noxious and innocuous stimuli (Procter et al., 1998), (Stanfa and Dickenson, 1999). These receptors mediate the majority of monosynaptic excitatory postsynaptic potentials (EPSPs) in the dorsal horn evoked by C fibres. Activation of these receptors results in a change in the potential of the postsynaptic membrane towards depolarisation. Summation of a number of these EPSPs may lead to the triggering of an action potential in the target neuron.

1.6.1 Structure

The four AMPA R subunits were first sequenced in 1990 and termed the GluR-A-D subunits (Keinanen et al., 1990), (reviewed in (Hollmann and Heinemann, 1994)). The GluR-A molecule appeared to have 100% sequence homology with the putative kainate receptor (termed GluR-K1) sequenced the previous year (Hollmann et al., 1989). Evidence from X-ray crystallography of partly homologous bacterial potassium channels, and electrophysiological studies of mutant channel conductance suggests that the AMPA R is a tetramer of four subunits with their pore regions facing the centre to produce an ion channel (Robert et al., 2001), (references in (Bredt and Nicoll, 2003)). The complete receptor consists of combinations of subunits, termed GluR1-4. Each subunit consists of a large extracellular N-terminal region (400 amino acids) followed by a membrane-spanning α helix, then a second hydrophobic domain which dips into the membrane from the cytoplasmic side and forms the pore. Beyond the pore there is a further transmembrane section followed by extracellular loop, a final transmembrane α helix and the cytoplasmic C terminal tail. The two extracellular modules S1 and S2 may form the glutamate-binding domain (see Figure 1.1b).

AMPA Rs exist in two splice variants: flip and flop, causing sequence variation just before the fourth transmembrane domain. GluR2 mRNA is edited at the Q/R site (before the third transmembrane domain) by a sequence specific RNA editase, ADAR2. The unedited form (containing Q) is permeable to calcium; the edited form (containing R) is not. The majority of receptors undergo editing at this point. GluR2, 3 and 4 mRNA is edited at the R/G site immediately before the flip/flop sequence, resulting in an increase in the rate of recovery from desensitisation. The

majority of these three GluR subtypes are edited in this manner in the adult. Receptor desensitisation to a glutamate stimulus depends on the receptor subunit composition. The R/G editing site and the flip/flop cassettes in AMPA R subunits contribute to a great extent in receptor desensitisation and recovery rates. The flop forms of the subunits desensitise more rapidly in response to glutamate than flip forms (Dingledine et al., 1999), (see Figure 1.1a). Flip variants predominate before birth and remain expressed at high levels into adulthood. Flop variants increase in number from postnatal day eight, and reach the same levels as flip by adulthood in the rat.

1.6.2 Anatomical location

The AMPA R is found in most areas of the central nervous system. The subunit composition of each receptor varies with its location as well as synaptic activity. In rat neostriatal cells, for example, 50% of dendritic spines were immunopositive to GluR1 and GluR2/3 (Bernard et al., 1997).

The superficial dorsal horn of the spinal cord is of particular relevance to nociceptive processing and contains significant numbers of AMPA Rs, particularly GluR1 and GluR2. These receptor subunits have been shown to be expressed solely in neurons rather than glia (Todd et al., 1998), and were localised to synaptic densities in lamina II on electron micrography (Tachibana et al., 1994). Most studies have demonstrated that GluR1 labelling was generally more superficial than GluR2/3, which was found at low levels in lamina I and in larger amounts in lamina II and III (Tolle et al., 1993), (Popratiloff et al., 1996), (Coggeshall and Carlton, 1997). The GluR2 subunit protein and mRNA is present in greater amounts than GluR1 in the superficial dorsal horn, and tends to be more evenly distributed through the outer three laminae when investigated using in situ hybridisation (ISHH) and immunocytochemistry (ICC) (Furuyama et al., 1993). GluR1 subunits tend to be localised in neurons in lamina I and outer lamina II (Tolle and et al, 1993), (Yung, 1998). Subunits GluR1, 2/3 are also found in the spinothalamic tract cells, soma and dendrites (Ye and Westlund, 1996). In vivo, it would seem that GluR1 and GluR2 subunits are largely isolated to separate bands in the dorsal horn of the spinal cord, with some overlap within lamina II. Both subunits may occur within individual cells, however (Zhang et al., 1995a).

The other AMPA subunits, GluR3 and 4 are expressed at much lower levels in the superficial dorsal horn. GluR3 is expressed at moderate levels throughout the

outer three laminae, whereas GluR4 is almost undetectable. In the ventral horn, levels of GluR3/4 immunostaining were high in motor neurons, whereas GluR1/2 staining was moderate to weak (Furuyama et al., 1993), (Tolle and et al, 1993).

On a subcellular level, post embedding immunogold electron microscopy has revealed clustering of AMPA R subunits at synaptic sites in the dorsal horn (Popratiloff et al., 1996). GluR1 and GluR2/3 subunits were found between 30nm outside and 40nm inside the postsynaptic membrane of asymmetric synapses. The AMPA R subunits were post-synaptic to two types of central bouton, termed C1 and C2 (RibeiroDaSilva and Coimbra, 1982). The C1 terminals were associated with unmyelinated primary afferents and GluR1 subunits, and the C2 with small myelinated afferents and GluR2/3. Although the majority of AMPA Rs are postsynaptic, some presynaptic AMPA Rs have been demonstrated in lamina II of the dorsal horn, in a spinal cord slice preparation of developing post natal day 9 (P9) rats, playing an inhibitory role by triggering primary afferent depolarisation when bound to their ligand (Lee et al., 2002).

In other CNS regions, the distribution of AMPA R subunits has shed some light on their possible function. Using immunocytochemistry combined with electron microscopy, the subcellular distributions of different AMPA R subunits in the rat caudal nucleus of the solitary tract were found to be mostly distinct. GluR2/3 was found at pre- and postsynaptic sites, and in astrocytic glia; while GluR1 was found primarily in small dendrites and spines. Using subtype-specific antibodies, GluR1 and GluR2 were occasionally colocalised in large dendrites. This study demonstrates that within the nucleus of the solitary tract, single (large) neurons may contain both GluR1 and GluR2, but there is apparently differential trafficking of GluR1 to potential sites for synaptic plasticity (ie: small dendrites and dendritic spines) (Aicher et al., 2002). Unlike other AMPA R subunits, GluR2 undergoes glycosylation with high mannose content, possibly delaying its transit through the endoplasmic reticulum (ER). This ensures that there is a large intracellular pool of GluR2 subunits available for assembly (references in (Bredt and Nicoll, 2003)).

The subcellular location of AMPA Rs has been investigated in hippocampal cell culture using a number of biochemical techniques. Proteolysis of surface receptors by chymotrypsin, cross-linking of surface receptors, and biotinylation of surface receptors all demonstrated similar results. It was found that 60–70% of total

GluR1 protein and 40–50% of total GluR2/3 protein was expressed on the surface of hippocampal neurons (Hall and Soderling, 1997).

Although a high proportion of receptors may be expressed on the cell surface, they may not be located at dendritic sites where they would affect synaptic function. It has been shown that 71% of GluR1 subunits are localised intracellularly in dendritic shafts, 20% on the dendritic shaft surface, 8% in spines and only 3% in postsynaptic densities (Shi et al., 1999).

The movement of GluR2 subunits on the surface of cultured hippocampal cells was investigated in an elegant study using latex-bead labelling of AMPA Rs. Cell surface GluR2 subunits would appear to meander randomly, possibly as a result of diffusion, until anchored at peri-synaptic sites. Localised increases in calcium concentration, as found at sites of synaptic activity, resulted in the accumulation of receptors at the cell surface and their immobilisation (Borgdorff and Choquet, 2002). The subcellular location of GluR1 and GluR2 subunits has not been investigated in adult rat dorsal horn in vivo.

1.6.3 Developmental changes

The expression of AMPA R subunits alters during development. In the adult rat, the majority of GluR1 labelling was found in the neuropil of dorsal horn lamina II and in small cell bodies and proximal processes. In postnatal day 7 (P7) animals, numerous neurons were labelled with very long tapering processes in most regions of spinal grey matter (Jakowec et al., 1995). The subcellular location of receptor subunits also varies as the animal matures. In the neonatal rat hippocampus, most GluR1 is intracellular with little at spines or synapses (Shi et al., 1999). By postnatal day 9, 30-40% of GluR1 and 2 remains intracellular. In the adult, cell surface receptor expression is greater than 85% for GluR1 and greater than 80% for GluR2 (Grosshans et al., 2002). These figures suggest a greater proportion of receptors may be expressed on the cell surface of hippocampal cells than was previously thought, however, it is not known whether these figures reflect the surface expression of receptors in vivo in the dorsal horn (Hall and Soderling, 1997).

In early development AMPA Rs are diffusely distributed in the dendrite, but later become localised at post-synaptic sites. Within individual neurons, the density of AMPA Rs in dendritic spines has been shown to vary throughout spine development. Early small spines are rich in NMDA R but lack AMPA R, perhaps representing silent

synapses, which are electrophysiologically inactive (Durand et al., 1996). As the spines enlarge, the AMPA R content increases, allowing for depolarisation and activation of the NMDA calcium channels at these synapses, causing them to become functional (Takumi et al., 1999).

1.6.4 Calcium permeability

Calcium permeable AMPA Rs form a subgroup of receptors that may play a significant role in the synaptic plasticity involved in the development of chronic pain states (Sorkin et al., 2001). These receptors may potentially alter synaptic strength by providing the appropriate Ca^{2+} signal to activate second messenger systems (Gu et al., 1996). Using kainate induced cobalt uptake in spinal cord slices, Ca^{2+} permeable AMPA Rs can be identified (Pruss et al., 1991). The uptake of cobalt was prevented by selective AMPA antagonism (using GYKI 53655 a selective non-competitive AMPA antagonist) demonstrating that kainate was acting as a ligand at the AMPA R. In addition, treatment with Joro spider toxin (JsTx) a specific antagonist of calcium permeable AMPA Rs and kainate receptors, prevented cobalt loading. Taken together, these two findings demonstrate that cobalt loading is occurring through Ca^{2+} permeable AMPA Rs only (Engelman et al., 1999). In rat pups, Ca^{2+} permeable AMPA Rs were found in lamina I and outer lamina II with heavier labelling towards the lateral edge of the dorsal horn (Engelman et al., 1999).

The presence of the GluR2 subunit determines calcium permeability (Burnashev et al., 1992). AMPA Rs lacking GluR2 have permeability ratios approaching $P_{Ca}/P_{Na} = 3$. Inclusion of GluR2 subunits markedly decreases calcium permeability (Hollmann and Heinemann, 1994). This feature of the receptor maps to a single arginine residue in the pore-forming region of the GluR2 subunit, which is a glutamine in GluR1, 3 and 4. This residue is coded as glutamine (Q) in GluR2 genomic DNA, it is converted to arginine (R) by post-transcriptional mRNA editing. This editing of the 'Q/R' site occurs in 99% of GluR2 mRNAs (Seeburg et al., 1998), (Seeburg, 2002). AMPA Rs with high and low calcium conductivity have been demonstrated within a single retinal ganglion cell demonstrating that individual cells may express more than one type of AMPA R subunit combination (Zhang et al., 1995a). There is evidence that the subunit composition of the receptors can rapidly alter in an activity-dependant manner. Using cerebellar cells, Liu and Cull-Candy demonstrated a rapid and lasting change in the subunit composition and Ca^{2+}

permeability of AMPA Rs following stimulation. Repeated activation of Ca^{2+} permeable AMPA Rs caused a rapid reduction in Ca^{2+} permeability and increased incorporation of GluR2 into AMPA subunits (Liu and Cull-Candy, 2000).

The functional role of Ca^{2+} permeable AMPA Rs

Calcium permeable AMPA Rs would seem to play a role in central sensitisation in pathological pain states, as blockade of these receptors with the specific non-competitive agent Joro spider toxin (JsTx) prevented mechanical hyperalgesia following thermal injury (Sorkin et al., 1999). The mechanism of action of these receptors in modulating nociception is likely to be complex, however, as there is evidence supporting the expression of Ca^{2+} permeable AMPA Rs on inhibitory interneurons and neurons projecting to the brainstem. Cells containing GABA are generally taken to have an inhibitory role, whereas those expressing the SP receptor NK_1 include projection neurons to the spinothalamic tract, which may be pronociceptive. Some Ca^{2+} permeable AMPA Rs were expressed with GABA and some with NK_1 in embryonic dorsal horn cell culture (Albuquerque et al., 1999).

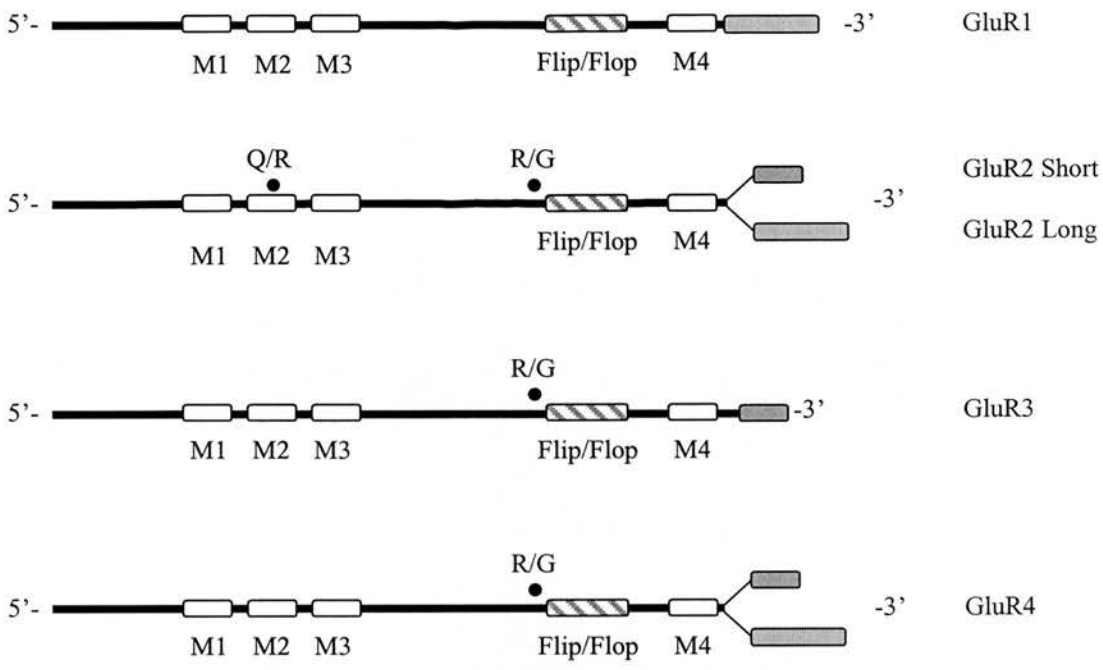
In situ studies have also demonstrated that some NK_1 positive dorsal horn cells coexpressed Ca^{2+} permeable AMPA Rs by cobalt uptake (13 of 38 cells surveyed) (Engelman et al., 1999). As 30% of lamina I neurons project to brainstem regions involved in pain processing (Bice and Beal, 1997) and approximately 80% of lamina I STT cells express NK_1 (Marshall et al., 1996), at least some projection neurons also express calcium permeable AMPA Rs (Engelman et al., 1999). This suggests that these receptors may play a role in the transmission and modulation of ascending nociceptive information.

In contrast to the above, Spike et al discovered that 78% of dorsal horn neurons with GluR1-immunoreactivity were GABA- immunoreactive, and some of these were also glycine- immunoreactive. Almost all of the GluR2/3- immunoreactive neurons were not GABA- or glycine- immunoreactive (Spike et al., 1998). This finding was supported by Albuquerque et al., who found that 39% of dorsal horn neurons expressing Ca^{2+} permeable AMPA Rs were also GABA-immunopositive. In the same paper, it was noted that the great majority of cells expressing the calcium binding proteins calbindin and calretinin, which were potentially excitatory interneurons, did not express Ca^{2+} permeable AMPA Rs (Albuquerque et al., 1999).

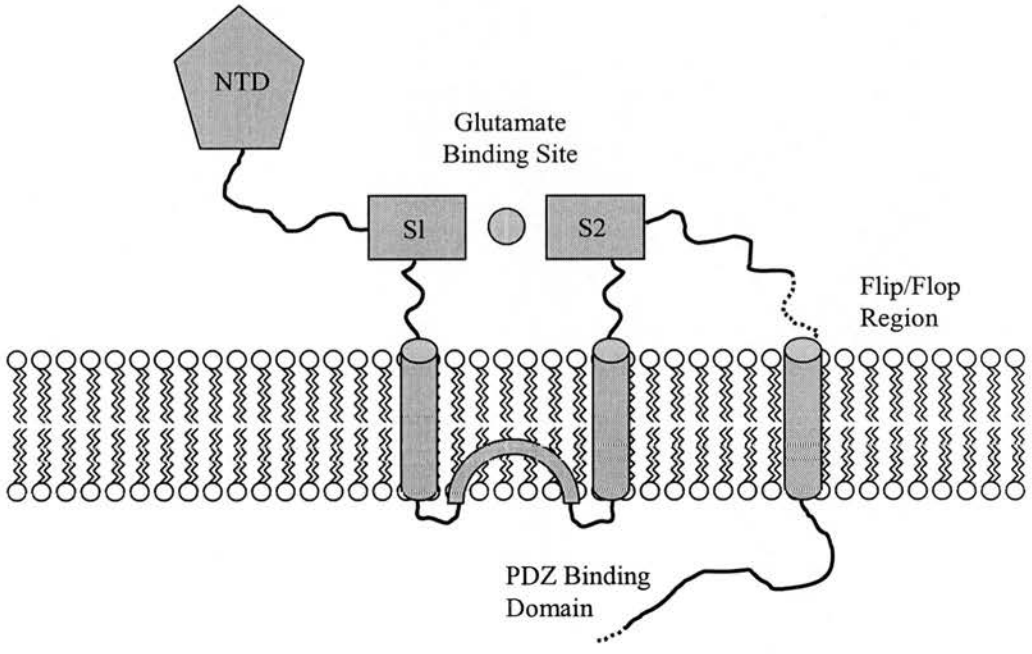
It would seem that a high proportion of GABAergic cells also express GluR1 subunits and Ca^{2+} permeable AMPA Rs. The majority of NK_1 positive cells would also appear to express calcium permeable AMPA Rs. However a significant number of potential excitatory interneurons expressing the calcium binding proteins calbindin and calretinin do not express Ca^{2+} permeable AMPA Rs. From the above evidence, the majority of cells expressing Ca^{2+} permeable AMPA Rs would seem to play an inhibitory role in the neural circuitry of the dorsal horn. However, a significant minority of neurons expressing this receptor subtype may have a modulatory or excitatory role in nociceptive transmission.

Figure 1.1 a) Alternative splicing and editing of AMPA R subunits. The flip/flop and C-terminal splice variants are depicted schematically. The transmembrane regions are shown as boxes M1 to M4. The Q/R and R/G editing sites are also shown. Adapted from (Dingledine et al., 1999). b) Diagrammatic representation of the molecular structure of an AMPA R subunit within the cytoplasmic membrane. The extracellular N-terminal domain is denoted NTD, and the two extracellular domains S1 and S2 are shown binding to the glutamate ligand. M1 to M4 domains are depicted as grey cylinders and an arc. Four such subunits combine to form a functional receptor. c) Diagrammatic representation of AMPA R cycling to and from the synaptic membrane. The cycling loop on the left represents the activity dependent insertion of 'long tailed' AMPA Rs containing the GluR1 subunit. The right hand loop represents the constitutive cycling of 'short tailed' receptors into synapses already containing AMPA Rs. Adapted from (Bredt and Nicoll, 2003).

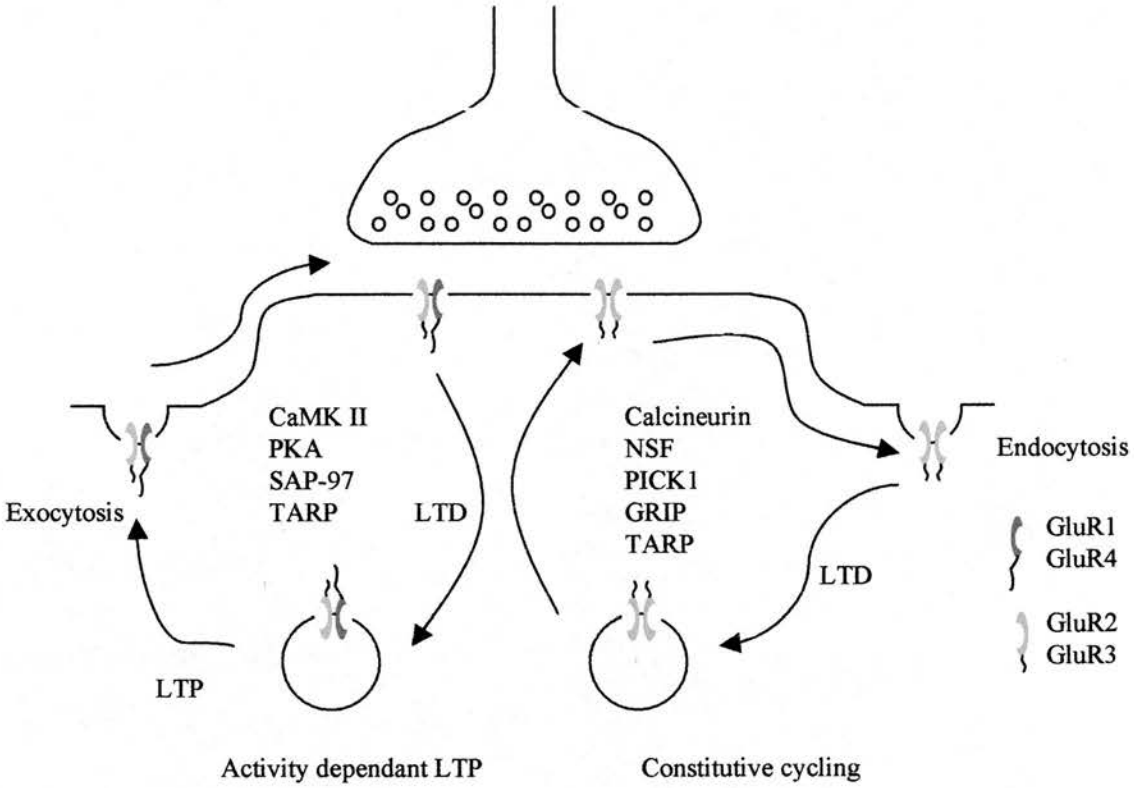
a)


5'-
-3'
GluR1
M1 M2 M3
Flip/Flop
M4
Q/R
R/G
GluR2 Short
GluR2 Long
R/G
GluR3
R/G
GluR4

b)


NTD
Glutamate
Binding Site
S1
S2
Flip/Flop
Region
PDZ Binding
Domain

c)


CaMK II
PKA
SAP-97
TARP
LTD
Calcineurin
NSF
PICK1
GRIP
TARP
Endocytosis
Exocytosis
LTD
GluR1
GluR4
GluR2
GluR3
LTP
Activity dependant LTP
Constitutive cycling

1.7 Neuronal plasticity in neuropathic pain

The neuronal plasticity occurring in chronic pain is the result of a process of peripheral and central sensitisation. The potential mechanisms involved in these processes are discussed below, with emphasis on the role of the AMPA R in neuropathic pain.

1.8 Peripheral sensitisation

Peripheral sensitisation is characterised by changes in nociceptor function including decreased threshold, increased response to supra-threshold stimuli and spontaneous activity. These changes in the primary afferent neuron are triggered by the release of inflammatory mediators at the site of injury (Dray et al., 1994). Arachidonic acid metabolites such as prostaglandin E2 (PGE2), bradykinin and nerve growth factor are released in inflammation and tissue injury and act on G-protein coupled receptors or tyrosine kinase receptors on nociceptor terminals. Second messenger systems may result in the phosphorylation of receptors on the nociceptor terminal altering their kinetics or increasing their sensitivity to ligand binding (Julius and Basbaum, 2001). This aspect of neuronal plasticity will not be considered further in this study.

1.9 Central sensitisation and the AMPA receptor

Central sensitisation has been defined as an immediate-onset activity-dependent increase in the excitability of nociceptive neurons in the dorsal horn as a result of and outlasting an intense peripheral noxious stimulus, tissue injury or nerve damage (Woolf, 1996), (Baranauskas and Nistri, 1998), (Ji et al., 2003). Changes occurring in spinal dorsal horn neurons include increased excitability, hyper responsiveness to afferent input and increased after discharges (Palecek et al., 1992), (Laird and Bennett, 1993), (Sotgiu et al., 1995).

AMPA ionotropic glutamate receptors have long been known to be involved in the transmission of acute nociceptive and innocuous signals (King and Lopez-Garcia, 1993). Iontophoretic treatment with AMPA results in depolarisation of dorsal horn wide dynamic range neurons in rats. These electrophysiological responses may be antagonised by systemically administered competitive and non-competitive AMPA antagonists (the quinoxalinedione NBQX and the 2,3-benzodiazepine GYKI 53655). Both NBQX and GYKI 53655 dose- dependently reduced responses to peripheral

innocuous touch and noxious heat stimuli and to AMPA (Budai and Larson, 1994), (Cumberbatch et al., 1994). Similar findings were also revealed in a study of STT cell responses in a primate model. Blockade of non-NMDA receptors resulted in a marked decrease in the responses of STT neurons to all stimuli (Dougherty et al., 1992).

There is also evidence for the involvement of AMPA Rs in mediating acute thermal pain. Intrathecal AMPA antagonist treatment resulted in an antinociceptive effect in a rat hot plate model of acute noxious thermal stimulation, which proved to be strongly synergistic with the antinociceptive effect of opioids (Nishiyama et al., 1998). There is evidence, therefore from several species and using electrophysiological and behavioural techniques to suggest that the AMPA R plays a role in acute nociceptive and non-nociceptive sensory transmission. The function of this receptor in mediating chronic pain and central sensitisation in the spinal cord is not as clear, however.

The wide range of functional and structural changes occurring in neurons and synapses in central sensitisation result from multiple intracellular signalling mechanisms activated by the neurotransmitter glutamate, and neuromodulators such as SP, brain-derived neurotrophic factor (BDNF) and other peptides. Once sensitisation has been established, even low levels of ongoing stimulation will allow it to persist (Gracely et al., 1992), (Liu et al., 2000).

Central sensitisation may occur immediately, as in electrophysiological stimulation studies (Cook et al., 1987). This form of sensitisation does not require protein synthesis and is therefore termed transcription-independent or classical activity-dependent central sensitisation. Other forms of immediate-onset, transcription-independent plasticity may occur in the spinal cord under certain conditions and these are termed wind-up and LTP. These processes are considered only briefly. Later stages of central sensitisation involving protein synthesis are termed transcription-dependent and may be further divided into activity-dependent and activity-independent (driven by inflammation, for example) (Ji et al., 2003).

There is strong evidence that a combination of loss of function of inhibitory mechanisms such as local GABAergic interneurons (Moore et al., 2002) and an increase in excitatory mechanisms, such as mGluR mediated activation of the NMDA R (Ji et al., 2003), occurs in central sensitisation. The AMPA R may play a role in both aspects of central sensitisation and is the focus of this study, particularly with reference to potential mechanisms of neuropathic pain.

It is known that NMDA antagonists reduce spontaneous pain and thermal hyperalgesia in a CCI model of peripheral neuropathy in the rat (Mao et al., 1992b). However, it was also found that intrathecal treatment with the non-NMDA R antagonists HA966 or CNQX beginning prior to nerve ligation (pre-injury treatment) reduced thermal hyperalgesia in CCI rats after nerve ligation. However, this treatment was ineffective once hyperalgesia was established. In contrast, a single treatment with the NMDA antagonist MK 801 was effective even when hyperalgesia had developed. These findings suggest that non-NMDA R may initiate thermal hyperalgesia following nerve injury, which is then maintained by NMDA R activation (Mao et al., 1992a). In addition to its role in neuropathic thermal hyperalgesia, co-activation of non-NMDA iGlu receptors and mGlu receptors has been shown to produce mechanical hyperalgesia in a study based on a behavioural paradigm (Meller et al., 1993).

In contrast to the NMDA receptor, the AMPA R has not been considered as a likely therapeutic target in the management of chronic pain, as non-specific AMPA R blockade results in loss of non-noxious somatosensory transmission as well as nociception. However, recent studies targeting two very different aspects of AMPA R pharmacology have revealed the potential of highly specific agents in alleviating hyperalgesia in models of neuropathic and inflammatory pain. The role of intracellular adapter proteins and calcium permeable subtypes of the AMPA R has been highlighted by these findings. Using a rat model of neuropathic pain (CCI, (Bennett and Xie, 1988)), thermal hyperalgesia was alleviated by blocking the interaction between the GluR2 receptor and the intracellular adapter proteins GRIP (glutamate receptor interacting protein) and PICK1 (protein interacting with C kinase 1) (Garry et al., 2003b). In addition, intrathecal administration of Joro spider toxin (JsTx), a calcium permeable AMPA R antagonist, blocked thermal injury-induced mechanical allodynia in a rat behavioural paradigm (Sorkin et al., 1999). This antagonist also prevented the induction of thermal hyperalgesia and mechanical allodynia given one hour after carrageenan injection into the rat hindpaw, providing evidence for the role of calcium permeable AMPA Rs in acute and chronic inflammatory pain. However, JsTx had no effect on spontaneous pain behaviour after subcutaneous formalin injection, on allodynia after spinal nerve ligation, or when given three hours after carrageenan (Sorkin et al., 2001).

It is clear, therefore, that AMPA Rs play a role in the central sensitisation underlying both inflammatory and neuropathic chronic pain. Receptors containing the GluR1 or GluR2 subunit are expressed at high levels in the superficial dorsal horn and would seem to be particularly important in these processes.

1.9.1 Activity dependent central sensitisation

In the initial stages of immediate onset central sensitisation, two processes are important: alteration in ion channel / receptor activity due to post-translational receptor modification and trafficking of receptors to the synaptic membrane (Woolf and Salter, 2000). These mechanisms are discussed below with reference to the AMPA R.

1.9.2 AMPA receptor phosphorylation

Evidence suggests that the post-translational processing of receptors, such as phosphorylation of key amino acids, is an important step in the development of central sensitisation (Woolf and Salter, 2000). The release of neurotransmitters and neuromodulators from the presynaptic cell results in the activation of ligand-gated ion channels (NMDA, AMPA and kainate), G-protein coupled mGluRs, the SP receptor NK_1 and tyrosine kinase receptors trkB and Eph. Activation of kinases leads to phosphorylation of various receptor proteins altering their structure and function. In addition, trafficking of AMPA Rs to and from the cell membrane has been shown to play an important role in LTP and potentially in central sensitisation in the spinal cord, but as yet there is no direct evidence to support this possibility.

Several protein kinases are involved in the development of central sensitisation, including protein kinases A, C, and G (PKA, PKC and PKG) and calcium / calmodulin-dependent protein kinase II (CaMK II). Blockade of PKC activity has been shown to inhibit the generation of a sensitised state after intradermal capsaicin injection, in primate STT cells (Lin et al., 1996). PKC may be activated by ligand binding to G-protein linked membrane receptors such as the mGluRs (Schoepp and Conn, 1993), (Schoepp et al., 1999). PKC may then act to increase NMDA channel opening and reduce the voltage dependant Mg^{2+} block of this receptor (Chen and Huang, 1992).

The central processes occurring in inflammation and neuropathic pain share some similarities, however significant differences exist. There is evidence to suggest a

differential role for protein kinase A (PKA) and protein kinase C (PKC) in inflammatory and neuropathic pain. Mutant mice lacking the RIβ subunit of PKA express normal behavioural responses to acutely noxious stimuli. However, pain behaviour in response to inflammation is markedly reduced in these animals. The PKCγ isoform of PKC is a calcium dependant kinase, found in the CNS. Mice lacking this kinase also demonstrate normal responses to acutely noxious stimuli, but do not develop thermal hyperalgesia or mechanical allodynia after partial sciatic nerve section. This would suggest a specific role for PKA in the development of central sensitisation in response to prolonged inflammatory stimulation, and for PKCγ in the development of neuropathic pain (references in (Petersen-Zeitz and Basbaum, 1999)).

CaMK II is found throughout the central nervous system. It regulates calcium signalling in synaptic transmission by phosphorylating various proteins, including neuronal membrane receptors and intracellular transcription factors. There is an increased expression and phosphorylation of CaMK II in rat spinal dorsal horn neurons after noxious stimulation by intradermal injection of capsaicin (Fang et al., 2002). Inhibition of this kinase by intrathecal administration of a specific blocker has been shown to prevent the enhanced electrophysiological responses of spinal nociceptive neurons and the typical changes in exploratory behaviour to capsaicin treatment (references in: (Ji et al., 2003)). Retrograde labelling techniques demonstrated that CaMK II is expressed in STT neurons, and was therefore likely to be involved in the transmission of nociceptive information (Fang et al., 2002).

There is evidence to suggest that CaMK II exerts its effects on the nociceptive pathway via the phosphorylation of the GluR1 subunit of the AMPA R. It has been demonstrated that CaMK II phosphorylation of GluR1 enhances AMPA-mediated synaptic transmission by increasing the single channel conductance of existing AMPA Rs and attracting new AMPA Rs to the synapse (Soderling and Derkach, 2000). Two phosphorylation sites have been discovered at the C-terminus of the GluR1 subunit. CaMK II phosphorylates GluR1 at Ser^{831} resulting in a 40% potentiation in the peak current through GluR1 homomeric channels, and PKA at Ser^{845} which accompanies the surface insertion of GluR1 (Roche et al., 1996), (Ehlers, 2000). However, insertion of GluR1 receptors at synapses has not yet been shown to contribute to central sensitisation in the spinal cord (Ji et al., 2003).

Rapid, early phosphorylation of GluR1 has been demonstrated after intradermal capsaicin treatment. Levels of phospho-GluR1 (at Ser^{845} and Ser^{831} sites)

in the spinal cord, as determined by Western Blot and immunohistochemical analysis, increased significantly from 5 minutes to at least 60 minutes post capsaicin treatment. The level of GluR1 protein itself did not alter after treatment (Fang et al., 2003). This very early activation of GluR1 phosphorylation in inflammation, mediated by PKA and CaMK II, may initiate many of the changes seen in the later stages of central sensitisation.

Recent studies have provided evidence to suggest that CaMK II may also play a role in neuropathic pain. Blockade of CaMK II activity in wild type mice prevented the development of behavioural reflex sensitisation following CCI injury (Garry et al., 2003a).

1.9.3 AMPA receptor trafficking

AMPA Rs mediate fast excitatory neurotransmission and it is clear that AMPA R concentration at the post-synaptic density (PSD) must be closely regulated, as the number of these iGluRs expressed at this site is a major determinant of postsynaptic excitability. Silent synapses in the dorsal horn do not transmit fast post-synaptic responses and may be activated by the recruitment of AMPA Rs (Wall, 1977), (Li and Zhuo, 1998). Activation of such synapses by AMPA R recruitment may play an important part in the generation of sensitisation in the spinal cord. After induction of LTP, it was noted that AMPA R-mediated EPSPs increased but NMDA R-mediated events did not, suggesting that the changes in neuronal excitability seen in this paradigm may to be due to the inclusion of increased functional AMPA Rs at the synapse (Muller et al., 1988).

AMPA Rs have been shown to be inserted and removed from the cell membrane by mechanisms common to many receptors, proteins and neurotransmitters (Man et al., 2000). There is currently little direct evidence to suggest that AMPA R trafficking occurs in central sensitisation in neuropathic pain, however it is likely that a similar process may take place in dorsal horn neurons.

Electrophysiological studies utilizing hippocampal CA1 pyramidal cell cultures have demonstrated that blocking membrane insertion of vesicles decreases AMPA R EPSPs, suggesting that active AMPA Rs were being removed from the synapse, but the insertion of new receptors had been blocked. Conversely, when endocytosis was prevented using GDPβS to block the GTPase activity of dynamin,

AMPA mediated EPSPs increased in amplitude by 100% over time. This suggests that functional AMPA Rs were being delivered to the synapse, but their removal by a process requiring endocytosis had been blocked. In both studies, NMDA currents were unaffected (Luscher et al., 1999).

