

**The role of N-methyl D-aspartate (NMDA) receptor antagonists in
neuropathic pain**

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For the degree of Doctor of Medicine

University of Edinburgh

2008

Dedicated to my wife Fiona, without whose encouragement at a difficult time this thesis would have not have been completed.

'Just because you find something difficult to do, don't think that it's humanly impossible. If something is humanly possible and appropriate, believe that it can also be attained by you'

- Marcus Aurelius AD 121-180

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Declaration

I composed this thesis. The research was conducted between March 2000 and December 2002, whilst a Research Fellow and subsequently a Clinical Lecturer in the Department of Anaesthesia, Critical Care and Pain Medicine, University of Edinburgh.

The first chapter is a personal review of the literature, with the subsequent chapters based on my own laboratory and clinical research.

The laboratory work was carried out in the Department of Preclinical Veterinary Sciences, The Royal (Dick) School of Veterinary Studies, University of Edinburgh. I personally carried out surgery and administered the pre-emptive memantine and ketamine to the animals. I carried out all of the behavioural testing and performed the intrathecal injections. Dr Fleetwood-Walker assisted me in placement of the intrathecal catheters. I carried out all the analysis of the data and prepared the data for presentation. Dr Rory Mitchell kindly prepared the drugs used for the intrathecal injections. Dr Emer Garry carried out the experiments with preemptive Ro 25-6891. I am grateful for permission to include the data from these experiments.

I prepared all of the animals for immunohistochemical analysis, retrieved and prepared the tissue samples. Ms Heather Anderson at the Department of Preclinical Veterinary Sciences carried out the immunohistochemical reactions and prepared the slides for analysis. I carried out all the quantification and analysis of the immunohistochemical staining. The specialist immunoblots were carried out by Dr Garry.

The clinical study was carried out in the Royal Infirmary of Edinburgh. I recruited all the subjects in the clinical study. I personally carried out all follow-up assessments and the quantitative sensory testing. I carried out all the data analysis and prepared the data for presentation.

Abstract

Neuropathic pain is pain caused by dysfunction in the nervous system. Current treatments have limited efficacy, often with intolerable side-effects. There is a need for therapies to prevent or reduce the severity of neuropathic pain. The N-methyl D-aspartate receptor (NMDAR) plays a key role in neuropathic sensitisation. In animal neuropathic models spinally administered NMDAR antagonists are effective in reducing behavioural changes associated with neuropathic pain. Clinical trials using NMDAR antagonists have shown them to be effective adjuncts in the treatment of acute pain and as a treatment option in established chronic pain. The clinical role of NMDAR antagonists in the development of neuropathic sensitisation is yet to be defined.

The aim of this thesis is to investigate the use of NMDAR antagonists in preventing neuropathic sensitisation. The chronic constriction injury (CCI) model of neuropathic pain is used to study the role of NMDARs in the development of neuropathic pain and subsequent modulation. Behavioural effects are assessed in association with changes in NMDAR subtypes. Memantine and ketamine (NMDAR antagonists) are shown to attenuate typical behavioural responses (thermal hyperalgesia and cold allodynia) to nerve injury. In addition, NMDAR antagonist pre-treatment is shown to effect the subsequent NMDAR subunit expression, with improved susceptibility to subsequent NMDAR antagonist treatment.

The clinical use of epidural ketamine as a preventative drug prior to lower limb amputation is investigated in a double blind randomised placebo controlled study. No significant effects on the incidence of post-amputation pain were found, although the overall incidence of pain was lower than in comparable studies. Ketamine is shown to improve peri-operative analgesia and have long lasting effects (up to one week) on sensory processing in the remaining stump.

In summary, NMDAR antagonists seem to be effective in attenuating neuropathic pain in animal models. The promise shown in these studies has not translated into a

reduction in post-amputation pain in a clinical study. Ketamine remains a clinically useful drug in peri-operative pain management but the role of ketamine and other clinically available NMDAR antagonists in the prevention of neuropathic sensitisation is still not clearly defined.

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Acknowledgements

I would like to acknowledge the immense support that I received from Dr Lesley Colvin who was my supervisor in this project. I am extremely grateful for her support and friendship.

I am grateful to the support from my friends and colleagues at the Royal Infirmary of Edinburgh. In particular I would like to thank Dr Alastair Nimmo, a co-researcher on the clinical project and Sister May Roseburgh without whom the project may never have got off the ground. I would also like to thank the other consultant anaesthetists working in the vascular unit; Dr John McClure, Dr Carl Moores and Dr Gerry Keenan.

In the Department of Preclinical Veterinary Sciences, I would like to thank Professor Sue Fleetwood-Walker, Dr Emer Garry, Heather Anderson and Dr Rory Mitchell who assisted me in parts of this research. In addition I am very grateful for the support and friendship from those working in the lab at that time; Andrew Moss, Francis O'Neill, Victoria Wallace and James Blakemore.

I am grateful to my friends and colleagues in the Department of Anaesthesia, Critical Care and Pain Medicine in the Royal Infirmary of Edinburgh in particular the support of Professor Ian Power and the University Department.

I would like to acknowledge Action Research UK who funded the bulk of my research and the support of a small projects grant from Lothian Universities Hospital Trust.

Finally I would like to thank my wife Fiona and my daughters Evie and Maddie, without whom none of this would have been worthwhile.

List of abbreviations

ACC	Anterior cingulate cortex
ADL	Activities of daily living
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxasolepropionic acid
ANOVA	One way analysis of variance
ASIC	Acid sensing ion channel
ATP	Adenosine triphosphate
BDI	Beck Depression Inventory
BPI	Brief Pain Inventory
BDNF	Brain derived neurotrophic factor
BOLD	Blood oxygen level dependent
CCK	Cholecystokinin
CCI	Chronic constriction injury
CGRP	Calcitonin gene related peptide
CNS	Central nervous system
CRPS	Complex regional pain syndrome
DH	Dorsal horn
DN	Diabetic neuropathy
DRG	Dorsal root ganglion
EAA	Excitatory amino acids
EEG	Electroencephalogram
ESPC	Excitatory post-synaptic current
GABA	Gamma-aminobutyric acid
GAL	Galanin
HADS	Hospital Anxiety and Depression Scale
HRP	Horse radish peroxidase
IASP	International Association for the Study of Pain
LANSS	Leeds Assessment of Neuropathic Symptoms and Signs
LTP	Long term potentiation
MEG	Magnetoencephalography
MPI	Multidimensional Pain Inventory

MPQ	Magill Pain Questionnaire
NGF	Nerve growth factor
NK-1	Neurokinin-1
NK-A	Neurokinin-A
NMDA	N-methyl D-Aspartate
NMDAR	N-methyl D-Aspartate receptor
NNT	Number-needed-to-treat
NO	Nitric oxide
NON-N	Non nociceptive
NPS	Neuropathic Pain Scale
NRS	Numerical rating scale
NT	Neurotrophin
NTS	Nucleus tractus solitarius
NS	Nociceptive specific
P2X3	Purinergic receptor subtype
PAG	Periaqueductal grey
PCP	Phencyclidine
PET	Positron emission tomography
PHN	Post herpetic neuralgia
PKC	Protein kinase C
PLP	Phantom limb pain
POMS	Profile of Mood States
PSNL	Partial spinal nerve ligation
PWL	Paw withdrawal latency
PWT	Paw withdrawal threshold
R-AP5	(2R)-amino-5-phosphonopentanoate
rCBF	Regional cerebral blood flow
R-CPP	3-(2-carboxypiperazin-4-yl) propyl-1-phosponic acid
RCT	Randomised controlled trial
RVM	Rostroventromedial medulla
SI	Somatosensory cortex
SII	Secondary somatosensory cortex

SG	Substantia gelatinosa
SNL	Spinal nerve ligation
SPECT	Single photon emission computerised tomography
TCA	Tricyclic antidepressant
Trk	Tyrosine kinase
TRP	Transient receptor subtype
TTX	Tetrodotoxin
SNRI	Serotonergic noradrenergic reuptake inhibitor
SPET	Suspended paw elevation time
Src	Protein tyrosine kinase
SSEP	Somatosensory evoked potential
WDR	Wide dynamic range
VAS	Visual analogue scale
VIP	Vasoactive intestinal polypeptide
VF	Von Frey

1.1 Nociception and Nociceptive Pathways

1.1.1 Peripheral Nociceptors

Sensory neurones responding to stimuli capable of producing tissue damage are termed nociceptors. Most body structures contain nociceptors. These are high threshold polymodal receptors, meaning they respond to a variety of noxious stimuli. Typically they respond to noxious heat, strong mechanical stimuli and chemical stimuli, both endogenous and exogenous (1). Cutaneous nociceptors can be divided into two main categories, A δ -mechanical nociceptors and C-polymodal nociceptors. These are named after the afferent fibres that innervate them and the stimuli that they respond to (2). In general terms, nociceptive processes begin with activation of the primary afferent nociceptor, a process known as transduction. Transduction results in the conversion of the noxious stimuli into electrochemical activity. Despite nociceptors normally having a high threshold for activation, repeated activation may reduce that threshold for activation, as can occur with peripheral sensitisation (3).

1.1.2 Primary afferents and cytoarchitecture of dorsal horn

Somatic primary afferent neurones are located in the dorsal root ganglia (DRG). They are unipolar neurones which conduct sensory inputs from peripheral nerve endings to the spinal cord. Transmission of nociceptive activity is via both small unmyelinated c-fibres (diameter $<2\ \mu\text{m}$; conduction speed $\sim 1\text{ms}^{-1}$) and small myelinated A δ -fibres (diameter $2\text{-}5\ \mu\text{m}$; conduction speed 15ms^{-1}). This contrasts with the much larger ($>10\ \mu\text{m}$) rapidly conducting ($>30\text{ms}^{-1}$) A β -fibres, which transmit light touch (2). Primary afferent fibres synapse in the dorsal horn of the spinal cord, where they form a highly organized somatotopic map (4). The spinal cord is divided into 10 laminae (2;5). The dorsal horn is made of laminae I-VI.

The first synapses where nociceptive information is processed are situated in the superficial dorsal horn. The superficial dorsal horn is made up from what is known as the marginal zone and the substantia gelatinosa (lamina I and II). Lamina I and II are the primary termination zone of C- and A δ -fibre primary afferents. Ascending output is mostly from Lamina I, though only about 10% of lamina I neurones are projection

neurones. The projection neurones are largely nociceptive responding to mechanical and noxious thermal stimulation. The vast majority of these neurones express NK-1 receptors. Immuno-staining for the NK-1 receptor can be used to define lamina I. Superficial dorsal horn projections are to the contralateral spinthalamic tracts and other areas such as the lateral periaqueductal grey matter (PAG) and the parabrachial area.

Nociceptive information is predominantly processed in lamina I, II, V, VI and X (the area around the canal). C-fibres and A δ -fibres largely terminate in the superficial laminae (I and II), while A β -fibres terminate in deeper laminae (III and IV) (6). Not only do primary afferent fibres synapse with second order neurones, but there is a complex interaction with local intrinsic spinal neurones and descending modulatory neurones coming from the brain.

Glutamate is the major excitatory neurotransmitter in the CNS and so, unsurprisingly, glutamate receptors are widespread in the spinal cord. These are both ionotropic and metabotropic. Immunohistochemical staining demonstrates ionotropic glutamate receptors (in the laminae I – III). There is evidence for the involvement of both α -amino-3-hydroxy-5-methyl-4-isoxasolepropionic acid (AMPA) and kainate receptors in sensory processing in the superficial dorsal horn (7). In addition the subunits of the N-methyl D-Aspartate (NMDA) receptor, NR1 and NR2B, are predominantly found in the laminae I-III).

1.1.3 Dorsal horn neurotransmitters

A wide range of neurotransmitters are involved in nociception. Examples of primary afferent and dorsal horn receptors and their ligands are shown in table 1.

Table 1.**Examples of primary afferent dorsal horn receptors and ligands**

Ionotropic receptor	Subtype	Ligand
TRP channels	TRPV1	Heat (>42°C), capsaicin, H ⁺
	TRPV2	Heat (>53°C)
	TRPA	Noxious cold (<17°C)
Acid sensing	DRASIC, ASIC	Protons
Purine	P2X3	ATP
Serotonin	5HT3	5HT
NMDA	NR1	glutamate
AMPA	iGluR1	glutamate
Kainate	iGluR5	glutamate
Metabotropic receptor	Subtype	Ligand
Metabotropic glutamate	mGluR1,2,4,5	glutamate
Prostanoids	EP1-4	PGE2
	IP	PGI2
Histamine	HI	HA
Serotonin	5HT1A	5HT
	5HT4	5HT
	5HT2A	5HT
Bradykinin	B1, B2	BK
Canabinoid	CB1-2	Anandamide
Tachykinin	NK-1	Substance P, neurokinin A
Opioid	mu, kappa, delta	enkephalin, dynorphin
		β-endorphin

Key

5HT	serotonin
ASIC	acid sensing ion channel
ATP	adenosine triphosphate
BK	bradykinin
DRASIC	subtype of acid sensing ion channel
iGluR	ionotropic glutamate receptor
P2X3	purinergic receptor subtype
PGE2	prostaglandin E2
PGI2	prostacyclin
TRP	transient receptor subtype
NK-1	neurokinin 1

Others (e.g. H1, EP1-4, TRPV2) are designated subtypes of receptors rather than abbreviations.

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Excitatory neurotransmitters

The predominant excitatory neurotransmitters are the amino acid glutamate and the neuropeptide, substance P, a tachykinin acting on NK-1 receptors. Glutamate acts on a variety of receptors, the slow G-protein linked metabotropic receptors and fast ligand-gated ion channels namely AMPA receptors, kainate receptors and the NMDA receptors (8) (see table 1). Fast excitatory synaptic transmission is mediated by glutamate acting on AMPA and kainate receptors, while NMDA receptors are thought to mediate the slower synaptic effects of glutamate. Co-release of substance P and another tachykinin, neurokinin A (NK-A) facilitate both the release of excitatory neurotransmitters and enhance glutamate mediated post-synaptic activity (9).

In addition to the excitatory amino acids and tachykinins described above, there are other neurotransmitters which act as pronociceptive mediators in the dorsal horn. These include calcitonin gene related peptide (CGRP) adenosine triphosphate (ATP), nitric oxide (NO), phospholipid metabolites, prostaglandins and various neurotrophins (10).

Inhibitory neurotransmitters

A δ and C fibres excite centrally projecting neurones. Dorsal horn activity can however be modulated by inhibitory DH neurones, acting both pre- and post-synaptically.

Many different neurochemical types have been identified including GABAergic inhibitory neurones, cholinergic inhibitory neurones and opioidergic inhibitory neurones. The majority of inhibitory neurones in the spinal cord use Gamma-aminobutyric acid (GABA) and glycine as their principle fast neurotransmitters. In the main GABA receptors are pre-synaptically situated (11). Electrophoretic application of GABA to the spinal cord alters the neuronal conductance to Cl⁻ resulting in an inhibitory effect (12). GABA and glycine open ligand gated ion channels which allow Cl⁻ and HCO₃⁻ across the cell membrane. The α 2 and α 3 subunits of the GABA_A receptor are most prominent in lamina II of the dorsal horn and are postulated to have a specific function in nociceptive transmission. Central inflammatory pain may originate from a disinhibition of dorsal horn neurones through a prostaglandin (PGE₂) mediated inhibition of glycine receptors.

Cholinergic inhibitory neurones act via muscarinic receptors while opioidergic inhibitory neurones containing enkephalins and/or dynorphin act via μ , δ and κ -opioid receptors (13;14).

1.1.4 Ascending Transmission

Spinal horn neurones are organised into 3 distinct groups; nociceptive specific neurones (NS), wide dynamic range neurones (WDR) and Non Nociceptive (NON-N). NS neurones respond exclusively to noxious stimuli and are found in laminae I,

II (external), V and VI. Afferent input is from A δ - nociceptive high threshold and heat nociceptive fibres and C polymodal nociceptive fibres. Their receptive fields are punctiform and show a degree of somatotropic organisation, largely lamina I. WDR neurones respond to mechanical thermal and chemical stimuli, with input from A δ , C and A β fibres. They are found in laminae I, II (external), IV, V, VI, X and the anterior horn. They are able to respond to a range of stimuli from innocuous to clearly noxious. NON-N respond to low intensity mechanical and thermal stimuli and proprioceptive inputs via both A δ and A β fibres, though these low threshold mechanical receptors may be involved on the mechanisms of allodynia (15).

Nociceptive information is transmitted cephalad via spinal sensory neurones. These are either nociceptive specific neurones (NS; responding to noxious stimuli only) or wide dynamic range neurones (WDR; responding to a range of stimuli from innocuous to clearly noxious). Both NS and WDR neurones will travel 2 or 3 segments in the tract of Lissauer and form connections in the substantia gelatinosa (SG) of adjacent segments (2). This may explain expansion of the receptive field after injury.

1.1.5 Projection of dorsal horn neurones

In general terms, the spinal cord has three major projections to the brain (16). They are

- Direct thalamic projections (Spinothalamic Tracts, STT)
- Direct projections to the reticular and homeostatic control regions of the brain stem (Spinoreticular and Spinomesencephalic Tracts)
- Direct projections to the hypothalamus and ventral forebrain (spinohypothalamic tracts)

Spinothalamic tracts

Cells projecting to the spinothalamic tract arise in three regions of the spinal cord (16).

The first of these projections is from lamina I, with input being predominantly from A δ and C-fibres. Lamina I cells are usually NS cells or polymodal nociceptive cells. The second deeper area is from laminae IV and V. Input here is predominantly from A β -fibres, with some smaller myelinated and unmyelinated fibre inputs. Cells here are largely NON-N or WDR. The third region is lamina VII and VIII, an intermediate/ ventral horn zone which receives large diameter afferents from the skin and deep inputs from muscles and joints.

Spinothalamic tracts usually cross within one or two segments rostral to their origins. Classically there are two distinct tracts, the lateral spinothalamic tract and the anterior spinothalamic tract (17). Input to the lateral tract is largely from superficial laminae, while the deeper laminae project to the anterior tract. The tracts are loosely somatographically organised, with caudal fibres more lateral and cephalad fibres more medial.

All nociceptive information reaching the cortex is processed and relayed by the thalamus. The spinothalamic tract terminates in 6 distinct regions of the thalamus. They are the ventral posterior nucleus, the ventroposterior inferior nucleus, the posterior portion of the ventral medial nucleus, the ventral lateral nucleus, the lateral central nucleus, the parafascicular nucleus and the ventral caudal portion of the medial dorsal nucleus. The thalamus in turn has cortical projections. The ventral posterior nucleus projects to SI, the ventroposterior inferior nucleus projects to SII, the posterior portion of the ventral medial nucleus projects to the insula and the ventral caudal portion of the medial dorsal nucleus projects to the anterior cingulate cortex (ACC). These are areas of the brain that have been shown to be active in functional imaging of the brain (18).

Spinoreticular and spinomesencephalic tract

The spinoreticular tract originates mainly from laminae V, VII and VIII. It is made up mostly from NS and WDR neurones, although there is some NON-N neuronal involvement (19). This tract has two main projections to the brain stem. One is to the precerebellar nucleus in the lateral reticular formation and the other to the medial pontobulbar reticular formation (10). The spinoreticular tract neurones are involved in the motivational-affective and neuro-vegetative responses to pain. It activates brain stem structures involved in descending suppression.

The spinomesencephalic tract is made from NS, WDR and NON-N neurones. They are arranged in a somatotopic way like the STT.

Spinohypothalamic tracts

The spinohypothalamic tract is composed of NS, WDR and NON-N neurones. The tract originates from laminae I, V, X, the lateral nucleus, nucleus caudalis and regions around the central medullary canal. Neurones in this tract respond to stimuli (noxious and non-noxious) from muscles, tendons, joints, skin and viscera. It has projections to various parts of the hypothalamus. Projections are thought to contribute to the neuroendocrine autonomic and motivational-affective components of pain

1.1.6 Descending modulatory systems

Descending pathways from the brainstem and other cerebral structures play an important role in modulation and integration of afferent activity in the dorsal horn. Modulation may be either inhibitory or facilitatory. Sources of modulation include the cerebral cortex, the hypothalamus, the periaqueductal grey (PAG), the rostroventromedial medulla (RVM), the dorsal reticular nucleus of the medulla, the parabrachial nucleus and the nucleus tractus solitarius (NTS) (20). Modulatory activity can be via a pre/post-synaptic action or intrinsic interneurones within the dorsal horn.

In the main inhibitory modulation is via serotonergic and noradrenergic systems (14). Descending pathways generally inhibit nociceptive DH neurones either directly

or by inhibition of excitatory interneurons or excitation of inhibitory neurons. There are indications that there is preferential inhibition of excitation of WDR neurons to noxious as opposed non-noxious stimulation i.e. there is interference with the activation of neurons by A δ and C fibres rather than A β fibres (10). It is worth noting that although monoamines are considered the major neurotransmitters released by the descending inhibitory pathways, other neurotransmitters may be important such as acetylcholine, enkephalin and GABA (10).

Descending facilitation is a type of anti-analgesia and can be initiated in the RVM. Triggers include peripheral and visceral inflammation and injury to primary afferent nerves. Descending facilitation contributes to hyperalgesia and allodynia. CCK, Substance P, NMDA and NO receptors have been implicated in descending facilitation (20). There is some evidence that this pathway may predominate in chronic pain states (21).

1.1.7 Cortical pain processing

The IASP defines pain as “An unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage” (22). It follows therefore that central processing of pain must reflect both a sensory-discriminative component and an affective-motivational component. Sensory-discriminative aspects of pain parallel other sensory modalities like vision or olfaction while affective-motivational aspects of pain reflect ‘suffering’ with regard to emotion, arousal and behaviour.

The cerebral cortex plays an important role in the both pain perception and the affective-motivational aspects of pain. Cortical responses can be measured by single neurone recordings. Important areas that have been studied by single neuron studies are the SI, SII and the ACC (19). Modern imaging techniques such as positron emission tomography (PET) and functional magnetic imaging (fMRI) have also been used. These imaging modalities make indirect measures of function by measuring increases in regional cerebral blood flow (rCBF) Cortical areas of the brain showing

an increase in rCBF are the primary and secondary somatosensory cortices (SI and SII), the insula and the anterior cingulate cortex (18).

One current model is that there are two distinct nociceptive projections acting in parallel. These are divided into lateral and medial systems depending on whether they project through the medial or lateral thalamic nuclei. The sensory-discriminative aspects of pain are processed through the lateral nociceptive system; while affective-motivational aspects of pain are processed in the medial nociceptive system (23;24).

SI & SII

The main thalamic projections to SI and SII are from the ventral posterior lateral nuclei, the ventral posterior medial nuclei and the ventral posterior nuclei of the thalamus (19;25).

Both SI and SII have projections from the lateral systems. SI has the ability to topographically code noxious stimuli of variable intensities making it an important area in the sensory discriminative component of pain. SII neurones, in addition, code noxious stimuli in temporal terms (26;27). The medial system also has projections to SI and SII although these are less well defined.

Insula

The insula has multiple thalamic inputs as well as inputs from SI, SII and the parietal association cortex (25). Its projections are to the limbic system, mainly amygdala, and regions of the prefrontal cortex involved in both memory related to painful experiences and the affective-motivational component of pain.

Anterior cingulate cortex

The ACC is involved in the affective-motivational component of pain. Projections to the ACC are from the anterior thalamic nuclei, the medial dorsal nuclei and the central lateral and parafascicular nuclei (25). Strength of cingulate gyrus activity correlates with unpleasantness of stimuli. ACC activation is important in affective-motivational aspects of pain and is involved in behavioural and autonomic responses to pain (24).

1.1.8 Brain Imaging and Pain

The pain experience, as discussed above, is a complex integration of sensory-discriminative, cognitive-evaluative and affective motivational components. A large part of the body's response to pain occurs in supraspinal structures from the brain stem to forebrain. Brain imaging techniques allow brain structure and function to be studied. Studies can be carried out in a range of pain conditions from the experimental scenario through to chronic pathological states. Brain imaging studies allow evaluation not only of the brain's response to the sensory-discriminative aspect of pain, but the cognitive-evaluative and effective motivational components as well.

Brain imaging methodology

Brain imaging of pain uses a mixture of structural and functional imaging. Functional CNS imaging in effect is an attempt to visualise physiological activity at the synapses. Brain function is made up from neuronal and glial activity. It is not possible to measure this activity directly, but factors that correlate closely to synaptic activity such as changes in regional blood flow, blood oxygenation and energy metabolism can be. These factors are measured in brain imaging studies and used as markers of brain function. The relationship between neuronal and glial activity, metabolic activity and blood flow is known as neurometabolic and neurovascular coupling. This relationship is key to the principles behind modern brain imaging

Brain imaging modalities

There are several brain imaging modalities in common usage such a magnetoencephalography (MEG), high density electroencephalogram (EEG), single photon emission computerised tomography (SPECT), PET and fMRI.

There is a close relationship between glucose utilisation and synaptic activity. Imaging techniques such as PET and SPECT make use of this relationship. These techniques are based on the detection of the product of flurodeoxyglucose metabolism which is accumulates at active presynaptic terminals. These modalities, however, can be time consuming to acquire images and require the use of a radioactive isotope flurodeoxyglucose. The isotope has a half life of 110 minutes which again limits the ease of use.

fMRI does not image brain activity directly but is based on changes in blood volume, flow and oxygenation. This is usually by changes in blood oxygenation and the signal contrast generated, known as blood oxygenation level dependent (BOLD). BOLD fMRI studies are frequently used in brain imaging studies. Neural activity in the brain is associated with an increase in local blood flow to the local vasculature. There is in fact a reduction in deoxyhaemoglobin because the increase in blood flow exceeds the rate of oxygen extraction. Deoxyhaemoglobin is paramagnetic and alters the T2 weighted magnetic resonance image signal. Deoxyhaemoglobin acts as an endogenous contrast enhancing agent, meaning that human cortical function can be observed without the need for exogenous contrast.

fMRI can readily image brain activity related to a specific task or in terms of pain activity related to a particular sensory stimulus (Both noxious or non-noxious).

Advantages include; no requirement for radioactive isotopes, relatively short scan times of 1.5-2 minutes per run and resolutions of 1.5mm x 1.5mm (smaller resolution is possible, down to less than 1mm).

Brain imaging studies and pain

One of the first studies of immediate brain activity and pain was a PET study. This study showed that human perception of pain correlates with activity in S1 and S2 and the ACC (28). Subsequent studies confirm this with activity being most consistent in the medial mid-brain, thalamus, lentiform nucleus, cerebellum and the insular, prefrontal, parietal (inc. S1 and S2) and anterior cingulate cortices. These multiple areas represent the entire pain experience i.e. sensory, affective and cognitive. Pain imaging studies are able to identify functional specificity of different parts of the brain. For example, areas of the brain correlating with heat pain unpleasantness can be separated from those correlating to heat pain intensity (29). Pain perception can be separated from pain anticipation (30). Pain imaging studies have been used to image neuropathic pain states. Using intradermal capsaicin models of neuropathic pain, researchers have demonstrated unique areas of activation of the brain in response to tactile allodynia. fMRI can show the importance of cortical networks (comprising areas such as S1, the parietal association cortex, S2/insula and inferior frontal cortex) in processing of dynamic allodynia (31).

Pain imaging studies have been also carried out in clinical chronic pain states, such as chronic low back pain, complex regional pain syndrome (CRPS) and in phantom pain (Discussed below, section 1.2.12). Alterations in cortical processing of somatosensory stimuli is not unique to phantom limb pain, but can be seen in other form of chronic pain including CRPS and chronic low back pain (32-34).

1.2 Neuropathic pain

Neuropathic pain is defined by the International Association for the Study of Pain (IASP) as “pain initiated or caused by a primary lesion or dysfunction in the nervous system” (22). The lesion can vary from transection of a major nerve to a subtle nerve lesion such as irritation of a nerve by inflammatory substances such as blood or infection. It typically has different characteristics from nociceptive pain and sufferers often use words such as burning, pulsing or stabbing. There is commonly pain in an area of sensory loss. Allodynia, hyperalgesia or dysaesthesia may be found.

1.2.1 Mechanisms of neuropathic pain

The mechanisms of neuropathic pain are complex. Understanding in this area is a rapidly changing and advancing field. Nerve injury causes neuroma formation in the nerve and increased excitability with ectopic discharge. There are changes in ion channel expression particularly sodium channels. The nerve phenotype changes with alteration in neuropeptide expression. Neurotrophins play a major part in these changes. There is evidence of involvement of the sympathetic system. In addition there are changes in central anatomy and electrophysiology leading to long term potentiation and central sensitisation. Dorsal horn inhibitory control is diminished and changes occur in both descending modulatory systems and the cerebral cortex.

1.2.2 Neuroma formation and ectopic discharges

Following peripheral nerve transection, damage to primary afferent fibres results in areas of demyelination at the injury site. Attempts at nerve regeneration by axonal sprouting results in a neuroma, a tangled axonal mass. Amputation of a limb may result in a neuroma. Experimental neuroma in a rat sciatic nerve have been shown to cause spontaneous discharges in the dorsal roots which could be augmented by mechanical and chemical stimulation (35). These frequent abnormal spontaneous discharges, termed “ectopic activity”, arise, not only from the neuroma, but in other parts of the nerve, including the dorsal root ganglion (DRG), where the cell bodies of the primary afferent fibres are located (36). Accumulation of sodium channels at the sites of ectopic discharges may result in altered membrane threshold leading to

spontaneous discharge (37). Ectopic activity in the nerve following injury can be prevented using systemic lignocaine, perhaps acting on TTX-resistant Na channels, at doses that do not block normal nerve conduction (38). In humans ectopic activity has been shown to correlate with pain in amputees and dysaesthesia associated with back pain and radicular pain (39;40). Cross-talk can occur in sensory neurones. This may result in amplification of sensory stimuli. Ephaptic (electrical) cross-talk can occur in regenerating neurones and may reflect the lack of glial insulation in the damaged axons (41;42). Ephapsis however, may not occur in the DRG following nerve injury (43).

1.2.3 Increased expression of sodium channels

Voltage-dependent sodium channels produce the inward currents that depolarise the cell membrane and thus are fundamental to conduction of the action potential in the nerve. As discussed above nerve injury leads to hyperexcitability in the peripheral nerve, therefore research has focussed on peripheral nerve sodium channels and what happens following nerve injury. There are almost a dozen molecularly distinct sodium channels in mammals, some of which are largely limited to the DRG (For review see Lai et al. (44)) Sodium channels are broadly classified into those that can be blocked by tetrodotoxin (TTX-sensitive) and those that cannot (TTX-resistant). See table 2 for classification of channels. Following injury to DRG axons there is accumulation and increased expression of sodium channels proximal to the site of injury (45). More recently research has been focusing on the types of sodium channel involved and how they are regulated following nerve injury. Following axotomy, there are changes in the sodium channel gene expression. Axotomy results in the upregulation of the Nav1.3 channel mRNA while Nav1.8 and Nav1.9 expression is down regulated (46-48). Nav 1.8 and Nav 1.9 encode for TTX-resistant channels. Axonal transection can be shown to result in loss of TTX-resistant currents. The TTX-sensitive channels switch properties, with the time constant for recovery increasing almost fourfold (49). The TTX sensitive channel Nav1.3 is thought to be responsible for this and can also be shown to accumulate in experimental neuromas. These changes in sodium channel expression may underlie hyperexcitability and spontaneous activity of DRG neurones (50).

Table 2 shown overleaf

Reproduced with permission from Neurology (51).

Key

GEFS(+)	Generalised epilepsy with febrile seizures plus
SMEI	Severe myoclonic epilepsy of infancy
DRG	Dorsal root ganglion
HyperKPP	Hyperkalaemic periodic paralysis
PMC	Paramyotonia congenita
PAM	Potassium aggravated myotonia.

Table 2 Voltage gated Na⁺ channels

Channel type α -subunit	Localisation	Kinetics and TTX sensitivity	Function	Clinical significance
Na _v 1.1	Cell bodies of CNS neurons, cardiac monocytes	Low threshold of activation, fast activation and inactivation, slow recovery from inactivation, TTX sensitive	Initiation of activation of action potential, repetitive firing	Point mutations and deletions cause GEFS+ and SMEI
Na _v 1.2	CNS unmyelinated axons	Low threshold of activation, fast activation and inactivation, slow recovery from inactivation, TTX sensitive	Conduction of action potential	Point mutation cause GEFS+
Na _v 1.3	Embryonic neurons of CNS and DRG	Fast activation and fast recovery from inactivation, TTX sensitive	Persistent depolarisation in response to subthreshold stimuli, increasing excitability, and repetitive firing	Up-regulated following axotomy and spinal cord injury, mediating pain in these conditions
Na _v 1.4	Skeletal muscle	Low threshold of activation, fast activation and inactivation, and slow recovery from inactivation, TTX sensitive	Muscle action potential and excitation – contraction coupling	Point mutations cause hyperKPP, PMC, or PAM
Na _v 1.5	Heart and embryonic neurons	Low threshold of activation, fast activation and inactivation, and slow recovery from inactivation, TTX resistant	Action potential in heart	Point mutations cause long QT syndrome
Na _v 1.6	Nodes of Ranvier in CNS and PNS	Low threshold of activation, fast activation and inactivation, slow recovery from inactivation, TTX sensitive	Fast (salutatory) conduction of action of potential	Point mutations in mice causes cerebellar ataxia or motor end-plate disease
Na _v 1.7	CNS, DRG and sympathetic neurones	Low threshold of activation, fast activation and inactivation, and slow recovery from inactivation, TTX sensitive	Initial depolarisation of action potential	Mutations cause primary familial erythromelalgia
Na _v 1.8	Small DRG neurones	High threshold and slow kinetics of activation and inactivation, fast recovery from inactivation, TTX resistant	Activated after depolarisation initiated by Na _v 1.7 and sustains repetitive firing	Upregulated by inflammatory mediators, involved in inflammatory pain and hyperalgesia
Na _v 1.9	Small DRG neurones	Very low threshold and slow kinetics of activation TTX resistant	Persistent subthreshold depolarisation	Upregulated by inflammatory mediators, involved in inflammatory pain and hyperalgesia

1.2.4 Neuropeptide changes (table 3)

Primary sensory neurones contain a significant amount of peptides. Some like substance P (SP) and calcitonin gene related peptide (CGRP) are present in the normal state, while others such as vasoactive intestinal polypeptide (VIP), galanin (GAL) and neuropeptide Y are normally expressed at low levels. Axotomy results in a phenotypical change in the neurone. In the DRG, following axotomy, there is down-regulation of several peptides that are normally present in the primary afferent cell bodies. This includes SP and CGRP (52;53). In contrast, there is an increase in VIP, GAL and NPY expression (54). Hokfelt has suggested that axotomy transfers the role of excitatory mediator from substance P and CGRP to VIP, with GAL having a protective effect. The sensory neurone in effect changes its phenotype with regard to messengers, receptor expression and function.

Table 3 Changes in neuropeptides in the DRG.

Neuropeptide	Large-diameter DRG cells	Small-diameter DRG cells
CCK	↑↑	↑
CGRP	↓↓	↓↓
GAL	↑	↑↑
NPY	↑↑	↑
PACAP	↑	↑↑
SOM		↓↓
SP		↓↓
VIP	↑	↑↑

Key

CCK	Cholecystokinin
CGRP	Calcitonin gene related peptide
GAL	Galanin
NPY	Neuropeptide Y
PACAP	Pituitary adenylate cyclase-activating polypeptide
SOM	Somatostatin
SP	Substance P
VIP	Vasoactive intestinal polypeptide

1.2.5 Neurotrophins and other signal molecules

Neurotrophic factors are molecules that play a role in the development, maintenance or regeneration of the nervous system. An important group of neurotrophic factors are the neurotrophins (55). Members of the neurotrophin family are nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). Adult sensory neurones have specific receptors for neurotrophins. These receptors are a family of transmembrane tyrosine kinases, trkA which binds NGF, trkB which binds BDNF and NT-4/5 and trkC which binds NT-3. NGF is important in inflammatory pain states. In addition NGF regulates the expression of BDNF which is released from activated nociceptors and acts as a central modulator of pain (56). NT-3 and NT-4/5 do not seem to have a major role in pain processing

NGF

NGF acts as an inflammatory mediator. NGF exerts a lot of influence via its regulation of gene expression in trkA containing neurones. NGF causes the upregulation of substance P and CGRP in neurones. The expression of BDNF is induced in almost all trkA expressing neurones following NGF treatment. Many ligand gated ion channels e.g TRPV1, P2X3, ASIC3 are regulated by NGF. NGF regulates voltage gated ion channels such as calcium, potassium and in particular sodium channels. NGF is required for maintenance of Nav1.8 expression in normal neurones (57). NGF delivered to DRG cell bodies causes a reduction in Nav 1.3 and an increase in Nav 1.8 expression, a reverse of what happens in the neuropathic state (58). It can be shown to partially restore TTX-R sodium currents in DRG cells (59). NGF, infused onto the ligated nerve has been shown to alleviate the painful neuropathy in a rat CCI model (60).

In vivo, NGF can cause sprouting of sympathetic post-ganglionic fibres. Many changes induced by axotomy such as down regulation of substance P, CGRP and down regulation of galanin and VIP can be prevented by a NGF infusion. BDNF is induced in nociceptors by NGF and may contribute to central pain processing. NGF acts as an inflammatory mediator. In inflamed tissues NGF levels increase,

both through increases in constitutive expression and de novo production (56). In human subjects subcutaneous or intramuscular NGF gives rise to pain and tenderness lasting for hours. Intravenous doses can cause pain lasting for days.

BDNF

BDNF is released in the dorsal horn by afferent fibre stimulation (61). BDNF increase is seen in typical models of neuropathic pain such as constriction injuries to the sciatic nerve. BDNF is found in vesicles in primary afferent terminals located in the superficial dorsal horn (lamina I and II) of the spinal cord (62). Noxious stimuli causes release of BDNF into these superficial laminae. BDNF causes activation of spinal *trkB* receptors which in turn causes intracellular signalling cascades and early gene and protein synthesis. An important result of BDNF activity is the modulation of glutamate receptor activity. BDNF can modulate spinal neurone responsiveness by potentiation of post-synaptic NMDA receptors and seems to potentiate glutamergic neurotransmission at the level of the spinal cord.

1.2.6 Sympathetic system

In the normal situation there is no connection between the sympathetic post-ganglionic neurones and the peripheral afferent neurones. In effect, small nociceptive fibres are physiologically and functionally distinct from efferent sympathetic tissues. After peripheral nerve injury, catecholamine containing perivascular axons sprout into the DRG and form basket-like structures around large-diameter axotomised sensory neurones (63;64). This sprouting may be triggered by cytokine-induced production of neurotrophins. Stimulation of sympathetic neurones innervating the DRG can alter activity (either excite or depress) in the spontaneously active DRG neurones. However the pattern of activity changes with time. At 2-44 days post nerve lesion sympathetic activation predominantly enhances activity of spontaneously active DRG cells, whereas by later time-points (110-171 days) depression of spontaneous activity becomes more prominent (65). In the clinical situation there is evidence of autonomic dysfunction that for certain types of neuropathic pain (e.g. phantom pain or CRPS (66;67). Indeed in CRPS, evidence of autonomic dysfunction is one of the diagnostic criteria (68). In CRPS patients, injection of adrenergic

agonists can provoke or increase pain, while increases in sympathetic arousal can be shown to increase pain (67;69). Sherman has shown that patients with burning type phantom pain have reduced surface blood flow to the stump (66). Despite the proposed link, treatment with sympathectomy has been disappointing. Systematic review of studies using intravenous sympathetic blockade using guanethidine, has not shown this treatment to be superior to placebo in the treatment of CRPS (70). Likewise, in the treatment of phantom pain, sympathectomy does not give long-term relief. Despite the association of autonomic dysfunction and certain types of pain, a causal link in humans has not been definitely established.

1.2.7 Central anatomical changes

It has been postulated that following peripheral nerve injury, there may be reorganisation of central terminals of myelinated primary afferents. Woolf et al have shown that after axotomy, large myelinated A β -fibre (and possibly A δ -fibres) terminals sprout from the deeper laminae to the more superficial laminae II (71). In this model, using Horse Radish Peroxidase (HRP) as a neuronal marker, sprouting was detectable by one week and persisted for over six months (72). A β -fibre activation of spinal neurones, normally activated by C-fibres, may contribute to touch-evoked pain (mechanical allodynia) (15). There are now, however doubts as to the validity of this method of labelling. Neuronal transport of HRP following axotomy may be altered with both large and small diameter neurones being labelled (73). More recent papers on central changes following axotomy seem to indicate that sprouting of central afferents is not as great as once thought (74;75).

There does however seem to be a functional reorganisation of sensory pathways in the dorsal horn. Following nerve injury, stimulation of A β afferents causes excitatory post-synaptic currents (EPSCs) in the substantia gelatinosa (76). It is unclear whether this functional connection is predominantly due to sprouting or via excitatory interneurones.

1.2.8 Long term potentiation (LTP)

Following peripheral nerve injury, there are alterations of central neuronal electrophysiology. Increased afferent input can lead to long-term potentiation, central sensitisation and increases in receptive fields.

LTP is a long-lasting increase in synaptic efficiency as result of tetanic stimulation. It is in effect the way the central nervous system stores information for a prolonged period of time, i.e. in learning or memory. However, LTP can also occur in nociceptive processing and may account for some of the features seen in chronic pain states e.g. allodynia and hyperalgesia (77).

LTP is the use dependent long-term increase in synaptic strength. This was first measured in hippocampal cells and forms the basis for learning and memory (78). LTP is, however, not confined to the hippocampus. It can occur in all parts of the brain and can occur in the spinal cord (79). Glutamatergic synapses; the most common excitatory synapse in the CNS has been extensively studied over the years. In essence high frequency stimulation (20-100Hz) for a few seconds can cause LTP lasting several hours. It is calcium dependent. In glutamate receptors there is calcium influx through glutamate-gated channels of the NMDA receptor. It also involves voltage gated Ca^{2+} channels, AMPA receptors or Ca^{2+} release from intracellular stores. Repetitive electrical stimulation of fine primary afferents (both A δ and C-fibres) can produce LTP. LTP of C-fibre evoked potentials is prevented by NMDA receptor blockade (80). Although LTP can be shown to occur in the spinal cord and can be blocked by NMDA antagonists it remains to be seen as to whether LTP underlies allodynia and hyperalgesia. In neuropathic rats neither electrical nor natural conditioning can be shown to produce a long-term increase in evoked neuronal firing (81). It may be that LTP is very rapidly induced following nerve injury and further increases are not possible.

1.2.9 Central Sensitisation

Central sensitisation is an important factor in the development of chronic pain states. Following sustained nociceptive input, whether from nerve damage or tissue injury results in sustained alteration in spinal cord neurones, with a prolonged increased output from the dorsal horn. A wide range of changes can be detected including, an

increase in receptive field (spatial changes), increased response to afferent activity (threshold changes), increased spontaneous neuronal activity and prolonged after discharges in response to transient stimuli (82;83).

The release of the excitatory amino acid glutamate and the subsequent activation of NMDA receptor are key to the development of central sensitisation. In the resting state the NMDA receptor is blocked in a voltage-dependent manner by extracellular magnesium such that it is not usually activated at normal resting membrane potentials. However if there is sufficient C-fibre activity, this leads to an increase in calcium in the C-fibre pre-synaptic terminal, resulting in increased release of substance P and glutamate. Post-synaptic membrane depolarisation occurs via activation of the AMPA receptor. This results in removal of the magnesium ion and allows receptor activation by glutamate. Consequently there is post-synaptic influx of both sodium and calcium ions. Increased cytosolic calcium levels result in activation of protein kinase C (PKC) and subsequently other protein kinases TrkB and protein tyrosine kinase (Src) with resultant phosphorylation of the NMDA receptor, resulting in reduced magnesium block and prolonged depolarisation (84). In the normal physiological state only C-fibres activation can induce central sensitisation. However in pathological states when there is loss of inhibitory GABA neurones stimulation via A-fibres can give rise to central sensitisation (85).

Clinical relevance of central sensitisation

The post-injury hypersensitive state leads to alterations in the sensory modality of low-threshold mechanoreceptors. Pain may occur following a normally innocuous stimulus (such as brushing), a condition known as allodynia. In addition the facilitated spinal processing may give rise to an exaggerated response to a normally painful stimulus, a condition known as hyperalgesia

1.2.10 Loss of GABAergic of inhibitory control

Processing of nociceptive activity in the spinal cord is a balance between excitatory neurones and inhibitory neurones. GABA along with glycine is one of the major inhibitory neurotransmitters in the central nervous system. The central nervous system is normally under a state of chronic inhibition largely due to the action of GABA containing neurones. In the spinal cord GABA is concentrated in the superficial laminae of the spinal cord, where the most of the A δ and C-fibres terminate. They make up between 24 and 33% of neurones in laminae I-III (86). At the time of nerve injury, an intense neuronal discharge from primary afferent neurones has been detected (87;88). This massive neuronal firing may cause excitotoxic cell death. These predominantly small neurones in the superficial layers of the dorsal horn, are more susceptible to depolarisation injury (89;90). Many of these small neurones are inhibitory and thus there is a reduction in presynaptic inhibition. Using animal models, partial peripheral nerve injury (but not axotomy), has been shown to cause a selective loss of GABAergic inhibition in the superficial dorsal horn (91). There seems to be a decrease in both presynaptic GABA release and a reduction in the GABA synthesising enzyme (glutamic acid decarboxylase). These alterations in GABAergic function may lead to a loss of tonic inhibition of both primary afferent neurones and post-synaptic spinal cord neurones resulting in increased afferent transmission.

1.2.11 Supraspinal modulation of pain

Descending input from supraspinal sites are important in modulating neuropathic pain. In an animal model of secondary hyperalgesia, spinal cord transection abolishes behavioural pain responses. This indicated that increased sensitivity to mechanical stimuli is also dependent on supraspinal factors. Cord transection on the side ipsilateral to nerve injury abolishes increased sensitivity to mechanical stimuli but not contralateral cord transection (92). This may mean that rather than afferent input promoting neuropathic pain being carried in the spinothalamic tracts, it may be carried via alternative routes e.g. the dorsal columns.

Areas of the brain that are considered important in modulating control are the PAG and the RVM. These two areas link with the spinal cord to produce a descending

pain modulating circuit (For review see Fields and Basbaum; Ren and Dubner (14;93). Tonic descending facilitation is an important driver in the maintenance of both thermal hypertalgesia and tactile allodynia (94). In addition the neuropeptide CCK may be a driver of tonic facilitation (95).

PAG

The PAG is sited in the midbrain and is important in descending inhibitory pathways. Electrical stimulation of the PAG produces potent antinociception (96). This is probably mediated by endogenous opioid systems as this can be mimicked by focal application of morphine and blocked by systemic naloxone (97;98).

RVM

The RVM is an important site for descending modulation of nociceptive signals. Like the spinal cord, activity-dependent plasticity takes place in the RVM. Both NMDA and AMPA receptors are involved, with changes in excitatory amino acid (EAA) gene/protein expression and increased phosphorylation of these receptors (99). While other CNS sites can enhance behavioural or spinal responses to a noxious stimulus, the final common facilitatory output seems to be the RVM (21). The RVM can, however, function in a bidirectional manner, i.e. inhibit or facilitate afferent activity. The RVM has three types of cells. These are 'On' cells, 'Off' cells and 'Neutral' cells (100). 'Off' cells attenuate nociceptive input and μ -opioid agonists increase their activity. In acute pain states 'On' cells accelerate firing before a nociceptive reflex and are seen to be a source of descending facilitation (100). Facilitation of 'On' cells in the RVM may account for maintenance of pain in chronic conditions. The function of neutral cells has yet to be clearly determined, but there seems to be evidence for phenotypic changes occurring in the RVM following inflammation, with cells changing their response profile with time (101;102).

