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An Investigation of Plasticity in Somatosensory Processing Following Early Life Adverse Events or Nerve Injury

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Thesis presented for the degree of Doctor of Philosophy

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Declaration

I hereby declare that the composition of this thesis and the work presented in it are entirely my own with the exception of the behavioural testing and 4.1N/GluA1 immunoprecipitation experiments, which were carried out as part of collaborative studies by Hayley Gooding, Andrew Allchorne and Emer Garry in our laboratory. Some of this work has been published in abstract form.

Liting Sun

Published Abstract

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Abstract

Chronic hypersensitive pain states can become established following sustained, repeated or earlier noxious stimuli and are notably difficult to treat, especially in cases where nerve injury contributes to the trauma. A key underlying reason is that a variety of plastic changes occur in the central nervous system (CNS) at spinal and potentially also supraspinal levels to upregulate functional activity in pain processing pathways. A major component of these changes is the enhanced function of excitatory amino acid receptors and related signalling pathways.

Here we utilised rodent models of neuropathic and inflammatory pain to investigate whether evidence could be found for lasting hypersensitivity following neonatal (or adult) noxious stimuli, in terms of programming hyper-responsiveness to subsequent noxious stimuli, and whether we could identify underlying biochemical mechanisms. We found that neonatal (postnatal day 8, P8) nerve injury induced either long lasting mechanical allodynia or shorter lasting allodynia that nonetheless was associated with hyper-responsiveness to a subsequent noxious formalin stimulus at P42 despite recovery of normal mechanical thresholds. By developing a new micro-scale method for preparation of postsynaptic densities (PSD) from appropriate spinal cord quadrants we were able to show increased formalin-induced trafficking of GluA1-containing AMPA receptors into the PSD of animals that had received (and apparently recovered from) nerve injury at P8. This was associated with increased activation of ERK MAP kinase (a known mediator of GluA1 translocation) and increased expression of the ERK pathway regulator, Sos-1. Synaptic insertion of GluA1, as well as its interaction with a key partner protein 4.1N, was also seen in adults during a nerve injury-induced hypersensitive pain state.

Further experiments were carried out to develop and optimise a new technological platform enabling fluorometric assessment of Ca^{2+} and membrane potential responses of acutely isolated CNS tissue; 30-100 μm tissue segments, synaptoneurosomes (synaptic entities comprising sealed and apposed pre- and post-synaptic elements) and 150 × 150 μm microslices. After extensive trials, specialised

conditions were found that produced viable preparations, which could consistently deliver dynamic functional responses. Responsiveness of these new preparations to metabotropic and ionotropic receptor stimuli as well as nociceptive afferent stimulant agents was characterised in frontal cortex and spinal cord.

These studies have provided new opportunities for assessment of plasticity in pain processing (and other) pathways in the CNS at the interface of in vivo and in vitro techniques. They allow for the first time, valuable approaches such as microscale measurement of synaptic insertion of GluA1 AMPA receptor subunits and ex vivo assessment of dynamic receptor-mediated Ca^{2+} and membrane potential responses.

Abbreviations

5-HT	5-hydroxytryptamine
ABP	AMPA receptor binding protein
ACB	allyl isothiocyanate, capsaicin, and bradykinin
ACC	anterior cingulate cortex
ACh	acetylcholine
ACPC	1-aminocyclopropane carboxylic acid
AITC	allyl isothiocyanate
AKAP	A kinase anchoring protein
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
AP-2	adapter protein-2
ATP	adenosine 5'-triphosphate
BAR	Bin/amphiphysin/Rvs
BK	bradykinin
BSA	bovine serum albumin
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase
CCI	chronic constriction injury
CFA	Complete Freund's Adjuvant
CHAPS	3-[(3-cholamidopropyl)dimethylammonium]-1-propanesulfonate
CGRP	calcitonin gene-related peptide
CNS	central nervous system
(R)-CPP	3-[(R)-2-carboxypiperazin-4-yl]-propyl-1-phosphonic acid
CREB	cAMP response element binding protein
CTD	C-terminal domain
CTZ	cyclothiazide
DLH	DL-homocysteic acid
DOI	2,5-dimethoxy-4-iodoamphetamine
DRG	dorsal root ganglion
EGFR	epidermal growth factor receptors
ERK	extracellular-regulated kinase

GABA	γ-aminobutyric acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDNF	glial cell line-derived neurotrophic factor
GEFs	Ras-specific guanine nucleotide exchange factors
GluR	glutamate receptor
GPCRs	G protein-coupled receptors
GRF	guanyl nucleotide-releasing factor
GRIP	glutamate receptor interacting protein
GRP	guanyl nucleotide-releasing protein
IB4	lectin B4
IP	immunoprecipitation
HBSS	Hanks' Balanced Salt Solution
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HRP	horseradish peroxidase
IASP	The International Association for the Study of Pain
IONO	ionomycin
JNK	c-Jun N-terminal kinase
KA	kynurenic acid
KCC2	K^+/Cl^- -cotransporter 2
LC	locus coeruleus
LII_i	inner zone of lamina II
LII_o	outer zone of lamina II
LTD	long-term depression
LTP	long-term potentiation
MAGUK	membrane-associated guanylate kinase
MAPK	mitogen-activated protein kinase
MES	2-(N-morpholino)ethanesulfonic acid
mGluR	metabotropic glutamate receptors
MOPS	3-morpholinopropane-1-sulfonic acid
NA	1-noradrenaline
NBQX	2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7 sulfonamide
NCF	nucleus cuneiformis

NGF	nerve growth factor
NK1	neurokinin-1
NMDA	N-methyl-D-aspartic acid
NSF	N-ethylamide-sensitive fusion protein
NSSP	non-synaptic synaptosomal proteins
NPY	neuropeptide Y
PAG	periaqueductal gray
PBS	phosphate-buffered saline
PBST	PBS-0.1%Tween-20
PI 3-K	phosphatidylinositol 3-kinase
PICK1	protein interacting with C-kinase-1
PKA	cyclic AMP-dependent protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PNS	peripheral nervous system
PRAZ	prazosin
PROP	1-propranolol
PSD	postsynaptic density
PSL	partial sciatic nerve ligation
PVDF	polyvinylidene difluoride
PWL	paw withdrawal latency
PWT	paw withdrawal threshold
RTKs	receptor tyrosine kinases
RVM	rostral ventromedial medulla
RFU	relative fluorescence unit
SAP	synapse-associated protein
SDS	sodium dodecyl sulfate
SMT	spinomesencephalic tract
SNL	spinal nerve ligation
SOM	somatostatin
Sos	Son of Sevenless
SP	substance P

SRE	serum response element
SRT	spinoreticular tract
STT	spinothalamic tract
TARPs	transmembrane AMPAR regulatory proteins
TBS	Tris-buffered saline
TRP	transient receptor potential
VIP	vasoactive intestinal polypeptide
VZV	varicella zoster virus
WDRs	wide dynamic range neurons

Chapter 1 Introduction

1.1 Pain

The International Association for the Study of Pain (IASP) has defined pain as “an unpleasant sensory and emotional experience associated with potential or actual tissue damage or described in terms of such damage” and added that “the inability to communicate in no way negates the possibility that an individual is experiencing pain and is in need of appropriate pain relieving treatment” (Merskey and Bogduk, 1994).

This definition indicates that pain is a complex experience of somatic mechanisms and psychological influences such as affect and cognition that may help humans or animals to best avoid further injury, but in some cases its maintenance as prolonged chronic pain states produces a continuous unpleasant sensory experience that causes suffering and distress.

Pain can be classified as acute (physiological) pain and chronic (pathological) pain, such as inflammatory pain and neuropathic pain (arising from nerve injury). The former appropriately signals the need for evasive or protective actions to avoid further damage, whereas the latter can represent inappropriately prolonged painful hypersensitivity even after resolution or healing from an acute injury.

1.2 Normal somatosensory processing

The somatosensory system is the part of the nervous system that is involved in the processing of many kinds of sensory modalities such as touch, pressure, proprioception, temperature, and nociception (pain) (Barker and Barasi, 2008c). It comprises the sensory receptors themselves, usually located on nerve terminals in the peripheral nervous system (PNS) and processing regions in the central nervous system (CNS). The full spectrum of events involved in somatosensory processing is still not completely understood. Briefly, a variety of sensory stimuli can be detected

by specialised endings of peripheral nerves and translated into electrical impulses, i.e. action potentials, which are carried along the axon towards to the spinal cord. There, the information is often influenced by interneurons before being relayed via ascending sensory pathways to higher centres of the brain including the cortex for cognitive awareness of that sensory input.

1.2.1 Sensory transmission in the PNS

Somatosensory stimuli can be detected by peripheral afferent nerve endings, which are distributed throughout the body to receive a variety of sensory information. The cell bodies of these afferents are located in the dorsal root ganglia of the spinal nerves for nerves originating in the body or in the trigeminal ganglia for nerves transmitting somatosensory information from the head. The central endings of their axons mainly terminate in the dorsal horn of spinal cord to form the first series of synapses before these inputs are relayed to higher levels of the CNS.

1.2.1.1 Peripheral afferent fibres

A peripheral sensory nerve is composed of afferent fibres that receive various stimuli in the periphery and transmit information as nerve impulses to CNS. The afferent fibres have been classified according to size, structure, function, and conduction velocity.

Aβ-fibres are large thick myelinated fibres (between 5-12 μm in diameter) that convey low-threshold stimuli, such as pressure, vibration and light touch, with conduction speeds of 30-70 metres/second. They normally do not contribute to pain sensation but are involved in the process of innocuous sensation. However a subpopulation of them that express the VGLUT1 subtype of vesicular glutamate transporter may have a role to play in injury-induced mechanical allodynia (Neumann *et al.*, 2008) Aδ-fibres are smaller, lightly myelinated fibres (1-5 μm in diameter) conveying high-threshold noxious stimuli at slower speeds of 2.5-30 metres/second and serving pain, temperature, and pressure sensation. C-fibres are unmyelinated fibres (0.1-1.3 μm in diameter) conveying high-threshold noxious

stimuli, such as thermal stimuli in the noxious range with lower speeds of 0.6-2.0 metres/second.

Generally there is a predictable correlation between the type of cutaneous stimulus detected and the type of afferent fibre involved; for example, faster, thicker, myelinated neurons are likely to conduct generalised touch sensation whereas slow, thin, unmyelinated neurons are likely to conduct nociceptive events.

1.2.1.2 Sensory receptors

There are many different kinds of sensory receptors in the skin. They can be divided into several broad groups, such as thermoreceptors, mechanoreceptors and a range of nociceptors.

Thermoreceptors

Thermoreceptors are represented by free endings of afferent fibres and encode a full range of innocuous thermal information. They are located in various tissues including cornea, urinary bladder and the skin (Darian-Smith *et al.*, 1979; Dhaka *et al.*, 2008). Cold-sensitive thermoreceptors give rise to the sensations of cooling and cold. They can be activated by temperatures below 27°C (Darian-Smith *et al.*, 1979; Duclaux *et al.*, 1980). Warm-sensitive thermoreceptors respond to a slight warming of the skin in the innocuous range, 30-37°C (Darian-Smith *et al.*, 1979; Moqrich *et al.*, 2005).

Mechanoreceptors

Mechanoreceptors are specialised for touch sensation and are found in the skin at particularly high density in the fingertips, whereas those subserving a proprioceptive role are found not only in the skin but also in the muscle and joints. Such mechanoreceptors can be further characterised by structure, location, receptive fields and speed of adaptation. Meissner corpuscles with very small, sharply defined receptive fields are packed in high density at the fingertips, palm of the hand, plantar surface of the foot, toes and lips. They provide accurate information about the exact

location of a stimulus, low-frequency flutter-vibration and dynamic touch stimuli (Fukuda *et al.*, 1982). In contrast, Pacinian corpuscles are rapidly adapting receptors and responsive to high-frequency vibration with a very short recovery cycle as they are only transiently responsive to vibration and quickly stop firing in the presence of continuous sensory stimuli (Weerakkody *et al.*, 2007). These receptors are distributed abundantly in the subcutaneous connective tissue of the hands and feet. Ruffini's corpuscles are found in the dermis of the skin and are widely located, especially in joint capsules. They are more slowly adapting receptors and associated with sensations of direction, magnitude, pressure, touch, and flexion and extension of joints (Grandis *et al.*, 2007). The discharge of these receptors is temperature-dependent, tending to increase with skin cooling and to decrease to skin warming.

Nociceptors

Nociceptors that detect potentially damaging levels of thermal, mechanical or chemical stimuli are specialised endings of peripheral afferents. They were discovered by Charles Scott Sherrington in 1906 and are found ubiquitously in the skin, cardiac muscles, visceral organs and associated blood vessels. Nociceptors encode the intensity, duration, location, and quality of the noxious stimulus and transduce this information into action potentials which are relayed to the CNS. Nociceptors are represented by two main types of peripheral afferents, which are thin myelinated Aδ-fibres and unmyelinated C-fibres. The Aδ- and C-fibres have been proposed to mediate rapid, sharp pain ('first pain') and dull, diffuse pain ('second pain') respectively (Wolf and Hardy, 1941). Several distinct types of nociceptors have been identified:

Thermal nociceptors are activated by noxious heat or cold. There is increasing evidence that Transient Receptor Potential (TRP) ion channels, which are expressed in nociceptive afferent fibres, may play a key role. TRP channels are non-selectively permeable to cations, including sodium, calcium, magnesium, and potassium. The first to be discovered was the transient receptor potential vanilloid 1 (TRPV1) receptor (Caterina *et al.*, 1997b), whose temperature activation threshold of around 42°C, coincides with that at which raised temperatures are considered to be noxious .

Other TRP channels such as TRPV2, TRPV3 and TRPV4 (Dhaka *et al.*, 2006), expressed by some afferents, can respond selectively to distinct ranges of elevated temperatures (Numazaki and Tominaga, 2004; Brauchi *et al.*, 2006). It is possible that monomers of these TRP channels may combine to form heteromeric (tetramer) complexes with potentially intermediate properties but there is little documentation of this at the present time. Moreover, the heat sensitivity primarily mediated by TRP channels may be modulated by heat-sensitive potassium channels like TREK-1 (Alloui *et al.*, 2006), whose activity could be reduced by increased heat.

Cool stimuli are sensed by other TRP channels such as TRPM8. Although this channel appears to be activated by cool stimuli, it is seems improbable that it contributes to a major extent in the detection of noxious levels of cold since TRPM8 knockout mice still respond to intense cold (Bautista *et al.*, 2007; Dhaka *et al.*, 2007). Other candidate channels such as transient receptor potential cation channel, subfamily A member 1, also known as TRPA1 may also contribute to the detection of intense cold (Bandell *et al.*, 2004b).

Chemical nociception can be mediated by individual TRP channels that respond to a wide variety of pungent plant-derived chemicals such as chili, garlic, mustard, and horseradish (Xu *et al.*, 2006). Other chemical stimulants are environmental irritants like formalin, which is thought to exert its noxious effects by molecular modification and activation of the TRPA1 channel (McNamara *et al.*, 2007). Moreover the TRPV1 channel for example, not only detects noxious heat stimuli and chemicals such as capsaicin, but also protons (Jancso, 1967) and some spider toxins (Siemens *et al.*, 2006). Nociceptors further show amplified responsiveness to such stimuli in the presence of endogenous inflammation-associated ligands like ATP, interleukin IL-1β, bradykinin, nerve growth factor (NGF) and certain fatty acid derivatives that arise from injury-induced changes in tissues (Geppetti, 2006).

There are also mechanical nociceptors that respond to excess pressure or mechanical deformation and also to incisions that break the skin surface (Kwan *et al.*, 2006). Many mechanical nociceptors have polymodal characteristics, whereby some forms of noxious thermal and/or chemical stimuli can also result in their activation.

Candidate molecular mediators of mechanical nociception, for example TRPA1 (Kwan *et al.*, 2006), can also respond to thermal and chemical stimuli.

Although most nociceptors are polymodal, some are unimodal and respond only to thermal or mechanical stimuli, for example Aδ-mechanonociceptors (Cooper and Bomalaski, 1994).

1.2.2 Sensory transmission in the spinal cord

As part of the CNS, the spinal cord is the gateway for information transfer between body and brain, as well as a centre for neuronal circuits that integrate and coordinate complex sensory, motor, and autonomic functions. The spinal dorsal horn is one of the sensory processing components and is the main target of investigation in this study.

The central terminals of sensory afferents form synapses with neurons in the spinal dorsal horn and release neurotransmitters and neuropeptides into the synaptic cleft. These agents diffuse from the presynaptic active zone to the postsynaptic membrane and bind to their receptors on the postsynaptic membranes especially at the postsynaptic density (PSD). The activated receptors in turn either gate ion movements or trigger intracellular signalling pathways to transmit the nociceptive message. This processing of nociceptive information can be diversely modulated by different types of interneurons or by descending neuronal tracts before transmission to higher centres of the CNS. As well as informing the brain of incoming sensory information, the circuitry of the dorsal horn processes different kinds of reflex circuits, such as nociceptive withdrawal responses and crossed extensor reflexes. However, many reflexes can be heavily modulated by other influences supplied from the brain. Behavioural reflex assessments of ‘pain’ sensitivity need careful design and handling because in many cases the animals can experience a learned component to responses with certain reflexes becoming extensively modulated across time with training. In addition, the regulation of some reflexes may not necessarily give a close reflection of influences over transmission processes to the brain, as it is possible that they may be exempt from some aspects of modulation. For example there may

hypothetically be inhibitory processes that set a gate to limit activity in transmission neurons but do not impinge on local reflexes.

1.2.2.1 Dorsal horn organisation

Axons of peripheral sensory afferents arriving from the body tissues end in different regions of spinal dorsal horn (Light and Perl, 1977), which can be broadly divided into five layers known as laminae I-V extending from the superficial to the deep dorsal horn (Molander *et al.*, 1984; Barker and Barasi, 2008) (Figure 1.1) according to the cytoarchitectonics, or cellular organisation, originally investigated in the cat (Rexed, 1952).

Figure 1.1: Laminar organization of lumbar spinal cord in rats

Source: Molander, Xu and Grant (1984)

The diagram shows a schematic drawing of the cytoarchitectonic subdivisions of rat spinal L5 segment. This is a transverse view of a hemisected spinal cord.

Abbreviations:

Liss- Lissauer's tract

Pyr- Pyramidal tract

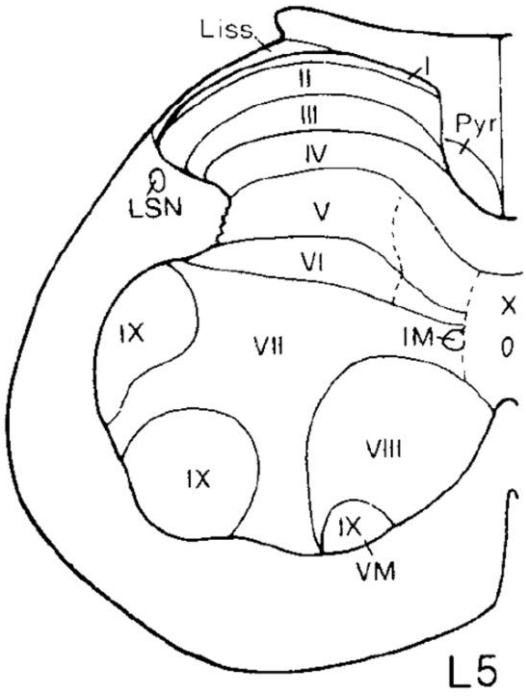
LSN- Lateral spinal nucleus

IM- Intermedio-medial nucleus

VM- Ventro-medial nucleus

I-X- Rexed's ten laminae

1.1



Lamina I (‘marginal zone’) is a thin layer extending in both rostrocaudal and mediolateral axes with loosely packed small and large cells. The marginal zone is functionally heterogeneous because many cells respond to a wide range of intense mechanical and thermal stimuli, receiving convergent inputs from Aδ- and C-fibres. Therefore, lamina I is considered to relay information related to nociception (pain) and temperature sensation. Indeed, a recent study gives support to the theory that lamina I may comprise part of a specific pain transmission system (Honore *et al.*, 2002), signalling pain intensity rather than finely resolved location, to autonomic and affective centres in the brain (Bernard *et al.*, 1996; Doyle and Hunt, 1999). Moreover, it has been reported that the cells in lamina I have two major axonal outputs, one projecting to different segments of the spinal cord via a propriospinal system (Sar *et al.*, 1978) and another contributing importantly to the contralateral Spinomesencephalic and Spinothalamic tracts (Ding *et al.*, 1995; Bester *et al.*, 2000) (see section 1.2.2.4 below).

Lamina II (‘substantia gelatinosa’) cells are located radially and oriented just ventral to the marginal zone. C-fibre thermoreceptors and nociceptors terminate in the most superficial substantia gelatinosa, which is referred as outer zone (LII_o). The deeper portions of lamina II receive input from C-fibre mechanoreceptors, whereas Aδ mechanoreceptors distribute to the deep substantia gelatinosa known as inner zone (LII_i) and lamina III immediately below this (Light and Perl, 1979; Sugiura *et al.*, 1986). Lamina II is the main termination site for unmyelinated C-fibres and is considered to be an important site for transmission of nociceptive information (Light and Perl, 1979; Sugiura *et al.*, 1986; Todd, 2002; Todd and Koerber, 2005). Only ~1% of LII neurons send projections to brainstem (Willis *et al.*, 1979) and are mostly intrinsic interneurons with extensive local integration.

Directly below these superficial layers are laminae III-V where cells are less tightly packed than in lamina II and are generally larger. They may be mainly concerned with exteroceptive and proprioceptive sensation, although cells in this layer are also responsive to noxious stimuli as well as non-noxious events. Many of the cells in laminae III-V contribute to the Spinocervical and Postsynaptic dorsal column tracts with also some participation in the Spinothalamic tract (Brown, 1981). The literature

broadly identifies the superficial laminae as receiving predominantly noxious information from the periphery, whilst the deeper layers of the dorsal horn appear to be involved in both non-nociceptive and nociceptive processing. It may well be that the more superficial laminae contribute more to sensory noxious awareness in terms of their trajectories to predominantly the brainstem, whereas the deeper layers of dorsal horn neurons appear to access more direct routes to the cortex (Hunt and Mantyh, 2001). These distinct trajectories may correspond to partially separate processing of the affective and discriminative properties of pain respectively. There are many different influences within the spinal dorsal horn that affect processing and neurotransmission towards higher levels of the CNS. These influences are mediated by the actions of neurotransmitters, through receptors involving numerous second messenger pathways, and also ion channels, all of which may modulate synaptic activation, which is the subject of this thesis.

1.2.2.2 Dorsal horn neuron types

The spinal cord dorsal horn contains various cell types ranging across nociceptive- and proprioceptive-specific neurons, wide dynamic range neurons, inhibitory and excitatory interneurons and glial cells. As mentioned before, nociceptive-specific cells are mostly found in superficial laminae I and II and form primary synapses with Aδ- and C-fibres only, being activated when a noxious stimulus is applied in the periphery (Cervero *et al.*, 1976). Proprioceptive-specific and non-nociceptive cells appear to be more often present in the deeper dorsal horn, responding to joint movement, touch and pressure (Iggo and Ramsey, 1974). Wide dynamic range neurons (WDRs), which are commonly found in laminae III-V are responsive to a broad range of intensity of stimuli from peripheral nerves including both noxious and innocuous events (Mendell, 1966). These cells in particular may be involved in the phenomenon of ‘wind up’, which is a kind of central sensitisation caused by repeated electrical activation of peripheral afferents at sufficient intensity to stimulate the C-fibres (Dickenson and Sullivan, 1987). In addition, numerous interneurons are located in the spinal superficial dorsal horn to provide both glutamatergic excitatory and GABAergic or glycinergic inhibitory influences. Subsets of interneurons may additionally contain various neuropeptides such as substance P. The interneurons

influence the output from projection cells located in lamina I or those in laminae III-V, which have dorsally directed dendrites in laminae I and II. Recently, the role of spinal glial cells such as astrocytes and microglia in nociceptive transmission especially in pathological conditions has been increasingly revealed (Meller *et al.*, 1994; Watkins *et al.*, 1997; Coyle, 1998; Scholz and Woolf, 2007).

1.2.2.3 Neurotransmitters and their receptors in dorsal horn

Neurons in the dorsal horn communicate with other neurons or glial cells by releasing chemical neurotransmitters at their synapses. A vast number of neurotransmitters have been identified in the superficial spinal dorsal horn including excitatory amino acids (for example glutamate) inhibitory amino acids(such as γ-aminobutyric acid (GABA) and glycine) as well as monoamines (such as serotonin, noradrenaline (NA) and dopamine) neuropeptides (such as substance P (SP) and neuropeptide Y (NPY)) purines, such as ATP and nitric oxide (NO) (von Bohlen und Halbach and Dermietzel, 2006).

Excitatory transmission by glutamate and glutamate receptors

Glutamate has long been established as an excitatory neurotransmitter at insect and crustacean neuromuscular junctions, and it has now become widely accepted that it is likely to represent the major excitatory neurotransmitter of the mammalian CNS (Wanaka *et al.*, 1987; Battaglia and Rustioni, 1988; Westlund *et al.*, 1989; Weinberg, 1999). At synapses within the spinal dorsal horn, glutamate is believed to be stored in vesicles of the terminals of many primary afferents regardless of whether they are myelinated or unmyelinated (Wanaka *et al.*, 1987). Nerve impulses are thought to trigger release of glutamate from the vesicles in the pre-synaptic active zone of these terminals (Curtis *et al.*, 1960; Davies *et al.*, 1979). It has been suggested that nociceptive inputs to the superficial laminae of spinal dorsal horn may release glutamate to transmit the nociceptive information and it was identified in early studies that glutamate receptor agonists both produce an increase in primary afferent-induced nociceptive responses and directly excite dorsal horn neurons (Curtis *et al.*, 1960). Electrophysiological investigation of sensory synaptic responses between

primary afferent fibres and dorsal horn neurons has provided evidence that glutamate is the principal fast excitatory transmitter, and synaptic responses are mediated by postsynaptic glutamate receptors, including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (Troca-Marin *et al.*) and N-methyl-D-aspartic acid (NMDA) receptors (Yoshimura and Jessell, 1990; Li and Zhuo, 1998). Both NMDA and AMPA receptors in the spinal cord appear to play a crucial role in the central sensitisation underlying chronic neuropathic pain (Garry *et al.*, 2000; Shiokawa *et al.*, 2010; Wang *et al.*, 2010).

Glutamate binding to its receptors localised in the postsynaptic membrane of CNS neurons causes postsynaptic excitation and synaptic plasticity which is important for communication, memory formation, learning and states of hyper-responsiveness (Asztely and Gustafsson, 1996; Debanne *et al.*, 2003; Perez-Otano and Ehlers, 2005). Glutamate receptors can either be ionotropic (which are ligand-gated ion channels) or metabotropic (involving second messenger production) (Palmada and Centelles, 1998). The present study has concentrated on the ionotropic variety. Ionotropic glutamate receptors contain an ion channel pore that allows the flux of Na^+ and sometimes Ca^{2+} and also K^+ in rapid response to glutamate binding. Several specific subtypes of ionotropic glutamate receptors with highly conserved structures have been identified. The main subtypes are designated by the pharmacological agonists capable of activating them; NMDA receptors, AMPA receptors and kainate receptors. The subunit monomers all have a common structure, with an extracellular N-terminus, four transmembrane domains (of which the second transmembrane domain kinks back on itself within the membrane and returns to the intracellular side) and an intracellular C-terminus (Hollmann *et al.*, 1994) (Figure 1.2). When the component monomers of the receptor come together, the second transmembrane domains form the wall of ion-permeable pore of the receptor.

Figure 1.2: General structure of AMPA receptor subunits

Source: modified diagram based on Song and Huganir, 2002

The different subtypes of AMPA receptor subunits follow the same basic structural pattern with an extracellular N-terminus and large loop that together form the glutamate binding domain. The C-terminus is intracellular, subject to mRNA splicing, creating functionally distinct variant forms and furthermore represents the interaction site with intracellular proteins.

Key:

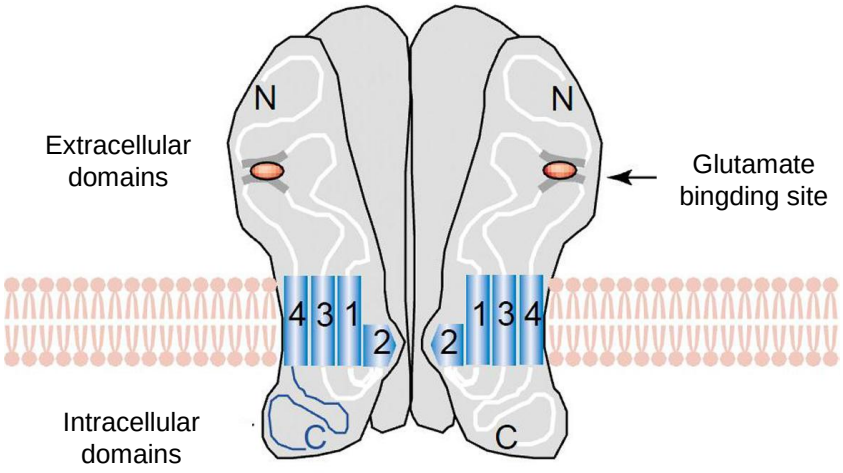
1- transmembrane domain I

2- transmembrane domain II

3- transmembrane domain III

4- transmembrane domain IV

1.2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N
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4 3 1
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1 3 4
Intracellular
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The NMDA receptor is a tetramer with the GluN1 subunit (represented twice) comprising the core functional unit and generally the GluN2 subunit subtypes (ranging from A-D) or the GluN3 ones (A/B) determining the specific channel characteristics (Schuler *et al.*, 2008). These subunits have been shown to be distributed throughout the spinal cord, particularly in the superficial dorsal horn, an area essential for the processing of nociceptive information in particular (Greenamyre *et al.*, 1984; Furuyama *et al.*, 1993; Wee *et al.*, 2008). NMDA receptors are usually located in an electron-dense thickening in the excitatory postsynaptic membrane, known as the PSD, which additionally contains a variety of scaffolding and signalling proteins such as membrane-associated guanylate kinase (MAGUK) proteins (which are multivalent adapter proteins), signal transduction molecules and other receptors (Ehlers *et al.*, 1996). In mammals, the MAGUK family includes PSD-95, synapse-associated protein-97, (SAP-97), PSD-93 and SAP-102. These proteins can interact with the intracellular terminal tails of GluN2A and GluN2B NMDA receptor subunits and are thought to play a role in the clustering and synaptic targeting of NMDA receptors (Scannevin and Huganir, 2000; Sheng and Sala, 2001). A unique property of the GluN2 subunit-containing NMDA receptor is voltage-dependent activation corresponding to removal of ion channel block by extracellular Mg^{2+}. Activation of NMDA receptors results in voltage-dependent nonselective cation flux of Na^{+} and Ca^{2+} ions into the cell and K^{+} out of the cell (Dingledine *et al.*, 1999; Liu and Zhang, 2000; Cull-Candy *et al.*, 2001; Paoletti and Neyton, 2007). In contrast, GluN1/3-containing NMDA receptors which are activated by glycine yet unaffected by glutamate or NMDA, are relatively impermeable to Ca^{2+}, and show low affinity for Mg^{2+} (Chatterton *et al.*, 2002), probably because the GluN3 subunit has a unique loop 1 structure which is different from that in all other glutamate ion channels (Yao *et al.*, 2008). It has been suggested that GluN3 subunit-containing NMDA receptors may play a role in synapse formation and long-term potentiation when NMDA receptors are thought to be functionally silent in the absence of AMPA receptors (Chatterton *et al.*, 2002). Little is still known of the potential functional roles of such receptors.

AMPA receptors are tetramers homomerically or heteromerically made up of combinations of four subunits: GluA1, GluA2 (these two generally being the most

abundant), GluA3, and GluA4 (alternatively known as GluRA-D or GluR1-4) (Keinanen *et al.*, 1990; Dingledine *et al.*, 1999; Collingridge *et al.*, 2009). All of the subunit proteins have an extracellular N-terminus, and four membrane-associated hydrophobic domains, an intracellular C-terminus, and generally including a PDZ domain-binding motif to associate with molecules such as MAGUK proteins. Different subunits are thought to bind with different members of the MAGUK protein family, for example GluA1 binds to SAP-97, while GluA2 binds to alternative partners, known as PICK1 (protein interacting with C-kinase-1) and GRIP/ABP (glutamate receptor-interacting protein)/AMPA receptor binding protein (Osten *et al.*, 2000; Dev *et al.*, 2004). The protein:protein interactions will be further addressed below (see section 1.3.2.4). However AMPA receptors are not thought to directly bind with high affinity to PSD-95 due to incompatible PDZ domains. A further link is provided by stargazin or other transmembrane AMPAR regulatory proteins (TARPs) that also co-assemble with AMPA receptors. They act as auxiliary subunits that are required for AMPA receptor maturation, trafficking, and channel function and also bind to PSD-95 (Chen *et al.*, 2000; Rouach *et al.*, 2005; Nicoll *et al.*, 2006; Ziff, 2007). The GluA1 subunit has been identified consistently as the main subunit mediating activity-dependent synaptic insertion and synaptic strengthening (Hayashi *et al.*, 2000). Immunohistochemical and *in situ* hybridization studies indicate that the four subunits of AMPA receptors are all expressed in spinal dorsal horn (Furuyama *et al.*, 1993) and have also been described in dorsal root ganglion cells and afferent terminals (where it has been suggested that they may function to inhibit glutamate release acting as autoreceptors (Lee *et al.*, 2002)) and also possibly in glia (Tachibana *et al.*, 1994). The GluA1 subunit appears to be predominantly expressed in the superficial dorsal horn of adults, whereas the GluA4 subunit is mainly expressed in younger animals, whilst the expression of GluA2 and GluA3 subunits shows little change during development (Brown *et al.*, 2002). AMPA receptors open and close quickly within a millisecond time-range and are thought to be responsible for most fast excitatory synaptic transmission in the CNS (Platt, 2007). AMPA receptor permeability to calcium ions is governed by the GluA2 subunit, with the presence of GluA2 subunit rendering the channel impermeable to calcium ions (Geiger *et al.*, 1995). The main influences of these two core glutamatergic ion

channels are the subject of much debate still within the nervous system in adult animals and have as yet been little addressed *in vivo* in neonatal animals.

Metabotropic glutamate receptors (mGluRs), which are G protein-coupled receptors (GPCRs), can indirectly modulate voltage-sensitive ion channels on the plasma membrane through signalling cascades over a longer time scale. They are members of the group C family of GPCRs containing seven transmembrane domains, an extracellular N-terminus and an intracellular C-terminus. Eight different types of mGluRs, named mGluR1 to mGluR8, are classified into groups I (mGluRs 1 and 5), II (mGluRs 2 and 3), and III (mGluRs 4, 6, 7 and 8) (Chu and Hablitz, 2000; Hinoi *et al.*, 2001; Endoh, 2004). They are found at various levels of the nervous system (Anwyl, 1999; Chu and Hablitz, 2000; Hinoi *et al.*, 2001). In dorsal root ganglia, mGluR1 and mGluR2/3 types are mainly expressed in small and medium diameter afferent neurons (Carlton and Hargett, 2007). Group I mGluRs are mainly expressed in postsynaptic membrane and coupled to Gq/11 proteins to facilitate nociceptive transmission (Conn and Pin, 1997). It is reported that group I mGluR expression is increased chronically in the dorsal horn following spinal cord injury (Gwak and Hulsebosch, 2005) and activation of group I mGluRs is necessary for activity-dependent central sensitisation mediated by C-fibres (Young *et al.*, 1997; Azkue *et al.*, 2003). Groups II and III mGluRs are found both in afferents and in superficial dorsal horn neurons where they are coupled to inhibitory Gi/o proteins to exert antinociceptive effects (Pin and Duvoisin, 1995; Proudfoot *et al.*, 2006; Zhang *et al.*, 2009a). mGluR2 and mGluR3 are strongly expressed in the inner part of lamina II, and are also detectable in lamina III-VI of spinal dorsal horn (Tang and Sim, 1999). mGluR 7 is reported to be localised in axon terminals of nociceptive afferents in laminae I and II of the spinal dorsal horn that are predominantly co-labelled with IB4 (Li *et al.*, 1997a). mGluR4 is expressed in lamina II of spinal dorsal horn and occasionally is reported as present in postsynaptic densities (Azkue *et al.*, 2001).

Activation of group II/III metabotropic glutamate receptors is consistently reported to exert antinociceptive influences in chronic neuropathic pain models (Fisher *et al.*, 2002; Simmons *et al.*, 2002; Chen and Pan, 2005), although a contrary role for inflammatory pain has been suggested (Zhang *et al.*, 2009b). In addition, group II

and III metabotropic glutamate receptors may modify the vulnerability of a neuron to excitotoxicity through their modulation of the outcome from NMDA receptor activation (Baskys and Blaabjerg, 2005). Consistently, agonists of group II and III mGluRs are reported to reduce NMDA receptor-induced activation and neurotoxicity (Ambrosini *et al.*, 1995; Wang and Brownell, 2007).

Inhibitory amino acids

The inhibitory amino acids, GABA and glycine, are the chief inhibitory neurotransmitters in CNS and contribute to the modulation of sensory processing. They bind to specific transmembrane receptors in the presynaptic membrane of peripheral afferents or postsynaptic membrane of dorsal horn neurons to regulate neuronal excitability. For instance, when GABA binds to its ionotropic $GABA_A$ receptor, which is a chloride channel (as opposed to $GABA_B$ which is a metabotropic, second messenger-coupled receptor), the flow of chloride ions into the cell causes hyperpolarisation. Such actions generally result in frank inhibition although it may be possible that influences are modified or even reversed during a noxious event or in chronic pain states following some kinds of damage (Coull *et al.*, 2003). GABA receptor activation may also be involved in descending modulatory pathways targeting the spinal cord (Pinto *et al.*, 2008), which could potentially attenuate chronic pain, for example after spinal cord injury (Gwak *et al.*, 2006). A complex profile of influence of GABAergic neurons may therefore be exerted in pain states. Glycine is another inhibitory neurotransmitter in the CNS, especially in the spinal cord and its receptor is a ligand-gated chloride channel (Cronin *et al.*, 2004; Rajalu *et al.*, 2009).

Neuropeptides

Diverse neuropeptides are also expressed in dorsal root ganglia and are released from the endings of peripheral afferent fibres in dorsal horn of the spinal cord. Here they may mediate or modulate neuronal communication by acting on cell surface receptors, generally metabotropic GPCRs, which means that cell signalling cascades are going to be instigated following activation. Neuropeptides including SP, calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP),

galanin, somatostatin (SOM) and neuropeptide Y (NPY) have been found in afferents and/or dorsal horn (Buck *et al.*, 1982; Merighi *et al.*, 1992; Rowan *et al.*, 1993; Wiesenfeld-Hallin and Xu, 2001). Many of these co-exist with conventional neurotransmitters such as glutamate in synaptic vesicles and their co-release is likely to result in complex conjoint actions on target cells (De Biasi and Rustioni, 1988).

Studies using pharmacological and behavioural approaches showed that neuropeptides (including substance P) can potentially act as nnociceptive transmitters in the spinal dorsal horn (Levine, 1993; Cao, 1998; De Felipe, 1998). Substance P is synthesised in a subset of small DRG cells and is axonally transported to the superficial laminae I and II of the dorsal horn, where it preferentially activates neurokinin-1 (NK1) receptors during responses to C-fibre mediated nociceptive inputs (Honore *et al.*, 2002; El-Nour *et al.*, 2006). Evidence for a role of SP in synaptic plasticity following synaptic activation of superficial dorsal horn neurons by capsaicin (which selective acts on C-fibre afferents) has been reported (Urban and Dray, 1992). In addition, increased expression of NK1 receptors in the superficial dorsal horn in both inflammatory and neuropathic pain models indicated that NK1 receptors may participate in nociceptive transmission (Abbadie *et al.*, 1996). It is reported that release of SP due to activation of presynaptic NMDA receptors located on the central terminals of small-diameter fibres facilitates and prolongs the neurotransmission of nociceptive messages (Liu *et al.*, 1997). Together, the co-release of glutamate and SP from fine afferent fibres may allow sufficient membrane depolarisation to allow for some NMDA receptor activation following prolonged peripheral inputs (Afrah *et al.*, 2001).

Small unmyelinated sensory neurons are classified into two populations; peptidergic and nonpeptidergic. The peptidergic population terminate in lamina LIIo of dorsal horn and predominantly express SP, CGRP and TrkA (the NGF receptor) while the non-peptidergic dorsal root ganglion (DRG) neurons, which terminate in LIIi of dorsal horn postnatally switch their dependency from NGF to glial cell line-derived neurotrophic factor (GDNF) and are identified by positive staining for lectin B4 (IB4) (Molliver *et al.*, 1997).

Purines

Adenosine 5'-triphosphate (ATP) is thought to be released from non-peptidergic small afferents (Hamilton and McMahon, 2000). $P2X_3$ ion-channel receptors, which are predominantly expressed by non-peptidergic afferents within the superficial dorsal horn are activated by ATP and play a role in primary afferent transmission and nociception (Lewis *et al.*, 1995; Bradbury *et al.*, 1998; Dowd *et al.*, 1998). Adenosine is a related neurotransmitter that is produced by metabolism of ATP and can exert a modulatory influence by coupling to metabotropic receptors that are widely distributed throughout the nervous system and generally exert inhibitory effects (Collo *et al.*, 1996).

Nitric oxide

Nitric oxide (NO) was identified as a signalling molecule in the body in 1987 (Palmer *et al.*, 1987). NO is very stable in low, physiological concentrations (~50 nM) and freely diffusible across cell membranes, so can ONLY ONE SPACE NOT TWO HERE LITINGmediate transcellular signalling (Garthwaite, 2008). NO is synthesised by three well-characterised isoforms of NO synthase (NOS): neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) in different cell types. In the nervous system the expression of nNOS is predominant and it plays a role in the development and maintenance of inflammatory and neuropathic pain (Chu *et al.*, 2005; Guan *et al.*, 2007). A recent study revealed that nNOS was present in 27% of neurons in laminae I-III of spinal dorsal horn, which is a key site for nociceptive processing. Expression of nNOS in spinal cord and DRG can be increased in inflammatory pain evoked by peripheral formalin or CFA injection (Chu *et al.*, 2005; Herdegen *et al.*, 1994; Lam *et al.*, 1996). However, following peripheral nerve injury, nNOS expression was increased only in DRG (Guan *et al.*, 2007). NMDA receptors may be important in recruiting nNOS to synaptic sites through the direct interaction with the NMDA receptor-interacting protein, PSD-95 (Brenman *et al.*, 1996a). The NO produced in pain states is thought to diffuse out from DRG neurons or interneurons in spinal cord to influence neighbouring DRG or dorsal horn neurons (Altis *et al.*, 2009). It activates the NO-sensitive guanylyl cyclase (NO-

GC)/cGMP signalling pathway. NO-GC is expressed in neurokinin 1 (NK1)-receptor-positive projection neurons in lamina I, in inhibitory interneurons in laminae II and III of the spinal cord (Ding and Weinberg, 2006) and in satellite non-neuronal cells in DRG (Schmidtko *et al.*, 2008). The NO-dependent production of cGMP in pain states may contribute to central sensitisation via activation of cGMP-dependent protein kinase (cGKI; more commonly known as PKG) in peripheral afferents to promote the release of glutamate and substance P or other factors that facilitate sensitisation in the spinal cord (Schmidtko *et al.*, 2009).

Interestingly, NO may also play a role in antinociception in spinal cord, depending on the concentration of NO (Luo and Cizkova, 2000; Sousa and Prado, 2001). The mechanical allodynia evoked by CCI could be reduced by low intrathecal doses of an NO donor but increased by high doses (Sousa and Prado, 2001). cGMP may be responsible for both effects of NO because intrathecal injection of the cell-permeable cGMP analogue, 8-Br-cGMP also elicits dose-dependent dual effects on nociceptive processing (Tegeder *et al.*, 2002).

1.2.2.4 Ascending sensory pathways

Most of the sensory output from the dorsal horn to higher centres in the brain is carried by spinal projection neurons terminating in the thalamus along such tracts as the Spinothalamic (STT), Spinoreticular (SRT), and Spinomesencephalic (SMT) tracts. In addition, further ascending pathways may include the Postsynaptic dorsal column, Spinocervical, Spinotectal, and Spinohypothalamic tracts, as well as an intersegmental system to pass innocuous or in some cases, noxious stimuli to other segments of spinal cord (propriospinal system) and/or other brain areas such as medulla, mesencephalon, pons, and hypothalamus. These tracts serve different purposes, which may depend on both their laminae of origin in the dorsal horn and final destination (Dostrovsky and Craig, 2006). Many of these ascending pathways may activate descending systems to heavily influence the reflex circuitry of the spinal cord.