Once delivered to the membrane, AMPA Rs would appear to move freely until they reach synaptic areas where they become tethered. In order to maintain a stable receptor population at the synapse, clathrin-dependant internalisation also occurs to balance the process of adding new receptors to the membrane. The free moving pool of receptors may act as a further reservoir for inclusion into synapses. Several proteins are involved in the processes of endocytosis, which may internalise AMPA Rs. These have been reviewed by Jarousse and Kelly and will not be considered in detail here (Jarousse and Kelly, 2001). The possible mechanisms of AMPA R trafficking are outlined in Figure 1.1c.

There is evidence to suggest that AMPA Rs are continuously cycled to and from the membrane without stimulation, some 7% of surface GluR1 were constitutively internalised within ten minutes. Binding of ligands to AMPA Rs causes a more rapid redistribution, with 46% of GluR1 subunits being internalised within 10 minutes of AMPA stimulation (Carroll et al., 1999), (Luscher and Frerking, 2001). Activation of the NMDA R has also been shown to trigger AMPA R internalisation in cultured kidney cells, via activation of calcineurin and dephosphorylation of GluR1 at a PKA phosphorylation site. Thus, the phosphorylation state of GluR1 at the PKA site may determine the fate of internalised AMPA RS (Luscher and Frerking, 2001). Calcineurin is likely to play a significant role in receptor internalisation, as it is known to dephosphorylate a number of proteins involved in endocytosis, including the key protein dynamin.

In addition to AMPA R internalisation by iGluR ligand binding, there is evidence to suggest that the group I mGluR agonist DHPG may trigger GluR1 and GluR2 subunit internalisation in cultured hippocampal neurons (Snyder et al., 2001). Mechanisms unrelated to glutamate receptors may also play a role in AMPA R internalisation. It has been demonstrated that AMPA Rs also undergo internalisation by a GluR2-dependant mechanism in response to insulin, suggesting that there may be more than one mechanism for receptor internalisation (Man et al., 2000). Finally, calcium entry into the cell is required for at least some forms of AMPA R

internalisation, as this process is markedly inhibited by the L-type calcium channel blocker nimodipine (Lin et al., 2000).

Study of the insertion of AMPA Rs into the post-synaptic membrane has proved to be difficult, as most stimulation protocols applied to cultured cells lead to receptor internalisation. In the resting state in cultured CA1 hippocampal neurons, the majority of GluR1 are not associated with synaptic sites. Shi et al demonstrated that tetanic stimulation of these cells leads to delivery of receptors to the spines and clustering of receptors within dendrites (Shi et al., 1999). Time-lapse two-photon laser scanning microscopy revealed that tetanic stimulation of cultured neurons caused delivery of fluorescent-tagged GluR1 subunits to the spines and clustering of receptors within the dendritic shaft. These events were prevented by NMDA R antagonism. This suggests a mechanism for delivery of GluR1-containing AMPA Rs to dendritic spines involving increased synaptic activity and activation of NMDA Rs.

From the above summary, it can be seen that AMPA Rs may control the effectiveness of synaptic transmission by trafficking into and out of the synapse. This may be of great relevance to the induction of sensitised states in the hippocampus and spinal cord. It is also clear that AMPA R trafficking involves interaction with a number of other key receptors and proteins, some of which are considered below.

1.9.4 Protein-protein interactions of the AMPA receptor

There is increasing evidence to suggest that numerous proteins interacting with AMPA R subunits play an important role in the generation of sensitised states such as neuropathic pain (Garry and Fleetwood-Walker, 2004). The most significant protein interaction module that can associate with the AMPA R is known as the PDZ (PSD-95, Discs-large, ZO-1) domain (see Figure 1.1a, b). The PDZ domain is a 90 amino-acid structure, which often binds to the extreme C-terminal end of proteins. Thus far, over 100 proteins within the human genome have been found to contain PDZ domains. The first to be discovered was postsynaptic density protein-95 (PSD-95), which associates with the extreme C-terminal end of the NR2 subunit of the NMDA R.

In the hippocampus, the majority of AMPA Rs contain either GluR1 / GluR2 subunits, or GluR2 / GluR3 subunits in combination. The subunits exist in two forms with long and short cytoplasmic C terminal tails, the former have a PDZ motif and the latter do not. GluR1 always has a long tail and GluR3 a short one. The tails of GluR2

and 4 may be either long or short, depending on alternative splicing. The length of the tail, and therefore the presence of a PDZ motif would seem to be important in determining the delivery of these receptors to synapses. Long-tail receptors (incorporating GluR1 subunits with a PDZ motif) require synaptic activity for delivery to spines, whereas short tailed AMPA Rs (consisting of the short form of GluR2) are continuously cycled to and from synapses (Shi et al., 2001), (references in (Bredt and Nicoll, 2003)).

The AMPA R subunits GluR1 and GluR2 interact with different protein groups, which may shed light on their specific roles in receptor trafficking. AMPA Rs containing the GluR1 subunit and lacking GluR2 may have a high degree of calcium permeability and their inclusion in active synapses may be important in synaptic strengthening and neuropathic sensitisation in the dorsal horn (Gu et al., 1996), (see section 1.6.4).

AMPA Rs containing GluR1 subunits are added to synapses during neuronal plasticity; requiring an interaction between GluR1 and PDZ domain proteins in a CaMK II-dependant mechanism (Hayashi et al., 2000). The GluR1 subunit binds via its C-terminal tail to the postsynaptic density protein SAP-97, and interacts with actin-associated protein 4.1N, which has been shown to increase AMPA R density at postsynaptic sites in cell cultures (Shen et al., 2000), (Rumbaugh et al., 2003). It has become apparent that trafficking of GluR1 subunits is highly regulated with distinct stages of exocytosis and synaptic insertion (Luscher and Frerking, 2001), (Passafaro et al., 2001), (Shi et al., 2001).

The GluR2 subunit is expressed at high levels in the superficial dorsal horn and interacts with a number of key proteins (Jakowec et al., 1995). Several of the proteins interacting with GluR2/3 have been the subjects of recent study, including: GRIP; PICK1; ABP (AMPA R binding protein) (Srivastava et al., 1998) and NSF (N-ethylmaleimide-sensitive fusion protein).

PICK1, originally identified as a protein kinase Cα binding protein, may serve to bring this kinase close to the AMPA R in the plasma membrane resulting in the removal of the GluR2 subunit from the synapse (Xia et al., 1999), (Perez et al., 2001).

Mutation of the GluR2 subunit at a single amino acid site (S880A) interferes with GRIP / ABP binding and results in decreased AMPA clustering at the membrane, suggesting that ABP and / or GRIP are required to stabilise the receptor at the PSD (Osten et al., 2000). This study supports the theory that phosphorylation of GluR2 at

the Ser 880 site by PKC results in a form of subunit that does not bind GRIP and is released from the membrane.

Recently, it has been demonstrated that blockade of the interaction between GRIP / PICK1 and GluR2 results in a marked attenuation of the thermal hyperalgesia seen after induction of reflex sensitisation by CCI. Specific blockade of PICK1 interactions had a similar effect. Both interventions had limited effect on mechanical allodynia, however. This study demonstrates the importance of GluR2 / PICK1 interaction in the maintenance of thermal hyperalgesia in neuropathic pain (Garry et al., 2003b).

Ligand binding to the AMPA R is known to cause receptor internalisation. Previously, GRIP has been demonstrated at the PSD and in the cytosol (Wyszynski et al., 1998), (Dong et al., 1999). Topical application of AMPA to the spinal cord caused GRIP and PICK1 to leave the membrane fraction and enter the cytosol. This was prevented by blockade of clathrin-mediated endocytosis, suggesting that GRIP, PICK1 and GluR2 are internalised in response to AMPA stimulation (Garry et al., 2003b).

The GluR2 and GluR4 subunits also bind NSF, an ATPase involved in membrane fusion, which may mediate trafficking or membrane fusion of vesicles containing GluR2. Binding to NSF stabilizes the AMPA R subunit at the membrane (Nishimune et al., 1998), (Osten et al., 1998). Blockade of the interaction between NSF and GluR2 resulted in a marked attenuation of the thermal hyperalgesia seen after CCI-induced reflex sensitisation (Garry et al., 2003b).

It has also been demonstrated that blockade of GluR2 / NSF interaction resulted in a decrease in AMPA R EPSCs. Ligand-induced internalisation of AMPA Rs was also increased by N-ethylmaleimide blockade of the NSF-GluR2 interaction (Luscher et al., 1999). This study suggests that AMPA Rs constantly cycle to and from the membrane. Interruption of either the endocytosis or insertion of vesicles appears to alter the number of functional AMPA Rs at the cell surface and hence the currents transmitted by AMPA R stimulation.

1.9.5 Additional AMPA R-interacting proteins

Some synaptic proteins interact with AMPA Rs irrespective of their subtype composition. In addition to the PDZ binding domains, AMPA Rs contain other binding sites, such as the N-terminal domain (NTD), which binds proteins such as the

extracellular neuronal activity-regulated pentraxin (Narp) (O'Brien et al., 1999). Binding of GluR1, 2 or 3 subunits to Narp has been shown to increase clustering and surface expression of AMPA Rs at excitatory synapses (O'Brien et al., 2002). The NTD may have other, as yet undiscovered functions, as over expression of this region of GluR2 subunits induces the formation of dendritic spines in cultured hippocampal cells (Passafaro et al., 2003).

Another synaptic protein known as stargazin, a member of a family of similar transmembrane AMPA R regulatory proteins (TARPs) interacts with GluR1, 2 and 4 (Tomita et al., 2003). This protein, first discovered in the ataxic mutant stargazer mouse binds via its C-terminal tail to PSD-95 in addition to AMPA Rs.

PSD-95 seems vital in determining the number of AMPA Rs at synaptic sites, whereas stargazin seems to determine their position relative to the cell surface. At least 50% of AMPA Rs containing the GluR2 subunit would appear to be localised beneath the cell membrane in hippocampal neurons, many within vesicles measuring 50-300nm containing GRIP and PICK1 (Lee et al., 2001). This pool of receptors can be recruited to the surface by over-expression of stargazin (Chen et al., 2000). Over expression of PSD-95 results in increased expression of AMPA Rs at the cell surface and increased AMPA transmission (El Hussein et al., 2000).

Recent evidence suggests that PSD-95 may have a specific role in the establishment and maintenance of sensitisation in neuropathic pain. Mutant mice expressing an abnormal variant of the multivalent adaptor protein PSD-95 in the dorsal horn of the spinal cord failed to develop the NMDA receptor-dependent hyperalgesia and allodynia seen in the CCI model of neuropathic pain. In contrast to this finding, normal inflammatory nociceptive behaviour was noted following the injection of formalin. In wild type mice, CaMK II associates with NMDA Rs in the generation of central sensitisation via a PSD-95 protein link. It would seem that disruption of CaMK II docking to the NMDA receptor may be important in the lack of neuropathic behavioural reflex sensitisation in PSD-95 mutant mice (Garry et al., 2003a).

1.10 AMPA RECEPTOR INVOLVEMENT IN OTHER FORMS OF ACTIVITY-DEPENDENT CENTRAL SENSITISATION: WIND-UP AND LONG TERM POTENTIATION

Glutamate receptors play a key role in the generation of wind-up. The ionotropic glutamate receptors have been extensively studied, particularly the NMDA

R. Treatment with NMDA R antagonists has been shown to attenuate wind-up (Woolf and Thompson, 1991), (King and Lopez-Garcia, 1993).

It has been suggested that co-activation of mGluRs and iGluRs may be required for wind-up. In vivo studies have demonstrated that induction of wind-up is enhanced by application of ACPD an mGluR group I agonist. In addition, electrically evoked wind-up was reduced by application of an mGluR group I antagonist. It was suggested that the mGluR acts to facilitate the response of iGluR to stimuli, as there was also an increase in the neuronal response to iGluR agonists (Budai and Larson, 1998). There are clearly some similarities between wind-up and central sensitisation in the spinal cord, but the two processes are distinct (Meller et al., 1993).

LTP refers to activity dependant changes in the efficacy of synaptic communication. Increases in postsynaptic $[Ca^{2+}]_i$ are sufficient to generate LTP. For the majority of glutamatergic synapses this can be achieved by calcium influx through NMDA Rs, but voltage-gated Ca^{2+} channels, calcium-permeable AMPA Rs or calcium release from intracellular stores (via activation of mGluRs) may all play a role (references in (Sandkuhler, 2000)). Enhanced excitatory transmission during LTP reflects greater responsiveness of post synaptic AMPA Rs caused by increased sensitivity of each receptor and / or insertion of more AMPA Rs into the postsynaptic membrane (Malenka and Nicoll, 1999). After induction of LTP, AMPA R responsiveness increases over about 30 minutes in the CA1 region of the hippocampus (Soderling and Derkach, 2000). It appears likely that this increase in the AMPA R response to glutamate is due partly to phosphorylation of GluR1 (Derkach et al., 1999).

It can be seen that LTP requires post-translational modification of AMPA Rs and an increase in AMPA R delivery to the synapse, very much like central sensitisation. Indeed, an LTP-like process has been demonstrated in the spinal cord. Following a brief period of very rapid C fibre stimulation (100Hz), the AMPA R mediated responses of a subgroup of lamina I neurons expressing the NK_1 R increased for tens of minutes. However, as the frequency of stimulation required to cause these changes is outside the physiological range, its functional significance is unclear (Ji et al., 2003), (Yang et al., 2004a).

It is important to appreciate the differences between LTP and central sensitisation when developing practical therapies to treat chronic pain. Treatments which are effective in blocking both LTP and central sensitisation, such as the NMDA

antagonist ketamine, tend to have unacceptable neuropsychiatric side effects (Hocking and Cousins, 2003). Ideally, central sensitisation in the spinal cord could be prevented, but memory formation left intact.

1.11 TRANSCRIPTION DEPENDENT CENTRAL SENSITISATION

In parallel with neuronal plasticity occurring via synaptic receptor recruitment and post-translational modification, long lasting changes in neuronal excitability occur which involve the activation of transcription factors via complex signal-transduction pathways, altering gene expression and protein synthesis in the DRG and DH (Hokfelt et al., 1994), (Ji and Woolf, 2001).

The dramatic effects of nerve damage on gene expression have been recently demonstrated using a cDNA microarray. Changes in levels of 14 channels, 25 receptors and 42 signal-transduction related molecules occurred in rat dorsal spinal cord 14 days after peripheral axotomy. These regulated genes include calcium and sodium channel subunits, AMPA R GluR3 and 4 subunits (however GluR1 and 2 were not tested), $GABA_A$ receptor subunits, nicotinic acetylcholine receptors, PKCα, β and δ isozymes, JNK1-3, ERK2-3, and p38 MAPK protein kinases (Yang et al., 2004b). Studies based on human DRG specimens following brachial plexus avulsion injury have revealed equally complex changes in gene expression. The expression of 91 genes were altered following injury, some of which are known to be involved in neurotransmission, trophism, cytokine functions, signal transduction, myelination, transcription regulation, and apoptosis (Rabert et al., 2004).

Peripheral nerve injury results in injury signals mediated by kinases, such as extracellular signal-regulated kinases (ERKs) and PKG, which regulate gene transcription (Sung and Ambron, 2004). Key among such transcription-regulating messengers are the mitogen-activated protein (MAP) kinase family of serine / threonine kinases (Widmann et al., 1999). The family has multiple members, 3 of which are considered here: the ERKs, c-Jun N-terminal kinase (JNK) and p38 MAPK.

The role of ERKs in a number of processes involving neuronal plasticity has been clearly demonstrated. Activation of these kinases is dependant upon NMDA R activation and Ca^{2+} influx in neurons and is required for LTP (Bading and Greenberg, 1991), (Rosen et al., 1994), (Impey et al., 1999). ERK activation by phosphorylation also occurs following central sensitisation by capsaicin and noxious thermal and

mechanical stimuli (Ji and Woolf, 2001). Some of these changes occur within 30-60mins and are therefore probably transcription independent.

In addition, some activated ERK translocates to the nucleus and phosphorylates key transcription factors such as the cAMP-response-element-binding protein (CREB) (Impey et al., 1998), (Widmann et al., 1999). This transcription factor has been implicated in the regulation of several genes involved in long-term neuronal plasticity (Bonni et al., 1995), (Impey et al., 1996). Other transcription factors such as c-Fos, c-Jun, Elk-1 may collaborate with CREB to regulate pain related gene transcription (Ji and Woolf, 2001).

JNK is also activated following peripheral axotomy in DRG. The delay in activation was dependant upon the distance of injury from DRG suggesting a retrogradely transported signal molecule was involved (Kenney and Kocsis, 1998). In addition, p38 MAPK is activated by peripheral inflammation and nerve injury in spinal microglia and has been shown to mediate NGF-induced CREB activation (Xing et al., 1998), (Kim et al., 2002). Several genes coding for proteins important in pain processing, such as TNF-α and IL-1β, are positively regulated by this kinase (Widmann et al., 1999).

As is clear from the above studies, transcription dependant central sensitisation represents a complex and expanding field of research, which will not be considered further here.

1.11.1 Neurotrophic factors and neuropathic pain

Neurotrophins constitute a family of molecules including nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) (Thoenen, 1991). Neurotrophins signal by binding to low and high affinity receptors. The p75 receptor binds all three neurotrophins, with low affinity, whereas the trkA (NGF), trkB (BDNF and NT4/5) and trkC (NT-3) tyrosine kinase receptors show high affinity and selective interactions with particular ligands (references in (Mendell et al., 2001)).

In a spinal nerve ligation model of neuropathic pain, intrathecal treatment with anti-BDNF antibody attenuated thermal hyperalgesia for 2 to 4 hours, and local application of anti-NGF antibody to the L4 spinal nerve beside the L5 spinal nerve-ligation site prevented the development of thermal hyperalgesia for 6 days following ligation. This study demonstrates the importance of both BDNF and NGF in the

development of thermal hyperalgesia after spinal nerve ligation (Fukuoka et al., 2001). NGF is thought to be critical for gene activation in the DRG, it induces an increased expression of SP, CGRP and BDNF (Lindsay and Harmar, 1989), (Michael et al., 1997). It has also been suggested that up regulation of BDNF expression in undamaged neurons following CCI may play an important role in the development of neuropathic pain (Obata et al., 2003).

1.11.2 The interaction between injured and non-injured axons in neuropathic pain

Central sensitisation may be described as a state of abnormal central processing maintained by continuing peripheral input (Gracely et al., 1992). It has been demonstrated that ectopic action potentials occur in damaged nerves which may contribute to central sensitisation (Sotgiu et al., 1994), (Liu et al., 2000). In addition to these abnormal stimuli, the importance of sensory input through undamaged axons and nerves has become apparent.

Using a spinal nerve ligation model of peripheral neuropathy, it has been found that noxious and innocuous sensory input from undamaged nerves is important in determining the severity of mechanical hyperalgesia resulting from the injury. In addition to tight ligation of the left L5 and L6 spinal nerves, the intact L4 spinal nerve was manipulated either by gentle repeated stretching of the L4 spinal nerve immediately after L5 and L6 SNL or by intermittent mechanical stimulation to the ipsilateral paw during the first week after SNL. It was found that both mild immediate stretch of L4 spinal nerve and application of mechanical stimuli to the ipsilateral paw significantly increased the development of mechanical allodynia after SNL. It may be that L4 stretch resulted in trauma to the nerve causing further ectopic discharges, but it is clear that moderate stimulation to skin served by undamaged adjacent nerve roots augmented the development of a sensitised state. Both these stimuli are mediated in part via AMPA Rs (Lee et al., 2003).

In the naïve animal, sciatic dorsal horn WDR neurons (L5/6) do not respond to stimulation of the saphenous nerve (L2). However, 4 to 14 days after sciatic CCI, 90% of sciatic (L5/6) neurons responded to saphenous (L2) stimulation ipsilateral to the injury. There was no L5/6 response to saphenous stimulation contralateral to injury (Sotgiu and Biella, 1995). This interesting finding suggests that input from adjacent undamaged spinal segments may impinge upon damaged neurons after nerve injury.

These abnormal inputs play a role in central sensitisation as stimulation of saphenous (L2) fibres increased the electrophysiological responses of sciatic neurons following sciatic CCI. Strengthening of such 'improper' neuronal connections is likely to be due to AMPA R recruitment and AMPA R activation by non-noxious and noxious stimuli. Abnormal sensory input from adjacent segments appears to be important in the development of electrophysiological changes typical of thermal hyperalgesia, as sectioning of the saphenous nerve prevented these changes (Sotgiu and Biella, 1997).

Interactions may also occur between damaged and undamaged axons within a single nerve. The type of sensory changes found after CCI would also seem to be dependent upon the proportion of damaged and undamaged neurons. Utilising activating transcription factor 3 (ATF3) expression as a marker of neuronal injury in DRG cells, Obata et al demonstrated that at least 12.5% of DRG neurons must be damaged for the development of thermal hyperalgesia. Mechanical hyperalgesia occurred only when 12.5%-25% of DRG neurons demonstrated damage. This equates to approximately 30-40% of sciatic axons undergoing axotomy. Interestingly, the same study revealed that the neurotrophic factor BDNF was upregulated in undamaged small and medium diameter neurons ipsilateral to injury whereas ATF3-IR was increase in large diameter cells. It had been suggested that small diameter DRG neurons mediate thermal hyperalgesia and large diameter mechanical allodynia (Ossipov et al., 2000), (Obata et al., 2003). These diverse lines of evidence all suggest that afferent input and neurochemical release from undamaged nerve fibres is important in the development of neuropathic pain after nerve injury.

In addition to interactions between adjacent nerve roots and within individual nerves, the activity of pairs of nociceptive and wide dynamic range neurons in the superficial (I-IIo) and deep (V) laminae of the dorsal horn, in response to supra-threshold electrical stimuli, was shown to be linked using cross-correlation techniques. Activity in one neuron was predictably linked to activity in the other (Biella et al., 1997). After CCI, there was no correlation between the electrophysiological activity of the pairs of cells, suggesting that the functional connections between superficial dorsal horn nociceptive neurons and deep dorsal horn WDR neurons are either inactive or rearranged in the nerve-injured rats. This change may underlie a reduced degree of functional coherence in the modulation of nociceptive spinal inputs in chronic pain.

1.11.3 Anatomical changes in neuropathic pain

In addition to the complex changes in neurotransmitter and receptor levels in neuropathic pain (Hokfelt et al., 1994), it had been suggested that significant anatomical changes also occur in the dorsal horn. In the resting state, the central arborisations of large myelinated Aβ cutaneous afferent fibres extend as far dorsally as the ventral part of lamina II in rat spinal cord. Woolf et al reported that six to twelve weeks after sciatic nerve axotomy approximately 30% of studied Aβ afferents sprouted into lamina I and the dorsal part of lamina II (Woolf et al., 1992). It would seem reasonable to assume that the sprouting of Aβ fibres, which normally transmit innocuous stimuli, into regions of the dorsal horn involved in nociceptive transmission would result in these innocuous stimuli being interpreted as painful, resulting in allodynia.

These findings are currently in doubt, however, as the evidence for sprouting was based on the use of cholera toxin B subunit (CTb) as a selective tracer for A fibres. The selectivity of this tracer has recently been disproved, and C fibres have also been shown to transport CTb after axotomy (Shehab et al., 2003). The original study has recently been repeated using intra-axonal Neurobiotin as a label for sciatic Aβ fibres in intact and axotomised nerves. No fibres were found to extend further dorsally than the ventral part of lamina II in either experimental group (Hughes et al., 2003).

Despite this lack of firm evidence for sprouting of Aβ fibres, it would seem that these neurons do undergo functional changes after sciatic nerve transection. It has been demonstrated in a cord slice preparation that large myelinated Aβ fibres establish functional synaptic contact with interneurons and transmit innocuous information to the substantia gelatinosa following sciatic nerve section. This represents a marked functional alteration in spinal cord circuitry and may account in part for the sensory abnormalities occurring in neuropathic pain (Okamoto et al., 2001). It would seem that new and inappropriate synaptic connections might be made between Aβ fibres and neurons in the superficial dorsal horn in neuropathic pain even if extensive fibre sprouting does not occur.

1.11.4 Neuronal disinhibition and central sensitisation

Inhibitory mechanisms are active in nociception, both locally within the dorsal horn and from descending pathways (Sotgiu, 1993), (see section 1.3.7). It has been suggested that loss of this inhibitory input onto excitatory neurons may play a role in central sensitisation. It is interesting to note that the majority of dorsal horn neurons expressing the GluR1 subunit co-express GABA and some may play an inhibitory role (Spike et al., 1998). In addition it has been suggested that at least some inhibitory neurons express calcium-permeable AMPA Rs (ie: lacking the GluR2 subunit) (Albuquerque et al., 1999), (see section 1.6.4).

There is evidence to suggest that changes in the expression of AMPA R subunits may be important in neuronal disinhibition in central sensitisation independent of GABAergic function. This possibility has been explored in a recent study of cultured dorsal horn neurons immunolabelled with GluR1, GluR2 and GAD 67 (glutamic acid decarboxylase, an enzyme essential for GABA synthesis). GluR1 and GAD 67 are often co-expressed within individual neurons, suggesting that GluR1 expressing cells also synthesize the inhibitory neurotransmitter GABA (Vikman et al., 2003). Cultured cells were treated with the pro-inflammatory cytokine interferon-γ (IFN-γ) for two weeks. This cytokine is known to trigger spontaneous pain when injected intrathecally (Robertson et al., 1997). Post treatment, it was found that the number of GluR1 immunofluorescent puncta associated with dendrites was significantly decreased, and that number of spontaneous EPSCs (excitatory postsynaptic currents) within the cultured cells was increased. This increase in excitation was shown to be independent of GABA function, suggesting that the down-regulation of GluR1 subunits was the cause of the disinhibition found after IFN-γ. This cytokine may also play a role in neuropathic pain, as knockout mice lacking the gene for IFN-γ did not display autotomy after sciatic nerve section (Robertson et al., 1997).

After prolonged noxious input, it has been suggested that excessive synaptic glutamate release may lead to the excitotoxic death of certain neurons, altering the balance between excitatory and inhibitory processes. Such stimulation-induced cell death may potentially contribute to the development of persistent pain. The region of the dorsal horn affected by cell death was shown to expand from lamina III in the naïve animal, to include lamina I and II after nerve injury (Coggeshall et al., 2001).

Indeed, there is evidence from several studies to suggest that the number of tonic inhibitory neurons containing GABA or glycine decreased significantly on both sides of the spinal cord after CCI. Bilateral loss of GABA-IR cells by the third day after CCI has been demonstrated (84% reduction in proportion of cells ipsilaterally and 25% reduction contralaterally) (Ibuki et al., 1997). This reduction in GABA-IR cell numbers was statistically significant bilaterally by week two following CCI. Interestingly, complete recovery was seen by week 7 contralateral to injury and partial recovery (to 45% of pre-injury levels) on the ipsilateral side. This recovery suggests that significant cell death was unlikely to be occurring, at least contralateral to injury.

Moore et al investigated excitatory and inhibitory postsynaptic potentials in lamina II neurons after sciatic nerve transection, sciatic nerve CCI and spared nerve injury (SNI) (Moore et al., 2002). This study demonstrated a significant decrease in the incidence, amplitude and duration of inhibitory postsynaptic currents following CCI and SNI. There was no change in excitatory potentials. Evidence was provided that the reduction in inhibition was due to a decrease in $GABA_A$ mediated currents. There was also a small but significant decrease in the expression of the GABA-synthesizing enzyme, GAD65 ipsilateral to CCI as measured by Western blotting by day 6 which returned to pre-injury levels by 4 weeks. It would seem that a subpopulation of inhibitory cells demonstrated a temporary decrease in GABA production as a result of partial nerve injury. Very few cells appeared to have undergone cell death ipsilateral to injury. These TUNEL-positive profiles with pyknotic nuclei were found at day 7 post SNI (1.5 profiles per section). Many of these were not neurons, as they did not express NeuN. However a very small number (approximately 0.16 per slice) of NeuN and TUNEL-positive profiles were seen in laminae I and II. The findings that GAD65 expression recovers with time and that very few neurons demonstrated signs of cell death following CCI suggest that loss of inhibitory neurons is not critical to the development of neuropathic pain.

Interestingly, a recent investigation of cell death following CCI has reported a dramatic cell loss of 38±5% of all neurons in lamina II. This cell loss was found ipsilateral to injury 3 days post CCI, and occurred bilaterally by day 7. This decrease in cell numbers was prevented in animals treated with MPEP (mGlu5 receptor antagonist, intra peritoneal twice daily) at day 3 post-injury, but this protective effect was lost by day 7. This study suggests that spinal cord neuron loss may be triggered by an apoptotic process involving glutamate mGlu5 receptors, and that these receptors

are involved transiently, as their blockade with MPEP was no longer cytoprotective by day 7 post injury (de Novellis et al., 2004). However, this study did not determine whether the lost cells were functional neurons. Such widespread loss of cells by day 3 post CCI does not correlate well with the development of behavioural signs in this model. Also, abnormal behaviour is only noted ipsilateral to the injury, so a major bilateral change in cell numbers would be difficult to explain (almost 50% loss bilaterally by day 7). Approximately 12 cells per spinal cord slice were TUNEL-positive ipsilateral to CCI, suggesting that these cells were undergoing apoptosis. It was not clear whether these cells were neurons. Also, the expression of several molecules thought to be involved in apoptosis did not change following CCI in this study, suggesting that they are not significantly involved in the process.

In contrast to the findings of these studies, work by Polgar et al has suggested that GABAergic cells are not lost during central sensitisation caused by CCI. This carefully conducted study revealed no decrease in the proportion of cells demonstrating GABA- and / or glycine-immunoreactivity in laminae I, II or III of the rat dorsal horn was found two weeks after CCI when thermal hyperalgesia was well developed (Polgar et al., 2003). It remains possible that cell loss was occurring in the above study, but GABA-immunopositive, glycine-immunopositive and other neurons would have to be lost in equal proportions. Also, several studies have demonstrated that GABA expression returns to pre-injury levels with recovery from CCI, suggesting that loss of entire cells is unlikely (Ibuki et al., 1997).

From the studies considered above, there is no clear consensus regarding the degree of loss of GABA expression and cell death in neuropathic pain. It would seem that cell death is less significant than was previously thought, but more work needs to be done to clarify the importance of this processes. However, it would seem likely that the balance between excitatory and inhibitory inputs into the dorsal horn might be disturbed in central sensitisation (Dickenson, 1996).

1.12 AIMS OF THE CURRENT STUDY

The current study was designed to investigate the role of key receptors in the generation and maintenance of central sensitisation underlying neuropathic pain. It is clear that several receptor types are involved in altering the sensitivity of dorsal horn neurons, including ionotropic and metabotropic glutamate receptors and peptidergic receptors.

Firstly, the roles of NMDA, AMPA, mGlu group I and $VPAC_2$ Rs were studied. The behavioural reflex responses of naïve rats to intrathecal administration of low doses of these receptor agonists, singly or in combinations were investigated. It is known that there are synergistic relationships between glutamate receptors in the generation of sensitised states, but the effects of activation of combinations of ionotropic and metabotropic glutamate receptors in addition to peptidergic receptors have not been investigated in a behavioural paradigm in vivo (Cerne and Randic, 1992), (Jones and Headley, 1995), (Meller et al., 1996).

The sciatic chronic constriction injury (CCI) model of neuropathic pain was adopted for the current study (Bennett and XIE, 1988). The expression of AMPA receptor GluR1 and GluR2 subunits in the superficial dorsal horn in neuropathy was studied using confocal immunofluorescence. The effects of CCI neuropathy on the proportion of cells expressing these AMPA R subtypes and the degree of colocalisation of GluR1 and GluR2 receptor subtypes within individual neurons was investigated. It is known that levels of GluR1 and GluR2 expression in the dorsal horn vary in a number of pain paradigms, but the anatomical location of these changes has not been investigated in the intact spinal cord using immunofluorescence (Harris et al., 1996), (Grossman et al., 1999), (Helgren et al., 1999), (Garry et al., 2003b).

The relationship between the presynaptic marker protein, Bassoon and GluR1-immunopositive cells following CCI was studied as a measure of the inclusion of GluR1 subunits into synapses in neuropathic pain. This protein has not been previously used to assess this process in the spinal cord (Friedman et al., 2000).

The subcellular distribution of AMPA R subunits was also investigated in the superficial dorsal horn of intact rats following CCI and in response to acute intrathecal AMPA treatment. The effects of AMPA R antagonists and a combination of other key receptor antagonists on the subcellular location of AMPA Rs were investigated in animals with established neuropathy.

The study was designed to add to understanding of the interactions between key receptors in neuropathic pain, and to shed light on the differential role of AMPA R subtypes in this process. It is becoming clear that AMPA Rs play an important role in the development of abnormal pain states (Mao et al., 1992a), (Garry et al., 2003b). This study may aid the development of novel therapies, designed to target either specific receptor subtypes or a combination of receptors at low dose in order to minimise side effects in the treatment of neuropathic pain.

CHAPTER 2: MATERIALS

2.1 ANAESTHETIC AGENTS

- Halothane (Zeneca Ltd., Cheshire, UK)

2.2 SCIATIC NERVE CONSTRICTION SURGERY

- 4/0 chromic gut (Ethicon Ltd., Edinburgh UK)
- 5/0 Vicryl (Ethicon)
- Hibitane (Zeneca Ltd., Cheshire, UK)
- Sterile gowns, masks, drapes and gloves (Hospital Management and Supplies, Glasgow, UK)

2.3 INTRATHECAL DRUG INJECTIONS

- Syringe, 1ml (Becton Dickinson, Plymouth, UK)
- Needle 25G (Terumo, Belgium)

2.3.1 Drugs used

Vehicle

- Sterile saline 0.9% (Sigma Chemical Co, Poole, UK) used as vehicle for all drugs other than PG 99-465, which was prepared in 0.06% dimethylformamide in saline.

Agonists

- α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (Tocris Cookson Inc., Bristol, UK)
- 3,5-dihydroxyphenylglycine ((±) DHPG), a metabotropic glutamate receptor subgroup 1 and 5 agonist (mGluR1/5) (Tocris Cookson Inc.)
- N-methyl-D-aspartate (NMDA) (Sigma)
- Ro 25-1553, a $VPAC_2$ R agonist (gift from P. Robberecht, Free University of Brussels, Belgium).

Antagonists

- (R)-CPP (NMDA R antagonist) (Tocris Cookson Inc.)
- PG 99-465 ($VPAC_2$ R antagonist) (gift from P. Robberecht, Free University of Brussels, Belgium)

- MPEP (mGluR5 antagonist) (Tocris Cookson Inc.)
- LY 376385 (mGluR1 antagonist) (Tocris Cookson Inc.)
- NBQX (1,2,3,4-Tetrahydro-6-nitro-2, 3-dioxo-benzo[f]quinoxaline-7-sulfonamide, AMPA R antagonist) (Tocris Cookson Inc.)

2.4 Behavioural reflex testing

- Thermal stimulus (Hargreaves-Plantar apparatus, Ugo Basile, Italy)
- Von Frey filaments (Stoelting Co., Wood Dale, Illinois, USA)

2.5 Immunohistochemistry

- Agar 0.25% (Becton Dickinson)
- Di-sodium hydrogen orthophosphate dihydrate (Merck, VWR, Poole, UK)
- Fish skin gelatin (Diagnostics Scotland, Edinburgh, UK)
- Freezing microtome: Leitz Kryomat 1700 (Leitz, Germany)
- Glass slides (Merck)
- Glutaraldehyde (Sigma)
- Heparin (Sigma)
- Leica confocal microscope (Leica Microsystems Semiconductor GmbH, Wetzlar, Germany)
- Normal goat serum (Diagnostics Scotland, Edinburgh, UK)
- Paraformaldehyde (Sigma)
- Saturated solution of picric acid in water (Becton Dickinson)
- Perfusion pump (Watson-Marlow Ltd., UK)
- Scalpel blades size 10 (Swann-Morton, Sheffield, UK)
- Sodium chloride (Merck)
- Sodium dihydrogen orthophosphate dihydrate (Merck)
- Sucrose (Sigma)
- To-Pro3 cyanine nucleic acid stain (Molecular Probes Europe BV, The Netherlands)
- Triton X-100 (Sigma)
- Vecta-Shield (Vector Laboratories, Burlingame, CA)

2.6 Stock solutions

- 0.2M Phosphate buffer (PB): Approximately 470mls 0.2M sodium dihydrogen orthophospahte dihydrate was added to 1500mls 0.2M di-sodium hydrogen orthophosphate dihydrate, yielding a buffer mixture of pH 7.4.
- 0.1M Phosphate buffered saline (PBS): 0.2M PB was diluted with an equal volume of distilled water (dH_2O) and 9g/l sodium chloride added
- Heparinised vascular flush: 0.6mg/ml heparin (Sigma) was added to 0.9% saline solution to give 100 iu/ml heparin.
- Fixative, 4% paraformaldehyde in 0.1M PB with 12.5% picric acid and 0.1% gluteraldehyde: 48g paraformaldehyde was dissolved in 400mls dH_2O at 60°C. The solution was cleared with a drop of molar sodium hydroxide and then filtered. To this solution, 600mls of 0.2M PB was added. After cooling, this was combined with150mls saturated picric acid. 50mls were removed for post-fixation prior to the addition of 4.8mls of gluteraldehyde (25%). The solution was then made up to 1200mls with dH_2O.
- Buffered sucrose solutions: 5%, 10% and 30% sucrose solutions were made up by adding 2.5g, 5g or 15g sucrose to 50mls 0.1M PBS.
- Blocking serum: Specimens were blocked in 4% fish skin gelatin, 10% normal goat serum and 0.2% Triton X-100 in 0.1M PBS. This solution was made up using 17.2mls PBS, 2mls normal goat serum, 0.8mls fish skin gelatin solution and 40µl Triton X-100.
- Antibody solutions: Primary and secondary antibodies were made up in a similar solution, containing Triton X-100 and fish skin gelatin as above, with 4% normal goat serum.