CCK

CCK is an endogenous neuropeptide which reduces the antinociceptive effect of opioids. Anatomically there is overlap in the distribution of CCK, endogenous opioids and their receptors, suggesting a functional link (103). CCK, its receptors

and the level of its release exhibit considerable plasticity in the chronic pain state. CCK is thought to be involved in the supraspinal facilitation of neuropathic pain. Using a spinal nerve ligation model, microinjection of the CCK-B antagonist L365,260 into the RVM reverses tactile allodynia and thermal hyperalgesia (95). The application of CCK-8, an agonist, to the RVM of naive animals produces behaviour suggestive of neuropathic pain (95). In nerve injured animals the effect of injection of morphine into the PAG is attenuated but can be restored by L365,260. This adds weight to the idea that CCK is, at least in part, responsible for the poor response of neuropathic pain to opioid analgesia (95). Despite the promise of animal studies, L365,260 did not potentiate the effects of morphine in humans with neuropathic pain (104). This disappointing result may be due to the fact that, in primates, CCK-A and not CCK-B seems to be the predominant receptor particularly in the DRG (105).

1.2.12 Cortical changes and Pain

Melzack has proposed the concept of the neuromatrix (106). This is made up from central neural networks linking certain areas of the brain including the thalamus, the somatosensory cortex, reticular formation, limbic system and posterior parietal cortex. These linked areas create the concept of self. Melzack has proposed that the neuromatrix is effect a neurosignature i.e a characteristic pattern of neuronal activity caused by cyclical processing and synthesis of nerve impulses. The neuromatrix is created genetically but it can evolve in response to the external environment. Particular alterations in central neuronal input may do this i.e. from an increase in afferent activity from a damaged nerve or perhaps decreased activity from, for example, an amputated limb. This concept is hard to prove. In certain types of pain e.g. CRPS or phantom pain, alterations in somatosensory processing can be demonstrated in both animal and human studies. Phantom limb pain gives in-sight to the concept of central neural plasticity. Much research has focussed on plasticity of the somatosensory cortex (107). The primary somatosensory area is located in the post central gyrus. This area is organised into a specific somatotopic map, known as the Penfield homunculus. Following denervation it has been shown that the cortical representation of a body part may expand into that part of the cortex supplying the deafferented area. In the adult macaque monkey, as in humans, the sensory area for

the hand is located adjacent to the area corresponding to the face. After upper limb amputation, tactile stimulation to the face resulted in cortical activity in the area which normally corresponded to the hand (108). Cortical reorganisation was found to occur over distances up to 14mm, ten times the distance that was previously thought possible. Similar cortical remapping has been demonstrated in human. In subjects with previous upper limb amputation, stimulation of areas of the face ipsilateral to the amputation results in sensation in the phantom hand, especially the digits (109). There seems to be an over-representation of the thumb and little finger, perhaps reflecting the greater cortical magnification of these areas. The relationship between the area of the face stroked and the region of the phantom where the sensation was felt was very consistent. Stimulation to contralateral areas of the body did not usually give rise to phantom sensations. This phenomenon was also consistent in other sensory modalities, i.e. the subject felt heat and vibration in the phantom if stimulated on the face. With reference to the Penfield homunculus, this would be exactly as expected, the arm and the face being topographically adjacent to the lower limb. A study by Knecht et al supports the overall theme of cortical shift and perceptual changes but observed that the mislocalisation of pain or non-painful modality specific sensation was not as exact as Ramachandran would have us believe (110). This gives rise to the question: Is cortical reorganisation related in some way to phantom pain? Using brain imaging techniques, cortical remapping has been demonstrated in patients with upper limb deafferentation (111). The magnitude of cortical remapping was correlated with the severity of phantom pain but not phantom sensations (112). The mechanism behind cortical reorganisation may be multifactorial, perhaps involving potentiation of synapses, unmasking latent connections or by growth of new neural connections. There is evidence to suggest that plasticity in the somatosensory cortex changes can occur very rapidly. Referred sensations from the face have been demonstrated within 24 hours of amputation of the upper limb and cortical remapping confirmed using fMRI at one month (113). Plasticity is not only rapid but in patients with painful upper limb phantoms has been shown to be rapid and reversible (114). This study of upper limb amputees with phantom pain found that cortical remapping was rapidly reversed with a brachial plexus block only when the phantom pain was relieved. In patients in whom phantom

pain persisted, despite a successful block, no reversal in cortical remapping was found. These rapid reversible changes in central neural plasticity suggest the mechanism, in part at least, to be due to unmasking of latent pathways rather than neuronal sprouting. Regrowth of new neural connections would take many months and traditionally very little new growth in the central nervous system was thought to occur. There is now some evidence for both new cortical connections and brainstem connections developing in monkeys following deafferentation. Markedly expanded cortical connections in macaque monkeys have been demonstrated many years following loss of the upper limb (115). There was no evidence for new thalamocortical connections. The same group went on to study the brain stem in monkeys who had either suffered loss of their upper limb or had lesions in their dorsal columns (116). Connections in the brain stem between the trigeminal nucleus (the area for afferent sensory input to the chin) and the caudate nucleus (where sensory information from the denervated area is processed) were studied. At lower brainstem levels, the chin representation in the trigeminal nucleus lies adjacent to the hand representation in the cuneate nucleus. They were able to demonstrate that the face afferents from the trigeminal nucleus of the brain stem sprouted and grew into the cuneate nucleus. It is evident therefore that although some of the changes in the cortex may be rapid and plastic, as a result of alterations in synaptic function or unmasking latent pathways, there may also be growth of new connections both in the cortex and the brain stem.

1.3 Models of neuropathic pain

There is no human experimental model that directly mimics neuropathic pain in the clinical setting, i.e a model that involves direct injury to a nerve. Human experimental models of neuropathic pain are thus surrogate models and as such have some limitations.

1.3.1 Human experimental models of neuropathic pain

Human models of neuropathic pain focus on sensory signs and symptoms. Human models have the advantage that verbal communication can be used to convey the various qualities of the pain using descriptive language and is able to describe location and temporal qualities of the pain. Evoked signs can also be elicited. Patients with neuropathic pain often complain of burning or paroxysmal pain described like 'electricity'. Pain often occurs in an area of sensory loss. Models that have been developed try to mimic these situations. Examples include the application of topical irritants such as mustard oil or capsaicin (both topically and intradermally). These produce burning type pain and can model hyperalgesia. There is some overlap with nociceptive pain. Electrical stimulation has also been used, both direct stimulation of nerve trunks and intracutaneous electrodes. Attempts have been made to mimic paraesthesia using direct nerve pressure to cause nerve ischaemia. These models by definition cannot mimic the long lasting effects of denervation or nerve injury.

1.3.2 Animal models of neuropathic pain

Animal models of neuropathic pain have been developed with signs consistent with the clinical syndromes. Most of our current understanding of the neurobiology of neuropathic pain is based on study of these models. In research terms, animal models have the advantage that they are highly reproducible in experienced hands and consistently produce behavioural signs of allodynia and hyperalgesia to both thermal and mechanical stimuli. However it is worth noting that the majority of humans, despite clear evidence of nerve injury, do not go on to develop neuropathic

pain. Common models in current use invariably use partial damage to a peripheral nerve (usually sciatic).

The first widely used animal model used total transection of the sciatic nerve (117). This results in a denervated limb with no sensory or motor function and was thought to reproduce 'Anaesthesia Dolorosa' that is seen in humans. This model however has high levels of self mutilation (known as autotomy). Autotomy may occur as a result of having a denervated limb rather than as a result of pain. In addition, for ethical reasons, autotomy is considered undesirable. Current models have a low incidence of autotomy.

Most subsequent models use partial damage to the sciatic nerve. The three most commonly used are the chronic constriction injury (CCI), partial sciatic nerve ligation (PSNL) and spinal nerve ligation (SNL). The CCI model was described by Bennett and Xie (118). It involves tying 4 loose chromic ligatures around the sciatic nerve. The resultant inflammation and swelling results in neuronal loss, largely of A-fibres but also some c-fibres. Within three days the animal displays behavioural signs of spontaneous pain, allodynia and hyperalgesia. The PSNL model ligates only 33-50% of the sciatic nerve at the mid thigh level (119). Animals develop similar changes to the CCI model. The third commonly used model is the spinal nerve ligation model of Kim and Chung (120). This model uses tight ligation of the spinal nerves of L5 and L6. In contrast to the first two models described, this model only affects only the sensory afferents and also spares the spinal nerve of L4, which means that changes in the DRG of the undamaged afferent can be studied. A direct comparison of the three models has been carried out (121). This shows the models largely comparable, but that they have subtle differences. For example, mechanical allodynia as measured by an 8.4mN Von Frey hair was greatest in the SNL model and least in the CCI model. In ongoing pain the reverse was true. The greatest change post sympathectomy was seen in the SNL model. The authors suggest that the choice of model may depend on which is the most important outcome measure. A more recent development of the sciatic nerve damage models is the spared nerve injury model (122). This produces profound and persistent behavioural changes suggesting mechanical allodynia, cold allodynia and hyperalgesia to pinprick. This model

involves a tight ligation of the tibial and common peroneal nerves leaving the sural nerve intact. This model has the advantage of paw testing involving areas with both nerve damage and areas with an intact nerve supply.

1.4 Glutamate and Glutamate receptors

Glutamate is the major excitatory neurotransmitter released in the central nervous system in response to noxious stimuli. Glutamate has many target receptors including metabotropic and ionotropic receptors. Metabotropic receptors are G-protein linked and modulate production of intracellular messengers, while ionotropic receptors are cation specific channels. Ionotropic glutamate receptors are divided into AMPA, kainate and NMDA receptor channels (For review of glutamate receptors see Ozawa et al. 1998 (8)). The N-Methyl D-Aspartate (NMDA) receptor is key to the development of synaptic plasticity and maintenance of chronic pain states. Studies from both animal and human studies suggest that NMDA receptor antagonists may have a useful role in the management of chronic pain states. The NMDA receptor, however, is widely distributed throughout the CNS and, in addition to its involvement in spinal sensory processing, has involvement in memory, motor-function and vision (For review see Mayer & Westbrook 1987 and McBain & Mayer 1994(123;124)). This can restrict the use of NMDA antagonists in the clinical setting due to unwanted side effects (125).

1.4.1 NMDA receptors

NMDA-sensitive ionotropic glutamate receptors are widely distributed in the CNS. The NMDA receptor has three major properties. Firstly, the receptor controls a cation channel permeable to monovalent ions and calcium. Secondly, for optimal efficiency the simultaneous binding of glycine (a co-agonist) with glutamate is required and thirdly NMDA receptors are blocked in a voltage dependent manner by magnesium which means depolarisation of the post-synaptic membrane is required for activation (e.g. by activation of post-synaptic AMPA receptors) (8).

NMDA receptor complexes are probably tetrameric structures (126). Functional receptor complexes are made up from combinations of NR1 subunits and NR2

subunits. Formation of functional NMDA receptor channels requires coexpression of NR1 and at least one of the NR2 subunits. The NR1 subunit contains the glycine recognition site while the NR2 subunit contains the glutamate recognition site. There are 8 isoforms of the NR1 subunit and four individual (NR2A-NR2D) subunits of the NR2 subfamily. (127;128). The NMDA receptor is associated with scaffolding protein complexes such as PSD-95. PSD-95 is specifically restricted to lamina II of the spinal cord and overlaps particularly the NR2B subunit expression in the lumbar and thoracic cord (129). PSD-95 / NMDA receptor interaction seems essential to the development of NMDA associated sensitivity (130).

NMDA receptor distribution studies (using binding of radioligands) show the receptor to be distributed throughout the brain, predominantly the forebrain, with highest levels being found in the hippocampus (131). NR1 subunits are widely distributed in contrast to the NR2 subunits that have regional patterns of distribution. The NR2B subunit, for example, is largely found in the forebrain and in the dorsal horn of the spinal cord (laminae I and II), probably presynaptically (132). The NR2B subunit is the most commonly expressed subunit in the dorsal horn of the rat. L5 spinal nerve ligation results in a reduction in NR2A mRNA expression with a resultant relative increase in NR2B expression (133). This may be important in neuropathic pain. The NR2B receptor is of particular interest because not only are there are commercially available antagonists to the subunit, such as ifenprodil and its derivatives. NR2B antagonists have been shown to be efficacious in an animal model of neuropathic pain with a reduced side-effect profile (132).

NMDA receptors and downstream messengers may be involved in opiate tolerance, sensitisation and physical dependence. Exogenous opioids such as morphine, by binding postsynaptically, activate PKC, which via NO results in the removal of the magnesium blockade and allows activation of the NMDA receptor. Increasing levels of Ca²⁺ results in activation of additional PKC which results in critical changes in opioid-responsive neurons leading to subsequent morphine tolerance (134).

1.4.2 Pre-emptive analgesia using NMDA antagonists in animal models

In animal models of neuropathic pain, the NMDA antagonists, memantine and ketamine, were both effective via spinal administration in reducing mechanical allodynia, with memantine being of higher efficacy (135;136). Systemic or intrathecally administered ketamine has been shown to reduce the neuropathic sensitisation of dorsal horn neurons (137). Several animal studies have investigated whether administration of NMDA antagonists, around the time of nerve injury can prevent the establishment of a sensitised neuropathic pain state. Evaluation of the effectiveness of pre-emptive NMDAR antagonists in preventing sensitisation following nerve injury has been hampered since different sensory tests were assessed in different animal models.

In the CCI model, administration of the NMDAR antagonists (memantine, MK801 or ketamine) for 3-8 days from the time of sciatic nerve injury resulted in reduced development of thermal hyperalgesia (138-141). Similar observations have been made for mechanical hyperalgesia (142;143), but in a different model, the spinal nerve ligation (SNL) model. Single bolus administration of intrathecal or systemic ketamine or memantine prior to nerve injury is reported to attenuate mechanical allodynia from 6 hours to two weeks in the SNL model (143;144), or delay its development for 3 days in the CCI model (145). The timing of administration is important, with attenuation of mechanical allodynia only being observed when MK-801 was administered pre-, but not post-operatively (146).

1.4.3 NMDA antagonists in clinical practice

Clinically available NMDA antagonists

As discussed NMDA receptor mediated increases in synaptic excitability are key factors in the development of neuronal plasticity and chronic pain states. NMDA antagonists can reverse central sensitisation and as a result clinically available NMDA antagonists have been studied in chronic pain states. There are four clinically available NMDA antagonists

- Ketamine
- Memantine
- Dextromethorphan
- Amantidine

Functional inhibition of NMDA receptors can be achieved through actions at different recognition sites. They are the primary transmitter site (competitive), the strychnine –insensitive glycine site (glycine_B), the polyamine site (NR2B selective) and phencyclidine site located in the cationic channel. These four drugs are all non-competitive antagonists with moderate affinity for the NMDA receptor. This means that they block the open channel. It is thought that this may produce a better clinical profile and avoid some of the numerous side effects that the NMDA antagonists have such as memory impairment, psychotomimetic effects, ataxia and motor in coordination.

Ketamine

The first clinical report of ketamine use was in 1965 (147). It is a racemic compound with complex pharmacology (see Hirota and Lambert; Kohrs and Durieux (148;149)). It is known to have effects at glutamate receptors, including NMDA, AMPA and kainite receptors. It also acts at nicotinic and muscarinic cholinergic receptors, monaminergic receptors and opioid receptors. In addition it interacts with voltage gated Ca²⁺ and Na⁺ channels. Its action on Na⁺ channels gives it a local anaesthetic action (150). Ketamine exists in both (R) and (S) forms. (S)-ketamine has

a more specific (four times) affinity for the NMDA receptor and is clinically more potent. S-ketamine has been postulated to have lower incidences of neuropsychiatric disturbances and thus might be preferred to the racemic compound. Research in this area seems to indicate that the compounds have equivalent psychopathological and neuropsychological disturbances as the racemic mixture (151).

At the NMDA receptor, ketamine is a non-competitive antagonist of the NMDA receptor Ca^{2+} pore. It also binds to the phencyclidine (PCP) channel of the NMDA receptor leading to significant inhibition of the NMDA receptor (148).

Ketamine has been used clinically in both acute and chronic pain (152;153). A Cochrane database systematic review identified 37 studies, including studies involving the epidural administration of ketamine (154). The authors concluded that subanaesthetic doses of ketamine in the peri-operative period are effective in reducing opioid requirements in the first 24 hours following surgery. There was a reduction in post-operative nausea and vomiting with little adverse effects. Another systematic review of ketamine peri-operative ketamine showed a reduction in pain and morphine usage. The clinical significance as opposed to statistical significance was questioned so its use in everyday practice is not established (155).

Hocking and Cousins produced an evidence-based review of its use in chronic pain (153). 24 studies were identified. These covered a range of chronic pain conditions including central pain, fibromyalgia, complex regional pain syndrome, orofacial pains and various types of neuropathic pain including phantom pain and post herpetic neuralgia. In the last 10 years or so, the use of ketamine in chronic pain states has become well established; however there is still no Level 1 evidence to support its use.

Memantine

Memantine was first synthesised in 1963 by Eli Lilly as a hypoglycaemic agent. It had no activity in this regard. It was noted to have CNS activity and a patent was applied for in Germany in 1972 for its use as a potential treatment for Parkinson's disease and other CNS disorders. Subsequently it was determined that memantine is an NMDA receptor antagonist. Parsons et al have extensively reviewed its pharmacology and preclinical data (156).

In brief, memantine seems to bind at the MK-801 site of the NMDA receptor complex. It is a well-tolerated uncompetitive NMDA antagonist with strong voltage dependency and rapid blocking and unblocking kinetics. In current UK clinical practice memantine is licensed for treating moderate to severe Alzheimer's disease and the majority of clinical trials reflect this. There have to date been six clinical trials using memantine in chronic pain patients (see table 4) (157-162). These studies have used doses ranging from 20-55mg per day of memantine and looked at several chronic pain conditions (Diabetic neuropathy, post-herpetic neuralgia, post-amputation pain and other nerve injuries). Despite the promise of pre-clinical studies, none of these studies have shown any benefit of memantine over placebo in the reduction of pain. This may reflect the difficulties in translating animal models into clinical situations(163;164).

Table 4 overleaf

Key

PLP	Phantom limb pain
DB	Double blind
Mem.	Memantine
Dext.	Dextromethorphan
PHN	Post herpetic neuralgia
DN	Diabetic neuropathy

Table 4 RCTs using memantine in the treatment of chronic pain

Authors	Pain Condition	Design	Dose / Duration	PLP measure	Outcome
Wiech et al (162)	PLP	DB X-over (n=8)	Mem 30mg 4 weeks	PLP intensity Cortical reorganisation	No change in either
Schwenkreis et al. (161)	PLP	DB placebo controlled (n=16)	Mem 30mg 3 weeks	PLP intensity Cortical excitability	No decrease pain. Cortical excitability decreases as do normal subjects
Maier et al. (160)	PLP	DB placebo controlled (n=36)	Mem 30mg 4 weeks	PLP intensity	No clinical sig, Mem had higher adverse events
Sang et al (159)	DN & PHN	Dext vs Mem vs Lorazepam (active control) (n=45)	Dext up to 960mg max Mem 55mg max 9 weeks	N/A	Mem not effective Dex useful in DN only
Nikolajsen et al. (158)	Amputees and nerve injury	DB X-over (n=19)	Mem up to 20mg 5 weeks	Spontaneous and evoked pain	No clinical significance
Eisenberg et al (157)	PHN	DB placebo controlled (n=24)	Mem 20mg 5 weeks	N/A	No clinical significance

Dextromethorphan

Dextromethorphan was originally synthesised as an alternative to morphine. It is the d-isomer of the opioid agonist levomethorphan. However, it was found to have no action on opioid receptors and for many years it was used as an anti-tussive in cough remedies. Pharmacologically it blocks the open NMDA channel, blocks the dopamine reuptake site, binds to PCP and PCP2 sites and binds to the sigma receptor (165-167).

Dextromethorphan has been used in several studies of chronic pain states (see table 5). Doses up to 920mg/day have been used. Results are mixed. In studies of general neuropathic pain, chronic pain, facial neuralgias and post-herpetic neuralgia (PHN), there was no difference compared with placebo. However, in studies of patients with diabetic neuropathy (DN) and phantom limb pain (PLP) in cancer patients, dextromethorphan has been shown to effectively reduce pain compared with placebo.

Table 5 overleaf

Key

PLP	Phantom limb pain
DB	Double blind
Mem.	Memantine
Dext.	Dextromethorphan
PHN	Post herpetic neuralgia
DN	Diabetic neuropathy

Table 5 RCTs using dextromethorphan in the treatment of chronic pain

Authors	Pain	Design	Treatment	Measures	Outcome
Carlsson et al (168)	Neuropathic traumatic pain	DB X-over placebo controlled (n=15)	Single dose Dex 270mg or placebo	Pain Intensity	Dext superior to placebo
Ben Abraham et al Ann (169)	PLP (cancer amputees)	DB X-over followed by open label (n=10)	Dext up to 90mg TDS	PLP intensity	>50% reduction in pain intensity
Sang et al (159)	DN & PHN	Dext vs Mem vs Lorazepam (active control) (n=45)	Dext up to 960mg max Mem 55mg max 9 weeks	Pain intensity	Mem not effective Dext useful in DN only
Heiskanen et al (170)	Chronic pain	DB placebo controlled (n=20)	100mg Dext prior to 15mg morphine infusion	Pain intensity	No significant difference
Gilron et al. (171)	Facial neuralgia	DB placebo controlled (active lorazepam; n=19)	Up to 920mg day	Pain Intensity	No significant difference
Nelson et al (172)	DN & PHN	Double blind X-over trial DN PHN (n=18)	Mean dose 381mg DN Mean dose 439mg PHN	Pain Intensity	Significant reduction in pain in DN but not PHN
McQuay et al. (173)	Neuropathic pain	Double blind X-over n-of-1 design (n=19)	Up to 81mg Dext per day	Pain intensity, mood, sleep etc.	No significant difference on any outcome measure

Amantadine

Amantadine is another low affinity non-competitive NMDA antagonist, clinically used in the treatment of Parkinson's disease or drug induced extra-pyramidal side effects. It has lower psychomimetic properties compared with other non-competitive NMDA antagonists. Amantadine binds to the PCP binding site of the NMDA receptor, the sigma₁-binding site and at higher concentrations to other receptors including the nicotinic acetylcholine receptor (174;175). There are a least two RCTs using amantadine in clinical practice (176;177). Both studies used i.v. infusions at a dose of 200mg weekly. A positive effect from the drug was seen in both studies. This is encouraging, but further RCTs with larger numbers and using oral preparations are needed.

1.5 Phantom limb pain

1.5.1 Background

“Truly it is a thing wondrous, strange, and prodigious which will be scarce be credited, unless by such have seen with their own eyes and heard with their own ears, the patients who many months after cutting away the leg, grievously complained that they felt exceeding great pain in the leg cut off”. Ambroise Paré, a sixteenth century French military surgeon, wrote this text. Clearly, the concept of phantom limb and phantom limb pain has been recognised for centuries. The actual term “phantom limb”, however, was first coined by Silas Weir Mitchell in 1871 (178). Mitchell was a US army surgeon working in the stump hospital in Philadelphia at the time of the American Civil War. His first report appeared in Atlantic Monthly, 1866, as a work of fiction, “The Case of George Dedlow.” This anonymously penned story was of an army physician who had lost all four limbs. There was, at that time, a widespread scepticism as to the existence of phantom phenomena, and it wasn’t until Mitchell’s 1871 scientific article on post-amputation pain that term “phantom pain” was born. Phantom limb pain is now accepted as occurring not only from the many causes of traumatic amputation, but following limb amputations due other causes, such as peripheral vascular disease and malignant disease.

1.5.2 Epidemiology

The incidence of phantom pain seems to be anything between 30 and 80% (179;180). It is estimated that 5-10% of patients will suffer from chronic severe pain, scoring greater than 5/10 on a NRS (180). Literature on the subject prior to 1978 quotes a much lower incidence of around 5-30% (see table 7). The low incidences reported in earlier studies are thought to be because prevalence was based on requests for treatment rather than true prevalence (179).

The majority of studies on the incidence of phantom pain are retrospective (table7). There is always a danger that retrospective studies may overestimate prevalence as they often use a questionnaire format, which results in those with the condition being

more likely to take part. Good prospective data does exist, showing similar prevalence of 72% at 8 days and 67% at 6 months (181).

Most studies on phantom limb have looked at either traumatic amputees or amputation due to disease (mostly vascular), with almost all looking at adult populations. Traditionally phantom sensations were thought to be rare in children. However, a study of 28 child and adolescent amputees found a phantom sensation rate of 92% and an 83% incidence of phantom pain (182). The incidences in upper limb amputees may be lower with estimated incidences of between 41 and 51% (183-185). Phantom pain can occur even if there is a congenital absence of a limb. In a comparative study of children and adolescents with either congenital limb absence or surgical limb loss, the surgical amputees had an incidence of phantom pain of 48.5% while, despite a congenital absence of the limb, the subjects born with an absent limb had an incidence of phantom sensation of 7.4% and phantom pain occurred in 3.7% (186). Phantoms can occur without limb loss, following a peripheral or central nerve lesion (e.g. a brachial plexus avulsion) (187). Phantoms sensation and pain do not only occur following limb loss or denervation, but have been described in other body areas with dense innervation such as the bladder, rectum, genitalia, breast, teeth and tongue (188).

1.5.3 Presentation and natural history

When discussing post amputation pain, it is important to be clear as to what is meant by the terms “stump pain”, “phantom sensation” and “phantom pain”.

- **Stump pain** refers to pain that is felt in the residual region, adjacent to the amputated area.
- **Phantom sensation** refers to the general sensation that the amputated part is still present in some form, but is not painful.
- **Phantom pain** is defined, as pain perceived to be coming from the amputated part.

The cause of these post amputation phenomena is complex is brought about by the previously discussed pathophysiological response of the neuraxis (peripheral and central nervous system) to peripheral nerve injury.

1.5.4 Phantom sensation

The phantom limb is perceived as being very real by the patient especially in the early weeks following amputation. The phantom may be sensed in the same way as a normal limb. If the patient has lost an arm, the patient can be aware of the arm swinging from side to side in the normal manner when walking. They may feel that they are able to reach out and grasp objects with the phantom hand. If the lower limb has been removed, the patient is aware that the limb bends normally when they are seated and will straighten when they stand up.

It is important to stress that this perception is an illusion rather than a delusion. This means that although the patient senses strongly that the limb is there, they know that it is not. If it were a delusion, the patient would, in addition to strongly sensing the limb, persist in an erroneous belief that the limb still was there. It is not necessary to amputate the limb in order to give rise to phantom limb sensations. Following amputation, phantom limb sensations are almost universal with an incidence of over 90%, but do tend to change with time in duration and frequency (181;189;190). The incidence of non-painful phantom phenomena at eight days, six months and two years was found to be 84%, 90% and 71% respectively (190). Around 75% of patients had kinaesthetic sensations (feelings of length, volume or other spatial sensations) in the first six months following amputation. This fell to less than 50% later in the time course. Between one and two thirds of amputees have been reported to experience shortening of the virtual limb with time, a concept known as 'telescoping' (181;191) It may be that because the foot and hand has such a large area of cortical representation that it persists much longer than the rest of the phantom and telescoping is a manifestation of this. There does not seem to be a difference in rate of telescoping, when comparing painful and non-painful phantoms (183).

1.5.5 Post amputation pain

Post amputation pain may manifest itself as either stump pain, phantom pain or both.

Stump pain has an incidence of anything between 7 and 76% (table 6); while as discussed phantom pain occurs in around two thirds of amputees.

Phantom pain can occur almost immediately following amputation with 75% of amputees developing pain within a few days (181;182;189;192). In 10-33% of patients however the onset of pain may not be for at least a year and in one individual case pain occurred after 30 years (193).

The incidence of phantom pain is highest immediately following amputation and tends to fall with time, at least in the first six months. Jensen et al found that the incidence at one week, six months and two years was 72, 65 and 59% (194). Parkes also found a decrease in pain at one year from 85% to 61% (195). Nikolajsen et al found the incidence to be constant during follow-up although frequency and duration of attacks decreased with time (192). Houghton et al found that the median phantom pain score tended to fall with time (1 at five years following amputation) (196).

The pain experienced is extremely variable ranging from occasional mild pain to continuous severe pain. In general terms phantom pain tends to be located to the distal part of the amputated limb (192;194). The majority of patients describe intermittent pain (184;197). The sensation described is variable but typically they feel burning, cramping, shooting or crushing pains. Knife-like piercing pains are reported in the early period following amputation with squeezing burning pains experienced as time progressed (194).

Table 6 Incidence of phantom pain

Authors	Date	Study type	No.	Phantom Sensation %	Phantom Pain %	Stump Pain %	Notes
Bailey & Moersch (198)	1941	R	137	87	34	NR	
Hermann & Gibbs (199)	1945	P	120	NR	6	NR	Incidence of PLP in vascular patients 20-25%
Henderson & Smyth (200)	1948	R	300	98	4	NR	Original notes lost in war, based on memory
Carlen et al. (189)	1978	R	73	100	67	43	
Finch et al (201)	1980	R	133	54	30	10	
Steinbach et al. (202)	1982	R	42	86	73	NR	
Jensen at al. (181)	1983	P	58	90	67	22	Measured at 6 mths post amputation
Sherman et al. (203)	1984	R	2694	NR	78	NR	
Buchanan & Mandel (204)	1986	R	716	84	62	NR	
Pohjolainen (205)	1991	R	155	41	59	7	
Houghtom at al. (196)	1994	R	338	82	78	NR	
Krane and Heller (182)	1994	R	28	100	92	NR	Child and adolescent amputees
Wartan et al. (197)	1997	R	590	67	55	56	
Montoya (183)	1997	R	32	81	50	40	Upper limb amputees
Smith at al. (206)	1999	R	92	80	63	76	
Kooijman (184)	2000	R	124	76	51	46	Upper limb amputees
Dijkstra et al (185)	2002	R	632	80	72	63	Phantom pain 41% in upper limb amputees

This table shows some of the epidemiological papers on phantom limb pain

R – Retropective

NR – not recorded

P - Prospective

1.5.6 Factors influencing pain

Occurrence of phantom pain is independent of age, level or side of amputation, reason for amputation and prosthesis use (see table 7). It is probably independent of gender but one study has suggested an increased incidence in females (207).

Phantom sensations, stump pain and preamputation pain, however, do have an association with phantom pain (see table 7).

Phantom limb pain sufferers frequently complain of pains similar to that before amputation (208). Pains reported have included cutaneous lesions, deep tissue injuries, bone and joint pain and painful pre-amputation postures. These have been termed 'pain memories' (208). Katz and Melzack hypothesise that pre-amputation pain may induce persistent cortical changes resulting in a somatosensory memory, which manifests itself as phantom pain following amputation. Katz and Melzack found that 44 out of 68 subjects reported pain at the time of injury and 29 reported phantom phenomena, including pain, which they had had prior to amputation (208). These somatosensory sensations were less common if there was no pain prior to amputation or if there was a pain-free period. Jensen et al. found that 75% of patients, mostly suffering from peripheral vascular disease, had pain prior to amputation lasting greater than one month (194). Six months following amputation, phantom limb pain was significantly more frequent in patients who had pre-amputation pain that had lasted more than one month or pain in the limb on the day before surgery. 60% of these patients, however, had pain that differed in type and distribution from their pre-amputation pain, suggesting that although "pain memories" are important, they are not the only factor.

Traditionally it was thought that psychological factors played a part in patients who experienced phantom limb pain. Rather than being a physiological phenomenon, phantom pain might in some way be a somatisation of a grief process, say for the loss of the limb or even being a manifestation of grief for loss of a spouse. A review of the literature concerning psychological factors and phantom pain concluded that there is no excess of psychiatric or psychological pathology in these patients, but in common with other chronic pain patients, they do suffer from stress, anxiety and

depression (209). Depressive symptoms do seem to be associated with post-amputation pain (210).

Table 7 Risk factors in the development of phantom pain

Risk Factor	Yes	No
Sex	Weiss & Lindell 1996 (increased report in females) (207)	Jensen et al 1985 (194), Buchanan & Mandel 1986 (204), Katz & Melzack 1990 (208), Kooijman et al 2000 (184), Hanley et al. 2007 (211)
Age		Buchanan & Mandel 1986 (204), Katz & Melzack 1990 (208), Kooijman et al 2000 (184), Hanley et al. 2007 (211)
Level of amputation		Jensen et al 1985 (194), Buchanan & Mandel 1986 (204), Katz & Melzack 1990 (208), Houghton et al. 1994 (196), Kooijman et al 2000 (184), Hanley et al. 2007 (211)
Side of amputation		Buchanan & Mandel 1986 (204), Jensen et al 1985 (194), Kooijman et al 2000 (184).
Previous amputation		Jensen et al 1985 (194)
Unilateral or bilateral amputation		Katz & Melzack 1990 (208)
Prosthesis use		Katz & Melzack 1990 (208), Watan et al 1997 (197)
Aetiology		Buchanan & Mandel 1986 (204), Katz & Melzack 1990(208) , Houghton et al. 1994 (196), Hanley et al. 2007 (211)
Time since amputation	Buchanan & Mandel 1986 (204)	Katz & Melzack 1990 (208)
Pain day before amputation	Jensen et al. 1985 (194) (only at six months)	
Pain lasting one month before amputation	Jensen et al. 1985 (194) (only at six months)	
Preoperative pain	Houghton et al. 1994 (196) Krane and Heller 1995 (182), Hanley et al. 2007 (211)	Kooijman et al 2000 (184)
Stump pain	Jensen et al. 1985 (194) (only at two years) Kooijman et al 2000 (184)	
Phantom sensations	Nikolajsen et al. 1997 (192), Kooijman et al 2000 (184)	
Coexisting DM		Jensen et al 1985 (194)
Personality		Katz & Melzack 1990 (208)
Depression	Ephraim et al. 2005 (210)	Katz & Melzack 1990 (208)
Anxiety		Katz & Melzack 1990 (208)

1.5.7 Treatment of phantom pain

A survey was carried out in US war veterans looking at treatments in use or treatments that they had tried (212). The fact that in excess of 50 different treatments were described suggests that there is no one accepted treatment and success is poor. Phantom pain is a type of neuropathic pain, and pharmacological treatment focuses largely on accepted treatments for neuropathic pain. Several guidelines and evidence based practice have been produced (213-216). These are for neuropathic pain in general. First line treatments tend to be tricyclic antidepressants (TCAs), serotonergic noradrenergic reuptake inhibitors (SNRIs; e.g. venlafaxine, duloxetine) or anticonvulsants such as the gabapentinoids. Topical lidocaine can be used for discrete areas of allodynia. Opioids and tramadol are used as second line agents. NMDA are usually 3rd line agents. League tables of efficacy have been produced using the concept of numbers needed-to-treat (NNT)* for neuropathic pain, rather than specifically phantom pain (217). Tricyclic antidepressants have NNTs of around 2.3-3.0, Anticonvulsants have NNTs of in the range of 2.1-3.6 and i.v.lidocaine has an NNT of 3.0 (218;219). Although these drugs have good NNT values in neuropathic pain, they are largely based on studies of patients with diabetic neuropathy or post-herpetic neuralgia. Whether these NNTs can be extrapolated to phantom limb pain is debatable, but there is no doubt that these drugs are popular first line therapies in phantom pain. The evidence base for phantom pain treatment is sparse. Up until relatively recently many of the treatments in use were largely based on anecdote and case reports. There is a case report supporting the use of carbamazepine, an open label study of mexiletine (an oral analogue of lidocaine), reports of opioids (oral and intrathecal) and one small study of ketamine with cases reports (220-226). These have been superseded by several small scale randomised controlled trials (RCTs) in the treatment of phantom pain, mostly published over the last five years. Despite a proven track record in other types of neuropathic pain, amitriptyline in doses up to 125mg in a RCT (n=39) did not show any benefit over placebo (227). NMDA antagonists have also been studied. There are several studies

* The NNT is a measure of clinical significance derived from a quantitative systematic review. It is the number of patients that have to be given a drug in order for one to have a 50% reduction in pain score compared with placebo (Cook, R. J. and Sackett, D. L. BMJ 1995; 310: 452-454)

using memantine (20-30mg daily), none of which have shown any benefit over placebo (158;160-162). This is in contrast to another NMDA antagonist dextromethorphan which has been shown to be effective in one small study of cancer patients who have undergone amputation (169). Gabapentin, an anticonvulsant, has shown to be superior (doses up to 2400mg) to placebo in one small RCT (228). In summary although TCAs have a proven track record in many types of neuropathic pain, they have not yet been shown to work in phantom pain. NMDA antagonists, despite many promising pre-clinical studies, have had mixed results. Memantine has at least 4 RCTs now, none of which have shown efficacy. Dextromethorphan and ketamine have one small study to support their use. The only RCT using an anticonvulsant is with gabapentin and it had a positive result compared to inactive placebo. In short the evidence base is still very sparse.

1.5.8 Prevention of phantom pain

As discussed, phantom pain is very difficult to treat. This has led researchers to focus on the prevention of phantom pain

The term “pre-emptive analgesia” is much used in the literature, often incorrectly. Igor Kissin has clearly defined the concept of pre-emptive analgesia in clinical practice: pre-emptive analgesia must start before the surgery; prevent central sensitisation as caused by the surgical stimulation continue into the post-operative period to further prevent the establishment of the central sensitisation caused by ongoing pain and inflammation (229). Many of the studies purporting to study pre-emptive analgesia do not in fact meet this definition. It may be more correct to use the term “pre-treatment” unless the above three criteria can be met.

As discussed above, there are few predictive features to suggest who will get post-amputation pain. Consistently though pre-amputation pain is associated with post-amputation pain. Severe acute pain is known to be a risk factor for chronic pain (230).

Severe pre and post-operative pain may lead to a lasting somatosensory change or ‘memory’ resulting in post-amputation pain. The observation that patients with little or no pain immediately prior to surgery were at a lower risk of post-amputation pain

led to the hypothesis that if patients could be made pain free prior to surgery there might be a reduction in post-amputation pain. In addition the fact that prevention of central sensitisation, particularly by using NMDA receptor blockade, might be important in preventing chronic neuropathic pain needs to be considered.

Researchers have therefore focused on techniques that involve continuous peripheral nerve blockade, continuous epidural analgesia and techniques which use NMDA receptor blockade with ketamine. On trial has investigated the use of gabapentin for 30 days following amputation with no significant result (231). Table 8 shows summary of these studies

Continuous peripheral nerve blockade

Several studies have looked at this. Two randomised double blind studies demonstrated the technique to provide effective post-operative analgesia but not to prevent chronic post-amputation pain (232;233). A third study looked at the early addition of the NMDA antagonist memantine (20-30mg daily) in addition to a continuous ropivacaine brachial plexus block in upper-limb amputees. There was a short term effect on the incidence of phantom pain but this was not sustained at 12 months (234).

Epidural analgesia for prevention of post-amputation pain

Bach et al were the first to study this in 1988 (235). This study compared prolonged epidural analgesia (using bupivacaine and morphine) for 72 hours prior to surgery compared with a more standard opioid-based analgesic regime. All patients in the epidural group were pain free at one year. This study was small (n=25) and unblinded. A similar prospective randomised double-blind study was carried out by Nikolajsen et al. This study looked at 56 patients. The study group had preoperative epidural analgesia for 18 hours compared with a more standard intramuscular morphine technique (236). Both groups had post-operative epidural analgesia. The incidence of phantom pain at 12 months was similar to previous studies at round 70% with no difference between groups.

Table 8
Preventative studies in phantom pain

Authors	Year	Study type	No	Treatment	Outcome
Bach et al (235)	1988	Prospective randomised unblinded	25	Epidural bupivacaine and morphine compared with conventional analgesia	No phantom pain in treatment group at one year
Fischer and Meller (237)	1991	Non randomised, unblinded, retrospective controls	11	Continuous Perineural infusion of bupivacaine	No phantom pain at one year
Elizaga et al (238)	1994	Retrospective unblinded	21	Perineural infusion of bupivacaine vs conventional analgesia	No difference between groups
Jahangiri et al (239)	1994	Prospective randomised unblinded	24	Epidural infusion of bupivacaine, clonidine and diamorphine vs in demand opioid	Significant decrease in phantom pain in the study group
Katsuly Liapis et al (240)	1996	Randomised	45	Group A - epidural bupivacaine before and after surgery Group B - Opioids and NSAIDs before surgery and epidural after Group C Opioids and NSAIDs before and after surgery	No phantom pain in Group A at one year Published in abstract form only
Pinzur et al (232)	1996	Randomised double-blind	21	Perineural infusion of bupivacaine 0.5% vs saline	No difference in incidence of phantom pain between the two groups
Nikolajsen et al (236)	1997	Randomised double-blind	56	Epidural bupivacaine and morphine vs oral or im morphine	No difference in incidence of phantom pain between the two groups
Lmabert et al (233)	2001	Randomised prospective	30	Epidural bupivacaine and diamorphine vs perineural bupivacaine	Lower incidence of stump pain at 3 days. No difference in PLP at 3 days 6 months and 12 months
Hayes et al. (241)	2004	Randomised double blind	45	Bouls ket 0.5mg/Kg followed by 3 days infusion vs placebo Both groups received GA and morphine	Ket had higher SP at 3 days otherwise no difference at up to 6 months
Nikolajsen et al. (231)	2006	Randomised double blind	46	Gabapentin up to 2400mg for 30 days vs placebo	No difference in incidence of phantom pain between the two groups
Schley et al. (234)	2007	Randomised double blind Upper limb	19	Continuous brachial plexus block with ropivacaine for 7 days with either memantine (20-30mg) or placebo for 30 days	Decreased incidence of phantom pain at 6 months in the memantine group. No long term effect at 12 months

The use of ketamine to prevent phantom pain

As discussed above, animal studies suggest that the preventative use of NMDA antagonists may reduce the incidence of chronic pain. Ketamine has also been shown to be effective in the short term relief of post-amputation pain. A small observational study has suggested that a bolus of ketamine followed by perioperative infusion reduces the severity but not incidence of phantom pain. A randomised controlled trial of a ketamine bolus (0.5mg/Kg) followed by 72 hour infusion compared with placebo showed no significant reduction in the incidence of phantom pain (241). Sensory testing of the stump also showed no difference between groups. This study however did not use any form of central neuraxial blockade.

1.6 Thesis aims and synopsis

The aim of this thesis is to review the mechanisms of pain, neurothtic pain and in particular phantom pain. The concepts of pre-emptive analgesia are explored. The importance of glutamate and subsequently NMDA antagonists in this area are investigated using an animal model of neuropathic pain complemented by a clinical study of patients undergoing lower limb amputation.

This thesis begins by reviewing the basic physiology of pain. The current thinking about the mechanisms of neuropathic pain is discussed. The key role of glutamate as a one of the major excitatory nociceptive neurotransmitters is examined along with the concept of pre-emptive analgesia using NMDA antagonists. Using an animal model of neuropathic pain, the thesis aims to demonstrate the protective effect that NMDA antagonists can have following nerve injury. The effect of NMDA antagonists on the development of typical pain behaviours following nerve injury has been established but the effects of pre-treatment with NMDA receptor antagonists on subsequent NMDA receptor subunit expression has not.

The model of pre-treatment with an NMDA antagonist prior to nerve injury is translated into a clinical study in chapter 4. This study examines the use of the clinically used NMDA antagonist ketamine in patients undergoing lower limb amputation. Limb amputation was chosen as it is one of the few clinical situations

that subjects can be identified before nerve injury occurs. The study uses pre-treatment with epidural ketamine and examines whether it has any effect on the prevention of phantom limb pain. In addition the effects on the drug on sensory processing as studied, using quantitative sensory techniques which parallels those used in the animal model.

The final chapter discusses the findings of both these studies. The difficulties of translating promising basic science studies into clinical practice is discussed.

Chapter 2 Assessment techniques

2.1 Introduction

The present studies aim to investigate the role of NMDAR antagonists in the prevention of neuropathic pain. A translational approach was used linking an animal model of neuropathic pain with a difficult clinical problem – post amputation pain. As pain perception is subjective, comprehensive assessment of pain in both animals and humans presents some challenges. The methodology used and the rationale behind it is discussed.

2.2 Methods of nociception assessment in animal models of pain

Several rodent models of neuropathic pain have been developed (see chapter 1 for descriptions of the commonly used models). Animals are unable to vocalise and thus observations of animal behaviour becomes a surrogate measure of pain in the animal.

2.2.1 Behavioural testing

Commonly models of neuropathic pain use standardised limb withdrawal measures as a surrogate measure of sensitivity to various mechanical and thermal stimuli (Both noxious and innocuous). Tests must be reproducible and thus testing needs to be carried out in a situation that the animal is familiar with. Ideally the animal should be unrestrained. This will minimise stress to the animal which can otherwise alter withdrawal thresholds. The main advantages of these methods are that they seem to mimic the changes seen in human pain conditions i.e. sensitivity such as allodynia and hyperalgesia, they are relatively easy to measure and can be reliably reproduced from day to day. On the downside, these measures do not tell us anything about spontaneous pain, a feature often seen in human conditions. One study found that in 421 papers (published in *Pain*) using an animal model of pain, 90% used exclusively tests of hypersensitivity (163). There does seem to be a trend to including measures of spontaneously occurring pain.

2.2.2 Responses to mechanical stimuli

Early measures of mechanical allodynia used devices such as a Randall-Selitto device (242). This requires restraint or handling of the animal to measure withdrawal thresholds or elicit vocalization. This produces stress in the animal and can affect the measurements (243). Pressures required to elicit a response are high and thus responses to deep pressure rather than light touch (tactile allodynia) was being measured. This is important as deep pressure is probably C/A δ -fibre mediated, while light touch is A β -fibre mediated (244). Sensitivity is typically measured using the application of graded nylon monofilaments e.g. Von Frey filaments. Each filament is of a standard length and diameter. The filament is applied to the skin until bending occurs. This results in the application of a standardised reproducible force. A standard set of filaments typically contains 20 probes with force applied (in a logarithmic scale) ranging from 0.008g to 300g. These have the advantage of using low pressures, not requiring animal restraint, being reproducible and widely used.

In the animal experiments in this thesis, mechanical allodynia was measured as the threshold for paw withdrawal in response to a graded mechanical stimuli applied to the mid-plantar glabrous surface of the hind paw using calibrated von Frey filaments (Stoelting, Wood Dale, IL). The method of testing was based on standard laboratory protocol. Animals were removed from the cage and placed on a wire cage lid. This enabled easy application of the filaments to the paw. The filaments were applied in ascending order of magnitude from a fine filament upwards. The methodology was based on the method of limits. Threshold was defined as the pressure (force per unit area) that caused foot withdrawal five times in every ten applications, repeated at 1-2 second intervals. The pressure applied to the hind paw by the von Frey filaments is calibrated as force (milliNewtons) divided by the area over which it is applied (millimetres squared).

2.2.3 Thermal hyperalgesia

Nerve injury results in lowered thresholds following the application of a thermal stimulus. Nociceptive thermal thresholds were traditionally measured using techniques such as the tail flick or hot-plate methods. Although widely used, both

techniques have limitations. The tail flick test stimulates an atypical (scaly) area of skin and there is no scope for intersubject comparison. The hot-plate has no automated end-point detection. The Hargreaves apparatus was designed to overcome some of these limitations(245). The animal is unrestrained and free to move about on a frosted glass plate. Radiant heat is can be applied to a paw, with the device automatically measuring paw withdrawal by means of a photoelectric cell. The contralateral paw can be used to provide intersubject control measurements.

In my experiments, thermal hyperalgesia was monitored using radiant heat (30-55°C; Hargreaves' thermal device, Linton Instruments, Diss UK) applied to the mid-plantar glabrous surface of the hind paw. The animals were placed a specifically designed Perspex box, which allowed three animals at once on top of the frosted glass plate of the apparatus. Animals were allowed to settle and become accustomed to their surroundings before testing began. Using the apparatus, radiant heat is shone onto the paw under test. The withdrawal response latency was characterised as a brief paw flick recorded to the nearest 0.1 sec; a standard cut-off latency of 20 seconds prevented tissue damage.

2.2.4 Cold allodynia

Pain can be elicited by cold temperatures. In neuropathic pain states innocuous cold temperatures may cause pain (cold allodynia).The TRPM8 (transient receptor potential melastatin 8) receptor seems important in this area with evidence that expression of the receptor rises following nerve injury and that the cold allodynic response can be attenuated by TRPM8 blockers (246;247). This receptor and other receptors are likely targets for new treatments of neuropathic pain (248).

