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Morphine modulation of excitatory transmission in the rat spinal cord

Thesis submitted to the University of London for the degree of
Doctor of Philosophy by

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To my wonderful parents, Hadla and Mustafa Karam, to whom I owe everything in my life. Thank you for making all my dreams come true. I hope that I can make one of your dreams come true with this thesis.

And to my husband Khalid, none of this would have been possible without you. Thank you for sharing my parents' dream.

I, Shaima Mustafa Karam, confirm the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

The transmission of painful messages from the periphery involves complex interactions between excitatory and inhibitory neurotransmitter systems in the spinal dorsal horn. The balance between these receptor events determines the level of pain transmission. Within the spinal cord, neuronal sensitization plays a key role in altering the ascending messages to the brain.

Morphine, widely used in pain control, acts on inhibitory mu opioid receptors located both on small diameter primary afferent fibres and postsynaptic sites. The aim of my studies was to determine how various excitatory transmitter systems at spinal levels are modulated by morphine in rats.

The activation of the receptor for substance P, the NK-1 receptor, and the NMDA receptor for glutamate, on dorsal horn neurons are thought to contribute to wind-up pain and central sensitisation underlying a number of abnormal clinical pain states.

Using in vivo electrophysiological studies and behavioural measures, I have studied the relations between peripheral inputs and central sensitisation using NK-1 and NMDA receptor agonists and further used a surgical neuropathic model and investigated the actions of morphine on the neuronal activity and pain behaviours.

I have also investigated NMDA receptors located on peripheral endings of sensory nerves, and found a lack of functional effects of these receptors in pathophysiological states.

Finally, the role of the excitatory 5HT3 receptor was assessed using the antagonist ondansetron in the neuropathic pain model, as was the combination of morphine and ondansetron which was more effective in inhibiting some of the pain behaviour seen in neuropathic pain than ondansetron alone.

The actions of morphine, at a non-sedating dose, could overcome central sensitisation, the actions of ondansetron confirm the role of descending facilitations after nerve injury and the results demonstrate how the balance between excitation and inhibition can alter the level of pain and its control.

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Contents

Chapter 1 Introduction 16

1.1 Pain transmission, neuropathic pain and aim 16
1.1.1 Pain transmission 16
1.1.2 Neuropathic pain 16
1.1.3 Hyperalgesia and allodynia 17
i) Definitions 17
ii) Thermal hyperalgesia 18
iii) Mechanical hyperalgesia/allodynia 18
Figure 1.1 19
1.1.4 Animal models of neuropathic pain and what they measure 19
1.1.5 Pharmacological treatments in neuropathic pain 20
1.1.6 Morphine 21
1.1.7 General aim 22
1.2 Primary afferent fibres 22
1.3 Nociceptors 23
1.4 Stimulus modality conduction in unmyelinated and myelinated fibres 24
1.5 Primary afferent fibres may be grouped using different molecular markers 25
1.6 Peripheral terminal 26
1.6.1 Channels and receptors at the periphery 26
1.6.2 Signal transduction receptors/channels 26
i) TRP channels 26
ii) Asic channels 28
iii) P2X channels 28
iv) P2X channels and microglia in neuropathic pain 28
1.6.3 Signal transduction due to thermal versus mechanical stimuli 29
1.6.4 Mechanisms involved in peripheral tissue damage and axonal reflex 29
i) Inflammatory mediators 29
ii) Substance P, calcitonin gene-related peptide and the axon reflex 30
Figure 1.2 31
1.6.5 Peripheral mechanisms in neuropathic pain 31
1.6.6 Channels involved in signal propagation 32
i) Na channels 32
ii) Na channels in neuropathic pain 33
iii) K channels 34
iv) K channels in neuropathic pain 35
1.6.7 Channels involved in neurotransmitter release 35
i) Ca channels 35
ii) Ca channels in neuropathic pain 36

1.7 Spinal cord dorsal horn 37
1.7.1 Dorsal horn anatomy 37
i) Lamina I 39
ii) Lamina II 39
iii) Laminae III and IV 40
iv) Lamina V and VI 40
1.7.2 Dorsal horn pharmacology 40
1.7.3 Excitatory transmitter systems 41
1.7.3.1 Glutamate 41
i) Actions of glutamate at the NMDA receptor 42
a) NMDA receptor location 42
b) NMDA receptor activation and wind-up 42
c) NMDA receptor subunits 43
d) The role of the NMDA receptor in pain states 44
e) The behaviour following NMDA receptor activation in animal studies 45
ii) Actions of glutamate at other receptors 45
a) The AMPA receptor 46
b) Peripheral receptors 46
iii) Peripheral NMDA receptors and neuropathic pain 48
1.7.3.2 Substance P 49
i) SP is co-released with other peptides from primary afferent fibres 49
ii) The NK-1 receptor composition and internalisation 50
iii) The NK-1 receptor location and projections 51
iv) The role of the NK-1 receptor in pain states 51
v) The behaviour which follows the activation of the NK-1 receptor in animal studies 52
vi) Ablating the NK-1-expressing neurons 53
1.7.4 The postulated role of the NMDA and NK-1 receptors in central sensitisation 53
1.7.5 The postulated role of the NK-1 and the NMDA receptors in neuropathic pain 54
1.7.6 Inhibitory transmitter systems 55
1.7.6.1 GABA 55
1.7.6.2 Opioids 56
i) The location of opioid receptors 57
ii) The mechanisms of action of opioid receptors 57
iii) The efficacy of opioids in different pain models 58
1.7.7 Morphine and neuropathic pain 60
i) Clinical studies 62
ii) Dynorphin and cholecystokinin 62
a) Dynorphin 63
b) CCK 63
iii) Dynamic/static allodynia, thermal hyperalgesia and morphine 64
iv) Route of administration of morphine 65
Figure 1.3 66
1.8 Ascending and descending systems 66