Spinothalamic tract (STT)

The axons of spinal nociceptive-specific neurons (that have received synaptic inputs from primarily Aδ-fibres in laminae I and V) traverse across the midline to the contralateral side of the spinal cord in the anterior white commissure to form the STT. Then, the nociceptive input is transmitted through the STT to the contralateral ventroposterior lateral nucleus of thalamus (Barker and Barasi, 2008), whereas a large proportion of STT cells with projections to the intralaminar nuclei of the medial thalamus originate from more ventral laminae of the spinal cord (laminae VI-VIII) in the rat (Matsuzaki *et al.,* 1993). The STT also sends some collateral projections to the reticular formation when it ascends through the brainstem. An average of 15% of STT cells project to both the thalamus and the reticular formation of brainstem (Matsuzaki *et al.*, 1993).

Spinoreticular tract (SRT)

C-fibres terminate on nociceptive-specific neurons in laminae II and III of dorsal horn. The axons of some neurons that synapse with these nociceptive-specific neurons in laminae V-VIII partially form the SRT (Willis, 1985; Zhang *et al.*, 1999). SRT neurons are also located in the lateral part of dorsal horn, in or around the nucleus proprius, or sparsely in lamina I or III of lumbar dorsal horn (Menetrey *et al.*, 1980). Many of the axons ascend ipsilaterally, however a small portion of axons cross to the opposite side of the spinal cord in the anterior white commissure. Approximately 86% of SRT neurons in the rat have clear cutaneous sensitivity and generally large receptive fields: 40% respond to innocuous and noxious mechanical cutaneous stimuli and radiant heat, 26% are exclusively excited by light tactile stimuli and 20% require noxious cutaneous mechanical stimulation for activation, whereas the remaining 14% have more complex receptive fields associated with both excitatory and inhibitory inputs originating from a single peripheral area (Menetrey *et al.*, 1980). The SRT conveys sensory information to the brainstem reticular formation, the region responsible for producing arousal and wakefulness, thus alerting the organism following an injury. Then the signals are relayed predominantly through contralateral or bilateral pathways in cervical segments to the intralaminar

nuclei of the thalamus through reticulothalamic fibres (Menetrey *et al.*, 1980; Kevetter *et al.*, 1982).

Spinomesencephalic tract (SMT)

Spinomesencephalic tract cells are located throughout spinal gray matter including marginal zone, lateral neck of the dorsal horn, nucleus proprius, the region around the central canal, the lateral cervical and spinal nuclei, and the ventral horn (Yezierski and Mendez, 1991). Most SMT cells in lumbar cord project to the contralateral midbrain containing the parabrachial nucleus, the periaqueductal gray matter (Zhang *et al.*, 1990) and the Raphe nuclei, whilst 5% of the cells have both ascending and descending projections (see descending modulatory pathways below). Of note, the projection cells originating in lamina I mainly terminate in the parabrachial nucleus of the midbrain to form the Spinoparabrachial tract, which relays nociceptive signals to the thalamus (Kitamura *et al.*, 1993). About 80% of the projection neurons in lamina I express NK1 receptors (Ding *et al.*, 1995; Todd *et al.*, 2000). This indicates that the Spinoparabrachial tract may well be an important tract for nociceptive transmission. It has been shown that NK1-positive lamina I neurons with projections to parabrachial area of the brainstem respond to a variety of types of noxious stimuli (Doyle and Hunt, 1999).

Postsynaptic dorsal column tract (PSDC)

The postsynaptic dorsal column tract represents a component of the Dorsal Column-Medial Lemniscus (DCML) pathway, which is generally responsible for the transmission of innocuous touch, vibration and proprioceptive information to higher parts of the CNS. Most neurons of the DCML pathway are afferents that do not relay in the spinal cord but pass directly up the dorsal column to synapse in the gracile and cuneate nuclei of the medulla. From there, projections pass to the medial lemniscus. In a small proportion of cases the afferents synapse in the dorsal horn with neurons known as postsynaptic dorsal column cells that then contribute their ascending axons to the DCML pathway.

Postsynaptic dorsal column cells are concentrated in lamina III or IV of spinal cord and project ipsilaterally through the dorsal column (Bennett *et al.*, 1983; Giesler *et al.*, 1984). They contain two types of fibres, some of which are specifically responsive to gentle stimulation such as hair movement, pressure, pinching and cooling, while others are vigorously responsive to noxious cutaneous stimulation (Brown *et al.*, 1983).

Other pathways

Many kinds of pathways transmit pain information to the brain. Spinocervical tract neurons are mainly located in laminae III, IV, and V and generally display a wide-dynamic range (WDR) profile of responsiveness. Many respond to low threshold tactile stimuli, although some are activated by intense cutaneous pressure, pinch and thermal nociceptive stimulation. Spinotectal fibres are predominantly large diameter fibres in the spinal cord (Antonetty and Webster, 1975) that end in the deep layers of the superior colliculus and have the reflex function of turning the upper body, head and eyes in the direction of a painful stimulus (Antonetty and Webster, 1975; Rhoades, 1981). Spinohypothalamic neurons are mainly found within the deep dorsal horn, while some are also found within the lateral spinal nucleus, the superficial lamina I of the dorsal horn, in the gray matter surrounding the central canal, and in ventral horn (Burstein *et al.*, 1990). Their fibres ascend to the hypothalamus where they synapse with neurons that give rise to the Hypothalamospinal tract. Spinohypothalamic neurons are also NK1-positive and responsive to SP (Li *et al.*, 1997b). Moreover this tract is also associated with autonomic nociception (Zhang *et al.*, 1999). The intersegmental propriospinal system consists of axons that arise from dorsal horn neurons and the dorsolateral fasciculus proprius. Neurons of both laminae I and II contribute axons that ascend or descend for up to five to six segments before terminating in the spinal grey matter (Christensen and Perl, 1970; George and Austin, 1983).

Overall, about 15% of nociceptive-specific neurons from the spinal cord carrying nociceptive information terminate directly in the thalamus, while approximately 85% terminate in the brainstem reticular formation, from where the information is then

relayed to eventually reach the thalamus via what must be multiple additional synaptic relays (Patestas and Gartner, 2006). The brainstem reticular formation sends fibres not only to the thalamus but also to the hypothalamus and the limbic system, which may each contribute to the affective and autonomic events surrounding nociception. From the thalamus, nociceptive information is transmitted to cortical regions including primary and secondary somatosensory, insular, anterior cingulate, and prefrontal cortices, which have been proposed to function together in what has been described as a 'pain matrix'(Tracey and Mantyh, 2007).

1.2.2.5 Spinal and supraspinal modulation of sensory processing

Presynaptic inhibition in the spinal dorsal horn

It was proposed some years ago that nociceptive signals from the periphery are filtered by modulation in the lamina II of the dorsal horn by a process of presynaptic inhibition known as 'Gate Control' (Woolf and Wall, 1982). In this theory it was proposed that cutaneous stimulation of large Aβ-fibres selectively inhibits C-fibre- and noxious stimulation-evoked excitation of dorsal horn neurons. Although there is some evidence to support this concept, the precise operation of any gating process may be much more complex than originally configured. The basis has never been fully resolved experimentally, but it does still provide a target for endeavour with modern approaches to address how balanced regulation of sensory processing within the dorsal horn may occur.

Descending modulatory pathways

The spinal cord is thought not only to be a processing area before transmission to the brain but it also importantly receives feedback from the brainstem in particular, via descending modulatory pathways. The descending pathways from the brainstem may in turn specifically target spinal dorsal horn cells, thus forming a spino-bulbo-spinal loop. In addition, a variety of brain regions including the frontal lobe, anterior cingulate cortex (ACC), insular cortex and amygdala are involved in contributing to the descending modulation exerted on the dorsal horn that is channelled through

brainstem structures including the periaqueductal gray (PAG), nucleus cuneiformis (NCF), and rostral ventromedial medulla (RVM). The PAG plays a pivotal role in the regulation of nociceptive processing by direct links to serotonergic or a few GABAergic and enkephalin-containing neurons in the RVM and to noradrenergic neurons in the locus coeruleus (LC) of the medulla (Bajic *et al.*, 2001; Odeh and Antal, 2001; Lu *et al.*, 2010). Generally, the descending fibres arising from the brainstem may contain serotonin or noradrenaline and many appear to terminate in superficial lamina II of the dorsal horn, in broad terms to facilitate or inhibit nociceptive transmission to the brain, respectively.

The descending pathway from the RVM is generally facilitatory and has been reported to play a role in maintaining chronic neuropathic pain (Burgess *et al.*, 2002). A growing body of evidence indicates that these descending influences may have important facilitatory gating influences and recent evidence indicates that they may act via 5-hydroxytryptamine (5-HT) receptors such as 5-HT_3 within the dorsal horn. These 5-HT_3 receptors are mainly expressed on nerve terminals of thinly myelinated afferents Aδ- and C-fibre afferents (Zeitz *et al.*, 2002) and exert pro-nociceptive effects at the spinal level (Green *et al.*, 2000). An active role of descending serotonergic facilitation in maintaining neuropathic pain was suggested by its attenuation following pharmacological inhibition of spinal 5-HT_3 receptors (Suzuki *et al.*, 2004). In addition, inhibition may be achieved by serotonin acting on other receptor types such as 5-HT_{1B} which exerts a direct inhibitory influence (El-Yassir *et al.*, 1988; Sommer, 2004). The descending serotonergic input could also suppress incoming nociceptive impulses by the excitation of inhibitory interneurons in the superficial laminae I-II of the dorsal horn, which may release the opioids, enkephalin or dynorphin. Enkephalinergic neurons in the spinal dorsal horn are closely associated with small diameter nociceptive afferents and may modulate the excitability of fine nociceptive fibres through pre- and postsynaptic mechanisms (Glazer and Basbaum, 1983).

Descending noradrenergic fibres originating from the LC in the brainstem release noradrenaline in the spinal cord, where it appears to act predominantly at α_2-adrenoceptors, which may inhibit transmitter release from peripheral afferent

terminals and suppress activity of projection neurons in the dorsal horn (Fleetwood-Walker and Coote, 1981; Millan, 2002). It is reported that application of α_2-adrenoceptor antagonists to the spinal cord promotes responses of dorsal horn neurons to peripheral inflammation (Green *et al.*, 1998) and further that an α_2-adrenoceptor agonist can inhibit capsaicin-induced glutamate release into the spinal cord (Li and Eisenach, 2001). The descending pathways are important to ensure that a balanced profile of somatosensory messages reaches the brain and so that the resultant pain experienced is appropriate for any particular circumstance. It has been suggested that in chronic pain states however, the modulatory effects of descending pathways may serve a maladaptive role, causing exacerbation of the pain state (see reviews (Zambreanu *et al.*, 2005; Bingel and Tracey, 2008; Mello and Dickenson, 2008)) and thereby represent a potential target for therapeutic intervention.

1.3 Chronic pain states

Chronic pain states can develop when injuries bring about a sensitisation of nociceptive processing pathways, which then inappropriately register noxious stimuli as intensely noxious (hyperalgesia) and normally innocuous stimuli as noxious (allodynia). These changes can long outlast the acute injury and are therefore seen as functionally maladaptive. In the clinical setting, chronic pain states represent an added level of difficulty for treatment. Various types of injury can induce chronic pain states but the focus of the current work, because of its special characteristics, is that brought about by nerve injury, i.e. neuropathic pain.

1.3.1 Neuropathic pain

Neuropathic pain is a major clinical problem. It arises from traumatic or degenerative damage to the nervous system and manifests as a chronic state of hypersensitivity to somatosensory stimuli. Neuropathic pain may result from disorders of the peripheral or central nervous system. Peripheral neuropathic pain is common in cancer as a direct result of the physical or chemical impact of the cancer on peripheral nerves or as a side effect of chemotherapy, radiation injury or surgery. Apart from cancer, it has many other causes such as traumatic injuries, diabetes, nutritional deficiencies, toxins,

HIV infection, genetic and immune-mediated disorders. Central neuropathic pain is found in spinal cord injury, multiple sclerosis, and some strokes (Schestatsky and Nascimento, 2009).

The common symptoms of neuropathic pain are weakness, numbness, paraesthesias (abnormal sensations such as tickling, pricking) and pain manifesting as increased response to a stimulus which is normally painful (hyperalgesia), painful responses to normally innocuous stimuli (allodynia) and spontaneous pain. The core mechanisms underlying neuropathic pain remain to be fully elucidated.

1.3.2 Animal models of neuropathic pain

The first developed animal model of peripheral neuropathic pain was chronic constriction injury (Bennett and Xie, 1988) in which the application of loose ligatures soaked in chromic salts around the sciatic nerve in rats brought about protective behaviour and lowered thresholds for reflex withdrawal responses to heat, cooling, and mechanical stimuli. The chromic salts are thought to enhance local neuroinflammation. Similar behaviours were also found in the model of partial sciatic nerve ligation (PSL), involving tightly ligating around 33%-50% of the sciatic nerve (Seltzer *et al.*, 1990). Another widely used neuropathic pain model is spinal nerve ligation (SNL), which involves tightly ligating one (L5) or two (L5 and L6) segmental spinal nerves in the rat resulting in long-lasting behavioural signs of mechanical allodynia, thermal hyperalgesia and spontaneous pain (Kim and Chung, 1992). In these three animal models of neuropathic pain, only a portion of afferents from the hind paw are injured. Aside from the trauma models, Ahlgren and Levine developed a metabolic model of neuropathic pain mimicking diabetic neuropathy in human by injection of streptozotocin in the rat, inducing decreased nociceptive thresholds (Ahlgren and Levine, 1993). The model used in this study was the Bennett and Xie 1988, CCI model.

1.3.3 Alterations in somatosensory processing in chronic pain: peripheral mechanisms

A number of nociceptive small diameter afferents can be activated by (or their activation thresholds lowered by) a variety of endogenous chemical mediators for instance histamine, cytokines, prostaglandins, growth factors, H^+, K^+, ATP and enzymes, especially in conditions of tissue inflammation (Kidd and Urban, 2001). These chemical mediators can be released from damaged cells, peripheral sensory neurons themselves and inflammatory cells to interact with and sensitise high-threshold nociceptors (i.e. peripheral sensitisation) and thereby promote primary hyperalgesia. Peripheral sensitisation accounts for the heat hyperalgesia that develops in the area of primary hyperalgesia within the injured skin area (Meyer and Campbell, 1981; LaMotte *et al.*, 1983). Alterations in the properties of voltage-gated sodium and potassium channels expressed in nociceptive neurons make them more electrically excitable. Inflammatory mediators, including NGF, regulate functional expression of the sodium $Na_v1.8$ channel, which is one of the tetrodotoxin-resistant subtypes of sodium channel that is predominantly expressed in small sensory afferents, especially nociceptors. Both antisense and knockout studies support a key role for the channel in contributing to mechanical, cold and inflammatory pain but not noxious heat or nerve injury-induced pain (Khasar *et al.*, 1998; Akopian *et al.*, 1999). For example, phosphorylation of $Na_v1.8$ residues S551 and S556 by p38 mitogen-activated protein kinase (MAPK) increases current density in dorsal root ganglion neurons, implying altered insertion or retention of channels at the plasma membrane (Hudmon *et al.*, 2008). In addition, tyrosine phosphorylation of potassium channel Kir3.1 in superficial layers of spinal dorsal horn in inflammatory or neuropathic pain states results in reduction of potassium channel activity that may elevate neuronal excitability to contribute to the hyper-responsiveness to noxious stimuli, possibly through a role of glial Kir3.1 in potassium buffering following intense pain-induced activity (Ippolito *et al.*, 2005). A variety of primary transducer molecules, such as the heat sensor TRPV1, are further regulated by inflammatory mediators to alter the gain of the sensory system, for example through phosphorylation by kinases such as protein kinase A (PKA) (Bhave *et al.*, 2002).

Furthermore, there is evidence for increased ATP receptor $P2X_3$ immunoreactivity in both ipsilateral L4-5 dorsal root ganglia and the associated level of spinal dorsal horn following CCI injury in rat (Novakovic *et al.*, 1999).

In overall terms, following nerve injury there are a number of distinct peripheral processes that could contribute peripherally to sensitisation of nociceptive processing; (1) direct injury to afferents could lead to spontaneous activity in Aδ-fibres (Boucher *et al.*, 2000; Liu *et al.*, 2000) and ectopic sensitivity due to the accumulation and clustering of sodium channels around the damaged axons (Lyu *et al.*, 2000; Roza *et al.*, 2003), (2) intact Aδ-fibres or adjacent C-fibres that share the same innervation territory of the injured fibres could develop spontaneous activity. For instance, A-fibres of the L5 spinal nerve and approximately half of C-fibres in the L4 spinal nerve developed spontaneous activity after ligation and transaction of the L5 spinal nerve using single nerve fibre recording (Wu *et al.*, 2001). Spontaneous activity in intact L4 C-fibres in both neuropathic and inflammatory pain states may result from peripheral inflammation (Djouhri *et al.*, 2006). Unmyelinated mechanoreceptors may display hypersensitivity by cross-activation with A-fibres after peripheral injury (Neumann *et al.*, 2008; Seal *et al.*, 2009), (Gillinov *et al.*) inflammation around the distal part of the injured nerve may expose the surviving nerve fibres from uninjured portions of the nerve to cytokines and growth factors (Scholz and Woolf, 2007) to sensitise nociceptors and activate silent nociceptors, (those acting through synapses with low basal AMPA receptor content or subject to high levels of tonic inhibition), (4) changes in gene expression (including genes for various peptides and receptors). The expression of NGF in dorsal root ganglion neurons of both the injured nerve and adjacent intact nerve is up- or down-regulated, reflecting the loss of trophic support from the periphery (Griffin, 2006), (5) then cross-excitation of afferents via ephaptic (non-synaptic, electric field-induced) transmission might occur in dorsal root ganglion where spontaneous activity has been observed in uninjured, myelinated afferents (Boucher *et al.*, 2000), (6) sympathetic activity may contribute to activation of nociceptor afferents; potentially by the sprouting of sympathetic efferents into more superficial areas of skin to activate sensory afferents which are induced to express adrenergic receptors following damage (Yen *et al.*, 2006).

1.3.4 Alterations in somatosensory processing in chronic pain: central mechanisms

The persistent nociceptive inputs from the peripheral nerves that are generated by injury and noxious stimuli are thought to induce functional reorganisation and central sensitisation in spinal cord, triggering an increased excitability of nociceptive neurons in the dorsal horn of the spinal cord (Woolf and Mannion, 1999; Ji and Woolf, 2001). Some degree of structural organisation may occur (Woolf *et al.*, 1995). However this hypothesis is now questioned by more recent data (Bao *et al.*, 2002; Hughes *et al.*, 2003). An understanding of the molecular and cellular mechanisms underlying pain transmission and modulation is essential to the development of clinical strategies aimed at alleviating chronic pain. Studies have shown that the activation of NMDA and AMPA receptors is crucial to the generation of spinal hyper-excitability and pain-related behaviours as a result of nerve injury. In the present study, I aim to specifically address the involvement of the GluA1 subunit of AMPA receptors and associated proteins in dorsal horn in the development of neuropathic pain in rats, since the GluA1 subunit appears to be the main AMPA receptor subunit that is recruited to synapses in circumstances of potentiated responsiveness in other parts of the brain (Shi *et al.*, 2001; Malinow, 2003).

1.3.4.1 Spinal dorsal horn re-organisation

Large myelinated Aβ-fibres normally terminate in the deep laminae III-IV of dorsal horn. However, there is a hypothesis that structural changes occur following repetitive discharge from nociceptive afferents. It is reported that Aβ-fibres may sprout into the superficial laminae I and II after peripheral axotomy (Woolf *et al.*, 1992; Shortland and Woolf, 1993; Koerber *et al.*, 1994). The consequence would be that the dorsal horn neurons which normally synapse with high-threshold nociceptive afferents begin to receive physical connections from innocuous inputs from low-threshold mechanoreceptors, providing an explanation for the emergence of allodynia after peripheral nerve injury. Recently, the tracing methods used by Woolf *et al.* (1992) have been questioned as the markers may be transported trans-synaptically.

This hypothesis remains largely conjectural because it is not fully supported by recent studies which showed that Aβ-fibres sprouted only to the lamina IIi and not further dorsally further into the superficial laminae I and IIo following peripheral axotomy (Bao *et al.*, 2002; Hughes *et al.*, 2003).

Other functional re-organisation of the dorsal horn was reported in peripheral neuropathy. Coull and colleagues demonstrated that a depolarising shift in chloride ion reversal potential occurred in GABA interneurons in the superficial lamina I of dorsal horn that was due to a reduction in KCC2 expression in these cells following chronic nerve constriction injury (Coull *et al.*, 2003). This could result in $GABA_A$ receptor-mediated responses becoming excitatory rather than their normal inhibitory influence.

1.3.4.2 Mechanisms potentially responsible for spinal hyper-excitability

Modification of neuronal excitability is one of the main ways with which neurotransmission can be regulated at spinal and supraspinal levels such as the brainstem. The spinal dorsal horn is a prime site contributing to mechanisms of chronic pain. Neurons in the spinal dorsal horn and related areas are sensitised by noxious stimuli carried by nociceptive fibres (Aδ-fibres and C-fibres) and convey up-regulated signals to supraspinal structures (Zhuo, 2000). This central sensitisation has been experimentally characterised as an increased excitability in response to sensory inputs, a prolonged neuronal afterdischarge to repeated stimulation, and expansion of peripheral receptive fields (Treede *et al.*, 1992; Yaksh *et al.*, 1999). Elsewhere in the CNS, repetitive activity of synapses can induce maintained alterations in synaptic strength observed as either long-term potentiation (LTP) or long-term depression (LTD) in hippocampus, for example, that may be reflective of changes in dendritic spine structure and glutamate receptor clustering at synapses (Hayashi *et al.*, 2000; Makino and Malinow, 2009). Some of the mechanisms involved in central sensitisation in spinal cord may be comparable. Central sensitisation can be brought about by multiple mediators from nociceptive inputs and does not simply represent altered strength of homosynaptic inputs. LTP as such has

been described in dorsal horn (Liu and Sandkuhler, 1995) and is likely to play some part in spinal excitability changes in chronic pain (Ji *et al.*, 2003). In both cases, long-lasting changes occur in the processing of neuronal inputs, altering the organism's perception of future sensory stimuli (Woolf and Salter, 2000b; Zhuo, 2000). Recent electrophysiological and pharmacological studies demonstrate that stimulus- or injury-induced release of glutamate contributes to spinal sensitisation (McAdoo *et al.*, 2005; Nguyen *et al.*, 2009).

The NMDA receptor is thought to act as a synaptic enhancer by reinforcing postsynaptic responses to glutamate. NMDA receptors are voltage- and ligand-dependent (Brown *et al.*, 1988; Daw *et al.*, 1993; Newpher and Ehlers, 2008). At resting membrane potentials, NMDA receptors are inactive because of pore blockade by extracellular Mg^{2+}, even in the presence of glutamate. Thus, to activate NMDA receptors at synapses, two events need to happen simultaneously. First, glutamate and its obligatory co-agonist, glycine need to be released and then bind to the GluN2 subunit and GluN1 subunit respectively; second, the postsynaptic membrane needs to be depolarised so that the extracellular Mg^{2+} blockage can be removed. Activation of NMDA receptors appears to be important for initiating long-lasting changes in synapses, such as 'wind-up' (the increased responsiveness of dorsal horn neurons) seen after repeated stimulation of afferents at high intensity), but does not play an extensive role in normal non-nociceptive or nociceptive transmission (Davies *et al.*, 1979; Dickenson and Sullivan, 1987). In various chronic or subacute pain states evoked by Complete Freund's Adjuvant (CFA), formalin, surgery, varicella zoster virus (VZV), diabetic neuropathy or mustard oil, nociceptive inputs are sensitive to NMDA receptor antagonists (Yaksh *et al.*, 1995). There is some evidence that GluN2B subunit levels are increased in the superficial dorsal horn following nerve injury (Boyce *et al.*, 1999), suggesting a possible role for this subunit in nociceptive processing. Moreover, the synaptic expression of the GluN1 subunit in spinal dorsal horn is elevated after injection of CFA (Yang *et al.*, 2009). Activation of NMDA receptors results in Ca^{2+} influx, elevating intracellular Ca^{2+} levels, which can have many cellular effects, such as the activation of Ca^{2+}/calmodulin-dependent kinase II (CaMKII). This was highlighted in a recent study where the NMDA receptor (and the MAGUK binding partner, PSD-95) showed increased binding to CaMKII following

a neuropathic injury (Garry *et al.*, 2003a) (Figure 1.3a). Furthermore, the sensitisation was inhibited by a selective CaMKII inhibitor and was not apparent in PSD-95 mutant mice lacking the GluN2-binding region. This provides a clear demonstration that receptor-associated protein complexes may play a key role in the sensitisation of dorsal horn neurons in neuropathic pain states. In addition, a point mutation of the SH3 domain of PSD-95 disrupts recruitment of (and functional signalling through) phosphatidylinositol 3-kinase C2α (PI 3-K C2α) to the NMDAR:PSD-95 complex in inflammatory pain models (Arbuckle et al., 2010) (Figure 1.3b). Moreover, NMDA receptors may play a structural role in controlling spine and synaptic stability mediated by C-terminal interactions with anchoring proteins such as PSD-95 (Alvarez *et al.*, 2007).

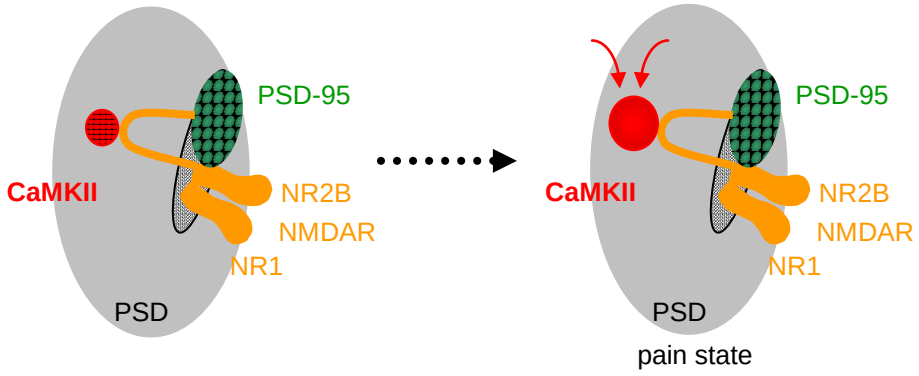
An increased involvement of AMPA receptors has also been shown in established neuropathic and inflammatory pain (Morales-Alcelay *et al.*, 2003; Garry *et al.*, 2003b; Galan *et al.*, 2004; Vikman *et al.*, 2008; Park *et al.*, 2009). A number of studies have tested the hypothesis that silent synapses that contain Mg^{2+}-blocked NMDA receptors and lack AMPA receptors can become active in chronic sensitised states such as LTP because AMPA receptors are rapidly delivered to synapses (Petralia and Wenthold, 1992; Molnar *et al.*, 1993; Baude *et al.*, 1995; Petralia *et al.*, 1999). These studies indicated the existence of pools of non-synaptic AMPA receptors on both the plasma membrane and intracellular regions of dendrites and soma. The trafficking of AMPA receptors into synapses may contribute to exciting the cells and may contribute importantly to long-lasting changes in synaptic strength (Shi *et al.*, 1999). The subunit composition of the AMPA receptor may be crucially important. Potentiation of synaptic transmission may occur as a result of delivery of GluA1 subunit-containing AMPA receptors to synapses, from reserve pools. In addition, maintenance of potentiated synaptic transmission may require that GluA2 subunit-containing AMPA receptors be constitutively exchanged with existing synaptic AMPA receptors (Shi, 2001).

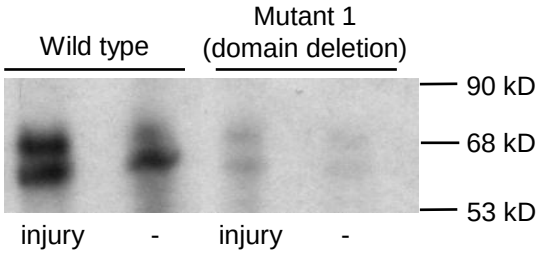
Figure 1.3: Roles of specific domains of PSD-95 in recruiting signalling enzymes to NMDA receptor complexes during pain states.

The MAGUK protein, PSD-95 is an abundant protein in postsynaptic densities of excitatory synapses where it serves as an adapter protein to recruit a variety of signalling and structural proteins to NR2 subunits of the NMDA receptor. PSD-95 has a modulator domain structure consisting of 3 PDZ domains, an SH3 domain and a GK domain. PDZ domains 1 and 2 mediate interaction with the NMDA receptor.

Diagrams a) and b) summarise data from PSD-95 mutant mice showing (a) that PDZ1/2 domain deletion disrupts neuropathic pain-induced recruitment of CaM kinase II to the NMDA receptor (NR2A/B immunoprecipitates probed for CaM kinase II) and (b) that W470L mutation in the SH3 domain disrupts inflammatory pain-induced recruitment of PI 3-K C2α to PSD-95 (PSD-95 immunoprecipitates probed for PI 3-K C2α). In each case the hypersensitivity of reflex pain behaviour responses was attenuated in the mutant mice compared to wild type litter mates. Summarised from Garry *et al.*, 2003; Arbuckle *et al.*, 2010.

1.3 a)

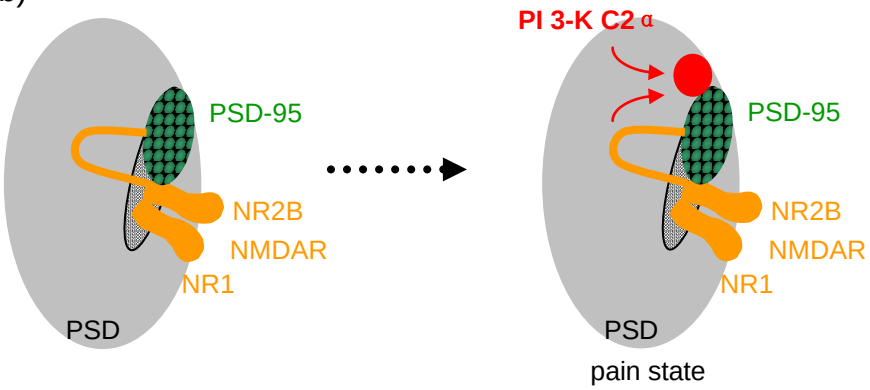


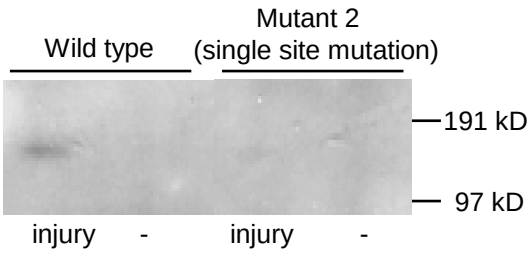


	injury	-	injury	-
Pain sensitivity	++++	+	+	+

(CCI neuropathic pain model)

1.3 b)





	injury	-	injury	-
Pain sensitivity	++++	+	+	+

(Formalin/CFA inflammatory pain model)

1.3.4.3 GluA1 subunit-containing AMPA receptor trafficking

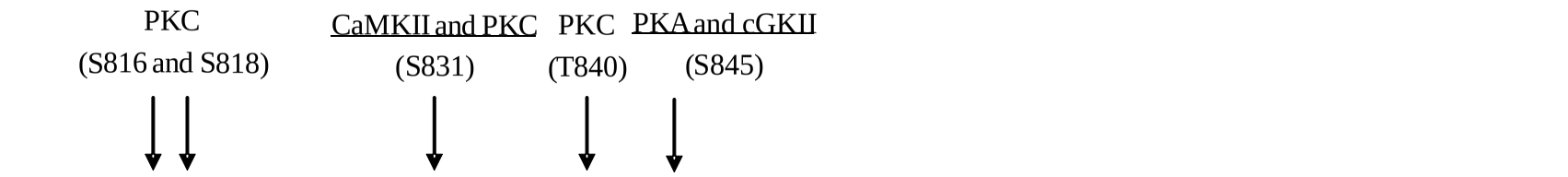
The current consensus view of the insertion of GluA1 subunit-containing AMPA receptors into excitatory synapses to strengthen synaptic plasticity during LTP is considered to have two main steps which are: extra-synaptic exocytosis to the dendritic spine surface and their lateral diffusion into synapses (Makino and Malinow, 2009). Extra-synaptic insertion is thought to replenish the plasma membrane GluA1 pools near synapses for subsequent bouts of activity-induced plasticity, while lateral diffusion may actually increase the number of GluA1 subunit-containing AMPA receptors within synapses. Thus the evoked multisteps AMPA receptor clustering at the postsynaptic density may represent a key determinant of long-term changes in synaptic excitability.

GluA1 mRNA and protein are strongly expressed in laminae I and II of the spinal dorsal horn in adult rats (Jakowec *et al.*, 1995b; Garry *et al.*, 2004; Polgar *et al.*, 2008) and this may be increased following nerve injury, but possibly not inflammation (Harris *et al.*, 1996). The intracellular carboxy-terminal tail of GluA1 (containing 82 amino acids from I808 to L889) is thought to be important in determining GluA1 trafficking by protein-protein interactions and phosphorylation events (Ruberti and Dotti, 2000). The extracellular N-terminus may also contribute to the trafficking (Anand, 2000). The GluA1 carboxy-terminal tail can bind to intracellular proteins through its PDZ target motif and membrane proximal region to facilitate trafficking (Ruberti and Dotti, 2000; Shen, 2000; Hirbec *et al.*, 2002; Douyard *et al.*, 2007). For example, the PDZ target motif at the carboxy-terminus of GluA1 binds SAP-97 (Leonard *et al.*, 1998), another MAGUK family member, whilst protein 4.1 and the p85 subunit of phosphatidylinositol 3-kinase (PI 3-K) have been reported as binding to GluA1 at proximal regions of the carboxy-terminal tail (Figure 1.4).

Figure 1.4: Sequence of the rat GluA1 intracellular C-terminus

The diagram represents the residues (808-889) of the C-terminal tail of the GluA1 subunit where binding sites for potential partner proteins (4.1N, p85 PI 3-kinase, AP-2 and SAP97) (grey shading) and confirmed phosphorylation sites (arrowed) are highlighted.

1.4


PKC
(S816 and S818)
CaMKII and PKC
(S831)
PKC
(T840)
PKA and cGKII
(S845)
EFCYKSRSESKRMKGFCLIPQQSINEAIRTSTLPRNSGAGASGGGGSGENGRVVSQDFPKSMQSIPCMSHSSGMPLGATGL
4.1N (812-823)
p85 PI 3-K (808 - 830)
AP-2 (823 - 829)
SAP97 (8862-889)

SAP-97 not only binds to the GluA1 carboxy-terminal tail but also interacts with the actin-associated protein 4.1N and A-kinase anchoring protein (AKAP) 79/150 (Lue *et al.*, 1994; Colledge *et al.*, 2000). 4.1N interaction with SAP-97 may be important for synaptic insertion of GluA1 (Rumbaugh *et al.*, 2003), but synaptic clustering of GluA1 appears not to be prevented in SAP-97 knockout mice (Klocker *et al.*, 2002). GluA1 and PDZ domain interactions however, are required to drive AMPA receptors into synapses by LTP or CaMKII in hippocampal neurons (Hayashi *et al.*, 2000). It is possible that other PDZ domain proteins may be involved in addition to SAP-97 (Hirbec *et al.*, 2003; Dev *et al.*, 2004).

Protein 4.1N can also bind directly to GluA1 in a proximal domain of the C-terminal tail between amino acids 812 and 823 (Shen *et al.*, 2000). 4.1N and 4.1G are homologs of the erythrocyte membrane cytoskeletal protein 4.1R, which comprises a 30 kDa N-terminal domain that interacts with glycophorin C, calmodulin, and p55 (Han *et al.*, 2000), a 16 kDa domain critical for membrane association, a 10 kDa domain containing the binding site for spectrin and actin complexes (Correas *et al.*, 1986) and a 22-24 kDa C-terminal domain (CTD) whose function is for the most part unknown. 4.1N is enriched in neurons and shows homology to the other 4.1 family members at the 10, 16, and 22-24 kDa domains, but is distinct at its N-terminal domain (Walensky *et al.*, 1999). More recently, these proteins have also been shown to interact with the GluA4 subunit in a similar manner, but not with GluA2 and GluA3 subunits (Coleman *et al.*, 2003). In unpublished work, S. Fleetwood-Walker, E. Garry and I have recently found that following nerve injury, the thermal hyperalgesia and mechanical allodynia were attenuated after injection of a myristoylated peptide, which was designed as a decoy blocker of the proposed binding region for 4.1N on the carboxy-terminal tail of GluA1. The amount of 4.1N bound to GluA1 in the spinal cord was correspondingly decreased after treatment with this agent. In addition, there may well be an increased association between 4.1N and GluA1 following the injury at the peak of sensitisation. Due to its other reported

interactions, 4.1N could potentially link GluA1 to actin in the vicinity of the synapse. Recent evidence has further demonstrated that 4.1N is required for activity-dependent extra-synaptic insertion of GluA1 subunit, thereby supporting the idea of the importance of lateral diffusion in supplying AMPA receptors to synapses from extra-synaptic pools (Lin *et al.*, 2009).

An additional GluA1 binding protein is the p85 subunit of Class 1A PI 3-K; one of a number of lipid kinases that can phosphorylate PtdIns(4, 5)P_2 to PtdIns(3, 4, 5)P_3 (PIP_3), which is an important signalling molecule involved in many membrane trafficking and recruitment processes (James and Piper, 1994; Carpenter and Cantley, 1996; De Camilli *et al.*, 1996; Rapoport *et al.*, 1997; Cheever *et al.*, 2001). Moreover, PI 3-K is required for selective AMPA receptor insertion into activated synapses during LTP in the hippocampus through a process that may involve the p85/GluRA1 interaction (Man *et al.*, 2003). A recent study illustrated that postsynaptic PIP_3 was necessary for LTP and required for cycling and synaptic retention of AMPA receptors, perhaps through accumulation of PSD-95 at dendritic spines (Arendt *et al.*, 2010).

PI 3-K, together with its downstream kinase, protein kinase B (PKB/Akt), has been implicated in a number of chronic pain states (Pezet *et al.*, 2008) and PI 3-K can be activated by a complex of Ca^{2+} and calmodulin through direct interaction with the SH2 domain of the PI 3-K p85 subunit during inflammatory hyperalgesia (Joyal *et al.*, 1997). A recent report indicated that activation of a PI 3-K, extracellular-regulated kinase (ERK), PKB signalling pathway was required to drive GluA1 into synapses in alerted animals (Qin *et al.*, 2005). Overlapping binding sites in GluA1 have been reported for 4.1N, PI 3-K and adapter protein-2 (AP-2), which may play a role in the regulated exocytosis of GluA1 (Lin *et al.*, 2000), but it is not yet clear whether these interactions are mutually exclusive. Stabilisation of AMPA receptor localisation at synaptic sites requires the interaction between stargazin-like TARPs and PSD-95 (Bats *et al.*, 2007), whereas stargazin interaction alone failed to increase

synaptic GluA1 levels in rat hippocampal neurons (Kessels *et al.*, 2009). Thus the acknowledged consensus view is that AMPA receptor trafficking into synaptic entities is regulated by a number of different protein-protein interactions.

Apart from protein-protein interactions, phosphorylation of GluA1 is also important for its clustering at excitatory synapses. The GluA1 carboxy-terminal tail can be directly phosphorylated on at least 5 distinct sites including serine 816 (S816), S818, S831and S845 as well as threonine 840 (T840) (Fang *et al.*, 2003a; Lee *et al.*, 2007; Lin *et al.*, 2009). Protein kinase C (PKC) phosphorylation of the S816 residue increased GluA1 binding with protein 4.1N and facilitated GluA1 trafficking to extra-synaptic plasma membrane of the soma and dendritic shaft, but not on spines (Lin *et al.*, 2009). Phosphorylation of the two major sites S831 and S845 on GluA1 in spinal dorsal horn contributes to central sensitisation and the increased in the number of AMPA receptors in excitatory synapses. After capsaicin injection, which activates TRPV1 channels in nociceptors, a significant up-regulation of GluA1 phosphorylation at both S831 and S845 was detected on the ipsilateral side of the spinal dorsal horn (Fang *et al.*, 2003a). Pain state-induced phosphorylation of GluA1 at Ser831 can be achieved by CaMKII and PKC (Fang *et al.*, 2002). Cyclic AMP-dependent protein kinase A (PKA) bound to GluA1-associated SAP-97 and AKAP 79/150 can phosphorylate GluA1 at S845 and control the synaptic trafficking underlying plasticity (Colledge *et al.*, 2000). The state of phosphorylation at the GluA1 PKA site can also affect channel open-time (Banke *et al.*, 2000) and has been correlated with changes in synaptic strength (Lee *et al.*, 2000). The PKA signalling pathway can be triggered by Ca^{2+} entry through NMDA receptors causing the activation of Ca^{2+}–dependent adenylate cyclase isoforms (Chetkovich and Sweatt, 1993; Roberson and Sweatt, 1996). In addition, GluA1 recycling between the plasma membrane and endosomal compartments is controlled by PKA phosphorylation (Ehlers, 2000). However, the role of phosphorylation of GluA1 may differ between different chronic pain models, as S845 and S831 phosphorylation may be associated

with inflammatory but not SNL-induced neuropathic pain (Lu *et al.*, 2008). Interestingly, GluA4 contains a corresponding PKA site at S842, at which phosphorylation is necessary and sufficient to drive receptors into synapses in early postnatal animals (Roche *et al.*, 1996; Carvalho *et al.*, 1999). In more mature animals, other factors such as CaMKII play a greater role, with CaMKII being required for LTP only after approximately P14-20 (Yasuda *et al.*, 2003).

1.3.4.4 The role of GluA2 subunit-containing AMPA receptors

GluA2 is also a critical subunit in determining mammalian AMPA receptor function. The great majority of AMPA receptors include GluA2 subunits. Although during early postnatal development expression of GluA2 is low compared with that of GluA1, it increases rapidly during the first postnatal week (Monyer *et al.*, 1991; Wisden and Seeburg, 1993). AMPA receptors containing GluA2 subunits have a linear current-voltage relationship (Boulter *et al.*, 1990), are impermeable to Ca^{2+}, and exhibit a relatively low single-channel conductance, while those lacking GluA2 are Ca^{2+}-permeable, and of higher conductance. There is also evidence that even in neurons expressing high levels of GluA2, a functionally relevant population of GluA2-lacking AMPA receptors can be expressed on the surface under certain conditions (Ju *et al.*, 2004; Clem and Barth, 2006). This may reflect GluA1 homomers, produced by local dendritic synthesis of this subunit (Ju *et al.*, 2004; Sutton *et al.*, 2006), potentially suggesting a differential assembly and trafficking for local dendritically synthesized AMPA receptors.

The best functionally characterised GluA2 interactions are at the intracellular tails, which contain a proximal NSF/AP-2 site and a distal PDZ binding site. The interaction of NSF with GluA2 may contribute to stabilising excitatory currents in hippocampal neurons (Song *et al.*, 1998), to the movement of AMPA receptors to the cell surface (Noel *et al.*, 1999) and to LTP (Lledo *et al.*, 1998). The PDZ domain-

containing proteins that can dock to GluA2 include GRIP/ABP and PICK1. GRIP and ABP are structurally very similar and contain multiple PDZ domains, so they are capable of interacting with many individual postsynaptic proteins. GRIP and ABP anchor AMPA receptors at the synapses in a phosphorylation-dependent manner (Hirbec *et al.*, 2002). PICK1 contains a single PDZ domain, but can dimerise via a separate Bin/amphiphysin/Rvs BAR domain and PICK1 also interacts with PKC through its PDZ domain (Perez *et al.*, 2001). It has been proposed that PICK1 may translocate GluA2/3 together with PKC and contribute to synaptic clustering (Xia *et al.*, 1999). In addition, the BAR domain of PICK1 forms a curved structure on dimerisation that can deform membranes or stabilise already curved membranes, suggesting that PICK1 may participate in AMPA receptor endocytosis (Perez *et al.*, 2001; Madsen *et al.*, 2008). A possible role for NSF, GRIP and PICK1 in neuropathic sensitisation was suggested in experiments with cell-permeable blocking peptides acting as decoys for interaction motifs on GluA2. Differential changes in expression of these proteins were also demonstrated following peripheral nerve injury (Garry *et al.*, 2003b). Association of NSF/GRIP or PICK1 with GluA2 may act to determine recruitment to the PSD (Hanley, 2008). PKC regulation of these interactions may be important in the plasma membrane turnover of GluA2 during the maintenance of inflammatory pain (Tao *et al.*, 2003; Park *et al.*, 2009).