Simple methods of cooling an animal's skin have been used such as application of topical acetone or ethyl chloride spray to the hind limb of an animal. Paw elevation is used as a measure of cold allodynia. Influencing factors can be ambient temperature and animals skin temperature which can affect the rate of evaporation and hence cooling of the skin. The substance its self, via smell or by mechanical stimulus, can affect paw lifting. More sophisticated models have been developed which involve

placing the hind paw of a restrained animal in a cold water bath. This is stressful to the animal and thus may influence the nociceptive responses. Models using an ice cooled plate or an ice cooled water bath and unrestrained animals have been developed. These are well tolerated but can only measure behaviour at one temperature (4°C) (249).

The protocol followed in my experiments followed established lab protocol (250). The model used was the shallow ice cooled water bath. Animals were placed in a perspex box with an elevated aluminium floor covered with iced water, sufficient to immerse both glabrous and hairy skin of the hind paw (3-4°C). Once placed in the box, rats were allowed 10 seconds to acclimatise. The number of seconds the animal raises its hind paw above the water over a 20 second period was recorded. This was repeated four times at 10 minute intervals to establish a mean suspended paw elevation time (SPET) for each rat.

2.3 Immunoblotting and Immunohistochemistry

The thesis investigates the role of NMDAR antagonists in the prevention of neuropathic pain. In addition to investigating drug action, an important part of the research was to investigate the effects of the drug on the NMDARs at the proposed site of action of the drug i.e the lumbar spinal cord. As discussed, in chapter 1, the NMDAR is made from a combination of subunits. Functional receptor units are made up of an NR1 subunit and at least one of the NR2 subunits. The NR2B subunit is relatively restricted to the superficial spinal cord and thought to be important in pain transmission and sensitisation (132). This thesis investigates the effect of NMDAR antagonists on the expression of the NR1 and NR2B subunit in the spinal cord. The two techniques that were used were immunoblotting and immunohistochemistry. Immunoblotting allows detection of specific antigen in a sample of tissue and can provide information on molecular weight and quantity. The technique allows quantification of the amount of a particular receptor in a given sample of tissue i.e the specific receptor of interest can be labelled and then quantified. Immunoblotting does not however localise a protein in a sample. To do this, proteins need to be labelled in situ using the technique of immunohistochemistry.

I am very grateful to have had the assistance of Dr Emer Garry and Heather Anderson (Centre for Neuroscience Research, University of Edinburgh) who helped in this part of the research. Dr Garry performed the immunoblotting experiments and Heather Anderson carried out the immunohistochemistry reactions and prepared the slides for analysis.

The concepts behind immunoblotting and immunohistochemistry are discussed below. Standard established laboratory protocols in the Centre for Neuroscience Research were used and these are described.

2.3.1 Immunoblotting

Immunoblotting (Western blotting) is used to detect a specific protein in a sample of tissue. The technique typically uses gel electrophoresis to separate proteins based on length of polypeptide. Proteins are transferred to a nitrocellulose membrane.

Nitrocellulose is a sticky membrane used to immobilise proteins. It has a non specific affinity for amino acids. Specific antibodies to the protein of interest are used to 'probe' the proteins and detect the protein of interest

General technique

The sample of tissue is first of all homogenised. A buffer is typically used to aid cell lysis and to solubilise the proteins. Protease and phosphatase inhibitors are added to prevent digestion of the sample by its own enzymes.

Gel electrophoresis is used to separate the proteins. A membrane fraction (i.e a fraction of cells including membranes and plasma) is loaded in the gel and an electric current used to separate the proteins. The proteins are then transferred to on to the nitrocellulose membrane. In order to get clearer samples the membrane is exposed to a dilute protein solution which attaches in the places other than where the target proteins are.

The membrane is then incubated with a dilute solution of the primary antibody. The membrane is then rinsed to remove unbound primary antibody and exposed to secondary antibody. The secondary antibody is typically peroxidase linked and used in conjunction with a chemiluminescent agent which then produces luminescence in proportion to the protein present. The membrane can then be exposed to a photographic film and an image obtained. The image can then be analysed by densitometry which quantifies the protein in terms of optical density.

Technique used

Western blotting was carried out to assess NMDA receptor subunit expression in the lumbar spinal cord of rats. Spinal cord segments (L3-6) were removed from anaesthetised animals and quickly homogenised in ice-cold buffer containing 5% glycerol with peptidase and phosphatase inhibitors. A membrane fraction was then solubilized in 1% deoxycholate. Blots were probed with rabbit or goat polyclonal primary antibodies to NR1 (SC-1467), NR2A (Cat No 06-313, Upstate Biotech) or NR2B (Cat No: AB1557P, Chemicon) and detected by peroxidase-linked secondary

antibody enhanced chemiluminescence. The ubiquitous housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was monitored as a control, for protein level normalisation using mouse monoclonal antibody (cat no MAB374, 1:750 from Chemicon International Ltd, Harrow, UK). Following quantitative densitometry, arbitrary grey scale values for receptor subunits were calculated as a percentage of that for GAPDH in each case and the statistical significance of injury induced changes between the ipsilateral and the contralateral side, sham or naïve controls was assessed by the Wilcoxon test.

2.3.2 Immunohistochemistry

Immunohistochemistry, in contrast, is the process which can localise exactly where an antigen is localised a tissue section. In a similar fashion to immunoblotting antibodies bind to the antigen of interest in the tissue section. The antibody is again conjugated to a peroxidase enzyme which catalyses a colour producing reaction enabling visualisation of the antibody-antigen complex.

General technique

The tissue of interest is typically fixed by rapidly transcardially perfusing the animal with paraformaldehyde. Post-fixation is carried out in paraformaldehyde with a buffer.

Following fixation, the sample of interest is mounted in wax and then can be sliced into thin slices using a microtome. The tissue slices are then mounted on a slide.

A two stage technique is used. The tissue is exposed firstly to a primary antibody which attaches to the antigen of interest. A second antibody against the IgG of the animal used (e.g goat) to create the first antibody is then used. This antibody is labelled with an enzyme or florescent dye. Typically the enzyme is coupled with a horse radish peroxidase which can be reacted with 3,3' diaminobenzidine (DAB) to produce a brown colour where the antibody is attached, a process known a DAB staining. For a detailed review of the technical aspects of immunohistochemistry see review article by Ramos-Vara (251).

Technique used

Under deep anaesthesia, rats were perfused transcardially with saline containing 100 units heparin/ml, followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Lumbar spinal segments (L3-6) were post-fixed in 4% paraformaldehyde/0.1M phosphate buffer for 4-5 hr at 4°C, before being stored in 0.1M phosphate buffer at 4°C. 70 µm transverse sections were initially incubated in normal serum from the species which donated the secondary antibody, to block non-specific binding. They were then incubated overnight at 4°C with either rabbit anti-NMDAR1 polyclonal antibody (Chemicon, 1:500) or rabbit anti-NMDAR2B polyclonal antibody (Chemicon, 1:750). Control sections were processed without the addition of primary antibody and incubated overnight at 4°C in 0.1 M phosphate buffer. Simultaneous staining was carried out for all treatments. All sections were then incubated in biotinylated donkey-anti-rabbit IgG (1:200, 60 min; Chemicon), followed by incubation for a further 60 min with an avidin-biotin complex solution (ABC Elite, Vector Laboratories, UK, 1:50). Following wash, sections were exposed to 3,3'-diaminobenzidine tetrachloride (DAB; 0.2mg/ml; Sigma, UK) in the presence of 3% hydrogen peroxide (1µl/ml) to enable visualisation of the precipitate, washed and mounted onto poly-L-lysine-coated microscope slides (BDH, UK), air-dried, dehydrated through ascending concentrations of alcohol, cleared in xylene (Sigma) and mounted in DePeX (BDH, UK).

For relative quantification of immunoreactivity, an Improvision 1.44 Image Analysis Package (NIH) was used at a magnification of x 40. Each image field was captured, using a CCD video camera (Sony, Japan), mounted on a Zeiss Axioscope microscope. The images were blank field-adjusted to compensate for any artefacts in the camera apparatus. A standardised region of interest (ROI; 80 µm diameter circle) cursor was aligned and consecutively centred on the mediolateral region of laminae I-II or III. Arbitrary grey scale units (in the range of 1-200) were assigned to make optimal use of the range for the given sample. Non-specific background levels were recorded from the dorsal column region for each section, and the net density was calculated by subtraction.

Alternative techniques

In addition to the techniques described above, there are other laboratory techniques that can be used to determine the presence and/or quantity of a specific antigen. Enzyme Linked ImmunoSorbent Assay (ELISA) can be used to detect specific antigens in a sample of tissue or serum (252). ELISA commonly used as diagnostic tool in medicine and is used to detect antigens such as HIV in a serum sample. The basic principle involves fixing the sample of interest onto a surface (usually a multiwell plate) and using antibodies to probe the sample. Antibody can be visualised using colourimetry or chemiluminescence. Techniques such as flow cytometry and immuno-electron microscopy can be used. These do require expensive and specialised equipment.

2.4 Assessment of pain in clinical practice

2.4.1 Introduction to clinical pain assessment

Pain is a subjective experience, with several different components including cognitive and affective components. The pain experience is a personal experience which can be influenced by the patient's beliefs, mood, personality, prognosis and culture. Assessment tools have been developed to either give a global assessment or to characterise the pain experience in more detail. When considering any assessment tool it is important to consider its reliability, how it has been validated and its applicability to the clinical situation being studied. In an ideal situation one observer should assess all the patients in a study.

The simplest measure of pain is that of pain intensity which is typically measured using a unilateral scale. Examples of scales used are verbal rating scales (VRS), numerical rating scales (NRS) and visual analogue scales (VAS). These are discussed below. Unidimensional scales are ideal in studies of peri-operative pain. The complete multidimensional pain experience is not however captured by these scales and thus pain intensity alone is insufficient alone in studies of chronic pain. Outcomes are more complex and measures must reflect the multidimensional nature of pain focusing on other outcome variables such as quality of life, changes in function, sleep patterns and affect.

There are many scales in used in the assessment of outcome in chronic pain studies. In clinical practice, especially in chronic pain states, outcome measures must reflect the whole pain experience. This means measuring not only the sensory component, but the affective and cognitive components as well. Consensus guidelines have been produced by the IMMPACT group (Initiative on Methods, Measurement and Pain Assessment in Clinical Practice) which recommend 6 core outcome domains (253). The first three of these are pain, physical functioning and emotional functioning. There is little evidence to suggest that one scale is superior to another in any particular domain (254). Pain intensity is generally measured on a unilateral pain

scale, while the physical and emotional functioning usually requires some type of multidimensional scale. Changes in sensory processing can be measured and quantified, a process known as quantitative sensory testing (QST).

2.4.2 Unilateral Pain Scales

Verbal Rating Scale (VRS)

The VRS measures pain in distinct categories, e.g. a four point scale such as no pain, mild, moderate or severe pain. It is very easy to use in clinical practice. The main downside of the VRS is that change between each point cannot be assumed to be the same, so it is difficult to record reductions in pain accurately. In research terms this may mean that small reductions in pain are not accurately detected. In the clinical setting the VRS may be a more appropriate scale for use in elderly patients who may have more difficulty understanding numerical rating and visual analogue scales (255).

Numerical Rating Scale (NRS)

The NRS uses a numeric scale ranging typically from 0-100 or 0-10. Zero represents no pain while the highest number in the maximum pain possible. A NRS unlike the VRS possesses ratio properties, i.e. a reduction in pain from 6 to 3 would indicate a 50% reduction in pain. In clinical practice a 0-10 point scale is as good as the longer scale. There seems to be no loss of sensitivity by reducing to the shorter scale and the 11 point scale is as reliable at detecting clinically meaningful reductions in pain compared with a 0-100 scale (256). The NRS is easy to apply even when a patient cannot use pencil and paper. It can be used over the telephone or using a computer data collection system.

Visual analogue scale (VAS)

The VAS is plain ruled line usually 0-100 mm long, the left hand side being no pain and the right the worst pain imaginable. The patient is asked to mark on the line a point corresponding to the severity of their pain. The pain score is then the distance in millimetres from the left hand side. It is easy to use but the paper version does

require the ability to use a pencil. Slide rule and computerised versions can get round this. It has been widely used in pain research since the 1960s and is reliable, valid and internally consistent (257). Like the NRS it has ratio properties and can measure percentage reductions in pain.

Choice of scale

In the research setting the unidimensional scale of choice should either be the NRS or the VAS. These scales correlate very well and are easy to administer (256). The VRS is probably the easiest to use but can underestimate severe pain and is only recommended as a coarse screening tool (258).

2.4.3 Multidimensional scales

As discussed the IMMPACT guidelines suggest that pain in addition to pain severity other outcome, measure of physical and emotional functioning should be used (259). At its most simple The VAS or NRS scales can be used to measure ‘unpleasantness’ of pain or of a particular stimuli. In addition the IMMPACT guidelines suggest the inclusion of a questionnaire designed to measure the affective and sensory component of the pain e.g. the SF-Magill Pain Questionnaire or the Magill Pain Questionnaire(260;261). Measures of physical functioning should ideally be included. Questionnaires of physical functioning focus on areas such as activities of daily living (ADL) and sleep. Patients with chronic pain often complain of interference with ADLs and sleep. Physical function, however, does not correlate well with pain giving rise to need for a separate assessment (262). In the absence of a disease specific questionnaire the guidelines suggest using either the Multidimensional Pain Inventory (MPI) or the Brief Pain Inventory (BPI) (263;264). These are both reliable and validated measures of the effect of pain on daily life. The recommended scales for measurement of emotional functioning are the Beck Depression Inventory (BDI) or the Profile of Mood States (POMS)(265;266). Like the measures of physical function, these are both reliable and validated. The impact guidelines were produced after the clinical study was complete and I was unable to benefit from these recommendations in the study design.

There are increasing numbers of scales designed to assess neuropathic pain becoming available. There are designed either to screen for the presence or absence of neuropathic pain to assess pain severity in those suffering from neuropathic pain. Scales designed to screen for the presence of neuropathic pain include the Leeds Assessment of Neuropathic Symptoms and Signs (LANSS) pain scale and the DN4 questionnaire (267;268). The LANSS pain scale is based on analysis of sensory description coupled with a bedside sensory function. A score of greater than 12 suggest that the patient is likely to have neuropathic pain. The initial validation study shows that it correctly identified 85% of the patients with neuropathic pain in a study population. The DN4 used 7 questions relating to sensory symptoms and 3 bedside examinations of signs. A score of 4 or greater out of 10 suggests that the patient may have neuropathic pain. The development study showed the DN4 to have 83% sensitivity and 90% specificity compared with clinical diagnosis (268). In addition to the LANSS and the DN4 there are other screening questionnaires in clinical use such as the Neuropathic Pain Questionnaire, PainDETECT and ID-pain (For a review of these scales and the use of screening questionnaires in neuropathic pain see Bennett et al. (269)). In addition to screening questionnaires there are scales designed and validated to measure neuropathic pain severity. Examples of these are the Neuropathic Pain Scale (NPS) or the Neuropathic Pain Symptom Inventory (NPSI) (270;271). I used the NPS and this is discussed below.

Assessment Questionnaires used

This thesis used a specifically designed data collection questionnaire which was a modification of that used by Shermann et al (Appendix 1). In addition the Magill Pain Questionnaire was used along with Hospital Anxiety and Depression Scale and the Neuropathic Pain Scale. These are discussed in detail below.

McGill Pain Questionnaire (MPQ)

The MPQ was first described in 1975 (261). It is a multidimensional assessment tool designed to separate and quantify the three distinct components of pain, sensory-discriminative, motivational-affective and cognitive evaluative. It consists of 78 words grouped into groups, which are further arranged into 4 categories, a sensory,

affective, evaluative and a miscellaneous category. There is also a 6-point verbal rating scale (Present Pain Index; PPI). The Questionnaire is self administered by the patient. A word from each category is selected that best describes the pain. Not all categories need be marked. The total score (known as the Pain Rating Index; PRI) is made up from adding the rank in the category of each word selected. The PPI score can be used as a response to treatment. The initial idea was that the three different components of pain making up the PRI could be assessed separately. The problem is that the sensory, affective and evaluative factors are highly interlinked rather than independent variables, therefore it has been suggested that the total PRI should be used when assessing pain with this tool (272). The MPQ has been extensively validated over the years and has good predictive validity, construct validity and test retest reliability (257).

The Hospital Anxiety and Depression Scale (HADS)

The HADS was devised by Zigmond and Snaith in 1983 (273). The purpose of the scale is to identify the probable and possible cases of anxiety and depression in patients in the non-psychiatric clinical setting. It specifically avoids symptoms such as dizziness, fatigue, insomnia or headache that may be associated with somatic disorders. It has been used in hundreds of studies since its introduction (274). In addition it has been used in studies of chronic pain such as musculoskeletal pain, low back pain and importantly post-amputation pain (275-277).

The questionnaire consists of 14 questions, seven concerning anxiety symptoms (HADS-A) and seven concerning depression symptoms (HADS-D). Each question has 4 possible answers, marked 0-3, thus making the maximum score for each subscale of 21.

A recent detailed review of 747 papers using the HAD scale suggests that the optimal balance between sensitivity and specificity is achieved with a cut off of 8 or greater for both HADS-A and HADS-D (274). In addition the authors conclude that the scale performs well in the assessment of symptom severity and helps to identify cases of anxiety and depression in hospital, primary care and the general population. However the scale can only act as a case finder and thus more detailed assessment is required to make a specific psychiatric diagnosis.

The Neuropathic Pain Scale (NPS)

The NPS was described in 1996 (270). This was designed to complement existing pain questionnaires such as those described above. The NPS has been designed to assess the distinct pain qualities associated with neuropathic pain. The scale uses 10 descriptors of pain such as sharp, hot, cold etc. and the subject rates each descriptors on a scale of 0-10. Preliminary validation suggest that the descriptors are for the most part statistically distinct may have some value in distinguishing one type of neuropathic pain from another (270). The NPS main strength is as a severity measure of neuropathic pain, rather than as a screening tool. At the time of study design and recruitment it was the one of the few scales available to assess neuropathic pain. That said, the NPS has however been shown to discriminate between neuropathic pain and non-neuropathic pain (278).

2.4.4 Quantitative Sensory Testing (QST)

Introduction

As discussed above, pain research has traditionally focussed outcome measures on qualitative responses to pain, such as VAS or NRS. Pain classification is now moving towards a mechanism-based approach (279). This may mean more accurate understanding and subsequent targeting of pain treatment therapies. QST can be used to detect modality specific disturbances in sensory processing. In essence it measures the intensity of stimuli needed to produce specific sensory perceptions and thus measures the function of the somatosensory system.

QST techniques use psychophysical techniques, with different stimulus modalities to assess functional changes in primary afferent fibres, central spinal connections and subsequent sensory projections. In effect using different sensory modalities to determine at what intensity they produce specific sensory perceptions. Detailed QST is time consuming and does require a cooperative, fully alert patient who can understand the, sometimes, complex tasks required.

Sensory information is carried in the large myelinated A β -fibres, the smaller myelinated A δ -fibres and the unmyelinated C-fibres. In normal physiological states the A β -fibres carry mechanosensitive modalities such as light touch, vibration and pressure while A δ -fibres and C-fibres can respond to both noxious and non-noxious stimuli. QST must then use targeted sensory tests to detect dysfunction in these pathways. On a practical level simple bedside tests may be used, such as an artist's paintbrush (measures dynamic allodynia; A β -fibre mediated), hot and cold rollers (temperature related pain; A δ and C-fibres) and a pin (painful pinprick; A δ -fibres) (280).

In a lab based research setting more detailed testing methods may be employed. These include the use of Von Frey hairs to measure tactile and pain thresholds, vibration and pressure thresholds and the use of a thermode to measure warm/ cold thresholds and heat/cold thresholds.

A comprehensive protocol now exists which was agreed after the completion of our study. The German Research Network on Neuropathic Pain have published a suggested battery of tests comprising seven tests covering 13 parameters (244). The group has also published reference values using the suggested protocol (281). The protocol suggests the following tests.

- Thermal detection thresholds for the perception of cold, warm and paradoxical heat sensations
- Thermal pain thresholds for cold and hot stimuli
- Mechanical detection thresholds for touch and vibration
- Mechanical pain sensitivity including thresholds for pinprick and blunt pressure, stimulus/response-functions for pinprick sensitivity, and dynamic mechanical allodynia, and pain summation to repetitive pinprick stimuli (wind-up type pain)

This battery of tests can be done mostly using simple bedside devices, although no easily portable device exists to measure thermal thresholds. Unlike standard

neurophysiology this battery of QST testing enables assessment of both small and large fibre dysfunction. Although the tests are comprehensive in nature they are still not the stage of producing a complete mechanistic approach to pain.

QST in clinical trials of neuropathic pain

There are increasing numbers of trials in patients with neuropathic pain showing the usefulness of the measure in these patients. In the same way sensory testing is used in animal models of pain, QST can be used in human models of central sensitisation (282). In clinical studies designed to prevent central sensitisation, QST has been shown to be a valuable outcome measure. A study of patients undergoing abdominal surgery was able to use Von Frey filaments to map out punctuate hyperalgesia around an abdominal wound to show a long term effect of ketamine on long term sensitisation (283). QST has also been used in patients with peripheral vascular disease showing sensory dysfunction is already established in these patients and dysfunction can be detected in the affected leg (284). QST has been also been used in groups of patients undergoing limb amputation (285;286). Although QST is becoming a vital tool in assessment and outcome in patients with neuropathic pain it has not yet really been able to predict likely responders to available therapeutic options (287;288).

QST protocol used

In designing the QST protocol for this study, a translational approach was used. The design of the QST protocol tries to reflect, at least in part, the testing modalities used in the animal studies, especially as some of the hypotheses generated come from the laboratory experiments. In this thesis the sensory testing modalities chosen were sensitivity to stroking light touch ($A\beta$ -fibres), tactile threshold using Von Frey filaments ($A\beta$ -fibres), mechanical sensitivity to painful pinprick ($A\delta$ -fibres), vibration ($A\beta$ -fibres) and sensitivity to cold using drops of topical acetone ($A\delta$ -fibres). The tests chosen needed to be easy to apply, easy for elderly patients to understand and be portable as some follow-up assessments took place outside the hospital setting.

The protocol designed attempted to delineate any areas of abnormal sensation using a soft artist's paint brush. This was applied to the stump to try and identify any areas of abnormal sensitivity. Further sensory testing was then carried out on the stump using the contralateral limb as a control. Rolke et al. demonstrated that although QST parameters are age and site specific, there is a high side to side correlations of all QST parameters, suggesting that the opposite side of the body is an ideal control (244).

Tactile threshold was measured using von Frey filaments (North Coast Medical, Inc., San Jose, California), described above. The methodology used in this thesis was to apply them in ascending and descending force magnitude until the subject was able to perceive four out of six stimuli. This is known as the method of limits, which is the commonly used method of testing. The method of limits involves a step wise increase (or decrease) in stimulus intensity. The stimulus is started at a level below which the subject is not able to perceive the stimulus. As the stimulus increases the subject will be able to perceive the stimulus and the threshold is measured when the subject perceives greater than 50% of the applied (six) stimuli, i.e. 4/6 applications. The main disadvantages to this method are that the subject may start to anticipate the detection or disappearance of the threshold (error of expectation). The alternative to the method of limits is to use a fixed choice method which asks the subject to choose between two different stimuli. This method is relatively slower (289).

Mechanical sensitivity to painful pinprick was used by the application of standardised blunt neurology pain (Neurotips™; Owen Mumford). These were applied to the stump and control areas, with the subject recording the pain induced on a visual analogue scale.

A standard battery powered electrical tooth brush was applied to the stump to see if vibration elicited any painful sensations. The brush was switched on and then applied to the area under test. The patient was then asked to record a VAS scale for both unpleasantness and pain.

Finally the response to topical cold was assessed using acetones dripped form a dropper onto the area under test. Any pain elicited was recorded using a VAS score.

Chapter 3

Investigation of the effects of pre-treatment with NMDAR antagonists using an animal model of neuropathic pain

3.1 Introduction

NMDA receptors (NMDAR) are widely distributed in the CNS and involved in excitatory neurotransmission(290). They have an important role in cognitive functions (eg learning and memory) (291). They also play a key role in the central sensitisation that occurs in the spinal cord in many chronic pain states, such as neuropathic pain (292-294). NMDAR antagonists have a potential therapeutic role in pain as well as many CNS disorders such as brain ischaemia, neurodegenerative disease, epilepsy and psychiatric disease (295-302).

3.1.1 NMDA receptors

As outlined in more detail in Chapter 1, NMDARs are heteromeric complexes made up from combinations of three subunits, NR1, NR2 and NR3. Functional receptors are made up from expression of at least one NR1 subunit and an NR2 subunit (A,B,C or D). In the functional receptor, the NR1 subunit binds the co-agonist glycine, while the NR2 subunit binds glutamate. Activation of the NMDAR requires simultaneous binding of these two co-agonists (303;304). NR1 subunits are widely distributed in the CNS. In the spinal cord, NR2A receptors are found in lamina III and IV while NR2B receptors are found lamina I and II (132;305). In addition NR2B receptors are found in the forebrain, cortex, hippocampus, striatum, thalamus and olfactory bulb (306;307). They are also found in DRG cells (308;309). Within the spinal cord, NR2B receptors are relatively restricted to the superficial laminae, known to be important in nociceptive transmission. This makes them a potentially important therapeutic target in chronic pain states where NMDA receptors are activated, such as occurs in neuropathic pain.

Other subunits may also be important, with evidence for the role of NR2A in inflammatory pain and NR2D in neuropathic pain (310) The role of the NR2A subunit in central sensitisation is not clearly determined but it does seem to be

involved in the development of analgesic tolerance (311). It has also been suggested that NR2A receptors are critically important in LTP induction (312).

The two principle NMDA receptor subunits, NR1 and NR2, show differential expression in sensory pathways in spinal dorsal horn (7;132;313-315). Single dorsal horn neurones may express multiple NMDA receptor subunit combinations (133). There is evidence that NR2B receptors are situated both pre and post-synaptically and (in rats) expressed on astrocytes (316). The contribution made by specific subunits to neuropathic sensitisation remains, however unclear.

3.1.2 Pharmacology of NMDARs

The NR2 subunits (A-D) determine the pharmacology and function of the receptor. NR2A and NR2B are pharmacologically distinct (317).

Most sub-type receptor specific antagonists are for the NR1/NR2B subunit. These are based on ifenprodil and its derivatives. Ifenprodil binds to the NR2B subunit and acts as a non-competitive antagonist in a use-dependent voltage-independent manner. Ro 25-6981 and CP-101,606 (traxoprodil) are derivatives of ifenprodil and also act as antagonists at NR1/NR2B receptors, both with a higher preference than ifenprodil. Specific NR2A receptor antagonists are less common although zinc and NVP-AAM077 (Novartis) do have specificity for the channel (318;319).

3.1.3 NMDAR antagonists (Table 9)

Non specific NMDAR antagonists can be divided into competitive or non-competitive antagonists. Competitive antagonists bind to the glutamate-binding site on the NR2 subunit. Examples of competitive antagonists include (R)-2-amino-5-phosphonopentanoate ((R)-AP5) and (R)-4-(3-phosphonopropyl) piperazine-2-carboxylic acid ((R)-CPP). Available compounds do show some NR2 subunit selectivity but currently there are no truly selective compounds that are competitive antagonists. Non-competitive antagonists act by occluding the pore of the ion channel. These compounds require prior receptor activation (pore opening). All are positively charged and act in a voltage dependent manner. Examples include

dizolcipine (MK-801) and clinically used compounds such as ketamine, memantine and amantidine (see Paoletti and Neyton 2007 for review (320)).

3.1.4 NR2 subunit selective compounds (Table 9)

Ifenprodil (NR2B) and its derivatives have NMDAR subtype selectivity. They are voltage dependent and non-competitive. Traxoprodil (CP 101,606) has been used in clinical trials of ischaemic brain injury but there are no published studies of its use in chronic pain states (321-323). The clinically available drug haloperidol is an NR2B selective antagonist but there is very little research in terms of an analgesic adjunct, with no trials in the treatment or prevention of neuropathic pain (324).

Selective NR2B antagonists have been shown to reverse neuropathic pain behaviour (132).

Table 9 Examples of NMDAR and NMDAR subtype specific antagonists

Compound	Action	Reference
(R) – AP5	Competitive NMDAR antagonist Poor subunit selectivity	Olverman et al 1984 (325)
(R) - CPP	Competitive NMDAR antagonist Poor subunit selectivity	Davies et al 1986 (326)
MK-801	Non-competitive ion channel blocker	Wong et al. 1986 (327)
Ketamine	Non-competitive ion channel blocker	Hirota and Lambert 1996 (148)
Memantine	Non-competitive ion channel blocker	Parsons et al. 1999 (156)
Ifenprodil	NR2B selective antagonist (non-competitive)	Williams et al. 1993 (328)
Ro 25-6891	NR2B selective antagonist (non-competitive)	Grimwood et al. 2000 (329)
CP 101,606	NR2B selective antagonist (non-competitive)	Brimecombe et al. 1997 (330)
Zinc	NR2A selective antagonist	Erreger and Traynelis 2008 (318)
NVP AAM077	NR2A selective antagonist	Wu et al. 2007 (319)

3.1.5 NMDAR antagonist in animal models of pain

In rodent neuropathic pain models, spinally administered NMDAR antagonists are effective in reducing mechanical allodynia (135;136) and reducing spinal neuronal sensitisation (137). Evaluation of the effectiveness of the pre-emptive NMDA receptor antagonists in preventing sensitisation following nerve injury has been

hampered since different sensory tests were assessed in different animal models. As discussed in chapter 1, administration of NMDA receptors antagonists (memantine, MK-801 or ketamine) for 3-8 days from chronic constriction injury (CCI), reduced development of thermal hyperalgesia (138-141). Similar observations have been made for mechanical hyperalgesia in the spinal nerve ligation (SNL) model (142;143). Single bolus administration of intrathecal or systemic ketamine prior to nerve injury attenuates mechanical allodynia from 6 h to 2 weeks (143;144), or delays its development for 3 days in the CCI model (145). The timing of the administration might also be important, attenuation of mechanical allodynia only being observed with pre-, not post-operative MK-801 (146).

The primary aim of this study was to clearly define the effects of pre-treatment with NMDAR antagonists on the development of behavioural signs of neuropathic pain following nerve injury (mechanical allodynia, cold allodynia and thermal hyperalgesia). , Secondly, the effect of nerve injury and nerve injury, modified by NMDAR antagonist pre-treatment, on spinal NMDA subunit expression was investigated. Finally the pharmacological properties of residual mechanical and cold allodynia that remained after pre-emptive treatment with NMDAR antagonists were investigated.

3.2 Materials and Methods

3.2.1 Partial chronic constriction injury to the sciatic nerve

Experiments were carried out in accordance with the U.K. Animals (Scientific Procedures) Act 1986 and followed IASP guidelines. Adult male Wistar rats (200-350g, Charles River, U.K.) were anaesthetised with intraperitoneal (i.p.) sodium pentobarbital (0.06ml/100g, Rhône Merieux, U.K.), supplemented with halothane/O₂ (Astra-Zeneca, U.K.). Under aseptic conditions, the right sciatic nerve was exposed proximal to the trifurcation and four 4/0 chromic gut ligatures (Ethicon, Edinburgh, UK) were tied to loosely constrict the tibial and peroneal nerves, but leaving the smallest, sural nerve intact (modification of the chronic constriction injury model (CCI))(118).

3.2.2 Testing of behavioural reflex responses to mechanical, thermal and cold stimuli

In order to ensure an accurate baseline, animals were exposed to the behavioural tests for several days prior to surgery. This ensured that the animals were habituated to the testing. Pre-surgical testing continued until there was no day to day variation in the values measured. Testing recommenced on day 5 following surgery and continued every two or three days until evidence of recovery was seen. In the experiments involving intrathecal injections, only nerve-injured animals that showed clear signs of thermal hyperalgesia, mechanical and cold allodynia were used for further study. Behavioural tests were carried out using quantified, sensory stimuli delivered to the mid-plantar glabrous surface of the hind paw. Reflex behavioural tests were performed to measure mechanical allodynia, thermal hyperalgesia and cold allodynia. The tests are described in detail below.

Mechanical allodynia was measured as the threshold for paw withdrawal in response to a graded mechanical stimuli applied to the mid-plantar glabrous surface of the hind paw using calibrated von Frey filaments (Stoelting, Wood Dale, IL). Threshold was defined as the pressure (force per unit area) that caused foot withdrawal five times in every ten applications, repeated at 1-2 second intervals. The

pressure applied to the hind paw by the von Frey filaments is calibrated as force (milliNewtons) divided by the area over which it is applied (millimetres squared).

Thermal hyperalgesia was monitored using noxious radiant heat (30-55°C; Hargreaves' thermal device, Linton Instruments, Diss UK) applied to the mid-plantar glabrous surface of the hind paw. The withdrawal response latency was characterised as a brief paw flick recorded to the nearest 0.1 sec; a standard cut-off latency of 20 seconds prevented tissue damage.

Cold allodynia was measured by placing the rats in a perspex box with an elevated aluminium floor covered with iced water, sufficient to immerse both glabrous and hairy skin of the hind paw (3-4°C). Once placed in the box, rats were allowed 10 seconds to acclimatise. The number of seconds the animal raises its hind paw above the water over a 20 second period was recorded. This was repeated four times at 10 minute intervals to establish a mean suspended paw elevation time (SPET) for each rat.

3.2.3 Statistical analysis

Mean behavioural data were analysed by SigmaStat software (version 2.03). In tests of thermal hyperalgesia and cold allodynia, the ipsilateral value was compared to the contralateral value at each time point using a paired Student's t-test then each compared to pre-surgery or pre-drug baseline using a one-way repeated measures ANOVA, with post-hoc analysis using Dunnett's test. The ANOVA test assumes normal distribution and identical variances but possible different means. It can be used to compare means of different groups. Dunnett's test is used in situations where the all groups are referred to a reference group and is used to identify which groups differ from the reference group (in this case baseline data). In tests of mechanical allodynia, the ipsilateral value was compared to the contralateral value at each time point using a Wilcoxon signed rank test and each compared to baseline using a repeated measures ANOVA on ranks, with post-hoc analysis using Dunn's test. These were used for the reasons above but assume non-parametric distribution.

3.2.4 Dose estimation for memantine and ketamine.

Memantine and ketamine are both clinically available uncompetitive antagonists which act by occluding the channel of the pore. Three studies using a rat model of pain and using pre-treatment with memantine were identified (136;141;143). The studies varied in design and technique of drug administration. Animals showed side-effects at doses $\geq 20\text{mg/Kg}$ (i.p.). Eisenberg et al used 15mg/Kg i.p. without side-effects, while Carlton and Hargett noted increased motor activity at 20mg/Kg (i.p.) (141;143). 15mg/Kg was chosen as the maximum dose that would be unlikely to produce side-effects in the animals. Side-effects (by behavioural observation) were not seen in animals at this dose. Two studies used a single dose of preemptive ketamine at 1mg/Kg in a neuropathic model of pain (144;145). One study used a continuous intrathecal infusion in a rat model of visceral and somatic pain, although not a nerve injury model (331). The dose used was $250\mu\text{g/Kg/hour}$. No study discussed side-effects. A dose of $150\mu\text{g/Kg/hour}$ was chosen, giving a dose of 3.6mg/Kg over 24 hours. No side-effects (by behavioural observation) were seen at these doses.

3.2.5 Pretreatment protocol for NMDAR antagonists

Memantine (15mg/kg , 0.5 ml , $n=10$; Sigma-RBI, Poole, Dorset, UK), Ro 25-6891 (10mg/Kg in 0.5ml saline with 45% hydroxypropyl-beta-cyclodextrin, $n=8^*$; Sigma-RBI and Tocris, Bristol, UK) or vehicle (0.9% saline, 0.5 ml , $n=10$) was administered by intraperitoneal (i.p.) injection immediately prior to surgery and repeated at the end of the first day and then twice a day for two days following surgery. Previous experiments showed no discernible effect of the vehicle used with Ro 25-6891 on nociceptive reflex responses.

Continuous lumbar intrathecal infusion of ketamine (Sigma-RBI, Poole, Dorset, UK), was provided by an implanted osmotic minipump (Alzet, $0.25\ \mu\text{l}$ per hour, total capacity 28 days, Alza Corporation, Palo Alto, CA, USA). Ketamine was dissolved in sterile 0.9% saline, pH 7.4, to give an infusion rate of $150\ \mu\text{g/kg/hour}$ over 3 days.

* Pre-treatment experiments using Ro 25-6891 were performed by Dr Garry. Included with permission.

The day 3 volume of ketamine solution in the cannula was separated by a small volume of paraffin from saline filling the remainder of the cannula connected to the minipump. In this way, the infusion was switched from ketamine to saline after 3 days. The minipump was inserted under sterile conditions. Anaesthesia was provided by i.p. sodium pentobarbital (0.06ml/100g, Rhône Merieux, U.K.), supplemented with halothane in oxygen (Astra-Zeneca, U.K.). The minipump was placed intramuscularly at an interscapular site. The catheter was threaded through muscle close to the exposed region of the spinal column and directed caudally. A small area of the muscle and vertebral bone was cleared from two dorsal thoracic segments (T10-T12), and the tip of the cannula was placed through a small incision, under the dura, and eased down the dorsal spinal cord by a premeasured distance to lie in the region of the lumbar segments L3-L6. Only data from animals with L3-6 cannula placement (ascertained at the end of the experiment) and showing normal gait were used in analysis.

3.2.6 Intrathecal injection NMDAR antagonists

All intrathecal injection experiments were carried out on CCI animals, 16 days following surgery at peak behavioural change. These animals had received pre-treatments with either vehicle (n=8), memantine (n=10) or the NMDA NR2B subunit selective antagonist, Ro 25-6981 (n=8) just prior to nerve injury and for 3 days after. Baseline measurements for thermal hyperalgesia, mechanical and cold allodynia were recorded both ipsilateral and contralateral to nerve injury over 2 hours prior to injection and mean values calculated. The effects of intrathecal administration of the highly selective NMDA R antagonist, 3-(2-carboxypiperazin-4-yl) propyl-1-phosphonic acid, ((R)-CPP), memantine, and the NMDA NR2B subunit selective antagonists, Ro 25-6981 and ifenprodil, or vehicle (saline) were examined on behavioural reflex responses.

Rats were briefly anaesthetised with halothane/O₂ and injected intrathecally at the L4-5 spinal segments with either (R)-CPP (1, 3 or 10nmol, where 10nmol was used as the standard dose in all figures; Tocris), memantine (150 nmol; Sigma RBI), Ro 25-6981, (50 nmol; Tocris) or ifenprodil (25nmol; Sigma RBI), all in 50µl saline,

using a 25 guage needle microsyringe. The vehicle for ifenprodil was 0.3% dimethylformamide in saline (previous experiments have shown that this has no effect on ipsilateral and contralateral nociceptive reflexes, following nerve injury (250)). Behavioural reflex testing began 20 minutes post-injection (following recovery from anaesthetic) and was carried out until recovery to pre-injection values was observed.

In the extensive tests comparing the acute sensitivity of mechanical allodynia to NMDA R antagonists in control or pre-treated rats, the percentage reversal of the pre-drug ipsilateral: contralateral difference in responses was calculated for each time point following drug injection. Statistical significance of differences in these values between control and pre-treated rats was assessed by Wilcoxon test.

3.2.7 Assessment of NMDA receptor subunit expression levels by Western immunoblotting

Western blotting was carried out to assess NMDA receptor subunit expression in the lumbar spinal cord of sham operated or naïve animals (n=6 in each case). At peak behavioural change (day 16) and while under deep anaesthesia (halothane/O₂), lumbar spinal cord segments (L3-6) were removed and quickly homogenised in ice-cold buffer containing 5% glycerol with peptidase and phosphatase inhibitors and a membrane fraction was solubilized in 1% deoxycholate.

The blot experiments were all carried by Dr Emer Garry. The methodology for these is described in chapter 2. I am very grateful to Dr Garry for her assistance.

3.2.8 Immunohistochemical studies on NMDA receptor subunit expression

Immunohistochemistry was carried out to assess the expression and localisation of NR1 and NR2B NMDA R subunits in the lumbar spinal cord following memantine pre-treatment

Rats with either vehicle (n=3) or memantine (n=3) pretreatment (at the time of surgery for the partial CCI injury) were studied at peak behavioural change (16 days

later). Under deep anaesthesia (Pentobarbital (Sagatal) 60mg/kg, i.p.), rats were perfused transcardially with saline containing 100 units heparin/ml, followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Lumbar spinal segments (L3-6) were post-fixed in 4% paraformaldehyde/0.1M phosphate buffer for 4-5 hr at 4°C, before being stored in 0.1M phosphate buffer at 4°C. The fixed specimens were then mounted in liquid wax, which was allowed to harden. The sections of spinal cord were then sliced using a microtome. Slices of 70 µm were made and placed in 0.1M phosphate buffer prior to preparation.

The methodology used in the immunohistochemical studies is described in chapter 2. I am very grateful to Heather Anderson (Centre for Neuroscience Research, University of Edinburgh) who prepared the slides for analysis.

3.3 Results

3.3.1 Neuropathic behavioural hyperalgesia and allodynia

Neuropathic sensitisation of behavioural reflexes, characteristic of the CCI model, began to appear at 7-9 days following surgery, with maximal changes in thermal hyperalgesia, cold allodynia and mechanical allodynia being observed at around 16 days (followed by plateau) in vehicle-treated animals (Figs 1a & c; 2a & c; 3a & c). No consistent changes were detected contralateral to nerve injury. In each case the changes recovered to baseline levels by 43 days post treatment. Rats that had undergone sham surgery, showed no change from baseline.

3.3.2 Effect of NMDAR pre-treatment on behavioural responses following CCI

Pre-treatment with non-competitive NMDA receptor ion channel blockers, memantine or ketamine completely prevented the reduction in paw withdrawal latency to noxious heat that occurs ipsilateral to nerve injury in saline-treated CCI rats (Figure 1).

Cold allodynia responses which are observed only in neuropathic animals, ipsilateral to nerve injury, were significantly reduced after both memantine and ketamine treatment to approximately half the magnitude of those in vehicle-treated animals through the first 30 days following CCI (Figure 2).

In contrast, memantine or ketamine pretreatment caused little alteration in the reduced paw withdrawal threshold to von Frey filaments that occurs ipsilateral to nerve injury (Figure 3).

Interestingly, pre-treatment with the selective NR2B-selective antagonist, Ro 25-6891, given systemically over 3 days, beginning immediately prior to exposure of the sciatic nerve, also showed similar effects to memantine and ketamine, with the main residual sensitisation being mechanical allodynia.

Figure 1. The effect of memantine and ketamine pre-treatment on the development of thermal hyperalgesia

Data shows the mean \pm SEM responses before (time zero) and following the induction of CCI for both ipsilateral and contralateral hindlimbs (n=10).

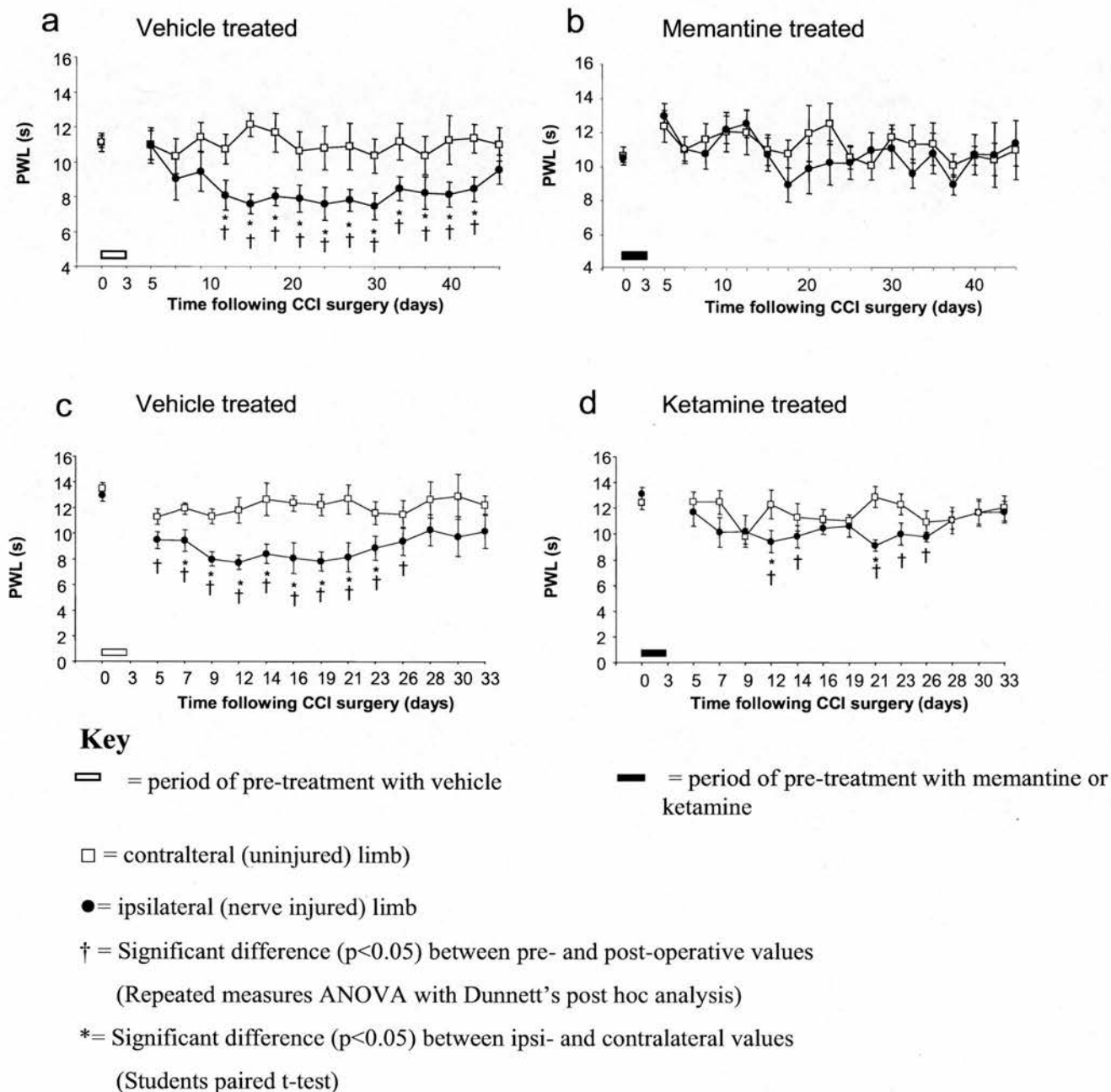


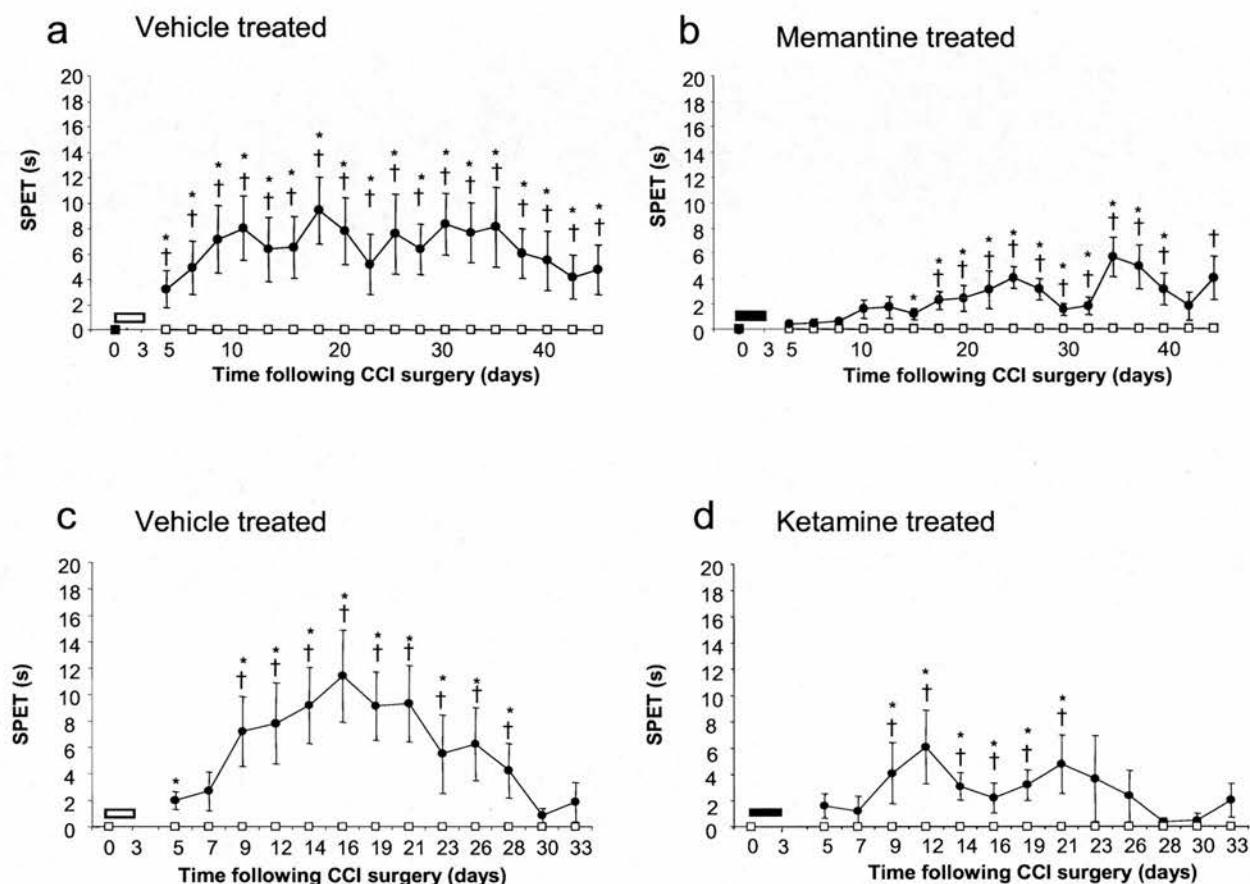
Figure 1 shows the effect of pre-treatment with both intraperitoneal memantine (a & b) and intrathecal ketamine (c & d) on the paw withdrawal latency (PWL) to a thermal stimulus.