2.7 Antibodies

2.7.1 Primary antibodies

- Rabbit polyclonal anti-GluR1 (Upstate Ltd., Milton Keynes, UK)
- Mouse monoclonal anti-GluR2 (Chemicon International Ltd., Harlow, UK)
- Mouse monoclonal anti-synaptophysin (Becton-Dickinson)
- Mouse monoclonal anti-Bassoon (Stressgen Biotechnologies Inc., San Diego, USA)

2.7.2 Secondary antibodies

- Alexa Fluor 488 goat anti mouse (Molecular Probes)
- Alexa Fluor 568 goat anti rabbit (Molecular Probes)

2.8 Miscellaneous

2.8.1 Image analysis and statistical analysis

- Image Tool for Windows v3.00 for cell counting (The University of Texas Health Science Centre, US).
- Leica Confocal Software v2.00 for cell length and brightness measurements (Leica Microsystems, Heidelberg, GmbH, Germany).
- Minitab Statistical Software v13.1 (Minitab Inc.) and SigmaStat for windows v2.03 (SPSS Inc.) for statistical analysis.

CHAPTER 3: THE EFFECTS OF INTRATHECAL ADMINISTRATION OF SPECIFIC RECEPTOR AGONISTS ON SOMATOSENSORY BEHAVIOURAL REFLEXES

3.1 AIMS

This study was designed to investigate the role of AMPA, NMDA, mGlu group I and VPAC2 Rs in the generation of the state of behavioural reflex hypersensitivity, thought to parallel the process of central sensitisation in the spinal cord in neuropathic pain. Low doses of specific receptor agonists were administered to naïve rats via the intrathecal route. The effects of individual agonists, and combinations of multiple agents on the behavioural reflex responses to thermal and mechanical stimuli were investigated.

3.2 METHODS

3.2.1 Experimental animals

All studies were carried out on adult male Wistar rats (Charles River, UK). Animals were housed in groups of three to five per cage. The cages contained bedding and a cardboard tube to provide environmental enrichment. Free access to water and food pellets was provided. Animal room temperature was controlled to 22-24°C with a twelve-hour light / dark cycle. All experiments were carried out in accordance with local ethical policy and within the constraints of the Animals (Scientific Procedures) Act (1986).

3.2.2 Behavioural reflex testing

To investigate the effects of the drug combinations on thermal hyperalgesia and mechanical allodynia, behavioural testing was carried out as described below. Following a 20-minute recovery period post injection, animals were tested every ten minutes for seventy minutes.

Thermal hyperalgesia

Animals were placed on a glass table in Perspex cages with adequate ventilation holes and permitted to acclimatise to their environment. Hargreaves' apparatus (Ugo Basile plantar test device, Italy) was used to determine paw withdrawal latency (PWL) (Hargreaves et al., 1988) (see Figure 3.1a). This consists of a moveable infra red heat source beneath the table, connected to a thermal detector

and electronic timer. The heat source was placed beneath the animal's hind paw and activated, causing the timer to start and heat source to activate. As soon as the animal lifts its paw from the hot surface, radiation is no longer reflected back onto the detector and the timer and heat source are cut off. This allows an objective measurement of paw withdrawal latency to the nearest 0.1 second. The heat source was cut off at a 20 second limit to prevent tissue damage in the event that the animal did not respond. Testing was carried out on each paw alternately, with the mean of three consecutive measurements noted. A minimum of five minutes was allowed between tests on the same paw. Mean latency was recorded every ten minutes during intrathecal drug injection studies, or daily to assess the development of hyperalgesia in animals following chronic constriction injury (CCI) surgery.

Mechanical allodynia

Mechanical allodynia was assessed using von Frey filaments (Semmes-Weinstein Anesthesiometer Kits, Stoelting Ltd., Wood Dale, Illinois, USA), (Chaplan et al., 1994). Each nylon filament is calibrated to exert a fixed force on the plantar surface of the animal's hind paw before flexing. The pressure exerted depends on the diameter of the filament and varied from 8.41 - 4831 mN/mm^2. Animals were placed on a metal mesh surface covered by a transparent cage (see Figure 3.1b). This allowed the experimenter to apply the von Frey filaments to the mid-plantar surface of the paw until bending occurred while the animal was able to move freely. Filaments were applied, finest first, to alternate hind paws at a 1Hz rate with ten applications per paw. Rapid elevation of the paw with or without shaking represented a nocifensive withdrawal to a noxious stimulus. Paw withdrawal to at least fifty percent of applications was taken as a threshold response. The test was repeated at ten-minute intervals for intrathecal studies and daily post CCI surgery.

Statistical analysis

The effects of intrathecal agonists were assessed using a number of statistical tests. The drug effect was calculated as the difference between the baseline and the measured parameter at each time point. The statistical significance of the difference between baseline and the measured parameter was assessed at each time point using a paired Student's t-test for thermal hyperalgesia and a Wilcoxon matched pairs signed rank sum test for mechanical allodynia (* $p \leq 0.05$, ** $p \leq 0.01$).

As these tests do not take into account the serial nature of these data, thermal and mechanical data were also assessed for statistical significance († $p \leq 0.05$, †† $p \leq 0.01$) of any differences between post-injection and pre-injection baseline values using a Friedman repeated measures ANOVA on ranks followed by Dunn's *post hoc* analysis (n=6-11) (see Figures 3.2 – 3.5). Thermal results were analysed using a conservative non-parametric statistical test, as the data set was not normally distributed on testing (see Figure 3.2).

When considering summary statistics of the effects of each agonist combination, the peak effect and the area under the effect-time curve (AUC), representing the cumulative response to the drug, were considered important features to compare (Altman, 1999). The peak effect of each drug combination was recorded for thermal and mechanical stimuli as the greatest decrease from baseline PWL at any time point, (Figures 3.3a and 3.5), together with cumulative drug effect as measured as the area of the graph between baseline PWL and measured values over the 70-minute experimental period for thermal stimuli (see Figure 3.3b).

3.2.3 Intrathecal injection of drugs

Male Wistar rats were used for intrathecal injection of combinations of agonists to ionotropic and metabotropic glutamate receptors and the $VPAC_2$ R. These animals had not undergone CCI surgery and are referred to as 'naïve' in the current study. Behavioural tests of thermal hyperalgesia and mechanical allodynia were carried out daily to achieve stable baseline values prior to drug administration. The experimental animals were injected intrathecally at the L4 level under halothane anaesthesia with 0.05mls of drug or saline vehicle using a 25G needle. The experimenter was blind to the pharmacological agent or vehicle to be injected in the majority of cases. There were a minimum of six animals in each experimental group, no one animal being used for more than three injections. In cases where animals were injected on more than one occasion, the experiments were carried out with a minimum 48-hour interval. Pre-injection behavioural measurements were taken each time to ensure that there were no residual effects from previous drug administration. Following intrathecal injection of a drug, behaviours were monitored until recovery to baseline values was observed following the drug effect.

3.2.4 Drug combinations for intrathecal administration

All drugs were made up in sterile saline (0.9%). 50μl was injected intrathecally and the rats ranged between 250-330g weight. The concentrations at which drug solutions were made were estimated according to the known relative affinity of the drugs for their target receptors. Every attempt was made to avoid excessive concentrations where overlap onto other non-specific targets might occur. Throughout this thesis, drug doses are given as the concentration of the solution used and the volume of solution injected. Unless otherwise stated, 50μl of solution was used. The absolute doseage may then be calculated from these figures. The AMPA R agonist was AMPA (50μM). NMDA (25μM) was used as the NMDA R agonist. The mGluR1/5 agonist used was DHPG, (30mM). The $VPAC_2$ R agonist was Ro25-1553, (6μM). Normal saline (0.9%) was the vehicle solution. Combinations of multiple drugs were given at the same doses in a 50μl volume. Agonists were given singly and then subsequently in double, triple and quadruple combinations. All possible combinations of one, two and three drugs were given, other than those including both AMPA and NMDA. The study was designed to compare the development of behavioural reflex sensitisation via the action of other iGluRs, mGluRs and peptide receptors on NMDA Rs, versus direct activation of the NMDA R itself. For this reason, the drug combinations were divided into two groups, one with NMDA R activation by NMDA and one with AMPA R activation. The relative efficacy of these two groups of agonists in triggering behavioural reflex sensitisation could then be compared. Finally, the quadruple combination contained all four agonists, giving 13 drug combinations in total.

Figure 3.1 Shows the behavioural testing apparatus used. a) Hargreaves' apparatus for measuring paw withdrawal latencies to noxious cutaneous thermal stimuli. b) von Frey filaments and testing apparatus for measurement of filament indentation pressure thresholds for paw withdrawal to mechanical stimuli. c) Tank for measurement of suspended paw elevation time (SPET) to a cold water stimulus. d) Animal exhibiting paw withdrawal response to cold (Section 3.2.2).

a)

b)

c)

d)

3.3 RESULTS

3.3.1 General considerations

Dose-finding studies were carried out with individual agonists to achieve a small but significant reduction in PWL to a thermal stimulus assessed using Hargreave's apparatus (see Figure 3.1a). The aim was to use as low a dose as possible to achieve a measurable, sub maximal, statistically significant effect. When combinations of several drugs were used, this approach was intended to minimise the possibility of ceiling effects being observed and obscuring the evidence for synergistic effects. The effect of adding each agonist to a combination would be apparent, as each drug alone produced only a small behavioural change. Doses were selected using tests of thermal hyperalgesia only, since the drugs generally produced less consistent effects on mechanical allodynia.

All drug combinations were investigated for development of thermal hyperalgesia using behavioural reflex testing (n=6-11). The majority of drug combinations were also investigated for the development of mechanical allodynia. However, because the results were generally less clear and consistent, the number of animals used in each mechanical allodynia experiment was low for some drug combinations (n=2-7) and statistical testing was not always appropriate.

3.3.2 Drug induced changes in behavioural reflex responses

Thermal hyperalgesia

a) Single agonists

After intrathecal injection of individual agonists under brief halothane anaesthesia, animals were allowed to recover for 20 minutes prior to behavioural testing (n=7-9). The saline treated group demonstrated a small (2.06 ± 0.58secs) but significant (* $p \leq 0.05$) increase in PWL at 20 minutes post-injection. None of the other single agonists had an effect at the 20-minute time point. These findings suggest an artifactual increase in the first PWL measurement, possibly due to residual anaesthesia or the disturbance of transferring the animals to the experimental apparatus.

Thermal hyperalgesia developed to a varying degree with all other agonist drugs at one or more time points from 30 minutes post-injection (* $p \leq 0.05$, ** $p \leq 0.01$, paired t-test, † $p \leq 0.05$, †† $p \leq 0.01$, Friedman repeated measures ANOVA on

ranks followed by Dunn's *post hoc* analysis) (see Figure 3.2a - e). The peak reduction in mean PWL caused by these drugs was not dramatic, and varied from 2.11 ± 0.69sec for AMPA (see Figure 3.2b, Figure 3.3) to 3.67 ± 0.59secs for Ro 25-1553 (see Figure 3.2d, Figure 3.3). Compared to a mean pre-drug baseline of 12.00 ± 0.15sec. The mGluR1/5 agonist, DHPG (peak effect DHPG, 3.54 ± 0.64secs) and the $VPAC_2$ R agonist Ro 25-1553 (peak effect Ro 25-1553, 3.67 ± 0.59secs) had a more marked effect than the other two drugs up to the 60-minute time point (see Figure 3.2c,d). The measurements ceased at 90 minutes post injection, as all PWLs were returning towards baseline. At this time point Ro 25-1553 (* $p \leq 0.05$), NMDA + Ro 25-1553 (* $p \leq 0.05$), and NMDA + Ro 25-1553 + DHPG (** $p \leq 0.01$) continued to cause a significant reduction in PWL (see Figure 3.2e).

b) Combinations of two agonists

Combinations of two of the agonist drugs were administered to five groups of naïve rats (n=7-11) (see Figure 3.2f - j). Of the double combinations, adding DHPG (peak effect NMDA + DHPG, 3.56 ± 0.48secs) or Ro 25-1553 (peak effect NMDA + Ro 25-1553, 3.46 ± 0.45secs) to NMDA demonstrated an increase in peak effect over NMDA alone (peak effect NMDA, 2.70 ± 0.58secs) (see Figure 3.2f, g). These combined effects were not greater than additive, and *post hoc* analysis did not reveal any statistically significant differences from single drug effects (Friedman repeated measures ANOVA on ranks followed by Dunn's *post hoc* analysis).

Similar changes occurred when DHPG (peak effect AMPA + DHPG, 3.08 ± 0.56secs) or Ro 25-1553 (peak effect AMPA + Ro 25-1553, 3.15 ± 0.40secs) were added to AMPA (peak effect AMPA, 2.11 ± 0.69secs) (see Figure 3.2h, i). Overall, the peak and cumulative effect (measured as the area of the graph between baseline PWL and measured experimental values over a 70-minute period for thermal stimuli) of double drug combinations did not differ significantly from single agonists, and there were no significant differences between the combinations (Friedman repeated measures ANOVA on ranks followed by Dunn's *post hoc* analysis) (see Figure 3.3a, b).

c) Combinations of three or four agonists

Combinations of three or four of the agonist drugs were administered to three groups of naïve rats (n=6–10) (see Figure 3.2k - m). The combination of NMDA + DHPG + Ro 25-1553 had no greater peak or cumulative effect than double drug combinations with NMDA as a component (see Figure 3.2k, 2).

In contrast, adding AMPA to the double combination of DHPG + Ro 25-1553 had a dramatic effect (see Figure 3.2l, Figure 3.3a). Intrathecal injection of AMPA + DHPG + Ro 25-1553 was the only experimental intervention to cause a significant decrease in PWL from baseline at the 20 minute time point (** $p \leq 0.01$).

Adding AMPA (peak effect 2.11 ± 0.69secs) to DHPG + Ro 25-1553 (peak effect 2.97 ± 0.67secs) resulted in a greater than additive increase in peak effect (AMPA + DHPG + Ro 25-1553 peak effect 6.80 ± 0.56secs). The sum of the mean peak effects of AMPA alone and DHPG + Ro 25-1553 is, therefore 5.08secs versus 6.80 ± 0.56secs for the triple combination. It was not possible to compare these findings using statistical tests, as experimental animals were not matched for the individual treatments. However, AMPA + DHPG + Ro 25-1553 had a significantly greater peak effect than all other combinations of drugs, excepting the quadruple combination (†† $p \leq 0.01$, Student-Newmann-Keuls ANOVA) (see Figure 3.3a).

Interestingly, the peak effect of the quadruple combination of agonists (peak effect 6.33 ± 0.55secs) was no greater than that of the AMPA + DHPG + Ro 25-1553 triple combination (see Figure 3.2m, Figure 3.3). The quadruple combination had a significantly greater peak effect than saline, NMDA, AMPA, AMPA + DHPG (§§ $p \leq 0.01$), and DHPG + Ro 25-1553, NMDA + Ro25-1553 and AMPA + Ro 25-1553 (§ $p \leq 0.05$, Student-Newmann-Keuls ANOVA).

When comparing cumulative drug effects, all combinations were significantly more effective than saline control (** $p \leq 0.01$, Student-Newmann-Keuls ANOVA vs. saline control). Comparing cumulative effects of drug combinations to each other revealed a significantly greater effect for AMPA + DHPG + Ro 25-1553 and the quadruple combination over all other combinations († $p \leq 0.05$, Student-Newmann-Keuls ANOVA) (see Figure 3.3b).

Mechanical allodynia

The marked changes in reflex response to thermal stimuli, after administration of these drugs, were not mirrored by changes in response to mechanical stimuli (see Figure 3.4). Analysis of data for intrathecal NMDA alone (peak effect 859 ± 206mN/mm^2) and AMPA alone (peak effect 1130 ± 264mN/mm^2) revealed a significant effect of each drug at one or more time points (* $p \leq 0.05$, ** $p \leq 0.01$).

Of the single agonists, DHPG (peak effect 1400 ± 309mN/mm^2) was the most effective drug, resulting in a significant decrease in filament indentation pressure for paw withdrawal thresholds (PWT) to cutaneous mechanical stimulation from 30 to 50minutes post injection (* $p \leq 0.05$, ** $p \leq 0.01$ Wilcoxon test, †† $p \leq 0.01$, Friedman repeated measures ANOVA on ranks, Dunn's *post hoc* analysis) (see Figure 3.4c). Administration of Ro 25-1553 or saline control had no discernable effect on filament indentation pressure thresholds.

Of the combinations of two agonists, NMDA + DHPG (peak effect 1400 ± 564mN/mm^2) caused a significant decrease in mechanical threshold values (* $p \leq 0.05$). The effects of AMPA + DHPG (peak effect 1250 ± 250mN/mm^2) did not reach statistical significance (see Figure 3.4f, g). The effects of NMDA + DHPG and AMPA + DHPG were not significantly greater than the effect of DHPG alone.

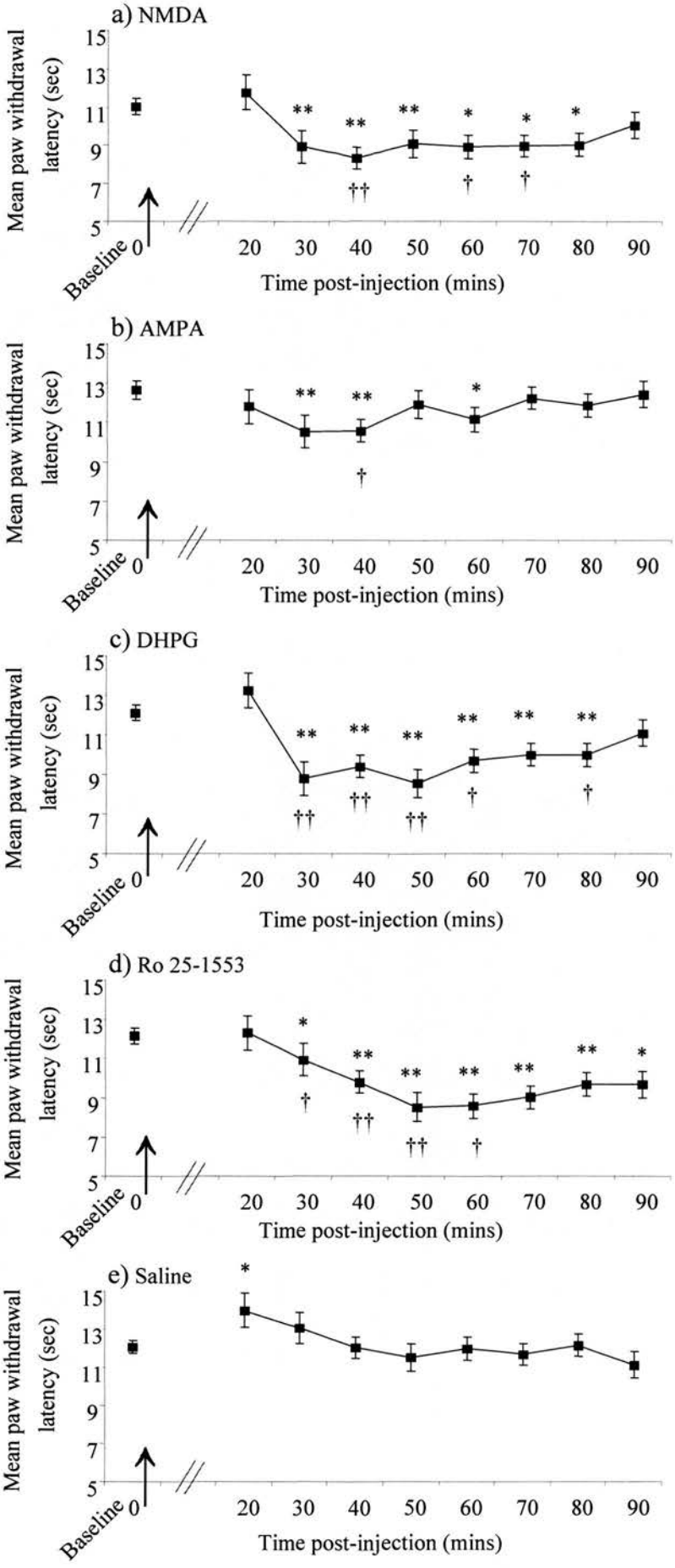
AMPA + Ro 25-1553 and NMDA + Ro 25-1553 had no discernable effect on filament indentation pressure thresholds (see Figure 3.4h, i). It is notable that single and double combinations of Ro 25-1553 had no discernable effect on mechanical sensitisation, whereas the $VPAC_2$ R agonist alone, and in combination with other agonists had a marked effect on thermal sensitisation.

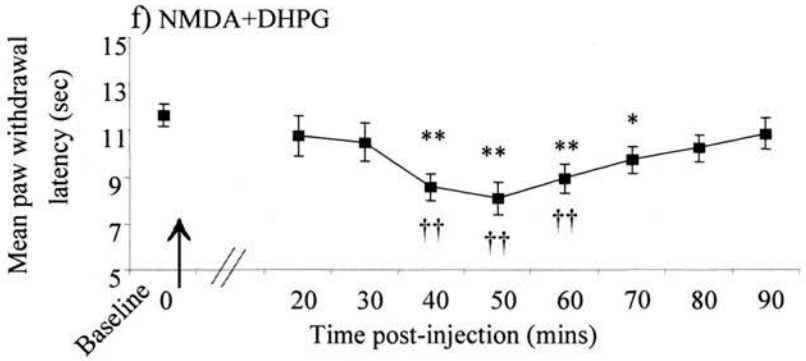
The triple combination of AMPA + DHPG + Ro 25-1553 (peak effect 1320 ± 188mN/mm^2) also caused a significant decrease in mechanical threshold (* $p \leq 0.05$ Wilcoxon test) (see Figure 3.4j). The quadruple combination of agonists caused a gradual reduction in mechanical threshold lasting approximately 30 minutes (peak effect 1320 ± 282mN/mm^2) with statistically significant reductions in PWT recorded at five time points (** $p \leq 0.01$, * $p \leq 0.05$ Wilcoxon test, †† $p \leq 0.01$, Friedman repeated measures ANOVA on ranks followed by Dunn's *post hoc* analysis) (see Figure 3.4k). The peak effect of AMPA + DHPG + Ro 25-1553 was almost identical to that of the quadruple combination (1315 versus 1318mN/mm^2), but the quadruple combination was clearly more effective.

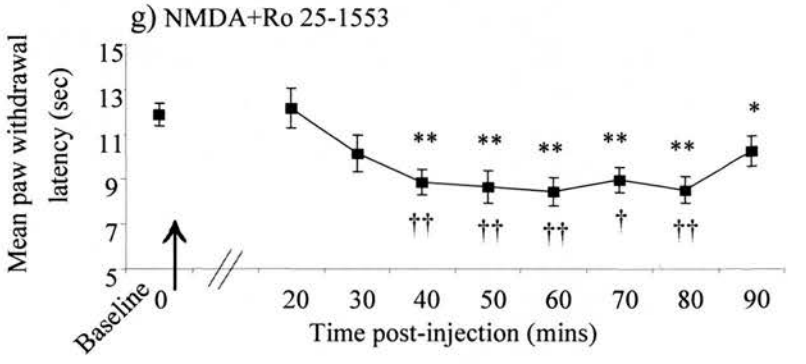
Peak effects of drug combinations on mechanical threshold were also compared to saline control to reveal whether any one combination was significantly more effective than the others (see Figure 3.5). Of the drug combinations tested AMPA, DHPG, AMPA + DHPG, AMPA + DHPG + Ro 25-1553 and the quadruple combination had a significantly greater effect than saline control at peak (** $p \leq 0.01$, * $p \leq 0.05$, Mann-Whitney rank sum test). The combination of AMPA + DHPG + Ro 25-1553 achieved a higher level of statistical significance (** $p \leq 0.01$) than other combinations.

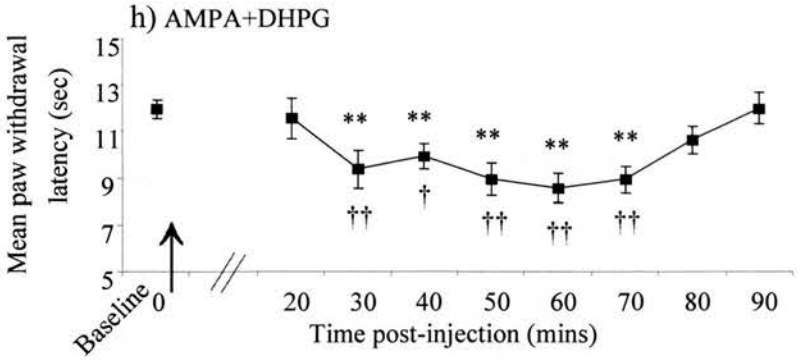
Due to the small sample size of the saline control group, the peak effect of NMDA alone ($p = 0.058$) (peak effect $859 \pm 206 mN/mm^2$) and NMDA + DHPG (peak effect $1400 \pm 564 mN/mm^2$) did not differ significantly from control when comparing peak values. Intrathecal Ro 25-1553, Ro 25-1553 + AMPA and Ro 25-1553 + DHPG had no discernable effect on von Frey response thresholds.

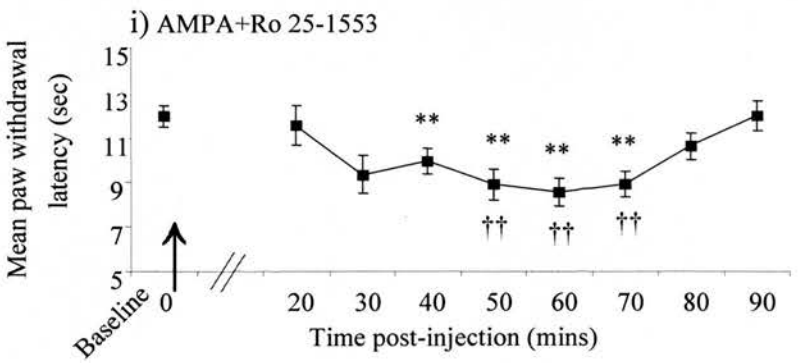
Figure 3.2 Effects of intrathecal administration of ionotropic glutamate (NMDA or AMPA), metabotropic glutamate (group I) and $VPAC_2$ R agonists, either individually or in combination, on paw withdrawal latencies (PWL) to cutaneous noxious thermal stimulation (a-m). PWL was measured in naïve animals before and at ten-minute intervals after intrathecal administration of single or combinations of two to four drugs. Data are shown as mean ± SEM. Statistical significance of any differences between pre-injection values and post-injection values and was determined at each time point by paired Student's t-test (* $p \leq 0.05$, ** $p \leq 0.01$), and Friedman repeated measures ANOVA on ranks followed by Dunn's post hoc analysis († $p \leq 0.05$, †† $p \leq 0.01$) (n=6-11). All single agonists and combinations of two agonists had a significant effect on behavioural reflex sensitivity at the low doses used in this study. The mGlu1/5R agonist DHPG (c), and the $VPAC_2$ R agonist Ro 25-1553 (d) had the greatest effects of single agonists. Combinations of two agonists had a greater effect, although this was not synergistic ie: not significantly greater than single agonists or the sum of single agonist effects. The saline control group demonstrated an increase in PWL at 20mins (* $p<0.05$) (e). The triple combination of NMDA, DHPG and Ro25-1553 (k) had a similar effect to combinations of two agonists. However, triple and quadruple combinations containing AMPA caused a significantly greater peak reduction in paw withdrawal latency than single and double combinations (l, m).

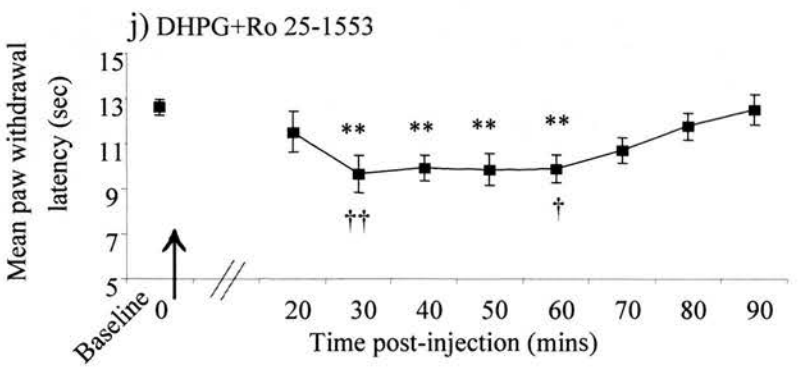

a) NMDA
b) AMPA
c) DHPG
d) Ro 25-1553
e) Saline
Mean paw withdrawal latency (sec)
15
13
11
9
7
5
Baseline
0
20
30
40
50
60
70
80
90
Time post-injection (mins)
**
*
†
††

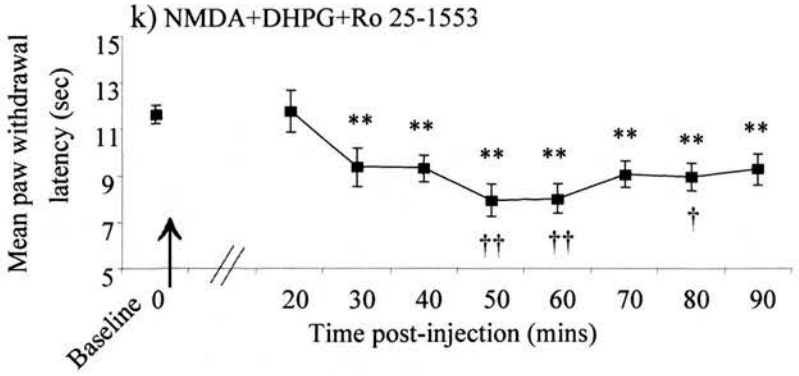

f) NMDA+DHPG
Mean paw withdrawal latency (sec)
15
13
11
9
7
5
Baseline
0
20
30
40
50
60
70
80
90
Time post-injection (mins)

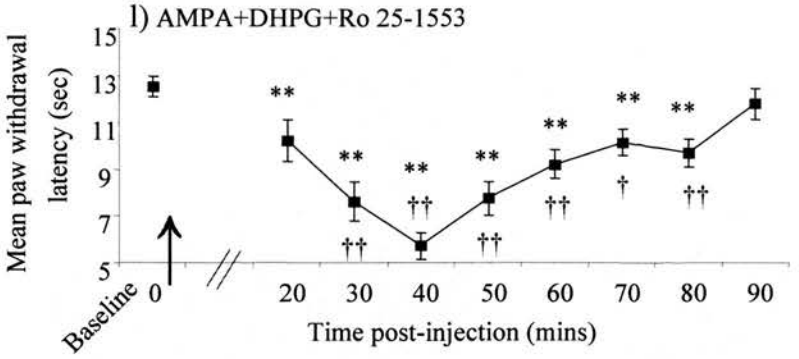

g) NMDA+Ro 25-1553
Mean paw withdrawal latency (sec)
15
13
11
9
7
5
Baseline
0
20
30
40
50
60
70
80
90
Time post-injection (mins)


h) AMPA+DHPG
Mean paw withdrawal latency (sec)
15
13
11
9
7
5
Baseline
0
20
30
40
50
60
70
80
90
Time post-injection (mins)


i) AMPA+Ro 25-1553
Mean paw withdrawal latency (sec)
15
13
11
9
7
5
Baseline
0
20
30
40
50
60
70
80
90
Time post-injection (mins)


j) DHPG+Ro 25-1553
Mean paw withdrawal latency (sec)
15
13
11
9
7
5
Baseline
0
20
30
40
50
60
70
80
90
Time post-injection (mins)


k) NMDA+DHPG+Ro 25-1553
Mean paw withdrawal latency (sec)
15
13
11
9
7
5
Baseline
0
20
30
40
50
60
70
80
90
Time post-injection (mins)


l) AMPA+DHPG+Ro 25-1553
Mean paw withdrawal latency (sec)
15
13
11
9
7
5
Baseline
0
20
30
40
50
60
70
80
90
Time post-injection (mins)

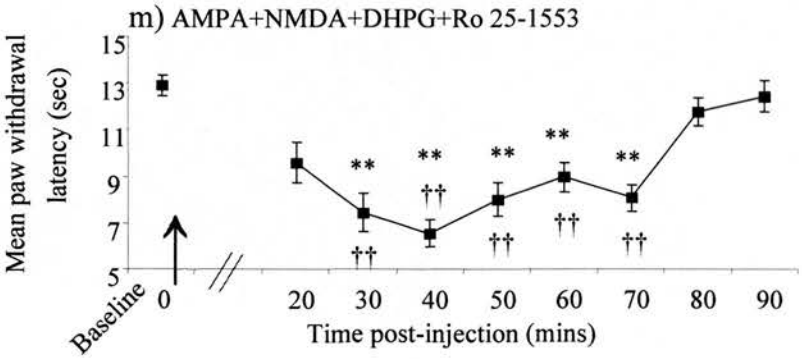
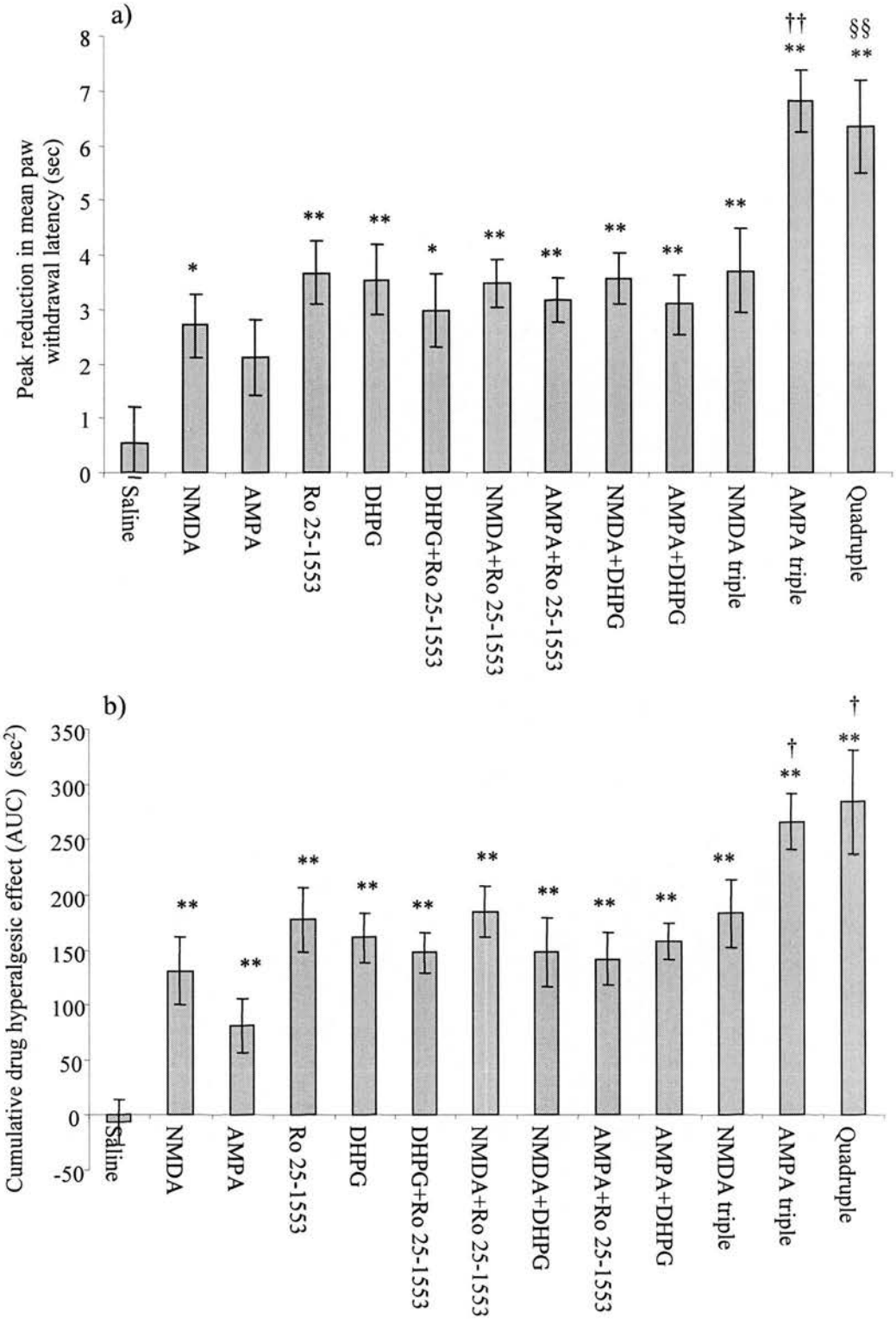

m) AMPA+NMDA+DHPG+Ro 25-1553
Mean paw withdrawal latency (sec)
15
13
11
9
7
5
Baseline
0
20
30
40
50
60
70
80
90
Time post-injection (mins)

Figure 3.3 a) Histogram showing the peak effects of intrathecal administration of combinations of ionotropic glutamate (NMDA or AMPA), metabotropic glutamate group I and $VPAC_2$ R agonists. Peak reduction in paw withdrawal latency to cutaneous noxious thermal stimulation was measured in naïve animals after intrathecal injection of drug combinations. Data are shown as mean ± SEM and statistical significance (* $p \leq 0.05$, ** $p \leq 0.01$) of any differences between peak reduction in PWL and saline control values were determined by Student's t test for each combination of agonists. The triple combination of AMPA + DHPG + Ro 25-1553 caused a significantly greater peak reduction in PWL than all other combinations excepting the quadruple combination of agonists (†† $p \leq 0.01$ Student-Newman-Keuls one-way ANOVA). The combination of all four agonists showed no greater peak effect than the triple combination of AMPA + DHPG + Ro 25-1553 (§§ see Section 3.3.2 Student-Newman-Keuls one-way ANOVA). b) Histogram showing cumulative effects of intrathecal injection of combinations of ionotropic glutamate (NMDA or AMPA), metabotropic glutamate group I and $VPAC_2$ R agonists. Cumulative effect was calculated as the area under the mean PWL versus time graph from 20 to 90mins after drug administration. The data are shown as mean ± SEM. Statistical significance of any differences between total reduction in PWL and saline control values were determined by Student's t test for each combination of agonists (* $p \leq 0.05$, ** $p \leq 0.01$). AMPA triple and quadruple combinations of agonists had a significantly greater cumulative effect than all other combinations († $p \leq 0.05$ Kruskal-Wallis ANOVA on ranks followed by Dunn's post hoc analysis).