1.3.4.5 Involvement of intracellular kinases in nociceptive sensitisation

During nociceptive transmission, a variety of intracellular kinases become activated following stimulation of excitatory synapses containing NMDA receptors, AMPA receptors and possibly also group I mGluRs. The elevation of intracellular Ca^{2+} concentration as a result of influx through ionotropic glutamate receptors and voltage-sensitive Ca^{2+} channels as well as release from endoplasmic reticulum is a key determinant of central sensitisation. A number of Ca^{2+}-dependent and other

kinases are responsible for critical regulatory phosphorylation events, both those discussed above and other key events in sensitisation.

CaMKII is a multifunctional, widely distributed kinase. It can be activated following NMDA receptor stimulation and is thought to be a critical mediator of neuronal plasticity. For example, activation of the CaMKII cascade may evoke alterations in gene expression in dorsal horn neurons, thereby producing long-lasting changes in function (Ji and Woolf, 2001). Nerve injury increases the assembly of CaMKII in a postsynaptic complex with GluN1/2 subunits in a manner dependent on domain-specific interactions with PSD-95 and furthermore, local administration of an inhibitor of CaMKII significantly attenuates nerve injury-induced sensitisation (Garry *et al.*, 2003a).

PKA and PKC are two major intracellular kinases that contribute to activity-dependent central sensitisation. PKA not only phosphorylates S845 within the intracellular terminus of the GluA1 subunit of AMPA receptors to drive them into synapses, but also phosphorylates the GluN1 subunit of NMDA receptors at S879 to enhance their response to glutamate (Raman *et al.*, 1996; Zou *et al.*, 2002). PKC also plays several different roles in hyper-excitability of nociceptive neurons. In addition to its effects on AMPA receptors such as phosphorylation of S816, S818, S831 and T840 in the GluA1 subunit, PKC phosphorylates GluA1 at S890 and S896 and enhances NMDA receptor activation (Lan *et al.*, 2001) as well as decreases inhibition arising from spinal GABA and glycine interneurons or from descending pathways originating from PAG (Lin *et al.*, 1996b).

A further kinase of major importance in synaptic plasticity is ERK MAP kinase. ERK increases AMPA receptor and NMDA receptor currents by contributing to the recruitment of AMPA receptors to the membrane, the phosphorylation of GluN1 subunit of the NMDA receptor, and reduces outward currents by phosphorylation of $Kv_{4.2}$ at S616 (Hu *et al.*, 2007). In addition, ERK activates transcription factors such

as cAMP-response element binding protein (CREB) to regulate expression of several genes including c-Fos, NK1 and COX-2, which may help to bring about a long-lasting synaptic strengthening (in the case of COX-2, indirectly by way of increased prostaglandin synthesis) (Ji *et al.*, 2009). The role of ERK is a focus of interest in the current work.

1.3.4.6 Regulation of the ERK MAP kinase pathway in the nervous system by the small G protein Ras

The Ras subfamily of small GTP-binding proteins is importantly involved in cell growth, differentiation and survival through regulation of gene transcription and other signalling events (Young *et al.*, 2009). Activation of the Ras pathway is mediated by various Ras-specific guanine nucleotide exchange factors (GEFs) which include the highly homologous guanyl nucleotide-releasing factors (GRF) 1/2 (Shou *et al.*, 1992), the closely related Son of Sevenless (Sos) 1/2 (Bowtell *et al.*, 1992), and guanyl nucleotide-releasing protein (GRP) (Ebinu *et al.*, 1998). In neurons, the three types of Ras-specific GEFs can be activated by diverse receptor systems including receptor tyrosine kinases (RTKs) for growth factors, GPCRs for neurotransmitters, and intracellular Ca^{2+} released from endoplasmic reticulum or Ca^{2+} influx through voltage-gated or ligand-gated Ca^{2+} channels (for example, NMDA receptors). Activated Ras can enhance AMPA receptor-mediated synaptic transmission in LTP by driving more AMPA receptors into synapses (Zhu *et al.*, 2002).

Ras-GRF1 and Ras-GRF2 are present predominantly in the adult, where they are highly expressed in neuronal cells but not in glial cells (Zippel *et al.*, 1997). Both of them can be activated by Ca^{2+} via direct interaction with Ca^{2+}/calmodulin in combination (Farnsworth *et al.*, 1995). It has been reported that Ras-GRF1 binds directly to the GluN2B subunit of the NMDA receptor as a regulator of the ERK

pathway (Krapivinsky *et al.*, 2003). In addition, experiments with Ras-GRF knock-out mice indicate that Ras-GRF2 contributes to the induction of NMDA receptor-dependent LTP in the hippocampus (Li *et al.*, 2006). Furthermore, Ras-GRF proteins appear to play an age-dependent role in synaptic recruitment of AMPA receptors. Their function is largely limited to mature neurons because their level of expression at least in cortical neurons is very low in normal neonatal animals, where interestingly, NMDA receptors signal through Son of Sevenless (Sos) (Tian *et al.*, 2004). Sos is ubiquitously present in tissues (Guerrero *et al.*, 1996) and highly expressed in young animals (Tian *et al.*, 2004). Sos-1 is stable and participates in short- and long-term signalling events, whereas Sos-2 appears to be degraded by a ubiquitin-dependent process and is predominantly involved in short-term signalling events (Qian *et al.*, 2000). Sos plays a crucial role in the coupling of RTKs to Ras-dependent signalling via binding through the adapter protein Grb2 (Chardin *et al.*, 1993). Similarly, it contributes to the Ras activation that occurs following GPCR transactivation of RTKs (Marinissen and Gutkind, 2001).

The ERK MAP kinase pathway is the best understood downstream cascade initiated by Ras activation. Ras is attached to plasma membrane because of its lipid modification by prenyl moieties and when activated leads to sequential activation of Raf (MAPK kinase kinase), MEK 1/2 (MAPK kinase) and MAPK/ERK1/2. ERK1 and 2 act as critical transducers of growth factor signalling to the nucleus where they act as important regulators of gene transcription. Among the major substrates of MAPKs are the ribosomal S6 kinases (RSKs) which directly phosphorylate the cAMP response element (CRE)-binding factor CREB, inducing expression of many immediate-early genes (IEGs) such as Fos (De Cesare *et al.*, 1998; Frodin and Gammeltoft, 1999). In addition, MAPKs can directly phosphorylate and activate serum response element (SRE)-binding proteins, such as p90RSK and Elk1, thus contributing to the control of gene transcription (Sutton *et al.*, 2004). ERK1/2 also

have effects on cytosolic proteins by phosphorylation of a wide range of substrates (see section 1.3.2).

1.4 Aims and objectives of this study

The overall aim of this work was to assess the biochemical events that may underlie the changes in synaptic function occurring in chronic pain states brought about following nerve injury both in adult animals and in neonates, where particularly long-lasting sensitisation may ensue.

The study should help to identify new molecular targets for potential intervention in otherwise largely intractable chronic neuropathic pain in adults and neonates. The outcomes may be valuable for future therapeutic developments in both clinical and veterinary medicine.

The experiments necessitated the development of new or greatly enhanced experimental procedures, a key aspect of this thesis.

Specifically, I asked:

1) In neonatally nerve-injured animals (where lasting facilitation of nociceptive responsiveness is observed), can I identify changes in expression, activation or synaptic insertion of key signalling/transduction proteins that may explain the lasting upregulation of sensitivity? These studies involved the unprecedented development of techniques for micropreparation of postsynaptic densities and subsequent quantitative Western blotting from injured/uninjured spinal dorsal horn.

2) In adult animals, in the first instance, can I develop a new technological platform that enables assessment of dynamic receptor-mediated responses in *ex vivo* tissue in a variety of formats (30-100 μm tissue segments, synaptoneurosomes and 150 × 150 μm microslices)? These studies, utilising novel no-wash Ca^{2+}- and membrane

potential- fluorophores in both cortex and spinal cord, establish for the first time the ability to measure dynamic responses to both metabotropic and ionotropic receptor activation. This will potentially enable deployment of the approach for assessing alterations in responsiveness in chronic pain and other states of hypersensitivity at different levels of the CNS.

Chapter 2: Materials and methods

2.1 Material information

Company	Product
Ascent Scientific Ltd. Unit 3 Avon Riverside Estate, Victoria Road, Avonmouth, Bristol BS11 9DB, UK	Substance P (SP)
Astra-Zeneca Ltd. Hurdsfield Industrial Estate, Macclesfield, Cheshire SK10 2NA, UK	Fluothane, Isoflurane, Hibitane
Becton Dickinson (BD) Biosciences The Danby Building, Edmund Halley Road, Oxford Science Park, Oxford , OX4 4DQ, UK	Mouse anti-protein 4.1N monoclonal IgG
Cell Signalling Technology, Inc. 3 Trask Lane, Danvers, MA 01923, USA	Goat anti-rabbit horseradish peroxidase (HRP)-linked IgG, Rabbit anti-ERK polyclonal IgG, Rabbit anti-phospho-ERK (Thr202/Tyr204) polyclonal IgG
Emmanuel Merck, Darmstadt (EMD) Biosciences Inc. 10394 Pacific Center Court San Diego, CA 92121, USA	3-[(3-Cholamidopropyl)dimethylammonium]-1–propanesulphonate (CHAPS), Protease Inhibitor Cocktail Set III
GE Healthcare Ltd. Pollards Wood, Nightingales Lane, Little Chalfont, Buckinghamshhire, HP8 4SP, UK	Amersham Hyperfilm™ ECL, Percoll, Phastgel™ Blue R

Genus Xpress (Ethicon) 10 Castings Court, Falkirk, KK2 9HJ, UK	Coated vicryl, 13mm curved needle 4/0 (5/0), 75cm
Invitrogen Corporation 5791 Van Allen Way P.O. Box 6482, Carlsbad, CA 92008, USA	4-12% Bis-Tris Nu-PAGE gels, Hanks' Balanced Salt Solution (HBSS), 2-(N-morpholino)ethanesulfonic acid (MES) running buffer, 3-Morpholinopropane-1-sulfonic acid (MOPS) running buffer, Sodium pyruvate, Blot transfer buffer, Tris-acetate running buffer
Santa Cruz Biotechnology, Inc. 2145 Delaware Avenue, Santa Cruz, CA 95060, USA	Mouse anti-GluA1 (E-6) monoclonal IgG, Mouse anti-GluA1 (G-12) monoclonal IgG, Rabbit anti-Sos1 (H-122) polyclonal IgG, Rabbit anti-Synaptophysin (H-93) polyclonal IgG
Sigma-Aldrich Corporation 3050 Spruce Street, St. Louise, MO 63103, USA	2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid (HEPES), 2,5-dimethoxy-4-iodoamphetamine (DOI), Acetylcholine chloride, Allyl isothiocyanate (AITC), Bovine serum albumin (BSA), Bradykinin (BK), Collagen, Cyclothiazide (CTZ), DL-homocysteic acid (DLH), Glutathione, Isoleucine, Krebs-Henseleit buffer, Kynurenic acid, Mouse anti-actin monoclonal IgG,

	N-methyl-D-aspartic acid (NMDA), 1-Noradrenaline hydrochloride, Phosphocreatine disodium salt, Prazosin, 1-Propranolol, Protein-G Sepharose beads
Thermo Scientific 3747N, Meridian Road, Rockford, IL 61101, USA	Pierce 3-Colour Protein Molecular Weight Marker mix
Tocris Cookson Ltd. Churchill Building, Langford House, Langford, Bristol, BS18 7DY, UK	1-Aminocyclopropane carboxylic acid (ACPC), α-Amino-3-hydroxyl-5-methyl-4-isoxazole-propionate hydrobromide (AMPA), Capsaicin
UC Davis/NIH NeuroMab Facility Department of Neurobiology, Physiology and Behavior, UC Davis, Davis CA 95616, USA	Mouse anti-PSD95 monoclonal IgG
Whatman Ltd. Whatman GMBH, Hahnestraβe 3 D-37586, Dassel, Germany.	Nitrocellulose transfer membrane, Filter paper

2.2 Methods

2.2.1 Animals

Sprague-Dawley rats were used throughout this study and were given access to food and water *ad libitum*. Animals were housed in accordance with Home Office guidelines. The temperature and humidity of the animal house were controlled at 21°C and 50% respectively. Animals were kept in 12h:12h scheduled lighting with lights on from 7am to 7pm. The experiments described were all carried out on female animals as they are reported to show greater long term consequences of early life inflammatory pain than males (LaPrairie and Murphy, 2007) and also exhibit greater nocifensive responses following formalin injection (S. Fleetwood-Walker, unpublished observations; T.L Yaksh, personal communication). Animals were used at various ages from postnatal day 8 (P8), the earliest time at which surgery to produce nerve-injury was feasible, then P9, P18, P28, P42 and P68 (spanning postnatal development into early adulthood) as well as adult animals of at least 180g in weight. Pain model surgery on all the neonates and adults was carried out by Andrew Allchorne, the experienced technical officer of the group, to ensure the greatest possible consistency between animals. Animal behavioural tests were carried out by Andrew Allchorne and Hayley Gooding with my technical and analytical assistance. I contributed to the design and planning of these experiments, provided a technical support role for the combined behavioural and tissue harvest procedures, helping with preparation of the apparatus, tubes and solutions, participated in tissue removal, homogenization and subsequent processing and carried out data analysis enabling interpretation of the behavioural and biochemical findings. In further experiments to obtain spinal cord tissue for *ex vivo* Ca^{2+} fluorescence measurements or Western blotting I carried out surgery on animals terminally anaesthetised with 2.5-3.5% isoflurane in oxygen under the supervision of Sue Fleeetwood-Walker, involving laminectomy, dissection and rapid removal of appropriate spinal cord

segments. In these studies I carried out all experimental design, biochemical and fluorescence procedures, data analysis and interpretation. All animal experiments were carried out in accordance with the UK Animal (Scientific Procedures) Act 1986 by staff with Home Office Personal Licences under the range of procedures covered by S.Fleetwood-Walker's Project Licence and approved by the University of Edinburgh Ethical Committee.

2.2.2 Neuropathic pain model

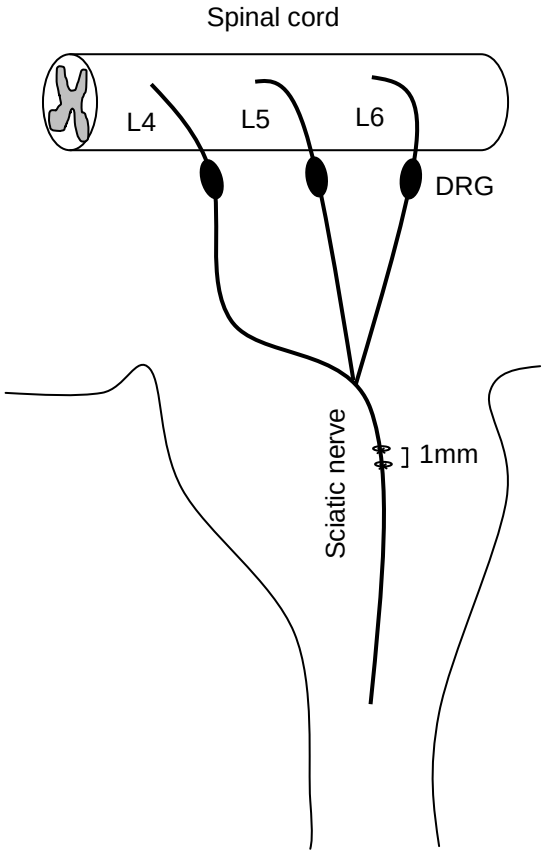
P8 litters or adult animals were divided into experimental and control groups. Chronic constriction injury (CCI) of the sciatic nerve was performed on experimental groups of P8 and adult rats as a neuropathic pain model. The procedure is well characterised and has previously been described in detail (Bennett and Xie, 1988). Briefly, rats were anaesthetised by inhalation of an isoflurane/O_2 mixture (Astra-Zeneca, Cheshire, UK), 4-5% for induction and 1.5-3% for maintenance. Animals were carefully monitored during surgery until recovery from anaesthesia and for a short time afterwards and were all marked using permanent marker pens (whilst anaesthetised) for identification. Following hair removal and sterilisation of the area with 10% Hibitane (Astra-Zeneca, UK), a small incision was made below the pelvis and the right biceps femoris and the gluteus superficialis were carefully separated to expose the sciatic nerve. Upon isolation of the nerve, two loose ligatures were tied around it proximal to the trifurcation using 4-0 chromic catgut in adults (SMI AG, Hunningen, Belgium) (or 5-0 grade in the neonates – Medgut, South Africa) with a 1 mm separation between ligatures (Figure 2.1). This is a modification of the classic CCI model for adults, which usually consists of four ligatures. Two were used instead here in both adult and neonates to standardise the injury, as four ligatures are difficult to place around the sciatic nerve of P8 animals. Earlier pilot experiments in the laboratory had shown no discernible difference in sensitised pain behaviours of adults with two- or four-ligature CCI. The procedure was standardised by checking

the extent of nerve constriction under a microscope, ensuring that the ligatures were just tight enough to prevent sliding along the nerve. The nerve was then carefully placed back into position and the wound was closed. Vetbond (Vetbond, 3M, Loughborough, UK) was used to close the wound in P8 animals. Control animals did not undergo any surgical procedure.

Figure 2.1: Schematic outline of procedure for Chronic Constriction Injury (CCI) applied to the sciatic nerve

The diagram shows an outline of the spinal cord at the lumbar level with dorsal roots arising from lumbar (L) segments 4, 5 and 6 on the left side. Each root passes through a dorsal root ganglion (DRG) before merging to form the sciatic nerve, which innervates the hind limb. The location of two loose ligatures, approximately 1 mm apart is indicated.

2.1



2.2.3 Subsequent acute noxious stimulus from intraplantar formalin

At P42 (34 days after surgery and a time at which to mechanical stimulation with von Frey filaments within the sciatic receptive field had fully returned to normal), an injection of formalin (40 μl of 4% formalin in 0.9% saline) was given under isoflurane anaesthesia into the hind paw ipsilateral to the original injury at P8 (Figure 2.2). Adult animals were also subjected to this formalin injection after behavioural recovery from previous nerve-injury. The two control groups for the neonates and adults received an equal volume of 0.9% saline. Rats were then placed in clear observation boxes to recover from the anaesthesia before recording. Each animal was observed for 60 seconds within a 5 minute window and nocifensive behaviours including flinching, flicking, licking or biting of the injected paw were recorded during this time using a stopwatch. The cumulative time in seconds was recorded every 5 minutes over 60 minutes or until the termination of nocifensive responses to formalin. At the end of the test, animals were euthanized by carbon dioxide asphyxiation. Tissue was collected immediately after death (within 5-6 minutes, see section 2.2.5).

2.2.4 Pain-related behavioural tests

Animals were weighed on every day of behavioural testing to ensure that pups were all gaining weight and therefore not in a distressed state which may be accompanied by weight loss. Behavioural tests were carried out to assess the development and progression of the neuropathic pain sensitivity characteristic of the CCI model, in both P8 and adult rats. Measurements of reflex responses to graded sensory stimuli were recorded in conscious animals prior to injury (not possible for P8 injured animals) and regularly post injury to establish a time-course of sensitivity. The assessment generally used to measure hypersensitivity was the presence of mechanical allodynia in response to testing with von Frey filaments although thermal hyperalgesia was also assessed in a small number of experiments.

Spontaneous pain as indicated by spontaneous nocifensive behaviours was also observed following noxious challenge induced by formalin injection to hind paws ipsilateral to CCI in both P8 and adult rats.

2.2.4.1. Thermal hyperalgesia

Animals were monitored using the Hargreaves' thermal stimulator which consists of an infrared beam, directed to the underside of the hind paw to cause local increasing level of warming (Figure 2.2a). Animals were placed on the plate of the Hargreaves' stimulator and covered with a clear plastic box to contain the animal. They were given 5-10 minutes in their new test environment to allow them to become habituated before testing. To measure the paw withdrawal latency (PWL), the infrared beam was put under the middle of the hind paw until the withdrawal occurred, at which point the beam was automatically terminated and the timer stopped. The latency for withdrawal was recorded for the ipsilateral and the contralateral hind paws. Responses were recorded at 5 minute intervals and mean values taken (using a set cut off point to avoid excessive heating).

2.2.4.2. Mechanical allodynia

Mechanical allodynia was measured as the withdrawal threshold to calibrated Semmes-Weinstein von Frey filaments (Stoelting, Illinois, US). It is only possible to begin testing the neonate on the plantar surface by around P14; before this time pups are too small for the mesh table used and are not able to produce the necessary response as testing depends on a fully developed foot withdrawal reflex and maturity of the motor system, as noted by other groups (Lee and Chung, 1996; Fitzgerald, 2005)

Animals were placed on a raised mesh grid and covered with a clear plastic box to contain the animal. Von Frey filaments were used to determine the threshold force for withdrawal of the ipsilateral paw compared to the contralateral paw. Filaments were applied to the middle of the plantar surface of each paw from below, (Figure 2.2b) until the filament bent; this was done 6-8 times per filament, in order of increasing force. The mean threshold required to elicit a withdrawal response in 50% of

applications was recorded. Data were expressed as the threshold indentation pressure (i.e. the bending force per cross-sectional area at the tip of the filament, mN/mm^2).

Figure 2.2: Illustrations of the apparatus used for a) the Hargreaves' (noxious heat) test and b) the Von Frey filament (mechanical threshold) test

a) Thermal test

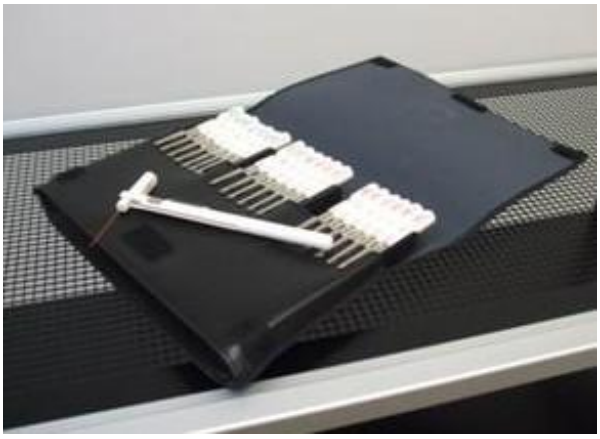
The latency for paw withdrawal from a noxious heat source was recorded using Hargreaves' apparatus. Animals were placed within plastic boxes on a glass plate and the plantar surface of a hind paw was heated to a set noxious level by the infra-red unit which cuts out upon paw withdrawal, automatically recording the latency.

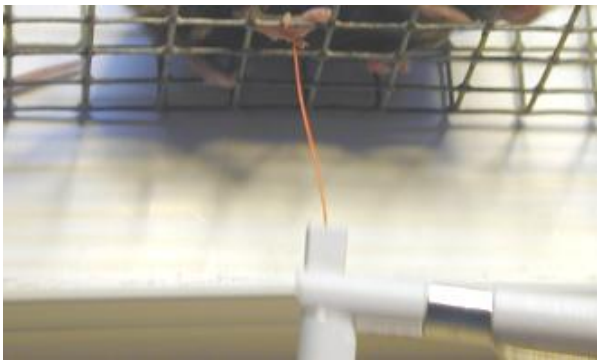
b) Mechanical test

The threshold for paw withdrawal from a punctate mechanical stimulus was recorded using Von Frey filaments. Animals were placed within plastic boxes on a raised metal grid and the plantar surface of a hind paw was probed with filaments of increasing (defined) bending pressures to determine the filament causing paw withdrawal in 50% of tests.

2.2 a)

2.2 b)





2.2.5 Tissue collection

The rats were euthanized by carbon dioxide asphyxiation (Schedule 1) or by terminal anaesthesia and the lumbar 4-6 (L4-6) spinal cord segments were removed quickly and hemisected into half cords containing ipsilateral and contralateral sides or quarter cords containing ipsilateral and contralateral dorsal horns.

Half cords were homogenised in ice-cold Laemmli buffer (Table 2.1) using 1 ml homogenisers kept on ice. The homogenates were boiled in sealed tubes at 100°C for 5 minutes to denature proteins and centrifuged at 12,000 g, 4°C for 10 minutes. The supernatant from this whole tissue lysate was stored at -20°C and used for Western blot studies or controls in co-immunoprecipitation experiments.

Quarter cords were collected and stored in sucrose storage/homogenising buffer at -20°C (Table 2.2) for later synaptic fraction preparation.

Table 2.1: Laemmli buffer

Component	Concentration
SDS	2%
Mercaptoethanol	5%
In: 50mM, Tris-HCl pH 7.40	

Table 2.2: Sucrose-containing storage/homogenising buffer for synaptic fraction preparation

Component	Concentration
Sucrose	0.32 M
$NaHCO_3$	1 mM
$MgCl_2$	1 mM
$CaCl_2$	0.5 mM
Sodium orthovanadate (phosphatase inhibitor)	1 mM
NaF (phosphatase inhibitor)	1 mM
Protease inhibitors (Type III, Calbiochem, EMD)	0.1%
In: UHP water	

2.2.6 Preparation of subcellular fractions

A modified subcellular fractionation technique for CNS tissue (Phillips *et al.*, 2001; Feligioni *et al.*, 2006) was used to allow simultaneous analysis of GluA1 AMPA receptor subunit translocation into the postsynaptic density (PSD) and the levels of signalling proteins in non-synaptic synaptosomal protein (NSSP) fractions under normal and CCI pain states (Figure 2.3). NSSP fractions include all the synaptosomal proteins excluding the PSD-associated proteins and the closely apposed presynaptic specialisation (Feligioni *et al.*, 2006). To obtain sufficient fractions for reliable and consistent results, 8 spinal cord quadrants from spinal segments L4-L6 were pooled together as one set. I collected 4 complete sets of tissues for the analysis of GluA1 subunit translocation. This is the first time that such a technique has ever been used to assess pain model-induced responses in the dorsal horn of spinal cord.

Before the procedure, sucrose/Percoll gradients were prepared and kept on ice. Homogenisers, buffers and centrifuges were cooled down to 4°C. Only ipsilateral and contralateral dorsal spinal cord quadrants were collected, so ventral spinal cord tissues were not assessed in the current study. Tissue samples had been collected in homogenising buffer and stored at -20°C. They were thawed, superficially dried with filter paper and weighed before being homogenised in 10 volumes of homogenising buffer for 10 further strokes after tissue disruption with a motorised teflon-glass homogeniser. The homogenate was centrifuged at 1000 g for 12 minutes at 4°C to remove nuclei and debris. The supernatant, which contains crude synaptosomes was gently loaded onto the surface of prepared sucrose/Percoll density gradients (Table 2.3) and centrifuged at 32,500 g, at 4°C, for exactly 6 minutes. The white bands, which include purified synaptosomes, present between the 3% and 23% Percoll layers were removed to clean tubes and washed twice with 10ml HEPES washing buffer (Table 2.4), by centrifugation at 40,000 g, for 22 minutes, at 4°C. The pellets were suspended in 2.5 ml 0.032 M sucrose containing 0.01 mM $CaCl_2$ and incubated with 2.5 ml 40 mM Tris, pH 7.40 containing 1% Triton X-100 for 30 minutes at 4°C. The suspension was centrifuged at 40,000 g, for 30 minutes, at 4°C. The supernatant, representing the NSSP fraction, was stored at -20°C and was precipitated later. The pellets containing the PSD, which is insoluble in 1% Triton X-100, were dissolved in

Laemmli buffer and boiled for 5 minutes. The PSD fractions were kept at -20°C. The supernatant containing NSSP was subsequently precipitated using 5 volumes of -20°C acetone for 1 hour at -20°C and centrifuged at 18,000 g, for 30 minutes, at -15°C to collect NSSP pellets, which were then dissolved in Laemmli buffer as above.

Figure 2.3: Scheme for micropreparation of postsynaptic densities (PSD) from spinal cord quadrants

Ipsilateral and contralateral dorsal lumbar spinal cord quadrants were collected and homogenised in ice-cold buffer, before centrifugation to remove nuclei and debris. Synaptosomes were isolated from the supernatent by a rapid discontinuous Percoll gradient method before incubation with Triton X-100 to obtain the insoluble PSD fraction and the soluble NSSP (non-synaptic synaptosomal protein) fraction. These were then dissolved in Laemmli buffer and stored at -20 ^{o}C.

Key:

ID: ipsilateral dorsal spinal cord CD: contralateral dorsal spinal cord

IV: ipsilateral ventral spinal cord CV: contralateral ventral spinal cord

PSD: postsynaptic density NSSP: non-synaptic synaptosomal proteins

2.3

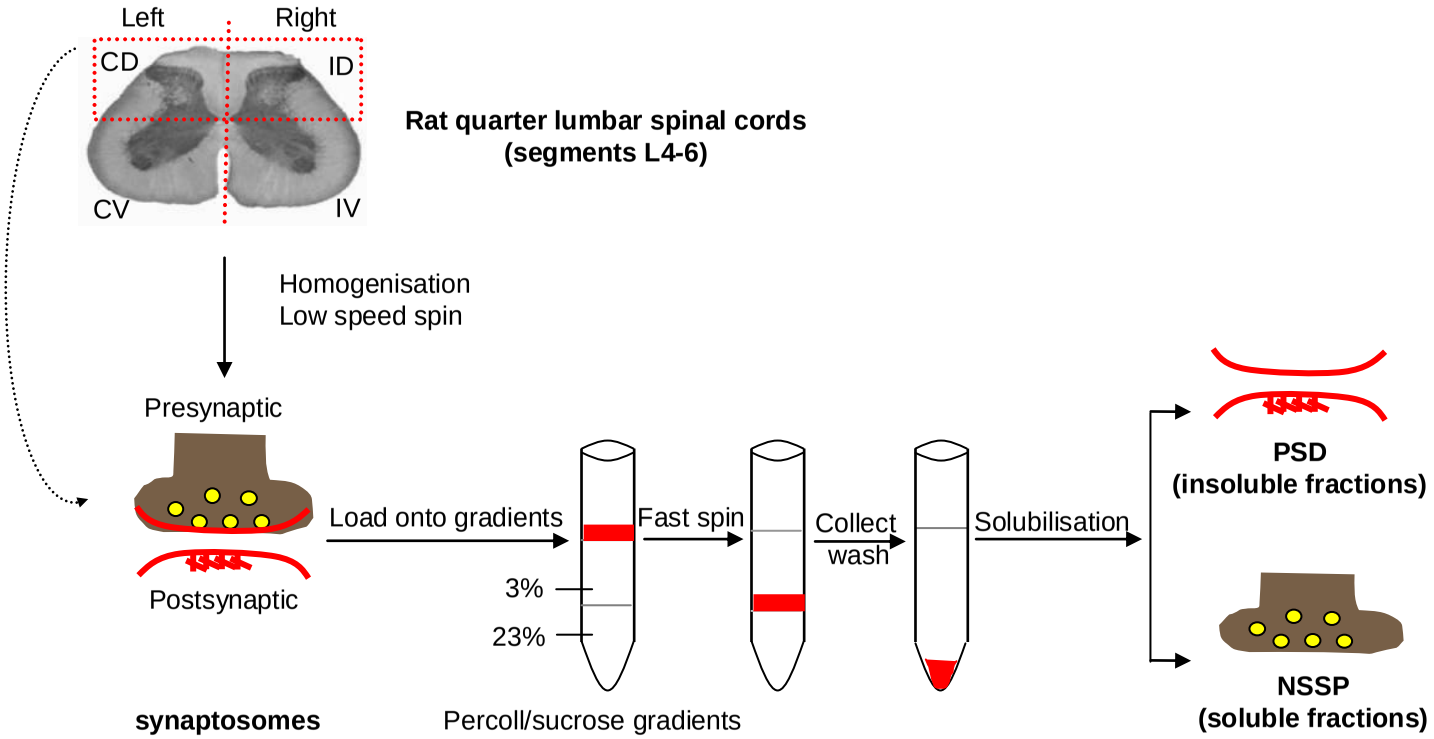

Left
Right
CD
ID
CV
IV
Rat quarter lumbar spinal cords
(segments L4-6)
Homogenisation
Low speed spin
Presynaptic
Postsynaptic
synaptosomes
Load onto gradients
3%
23%
Percoll/sucrose gradients
Fast spin
Collect
wash
Solubilisation
PSD
(insoluble fractions)
NSSP
(soluble fractions)

Table 2.3: Sucrose/Percoll gradients

Component		Concentration
3% Sucrose/Percoll solution	Sucrose	0.32 M
	$NaHCO_3$	1 mM
	Percoll	3%
	In: UHP water, pH adjusted to 7.40	
23% Sucrose/Percoll solution	Sucrose	0.32 M
	$NaHCO_3$	1 mM
	Percoll	23%
	In: UHP water, pH adjusted to 7.40	

Table 2.4: HEPES washing buffer

Component	Concentration
NaCl	137.9 mM
KCl	5.3 mM
$MgCl_2$	1 mM
$CaCl_2$	1 mM
Na_2HPO_4	0.3 mM
KH_2PO_4	0.4 mM
$NaHCO_3$	4.2 mM
D-Glucose	5.6 mM
HEPES	10 mM
In: UHP water, pH adjusted to 7.40	

2.2.7 SDS-PAGE and Western blots

SDS-PAGE and Western blots were used to examine the expression of GluA subunits and potential signalling proteins in the spinal cord of neonatal and adult nerve-injured rats. NuPage 4-12% Bis-tris 10- or 12-well gels and MOPS running buffer from Invitrogen were used throughout the experiments. Proteins were loaded evenly into the NuPage gels and then separated by SDS-PAGE for 50 minutes at 200 V. The proteins were then transferred from gels to PVDF membranes for 120 minutes at 30 V or to nitrocellulose membranes (for phosphorylated proteins) for 90 minutes at 30 V. The uniformity and overall effectiveness of transfer of proteins from gels to PVDF membranes was checked by staining the membranes with Coomassie Blue. The membranes were blocked with 5% non-fat dried milk or 5% BSA in PBS or TBS with 0.05% Tween-20 for 90 minutes at room temperature with shaking. Blots were probed with primary antibodies to 4.1N, GluA1, phospho-ERK, ERK, PSD-95 or synaptophysin at 4°C overnight with rotation. After three washes of 10 minutes with PBS-0.1%Tween (PBST), blots were incubated in appropriate HRP-linked secondary antibodies (Table 2.5) at room temperature for 50 minutes with mixing by rotation. After a further three washes with PBST, the immunoreactive bands were detected by enhanced chemiluminescence and visualised by exposure to Amersham Hyperfilm. The ubiquitous housekeeping enzyme GAPDH or actin was used as control, for protein level normalisation.

Following high resolution scanning of films, the density of immunoblot bands was quantified by Image J 7.0 software. Background grey values were obtained from appropriately matched regions of the film and subtracted from the total value. The data were analysed by comparing to GAPDH or actin levels on the same blot.

2.2.8 Co-immunoprecipitation

In addition, the interaction between GluA1 and its binding partners such as 4.1N in spinal cord of adult CCI rats was examined in comparison to that in naïve animals. The co-immunoprecipitation procedure used briefly involves: i) homogenising tissue in cold buffer containing non-denaturing detergents and protease inhibitors; ii) pre-

clearing supernatant fractions from the homogenates with protein-G Sepharose beads to deplete non-specific binding of tissue proteins; iii) incubation of the cleared extracts with specific anti-rat GluA1 subunit antibody (or control); iv) precipitation of the antibody-protein complex using protein-G Sepharose beads (if there are any protein molecules that bind to the GluA1, they will also be precipitated); v) identification of co-precipitated proteins by Western blot analysis.

Spinal cord segments L4-6 were collected rapidly, hemisected and homogenised gently in 10 volumes cold immunoprecipitation (IP) buffer (Table 2.6). They were kept at 4°C for 1.5 hours on a roller to allow mixing and solubilisation of membrane-bound protein complexes and then centrifuged at 12,000 g for 15 minutes at 4°C. The supernatant was removed and added to Eppendorf tubes. Protein G-coupled Sepharose beads were washed in cold IP buffer and left with an equal volume of buffer after the final wash. The samples were pre-cleared with the beads (20 µl of 50 % suspension per ml of supernatant) for 1 hour at 4°C to remove non-specifically binding endogenous proteins. The supernatant was removed to new tubes after centrifugation of the samples at 12,000 g for 10 minutes at 4°C and incubated with GluA1 antibody (Santa Cruz, sc-13152, 1:50) and protein G-Sepharose beads (40 µl of 50% suspension per ml of supernatant) overnight at 4°C on a roller. An aliquot of supernatant was incubated with non-immune IgG of the same species as the antibody, instead of the GluA1 antibody, as a control. On the second day, samples were centrifuged at 12,000 g for 15 minutes at 4°C and the beads were washed once with 1 ml IP buffer and twice with PBS. After the final PBS wash, all remaining liquid was removed with thin strips of filter paper. An equal volume of Laemmli buffer containing sodium dodecyl sulphate and mercaptoethanol to solubilise and denature the proteins was added to beads and the mixture was boiled for 5 minutes in a sealed tube, briefly centrifuged at 12,000 g and stored at -20°C. Western blots were carried out to probe for GluA1 binding proteins and the levels of GluA1 itself collected in the pull-down were assessed by a different GluA1 antibody raised in another species.

Table 2.5: Antibodies used for Western blots and co-immunoprecipitation

Primary antibody	Catalog number	Dilution	Secondary antibody	Catalog number	Dilution
Mouse anti-protein 4.1N monoclonal IgG (BD)	611836	1:200	Donkey anti-Mouse HRP-linked IgG (Millipore)	AP192P	1:40,000
Mouse anti-GAPDH monoclonal IgG (Millipore)	MAB374	1:1000			
Mouse anti-GluR1 (E-6) monoclonal IgG (Santa Cruz)	sc-13152	1:100 IP: 4 μg/ml			
Mouse anti-GluR1 (G-12) monoclonal IgG (Santa Cruz)	sc-55509	1:100			
Mouse anti-RhoA monoclonal IgG (Santa Cruz)	sc-418	1:100			
Mouse anti-PSD95 monoclonal IgG (Neuromab)	75-028	1:400			
Mouse anti-actin monoclonal IgG (Sigma)	A4700	1:400			
Rabbit anti-Sos1 (H-122) polyclonal IgG (Santa Cruz)	sc-10803	1:200	Goat anti-Rabbit HRP-linked IgG (Millipore)	AP307P	1:40,000
Rabbit anti-Synaptophysin (H-93) polyclonal IgG	sc-9116	1:10,000			

(Santa Cruz)					
Rabbit anti-GluR1 polyclonal IgG (Millipore)	AB1504	1:1500			
Rabbit anti-ERK polyclonal IgG (Cell Signalling)	9102	1:1000	Goat anti-Rabbit HRP-linked IgG (Cell Signalling)	7074	1:4,000
Rabbit anti-phospho(Thr202/Tyr204)-ERK polyclonal IgG (Cell Signalling)	9101S	1:1000			

Table 2.6: Immunoprecipitation (IP) buffer

Component	Concentration
CHAPS	16.3 mM
Deoxycholate	11.6 mM
Sodium molybdate (phosphatase inhibitor)	5 mM
Sodium orthovanadate (phosphatase inhibitor)	1 mM
NaF (phosphatase inhibitor)	1 mM
Protease inhibitor mix (Type III, Calbiochem, EMD)	1%
Glycerol	10%
In: PBS, pH 7.40	

2.3 Methods specific to Chapter 4

2.3.1 Preparation of freshly isolated neural tissue segments, synaptoneurosomes and microslices

A number of reports in the literature describe the preparation from gently homogenised CNS tissue of subcellular fractions enriched in entities referred to as synaptoneurosomes (Hollingsworth *et al.*, 1985; Moring *et al.*, 1990; Johnson *et al.*, 1997; Villasana *et al.*, 2006; Troca-Marin *et al.*, 2010). These represent resealed presynaptic and apposed postsynaptic elements, presenting at the electron microscopy level as classic 'snowman-shaped profiles' (Hollingsworth *et al.*, 1985) with reportedly a greater inclusion of postsynaptic material than conventional synaptosomes. As part of the preparative procedure for synaptoneurosomes involves filtration through net and matrix filters of decreasing pore size, I took the opportunity to investigate for the first time, responsiveness in 30-100 μm diameter tissue segments as well as the sub-5 μm synaptoneurosome fraction. In addition, I explored whether it was possible to record dynamic cellular responses in tissue microslices, a preparation used successfully in the past for neurotransmitter release studies (Mitchell and Martin, 1978).

Näive adult Sprague-Dawley rats were euthanised and brains were removed swiftly, normally within 60 seconds, dissected and homogenised gently in ice-cold, well oxygenated homogenising buffer (8 ml per frontal cortex) using a large clearance glass/glass homogeniser by hand for 15 seconds. For experiments on spinal cord, lumbar segments were removed following laminectomy from rats under terminal isoflurane anaesthesia. To prepare microslices, frontal cerebral cortex or lumbar spinal cord was rapidly removed from adult Sprague-Dawley rats and immersed in ice-cold, oxygenated homogenisation medium (as above). Perpendicular microslices (150 × 150 μm) were prepared as quickly as possible using a McIlwain tissue slicer (Mitchell and Martin, 1978) and washed twice in fresh homogenising buffer before preparation for assay.

Krebs-Henseleit buffer (Krebs and Henseleit, 1932) (Table 2.7) was modified to make the homogenising buffer. The modifications included the addition of HEPES (10 mM, to ensure secure pH buffering), glutathione (5 μM, as a sulphydryl group protectant), protease inhibitors (0.1% Calbiochem cocktail III, to minimise proteolysis), sodium pyruvate (0.5 mM, as a glycolytic intermediate), and creatine phosphate (2 mM, as a high energy source). 5 mM $MgCl_2$ (an inhibitor of Ca^{2+} entry through voltage-sensitive Ca^{2+} channels and NMDA receptors) and 0.3 mM kynurenic acid (a broad spectrum blocker of glutamate receptors and the α7-nicotinic acetylcholine receptor); (Rozsa *et al.*, 2008) were added into the basic solution. Finally, the pH value of the homogenising buffer was adjusted to 7.40 and the solution on ice was bubbled for at least 1 hour with 95% O_2, 5% CO_2.

For the preparation of neural tissue segments and synaptoneurosomes, homogenate was loaded into 5 ml syringes and passed successively under modest manual pressure through 100 μm and 30 μm nylon net filters and then a 5 μm pore size cellulose fibre matrix filter (Millipore) (Hollingsworth *et al.*, 1985). A synaptoneurosome-enriched pellet was obtained by centrifugation of the sub - 5 μm filtrate (1500 g, 4°C, 8 minutes). The tissue segments captured on the 30 μm nylon net filters (i.e. 30-100 μm range) were eluted by gentle washing. The medium used for resuspension of tissue segments, synaptoneurosome and microslices was the same modified Krebs-Henseleit with the omission of $MgCl_2$ and kynurenic acid but the addition of 2 mM $CaCl_2$ and was similarly kept oxygenated on ice. Aliquots of the tissue segment, synaptoneurosomes or microslice suspensions were loaded into wells of 24-well cell culture plates that had been pre-coated with 5% collagen (Sigma) (Figure 2.4).