1a and c show the development of thermal hyperalgesia following CCI in the vehicle treated animals

1b and d shows the attenuation of the development of thermal hyperalgesia caused by pre-treatment with a NMDA antagonist (Memantine (b) or ketamine (d)).

Figure 2. The effect of memantine and ketamine pre-treatment on the suspended paw elevation time (SPET) in seconds (response to cold (4°C))

Data shows the mean \pm SEM responses before (time zero) and following the induction of CCI for both ipsilateral and contralateral hindlimbs (n=10).



Key

□ = period of pre-treatment with vehicle

■ = period of pre-treatment with memantine or ketamine

□ = contralateral (uninjured) limb

● = ipsilateral (nerve injured) limb

† = Significant difference ($p < 0.05$) between pre- and post-operative values

(Repeated measures ANOVA with Dunnett's post hoc analysis)

* = Significant difference ($p < 0.05$) between ipsi- and contralateral values

(Students paired t-test)

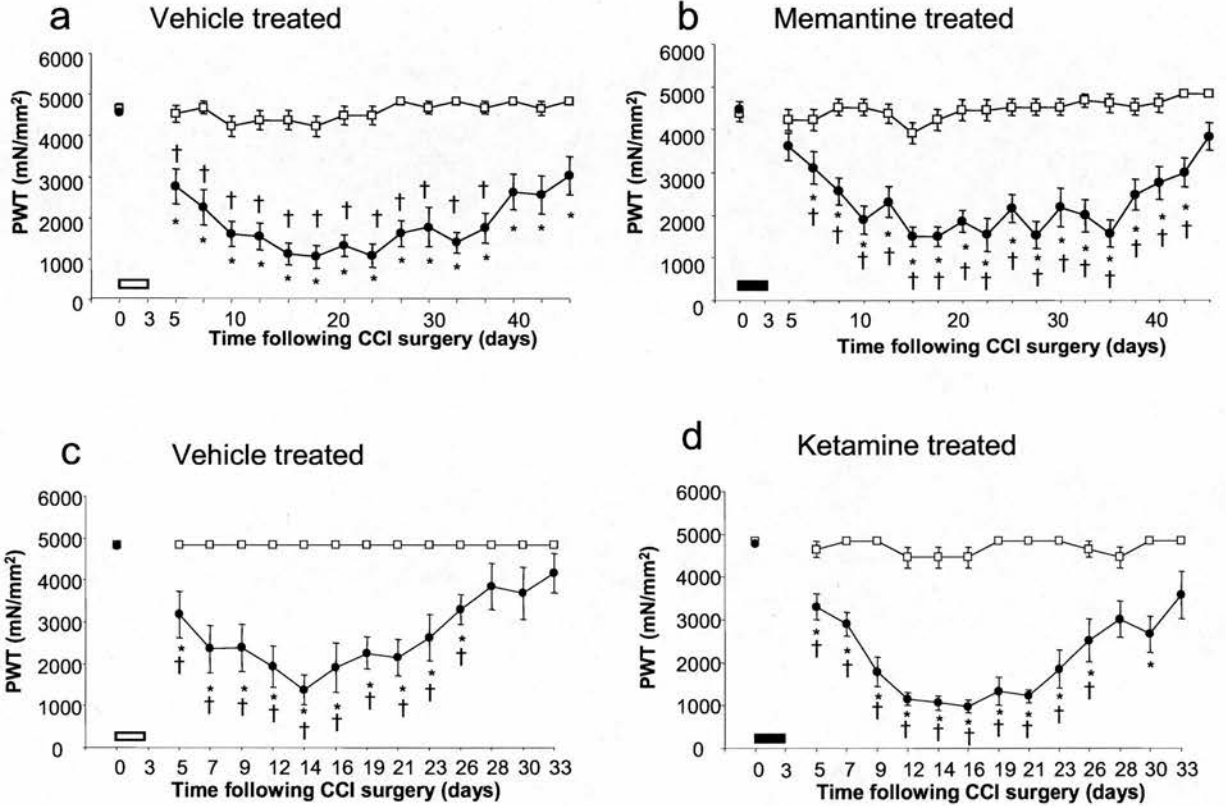
Figure 2 the suspended paw elevation time (SPET, number of lifts) in response to a cold (4°C) stimulus

2 a & c shows the SPET to cold following CCI in the vehicle treated animals

2b & d shows the effects on the SPET caused by pre-treatment with a NMDA antagonist (Memantine (b) or ketamine (d)).

Figure 3 The effect of memantine and ketamine pre-treatment on paw withdrawal thresholds to Von Frey filaments (PWT, mN/mm³).

Data shows the mean \pm SEM responses before (time zero) and following the induction of CCI for both ipsilateral and contralateral hindlimbs (n=10).



Key

☐ = period of pre-treatment with vehicle

▬ = period of pre-treatment with memantine or ketamine

□ = contralateral (uninjured) limb

● = ipsilateral (nerve injured) limb

† = Significant difference (p<0.05) between pre- and post-operative values

(Repeated measures ANOVA on ranks, Friedmanns test with Dunn's post hoc analysis)

* = Significant difference (p<0.05) between ipsi- and contralateral values

(Wilcoxon test)

Figure 3 shows the development of mechanical allodynia as measured by PWT (mN/mm³) following CCI

3 a & c shows PWT following CCI in the vehicle treated animals

3b & d shows PWT following pre-treatment with a NMDA antagonist (Memantine (b) or ketamine (d)).

It can be seen that there is no discernable difference between groups.

3.3.3 Effects of subsequent intrathecal NMDAR and NMDA NR2B selective antagonists in pre-treated animals

Although pre-treatment with the NMDAR antagonists blocks the development of thermal hyperalgesia, it is unknown whether any residual sensitisation, such as cold allodynia, displays altered susceptibility to pharmacological interventions using acute spinally administered NMDAR antagonists.

Here we assessed whether there were any alterations in the subsequent sensitivity to NMDAR antagonists with particular interest in the NR2B subunit, since selective antagonists exert marked analgesic effects on sensitised behaviours following CCI. The effects of memantine pre-treatment on subsequent sensitivity of mechanical allodynia to acute challenge with different types of NMDAR antagonist (either the general NMDAR antagonists, (R)-CPP, memantine, or the NR2B sub-unit-selective antagonists, Ro 25-6891 or ifenprodil) can be seen on Figs. 4a, b and 5a, b.

Although the degree of mechanical allodynia was essentially unaffected by memantine pre-treatment, there was a significantly greater extent and/or duration of reversal of mechanical allodynia by acute intrathecal administration of (R)-CPP, memantine, ifenprodil or Ro 25-6891. In vehicle pre-treated rats acutely administered (R)-CPP, memantine and ifenprodil caused significant attenuation of ipsilateral allodynia and the extent of this reversal was significantly greater in memantine pre-treated rats (Fig. 4a, b). Facilitation of the acute reversal of allodynia following memantine pre-treatment was seen with (R)-CPP doses of 10 nmol (Fig. 4) and also 3 nmol, but not 1 nmol (data not shown). Ro 25-6891 did not cause significant attenuation of ipsilateral allodynia in vehicle pre-treated rats, but did in memantine pre-treated animals. This reversal was sustained for up to 65 min following injection (Fig. 5a) such that the Ro 25-6891 –induced reversal of allodynia in the 35-50 min period of memantine pre-treated rats was significantly greater than controls. None of the acutely administered drugs had any significant effect on contralateral responses. Acute intrathecal injection of saline vehicle, in both groups of rats, had no discernible effect on behavioural reflexes. It was not possible to test thermal hyperalgesia as this was effectively absent following memantine pre-treatment.

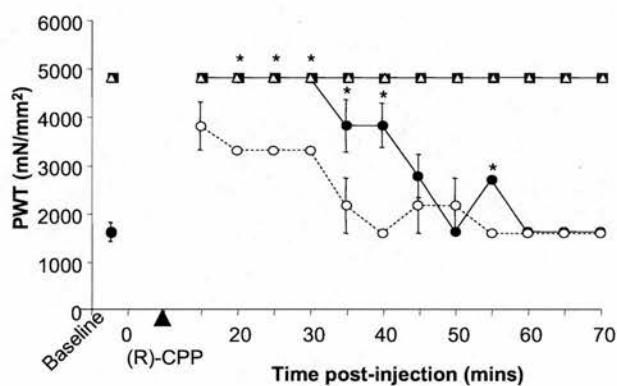
Ro 25-6891 also inhibited cold allodynia with the partially reduced responses (SPET scores) after memantine pre-treatment being significantly inhibited for 80 min by acute Ro 25-6891 injection, whereas in vehicle pre-treated controls there was no significant effect of Ro 25-6891 beyond 50 min (Fig 5b). Contralateral SPET scores were zero in every case. It is notable that the 2B-selective agent can substitute effectively for memantine in the pre-treatment paradigm with subsequent reversal of CCI-induced mechanical allodynia by acute (R)-CPP or ifenprodil, both being significantly facilitated following the pre-emptive treatment (Fig. 5c).

Figure 4.

The effect of memantine pre-treatment on subsequent sensitivity of allodynia to different types of NMDA receptor antagonist.

Data is all shown as mean \pm SEM
(Legend overleaf)

a



b

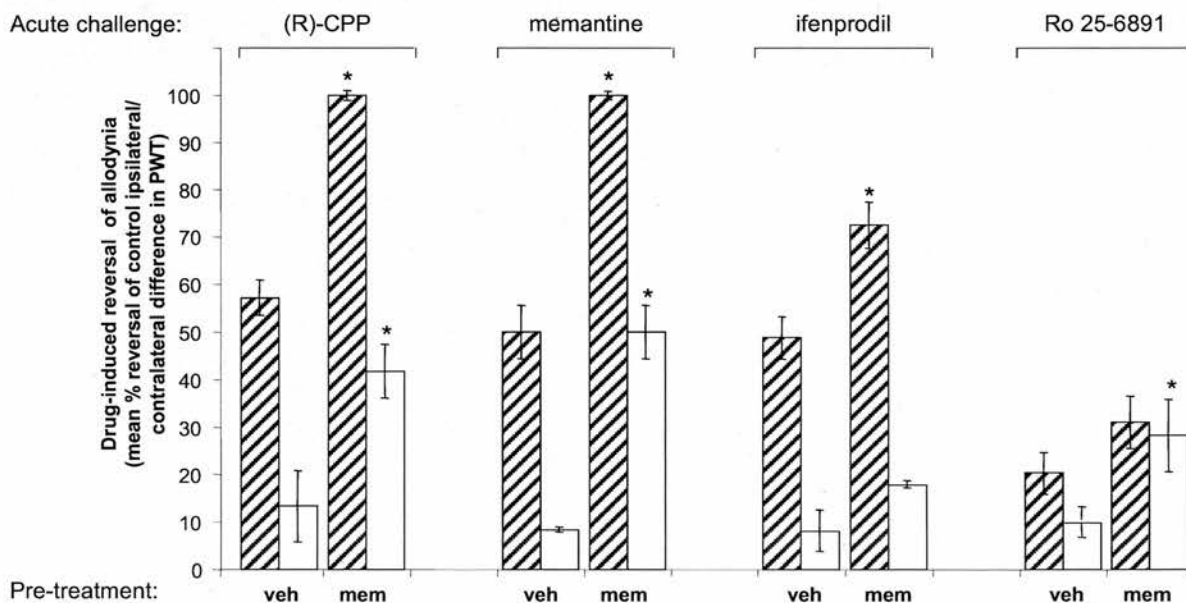


Figure 4. (previous page)

The effect of memantine pre-treatment on subsequent sensitivity of allodynia to different types of NMDA receptor antagonist.

Data in (a) and (b) reveal differences in the acute, spinally administered drug-induced reversal of mechanical allodynia (expressed as mean percentage reversal of the ipsilateral/contralateral difference in paw withdrawal threshold; PWT \pm SEM, n=8) in vehicle or memantine pre-treated animals. (a) shows enhanced reversal by (R)-CPP of mechanical allodynia ipsilateral to injury following following memantine pre-treatment compared to vehicle pre-treatment in nerve injured animals. *indicates statistically significant differences between control and memantine pre-treated animals in the extent of allodynia reversed by (R)-CPP, $p < 0.05$ by Wilcoxon test. There were no detectable effects of (R)-CPP on the contralateral hindlimb mechanical PWT. (b) Compares the effect of NMDA R antagonists, (R)-CPP, memantine and NR2B subunit-selective antagonists, Ro 25-6891 or ifenprodil on residual mechanical allodynia (either 15-30 min (stripped bars) or 35-50 minutes (white bars) following intrathecal injection of drug), following either vehicle (veh) or memantine (mem) pre-treatment. The residual mechanical allodynia was more sensitive to acute injection of NMDA R antagonists in memantine pre-treated animals. Following memantine pre-treatment all drugs showed significantly enhanced extent or duration of reversal of mechanical allodynia (expressed as mean percentage reversal of the control ipsilateral/contralateral difference in paw withdrawal threshold (PWT) \pm SEM) compared to vehicle pre-treatment (n=8 in each case). *represents a significant ipsilateral to contralateral difference at each time point, $p < 0.05$ by Wilcoxon test.

Figure 5.

A highly selective NR2B antagonist elicits inhibition of both mechanical and cold allodynia after memantine pre-treatment and can effectively substitute for memantine in the pre-treatment paradigm.

Data is all shown as mean \pm SEM

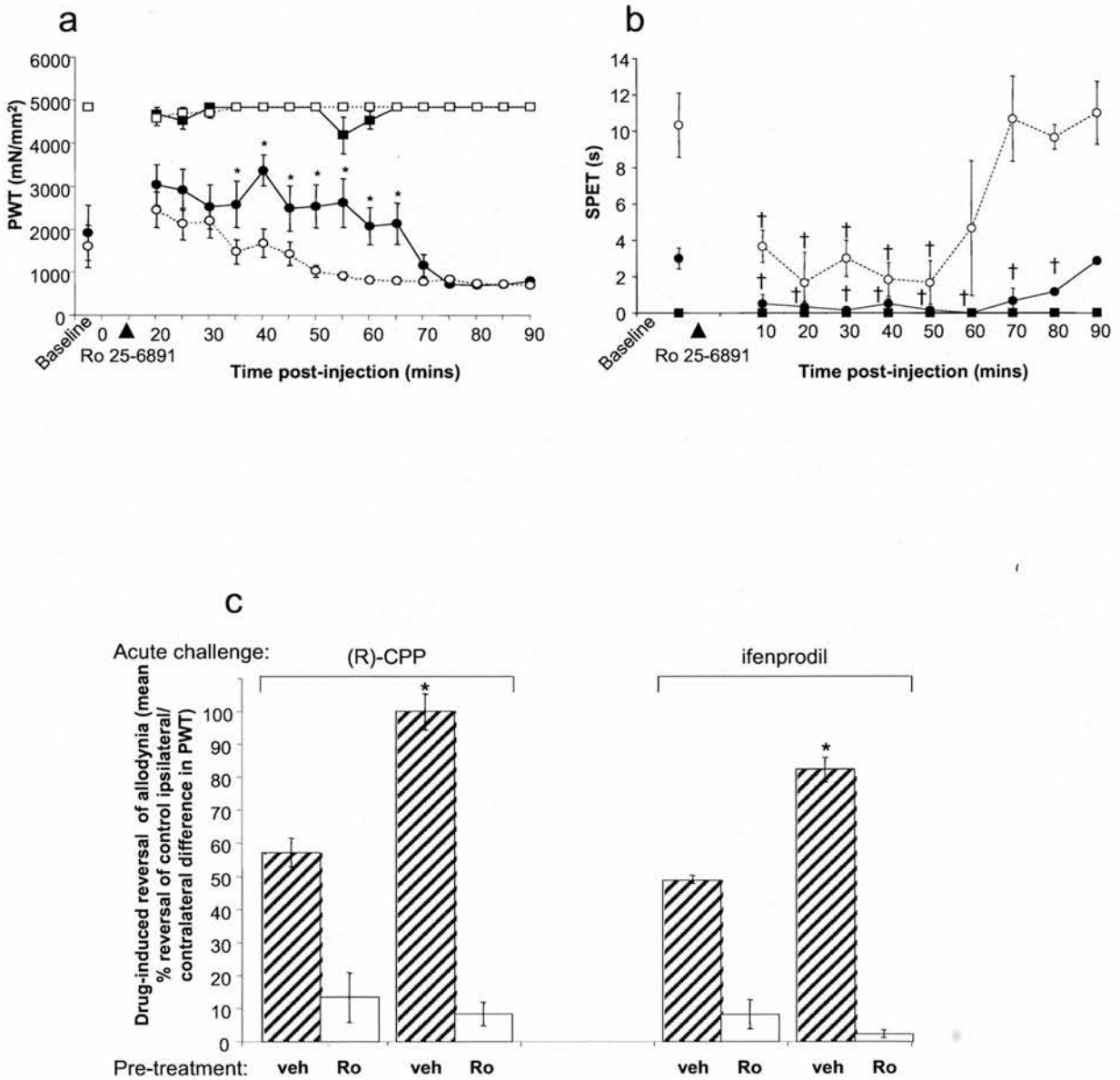


Figure 5. (previous page)

A highly selective NR2B antagonist elicits inhibition of both mechanical and cold allodynia after memantine pre-treatment and can effectively substitute for memantine in the pre-treatment paradigm.

Data in (a) are presented as mean paw withdrawal threshold (PWT) responses to graded mechanical (von Frey filaments). In (a), memantine pre-treated animals showed a marked prolongation of the Ro 25-6891-induced reversal of ipsilateral mechanical allodynia (●) compared to vehicle treated animals (○). Statistically significant differences between memantine pre-treatment and vehicle pre-treated of reversal of allodynia are shown as * $p < 0.05$ (by Wilcoxon test). In (b), memantine pre-treated animals (●) showed a clear prolongation (up to 30min longer) of the Ro 25-6891-induced reversal of cold allodynia, compared to vehicle-treated animals (○; $n=8$). Statistically significant differences between ipsilateral pre-drug and post-drug values are shown, † $p < 0.05$, repeated measures ANOVA with Dunnett's post analysis. (c) When Ro 25-6891 (Ro) was substituted as a pre-treatment instead of memantine, acute intrathecal administration of (R)-CPP of infenprodil caused enhanced reversal of mechanical allodynia, ipsilateral to nerve injury (expressed as mean percentage reversal of the control ipsilateral/contralateral difference in paw withdrawal threshold (($PWT \pm SEM$, $n=8$) compared to vehicle (veh) pre-treated animals. The striped and white bars show mean data from 15-30 min and 35-50 min periods following drug injection respectively. *represents a significant ipsilateral to contralateral difference at each time point, $p < 0.05$ by Wilcoxon test.

3.3.4 Effects of NMDAR antagonist pre-treatment on NMDAR receptor subunit expression in the spinal dorsal horn.

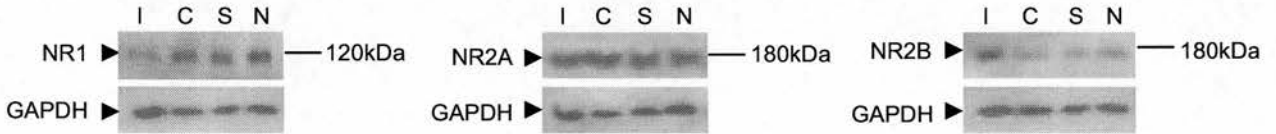
In animals 16 days after nerve injury, at peak behavioural sensitisation, or in sham operated or naïve animals, the levels of NR1 and NR2 subunit expression were examined by Western immunoblotting (Fig. 6) and their localisation was examined by immunohistochemistry (Fig.7). In Fig. 6 the levels of NR1, NR2A and NR2B immunoreactivity in L3-6 spinal cord were compared between animals ipsilateral/contralateral to partial CCI, sham surgery or naïve controls. Expression levels were compared (on the same blots) with those of the ubiquitous housekeeping enzyme GAPDH, which undergoes no discernable change in expression following a wide range of afferent manipulations. Figure 6 shows examples of immunoblots showing a reduction in NR1 subunit expression ipsilateral to CCI, no change in NR2A, and an increase in NR2B subunit expression. These results were typical of at least six separate experiments. Quantitative densitometry and image analysis relative to GAPDH expression were used to calculate values for the relative changes in NR1 and NR2B expression levels (Figure 6). Immunohistochemistry and subsequent image analysis of grey scale densities confirmed that NR1 expression was decreased in spinal dorsal horn and further that this change took place selectively in ipsilateral laminae I-II (but not laminae III or ventral horn) in CCI animals pretreated with only vehicle (Figure 7a, c, d, e). This reduction in NR1 expression represented a change of -7.3 ± 0.96 grey scale units ipsilateral to CCI compared to the contralateral side, which had a grey scale score of 28.2 ± 1.54 ; mean \pm SEM; one ROI per side; n=45 sections (Figures 7 a, c). In laminae I-II of naïve animals, the mean ipsilateral:contralateral difference was 0.0 ± 0.52 grey scale units. In contrast, in memantine pretreated animals there was a relative increase in laminae I-II NR1 immunoreactivity ipsilateral to CCI ($+7.1 \pm 2.2$ grey scale units ipsilateral to CCI compared to the contralateral side of 23.6 ± 1.61 ; mean \pm SEM). Both of these ipsilateral/contralateral differences in NR1 immunoreactivity in dorsal horn laminae I/II were statistically significant ($p < 0.05$; Student's t-test). There was no change in laminae III or ventral horn NR1 immunoreactivity (Figure 7d, e). Equivalent analysis of NR2B immunoreactivity (Figure 7b-e) showed a relative increase in NR2B immunoreactivity on the ipsilateral side relative to the contralateral side in vehicle-

pretreated CCI rats. In laminae I-II the increment in NR2B immunoreactivity represented $+9.6 \pm 1.3$ grey scale units ipsilateral to CCI compared to the contralateral side, which had a grey scale score of 16.5 ± 1.25 ; mean \pm SEM; Figures 7b, c, $p < 0.05$, Student's t-test. Corresponding values for ipsilateral/contralateral differences from naïve rats were 0.61 ± 0.85 (not significant). In CCI animals that had been pre-treated with memantine, NR2B expression appeared similar in laminae I-II of both dorsal horns (with an excess grey scale score of only 0.6 ± 1.2 units ipsilaterally compared to a contralateral score of 17.1 ± 1.45 ; mean \pm SEM; Figures 7c).

NR2B immunoreactivity was also increased in laminae III ipsilateral compared to the contralateral side in vehicle-pre-treated CCI rats (Figure 7d). The increment was $+8.3 \pm 1.4$ grey scale units above the contralateral side, which had a grey scale score of 19.4 ± 1.3 , mean \pm SEM, ($p < 0.05$, Student's t-test). However, in contrast to the results in more superficial laminae, memantine-pretreatment appeared to have little effect (Figure 7d). Ipsilateral NR2B expression in lamina III of memantine pretreated CCI rats was 24.1 ± 2.1 grey scale units greater than that on the contralateral side, which had a score of 17.9 ± 1.9 units, mean \pm SEM, ($p > 0.05$, Student's t-test). Ventral horn values for NR2B immunoreactivity were not discernably different ipsilateral/contralateral to CCI, nor were they detectably altered by memantine pretreatment (Figure 7e).

Figure 6.

Assessment of injury induced changes in spinal cord expression of NMDA receptor subunits by immunoblotting.



Expression of protein in the spinal cord as % of GAPDH expression				
	Ipsilateral CCI	Contralateral CCI	Sham	Naïve
NR1	30.7 ± 1.9 *	41.8 ± 1.5	44.3 ± 2.0	40.9 ± 4.0
NR2A	48.6 ± 2.7	51.3 ± 3.1	47.2 ± 1.6	50.0 ± 2.6
NR2B	72.8 ± 5.3 *	47.3 ± 4.2	48.5 ± 3.9	53.8 ± 6.0

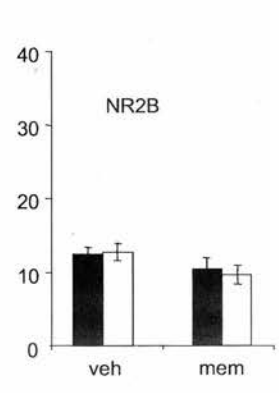
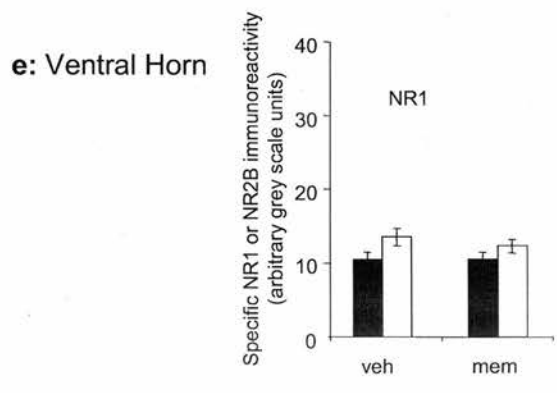
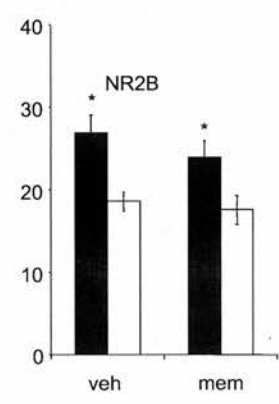
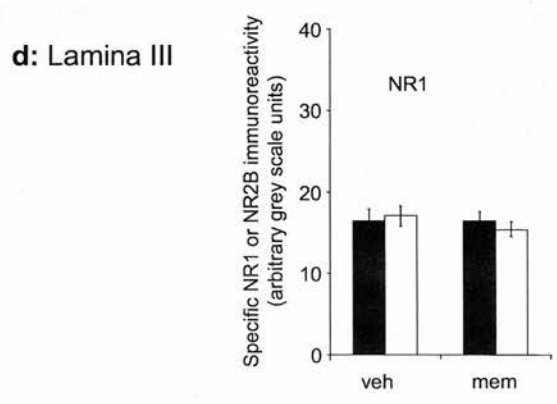
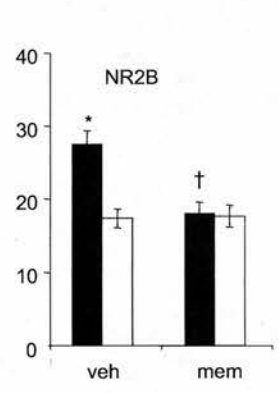
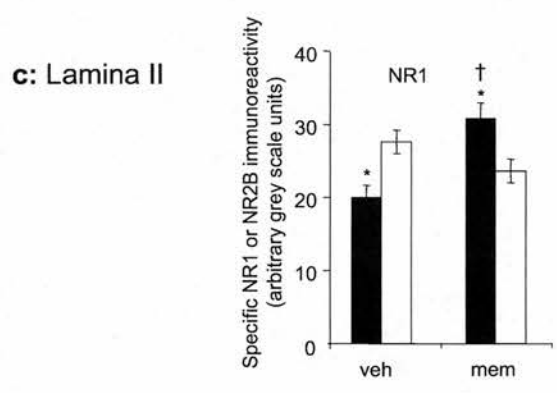
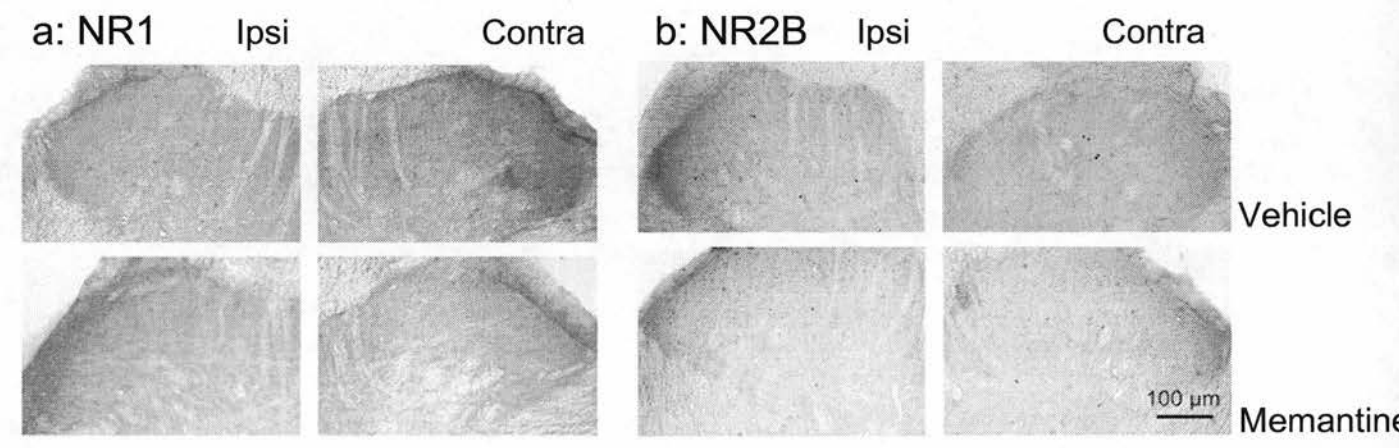
Immunoblots for the levels of NR1, NR2A and NR2B NMDA receptor subunit expression in L3-6 spinal cord from the ipsilateral and contralateral pCCI, as well as sham-operated or naïve rats showed that NR1 subunit expression was reduced and NR2B subunit expression was increased ipsilateral to pCCI. NR2A expression appeared to be unchanged. The table shows mean ± SEM values (n=6-8) derived from quantitative densitometry of co-processed immunoblots from NR1, NR2A and NR2B in the spinal cord following pCCI. Differences in the levels of protein expression (arbitrary grey scale values) given as a percentage of GAPDH levels, were assessed between ipsilateral and contralateral pCCI, sham operated or naïve spinal cord (as shown in blots above the table) and statistically significant changes are shown as *p<0.05 by Wilcoxon test.

Figure 7.

Immunohistochemical localisation and image analysis of NR1 and NR2B NMDA receptor subunit expression in the superficial dorsal horn, following nerve injury and the effects of pre-treatment with memantine.

Typical examples are shown. Of the expression of NR1 (a) or NR2B (b) subunits of the NMDA receptor in the ipsilateral and contralateral dorsal horn of pCCI animals pre-treated with either vehicle (upper panels; n=3) or memantine (lower panels; n=3). In vehicle pre-treated animals (a) shows relatively lower levels of NR1 subunit, ipsilateral to CCI compared to the contralateral side. Memantine pre-treatment however, results in relatively higher levels of NR1 expression ipsilateral to CCI compared to the contralateral side. (b) shows that in contrast to NR1, higher levels of the NR2B subunit are expressed ipsilateral to CCI compared to the contralateral side (white bar) side. Following memantine pre-treatment, this ipsilateral; contralateral difference in the NR2B subunit are no longer apparent. Scale bar represents 100 μm in each panel. In (c-e) data from the immunohistochemistry experiments were analysed by quantitative densitometry and expressed as arbitrary grey scale values (mean \pm SEM) in vehicle- (veh; n=3) and memantine- pre-treated animals (mem; n=3). Relative expression of NR1 subunit (on the left hand side) or NR2B subunit (on the right hand side) is shown for ipsilateral (black bar) or contralateral (white bar). Statistically significant differences between ipsilateral (black bar) and contralateral (white bar) within a treatment group are represented by * $p < 0.05$, Student's matched pair t-test, † $p < 0.05$ represents statistically significant differences between vehicle and memantine pre-treated animals (One-Way ANOVA with Dunnett's post hoc analysis).

Figure 7.
Immunohistochemical localisation and image analysis of NR1 and NR2B NMDA receptor subunit expression in the superficial dorsal horn, following nerve injury and the effects of pre-treatment with memantine.



■ Ipsilateral
 □ Contralateral

3.4 Discussion

Peripheral nerve injury leads to central sensitisation, where increased excitability of the neurones in the spinal cord is thought to underlie chronic hyperalgesia and allodynia (82;332-334). Pre-emptive analgesia attempts to reduce or prevent the spinal plasticity associated with the development of chronic pain and would be of clear clinical value. The spinal NMDAR plays a crucial role in sensitisation, however previous studies investigating the potential of pre-emptive NMDAR antagonist treatment show little direct comparison of relative effectiveness on different sensory modalities of neuropathic pain.

Using a comprehensive range of sensory tests, it was found that pre-emptive NMDAR antagonist treatment over 3 days from nerve injury caused differential effects on the development of neuropathic pain behaviours to different sensory stimuli. There was a virtual abolition of thermal hyperalgesia and marked attenuation of cold related pain behaviours, contrasting with minimal effects on mechanical allodynia in the same animals. Similar results were obtained with pre-emptive use of several different NMDAR antagonists including memantine, ketamine and the NR2B- selective antagonist, Ro 25-6891, and several routes of administration (systemic memantine, Ro 25-6891 or intrathecal ketamine). The marked preventative effect on thermal hyperalgesia is consistent with previous pre-emptive NMDAR antagonists studies in rodents, using chronic administration, where only noxious thermal heat responses were measured following CCI, using either memantine (i.p. for 7 days (141)) or MK801 (i.p. 7-15 days (138;139)). Shorter duration NMDAR antagonist administration (MK801, HA 966, ketamine or dextrorphan, either i.p. or i.t for 4 days), yielded only partial attenuation of thermal hyperalgesia (139;140;335).

The effects of pre-emptive NMDAR antagonists on the degree and duration of mechanical allodynia in previous studies have been more variable. Single dose intrathecal ketamine pre-treatment at CCI surgery delayed mechanical allodynia until post-surgery day 3 (145), although prolonged intrathecal ketamine (144) or

intravenous MK-801 (146) administration gave attenuation up to 14 days after surgery in the SNL model. MK-801 administration for 8 days (subcutaneously) following CCI was reported to attenuate mechanical allodynia at 27 days (142), although 8 days of memantine treatment (i.p.) following SNL attenuated mechanical allodynia for only 3 days (143). Our results establish that the sensitisation of different sensory modalities is differentially affected by early administration of NMDAR antagonists following nerve injury.

This study showed that pre-emptive systemic or intrathecal administration of NMDAR antagonists attenuated the development of thermal hyperalgesia to a greater extent than that of mechanical allodynia. A similar profile of mechanical allodynia resistant to pre-treatment with NMDA receptor antagonists has been reported in a neuropathic pain model involving transient cryoneurolysis injury to the peripheral nerve (336). It has also been found that acutely administered NMDA antagonists reversed thermal hyperalgesia, but not mechanical allodynia following CCI (337) or nerve root ligation (338). There is contradictory evidence with some rodent studies describing robust reversal of thermal hyperalgesia and mechanical allodynia by NMDAR antagonists (129;135;136).

The reason for this differential between thermal and mechanical stimuli is not clear, but may reflect the fibres and the pathways involved in these sensitivities and perhaps the model used. CCI results in extensive demyelination of the nerve with a preferential loss of large myelinated axons which is associated with heat hyperalgesia (118;339). Spontaneous activity, with lower response threshold has been shown to occur in both C-fibres and A δ -fibres following nerve injury (340). The afferent fibres responsive to heat stimulus were all C-fibres, which may be relatively spared (340). In addition, there is evidence that mechanical allodynia may in fact be mediated by ascending spinal dorsal column projection and not in the dorsal horn, the proposed site action on the NMDAR antagonists (92). The specific channels involved in noxious heat might also be relevant.

TRPV1 is a sensory neuron specific channel sensitive to noxious heat (341). TRPV1 primary afferents use glutamate as a neurotransmitter and target post synaptic NMDA/NK-1 receptors (342). In nerve injury models of neuropathic pain TRPV1

expression rises in fibres with undamaged sensory connections, while decreasing in damaged axons (343). Using a partial nerve injury model will result in intact fibres and an increase in TRPV1 which may account for an increased sensitivity to noxious heat.

Capsaicin sensitive primary afferents (CSPAs) express TRPV1 and are involved in central sensitisation. Spinal NR1 is up regulated in response to nerve injury such as CCI. Depletion of CSPAs prevents NR1 upregulation and importantly prevents thermal hyperalgesia but not mechanical allodynia (344)

This study showed for the first time that the expression of NR1 and NR2 R subtypes in the spinal dorsal horn changed radically and differentially over the time of development of neuropathic pain behaviours following CCI and that this was prevented by NMDAR antagonist pre-treatment. Novel evidence is presented showing that the sensitivity of CCI-induced mechanical allodynia to acute, intrathecal of NMDAR antagonists was greater or longer lasting in animals that had been pre-treated with an NMDAR antagonist at the time of nerve injury. The substantial time after injury (16 days) ensures there is no significant concentration of the original drug still present, suggesting the pre-treatment regime may enable more effective analgesic treatment for any residual pain development in human pain patients. In clinical trials, the NMDAR antagonists, ketamine and dextromethorphan have been shown to have a preventative effect, with effects seen more than 5 half lives after the administration of the drug (345). While this long term effect has been demonstrated, there is no research on human subjects investigating the hypothesis that pre-treatment with a NMDAR antagonist will result in an improved susceptibility to subsequent treatment with a NMDAR antagonist.

The NMDA R subunits, NR1 and NR2 are important candidates in the cellular mechanisms underlying neuropathic sensation after nerve injury. Immunoblotting and immunohistochemical studies found an alteration in expression levels of NR1 and NR2B, but not NR2A in animals with established CCI sensitisation. NR1 expression appeared to be reduced ipsilateral to CCI consistent with previous reports (129;346). Although we found no change in NR2A expression in dorsal horn tissue

following CCI, a single cell RT-PCR study on acutely dissociated dorsal horn neurones reported a relative decrease following L5 spinal nerve transaction (133). The neuronal population from dissociated dorsal horn neurones and that assayed here by immunoblot and immunohistochemistry are unlikely to be equivalent. Moreover, no overt changes in pain-related behaviours were observed in NR2A subunit knock-out mice (347). Nevertheless, our data do not address directly a role for NR2A, which may still be functionally important, either alone in complexes with NR1 or in mixed complexes with NR1 and other NR2 subunits such as NR2B.

In contrast, the expression of NR2B was elevated ipsilateral to CCI. It might be expected that nerve-injury-induced pain states would show a relatively greater sensitivity to selective NR2B antagonists than other forms of pain. Although some previous reports have described only scarce expression of NR2B in dorsal horn (313;314;348), single cell RT-PCR found NR2B to be the most commonly expressed variant in dissociated dorsal horn neurones (133). Recent immunohistochemical evidence suggests that NR2B protein shows a distribution associated with small diameter fibres in laminae I and II, which are postulated to be of primary afferent origin (132;349) and post-synaptically on dorsal horn neurones (7;350). Several NR2B-selective antagonists show good efficacy and a particularly low incidence of side-effects upon acute administration in animal models of inflammatory and neuropathic hyperalgesia (132;351). In these studies mechanical allodynia and hyperalgesia were both shown to be sensitive to NR2B-selective antagonists.

Following NMDA R antagonist pre-treatment, we found that CCI-induced thermal hyperalgesia was essentially prevented, while cold allodynia was reduced and mechanical allodynia was apparently unaffected. This would be consistent with the idea that NMDA R complexes are involved to different degrees in these different types of sensitised sensory reflexes. In view of these findings, we further assessed the analgesic effectiveness of NMDA R antagonists (including NR2B-selective agents) on the CCI-induced mechanical allodynia that remained following pre-treatment with NMDA R antagonist around the time of injury.

Sensitivity to all acutely administered antagonists (including agents selective to a greater or lesser degree for NR2B subunit-containing complexes) was increased in nerve-injured animals following NMDA R antagonist pre-treatment. This suggests an important role of NMDARs, especially the NR2B subunit, not only in the untreated neuropathic pain state but notably in the case of residual pain following pre-emptive NMDAR antagonist treatment. When injury-induced changes in NMDAR subunit expression were investigated, the increased expression of NR2B in lamina II was prevented following memantine pre-treatment, whereas the similar increment in lamina III was unaffected.

In summary these studies provide further evidence for a role for the NMDAR in the development of neuropathic pain and suggest that the NMDAR may normally play a greater role in the development and expression of thermal hyperalgesia rather than mechanical allodynia. Correspondingly, the lasting attenuation of neuropathic behavioural sensitisation caused by NMDAR antagonist pre-treatment is more marked for thermal hyperalgesia than mechanical allodynia. The reasons for this are discussed above and amongst other things may reflect the importance of other receptors such as TRPV1 which use glutamate as a neurotransmitter. This study did not look at other receptor sub-types but future studies might further investigate the links between NMDA receptors, TRPV1 and thermal hyperalgesia.

The timing of pre-emptive drug administration may also be relevant, with prolonged delivery being more effective. The increased expression of NR2B relative to NR1 that we observed in spinal dorsal horn laminae II and III may contribute to the sensitisation of behavioural reflexes following CCI. Since pre-treatment with the NMDAR antagonist memantine blocks both NR2B expression in laminae II and thermal hyperalgesia, these may be linked events. In contrast, CCI-induced NR2B expression in lamina III was undiminished by memantine pre-treatment, although the residual CCI-induced mechanical allodynia showed increased sensitivity to NMDAR or NR2B-selective antagonists. These findings suggest that NMDAR antagonist pre-treatment is an effective analgesic therapy for thermal aspects of neuropathic pain

and that it increases the subsequent sensitivity of residual mechanical allodynia to treatment with NMDAR and NR2B-selective antagonists.

There is however great difficulty in translating these findings to clinical research. In terms of clinically available NMDAR antagonists, ketamine is the most commonly used in the peri-operative period. The effects on long term clinical sensitisation have been researched (283), but no research has yet looked re-challenging these patients with an NMDAR receptor antagonist at a later date. There may be opportunities to treat patients suffering from herpes zoster infection with NMDA antagonists and hope that in those unfortunate to develop pain there may at least an increased susceptibility to treatment with NMDAR antagonists. In terms of clinically available NR2B antagonist, Traxoprodil and haloperidol can be used. Little or no clinical research has been conducted with these drugs in the prevention of treatment of neuropathic pain. Infusions of traxoprodil have, however been used in patients with serious head injury and seem to be well tolerated (321;323). Haloperidol is an extensively researched drug in terms of its anti-emetic and anti-psychotic properties but not in the area of chronic pain. This may well reflect its high risk of adverse events, in particular movement disorders (352). Future research in the treatment and prevention of neuropathic pain, may address the use of these drugs. Increasing numbers of clinical studies are using quantitative sensory testing and this study reinforces the relevance of these to outcomes and in particular to address both thermal and tactile hyperalgesia.

Chapter 4

Investigation into the effects of peri-operative epidural ketamine on the development of post-amputation pain following lower limb amputation

4.1 Introduction

Up to 80% of patients may experience persistent pain after lower limb amputation (179;210).

Post amputation pain may either be stump pain (pain at the site of amputation that may have neuropathic elements) or phantom pain, which is a form of neuropathic pain, perceived where the limb was previously. Post-amputation pain is an important clinical problem as strong evidence for its prevention and treatment is lacking (353). The mechanisms of pain following amputation are not fully understood however the neuronal plasticity that is seen following the inevitable nerve transaction is important.

It is difficult to determine the true incidence of post amputation pain, with considerable differences from study to study, as summarised in Nikolajsen and Jensen's paper and in chapter 1 (table 7) (179). Kehlet et al estimate the figure to be lower at up to 50% with chronic severe disabling pain occurring in 5-10% of patients (180). The figure quoted in Kehlet et al.'s paper is derived from the review of Nikolajsen and Jensen and it is not clear how these lower figures are arrived at. Nikolajsen and Jensen reviewed the original papers and demonstrate an incidence in the region of 50-80%. Pain severity is not commented on.

Following amputation major changes occur in the peripheral and central nervous system in response to peripheral nerve injury and subsequent alterations in peripheral sensory input (187;354). Central sensitisation, occurring at the level of the spinal cord, is likely to play a key role in ongoing pain (355). Melzack et al hypothesise that if central sensitisation is to be reduced or avoided then blockade of the neuronal barrage at the time of surgery using either a regional anaesthetic technique and/or pre-emptive opioids is required (356). Spinal anaesthesia, in contrast to epidural anaesthesia, can block somatosensory evoked potentials (SSEPs) completely and

thus will (at least for a time) completely suppress afferent inputs to the spinal cord (357;358). As shown and discussed in chapter 3, animal models of peripheral nerve injury have shown that activation of the ionotropic glutamate receptor, the N-methyl D-aspartate (NMDA) receptor is integral to the process of central sensitisation (83). In animals, NMDA antagonists given before nerve injury can reduce behavioural signs of neuropathic pain and associated neurochemical changes (144;359).

There is a need for early intervention as the onset of post-amputation pain can be rapid, with approximately 30% of patients experiencing phantom pain within the first 24 hours (189). In keeping with this cortical remapping, which is associated with phantom pain is equally rapid and can also occur within the first 24 hours (113). As discussed the mechanism of pain is complex but nerve injury results in an excitotoxic discharge with glutamate release leading to sensitisation (87-90).

The aim of this study was to determine the role of the NMDA receptor in the development of the different components of post-amputation pain. An NMDAR antagonist, ketamine, was administered close to the proposed site of action. Spinal anaesthesia at the time of nerve transaction ensured that maximal suppression of afferent sensitising neuronal barrages occurred. A post-operative epidural infusion was given to try and suppress central sensitisation using both local anaesthetic and an NMDAR antagonist.