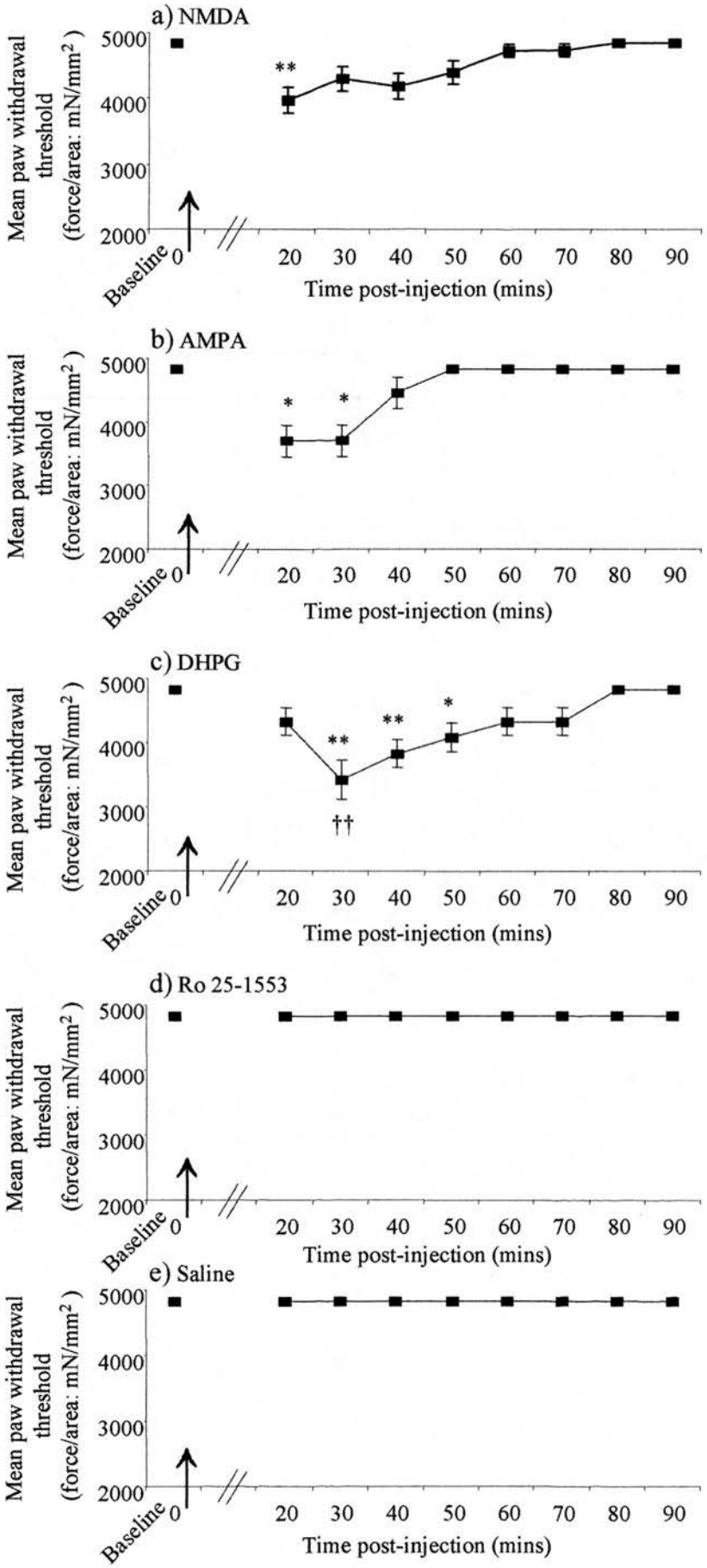


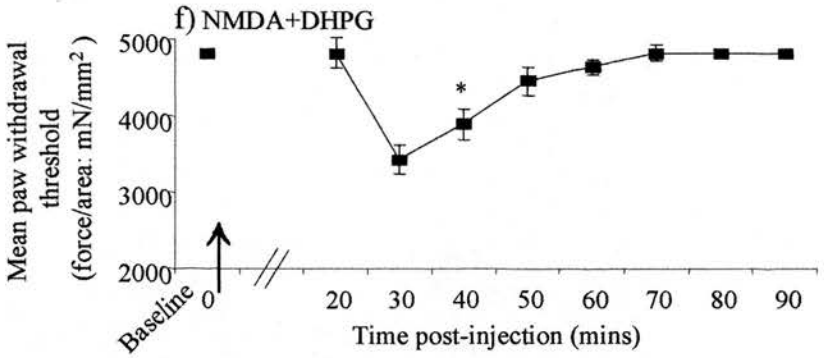
NMDA triple = NMDA+DHPG+Ro 25-1552

AMPA triple = AMPA+DHPG+Ro 25-1553

Quadruple = NMDA+AMPA+DHPG+Ro 25-1553

Figure 3.4 Effects of intrathecal administration of combinations of ionotropic glutamate (NMDA or AMPA), metabotropic glutamate group I and $VPAC_2$ R agonists on paw withdrawal threshold (PWT) responses to cutaneous mechanical stimulation using von Frey filaments (a-k). Thresholds were measured in naïve animals before and at ten-minute intervals after intrathecal administration of single or combinations of two to four drugs. Data are shown as mean ± SEM and statistical significance of any difference between pre-injection values and post-injection values was determined by Wilcoxon matched pairs signed rank sum test (** $p \leq 0.01$. * $p \leq 0.05$) and Friedman repeated measures ANOVA on ranks with Dunn's post hoc analysis (†† $p \leq 0.01$ see Section 3.3.2). Saline control had no effect on paw withdrawal threshold (e). All single agonists except Ro 25-1553 exhibited a statistically significant reduction in PWT. NMDA + DHPG had a similar effect, but AMPA + DHPG and double combinations containing Ro 25-1553 failed to show statistical significance. The AMPA + DHPG + Ro 25-1553 and quadruple combinations produced statistically significant reductions in PWT. The quadruple combination was particularly effective, with statistically significant reductions in PWT at five time points (k).


a) NMDA
b) AMPA
c) DHPG
d) Ro 25-1553
e) Saline
Mean paw withdrawal threshold (force/area: mN/mm²)
5000
4000
3000
2000
Baseline
0
20
30
40
50
60
70
80
90
Time post-injection (mins)
**
*
*
**
**
*
††


f) NMDA+DHPG
Mean paw withdrawal threshold (force/area: mN/mm²)
5000
4000
3000
2000
*
Baseline
0
20
30
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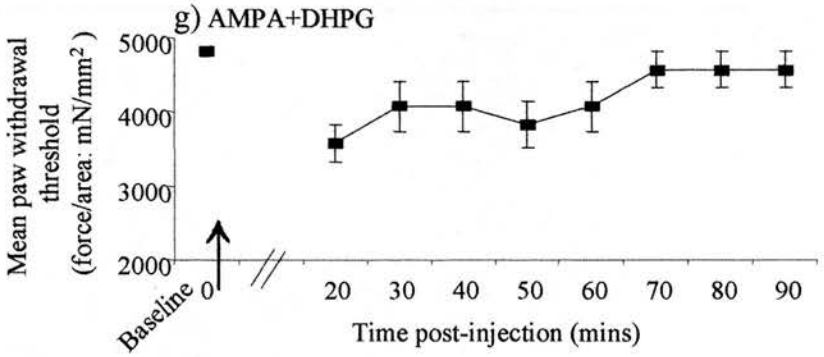
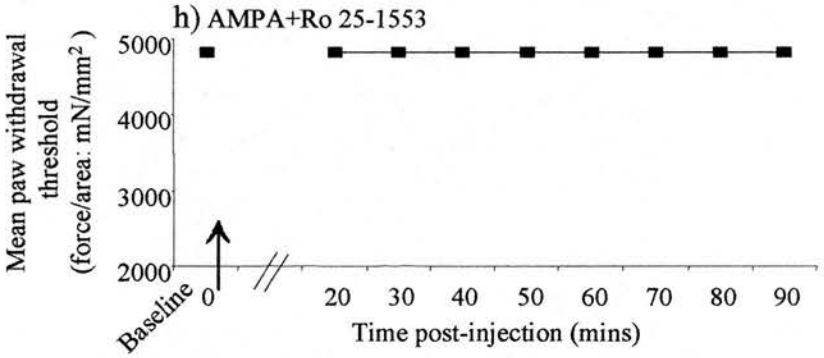
50
60
70
80
90
Time post-injection (mins)


g) AMPA+DHPG
Mean paw withdrawal threshold (force/area: mN/mm²)
5000
4000
3000
2000
Baseline
0
20
30
40
50
60
70
80
90
Time post-injection (mins)


h) AMPA+Ro 25-1553
Mean paw withdrawal threshold (force/area: mN/mm²)
5000
4000
3000
2000
Baseline
0
20
30
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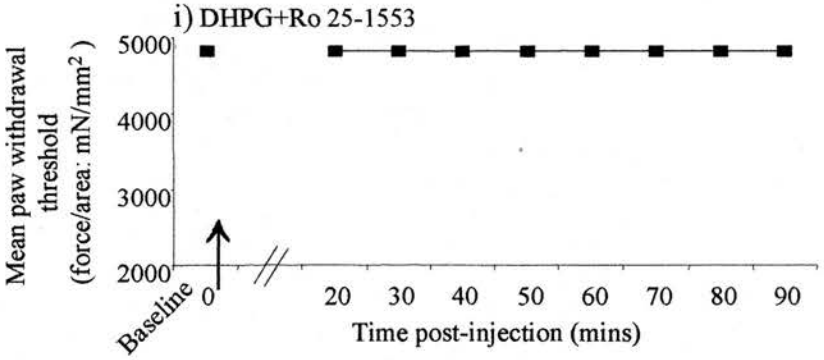
50
60
70
80
90
Time post-injection (mins)


i) DHPG+Ro 25-1553
Mean paw withdrawal threshold (force/area: mN/mm²)
5000
4000
3000
2000
Baseline
0
20
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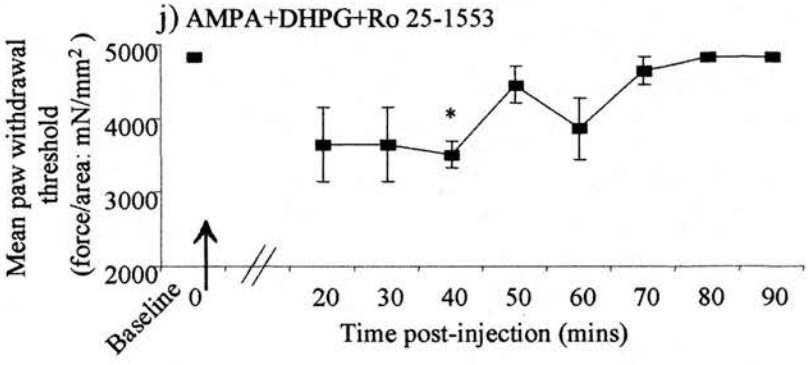
40
50
60
70
80
90
Time post-injection (mins)


j) AMPA+DHPG+Ro 25-1553
Mean paw withdrawal threshold (force/area: mN/mm²)
5000
4000
3000
2000
Baseline
0
20 30 40 50 60 70 80 90
Time post-injection (mins)
*

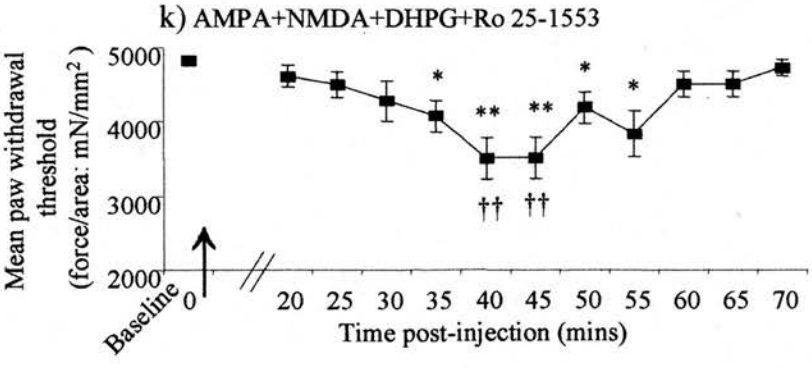
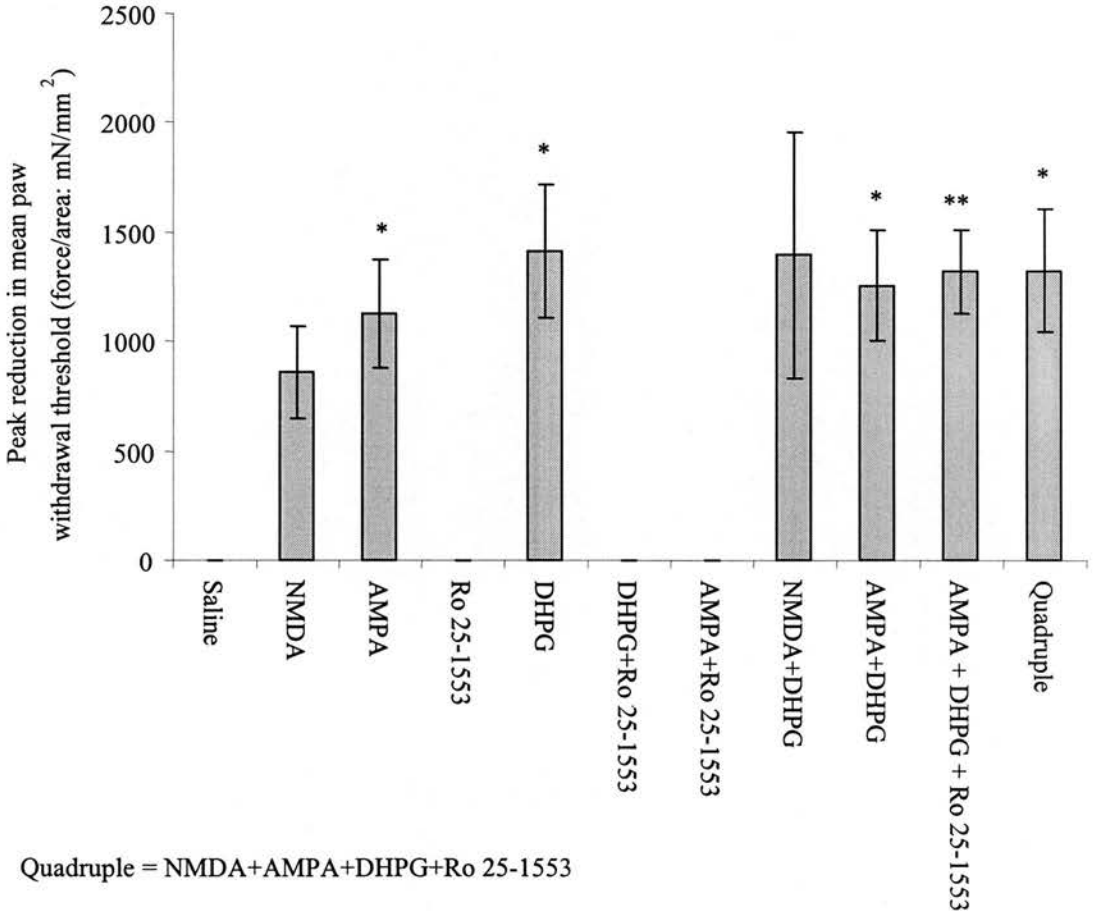

k) AMPA+NMDA+DHPG+Ro 25-1553
Mean paw withdrawal threshold (force/area: mN/mm²)
5000
4000
3000
2000
Baseline
0
20 25 30 35 40 45 50 55 60 65 70
Time post-injection (mins)
*
**
**
*
*
††
††

Figure 3.5 Histogram showing peak effects of single intrathecal injections or combinations of two to four ionotropic glutamate, metabotropic glutamate and $VPAC_2$ receptor agonists on PWT responses to cutaneous mechanical stimulation using von Frey filaments in naïve animals. Data shown as mean ± SEM and statistical significance of any differences between peak reduction in mechanical threshold and saline control values for each combination of agonists were determined using a Mann-Whitney rank sum test. The peak effects of combinations including DHPG, AMPA, AMPA + DHPG, AMPA + DHPG + Ro 25-1553 and AMPA + DHPG + Ro 25-1553 + NMDA were significantly greater than the effect of saline control (* $p \leq 0.05$, ** $p \leq 0.01$).



Quadruple = NMDA+AMPA+DHPG+Ro 25-1553

3.4 Discussion

There is a great deal of evidence to suggest that the activation of multiple receptor types occurs in neuropathic pain, resulting in central sensitisation and abnormal pain sensations such as hyperalgesia and allodynia.

Co-activation of iGluRs and mGluRs has been shown to generate behavioural responses suggestive of central sensitisation in naïve rats and mice. The non-selective mGluR agonist trans-ACPD potentiates the effects of NMDA R agonists in producing thermal hyperalgesia in rats (Meller et al., 1996). In vivo electrophysiological studies have revealed that mGluR activation by the non-selective agonist trans-ACPD or the group I mGluR agonist DHPG potentiated the electrophysiological responses of rat dorsal horn cells to AMPA, NMDA and kainate (Jones and Headley, 1995). However, the level of enhancement was identical for all three agonists and was thought to be due to a non-specific increase in cell excitability, probably due to mGluR group I - mediated depolarisation, rather than any specific functional interaction with the role of iGlu Rs.

Activation of mGluRs resulted in similar effects on acutely isolated DH neurons using whole-cell voltage clamp techniques. ACPD-induced mGluR activation potentiated the effects of glutamate, AMPA, and NMDA but did not alter the effect of kainate on dorsal horn neurons (Cerne and Randic, 1992). It is difficult to explain how ACPD had a selective effect on AMPA and NMDA, but not kainate, without postulating that there is a specific interaction between mGluR and iGluR, unless perhaps the kainate-responsive iGluRs are located substantially on different cells from the AMPA and NMDA Rs.

There is also evidence for the interaction of iGluRs and mGluRs in the generation of central sensitisation due to noxious inflammatory peripheral stimuli. It has been demonstrated that intrathecal pre-treatment with NMDA and ACPD but not AMPA enhanced the spontaneous pain behaviour following intraplantar formalin injection in the rat. Although AMPA given alone had no effect on spontaneous pain behaviour following formalin injection, the effect of NMDA was enhanced by combined administration with AMPA or ACPD, suggesting that AMPA and mGluRs may increase the activity of NMDA Rs in inflammatory pain (Coderre and Melzack, 1992). The role of non-NMDA iGluRs and mGluRs in activation of the NMDA R has been discussed elsewhere in this thesis (see section 1.5.1). In a rat model of inflammatory hyperalgesia, the NMDA R antagonist (D-AP5) prevented the

additional hyperalgesic effect of a mGluR agonist (trans-ACPD), suggesting that mGluR may also modulate the effect produced by activated NMDA R in inflammation (Boxall and Tolle, 1998). In addition to the previously demonstrated interactions between mGluR and iGluR in the induction and maintenance of central sensitisation, it is clear that peptide neurotransmitters also play a significant role (Cumberbatch et al., 1995), (Dickinson and Fleetwood-Walker, 1999).

Following nerve injury the expression of certain peptide neurotransmitters is markedly altered, and it would appear that they play a significant role in the transduction of prolonged noxious stimuli (Hokfelt et al., 1994), (Dickinson et al., 1999). For example, levels of SP, CGRP and somatostatin decrease, whereas VIP and PACAP increase in small diameter, nociceptive neurons in the DRG following nerve injury (WiesenfeldHallin et al., 1990). The expression of VIP increases gradually after injury in the superficial dorsal horn of the rat and remains elevated for several weeks (Nahin et al., 1994), (Zhang et al., 1995b). Following CCI, the electrophysiological response of DH neurons to the iontophoretic application of the $VPAC_2$ R agonist Ro 25-1553 was dramatically augmented (Dickinson et al., 1999). These findings suggest that the $VPAC_2$ R may play a specific role in the transmission of noxious stimuli in neuropathic pain. As this peptide plays relatively little role in the transmission of acute noxious and non-noxious stimuli, and has a marked increase in functional significance following peripheral mononeuropathy, it would seem to be an ideal target for investigation of the induction of a sensitised state in naïve animals. The effect of co-activation of mGluR, iGluR and peptide receptors, on nociceptive behavioural reflexes, has not yet been described in naïve animals.

3.4.1 AMPA and NMDA receptors

It is known that AMPA R activation plays a role in initiating thermal hyperalgesia seen following nerve injury, which may then be maintained by NMDA R activation (Mao et al., 1992a). In addition to its role in neuropathic thermal hyperalgesia, co-activation of non-NMDA iGluRs and mGluRs has been shown to produce mechanical hyperalgesia (Meller et al., 1993).

The current study revealed that all agonist combinations resulted in significant thermal hyperalgesia. This was to be expected as this sensory modality was used in the initial dose-finding studies. The effect of the agonists on mechanical hyperalgesia

was less marked, however. It is known that different neurotransmitter systems may be involved in differing aspects of sensory sensitisation.

Previous work suggested that thermal hyperalgesia may be mediated by NMDA R activation and AMPA appeared not to play a role (Meller et al., 1996). However, this study utilised lumbar intrathecal catheters inserted via the atlanto-occipital membrane, used very small doses of drug (1 pmol NMDA) and found only transient (less than 5 minutes) effects. In addition, when larger doses of AMPA (100-200pmol) were given, spontaneous nociceptive behaviour was noted for up to 30mins, suggesting that a narrow dose range would be required for the analysis of the effects of AMPA in this experimental paradigm. Similar effects of higher doses of AMPA were noted in the current study (dose finding study, data not shown). There is evidence that AMPA Rs may be important in thermal hyperalgesia in a CCI model of neuropathy, with a greater role in its initiation than in its maintenance (Mao et al., 1992a). This may explain why the AMPA agonist produced only modest thermal hyperalgesia at the doses used (2.5nmol), although additional factors are likely to be important.

Further evidence for a significant role for AMPA Rs in the spinal cord in mediating the sensitised nociceptive transmission that occurs following peripheral nerve damage has been provided by a recent study (Garry et al., 2003b). The effect of intrathecal injection of the AMPA/kainate receptor antagonist NBQX and the highly selective AMPA R antagonists NS-257 and SYM 2206 on rats at the peak of behavioural reflex sensitisation by CCI was investigated. It was demonstrated that these antagonists attenuated the ipsilaterally sensitised behavioural measures of thermal hyperalgesia and had similar but less marked effects on mechanical allodynia following CCI. This suggests that AMPA Rs may play a significant role in both thermal hyperalgesia and mechanical allodynia following CCI.

In the current study, the effects of AMPA on mechanical allodynia were less marked than those on thermal hyperalgesia. This is perhaps surprising in the light of previous studies, which suggest that AMPA Rs play an important role in the generation of acute mechanical hyperalgesia to noxious stimuli (stimulation of rat tail by von Frey filaments). These sensory changes have been demonstrated following intrathecal delivery of the non-NMDA iGluR and mGluR agonist quisqualate via an indwelling intrathecal catheter (Meller et al., 1993). The induction of mechanical hyperalgesia in this model was blocked by inhibition of either AMPA R or mGluR

with selective antagonists. This suggests that mechanical hyperalgesia requires the activation of both AMPA Rs and mGluRs. However, this study used very small drug doses (AMPA 1-100pmol) and found a transient effect (less than 5 minutes).

However, the findings of the current study are consistent with a recent study demonstrating that AMPA R blockade resulted in a less marked reduction in mechanical allodynia than thermal hyperalgesia in rats at peak behavioural reflex sensitisation by CCI (Garry et al., 2003b).

It has also been demonstrated that intrathecal pre-treatment with Joro spider toxin, a specific antagonist at Ca^{2+} permeable AMPA Rs prevented the development of mechanical allodynia following a mild burn (Sorkin et al., 1999). This finding raises the possibility that AMPA R subunit composition may be important in determining the role of this receptor in nociception.

The above studies and the results of the current investigation suggest that NMDA and AMPA Rs are involved in both mechanical allodynia and thermal hyperalgesia following central sensitisation due to prolonged noxious stimuli. However, the significance of each receptor type and its associated intracellular consequences in the induction of abnormal pain states may vary with the mechanism of induction (inflammation versus nerve damage) and the sensory modality involved (mechanical allodynia versus thermal hyperalgesia).

3.4.2 Group I mGlu receptors

It has been demonstrated that intrathecal injection of high doses of the mGluR group I agonist DHPG (25nmol) resulted in SNB for 30minutes. Although the dose used in the current study was much smaller (1.5nmol), activation of mGluR group I resulted in the most marked decrease in paw withdrawal threshold of the single agonists, with significant effects up to 50minutes after injection (see Figure 3.4c).

3.4.3 $VPAC_2$ receptors

The $VPAC_2$ R agonist Ro 25-1553 (0.3nmol) had a prolonged, significant effect on PWL to a noxious thermal stimulus but no discernable effect on paw withdrawal threshold to a mechanical stimulus. Indeed, the double combinations of AMPA + Ro 25-1553 and DHPG + Ro 25-1553 also had no effect on mechanical allodynia, whereas AMPA alone and DHPG alone caused a significant decrease in paw withdrawal thresholds. The triple combination of AMPA + DHPG + Ro 25-1553

reduced paw withdrawal threshold significantly at a single time point only. These findings suggest that activation of the $VPAC_2$ R agonist Ro 25-1553 may have an antiallodynic effect to mechanical stimuli, but causes hyperalgesia to noxious thermal stimuli.

This apparent differential effect of the $VPAC_2$ R has been demonstrated using VIP as an agonist at VPAC Rs. In the rat tail flick test to a thermal stimulus, intrathecal VIP (6.5nmol) had a pronociceptive effect, decreasing reaction time to 37% of control value at 1min after injection (Cridland and Henry, 1988). In agreement with the current study, VIP did not result in mechanical allodynia after intrathecal administration.

The PAC and VPAC R agonist PACAP has been shown to have differing effects depending on the dose. Low dose PACAP applied intrathecally (10-100pmol) has been shown to increase mouse tail flick latency in a hot plate test, whereas high dose regimes (200-2000pmol) resulted in scratching and biting pain related behaviour (Narita et al., 1996). In addition, electrophysiological studies have demonstrated that the $VPAC_2$ R plays relatively little role in sensory transmission in the normal animal, but is important in the transmission of prolonged noxious stimuli (such as mustard oil application) following nerve injury (Dickinson et al., 1999). These studies suggest that the PAC and VPAC Rs may have complex roles in the generation of sensitised states, depending upon the dose and sensory modality involved.

3.4.4 Synergy between AMPA, DHPG and Ro 25-1553

The combined effects of DHPG, AMPA and Ro 25-1553 on thermal hyperalgesia revealed a degree of interaction between these receptor agonists that has not been previously demonstrated (see Figure 3.2l and Figure 3.3a and b). Adding AMPA to DHPG + Ro 25-1553 resulted in a greater than additive increase in peak effect on thermal hyperalgesia. AMPA + DHPG + Ro 25-1553 had a significantly greater peak effect than all other combinations of drugs, excepting the quadruple combination (†† $p \leq 0.01$, Student-Newmann-Keuls ANOVA) (see Figure 3.3a). When comparing the cumulative effect of drug combinations, AMPA + DHPG + Ro 25-1553 and the quadruple combination had a significantly greater effect on thermal hyperalgesia than all other combinations († $p \leq 0.05$, Student-Newmann-Keuls ANOVA) (see Figure 3.3b). These findings suggest that activation of AMPA Rs in

addition to group I mGluRs and peptide $VPAC_2$ Rs results in a synergistic interaction in the induction of thermal hyperalgesia.

The addition of NMDA to the triple combination of AMPA + DHPG + Ro 25-1553 made no significant difference to the peak or cumulative effect of the drug combination on thermal hyperalgesia (see Figure 3.3a and b). This suggests that activation of the other receptor subtypes is sufficient to create a sensitised state, and that the NMDA R may have been activated by removal of the magnesium ion block as a consequence of depolarisation resulting from activating these receptors. The NMDA R would then be responsive to glutamate released from primary sensory neurons by innocuous and noxious peripheral stimuli.

3.4.5 Mechanical allodynia

The effect of drug combinations on mechanical allodynia was less marked than that on thermal hyperalgesia. In a practical sense this may have been due to the use of thermal hyperalgesia in determining the optimal dose of the agonist drugs. It is possible that a dose adequate to produce mild thermal hyperalgesia would not produce mechanical allodynia. Also, although there is no simple division of function, it is clear that certain receptors would seem to be involved to differential extents in either thermal hyperalgesia or mechanical allodynia, as was demonstrated by Ro 25-1553 (Cridland and Henry, 1988). It is possible that AMPA R play a less significant role in mechanical allodynia than in thermal hyperalgesia. In support of this suggestion, it has recently been demonstrated that specific AMPA R antagonists are less effective in reversing mechanical allodynia than thermal hyperalgesia in a CCI model of neuropathic pain (Garry et al., 2003b). Finally, the use of von Frey filaments to test the threshold for paw withdrawal involved discrete, large changes in stimulus intensity rather than the continuous scale used for PWL from a noxious thermal stimulus. This technique may render subtle changes in paw withdrawal threshold undetectable.

It proved impractical to test animals for cold allodynia following intrathecal injection, as the effects of the agonist combinations were bilateral and did not result in a clear pattern of behavioural change in the cold-water tank. There was also no apparent effect of acetone application to either hindpaw (data not shown).

3.4.6 Summary

It is clear from these results that AMPA Rs play an important role in thermal hyperalgesia and mechanical allodynia in the intact rat. Activation of AMPA Rs in addition to group I mGluRs and peptide $VPAC_2$ Rs results in a synergistic interaction in the induction of thermal hyperalgesia, possibly by inducing activation of NMDA Rs. In order to study the effect of neuropathic pain on the expression and subcellular location of AMPA GluR1 and GluR2 Rs, a well-characterised model of peripheral mononeuropathy in the rat was utilised (see Chapter 4).

CHAPTER 4: NEUROPATHIC PAIN AND LOCALISATION OF AMPA RECEPTOR EXPRESSION IN THE SPINAL DORSAL HORN

4.1 AIMS AND INTRODUCTION

These experiments were carried out to investigate the effect of an animal model of chronic, neuropathic pain due to peripheral nerve injury on the expression and subcellular location of AMPA R GluR1 and GluR2 subunits in the dorsal horn of spinal cord. The model used in this study was the chronic constriction injury (CCI) model of Bennett and Xie (Bennett and Xie, 1988), which involved loose ligation of the common sciatic nerve in the rat. Neuropathy developing after surgery was quantified using tests of behavioural reflex function.

In the current study AMPA R GluR1 and GluR2 subunit expression was assessed using immunofluorescence techniques under a number of different experimental conditions. The proportion of neurons expressing these subunits in the superficial dorsal horn, and the subcellular distribution of subunits was investigated in naïve animals, animals demonstrating peak behavioural reflex changes following CCI, and animals after sham surgery and after recovery from CCI. A second group of naïve animals were treated with AMPA 50μM in 0.2mls saline (0.9%) or 0.2mls saline (0.9%) intrathecally, 35 minutes prior to perfusion. In addition to the subcellular location of AMPA R subunits, the co-expression of GluR1 and GluR2 by superficial dorsal horn neurons was investigated following nerve injury.

In addition to changes in AMPA subunit expression, levels of key synaptic proteins also change in models of neuropathic pain, suggesting that synaptogenesis may be occurring in this abnormal pain state. The presynaptic vesicle protein synaptophysin was first isolated in 1985 (Wiedenmann and Franke, 1985) and has proved useful in the identification of synaptogenesis in several areas of the CNS (Bergmann et al., 1997).

Bassoon is a presynaptic nerve terminal protein, which shares features with both cytoskeleton-associated and peripheral membrane proteins (Sanmarti-Vila et al., 2000). In the current study, both synaptophysin and Bassoon were utilised as markers for regions of synaptic activity in dorsal horn neurons. To investigate the inclusion of AMPA Rs into synapses in the CCI model of neuropathic pain, the relationship

between the subcellular location of synaptic proteins Bassoon and synaptophysin and GluR1 subunits was assessed in dorsal horn cells.

The effect of intrathecal treatment with specific antagonists on the subcellular location of AMPA R GluR1 subunits was studied using animals with well-established behavioural reflex changes following nerve injury. Antagonists to AMPA R alone, and to NMDA R, mGluR and $VPAC_2$ R in combination were given intrathecally. This study was designed to investigate the relative importance of different receptor signals in GluR1 subunit trafficking.

4.2 Methods

4.2.1 Chronic constriction injury surgery

Surgical procedures were carried out in an aseptic manner, operators wearing gowns, filter masks and sterile gloves. Surgical instruments were autoclaved prior to use. Induction and maintenance of anaesthesia was provided by vaporised halothane at 2-4%. The carrier gas was compressed medical oxygen at a flow rate of 2-4l/min. Depth of anaesthesia was assessed using respiratory rate and pattern and response to surgery. After shaving the right hind limb, the skin was cleaned with chlorhexidine (0.5% in 70% alcohol), and a small (4-5mm) skin incision made over the sciatic nerve. The nerve was identified by blunt dissection and freed from surrounding tissues. Using a x40 operating microscope, four 4.0 chromic gut sutures were loosely tied around the nerve at 1mm intervals, proximal to its trifurcation. The sutures were tightened sufficiently to prevent slippage but not to occlude the vasa nervorum. The wound was sutured with 5.0 vicryl using a continuous subcutaneous technique with the suture ends buried. Postoperatively, adequate recovery from surgery and anaesthesia was ensured prior to return to their original cages. The animals were checked each day for three to five days before behavioural testing commenced (n = 15 animals). During sham surgery the nerve was exposed before wound closure without sciatic ligation (n = 3 animals).