Table 2.7: Buffers for experiments with acutely prepared neural tissue samples

Basic buffer:

Component	Concentration
D-glucose	11.1 mM
$MgSO_4$	1.2 mM
KH_2PO_4	1.2 mM
KCl	4.7 mM
NaCl	118.5 mM
$NaHCO_3$	25 mM
HEPES	10 mM
Sodium pyruvate	0.5 mM
Glutathione	5 μM
Phosphocreatine disodium	2 mM
Protease inhibitors (Type III, Calbiochem)	0.1%
In: UHP water, pH adjusted to7.40	

Additions to make homogenising buffer:

$MgCl_2$	5 mM
kynurenic acid (KA) (as sodium salt)	0.3 mM

Additions to make assay buffer:

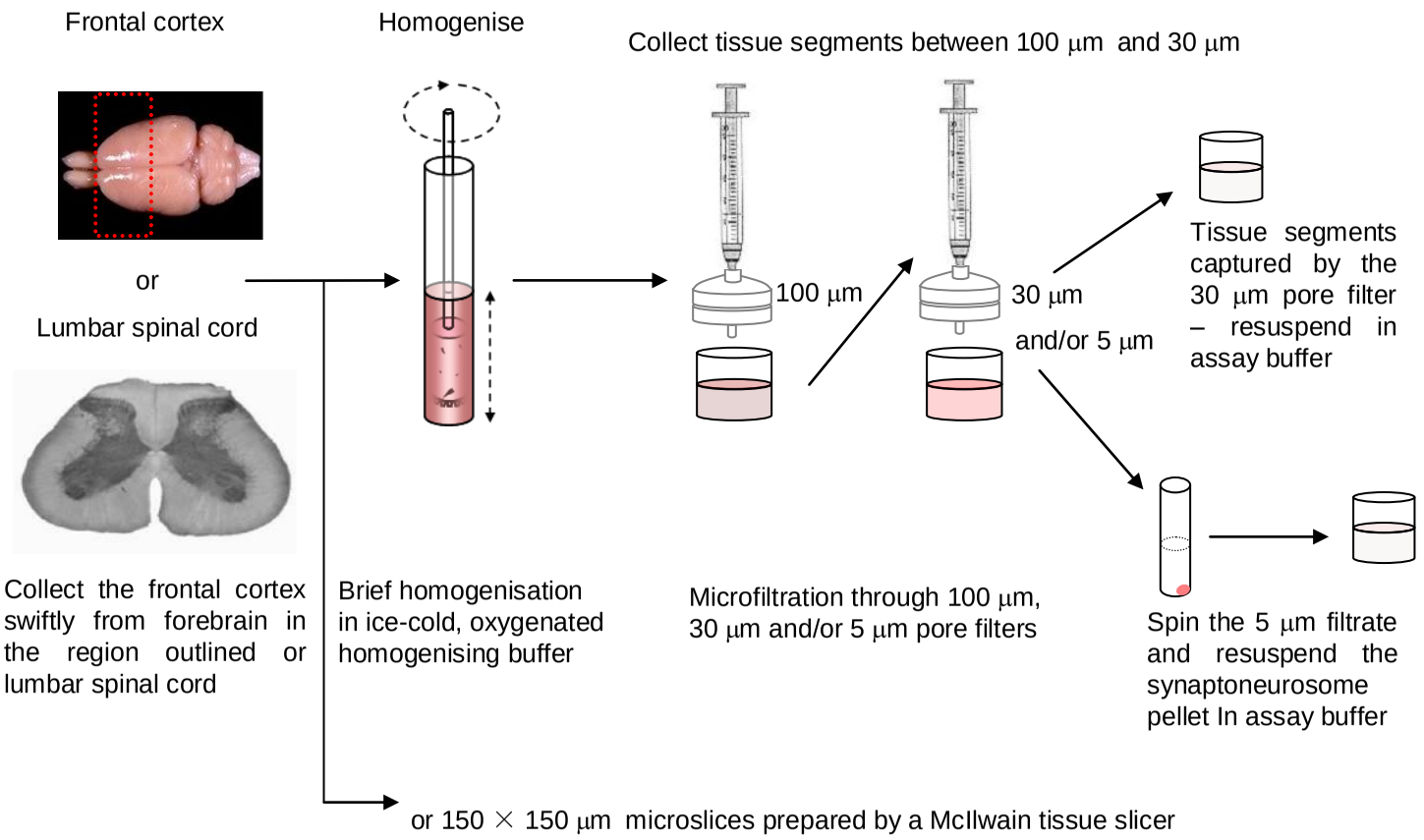
$CaCl_2$	2 mM

Figure 2.4 Preparation of freshly isolated neural tissue segments, synaptoneurosomes and microslices

Rats were euthanised and brains were removed rapidly, dissected and homogenised gently in ice-cold, well-oxygenated buffer containing $MgCl_2$ and kynurenic acid but lacking $CaCl_2$, using a large clearance glass/glass homogeniser. For experiments on spinal cord, lumbar segments were removed following laminectomy from rats under terminal isoflurane anaesthesia.

For the preparation of neural tissue segments and synaptoneurosomes, homogenate was syringe-filtered through 100 μm and 30 μm nylon net filters and then a 5 μm pore size cellulose fibre matrix. A synaptoneurosome-enriched pellet was obtained by centrifugation of the sub - 5 μm filtrate and the tissue segments captured on the 30 μm nylon net filters (i.e. 30-100 μm range) were eluted by gentle washing. Each was resuspended in buffer containing $CaCl_2$ but lacking $MgCl_2$ and kynurenic acid, for assay. To prepare microslices, frontal cerebral cortex or lumbar spinal cord was rapidly immersed in ice-cold, oxygenated homogenisation medium. Perpendicular microslices (150 × 150 μm) were prepared as quickly as possible using a McIlwain tissue slicer and immediately washed twice in fresh buffer before assay.

2.4


Frontal cortex
Homogenise
Collect tissue segments between 100 μm and 30 μm
or
Lumbar spinal cord
100 μm
30 μm
and/or 5 μm
Tissue segments captured by the 30 μm pore filter – resuspend in assay buffer
Collect the frontal cortex swiftly from forebrain in the region outlined or lumbar spinal cord
Brief homogenisation in ice-cold, oxygenated homogenising buffer
Microfiltration through 100 μm, 30 μm and/or 5 μm pore filters
Spin the 5 μm filtrate and resuspend the synaptoneurosome pellet In assay buffer
or 150 × 150 μm microslices prepared by a McIlwain tissue slicer

2.3.2 Fluorometric measurements on 30-100 μm neural tissue segments, synaptoneurosomes and tissue microslices

The general strategy adopted was to try and achieve quantitative fluorometric measurements from the *ex vivo* preparations that would reflect dynamic responses to receptor stimuli and then ultimately might be able to reveal long term changes in responsiveness brought about by earlier *in vivo* events (such as the central sensitisation in chronic pain models). It was considered that the better approach (if it proved possible) would be to use a plate-reader format, where several aliquots of the same preparation could be subjected to different drug treatments and measured in parallel. The alternative would be to assess samples in suspension in a cuvette fluorometer but this would have meant samples could only be assessed one at a time and that the preparations would require constant mixing, which might cause deterioration of the tissue. An important consideration with the fluorometric plate reader is that the most robust readings are generally obtained in bottom reading mode from cells coating the bottom of the well rather than from above where the light is dispersed by passage through buffer. It therefore seemed that allowing samples time to settle to the bottom of wells would most likely be essential as would avoiding movements that would create turbulence, especially as the preparations were not anchored in any way to the well surface. Indeed it was found that automatic injection of drug solutions into the wells (a facility available in real-time recording mode) caused so much turbulence that fluorescence readings became too variable to be useful. A disadvantage of this was that any drugs (or vehicle controls) had to be very carefully added by hand to parallel wells before reading could be commenced.

As a large proportion of cellular stimuli lead either directly or indirectly to elevation of intracellular Ca^{2+} concentrations and a wide range of Ca^{2+} indicator fluorophores with excellent spectral and signal-to-noise properties are available, the primary approach taken was to assess Ca^{2+} fluorescence of the tissue preparations. Some experiments were carried out in addition using a membrane potential-sensitive fluorophore. In each case, it was considered that extensive washing of the

preparations, after loading the dyes in ester form, (normally needed to remove any extracellular dye which could contribute to a high basal fluorescence) would be a disadvantage as it could damage the preparations and would certainly disrupt the settled layer of tissue fragments. Fortunately several appropriate fluorophores are available in 'no-wash' formulations where such procedures are completely unnecessary because they contain proprietary impermeant quenching agents, which suppress any unwanted basal fluorescence from extracellular dye. The use of these agents was considered to be an important factor in gaining usable quantitative data.

For Ca^{2+} fluorometric studies the preparations were loaded with an intracellular Ca^{2+} fluorometric dye (Calcium 4, Molecular Devices) according to the manufacturer's instructions by addition of 100 μl of ice-cold 4 × concentrated solution in assay buffer to the wells. Importantly, Calcium 4 is a no-wash dye, meaning that samples do not have to be washed to avoid non-specific fluorescence from dye outside the cells responding to extracellular Ca^{2+}. The dye functions in principle like most Ca^{2+} fluorophores such as Fluo-4 (Gee *et al.*, 2000), being loaded as an inert acetoxymethoxy (AM) ester which is cleaved by intracellular esterases to the active form, but additionally contains a proprietary extracellular quenching agent that suppresses extracellular fluorescence. Calcium 4 otherwise displays very similar spectral properties and Ca^{2+} affinity to Fluo-4. After dye addition, the 24-well plate was then incubated at 37°C in a humidified 95% O_2, 5% CO_2 atmosphere for 45 minutes, before careful transfer to a Varioskan Flash fluorometric plate reader (Thermo Scientific). The plate was then left undisturbed in the thermostatically controlled (28^{o}C) carrier stage of the fluorometer to allow for settlement of the tissue preparations after the transfer. Drugs or vehicle as appropriate (Table 2.8) were then added very carefully (in 10% of the well volume; i.e. 40 μl) from stock solutions that contained 10 × the final required concentration. The programme controlling the well reading cycle was then initiated as soon as possible and ran for up to 15 minutes. Readings were taken from each well on a 30 seconds-repeating cycle with excitation and emission wavelengths set at 488 and 519 nm respectively (bottom reading mode). Readings were collected through the complete time-period of the programme but particular time points were selected for subsequent quantitative analysis of data that

seemed to best reflect the stable plateau values of fluorescence that were reached. The data collection period for synaptoneurosomes and microslices was routinely begun at 3 minutes into the fluorescence reading cycle and that for 30-100 μm tissue segments was begun at 6 minutes because of an impression that they were more mobile in the wells and more easily disturbed. Retrospective analysis of time-course data however (Chapter 4), indicated that this distinction did not make any clear difference to the results and so was not really necessary.

Pilot experiments were also carried out to assess changes in membrane potential using a no-wash voltage-sensitive fluorescent dye (R8042, MP Blue, Molecular Devices). The experimental protocol was identical to that for the above Ca^{2+} fluorometric studies, apart from the excitation and emission wavelengths, which were set at 530 and 565 nm, respectively.

Table 2.8: Compounds used for calcium fluorometry

Compound	Activity	Concentration
1-Aminocyclopropane carboxylic acid (ACPC)	An activator of the glycine site of the NMDA receptor	10-200 μM
1-Noradrenaline	Mediator released upon sympathetic activation that can act on Gq-coupled α-adrenergic receptors to cause elevation of intracellular Ca^{2+} concentration	0.01-300 μM
1-Propranolol	Selective β–adrenoreceptor antagonist	6 μM
2,5-Dimethoxy-4-iodoamphetamine [(R)-DOI]	A selective 5-HT_{2A} receptor agonist	1-10 μM
α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA)	Agonist at AMPA-type ionotropic glutamate receptors	10-200 μM
Acetylcholine	Acetylcholine receptor agonist	30 μM
Cyclothiazide	Inhibitor of desensitisation at activated AMPA receptors.	30 μM
Ionomycin	Calcium ionophore	5 μM
N-methyl-D-aspartic acid (NMDA)	Agonist at NMDA-type glutmate receptors	10-200 μM
Prazosin	Selective α_1–adrenoreceptor antagonist	6 μM

2.3.3 Morphological examination of ex vivo tissue preparations by light microscopy and electron microscopy

For assessment by light microscopy, 30-100 μm tissue segments or synaptoneurosomes were loaded with Calcium 4 in chambers based with collagen-coated coverslips and incubated as normal for 45 minutes at 37^{o}C in a 95%O_2:5%CO_2 atmosphere. Samples were transferred, without washing, to a Zeiss Axiovert LSM510 microscope for imaging using a × 10 objective lens for 30-100 μm segments and a × 40 lens for synaptoneurosomes. The basal levels of tissue Ca^{2+} fluorescence at 488 nm excitation/519 nm emission were used to identify the morphology of the preparations. It was not possible to add stimuli to the coverslips under these conditions without causing unacceptable disruption of the field of view.

For electron microscope assessment, aliquots of synaptoneurosome suspension were pelleted by centrifugation (12,000 g, 15 minutes, 4°C) and resuspended in HEPES-buffered 0.32 M sucrose with protease inhibitors before fixing in 2.5 % glutaraldehyde in 0.1 M cacodylate/0.1 M phosphate buffer (pH 7.40) for 30 minutes on ice. Samples were washed 3 times for 3 minutes with 0.1 M cacodylate buffer containing 2 mM $CaCl_2$, fixed for 30 minutes in 1% OsO_4 in 0.1 M cacodylate buffer and washed twice for 5 minutes with 0.1 M cacodylate buffer containing 2 mM $CaCl_2$. Samples were gradually dehydrated with two 3 minute washes of 25% and 50% ethanol and then stained *en bloc* with filtered 2% ethanolic uranyl acetate for 30 minutes. The samples were dehydrated further in a series of 70, 95, and 100% ethanol washes. Durcupan ACM Epoxy Resin was used to embed the samples and cured at 55°C for 2–3 days. Eighty nanometre thin sections were cut using a MT6000-XL ultramicrotome and then stained in uranyl acetate and lead citrate. Samples were processed with the kind help of Heather Anderson and Steven Mitchell from the stage of post-fixing. Ultrathin sections (10 nm) were examined using a Phillips Biotwin electron microscope (School of Biology, University of Edinburgh) and images were acquired with a CCD camera.

2.4 Statistical analysis

2.4.1 Analysis of behavioural data

In experiments investigating drug effects on behavioural responses to a noxious thermal stimulus, differences from pre-drug baseline were analysed by One-way Repeat-Measures ANOVA followed by Dunnett's post-hoc test and ipsilateral:contralateral differences at each time point were assessed by Student's t-test. For mechanical von Frey responses, differences from pre-drug baseline were analysed by Friedman test followed by Dunn's post-hoc test and ipsilateral:contralateral differences at each time point were assessed by Wilcoxon test. Ipsilateral:contralateral differences in von Frey paw withdrawal threshold following nerve injury at P8 or in adults were analysed by Mann-Whitney U-test. Non-parametric tests were used for the Von Frey data because the data from discrete filaments are at non-linear intervals. Comparisons at different time points of formalin-induced nocifensive behaviours between P8 nerve-injured animals assessed at P42 and adult nerve-injured animals, assessed correspondingly 34 days later, were made by Student's t-test. In all cases p values less than 0.05 were considered significant.

2.4.2 Analysis of immunoblots

After high resolution scanning of films at submaximal development times, the grey scale density of immunoblot bands was quantified by Image J 7.0 software. Background grey values were obtained from appropriately matched regions of the film and subtracted from the total value. In each experiment, specific image density for the protein of interest was normalised to that for a protein that is generally considered to be unchanged by pain or activity-related stimulation, either actin (in the case of synaptic preparations) or GAPDH (for whole tissue analysis), from the same lane of the same gel. Statistical significance of ipsilateral:contralateral differences in the ratios of GluA1 or phospho-ERK normalised to actin was assessed using the Ratio t-test. This test is recommended where a comparison of the relative

values of two measures might be predicted to provide a more appropriate assessment than a comparison of absolute values (Motulsky, 2003). Here this is indicated when comparing relative GluA1:actin (or phospho-ERK:actin) ratios for individual datasets which were run on a single gel, as such relative values will represent a more appropriate comparison than absolute grey scale values, which will vary from gel to gel. Comparisons of individual ipsilateral:contralateral ratios for GluA1 (or phospho-ERK) normalised to actin between two treatment groups (formalin-injection either following or not following previous nerve-injury) were made by Student's unpaired t-test. In all cases p values of less than 0.05 were considered significant.

2.4.3 Analysis of fluorometric data

All relative fluorescence values were saved both within the fluorometer driver software and exported as Excel spreadsheets. The specific drug-induced increases in fluorescence values were obtained by subtraction of corresponding basal values from parallel control wells. Increases in fluorescence values specifically induced by benchmark positive control stimuli (5 μM ionomycin or 50 mM K^+, for Ca^{2+} fluorescence or membrane potential fluorescence experiments, respectively) were similarly obtained by subtraction of corresponding basal values. In many parts of the study, the specific drug-induced increase was then divided by the specific positive control-induced increase to normalise the data and enable easy and rigorous comparisons between experiments. Statistical analysis of differences between groups was generally carried out by Student's t-test for comparisons between two groups or One-way ANOVA (with an appropriate post-test such as Tukey's test) for comparisons between 3 or more groups. Comparisons of time-course curves were made by Repeated-Measures One-way ANOVA with Tukey's post-test. Individual comparisons between matched groups with or without addition of a further drug were made by Student's paired t-test. In all cases p values of less than 0.05 were considered significant.

Chapter 3: Translocation of GluA1 AMPA receptor subunits to spinal postsynaptic densities in pain states

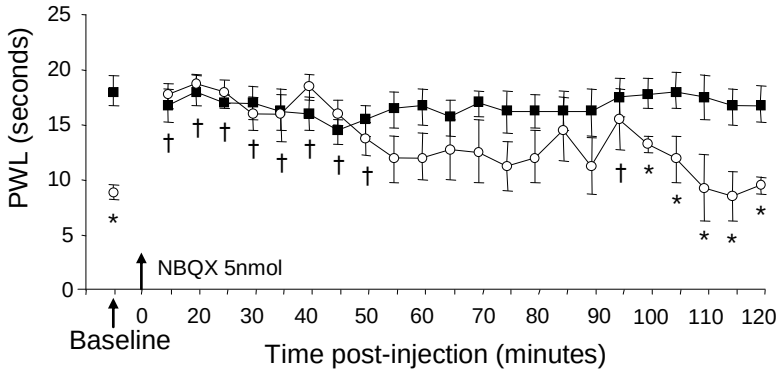
3.1 Introduction

As mentioned in the general introduction, AMPA receptors mediate fast excitatory transmission in the dorsal horn and are thought to be required in spinal sensory processing of both acute and chronic nociceptive inputs. There is some evidence to suggest that AMPA receptors may play a greater role in sensitised pain states, as nociceptive withdrawal responses in sensitised pain states are more readily reversed by AMPA receptor antagonists than responses in naïve animals (Garry *et al.*, 2003b; Larsson and Broman, 2008)(Figure 3.1). AMPA receptors are tetrameric structures made up from four subunits, GluA1-4. The GluA1 subunit of AMPA receptors has received particular attention due to evidence that NMDA receptor-dependent GluA1 incorporation to synapses contributes to the enhanced synaptic transmission in LTP in hippocampal neurons (Shi *et al.*, 1999). Such signal-directed trafficking to synapses is most clearly established for AMPA receptors containing GluA1 subunits rather than other subtypes (Shi *et al.*, 1999). As many AMPA receptors contain GluA1 subunits and they are strongly expressed in superficial dorsal horn, I hypothesised here that GluA1 trafficking to synapses may be important in bringing about the central sensitisation in spinal cord in chronic pain states.

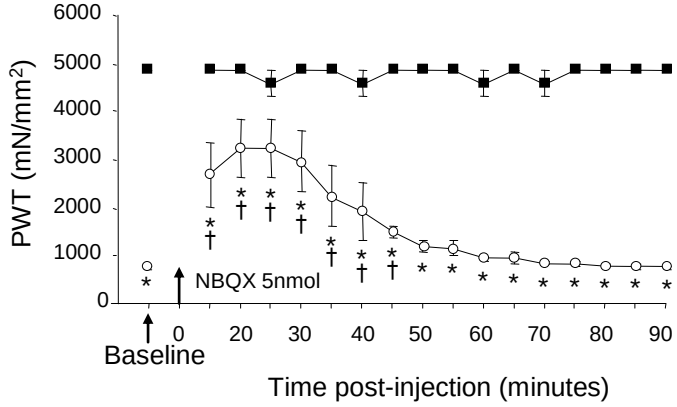
Figure 3.1: Intrathecal administration of the selective AMPA receptor antagonist NBQX inhibits sensitised reflex responses ipsilateral to CCI nerve injury in adult rats.

Thermal and mechanical reflex withdrawal responses were measured in adult animals with established CCI-induced sensitisation. Paw withdrawal latency (PWL, seconds) to a noxious thermal stimulus a) or paw withdrawal threshold (PWT, mN/mm^2) to mechanical von Frey stimulation b) are shown prior to and following the intrathecal injection (at arrow) of 5 nmol NBQX (50 μl of 100 μM solution of NBQX, sodium salt, in 0.9% saline). For thermal sensitivity, significant differences between ipsilateral (○) and contralateral (■) responses are indicated by * ($p<0.05$, Student's t-test) and differences from pre-drug baseline are indicated by † ($p<0.05$ One-way Repeated Measures ANOVA followed by Dunnett's post-hoc test). For mechanical sensitivity, significant differences between ipsilateral (○) and contralateral (■) responses are indicated (*, $p<0.05$, Wilcoxon rank test). Significant differences from pre-drug baseline are indicated (†, $p<0.05$, Friedman test followed by Dunn's post-hoc test). Data are shown as mean ± SEM (n=9), in each case. NBQX had no discernible effect on contralateral responses in CCI animals at a dose of 5 nmol or on responses of control animals at doses up to 10 nmol (data not shown), suggesting a greater involvement of AMPA receptors in the sensitised pain state. These experiments were carried out by Emer Garry as part of our joint research programme within the laboratory.

3.1 a)


PWL (seconds)
25
20
15
10
5
0
NBQX 5nmol
Baseline
0
20
30
40
50
60
70
80
90
100
110
120
Time post-injection (minutes)

3.1 b)


PWT (mN/mm2)
6000
5000
4000
3000
2000
1000
0
NBQX 5nmol
Baseline
0
20
30
40
50
60
70
80
90
Time post-injection (minutes)

3.1.1 GluA1 subunits in spinal dorsal horn

The expression and localisation of the GluA1 subunit of AMPA receptors in spinal cord are age-dependent. Differential changes in expression of GluA1 mRNA in spinal cord occur over postnatal development; it is widely expressed across the whole spinal cord for the first few days after birth and then diminishes gradually to leave limited expression in the superficial dorsal horn in adult (Jakowec *et al.*, 1995b). A quantitative immunostaining study indicated that almost 65% of spinal GluA1 locates in laminae I–II and 34% in lamina III of adult rat dorsal horn (Polgar *et al.*, 2008), which are known as the main nociceptive processing sites in the spinal cord. There are several lines of evidence to suggest that this subunit plays a role in synaptic plasticity in the dorsal horn in pain states. For example, a study of inflammatory pain showed a rapid up-regulation of GluA1 mRNA in the lumbar dorsal horn following injection of Complete Freund's Adjuvant (CFA) into the hindpaw of rats (Zhou *et al.*, 2001). Another report describes phosphorylation of the GluA1 subunit at both S831 and S845 sites in spinal cord tissue after intradermal capsaicin injection, as shown by Western blot (Fang *et al.*, 2003a). More specifically, S845 phosphorylation of GluA1 at synapses in laminae I–II of the ipsilateral dorsal horn has also been demonstrated after capsaicin injection (Nagy *et al.*, 2004). In addition, it has been reported that GluA1-containing receptors can be recruited to a membrane fraction in lumbar spinal cord following a noxious visceral stimulus (Galan *et al.*, 2004). Synaptic plasticity involving GluA1 could therefore potentially occur in nociceptive processing neurons in the superficial dorsal horn.

3.1.2 GluA1 subunit mobility and synaptic activity

AMPA receptors containing GluA1 subunits are most probably assembled in the rough endoplasmic reticulum, sorted in the Golgi and then transported by vesicles to dendrites, where they are inserted and concentrated into synapses (Makino and Malinow, 2009). However, local protein synthesis may occur, since the mRNAs coding for AMPA receptor subunits are found in dendrites (Grooms *et al.*, 2006) and transfected tagged GluA1 subunit can be synthesised in a dendritic compartment independent from the cell body in cultured hippocampal neurons (Ju *et al.*, 2004).

The full range of molecular mechanisms that underlies the synaptic targeting is not clear. A growing body of studies focuses on the relationship between the GluA1 subunit mobility and synaptic activity, which may be helpful in understanding the role of GluA1-containing AMPA receptors in neuropathic pain mechanisms.

There is increasing understanding of the dynamic process that controls AMPA receptor trafficking to and from the synapses. Using a single-cell genetic approach, it was found that approximately 80% of AMPA receptors contain the GluA1 subunit at CA1 hippocampal synapses under conditions of basal synaptic transmission (Lu *et al.*, 2009). In contrast, almost all (95% of) AMPA receptors contained GluA1 subunits in the extrasynaptic compartment (Lu *et al.*, 2009). Moreover, it has been well established that AMPA receptors are highly mobile on the surface of neurons and that there is a dynamic exchange between synaptic and extrasynaptic receptors (Borgdorff and Choquet, 2002). Indeed, GluA1-containing AMPA receptors actively shuttle between synaptic and extrasynaptic sites (Ehlers *et al.*, 2007).

Synaptic activity may regulate the postsynaptic accumulation of AMPA receptors through diverse mechanisms such as control of cytoskeletal organisation, the affinity of interactions with PSD scaffold proteins or other intracellular proteins such as 4.1N, and spine morphology (Garry and Fleetwood-Walker, 2004; Ashby *et al.*, 2006; Kopec *et al.*, 2006). Indeed, it has been shown using single-molecule tracking and fluorescence photobleaching that postsynaptic accumulation of GluA1 is induced by local synaptic activity through stabilisation of GluA1 at active synapses and reduction of diffusional exchange of GluA1 between synaptic and extrasynaptic domains (Ehlers *et al.*, 2007). The study illustrated that the mobility of GluA1 subunits is reduced within active synapses, resulting in postsynaptic accumulation of GluA1 (Ehlers *et al.*, 2007). Changes in mobility of GluA1 subunit-containing AMPA receptors are important for long-term forms of synaptic plasticity. As described in Chapter 1, synaptic insertion of GluA1 subunits is known to play an important role in mediating the enhancement in synaptic strength during LTP or central sensitisation through interactions with intracellular partner proteins and/or phosphorylation of a large number of molecules and kinases. 4.1N is one of the best-studied partner proteins. It regulates the surface expression of GluA1 subunit-

containing AMPA receptors by association with the cytoskeletal protein actin (Shen *et al.*, 2000) and is proposed to play a role in delivering more GluA1 subunits to synapses through 4.1N-actin-actin-CaMKII-NMDA receptor protein:protein interactions (Lisman and Zhabotinsky, 2001). In addition, the synaptic incorporation of GluA1 is also required to stabilise the increase in spine size and its C-terminal domain has been shown to play a key role in spine enlargement in hippocampal neurons (Kopec *et al.*, 2007).

Overall, GluA1 subunit translocation to synapses is widely considered to be crucial for long-term synaptic plasticity. It may be also critical for central sensitisation in spinal cord and contribute to the mechanisms underlying chronic pain states.

3.1.3 Alterations in nociceptive circuitry in neonatal pain states

The neonatal period is considered to be a time of continuing development for structural and functional organisation of spinal sensory systems (Fitzgerald and Beggs, 2001), and for expression of molecules, receptors, and channels associated with pain transmission (Alvares and Fitzgerald, 1999). The study of neonatal pain is not only beneficial for understanding the development of the nervous system but also helpful for investigating the mechanisms involved in the generation of chronic sensitised pain states. As the synaptic development of spinal sensory pathways is activity-dependent, inflammation related to pain or nerve injury in early life may affect normal synaptic development and lead to alterations in nociceptive circuits in later life (Ruda *et al.*, 2000; Beggs *et al.*, 2002). An important implication of such plasticity is that pain related to surgery or repeated procedural interventions in human neonates or neonatal husbandry procedures in farm animals or companion animals, such as tail-docking in pigs or dogs respectively, may have long-term consequences.

Nociceptive primary afferents are changed in adult rats exposed to peripheral inflammation in neonatal life. Using wheat germ agglutinin–horseradish peroxidase (WGA-HRP) labelling, the density of nociceptive afferents including fine-myelinated and unmyelinated fibres was shown to increase dramatically on the ipsilateral side

compared to the contralateral side following CFA injection into the hind paw on postnatal day 1 (Ruda *et al.*, 2000). Pain-related behaviours were also changed ipsilaterally in adult rats that had previously experienced neonatal inflammation caused by injection of CFA or formalin into the paw. The rats with neonatal inflammation showed corresponding increased spontaneous sensitivity or decreased mechanical threshold in later life. Consistent with these findings, another study on the effects of neonatal inflammation on the development of nociceptive processing showed that the expansion of afferent terminals in lamina II of dorsal horn following neonatal CFA inflammation was maintained into adulthood in a dose-dependent manner (Walker *et al.*, 2003). Nevertheless, there are conflicting reports of what long-term effects are exerted upon adult pain responses when different inflammatory agents and injection volumes are used or the insult is applied at different developmental stages (Buck and Burks, 1986; Alvares and Fitzgerald, 1999; Ruda *et al.*, 2000; Bhutta *et al.*, 2001; Lidow *et al.*, 2001). Thus long-term changes in sensory and pain processing may critically depend upon the degree, duration and timing of the neonatal inflammatory insult.

The ability of early life injury to produce long-term changes in somatosensory structure and function depends upon the nature of the injury. Neonatal nerve damage is associated with accentuated nociceptive behavioural responsiveness, peripheral afferent rearrangement and alterations of their central termini as well as changes in the interneurons within the spinal dorsal horn. In animal pain models, infant rats show different and more lasting behaviours evoked by peripheral nerve injury compared to adults (Lee and Chung, 1996). The duration of behaviours elicited by nociceptive stimulation is age-dependent and may vary in different neuropathic pain models. Mechanical allodynia induced by partial ligation of L4-6 spinal nerves does not last longer than 4-5 weeks if the nerves are injured at an age of 2 weeks or younger and this finding points out that several factors may be important for maintenance of neuropathic pain behaviours, which may involve the development of altered C-fibre terminal distribution, changes in descending inhibition or sympathetic innervation and ectopic discharge in nociceptive afferents (Lee and Chung, 1996). A recent study further indicated that mechanical allodynia is not detectable at 4 weeks of age in rats that had been subjected to a neuropathic pain injury (SNI, spared nerve

injury) at ages younger than 3 weeks (Howard *et al.*, 2005). Surprisingly, postnatal day 10 rats that are subjected to chronic constriction injury (CCI), which evokes a significant mechanical allodynia in adult rats, are reported to show no change in nociceptive sensitivity compared to the sham group (Howard *et al.*, 2005). A histochemical study has found that following neonatal peripheral nerve injury adjacent intact myelinated and unmyelinated primary afferents sprout into the central denervated terminal area in the dorsal horn of adult animals (Shortland and Fitzgerald, 1994). Neonatal changes in nociceptive circuitry may occur following peripheral nerve injury although this area is largely unexplored. Enhanced responsiveness of dorsal horn neurons that can be observed following early life injury may translate into a long-lasting facilitated response to noxious stimulation.

The level of expression of excitatory glutamate receptors in the superficial laminae of spinal dorsal horn may be important in determining the alterations in behaviours induced by early life injury. There is some evidence for expression of AMPA and NMDA receptor subunits in spinal dorsal horn changing through postnatal development on a long-term basis. The expression of GluA1-4 mRNA in lamina II of the dorsal horn in rats is significantly reduced to varying degrees during postnatal development as assessed by immunoblotting and immunohistochemistry (Jakowec *et al.*, 1995b). With regard to NMDA receptor subunits, diminished expression of GluN1 and GluN2B and slightly increased GluN2A mRNA expression in spinal dorsal horn of rats was detected by *in situ* hybridisation during postnatal development (Stegenga and Kalb, 2001). However, it is largely unknown whether the normal expression of the glutamate receptor subunits is disturbed by early life injury or not.

3.1.4 Activation of the Ras/MAPK pathway in pain states

If lasting changes in nociceptive responsiveness following early life injury were to involve the trafficking and synaptic insertion of AMPA receptors, in particular those containing GluA1 subunits (Hayashi *et al.*, 2000; Shi *et al.*, 2001; Makino and Malinow, 2009), the signals responsible may, at least in part, correspond to those

reported in events of neuronal plasticity in other parts of the CNS. In hippocampal neurons, NMDA receptors and CaMKII are important in driving synaptic delivery of AMPA receptors during LTP (Hayashi *et al.*, 2000) and the contributions of both are dependent on the small G protein, Ras (Zhu *et al.*, 2002; Kim *et al.*, 2005). Ras is localised in the inner face of the plasma membrane and controls a variety of intracellular signalling cascades, which regulate cellular proliferation, differentiation, growth, and survival. Ras is highly expressed in the developing and adult nervous system where it acts as a master regulator of growth factor signalling to downstream ERK MAP kinase cascades (Nakafuku *et al.*, 1992; Seger and Krebs, 1995). Moreover, it can also be activated by membrane depolarisation and voltage-dependent Ca^{2+} influx such as that due to NMDA receptor activation (Rosen *et al.*, 1994; Zhu *et al.*, 2002; Kim *et al.*, 2005) and plays a key role in the regulation of neuronal plasticity and memory formation (Martinez and Derrick, 1996). Thus the Ras/ERK pathway may play a role in modulation of nociceptive transmission in spinal neurons during chronic pain states or following early life injury.

The activation status of Ras is controlled by GTP-exchange factors (GEFs), notably those of the Sos and Ras-GRF families, both of which can be activated through Ca^{2+}-dependent processes (Farnsworth *et al.*, 1995; Takai *et al.*, 2001). Prior to cytosolic Ca^{2+} elevation or growth factor stimulation, Ras binds GDP in its inactive conformation. In addition to Ca^{2+} activation of the Ras-GEFs, Ca^{2+} influx through NMDA receptors or L-type voltage-dependent channels induces a rapid tyrosine phosphorylation of epidermal growth factor receptors (EGFRs) and increases the association of the EGFR with Grb2, an adapter protein, that binds and enables activation of Sos (Rosen and Greenberg, 1996). The Ca^{2+}-dependent kinase that mediates EGFR phosphorylation and activates the Ras/ERK cascade is unknown, but could be tyrosine kinases Pyk2 ONLY ONE SPACE HERE LITING NOT TWOor Src (Lev *et al.*, 1995; Rusanescu *et al.*, 1995). Sos is thought be the major Ras-GEF for Ras activation in neonatal animals and differential changes in Sos and Ras-GRF expression occur through early life development (Tian *et al.*, 2004), so the respective roles of these Ras-GEFs in ERK activation in hypersensitive pain responses may differ between neonates and adults.

MAPKs such as ERK are important for intracellular signal transduction and play critical roles in regulating neural plasticity and inflammatory responses. The MAPK family consists of three major and distinct subgroups: ERK, p38 and c-Jun N-terminal kinase (JNK). Activation of MAPKs under different persistent pain conditions results in the induction and maintenance of pain hypersensitivity via transcriptional and post-translational regulation. ERK itself is a major regulator of gene transcription with important additional post-translational roles in neuronal plasticity and development (Seger and Krebs, 1995; Impey *et al.*, 1999; Widmann *et al.*, 1999; Sweatt, 2001). In particular, pain-induced ERK activation in spinal cord dorsal horn neurons (via multiple neurotransmitter receptors, and using different second messenger pathways) plays a critical role in central sensitisation by regulating the activity of glutamate receptors and potassium channels and inducing gene transcription (Ji *et al.*, 1999; Ji *et al.*, 2002; Kawasaki *et al.*, 2004). ERK was shown to be activated (phosphorylated by the upstream kinase MEK) in response to noxious chemical, thermal or electrical stimuli and the spinal administration of MEK inhibitors attenuated nociceptive behavioural responses. Interestingly, in the sensitised state following nerve injury even low threshold electrical stimulation elicits ERK activation in dorsal horn neurons (Wang *et al.*, 2004). The magnitude of ERK activation in response to a set stimulus may therefore provide a measure of the degree of central sensitisation that is present.

3.2 Results

3.2.1 Behavioural tests after CCI in neonatal animals

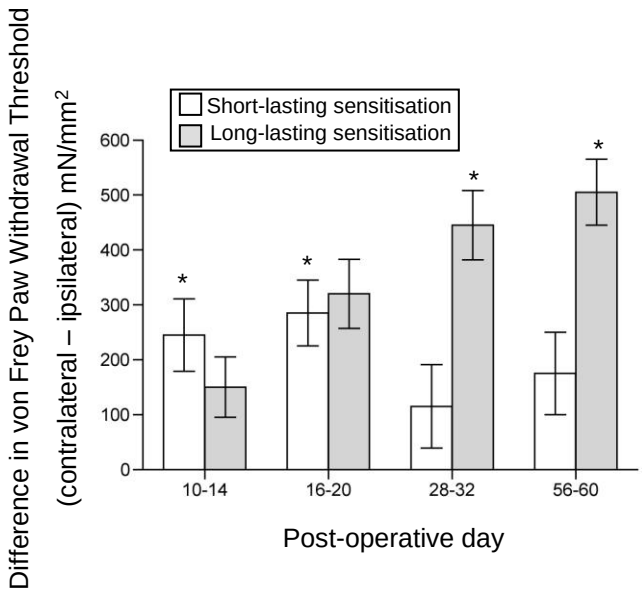
CCI surgery was performed in the right hind limbs of postnatal day 8 (P8) rats in a series of experiments carried out jointly with Hayley Gooding and Andrew Allchorne. The procedure was modified from that used for adult animals (Garry *et al.*, 2003b; Proudfoot *et al.*, 2006) as outlined in Chapter 2. Pain-related responses reflecting mechanical allodynia were assessed using von Frey filaments. The paw withdrawal threshold (PWT, mN/mm^2) of both ipsilateral and contralateral sides was recorded from post-operative day 10 up until 60. As in adults, no marked changes in von Frey

PWT were seen on the contralateral side whereas consistent reductions developed in ipsilateral PWT values, so the data were presented as the difference between contralateral and ipsilateral value. The sensitisation of mechanical reflex withdrawal responses caused by CCI is similar between males and females (previous unpublished observations within the laboratory). Here, the difference between contralateral and ipsilateral PWT is shown in females, which were the animals available for this study (Figure 3.2).

Figure 3.2: Mechanical sensitisation in rats following CCI at postnatal 8 day (P8)

Time-courses of mechanical sensitisation (PWT, mN/mm^2) to calibrated von Frey filament testing were assessed following CCI surgery at P8. Data are plotted as mean ± SEM difference (Contralateral-Ipsilateral) in PWT from calibrated von Frey filaments. In each case, the statistical significance of differences between ipsilateral and corresponding contralateral responses is indicated as *: $p<0.05$ (Mann-Whitney Rank Sum Test on raw data). The white columns show results from the subgroup of animals (approximately half of the cohort) that showed rapid recovery from ipsilateral sensitisation, which displayed statistically significant ipsilateral: contralateral differences between days 10-20 following surgery (n=11). The grey columns show results from the subgroup of animals that displayed longer lasting sensitisation (n=9), which was statistically significant and continued to increase from beyond day 20 following surgery up to day 60 when the experiment was terminated. This subdivision of the cohort could be identified not only by the duration of sensitisation but also by an abnormal postural presentation of the affected hind paw in the long-lasting sensitised animals. An unusual flexed posture of the paw was observed, which correlated with the appearance of long-lasting hypersensitivity. It was considered that the most likely explanation for this was that the severity of the nerve injury had been greater in these cases (but the underlying basis was not further investigated here). The reflex response measurements were made by Hayley Gooding and Andrew Allchorne with my assistance, as described in detail in Section 2.1.1.

3.2


Short-lasting sensitisation
Long-lasting sensitisation
Difference in von Frey Paw Withdrawal Threshold
(contralateral – ipsilateral) mN/mm2
600
500
400
300
200
100
0
*
*
*
*
10-14
16-20
28-32
56-60
Post-operative day

Interestingly, two different profiles of behavioural reflex sensitisation were observed in the P8 CCI animals. Approximately half of them showed short-lasting mechanical sensitisation (significant ipsilateral-contralateral differences in PWT) through post-operative days 10-20 and then recovered by day 30, beyond which there were no longer significant differences in PWT. The other half of the animals showed long-lasting mechanical sensitisation that appeared to develop more slowly and reach a greater magnitude. Statistically significant ipsilateral:contralateral differences were seen between post-operative days 28-60 that appeared to be continuing to increase even as the experiment was terminated at 60 days. The long-lasting group, in every case, also displayed postural abnormality in the relevant paw that was not seen in the short-lasting group. This presented as abnormal flexion of the paw and was interpreted as most likely being due to greater severity of injury in these animals. The underlying basis was not further investigated here. Nevertheless, these findings suggested that different molecular events evoked by peripheral nerve injury may occur in the rapidly recovering and slowly recovering series of P8 CCI rats. In the present study, I only focus on the recovered CCI rats in order to address the hypothesis that there may be an enduring hypersensitivity to subsequent challenges even after apparent resolution of the neonatal injury-induced pain state.

3.2.2 Behavioural assessment of formalin responses in animals that received neonatal CCI and had subsequently recovered

To assess the responsiveness of rats that had recovered from early life nerve injury to a noxious challenge in later life, female animals of age P42 that had either received early postnatal (CCI) nerve injury at P8 or controls were given a unilateral hindpaw intraplantar injection of formalin or saline under brief isoflurane anaesthesia. CCI induced mechanical allodynia around the time of injury in early life but in the cohort of animals tested here, von Frey paw withdrawal thresholds had fully recovered to the normal range before formalin testing at P42. Formalin-induced nocifensive behaviours exemplified by paw shaking, flicking and lifting were observed for 60 minutes. At 5 minute intervals the duration of paw shakes, flicks or lifts occurring in

1 minute was recorded using a stop-watch by the same observer who was blinded to the treatment history of the animals.

Interestingly, formalin-induced inflammation in the hind paw triggered a greater nocifensive behavioural response in the early life injury rats even though, in terms of von Frey PWT scores, they had apparently fully recovered from a hypersensitive state. The number of nocifensive behaviours in both pre-treated (n=8) and näive (n=15) rats showed a similar profile following formalin injection with a slow increase, a peak for few minutes, and a decrease back to normal levels. Notably, in the peak stage of response to formalin (25-35 minutes following injection) the number of nocicfensive behaviours in the rats that had received early life nerve injury was significantly greater than that in age-matched controls (Figure 3.3). This result indicated that when the nerve-injured neonates grew to adulthood, they were more sensitive to noxious stimuli such as formalin challenge. This implies that the neonatally nerve-injured rats that had apparently recovered at the level of acute behavioural reflexes may still have long-lasting or permanent alterations in the sensitivity of nociceptive processing. In the latter stages of the formalin response of neonatally injured animals there was a trend to lower nocifensive scores compared to age-matched controls but this did not reach statistical significance. It is possible that a more extensive study would expose a significant effect, which might reflect greater induction of endogenous analgesic processes in addition to the hypersensitivity seen in peak formalin responses. The animals available for this study were females, which provided an advantage in that their formalin nocifensive scores are consistently greater than in males, allowing easier discrimination of changes in sensitivity. The underlying basis for the difference is not known but it is consistently observed in this and other labs (Fleetwood-Walker, unpublished observations; Yaksh, T.L, personal communication). Animals that were uninjected or injected with saline displayed minimal nocifensive behaviour.

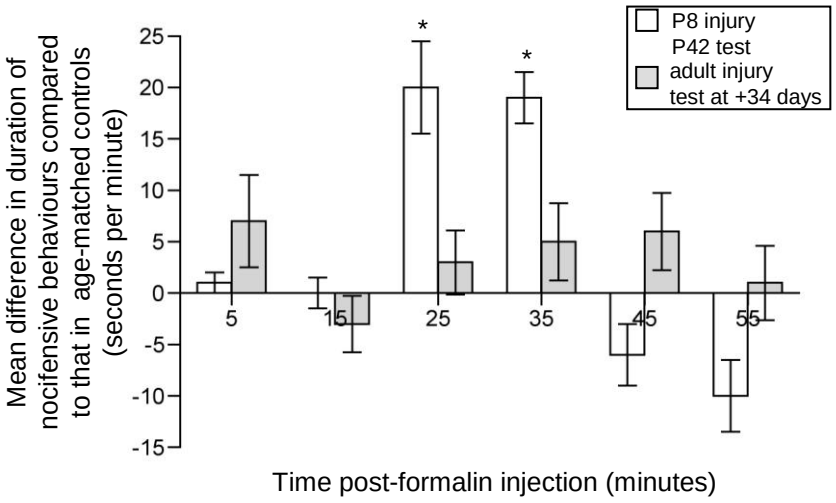
When adult rats were subjected to CCI and then tested with formalin 34 days later (equivalent to the P8/P42 time interval) they showed a broadly similar profile of nocifensive responses, with a peak around 25-35 minutes after injection. This profile is consistent with observations from this and other labs, which administer formalin

under brief anaesthesia and displays only the slowly developing peak of nocifensive behaviours without an initial acute pain response. The nocifensive response profile in previously CCI-injured adults (that had recovered normal von Frey PWT values) showed no discernible difference in nocifensive behaviour scores at any stage of the response, when compared to uninjured age-matched controls. Thus the lasting hypersensitivity to a subsequent noxious challenge was seen only in animals that received nerve injury in the neonatal period, not in adulthood.