4.1.1 The use of ketamine and epidural ketamine in the peri-operative period

Ketamine is in widespread use in the peri-operative period, with numerous studies and reports of its use in both children and adults with no adverse events. It can be given pre-, intra- or post-operatively, usually in combination with an opioid. The main side-effects seen are hallucinations, dreams, dizziness, blurred vision and nausea and vomiting. A Cochrane Database systematic review found it to have a morphine sparing effect, a decrease in nausea and vomiting and with minimal adverse events (154). The authors tried to identify an optimal morphine sparing effect. All trials used a subanaesthetic dose. Ketamine dose ranged from about 10mg

up to around 260mg (with twenty-four different doses). Although no conclusion on optimal dose was made, a dose of greater than 30mg/24hours seemed to have a greater morphine sparing effect. In a systematic review, Subramaniam et al identified thirty-seven RCTs in of ketamine as an adjuvant analgesic to opioids (360). Eight of these used epidural ketamine. Five studies had a beneficial effect. Importantly, although one study demonstrated increased sedation in the ketamine group, no major short or long term adverse events were described.

In the vascular surgical unit in the Royal Infirmary of Edinburgh, epidural ketamine is routinely used in adults in clinical practice. Previous experience has shown that a bolus of epidural ketamine larger than 500 mcg/kg was likely to be associated with increased sedation after surgery in some patients. A dose of 500 mcg/kg was therefore chosen as being the highest dose that was not likely to be associated with increased sedation in an elderly patient group. Experience of continuous infusions of subcutaneous ketamine in elderly patients found that doses above 5 mg/h were likely to be associated with sedation or psychological side-effects. We presumed that the same side effects would be likely with epidural infusion doses above 5 mg/h. Our previous experience of continuous local anaesthetic epidural infusions with ketamine between 2 mg/h and 4 mg/h found that analgesia appeared to be improved but sedation or psychological side effects were rare in this dose range. Therefore we chose a starting epidural infusion dose corresponding to 3mg/h in a 60 kg patient or 5 mg/h in a 100 kg patient as being doses likely to demonstrate an analgesic effect without being associated with significant sedation or psychological side effects

4.1.2 Ketamine and phantom pain

Two case reports suggest that the NMDA antagonist ketamine might be a useful drug in the treatment of established phantom pain (223;224). A small (n=11) double blind trial of an IV ketamine infusion found a reduction in stump and phantom pain as assessed by VAS and MPQ (361). In addition sensory testing found an increase in pressure threshold, a reduction in 'wind-up' type pain but no effect on pain evoked by repeated thermal stimuli. Side-effects were seen in 9 of the 11 patients. These

case reports and one small study were in established phantom pain. As discussed in pre-clinical models NMDAR receptors antagonists can not only reverse established sensitisation, but prevent or delay the onset of sensitisation when given in the peri-operative period.

4.1.3 Epidural analgesia and NMDAR antagonists in the prevention of phantom pain

Clinical studies of pre-emptive treatment interfering with spinal sensory input are inconsistent. One non-blinded, non-randomised study of 25 patients found a reduction in long-term phantom limb pain by using epidural analgesia with bupivacaine and morphine started prior to surgery and continued afterwards (235). A larger randomised controlled trial of 56 patients found an incidence of ~70% for phantom pain at one year regardless of whether epidural bupivacaine and morphine were commenced 18 hours before surgery or immediately after surgery (236).

A study of intravenous ketamine used around the time of amputation in combination with general anaesthetic and morphine, had no significant effect on the incidence of phantom limb pain (241). Administration of the oral NMDA antagonist memantine resulted in a reduction in phantom pain at 6 months which was not sustained at 12 months (234). The route of administration of NMDA receptor antagonists may therefore be clinically relevant in terms of efficacy. As discussed, the hypothesis behind our study is that it is specifically spinal NMDAR activation that is one of the crucial factors leading to post-amputation pain and sensitisation. The receptor ideally needs to be targeted before nerve injury. NMDAR antagonists do have significant side-effects so targeting the drug at the site of the spinal receptors thought to be crucial may result in an improved efficacy with a lower dose and a resultant reduction in side-effects. These two previous studies using NMDAR antagonists at the time of amputation have used parenteral ketamine and oral memantine respectively. Neither has specifically targeted the spinal NMDA receptor.

No previous study has tried to modulate spinal NMDA receptors at the time of amputation. While there may be some NMDA receptor activation as a result of pre-operative pain, the aim of this study was to focus on NMDA receptor blockade at the time of nerve injury. At this time a massive excitotoxic injury discharge may occur resulting in excessive glutamate release with subsequent central neuronal plasticity (87-90).

This study assesses the effect of pre-emptive treatment with an epidurally administered NMDA receptor antagonist, ketamine, in combination with local anaesthetic, on reducing spinal sensory transmission, acute central sensitisation and the development of persistent post amputation pain. Patients undergoing lower limb amputation for vascular reasons are usually elderly, often smokers and have a high risk of cardiovascular and cerebrovascular disease. Their life expectancy at surgery may be short with an expected around 30% one year mortality (362). There is a high risk of peri-operative morbidity particularly cardiac (10.2%), wound infection (5.5%) and pneumonia (4.5%) (362). Their pre-morbid condition coupled with pain and opioid exposure increases the need to use as 'clean' an anaesthetic technique as possible. The study was carefully designed to avoid any confounding variables on the peri-operative period. In particular the subjects did not have a general anaesthetic or opioid exposure in the peri-operative period. It was of course impossible to avoid opioids in the period leading up to surgery as the vast majority of patients undergoing limb amputation for vascular reasons do so because of severe pain. Nevertheless it was important to try and avoid peri-operative opioid exposure as repeated exposure to opioids can itself result in spinal cord neural plasticity in a mechanism which involves the NMDA receptor (363).

4.2 Methods

Approval was granted by the local research ethics committee and was in accordance with the Helsinki Declaration of 1975(revised 1983). International Standard Randomised Controlled Trial Number (ISRCTN) 48374927 was assigned to this trial.

4.2.1 Subjects

Patients scheduled to undergo lower limb amputation in the Vascular Surgery Unit, Royal Infirmary of Edinburgh, Scotland were approached regarding participation in the trial. After written informed consent was obtained they were then entered into the trial. Trial recruitment took place over a twenty month period (March 2000 – October 2001). Patients had to be able to participate in the questionnaire-based pain assessment and lower limb examination before they were invited to take part in the study. Exclusion criteria included: contraindication to spinal or epidural blockade; contraindication to the use of ketamine or bupivacaine; previous lower limb amputation. Patients who underwent further amputation during the follow-up period were excluded from further analysis and follow-up.

4.2.2 Study design

Patients who met entry criteria were randomly assigned to one of two treatment groups: Group K to receive epidural bupivacaine and racemic ketamine and Group S (control group) to receive epidural bupivacaine and saline around the time of amputation. The infusion was made up in theatre by a staff member unconnected to the study. Sufficient infusate was made up to cover the whole study period. Both patients and staff were blinded as to treatment group, but for safety reasons, one of the researchers held sealed records as to the group allocation. The researchers involved in assessments were blind to patient allocation until all subjects had completed their one-year assessment. Recruiting and post-operative analgesic adjustments were carried out by a single researcher (JW).

Randomisation was by GraphPad StatMate version 1.0 (GraphPad software Inc. San Diego) which allocated subjects to either group using computer generated pseudo-

random numbers. All patients underwent a detailed pain assessment, including bedside quantitative sensory testing (QST) of both lower limbs before surgery, and at eight days, six weeks, three months, six months and twelve months after surgery. If the stump was infected or not healed, no QST was performed at that visit.

4.2.3 Sample size and power calculation

The primary outcome measure was the incidence and severity of both phantom limb and stump pain. The incidence of phantom pain was chosen to calculate sample size. Secondary outcome measures were the effectiveness of post-operative analgesia and changes in sensory processing as measured by QST.

The incidence of phantom pain in most comparable studies is around 80% (179;210). We considered that a decrease to 40% would be clinically significant. For a power of 80%, an estimated sample size of 23 per group was needed (SigmaStat 2.03; Power = 0.8; $\alpha = 0.05$). A 30% one-year death rate would increase the sample size to 30 per group. This drop-out rate is comparable to that found in other studies in this patient population (362). Interim analysis (see below) by an observer not connected with the study was carried out at the midpoint of the study period, using Fisher's Exact Test, with the primary endpoint being rate of phantom pain occurrence. This was done to further refine the sample size and ensure that recruiting did not continue inappropriately.

4.2.4 Patient assessment and outcomes measures

The rationale and background to the assessments used have been discussed in chapter 2. In this study the primary end-points of the presence or absence of both phantom and stump pains were used. Pain severity was measured using a standard (0-100mm) visual analogue scale (VAS). In addition the characteristics of any phantom and stump pain were recorded, including frequency and severity of attacks. This was recorded using a data collection form, a modification of Sherman et al's phantom limb questionnaire (203) (Appendix 1). Current analgesic medications were also recorded, and classified in terms of the WHO analgesic ladder, i.e. simple analgesics, weak opioids, strong opioids and adjuvant drugs for neuropathic pain.

In addition other outcome measures were used namely the Magill Pain Questionnaire (MPQ), the Neuropathic Pain Scale (NPS) and Hospital Anxiety and Depression Score (HADS) (261;270;273). Quantitative sensory testing (QST) was also performed to see if subtle changes in sensory processing could be found. MPQ results were expressed as total Pain Rating Index (PRI) scores (272). Post-amputation pain is likely to have a neuropathic component therefore the NPS was used as another measure of pain severity and to characterize the neuropathic component in more detail. Results were expressed as a total NPS score out of 100. The HADS was chosen as a measure of psychological distress. It is widely used in the hospital setting and is both easy to administer and for the patient to understand. HAD scores were divided into HADS-anxiety (HADS-A) and HADS-depression (HADS-D).

4.2.5 QST

The background, methodology and rationale for QST are discussed in detail in chapter 2 (section 2.4.4). Only a few studies on post-amputation pain have used QST (285;364). In this study the patient acts as his or her control using the contralateral limb as a control. A study protocol was designed that frail, elderly patients would be likely to be able to complete, and that was also portable. This allowed assessments to be carried out in a community setting, if there were transport issues, thus maximising recruitment. The assessment was as follows:

- Soft brush

- Standard pinprick (Neurotips™; Owen Mumford)

- Von Frey filaments (North Coast Medical, Inc., San Jose, California)

- Vibration – using a standard electrical tooth brush

- Cold temperature – topical acetone was dripped a few drops at a time using a standard laboratory pipette

Bedside QST was carried out on the base of the stump, or where it was anticipated to be (for pre-operative testing), using the ventral aspect of the contralateral limb as a control. Testing on each limb was carried out equidistant from the corresponding iliac crest. Initially any area of altered mechanical sensation was mapped out using a soft artist's paintbrush. The brush was drawn over the skin from an area of normal sensation towards the stump and the patient asked to note any change (increase or decrease) in the stimulus perception. Further assessment was carried out in any area of altered sensation or, if none detected, on the base of the stump. Tactile and pain thresholds were assessed using von Frey (VF) filaments (North Coast Medical, Inc., San Jose, California). These were applied in ascending and descending force magnitude until the subject was able to perceive four out of six stimuli. VAS scores to pain and to unpleasantness were recorded following brush stimuli, vibration (using a standard electric toothbrush), pinprick, using neurological examination pins (Neurotips™; Owen Mumford) and cold (acetone drops).

4.2.6 Peri-operative management

The peri-operative period was from the time of consent for surgery up until 72 hours afterwards. No premedication was given. This was to keep the anaesthetic as clean as possible, avoiding benzodiazepines which act on GABAergic receptors. Any prescribed analgesics were continued up until the time of surgery. No patient received general anaesthesia or supplementary opioids over the period of epidural use. Both groups received combined spinal and epidural anaesthesia and intravenous sedation. Epidural catheters (16G Portex) were placed just before surgery (JW or Dr Alastair Nimmo (a co-researcher)). These were inserted through either the L2-L3 or L3-L4 interspace. A test-dose of 4mls of 2% lidocaine was given to exclude intravascular or intrathecal placement. Immediately after the epidural test dose, a spinal (intrathecal) anaesthetic was administered at an interspace below the epidural catheter (24G Sprotte needle; 2.5-3.0ml of bupivacaine 0.5%; AstraZeneca). Before starting surgery, an epidural bolus dose of bupivacaine 0.5% (1mg/kg) with either 0.5mg/kg preservative free racemic ketamine (Curamed Pharma, Germany) (Gp K) or an equivalent volume of NaCl 0.9% (Gp S) was given. The epidural infusion

mixture was prepared in theatre by an anaesthetist not involved in the study and labelled “study medication”. Additional study medication was prepared at this time to be available if required during the infusion period in order to maintain blinding. Neither ward staff nor the researcher carrying out the assessments was aware of the contents of the mixture.

Sedation was given as required using a target-controlled infusion of propofol.

Benzodiazepine sedation with drugs such as midazolam was avoided.

Intraoperatively all patients had standard monitoring, supplemental oxygen and intravenous fluids. During surgery the epidural infusion was commenced at 15ml/hour. Group K received an infusion of bupivacaine 0.125% with ketamine 3.3 mg/kg/l. Group S had bupivacaine 0.125% with an equivalent volume of NaCl 0.9%. Following surgery the epidural infusion was adjusted by a blinded observer within the range of 10-20ml /hour to ensure adequate pain relief ($VAS \leq 30$). If analgesia was inadequate, top-up boluses (10-15mls) were given from the infusion mixture.

Post-operative analgesia was supplemented with paracetamol 1g 6 hourly orally. No other analgesic medication was permitted during the period of epidural use. All epidurals ran for between 48 and 72 hours. Failure of the epidural to provide adequate analgesia required the removal of the patient from the study.

During the period of epidural analgesia, block height (blunt pin prick) and motor block using a modified Bromage scale (365), number of epidural bolus doses, average pain VAS scores, sedation score, respiratory rate and presence of nausea or vomiting (self report) were recorded twice-daily.

4.2.7 Statistics

Statistics were carried out using SigmaStat for Window version 2.03. The primary end point was the incidence of phantom pain. The statistical analysis is therefore asking the question; are two variables related? Data is laid out in a contingency table and the frequencies of the variables compared. Proportions were compared using Chi-squared analysis or Fisher’s exact test if the expected numbers in each group were small <5 . Other measures were compared using One Way Analysis of Variance (ANOVA) or Kruskal-Wallis ANOVA on Ranks if non parametric data. The rationale behind these tests have been discussed in chapter 3.

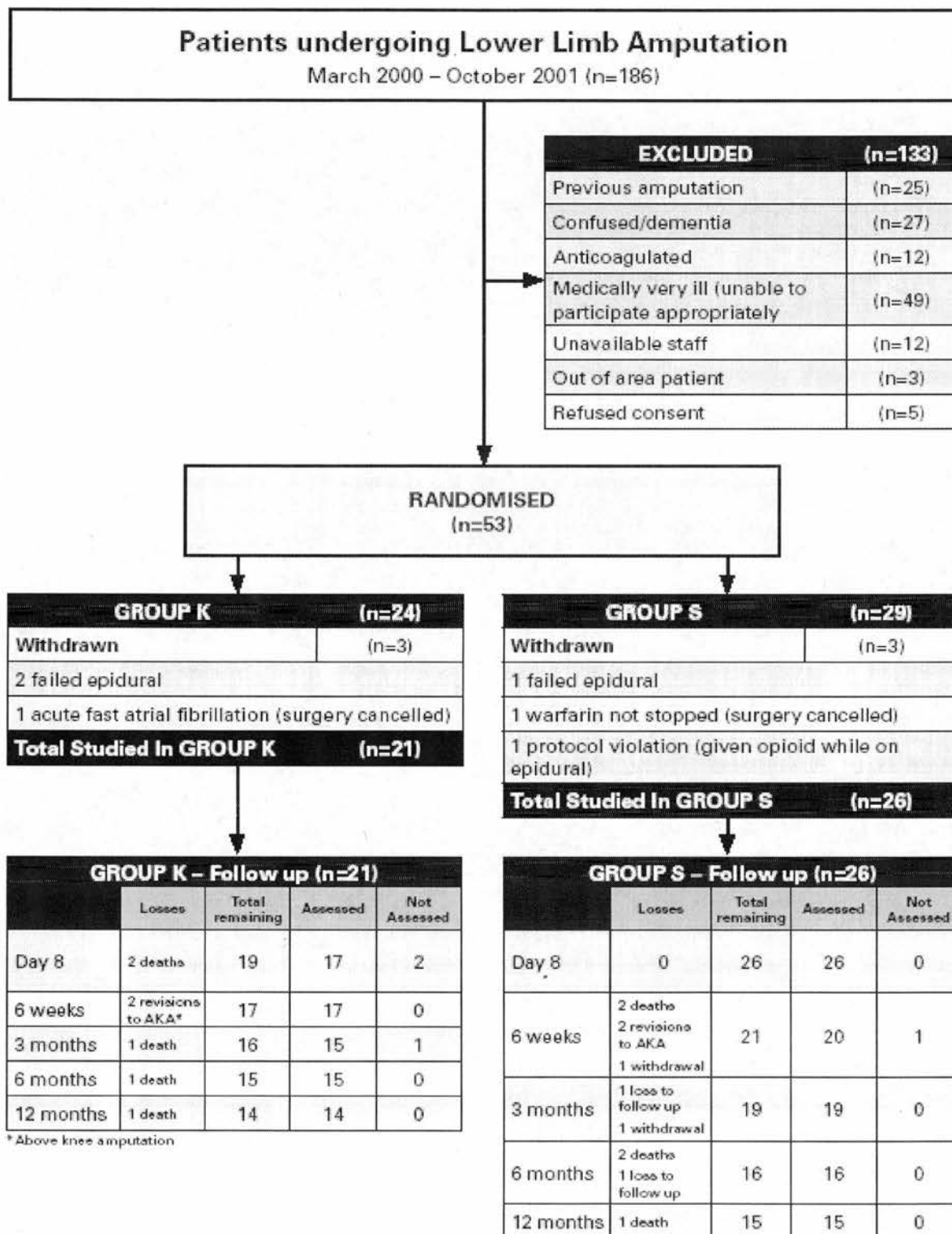


Figure 8

Consort diagram showing patient flow through the study

Results

4.3.1 Recruitment and baseline characteristics

Of 186 patients undergoing lower limb amputation over the 20-month period studied, 53 patients were suitable and consented to participate in the study. They were randomised such that 24 subjects were allocated to group K and 29 to group S. Six were withdrawn post randomisation. 47 patients were included in the analysis, with 21 subjects in group K and 26 subjects in group S. 4 subjects subsequently had a below knee amputation revised to an above knee and were removed from further analysis (Fig 8).

Table 10 **Characteristics of patients**

		Group S (n=26)	Group K (n=21)
Mean age years (n; SD)	Male	73 (16; 5.3)	69 (19; 9.4)
Mean age years (n; SD)	Female	78 (10; 5.5)	73 (2; 0.7)
Mean weight in Kg (SD)	Male	68.3 (13.1)	74.8 (20.0)
Mean weight in Kg (SD)	Female	51.6 (10.0)	67.0 (1.4)
Mean height in cm (SD)	Male	172.3 (6.1)	173.5 (9.0)
Mean height in cm (SD)	Female	163.9 (9.1)	161.5 (2.1)
Diabetes (n)		6	7
Median pre-op VAS score (IQ range)		70 (50-83)	50 (40-50)
Above knee amputation		11	5
Below knee amputation		15	16

Patient characteristics (table 10)

The subjects were comparable in age, weight, height, incidence of diabetes mellitus and type of amputation. The characteristics of each group are shown in Table 10. Of particular note, there was no difference in median (quartiles) pre-operative VAS pain scores (group K 50 (40-50), group S 70 (50-83)). The incidence of diabetes in group K and group S was 7 and 6 respectively. Similar numbers in both groups used pre-operative opioids (K11/21:S21/26; ns; chi squared $p=0.078$). No problems with opioid withdrawal were seen when opioids were stopped from the time of surgery onwards.

4.3.2 Interim analysis

The power of the study is an area that causes some difficulty in this type of study. Patients undergoing lower limb amputation for peripheral vascular disease are usually elderly, with many meeting the exclusion criteria, resulting in slower than expected recruitment. After taking independent statistical advice, a blinded interim analysis was recommended.

After the first 35 patients had been assessed at three-months, interim analysis (using Fishers exact test) was carried out for the primary end-point (presence or absence of phantom pain) by an independent assessor not involved in the study, with the results of this not being available to the researchers. The rate of phantom pain at 8 days, 6 weeks and 3 months in Group K was 3/11 (27%), 7/11 (64%) and 4/10 (40%) respectively compared with Group S where 9/18 (50%), 8/17 (47%) and 6/15 (40%) patients had phantom limb pain. There was no significant difference between groups at 8 days, 6 weeks and 3 months (with p-values of 0.273, 0.460 and 1.000 respectively). The study authors were blinded to the result details, but were advised that recruitment should cease, at which time 53 patients had entered the study.

4.3.3 Assessment during epidural infusion period (table 11)

There was no difference in median epidural infusion rates between the groups. Both groups had good postoperative analgesia with median total VAS scores of less than 30 mm. Group K had significantly lower pain scores than group S. Group K required a significantly lower number of epidural top-ups. Following establishment of epidural blockade, no subject was withdrawn due to inadequate analgesia. Mean epidural infusion rate and duration was the same for both groups.

Table 11 Peri-operative infusion data

	Group K	Group S	p-value
Median mean VAS (Quartiles)	0 (0-17)	17 (0-35)	0.031*†
Median Infusion rate; ml/hour (Quartiles)	15 (15-18)	15 (14-16)	0.397
Mean number of top-ups (SD)	1.8 (2.2)	3.5 (3.2)	0.044*‡

* = Significant difference † = Mann-Whitney rank sum test ‡ = t-test

There was no significant difference in motor block between the groups during the duration of epidural infusion. Median (IQ range) Bromage scores on day one were 1 (0-2) for group K and 0 (0-1) for group S. On day two median (IQ range) Bromage scores were 0 (0-1) in both groups. The incidence of nausea and vomiting (Group K: 4/21; Group S: 3/26; p=0.684), sedation (Group K: 2/21; Group S: 1/26; p=0.579) and confusion (Group K: 2/21; Group S: 0/26; p=0.194) were low and did not differ between groups (Fishers exact test). No patients suffered from hallucinations. There were no complications that required revealing the composition of the infusion.

4.3.4 Post-amputation pain

The rates of phantom and stump pain did not differ between the two groups at any of the assessments up to and including one year, when the rate of phantom pain was no more than 50% and stump pain was no more than 33% in either group (Table 12a & 12b). Neither stump pain nor phantom pain intensity, as measured using VAS, differed between groups at any time point (Figures 9 and 10). Frequency and duration of attacks are presented in graphical form at each assessment point (Figs 11-15). Numbers in each group were small. There was no visual trends identified and further analysis was not carried out as multiple analysis of this data would result in a significant chance of a type 1 error i.e a false positive.

Table 12a Stump Pain

	Group K	Group S	p
Time	n (%)	n (%)	
8 days	11/17 (65)	15/26 (58)	0.888
6 weeks	9/17 (53)	9/20 (45)	0.879
3 months	5/15 (33)	9/19 (43)	0.635
6 months	7/15 (47)	5/16 (32)	0.609
12 months	3/14 (21)	5/15 (33)	0.682†

Proportions compared using chi-squared analysis except as indicated by †.

†=Fisher's exact test.

Table 12b Phantom Pain

	Group K	Group S	p
Time	n (%)	n (%)	
8 days	6/17 (35)	13/26 (50)	0.525
6 weeks	10/17 (59)	9/20 (45)	0.611
3 months	6/15 (40)	7/19 (37)	0.867
6 months	6/15 (40)	3/16 (19)	0.252†
12 months	7/14 (50)	6/15 (40)	0.867

Proportions compared using chi-squared analysis except as indicated by †.

†=Fisher's exact test.

Figures 9-15 are shown on the next four pages

The intensity of stump pain (fig 9) and phantom pain (fig 10) at each time point following amputation.

Data is shown as box plots with medians represented by horizontal lines with the 75th percentile at the top and the 25th percentile at the bottom. The 10th and 90th percentiles are shown as whiskers.

Figures 11-15 show the frequency of phantom pain attacks (LEFT) and the length of attacks (RIGHT) for each group at each time point

Stump Pain VAS scores

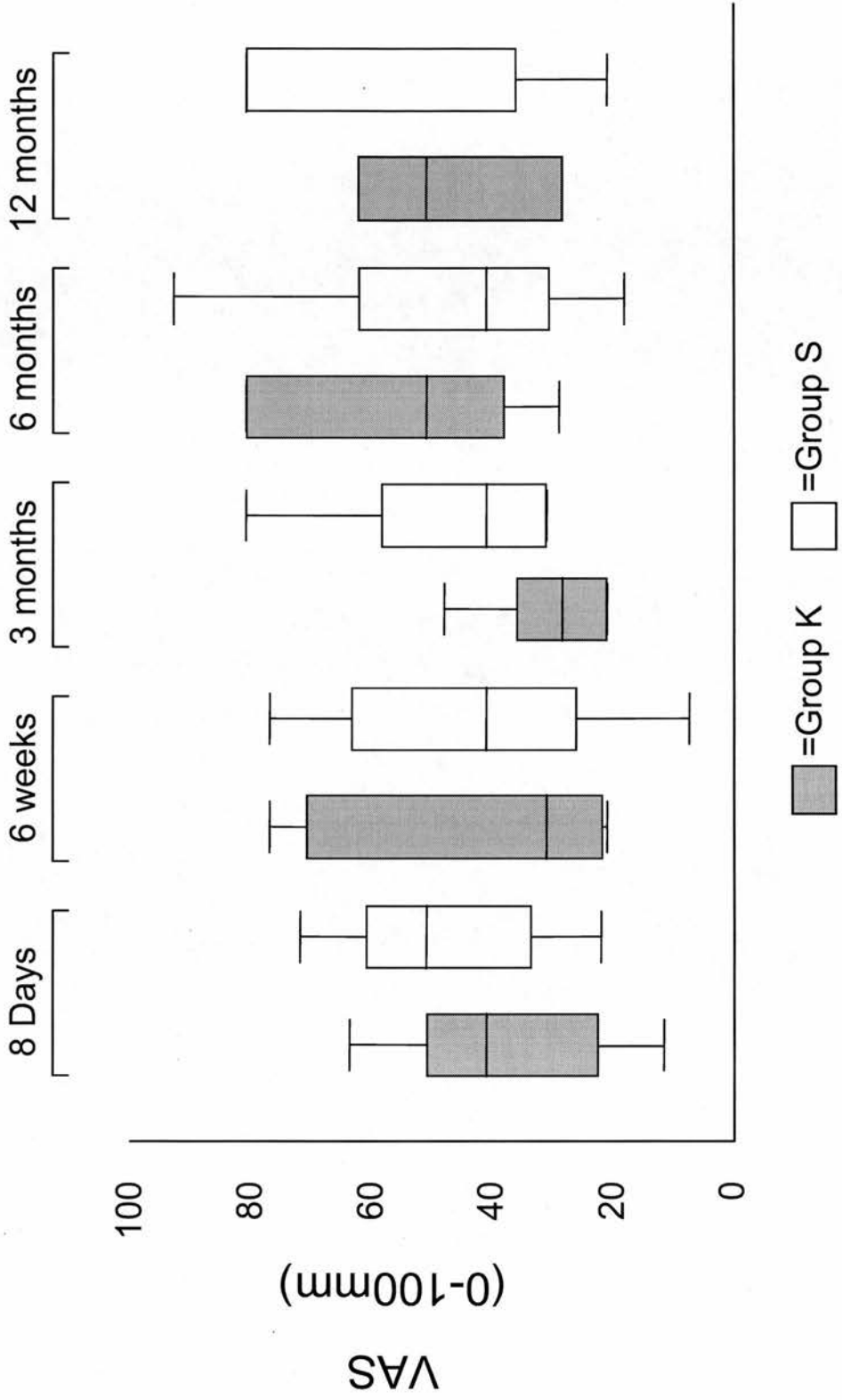


Figure 9

Phantom pain VAS scores

8 Days 6 weeks 3 months 6 months 12 months

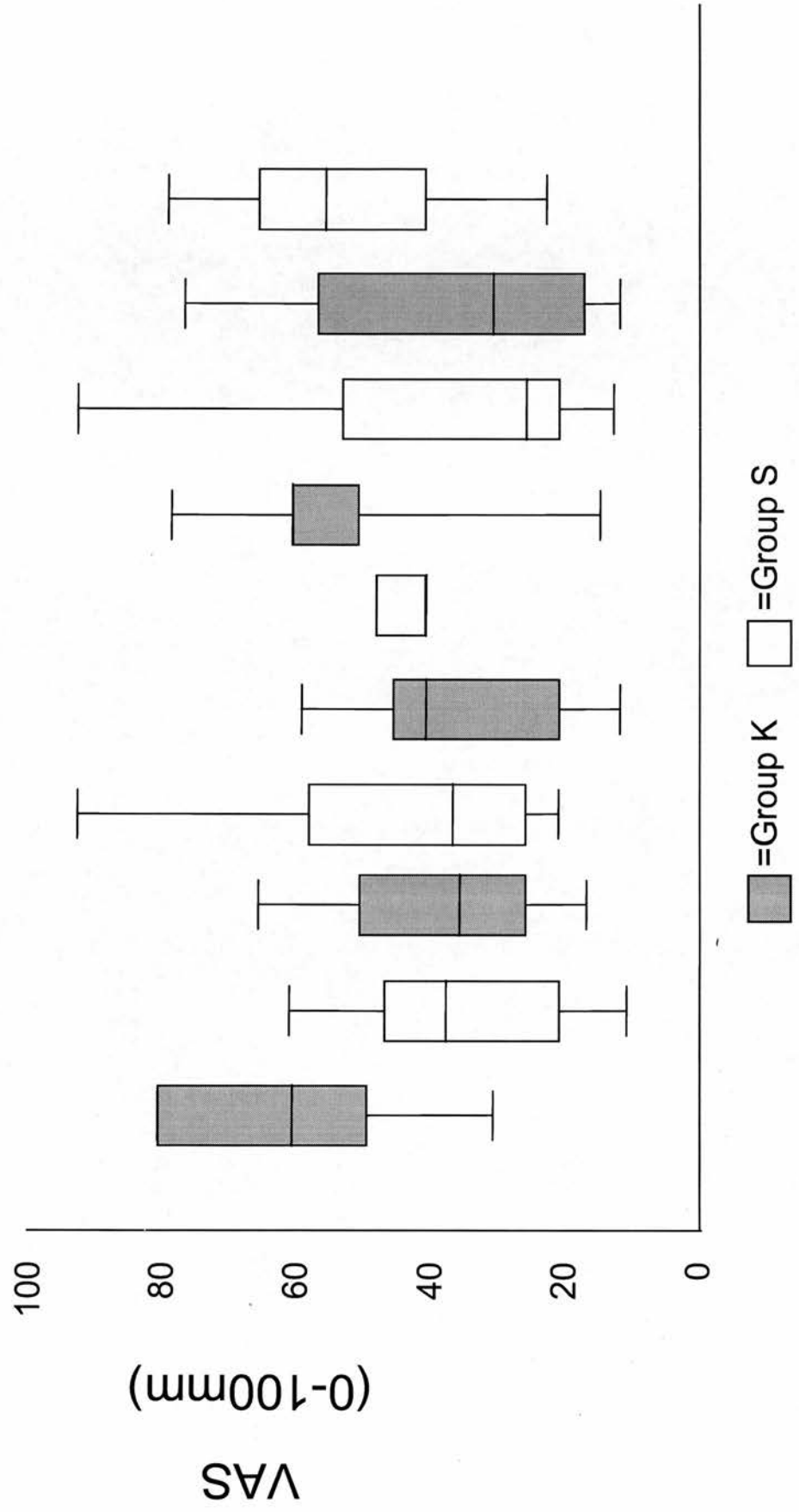


Figure 10

Fig 11

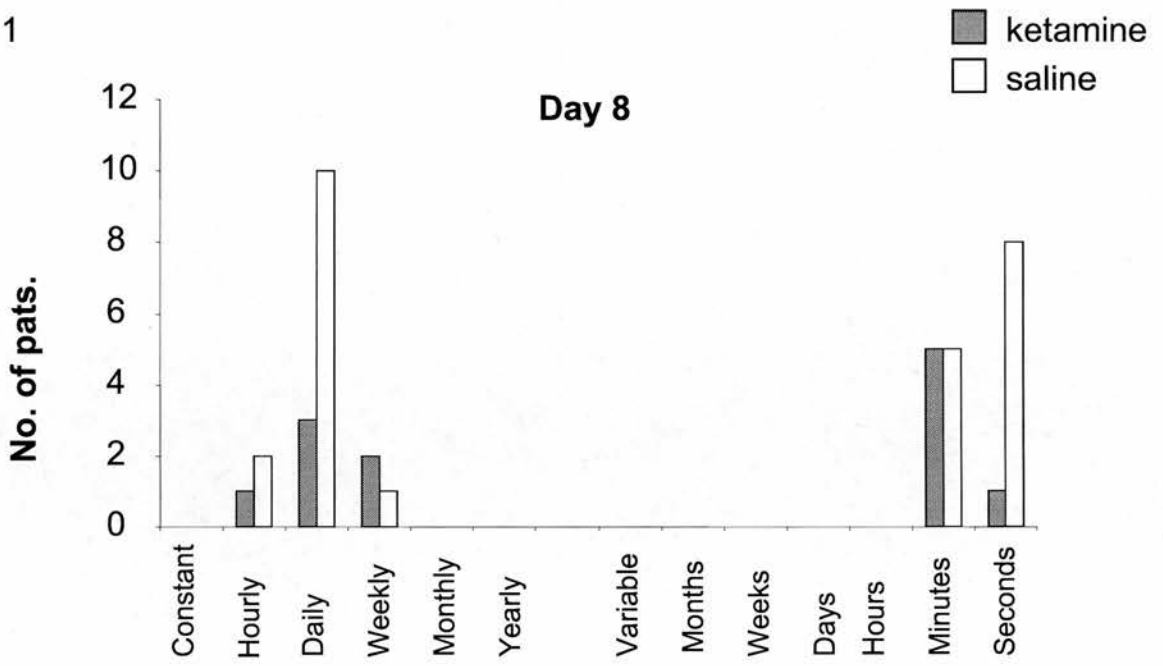


Fig 12

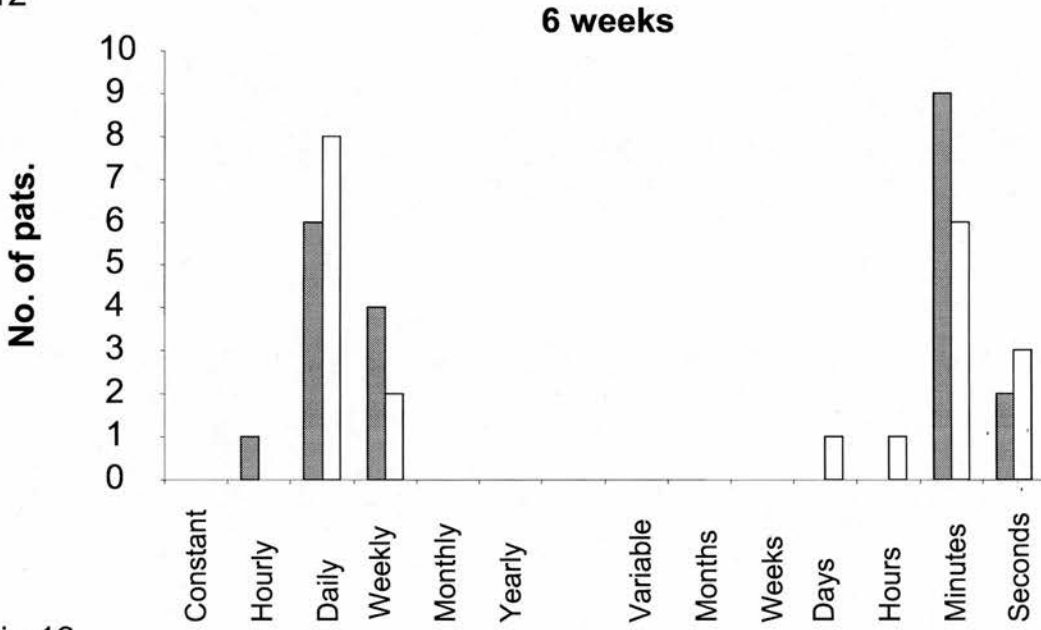
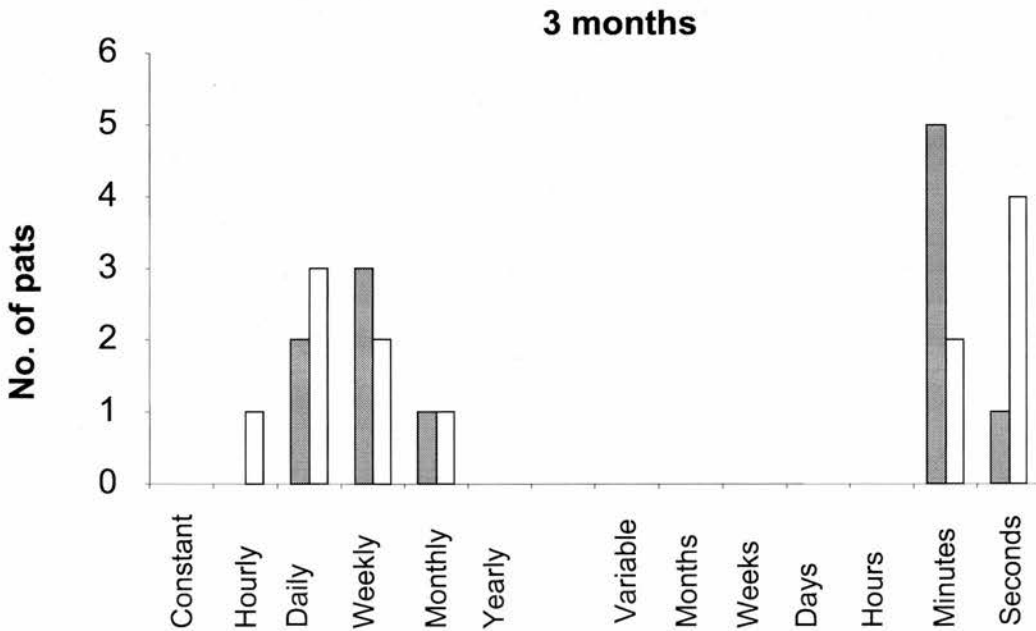


Fig 13



■ ketamine
□ saline

Fig 14

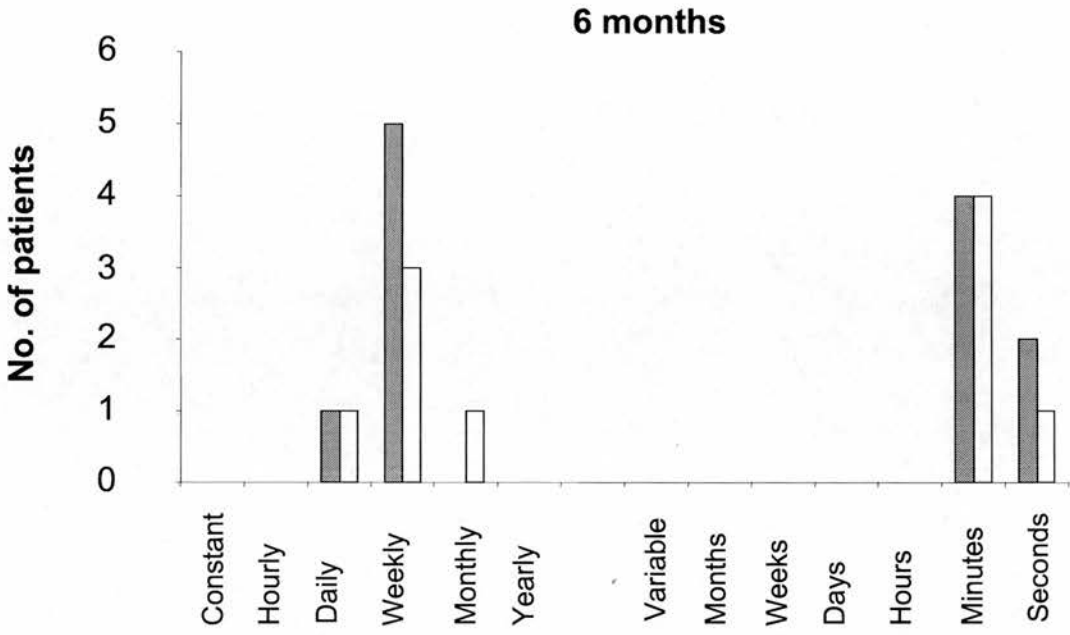
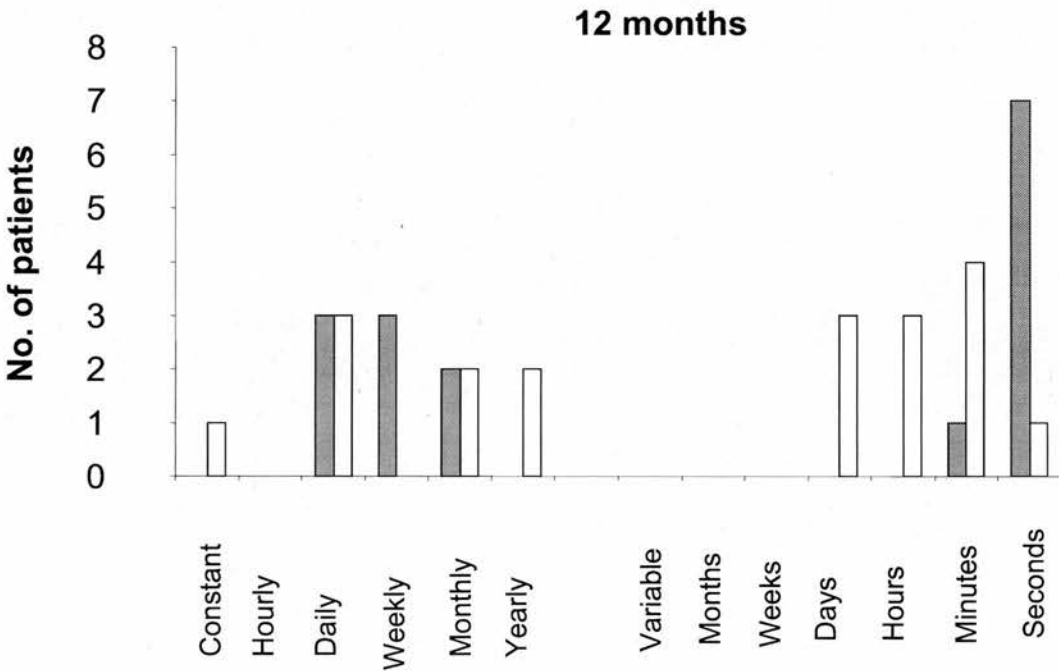


Fig 15



Figures 10-14 show the frequency of phantom pain attacks (LEFT) and the length of attacks (RIGHT) for each group at each time point

4.3.5 Questionnaire data

Total MPQ scores were highest before surgery, Group K: 23 (15-33), Group S: 28.5 (15.25-35.75); median (IQ range). These fell to 3 (0-8) in Group K and 3 (1-11) in Group S at 12 months. Scores in both groups were significantly less than pre-operative values by 6 weeks (Kruskal-Wallis ANOVA on Ranks with Dunn's post-hoc analysis, $p < 0.001$), but groups did not differ from each other at any time point (see Figure 16)

Median NPS scores started high (Figure 17) and dropped with time, pre-operatively scores were: Group K; 49 (40-59) vs Group S; 50 (37-66); median (IQ range). This fell to a median score of 4 (1-22) in Group K and 5 (0-16) in Group S at 12 months. There was a significant reduction from pre-operative values in both groups at all time points post-operatively, but with no difference between groups (Kruskal-Wallis ANOVA on Ranks with Dunn's post-hoc analysis, $p < 0.001$).

Levels of anxiety, as assessed by HADS scoring (expressed as mean (SD)) were: Group K: 8.4 (4.5) pre-operatively, decreasing to 5.4 (2.8) at 6 weeks post amputation, and remained significantly lower at all time points up to and including one year (4.3(2.9)) (one way ANOVA with Dunnett's post-hoc analysis; $p < 0.001$) and Group S: 8.2 (3.7) pre-operatively with no significant reduction any time point up to and including one year (4.7 (4.0))(one way ANOVA with Dunnett's post-hoc analysis; $p = 0.071$). Levels of depression for Group K were 8.1 (4.3) pre-operatively, reducing significantly at 3 months to 5.4 (3.8) and remaining significantly reduced up to and including one year (one way ANOVA with Dunnett's post-hoc analysis; $p = 0.003$). Pre-operative scores for depression in Group S were 6.0 (3.4), with no significant reduction at any time point up to and including one year (4.5(4.6)) (one way ANOVA $p = 0.829$). Figure 18 shows the mean HADS score at each time point divided into anxiety and depression scores. The comparison between groups and the fall with time can be clearly seen.