1.8.1 Ascending systems 67
i) The spinothalamic (STT) tract 67
ii) The spinomesencephalic tract 67
iii) The spinoreticular tract 68
iv) The spinoparabrachial tract 68
v) The spinocervical tract 69
vi) The postsynaptic dorsal column (PSDC-lemniscal) tract 69
vii) Labelling and convergence 69
1.8.2 Descending controls 70
i) Origins of descending controls 70
ii) The PAG 71
iii) The RVM 71
iv) ON, OFF and neutral cells 72
v) Descending inhibitions or descending facilitations? 73
vi) Descending controls in neuropathic pain 73
vii) Thermal versus mechanical hypersensitivity in neuropathic pain- from the periphery to supraspinal sites 74
a) Thermal hyperalgesia 74
b) Mechanical allodynia 75
1.9 Serotonin 76
1.9.1 5-HT receptor subtypes 76
1.9.2 5HT3 receptor location 77
1.9.3 5HT3 receptor- nociceptive or antinociceptive? 77
1.9.4 The 5HT3 receptor in neuropathic pain 79
Figure 1.4 81
1.10 Aim 81

Chapter 2 Methods 84

2.1 Animals 84
2.2 In vivo electrophysiology 84
2.3 Acute chemical model of nociception 86
2.3.1 Lumbar catheterisation 86
2.3.2 Biting, scratching and licking behaviour (BSL) 88
2.3.3 Thermal hypersensitivity 88
Figure 2.1 89
2.3.4 Paw withdrawal threshold assessment 89
Figure 2.2 91
2.3.5 Assessment of ambulation 91
2.4 Peripheral NMDA receptor study- Spinal nerve ligation model 92
2.5 Morphine and ondansetron in neuropathic pain study- Spinal nerve ligation and lumbar catheterisation 93
2.6 Drugs 93
2.6.1 In vivo electrophysiology drugs 93
2.6.2 Acute chemical model of nociception 94
2.6.3 Peripheral NMDA receptor study 96
2.6.4 Morphine and ondansetron in neuropathic pain 96

2.7 Statistical analysis _______________ 97

Chapter 3 A study investigating the effects of morphine, [Sar[9]Met (O_2)[11]]-Substance P and NMDA on rat deep wide dynamic range dorsal horn neurons, using in vivo electrophysiology_99

3.1 Introduction_______________ 99
3.2 Methods_______________ 100
3.3 Results_______________ 102
3.3.1 Characterisations of deep, wide dynamic range and nociceptive-specific neurons_______________ 102
Table 3.1 _______________ 102
Figure 3.1 _______________ 103
Figure 3.2 _______________ 103
Trace 1 _______________ 104
Table 3.2 _______________ 104
Figure 3.3 _______________ 105
Figure 3.4 _______________ 105
3.3.2 The effect of intrathecal morphine on the evoked responses of deep WDR neurons _______________ 105
Table 3.3 _______________ 106
i) Electrical parameters_______________ 106
Figure 3.5 _______________ 106
Figure 3.6 _______________ 107
Figure 3.7 _______________ 107
Figure 3.8 _______________ 108
Figure 3.9 _______________ 109
Figure 3.10 _______________ 109
Figure 3.11 _______________ 110
ii) Natural parameters _______________ 110
Figure 3.12 _______________ 111
Figure 3.13a _______________ 111
Figure 3.13b _______________ 112
Figure 3.14 _______________ 113
3.3.3 The effect of intrathecal Sar-SP on the evoked responses of deep WDR neurons_______________ 113
Table 3.4 _______________ 113
Table 3.5 _______________ 114
Figure 3.15 _______________ 114
Figure 3.16 _______________ 115
Figure 3.17 _______________ 115
Figure 3.18 _______________ 116
i) The effect of Sar-SP on the electrical evoked responses116
Figure 3.19 _______________ 117
Figure 3.20 _______________ 117
Figure 3.21 _______________ 118
Figure 3.22 _______________ 118
ii) The effect of Sar-SP on the natural evoked responses_118
Figure 3.23 _______________ 119