Behavioural reflex testing

Tests were designed to highlight the occurrence of behavioural changes thought to represent neuropathic pain in experimental animals. Animals were tested pre-operatively and every few days post-operatively from day three. Animals were examined daily for the presence of autotomy. Gait was inspected and the posture and

condition of the affected hind limb noted. Abnormal behaviours such as repeatedly licking the affected limb, jumping, biting, or vocalising were also noted. In all cases, the ipsilateral operated limb was compared to the contralateral non-operated side. Thermal hyperalgesia and mechanical allodynia were assessed as described above (section 3.2.3).

The sensory stimulus used to detect cold allodynia in animals post CCI surgery was ice-cold water. The testing apparatus consisted of a transparent Perspex box with an aluminium floor plate submerged in water in equilibrium with ice in the base of the cage to give a water temperature of 3-4°C. The water depth was approximately 1cm; sufficient to cover both the hairy and glabrous skin of the rats' paws. Once placed in the box, animals were given a ten second acclimatisation period before measurement began. After acclimatisation, the time spent with each paw elevated out of the water (suspended paw elevation time – SPET), was noted over a twenty second period. This produced a score of 0 to 20 for each paw. This test was shown to be suitable for animals post CCI surgery, as there was a clear difference between the limb ipsilateral to surgery (marked withdrawal responses) and the normal limb (where there was minimal tendency to raise the limb).

Statistical analysis

Behavioural data were acquired on days 3, 6, 9 and 14 after CCI injury. Data were pooled for each day and the mean and SEM values calculated (see Figure 4.1). Development of thermal hyperalgesia was assessed using a paired Student's t-test to determine the statistical significance of any differences between the mean ipsilateral and contralateral hind paw values at each time point (**$p \leq 0.01$, * $p \leq 0.05$, $n = 8$). Student-Newmann-Keuls repeated measures ANOVA was then used to determine the statistical significance of differences between group values at each time point when compared to pre-operative control values (†† $p \leq 0.01$, † $p \leq 0.05$, $n = 8$) (see Figure 4.1a). Mechanical allodynia and cold allodynia were investigated using the equivalent non-parametric tests. Ipsilateral and contralateral sides were compared at each time point using a Wilcoxon test (** $p \leq 0.01$, * $p \leq 0.05$, $n = 8$). Statistically significant differences from pre-operative baseline were determined using a Friedman repeated measures ANOVA on ranks followed by Dunn's *post hoc* test (†† $p \leq 0.01$, † $p \leq 0.05$, $n = 8$) (see Figure 4.1b and c).

4.2.2 General immunohistochemistry methods

Experimental animals were deeply anaesthetised with halothane before being transcardially perfused with 150mls heparinised vascular flush consisting of 0.9% saline with 0.6mg/ml heparin (Sigma) using a small rotary pump at 30mls/minute (Watson-Marlow Ltd., UK.). The heparinised flush solution was followed by 300mls fixative solution containing 4% paraformaldehyde (Sigma), 0.1% gluteraldehyde (Sigma) and 12.5% picric acid (Becton Dickinson, UK) in 0.1M phosphate buffer at pH 7.4. After perfusion, the lumbar spinal cord was dissected out under an operating microscope and placed in a post-fix solution containing 4% paraformaldehyde, and 12.5% picric acid in 0.1M phosphate buffered normal saline (PBS). The tissue was post-fixed at 4°C for 24hours before being transferred through increasing concentrations of sucrose to a 30% solution in PBS for cryoprotection overnight.

Tissue sectioning was carried out on a Leitz Kryomat 1700 freezing microtome. Blocks of tissue 2-3mm long were cut from the lumbar enlargement of the spinal cord approximately at the L4-L5 level, with a scalpel blade, care being taken to maintain the orientation of the tissue. The blocks were then placed on the freezing microtome and embedded in 0.25% agar (Becton Dickinson, UK) prior to freezing at –20°C. If the tissue was derived from a CCI animal, a small pinhole was made in the contralateral ventral horn to aid recognition of the ipsi- and contralateral dorsal horns. Sections 40μm thick were cut and stored briefly in 0.1M PBS at 4°C.

Spinal cord sections were washed in PBS prior to suspension in a blocking solution containing 10% normal goat serum (Diagnostics Scotland), 4% fish skin gelatin (Sigma) and 0.2% Triton X-100 (Sigma) in 0.1M PBS for 1 hour at room temperature. Both primary and secondary antibodies were diluted in a solution containing 4% normal goat serum, 4% fish skin gelatin, and 0.2% Triton X-100 in 0.1M PBS. After removing the blocking solution, the sections were incubated in primary antibody solution overnight at 4°C. The sections were then washed in PBS once more prior to incubation at room temperature with fluorescent secondary antibodies for two hours. During secondary antibody incubation, pots containing sections were gently agitated in the dark. After a final wash in PBS, sections were treated with To-Pro3, a cyanine nucleic acid stain, (Molecular Probes) 1:1000 for two minutes prior to cover-slipping with Vecta Shield (Vector Laboratories). Cover slips were sealed using clear varnish and the slides were stored at 4°C in the dark prior to

confocal microscopy. Control sections were processed as above omitting primary or secondary antibodies.

Antibodies

Primary antibodies were used at the following concentrations: rabbit polyclonal anti-GluR1 (1:100, Upstate Biotech Inc); mouse monoclonal anti-GluR2 (1:200, Chemicon). Secondary fluorescent antibodies were used at 1:1000: Alexa Fluor goat anti-mouse 488nm (green fluorescence) (Molecular Probes) and Alexa Fluor 568nm goat anti-rabbit (red fluorescence) (Molecular Probes).

4.2.3 GluR1 and GluR2 AMPA receptor subunit expression in the spinal dorsal horn in a neuropathic pain state

To investigate the effect of neuropathic pain on AMPA R subunit expression, four experimental groups were used (n = 3-6): naïve animals; those exhibiting peak sensitisation due to CCI surgery as assessed by behavioural reflex testing at post operative day 14; sham-operated animals and animals demonstrating behavioural recovery from CCI surgery.

Confocal microscopy and image analysis

Slides produced using the above technique were examined using a Leica TCS NT confocal system (Leica Microsystems Heidelberg GmbH, Germany). Digital images were stored on TDK CD-R media for display and analysis using 'LCS Lite', imaging software. Each image was labelled with a code name to facilitate blinding during analysis. Single optical section x20 images were acquired from both dorsal horns for each cord slice. Images were 500 x 500μm square, and included laminae I to III of the dorsal horn (see Figure 4.2a). Between six and twenty image slices were analysed for each experimental group. The lateral aspect of the dorsal horn was included on each image, where the superficial laminae curve steeply in a ventral direction. As a result, the extreme medial part of the dorsal horn was often omitted. Cells were counted from the medial cut-off of the dorsal horn to the start of the steeply curved section. In this manner, the region of afferent input from the hind limb was the focus of attention (Swett and Woolf, 1985). The experimenter was blinded to the treatment group and the side of dorsal horn for cell counting. GluR1-

immunopositive cells (red) and GluR2-immunopositive cells (green) were counted in each of the three outer dorsal horn laminae.

The definition of dorsal horn laminae was based around the location of lamina II for this study, as this region had the clearest neuropil labelling (Spike et al., 1998). Lamina II was taken as the region of dorsal horn with neuropil labelled with GluR2 in its inner- and mid-portion, and GluR1 in its mid- and outer-portion. This lamina comprised a band of dense neuropil and small somata running from approximately 60-120μm below the surface of the dorsal horn, measured midway through its mediolateral aspect. This defined a region of the dorsal horn which was consistent with that described by Molander et al (Molander et al., 1984). Lamina I was defined as the region from outer lamina II extending dorsally for 30μm. Lamina III was taken as the region 100μm ventrally from inner lamina II. Measurements of the thickness of the superficial laminae were based upon these criteria generated using a group of naïve animals (n=3, 3 slices per animal). Lamina I measured 29.6 ± 1.5μm in depth, and lamina II was approximately twice as deep, measuring 61.7 ± 3.2μm using the fixing protocol described. Lamina IV and V showed little GluR1/2 labelling and were not investigated further in the current study.

Where labelling was less clearly defined, immunofluorescent objects with greater than twice background intensity were recorded as cells, if they were typically cell-shaped and were associated with cyanine dye nuclear staining with To-Pro3. This nucleic acid-binding cyanine dye causes far-red fluorescence of nuclei when excited at 633nm, which has been artificially coloured blue in the current study. The total number of cells in each lamina was noted by counting all To-Pro3 stained nuclei. The proportion of GluR1- or GluR2-immunopositive cells was then calculated for each lamina by dividing the number of immunopositive cells by the total number of nuclei. Statistically significant differences between the mean percentage of GluR1- and GluR2-immunopositive cells on ipsilateral and contralateral sides of the dorsal horn were determined for each lamina using a chi-squared test (** $p \leq 0.01$). All experimental groups were then compared to the naïve control group (ANOVA followed by Dunnetts's *post hoc* analysis, †† $p \leq 0.01$) (see Figures 4.3 and 4.4).

4.2.4 Comparison of GluR1/2 localisation with that of presynaptic markers

Spinal cord tissue from naïve animals and animals at peak behavioural sensitisation due to CCI surgery was prepared as above (n=3) (Section 4.2.2). Sections were labelled with GluR1 primary antibodies and also mouse monoclonal anti-Bassoon (1:500, Stressgen) or mouse monoclonal anti-synaptophysin (1:200, Pharmingen). Alexa Fluor secondary antibodies were applied as described above (Section 4.2.2).

Confocal microscopy and image analysis

Stacks of confocal optical sections were acquired using a x63 oil immersion lens. Image stacks consisted of 7-12 150μm^2 optical slices, 1-2μm apart centred on the midpoint of the dorsal horn within lamina II. Single low magnification optical sections of dorsal horns labelled with anti-Bassoon antibodies were also acquired using a x10 lens.

Low magnification images were used to compare levels of Bassoon immunofluorescence between ipsilateral and contralateral dorsal horns after CCI surgery. Regions of interest in the medial, mediolateral and far lateral dorsal horn of lamina II measuring 50x50μm were analysed to give a mean fluorescence intensity value. The mean intensity values for each region of the dorsal horn ipsilateral and contralateral to CCI surgery were compared using a paired Student's *t* test (see Figure 4.5b, Figure 4.6b).

Bassoon immunofluorescence was punctate in appearance, with some puncta clustered near GluR1-immunopositive cells (see Figure 4.5b). For the purpose of analysis, a Bassoon 'punctum' was defined as a group of 9 or more pixels with a green fluorescence intensity level of 75%-100% of the maximum found on that image, provided that this was at least twice the background level of green fluorescence. The number of Bassoon immunopositive puncta per GluR1-immunopositive cell was determined by counting all puncta within 0.5μm of a cell. A single mid-cell optical slice was used for this analysis, and the cell marked to prevent double counting. The mean number of puncta per mid-cell slice was determined for naïve animals and ipsi- and contralateral to CCI injury. Statistical significance of any difference between ipsilateral and contralateral sides was determined by a Mann-Whitney U test (* $p \leq 0.05$). All three experimental groups were compared using a Kruskal-Wallis one-way

ANOVA on ranks followed by Dunn's *post hoc* analysis (** $p \leq 0.01$) (see Figure 4.5a).

Synaptophysin immunofluorescence was less clearly defined than that of the Bassoon antibody and it was not possible to define synaptophysin puncta as precisely. Any green synaptophysin labelling within 0.5μm of a GluR1-immunopositive cell was counted as a punctum. As for Bassoon immunohistochemical analysis, a single mid-cell optical slice was used for counting synaptophysin puncta. The mean number of puncta per mid-cell slice was determined for naïve animals and ipsi- and contralateral to CCI injury. Statistical significance of any difference between ipsilateral and contralateral sides in CCI animals was determined by a Mann-Whitney test (see Figure 4.7).

4.2.5 AMPA receptor subunit colocalisation in the spinal dorsal horn in a neuropathic pain state

Spinal cord tissue from naïve animals and animals at peak behavioural sensitisation post CCI (n = 3) was prepared using the technique outlined above (section 4.2.2). Confocal images of both dorsal horns from each spinal cord slice were obtained with a x40 objective lens (see Figure 4.8b). The images consisted of a stack of 7-12 optical slices (approximately 0.5μm thick), from a 40μm tissue section, each spaced 1-2μm apart and approximately 250μm^2 of an area centred on the central part of lamina II of the dorsal horn. This region is known to receive afferent input from branches of the sciatic nerve including the superficial peroneal and the sural nerves (Swett and Woolf, 1985). The images of the cord slices were scanned from the surface down to loss of antibody penetration. For each GluR1- or GluR2-immunopositive cell, red and green fluorescent intensity was measured using 'LCS Lite' software across a single mid-section optical slice on a scale of 0-255units. Peak red (GluR1) and green (GluR2) intensity along the longest axis was noted for each immunopositive cell. Background and peak levels of red GluR1 fluorescence and green GluR2 fluorescence were measured for each slice in the stack. Red and green fluorescence was graded as absent, low or high. Absent fluorescence was defined as being less than twice baseline levels. Low fluorescence was defined as twice baseline to 75% of maximum intensity for that slice. High fluorescence was defined as intensity from 75-100% of maximum for that slice. The selection of these levels of

fluorescence as 'high' and 'low', although arbitrary, resulted in a division of the cells into two discrete categories.

Comparisons between groups were made using Chi-Squared tests. The proportion of cells expressing GluR1 alone, GluR2 alone or both, was compared between experimental groups (see Figure 4.8a). Changes in the level of fluorescence expressed by cells were investigated for GluR1 and GluR2 separately (see Figure 4.9). The degree of colocalisation demonstrated by cells brightly labelled (>75% of maximal value for that image) with either antibody was also compared.

4.2.6 The influence of specific receptor agonists and CCI on the subcellular distribution of AMPA receptor subunits

To investigate the effect of neuropathic pain on AMPA R subunit subcellular distribution, the following experimental groups were used: naïve animals; those exhibiting peak behavioural sensitisation following CCI surgery (post-operative day 14); sham-operated animals (post-operative day 14) and animals demonstrating behavioural recovery from CCI surgery (n=3-6) (12 weeks post-operative). Two further groups of anaesthetised naïve animals were treated with either AMPA 50μM in 0.2mls saline (0.9%) or 0.2mls saline (0.9%) intrathecally, 35 minutes prior to perfusion (n=3). Spinal cord tissue from experimental animals was prepared as above (Section 4.2.2).

Confocal microscopy and image analysis

Confocal images of both dorsal horns from each spinal cord slice were obtained using a x40 lens (see Figure 4.2b). Image stacks were acquired, consisting of 7-12 250μm^2 optical slices, 1-2μm apart centred on the mediolateral dorsal horn within lamina II. The subcellular distribution of AMPA R subunits was investigated by measuring immunopositive cells in the dorsal horn using 'LCS Lite' software. The GluR1-immunopositive region of each cell was measured along its longest axis choosing the optical slice nearest to its midpoint. Each cell was marked after GluR1 measurement to prevent double counting. The nucleus was also measured and the data used to calculate the GluR1-immunopositive length to nuclear-diameter ratio. There were sufficient GluR1-immunopositive cells to analyse data for all experimental groups for laminae I, II and III (see Figures 4.10a-e, 4.6a). GluR2 immunofluorescence was less discrete and fewer cells were measurable, limiting

analysis of data to lamina II (see Figure 4.11). Statistically significant (** $p \leq 0.01$, * $p \leq 0.05$) differences between the median length of the GluR1-immunopositive regions of cells in each experimental group was investigated using a Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's *post hoc* analysis for lamina I, II and III. The same statistical tests were applied to GluR2 data for lamina II.

4.2.7 The effect of specific receptor antagonists on the subcellular distribution of AMPA receptor subunit in neuropathic pain

Male Wistar rats exhibiting peak behavioural sensitisation following CCI surgery (post-operative day 14) were used for this study. Animals were anaesthetised with halothane prior to slow intrathecal injection of either 0.2mls of AMPA R antagonist or 0.2mls of a combination of antagonists to NMDA R, mGluR1, mGluR5 and $VPAC_2$ R (n=3). These animals were kept warm and anaesthetised for 35minutes prior to perfusion. Spinal cord slices were labelled with antibodies to GluR1 and nuclei were stained with To-Pro3 as above (Section 4.2.2).

Specific receptor antagonists

All drugs were administered intrathecally to anaesthetized animals in 200µl sterile saline (0.9%) unless stated otherwise. The AMPA R antagonist was NBQX (sodium salt), 100µM. The combination of antagonists included: (R)-CPP (NMDA R antagonist), 10µM; LY 376385 (mGluR1 antagonist), 10mM; MPEP (mGluR5 antagonist), 10µM and PG 99-465 ($VPAC_2$ R antagonist), 2µM in 0.06% dimethylformamide in saline.

Confocal microscopy and image analysis

Confocal image stacks were acquired with a x40 lens and analysed as above (Section 4.2.6.1). GluR1-immunopositive cells from the mediolateral region of lamina II alone were analysed for this study (see Figure 4.12b). Statistically significant differences between the median linear extent of the GluR1-immunopositive regions of cells in each experimental group were investigated using a Mann-Whitney rank sum test (see Figure 4.12).

4.3 Results

4.3.1 Chronic constriction injury

General observations

The vast majority of animals developed behavioural changes characteristic of CCI injury (12 of 15animals) and any that did not were excluded from further analysis (n=3). Following CCI, the operated limb was held flexed and the animals tended to favour the contralateral hind paw in locomotion with the affected paw held inverted and in plantar flexion with claws tightly clasped. On some occasions animals held the affected paw away from the surface completely. Other than developing a 'hopping' gait, there were no other changes in the speed or pattern of locomotion. Post-operative animals appeared healthy and had no gross signs of weight loss or neglect and could be handled without any distress being evident. During the study, no animals developed autotomy. Sham-operated animals did not develop any behavioural signs of neuropathic pain.

Thermal hyperalgesia

Thermal hyperalgesia assessed using Hargreaves' apparatus (see Figure 3.1a) developed to a varying degree in all animals post-operatively. Mean pre-operative baseline PWL were measured as ipsilateral, 15.29 ± 0.74 secs and contralateral 14.77 ± 0.86 secs (all data mean ± SEM). At all time points from the third post-operative day the animals showed significantly reduced PWL ipsilateral to the injury and as compared to the contralateral side ($p \leq 0.01$, paired Student's *t* test) and to all other time points ($p \leq 0.01$, Student-Newmann-Keuls repeated measures ANOVA) (see Figure 4.1a). Although there was a trend for the contralateral PWL to decrease in parallel with the ipsilateral side, this was not statistically significant. The animals reached a peak of thermal hyperalgesia at varying times, the median being post-operative day 9 when the ipsilateral latency was 7.64 ± 0.78secs and the contralateral 12.50 ± 0.68secs.

The behaviour of the animals also altered qualitatively with the development of thermal hyperalgesia. In pre-operative animals the response to a noxious thermal stimulus consisted of a rapid flick of the hind paw with a return to the resting posture. After CCI surgery, animals tended to hold the limb elevated for a longer period of time, or shake or lick the affected paw.

Mechanical allodynia

Prior to CCI injury, animals displayed a mean paw withdrawal threshold pressure of 4830 mN/mm^2 (see Figure 4.1b). This value was consistent between both paws and for all animals. The majority of animals demonstrated an increased sensitivity to mechanical stimuli by post-operative day 3, and by post-operative day 6 these changes were universal. From post-operative day 9 onwards, there was a significant reduction in the withdrawal threshold of the ipsilateral hind paw (1413 ± 293.3 mN/mm^2) when compared with the contralateral hind limb (4830 ± 0 mN/mm^2; $p \leq 0.01$, Mann-Whitney rank sum test) or pre-operative values ($p \leq 0.01$, Kruskal-Wallis one way ANOVA on ranks). There were no significant differences between the threshold values of the contralateral hind limb in the nerve-injured animals compared to pre-surgery values at any time point. The rats were responding to previously innocuous stimuli with behaviour indicative of pain-related withdrawal reflex activation, suggesting that CCI injury leads to the development of mechanical allodynia on the nerve-injured side, which is in agreement with previous reports using this model.

Cold Allodynia

Cold allodynia was a marked feature of CCI injury. Naïve rats showed no change in observed behaviour when placed in the shallow cold water bath (see Figure 3.1c). Their general level of activity remained as before and they showed no aversive response to the cold stimulus. From post-operative day 3, nerve injured animals exhibited altered behaviour, lifting the affected hind paw out of the water and keeping it elevated or shaking or licking it. As the cold stimulus did not evoke any pain-related behaviour in naïve animals, but had a clear aversive paw withdrawal effect after nerve injury, it can be defined as a form of allodynia where a previously innocuous stimulus triggers pain behaviour following CCI.

Initially, the affected paw was only briefly elevated, but by post-operative day 9, animals were maintaining this abnormal posture for the majority of the time (see Figure 4.1c). Most animals demonstrated marked effects by post-operative day 14 (SPET 15.8 ± 1.99 secs), with 50% of the animals keeping the affected paw elevated for the full 20-second test period accompanied by occasional vocalising. Once removed from the bath after a total of 30sec, normal behaviour resumed almost

immediately, suggesting that the pain related behaviour was dependent on the cold stimulus and no continuing pain was experienced. As animals were used for perfusion experiments at this time point, it was not possible to collect recovery data.

4.3.2 AMPA receptor subunit expression in the spinal dorsal horn

General considerations

Both GluR1 and GluR2 were strongly expressed in the superficial dorsal horn (see Figure 4.2a), with a larger proportion of cells labelled with GluR2, in agreement with previous findings as reviewed in (Coggeshall and Carlton, 1997). Both antibodies labelled cell bodies and proximal processes, and neuropil between cells. The marked labelling of neuropil, consisting of fine cellular processes between cell bodies, has been noted in many previous studies (Coggeshall and Carlton, 1997). In general, GluR1-immunofluorescence was strongest in lamina I and outer-to-mid lamina II and GluR2-immunofluorescence was strongest in lamina II and less marked in lamina I and III. The distribution of GluR1 and GluR2 immunoreactivity matched that seen by Todd et al using immunoperoxidase staining and immunofluorescent labelling (Spike et al., 1998), and also Popratiloff using immunoperoxidase (Popratiloff et al., 1996). For each experimental group, 6 to 20 optical slices were analysed. Only the region of dorsal horn supplied by the sciatic nerve was analysed (see section 4.2.3). Within this region, each slice contained 37 ± 2.6 cells in lamina I, 70 ± 4.7 cells in lamina II and 48 ± 3.0 cells in lamina III (all data shown as mean ± SEM).

a) GluR1 expression

In naïve animals, 27.1 ± 1.9% of cells in lamina I were labelled with GluR1 antibody (see Figure 4.3). In lamina II, roughly the same percentage of cells was GluR1-immunopositive (24.6 ± 1.7%). Fewer cells were GluR1-immunopositive in lamina III (12.8 ±1.7%). There was no difference in the percentage of GluR1-positive cells in laminae I or III between any of the experimental groups (ANOVA).

The most notable finding was a marked difference in the percentage of GluR1-immunopositive cells between ipsilateral (13.4 ± 0.99%) and contralateral (23.62 ± 2.34%) sides of the spinal cord in lamina II found in animals at peak behavioural sensitisation following CCI surgery (** $p \leq 0.01$ Chi-squared test, See Figure 4.3c). When all experimental groups were compared to naïve animals, the percentage of

GluR1-immunopositive cells in lamina II of the dorsal horn ipsilateral to injury was significantly lower (†† $p \leq 0.01$ ANOVA versus naïve control followed by Dunnett's *post hoc* test). There was no difference between the ipsilateral and contralateral sides for recovered and sham animals in any of the three laminae investigated (Chi-squared test, See Figure 4.3).

b) GluR2 expression

In naïve animals, labelling of neuropil was virtually absent in lamina I. Compared to GluR1, there were 1/3 fewer cells being labelled in this lamina (18.3 ± 3.0% of all cells). GluR2 labelling of both cells and neuropil was more marked and more focussed in Lamina II. Some 26.9 ± 2.8% of cells were immunopositive, the majority being within mid- and inner part of lamina II. Fewer cells were GluR2-immunopositive in lamina III (15.6 ± 1.8%) (see Figure 4.4).

There was no detectable difference between the ipsilateral and contralateral sides for recovered animals, sham-operated animals and animals at peak behavioural sensitisation following CCI, as determined by behavioural sensory reflex testing, in any of the three laminae investigated (Chi-squared test, See Figure 4.4). None of the experimental conditions differed from control when compared with naïve animals (ANOVA).

4.3.3 Colocalisation with presynaptic markers

Bassoon and GluR1 expression in the spinal dorsal horn in a neuropathic pain state

Labelling with anti-Bassoon antibody revealed bright, punctate immunofluorescence throughout the superficial dorsal horn. Labelling was particularly marked in a band along mid- and inner lamina II. There appeared to be a complex network of Bassoon-immunopositive elements within the dorsal horn, surrounding the shadows of cells with small, bright puncta superimposed on the background immunofluorescence (see Figure 4.5b). GluR1-immunopositive cells were clearly visible within the dorsal horn and their appearance was not altered by the presence of Bassoon immunofluorescence.

There were significantly more puncta associated with each lamina II GluR1-immunopositive cell ipsilateral to injury (4.1 ± 1.0 per cell) as compared to the contralateral side (2.7 ± 0.7per cell) (* $p \leq 0.05$, Mann-Whitney test, See Figure 4.5a),

and as compared to naïve animals (2.1 ± 0.5 per cell) (†† $p \leq 0.01$, Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's *post hoc* analysis).

Overall Bassoon immunofluorescence was also compared for three regions of interest from lamina II of dorsal horns on each side of the spinal cord in animals showing peak behavioural sensitisation due to CCI (see Figure 4.5c). There was no significant difference in the intensity of Bassoon immunofluorescence between the two sides of the cord in sensitised animals, suggesting that Bassoon expression itself was not altered by nerve injury.

Synaptophysin and GluR1 expression in the spinal dorsal horn in a neuropathic pain state

Synaptophysin immunofluorescence was less discrete and less bright than Bassoon. The antibody did not penetrate well into the tissue slices, leaving only the upper few layers of each image stack for analysis. However, brighter puncta of synaptophysin were clearly visible, some of which were associated with GluR1-immunopositive cells (see Figure 4.7b). There was no significant difference in the number of synaptophysin puncta associated with each lamina II GluR1-immunopositive cell ipsilateral to injury (1.3 ± 0.22 per cell) as compared to the contralateral side (1.8 ± 0.27per cell) (Mann-Whitney test, See Figure 4.7a).

As synaptophysin immunofluorescence was incompletely developed through the surface layers of the spinal cord slices, it appeared patchy on any given optical slice. This prevented comparison of overall synaptophysin immunofluorescence between the ipsilateral and contralateral sides of the spinal cord.

4.3.4 Colocalisation of AMPA receptor subunits in the spinal dorsal horn

When considering all immunopositive cells in lamina II in naïve animals, 21.1 ± 2.7% appear to express GluR1 alone, 23.2 ± 3.1% appear to express GluR2 alone and the remainder express both subunits (55.8 ± 3.8%). The proportion of cells appearing either GluR1-immunopositive or GluR2-immunopositive, or co-expressing both GluR1 and GluR2 did not differ significantly between naïve animals and animals at peak behavioural sensitisation following CCI (Chi-squared test, n=3), suggesting that the subunit composition of AMPA Rs here is not markedly altered in response to CCI (see Figure 4.8a).

However, there was a significant difference in the proportion of cells expressing low and high levels of GluR1-fluorescence between experimental groups (see Figure 4.9a) (* $p \leq 0.05$ Chi-squared test). These two levels of fluorescence were defined as follows: low fluorescence was defined as twice background to 75% of peak fluorescence for GluR1 on that optical slice; high fluorescence was defined as 75% - 100% of peak fluorescence in arbitrary units (0-255) (see section 4.2.5). The same definitions of high and low fluorescence were applied to GluR2 expression. For naïve animals, a greater proportion of cells tended to express GluR1 at a high, rather than low, level (45.1 ± 5.0% high versus 29.7 ± 4.8% low). The same pattern was noted for cells contralateral to CCI injury (58.0 ± 2.0% high versus 18.0 ± 3.7% low). Ipsilateral to CCI injury, this finding was reversed, and the majority of GluR1 expression was at a low level (28.0 ± 4.9% high versus 40.0 ± 7.1% low) (n=3). This represents a significant reduction in the proportion of cells labelled with high levels of GluR1 immunofluorescence ipsilateral to CCI injury (* $p = 0.0377$, Chi-squared test, see Figure 4.9). No such suggestion of a change in the pattern of labelling intensity was seen for GluR2 fluorescence, and there was no apparent difference between the experimental groups in the proportion of cells expressing high and low levels of GluR2 (see Figure 4.9b).

4.3.5 Receptors influencing the subcellular distribution of AMPA receptor subunits

Subcellular GluR1 distribution

Several distinct patterns of GluR1 distribution occurred within immunopositive dorsal horn cells of naïve animals. In one population, the GluR1-immunopositive region closely surrounded the nucleus in a ring-shape, with occasional projections indenting it deeply (see Figure 4.10e, lamina I, CCI con). Often, the GluR1-immunopositive region was thicker towards one end of the cell (see Figure 4.10e, lamina II, naïve). Less commonly, GluR1-immunopositive cytoplasm was seen streaming away from the nucleus, towards processes, up to 40μm long (see Figure 4.10e, lamina II, AMPA or CCI ips). In a number of cells, labelling appeared punctate (see Figure 4.10e, lamina II, CCI ips), whilst in others the distribution of GluR1 immunoreactivity appeared more homogeneous (see Figure 4.10e, lamina I, naïve).

The ring-shaped perinuclear GluR1 pattern may represent the resting state, whereas redistribution of GluR1 subunits reflects trafficking of GluR1 further from the nucleus towards sites of synaptic plasticity during sensitisation. This change in the pattern of subcellular GluR1 immunoreactivity caused the linear extent of GluR1-immunopositive cytoplasm to increase. The mean linear extent of GluR1-immunopositive cytoplasm within the dorsal horn cell (as a multiple of nuclear diameters) was evaluated, potentially as a measure of the degree of redistribution of AMPA R subunits.

There was no significant difference in the mean length of GluR1-immunopositive cytoplasm in lamina I cells in dorsal horns of naïve animals, those treated with AMPA or those ipsilateral or contralateral to nerve injury (1.40 ± 0.02 nuclear diameters) (see Figure 4.10a).

Major changes in subcellular GluR1 subunit distribution occurred at peak behavioural sensitisation following CCI in lamina II. There was a marked increase in the mean length of GluR1-immunopositive cytoplasm in lamina II dorsal horn cells on the side ipsilateral to CCI injury when compared to the contralateral side (2.00 ± 0.06 nuclear diameters versus 1.62 ± 0.03 nuclear diameters). There was also a similar increase in GluR1-immunopositive cytoplasm length within the dorsal horn cells after intrathecal AMPA treatment (1.97 ± 0.04 nuclear diameters) (** $p \leq 0.01$ Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's *post hoc* test).

There was no difference in the mean length of GluR1-immunopositive cytoplasm between lamina II dorsal horn cells of naïve animals, those contralateral to injury and animals treated with intrathecal saline (mean GluR1-immunopositive length 1.54 ± 0.02 nuclear diameters) (see Figure 4.10b).

The mean length of GluR1-immunopositive cytoplasm within cells in lamina III was similar to that seen in lamina I (1.46 ± 0.04 nuclear diameters), and there was no significant difference between experimental groups (see Figure 4.10c).

Subcellular GluR2 distribution

The immunofluorescence associated with GluR2 was more diffuse than GluR1, and did not appear punctate. Cells were labelled in a perinuclear fashion and, unlike the GluR1 subunit, did not appear to include labelling of processes of any length (see Figure 4.11b). As fewer cells throughout the dorsal horn were labelled

clearly enough to allow accurate measurement of their length, data were collected for lamina II only. Unlike GluR1, there was no significant difference in the mean length of cells in lamina II of dorsal horns of naïve animals, those treated with AMPA or those ipsilateral or contralateral to nerve injury (Kruskal-Wallis one-way ANOVA on ranks) (see Figure 4.11a).

4.3.6 Effects of specific receptor antagonists on AMPA receptor subunit subcellular distribution in neuropathic pain

The appearance of the GluR1 immunopositive cells in this study did not differ from that noted previously (See Figure 4.12b). After administration of the AMPA R antagonist NBQX, overall appearances changed little. Some cells demonstrated labelling of GluR1 further from the centre of the cell along processes whereas others demonstrated a perinuclear pattern of fluorescence (see Figure 4.12b).

Intrathecal administration of antagonists to mGlu1/5R, NMDA R and $VPAC_2R$ in combination caused a reversal in the subcellular redistribution of GluR1 that was seen in animals at peak behavioural sensitisation following CCI ipsilateral to injury. The group receiving the combination of antagonists showed no difference in mean length of GluR1-immunopositive cytoplasm within dorsal horn cells in lamina II between the side ipsilateral to CCI injury and the contralateral side (1.45 ± 0.04 nuclear diameters versus 1.40 ± 0.02 nuclear diameters) (Mann-Whitney test) (see Figure 4.12a). However, the difference in mean length of GluR1-immunopositive cytoplasm between the two sides of the cord was not reversed by administration of an AMPA R antagonist alone. In this experimental group, the cells on the side ipsilateral to CCI injury (1.55 ± 0.04 nuclear diameters) had a greater mean spread of GluR1 immunopositive cytoplasm than those on the contralateral side (1.35 ± 0.03 nuclear diameters) (**$p \leq 0.05$, Mann-Whitney test).

The above findings suggest that the change in subcellular GluR1 distribution associated with sensitisation may be reversed by administration of antagonists to mGluR1/5, NMDA R and $VPAC_2$ R in combination, but not by an antagonist to the AMPA R alone. These data suggest that CCI-mediated activation of this combination of receptors is instrumental in causing subcellular re-distribution of GluR1 following nerve injury.

Figure 4.1 The time course of development of thermal hyperalgesia, mechanical and cold allodynia ipsilateral to CCI injury. a) The time taken for hindpaw withdrawal from a noxious thermal stimulus was measured before and at several time points after CCI surgery to the sciatic nerve (n=8). Data shown as mean ± SEM. Significant differences between the ipsilateral (○) and contralateral (■) paws were determined by paired Student's t tests (* $p \leq 0.05$, ** $p \leq 0.01$). Differences between pre-operative and post-operative values were determined by Student-Newmann-Keuls repeated measures ANOVA († $p \leq 0.05$, †† $p \leq 0.01$). b) Paw withdrawal responses to von Frey filaments (n=8) were measured at the same time points. Significant differences between ipsilateral (○) and contralateral (■) paws were determined by a Wilcoxon test (* $p \leq 0.05$, ** $p \leq 0.01$), and differences between pre- and post-operative values were determined by Friedman repeated measures ANOVA on ranks followed by Dunn's post hoc test († $p \leq 0.05$, †† $p \leq 0.01$). c) The suspended paw withdrawal time (SPET) to a cold water stimulus (n=8) was also noted. Significant differences between paws were determined by paired Student's t tests (* $p \leq 0.05$, ** $p \leq 0.01$). Differences between pre-operative and post-operative values were determined by repeated measures ANOVA followed by Dunnett's post hoc test (†† $p \leq 0.01$).