Figure 3.3: Formalin-induced nocifensive behaviours are increased in recovered neonatally-injured animals compared to controls but not in recovered adult-injured animals

Time-courses of differences in duration of nocifensive (spontaneous paw-shaking, flicking and lifting) behaviours (seconds per minute) between CCI-injured animals and age-matched controls following intraplantar injection of formalin under brief isoflurane anaesthesia. Animals were tested at 34 days following injury, or equivalent for controls, at which time the sensitisation of von Frey PWT values had recovered to normal. Female animals were used for these experiments because of their availability and consistently greater nocifensive responses to formalin, which facilitated discrimination of any changes in responsiveness. In all cases nocifensive behaviours increased to a peak around 25-35 minutes following formalin injection and then declined. Uninjected or saline-injected animals displayed minimal nocifensive behaviour. White columns display the differences in time-course of nocifensive behaviour between P8-injured animals (n=8) and age-matched controls (n=15) when formalin-injected at P42. Grey columns display the differences in time-course of nocifensive behavior between adult-injured animals (n=8) and age-matched controls (n=8) when formalin-injected 34 days later. Data showing the difference in nocifensive scores between injured and control groups are plotted as mean ±SEM. The statistical significance of differences between nerve-injured animals and corresponding controls was assessed by Student's unpaired t-test on the raw nocifensive score data (* indicates $p<0.05$). The reflex response measurements were made by Hayley Gooding and Andrew Allchorne with my assistance as described in detail in Section 2.1.1.

3.3


P8 injury
P42 test
adult injury
test at +34 days
Mean difference in duration of nocifensive behaviours compared to that in age-matched controls (seconds per minute)
25
20
15
10
5
0
-5
-10
-15
*
*
5
15
25
35
45
55
Time post-formalin injection (minutes)

3.2.3 Assessment of GluA1 expression in postsynaptic density of dorsal horn in animals that received early life nerve injury at P8 and a subsequent acute noxious challenge at P42

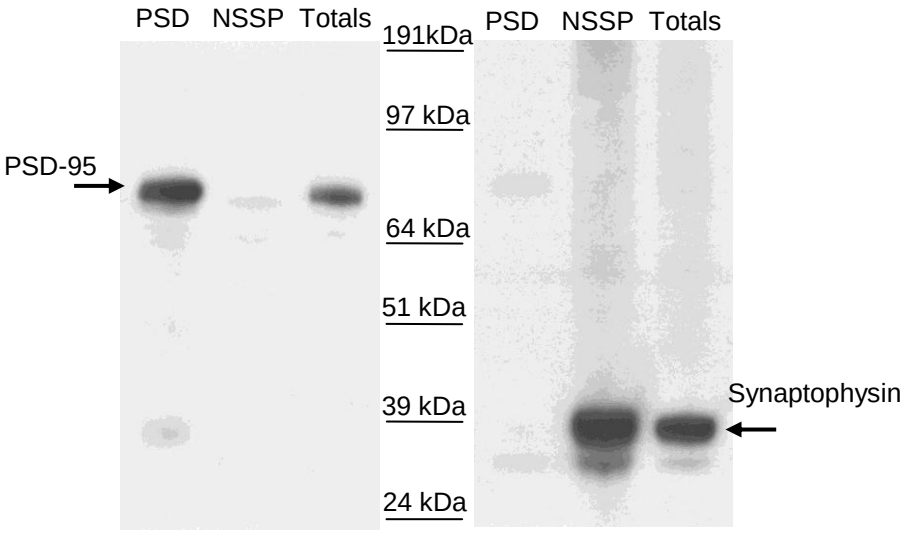
In order to optimise the procedure for preparation of postsynaptic density (PSD) and non-synaptic synaptosomal proteins (NSSP), a number of pilot experiments were carried out using forebrain and näive adult spinal cord tissue. Several minor procedural changes were made to the published methods for rapid Percoll/sucrose gradient separation of synaptosomes and for subsequent ultrafractionation of PSD and NSSP. This was essential before attempting to apply this kind of approach to the very small tissue samples of microdissected ipsilateral and contralateral dorsal horn following various pain-related interventions – a strategy that to my knowledge has not been attempted previously. This approach was especially challenging in young animals where the amounts of tissue are considerably smaller than in adults. A number of variants of the final protocol were explored, including for example attempts to separate pre- and post-synaptic components of the NSSP fraction using different pH buffers (Feligioni *et al.*, 2006) but were rejected because of inconsistent recovery of marker proteins with the very small tissue samples involved here. The final selected protocol was validated using näive adult spinal cord by probing the PSD and NSSP fractions for the marker proteins PSD-95 and synaptophysin, respectively, using immunoblot.

The PSD marker protein PSD-95 was very selectively recovered from the PSD fraction and not the NSSP fraction, whereas the synaptophysin overwhelmingly presented in the NSSP fraction of both frontal cortex and spinal cord of näive adults (Figure 3.4). This indicated that the two subcellular fractions of dorsal horn were successfully isolated by the final optimised protocol. GluA1 and a key GluA1-interacting protein, 4.1N, were highly concentrated in PSD rather than NSSP fractions of näive adult spinal cord (Figure 3.4).

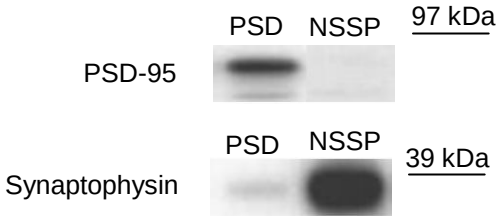
Figure 3.4: Validation of the protocol for preparation of postsynaptic density (PSD) and non-synaptic synaptosomal protein (NSSP) fractions and identification of GluA1 and 4.1N expression in the PSD fraction.

Marker proteins for subcellular fractions were probed in both the PSD fraction and the NSSP fraction prepared from CNS regions of naïve adult rats. Following SDS-PAGE electrophoresis, proteins were blotted to PVDF membrane and probed with specific anti-PSD-95 and anti-synaptophysin antibodies and HRP-conjugated secondaries before visualisation by enhanced chemiluminescence and X-ray film. PSD-95 is a postsynaptic marker, whereas synaptophysin is a marker for non-synaptic synaptosomal proteins and is localised to presynaptic terminals. Highly selective recovery of PSD-95 and synaptophysin in their predicted subcellular fractions from a) frontal cortex and b) spinal cord, served to validate the final optimised protocol before its use with microdissected tissues from animals with pain state interventions. c) Shows the relative distribution of GluA1 and a key GluA1-interacting protein, 4.1N, between PSD and NSSP fractions of spinal cord from naïve adult rats. Both GluA1 and 4.1N would be predicted to localise predominantly in the postsynaptic fraction and may be largely associated with the PSD. These data indicate that both GluA1 and 4.1N are highly concentrated in PSD compared to NSSP fractions.

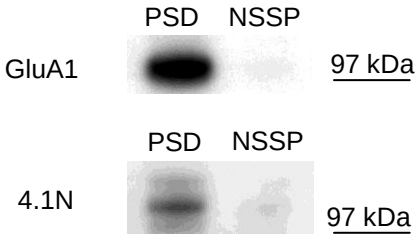
3.4 a) Frontal cortex (näive adult): marker proteins



3.4 b) Spinal cord (näive adult): marker proteins



3.4 c) Spinal cord (näive adult): AMPA receptor-related proteins



Following my successful validation of the subcellular preparation protocol, the PSD fractions were isolated from ipsilateral and contralateral dorsal quadrants of L4-L6 spinal cord in näive P42 rats (N), näive P42 rats with formalin challenge (N + F), P8 nerve-injured rats recovered at P42 and injected with saline (CCI) and P8 nerve-injured rats with formalin challenge at P42 (CCI + F) following the behavioural experiments as appropriate. The isolated PSD fractions were probed by immunoblot for content of GluA1 and actin (control protein for normalisation) (Figure 3.5). Female rats were used for these experiments because of their more robust nocifensive response profile. All animals that received early life nerve injury displayed initial mechanical sensitisation to von Frey filaments but had recovered to normal range PWT values before P42.

PSD fractions from dorsal horn ipsilateral to formalin injection in animals that had received early life nerve injury (CCI + F) showed a significantly greater GluA1:actin ratio than those from contralateral dorsal horn, as did those from formalin-treated animals that had not received early life injury (N + F) ($p<0.05$, Ratio t-test, n=4; Figure 3.5a). Animals that had received early life nerve injury but saline instead of formalin (CCI) did not show a significant side difference in GluA1:actin ratio. The group means for ipsilateral and contralateral values of GluA1:actin ratio are shown in Figure 3.5a, below typical example blots. The Ratio t-test is recommended where comparing the relative values of two measures (here the GluA1 and actin image density ratios for ipsilateral:contralateral sides from the same blots) might be predicted to provide a more appropriate assessment than a comparison of absolute values (Motulsky, 2003). This test further avoids any requirement for normal distribution of data sets. When individual ipsilateral:contralateral ratio values for GluA1 normalised to actin were compared (Figure 3.5b), those for the CCI + F group were significantly greater than those for the N + F group ($p<0.05$, Student's unpaired t-test, n=4). This finding demonstrates that formalin induced significantly greater incorporation of GluA1 subunits into the PSD of dorsal horn in animals that had received nerve injury in early life but had apparently recovered. Thus a long-lasting susceptibility to increased biochemical changes that could explain hypersensitivity in subsequent noxious challenges appears to have been set up by early life nerve injury.

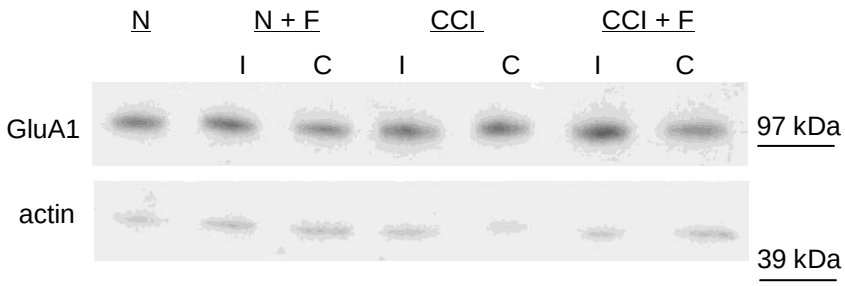
Figure 3.5: Early life nerve injury facilitates formalin-induced increases in GluA1 content in the PSD fraction in ipsilateral dorsal horn.

Immunoblots for GluA1 and actin (for normalisation of protein loading) in PSD fractions were prepared from ipsilateral (I) and contralateral (C) spinal dorsal horn of rats following various treatments: N: Näive at P42; N+F: Näive with formalin injection at P42; CCI: P8 CCI with saline injection at P42; CCI+F: P8 CCI with formalin injection at P42. Quantitative densitometry analysis of the results from 4 separate experiments was carried out using Image-J software.

a) Shows representative blots for GluA1 and actin from a typical example gel together with quantitative analysis of GluA1:actin ratios in each case. Data are shown as mean ± SEM (n=4). Statistical significance of differences in ipsilateral:contralateral ratios for GluA1 normalised to actin was assessed using the Ratio t-test, Section 2.4.2 (* $p<0.05$). Both N + F and CCI + F groups showed statistically significant increases in relative GluA1:actin content of PSD ipsilateral to formalin injection compared to the contralateral side.

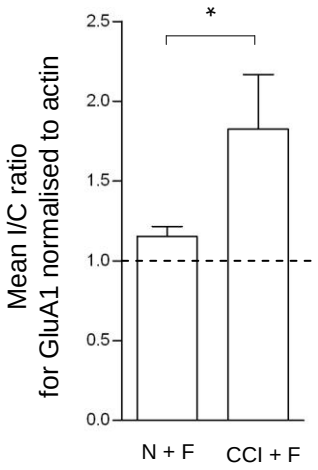
b) Shows a comparison of ipsilateral:contralateral ratios for GluA1 normalised to actin from each individual data set for the N + F and CCI + F groups. Data are shown as means ± SEM (n=4). Statistical analysis by Student's unpaired t-test indicated that the values for ipsilateral:contralateral ratio of GluA1 normalised to actin were significantly greater in the CCI + F group than in the N + F group (* $p<0.05$, n=4).

3.5 a)



GluA1:actin (n=4)	N	N + F		CCI		CCI + F	
		I	C	I	C	I	C
Mean	1.16	1.55	1.32	1.44	2.00	2.09	1.06
SEM	0.18	0.22	0.18	0.22	0.54	0.39	0.14
		*		ns		*	

3.5 b)



3.2.4 Assessment of phospho-ERK and Sos-1 expression in subcellular fractions of dorsal horn in animals that received early life nerve injury at P8 and a subsequent acute noxious challenge at P42

In order to further examine the intracellular signalling events that may be changed by early life nerve injury and potentially contribute to enabling both enhanced GluA1 translocation to PSD and also amplified nocifensive responses to later life noxious stimuli, phosphorylated (activated) ERK in the NSSP fraction and Sos-1 in the PSD fraction were assessed using immunoblot.

Animals of age P42 that had received either early life (P8) CCI nerve injury or age-matched controls were given a unilateral intraplantar injection of formalin. Full recovery from early life injury was achieved as assessed by von Frey paw withdrawal thresholds before formalin testing at P42. Following formalin injection (or saline control) both PSD and non-synaptic synaptosomal protein (NSSP) fractions were prepared from ipsilateral and contralateral dorsal quadrants of L4-L6 spinal cord and then probed by immunoblot for phospho-ERK (activated state), Sos-1 and actin (normalisation control protein).

NSSP fractions from dorsal horn ipsilateral to formalin injection in animals that had received early life nerve injury (CCI + F) showed a significantly greater phospho-ERK:actin ratio than those from contralateral dorsal horn ($p<0.05$, Ratio t-test, n=3; Figure 3.6a). Animals that received formalin but not early life nerve injury (N + F) or those that received a saline injection following early life nerve injury (CCI) did not show statistically significant differences in phospho-ERK:actin ratios between ipsilateral and contralateral sides. The group means for values of phospho-ERK:actin ratio are shown in Figure 3.6a below typical example blots. Similar but less clear-cut results were observed in the PSD fraction. When individual ipsilateral:contralateral ratio values for phospho-ERK normalised to actin were compared (Figure 3.6a), those for the CCI + F group were greater than those for the N + F group but the values were not significantly different (Student's unpaired t-test, n=3; Figure 3.6b).

The addition of further replicate observations may achieve a statistically significant result. In pilot experiments, evidence was provided for increased expression of the Ras-GEF, Sos-1 (a key regulator of ERK pathway activation) in the PSD following early life nerve injury (in both CCI and CCI + F groups; Figure 3.6c). Sos-1 expression relative to actin appeared to be increased on both ipsilateral and contralateral sides following earlier unilateral nerve injury and may have been further increased ipsilateral to formalin in the CCI + F group. This provides preliminary evidence to suggest that a key regulator of the ERK pathway shows increased expression in dorsal horn lasting over a prolonged period following early life nerve injury despite the recovery of von Frey thresholds to the normal range, but further work would be needed to corroborate this initial finding.

Figure 3.6: Early life nerve injury facilitates formalin-induced increases in phospho-ERK content of the NSSP fraction in ipsilateral dorsal horn and elicits apparently enduring increases in expression of Sos-1 in the PSD fraction.

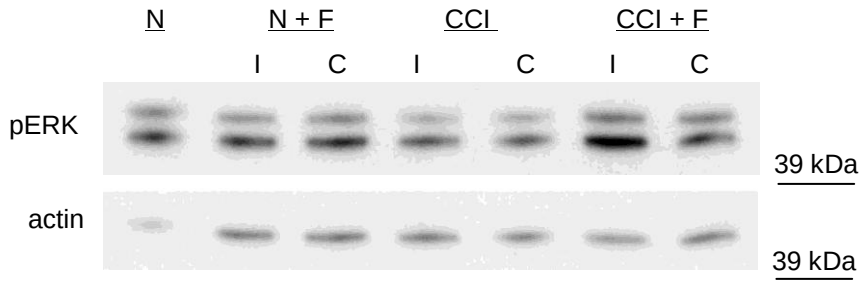
Immunoblots for phospho-ERK and actin (for normalisation of protein loading) in NSSP and PSD fractions were prepared from ipsilateral (I) and contralateral (C) spinal dorsal horn of rats following various treatments: N: Näive at P42; N+F: Näive with formalin injection at P42; CCI: P8 CCI with saline injection at P42; CCI+F: P8 CCI with formalin injection at P42. Quantitative densitometry analysis of the results from 3 separate experiments was carried out using Image-J software.

a) Shows representative blots for phospho-ERK and actin from a typical example gel together with quantitative analysis of phospho-ERK:actin ratios in each case. Data are shown as mean ± SEM (n=3). Statistical significance of differences in ipsilateral:contralateral ratios for phospho-ERK normalised to actin was assessed using the Ratio t-test, Section 2.4.2 (* $p<0.05$). The CCI + F group alone showed a statistically significant increase in relative phospho-ERK: actin content of NSSP ipsilateral to formalin injection compared to the contralateral side.

b) Shows a comparison of ipsilateral:contralateral ratios for phospho-ERK normalised to actin from each individual data set for the N + F and CCI + F groups. Data are shown as means ± SEM (n=3). Statistical analysis by Student's unpaired t-test indicated that the values for ipsilateral:contralateral ratio of phospho-ERK normalised to actin were not significantly greater in the CCI + F group than in the N + F group, given the current number of replicate observations.

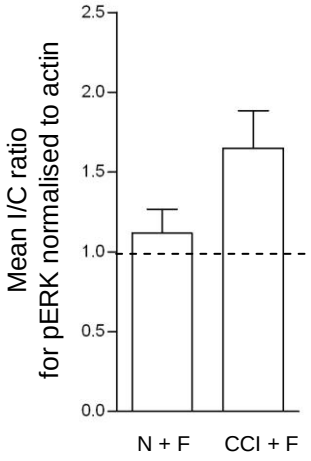
c) Shows representative blots for Sos-1 and actin in the PSD fraction from pilot experiments. These suggested that a long-lasting increase in Sos-1 expression was caused on both ipsilateral and contralateral sides of spinal dorsal horn following early life nerve injury and that this may have been further increased ipsilateral to formalin injection in the CCI + F group. Further experiments would be required to corroborate this finding.

3.6 a)

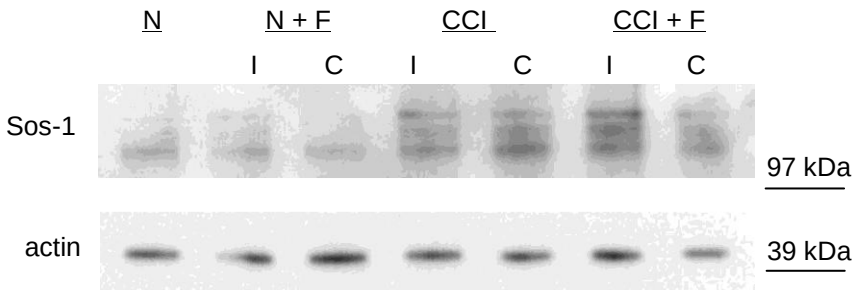


pERK:actin (n=4)	N	N + F		CCI		CCI + F	
		I	C	I	C	I	C
Mean	1.33	0.80	0.71	1.20	1.26	1.09	0.76
SEM	0.50	0.49	0.32	0.15	0.20	0.35	0.14
		ns		ns		*	

3.6 b)



3.6 c)



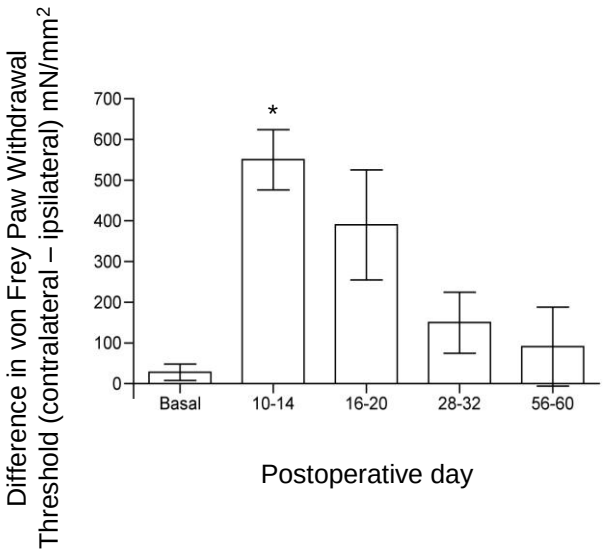
3.2.5 Assessment of behavioural reflex hypersensitivity and associated biochemical changes after CCI in adult rats

In order to investigate whether equivalent events contribute to neuropathic pain mechanisms in adult animals, CCI surgery was performed in right hindlimbs of adult rats. These animals were assessed initially by testing for mechanical allodynia using von Frey filaments. The paw withdrawal threshold (PWT, mN/mm^2) of both ipsilateral and contralateral sides was recorded for 60 days (Figure 3.7). As there were no marked changes in PWT on the contralateral side, the data were plotted as the difference between contralateral and ipsilateral values. In a cohort of 6 adult CCI rats, assessed in joint experiments with Hayley Gooding and Andrew Allchorne, significant differences between contralateral and ipsilateral PWT values were observed at post-operative days 10-14 ($p<0.05$, Mann-Whitney Rank Sum Test). The lowered ipsilateral mechanical threshold had recovered to normal levels from post-operative days 28-60. The data show that CCI injury can reduce the paw withdrawal threshold and cause mechanical allodynia in adult rats. However, in contrast to the two subgroups of response in P8-injured rats (either short or long-lasting; Figure 3.2), all adult CCI animals showed rapid recovery from the hypersensitive state. This might suggest that there is the possibility of accessing some different molecular signalling events during CCI in neonates that are no longer available in adults.

Figure 3.7: Sensitisation of mechanical reflex withdrawal responses in adult rats following CCI

The time-course of mechanical sensitisation (Paw Withdrawal Threshold; PWT, mN/mm^2) to von Frey filament testing was assessed following CCI surgery in adults. Data are plotted as mean±SEM difference (Contralateral-Ipsilateral) in PWT from calibrated von Frey filaments (*$p<0.05$, Mann-Whitney Rank Sum Test in the period 10-14 days following CCI, n=6). The reflex response measurements were made by Hayley Gooding and Andrew Allchorne with my assistance as described in detail in Section 2.1.1.

3.7


Difference in von Frey Paw Withdrawal Threshold (contralateral – ipsilateral) mN/mm²
700
600
500
400
300
200
100
0
*
Basal
10-14
16-20
28-32
56-60
Postoperative day

3.2.6 Measurement of nerve injury-induced changes in GluA1 content of postsynaptic density from dorsal horn of adult CCI rats

The PSD fractions were isolated from ipsilateral and contralateral dorsal quadrants of L4-L6 spinal cord from nerve-injured rats at post-operative day 10; the time point at which the greatest behavioural responses were detected. The dorsal horns of näive rats (bilaterally combined) were used to prepare PSD fractions for controls. All animals were within the weight range 180-250 g and their normal rate of weight gain was not discernibly affected by nerve injury. Proteins within the PSD fractions from these animals were separated by SDS-PAGE and then probed by immunoblot for content of GluA1 and actin (control protein for normalisation).

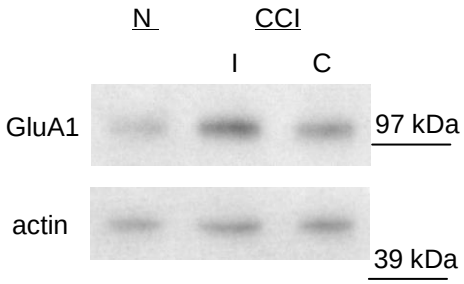
Figure 3.8 shows that PSD fractions from dorsal horn of animals ipsilateral to peripheral nerve injury showed a significantly greater GluA1:actin ratio than the contralateral side ($p<0.01$, Ratio t-test, $n=3$). This finding showed that peripheral nerve injury induced an increased GluA1 subunit content in the PSD of ipsilateral dorsal horn in adult animals.

Figure 3.8: CCI nerve injury induces increased GluA1 content in the PSD fraction of ipsilateral dorsal horn in adult animals.

Immunoblots for GluA1 and actin (for normalisation of protein loading) in PSD fractions were prepared from ipsilateral (I) and contralateral (C) spinal dorsal horn of adult CCI rats and naïve (N) controls. Quantitative densitometry analysis of the results from 3 separate experiments was carried out using Image-J software.

The Figure shows representative blots for GluA1 and actin from a typical example gel, together with quantitative analysis of GluA1:actin ratios in each case. Data are shown as mean ± SEM (n=3). Statistical significance of differences in ipsilateral:contralateral ratios for GluA1 normalised to actin was assessed using the Ratio t-test, Section 2.4.2 (** $p<0.01$). This test is recommended where comparison of the relative values of two measures might be predicted to provide a more appropriate assessment than a comparison of absolute values (Motulsky, 2003). Here this is indicated when comparing relative GluA1:actin ratios for the individual data sets, which were each run on a single gel, as such relative values will provide a more appropriate between-gel comparison than absolute grey scale values which will vary from gel to gel. Adult CCI animals showed statistically significant increases in relative GluA1:actin content of PSD ipsilateral to formalin injection compared to the contralateral side.

3.8



GluA1:actin (n=4)	N	CCI	
		I	C
Mean	0.86	1.62	1.11
SEM	0.05	0.10	0.05

**

3.2.7 Measurement of changes in GluA1 association with its interacting protein, 4.1N in adult spinal cord following CCI

Any changes in direct protein:protein interactions between GluA1 and its interacting protein/trafficking chaperone, 4.1N were investigated by co-immunoprecipitation and subsequent immunoblot for captured proteins in the spinal cord of adult CCI animals or naïve controls. Tissue samples were obtained from CCI animals at post-operative day 10 when the greatest sensitisation of mechanical responses was detected.

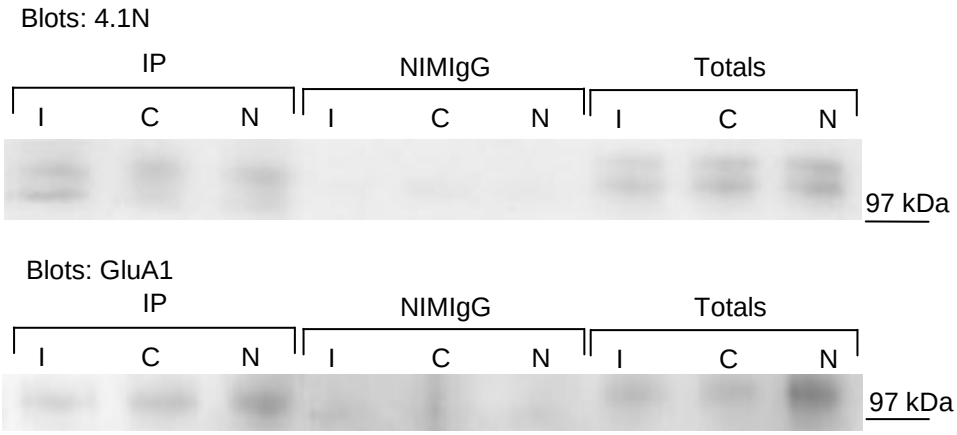
Following GluA1-directed immunoprecipitation, greater levels of its interacting protein, 4.1N, appeared to be captured ipsilateral to CCI (Figure 3.9a). These immunoprecipitations were carried out by Emer Garry as part of our joint experimental programme. I subsequently carried out a number of experiments to assess whether a reciprocal immunoprecipitation procedure, which used 4.1N as a pull-down target and evaluated capture of associated GluA1, was feasible. Although the 4.1N antibody used for Western blot detection was unsuitable for native immunoprecipitation, an immunoprecipitating 4.1N antiserum was sourced from Millipore. Despite a variety of optimisation steps including chemical crosslinking to protein G-Sepharose beads and extensive clean-up procedures, the reagent retained too much non-specific binding to allow demonstration of specific GluA1 capture associated with 4.1N pulldowns. The amounts of material required for co-immunoprecipitation of such proteins expressed at low levels precluded such experiments being carried out on the subcellular fractions. Thus, it was not possible to directly evaluate the synaptic or extrasynaptic location of the GluA1:4.1N complexes. However, I had been able to establish in Figure 3.4c that both GluA1 and 4.1N were expressed in a highly selective fashion in PSD rather than NSSP fractions of näive adult spinal cord. The detailed subcellular localisation of 4.1N within spinal cord neurons remains unclear as the available antibodies also proved unsuitable for immunohistochemistry. Current evidence suggests that 4.1N may play a part in the trafficking of GluA1-containing AMPA receptors towards synapses or help to anchor

them into place within synapses in order to bring about excitation at greater levels. My data suggest that GluA1:4.1N interactions and hence possibly also these processes may occur to a greater extent following nerve injury in adults. It is possible that similar events to these may occur following nerve injury in neonates but this was not investigated in the present study. In a further series of immunoblot experiments I addressed the developmental time-course of GluA1 and 4.1N expression in spinal cord. Figure 3.9b shows that expression of GluA1 increased from P9 to P28 whereas 4.1N showed the inverse profile of decreasing expression. However, both of these proteins appeared to show increased expression ipsilateral to P8 CCI when assessed at P28. These findings indicate that GluA1 and 4.1N appear to show reciprocal time-courses of developmental expression in spinal cord through the early postnatal period but also that expression of each is increased ipsilateral to injury in animals that received early life CCI.

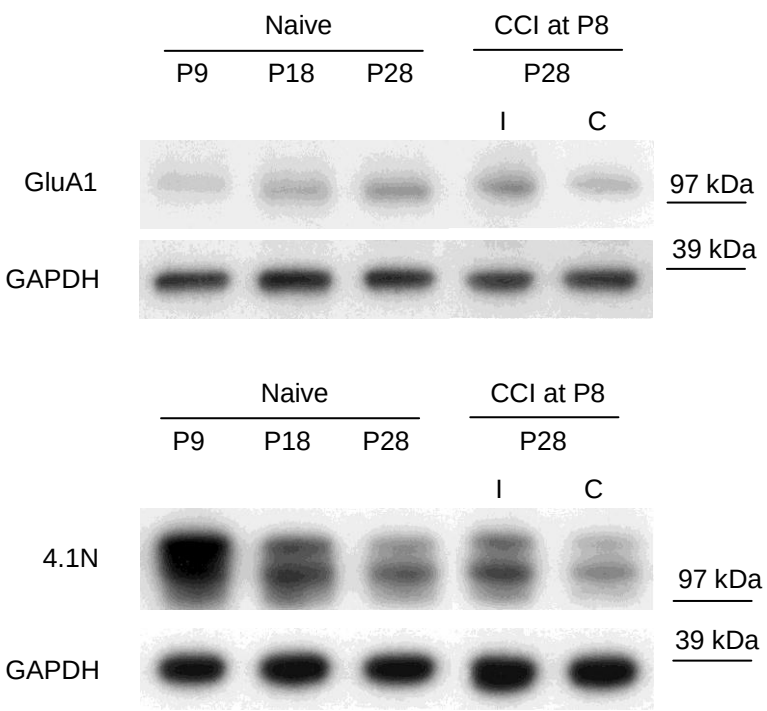
Figure 3.9: GluA1 and 4.1N show increased co-immunoprecipitation in adult spinal cord ipsilateral to CCI and nerve injury-induced expression in neonates despite distinct developmental time-courses of expression.

Immunoblots for GluA1, 4.1N and GAPDH (for normalisation of protein loading) were carried out on extracts from spinal cord ipsilateral (I) or contralateral (C) to CCI or from naïve animals (N). In a) samples from adult CCI or N animals were solubilised in a non-denaturing detergent-containing medium and immunoprecipitated using an anti-GluA1 antibody (or non-immune IgG: NIMIgG control) conjugated to protein G-Sepharose beads before washing and lysis of captured proteins in Laemmli buffer. Protein content of solubilised preparations prior to immunoprecipitation is shown in 'Total' lanes. The levels of 4.1N specifically associated with GluA1 immunoprecipitates appeared to be increased ipsilateral to CCI. b) Shows results from direct immunoblots on spinal cord lysates. Equivalence of protein loaded was monitored by immunoblotting for the constitutively expressed housekeeping enzyme, GAPDH. Expression of GluA1 appeared to increase and 4.1N to decrease through early post-natal development, although early life nerve injury at P8 appeared to selectively increase ipsilateral expression of both proteins.

3.9 a) IP: GluA1



3.9 b)



3.3 Discussion

In the presence of chronic stimulation such as inflammation or nerve injury, the CNS, in particular the spinal cord, has the protective capability to minimise injury by generating both an immediate reflex withdrawal from the harmful stimulus and an enhanced responsiveness to subsequent sensory inputs (i.e. hyperalgesia and allodynia). This hypersensitivity to further sensory inputs is maintained by changes in the peripheral and central nervous systems, which in the case of neuropathic pain states are largely dependent on central sensitisation.

Central sensitisation has been described as "an enhancement in the function of neurons and circuits in nociceptive pathways caused by increases in membrane excitability and synaptic efficacy as well as reduced inhibition and represents a manifestation of the remarkable plasticity of the somatosensory nervous system in response to activity, inflammation and neural injury" (Latremoliere and Woolf, 2009).

Mechanical allodynia is a typical feature of central sensitisation in neuropathic pain states, manifested as alterations in sensory pathways including those that do not normally mediate nociceptive inputs, such as large low-threshold myelinated mechanoreceptors, which now appear to adopt a role in mediating pain (Woolf and Salter, 2000a). In the present study, neonatally nerve-injured rats showed a significantly reduced withdrawal threshold to von Frey filaments in the hind paw ipsilateral to CCI compared to that in the contralateral paw. This demonstrated that mechanical allodynia could be elicited in very young animals by peripheral nerve injury. Behavioural reflex sensitisation evoked by CCI in adult rats has been reported widely, including by our group (Garry *et al.*, 2003a). However, the neuropathic pain mechanisms in young and adult animals may vary, as about half of the nerve-injured young animals showed much longer-lasting behavioural sensitisation. This might be due to greater severity of the lesion in the neonatal animals that displayed longer lasting sensitisation, although it should be noted that adult CCI animals never displayed such long-lasting changes. The underlying basis for the different behaviours apparent in young animals following nerve injury needs to be further investigated.

Mechanical allodynia in neuropathic pain states is attributed to alterations in the properties of dorsal root ganglion (DRG) neurons and in the responsiveness of central neurons in the spinal dorsal horn to sensory inputs. This may be because after nerve injury, at least a subpopulation of DRG cells change their phenotype and their activity is associated with tactile allodynia (Liu *et al.*, 2000). Meanwhile, further diverse alterations of spinal somatosensory processing occur, such as hypersensitivity of excitatory neurons, possible disinhibition through alterations in the influence of GABAergic interneurons, and changes in descending facilitatory influences on spinal neurons (Kim *et al.*, 1997; Suzuki *et al.*, 2004; Miraucourt *et al.*, 2009).

Spinal AMPA receptors have a well-established role in transmitting fast excitatory information under normal conditions and can contribute to acute nociceptive inputs (Yoshimura and Jessell, 1990). Injection of formalin (which may activate TRPA1 channels) is widely used as a pain model to assess the mediation and modulation of pain-related behaviours. Behavioural nocifensive responses to formalin in conscious animals contain two phases, a first acute phase for the first 10 minutes, followed by a transient decrease and a second tonic (centrally-mediated) phase from 30-60 minutes after injection (Abbott *et al.*, 1995; McNamara *et al.*, 2007). AMPA receptors in spinal cord are believed to contribute to the two phases of hypersensitivity. In the present studies, animals were briefly anaesthetised with isoflurane prior to formalin injection to avoid any additional influences of stress on response characteristics, which means that first and second phase responses are not so clearly delineated (Fleetwood-Walker, unpublished; Yaksh, T.L., personal communication). Intrathecal administration of the selective AMPA antagonist NBQX inhibited the acute phase of behavioural nociceptive response to formalin in adult rats (Hunter and Singh, 1994). Moreover, spinal AMPA receptors were shown to be crucial to the later phase behavioural response to formalin, which can be blocked by the AMPA receptor antagonist YM872 throughout development (King and Barr, 2007). In the present study, the P42 rats showed typical nocifensive behaviours including paw flinching, licking and lifting after formalin injection and the content of GluA1 subunits of AMPA receptors in the PSD of ipsilateral spinal dorsal horn was specifically increased ipsilateral to formalin stimulation, with significantly enhanced responses in animals that had received P8 nerve injury, matching behavioural hyper-

responsiveness in these conditions. Thus our results are consistent with previous reports that AMPA receptors, in particular GluA1 subunits, undergo stimulus-induced insertion into synapses and may play a role in formalin-induced pain. However, there is a controversial report that intrathecal pre-treatment with a (less-selective) AMPA receptor antagonist, CNQX, failed to reduce the formalin nociceptive responses (Coderre and Melzack, 1992). The differences may be attributable to the different specificity profiles of the drugs or a mismatch in the adequacy of doses administered.

Recent studies further support the idea that AMPA receptors in the spinal cord contribute to chronic pain. The expression of GluA2 subunits of AMPA receptors and of their associated interacting protein, GRIP in the spinal dorsal horn increased following peripheral nerve injury (Garry *et al.,* 2003b). A role of AMPA receptors in sensitised nociceptive processing is further supported by a pharmacological study which demonstrated that administration of the selective AMPA receptor antagonist NBQX abolished the mechanical allodynia and neural hyperexcitability resulting from spinal cord injury in rats (Gwak *et al.*, 2007). This accords with data from our laboratory (Figure 3.1). It has been reported that the expression of GluA1 subunits within the adult spinal dorsal horn is upregulated following nerve injury at post-operative day 14, corresponding to the presence of mechanical allodynia, but not before post-operative day 3 or after post-operative day 35 (Harris *et al.*, 1996; Lu *et al.*, 2008). In this study, half of the young rats with early life CCI injury appeared to recover rapidly, with their mechanical withdrawal threshold being within the normal range and no discernible difference in expression of spinal GluA1 subunits being observed between ipsilateral and contralateral sides by post-operative day 28. The other half of the young nerve-injured animals maintained their hypersensitivity for several months, perhaps because of more severe injury. In contrast, all of the adult rats with CCI showed short-lasting sensitisation with paw withdrawal thresholds returning to normal levels beyond post-operative day 20. Therefore, my data indicate that the time-courses of sensitised mechanical nociceptive behaviours and of increased expression of spinal AMPA receptors in neonatal rats following peripheral nerve injury are different from those in adult rats. In addition, I find that the recovered young rats have greater behavioural nocifensive responses to formalin challenge when they grow to adulthood compared to the uninjured ones. Animals that

had previously received (but subsequently recovered from) early life nerve injury showed a significantly greater formalin-induced incorporation of GluA1 into synapses compared to uninjured animals, suggesting a key role of synaptic GluA1 in pain hypersensitivity. The role of early life injury in any alterations of nociceptive circuits has been poorly understood to date but it may have a great formative effect on subsequent nociceptive processing or even cause long-term alterations throughout the whole of life. I find that such a lasting hyper-responsiveness despite apparent recovery of acute sensory response thresholds is only elicited following nerve injury in neonatal but not adult rats.

GluA1 subunit-containing AMPA receptors have been shown to play an important role in central neural plasticity, in particular long-term potentiation (LTP). GluA1 subunit-containing AMPA receptors specifically translocate to synapses in hippocampal neurons during LTP (Hayashi *et al.*, 2000; Boehm *et al.*, 2006; Makino and Malinow, 2009). Phosphorylation of the C-terminal tail of GluA1 and interaction with PDZ domain proteins are required for this AMPA receptor insertion into synapses (Hayashi *et al.*, 2000; Lee *et al.*, 2003). The proportion of phosphorylated GluA1 correlates with the surface expression of GluA1 and synaptic efficacy. It is reported that every 1% phosphorylation at S845 of GluA1 causes a 0.75% increase in GluA1 expression on the cell surface (Oh *et al.*, 2006). Phosphorylation of S818 of GluA1 by PKC is also required for AMPA receptor incorporation into synapses during LTP (Boehm *et al.*, 2006). The interaction of PDZ domain proteins such as PSD-95 with GluA1 appears to be important for synaptic insertion but may not required for synaptic plasticity *per se*. Point mutation at T887 of the GluA1 C-terminal tail (or deletion of the last 7 residues) both prevent PDZ-protein interactions of GluA1, whereas they have opposite effects on synaptic plasticity (Boehm *et al.*, 2006a). Therefore, the PDZ interaction at the GluA1 C-terminal tail may play more of a modulatory role in synaptic plasticity.

Based on the current literature, GluA1 subunit-containing AMPA receptors are the prime candidate for underpinning central sensitisation in chronic pain. An increased synaptic distribution of GluA1 subunits correlates with the efficacy of synaptic transmission. Incorporation of functional GluA1 subunits into synapses elevates the

synaptic strength within 5-10 minutes after LTP induction in anterior cingulate cortex (Wei *et al.,* 2006). In CFA-induced inflammatory pain, the expression of GluA1 subunits in dorsal horn is not changed but their subcellular distribution is altered. The level of GluA1 is significantly decreased in cytosolic fractions and increased in membrane fractions from the ipsilateral dorsal horn at 24 hours post-CFA injection (Park *et al.,* 2008). In the present study, purified subcellular fractions of dorsal spinal cord quadrants have been successfully prepared for the first time and further separated into PSD and NSSP fractions showing that both GluA1 and 4.1N in spinal cord are predominantly localised to the PSD rather than the NSSP fraction. It has been shown that under normal conditions the GluA1 subunit is predominantly expressed in the superficial laminae I-II of the spinal dorsal horn (Furuyama *et al.,* 1993; Engelman *et al.,* 1999; Polgar *et al.,* 2008). I report that in pathological conditions (i.e. CCI in adult rats), GluA1 subunits show increased expression in the ipsilateral dorsal horn PSD fraction at post-operative day 10. These results are consistent with the theory of incorporation of GluA1-containing AMPA receptors into synapses by lateral movement from an extrasynaptic pool (Makino and Malinow, 2009). The increased expression of spinal GluA1 subunits in PSD fractions of ipsilateral dorsal horn following early life CCI injury corresponds to the marked increase in mechanical sensitivity as assessed by behavioural reflexes. This matches results following intrathecal administration of the AMPA receptor antagonist NBQX, which attenuated the CCI-induced ipsilateral sensitisation of mechanical allodynia in adult rats (Figure 3.1). I conclude that the incorporation of spinal GluA1 subunit containing-AMPA receptors into the PSD of dorsal horn neurons may contribute to the mechanical allodynia evoked by peripheral nerve injury. Therefore spinal GluA1 subunits may well play a role in central sensitisation in neuropathic pain. Furthermore, I also find that formalin-induced expression of GluA1 subunits in PSD of spinal dorsal horn is significantly greater in animals that had received early life nerve injury (and since recovered) compared to naïve animals. Thus animals that had received nerve injury in early life have altered functional nociceptive circuits and some form of "memory of pain hypersensitivity" that renders them vulnerable to accentuated responses when faced with further noxious challenge in later life. I further demonstrated that while GluA1 showed decreasing expression in spinal cord

through early postnatal development, and 4.1N showed an inverse profile; CCI injury appeared to induce increased spinal cord expression of both proteins.

The Ras/MAPK (ERK) signalling pathway is one of the best-studied nociceptive pathways, which may play a role in bringing about sensitised states. The translocation of GluR1 subunit-containing AMPA receptors into the PSD following noxious stimulation may be attributed at least in part, to the activation of this pathway. The bulk of evidence shows that ERK participates in central sensitisation and contributes to the pain hypersensitivity following noxious inputs (Ji *et al.*, 1999; Karim *et al.*, 2001; Kawasaki *et al.*, 2004; Walker *et al.*, 2007). Many of the mediators of central sensitisation such as NMDA receptors, group I metabotropic glutamate receptors, TrkB or NK1 receptors converge to activate ERK (Ji *et al.*, 1999; Lever *et al.*, 2003; Ji *et al.*, 2009). Amongst the major activators, glutamate and substance P (two principal mediators of sensory inputs) activate ERK in the spinal dorsal horn through an elevation of intracellular Ca^{2+} concentration sufficient to drive a calmodulin-induced stimulation of adenylyl cyclases 1 and 8, whose cAMP production in turn activates PKA and subsequent cascades (Wei *et al.*, 2006). PKA and PKC signalling can converge on ERK to modulate neural excitability in superficial dorsal horn (Hu and Gereau, 2003). One of the effects ERK produces is the recruitment of AMPA receptors to the membrane (Galan *et al.*, 2004). In the present study, I have examined the levels of phospho-ERK in the NSSP (and PSD) fractions of the spinal dorsal horn, showing that this is enhanced on the ipsilateral side following formalin injection in animals that had received nerve injury in early life. This corresponds to the significantly increased content of GluA1 subunit-containing AMPA receptors in PSD fractions. These results are consistent with previous reports that ERK is involved in spinal hypersensitivity and may underlie GluA1 subunit-containing AMPA receptor trafficking to synapses responding to noxious stimuli.