Figure 16

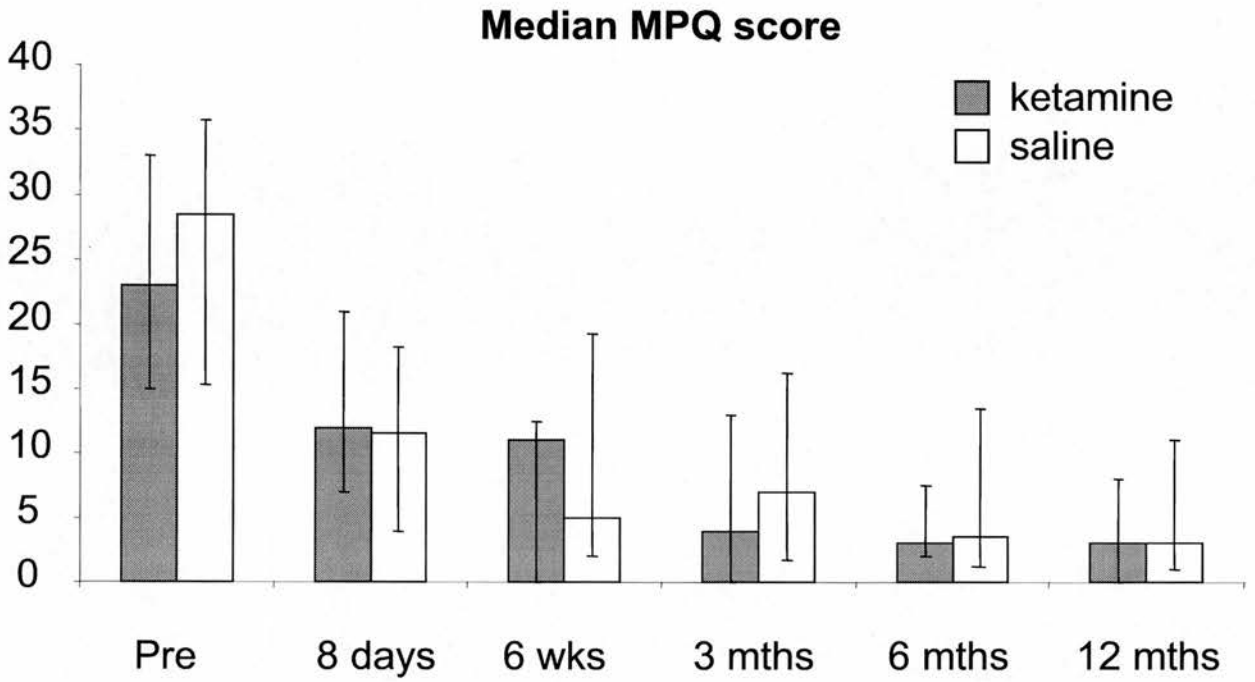
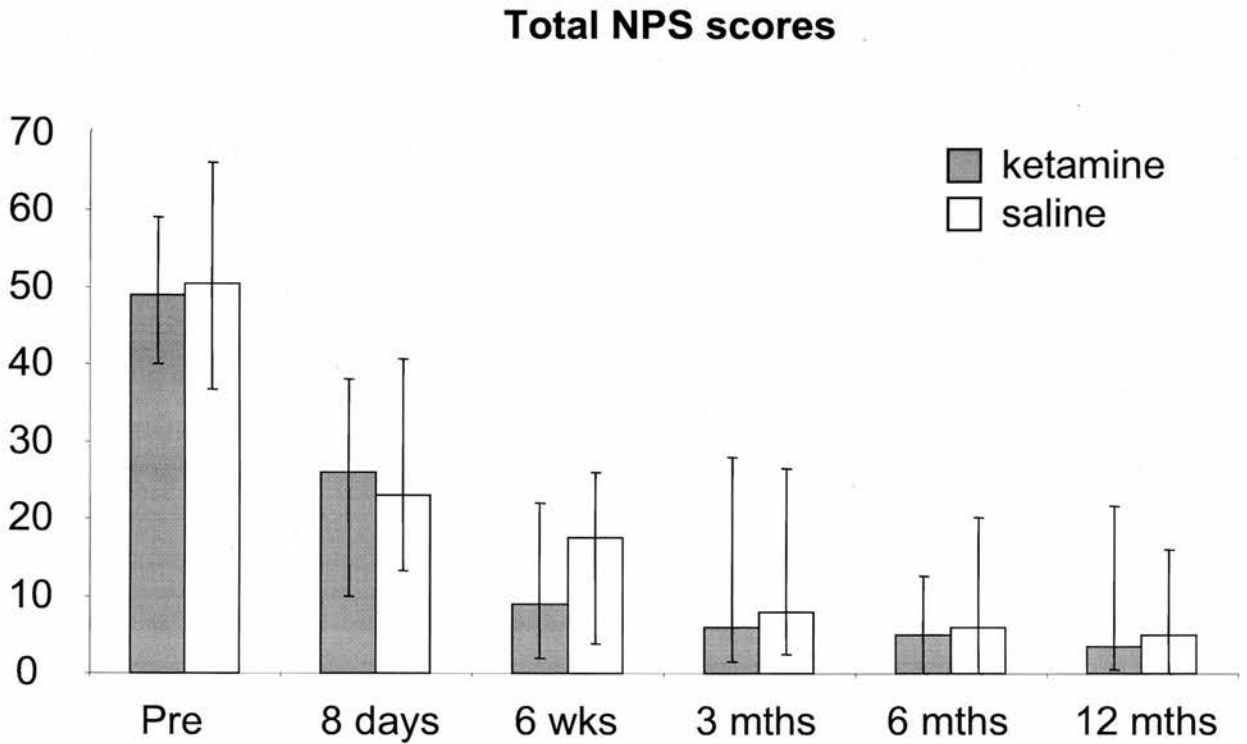
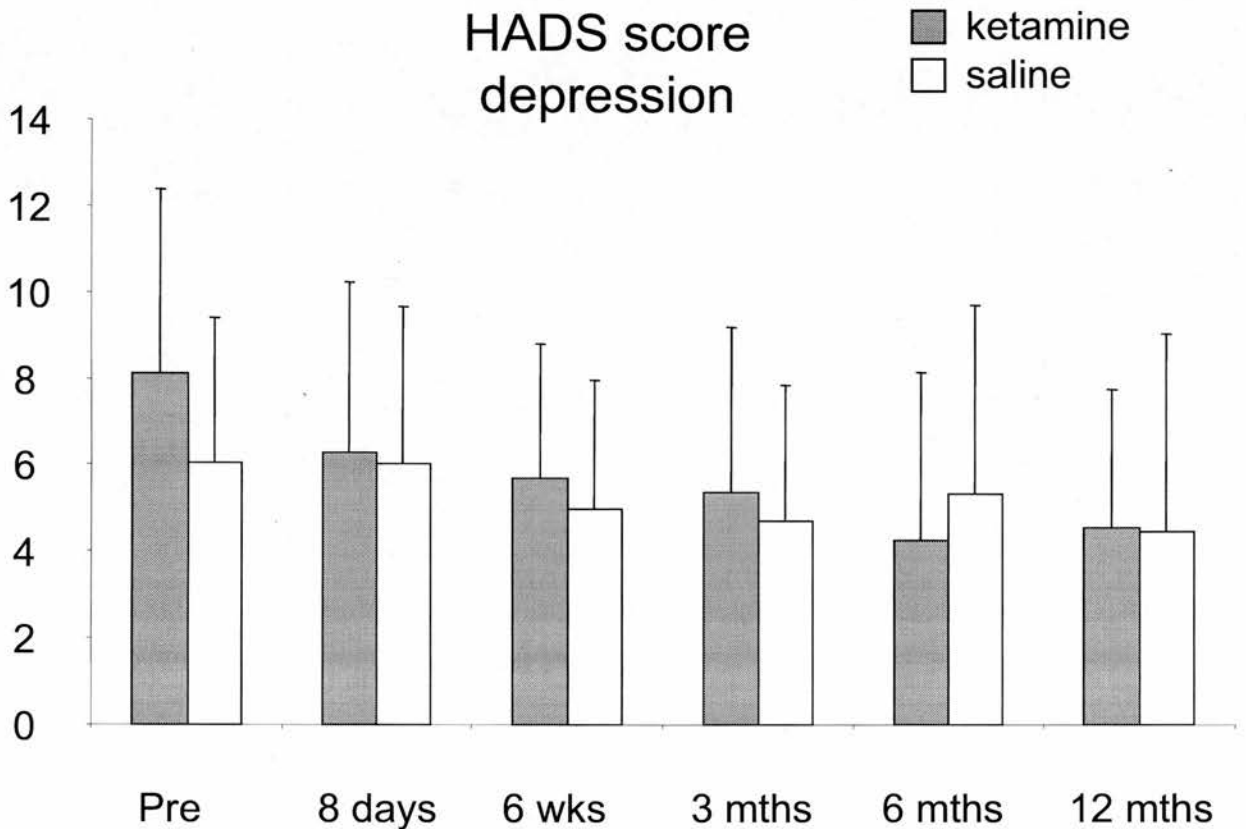
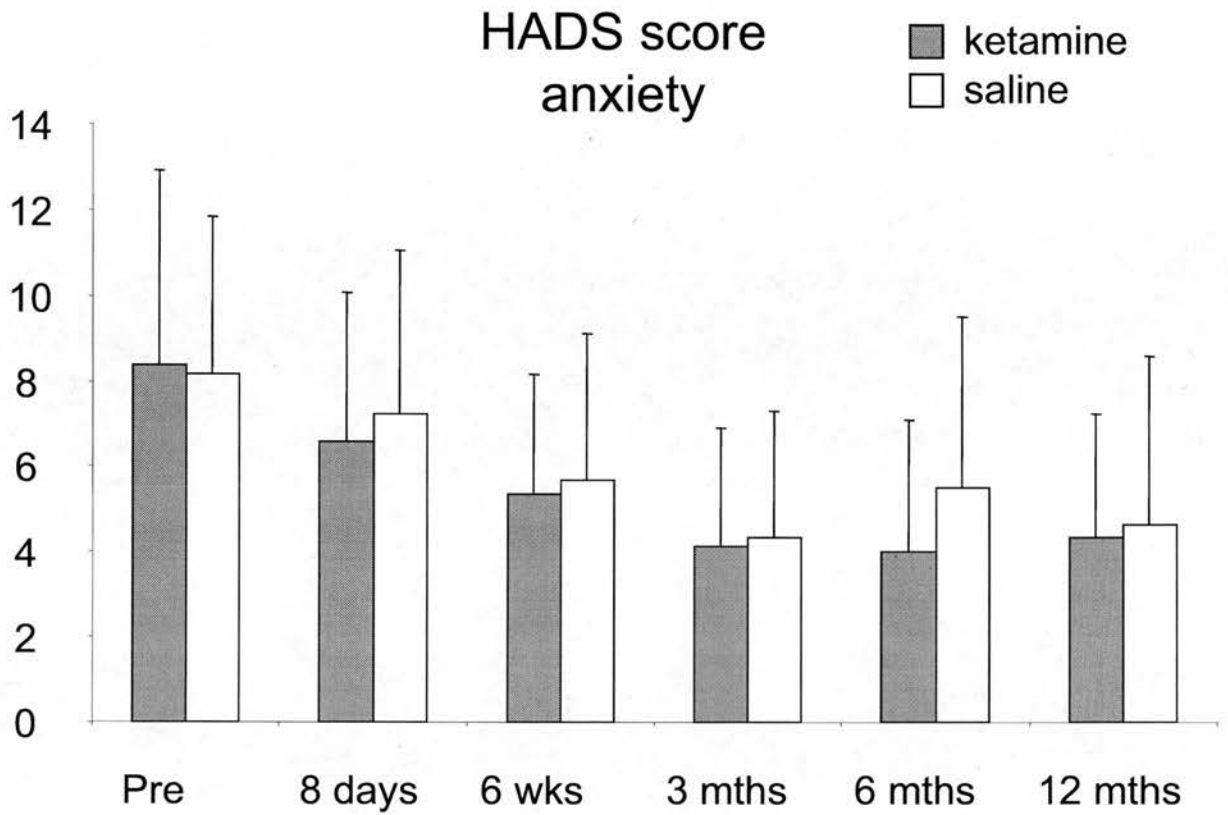


Figure 17



Data is shown as median scores with IQ ranges

Figure 18



Data is shown as mean HAD score (SD)

4.3.6 Sensory processing as measured by QST

Preoperatively there was a slightly higher tactile threshold in the ischaemic limb (206 mNmm⁻² (191)) than the contralateral limb: (139 mNmm⁻² (82)), t-test p=0.036. Ketamine did modify some aspects of sensory transmission, with an alteration in the tactile threshold of the stump on day 8 after surgery. In Group K, the tactile threshold was found to be significantly higher on the stump (589 mNmm⁻² (782); mean (SD)) compared to the contralateral limb (180 mNmm⁻² (160); mean (SD)) and to both limbs in Group S (stump; 250 mNmm⁻² (97); mean (SD)), contralateral, 208 mNmm⁻² (140); mean (SD)); (One way ANOVA with Tukey test.; p=0.005). This difference was not found at 6 weeks (see Table 13). There was no significant difference between sides at any other time point. There was no difference between the groups in the evoked response to pin prick at any time point (Table 14). Data for both tactile threshold and VAS score to painful pin prick has been displayed as a scatter plot for both groups at 12 months (Figures 19 and 20). The similarity between the two groups is clearly displayed. Data comparing those with phantom pain and those without has been plotted using a scatter graph showing tactile threshold for the stump and contralateral limb for both Group K and Group S (Figures 21 and 22) on Day 8. VAS scores for cold and vibration were 0 in the majority of patients (88-100%) at all time points. Further detailed analysis was not carried out.

4.3.7 Analgesic medications

The overall number of analgesic drugs prescribed did not vary between groups. The number of analgesic drugs prescribed pre-operatively was Group K; 2 (2-3) vs Group S 3 (2-3); median IQ range. The number of analgesic drugs prescribed was less in both groups after surgery. At 12 months the number prescribed was the same in both groups: 1 (1-2); median (IQ range). There was no difference between the two groups in the median numbers of drugs at any time point after surgery (Kruskal-Wallis ANOVA on ranks)

Table 13

Mean tactile threshold

Time	Group K Stump	Group K Con	Group S Stump	Group S Con	P
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	
8 days	589 (782)	180 (160)	250 (97)	208 (140)	0.005*
6 weeks	314 (412)	180 (156)	255 (173)	145 (68)	0.224
3 months	216 (225)	199 (224)	154 (106)	127 (77)	0.442
6 months	180 (145)	138 (61)	143 (72)	134 (70)	0.472
12 months	147 (103)	150 (102)	215 (123)	155 (90)	0.236

* = One way analysis of variance

Tactile threshold for the stump and the contralateral limb as measured by Von-Frey filaments

Data is shown as mean tactile threshold (force/unit area; g/mm²) with SD for both Group S and Group K, for up to 12 months following surgery.

Table 14

Mean VAS to pinprick

Time	Group K Stump	Group K Con	Group S Stump	Group S Con	P
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	
8 days	29 (29)	22 (25)	46 (29)	36 (32)	0.051
6 weeks	46 (37)	29 (31)	47 (33)	33 (23)	0.208
3 months	35 (34)	33 (37)	30 (23)	28 (25)	0.935
6 months	35 (31)	32 (34)	33 (34)	41 (25)	0.761
12 months	45 (30)	40 (32)	42 (31)	42 (26)	0.972

Data is shown as mean VAS score (0-100mm) with SD for both Group S and Group K, for up to 12 months following surgery.

Fig 19

VF 12 months

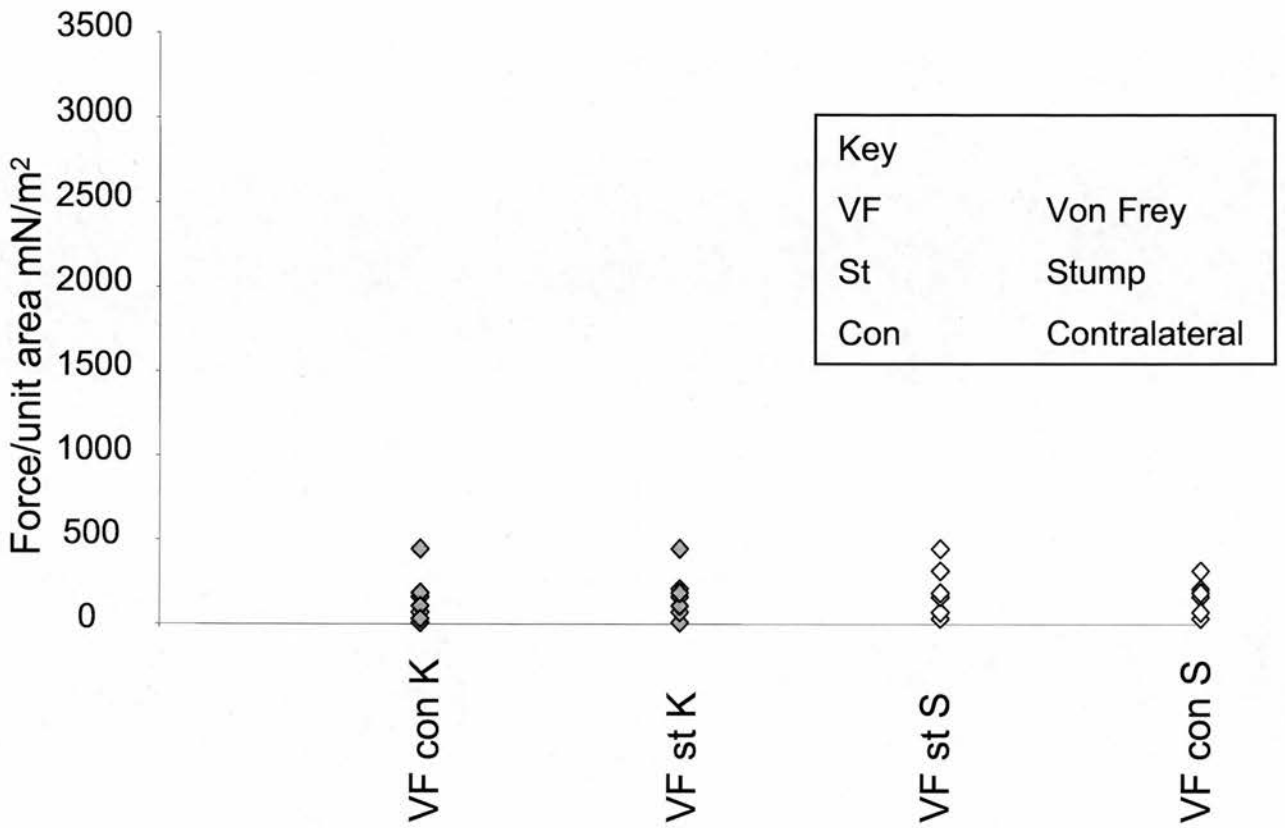
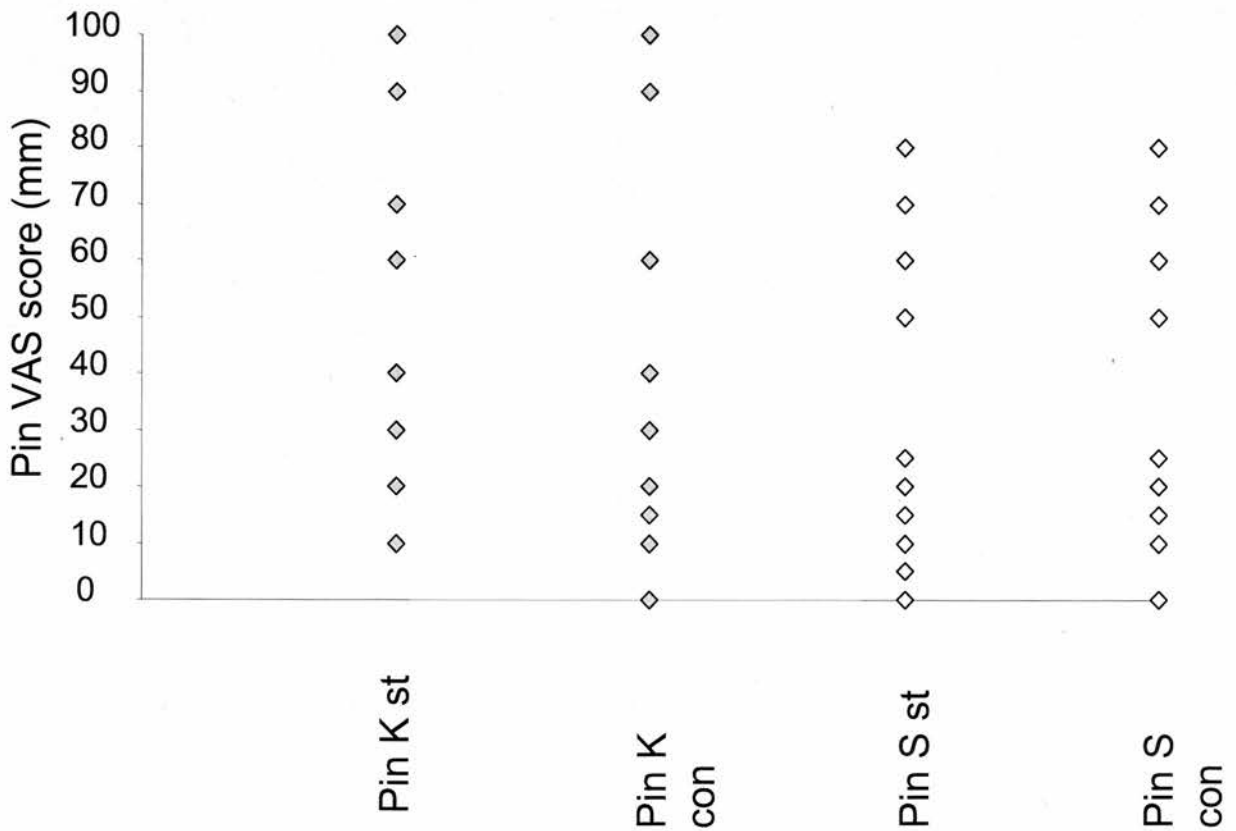


Fig 20

Pin 12 months



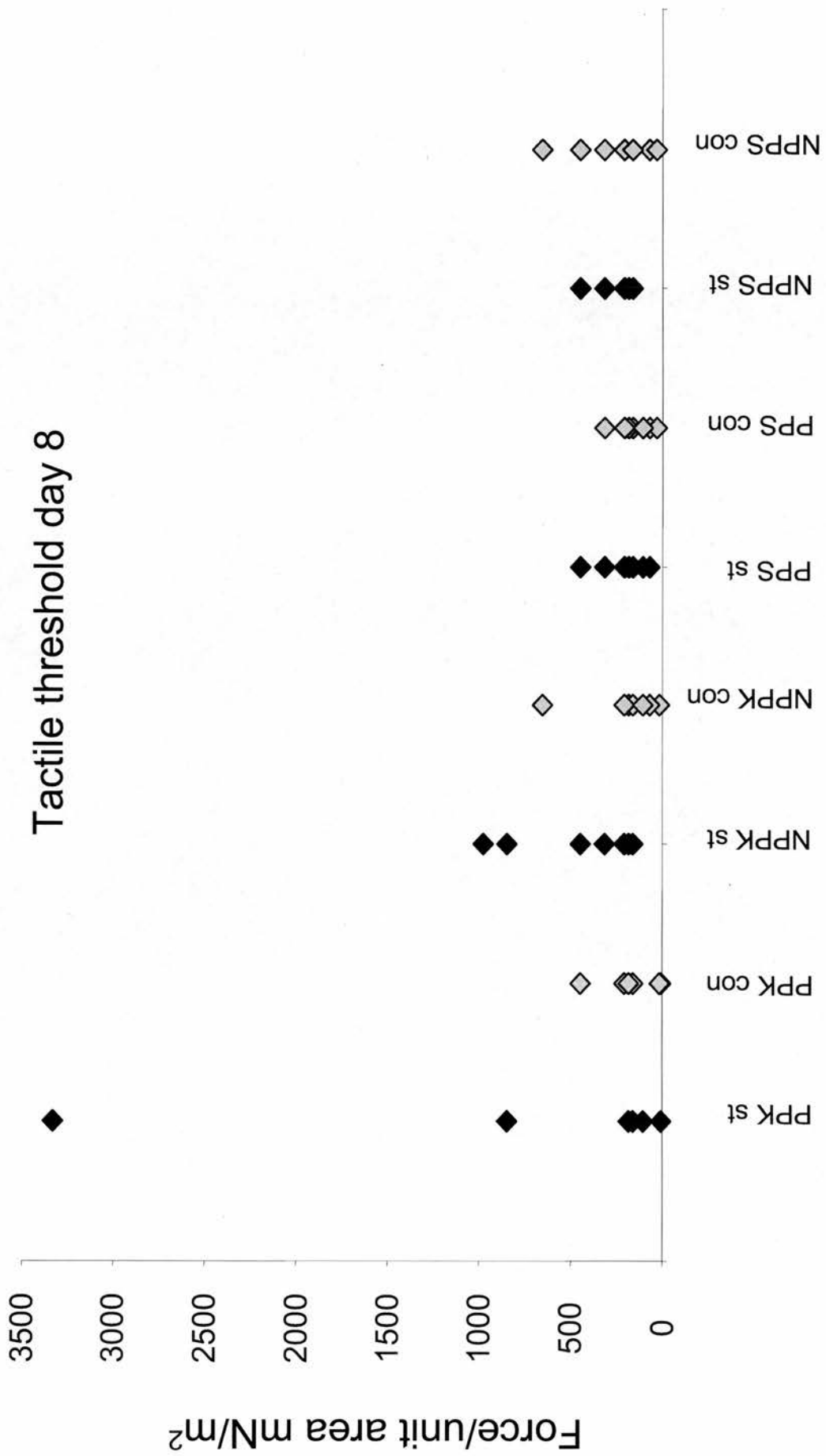


Figure 21

VAS Score pin 8 days

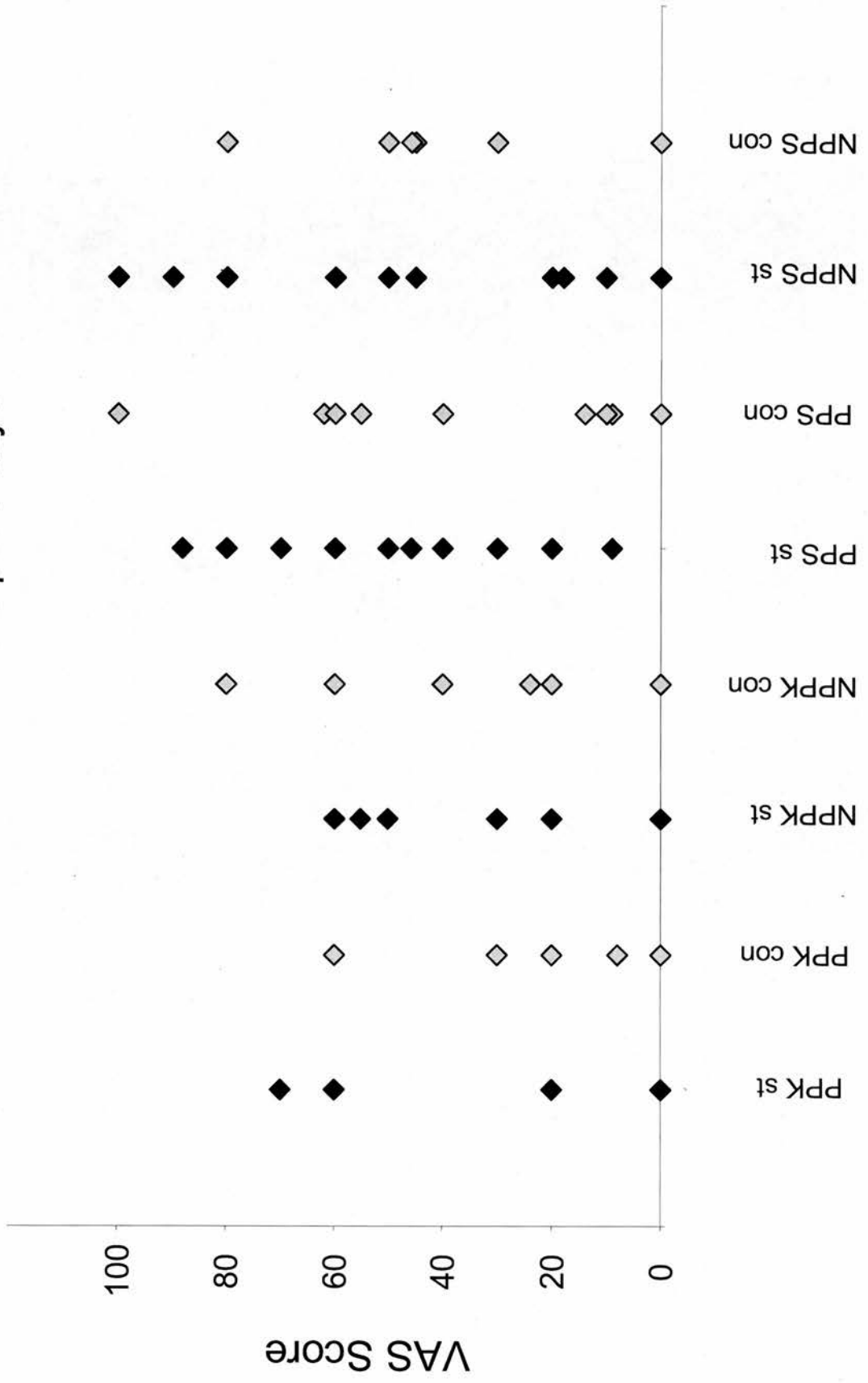


Figure 22

4.4 Discussion

This study has found that modulation of sensory input at the time of amputation may have both short and longer term effects on pain perception.

The anaesthetic/ analgesic technique used in our study has resulted in a lower incidence of persistent pain than other randomised controlled trials of lower limb amputation where around 70% of patients have persistent pain (236;241). A wide range of incidences for post-amputation pain has been found (179;210)), with lower incidences in particular types of study (retrospective or postal survey) and patient groups (paediatric or upper limb amputation) compared to lower limb amputation (183-185;197;366). Prospective data does not identify age, gender, level of amputation or reason for amputation as a risk factor for post-amputation pain (211).

A key difference in our study is the anaesthetic technique used compared to previous studies. This combined spinal/ epidural technique, without use of general anaesthesia or opioids produces a very dense block of sensory input that may provide a novel strategy for reducing persistent pain after amputation. No other published clinical trial has used either a spinal anaesthetic or a combined spinal epidural technique. The study was carefully designed to ensure that it was appropriately randomised and double blind. The sample size of the study is extremely important. Interim analysis was carried out to refine initial sample size. This was to see if the original sample was adequate or whether a small increase would be appropriate. It was important to not to miss a positive result due to an under powered study while at the same time it was going to be difficult to continue recruitment for much longer than two years using one researcher. In addition to primary outcomes the study design allowed for secondary outcome measures such as mood. The trial was designed and completed before the IMMPACT consensus guidelines in chronic pain study design were published (259). Of the six recommended outcome measure three were well addressed namely pain and pain severity, adverse incidents and patient deposition. The HADS score gives a useful measure of emotional distress. Despite this, in the categories of physical function and emotional function more information might have been collected to improve the quality of the study design. Questionnaires such as

Brief Pain Inventory (BPI; measuring physical functioning) or Beck Depression Inventory (BDI; emotional functioning), with retrospect, should have been used. The category of 'global impression of change' was not relevant to this study type and therefore appropriately not addressed. That said the study outcomes were well designed in keeping with similar studies at that time.

The initial hypothesis was that specific blockade of the NMDA receptor would be required to reduce persistent pain. This was based on evidence from basic science studies of the key role that the NMDA receptor, plays in central sensitisation and subsequent spinal events in pathological pain states (367;368). Failure of previous studies to reduce persistent pain may have been related to inadequate block of the effects of massive glutamate release and excitotoxic discharge at the time of nerve injury (91;369). No previous study has used a spinal anaesthetic which could be expected to completely block (at least at the time of nerve transaction) afferent neuronal input from the lower limb(357). Clinically, there is also some evidence for involvement of the NMDA receptor in central processing in patients with established phantom pain (161;370). Intravenous ketamine has been used peri-operatively at the time of lower limb amputation, but no acute effect on central processing was found, nor a statistically significant reduction in phantom limb pain at 6 months, although phantom pain was 47% in the ketamine group and 71% in the control group (241).

The potential neurotoxicity of spinal ketamine (either epidural or intrathecal) must be considered. Firstly, there is concern that preservatives in commercially available compound may be neurotoxic, as has been demonstrated in sub-human primates and rabbits (371-373). Secondly, although preservative free ketamine widely used in some European countries, there have been concerns both about a direct toxic effect specifically related to the action of NMDA antagonists and an increased rate of apoptosis during neuronal development. The direct toxic effect has only been found selectively certain cortical neurons in rodents (cingulate and retrosplenial cortices), with both toxicity and apoptosis only seen with high doses, unlikely to be used in clinical practice (374-376). Additionally, repeated intrathecal injections of up to 1%

of preservative-free ketamine (377) or repeat epidural boluses of ketamine in dogs (378) had no neurotoxic effect.

Recent systematic reviews of ketamine have found no clinical evidence of neurotoxicity from epidural use, with some benefits in analgesia (360).

One reason for our study not detecting an additional effect of ketamine, over and above local anaesthetic alone, may be that, due to the much lower overall incidence of persistent pain, the power of the study to answer the original hypothesis was reduced. It may be however, that the key element to reducing persistent pain is the blockade of spinal sensory transmission (either NMDA receptor-specific or non-specific blockade). Previous studies have found that neither general anaesthesia, acute opioid use, nor prolonged sensory block, with epidural analgesia before or after surgery, were sufficient to reduce persistent pain (236;241). In order to adequately reduce the massively increased peripheral input at the time of nerve injury, it would be necessary to reduce sensory input to such an extent that spinal responses were prevented. It has been shown previously that local anaesthetic administered via the epidural route does not completely suppress neuronal input to the spinal cord (358). There is also recent evidence from animal models that electrical stimuli well below that required to elicit spinal cord activity, may evoke neuronal activity within the brainstem, as assessed by fMRI (379). While electrophysiological recordings of spinal input were not made during our study, the combined spinal/ epidural technique did provide a significant block of sensory input such that amputation was performed without general anaesthesia or other analgesia such as strong opioids. This blockade of sensory input was associated with an incidence of persistent pain of 50% or less in both groups in our study.

The timing of NMDA receptor blockade may be important in its effect on the neurobiological response, as some studies have found that NMDA receptor antagonists did not affect phantom or stump pain, or responses to mechanical and thermal stimuli, and cortical changes, in established post amputation pain (158;162)), with one small study of traumatic upper limb amputates finding that acute administration of memantine (in addition to brachial plexus blockade) reduced

prevalence and severity of phantom limb pain up to 6 months but not one year afterwards (234). Other studies in animal models have also found an anti-allodynic effect of NMDA receptor antagonists that outlast their expected pharmacological duration of action (380), with selective effects on different aspects of pain behaviours (144;359)

There were limitations to the QST used in this study. The need for easy to use bedside tests limited what could be achieved. In addition QST methodology has advanced significantly since the study was designed. The developments in QST have been discussed in detail in chapter 2 (section 2.4.4). Few previous studies have attempted to use sensory testing to assess the alterations in sensory processing that might be seen following amputation. An attempt has been made to classify phantom phenomena according to changes in peripheral sensory function (286). Sensory abnormalities varied and no clear pattern of abnormalities in sensory processing emerged. This study measured tactile threshold using very similar methodology to our study. Tactile threshold did not differ, as in our control group, between the stump and the contralateral limb. Increased sensitivity was, however, found on the scar. A previous preventative study comparing epidural bupivacaine and morphine with conventional analgesia assessed post-operative sensory processing (285). This included pressure pain, VF hairs to detect touch and pressure thresholds, thermal pain, allodynia and wind up. No differences in sensory processing were detected between the groups. Our hypothesis was that, due to importance of the NMDA receptor in sensitisation, an NMDAR antagonist would be needed have an effect on sensory processing. Despite no additional reduction in persistent pain in the ketamine group, there were alterations in pain processing, with significant improvements in acute post-operative analgesia. Specific effects on sensory processing were also detected, with a selective decrease in mechanical sensitivity on the stump that outlasted the expected pharmacological action of ketamine (381), similar to that seen in phantom and stump pain (361). Ketamine can also alter sensory processing in chronic phantom pain (382). Ketamine treatment alters pressure pain thresholds but not affect electrical or thermal pain. As discussed above (chapter 3) we have found that treatment with NMDA antagonists prior to nerve

injury in a rodent model, resulted in an enhancement of subsequent responses to NMDA antagonists, particularly responses to mechanical stimuli (383). There is also evidence of inhibitory pathways mediated by metabotropic glutamate receptors type II/ III in neuropathy models, with a complex relationship between the ionotropic NMDA receptors and metabotropic (246;384). This may partly explain the reduced mechanical sensitivity, although the precise mechanism requires further study. These results would support a role for the NMDA receptor in acute central sensitisation as well as providing clinical evidence supporting the role of epidural ketamine in modifying pain processing acutely.

There is still debate as to the role of ketamine as part of multimodal post-operative analgesia. Systematic reviews of ketamine found that ketamine could reduce pain scores and opioid consumption, with minimal adverse effects (154;155). A systematic review of another clinically available NMDA antagonist, dextromethorphan, also found a reduction in opioid consumption, but no effect on post-operative pain scores (385). The route of administration may be important, with epidural being superior to systemic ketamine after thoracotomy, while another study found no effect of intravenous ketamine acutely or at 6 months post lower limb amputation (241;386). By giving ketamine, close to its putative site of action on nociceptive pathways in the spinal cord, an adequate dose may be given without significant adverse effects. Our study provides evidence of the efficacy of epidural ketamine as part of an acute analgesic strategy with some detectable effects on acute sensory responses. An additional benefit, particularly in an elderly population, is the lack of sedation or respiratory depression produced by using a technique without a requirement for opioid in the peri-operative analgesic regimen.

The questionnaire data did not reveal any useful differences between groups and score in all outcome measures fell with time. A study investigating coping strategies of amputees with phantom pain found mean (SD) total MPQ scores in two study groups of 31.2 (14.6) and 35.6 (15.1) (387). Similar mean values of about 30 were found in a study of back pain patients (388). This was similar in value to our preoperative values, though we have expressed them as median values. Nikolajsen et

al. found a pre-amputation median score of 18 with median values of 11.0, 11.5 and 13.6 at one week, three months and six months respectively following amputation (in patients who experienced phantom pain) (192). Our study found very low levels at 12 months in both groups. The neuropathic pain scale has mostly been used for monitoring responses to treatment to neuropathic pain rather than distinguishing between neuropathic and non-neuropathic pain. It has been recently shown to be effective in distinguishing between neuropathic and non-neuropathic pain (278). This study found total mean scores of 43.2 and 63.49 in non-neuropathic and neuropathic pain respectively. Median pain scores in both groups in our study were very low at all post-operative time points suggesting that neuropathic pain did not predominate. The scale might have been better used in a study that was monitoring the response to pain rather than as a global measure of post-operative pain. The HADS score is a useful screening tool in looking for emotional distress in a population of patients. The cut off score for both anxiety and depression is 8 (274). The mean scores in both groups in the post-operative period was below 8 at all time points for both groups suggesting that a low incidence of emotional distress occurring despite the loss of a limb.

Further study of the unexpected finding of ketamine in reducing anxiety and depression is warranted. The acute, and often dose-limiting, effects of ketamine on mood, such as dysphoria and increased anxiety, were not found in our study, which may relate to the route of administration. Improvement in mood has been found with ketamine after peri-operative use in depressed patients (389), and acutely in the treatment of major depression (390-392). No previous study has found a positive long-term effect of short-term ketamine administration on levels of anxiety and depression (393;394).

Future research in this area needs to focus further on the use of spinal anaesthesia at the time of amputation. The role of the NMDAR antagonists is not clear but memantine given for a pronged period does seem to produce a short term reduction in the incidence of post amputation pain (234). The length of time that the NMDAR antagonist is administered may be very important. In order to prevent or reduce

central sensitisation, any intervention must continue into the post-operative period (229). 48-72 hours of epidural ketamine may not be sufficient, but realistically this is as long as is practically possible in a clinical situation. Ketamine can be given by alternative routes e.g. using the oral or nasal route (395). It seems well tolerated and this might give an option to carry out continue the drug administration well into the post-operative period (4-6 weeks). Alternatively NMDARs such as dextromethorphan or amantidine, which exist as oral preparations could be tried as a long term suppressor of central sensitisation. Newer NR2B specific antagonists targeted at the spinal NMDAR receptors may have a role to play. Traxoprodil has NR2B subunit selectivity. It has been used in clinical trials of brain injury but no trials yet exist in pain treatment or prevention (323).

In conclusion, it has been suggested that a combination of general and regional techniques is needed to reduce central sensitisation (356). However, this may not be the case, with the key element being modulation of sensory transmission around the time of nerve injury. A combination of specific (ketamine) and non-specific agents (local anaesthetic) administered close to the spinal cord can alter acute and chronic pain processing. It may be that prolonged (several weeks) administration of the NMDAR antagonist is needed. The NMDA receptor has a defined role in the development of pain, but further study is needed to clarify the role of the NMDA receptor in persistent phantom limb pain.

Chapter 5

Summary and conclusions

This thesis demonstrates the importance of the NMDA receptor in neuropathic pain syndromes and explores its potential therapeutic uses.

Animal experiments, using a CCI model of neuropathic pain, have demonstrated the effectiveness of the NMDA antagonists, memantine and ketamine, in attenuating typical behavioural responses to nerve injury. In addition the NR2B selective compound Ro 25-6891 similarly attenuates these effects. Furthermore I have shown that in animals that have had pre-treatment with an NMDA antagonist, there is a subsequent increased susceptibility to treatment with NMDA antagonists. The expression of the NR1 and NR2B subunits alters following pre-treatment with NMDAR antagonists, with no change in NR2A expression. The expression of NR2B seems to be elevated ipsilateral to the CCI. Pre-treatment with NMDAR antagonists blocks this elevation and might account for the increased subsequent susceptibility to treatment seen with various NMDAR antagonists.

A clinical study was designed to mirror the animal experiments and investigated whether the promise shown by NMDA antagonists can be translated into clinical practice. Patients scheduled for lower limb amputation are a patient group that can be identified before nerve injury occurs making them an ideal group to study. Ketamine was given epidurally, close to its proposed site of action on spinal NMDARs, before the nerves were cut in surgery. Ketamine was shown to be an effective adjunct to plain local anaesthetic and to have a long lasting (up to one week) effect on sensory processing in the remaining stump. Disappointingly in clinical practice 48-72 hours of peri-operative ketamine made no difference to the primary end point of the incidence and severity of post-amputation pain.

One other RCT has used peri-operative ketamine in an attempt to reduce the incidence and/or severity of post-amputation pain. Hayes at al used an intravenous infusion of ketamine, preceded by a pre-induction bolus of ketamine (241). The infusion ran for 72 hours. No statistical difference was found in either incidence or

severity of post-amputation pain. The incidence of phantom pain was lower in the treatment group at 6 months which might indicate that the study was either insufficiently powered or perhaps the ketamine might need to be given for a longer period. Interim analysis on our own data indicated that it would not produce a positive result even if the sample size was substantially increased. In clinical practice it would be unrealistic to have parenteral drug administration for more than a few days and thus if the length of time is important then an alternative NMDAR antagonist designed for oral use might be better. Schley et al. treated traumatic upper limb amputees for four weeks with the orally available NMDAR antagonist memantine (20-30mg per day, in addition to 7 days of a continuous ropivacaine brachial plexus block). No long term effect on the prevalence and severity of phantom pain was found, although short term (up to 6 months) effects were demonstrated (234).

Our own study attempts to translate promising animal data into clinical practice. Our own basic science experiments demonstrate the potential usefulness of NMDAR antagonists on preventing neuropathic sensitisation and how important spinal NMDARs are. Ketamine was given epidurally, in the clinical study, based on the hypothesis that administration in the proximity of spinal NMDARs would optimise efficacy and translate into a clinically useful effect with minimal side-effects. No major side-effects were seen showing the technique to be well tolerated. Secondary outcome measures based on QST were chosen. These allowed demonstration of the prolonged effect (longer than pharmacological effect) that ketamine may have on sensory processing.

The failure to translate the promise shown in the basic science experiments was very disappointing. This is a problem often seen in pre-clinical experiments. There are a number of reasons why this may be, both in general terms and in our own particular studies. In the human pain is felt as a consequence of nociceptive activity in the nervous system, which is communicated as an emotional and sensory experience. Human brains differ considerably from rats' brains in the volume devoted to higher function. The human forebrain makes up 85% of the volume of the human CNS, with

the spinal cord making only 2%. In rats the percentages are 44 and 35% respectively (396). Human forebrains play a large part in the perception and in descending modulation of pain. This may not be mirrored in the rat model. Although nerve injury models are well validated and accepted they do differ from clinical practice in a number of regards. In the first instance they are all based on damage to the nerve rather than total nerve transection as occurs in the clinical situation. The animal models are very reliable and reproducible. In effect this means that most, if not all, animals subjected to the injury will develop the associated pain behaviours. If left long enough (several weeks) these changes resolve. In contrast not all humans develop neuropathic pain. This is regardless of the cause, i.e. amputation, post herpes infection, diabetic neuropathy etc. Importantly, although some cases do spontaneously resolve with time, the majority do not. Testing in animal models of neuropathic pain depends in large part on tests of sensitivity (allodynia/hyperalgesia) to a mechanical or thermal stimuli (163). Work is being done to try to focus on other measures of pain such as observed behaviours which may better reflect actual pain. Measurements of activity, such as vocalisation grooming, spontaneous paw lifting, analgesic administration etc. have been suggested (163). This may imply that although the models are very reliable, they are probably not exactly mirroring the clinical situation seen in humans. In the clinical situation, neuropathic pain symptoms such as spontaneously occurring burning pain are much more frequent and intense than evoked thermal pains suggesting that although the models are valuable they do not yet fully mimic human pain (397). Work is continuing to develop improve pain models and make them more like human conditions (164).

A major strength of this thesis is the combination of a preclinical model of pain and a clinical research project. The research investigates the use of NMDAR antagonists in the animal model and then tries to replicate this as far as possible in a clinical trial. Great care was taken to try and ensure that the two studies mirrored each other as far as possible. This type of translational research is becoming the more common but at the time of this research was rare.

The recruitment, study throughput and primary outcome of the presence and severity of post-amputation pain were well conducted. Even in big centres, recruitment of patients to this type of study is difficult. Patients are often elderly and have significant medical and cognitive problems that prevent inclusion. The rate of recruitment was good and overall compare very favourably with other larger trials in this area. The weaknesses in the thesis mostly stem from some of the secondary outcomes in the clinical trial. IMMPACT guidelines have focused clearly on the core outcome variables needed in trials of chronic pain. Every attempt was made to include outcomes other than pain, but with the benefit of hindsight, perhaps some refinement of the questionnaires chosen should have been done. The QST methodology was chosen to be easily and cheaply accomplished at the bedside or even the patients home. QST guidelines are constantly developing in my own centre and others. Further refinement of the protocol would be done if the study was to be repeated or a similar trial contemplated. The deficiencies identified here do not detract from the primary outcome, which was the incidence and severity of post-amputation pain.

In conclusion this thesis demonstrates not only the significant role that ionotropic NMDA receptor antagonist has in the establishment of neuropathic pain states but the potential therapeutic roles that they might deliver. The pre-emptive effect of NMDAR antagonists in a preclinical model is clearly demonstrated. The promise shown in these studies fails to translate into clinical practice. Although there was a failure to prevent post-amputation pain, there was a prolonged effect on sensory processing which warrants further research.

Future research needs to focus on development and improvement of animal models. There is likely to be a move away from using evoked responses as the sole outcome. Efforts will (and are) being made to measure spontaneous pain using behavioural observations in addition to evoked responses. There is opportunity to develop less observer dependent outcomes such as electroencephalogram (EEG) or functional MRI in animal models (164). Clinical studies often use analgesic consumption as a surrogate measure of pain and this can be used in the preclinical setting (398).

Analgesic drug can be given in 'on demand' fashion using a lever and give a similar outcome measure to the clinical situation. Emotional functioning is a key outcome measure in clinical trials of chronic pain (259). Emotional outcome can in fact be measured in animal models and this may become a relevant area of development (399).

QST as an outcome measure will become more refined. The German neuropathic pain network has made great strides in this direction, suggesting detailed protocols and reference values (244;281). There has been a long identified need to push forward on a more mechanistic approach to pain (279). QST has the potential to deliver in this area, but at present QST protocols are time consuming (often one hour or more) and there is still a way to go before detailed QST becomes a relevant 'bedside' test in the pain clinic (400).

Future research projects, using clinically available NMDAR antagonists, might include the long term (up to 6 weeks) use of these drugs at the time of nerve damage in conditions such as herpes zoster. This may lead to a reduced incidence of persistent pain but also may make those with established pain more susceptible to subsequent treatment with NMDAR antagonists. There is a need to investigate the use of clinically available NR2B subunit receptor antagonists. Traxoprodil has been used in clinical studies of brain injury but no data has been yet published in pain conditions. This is despite it being apparently well tolerated and could be taken orally (321;323;401). Haloperidol, a clinically available NR2B antagonist, is more likely to have significant side-effects and this may, as in the case of ketamine, limit its potential therapeutic use.

Finally there is a need for greater collaboration between basic science researchers and clinicians. Large translational research networks need to be set up that can rapidly move promising new compounds into clinical trials, while at the same time using clinical experience to refine laboratory methodology so that compounds more likely to deliver in the clinical setting are identified. Governments, nationally and internationally need to promote these collaborations and ensure a coordinated

approach to research, ensuring good clinical outcomes, patient safety and cost-effectiveness (402).

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Appendix 1

Phantom limb study, Questionnaire

Name:
Address:
.....
.....
Affix label

DOB Age:

Height:cm Weight:Kg

Leg to be amputated: L / R

Do you have pain in the limb? Y / N

Where is the pain in the limb?
.....

Verbal description of the pain
.....

VAS score:

a) How long have you had pain in the limb?

Current medications, Both analgesic and non analgesic

List of medications

Post operative questions

Time following surgery?

Do you get pain in the stump? Y / N

If so, how often does it occur?

- i. Few times per hour
- ii. Few times per day
- iii. Few times per week
- iv. Few times per month
- v. Few times per year
- vi. All the time

VAS score for stump pain

Treatments or medicines for pain in your stump? Y / N

If so, give details

.....
.....
.....

Do you have sensations from the part of the limb that has been amputated, i.e. phantom sensations (not pain)

If these sensations were painful , how strong would you say that these were on a scale of 0-10?

Do you have pain in the any pain in the part of the limb that has been removed?

If so, how often does it occur?

- i. Few times per hour
- ii. Few times per day
- iii. Few times per week
- iv. Few times per month
- v. Few times per year
- vi. All the time

When the pain comes how long does it last?

- i. Seconds
- ii. Minutes
- iii. Hours
- iv. Days
- v. Weeks
- vi. Months
- vii. Variable

From which part of the phantom do these pains seem to come from?

What do they feel like?

VAS score worst pain :.....
VAS score least pain :.....
Vas score usual pain :.....

Have you seen a doctor regarding your pain? Y / N

What treatment did you receive

.....
.....