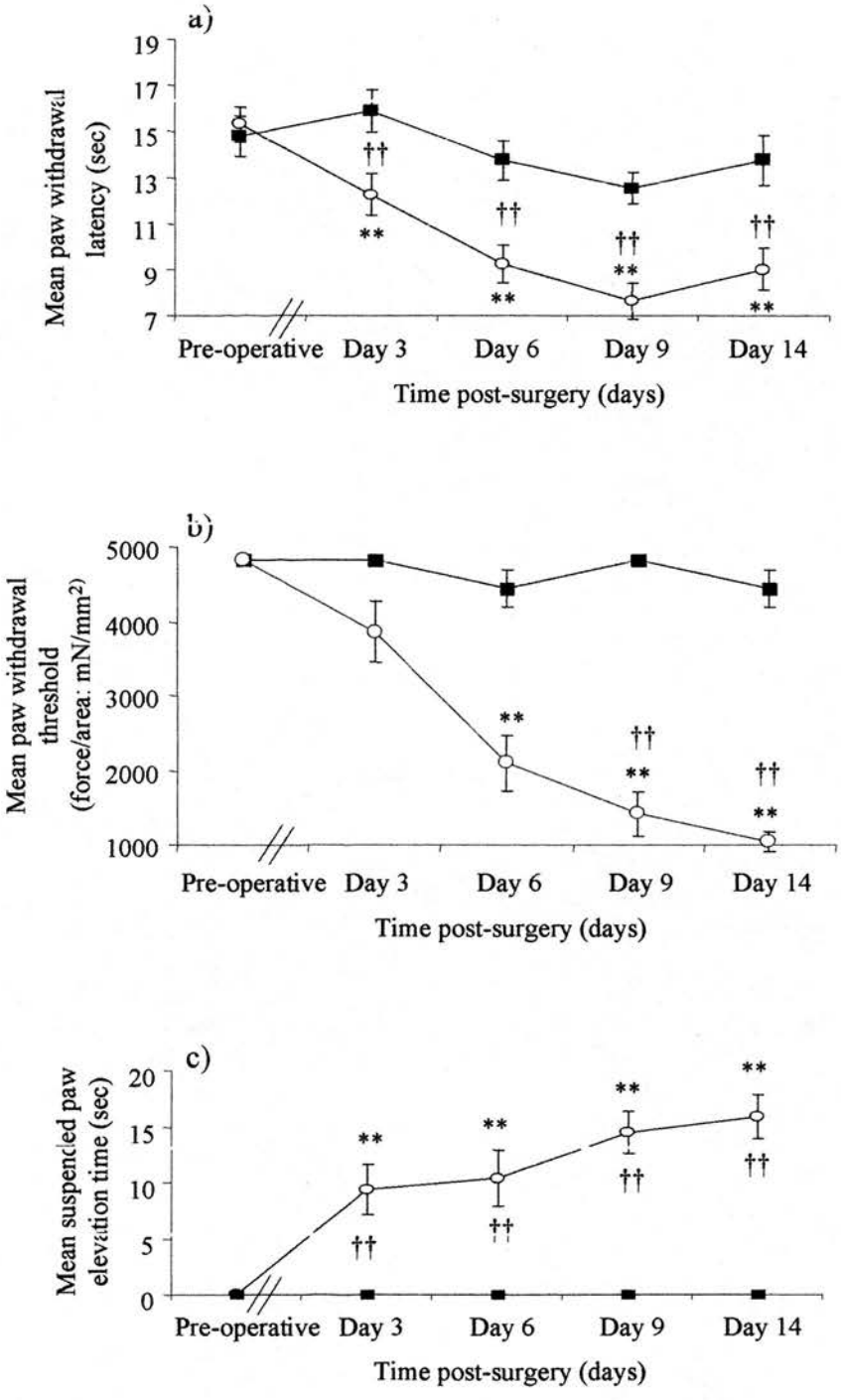
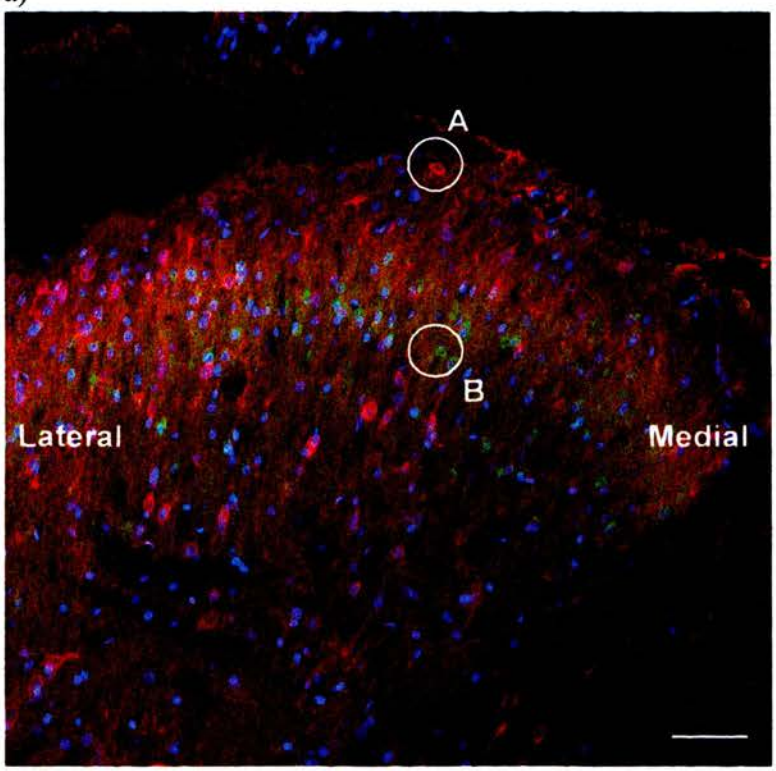

a)
Mean paw withdrawal latency (sec)
19
17
15
13
11
9
7
Pre-operative
Day 3
Day 6
Day 9
Day 14
Time post-surgery (days)
b)
Mean paw withdrawal threshold (force/area: mN/mm²)
5000
4000
3000
2000
1000
Pre-operative
Day 3
Day 6
Day 9
Day 14
Time post-surgery (days)
c)
Mean suspended paw elevation time (sec)
20
15
10
5
0
Pre-operative
Day 3
Day 6
Day 9
Day 14
Time post-surgery (days)

Figure 4.2 Confocal microscope image of the dorsal horn of a naïve animal, using x20 (a) or x40 (b) lens to show GluR1-immunopositive cells labelled with fluorescent secondary antibody (rabbit polyclonal anti-GluR1, Alexa Fluor 568 goat anti rabbit - red), GluR2-immunopositive cells labelled with fluorescent secondary antibody (mouse monoclonal anti-GluR2, Alexa Fluor 488 goat anti mouse - green) and To Pro3 nuclear marker (blue). A shows a GluR1-immunopositive cell (circled) and B shows a GluR2-immunopositive cell (circled). Scale bar 50μm.

a)



b)

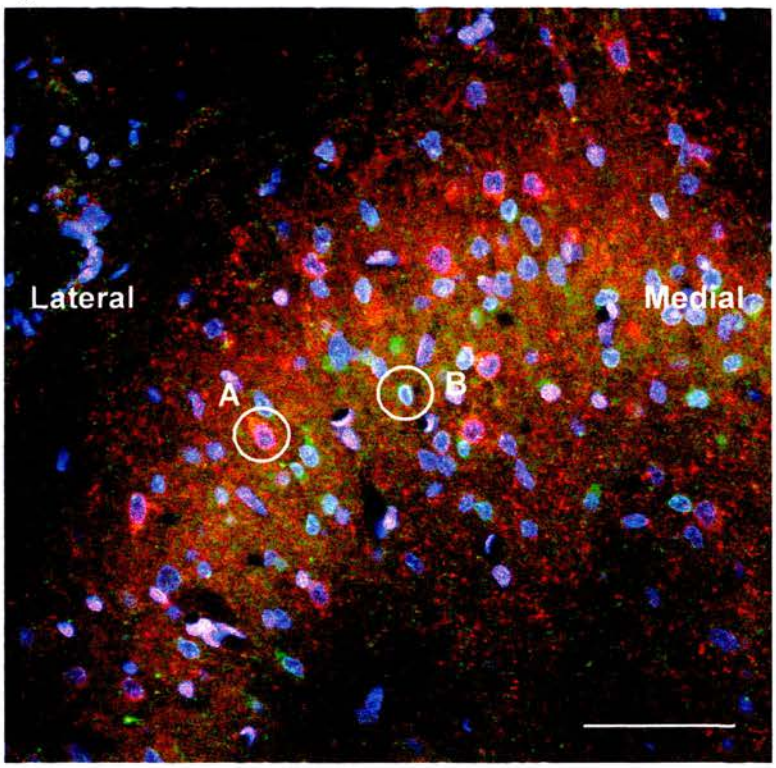
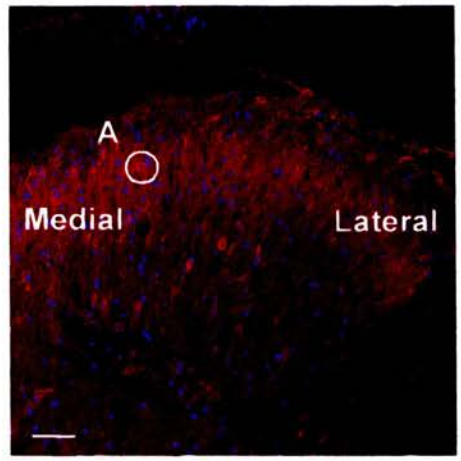
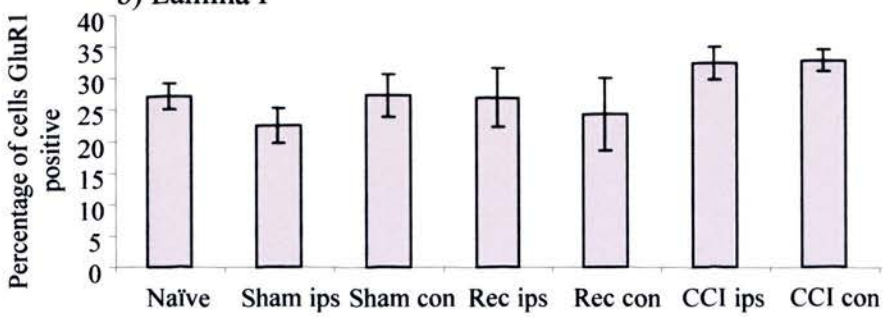


Figure 4.3 a) Shows GluR1-immunopositive cells labelled with fluorescent secondary antibody (red) and To Pro3 nuclear marker (blue) in the dorsal horn of a naïve animal (x20). A GluR1-immunopositive cell (circled). Scale bar 50μm. b)-d) GluR1-immunopositive cells were counted and the ratio of immunopositive cells to the total number of To Pro3 positive nuclei in laminae I, II and III was calculated as a percentage (b-d). Data are shown as mean ± SEM (n=3-6). At peak sensitisation, as determined by behavioural sensory reflex testing, chronic constriction injury results in a marked relative decrease in the number of GluR1-immunopositive cell bodies in lamina II (but not I or III) of the dorsal horn ipsilateral to the injury, compared to the contralateral side (** $p \leq 0.01$, Chi-squared test), and to recovered animals, sham surgery animals and naïve controls (†† $p \leq 0.01$, ANOVA versus naïve control followed by Dunnett's post hoc test).

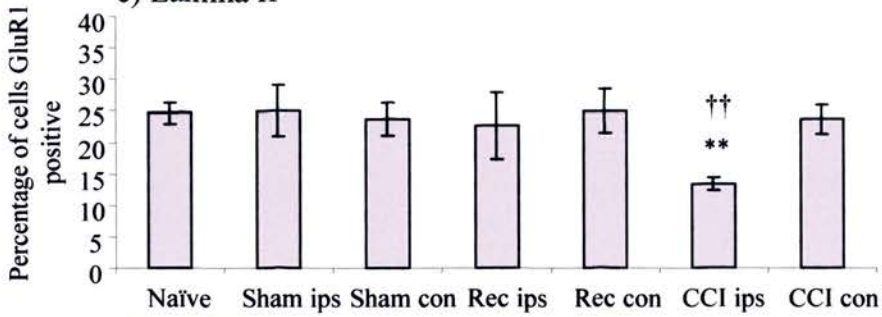
a)



b) Lamina I



c) Lamina II



d) Lamina III

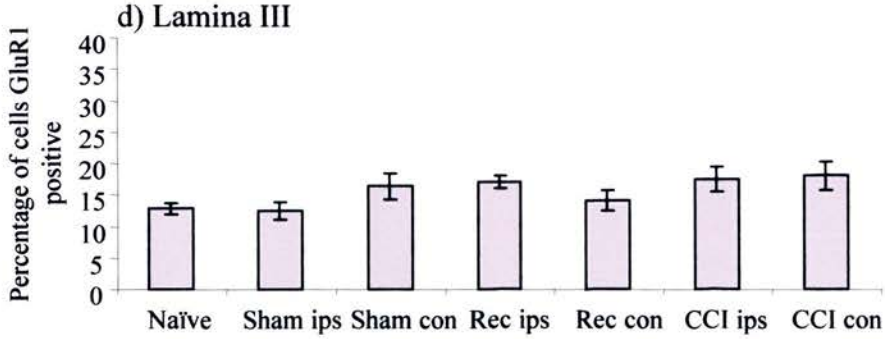
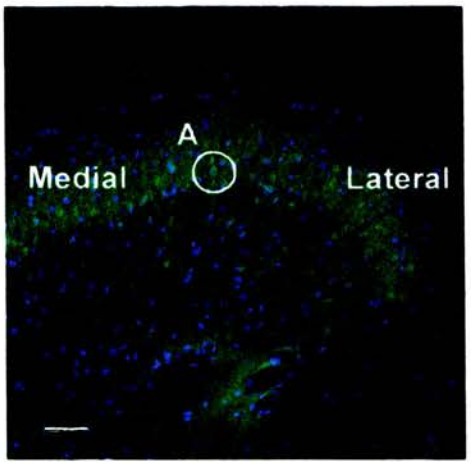
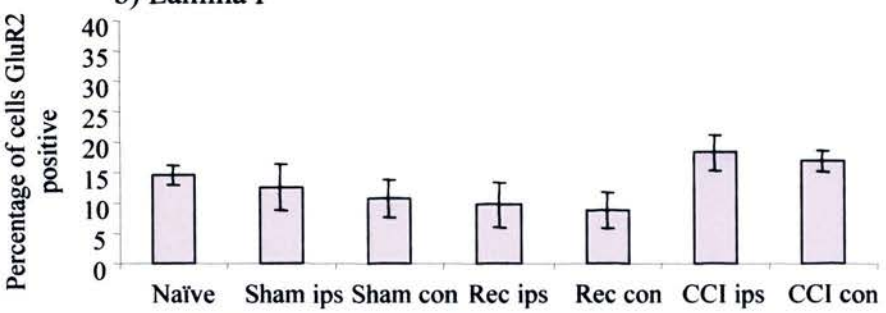


Figure 4.4 a) Shows GluR2-immunopositive cells labelled with fluorescent secondary antibody (green) and To Pro3 nuclear marker (blue) in the dorsal horn of a naïve animal (x20). A indicates a GluR2-immunopositive cell (circled). Scale bar 50μm. b)-d) GluR2-immunopositive cells were counted and the ratio of immunopositive cells to the total number in laminae I, II and III calculated as a percentage (b-d). Data shown as mean ± SEM (n=3-6). At peak sensitisation, as determined by behavioural sensory reflex testing, chronic constriction injury did not result in any significant change in the number of GluR2-immunopositive cells in laminae I to III of the dorsal horn when compared to naïve animals, animals showing behavioural recovery from CCI or animals after sham CCI surgery (Chi-squared test was used to compare ipsilateral and contralateral sides. ANOVA was used to compare all groups to naïve controls).

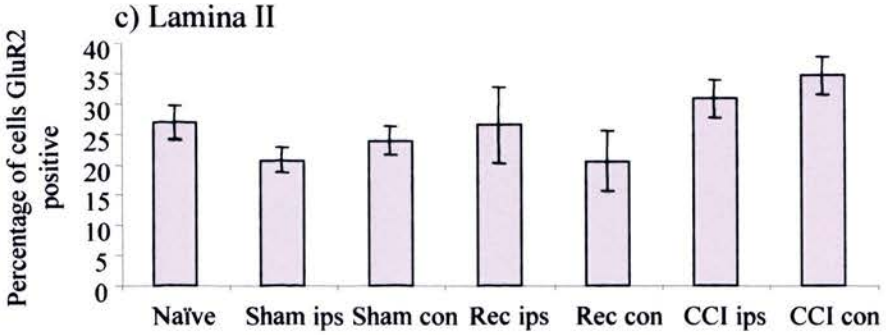
a)



b) Lamina I



c) Lamina II



d) Lamina III

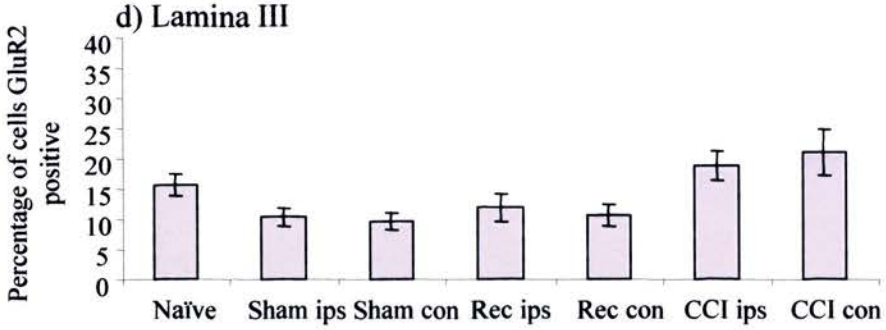


Figure 4.5 The effect of sensitisation induced by chronic constriction injury on the expression of Bassoon, a presynaptic nerve terminal protein, associated within GluR1 immunopositive cells (defined as the number of Bassoon immunopositive puncta within 0.5μm of a GluR1-immunopositive cell). a) At peak sensitisation due to CCI, there was a significant increase in the number of Bassoon-immunopositive puncta (defined as a group of 9 or more pixels with a green intensity level of at least 75% of the maximum found on that image) associated with each GluR1-immunopositive cell in lamina II of the dorsal horn ipsilateral to injury when compared to the contralateral side (* $p = 0.0372$, Mann-Whitney test). Data are shown as mean ± SEM. There were significantly more Bassoon puncta per cell ipsilateral to CCI injury than in naïve animals on comparing all three experimental groups (†† $p \leq 0.01$, Kruskal-Wallis one-way ANOVA on ranks and Dunn's post hoc analysis). b) Images of typical cells within lamina II ipsilateral and contralateral to CCI injury. The images show GluR1-immunopositive cells labelled with fluorescent secondary antibody (red), Bassoon-immunopositive puncta labelled with fluorescent secondary antibody (mouse monoclonal anti-Bassoon, Alexa Fluor 488 goat anti mouse - green), and To Pro3 nuclear marker (blue). White arrows indicate Bassoon puncta associated with each GluR1-immunopositive cell (within 0.5μm of a GluR1-immunopositive cell, scale bars 10μm). c) Differences in overall Bassoon immunofluorescence between ipsilateral and contralateral dorsal horns at peak sensitisation due to CCI were investigated in three regions of lamina II (x10) (n=3). There was no significant difference in Bassoon immunofluorescence between the ipsilateral and contralateral sides in any of the regions of interest (Mann-Whitney test, data shown as mean ± SEM).

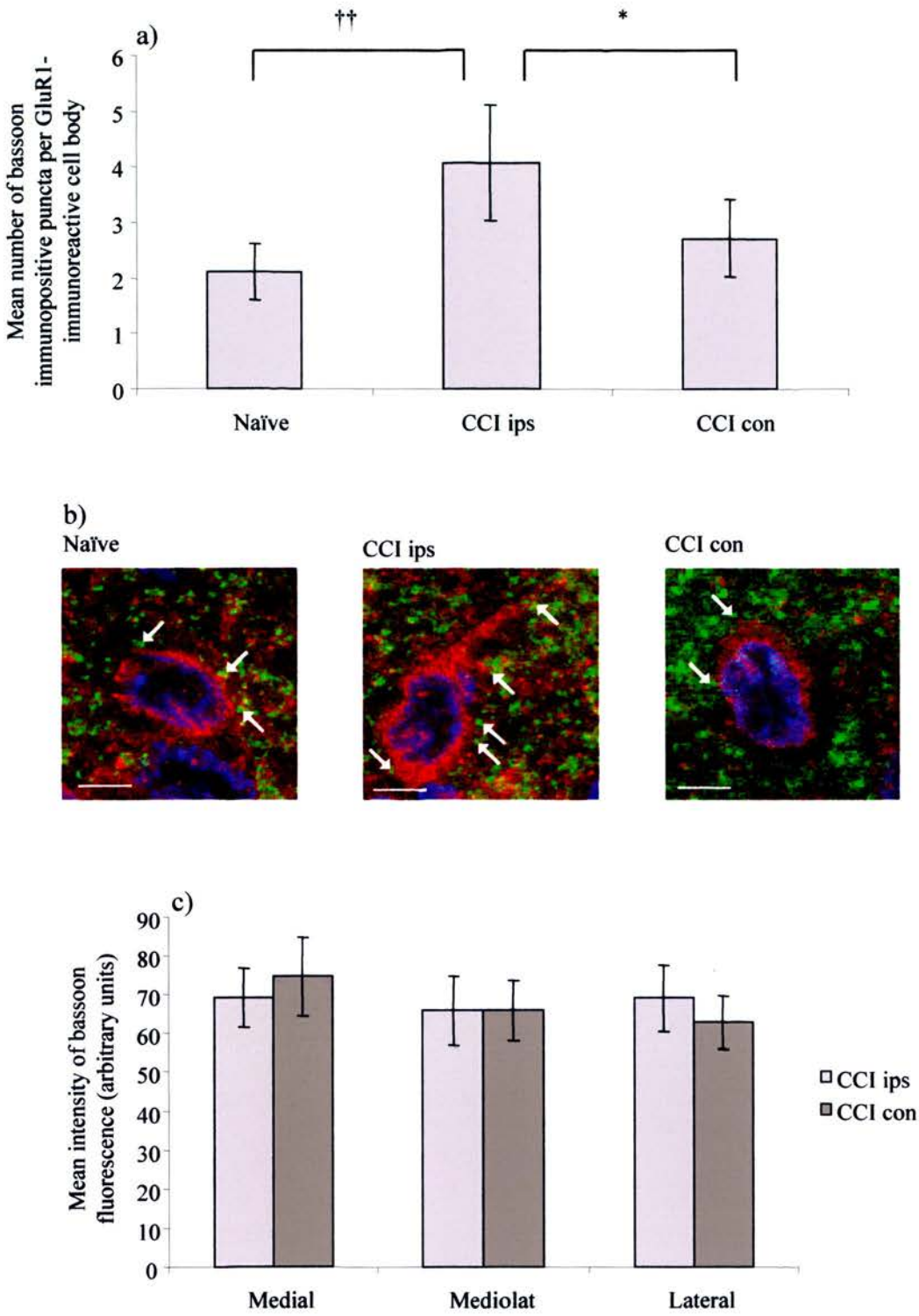
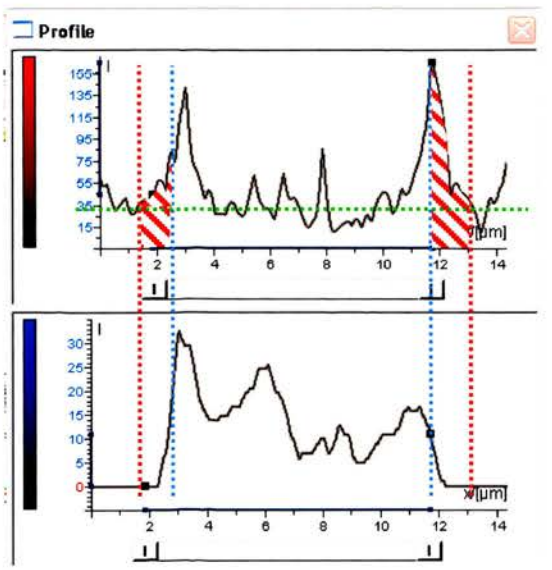
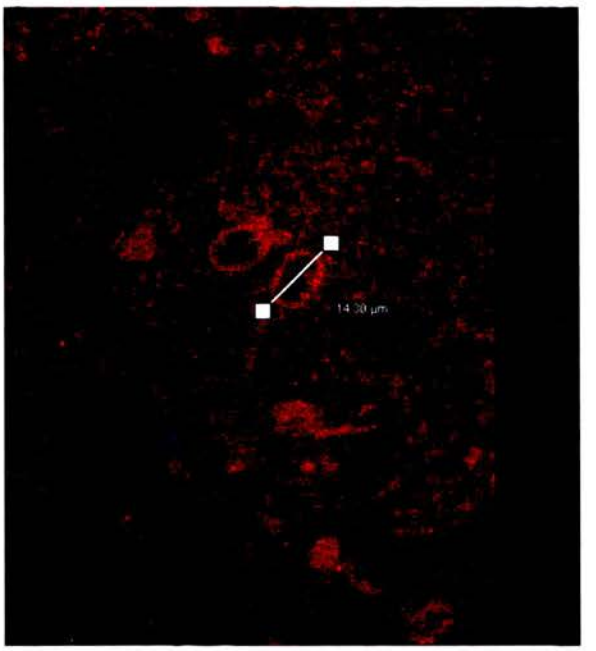

a)
††
*
Mean number of bassoon immunopositive puncta per GluR1-immunoreactive cell body
0
1
2
3
4
5
6
Naïve
CCI ips
CCI con
b)
Naïve
CCI ips
CCI con
c)
Mean intensity of bassoon fluorescence (arbitrary units)
0
10
20
30
40
50
60
70
80
90
Medial
Mediolat
Lateral
CCI ips
CCI con

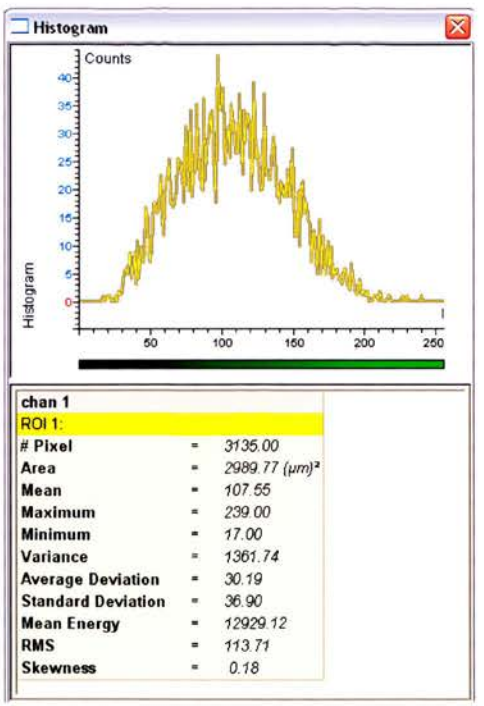
Figure 4.6 Analysis of the fluorescence intensity profile of images from dorsal horn. Leica Confocal Software lite was used to analyse all images. a) The profile tool allows measurement of fluorescent intensity on a scale of 0 to 255 for each red, green or blue colour channel along a given axis. This application was used to measure the linear extent of immunofluorescent cytoplasm within cells and the nuclear densities along the same axis. The red dashed line indicates the extent of GluR1 immunofluorescence above baseline. The blue dashed line indicates the extent of To Pro3 nuclear DS DNA labelling – taken as 50% of peak blue fluorescence. The red striped area represents the extent of GluR1 immunofluorescence within the cell body (in this case 1.2μm). b) The histogram tool measures various parameters within a selected region of interest (ROI). The region of interest is shown on a typical x10 view of a dorsal horn labelled with anti-Bassoon antibody and fluorescent secondary antibody (green). This tool was used to measure the mean fluorescence within each ROI for statistical comparison.

a)


Profile
I
155
135
115
95
75
55
35
15
2
4
6
8
10
12
14
x [µm]
I
30
25
20
15
10
5
0
2
4
6
8
10
12
14
x [µm]


14.20 µm

b)


Histogram
Counts
Histogram
40
35
30
25
20
15
10
5
0
50
100
150
200
250
I
chan 1
ROI 1:
# Pixel = 3135.00
Area = 2989.77 (µm)²
Mean = 107.55
Maximum = 239.00
Minimum = 17.00
Variance = 1361.74
Average Deviation = 30.19
Standard Deviation = 36.90
Mean Energy = 12929.12
RMS = 113.71
Skewness = 0.18

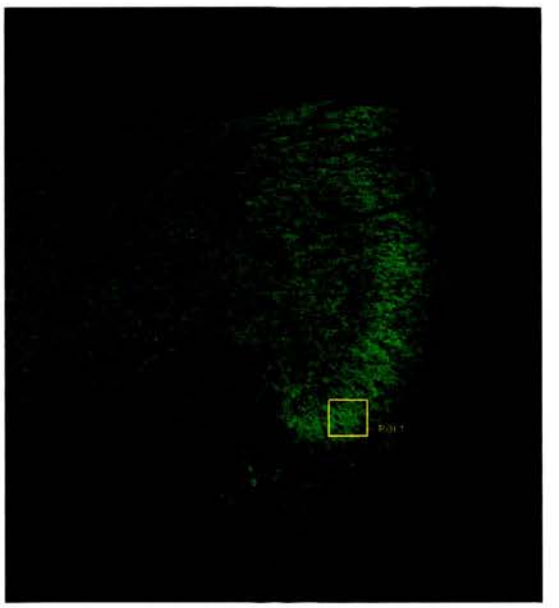
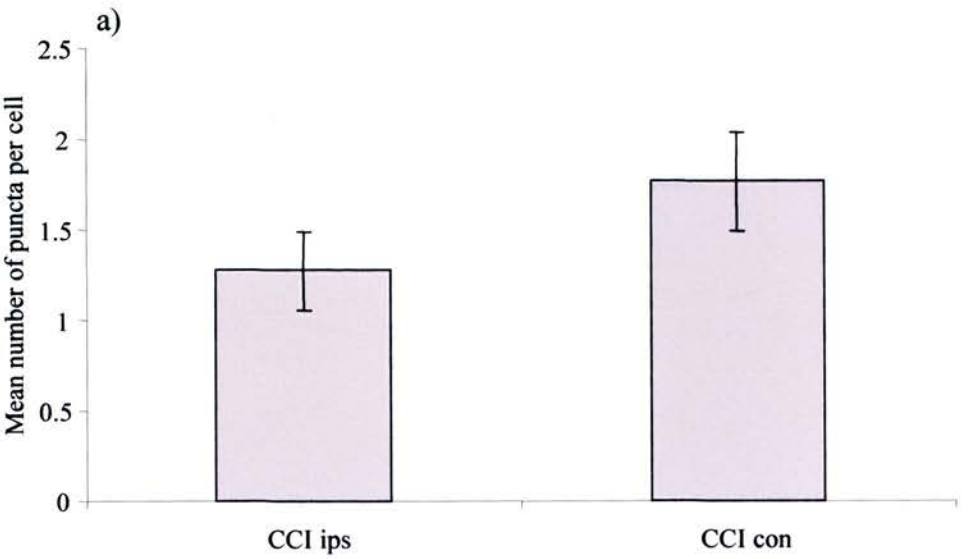

ROI 1

Figure 4.7 The effect of sensitisation induced by chronic constriction injury on the expression of synaptophysin, an integral membrane glycoprotein concentrated in presynaptic vesicles in apposition to GluR1-immunopositive cells. a) At peak sensitisation due to CCI, there was no difference in the number of synaptophysin-immunopositive puncta associated with each GluR1-immunopositive cell in lamina II of the dorsal horn ipsilateral to injury when compared to the contralateral side (Mann-Whitney test). Data are shown as mean ± SEM. b) Images of typical cells within lamina II ipsilateral and contralateral to CCI injury. The images show GluR1-immunopositive cells labelled with fluorescent secondary antibody (red), synaptophysin-immunopositive puncta labelled with fluorescent secondary antibody (green), and To Pro3 nuclear marker (blue). White arrows indicate synaptophysin puncta associated with each GluR1-immunopositive cell. Scale bar 10μm.


a)
2.5
2
1.5
1
0.5
0
Mean number of puncta per cell
CCI ips
CCI con

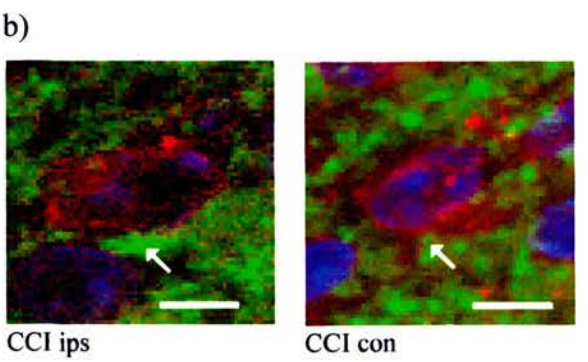
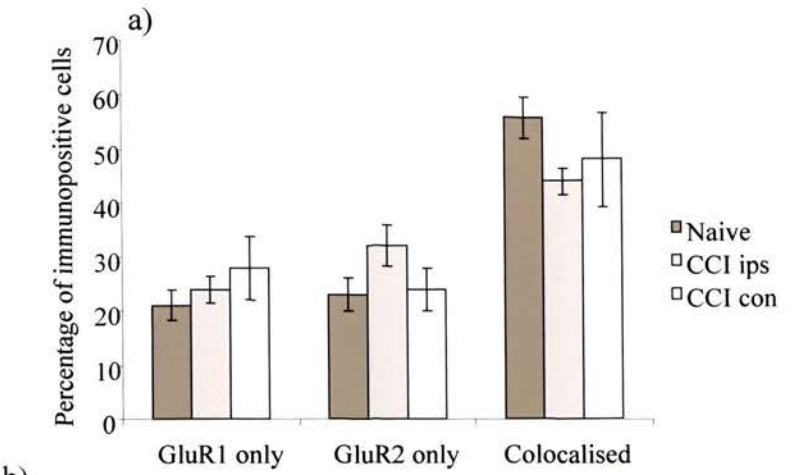
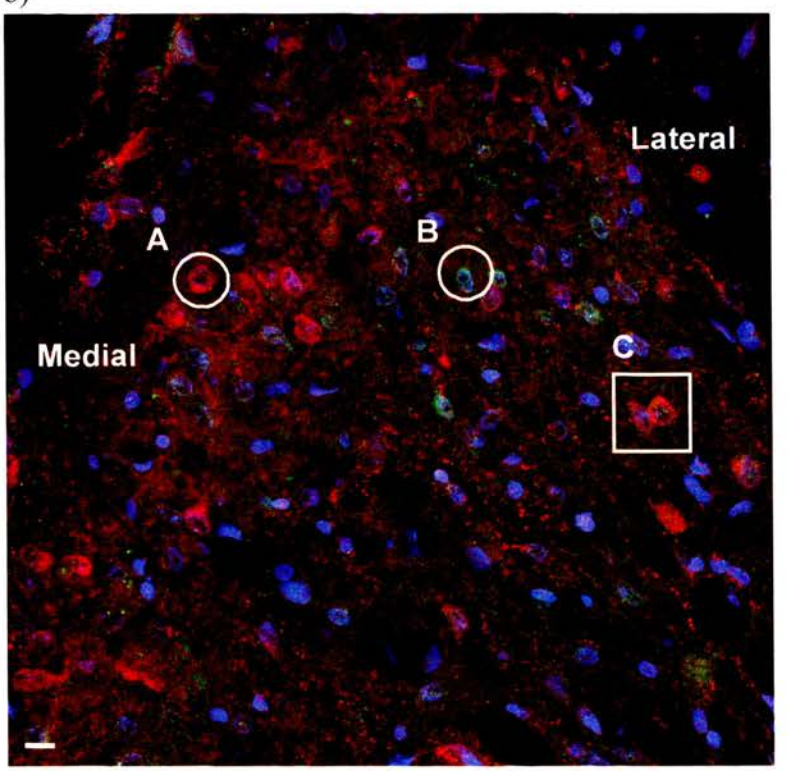

b)
CCI ips
CCI con

Figure 4.8 a) The proportion of GluR1-immunopositive or GluR2-immunopositive cells and cells co-expressing both receptor subtypes in lamina II was calculated for each dorsal horn for naïve animals and animals at peak sensitisation due to chronic constriction injury, as determined by behavioural sensory reflex testing. The proportion of each pattern of receptor expression (ie: GluR1 only, GluR2 only or both) did not change at peak sensitisation due to chronic constriction injury (each value is the mean ± SEM. Chi-Squared test with four degrees of freedom (n=3) b) Shows GluR1-immunopositive cells labelled with fluorescent secondary antibody (red), GluR2-immunopositive cells labelled with fluorescent secondary antibody (green) and To Pro3 nuclear marker (blue). (A) Shows a GluR1-immunopositive cell (circled), (B) shows a GluR2-immunopositive cell (circled), (C) shows a cell strongly expressing both GluR1 and GluR2 (boxed). Scale bar 50μm (x40). c) Region (C) at 100x magnification. Scale bar 10μm.


a)
Percentage of immunopositive cells
70
60
50
40
30
20
10
0
GluR1 only
GluR2 only
Colocalised
Naive
CCI ips
CCI con

b)


Lateral
A
B
Medial
C

c)