Ras is considered to be a critical upstream signalling regulator of ERK and to play a key role in regulating synaptic trafficking of AMPA receptors in synaptic plasticity (Zhu *et al.*, 2002; Qin *et al.*, 2005). Sos-1, a Ras-GEF protein, acting as key controller of Ras activation, can be stimulated by receptor tyrosine kinases (RTKs)

when they bind growth factors or by GPCR transactivation of RTKs (Bonfini *et al.*, 1992; Buday and Downward, 1993; Aronheim *et al.*, 1994). Furthermore, Sos-1 has been proved to play a role in transmission of hypersensitivity-associated signals from NMDA receptors in cortical neurons of neonatal animals (Tian *et al.*, 2004). The effects of Sos-1 on nociceptive processing in neuropathic pain remain speculative. The current data suggest that enduring high expression of Sos-1 is found in the PSD fractions of the spinal dorsal horn in recovered early life CCI rats, but not in rats without early life nerve injury. The activation of ERK is a consequence of integrated modulation by multiple signalling events including Sos-1. However, Sos-1 in particular appears to be a marker for "pain memory" since it is still expressed at a high level in the early life-injured animals versus näive animals, even though the behavioural sensitivity of the nerve-injured animals had returned to normal. Early life injury may serve to influence the expression of Sos-1 in dorsal horn and thus alter the nociceptive processing of spinal circuits in young animals in a lasting way.

Although the current results represent significant advances into new territory, establishing a likely molecular basis for the lasting hypersensitivity following early life pain, it is important to consider that the approaches used here have some limitations: These include: 1) Large amounts of tissue were required to achieve enough post-synaptic density fractions for assessment of protein alteration in synapses. Significant amounts of material were lost through the stages of PSD preparation. The reported protocol for PSD isolation was modified by simplifying Percoll/sucrose gradient layers and the times of centrifugation to reduce loss of material (Kennedy, 1997; Villasana *et al.*, 2006). More extensive separation procedures may achieve more precise subfractionation but would have required even more starting material. 2) The interpretation of results from immunoblot and immunoprecipitation experiments is entirely dependent on the specificity of antibodies (despite the inclusion of appropriate control procedures). 3) The usable quantitative range of chemiluminescence for HRP-conjugated secondary antibodies in immunoblots is relatively limited. Furthermore the image density does not give a directly linear measure of the amount of target protein because the secondary antibody may not bind to the primary in a 1:1 ratio and its HRP conjugate is catalytic rather than being a directly measurable chemical tag. An improved linearity and

dynamic range may be obtained with antibodies directly tagged with labels such as near-infrared dye if they were available. As the X-ray films exposed to the chemiluminescence signal could be saturated in grey scale by a strong signal, a control lane was loaded for each blot to estimate whether the exposure time was appropriate or not and I always carried out a range of development times to ensure that data were obtained from submaximal exposures. While comparison to levels of a 'housekeeping protein', (whose levels generally do not alter) improves confidence in the specificity of changes, it is always possible that the conditions here could elicit previously unreported changes in the 'housekeeping protein'. In addition, there is always an open question of whether the running of each gel lane, electrotransfer to PVDF and access of antibodies, chemiluminescent reagents and film development chemicals is identical between lanes. Although these factors may contribute to variability, it is likely that they will not impact in a biased manner on the results.

Despite these caveats, the results indicate that nerve injury induces synaptic incorporation of GluA1 following adult CCI together with its increased association with partner protein 4.1N that may underlie pain hypersensitivity. In addition, early life nerve injury can produce temporary or very long-lasting hypersensitivity. In animals with short-lasting (recovered) hypersensitivity after early life nerve injury, an acute noxious challenge with formalin elicited accentuated pain behaviours and increased incorporation of GluA1 into synapses; a potential basis for the functional hypersensitivity. Increases in levels of phospho-ERK (a potential driver of GluA1 incorporation into synapses) in the non-synaptic synaptoneurosomal fraction were also induced by formalin challenge following early life CCI, together with a long-lasting increase in spinal cord expression of the ERK pathway regulator, Sos-1. These findings suggest that not only adult, but in particular early life nerve injury induces increased synaptic incorporation of GluA1 that may be in part dependent on lasting changes in the ERK MAP kinase pathway and that the effects of early life nerve injury may cause long-lasting hyper-responsiveness at both behavioural and biochemical levels.

Chapter 4: Fluorometric measurement of dynamic responses to receptor stimulation in freshly isolated neural tissue samples

The processes underlying neuronal hyperexcitability are poorly understood and many different signalling events may contribute to this. The previous chapter described a multidisciplinary approach, bringing together *in vivo* behavioural assessments and *in vitro* analysis of synaptic proteins, to address the events underlying hypersensitivity in somatosensory processing. However to date, only a limited range of techniques has been available to explore dynamic aspects of such cellular mechanisms, especially at the interface between biochemical and physiological assessments. In order to directly address this deficit I sought to develop new approaches that would enable quantitative assessment of dynamic receptor-mediated responses in *ex vivo* neural tissue samples. Such approaches can potentially provide a key way forward for elucidation of the mechanisms underlying neuronal hyperexcitability, as for example in chronic pain states.

The general strategy taken aimed for a consensus view of dynamic changes that could be associated with hyperexcitability rather than assessing individual neurons by electrophysiological or microscopy techniques. To obtain finely divided preparations of *ex vivo* neural tissue, two approaches were taken. In the first, tissue was homogenised in protective medium (ice-cold, highly oxygenated medium containing high Mg^{2+} and glutamate receptor antagonist to minimise unwanted Ca^{2+} entry during preparation, protease inhibitors and metabolic supplements). These preparations were then passed through a succession of defined pore microfilters to obtain tissue segments in the 30-100 μm range and a sub - 5 μm fraction enriched in synaptoneurosomes (closely apposed resealed pre- and postsynaptic elements) (Hollingsworth *et al.*, 1985). Each of these types of preparation was subsequently

assessed. In the second approach, 150 μm × 150 μm microslices of tissue were rapidly made using a McIlwain tissue slicer and resuspended in the protective medium.

In order to provide a dynamic measure of excitability in *ex vivo* tissue samples once they were returned to normal extracellular medium, I investigated fluorometric dyes that indicate cytosolic Ca^{2+} concentration or membrane potential, focusing on the former because of their greater signal-to-noise ratio. The use of proprietary "no wash" dyes from Molecular Devices, with which external fluorescence is suppressed by an impermeant quenching agent, gave the advantage that extensive washing of the tissue samples was unnecessary. In order to facilitate parallel comparisons with a number of pharmacological agents it was decided to adopt a plate-reader format where aliquots of tissue preparations were dispensed into wells rather than a cuvette-based assay where measurements would need to be made one at a time. It was initially unclear whether the tissue preparations would be in sufficiently good structural and metabolic condition to produce dynamic responses to stimuli, whether there would be sufficient signal-to-noise ratio to enable useful measurements and whether the tissue preparations would need to be maintained in suspension or allowed to settle to the bottom of the well in order to give better results. The experiments described here show how I developed an optimised procedure that achieved successful outcomes in each of these aspects and then used it in pilot studies to show dynamic responses to ionotropic and metabotropic receptor stimuli and their possible interaction.

A number of the experiments carried out to develop and optimise these techniques utilised cerebral cortex tissue, where as proof-of-concept studies I have addressed responses of example metabotropic and ionotropic receptors. Having successfully developed and validated these approaches, pilot experiments were carried out to establish feasibility in spinal cord, prior to their future application in addressing changes in responsiveness underlying chronic pain mechanisms.

4.1 Introduction

4.1.1 The organisation and functional interaction of cerebral cortex and brainstem

The cerebral cortex is classically described as consisting topographically of frontal, parietal, temporal and occipital cortex or consisting functionally of many specific areas such as somatosensory, auditory, visual, and motor areas (Barker and Barasi, 2008a). Cortical areas are richly interconnected with each other and with subcortical structures. In microanatomy, the cortex is vertically divided into six layers I-VI with diverse cellular subpopulations and the layering of the cortex reflects the radial organisation of all the input-output relationships. For example, the primary target of the ascending input, layer IV, is defined cytoarchitecturally by the dense packing of small cells which contains pyramidal and stellate cells and is also known as the inner 'granular layer'. Layer IV includes a majority of excitatory synapses, of which 95% represent intrinsic connections between layer IV pyramidal cells and 5% are contributed by the ascending input. Ascending inhibitory synapses are far less numerous, forming only about 10 – 20% of all contacts made by the ascending axon terminals (Shipp, 2007).

Structurally, brainstem comprises the medulla, pons, midbrain and diencephalon and forms the connection of the motor and sensory systems from the main part of brain to the rest of the body, in addition to multiple other functions. As described in Chapter 1, the brainstem plays a crucial role in relaying the inputs and outputs along the ascending and descending pathways. For example, the thalamic afferent fibres, relaying sensory inputs, largely project to cortical layers I and IV, often with a smaller input to layer VI, and terminate in discrete patches thus ensuring that sensory information from a specific location and/or receptor type is relayed to a specific area of cortex (Kharazia and Weinberg, 1994; Rubio-Garrido *et al.*, 2009). Interneurons in layer VI relay the thalamic input vertically to layers II, III, and V, which in turn project to other cortical and subcortical sites.

4.1.2 Central noradrenaline pathways in the brain

Noradrenergic fibres innervate structures throughout the brain and are thought to have important modulatory effects on a variety of brain functions (Moore and Bloom, 1979). The noradrenaline pathways in the brain have been mapped by fluorescence histochemistry in conjunction with selective surgical and pharmacological lesions (Dahlstrom and Fuxe, 1964; Ungerstedt, 1971). Noradrenergic fibres with cell bodies in the pons and medulla give rise to large ascending and descending tracts, terminating at discrete sites in brain and spinal cord respectively.

The noradrenergic fibres innervating the cerebral cortex originate from the locus coeruleus (LC, noradrenergic cell group A6) in the pons and run vertically into the layers of cortex with extensive collateralisation, then turn horizontally to run parallel to the surface as the vertical fibres reach layer I where the innervation is much greater than that of other layers of cortex (Levitt and Moore, 1978). Other noradrenergic fibres taht originate in the cell groups A1, A2, A5, A6 and A7 in the pons and medulla give rise to the ascending noradrenaline pathways innervating the hippocampus, cerebellum, hypothalamus, limbic system and the lower brainstem (Livett, 1973; Longstaff, 2000) (Figure 4.1a and 4.1b). Descending pathways comprise two systems: one arises from LC and terminates in the lower brainstem nuclei, whereas the other arises from the cell group A1 and innervates the ventral horn and other sites in the lateral column and dorsal horn of the spinal cord (Levitt and Moore, 1978; Martin *et al.*, 1978; Fleetwood-Walker and Coote, 1981).

Noradrenergic transmission is believed to be important in numerous centrally regulated processes, including alerting responses, long-term memory and blood pressure regulation (Cahill *et al.*, 1994; Morgan *et al.*, 1997). There are two subgroups of noradrenaline receptors, α-adrenergic receptors and β-adrenergic receptors. It has been shown that noradrenaline acts through β-adrenergic receptors to inhibit protein phosphatases which oppose the induction of long-term potentiation (LTP) and thereby facilitates the induction of LTP in CA1 hippocampal neurons (Thomas *et al.*, 1996). Intracellular signalling enzymes such as adenylyl cyclase, PKA and in some cases CaMKII, can be activated by noradrenaline via β-adrenergic

receptors (Longstaff, 2000; Hall, 2004; Wang *et al.*, 2004). Moreover, there is evidence for the involvement of AMPA receptors in noradrenaline-regulated LTP in that the synaptic delivery of GluA1 subunits is facilitated by noradrenaline through GluA1 phosphorylation at S831 and S845 sites (Hu *et al.*, 2007). In some situations, noradrenaline has an excitatory effect mediated by α-adrenergic receptors. Activation of α-adrenergic receptors contributes to the disinhibitory influence of noradrenaline over synaptic transmission in rat hippocampal neurons (Madison and Nicoll, 1988). Furthermore, it has been reported that noradrenaline increases cytosolic Ca^{2+} concentrations and mobilisation through binding to α1-adrenergic receptors in cultured local interneurons of mouse olfactory bulb (Tani *et al.*, 1992). Taking together, noradrenaline has been suggested to play a number of key roles in biochemical and physiological events associated with long-term modification of neuronal excitability and synaptic transmission.

Figure 4.1: Central noradrenaline pathways in rat brain

a) Source: taken from Longstaff A. (2000), Neuroscience

Saggital representation of the rat brain shows the major noradrenergic pathways. A6 is the Locus Coeruleus.

Abbreviations:

MFB- Medial Forebrain Bundle
ST- Stria Terminalis.

b) Source: taken and modified from Livett (1973), Histochemical visualisation of peripheral and central adrenergic neurons

Horizontal representation of ascending and descending noradrenaline pathways in rat brain.

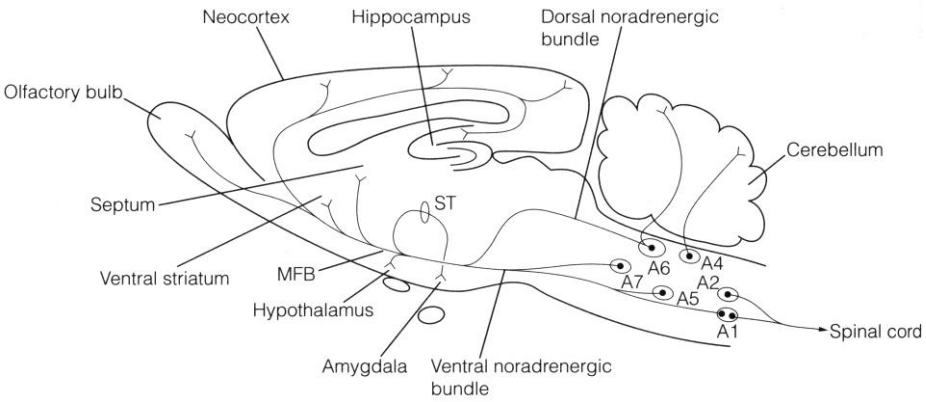
Key:

- - - - - - - : Noradrenergic fibres originating from the A6 cell group in the brainstem

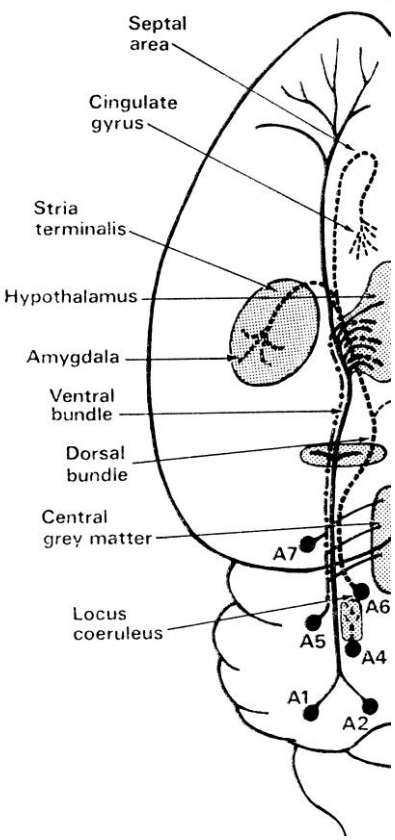
_____ : Noradrenergic fibres originating from A1/A2 cell groups

. . . . . : Noradrenergic fibres originating from A7/A5 cell groups

4.1 a)


Neocortex
Hippocampus
Dorsal noradrenergic bundle
Olfactory bulb
Cerebellum
Septum
ST
Ventral striatum
MFB
Hypothalamus
A6
A4
A7
A2
A5
A1
Spinal cord
Amygdala
Ventral noradrenergic bundle

4.1 b)


Septal area
Cingulate gyrus
Stria terminalis
Hypothalamus
Amygdala
Ventral bundle
Dorsal bundle
Central grey matter
A7
A6
Locus coeruleus
A5
A4
A1
A2

4.1.3 Central acetylcholine pathways in the brain

Acetylcholine is very widely distributed in the brain, occurring in all parts of the forebrain, midbrain and brainstem, though there is little in the cerebellum. The key acetylcholine pathways in the brain are shown in Figure 4.2. There are two major pathways: the one with cell bodies located in the magnocellular forebrain nuclei in the basal forebrain gives rise to the main acetylcholine pathways which innervate many parts of the cortex, while the other one with cell bodies located in the septohippocampal nucleus sends projections to the hippocampus (Rang *et al.*, 2003). Apart from these there are other projection pathways, originating in lower brainstem and many local cholinergic interneurons particularly in the cortex, corpus striatum and spinal cord (Sherriff *et al.*, 1991; Woolf, 1991).

The functional roles of acetylcholine are related to cognitive processes such as arousal, attention, fear, learning and memory, and motor control via cholinergic pathways mediated by various subtypes of receptors which are classified into two major types, muscarinic (G protein-coupled) and nicotinic (ionotropic) (Phillis and Chong, 1965; Phillis, 2005; Raybuck and Gould, 2010). Five subtypes of muscarinic receptors (M1-5) and their transduction pathways, localisation and functional responses have been characterised (Caulfield and Birdsall, 1998; Jones *et al.*, 1999). M1, M3 and M5 receptors couple through Gq/11 to phospholipase C activation and function predominantly as postsynaptic receptors, while M2 and M4 receptors, which couple through Gi/o to inhibit adenylyl cyclase and regulate K^+ and Ca^{2+} channels, often function as presynaptic receptors, to regulate excitatory and inhibitory neurotransmitter release in the central nervous system (Caulfield and Birdsall, 1998; Rouse *et al.*, 1998; Whalley and Constanti, 2006). In addition, it has been shown that muscarinic receptors auto-inhibit acetylcholine release from cholinergic neurons in the cortex of rats (Raiteri *et al.*, 1984). Recent experiments suggest that M1 and M2 subtypes in the cortex are involved in neuropathic pain (Ortega-Legaspi *et al.*, 2010). Neuronal nicotinic receptors are pentameric, being assembled from α- and β-subunits as a heteromeric receptor, or five α-subunits as a homomeric receptor (Dani, 2001; Leonard and Bertrand, 2001). The main varieties occurring in the cortex and

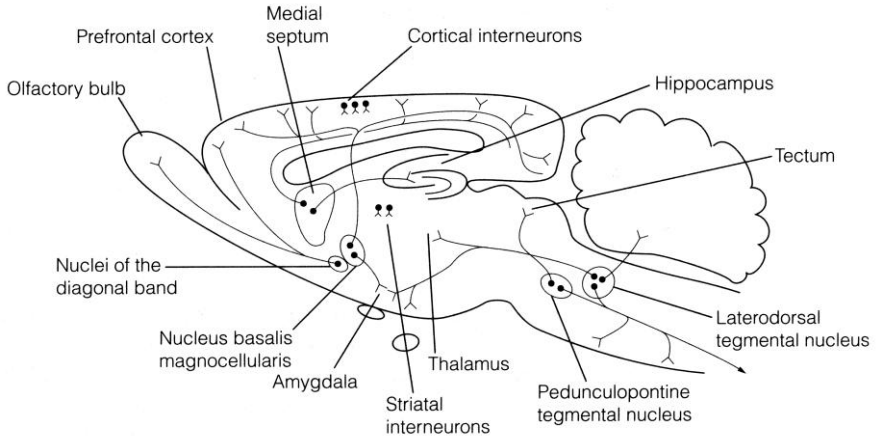
the hippocampus are thought to be the heteropentameric α4β2 subtype and homopentameric α7 subtype respectively (Rollema *et al.*, 2009; Loughead *et al.*, 2010). For the most part (but not exclusively), nicotinic acetylcholine receptors locate presynaptically and act to facilitate the release of other transmitters, such as glutamate, dopamine and GABA (Metherate and Hsieh, 2003; Anderson *et al.*, 2009; McClure-Begley *et al.*, 2009). Activation of nicotinic receptors causes changes in synaptic transmission and in turn modulates the cognitive responses of the brain to external stimulation. For instance, α7 subtype nicotinic receptors can bring about postsynaptic excitation by enhancing NMDA currents and functionally co-operate with AMPA receptors in rat auditory cortex (Levy and Aoki, 2002). Activation of β-subunit-containing nicotinic receptors induces the release of GABA from synaptosomes isolated from the mouse brain (Lu *et al.*, 1998). However, some nicotinic receptors function in a postsynaptic role by mediating fast synaptic transmission in the hippocampus and in the sensory cortex (Zhang *et al.*, 1993). The changes mediated by presynaptic and postsynaptic nicotinic acetylcholine receptors in the efficacy of synaptic transmission could underlie the proposed functions of acetylcholine in cognitive responses of the cerebral cortex and the hippocampus.

Figure 4.2: Central acetylcholine pathways in rat brain

Source: taken from Longstaff A. (2000), Neuroscience

The diagram shows major cholinergic pathways in a sagittal section of the rat brain.

4.2


Medial
septum
Prefrontal cortex
Cortical interneurons
Olfactory bulb
Hippocampus
Tectum
Nuclei of the
diagonal band
Nucleus basalis
magnocellularis
Amygdala
Thalamus
Striatal
interneurons
Pedunculopontine
tegmental nucleus
Laterodorsal
tegmental nucleus

4.1.4 Central 5-hydroxytryptamine pathways in the brain

5-Hydroxytryptamine (5-HT) neurons are concentrated in the mid-line Raphe nuclei in the pons and medulla, projecting diffusely to the cortex, hypothalamus, limbic system and spinal cord, in a similar way to the noradrenergic projections (Barnes and Sharp, 1999) (Figure 4.3). 5-HT pathways play a variety of roles in behavioural responses and control of mood, emotion, sleep, and sensory (nociceptive) pathways, which have been mentioned in the descending pathways section of Chapter 1. Fourteen subtypes of 5-HT receptors have been identified ($5\text{-}HT_{1\text{-}7}$ are the main variants and some of these exist as further distinct isoforms) and all are expressed in the central nervous system (Mengod *et al.*, 1996). $5\text{-}HT_2$ receptors, in particular $5\text{-}HT_{2A}$ receptors, are abundant in the cerebral cortex and exert an excitatory postsynaptic effect through coupling via Gq/11 to phospholipase C (Marek and Aghajanian, 1999).

Figure 4.3: Central 5-HT pathways in rat brain

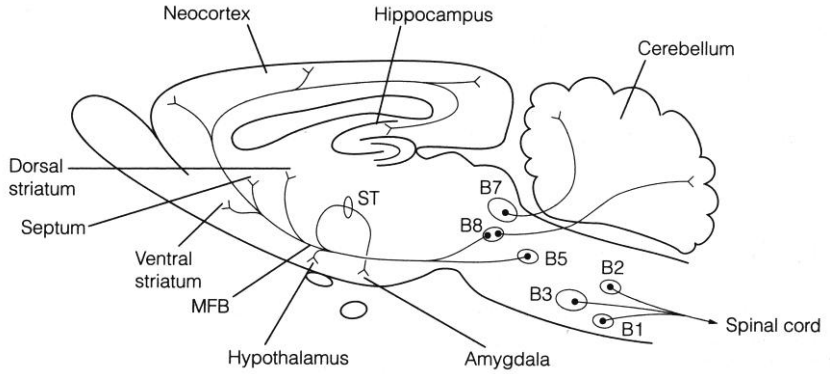
Source: taken from Longstaff A. (2000), Neuroscience

The diagram shows major serotonin pathways in a sagittal section of the rat brain. The cell groups B1-B8 corresponding to the 5-HT-containing Raphe nuclei (except B4 and B6). The major B7 and B8 groups correspond to the Dorsal and Median Raphe Nuclei, respectively (Hillarp et al. 1966).

Abbreviations:

MFB: Medial Forebrain Bundle;
ST: Stria Terminalis.

4.3


Neocortex
Hippocampus
Cerebellum
Dorsal striatum
Septum
Ventral striatum
MFB
ST
B7
B8
B5
B2
B3
B1
Spinal cord
Hypothalamus
Amygdala

4.2 Results

The morphological characteristics of the different preparations were examined microscopically and found to be consistent with previous reports. In order to try and directly measure the ability of noradrenergic, cholinergic and serotonergic metabotropic receptors, as well as ionotropic glutamate receptors, to cause activation in cortical tissue, I used freshly isolated rat frontal cortex segments (and in some cases synaptoneurosomes) to examine changes in the neuronal excitability using fluorescent indicators of intracellular Ca^{2+} concentration and voltage. The dyes enabling such successful measurements were no-wash formulations with external quenching agents to suppress non-specific extracellular fluorescence (supplied by Molecular Devices). In addition, I used the Ca^{2+} indicator to investigate the excitability of freshly isolated spinal cord samples in several different formats (30-100 μm tissue segments, synaptoneurosomes or microslices) following stimulation with AMPA or nociceptor-associated agonists.

4.2.1 Morphological characteristics of acute *ex vivo* tissue preparations

Frontal cortex or spinal cord tissue samples were prepared as described in Chapter 2 and their morphology assessed at light microscopy or electron microscopy levels as appropriate. Figure 4.4 shows fluorescence microscopy images of basal Calcium 4 fluorescence taken with × 10 (a), and × 40 objective lenses (b) and (c), using a Zeiss LSM510 Axiovert microscope. Samples were aliquoted into collagen-coated cover-slip-based chambers and loaded with Ca^{2+} indicator as normal before assessment without washing. Typical profiles are illustrated of 30-100 μm tissue segments, occasionally observed single cell bodies and synaptoneurosomal elements with dual apposed vesicular structures. The morphology of the synaptoneurosome elements corresponds closely to those described and illustrated in previous studies (Hollingsworth *et al.*, 1985; Johnson *et al.*, 1997). A number of attempts were made to add activating agents to the medium to assess responses in real time but in each case turbulence disturbed the optical field of view. Further experiments with

coverslips coated with cell-adhesion agents to promote anchorage of the samples may enable imaging of single tissue elements before and after drug addition, but this was not further investigated here.

Figure 4.5 shows a high-resolution electron microscopy image of a typical synaptoneurosome profile (with presynaptic and postsynaptic sealed vesicular structures, as well as the electron-dense postsynaptic density). While the light microscopy image of a presumed synaptoneurosome profile in Figure 4.4(c) shows little detail as the size of the structure is approaching the limits of resolution for visible light, it does appear in terms of both size and the overall dual vesicular appearance to match closely with the electron microscopy image in Figure 4.5.

Figure 4.4: Microscopic assessment of 30-100 μm tissue segments, neuronal cell bodies and synaptoneurosomes from rat lumbar spinal cord

Light microscopy in Calcium 4-loaded samples was used to show basal intracellular Ca^{2+} fluorescence in a) 30-100 μm tissue segments, b) occasionally observed neuronal cell bodies within the synaptoneurosome fraction and c) apparent synaptoneurosomal elements with a dual vesicular structure, corresponding closely to those described originally by Hollingsworth et al (1985). Samples in collagen-coated coverslip-based chambers were loaded with dye for 45 minutes at 37^{o}C under standard conditions (Chapter 2) and then transferred (without washing) to a Zeiss Axiovert LSM510 microscope for imaging using × 10 (a) or × 40 objective lenses (b) and (c). These images show basal Calcium 4 fluorescence under unstimulated conditions. Similar observations were made with samples from frontal cortex. Attempts to carry out real-time imaging of the same tissue elements before and after drug addition were unsuccessful due to turbulence affecting the field of view. Possible approaches to overcome this were not further investigated in the present study.

4.4

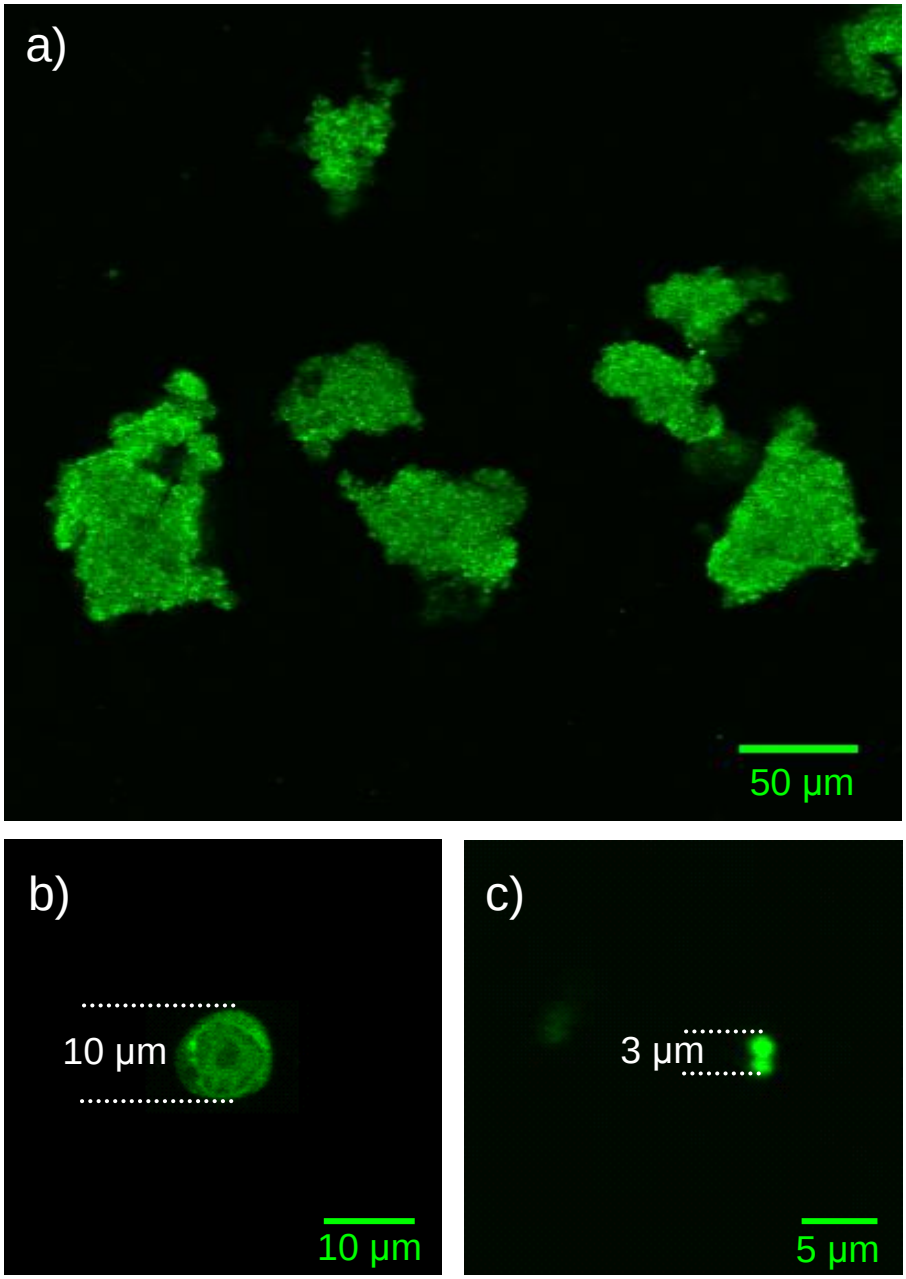
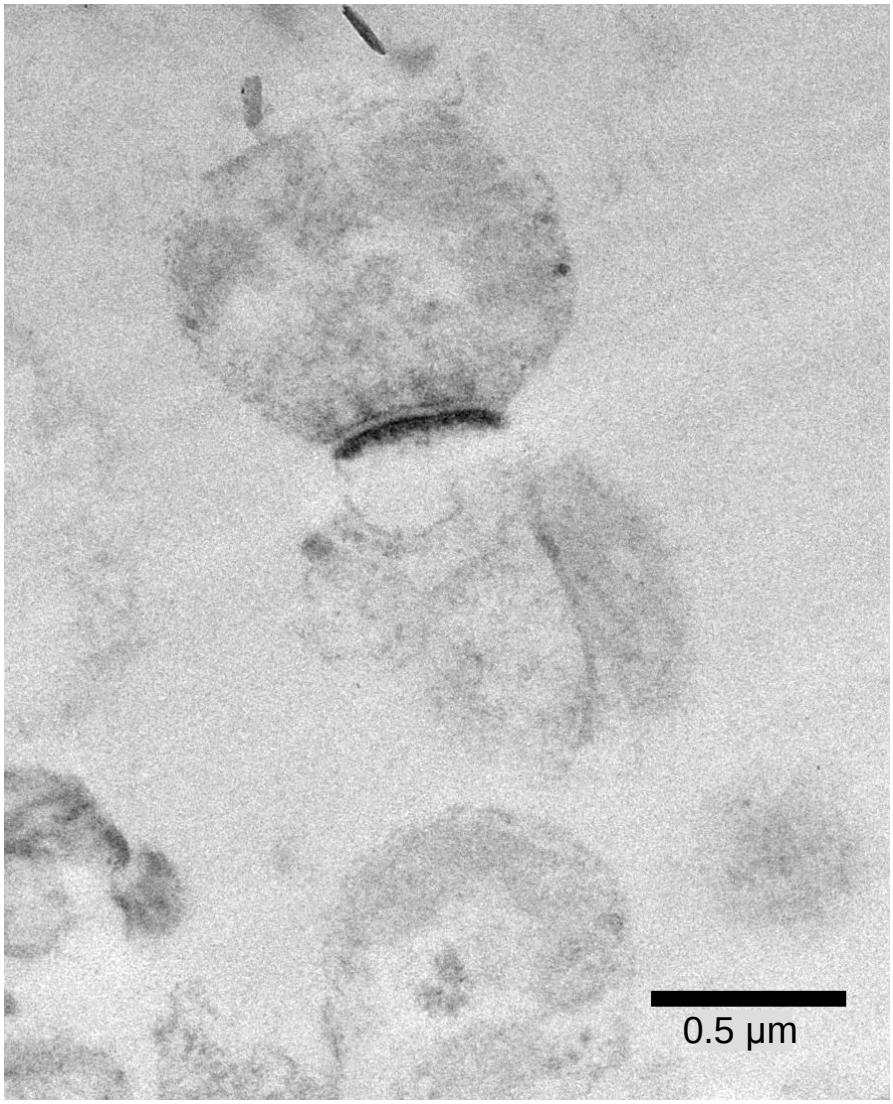


Figure 4.5: Electron microscopic assessment of the morphology of synaptoneurosomes from rat lumbar spinal cord

Electron microscopy of the synaptoneurosome fraction from rat spinal cord revealed the presence of composite structures with sealed presynaptic and postsynaptic membrane elements and electron-dense postsynaptic densities, which were morphologically identified as synaptoneurosomes matching closely to the structures described by Hollingsworth et al. 1985. Samples were fixed in glutaraldehyde/osmium tetroxide and stained with uranyl acetate and lead citrate. Ultrathin (10 nm) sections were imaged using a Phillips Biotwin electron microscope. Similar observations were made with samples from frontal cortex.

Scale bar: 0.5 μm

4.5



4.2.2 Ca^{2+} fluorescence responses of *ex vivo* tissue segments and synaptoneurosomes from frontal cortex

Frontal cortex segments of 30-100 μm diameter and synaptoneurosomes were rapidly prepared in oxygenated media, aliquoted to 24-well plates, and loaded with no-wash fluorometric Ca^{2+} indicator, Calcium 4 (Molecular Devices). Plates were then transferred to a fluorometric plate reader with the plate-carrier stage maintained at 28^{o}C. The samples were allowed to settle for 3 minutes following the turbulence associated with manual transfer of the 24-well plate from the incubator. CLOSE TWO GAPS LITINGDrugs or appropriate vehicle were then added as 40 μl per well (total well volume of 400 μl) from stock solutions at 10 fold higher concentrations. Immediately following drug addition and initiation of the plate-reader programme, sequential fluorescence readings were made from every well every 30 seconds with excitation set at 488 nm and emission at 519 nm for periods of up to 15 minutes. Data for the purpose of quantifying drug effects was generally obtained by averaging 6 sequential readings after responses had reached stable plateau values. For 30-100 μm tissue segments data collection was routinely begun after 6 minutes, whereas for synaptoneurosomes (and microslices, see below) data collection was started after 3 minutes. This difference in protocol was due to the impression that the tissue segments were more mobile and susceptible to disturbance in the wells than synaptoneurosomes or microslices which tended to settle more quickly. However extended time-course observations from each type of preparation showed that responses had reached relatively stable plateau values in each case throughout either of these time periods. In general terms the strategy of assessing multiple samples in parallel by plate-reader is not ideally suited to measuring early phase responses with sequential observations only being possible in the tens of seconds time-scale (here using a 30 seconds reading cycle). Real time (but individual sample) observations made by cuvette fluorometer or fluorescence microscopy may be more suitable for this but each would involve its own technical limitations. Some distinctions between the early phase responses to different stimuli were observed when investigated but

these were not as marked or consistent as distinctions in plateau-phase responses, so were not routinely assessed.

4.2.2.1 Ionomycin and Noradrenaline

Initial experiments were carried out to evaluate whether the protocol developed could deliver consistent results with sufficient signal to noise ratio to enable quantitative analysis. 30-100 μm tissue segments or synaptoneurosomes from frontal cortex were aliquoted into wells and loaded with the fluorescent Ca^{2+} indicator, before the following additions were made: ionomycin (5 μM final concentration), l-noradrenaline (100 μM) or vehicle. The Ca^{2+} ionophore ionomycin was used as a positive control in each experiment as it generates Ca^{2+}-permeable pores in membranes and would provide a maximal signal, allowing equilibration of extracellular Ca^{2+} with the intracellular cytosol which would be maintaining a lower Ca^{2+} concentration if the preparations were metabolically viable. l-noradrenaline was used as an agonist for adrenergic receptors, an example class of G protein-coupled receptors that are expressed in cerebral cortex. The vehicle was 0-0.5% dimethylformamide in assay medium, as appropriate because some drugs (such as ionomycin) needed to be dissolved in solvent before dilution to the aqueous solutions used for addition to the wells. The addition of vehicle had no discernible effects on fluorescence readings and vehicle control wells were taken to represent baseline signals. Using 30-100 μm tissue segments, 5 μM ionomycin rapidly caused a marked increase in Ca^{2+} fluorescence, reaching fluorescence values 2.5-3 fold those of basal (vehicle control) wells within about 3 minutes that were then maintained for up to 15 minutes with little apparent change. A typical example set of data is shown in Figure 4.6a together with an indication of the standard data collection period in the established response phase of 30-100 μm tissue segments that was normally used for quantitative comparisons. 100 μM l-noradrenaline also increased Ca^{2+} to about 30-40% of the increment above basal shown by ionomycin. Fluorescence of the basal (vehicle control) wells and wells to which no addition was made showed an increase at the start of the recording period, which was considered likely to be an instrumentation artifact due to the movement of the sample within the wells as the

plate was moved into its recording position. Experiments were not carried out to determine absolute molar concentrations of Ca^{2+}, using ratiometric dyes (which would require washing after loading) or detergent and defined Ca^{2+}:EGTA buffers. Instead the comparison of responses to basal and ionomycin responses provided relative quantitation that was fully adequate for the purposes of the present study. Figure 4.6b shows mean fluorescence values (in relative fluorescence units) taken from readings between 6.0 and 8.5 minutes for basal, 100 μM l-noradrenaline and 5 μM ionomycin in 3 separate experiments. Statistical analysis by One-way ANOVA with Tukey's post-test indicated significant differences between the mean values for basal, l-noradrenaline and ionomycin groups. The results indicate that the freshly isolated neuronal tissue segments were metabolically functional in terms of being able to maintain lowered cytosolic Ca^{2+} concentrations and that consistent elevation of intracellular Ca^{2+} concentrations was elicited by l-noradrenaline but to a lesser extent than the positive control ionomycin. Further validation of specificity was provided by the demonstration that the Ca^{2+} response to l-noradrenaline was concentration-dependent (Figure 4.6c). These data were plotted as the ratio of the l-noradrenaline-induced response above basal relative to the ionomycin-induced response above basal, an approach, which was widely utilised in further experiments. This indicated that a large part of the response to l-noradrenaline occurred with a high potency of less than 1 μM and that an additional component (that was perhaps not mediated by adrenoreceptors) may have been evoked at high concentrations (30 μM and above). Further experiments characterising the Ca^{2+} fluorescence response to l-noradrenaline were carried out using frontal cortex synaptoneurosomes. The time-course responses of a typical example data set are shown in Figure 4.6d together with an indication of the standard data collection period in the established response phase of synaptoneurosomes that was normally used for quantitative comparisons. 3 μM l-noradrenaline induced an increase in Ca^{2+} fluorescence to around 50% of the extent shown by 5 μM ionomycin. Figure 4.6e further validates the receptor specificity of the l-noradrenaline response, showing that it was significantly attenuated (almost entirely prevented) in the presence of a combination of selective antagonists at α_1- and β-subtype adrenoreceptors, prazosin and l-propranolol, 6 μM in each case ($p<0.001$, One-way ANOVA, with Tukey's post-test, n=4). Noradrenaline could

cause elevation of cytosolic Ca^{2+} concentrations by mobilisation of intracellular Ca^{2+} stores and receptor-operated Ca^{2+} entry through activating α_1-receptors. Alternatively β–adrenoreceptor activation of adenylyl cyclase and PKA could lead to phosphorylation facilitating the operation of voltage-sensitive Ca^{2+} channels.

4.2.2.2 Comparative responses to noradrenaline and different ionotropic glutamate receptor agonists

The actions of metabotropic adrenoceptors and ionotropic glutamate receptors in frontal cortex segments were examined through the simultaneous application of noradrenaline and different glutamate receptor agonists. Figure 4.7a shows time-courses of responses to 100 μM l-noradrenaline, 100 μM AMPA, 100 μM NMDA plus 100 μM ACPC (an agonist at the glycine co-agonist recognition site of NMDA receptors (Watson and Lanthorn, 1990)) or 5 μM ionomycin. Responses are plotted as mean ± SEM at each time point from 4 separate experiments and illustrate the reproducibility of the technique. The adrenoreceptor and glutamate receptor agonists each induced Ca^{2+} fluorescence responses of approximately 50% of the extent shown by ionomycin. Figure 4.7b shows these responses after subtraction of corresponding basal data in each case, (i.e. revealing purely the evoked responses) and indicates the standard data collection period in the established response phase that was normally used for quantitative comparisons. The responses shown were maintained at similar levels for up to 15 minutes.

When the earliest part of the responses was examined in detail it was possible to distinguish some difference in their properties. Figure 4.7c shows that when the responses were each normalised to their own first data point the rate of rise for ionomycin and AMPA was greater than that for NMDA/ACPC or l-noradrenaline. Over the first two minutes of response Repeated-Measures ANOVA with Tukey's post-test indicated a significant difference between the relative curves for ionomycin or AMPA and that for l-noradrenaline. This could be consistent with rapid Ca^{2+} entry through ionomycin pores or a Ca^{2+}-permeable (GluA2 subunit-lacking) population of AMPA receptors compared to slower, second messenger-mediated effects through adrenoreceptors mobilising intracellular Ca^{2+} stores or acting indirectly on voltage-

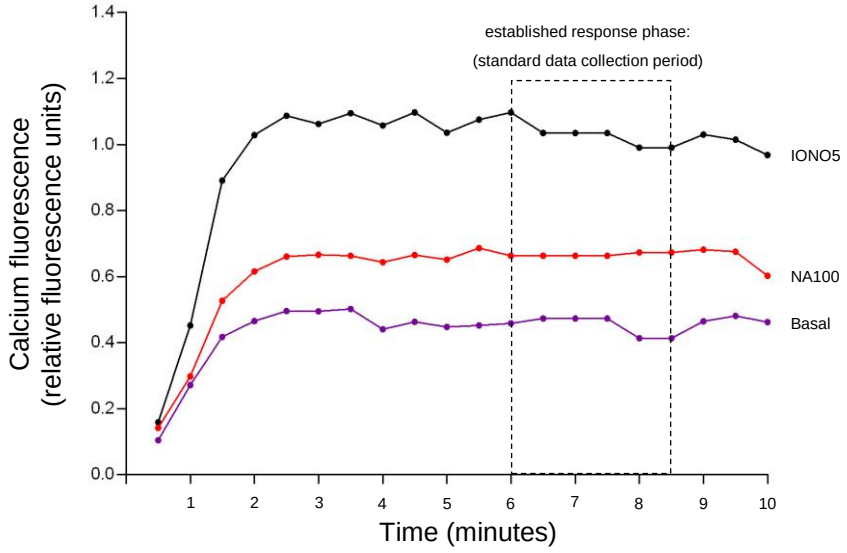
sensitive ion channels. NMDA/ACPC responses may be slowed because of the need for concurrent depolarisation to remove the basal block of the NMDA receptor channel by Mg^{2+}. Although of interest, these apparent differences were much less clear-cut and reproducible than the differences in plateau phase responses so were not further studied.