Was it successful Y / N

Appendix 2

Papers published from this research

Wilson JA, Garry EM, Anderson HA, Rosie R, Colvin LA, Mitchell R, Fleetwood-Walker SM.

NMDA receptor antagonist treatment at the time of nerve injury prevents injury-induced changes in spinal NR1 and NR2B subunit expression and increases the sensitivity of residual pain behaviours to subsequently administered NMDA receptor antagonists. *Pain* 2005 117:421-32.

Wilson JA, Nimmo AF, Fleetwood-Walker FM and Colvin LA

A randomised trial of the effect of pre-emptive epidural ketamine on persistent pain after lower limb amputation *Pain* 2008 135:108-18



NMDA receptor antagonist treatment at the time of nerve injury prevents injury-induced changes in spinal NR1 and NR2B subunit expression and increases the sensitivity of residual pain behaviours to subsequently administered NMDA receptor antagonists

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Received 2 May 2005; accepted 12 July 2005

Abstract

Spinal NMDA receptors (NMDA R) are important in neuropathic sensitisation and acute administration of antagonists can provide temporary attenuation of sensitisation. If establishment of the chronic pain state could be prevented by brief administration of such agents at or around the time of nerve injury (pre-emptive analgesia) it might be possible to avoid many of the unacceptable side effects associated with repeated administration of these or other antagonists. Several reports describe aspects of effective pre-emptive analgesia from NMDA R antagonists in animal models of neuropathic pain. The first aim of the present study was to make a direct comparison of changes in mechanical allodynia, cold allodynia and thermal hyperalgesia following nerve injury, demonstrating their increasing degree of susceptibility to pre-emptive NMDA R antagonist treatment. Secondly, we used immunoblotting and immunohistochemistry to investigate the effects of nerve injury on NMDA receptor subunit expression, revealing increased expression of NR2B, but not NR2A and reduced NR1 in the superficial dorsal horn. These changes were attenuated following NMDA receptor antagonist pre-treatment. Thirdly, we investigated the pharmacological properties of residual mechanical allodynia and cold allodynia that remained after pre-emptive treatment and revealed a greater sensitivity to NMDA R antagonists. These findings indicate that in addition to a marked suppression of thermal hyperalgesia and cold allodynia, pre-emptive treatment with NMDA R antagonist causes a lasting change in spinal NMDA R complexes such that remaining mechanical allodynia should be more effectively targeted by NMDA R antagonists.

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Keywords: NMDA receptor; Pre-emptive analgesia; NR1 and NR2B subunits; Neuropathic pain; Spinal

1. Introduction

Peripheral nerve injury often results in neuropathic pain, characterised by hyperalgesia (increased pain elicited by noxious stimuli), allodynia (pain from normally innocuous

stimuli) and spontaneous pain, which are difficult to treat (Arner and Meyerson, 1988; Galer, 1995; Kingery, 1997). The mechanisms underlying neuropathic pain are unclear, involving changes in afferent and central spinal sensory relays, leading to neuronal hyperexcitability (Kohama et al., 2000; Laird and Bennett, 1993; Takaishi et al., 1996; Woolf, 1983). Central sensitisation crucially involves spinal *N*-methyl *D*-aspartate receptors (NMDA R) (Coderre and Melzack, 1992; Davies and Lodge, 1987; Dickenson and Sullivan, 1987; Leem et al., 1996; Sotgiu and Biella, 2000;

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Woolf and Thompson, 1991). NMDA R antagonists reverse neuropathic pain behaviours (Chaplan et al., 1997; Davar et al., 1991; Garry et al., 2003; Mao et al., 1992a,b, 1993; Tal and Bennett, 1993; Yamamoto and Yaksh, 1992), dorsal horn neuronal hyper-responsiveness (Carlton et al., 1998; Dougherty and Willis, 1991; Laurido et al., 2001; Leem et al., 1996; Suzuki et al., 2001) and human neuropathic pain (Kingery, 1997). Side effects can limit the use of NMDA R antagonists, although ketamine is effective (De Kock et al., 2001; Felsby et al., 1996).

In animal neuropathic pain models, spinally administered NMDA R antagonists are effective in reducing mechanical allodynia (Chaplan et al., 1997; Yamamoto and Yaksh, 1992) and reducing spinal neuron sensitisation (Laurido et al., 2001).

Evaluation of the effectiveness of pre-emptive NMDA R antagonists in preventing sensitisation following nerve injury has been hampered since different sensory tests were assessed in different animal models. Administration of NMDA R antagonists (memantine, MK801 or ketamine) for 3–8 days from chronic constriction injury (CCI), reduced development of thermal hyperalgesia (Davar et al., 1991; Eisenberg et al., 1995; Mao et al., 1992a; 1993). Similar observations have been made for mechanical hyperalgesia in the spinal nerve ligation (SNL) model (Carlton and Hargrett, 1995; Smith et al., 1994). Single bolus administration of intrathecal or systemic ketamine or memantine prior to SNL nerve injury attenuates mechanical allodynia from 6 h to 2 weeks (Burton et al., 1999; Carlton and Hargrett, 1995), or delays its development for 3 days in the CCI model (Hartrick et al., 1998). The timing of administration is important, attenuation of mechanical allodynia only being observed with pre-, not post-operative MK-801 (Wei and Pertovaara, 1999).

The two principal NMDA R subunits, NR1 and NR2, show differential expression in sensory pathways in spinal dorsal horn (Boyce et al., 1999; Luque et al., 1994; Petralia et al., 1994; Tolle et al., 1993; Yung, 1998). Single dorsal horn neurons may express multiple NMDA R subunit combinations (Karlsson et al., 2002), although the contribution made by specific subunits to neuropathic sensitisation remain unclear, despite selective NR2B antagonists reversing neuropathic pain behaviour (Boyce et al., 1999).

We compare the effects of pre-emptive NMDA R antagonists on different sensory reflex responses, on expression of spinal NMDA R subunits and explore whether there is altered sensitivity of residual pain behaviours to acute, spinal NMDA R antagonists in a neuropathic pain model.

2. Materials and methods

2.1. Partial chronic constriction injury (pCCI) to the sciatic nerve

Experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and follow IASP

guidelines. Adult male Wistar rats (200–350 g, Charles River, UK) were anaesthetised with intraperitoneal (i.p.) sodium pentobarbital (0.06 ml/100 g, Rhône Merieux, UK), supplemented with halothane/O₂ (Astra-Zeneca, UK). Under aseptic conditions, the right sciatic nerve was exposed in the region of the trifurcation and four 4/0 chromic gut ligatures (Ethicon, Edinburgh, UK) were tied to loosely constrict the tibial and peroneal nerves, but leaving the smallest, sural nerve intact (ie partial chronic constriction injury (pCCI), a variation of the chronic constriction injury, (CCI) (Bennett and Xie, 1988), as adapted previously for partial nerve injury (Garry et al., 2003). Sham-operated animals underwent the same surgical procedure, but no ligatures were placed around the nerve.

2.2. Assessment of behavioural reflex responses to mechanical, thermal and cold stimuli

Behavioural testing began prior to surgery, recommenced on day 5 following surgery and continued every 2 or 3 days until evidence of recovery was seen. In the experiments involving intrathecal injections, only nerve-injured animals that showed clear signs of thermal hyperalgesia, mechanical and cold allodynia were used for further study. Behavioural tests were carried out as previously described (Moss et al., 2002), using quantified, sensory stimuli delivered to the mid-plantar glabrous surface of the hind paw. Mechanical allodynia was assessed using calibrated von Frey filaments (Stoelting, Wood Dale, IL), measuring the threshold for paw withdrawal (PWT, mN/mm²) in response to graded mechanical stimuli. Thermal hyperalgesia was monitored as paw withdrawal latency (PWL, s) from noxious radiant heat (48 °C; Hargreaves' thermal device, Linton Instruments, Diss, UK). Cold allodynia was assessed as the suspended paw elevation time (SPET, s) in a shallow (4 °C) iced water bath (Moss et al., 2002). The behavioural responses obtained at each time point were calculated as the mean \pm SEM for each test and data were analysed by SigmaStat software (version 2.03). In tests of thermal hyperalgesia and cold allodynia, the ipsilateral value was compared to the contralateral value at each time point using a paired Student's *t*-test, then each compared to pre-surgery or pre-drug baseline using a One-Way repeated measures ANOVA, with post hoc analysis using Dunnett's test. In tests of mechanical allodynia, the ipsilateral value was compared to the contralateral value at each time point using a Wilcoxon test and each compared to pre-surgery or pre-drug baseline using a repeated measures ANOVA on ranks (Friedman's test) with Dunn's post hoc analysis.

2.3. Pre-treatment protocol for NMDA receptor antagonists

NMDA R antagonists, memantine (15 mg/kg in 0.5 ml saline, *n* = 10; Sigma-RBI, Poole, Dorset, UK), Ro 25-6891 (10 mg/kg in 0.5 ml saline with 45% hydroxypropyl-beta-cyclodextrin, *n* = 8; Sigma-RBI and Tocris, Bristol, UK), or vehicle (0.9% saline, 0.5 ml, *n* = 10; Sigma-RBI, Poole, Dorset, UK) were administered by intraperitoneal (i.p.) injection immediately prior to exposure of the nerve and repeated at the end of the first day and then twice a day for a further two days following surgery. Previous experiments showed no discernible effect of the vehicle used with Ro 25-6891 on nociceptive reflex responses.

Continuous lumbar intrathecal infusion of ketamine (Sigma-RBI), was provided by an implanted osmotic minipump (Alzet, 0.25 μ l per hour, total capacity 28 days, Alza Corporation, Palo Alto, CA, USA). Ketamine was dissolved in sterile 0.9% saline, pH 7.4, to give an infusion rate of 150 μ g/kg/h over 3 days. The day 3 volume of ketamine solution in the cannula was separated by a small volume of paraffin from saline filling the remainder of the cannula connected to the minipump. In this way, the infusion was switched from ketamine to saline after 3 days. Under sterile conditions, the minipump was placed between the interscapular muscles. The silicone cannula (12 cm) tip (Degania, Merck, UK) was passed through a small dural incision into the subarachnoid space to lie in the lumbar L3–L6 region, as previously described (Young et al., 1998). Only data from animals with L3–6 cannula placement (ascertained at the end of the experiment) and showing normal gait were used in analysis.

2.4. Acute intrathecal administration of NMDA receptor antagonists

All intrathecal injection experiments were carried out on pCCI animals, 16 days following surgery, at peak behavioural change. These animals had received pre-treatments of either vehicle ($n=8$), memantine ($n=10$) or the NMDA NR2B subunit selective antagonist, Ro 25-6981 ($n=8$) just prior to nerve injury and for the next 3 days. Baseline reflex withdrawal responses were recorded both ipsilateral and contralateral to nerve injury over 2 h prior to injection and mean values calculated. The effects of intrathecal administration of the highly selective NMDA R antagonist, 3-(2-carboxypiperazin-4-yl) propyl-1-phosphonic acid, ((R)-CPP), memantine, the NMDA NR2B subunit selective antagonists, Ro 25-6981 and ifenprodil, or vehicle (saline) were examined on behavioural reflex responses. Rats were briefly anaesthetised with halothane/O₂ and injected intrathecally at the L4–5 spinal segments with either (R)-CPP (1, 3 or 10 nmol, where 10 nmol was used as the standard dose in all figures; Tocris), memantine (150 nmol; Sigma-RBI), Ro 25-6981, (50 nmol; Tocris), or ifenprodil (25 nmol; Sigma-RBI), all in 50 μ l saline, using a 25 gauge needle microsyringe. The vehicle for ifenprodil was 0.3% dimethylformamide in saline (previous experiments have shown this has no effect on ipsilateral or contralateral nociceptive reflexes, following nerve injury). Behavioural reflex testing began 15–20 min post-injection (following recovery from anaesthetic) and was carried out until recovery to pre-injection values was observed. In the extensive tests comparing the acute sensitivity of mechanical allodynia to NMDA R antagonists in control or pre-treated rats, the percentage reversal of the pre-drug ipsilateral:contralateral difference in responses was calculated for each time point following drug injection. Statistical significance of differences in these values between control and pre-treated rats was assessed by Wilcoxon test.

2.5. Assessment of NMDA receptor subunit expression levels by Western immunoblotting

Immunoblotting was carried out using methodology described previously on tissue taken from animals under deep anaesthesia (halothane/O₂) at peak behavioural change (16 days later; Garry et al., 2003) or from sham operated or naïve animals ($n=6$ in

each case). Blots were probed with rabbit or goat polyclonal primary antibodies to NR1 (Cat No: SC-1467, Santa Cruz Biotech, CA, USA), NR2A (Cat No: 06-313, Upstate Biotech, Milton Keynes, UK) or NR2B (Cat No: AB1557P, Chemicon) and detected by peroxidase-linked secondary antibody enhanced chemiluminescence. The ubiquitous housekeeping enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was monitored as a control for protein level normalisation, using mouse monoclonal antibody (Cat No: MAB374, 1:750, Chemicon). Following quantitative densitometry, arbitrary grey scale values for receptor subunits were calculated as a percentage of that for GAPDH in each case and the statistical significance of injury-induced changes between the ipsilateral and the contralateral side, sham or naïve controls was assessed by Wilcoxon test.

2.6. Immunohistochemical studies on NMDA receptor subunit expression

Immunohistochemistry was carried out using a protocol similar to that described previously (Young et al., 1998), to assess the expression and localisation of NR1 and NR2B NMDA R subunits in the lumbar spinal cord. Rats with either vehicle ($n=3$) or memantine ($n=3$) pre-treatment (at the time of surgery for the pCCI injury) were studied at peak behavioural change (16 days later). Under deep anaesthesia (halothane/O₂), rats were perfused transcardially with saline containing 100 units heparin/ml, followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Lumbar spinal segments (L3–6) were post-fixed in 4% paraformaldehyde/0.1 M phosphate buffer for 4–5 h at 4 °C, before being stored in 0.1 M phosphate buffer at 4 °C. Transverse sections (70 μ m) were initially incubated in normal serum from the species which donated the secondary antibody, to block non-specific binding. They were then incubated overnight at 4 °C with either rabbit anti-NMDAR1 polyclonal antibody (Chemicon, 1:500) or rabbit anti-NMDAR2B polyclonal antibody (Chemicon, 1:750). Control sections were processed without the addition of primary antibody and incubated overnight at 4 °C in 0.1 M phosphate buffer. Simultaneous staining was carried out for all treatments. All sections were then incubated in biotinylated donkey-anti-rabbit IgG (1:200, 60 min; Chemicon), followed by incubation for a further 60 min with an avidin-biotin complex solution (ABC Elite, Vector Laboratories, UK, 1:50). Following wash, sections were exposed to 3,3'-diaminobenzidine tetrachloride (DAB; 0.2 mg/ml; Sigma, UK) in the presence of 3% hydrogen peroxide (1 μ l/ml) to enable visualisation of the precipitate, washed and mounted onto poly-L-lysine-coated microscope slides (BDH, UK), air-dried dehydrated through ascending concentrations of alcohol, cleared in xylene (Sigma) and mounted in DePeX (BDH, UK).

For relative quantification of immunoreactivity, an Improvision 1.44 Image Analysis Package (NIH) was used at a magnification of $\times 40$. Each image field was captured, using a CCD video camera (Sony, Japan), mounted on a Zeiss Axioscope microscope. The images were blank field-adjusted to compensate for any artefacts in the camera apparatus. A standardised region of interest (ROI; 80 (m diameter circle) cursor was aligned and consecutively centred on the mediolateral region of laminae I–II or III. Arbitrary grey scale units (in the range of 1–200) were assigned to make optimal use of the range for the given sample. Non-specific

background levels were recorded from the dorsal column region for each section, and the net density was calculated by subtraction. ROIs were taken in lamina I/II and lamina III from contralateral and ipsilateral sides and ventral horn motoneurons from 45 sections, for each experimental condition. Immunohistochemical data for NR1 and NR2B subunits were pooled for each animal and presented as the mean \pm SEM. Ipsilateral values were compared with the contralateral side in pCCI animals that had been pre-treated with either NMDA R antagonist or vehicle and statistical significance assessed by matched pair Student's *t*-test. Between treatment significance, was assessed by One-Way ANOVA with Dunnett's post hoc test.

3. Results

3.1. Neuropathic behavioural hyperalgesia and allodynia

Neuropathic sensitisation of behavioural reflexes, characteristic of the pCCI model (Garry et al., 2003), began to appear at 7–9 days following surgery, with peak changes in thermal hyperalgesia, cold allodynia and mechanical allodynia being observed by 16 days in all cases in vehicle-treated animals (Figs. 1 and 2 a, c, e). No consistent changes were detected contralateral to nerve

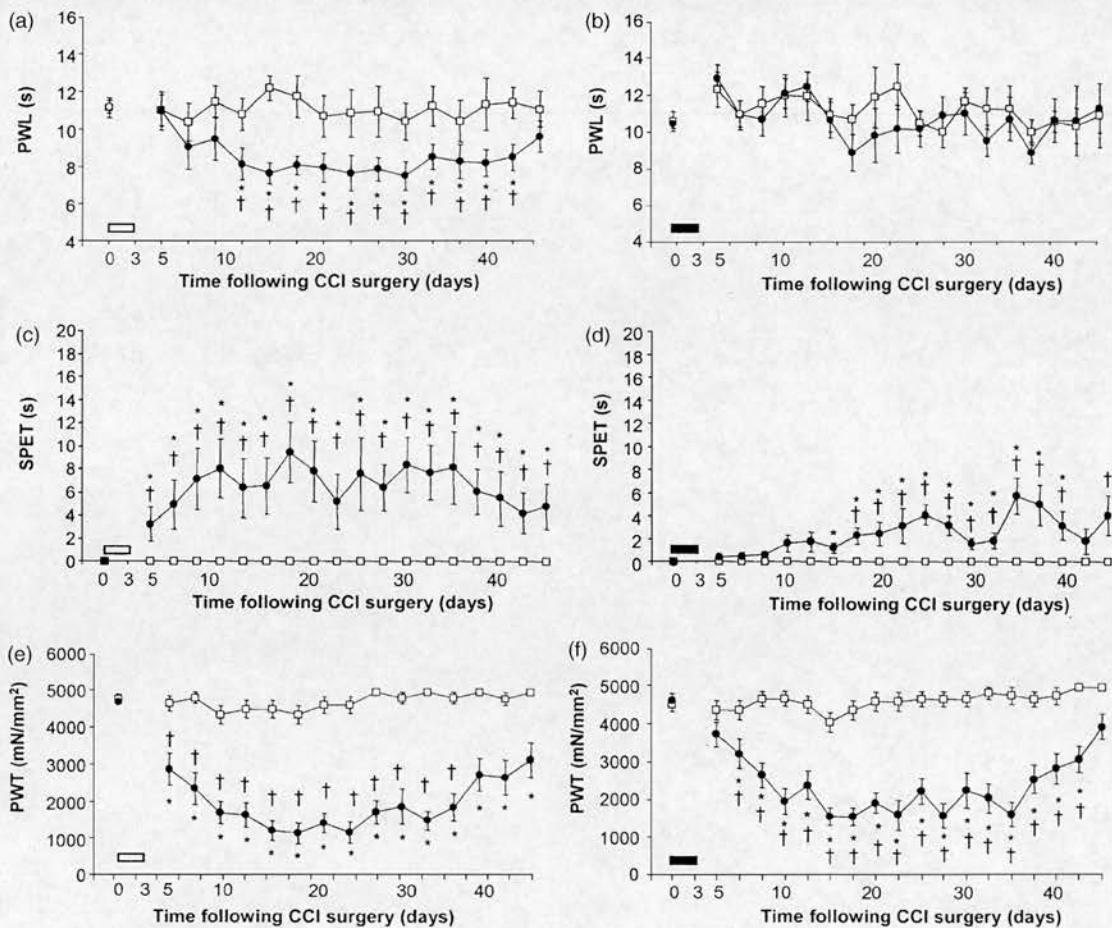


Fig. 1. The effect of memantine pre-treatment on the development of sensitised reflex withdrawal responses to sensory stimuli after nerve injury. Data show mean \pm SEM responses before (time zero) and following the induction of pCCI for both ipsilateral (\bullet) and contralateral (\square) hindlimbs ($n=10$). (a) In vehicle-pre-treated rats (\square), paw withdrawal latency (PWL, s) from a noxious thermal stimulus (Hargreaves' thermal stimulator) ipsilateral to pCCI showed significant differences between pre- and post-operative values ($\dagger p < 0.05$, Repeated measures ANOVA with Dunnett's post hoc analysis) and from post-operative, contralateral values ($*p < 0.05$ by Student's paired *t*-test). Memantine pre-treatment (\bullet), however, prevented the development of thermal hyperalgesia ipsilateral to nerve ligation (b). Memantine pre-treatment also significantly attenuated the development of responses to a noxious cold (4 °C water) stimulus. The Suspended Paw Elevation Time (SPET, number of paw lifts) in response to the cold stimulus is shown for the ipsilateral and contralateral paws in (c) vehicle-pre-treated and (d) memantine-pre-treated rats. Following nerve ligation, SPET scores for the contralateral paw was always zero for both vehicle and memantine pre-treated rats. Statistically significant differences are shown between pre- and post-surgery ipsilateral values ($\dagger p < 0.05$, Repeated measures ANOVA with Dunnett's post hoc analysis) and between post-surgery ipsilateral and contralateral responses, ($*p < 0.05$ by Student's paired *t*-test). However, some attenuation of SPET scores can be seen following memantine pre-treatment (c) compared to vehicle pre-treatment (d). In (e) paw withdrawal thresholds (PWT, mN/mm²) from calibrated von Frey filaments in vehicle-pre-treated rats showed significant differences between post-operative and pre-operative values on the side ipsilateral to pCCI ($\dagger p < 0.05$; Repeated measures ANOVA on ranks, Friedman's test with Dunn's post hoc analysis) and between post-operative ipsilateral and contralateral values ($*p < 0.05$ by Wilcoxon test). No significant differences were seen on the contralateral side following nerve ligation in either vehicle- or in memantine pre-treated rats. (f) Pre-treatment with memantine, caused no discernible change in the development of mechanical allodynia ipsilateral to nerve ligation compared to vehicle pre-treatment.

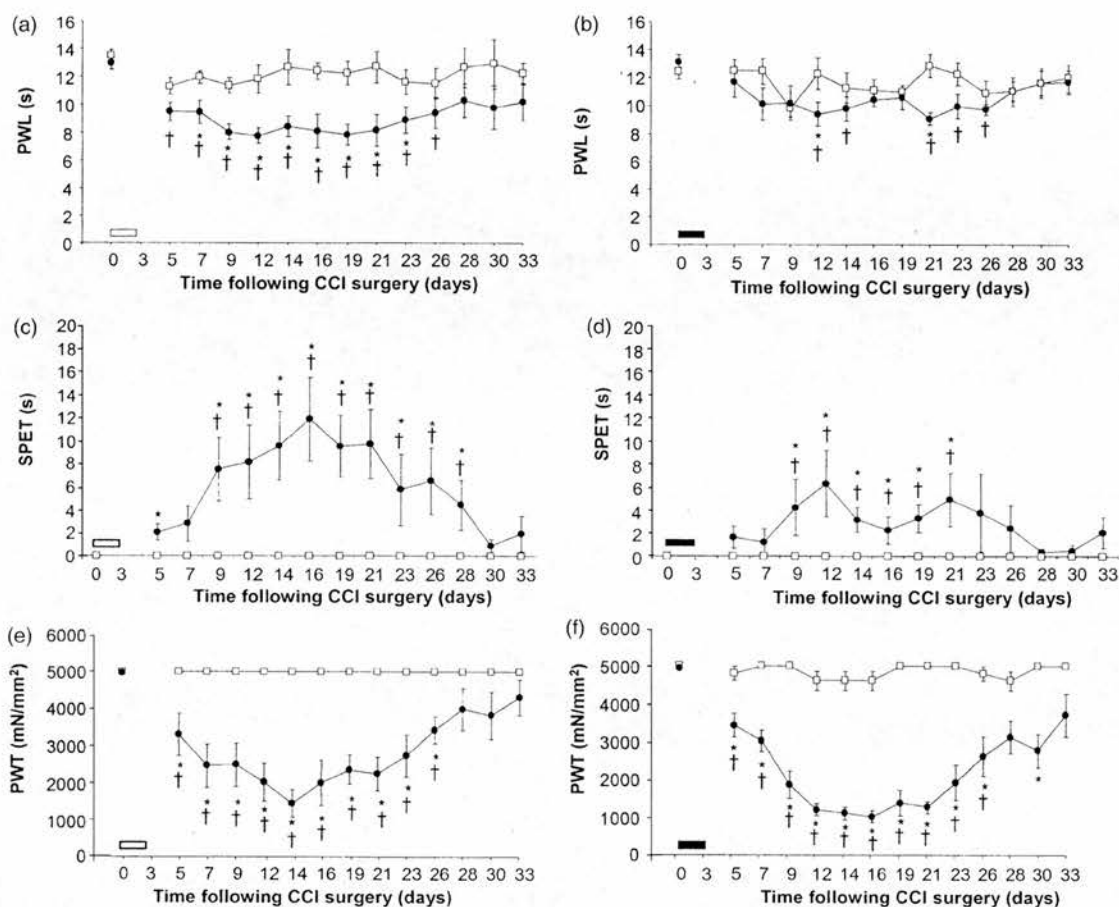


Fig. 2. The effect of ketamine pre-treatment on the development of sensitised reflex withdrawal responses to sensory stimuli after nerve injury. Data show mean \pm SEM values for PWL responses to a noxious thermal (Hargreaves' thermal stimulator) in (a, b), SPET to noxious cold 4 °C water in (c, d) and PWT to calibrated mechanical von Frey (e, f) stimuli before and following the induction of pCCI for intrathecal vehicle (a, c, e) or ketamine (b, d, f) administration over 3 days from the time of pCCI surgery. Statistical analyses were carried out as in Fig. 1.

injury. In each case the changes recovered to baseline levels by 43 days post-treatment. Rats that had undergone sham surgery showed no change from baseline.

3.2. Neuropathic thermal hyperalgesia and cold allodynia, but not mechanical allodynia, are substantially attenuated by NMDA receptor antagonist pre-treatment

Pre-treatment with non-competitive NMDA receptor ion channel blockers memantine or ketamine or a NR2B-selective antagonist, Ro 25-6891, gave similar results. Memantine (15 mg/kg, i.p.) given systemically twice daily over 3 days, beginning immediately prior to exposure of the sciatic nerve, completely prevented the reduction in paw withdrawal latency to noxious heat that occurs ipsilateral to nerve injury in saline-treated pCCI rats (Fig. 1b). Cold allodynia responses which are observed only in neuropathic animals, ipsilateral to nerve injury, were significantly reduced after memantine treatment to approximately half the magnitude of those in

vehicle-treated animals through the first 30 days following pCCI (Fig. 1d). In contrast, memantine pre-treatment caused little alteration in the reduced paw withdrawal threshold to von Frey filaments that occurs ipsilateral to nerve injury (Fig. 1f).

Fig. 2 shows similar studies in which another NMDA R antagonist, ketamine was administered locally, by intrathecal infusion over a 3-day period encompassing the time of nerve injury. Results were very similar to those with systemic memantine, with behavioural reflexes at 16 days after surgery showing abolition of thermal hyperalgesia (Fig. 2b), significant attenuation of cold allodynia (Fig. 2d) and no discernible effect on mechanical allodynia (Fig. 2f).

Interestingly, pre-treatment with the NR2B-selective antagonist, Ro 25-6891, given systemically over 3 days, beginning immediately prior to exposure of the sciatic nerve, also showed similar effects to memantine and ketamine, with the main residual sensitisation being mechanical allodynia (data not shown).

3.3. NMDA receptor antagonist pre-treatment selectively enhances the reversal of nerve injury-induced mechanical allodynia and cold allodynia caused by acute spinal administration of NMDA receptor antagonists, (R)-CPP, memantine or the NR2B subunit-selective antagonists, Ro 25-6891 or ifenprodil

Although pre-treatment with NMDA R antagonists blocks the development of thermal hyperalgesia, it is unknown whether any residual sensitisation, such as some cold allodynia and mechanical allodynia, displays altered susceptibility to pharmacological intervention using acute, spinally administered NMDA R antagonists. Here we assessed whether there were any alterations in the subsequent sensitivity to NMDA R antagonists with particular interest in the NR2B subunit, since selective

antagonists exert marked analgesic effects on sensitised behaviours following CCI (Boyce et al., 1999). The effects of memantine pre-treatment on subsequent sensitivity of mechanical allodynia to acute challenge with different types of NMDA R antagonist (either the general NMDA R antagonists, (R)-CPP, memantine, or the NR2B subunit-selective antagonists, Ro 25-6891 or ifenprodil) can be seen on Figs. 3a, b and 4a, b.

Although the degree of mechanical allodynia was essentially unaffected by memantine pre-treatment, there was a significantly greater extent and/or duration of reversal of mechanical allodynia by acute intrathecal administration of (R)-CPP, memantine, ifenprodil or Ro 25-6891. In vehicle pre-treated rats acutely administered (R)-CPP, memantine and ifenprodil each caused significant attenuation of ipsilateral allodynia and the extent of this reversal

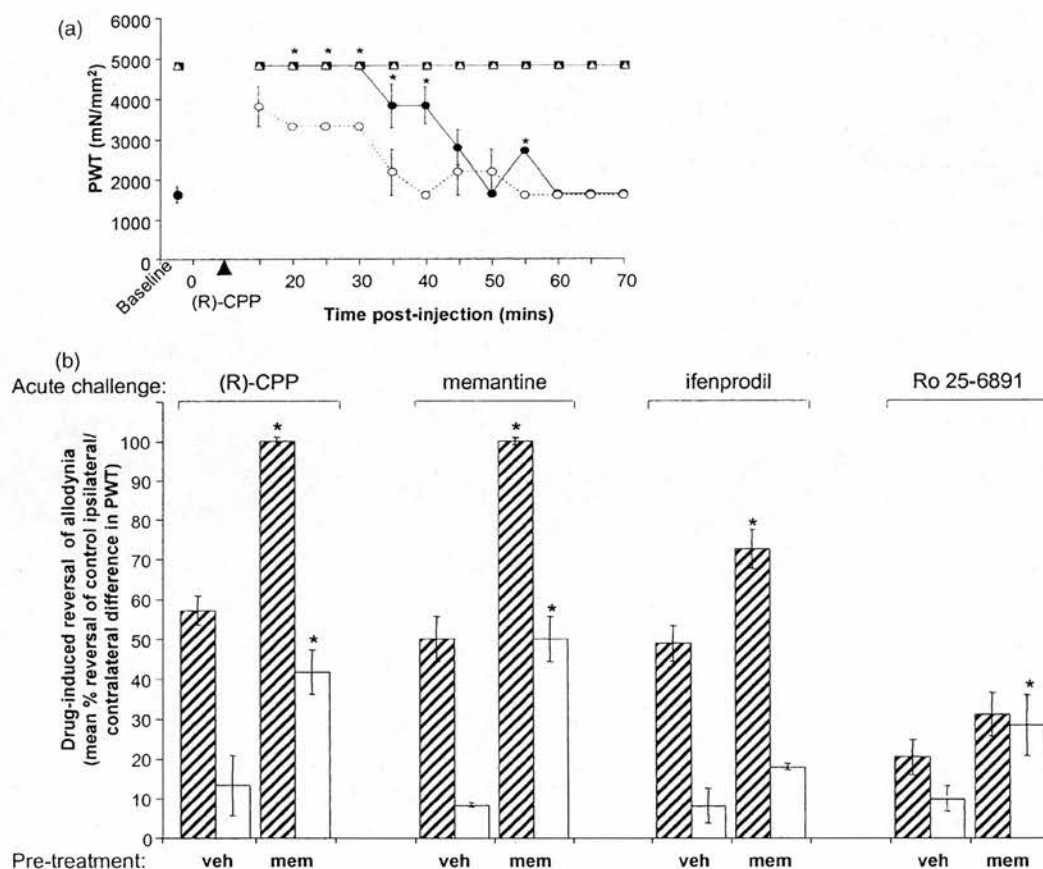


Fig. 3. The effect of memantine pre-treatment on subsequent sensitivity of allodynia to different types of NMDA receptor antagonist. Data in (a) and (b) reveal differences in the acute, spinally administered drug-induced reversal of mechanical allodynia (expressed as mean percentage reversal of the ipsilateral/contralateral difference in paw withdrawal threshold (PWT) \pm SEM, $n=8$) in vehicle or memantine pre-treated animals. (a) shows enhanced reversal by (R)-CPP of mechanical allodynia ipsilateral to injury following memantine pre-treatment compared to vehicle pre-treatment in nerve injured animals. *indicates statistically significant differences between control and memantine pre-treated animals in the extent of allodynia reversal by (R)-CPP, $p < 0.05$ by Wilcoxon test. There were no detectable effects of (R)-CPP on the contralateral hindlimb mechanical PWT. (b) Compares the effects of NMDA R antagonists, (R)-CPP, memantine and NR2B subunit-selective antagonists, Ro 25-6891 or ifenprodil on residual mechanical allodynia (either 15–30 min (striped bars) or 35–50 min (white bars) following intrathecal injection of drug), following either vehicle (veh) or memantine (mem) pre-treatment. The residual mechanical allodynia was more sensitive to acute injection of NMDA R antagonists or NR2B-selective antagonists in memantine pre-treated animals. Following memantine pre-treatment all drugs showed significantly enhanced extent or duration of reversal of mechanical allodynia (expressed as mean percentage reversal of the control ipsilateral/contralateral difference in paw withdrawal threshold (PWT) \pm SEM) compared to vehicle pre-treatment ($n=8$ in each case). *represents a significant ipsilateral to contralateral difference at each time point, $p < 0.05$ by Wilcoxon test.

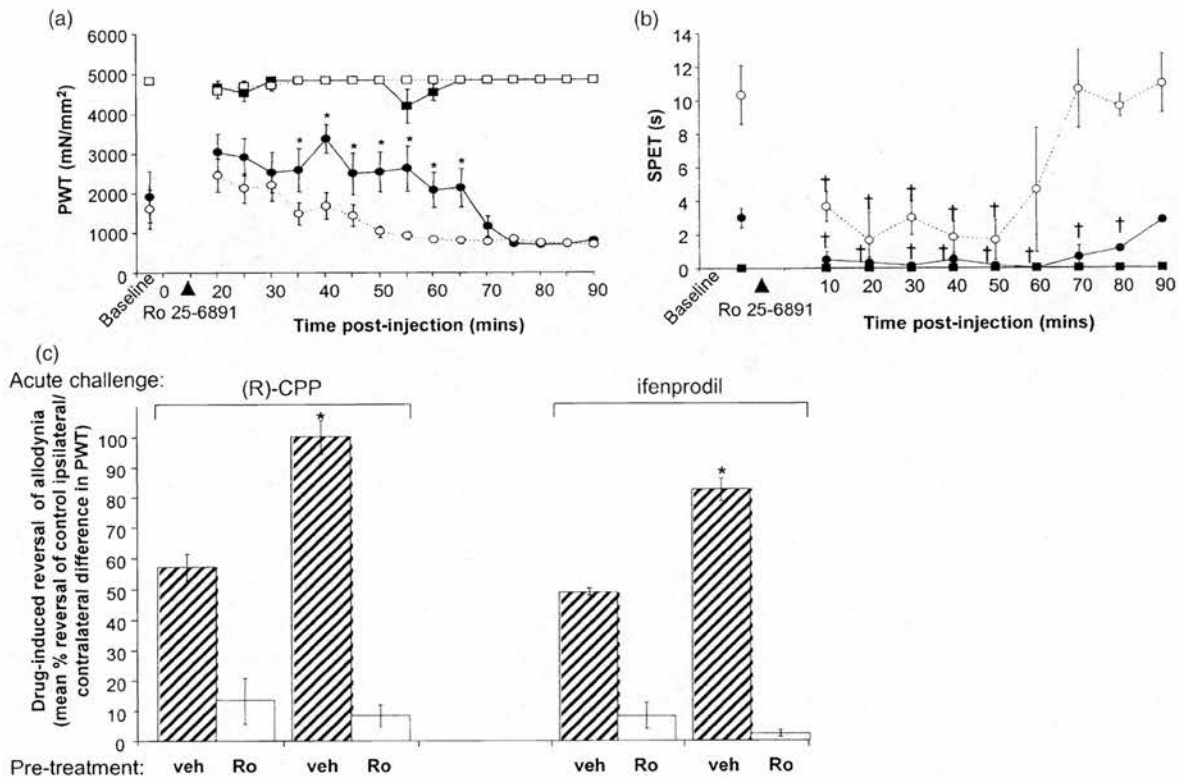


Fig. 4. A highly selective NR2B antagonist elicits inhibition of both mechanical and cold allodynia after memantine pre-treatment and can effectively substitute for memantine in the pre-treatment paradigm. Data in (a) are presented as mean paw withdrawal threshold (PWT) responses to graded mechanical stimuli (von Frey filaments). In (a), memantine pre-treated animals showed a marked prolongation of the Ro 25-6891-induced reversal of ipsilateral mechanical allodynia (●) compared to vehicle-treated animals (○). Statistically significant differences between memantine pre-treated and vehicle pre-treated animals in the extent of reversal of allodynia are shown as * $p < 0.05$ (by Wilcoxon test). In (b), memantine pre-treated animals (●) showed a clear prolongation (up to 30 min longer) of the Ro 25-6891-induced reversal of cold allodynia, compared to vehicle-treated animals (○; $n = 8$). Statistically significant differences between ipsilateral pre-drug and post-drug values are shown, † $p < 0.05$, Repeated measures ANOVA with Dunnett's post-hoc analysis. (c) When Ro 25-6891 (Ro) was substituted as a pre-treatment instead of memantine, acute intrathecal administration of (R)-CPP or ifenprodil caused enhanced reversal of mechanical allodynia, ipsilateral to nerve injury (expressed as mean percentage reversal of the control ipsilateral/contralateral difference in paw withdrawal threshold ((PWT) ± SEM, $n = 8$) compared to vehicle (veh) pre-treated animals. The striped and white bars show mean data from 15–30 min and 35–50 min periods following drug injection respectively. *represents a significant ipsilateral to contralateral difference at each time point, $p < 0.05$ by Wilcoxon test.

was significantly greater in memantine-pre-treated rats (Fig. 3a, b). Facilitation of the acute reversal of allodynia following memantine pre-treatment was seen with (R)-CPP doses of 10 nmol (Fig. 3) and also 3 nmol, but not 1 nmol (data not shown). Ro 25-6891 did not cause significant attenuation of ipsilateral allodynia in vehicle pre-treated rats, but did in memantine pre-treated animals. This reversal was sustained for up to 65 min following injection (Fig. 4a) such that the Ro 25-6891-induced reversal of allodynia in the 35–50 min period of memantine pre-treated rats was significantly greater than in controls. None of the acutely administered drugs had any significant effect on contralateral responses. Acute intrathecal injection of saline vehicle, in both groups of rats, had no discernible effect on behavioural reflexes. It was not possible to test thermal hyperalgesia as this was effectively absent following memantine pre-treatment.

Ro 25-6891 also inhibited cold allodynia with the partially reduced responses (SPET scores) after memantine

pre-treatment being significantly inhibited for 80 min by acute Ro 25-6891 injection, whereas in vehicle pre-treated controls there was no significant effect of Ro 25-6891 beyond 50 min (Fig. 4b). Contralateral SPET scores for cold allodynia were zero in every case. It is notable that the 2B-selective agent can substitute effectively for memantine in the pre-treatment paradigm with subsequent reversal of pCCI-induced mechanical allodynia by acute (R)-CPP or ifenprodil, both being significantly facilitated following the pre-emptive treatment (Fig. 4c).

3.4. NMDA receptor antagonist pre-treatment reverses nerve injury-induced changes in NR1 and NR2B NMDA receptor subunit expression in superficial spinal dorsal horn

In animals 16 days after nerve injury, at peak behavioural sensitisation, or in sham operated or naïve animals, the levels of NR1 and NR2 subunit expression were examined by Western immunoblotting (Fig. 5) and



Expression of protein in the spinal cord as % of GAPDH expression				
	Ipsilateral CCI	Contralateral CCI	Sham	Naïve
NR1	30.7 ± 1.9 *	41.8 ± 1.5	44.3 ± 2.0	40.9 ± 4.0
NR2A	48.6 ± 2.7	51.3 ± 3.1	47.2 ± 1.6	50.0 ± 2.6
NR2B	72.8 ± 5.3 *	47.3 ± 4.2	48.5 ± 3.9	53.8 ± 6.0

Fig. 5. Assessment of injury-induced changes in spinal cord expression of NMDA receptor subunits by immunoblotting. Immunoblots for the levels of NR1, NR2A and NR2B NMDA receptor subunit expression in L3–6 spinal cord from ipsilateral and contralateral pCCI, as well as sham-operated or naïve rats showed that NR1 subunit expression was reduced and NR2B subunit expression was increased ipsilateral to pCCI. NR2A expression appeared to be unchanged. The table shows mean \pm SEM values ($n=6-8$) derived from quantitative densitometry of co-processed immunoblots for NR1, NR2A and NR2B in the spinal cord following pCCI. Differences in the levels of protein expression (arbitrary grey scale values) given as a percentage of GAPDH levels, were assessed between ipsilateral and contralateral pCCI, sham operated or naïve spinal cord (as shown in blots above the table) and statistically significant changes are shown as $*p < 0.05$ by Wilcoxon test.

their localisation was examined by immunohistochemistry (Fig. 6). In Fig. 5 the levels of NR1, NR2A and NR2B immunoreactivity in L3–6 spinal cord were compared between animals ipsilateral/contralateral to pCCI, sham surgery or naïve controls. Expression levels were compared (on the same blots) with those of the ubiquitous housekeeping enzyme GAPDH, which undergoes no discernible change in expression following a wide range of afferent manipulations. Fig. 5 shows examples of immunoblots showing a reduction in NR1 subunit expression ipsilateral to pCCI, no change in NR2A, and an increase in NR2B subunit expression. These results were typical of at least six separate experiments. Quantitative densitometry and image analysis relative to GAPDH expression were used to calculate values for the relative changes in NR1 and NR2B expression levels (Fig. 5). Immunohistochemistry and subsequent image analysis of grey scale densities compared to GAPDH confirmed that NR1 expression was decreased in spinal dorsal horn and further that this change took place selectively in ipsilateral laminae I–II (but not laminae III or ventral horn) in pCCI animals pre-treated with vehicle (Fig. 6a, c, d, e). This reduction in NR1 expression represented a change of -7.3 ± 0.96 grey scale units ipsilateral to pCCI compared to the contralateral side, which had a grey scale score of 28.2 ± 1.54 ; mean \pm SEM; one ROI per side; $n=45$ sections (Fig. 6a, c). In laminae I–II of naïve animals, the mean ipsilateral:contralateral difference was 0.0 ± 0.52 grey scale units. In contrast, in memantine pre-treated animals there was a relative increase in laminae I–II NR1 immunoreactivity ipsilateral to pCCI ($+7.1 \pm 2.2$ grey scale units ipsilateral to pCCI compared to the contralateral side of 23.6 ± 1.61 ; mean \pm SEM). Both of these ipsilateral/contralateral differences in NR1

immunoreactivity in dorsal horn laminae I/II were statistically significant ($p < 0.05$; Student's *t*-test). There was no change in laminae III or ventral horn NR1 immunoreactivity (Fig. 6d, e). Equivalent analysis of NR2B immunoreactivity (Fig. 6b–e) showed a relative increase in NR2B immunoreactivity on the ipsilateral side relative to the contralateral side in vehicle-pre-treated pCCI rats. In laminae I–II the increment in NR2B immunoreactivity represented $+9.6 \pm 1.3$ grey scale units ipsilateral to pCCI compared to the contralateral side, which had a grey scale score of 16.5 ± 1.25 ; mean \pm SEM; Fig. 6b, c, $p < 0.05$, Student's *t*-test. Corresponding values for ipsilateral/contralateral differences from naïve rats were 0.61 ± 0.85 (not significant). In pCCI animals that had been pre-treated with memantine, NR2B expression appeared similar in laminae I–II of both dorsal horns (with an excess grey scale score of only 0.6 ± 1.2 units ipsilaterally compared to a contralateral score of 17.1 ± 1.45 ; mean \pm SEM; Fig. 6c).

NR2B immunoreactivity was also increased in laminae III ipsilateral compared to the contralateral side in vehicle-pre-treated pCCI rats (Fig. 6d). The increment was $+8.3 \pm 1.4$ grey scale units above the contralateral side, which had a grey scale score of 19.4 ± 1.3 , mean \pm SEM, ($p < 0.05$, Student's *t*-test). However, in contrast to the results in more superficial laminae, memantine-pre-treatment appeared to have little effect (Fig. 6d). Ipsilateral NR2B expression in lamina III of memantine pre-treated pCCI rats was 24.1 ± 2.1 grey scale units greater than that on the contralateral side, which had a score of 17.9 ± 1.9 units, mean \pm SEM, ($p > 0.05$, Student's *t*-test). Ventral horn values for NR2B immunoreactivity were not discernibly different ipsilateral/contralateral to pCCI, nor were they detectably altered by memantine pre-treatment (Fig. 6e).

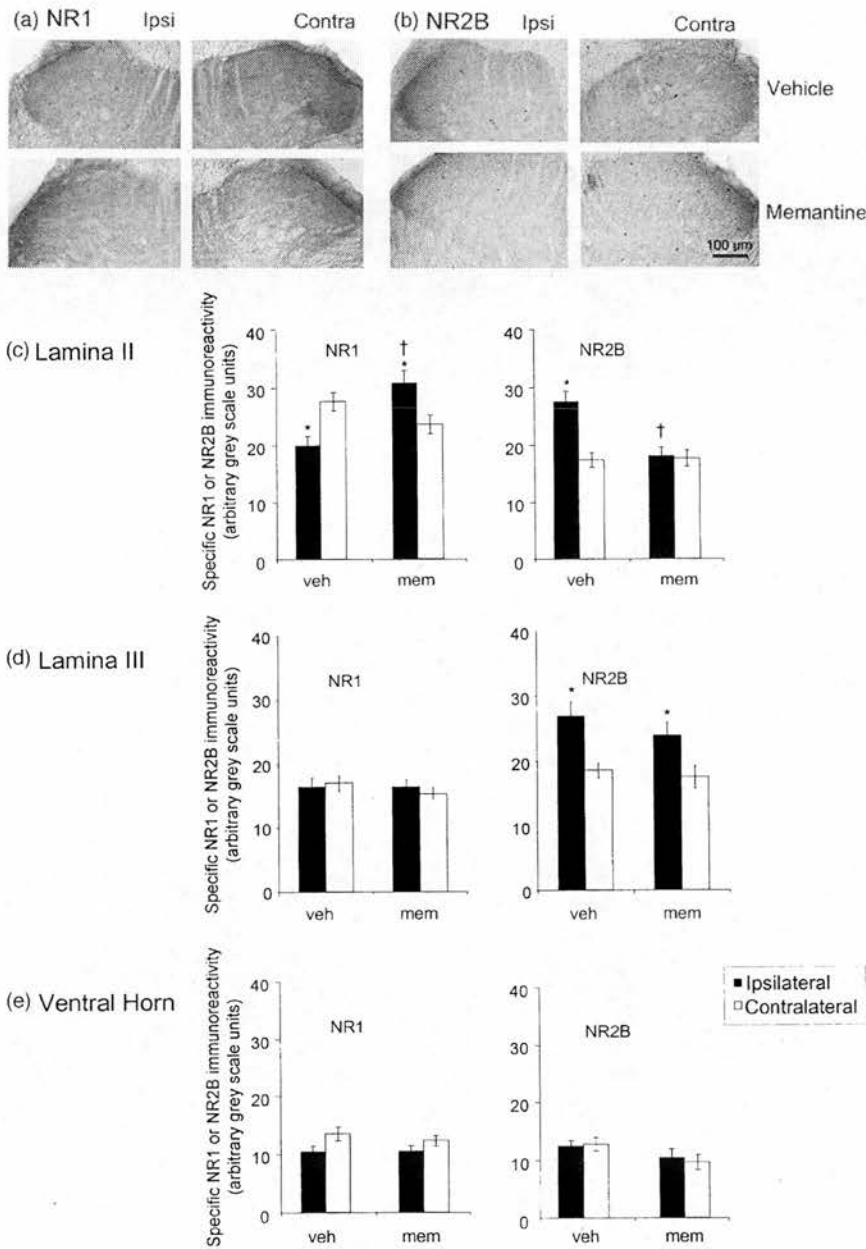


Fig. 6. Immunohistochemical localisation and image analysis of NR1 and NR2B NMDA receptor subunit expression in the superficial dorsal horn, following nerve injury and the effects of pre-treatment with memantine. Typical examples are shown of the expression of NR1 (a) or NR2B (b) subunits of the NMDA receptor in the ipsilateral and contralateral dorsal horn of pCCI animals pre-treated with either vehicle (upper panels; $n=3$) or memantine (lower panels; $n=3$). In vehicle pre-treated animals (a) shows relatively lower levels of NR1 subunit, ipsilateral to pCCI compared to the contralateral side. Memantine pre-treatment however, results in relatively higher levels of NR1 expression ipsilateral to pCCI, compared to the contralateral side. (b) Shows that in contrast to NR1, higher levels of the NR2B subunit are expressed ipsilateral to pCCI compared to the contralateral (white bar) side. Following memantine pre-treatment, this ipsilateral:contralateral difference in the NR2B subunit expression is no longer apparent. Scale bar represents 100 μ m in each panel. In (c–e) data from the immunohistochemistry experiments were analysed by quantitative densitometry and expressed as arbitrary grey scale values (mean \pm SEM) in vehicle- (veh; $n=3$) and memantine-pre-treated animals (mem; $n=3$). Relative expression of NR1 subunit (on left hand side) or NR2B subunit (on right hand side) is shown for ipsilateral (black bar) or contralateral (white bar). Statistically significant differences between ipsilateral (black bar) and contralateral (white bar) within a treatment group are represented by * $p < 0.05$, Student's matched pair t -test; † $p < 0.05$ represents statistically significant differences between vehicle and memantine pre-treated animals (One-Way ANOVA with Dunnett's post hoc analysis).