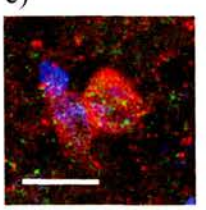
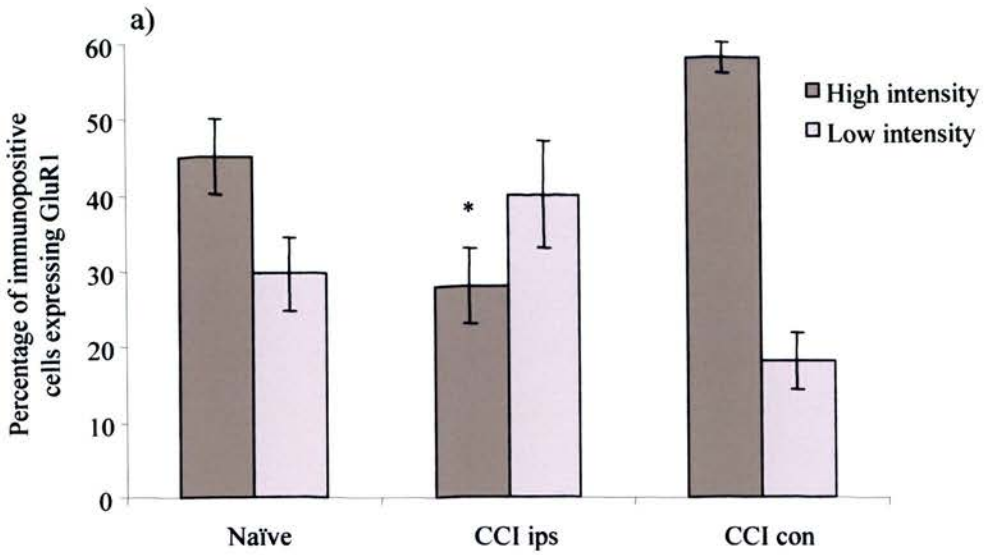
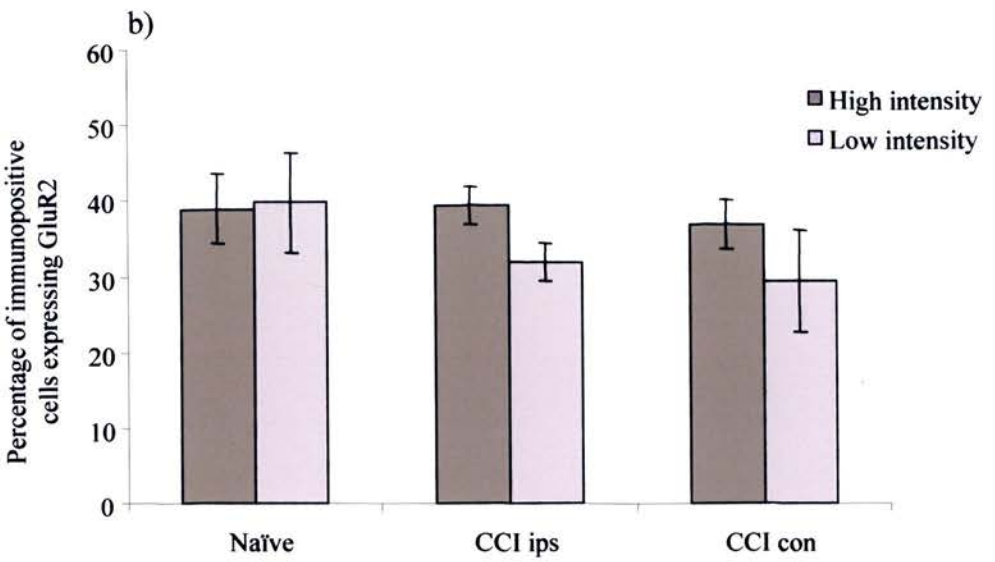


Figure 4.9 a) The proportion of GluR1 and GluR2-immunopositive cells expressing high and low levels of fluorescence (low fluorescence defined as twice baseline to 75% of maximum intensity for that slice. High fluorescence was defined as intensity from 75-100% of maximum for that slice) (Section 4.2.5) was noted in lamina II of the dorsal horn of naïve animals and animals at peak sensitisation due to chronic constriction injury, as determined by behavioural sensory reflex testing. Each value is the mean ± SEM. (n=3). The proportion of GluR1-immunopositive cells expressing high levels of fluorescence in lamina II of the dorsal horn decreased significantly at peak sensitisation due to chronic constriction injury, as determined by behavioural sensory reflex testing, ipsilateral to injury ($p \leq 0.05$, Chi-Squared test.). No significant difference was noted between the experimental groups for GluR2-immunopositive cells. b) Typical cells demonstrating high and low levels of GluR1 (red) and GluR2-immunofluorescence (green). Scale bars 10μm.


a)
Percentage of immunopositive cells expressing GluR1
60
50
40
30
20
10
0
*
High intensity
Low intensity
Naïve
CCI ips
CCI con


b)
Percentage of immunopositive cells expressing GluR2
60
50
40
30
20
10
0
High intensity
Low intensity
Naïve
CCI ips
CCI con

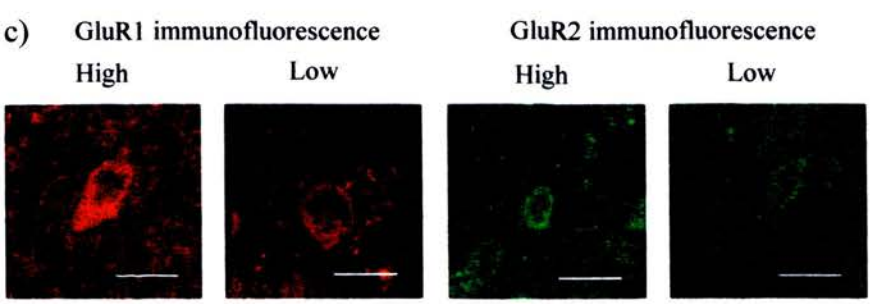
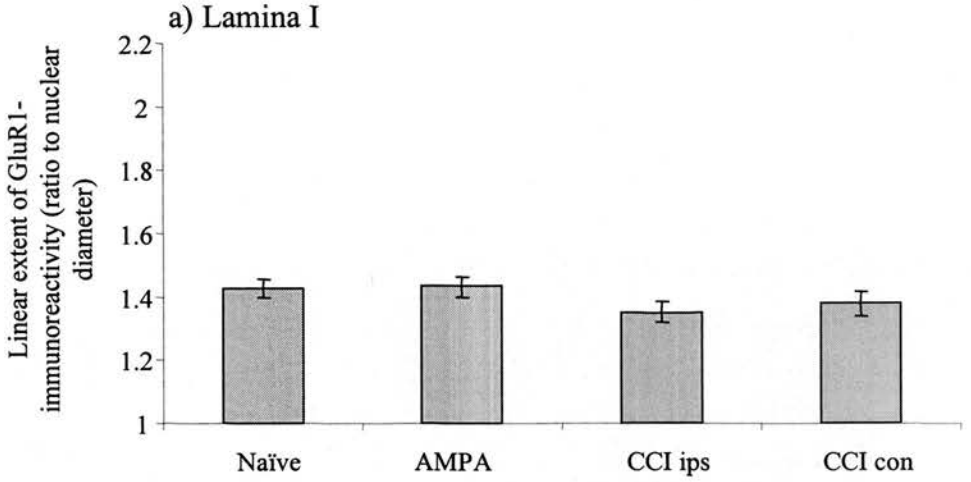
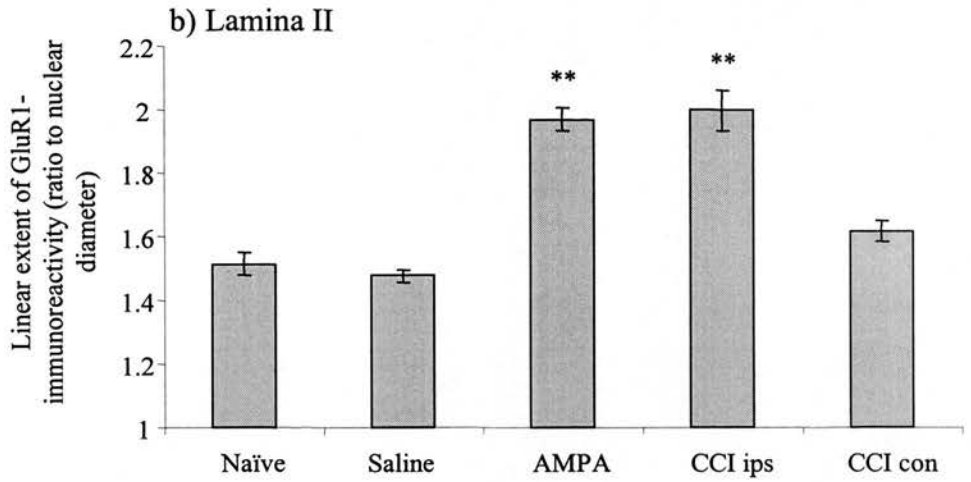
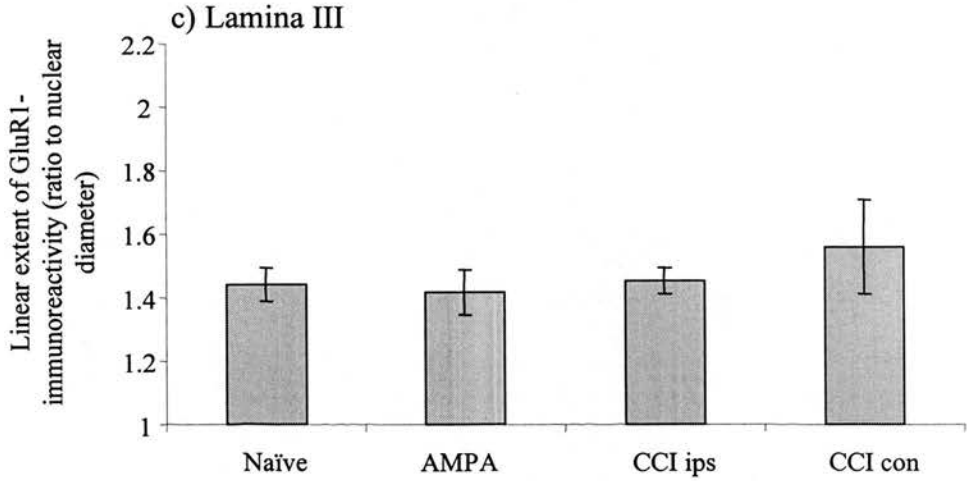
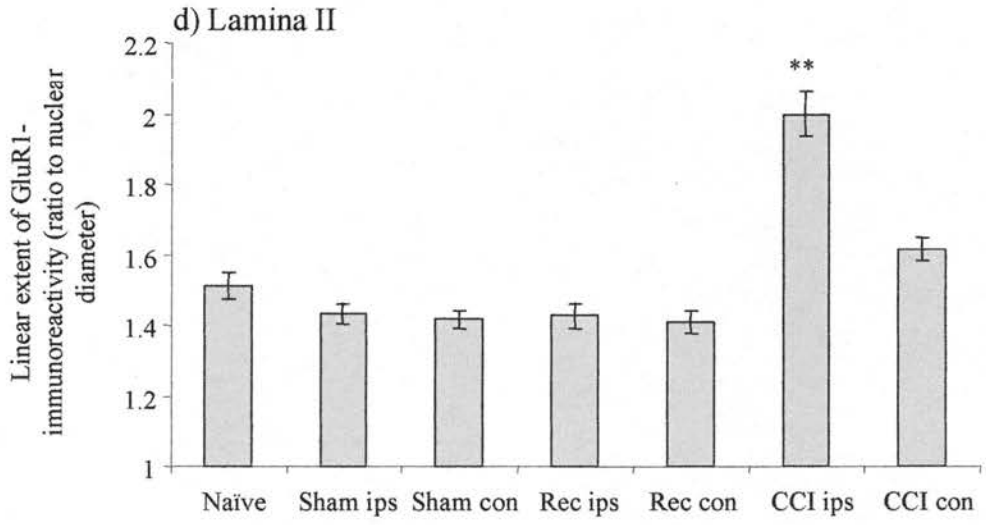

c)
GluR1 immunofluorescence
High
Low
GluR2 immunofluorescence
High
Low

Figure 4.10 a)-c) The linear extent of GluR1-immunopositive cytoplasm within cells in the dorsal horn was measured as a multiple of the nuclear diameter for each immunopositive cell at x40 or x63 magnification within laminae I-III (a-c). GluR1-immunofluorescence was measured above background, and the nuclear density was measured as 50%-100% of maximum blue fluorescence as in previous experiments (see Figure 4.6). Peak sensitisation induced by chronic constriction injury or intrathecal AMPA administration resulted in a marked change in the subcellular distribution of GluR1 AMPA R subunits within immunopositive cells in lamina II, compared to naïve animals and controls treated with intrathecal saline (** $p \leq 0.01$, Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's post hoc test. Data are shown as the mean ± SEM (n=3)). There were no significant differences between experimental groups in lamina I or III. d) Redistribution of GluR1 subunits was not seen in naïve animals, animals showing behavioural recovery from CCI or animals after sham CCI surgery in lamina II (Kruskal-Wallis one way ANOVA on ranks followed by Dunn's post hoc test). e) To show typical cells from lamina I-III (x40). GluR1-immunopositive cells were labelled with fluorescent secondary antibody (red) and To Pro3 nuclear marker (blue). Scale bars 10μm.


a) Lamina I
Linear extent of GluR1-immunoreactivity (ratio to nuclear diameter)
2.2
2
1.8
1.6
1.4
1.2
1
Naïve
AMPA
CCI ips
CCI con


b) Lamina II
Linear extent of GluR1-immunoreactivity (ratio to nuclear diameter)
2.2
2
1.8
1.6
1.4
1.2
1
**
**
Naïve
Saline
AMPA
CCI ips
CCI con


c) Lamina III
Linear extent of GluR1-immunoreactivity (ratio to nuclear diameter)
2.2
2
1.8
1.6
1.4
1.2
1
Naïve
AMPA
CCI ips
CCI con


d) Lamina II
Linear extent of GluR1-immunoreactivity (ratio to nuclear diameter)
2.2
2
1.8
1.6
1.4
1.2
1
**
Naïve
Sham ips
Sham con
Rec ips
Rec con
CCI ips
CCI con

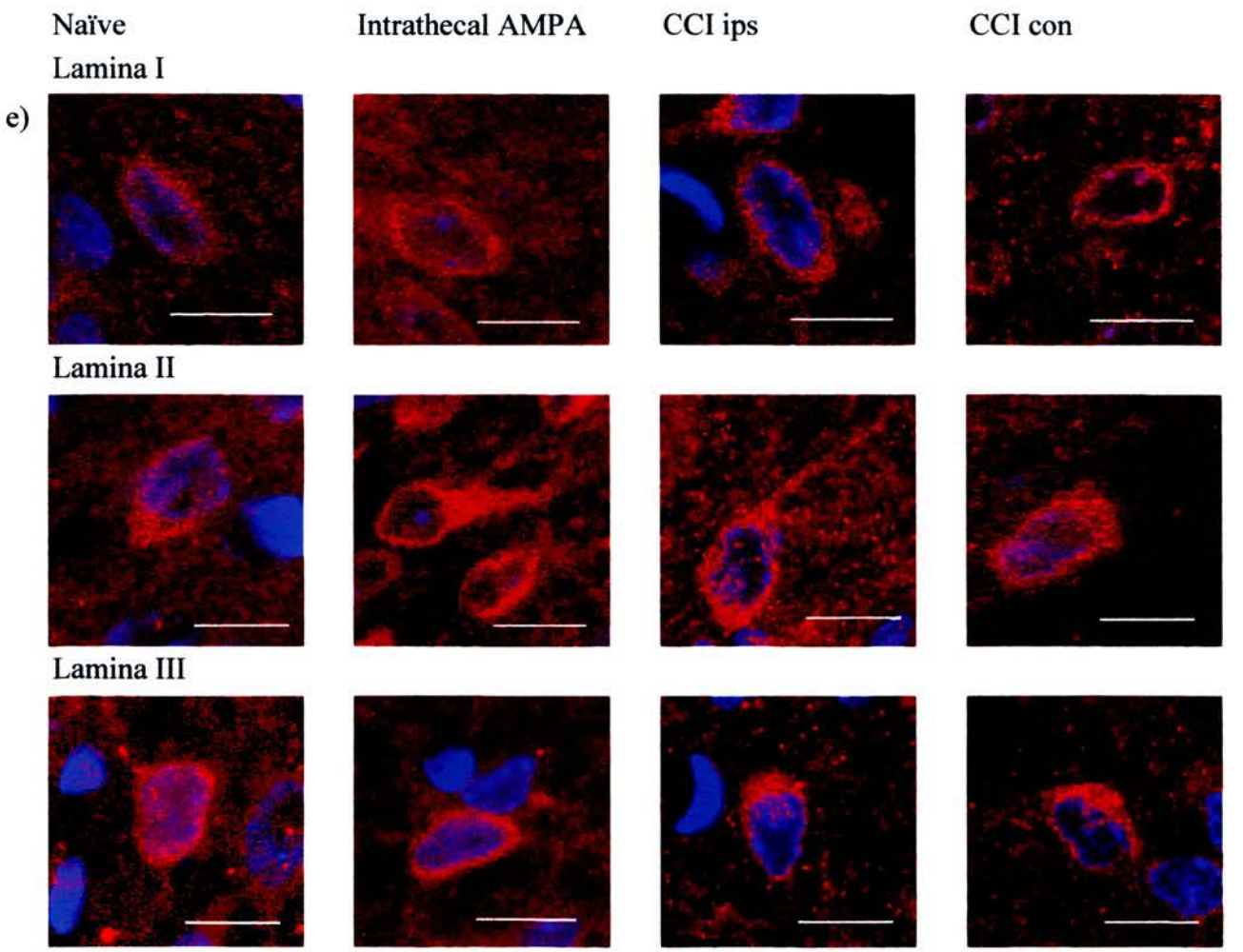
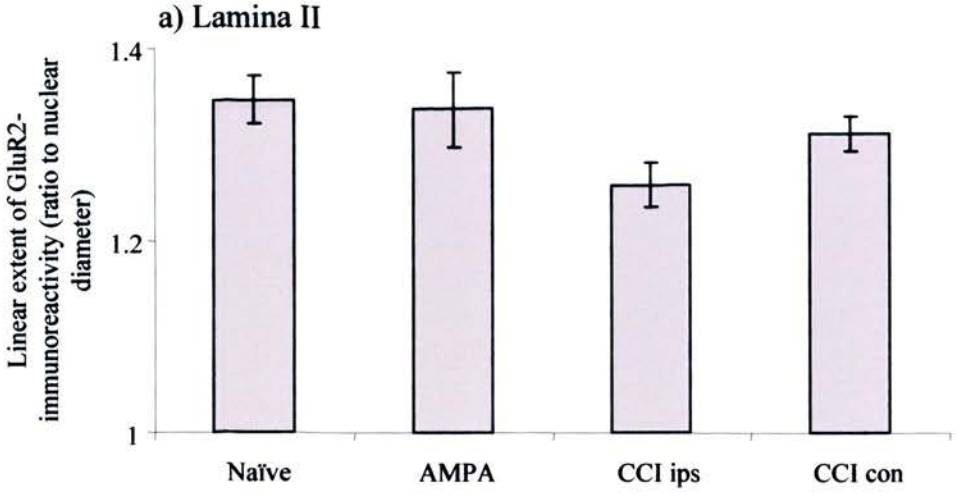

Naïve
Intrathecal AMPA
CCI ips
CCI con
e)
Lamina I
Lamina II
Lamina III

Figure 4.11 a) The linear extent of GluR2-immunopositive cytoplasm within cells in lamina II of the dorsal horn was recorded for each immunopositive cell, as a multiple of nuclear diameters at x40 or x63 magnification. Peak sensitisation induced by chronic constriction injury or intrathecal AMPA administration did not result in any significant change in the subcellular distribution of GluR2 AMPA R subunits in immunopositive cells in lamina II, compared to animals naïve to any treatment and control animals given saline intrathecally (Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's post hoc test. Data are shown as mean ± SEM (n=3)). b) Shows typical cells from lamina II (x40). GluR2-immunopositive cells were labelled with fluorescent secondary antibody (green) and To Pro3 nuclear marker (blue). Scale bars 10μm.


a) Lamina II
Linear extent of GluR2-immunoreactivity (ratio to nuclear diameter)
1.4
1.2
1
Naïve
AMPA
CCI ips
CCI con

b)

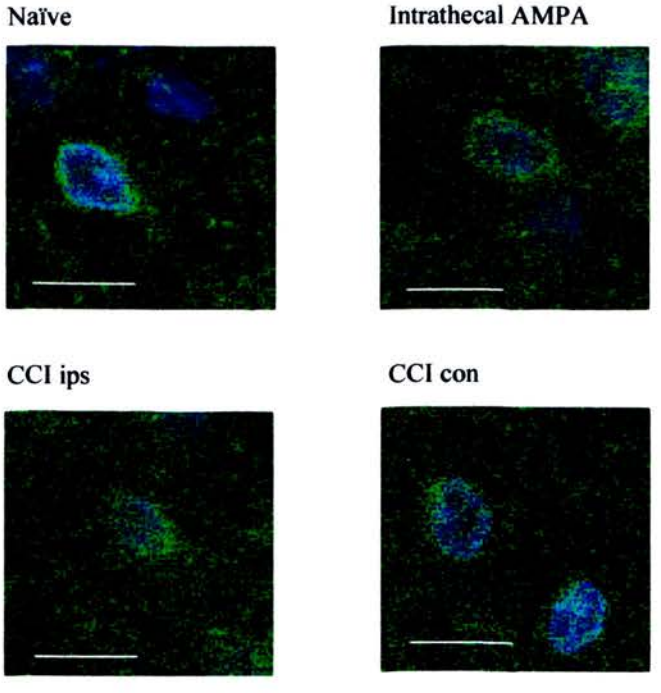
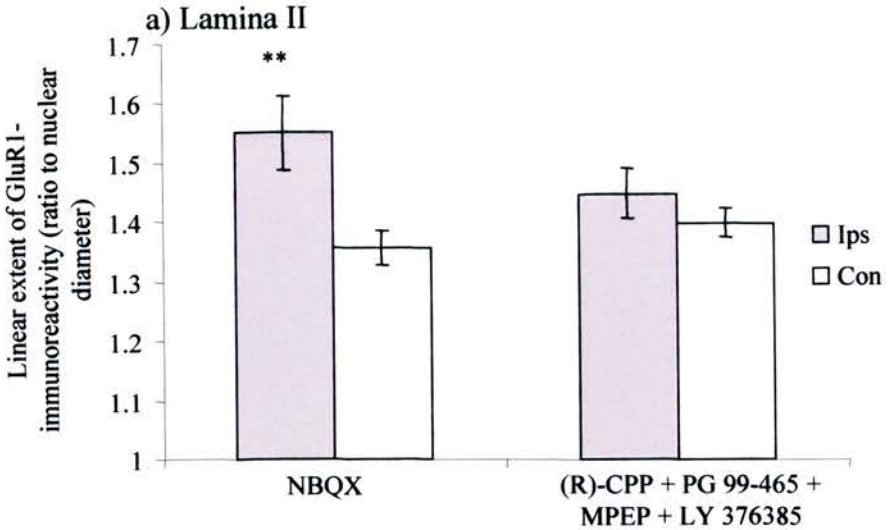
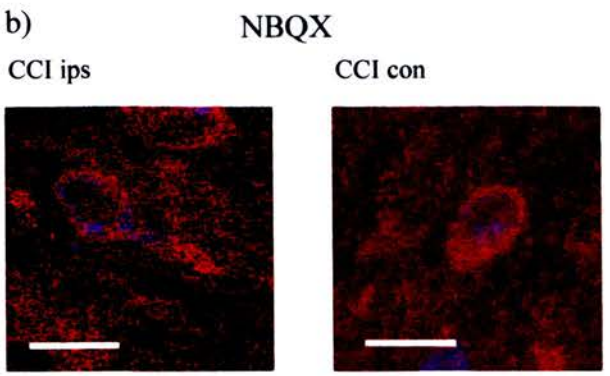
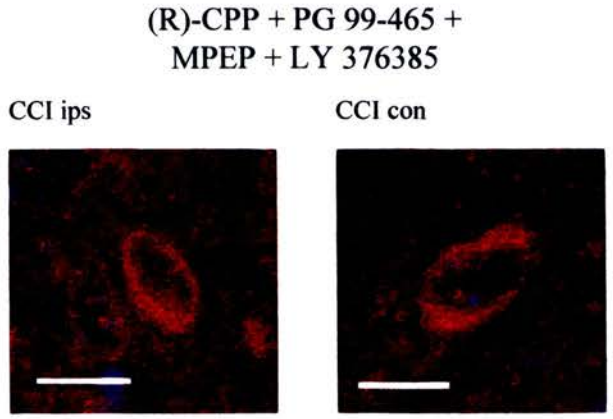

Naïve
Intrathecal AMPA
CCI ips
CCI con

Figure 4.12 a) The linear extent of GluR1-immunopositive cytoplasm within cells in lamina II was recorded as a multiple of the nuclear diameter for animals at peak sensitisation due to CCI, after treatment with various key antagonist drugs at x40 magnification (n=3). The difference in mean linear extent of immunoreactivity between ipsilateral and contralateral sides was compared after combined blockade of the mGlu1/5R, NMDA R and $VPAC_2R$ and after AMPA R blockade alone. Blockade of all the non-AMPA Rs reversed the subcellular redistribution of AMPA R GluR1 subunits demonstrated in cells in lamina II of the dorsal horn ipsilateral to CCI injury (Mann-Whitney rank sum test. No difference could be detected between ipsilateral and contralateral sides $p=0.88$). Blockade of the AMPA R alone did not reverse this redistribution (a significant difference remained between ipsilateral and contralateral sides $p=0.003$). b) Confocal images of typical lamina II dorsal horn cells (x40). The images show GluR1-immunopositive cells labelled with fluorescent secondary antibody (red) and To Pro3 nuclear marker (blue). Scale bars 10μm.


a) Lamina II
**
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
Linear extent of GluR1-immunoreactivity (ratio to nuclear diameter)
Ips
Con
NBQX
(R)-CPP + PG 99-465 + MPEP + LY 376385


b)
NBQX
CCI ips
CCI con


(R)-CPP + PG 99-465 + MPEP + LY 376385
CCI ips
CCI con

4.4 Discussion

These experiments were carried out to investigate the effect of an animal model of neuropathic pain induced by chronic, peripheral nerve constriction on the expression and subcellular location of AMPA R GluR1 and GluR2 subunits.

Studies of neuropathic pain have focused on traumatic peripheral nerve injury as a trigger for generation of central sensitisation, usually involving the rat sciatic nerve (Boucher and McMahon, 2001). At mid-thigh level, this nerve consists of some 27000 axons consisting of 6% motor, 23% myelinated sensory, 48% unmyelinated sensory and 23% unmyelinated sympathetic fibres (Schmalbruch, 1986). It is ideally placed for generating models of neuropathic pain as it is accessible in the hind limb and is large enough for easy handling. It arises from the L4 - S2 spinal roots and contains four subdivisions (the sural, lateral sural, superficial peroneal and tibial nerves), which may be manipulated independently. Also, the contralateral nerve may serve as an intrinsic control in certain studies. Some models of neuropathic pain such as the Seltzer technique of partial sciatic nerve damage result in behavioural changes, that are partly bilateral, however (Seltzer et al., 1990).

The model used in this study was the chronic constriction injury (CCI) model of Bennett and Xie (Bennett and Xie, 1988). This model relies on partial nerve damage rather than complete transection, with the intact axons providing peripheral skin sensation and also acting to increase the intensity of sensitisation (Lee et al., 2003). The pharmacological profile of sciatic CCI is similar to that seen in human neuropathic pain and it may be compared to direct nerve trauma in humans. The frequency of autotomy is lower in this model than following sciatic transection, and there was no evidence of its occurrence in the current study (Bennett and Xie, 1988), (Attal et al., 1990). The sympathetic nervous system (SNS) appears to be affected in CCI, as sympathectomy often improves the thermal hyperalgesia seen in this model whereas mechanical and cold allodynia are not affected (Desmeules et al., 1995), (Perrot et al., 1993). This model may result in thermal, mechanical and cold allodynia to a variable extent, which parallels the variable responses of human subjects to seemingly similar nerve injuries (see Figure 4.1).

It is known that there are changes in the spinal expression of GluR1 and GluR2 subunits in various pain paradigms including CCI, many of which may involve central sensitisation. Decreases in GluR1 and GluR2/3 were found in a model of spinal cord injury (Grossman et al., 1999). In a model of peripheral inflammation, an

early decrease in GluR1 and GluR2/3 was demonstrated in the trigeminal nucleus (Florenzano and De Luca, 1999). Three days after dorsal rhizotomy, GluR1 and GluR2/3 immunostaining was found to decrease in the superficial dorsal horn. This finding may have been due to loss of receptors from primary afferents or to loss of expression in intrinsic dorsal horn neurons (Carlton et al., 1998). Following partial sciatic deafferentation using pronase, an ipsilateral decrease in GluR1 was demonstrated in the sciatic nerve territory of the superficial dorsal horn (Helgren et al., 1999). In joint inflammation a bilateral decrease in GluR1 was noted (Pellegrini-Giampietro et al., 1994).

Some studies noted an increase in AMPA R subunit expression. Following spinal cord section, GluR2/3 expression increased in the dorsal horn (Popratiloff et al., 1998). Harris et al reported a small but significant increase in GluR1 and GluR2/3 expression in the superficial dorsal horn 14 days after CCI ipsilateral to the injury using an avidin-biotin peroxidase technique (Harris et al., 1996). However, a similar increase was also found after treatment with the NMDA antagonist MK-801 given in a regime known to prevent thermal hyperalgesia in animals after CCI surgery. As the increase in receptor expression did not alter with NMDA blockade, its functional significance remains unclear. Recent studies have also revealed an ipsilateral increase in GluR2 mRNA in lamina I and II dorsal horn cells in CCI, with no change in the number of cells expressing this subunit (Garry et al., 2003b).

4.4.1 Immunohistochemisty of the superficial dorsal horn

The cytoarchitecture of the dorsal horn, and in particular lamina II is very complex. Axons, dendrites, neuronal cell bodies and glia are closely apposed and overlap. Quantitative analysis of confocal images of the dorsal horn using immunofluorescent antibodies proved to be difficult as the axons and dendrites forming the neuropil around neurons was also brightly labelled. A number of strategies were tried to delineate the extent of individual neurons with greater clarity. Firstly, we attempted to label the cell membrane with an antibody to the $\alpha 3$ subunit of the ubiquitous Na^+/K^+ ATPase (rabbit anti-Na^+/K^+ ATPase, Upstate Ltd.). This was unsuccessful as background labelling was excessively high. The cytoskeletal protein MAP2 (microtubule associated protein 2, mouse anti-MAP2, Sigma Aldrich Ltd.) was labelled in another attempt to identify the axons and dendrites of individual cells. This technique was effective in demonstrating neuronal processes and revealed a complex

system of fibres running perpendicular to lamina II, with occasional processes passing dorsally into lamina I (see Figure 4.13). However, it was not generally possible to follow the processes of individual cells any further from the nucleus using this technique, due to overlapping of fibres from neighbouring cells.

The antibodies used to label GluR1 and GluR2 have been used in many other studies and their specificity has been confirmed (Hollmann and Heinemann, 1994), (Agrawal and Fehlings, 1997), (Kumar et al., 2002). It has also been demonstrated that the anti-GluR1 antibody used binds both unphosphorylated and phosphorylated forms of the receptor (Fang et al., 2003). It is clear that quantitative analysis of immunofluorescent studies of intact spinal cord sections proved difficult, and visualisation of individual small neuronal processes impossible. However, large processes were visible in a number of cells.

4.4.2 AMPA receptor subtype expression in the dorsal horn

These results provide new insight into the expression and distribution of AMPA R GluR1/2 subtypes in the dorsal horn of the rat in an experimental model of neuropathic pain. The most notable finding was a marked decrease in the proportion of cell bodies seen in lamina II of the DH that were GluR1 immunoreactive and a redistribution of GluR1 on a subcellular level. The mean linear extent of GluR1 immunopositive cytoplasm in the longest axis of the cells was shown to be significantly greater in cells ipsilateral to CCI in lamina II, and following AMPA treatment (see Figure 4.10). This finding suggests that GluR1 subunits may be redistributing away from the nucleus towards more distant parts of the cell. The proportion of GluR1 immunopositive cell bodies and their subcellular distribution both returned to normal on recovery from CCI. There was no apparent change in the proportion of GluR1 immunoreactive cell bodies in lamina I or III, following CCI.

The relative lack of changes in lamina I or III may reflect their differing roles in sensory processing. Lamina I contains many projection neurons, which connect to higher centres via the STT and SMT (Todd et al., 2000), (Todd, 2002). It has been demonstrated that 75% of lamina I neurons are nocispecific, the remainder being type II WDR neurons (Seagrove et al., 2004). Cells in lamina I may become sensitised by increased excitatory input from altered neuronal responses in lamina II, leading to central sensitisation (Willis et al., 1979). Unmyelinated nociceptor C fibres terminate

predominantly in lamina II, so it is unsurprising that changes resulting from peripheral nerve injury occur here (Willis and Coggeshall, 1991).

Lamina III receives the primary afferents (mainly large Aβ fibre) of hair follicles, Pacinian corpuscles and rapidly adapting low threshold mechanoreceptors. Second order neurons send dendrites from lamina III to laminae I and IV, in addition to ascending tracts (Brown et al., 1981). Aβ fibre mechanoreceptor neurons undergo functional changes after sciatic nerve transection, establishing functional synaptic contact with interneurons in lamina II and transmitting innocuous information to the substantia gelatinosa (Okamoto et al., 2001). This represents a marked functional alteration in spinal cord circuitry and may account in part for the sensory abnormalities occurring in neuropathic pain (see section 1.11.3). It is possible that these changes occur via mechanisms which do not involve redistribution of AMPA Rs. The somatosensory output from lamina III neurons may remain relatively unchanged in neuropathic pain, and abnormal signal modification via sensitised lamina I neurons, or novel connections within lamina II, result in the interpretation of innocuous stimuli as noxious and the sensation of allodynia.

In contrast to the changes seen in GluR1, the expression of GluR2 subunits appeared unaltered by CCI. These findings are open to a number of possible interpretations. There may be a decrease in overall expression levels of GluR1 within cells, resulting in the cytoplasm becoming immunonegative. It is know that neurons may rapidly change their subunit composition in response to stimulation, becoming less Ca^{2+} permeable and incorporating more GluR2 into the receptors (Liu and Cull-Candy, 2000). Any reduction in GluR1 expression would result in a shift in the ratio of receptor subtype expression towards GluR2, as we have shown that approximately 50% of DH neurons coexpress GluR1 and GluR2. This change may lead to an overall decrease in AMPA R Ca^{2+} permeability. Since there is some evidence that a major proportion of GluR1 is expressed in inhibitory cells (Spike et al., 1998), this may represent a loss of function of inhibitory cells or perhaps a compensatory decrease in the function of excitatory cells in response to CCI (see section 1.6.4). Either of these potential mechanisms may occur, as the majority of cells expressing Ca^{2+} permeable AMPA Rs would seem to play an inhibitory role but a significant minority of neurons expressing this receptor subtype have a modulatory or excitatory role in nociceptive transmission. The possibility that the apparent decrease in GluR1 represents a loss of inhibitory function is supported by some studies (see section 1.11.4) (Robertson et al.,

1997). The apparent decrease in GluR1 expression could be investigated further using Western Blotting techniques. Contrary to the above hypothesis, some studies have reported an increase in GluR1 expression ipsilateral to CCI, although this finding could not be reproduced in our lab (unpublished data) (Harris et al., 1996).

Another possibility is that it is loss of entire cells that causes disinhibition. GluR1 has been shown to be coexpressed with GABA on inhibitory cells in the DH (Spike et al., 1998), and loss of GABA IR has been recorded in CCI (Ibuki et al., 1997). It has been suggested that apoptotic cell death in response to prolonged noxious stimuli may underlie the loss of inhibitory function (Coggeshall et al., 2001). The role of cell death is far from clear, however, and although Moore et al demonstrated a significant decrease in $GABA_A$ mediated currents and a small but significant decrease in the expression of a GABA synthesizing enzyme (GAD65), less than two cells per spinal dorsal horn were found to have undergone apoptosis (Moore et al., 2002). In addition, a recent study did not demonstrate any evidence of cell loss or of decreasing GABA expression in CCI (Polgar et al., 2003).