Figure 4.7d illustrates the low level of variability of established-plateau phase responses to 100 μM l-noradrenaline, 100 μM AMPA, 100 μM NMDA plus 100 μM ACPC and 5 μM ionomycin, shown as mean ± SEM values at each time point through the 6.0 to 8.5 minutes recording period (n=4). Figure 4.7e compares the mean values for these evoked responses throughout this period and shows that approximately similar sized responses were elicited by each of the agonists which were around half the magnitude of the ionomycin response. One-way ANOVA with Tukey's post-test indicated that each of the agonist responses was significantly smaller than that due to ionomycin ($p<0.001$, n=4).

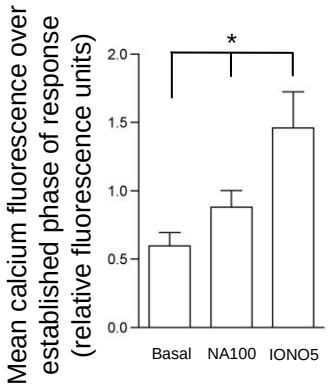
Figure 4.6: Ca^{2+} fluorescence responses of *ex vivo* tissue segments and synaptoneurosomes from frontal cortex: noradrenaline and ionomycin

Tissue segments of 30-100 μm diameter or synaptoneurosomes in 24-well plates were loaded with the fluorometric Ca^{2+} indicator, Calcium 4. After transfer to a fluorometric plate reader, three minutes were allowed for settlement and then drugs were added before sequential fluorescence readings were made every 30 seconds from each well. a) Shows representative records from individual wells in a typical example experiment with 30-100 μM tissue segments stimulated with l-noradrenaline 100 μM (red, NA10), ionomycin 5 μM（black, IONO5) or control (purple). The standard data collection period in the established phase of responses that was generally used for quantitative analysis is outlined. b) Shows mean fluorescence values averaged over the 6.0-8.5 minutes time range, from traces as in part a) with data taken from 3 separate experiments and shown as mean ± SEM. Statistical analysis by One-way ANOVA with Tukey's post-test indicated significant differences between the mean values for basal, l-noradrenaline (NA100) and ionomycin (IONO5) groups (*, $p<0.05$). Consistent elevation of intracellular Ca^{2+} concentrations was elicited by l-noradrenaline but to a lesser extent than the positive control ionomycin. c) Shows concentration-dependence of responses to l-noradrenaline (0.01-300 μM) in tissue segments with values representing mean ± SEM from 3-4 separate determinations expressed as evoked responses normalised to ionomycin-evoked responses (ionomycin responses being designated a value of 1.0) in each case.

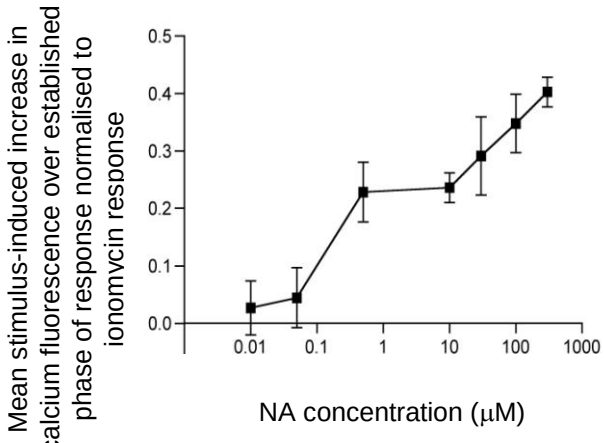
4.6 a)


established response phase:
(standard data collection period)
Calcium fluorescence
(relative fluorescence units)
IONO5
NA100
Basal
Time (minutes)

4.6 b)

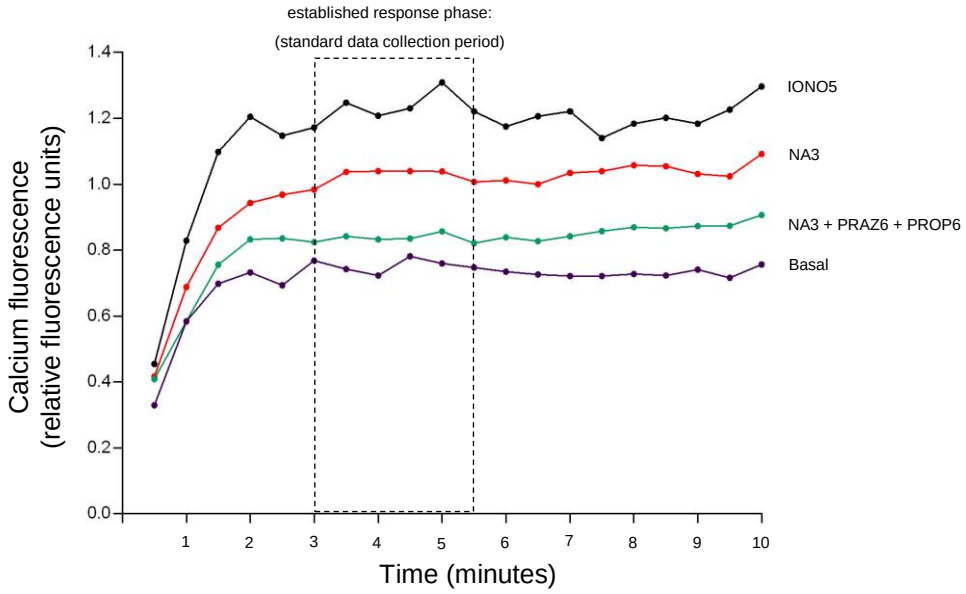

Mean calcium fluorescence over
established phase of response
(relative fluorescence units)
*
Basal
NA100
IONO5

4.6 c)


Mean stimulus-induced increase in
calcium fluorescence over established
phase of response normalised to
ionomycin response
NA concentration (μM)

d) Shows representative records from individual wells in a typical example experiment with synaptoneurosomes, stimulated with l-noradenaline 3 μM (red, NA3), l-noradrenaline 3 μM plus prazosin 6 μM and l-propranolol 6 μM (green; NA3 + PRAZ6 + PROP6), ionomycin 5 μM (black, IONO5) or basal control (purple). The standard data collection period in the established phase of responses that was generally used for quantitative analysis is outlined. e) Shows mean evoked responses over the 3.0-5.5 minutes recording period from traces as in part d) (after subtraction of basal values) with data taken from 4 separate experiments. The evoked responses are normalised to ionomycin-evoked responses and shown as mean ± SEM. Mean evoked responses are shown to l-noradrenaline 3 μM (NA), l-noradrenaline plus prazosin 6 μM (NA3 + PRAZ6), l-noradrenaline plus l-propranolol 6 μM (NA3 + PROP6) and l-noradrenaline plus both prazosin and propranolol (NA3 + PRAZ6 + PROP6). Statistical analysis by One-way ANOVA with Tukey's post-test indicated that the NA3 + PRAZ6 + PROP6 response was significantly reduced compared to the NA response (***, $p<0.001$) and compared to the NA3 + PRAZ6 or NA3 + PROP6 responses (**, $p<0.01$), n=4 in each case.

4.6 d)


established response phase:
(standard data collection period)
Calcium fluorescence
(relative fluorescence units)
Time (minutes)
IONO5
NA3
NA3 + PRAZ6 + PROP6
Basal

4.6 e)

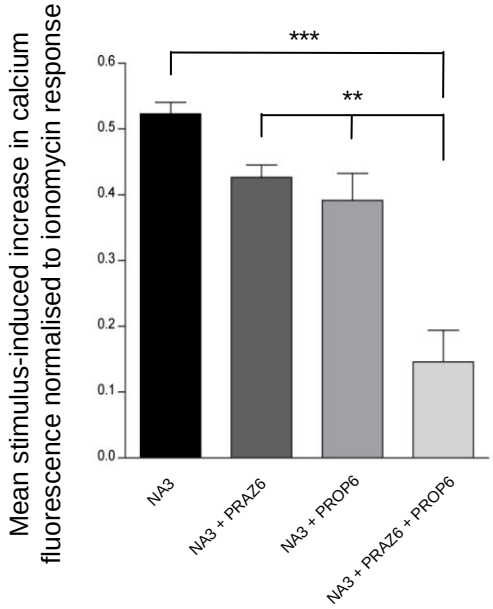
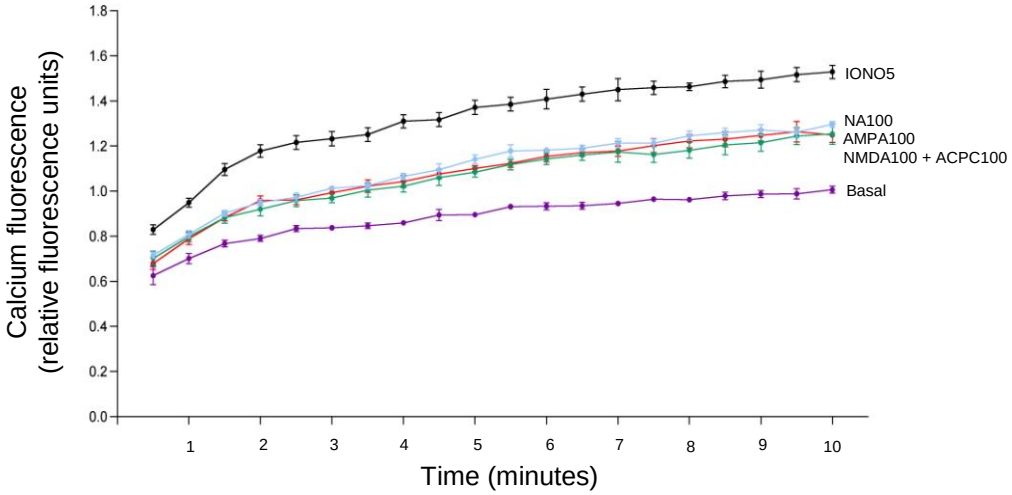

Mean stimulus-induced increase in calcium
fluorescence normalised to ionomycin response
***
**
NA3
NA3 + PRAZ6
NA3 + PROP6
NA3 + PRAZ6 + PROP6

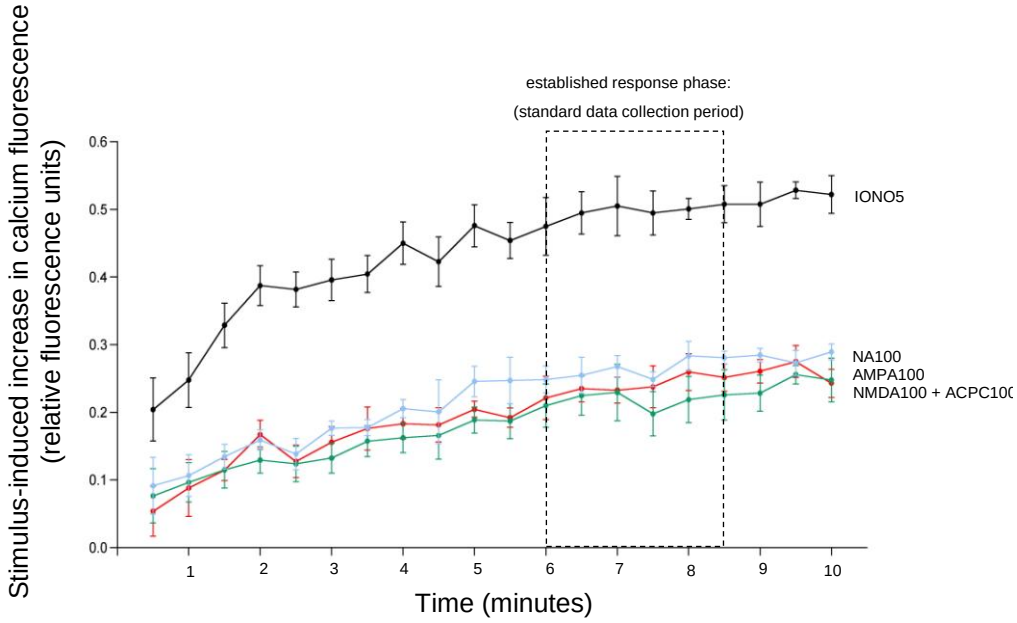
Figure 4.7: Ca^{2+} fluorescence responses of ex vivo tissue segments from frontal cortex: noradrenaline in comparison with different ionotropic glutamate receptor agonists

Tissue segments of 30-100 μm diameter or synaptoneurosomes in 24-well plates were loaded with the fluorometric Ca^{2+} indicator, Calcium 4. After transfer to a fluorometric plate reader, three minutes were allowed for settlement and then drugs were added before sequential fluorescence readings were made every 30 seconds from each well. a) Shows time-course data expressed as mean ± SEM values from 4 separate experiments in which tissue segments were stimulated with ionomycin 5 μM (black, IONO5), l-noradrenaline 100 μM (pale blue, NA100), AMPA 100 μM (red, AMPA100), NMDA 100 μM plus ACPC 100 μM (green, NMDA100 + ACPC100) or basal control (purple). b) Shows the stimulus-evoked components of the responses in part a), obtained by subtraction of relevant basal values in each case and indicates the time period through the established plateau phase of responses from which data were normally taken for subsequent quantitative analyses. Symbols show ionomycin (black, IONO5), l-noradrenaline (pale blue, NA100), AMPA (red, AMPA100) and NMDA + ACPC (green, NMDA100 + ACPC100).

4.7 a)


Calcium fluorescence
(relative fluorescence units)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
2
3
4
5
6
7
8
9
10
Time (minutes)
IONO5
NA100
AMPA100
NMDA100 + ACPC100
Basal

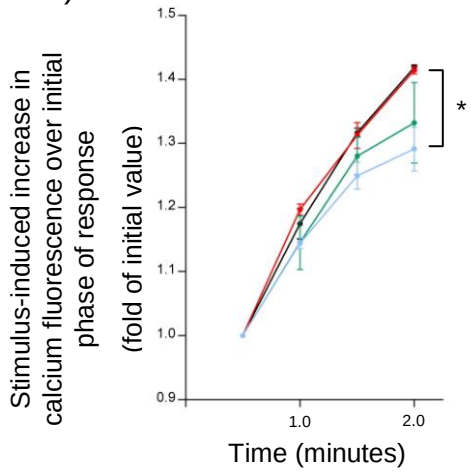
4.7 b)


Stimulus-induced increase in calcium fluorescence
(relative fluorescence units)
established response phase:
(standard data collection period)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1
2
3
4
5
6
7
8
9
10
Time (minutes)
IONO5
NA100
AMPA100
NMDA100 + ACPC10

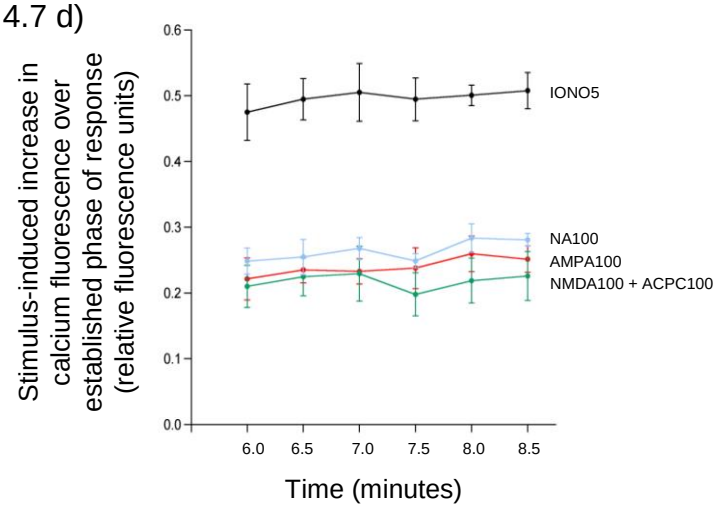
c) Shows evoked responses through the initial 2 minutes period, which have been normalised to their own initial data point in each case. Responses to ionomycin (black) and AMPA (red) appeared to increase with a steeper initial gradient than those to NMDA plus ACPC (green) or l-noradrenaline (pale blue). Statistical analysis of curves through the first 2 minutes (readings 2-4) by Repeated-Measures ANOVA with Tukey's post-test indicated a significant difference between the curve for l-noradrenaline and those for either AMPA or ionomycin (*, $p<0.05$, n=4 in each case).

d) Shows the mean ± SEM values for evoked increases in Ca^{2+} fluorescence through the 6.0-8.5 minutes time period of the established plateau phase of responses (shown in part b) from which data were normally collected for subsequent quantitative analysis. Symbols show ionomycin (black, IONO5), l-noradrenaline (pale blue, NA100), AMPA (red, AMPA100) and NMDA plus ACPC (green, NMDA100 + ACPC100). e) Shows the overall mean values for the evoked responses shown in part d) averaged over the 6.0-8.5 minutes time points in each case and then displayed as the mean ± SEM values from 4 separate experiments. Statistical analysis by One-way ANOVA with Tukey's post-test showed that responses to AMPA (AMPA100), NMDA plus ACPC (NMDA100 + ACPC100) and l-noradrenaline (NA100) were all significantly less than responses to ionomycin (IONO), ***, $p<0.001$, n=4 in each case.

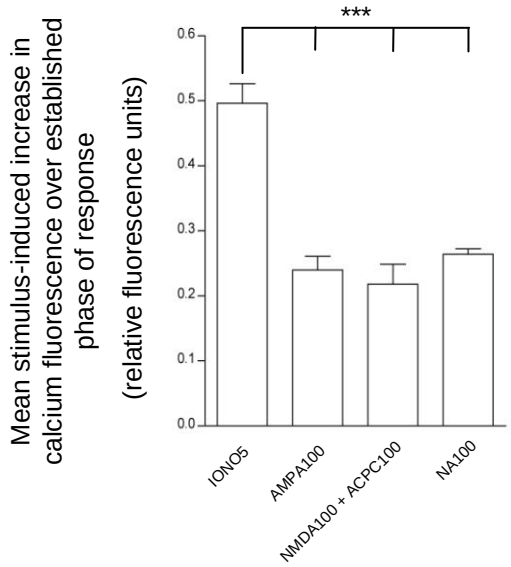
4.7 c)


Stimulus-induced increase in calcium fluorescence over initial phase of response (fold of initial value)
1.5
1.4
1.3
1.2
1.1
1.0
0.9
*
1.0
2.0
Time (minutes)

4.7 d)


Stimulus-induced increase in calcium fluorescence over established phase of response (relative fluorescence units)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
IONO5
NA100
AMPA100
NMDA100 + ACPC100
6.0
6.5
7.0
7.5
8.0
8.5
Time (minutes)

4.7 e)


Mean stimulus-induced increase in calcium fluorescence over established phase of response (relative fluorescence units)
***
0.6
0.5
0.4
0.3
0.2
0.1
0.0
IONO5
AMPA100
NMDA100 + ACPC100
NA100

4.2.2.3 Noradrenaline in combination with ionotropic glutamate receptor agonists at different concentrations

In order to further explore the types of analysis that could be achieved with these newly developed protocols and assess whether there was any evidence for interactions between l-noradrenaline- and AMPA- or NMDA/ACPC-evoked Ca^{2+} fluorescence responses, experiments were carried out with combinations of agonists on 30-100 μm tissue segments from frontal cortex. To achieve this the glutamate receptor agonists were initially added in combination (AMPA plus NMDA plus ACPC; ANA) at a range of concentrations (10, 30 and 100 μM for each component in each case), establishing concentration-dependence of the mixed ionotropic glutamate receptor response with small evoked increases at 10 μM and 30 μM ANA and a much larger response at 100 μM (Figure 4.8). 100 μM l-noradrenaline (NA100), was then tested alone and in combination with ANA (10-100 μM). Data were collected from the 6.0-8.5 minutes recording period in each case, averaged, and then mean values from 4 separate experiments calculated, with results being expressed as mean ± SEM of the evoked response normalised to the evoked response from 5 μM ionomycin (which was designated a value of 1.0). l-noradrenaline alone induced a clear increase in Ca^{2+} fluorescence (as seen in Figures 4.6 and 4.7) and increased the size of responses to ANA at submaximal concentrations of 10 μM and 30 μM but not at 100 μM, where the response may be approaching its maximum extent. Statistical analysis comparing the responses to each concentration of ANA, with or without l-noradrenaline LITING CLOSE GAP100 μM indicated that responses were significantly greater in the presence of l-noradrenaline at ANA concentrations of 10 μM and 30 μM but not 100 μM ($p<0.05$, Student's paired t-test, n=4 in each case; Figure 4.8). However, l-noradrenaline alone produced Ca^{2+} fluorescence responses greater than the magnitude of increment it caused in ANA responses, so while these data certainly add to the characterisation of both metabotropic and ionotropic receptor responses assessed by the new assay they do not provide any evidence for synergistic interactions (although it may be possible that this could be seen at different combinations of agonist concentrations). Nevertheless, the precise interrelations between the effects of adrenergic, AMPA and

NMDA receptor activation were not further investigated here as the aim was simply to establish that dynamic Ca^{2+} responses to each could be successfully monitored with these protocols.

4.2.2.4 The effect of acetylcholine on frontal cortex Ca^{2+} fluorescence levels, its effect on responses to glutamate receptor agonists and the modulatory influence of noradrenaline on these responses

The role of acetylcholine in the regulation of the intracellular Ca^{2+} concentration of *ex vivo* 30-100 μm frontal cortex segments was assessed alone and then in combination with mixed glutamate receptor agonists and subsequently with the addition of noradrenaline applied either acutely or following pre-incubation. Figure 4.9 shows mean overall stimulus-evoked responses from these data normalised to ionomycin-evoked responses. Acetylcholine 20 μM evoked a small increase in Ca^{2+} fluorescence of 30-100 μm tissue segments from frontal cortex and when administered in conjunction with a combined stimulus from AMPA 20 μM, NMDA 20 μM and ACPC 20 μM (ANA 20 μM) appeared to amplify the response, although there was no evidence that this was to a greater than additive extent. When l-noradrenaline was added acutely to the ANA plus Ach stimulus, responses to acetycholine plus glutamate receptor agonists appeared to be increased but this did not reach statistical significance by One-way ANOVA with Tukey's post-test. If l-noradrenaline was pre-incubated with the samples for 45 minutes, rather than acutely applied, the response to ANA 20 μM plus acetylcholine 20 μM was significantly amplified ($p<0.01$ by One-way ANOVA with Tukey's post-test, $n=4$ in each case). This may imply that optimal facilitation of ionotropic glutamate receptor responsiveness in the presence of acetylcholine is engendered by some relatively slowly developing signalling influence elicited by noradrenaline (perhaps involving indirect processes such as phosphorylation of voltage-sensitive ion channels occurring over a tens of minutes time-scale).

Figure 4.8: Ca^{2+} fluorescence responses of *ex vivo* tissue segments from frontal cortex: noradrenaline in combination with ionotropic glutamate receptor agonists at different concentrations

Tissue segments of 30-100 μm diameter or synaptoneurosomes in 24-well plates were loaded with the fluorometric Ca^{2+} indicator, Calcium 4. After transfer to a fluorometric plate reader, three minutes were allowed for settlement and then drugs were added before sequential fluorescence readings were made every 30 seconds. Data were collected and averaged from the 6.0-8.5 minutes period of plateau phase responses following drug addition, basal values were subtracted and the mean evoked responses were normalised to responses evoked by 5 μM ionomycin (which were designated a value of 1.0). These values were expressed as mean ± SEM from 4 separate experiments. The evoked responses are shown to AMPA plus NMDA plus ACPC (ANA, with each component at either 10 μM, 30 μM or 100 μM; ANA10, ANA 30, ANA100), to l-noradrenaline 100 μM (NA100) and to combinations of these stimuli. Statistical analysis of responses to different concentrations of ANA, with or without l-noradrenaline indicated significantly greater responses to ANA 10 μM and 30 μM but not 100 μM in the presence of l-noradrenaline (*, $p<0.05$, Student's paired t-test, n=4 in each case).

4.8

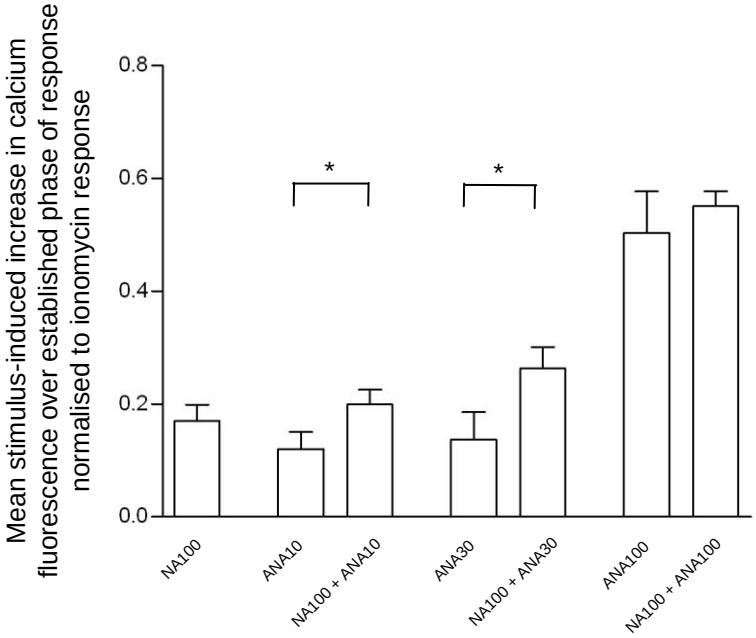
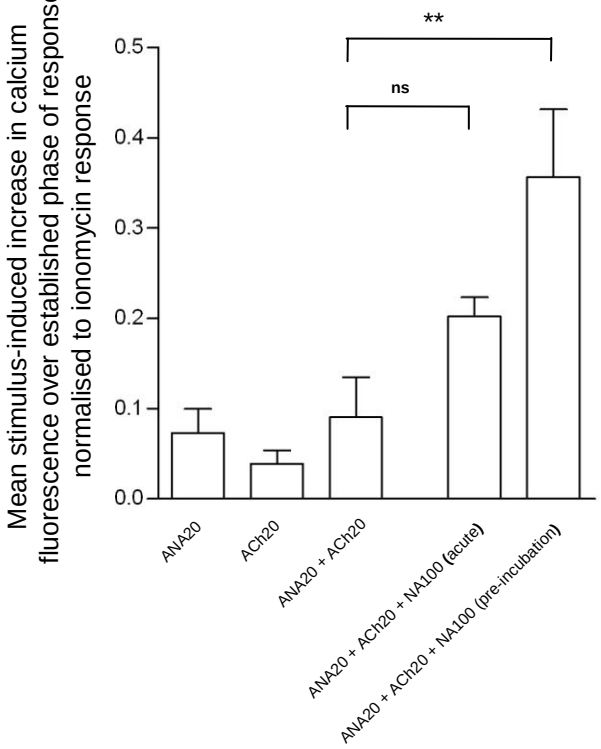

Mean stimulus-induced increase in calcium fluorescence over established phase of response normalised to ionomycin response
0.8
0.6
0.4
0.2
0.0
*
*
NA100
ANA10
NA100 + ANA10
ANA30
NA100 + ANA30
ANA100
NA100 + ANA100

Figure 4.9: Ca^{2+} fluorescence responses of *ex vivo* tissue segments from frontal cortex: effect of noradrenaline pre-incubation on responses to acetylcholine and glutamate receptor agonists

Tissue segments of 30-100 μm diameter or synaptoneurosomes in 24-well plates were loaded with the fluorometric Ca^{2+} indicator, Calcium 4. After transfer to a fluorometric plate reader, three minutes were allowed for settlement and then drugs were added before sequential fluorescence readings were made every 30 seconds. Data were collected from 6.0-8.5 minutes time points in the established plateau phases of responses, basal values were subtracted, then the average evoked response in each case was normalised to the averaged ionomycin-evoked response from the same plate (which was designated a value of 1.0). Results were plotted as mean ± SEM values from 4 separate experiments. Tissue segments were stimulated with a combination of glutamate receptor agonists, AMPA 20 μM, NMDA 20 μM, ACPC 20 μM (ANA20), acetylcholine 20 μM (ACh20) or these added together (ANA20 + ACh20). In addition, some of these samples were stimulated with 100 μM l-noradrenaline either acutely (ANA20 + ACh20 + NA100 acute) or following pre-incubation throughout the 45 minutes dye-loading period (ANA20 + ACh20 + NA100 pre-incubation). ANA20 and ACh20 produced small increases in Ca^{2+} fluorescence. The combination ANA20 + ACh20 appeared to produce a larger response but not to the extent of being greater than additive. The further addition of 100 μl l-noradrenaline (which would produce clear rises in Ca^{2+} fluorescence alone, Figures 4.6, 4.7, 4.8) resulted in a larger response when added acutely (ANA20 + ACh20 + NA100 acute) but this was clearly enhanced if the noradrenaline was applied with pre-incubation (ANA20 + ACh20 + NA100 pre-incubation). Statistical analysis by One-way ANOVA with Tukey's post-test indicated that responses to the ANA20 + ACh20 + NA100 pre-incubation stimulus were significantly greater than those to ANA20 + ACh20, whereas responses to ANA20 + ACh20 + NA100 acute, were not (**, $p<0.01$, $n=4$ in each case). Pre-incubation with noradrenaline amplified responses to acetycholine and glutamate receptor agonists to a greater extent than when added simultaneously.

4.9


Mean stimulus-induced increase in calcium fluorescence over established phase of response normalised to ionomycin response
0.5
0.4
0.3
0.2
0.1
0.0
**
ns
ANA20
ACh20
ANA20 + ACh20
ANA20 + ACh20 + NA100 (acute)
ANA20 + ACh20 + NA100 (pre-incubation)

4.2.3 Membrane potential fluorescence responses of *ex vivo* tissue segments from frontal cortex

Apart from the intracellular Ca^{2+} fluorescence responses, membrane potential fluorescence responses of the tissue segments were examined to confirm that they were able to maintain a membrane potential difference and that the effects of ionotropic glutamate receptor stimulation involved a depolarisation of this membrane potential.

Frontal cortex segments of 30-100 μm diameter were rapidly prepared in oxygenated media, aliquoted to 24-well plates, and loaded with no-wash fluorometric voltage-sensitive dye (R8042, MP Blue; Molecular Devices). After transfer to a fluorometric plate reader, 3 minutes were allowed for settlement and then drugs or appropriate vehicle were added in one tenth of the well volume from stocks at ten-fold higher concentrations. Sequential fluorescence readings were made every 30 seconds from each well with excitation set at 530 nm and emission at 565 nm.

As a trial, I investigated co-activation of 5-HT and glutamate receptors in these experiments (Figure 4.10). The selective 5-HT_{2A} receptor agonist DOI was added together with AMPA and cyclothiazide (CTZ), which acts to minimize AMPA receptor desensitisation (Kovacs *et al.*, 2004). Medium containing an additional 50 mM concentration of K^+ was used as a strongly depolarising positive control. Figure 4.10a shows the time-course of responses to 50 mM K^+ and both metabotropic and ionotropic receptor stimuli. Clear and reproducible increases above basal fluorescence were seen in each case, with a similar temporal profile to the Ca^{2+} fluorescence responses studied with this assay system. Stable plateau responses were reached within the first few minutes and then maintained for up to 15 minutes. Although adequate for quantification of drug-induced responses, the relative degree of increase over the basal fluorescence (only in the order of 20-25%) was not as great as with the Ca^{2+} fluorophore. As this was a pilot proof-of-concept study and the ability to quantify and compare relative responses was sufficient for current purposes, I did not attempt to calibrate the dynamic range of the assay with a range of K^+ concentrations and ionophore. Data were collected from the 6.0-8.5 minutes time

points, background was subtracted and average values were expressed as evoked responses normalised to 50 mM K^+-evoked responses. These results are shown in Figure 4.10b as mean ± SEM values from 4 separate experiments. DOI 3 μM alone evoked a clear membrane potential fluorescence response and a greater response was elicited by AMPA 100 μM in the presence of CTZ 30 μM. The combination of DOI 3 μM with AMPA 100 μM plus CTZ 30 μM however, evoked a response that was no greater than that due to AMPA plus CTZ alone. Statistical analysis by One-way ANOVA with Tukey's post-test indicated that both AMPA plus CTZ and DOI plus AMPA plus CTZ responses were significantly greater than those due to DOI alone ($p<0.01$, $n=4$). These results showed that DOI, AMPA plus CTZ (in the absence or presence of DOI) and high K^+ medium each elicited clear changes in fluorescence of the membrane potential-sensitive dye, establishing the idea that this kind of preparation could be used to assess stimulus-induced changes in membrane potential in addition to changes in cytosolic Ca^{2+} concentration.

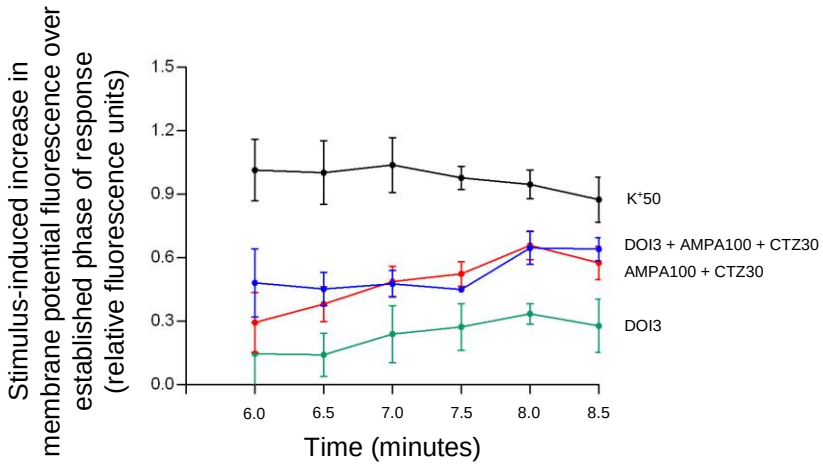
Figure 4.10: Membrane potential fluorescence responses of *ex vivo* tissue segments from frontal cortex: effects of a selective 5-HT_{2A} receptor agonist, DOI and AMPA in the presence of cyclothiazide compared to depolarisation with high K^+ medium

Tissue segments of 30-100 μm diameter or synaptoneurosomes in 24-well plates were loaded with the fluorometric Ca^{2+} indicator, Calcium 4. After transfer to a fluorometric plate reader, three minutes were allowed for settlement and then drugs were added before sequential fluorescence readings were made every 30 seconds. Tissue segments were stimulated with 50 mM K^+ (black, K^+50), DOI 3 μM (green, DOI3), AMPA 100 μM plus an inhibitor of AMPA receptor desensitisation, cyclothiazide, CTZ 30 μM (red, AMPA100 + CTZ30), the combination of DOI plus AMPA and CTZ (blue, DOI3 + AMPA100 + CTZ30) or basal. Values were plotted as mean ± SEM from 4 separate experiments.

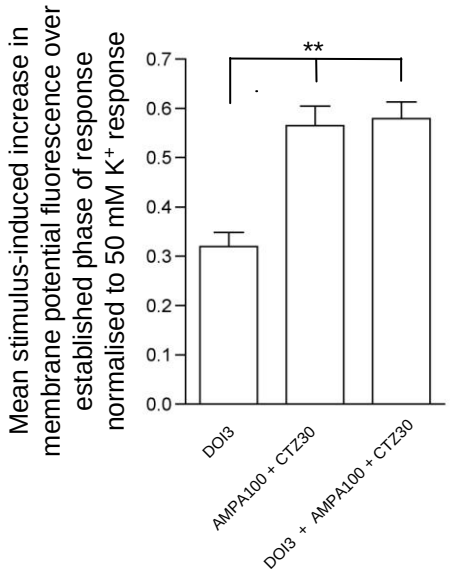
a) Shows the time-course of membrane potential fluorescence responses through the 6.0-8.5 minutes recording period expressed as evoked responses (i.e. following subtraction of basal fluorescence values) from the 4 separate experiments, revealing the good reproducibility of responses.

b) Shows data collected from the 6.0-8.5 minutes time points in each case expressed as evoked responses normalised to the 50 mM K^+-evoked responses in each experiment, (which were designated a value of 1.0) then calculated as mean ± SEM, from the 4 separate experiments. Statistical analysis by One-way ANOVA with Tukey's post-test indicated that the membrane potential fluorescence responses to AMPA100 + CTZ30 and DOI3 + AMPA100 + CTZ30 were both significantly greater than responses to DOI3 alone (**, $p<0.01$, $n=4$). DOI alone, AMPA plus CTZ (in the absence or presence of DOI) or high K^+ medium each elicited clear changes in fluorescence of the membrane potential-sensitive dye.

4.10 a)


Stimulus-induced increase in membrane potential fluorescence over established phase of response (relative fluorescence units)
1.5
1.2
0.9
0.6
0.3
0.0
6.0
6.5
7.0
7.5
8.0
8.5
Time (minutes)
K+50
DOI3 + AMPA100 + CTZ30
AMPA100 + CTZ30
DOI3

4.10 b)


Mean stimulus-induced increase in membrane potential fluorescence over established phase of response normalised to 50 mM K+ response
**
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
DOI3
AMPA100 + CTZ30
DOI3 + AMPA100 + CTZ30

4.2.4 Ca^{2+} fluorescence responses of *ex vivo* tissue segments from lumbar spinal cord

Lumbar spinal cord tissue was dissected following laminectomy under isoflurane anaesthesia as there would otherwise have been an excessive delay after death before the tissue was prepared in oxygenated medium compared to the cerebral cortex tissue. Surgery was carried out by S. Fleetwood-Walker and myself as described in detail in Section 2.2.1. Subsequent procedures with spinal cord tissue segments were the same as those for cerebral cortex segments.

Time-courses of Ca^{2+} fluorescence responses evoked by AMPA 100 μM, AMPA plus CTZ 30 μM, ionomycin 5 μM and basal were recorded. In Figure 4.11a results are shown as the mean ± SEM evoked responses (i.e. after subtraction of corresponding basal values) at each time through the 6.0-8.5 minutes period of established responses, with data from 4 separate experiments. The temporal profile of Ca^{2+} fluorescence responses from spinal cord tissue segments was very similar to that seen with cerebral cortex tissue. Stimulation with AMPA produced a small increase in Ca^{2+} fluorescence which appeared to be greater in the presence of CTZ, although as expected ionomycin produced a greater response than either.

Figure 4.11b shows the mean evoked responses (averaged through the 6.0-8.5 minutes time points), normalised to the ionomycin-evoked responses from each experiment (which were designated a value of 1.0), then calculated as mean ± SEM from 4 separate experiments. Stimulus-induced responses are shown to AMPA 100 μM and AMPA 100 μM plus CTZ 30 μM. Statistical analysis by Student's paired t-test indicated that AMPA-induced Ca^{2+} fluorescence responses in the presence of CTZ were significantly greater than in its absence ($p<0.05$, $n=4$). These results indicated that Ca^{2+} fluorescence responses to AMPA, AMPA plus CTZ and ionomycin could be elicited from spinal cord tissue segments with the newly developed methodology and paved the way forward for future studies on altered responsiveness of *ex vivo* spinal cord tissues in states of chronic pain or hypersensitivity to incoming stimuli.

4.2.5 Ca^{2+} fluorescence responses of *ex vivo* synaptoneurosomes from lumbar spinal cord

Lumbar spinal cord tissue was dissected under isoflurane anaesthesia and synaptoneurosomes prepared as described in 2.3.1. Synaptoneurosomes were aliquoted into 24-well plates and loaded with no-wash fluorometric Ca^{2+} indicator, Calcium 4. After transfer to a fluorometric plate reader, 3 minutes were allowed for settlement before starting the recording of fluorescence values. Samples were stimulated with the broad spectrum agonist of glutamate receptors, DL-homocysteic acid (DLH) 400 μM (Curtis *et al.*, 1961; Mayer and Westbrook, 1984), and agonists of receptors selectively localised to nociceptive primary afferents, TRPA1, TRPV1, and bradykinin (allyl isothiocyanate (AITC) 20 μM (Bandell *et al.*, 2004a), capsaicin 10 μM (Caterina *et al.*, 1997a) and bradykinin 10 μM respectively), ionomycin 5 μM or vehicle as basal control. The recorded Ca^{2+} fluorescence readings from the 3.0-5.5 minutes time points were plotted against time as the mean ± SEM values from 5 separate experiments (Figure 4.12a). The results indicated that the isolated spinal cord synaptoneurosomes were functional and consistent Ca^{2+} fluorescence responses could be elicited by ionomycin, DLH or the mixture of agents acting on nociceptive afferents (ACB). Figure 4.12b shows the mean Ca^{2+} fluorescence values averaged throughout this time period as mean ± SEM from the 5 separate experiments. Statistical analysis by Student's paired t-test indicated that DLH and ACB each elicited significant increases in Ca^{2+} fluorescence above basal levels. While mediators of some of these responses are likely to be present on postsynaptic elements (such as those responding to DLH), the AITC/capsaicin/bradykinin (ACB) responses may originate (in substantial part) from presynaptic elements of primary afferents.

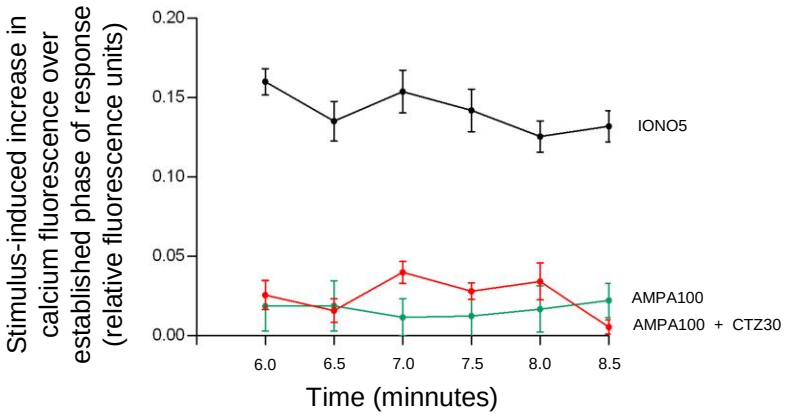
Figure 4.11: Ca^{2+} fluorescence responses of *ex vivo* tissue segments from lumbar spinal cord: effects of AMPA, AMPA plus CTZ and ionomycin

Tissue segments of 30-100 μm diameter or synaptoneurosomes in 24-well plates were loaded with the fluorometric Ca^{2+} indicator, Calcium 4. After transfer to a fluorometric plate reader, three minutes were allowed for settlement and then drugs were added before sequential fluorescence readings were made every 30 seconds. Tissue segments were stimulated with AMPA 100 μM (green, AMPA100), AMPA 100 μM plus CTZ 30 μM (red, AMPA100 + CTZ30), ionomycin 5 μM (black, IONO5) or basal.

a) Shows evoked Ca^{2+} fluorescence responses (i.e. after subtraction of corresponding basal values) expressed as the mean ± SEM at each time point through the established response phase at 6.0-8.5 minutes, from 4 separate experiments. Each of the stimuli produced reproducible responses with a similar temporal profile to responses in cerebral cortex tissue.

b) Shows mean evoked Ca^{2+} fluorescence responses from data averaged through the 6.0-8.5 minutes recording period, normalised to ionomycin-evoked responses from the same experiment (which were designated a value of 1.0) and then calculated as mean ± SEM from the 4 separate experiments. Statistical analysis by Student's paired t-test indicated that responses to AMPA in the presence of CTZ were significantly greater than those in its absence (*, $p<0.05$, $n=4$). Thus, consistent Ca^{2+} fluorescence responses to AMPA, AMPA plus CTZ (and also to ionomycin) could be achieved from spinal cord tissue segments and the presence of CTZ appeared to amplify responses to AMPA.