4. Discussion

Peripheral nerve injury leads to central sensitisation, where increased excitability of neurons in the spinal dorsal

horn is thought to underlie chronic hyperalgesia and allodynia (Chapman et al., 1998; Laird and Bennett, 1993; Takaishi et al., 1996; Woolf, 1983). Pre-emptive analgesia attempts to reduce or prevent the spinal plasticity associated

with the development of chronic pain and would be of clear clinical value. The spinal NMDA R plays a crucial role in sensitisation, however previous studies investigating the potential of pre-emptive NMDA R antagonist treatment show little direct comparison of relative effectiveness on different sensory modalities of neuropathic pain.

Our results show clearly that pre-emptive NMDA R antagonist treatment over 3 days from nerve injury caused differential effects on the development of neuropathic pain behaviours to different sensory stimuli. We found a virtual abolition of thermal hyperalgesia and marked attenuation of cold-related pain behaviours, contrasting with minimal effects on mechanical allodynia in the same animals. Similar results were obtained with pre-emptive use of several different NMDA R antagonists, including memantine, ketamine and the NR2B-selective antagonist, Ro 25-6891, and several routes of administration (systemic memantine, Ro 25-6891 or intrathecal ketamine). The marked preventative effect on thermal hyperalgesia is consistent with previous pre-emptive NMDA R antagonist studies in rodents, using chronic administration, where only noxious thermal heat responses were measured following CCI, using either memantine (i.p. for 7 days; Eisenberg et al., 1995) or MK801 (i.p. 7–15 days; Davar et al., 1991; Mao et al., 1992b). Shorter duration NMDA R antagonist administration (MK801, HA966, ketamine or dextrophan, either i.p. or i.t. for 4 days), yielded only partial attenuation of thermal hyperalgesia (Mao et al., 1992a,b, 1993).

The effects of pre-emptive NMDA R antagonists on the degree and duration of development of neuropathic mechanical allodynia in previous studies have been more variable. Single dose intrathecal ketamine pre-treatment at CCI surgery delayed mechanical allodynia until post-surgery day 3 (Hartick et al., 1998), although prolonged intrathecal ketamine (Burton et al., 1999) or intravenous MK-801 treatment (Wei and Pertovaara, 1999) gave attenuation up to 14 days after surgery in the SNL model. MK-801 administration for 8 days (subcutaneously) following CCI was reported to attenuate mechanical allodynia at 27 days (Smith et al., 1994), although 8 days of memantine treatment (i.p.) following SNL attenuated mechanical allodynia for only 3 days (Carlton and Hargett, 1995). Our results establish that the sensitisation of different sensory modalities is differentially affected by early administration of NMDA R antagonists following nerve injury.

The present study shows directly that pre-emptive systemic or intrathecal administration of NMDA R antagonists attenuates the development of thermal hyperalgesia more than that of mechanical allodynia, indicating that there may be different molecular mechanisms underlying thermal and mechanical sensitisation following nerve injury. A similar profile of allodynia resistant to pre-treatment with NMDA receptor antagonists has been reported in a neuropathic pain model that involves transient cryoneurolysis injury to the peripheral nerve

(Wagner and Deleo, 1996). In agreement, acutely administered NMDA R antagonists reversed thermal hyperalgesia, but not mechanical allodynia following CCI (Tal and Bennett, 1994) or nerve root ligation (Wegert et al., 1997). However, other animal studies describe robust reversal of thermal hyperalgesia and mechanical allodynia by NMDA R antagonists (Chaplan et al., 1997; Garry et al., 2003; Yamamoto and Yaksh, 1992).

The mechanisms responsible for sensitisation of sensory responsiveness are complex, with changes occurring in the peripheral and central nervous system. Centrally, the NMDA R plays a pivotal role in injury-induced sensitisation in the spinal cord (Coderre and Melzack, 1992; Davies and Lodge, 1987; Dickenson and Sullivan, 1987; Woolf and Thompson, 1991).

This study shows for the first time that the expression of glutamate NR1 and NR2 R subtypes in the spinal dorsal horn changes radically and differentially over the time of development of neuropathic pain behaviours following pCCI and that this is prevented by NMDA R antagonist pre-treatment. We also provide novel evidence that the sensitivity of pCCI-induced mechanical allodynia to acute, intrathecal administration of NMDA R antagonists or NR2B-selective antagonists was greater or longer lasting in animals that had been pre-treated with an NMDA R antagonist at the time of injury. The substantial time after injury (16 days) ensures there is no significant concentration of the original drug still present, suggesting that the pre-treatment regime may enable more effective analgesic treatment for any residual pain development in human pain patients.

The NMDA R subunits, NR1 and NR2 are important candidates in the cellular mechanisms underlying neuropathic sensitisation after nerve injury. Immunoblotting and immunohistochemical studies show an alteration in expression levels of NR1 and NR2B, but not NR2A, in animals with established pCCI sensitisation. NR1 expression appeared to be reduced ipsilateral to pCCI, consistent with previous reports (Garry et al., 2003; Siegan and Sagen, 1995). Although we found no change in NR2A expression in dorsal horn tissue following pCCI, a single cell RT-PCR study on acutely dissociated dorsal horn neurons reported a relative decrease following L5 spinal nerve transection (Karlsson et al., 2002). The neuronal population sampled from dissociated dorsal horn neurons and that assayed here by immunoblot and immunohistochemistry are unlikely to be equivalent. Moreover, no overt changes in pain-related behaviours were observed in NR2A subunit knockout mice (Petrenko et al., 2003). Nevertheless, our data do not address directly a role for NR2A, which may still be functionally important, either alone in complexes with NR1 or in mixed complexes with NR1 and other NR2 subunits such as NR2B.

In contrast, the expression of NR2B was elevated ipsilateral to pCCI. It might therefore be expected that nerve-injury-induced pain states would show a relatively

greater sensitivity to selective NR2B antagonists than other forms of pain. Although some previous reports have described only scarce expression of NR2B mRNA in dorsal horn (Luque et al., 1994; Portera-Cailliau et al., 1996; Tolle et al., 1993), single cell RT-PCR found NR2B to be the most commonly expressed variant in dissociated dorsal horn neurons (Karlsson et al., 2002). Recent immunohistochemical evidence suggests that NR2B protein shows a distribution associated both with small diameter fibres in laminae I and II, which are postulated to be of primary afferent origin (Boyce et al., 1999; Ma and Hargreaves, 2000) and post-synaptically on dorsal horn neurons (Moriyama, 2000; Yung, 1998). Several NR2B-selective antagonists show good efficacy and a particularly low incidence of side effects upon acute administration in animal models of inflammatory and neuropathic hyperalgesia (Boyce et al., 1999; Taniguchi et al., 1997). In these studies mechanical allodynia and mechanical hyperalgesia were both shown to be sensitive to NR2B-selective antagonists.

Following NMDA R antagonist pre-treatment, we found that pCCI-induced thermal hyperalgesia was essentially prevented, while cold allodynia was reduced and mechanical allodynia was apparently unaffected. This would be consistent with the idea that NMDA R complexes are involved to different degrees in these different types of sensitised sensory reflexes. In view of these findings, we further assessed the analgesic effectiveness of NMDA R antagonists (including NR2B-selective agents) on the pCCI-induced mechanical allodynia that remained following pre-treatment with NMDA R antagonist around the time of injury. Sensitivity to all acutely administered antagonists (including agents selective to a greater or lesser degree for NR2B subunit-containing complexes) was increased in nerve-injured animals following NMDA R antagonist pre-treatment. This suggests an important role of NMDA Rs, especially the NR2B subunit, not only in the untreated neuropathic pain state but notably in the case of residual pain following pre-emptive NMDA R antagonist treatment. When injury-induced changes in NMDA R subunit expression were investigated, the increased expression of NR2B in lamina II was prevented following memantine pre-treatment, whereas the similar increment in lamina III was unaffected.

In summary these studies provide further evidence for a role for the NMDA R in the development of neuropathic pain and suggest that the NMDA R may normally play a greater role in the development and expression of thermal hyperalgesia than of mechanical allodynia. Correspondingly, the lasting attenuation of neuropathic behavioural sensitisation caused by NMDA R antagonist pre-treatment is more marked for thermal hyperalgesia than mechanical allodynia. The timing of pre-emptive drug administration may also be relevant, with prolonged delivery being more effective. The increased expression of NR2B relative to NR1 that we observed in spinal dorsal horn laminae II and

III may contribute to the sensitisation of behavioural reflexes following pCCI. Since pre-treatment with the NMDA R antagonist, memantine blocks both NR2B expression in lamina II and thermal hyperalgesia, these may be linked events. In contrast, pCCI-induced NR2B expression in lamina III was undiminished by memantine pre-treatment, although the residual pCCI-induced mechanical allodynia showed increased sensitivity to NMDA R or NR2B-selective antagonists. These findings suggest that NMDA R antagonist pre-treatment is an effective analgesic therapy for thermal aspects of neuropathic pain and that it increases the subsequent sensitivity of residual mechanical allodynia to treatment with NMDA R and NR2B-selective antagonists.

Acknowledgements

We thank staff at WARU and MFAA facilities for animal husbandry. This work was funded by Action Medical Research UK and The Wellcome Trust.

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A randomised double blind trial of the effect of pre-emptive epidural ketamine on persistent pain after lower limb amputation

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Received 10 May 2006; received in revised form 9 May 2007; accepted 14 May 2007

Abstract

Persistent pain has been reported in up to 80% of patients after limb amputation. The mechanisms are not fully understood, but nerve injury during amputation is important, with evidence for the crucial involvement of the spinal *N*-methyl *D*-aspartate (NMDA) receptor in central changes. The study objective was to assess the effect of pre-emptively modulating sensory input with epidural ketamine (an NMDA antagonist) on post-amputation pain and sensory processing. The study recruited 53 patients undergoing lower limb amputation who received a combined intrathecal/epidural anaesthetic for surgery followed by a randomised epidural infusion (Group K received racemic ketamine and bupivacaine; Group S received saline and bupivacaine). Neither general anaesthesia nor opioids were used during the peri-operative period. Pain characteristics were assessed for 12 months. The primary endpoint was incidence and severity of post-amputation pain. Persistent pain at one year was much less in both groups than in comparable studies, with no significant difference between groups (Group K = 21% (3/14) and 50% (7/14); and Group S = 33% (5/15) and 40% (6/15) for stump and phantom pain, respectively). Post-operative analgesia was significantly better in Group K, with reduced stump sensitivity. The intrathecal/epidural technique used, with peri-operative sensory attenuation, may have reduced ongoing sensitisation, reducing the overall incidence of persistent pain. The improved short-term analgesia and reduced mechanical sensitivity in Group K may reflect acute effects of ketamine on central sensitisation. Longer term effects on mood were detected in Group K that requires further study.

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Keywords: Phantom limb; Epidural analgesia; Ketamine; NMDA receptors; RCT

1. Introduction

Persistent pain after amputation is an important clinical problem with no reliably effective treatment (Halbert et al., 2002). Up to 80% of patients may

experience persistent pain after lower limb amputation (Nikolajsen and Jensen, 2001; Ephraim et al., 2005). This may either be stump pain (pain at the site of amputation that may have neuropathic elements) or phantom pain, which is a form of neuropathic pain, perceived where the limb was previously.

The underlying mechanisms are not understood, but it is clear that major changes occur in the peripheral and central nervous system in response to

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peripheral nerve injury and subsequent alterations in peripheral sensory input (Woolf and Mannion, 1999; Flor, 2002). Central sensitisation, occurring at the level of the spinal cord, is likely to play a key role in ongoing pain (Woolf, 1995). Animal models of peripheral nerve injury have shown that activation of the ionotropic glutamate receptor, the *N*-methyl *D*-aspartate (NMDA) receptor, is integral to the process of central sensitisation, particularly in nerve injury models (Woolf and Thompson, 1991). Laboratory studies implicate sensory input at the time of nerve injury with acute central neural plasticity leading to persistent neuropathic pain. In animals, NMDA antagonists given before nerve injury can reduce behavioural signs of neuropathic pain and associated neurochemical changes (Burton et al., 1999; Munglani et al., 1999).

Clinical studies of pre-emptive treatment interfering with spinal sensory input are inconsistent. One non-blinded, non-randomised study found a reduction in long-term phantom limb pain by using epidural analgesia with bupivacaine and morphine prior to surgery (Bach et al., 1988). A larger randomised controlled trial found an incidence of ~70% for phantom pain at one year regardless of whether epidural bupivacaine and morphine were commenced 18 h before surgery or immediately after surgery (Nikolajsen et al., 1997). Neither of those studies aimed to specifically modulate spinal NMDA receptors, activated at the time of nerve injury and likely to be important in central sensitisation. Additionally, the route of administration of NMDA receptor antagonists may be clinically relevant in terms of efficacy. Superior analgesia after thoracotomy was achieved with epidural administration of ketamine, an NMDA antagonist, compared to intramuscular administration (Ozyalcin et al., 2004). Intravenous ketamine used around the time of amputation in combination with general anaesthetic and morphine had no significant effect on the incidence of phantom limb pain (Hayes et al., 2004). Modulation of sensory input to the spinal cord around the time of nerve injury may play a key role in altering neuronal plasticity. While there may be some NMDA receptor activation as a result of pre-operative pain, the aim of this study was to focus on NMDA receptor blockade at the time of nerve injury. At this time a massive excitotoxic injury discharge may occur, with excessive glutamate release.

In this study we assessed the effect of pre-emptive treatment with an epidurally administered NMDA receptor antagonist, ketamine, in combination with local anaesthetic, on reducing spinal sensory transmission, acute central sensitisation and the development of persistent post-amputation pain.

2. Methods

2.1. Overview

The study was approved by the local Research Ethics Committee and was in accordance with the Helsinki Declaration of 1975 (revised 1983). International Standard Randomised Controlled Trial Number (ISRCTN) 48374927 was assigned to this trial.

2.2. Subjects

Patients scheduled to undergo lower limb amputation in the Vascular Surgery Unit, Royal Infirmary of Edinburgh, Scotland, were approached regarding participation in the trial. After written informed consent was obtained they were then entered into the trial. Trial recruitment took place over a 20-month period. Patients had to be able to participate in the questionnaire-based pain assessment and lower limb examination before they were invited to take part in the study. Exclusion criteria included: contraindication to spinal or epidural blockade; contraindication to the use of ketamine or bupivacaine; previous lower limb amputation. Patients who underwent further amputation during the follow-up period were excluded from further analysis. Written informed consent was obtained for each patient.

2.3. Study design

Patients who met entry criteria were randomly assigned to one of two treatment groups: Group K to receive epidural bupivacaine and racemic ketamine and Group S (control group) to receive epidural bupivacaine and saline around the time of amputation.

Both patients and staff were blinded as to treatment group, but for safety reasons, one of the researchers (AN) held sealed records as to the group allocation. The researchers involved in assessments were blind to patient allocation until all subjects had completed their 1-year assessment.

Recruiting and post-operative analgesic adjustments were carried out by a single researcher (J.W.).

Randomisation was by GraphPad StatMate version 1.0 (GraphPad software Inc., San Diego) which allocated subjects to either group using computer generated pseudo-random numbers.

All patients underwent a detailed pain assessment, including bedside quantitative sensory testing (QST), of both lower limbs before surgery, and at 8 days, 6 weeks, 3 months, 6 months and 12 months after surgery. If the stump was infected or not healed, no QST was performed at that visit.

2.3.1. Pain assessment

This consisted of four structured questionnaires:

1. A modification of Sherman et al.'s phantom limb questionnaire (Sherman et al., 1984) (Appendix 1; Supplementary information on the web). The characteristics of any phantom and stump pain were recorded, including frequency

- and severity of attacks. Current analgesic medications were also recorded, and classified in terms of the WHO analgesic ladder, i.e., simple analgesics, weak opioids, strong opioids and adjuvant drugs for neuropathic pain.
2. The McGill pain questionnaire (MPQ) (Melzack, 1975). Results were expressed as total Pain rating index (PRI) scores (Turk et al., 1985).
 3. The Neuropathic pain scale (NPS) (Galer and Jensen, 1997). Results were expressed as a total NPS score out of 100.
 4. The Hospital anxiety and depression scale (HADS) (Zigmond and Snaith, 1983). HAD scores were divided into HADS-anxiety (HADS-A) and HADS-depression (HADS-D).

The examination used bedside QST carried out on the base of the stump, or where it was anticipated to be (for pre-operative testing), using the ventral aspect of the contralateral limb as a control. Testing on each limb was carried out equidistant from the corresponding iliac crest. Initially any area of altered mechanical sensation was mapped out using a soft artist's paintbrush. The brush was drawn over the skin from an area of normal sensation towards the stump and the patient asked to note any change (increase or decrease) in the stimulus perception. Further assessment was carried out in any area of altered sensation or, if none detected, on the base of the stump. Tactile and pain thresholds were assessed using von Frey (VF) filaments (North Coast Medical, Inc., San Jose, CA). These were applied in ascending and descending force magnitude until the subject was able to perceive four out of six stimuli. VAS scores to pain and to unpleasantness were recorded following brush stimuli, vibration (using a standard electric toothbrush), pinprick, using neurological examination pins (Neurotips™; Owen Mumford) and cold (acetone drops). For a review of QST methodology, see Jorum and Arendt-Nielsen (2003).

2.3.2. Peri-operative management

The peri-operative period was from the time of consent for surgery up until 72 h afterwards. No premedication was given. Any prescribed analgesics were continued up until the time of surgery. No patient received general anaesthesia or supplementary opioids over the period of epidural use. Both groups received combined spinal and epidural anaesthesia and intravenous sedation. Epidural catheters (16G Portex) were placed just before surgery through either the L2–L3 or L3–L4 interspace. A test-dose of 4 ml of 2% lidocaine was given to exclude intravascular or intrathecal placement. Immediately after the epidural test-dose, a spinal (intrathecal) anaesthetic was administered at an interspace below the epidural catheter (24G Sprotte needle; 2 × 5–3 × 0 ml of bupivacaine 0.5%; AstraZeneca). Before starting surgery, an epidural bolus dose of bupivacaine 0.5% (1 mg/kg) with either 0.5 mg/kg preservative free racemic ketamine (Curamed Pharma, Germany) (Gp K) or an equivalent volume of NaCl 0.9% (Gp S) was given. The epidural infusion mixture was prepared in theatre by an anaesthetist not involved in the study and labelled "study medication". Additional study medication was prepared at this time to be available if required during the infusion period in

order to maintain blinding. Neither ward staff nor the researcher carrying out the assessments was aware of the contents of the mixture.

Sedation was given as required using a target-controlled infusion of propofol. Intraoperatively all patients had standard monitoring, supplemental oxygen and intravenous fluids. During surgery the epidural infusion was commenced at 15 ml/h. Group K received an infusion of bupivacaine 0.125% with ketamine 3.3 mg/kg/l. Group S had bupivacaine 0.125% with an equivalent volume of NaCl 0.9%. Following surgery the epidural infusion was adjusted by a blinded observer within the range of 10–20 ml/h to ensure adequate pain relief (VAS ≤ 30). If analgesia was inadequate, top-up boluses (10–15 ml) were given from the infusion mixture.

Epidural ketamine is used in routine clinical practice in our centre. The doses chosen for this study were based on previous experience that had found that bolus doses of greater than 500 mcg/kg were likely to be associated with increased sedation after surgery. We have found previously that epidural ketamine by infusion at between 2 and 4 mg/h rarely resulted in side effects such as sedation and dysphoria.

Post-operative analgesia was supplemented with paracetamol 1 g 6 h orally. No other analgesic medication was permitted during the period of epidural use. All epidurals ran for between 48 and 72 h. Failure of the epidural to provide adequate analgesia required the removal of the patient from the study.

During the period of epidural analgesia, block height (blunt pin prick) and motor block using a modified Bromage scale (Bromage, 1965), number of epidural bolus doses, average pain VAS scores, sedation score, respiratory rate and presence of nausea or vomiting (self-report) were recorded twice-daily.

2.4. Statistics – design and analysis

The primary outcome measure was the incidence and severity of both phantom limb and stump pain. The incidence of phantom pain was chosen to calculate sample size. Secondary outcome measures were the effectiveness of post-operative analgesia and changes in sensory processing as measured by QST.

The incidence of phantom pain in most comparable studies is around 80% (Nikolajsen and Jensen, 2001; Ephraim et al., 2005). We considered that a decrease to 40% would be clinically significant. For a power of 80%, an estimated sample size of 23 per group was needed (SigmaStat 2.03; Power = 0.8; $\alpha = 0.05$). A 30% 1-year death rate would increase the sample size to 30 per group. This drop-out rate is comparable to that found in other studies in this patient population (Aulivola et al., 2004). Interim analysis by an observer not connected with the study was carried out at the midpoint of the study period, using Fisher's exact test, with the primary endpoint being rate of phantom pain occurrence. This was done to further refine the sample size and ensure that recruiting did not continue inappropriately.

Statistics were carried out using SigmaStat for Windows version 2.03. Proportions were compared using Chi-squared analysis or Fisher's exact test as appropriate. Other measures were compared using one way Analysis of Variance (ANOVA) or Kruskal–Wallis ANOVA on Ranks if non-parametric data.

3. Results

3.1. Recruitment and baseline characteristics

Of 186 patients undergoing lower limb amputation over the 20-month period studied, 53 patients were suitable and consented to participate in the study. They were randomised such that 24 subjects were allocated to Group K and 29 to Group S. Six were withdrawn post-randomisation. Forty-seven patients were included in the analysis, with 21 subjects in group K and 26 subjects in group S. Four subjects subsequently had a below knee amputation revised to an above knee and were removed from further analysis (Fig. 1).

The subjects were comparable in age, weight, height, incidence of diabetes mellitus and type of amputation. Group K consisted of 19 males and 2 females. Males had a mean (SD) age, weight and height of 69 (9.4), 74.8 kg (20.0) and 173.5 cm (9.0), respectively. Females had a mean (SD) age, weight and height of 73 (0.7), 67.0 kg (1.4) and 161.5 cm (2.1), respectively. Group S consisted of 16 males and 10 females. Males had a mean (SD) age, weight and height of 73 (5.3), 68.3 kg (13.1) and 172.3 cm (6.1), respectively. Females had a mean (SD) age, weight and height of 78 (5.5), 51.6 kg (10.0) and 163.9 cm (9.1), respectively. Sixteen subjects (5 Group K; 11 Group S) underwent an above knee amputation and 31 (16 Group K; 15 Group S) a below knee amputation. There was no difference in median (quartiles) pre-operative VAS pain scores (Group K 50 (40–50), Group S 70 (50–83)). The incidence of diabetes in Group K and Group S was 7 and 6, respectively. Similar numbers in both groups used pre-operative opioids (K11/21:S21/26; ns; Chi-squared, $p = 0.078$). No problems with withdrawal were seen when opioids were stopped from the time of surgery onwards.

3.2. Interim analysis

After the first 35 patients had been assessed at 3 months, interim analysis (using Fisher's exact test) was carried out for the primary end-point (presence or absence of phantom pain) by an independent assessor not involved in the study. The rate of phantom pain at 8 days, 6 weeks and 3 months in Group K was 3/11 (27%), 7/11 (64%) and 4/10 (40%), respectively, compared with Group S where 9/18 (50%), 8/17 (47%) and 6/15 (40%) patients had phantom limb pain. There was no significant difference between groups at 8 days, 6 weeks and 3 months (with p -values of 0.273, 0.460 and 1.000, respectively). The study authors were blinded to the result details, but were advised that recruitment should cease.

3.3. Outcomes

3.3.1. Assessment during epidural infusion period

There was no difference in median (Quartiles) epidural infusion rates between the groups (Group K 15 ml/h (15–18) and Group S 15 ml/h (14–16)), Mann–Whitney rank sum test; $p = 0.397$. Both groups had good post-operative analgesia with median total VAS scores of less than 30 mm. Group K had significantly lower pain scores than Group S. Median mean VAS (quartiles) Group K 0 mm (0–17), Group S 17 mm (0–35) (Mann–Whitney rank sum test; $p = 0.031$). Group K required a significantly lower number of epidural top-ups. Group K 1.8 (2.2), mean (SD) and Group S 3.5 (3.2), mean (SD) (Student's t -test; $p = 0.044$). Following establishment of epidural blockade, no subject was withdrawn due to inadequate analgesia. Mean epidural infusion rate and duration was the same for both groups.

There was no significant difference in motor block between the groups during the duration of epidural infusion. Median (IQ range) Bromage scores on day one were 1 (0–2) for Group K and 0 (0–1) for Group S. On day two median (IQ range) Bromage scores were 0 (0–1) in both groups. The incidence of nausea and vomiting (Group K: 4/21; Group S: 3/26; $p = 0.684$), sedation (Group K: 2/21; Group S: 1/26; $p = 0.579$) and confusion (Group K: 2/21; Group S: 0/26; $p = 0.194$) were low and did not differ between groups (Fisher's exact test).

No patients suffered from hallucinations. There were no complications that required revealing the composition of the infusion.

3.3.2. Post-amputation pain

The rates of phantom and stump pain did not differ between the two groups at any of the assessments up to and including one year, when the rate of phantom pain was no more than 50% and stump pain was no more than 33% in either group (Table 1). Neither stump pain nor phantom pain intensity, as measured using VAS, differed between groups at any time point (Figs. 2 and 3). There was no detectable difference in the frequency of attacks between the two groups (Table 2).

3.3.3. Questionnaire data

Total MPQ scores were highest before surgery, Group K: 23 (15–33), Group S: 28.5 (15.25–35.75); median (IQ range). These fell to 3 (0–8) in Group K and 3 (1–11) in Group S at 12 months. Scores in both groups were significantly less than pre-operative values by 6 weeks (Kruskal–Wallis one way Analysis of Variance on Ranks with Dunn's post-hoc analysis, $p < 0.001$), but groups did not differ from each other at any time point.

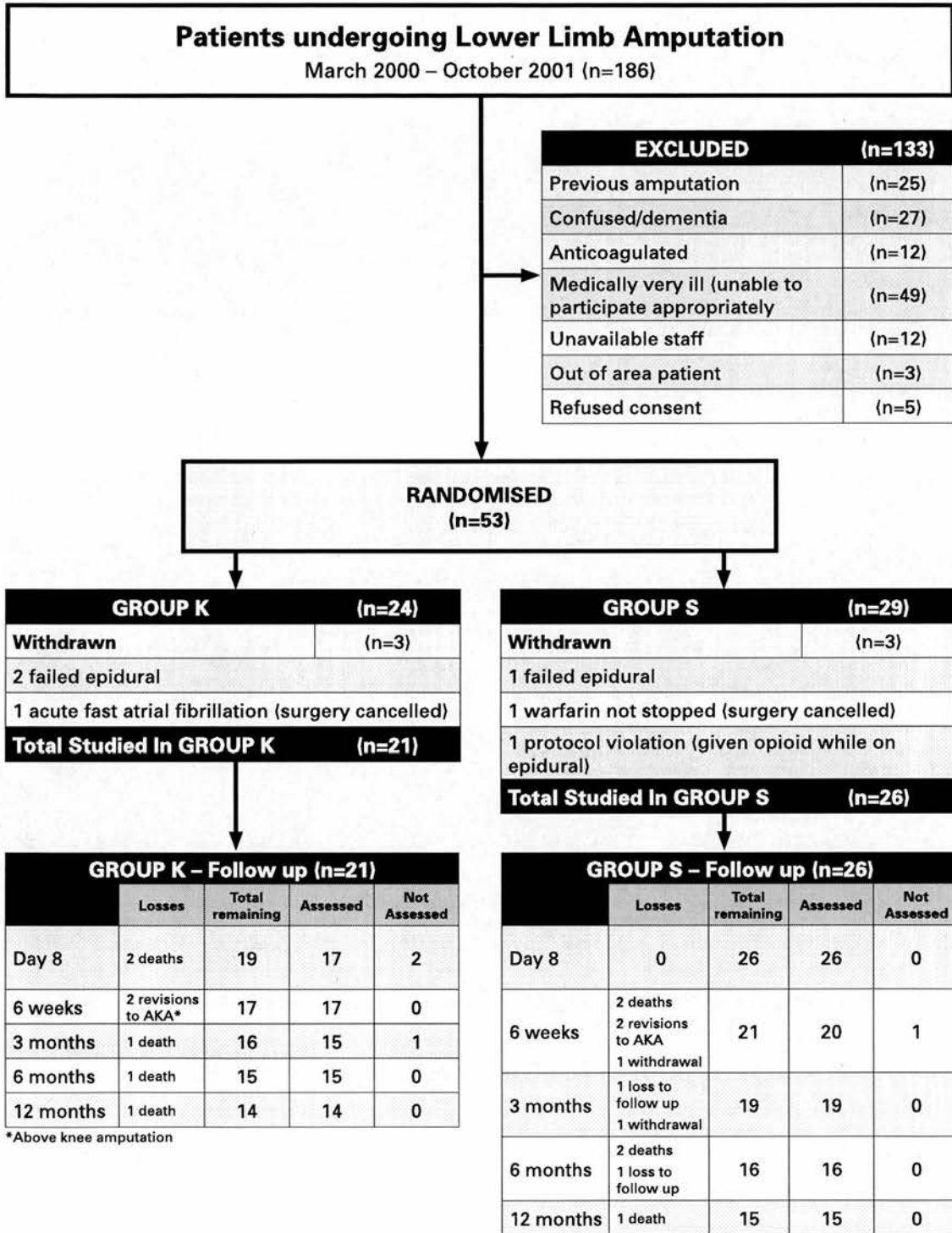


Fig. 1. Consort diagram.

Median NPS scores started high and dropped with time, pre-operatively scores were: Group K; 49 (40–59) vs Group S; 50 (37–66); median (IQ range). This fell to a median score of 4 (1–22) in Group K and 5 (0–16) in Group S at 12 months. There was a significant reduction from pre-operative values in both groups at all time

points post-operatively, but with no difference between groups (Kruskal–Wallis ANOVA on Ranks with Dunn’s post-hoc analysis, $p < 0.001$).

Levels of anxiety, as assessed by HADS scoring (expressed as mean (SD)), were: Group K: 8.4 (4.5) pre-operatively, decreasing to 5.4 (2.8) at 6 weeks

Table 1
Incidence of stump and phantom pain at each time point

Time	Stump pain		p	Phantom pain		p
	Group K n (%)	Group S n (%)		Group K n (%)	Group S n (%)	
8 days	11/17 (65)	15/26 (58)	0.888	6/17 (35)	13/26 (50)	0.525
6 weeks	9/17 (53)	9/20 (45)	0.879	10/17 (59)	9/20 (45)	0.611
3 months	5/15 (33)	9/19 (43)	0.635	6/15 (40)	7/19 (37)	0.867
6 months	7/15 (47)	5/16 (32)	0.609	6/15 (40)	3/16 (19)	0.252†
12 months	3/14 (21)	5/15 (33)	0.682†	7/14 (50)	6/15 (40)	0.867

Proportions compared using Chi-squared analysis except as indicated by †.

†Fisher’s exact test.

Data are shown as numbers experiencing pain over total number in the group at each time point for both Group K and Group S (percentages in brackets) for up to 12 months following surgery.

post-amputation, and remained significantly lower at all time points up to and including one year (4.3(2.9)) (one way ANOVA with Dunnett’s post-hoc analysis; $p < 0.001$) and Group S: 8.2 (3.7) pre-operatively with no significant reduction any time point up to and including one year (4.7 (4.0)) (one way ANOVA with Dunnett’s post-hoc analysis; $p = 0.071$).

Levels of depression for Group K were 8.1 (4.3) pre-operatively, reducing significantly at 3 months to 5.4 (3.8) and remaining significantly reduced up to and including one year (one way ANOVA with Dunnett’s post-hoc analysis; $p = 0.003$). Pre-operative scores for depression in Group S were 6.0 (3.4), with no significant reduction at any time point up to and including one year (4.5 (4.6)) (one way ANOVA $p = 0.829$).

3.3.4. Sensory processing as measured by QST

Pre-operatively there was a slightly higher tactile threshold in the ischaemic limb (206 mN mm⁻² (191))

than the contralateral limb: (139 mN mm⁻² (82)), *t*-test $p = 0.036$. Ketamine did modify some aspects of sensory transmission, with an alteration in the tactile threshold of the stump on day 8 after surgery. In Group K, the tactile threshold was found to be significantly higher on the stump (589 mN mm⁻² (782); mean (SD)) compared to the contralateral limb (180 mN mm⁻² (160); mean (SD)) and to both limbs in Group S (stump; 250 mN mm⁻² (97); mean (SD)), contralateral, 208 mN mm⁻² (140); mean (SD)); (one way ANOVA with Tukey test; $p = 0.005$). This difference was not found at 6 weeks (see Table 3). There was no significant difference between sides at any other time point.

There was no difference between the groups in the evoked response to pin prick at any time point (Table 4).

VAS scores for cold and vibration were 0 in the majority of patients (88–100%) at all time points. Further detailed analysis was not carried out.

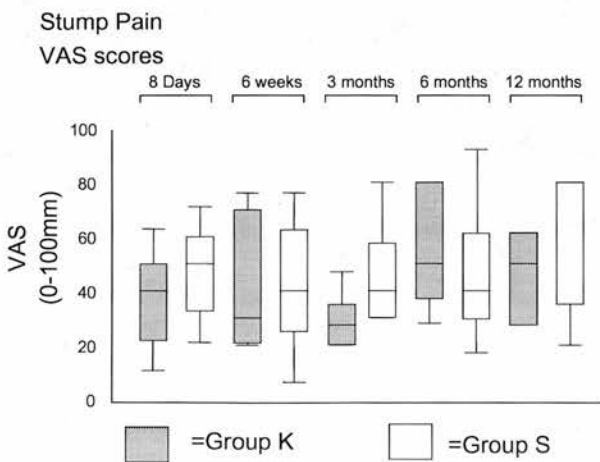


Fig. 2. Intensity of stump pain at each time point following amputation. Data are shown as box plots with medians represented by horizontal lines with the 75th percentile at the top and the 25th percentile at the bottom. The 10th and 90th percentiles are shown as whiskers.

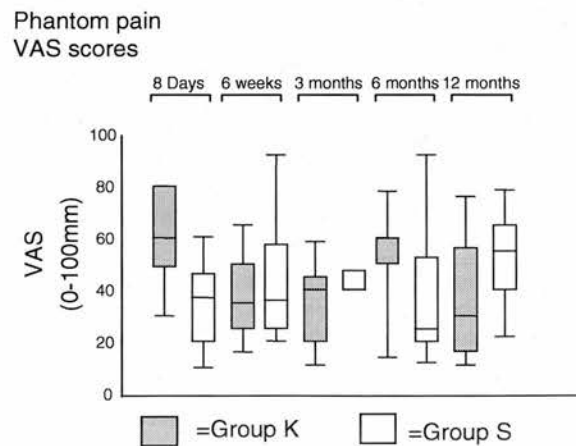


Fig. 3. Intensity of phantom pain at each time point following amputation. Data are shown as box plots with medians represented by horizontal lines with the 75th percentile at the top and the 25th percentile at the bottom. The 10th and 90th percentiles are shown as whiskers.

Table 2
Frequency of phantom pain attacks in each group

		Group K	Group S
Day 8	Phantom pain (n)	6	13
	Constant	0	0
	Hourly	1	2
	Daily	3	10
	Weekly	2	1
	Monthly	0	0
	Yearly	0	0
6 months	Phantom pain (n)	6	3
	Constant	0	0
	Hourly	0	0
	Daily	1	0
	Weekly	5	2
	Monthly	0	1
	Yearly	0	0
12 months	Phantom pain (n)	7	6
	Constant	0	1
	Hourly	0	0
	Daily	3	2
	Weekly	2	0
	Monthly	2	1
	Yearly	0	2

Data are shown at 8 days, 6 months and 12 months. The data show the number of patients experiencing pain at each time point. The frequency of the pain attacks is shown at each time point. Due to the small numbers involved meaningful analysis is not possible.

3.3.5. Analgesic medications

The overall number of analgesic drugs prescribed did not vary between groups. The number of analgesic drugs prescribed pre-operatively was Group K; 2 (2–3) vs Group S 3 (2–3); median IQ range. The number of analgesic drugs prescribed was less in both groups after surgery. At 12 months the number prescribed was the same in both groups: 1 (1–2); median (IQ range). There was no difference between the two groups in the median numbers of drugs at any time point after surgery (Kruskal–Wallis ANOVA on ranks).

4. Discussion

This study has found that modulation of sensory input at the time of amputation may have both short and longer term effects on pain perception.

The anaesthetic/analgesic technique used in our study has resulted in a lower incidence of persistent pain than other randomised controlled trials of lower limb amputation where around 70% of patients have persistent pain (Nikolajsen et al., 1997; Hayes et al., 2004). A wide range of incidences for post-amputation pain has been found (Nikolajsen and Jensen, 2001; Ephraim et al., 2005), with lower incidences in particular types of study (retrospective or postal survey) and patient groups (paediatric or upper limb amputation) compared to lower limb amputation (Montoya et al., 1997; Wartan et al., 1997; Kooijman et al., 2000; Wilkins et al., 2004).

A key difference in our study is the anaesthetic technique used compared to previous studies. This combined spinal/epidural technique, without use of general anaesthesia or opioids, produces a very dense block of sensory input that may provide a novel strategy for reducing persistent pain after amputation.

The initial hypothesis was that specific blockade of the NMDA receptor would be required to reduce persistent pain. This was based on evidence from basic science studies of the key role that the NMDA receptor plays in central sensitisation and subsequent spinal events in pathological pain states (Davies and Lodge, 1987; Torsney and MacDermott, 2006). Failure of previous studies to reduce persistent pain may have been related to inadequate block of the effects of massive glutamate release and excitotoxic discharge at the time of nerve injury (Li and Stys, 2000; Moore et al., 2002). Clinically, there is also some evidence for involvement of the NMDA receptor in central processing in patients with established phantom pain (Larbig et al., 1996; Schwenkreis et al., 2003). Intravenous ketamine has been used peri-operatively at the time of lower limb amputation, but no acute effect on central processing was found, nor a statistically significant reduction in phantom limb pain at 6 months, although phantom pain was 47% in the ketamine group and 71% in the control group (Hayes et al., 2004).

One reason for our study not detecting an additional effect of ketamine, over and above local anaesthetic alone, may be that, due to the much lower overall incidence of persistent pain, the power of the study to answer the original hypothesis was reduced. It may be, however, that the key element to reducing persistent

Table 3
Tactile threshold for the stump and the contralateral limb as measured by von Frey filaments

Time	Group K stump Mean (SD)	Group K con Mean (SD)	Group S stump Mean (SD)	Group S con Mean (SD)	p
8 days	589 (782)	180 (160)	250 (97)	208 (140)	0.005*
6 weeks	314 (412)	180 (156)	255 (173)	145 (68)	0.224
3 months	216 (225)	199 (224)	154 (106)	127 (77)	0.442
6 months	180 (145)	138 (61)	143 (72)	134 (70)	0.472
12 months	147 (103)	150 (102)	215 (123)	155 (90)	0.236

*One way Analysis of Variance.

Data are shown as mean tactile threshold (force/unit area; g/mm²) with SD for both Group S and Group K, for up to 12 months following surgery.

Table 4
Mean VAS score to pinprick for the stump and the contralateral limb

Time	Group K stump Mean (SD)	Group K con Mean (SD)	Group S stump Mean (SD)	Group S con Mean (SD)	<i>p</i>
8 days	29 (29)	22 (25)	46 (29)	36 (32)	0.051
6 weeks	46 (37)	29 (31)	47 (33)	33 (23)	0.208
3 months	35 (34)	33 (37)	30 (23)	28 (25)	0.935
6 months	35 (31)	32 (34)	33 (34)	41 (25)	0.761
12 months	45 (30)	40 (32)	42 (31)	42 (26)	0.972

Data are shown as mean VAS score (0–100 mm) with SD for both Group S and Group K, for up to 12 months following surgery.

pain is the blockade of spinal sensory transmission (either NMDA receptor-specific or non-specific blockade). Previous studies have found that neither general anaesthesia, acute opioid use, nor prolonged sensory block, with epidural analgesia before or after surgery, was sufficient to reduce persistent pain (Nikolajsen et al., 1997; Hayes et al., 2004). In order to adequately reduce the massively increased peripheral input at the time of nerve injury, it would be necessary to reduce sensory input to such an extent that spinal responses were prevented. It has been shown previously that local anaesthetic administered via the epidural route does not completely suppress neuronal input to the spinal cord (Loughman et al., 1995). There is also recent evidence from animal models that electrical stimuli well below that required to elicit spinal cord activity may evoke neuronal activity within the brainstem, as assessed by functional Magnetic resonance imaging (Lilja et al., 2006).

While electrophysiological recordings of spinal input were not made during our study, the combined spinal/epidural technique did provide a significant block of sensory input such that amputation was performed without general anaesthesia or other analgesia such as strong opioids. This blockade of sensory input was associated with an incidence of persistent pain of 50% or less in both groups in our study.

Despite no additional reduction in persistent pain in the ketamine group, there were alterations in pain processing, with significant improvements in acute post-operative analgesia. Specific effects on sensory processing were also detected, with a selective decrease in mechanical sensitivity on the stump that outlasted the expected pharmacological action of ketamine (Clements et al., 1982), similar to that seen in established phantom and stump pain (Nikolajsen et al., 1996). The timing of NMDA receptor blockade may be important in its effect on the neurobiological response, as some studies have found that NMDA receptor antagonists did not affect phantom or stump pain, or responses to mechanical and thermal stimuli, and cortical changes, in established post-amputation pain (Nikolajsen et al., 2000; Wiech et al., 2004), with one small study of traumatic upper limb amputees finding that acute administration of memantine (in addition to brachial plexus blockade)

reduced prevalence and severity of phantom limb pain up to 6 months but not one year afterwards (Schley et al., 2007).

Other studies in animal models have also found an anti-allodynic effect of NMDA receptor antagonists that outlast their expected pharmacological duration of action (Christoph et al., 2006), with selective effects on different aspects of pain behaviours (Burton et al., 1999; Munglani et al., 1999). Additionally we have found that treatment with NMDA antagonists prior to nerve injury in a rodent model resulted in an enhancement of subsequent responses to NMDA antagonists, particularly responses to mechanical stimuli (Wilson et al., 2005). There is also evidence of inhibitory pathways mediated by metabotropic glutamate receptors type II/III in neuropathy models, with a complex relationship between the ionotropic NMDA receptors and metabotropic (Garry and Fleetwood-Walker, 2004; Proudfoot et al., 2006). This may partly explain the reduced mechanical sensitivity, although the precise mechanism requires further study.

These results would support a role for the NMDA receptor in acute central sensitisation as well as providing clinical evidence supporting the role of epidural ketamine in modifying pain processing acutely.

There is still debate as to the role of ketamine as part of multimodal post-operative analgesia. Systematic reviews of ketamine found that ketamine could reduce pain scores and opioid consumption, with minimal adverse effects (Elia and Tramer, 2005; Bell et al., 2006). A systematic review of another clinically available NMDA antagonist, dextromethorphan, also found a reduction in opioid consumption, but no effect on post-operative pain scores (Duedahl et al., 2006). The route of administration may be important, with epidural being superior to systemic ketamine after thoracotomy, while another study found no effect of intravenous ketamine acutely or at 6 months post lower limb amputation (Hayes et al., 2004; Ozyalcin et al., 2004). By giving ketamine, close to its putative site of action on nociceptive pathways in the spinal cord, an adequate dose may be given without significant adverse effects. Our study provides evidence of the efficacy of epidural ketamine as part of an acute analgesic strategy with some detectable effects on acute

sensory responses. An additional benefit, particularly in an elderly population, is the lack of sedation or respiratory depression produced by using a technique without a requirement for opioid in the peri-operative analgesic regimen.

The potential neurotoxicity of spinal ketamine (either epidural or intrathecal) must be considered. First, there is concern that preservatives in commercially available compound may be neurotoxic, as has been demonstrated in sub-human primates and rabbits (Brock-Utne et al., 1982; Malinovsky et al., 1993; Borgbjerg et al., 1994). Second, although preservative free ketamine widely used in some European countries, there have been concerns both about a direct toxic effect specifically related to the action of NMDA antagonists and an increased rate of apoptosis during neuronal development. The direct toxic effect has only been found selectively, certain cortical neurons in rodents (cingulate and retrosplenial cortices), with both toxicity and apoptosis only seen with high doses, unlikely to be used in clinical practice (Olney et al., 1989; Ikonomidou et al., 1999; de Lima et al., 2000). Additionally, repeated intrathecal injections of up to 1% of preservative free ketamine (Dalens, 2006) or repeat epidural boluses of ketamine in dogs (Acosta et al., 2006) had no neurotoxic effect.

Recent systematic reviews of ketamine have found no clinical evidence of neurotoxicity from epidural use, with some benefits in analgesia (Subramaniam et al., 2004).

Further study of the unexpected finding of ketamine in reducing anxiety and depression is warranted. The acute, and often dose-limiting, effects of ketamine on mood, such as dysphoria and increased anxiety, were not found in our study, which may relate to the route of administration.

Improvement in mood has been found with ketamine after peri-operative use in depressed patients (Kudoh et al., 2002), and acutely in the treatment of major depression (Berman et al., 2000; Sprenger et al., 2006; Zarate et al., 2006). No previous study has found a positive long-term effect of short-term ketamine administration on levels of anxiety and depression (Zarate et al., 2003; Goodman, 2004).

It has been suggested that a combination of general and regional techniques is needed to reduce central sensitisation (Melzack et al., 2001). However, this may not be the case, with the key element being modulation of sensory transmission around the time of nerve injury. A combination of specific (ketamine) and non-specific agents (local anaesthetic) administered close to the spinal cord can alter acute and chronic pain processing. The NMDA receptor has a defined role in the development of pain, but further study is needed to clarify the role of the NMDA receptor in persistent phantom limb pain.

Acknowledgements

Funding by Action Medical Research.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pain.2007.05.011.

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