It is important to note that the proportion of immunopositive cell bodies was found to return to pre-operative levels on recovery from CCI. This finding links changes in GluR1 expression to the behavioural changes found following CCI, and suggests that it is unlikely that a large proportion of cells are undergoing apoptosis in this process.

Although there was an apparent decrease in the proportion of cells expressing GluR1, there was no obvious decrease in the overall level of GluR1 immunofluorescence ipsilateral to CCI. This leads to an alternative interpretation of the experimental findings. The level of fluorescence of neuropil in the superficial laminae of the DH did not appear to differ markedly between both sides of the cord. This was not measured quantitatively here, but protein expression levels could be assessed using Western Blotting. It is possible that overall expression of GluR1 did not change, and that the apparent decrease in the proportion of GluR1 immunopositive cell bodies was due to the redistribution of GluR1 subunits to areas of synaptic plasticity in the axons and dendrites of the neuron and away from the perinuclear region. Once redistributed to peripheral parts of the cell, the GluR1 immunofluorescence may have been lost against the background neuropil, leaving the perinuclear region GluR1 immunonegative and dark.

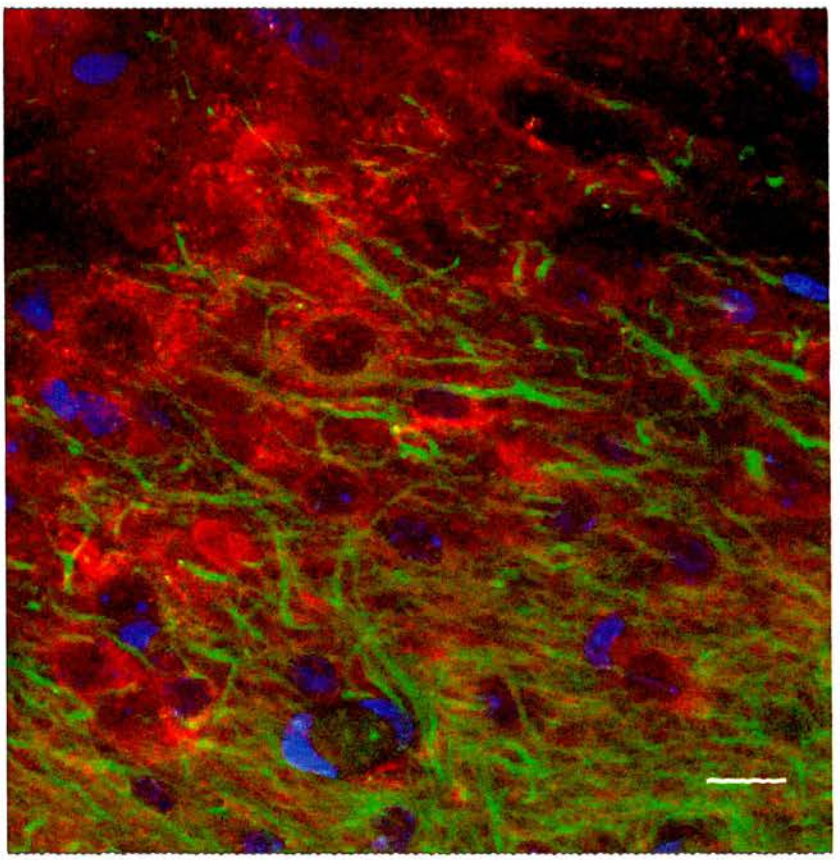
The results of the current study demonstrate that there are significant changes in GluR1 distribution within cells in the CCI model of neuropathic pain. Redistribution of mGluRs in the dorsal horn following application of DHPG has been previously reported (Yashpal et al., 2002), but the subcellular redistribution of GluR1 in the CCI model of neuropathic pain is a novel finding.

Changes in the subcellular distribution of GluR1 subunits were also apparent following intrathecal administration of AMPA (see Figure 4.10). These changes were very similar in appearance and extent to those found following CCI, with the mean linear extent of GluR1 immunopositive cytoplasm shown to be significantly greater in lamina II neurons following AMPA treatment, suggesting that GluR1 subunits may be redistributing away from the nucleus. It is known that activation of AMPA Rs causes spontaneous nociceptive behaviour at higher doses, but it has not been previously demonstrated that this finding is associated with a subcellular redistribution of GluR1 in vivo (Meller et al., 1996).

In contrast to GluR1 subunits, GluR2 appeared relatively inert. No change in the number of cells expressing GluR2 was noted in the current study, nor was there any discernable change in the subcellular location of this subtype in neurons in lamina II These differences in GluR1 and GluR2 expression in CCI may be explained by their differing mechanisms of trafficking to the membrane. GluR1 is known to move to sites of synaptic plasticity (Aicher et al., 2002), in response to specific stimuli and is inserted into synapses in a tightly controlled manner (Hayashi et al., 2000), (Passafaro et al., 2001). In contrast, GluR2 is known to cycle constitutively (Shi et al., 2001), (Borgdorff and Choquet, 2002), and to be inserted directly into synapses from an intracellular pool beneath the membrane (references in (Bredt and Nicoll, 2003)). Recent studies have shown an increase in the expression of GluR2 following CCI, with no increase in the number of cells expressing this subunit. It was also found that GluR2 moved from the membrane compartment into the intracellular compartment ipsilateral to nerve injury (Garry et al., 2003b). This may represent GluR2 trafficking over very small distances, resulting in an important functional redistribution of AMPA Rs, which was not visible on confocal microscopy at the magnification used in the current study.

Figure 4.13 a) Shows GluR1-immunopositive cells labelled with fluorescent secondary antibody (red) and To Pro3 nuclear marker (blue) in the dorsal horn of a naïve animal (x63). Axons, cytoplasm and dendrites are labelled with MAP2 (microtubule associated protein 2) antibody and fluorescent secondary antibody (green). The image is centred on lamina II, with lamina I towards the top left and lamina III towards the bottom right. Scale bar 10μm.

a)



4.4.3 Expression of Bassoon and synaptophysin in the dorsal horn

These results demonstrate that there is an increased association between GluR1 receptors and Bassoon puncta ipsilateral to nerve injury (see section 4.3.3). This may represent increased GluR1 density at synaptic sites. Bassoon is thought to be involved in the structural organisation of the neurotransmitter release site (Wilson, 2003). In situ hybridisation has revealed that Bassoon transcripts are widely distributed in the brain and occur primarily in excitatory neurons, but examples of GABAergic neurons expressing Bassoon have also been detected. In cultured hippocampal neurons, Bassoon was found to colocalise with $GABA_A$ and glutamate (GluR1) receptors. These data indicate that Bassoon is a component of the presynaptic apparatus of both excitatory and inhibitory synapses (Righter et al., 1999). Studies of synaptogenesis have revealed that Bassoon would appear to cluster in new synaptic boutons within 30minutes of their formation, suggesting that it would act as a marker of both newly formed and established synapses (Friedman et al., 2000).

The image magnification available using confocal laser microscopy did not allow examination of synaptic structures on a nanometre scale. Further studies using electron microscopy would be required to investigate the subcellular location of GluR1. It is also possible that many more Bassoon immunopositive puncta were associated with GluR1 on neuronal processes distant from the nucleus, which were not visible due to the complex cytoarchitecture of lamina II.

No change was noted in the overall level of Bassoon-immunofluorescence on either side of the cord in established neuropathy. Levels of protein expression could be assessed in a more quantitative fashion using techniques such as Western Blotting. Although we found no obvious change in Bassoon expression, there is evidence to suggest that other synaptic proteins may be upregulated following nerve injury. For example, synaptophysin levels were increased in the ipsilateral dorsal horn with a peak level on day 14 and returned to baseline on day 21 post injury using immunohistochemistry, Western immunoblotting and densitometry (Chou et al., 2002). Levels of this synaptic marker increased in parallel to the emergence of thermal hyperalgesia, but not mechanical allodynia in this study. The authors suggest that this may represent synaptogenesis occurring ipsilateral to nerve injury. In the current study, the immunofluorescent labelling of synaptophysin was not as consistent

as that for Bassoon and it was not possible to assess overall levels of expression of this protein.

4.4.4 Colocalisation of GluR1/2 in the dorsal horn

In addition to the subcellular location of AMPA R subunits, the co-expression of GluR1 and GluR2 by superficial dorsal horn neurons was investigated. It is known that GluR1 and GluR2 AMPA R subunits may be expressed within the same cell (Zhang et al., 1995a), (Aicher et al., 2002) but their expression in the superficial dorsal horn is generally limited to separate bands (Tolle et al., 1993), (Coggeshall and Carlton, 1997). AMPA Rs can also rapidly change their subunit composition in response to stimulation (Liu and Cull-Candy, 2000). The current study was designed to determine whether the co-expression of GluR1 and GluR2 in lamina II of the dorsal horn changes as a result of CCI.

On initial inspection, GluR1 and GluR2 appear to occur in different cell populations, divided into bands in the superficial dorsal horn (Furuyama et al., 1993), (Tolle and et al, 1993). However, the present study revealed that in lamina II of the spinal dorsal horn in naïve animals, only 21% of all immunopositive cells expressed GluR1 alone and 25% expressed GluR2 alone. The majority of cells (56%) coexpressed both receptor subtypes. This finding is in keeping with previous studies which revealed that some 50% of dendritic spines were immunopositive to both GluR1 and GluR2/3 in hippocampal cells (Bernard et al., 1997).

This finding is important as these cells are likely to demonstrate variable levels of calcium permeability, and no generalisation as to their function can be made. However, a significant proportion of cells do not express GluR2 and are likely to be calcium permeable. It is interesting to note that the proportion of cells expressing both GluR1 and GluR2 did not change in neuropathy. As these measurements were made from cytoplasmic regions near the nucleus, the possibility remains that changes in colocalisation may have occurred at regions of synaptic connectivity distant from the cell body. These results also show that the levels of GluR1 expression were lower in the cell bodies ipsilateral to CCI. This may represent GluR1 migration away from perinuclear areas.

4.4.5 Reversal of GluR1 redistribution

The current study revealed that the redistribution of GluR1 subunits seen in neurons ipsilateral to CCI injury in lamina II can be reversed following treatment with a combination of antagonists to the NMDA R, mGluR1/5 and $VPAC_2$ R. These antagonists have previously been shown to significantly attenuate neuropathic pain behaviour (see Figure 4.12). NMDA R blockade using systemically administered MK-801 reversed hyperalgesia following CCI (Mao et al., 1992a). Blockade of the $VPAC_2$ R suppressed neuronal responses to prolonged noxious input in both normal and nerve-injured rats (Dickinson et al., 1999). Ablation of group I mGluRs using antibody treatment reversed cold allodynia in a CCI model of neuropathy and blockade of mGluR5 using SIB1757 reversed thermal hyperalgesia in a SNL model (Dogrul et al., 2000). It is highly likely that administration of a combination of these three antagonists would result in a particularly effective reversal of behavioural reflex sensitisation in CCI. This finding suggests that the reversal of GluR1 redistribution would be associated with reversal of the behavioural changes seen in neuropathic pain.

Interestingly, intrathecal treatment with the AMPA R antagonist NBQX alone failed to reverse the redistribution of GluR1 subunits at peak behavioural change following CCI. It is known that various stimuli may cause trafficking of AMPA Rs to and from the cell membrane (Luscher and Frerking, 2001), and that blockade of AMPA Rs and related proteins may result in the reversal of behavioural reflex sensitisation that is thought to reflect neuropathic pain (Mao et al., 1992a), (Garry et al., 2003b).

The previously demonstrated reversal of thermal hyperalgesia using an AMPA R antagonist alone (NBQX 5nmol) was relatively short lived, lasting 15-30mins post injection (Garry et al., 2003b). It is possible that AMPA R blockade may reverse behavioural changes as it prevents continuing stimulus from entering the dorsal horn transiently, switching off sensitisation. It is known that AMPA R blockade may result in the prevention of transmission of both noxious and innocuous stimuli (Dougherty et al., 1992). The effect of AMPA R antagonism alone may be analogous to that of a local anaesthetic agent acting through sodium channel blockade. Peripheral nerve blockade may also be transiently effective in preventing neuropathic hyperalgesia and allodynia, but usually fails to demonstrate long term relief (Abram, 2000).

The spinal cord specimens used in the current study were obtained 35-40mins following intrathecal injection of NBQX (20nmol). At this time point, the behavioural effects of the drug found in a previous study had resolved (Garry et al., 2003b). It is possible that transient changes in the subcellular location of GluR1 may have occurred during this period, which would not have been apparent on analysis of tissue taken at 35mins post injection.

4.4.6 Summary

The current study has revealed significant changes in the apparent expression and subcellular location of GluR1 subunits within lamina II ipsilateral to CCI. It is clear that changes in the function of lamina II neurons may influence the processing of nociceptive and innocuous somatosensory information, resulting in central sensitisation. In the context of other reports in the literature, the results are consistent with the idea that the induction of reflex sensitisation may result from both an increase in excitatory function and a decrease in inhibitory function of lamina II neurons.

It was possible to reverse the redistribution of GluR1 subunits within lamina II cells demonstrated following CCI using a combination of antagonists known to attenuate behavioural reflex sensitisation, suggesting that trafficking of GluR1 subunits may play a significant part in neuropathic pain. Also, blockade of AMPA receptors alone was not sufficient to reverse these changes, suggesting that continued activation of NMDA, mGluR and $VPAC_2$ Rs is important in maintaining this sensitised state, but continued AMPA R activation is not essential.

There were no apparent changes in the expression or subcellular distribution of GluR2 subunits following nerve injury. This does not exclude significant functional changes in the role of this receptor subunit in neuropathic pain, however.

CHAPTER 5: SUMMARY AND CONCLUSIONS

Neuropathic pain is known to involve marked plastic changes in synaptic and neuronal structure and function including changes is neurotransmitter and receptor expression in the DRG and dorsal horn (Hokfelt et al., 1994). However, the role of AMPA Rs and their interaction with other neurotransmitter systems in this sensitised state has not been fully elucidated.

5.1 Behavioural studies

The use of behavioural reflex techniques provides valuable information about the response of the intact animal to drug treatment, potentially including the functioning of higher brain centres and intact inhibitory mechanisms. However, this paradigm is limited in the amount of information that can be obtained regarding the mechanism of the processes involved in generating specific behaviours.

The most marked behavioural finding was the synergistic effect on sensitisation of thermal nociceptive responses found between AMPA, Ro 25-1553 and DHPG. It is possible that the activation of AMPA Rs would reproduce the effects of ongoing noxious and innocuous sensory input thought to be required to maintain central sensitisation, as AMPA Rs mediate the majority of monosynaptic excitatory postsynaptic potentials (EPSP) in the dorsal horn evoked by C fibres (Gracely et al., 1992), (Liu et al., 2000) (see Figure 3.3). This AMPA-mediated stimulus may be sufficient to trigger central sensitisation in the spinal cord (Sotgiu et al., 1994), (Lee et al., 2003). In addition, activation of calcium permeable AMPA Rs may have a specific effect by triggering second messenger systems that activate many downstream processes including phosphorylation of NMDA Rs and AMPA Rs (see section 1.6.4 (Sorkin et al., 2001)).

Although the current behavioural study has revealed that combinations of receptors may be activated in central sensitisation, the changes occurring in the spinal dorsal horn during neuropathic pain are somewhat different. The duration of noxious stimulation is far longer following nerve injury, permitting transcription dependant changes in the expression of neurotransmitters and receptors to occur over hours to days (see section 1.11), (Hokfelt et al., 1994). Also, central sensitisation in neuropathic pain occurs following release of glutamate and co-transmitters from

primary sensory neurons, which would initially affect local dorsal horn neurons and activate many more receptor types than those considered in the current study.

The duration of reflex sensitisation was limited to 90minutes following drug treatment, in most cases, which is far shorter than the duration of sensory changes following damage to a peripheral nerve. It is likely that the effect of intrathecal agonist administration was terminated by local metabolism, redistribution within the CSF or absorption into the circulation. The low frequency ongoing afferent input from injured and uninjured axons required to maintain a neuropathic state is also lacking in drug treatment. The absence of this afferent barrage may have allowed more rapid recovery from sensitisation (Gracely et al., 1992), (Liu et al., 2000), (see section 1.11.2).

Long-lasting sensitisation could, potentially be triggered by increasing the duration of exposure to the agonist drugs. This could be achieved using intrathecal infusions of drugs delivered to experimental animals via an osmotic minipump and intrathecal catheter. However, the current study utilised a simple intrathecal injection technique. Halothane was used as the sole anaesthetic agent for all studies, as is does not interact with AMPA Rs at clinically relevant concentrations, whereas certain barbiturate anaesthetic agents may cause channel blockade (Joo et al., 1999), (Joo et al., 2001). The insertion of intrathecal catheters requires more invasive surgery and results in an inflammatory response that takes several days to recover (Dib, 1984). Catheter insertion may also affect levels of endogenous opioids and potentially cause long-lasting changes to the responsiveness of neurons to further stimuli (Dib et al., 1991).

5.2 Immunocytochemistry

The findings of the behavioural study suggested that further investigation of the role of the AMPA Rs in neuropathic sensitisation was warranted. AMPA Rs are involved in the fast transmission of noxious and innocuous stimuli, and mediate the majority of monosynaptic excitatory postsynaptic potentials (EPSP) in the dorsal horn evoked by C fibres (Procter et al., 1998), (Stanfa and Dickenson, 1999).

The GluR1 and GluR2 AMPA R subunits were chosen for further study for a number of reasons. Firstly, they are expressed significantly by neurons in the superficial dorsal horn of the spinal cord, and have been localised to synaptic densities in lamina II on electron micrography (Tachibana et al., 1994), (Popratiloff et al.,

1996), (Todd et al., 1998). Electron microscopy has revealed that these subunits were found between 30nm outside and 40nm inside the postsynaptic membrane of asymmetric synapses, associated with two types of nerve terminal. GluR1 subunits were associated with C1 terminal boutons and unmyelinated primary afferents, and GluR2/3 with C2 terminal boutons and small myelinated afferents (RibeiroDaSilva and Coimbra, 1982). It is therefore likely that these two receptor subtypes play an important role in nociception, and their distribution in the CCI model of neuropathic pain was investigated in detail using immunohistochemistry and confocal microscopy.

Initial experiments revealed an apparent marked decrease in the proportion of cell bodies expressing GluR1 in lamina II of the DH ipsilateral to CCI at peak neuropathic change in reflex responses (25% to 15%) (see Figure 4.3). It would seem likely that this is due to a redistribution of GluR1 distally into regions of synaptic connectivity or a reduction in expression rather than cell death (see section 4.4.2). This hypothesis is supported by the finding that there was no deficit in the proportion of cells expressing GluR1 in animals showing recovery from CCI (see Figure 4.3).

The current study revealed a significant subcellular redistribution of GluR1 subunits in the dorsal horn in neuropathy (see Figure 4.10 and section 4.4.2). Although it is known that 60–85% of total GluR1 protein and 40–80% of total GluR2/3 protein was expressed on the surface of hippocampal neurons, only a very small proportion of these receptors are localized at synapses (Hall and Soderling, 1997), (Grosshans et al., 2002). A study of apical dendrites in hippocampal cells revealed 71% of GluR1 subunits to be localised intracellularly in the dendritic shafts, 20% on the dendritic shaft surface, 8% in spines and only 3% in postsynaptic densities (Shi et al., 1999). This suggests that there is a large pool of receptors available for insertion into the synapse.

We also provide evidence to suggest that redistribution of GluR1 is functionally significant. Firstly, redistribution was demonstrated at peak CCI and absent in recovered animals – this suggests that there is a functional association between GluR1 redistribution and neuropathic pain, but it was not possible to prove causality. Receptor redistribution also occurred following high dose AMPA treatment, which has been shown to cause behavioural reflex sensitisation (see section 3.4) and which may be sufficient to trigger central sensitisation (see Figure 4.10).

Subcellular redistribution of GluR1 was also associated with an increase in the number of Bassoon-immunoreactive puncta in contact with each GluR1-

immunopositive neuron (see Figure 4.5). This may reflect the movement of GluR1-containing AMPA Rs towards sites of synaptic activity for insertion into the PSD. AMPA Rs containing GluR1 subunits are added to synapses during neuronal plasticity; requiring an interaction between GluR1 and PDZ domain proteins (Hayashi et al., 2000). The insertion of AMPA Rs into excitatory synapses would result in an increased number of EPSPs and play a role in the initiation of central sensitisation in the spinal cord. In addition, insertion of AMPA Rs consisting of tetramers of GluR1 into the synapse would increase Ca^{2+} permeability, permitting further depolarisation and activating numerous calcium dependant second messenger systems (Sorkin et al., 1999). Some 20% of lamina II neurons were found to express GluR1 only, so the contribution of Ca^{2+} permeable AMPA Rs to synaptic transmission in the DH may be highly significant. However, the AMPA R subunit colocalisation study revealed that 80% of immunopositive lamina II cells expressed GluR2, suggesting that the degree of calcium-permeability of the majority of lamina II cells is reduced. There was no change in the proportion of colocalised cells in a neuropathic state, so it was not possible to generalise as to which group of cells demonstrated GluR1 redistribution.

In addition to increased excitatory transmission due to GluR1 redistribution leading to increased AMPA R insertion into synapses in excitatory neurons, GluR1 expression may decrease within inhibitory interneurons, reducing their responsiveness. This hypothesis is supported by the finding that down regulation of GluR1 subunits following administration of the pro inflammatory cytokine IFN-γ resulted in a GABA-independent increase in spontaneous excitatory activity in neuronal culture (Robertson et al., 1997). GluR1 redistribution and down-regulation may result in an overall change in the balance of excitatory / inhibitory function resulting in central sensitisation (Dickenson, 1996).

The current study demonstrated the reversal of GluR1 redistribution in established neuropathy using combinations of antagonists. Whether this finding reflects long-term reversal of behavioural reflex sensitisation and long-term analgesia remains to be determined. Also, the current study does not provide evidence for a causal role for GluR1 redistribution in central sensitisation.

Blockade of AMPA Rs alone was not effective in restoring pre-injury GluR1 distribution (see Figure 4.12). It is possible that blockade of AMPA Rs alone is not sufficient to release AMPA Rs containing GluR1 subunits from the PSD, where they

are bound via the C-terminal tail to the postsynaptic density protein SAP-97, and actin-associated protein 4.1N, which has been shown to increase AMPA R density at postsynaptic sites in cell cultures (Shen et al., 2000), (Rumbaugh et al., 2003). AMPA Rs containing GluR2 are also stabilised at the PSD by ABP and GRIP (Osten et al., 2000). The interaction between GluR2 and PICK1 has also been shown to be important in the maintenance of thermal hyperalgesia in neuropathic pain (Garry et al., 2003b) (see section 1.9.4). It is possible that blockade of mGluR, $VPAC_2$ R and NMDA Rs is sufficient to release GluR1 and possibly GluR2 containing AMPA Rs from the PSD, reversing the redistribution of GluR1 in established neuropathy. This may reflect a long-term reversal of central sensitisation. This could be investigated further using the behavioural paradigm and administering antagonists to combinations of receptor subtypes to animals with established neuropathic sensitisation. It is possible that combinations of multiple antagonists would lead to a prolonged and extensive reversal of behavioural reflex sensitisation.

In contrast to the marked changes in GluR1 expression revealed in the current study, the expression and subcellular location of the GluR2 subunit did not appear to change in response to CCI or AMPA treatment. AMPA Rs incorporating the GluR2 subunit have been shown to constitutively cycle to and from synapses in hippocampal cells (Shi et al., 2001). Although no changes in GluR2 distribution were seen in the current study, trafficking of this receptor is likely to be important in neuropathic pain, as it has been recently demonstrated that blockade of GluR2 interaction with adapter proteins in the dorsal horn results in a reduction in thermal hyperalgesia in CCI (Garry et al., 2003b). It is known that at least 50% of AMPA Rs containing the GluR2 subunit are localised beneath the cell membrane within vesicles containing GRIP and PICK1 in hippocampal cells (Lee et al., 2001). It is possible that insertion of GluR2 AMPA Rs occurs from these intracellular stores located close to the synapse, which was not apparent using immunofluorescent antibodies and confocal microscopy. There may have been significant functional changes in the role of GluR2 at peak neuropathy, which could be more effectively investigated using electrophysiological techniques (see section 1.6.2).

Behavioural studies revealed a synergistic interaction between AMPA Rs and mGluR group I, and $VPAC_2$ Rs in the generation of thermal hyperalgesia. Behavioural sensitisation following CCI is accompanied by a redistribution of GluR1 AMPA R subunits towards sites of synaptic plasticity in lamina II of the dorsal horn.

This redistribution may also be triggered by treatment with AMPA. GluR1 redistribution is reversed on behavioural recovery from CCI or treatment with a combination of antagonists to NMDA Rs, mGluR group I, and $VPAC_2$ peptide Rs. It is suggested that reversal of GluR1 redistribution by a combination of antagonists may result in prolonged reversal of central sensitisation occurring in neuropathic pain.

In conclusion the current study provides evidence using behavioural and immunohistochemical techniques for the importance of AMPA Rs in the induction of central sensitisation, and the redistribution of GluR1 subunits in neuropathic pain.

5.3 Therapeutic implications

Further study is required before the therapeutic potential of these findings can be assessed. The behavioural responses of rats at peak neuropathic sensitisation, to combinations of the antagonists used in the current study would provide useful information as to the duration and effectiveness of reversal of neuropathic pain behaviour. Electron microscopy may provide further information regarding the subcellular redistribution of GluR1/2 in neuropathy. Functional investigation of the changes in dorsal horn neuron responsiveness using electrophysiological techniques may provide additional information as to the specific roles of GluR1 and 2 subunits in neuropathic pain.

The therapeutic potential of AMPA blockade has been assessed in a number of paradigms. Non-specific blockade of all AMPA Rs has been shown to result in loss of all sensory transmission and would initially be thought inappropriate in the management of chronic pain (Dougherty et al., 1992). However, it is clear that low doses of AMPA R antagonists can strongly inhibit neuropathic sensitisation at levels which have no detectable effect on naïve unsensitised responses (Garry et al., 2003b). As well as their possible value in neuropathic pain such AMPA R antagonists may have a role in suppressing excitotoxicity in other clinical situations such as head injury (Belayev et al., 2001).

It is possible that blockade of AMPA R subtypes may be effective in alleviating certain types of pain. For example, blockade of Ca^{2+} permeable AMPA Rs using JsTx reduced mechanical allodynia following a burn injury (Sorkin et al., 1999). Recently, the GluR2 subunit has been shown to be important in neuropathic pain. Blockade of the interaction between GRIP / PICK1 and GluR2 resulted in a marked

attenuation of the thermal hyperalgesia seen after induction of reflex sensitisation by CCI (Garry et al., 2003b), (Garry and Fleetwood-Walker, 2004).

The findings of the current study suggest that there may also be therapeutic potential in the use of multiple antagonist drugs in combination to treat neuropathic pain. Multiple antagonist treatment may result in unbinding of AMPA Rs from the PSD, potentially resulting in long-term pain relief. Combinations of antagonists could be given at lower doses, resulting in fewer systemic side effects. However, as many of the drugs used are peptides and unable to cross the blood-brain barrier, or survive enteral dosing intact, they would have to be administered via the intrathecal route. Non-peptide agents may of course be developed in the future. Although intrathecal injection results in high drug concentrations focussed at the required site, it is not a practical route of administration if long-term repeated dosing is required.

It is also of note that certain antagonists are likely to be more effective in treating differing modalities of sensory abnormality. Attempts have been made to rationalise the treatment of various neuropathic pain conditions. Treatments have been targeted at specific conditions, at individual symptoms and at underlying mechanisms. However, it would seem that there are often no clear associations between the presenting symptoms of the condition, its aetiology or response to treatment. On reviewing the effectiveness of drug therapies in neuropathic pain, there were no clear relations between the mechanism of action of the drugs and their effectiveness in distinct pain conditions, or for single drug classes and different pain conditions (Sindrup and Jensen, 1999). Treatment based on the specific mechanisms underlying various neuropathic pain conditions has been suggested, but it is not yet possible to establish the underlying changes in the CNS resulting in neuropathic pain in a clinical setting (Woolf and Mannion, 1999).

Attempts to treat specific symptoms, rather than underlying mechanisms, with tailored therapy in neuropathic pain have been largely unsuccessful to date, possibly because the drugs employed such as membrane stabilisers, tricyclic antidepressants, sodium and calcium channel blockers and opioids do not target receptors linked to specific aspects of central sensitisation. Potentially, treatment with combinations of antagonists to particular receptors, such as those shown to play a role here, may allow rational targeting of specific symptoms in neuropathic pain.

NOTES

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APPENDIX: PUBLICATIONS ARISING FROM RESEARCH

Abstract presented at the 10th World Congress on Pain August 2002 San Diego, California, United states. IASP Press.

EVIDENCE FOR SYNERGISM BETWEEN GLUTAMATE AND PEPTIDE RECEPTORS IN THE GENERATION OF HYPERALGESIC PAIN STATES

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Aim of investigation To determine the relationship between various key receptors in the development of an hyperalgesic pain state in the rat.

Methods Male rats were injected intrathecally, under brief halothane anaesthesia, with agonists to the ionotropic glutamate receptors N-methyl-D-aspartate (NMDAR), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPAR), the Group I metabotropic glutamate (mGlu) receptors and the vasoactive intestinal polypeptide ($VPAC_2$) peptide receptor. The drugs were administered individually, in pairs and in triple combinations. After recovery from anaesthesia, the latency of paw withdrawal (PWL) to a noxious thermal stimulus (Hargreaves) and the withdrawal threshold to mechanical stimuli (von Frey) was recorded every ten minutes for ninety minutes.

Results All individual drugs caused a statistically significant decrease in PWL for a period after injection. Injection of pairs of agonists had a similar effect. However, injection of a triple agonist combination of AMPA, Ro-25-1553; $VPAC_2$ receptor agonist, and DHPG (3,5-dihydroxyphenylglycine; mGlu Group I agonist) caused a more marked decrease in PWL than the sum of all other combinations

Conclusions The greater than additive effect of the triple AMPA, Ro-25-1553 and DHPG injection, suggests that there may be a synergistic interaction between these three receptors in the development of hyperalgesic states. This study suggests that there may be scope for developing therapies directed towards more than one receptor in the treatment of chronic pain states in humans.

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Poster presented at the 10th World Congress on Pain August 2002 San Diego, California, United states.

Evidence for a Facilitative Interaction Between Glutamate and Peptide Receptors in the Generation of Hyperalgesic Pain States

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Background

Chronic neuropathic pain is not fully understood and responds poorly to classical analgesics such as morphine. Neuropathic pain is characterised by allodynia (the perception of innocuous stimuli as painful), hyperalgesia (an exaggerated response to painful stimuli) and spontaneous pain (stimulus-independent pain). It is thought that some form of central sensitisation of neuronal responses underlies the clinical findings in this disorder. Previous evidence has indicated that the N-methyl-D-aspartate receptor (NMDAR) plays a key role in central sensitisation in models of neuropathic pain (Davies and Lodge 1987). Other glutamate receptors, including the metabotropic glutamate receptors (mGluR) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR) are also involved in the induction of hyperalgesic pain states (Meller, Dykstra and Gebhart 1996a, Sorkin and Yaksh 2001) . The role of the vasoactive intestinal polypeptide receptor ($VPAC_2R$) has also been investigated (Dickinson et al 1999).

Intrathecal Study

The aim of this study was to determine the relationship between various key receptors in the development of an hyper-sensitive pain state in the rat.

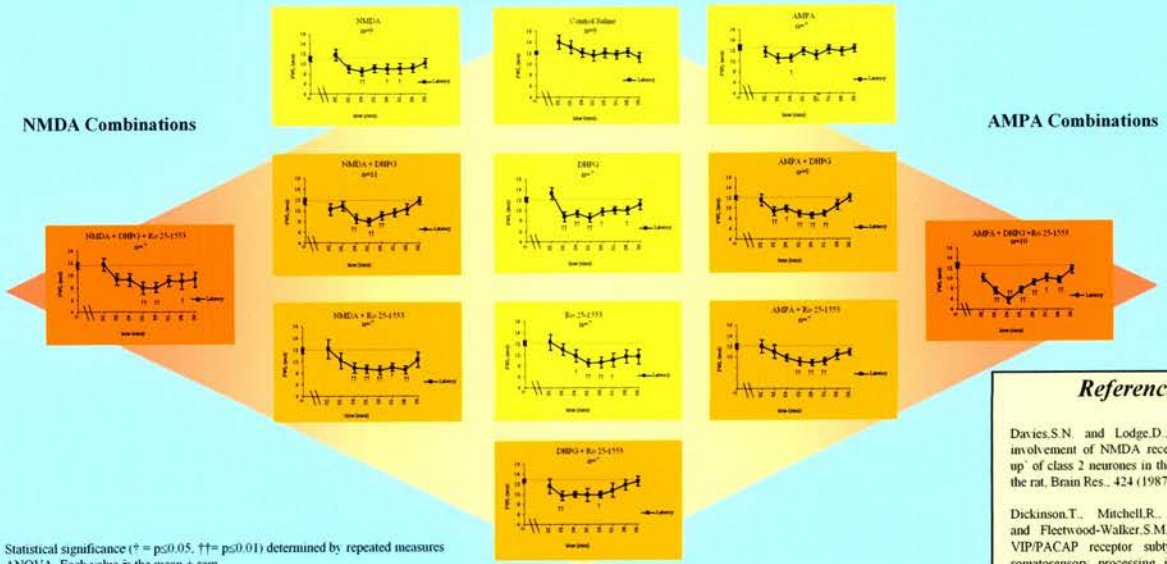
Methods

Male rats were injected intrathecally, under brief halothane anaesthesia, with agonists to the ionotropic glutamate receptors NMDAR and AMPAR, the mGluR Group I and the $VPAC_2$ R. The drugs were administered individually, in pairs and in triple combinations, in all cases in a volume of 50µl (Fig. 3). After recovery from anaesthesia, the latency of paw withdrawal (PWL) to a noxious thermal stimulus (Hargreaves 1988) was recorded every ten minutes for ninety minutes.

Results

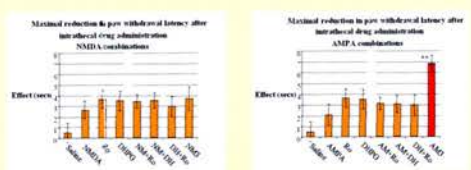
All individual drugs caused a statistically significant decrease in PWL for a period after injection compared to baseline measurements. Injection of pairs of agonists had a similar effect (Fig. 1). However, injection of a triple agonist combination of AMPA, Ro 25-1553 ($VPAC_2$ receptor-selective agonist), and DHPG (3,5-dihydroxyphenylglycine, an mGlu Group I receptor-selective agonist) caused a significantly greater decrease in PWL at maximum effect than all other combinations ($p \leq 0.015$ Fig. 2).

Fig. 1 Effects of intrathecal drug combinations on Hargreave's thermal test – paw withdrawal responses



Statistical significance († = p≤0.05, †† = p≤0.01) determined by repeated measures ANOVA. Each value is the mean ± sem.

Fig. 2 The maximum effect of intrathecal injection of 50µl drug on thermal paw withdrawal latency



Ro = Ro 25-1553, NMD = NMDA + DHPG + Ro 25-1553, AMD = AMPA + DHPG +Ro 25-1553. All data shown as mean + - sem
**Denotes significantly greater effect than all other NMDA & AMPA combinations at p ≤ 0.015

Fig. 3 Drugs and doses used

Drug	Concentration and dose
AMPA	50 µmolar 2.5 nmol per injection
Ro 25-1553	6 µmolar 0.3 nmol per injection
NMDA	25 µmolar 1.25 nmol per injection
DHPG	30 µmolar 1.5 nmol per injection
Saline	50 µl
Combinations	Same doses in 50 µl saline

Conclusions

The marked effect of the triple AMPA, Ro 25-1553 and DHPG injection suggests that there may be a positive interaction between these three receptors in the induction of thermal hyperalgesia. It is known that NMDA receptors play an important role in the maintenance of hyperalgesic pain states (Meller 1996b). The role of non-NMDA glutamate receptors in the induction of thermal hyperalgesia in a rat model of neuropathic pain has been previously demonstrated (Mao and Price 1992). This study suggests that there may be scope for developing combination therapies directed towards more than one receptor in the treatment of chronic pain states in humans, and in particular the role of AMPA receptors in the induction of hyperalgesic pain states has been highlighted.

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