4.11 a)


Stimulus-induced increase in calcium fluorescence over established phase of response (relative fluorescence units)
0.20
0.15
0.10
0.05
0.00
6.0
6.5
7.0
7.5
8.0
8.5
Time (minnutes)
IONO5
AMPA100
AMPA100 + CTZ30

4.11 b)

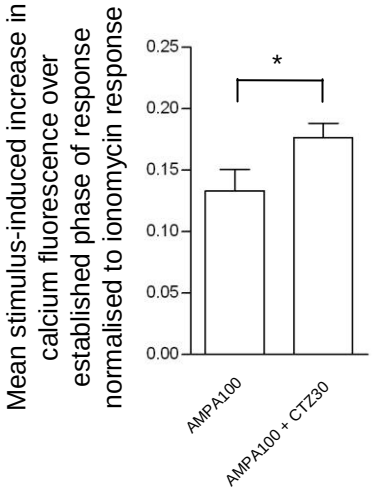

Mean stimulus-induced increase in calcium fluorescence over established phase of response normalised to ionomycin response
0.25
0.20
0.15
0.10
0.05
0.00
*
AMPA100
AMPA100 + CTZ30

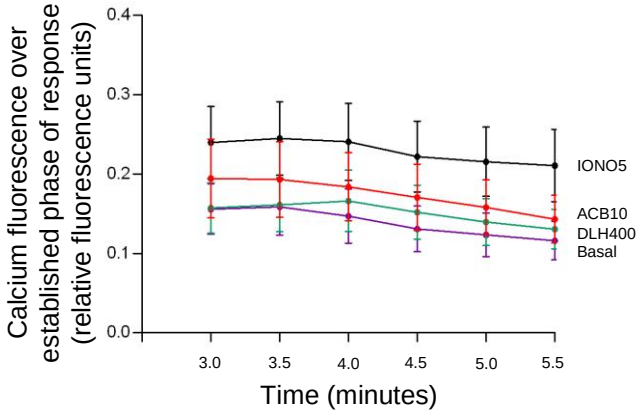
Figure 4.12: Ca^{2+} fluorescence responses of *ex vivo* synaptoneurosomes from lumbar spinal cord: effects of DLH, the combination of AITC, capsaicin and bradykinin (ACB), and ionomycin

Synaptoneurosomes in 24-well plates were loaded with the fluorometric Ca^{2+} indicator, Calcium 4. After transfer to a fluorometric plate reader, three minutes were allowed for settlement and then drugs were added before sequential fluorescence readings were made every 30 seconds. Synaptoneurosomes were stimulated with DLH 400 μM (green, DLH400), AITC 20 μM plus capsaicin 10 μM plus bradykinin 10μM (red ACB10), ionomycin 5 μM (black, IONO5) or basal vehicle control (purple, Basal).

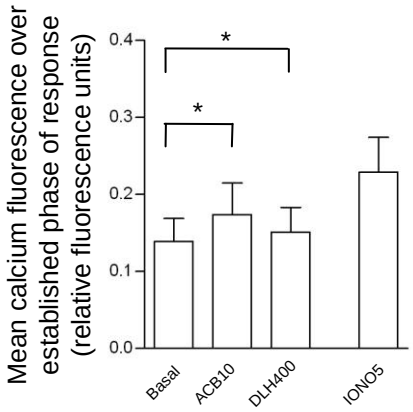
a) Shows evoked Ca^{2+} fluorescence records through the 3.0-5.5 minutes period of established plateau phase responses that was generally used to obtain data from quantitative analysis. Values at each time point represent the mean ± SEM from 5 separate experiments. DLH and ACB elicited small but consistent Ca^{2+} fluorescence responses from spinal cord synaptoneurosomes.

b) Shows mean Ca^{2+} fluorescence responses from data as in a) averaged through the 3.0-5.5 minutes data collection period and then expressed as mean ± SEM values from 5 separate experiments. Statistical analysis by Student's paired t-test indicated that DLH or ACB elicited significant increases in Ca^{2+} fluorescence above basal levels (*, $p<0.05$, $n=5$).

4.12 a)


Calcium fluorescence over established phase of response (relative fluorescence units)
0.4
0.3
0.2
0.1
0.0
3.0
3.5
4.0
4.5
5.0
5.5
Time (minutes)
IONO5
ACB10
DLH400
Basal

4.12 b)


Mean calcium fluorescence over established phase of response (relative fluorescence units)
0.4
0.3
0.2
0.1
0.0
*
*
Basal
ACB10
DLH400
IONO5

4.2.6 Ca^{2+} fluorescence responses of *ex vivo* microslices from lumbar spinal cord

In order to develop a protocol with samples that retained a degree of neuronal interconnectivity, lumbar spinal cord tissue was dissected under brief isoflurane anaesthesia and 150 × 150 μm microslices rapidly prepared as described in 2.3.1. The subsequent procedures with microslices from lumbar spinal cord were the same as those for spinal cord synaptoneurosomes. Microslices were suspended and washed in ice-cold oxygenated media, aliquoted to 24-well plates and loaded with no-wash fluorometric Ca^{2+} indicator, Calcium 4. After transfer to the plate reader, 3 minutes were allowed for settlement before drug additions and commencement of fluorescence readings. Microslices were stimulated with AMPA 100 μM plus CTZ 30 μM, AITC 20 μM, capsaicin 10 μM, ionomycin 5 μM or basal vehicle control. Figure 4.13a shows the time-course of Ca^{2+} fluorescence responses to AMPA plus CTZ, ionomycin and basal, expressed as mean ± SEM values at each time point, from 4 separate experiments. Both AMPA plus CTZ and ionomycin elicited clear and consistent increases in Ca^{2+} fluorescence. Figure 4.13b shows mean Ca^{2+} fluorescence values averaged over the 3.0-5.5 minutes data collection period (at which stable plateau phase responses were achieved) and then calculated as mean ± SEM values for the 4 separate experiments. Statistical analysis by Student's matched pair t-test indicated that AMPA plus CTZ caused significant increases in Ca^{2+} fluorescence above basal levels ($p<0.05$, $n=4$). Further experiments were carried out to investigate the effects of DLH 400 μM and the combined stimulus of nociceptive afferents ACB (AITC 20 μM plus capsaicin 10 μM plus bradykinin 10 μM) on Ca^{2+} fluorescence of spinal cord microslices. Figure 4.14a shows the time-course of responses to DLH, ACB, ionomycin and basal vehicle control through the 3.0-5.5 minutes period of stable plateau phase responses used to collect data for further analysis. Values are shown as mean ± SEM at each time point from 3 separate experiments and demonstrate that the responses were clear and reproducible. Figure 4.14b shows the mean Ca^{2+} fluorescence responses averaged through the 3.0-5.5 minutes period in each experiment and then expressed as the overall mean ± SEM from the 3 separate experiments. Statistical analysis by Student's paired t-test, or by

One-way ANOVA with Tukey's post-test, indicated that DLH or ACB each elicited significant increases in Ca^{2+} fluorescence above basal control levels ($p<0.05$, $n=3$). These results suggest that microslices could also represent a viable test platform to assess alterations in neuronal excitability in response to agonists that might additionally reflect indirect influences within functional neural micro-circuitry.

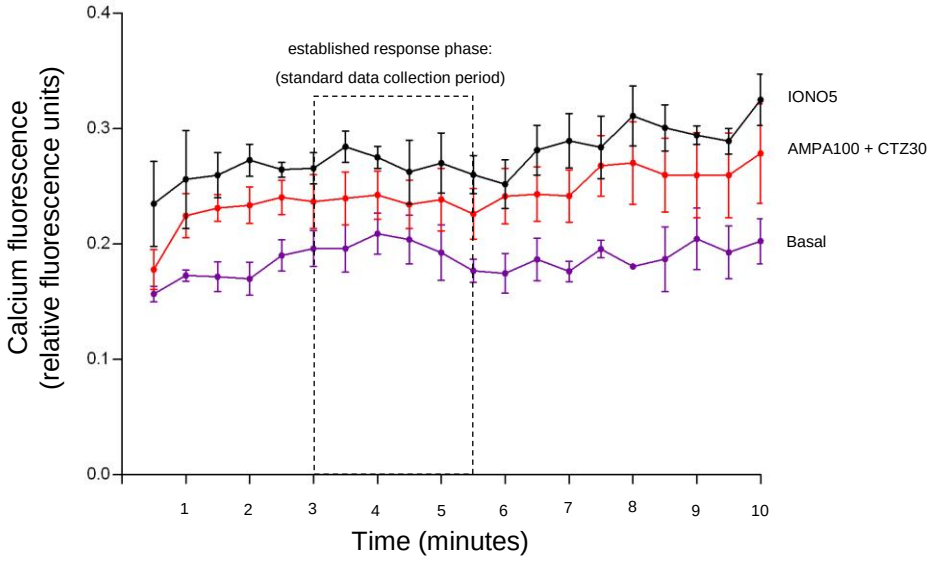
Figure 4.13: Ca^{2+} fluorescence responses of *ex vivo* microslices from lumbar spinal cord: effects of the combination of AMPA and CTZ, AITC, capsaicin or ionomycin

Spinal cord microslices in 24-well plates were loaded with the fluorometric Ca^{2+} indicator, Calcium 4. After transfer to a fluorometric plate reader, three minutes were allowed for settlement and then drugs were added before sequential fluorescence readings were made every 30 seconds. Spinal cord microslices were stimulated with AMPA 100 μM plus CTZ 30 μM (red, AMPA100 + CTZ30); AITC 20 μM (AITC20), capsaicin 10 μM（capsaicin10) and ionomycin 5μM (black, IONO5) or basal vehicle control (purple, Basal).

a) Shows time-courses of evoked Ca^{2+} fluorescence readings from spinal cord microslices stimulated with AMPA plus CTZ, ionomycin or basal expressed as mean ± SEM values at each time point from 4 separate experiments. Clear and reproducible responses to AMPA plus CTZ (and also to ionomycin) could be achieved from spinal cord microslices, although AITC or capsaicin alone only produced small increments in Ca^{2+} fluorescence.

b) Shows mean Ca^{2+} fluorescence readings averaged through the 3.0-5.5 minutes time period of plateau phase responses that was used to collect data for subsequent analysis, then calculated as overall mean ± SEM values from the 4 separate experiments. Statistical analysis by Student's paired t-test indicated that AMPA plus CTZ elicited a significant increase in Ca^{2+} fluorescence above that in basal controls (*, $p<0.05$, $n=4$).

4.13 a)


established response phase:
(standard data collection period)
IONO5
AMPA100 + CTZ30
Basal
Calcium fluorescence
(relative fluorescence units)
Time (minutes)
0.0
0.1
0.2
0.3
0.4
1
2
3
4
5
6
7
8
9
10

4.13 b)

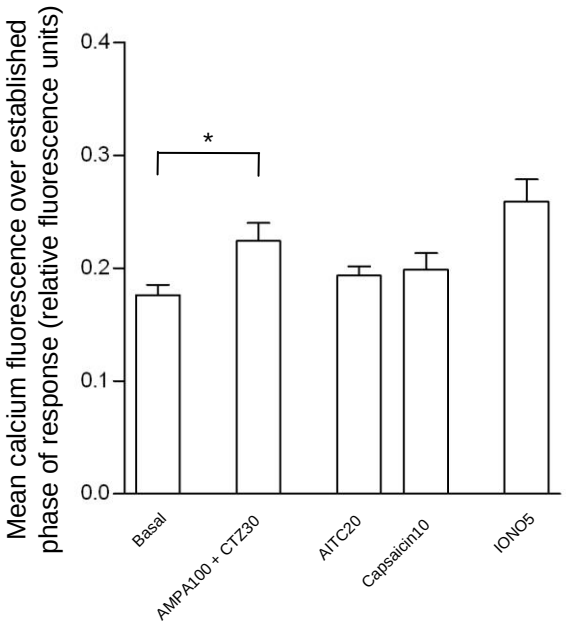

Mean calcium fluorescence over established phase of response (relative fluorescence units)
*
0.0
0.1
0.2
0.3
0.4
Basal
AMPA100 + CTZ30
AITC20
Capsaicin10
IONO5

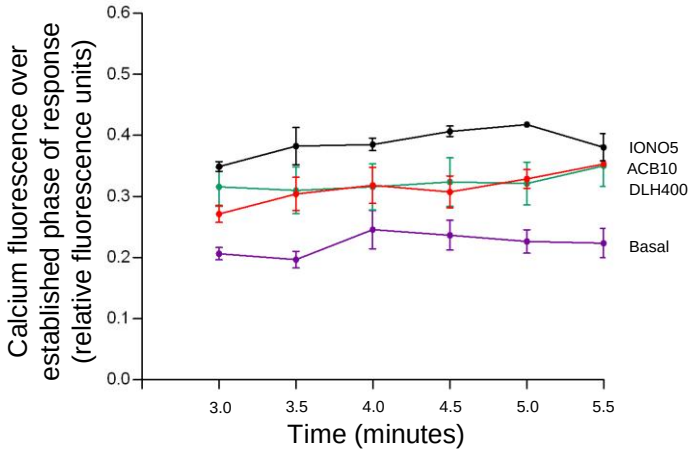
Figure 4.14: Ca^{2+} fluorescence responses of *ex vivo* microslices from lumbar spinal cord: effects of DLH, the combination of AITC, capsaicin and bradykinin (ACB) or ionomycin.

Spinal cord microslices in 24-well plates were loaded with the fluorometric Ca^{2+} indicator, Calcium 4. After transfer to a fluorometric plate reader, three minutes were allowed for settlement and then drugs were added before sequential fluorescence readings were made every 30 seconds. Spinal cord microslices were stimulated with DLH 400 μM (green, DLH400); AITC 20 μM plus capsaicin 10 μM plus bradykinin 10 μM (red, ACB10), ionomycin (black, IONO5) or basal vehicle control (purple, Basal).

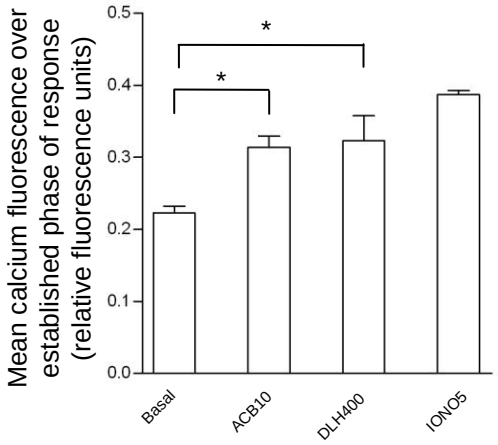
a) Shows Ca^{2+} fluorescence readings through the 3.0-5.5 minutes period of stable plateau phase responses used to collect data for subsequent analysis. Values at each time point are expressed as the mean ± SEM from 3 separate experiments. DLH, ACB and ionomycin each elicited clear and reproducible increases in Ca^{2+} fluorescence.

b) Shows mean Ca^{2+} fluorescence values averaged through the 3.0-5.5 minutes data collection period in each case and then expressed as the overall mean from the 3 separate experiments. Statistical analysis by Student's paired t-test (or One-way ANOVA with Tukey's post-test) indicated that DLH and ACB each caused a significant increase in Ca^{2+} fluorescence above basal control levels (*, $p<0.05$, $n=3$). These results show that clear Ca^{2+} fluorescence responses could be obtained from DLH and from the combined nociceptive afferent stimulus of AITC, capsaicin and bradykinin in spinal cord microslices.

4.14 a)


Calcium fluorescence over established phase of response (relative fluorescence units)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
3.0
3.5
4.0
4.5
5.0
5.5
Time (minutes)
IONO5
ACB10
DLH400
Basal

4.14 b)


Mean calcium fluorescence over established phase of response (relative fluorescence units)
0.5
0.4
0.3
0.2
0.1
0.0
*
*
Basal
ACB10
DLH400
IONO5

4.3 Discussion

Traditional approaches to the study of cell signalling events that may be applied to studying states of altered somatosensory processing often focus on single receptor or channel activation. The functions of such molecules are commonly examined using pharmacological, electrophysiological, genetic and behavioural techniques. While there is little doubt that these investigations have been of substantial utility in understanding cell signalling, it is widely realised that the signalling involves systems of interacting component molecules rather than individual molecules functioning independently. In the present study, a new methodological strategy was developed to enable dynamic assessment of the co-ordinated effects of stimulating multiple receptors and different signalling pathways in acutely isolated *ex vivo* neural tissue preparations. The demonstration of consistent Ca^{2+} fluorescence responses induced by noradrenaline or ionomycin in the 30-100 μm cortical tissue segments and synaptoneurosomes provided the first evidence that the newly developed method had been successfully set up. The responses to noradrenaline were reproducible, concentration-dependent and reversible (in synaptoneurosomes) by highly NO HYPHEN LITINGselective α_1-adrenoreceptor and β-adrenoreceptor antagonists, thus likely to represent receptor-mediated actions. Further experiments indicated that both AMPA- and NMDA-type ionotropic receptors for glutamate could increase Ca^{2+} fluorescence of cortical tissue segments, similarly to the effect of metabotropic adrenergic receptors.

Any functional interactions between influences of adrenergic and glutamate receptors could possibly reflect some aspect of the alerting response which is brought about by noradrenaline in higher levels of the CNS in animals. It has been reported that noradrenaline enhances NMDA receptor-mediated depolarisation in the cortex without affecting the AMPA receptor-mediated responses (Radisavljevic *et al.*, 1994). My results showed that cortical tissue segments showed a concentration-dependent elevation of Ca^{2+} in response to a combination of AMPA and NMDA receptor agonists and furthermore that noradrenaline increased responses to low levels of this

stimulus. However there was no evidence in these initial studies for greater than additive effects. There is also evidence that glutamate causes a concentration-dependent stimulation of noradrenaline release from rat prefrontal cortex slices (Russell and Wiggins, 2000). These findings could all be consistent with the idea that noradrenaline plays a facilitatory role in glutamatergic depolarisation in rat frontal cortex that may be subject to positive feedback enhancement. Noradrenergic cells that give rise to ascending sensory pathways innervate the cortex, where they may participate in cognitive responses such as alertness and pain perception. The current findings are consistent with the idea of co-operation of noradrenergic and glutamatergic inputs to cortex that could contribute to accentuated cognitive responses in rats.

Acetylcholine exerts its actions via nicotinic and muscarinic receptors in the brain. It has been proposed that nicotinic acetylcholine receptors expressed in the postsynaptic density of the somatosensory cortex work together with AMPA receptors to facilitate the activation of glutamatergic synapses in rats (Levy and Aoki, 2002). However, direct and specific evidence for modulatory effects of central acetylcholine on glutamatergic synapses is very limited. I applied acetylcholine together with combined ionotropic glutamate agonists to activate Ca^{2+} fluorescence responses of cortical tissue segments and then investigated the effect on that of acute or prior addition of noradrenaline. Pre-incubation with noradrenaline markedly increased the Ca^{2+} responses to acetylcholine and glutamate agonists to a significantly greater extent than acute application. This is consistent with the idea that excitability of cortical neurons may be increased by the influence of activity in noradrenaline-releasing fibres so that these cortical neurons may be ready to give enhanced responses to subsequent incoming stimuli, by way of relatively slow intracellular signalling changes taking place over a tens of minutes time-scale.

It is possible that measurements of raised intracellular Ca^{2+} levels may not directly represent the membrane excitability in that they could be due in part to release from intracellular endoplasmic reticulum stores by diverse calcium-mobilising receptors and Ca^{2+} entry through receptor-operated and voltage–sensitive channels. Thus a

membrane potential-sensitive dye was also evaluated as a more direct measure of excitability in trial experiments. I examined the effects of a 5-HT_{2A} receptor agonist and an ionotropic glutamate receptor agonist on the membrane potential-sensitive fluorescence of rat cortex segments. Clear membrane potential responses were detected following application of DOI or AMPA plus CTZ. This indicated that substantial depolarisation of the membrane was occurring either due to the metabotropic 5-HT_{2A} receptor agonist alone, or to a greater extent, in the presence of AMPA plus CTZ or the joint presence of the 5-HT_{2A} receptor agonist and AMPA/CTZ. Although I found no definitive evidence to prove the idea in these initial experiments, it is possible that the metabotropic receptor may work together with ionotropic glutamate receptors to facilitate transmission in the cortex and that this may be more readily revealed under different experimental conditions.

Additionally the approach of fluorometric assessment of *ex vivo* tissue responses was evaluated on lumbar spinal cord tissue. Small Ca^{2+} fluorescence responses to the glutamate receptor agonist, AMPA were detected in spinal cord tissue segments especially in the presence of the inhibitor of AMPA receptor desensitisation, CTZ. Moreover, synaptoneurosomes from lumbar spinal cord gave small but significant Ca^{2+} fluorescence responses to the broad spectrum glutamate receptor agonist DLH and to activation of nociceptive afferents by a combination of TRPA1, TRPV1 and bradykinin receptor agonists. This suggests that Ca^{2+} gradients can be maintained also in *ex vivo* spinal cord synaptoneurosomes. In addition, microslices from spinal cord tissue were prepared with the idea that these could maintain some level of neuron:neuron connectivity. Combined TRPA1, TRPV1 and bradykinin receptor agonists, as well as AMPA in the presence of CTZ, or DLH elicited significant increments in Ca^{2+} fluorescence from spinal cord microslices. These results further confirm that synaptoneurosomes and microslices could be successfully prepared from spinal cord in viable form and that they are functionally responsive to external receptor-mediated stimulation.

Together, these studies establish that the newly developed technological platform provides a valuable quantitative measure of neuronal responsiveness and yields clear

and interpretable results in both cortical and spinal cord *ex vivo* tissue with an array of different tissue preparations, 30-100 μm segments, synaptoneurosomes and 150 × 150 μm microslices. Furthermore, our pilot experiments illustrated that the excitability of isolated central neurons in response to stimulation could potentially be increased by the co-activation of several different monoaminergic neuronal systems, which may occur in animals under physiological and pathological circumstances, such as in chronic pain states. Such changes might be quantifiable following the further fine tuning of experimental conditions.

The experiments described here show that *ex vivo* CNS tissue samples prepared under the conditions used show consistent and quantifiable dynamic responses to receptor stimuli as assessed by Ca^{2+}- or voltage-sensitive fluorophores. 30-100 μm tissue segments, synaptoneurosomes and 150 × 150 μm microslices were all shown to be viable preparations for Ca^{2+} fluorometry. Synaptoneurosomes are of particular interest as they may reflect more closely any functional changes at synapses and microslices are of interest because they may retain some neuron:neuron interconnections. Further studies would be required to assess these aspects. In general terms, the present results suggest that the tissue preparations maintain competent plasma membranes and metabolic activity to a degree sufficient to show receptor-mediated increases in Ca^{2+} fluorescence and membrane potential fluorescence. In each case the development of ion gradients in basal conditions prior to stimulation would require sealed membranes and metabolic activity to drive Ca^{2+} extrusion or storage by Ca^{2+}-ATPases or Na^{+}/ K^{+} redistribution by Na^{+}/K^{+}-ATPase. Further studies would be required with precisely controlled ionic composition buffers and membrane permeabilisation or alternative ratiometric fluorophores in order to give absolute numerical estimates of intracellular Ca^{2+} concentrations or membrane potential. It may well be that these are not entirely normal as *in vivo* but the approach gives a model system in which dynamic receptor responses are quantifiable and readily analysed in relative terms to evaluate changes in responsiveness. Comparisons between experiments are made possible by the inclusion of a maximal (or very substantial) positive control for calibration each time (the Ca^{2+} ionophore

ionomycin for Ca^{2+} fluorescence and a substantially depolarising K^+ concentration for membrane potential fluorescence). Receptor-mediated responses above basal readings are then normalised to the positive control-mediated responses above basal, yielding values that are comparable between different experiments.

The affinity of the Ca^{2+} fluorophore for Ca^{2+} is not formally disclosed by the manufacturer as it is one of their proprietary no-wash reagents protected by patent. However they indicate that the fluorophore is similar to Fluo-4 from Invitrogen, which has an affinity (K_d, dissociation constant) for Ca^{2+} of 350 nM and is extensively used in the literature to assess receptor-mediated Ca^{2+} fluorescence responses in a range of cell types. Such an affinity is well suited for detection of receptor-mediated increases from normal cytosolic concentrations in the 100 nM range. As for Fluo-4, Calcium 4 is loaded as an inert but membrane-permeable acetoxymethoxy ester, which is then cleaved by intracellular esterases to yield the active form of the fluorophore to chelate Ca^{2+} and shift its fluorescence spectrum accordingly. The molecular identity of the MP Blue fluorophore used to assess membrane potential fluorescence is similarly not disclosed by the manufacturer but is indicated to be similar to bis-oxonol dyes of the DiBAC series, which selectively enter depolarised cells. The fluorescent signal increases in intensity during membrane depolarisation as dye follows positively charged ions moving inside the cell. During membrane hyperpolarisation, the fluorescent signal decreases in intensity as dye follows the positively charged ions out of the cell. The manufacturer describes a useful dynamic range of the dye through the range 5-60 mM external K^+, corresponding to membrane potentials of approximately -65 to -10 mV but this was not investigated in the present study. DiBAC series dyes redistribute relatively slowly over several tens of seconds and although MP Blue responds several fold faster than other DiBAC dyes, it would still not be fast enough to monitor action potentials. However such dyes can measure longer lasting changes due to receptor activation and may be able to follow slow rhythmic changes such as slow oscillations in cortical membrane potential, if these continue in the *ex vivo* preparations.

The assay was designed in plate-reader format to enable the parallel assessment of responses to aliquots of the same preparation. Stimuli were added separately to each well and responses followed for up to 15 minutes. Responses to receptor agonists and positive controls increased above basal through the first 2-3 minutes and then maintained an elevated plateau level until the end of the experiment where measured for Ca^{2+} or membrane potential fluorescence in each type of tissue preparation. None of the data suggested the presence of rapid but transient responses that had peaked before our measurements commenced. A limitation is that this format does not allow very short time scale measurement of responses, which would be ideal in modeling physiological responses. Real-time rapid measurements with programmed injection of drugs into wells are possible with plate readers but when this was investigated the turbulence caused by fluid addition caused unacceptable variability in readings. It may be possible to measure responses of samples in suspension in a cuvette fluorometer but this would have the disadvantage of processing samples only one at a time and it seems possible that continuous stirring may cause deterioration of the tissue. This approach was not investigated in the present study. The relative contribution of fluorescence from pre- or postsynaptic compartments is a question of considerable interest, especially with the synaptoneurosome preparation. Further study will be required to assess this and it is possible that new developments in super-resolution confocal imaging will enable progress to be made. The use of selective stimuli for nociceptive afferent fibres in spinal cord synaptoneurosomes represented a first step towards understanding pre- and postsynaptic contributions to the fluorescence signals recorded. One possible approach to assessing whether functional transmission could occur across the synaptic gap in stimulated synaptoneurosomes might involve the use of presynaptic release-modifying toxins such as botulinum or tetanus toxin.

There are clearly many further avenues of research to be followed to more fully characterise these models of dynamic functional responses in *ex vivo* CNS tissue preparations. The validation of the general approach provided in the current study represents the first proof-of-principle step in this process.

Chapter 5: General discussion

Many studies indicate that neurochemical and neurophysiological changes contribute to pain arising from injury to the nervous system. Tissue injury results in the release of pro-nociceptive mediators that sensitise peripheral nerve terminals (peripheral sensitisation), leading to neurochemical and phenotypic alterations of sensory neurons and increased excitability of spinal cord dorsal horn neurons (central sensitisation). In addition, the response of the nervous system to pain is not static, but is modulated by descending systems originating in the brain that can modulate pain thresholds.

Central sensitisation may involve functional changes in the influence exerted by central neurons, including inhibitory interneurons, anatomical changes in the connectivity of non-nociceptive terminals (although this is still a controversial area) and variations in the sensitivity or cellular influence of postsynaptic receptors. The excitatory ionotropic NMDA and AMPA receptors that respond to glutamate are important mediators of synaptic transmission in the central nervous system (CNS). AMPA receptors are widely distributed across the spinal dorsal horn and they not only mediate the fast excitatory information transmission in response to acute peripheral stimulation, but also play a pivotal role in transmission during chronic neuropathic pain (Furuyama *et al.*, 1993; Garry *et al.*, 2003b; Platt, 2007). The translocation of GluA1 subunit-containing AMPA receptors into synapses is believed to enhance synaptic transmission and contribute to synaptic plasticity in long-term potentiation (LTP) in hippocampal neurons (Shi, 2001). Activation of NMDA and AMPA receptors may directly or indirectly increase intracellular Ca^{2+} concentrations, thereby activating cascades serving to alter membrane excitability and protein expression as well as protein trafficking. Ca^{2+}-dependent proteins such as CaM kinase II and perhaps Ca^{2+}-dependent activators of the Ras/MAPK pathway (ERK) may play a crucial role in bringing about GluA1 translocation underlying LTP. Following nerve injury, phosphorylation of extracellular signal-regulated protein kinase (ERK), an important member of the MAPK family, is increased and this

increase is long-lasting in dorsal horn neurons (Ji *et al.*, 1999). It is further possible that the expression of Ras-GEFs such as Sos-1, key upstream regulators of ERK, may be altered in spinal neurons in response to peripheral noxious stimulation.

Here, attention is given to the synaptic alterations in expression of GluA1 subunit-containing AMPA receptors and phosphorylated ERK within spinal dorsal horn in the chronic constriction injury (CCI) model (Bennett and Xie, 1988), using biochemical methods to help elucidate neuropathic pain mechanisms. These studies in both adult and neonatal neuropathic pain re-challenge models necessitated considerable refinement and optimisation of procedures to allow their application to very small tissue samples. In addition, I developed entirely new protocols enabling the assessment of neurophysiological responses of the CNS including cortex and spinal cord to stimulation by multiple receptor systems in *ex vivo* neural tissue. This novel approach may lead to the discovery and development of novel pharmacological interventions that may have clinical utility, not necessarily limited to treating neuropathic pain.

5.1 Increased synaptic translocation of GluA1 subunits in spinal dorsal horn after noxious challenge

In the present study, synaptic translocation of GluA1 in spinal cord was increased by peripheral nerve injury (i.e. CCI) in adult rats or by formalin stimulation in young adult rats. Formalin caused greater synaptic insertion of GluA1 in animals that had received neonatal CCI and since recovered, compared to uninured controls. The findings were consistent with the hypothesis I had proposed, that increased translocation of spinal GluA1 subunit-containing AMPA receptors into postsynaptic densities may contribute to the central sensitisation of nociceptive processing in each case.

In young animals with nerve injury at P8, Western blots showed an increased expression of GluA1 subunits in ipsilateral spinal dorsal horn compared to the contralateral side, which is not generally reported in adults. In adult animals, although it has been reported that the expression of the GluA1 subunits was not changed in the superficial dorsal horn of the spinal cord after intradermal injection of capsaicin in adult rats, the subcellular location of the GluA1 subunits may be altered, since phosphorylation of GluA1 at S831 and S845, which modifies trafficking, is dramatically increased (Fang *et al.*, 2003b). Some studies support such an idea because GluA1 subunit-containing AMPA receptors in crude membrane fractions are elevated by CFA injection (Park *et al.*, 2008) or intra-colonic instillation of capsaicin (Galan *et al.*, 2004). In addition, translocation of GluA1 subunits to synapses is found following acute noxious stimulation and suggests that GluA1 trafficking may play a role in nociceptive transmission (Larsson and Broman, 2008). Taken together, I conclude that incorporation of GluA1 subunit-containing AMPA receptors to synapses is caused by nerve injury and may play a key part in underpinning the chronic hyper-responsive pain state.

GluA1 may additionally be present in other cell types such as glial cells. It has been reported that the expression of GluA1 in astrocytes is upregulated after spinal cord injury (Gomez-Soriano *et al.*, 2010). With the subcellular fractionation technique here, I reported for the first time that GluA1 levels are increased in neuronal postsynaptic densities of the spinal dorsal horn following peripheral nerve injury. Although I cannot be entirely certain that the increased GluA1 content in synapses represents fully functional receptors and contributes to the synaptic transmission in neuropathic pain state, this seems most likely to be the case.

If animals subjected to CCI at P8 (that had then recovered from the mechanical hypersensitivity caused by that injury) were subsequently challenged with intraplantar formalin, this acute stimulus became able to elicit a significantly greater synaptic incorporation of GluA1. This corresponded with a greater degree of formalin-induced nocifensive behaviours in these animals. No such enhancement of formalin responses was observed in adults subjected to CCI, which had then

recovered before a formalin challenge. Taken together, these findings indicate that increased levels of GluA1 in the PSD fraction correspond to the states of sensory hyper-responsiveness, either in a chronic situation, established adult CCI pain state, or an acute situation (formalin re-challenge) in neonatally injured and recovered rats. Neonates therefore display a lasting potential for hyper-responsiveness to later life noxious stimuli or 'pain memory' that is not observed after adult injury. Therefore, the expression and the distribution of GluA1 subunit in spinal dorsal horn appear to be key factors in nociceptive processing that are tightly regulated through the whole of life. Many additional factors may of course play a part in bringing about the lasting hyper-responsiveness following early life nerve injury, for example potential changes in neuronal circuitry, but little is yet known of such processes.

I then sought to explore the signalling events contributing to GluA1 trafficking and potentially also the lasting susceptibility to hyper-responsiveness observed in neonatally injured animals.

The origin of the increased level of GluA1 subunits in the postsynaptic density is not clear. Two potential mechanisms could account for such an upregulation of synaptic receptors: redistribution of existing membrane receptors to synaptic sites or translocation of intracellular receptors into synaptic membranes. Correspondingly, two major trafficking theories for GluA1 recruitment to synapses have been proposed. One theory supports the first mechanism, which the additional synaptic GluA1 subunits originate from extrasynaptic membrane of dendrites or spines. It has been reported that lateral movement of GluA1 subunit-containing AMPA receptors from non-synaptic spine plasma membranes enhances the number of these receptors at the synapse in hippocampal neurons (Makino and Malinow, 2009). The non-synaptic expression of GluA1 may serve as an extra-synaptic pool, which is replenished by exocytosis during subsequent plasticity. 4.1N may act as a regulator for GluA1 translocation to synapses since 4.1N can associate with the membrane proximal region of the C-terminal tail of GluA1 *in vivo* and disruption of the interaction of GluA1 with 4.1N decreased the surface expression of GluA1 and attenuated LTP in hippocampus (Shen *et al.*, 2000; Lin *et al.*, 2009). In the present study, I examined

the subcellular location in 4.1N of näive adult spinal dorsal horn, which was found to be predominantly in the PSD fraction, where it may co-localise with GluA1 subunits. The GluA1 co-immunoprecipitation results which showed increased association of GluA1 with 4.1N after nerve injury in adult spinal dorsal horn, may well support the idea of a facilitatory role for 4.1N in the synaptic insertion of GluA1. It has been suggested that the exocytosis of GluA1 does not directly increase the number of synaptic GluA1 subunits and may not occur at the same time as lateral movement (Makino and Malinow, 2009). An alternative, earlier theory suggests that GluA1 subunit-containing AMPA receptors are transported to synapses directly by exocytosis through secretory pathways originating from endoplasmic reticulum (Broutman and Baudry, 2001). The increased level of GluA1 subunits at synaptic sites is accompanied by decreased levels of this subunit at intracellular sites and can be inhibited by brefeldin A (BFA), which inhibits anterograde protein secretion from the Golgi apparatus (Broutman and Baudry, 2001). Therefore, the precise basis for GluA1 trafficking mechanisms remains incompletely understood and further investigation is needed.

5.2 Contribution of the Ras/MAP kinase pathway to the neuropathic pain

Multiple inflammatory mediators released from damaged tissue not only acutely excite primary sensory neurons in the peripheral nervous system, producing accentuated or ectopic discharge, but also lead to a sustained increase in their excitability. Hyperexcitability also develops in the central nervous system (for instance, in dorsal horn neurons), and both peripheral and spinal elements contribute to neuropathic pain, so that spontaneous pain may occur or normally innocuous stimuli may produce pain. Inflammatory mediators and aberrant neuronal activity activate several signalling pathways including Ras/MAP kinases in dorsal horn neurons that mediate the induction and maintenance of neuropathic pain through both post-translational and transcriptional mechanisms (Ji *et al.*, 2009).

In animals that had received early life nerve injury and had since recovered from the mechanical allodynia that this induced, intraplantar formalin evoked a greater (statistically significant) activation of ERK in the NSSP fraction. Similar but less marked observations were made in the PSD fraction. These observations are consistent with a role of ERK in contributing to the signals driving synaptic insertion of GluA1-containing AMPA receptors. One possible mechanism for enabling greater responsiveness to further noxious challenges (despite all overt signs of sensory hyper-responsiveness having recovered) might be a long-lasting increase in the expression of key regulators of such signalling pathways (such as GTP-exchange factors for the small G protein Ras) even though they are not active under quiescent recovered conditions. In the ERK pathway, such a rate-limiting regulator could be the Ras-GEF, Sos-1. Indeed the current experiments provide preliminary data to suggest exactly this; enduring elevated expression of Sos-1 maintained long after early life (now recovered) nerve injury and its possible further increase ipsilateral to a subsequent formalin challenge. My working hypothesis so far is therefore that this maintained elevation of Ras-GEF expression following neonatal injury may be responsible (at least in part) for the 'pain memory' ie lasting susceptibility to noxious hyper-responsiveness in later life.

5.3 Dynamic receptor responses in isolated *ex vivo* neural tissue

In Chapter 4 I set about trying to develop and validate a new methodological approach that would enable fluorometric measurements to be made on live acutely isolated neural tissue preparations. Given that I could isolate and sustain such samples under conditions that would preserve or allow the restoration of ion gradients through metabolic activity, I then hoped to be able to use no-wash fluorometric dyes to monitor changes in intracellular Ca^{2+} and membrane potential. Careful consideration was given to the tissue preparation procedure. In preparing the tissue samples, neural shock may follow because of the traumatic disruption of the tissue during its preparation. This may lead to a depressed neuronal activity. To

minimise this, I used special additions to buffers based around a classical Krebs-Henseleit buffer (Krebs and Henseleit, 1932). The special precautions involved rapid removal of tissues, rigorously maintaining oxygenation, initial cooling (to try to avoid toxic metabolite accumulation) and blockade of NMDA-type glutamate receptors and voltage-sensitive Ca^{2+} channels in high Mg^{2+} concentrations (hence reduced synaptic transmission and neurotoxic Ca^{2+} entry), together with metabolic supplementation with a glycolytic intermediate and energy source. The technique was tested by two different kinds of fluorescent dyes and consistent, concentration-dependent responses were detected to various receptor stimuli. These findings demonstrate that this technique had been successfully set up and validated for use in further studies.

The responses of noradrenergic, cholinergic, and serotonergic receptors in frontal cortex of rats were examined separately or in combination with ionotropic glutamate receptor responses using the new developed technique.

Noradrenaline is a catecholamine with multiple roles including as a stress-related neurotransmitter in the autonomic nervous system and the CNS. Noradrenaline increases excitability in parts of the brain where alertness and responses to danger are controlled, most likely through its receptor-mediated signalling pathways up-regulating excitatory processes or down-regulating inhibitory processes. Along with adrenaline secreted from the adrenal glands, noradrenaline also underlies the fight-or-flight response (Bracha *et al.*, 2004), increasing heart rate, triggering the release of glucose from energy stores, and increasing blood flow to skeletal muscle. As a neurotransmitter, noradrenaline can also suppress neural inflammation when released diffusely in the brain from the Locus Coeruleus (Heneka *et al.*, 2010). Furthermore, cortical noradrenergic pathways promoted by interleukin-1 (IL-1) release are involved in changes in sensory processing that involve NMDA receptors (Kamikawa *et al.*, 1998). The present work indicates that intracellular concentrations of Ca^{2+} are increased by activation of adrenergic receptors or ionotropic glutamate receptors frontal cortex segments. Although it was not possible to unequivocally demonstrate a synergistic interaction between adrenergic receptors and glutamate receptors under

the present conditions. I suspect that these receptors and their signalling pathways may be activated and work together in animals when they face noxious challenges in real life.

Acetylcholine is involved in synaptic plasticity by enhancing the amplitude of synaptic potentials following LTP in many regions in brain including dentate gyrus, hippocampus and cerebral cortex (Matsuyama *et al.*, 2000; Wu *et al.*, 2001; Lopes Aguiar *et al.*, 2008). It has been reported that Ca^{2+} entry through α7 subunit-containing nicotinic acetylcholine receptors is critical for the induction of NMDA receptor-independent LTP in hippocampus (Wu *et al.*, 2001; Jia *et al.*, 2010). Muscarinic cholinergic neurotransmission enhances the late phase of LTP in rat hippocampus *in vivo* (Lopes *et al.*, 2008), so both nicotinic and muscarinic acetylcholine receptors could contribute to enhanced transmission. Intracellular signals from muscarinic acetycholine receptors also modify the levels of productions of noradrenaline and serotonin in the CNS (Lopes *et al.*, 2008). A recent study showed that noradrenaline depletions facilitates the release of acetylcholine and serotonin in the hippocampus, illustrating a further level of functional interaction (Heneka, 2009). These findings suggest a monoaminergic network exists in the brain to regulate synaptic transmission, as exemplified by LTP. Here, I illustrated that intracellular Ca^{2+} concentrations increased following activation of cholinergic receptors in frontal cortex, especially in combination with activation of combined ionotropic glutamate receptors, and that this joint response was further amplified by prior noradrenaline stimulation.

Serotonin (5-hydroxytryptamine)-containing neurons originating from the brainstem Raphe nuclei also innervate diverse regions of the CNS, where they exert a variety of roles, for example, in affective and associative processing, sleep and at brainstem/spinal levels, in regulation of sensory processing. In frontal cortex, pyramidal cells, thalamic afferents and interneurons receive serotonergic inputs and respond (especially via $5\text{-}HT_{2A}$ receptors) with generally increased excitability to other inputs (Aghajanian and Marek, 1997; Jakab and Goldman-Rakic, 1998; Puig *et al.*, 2003). The present results are consistent with the idea of positive co-operation

between AMPA and 5-HT_{2A} receptor responses in increasing excitability levels within frontal cortex.

5.4 Management of neuropathic pain

Pain relief can be approached using a number of different therapies, which work by reducing the sensitivity of nociceptive receptors, interrupting nociceptive transmission, and activating inhibitory receptors in spinal cord or descending inhibitory pathways originating from the brainstem. For example, stimulation of non-nociceptive receptors can inhibit the transmission of nociceptive information in the dorsal horn of spinal cord, which means that painful stimuli can be 'gated' out using non-painful stimuli, as a basis of 'Gate Control' theory (see details in section 1.2). The proposed 'Gate Control' system is not directly targeted by current analgesic agents, which interact with a variety of distinct peripheral and central targets instead. One example study showed that ralfinamide, a sodium channel blocker, suppressed Na^+ currents in dorsal root ganglion and reduced pain responses in an animal model of neuropathic pain (Yamane *et al.*, 2007) pointing to Na^+ channel blockade as a potential therapeutic strategy. Similarly, gabapentin, a blocker of $\alpha_2\delta_1$ Ca^{2+} channel subunits in nociceptive afferents is an effective analgesic for neuropathic pain (Rose and Kam, 2002). However, the most common analgesic strategy is to manipulate the function of interneurons through opioid receptors by administration of exogenous morphine or its analogues to attenuate pain. Opioid analgesics are drugs that exert their influence through activation of opioid receptors to reduce pain transmission at synapses in the dorsal horn of spinal cord. Opioids also stimulate noradrenergic, serotoninergic and enkephalinergic neurons in the brainstem that descend to the spinal cord and further inhibit the relay neurons of the Spinothalamic tract. The side effects of opioid analgesics are that repeated doses may cause dependence so that a withdrawal syndrome may be seen after sudden termination. Therefore, they are not recommended as first line treatments. Favoured treatments are antidepressants, anticonvulsants (such as gabapentin and Na^+ channel blockers), and topical agents (generally local anaesthetics such as lidocaine, anti-inflammatory agents or agents to

desensitise nociceptive TRP channels). The mode of action of antidepressants such as selective serotonin-noradrenaline re-uptake inhibitors (SNRIs) is probably to re-inforce the activation of receptors that respond to descending noradrenergic and serotoninergic pathways and thereby limit pain signals in spinal cord and minimise their ascent to the brain.

With current therapeutic strategies for chronic neuropathic pain, only 40-60% of patients achieve relief, which in itself is only partial and is undermined by problematic side effects (Dworkin *et al.*, 2007). Novel specific drugs that relieve neuropathic pain but do not disturb normal somatosensory processing would represent the gold-standard target for the development of next-generation analgesics. It is anticipated that the developments made in the current work; ie the optimisation of quantitative biochemical techniques to a smaller scale than before, together with the newly developed techniques for assessing dynamic receptor responsiveness of living *ex vivo* neural tissue samples will play a future part in identifying new molecular targets for analgesia. Their contribution to this process would be by helping to elucidate the mechanisms that bring about hyper-responsiveness, which would highlight potential targets for intervention. Ultimately, such improved treatments may serve to fill the unmet need of efficacious analgesia for chronic pain states. The application of these approaches may benefit not only sufferers from neuropathic and other types of chronic pain states, but potentially also help in elucidating the events underlying other neurological and psychiatric dysfunctional states in the CNS.

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