Figure 3.24 ______ 119
Figure 3.25 ______ 120
Figure 3.26 ______ 120
Figure 3.27 ______ 121
Figure 3.28 ______ 121
3.3.4 The effects of Sar-SP on morphine's inhibitory action of the evoked responses of deep WDR neurons ______ 121
Table 3.6 ______ 122
i) Effects on the electrical evoked response of deep WDR neurons ______ 122
Figure 3.29 ______ 123
Figure 3.30 ______ 123
ii) Effects on the natural evoked response of deep WDR neurons ______ 124
Figure 3.31 ______ 124
Figure 3.32a ______ 125
Figure 3.32b ______ 126
Figure 3.33 ______ 126
3.3.5 The effect of intrathecal NMDA on the evoked responses of deep WDR neurons ______ 127
Table 3.7 ______ 127
Figure 3.34 ______ 128
Figure 3.35 ______ 128
Figure 3.36 ______ 129
Figure 3.37 ______ 129
Figure 3.38 ______ 130
3.4 Discussion ______ 130
3.4.1 The effects of morphine on electrical and natural evoked responses of deep WDR neurons ______ 130
i) The effect of morphine on the electrical evoked responses of deep dorsal horn neurons ______ 131
ii) The effect of morphine on the natural evoked responses of deep dorsal horn neurons ______ 133
iii) Conclusion ______ 135
3.4.2 The effects of each of Sar-SP and NMDA on electrical and natural evoked responses of deep WDR neurons ______ 135
i) The interaction between the NK-1 and the NMDA receptor ______ 135
ii) Sar-SP ______ 138
iii) NMDA ______ 141
iv) Conclusion ______ 142
3.4.3 The effects of morphine, in the presence of Sar-SP, on electrical and natural evoked responses of deep WDR neurons ______ 143
3.4.4 Conclusion ______ 144

Chapter 4 A study investigating the effectiveness of morphine in inhibiting the behaviour and nociceptive response which arise following the activation of each of the NMDA and NK-1

receptors 147

4.1 Introduction 147
4.2 Methods 148
4.3 Results 150
4.3.1 Biting, scratching and licking behaviour 150
i) Intrathecal saline 150
ii) Intrathecal NMDA 150
iii) Intrathecal Sar-SP 151
4.3.2 Behavioural response to thermal stimuli using radiant heat 151
i) Saline control experiments 151
Figure 4.1 152
ii) Morphine experiments 152
Figure 4.2 153
iii) NMDA experiments 153
Figure 4.3 154
Figure 4.4 155
Figure 4.5 156
Figure 4.6 157
iv) NK-1 experiments 157
Figure 4.7 158
Figure 4.8 159
Figure 4.9 160
Figure 4.10 161
Figure 4.11 162
Figure 4.12 163
Figure 4.13 164
4.3.3 Behavioural response to tactile stimuli using von Frey monofilaments 164
i) Saline control experiments 164
Figure 4.14 165
ii) Morphine experiments 165
Figure 4.15 166
iii) NMDA experiments 166
Figure 4.16 167
Figure 4.17 168
Figure 4.18 169
Figure 4.19 170
iv) NK-1 experiments 170
Figure 4.20 171
Figure 4.21 172
Figure 4.22 173
Figure 4.23 174
Figure 4.24 175
4.3.4 Rotarod experiments 175
i) Morphine experiment 175
Figure 4.25 176
ii) MK-801 experiment 176

Figure 4.26 ______ 177
4.4 Discussion ______ 177
4.4.1 Biting, scratching and licking behaviour ______ 177
i) NMDA receptor activation and BSL ______ 178
ii) The effect of morphine on BSL behaviour ______ 179
iii) The effect of the antagonists on the BSL behaviour ______ 179
iv) What does BSL behaviour show? ______ 181
v) Conclusion ______ 181
4.4.2 NMDA and NK-1 receptor activation and hypersensitivity response ______ 182
i) NMDA receptor activation ______ 183
ii) NK-1 receptor activation ______ 184
4.4.3 Antagonist studies ______ 184
4.4.4 Morphine modulation of the thermal and tactile hypersensitivity ______ 185
i) Thermal hypersensitivity ______ 185
ii) Tactile hypersensitivity ______ 186
4.4.5 NMDA versus NK-1 ______ 186
4.4.6 Conclusion ______ 188

Chapter 5 A study investigating whether peripheral NMDA receptors play a role in the hypersensitivity following peripheral nerve injury ______ 191

5.1 Introduction ______ 191
5.2 Methods ______ 193
5.3 Results ______ 195
5.3.1 Intraplantar injections in normal rats ______ 195
i) Intraplantar saline ______ 195
Figure 5.1 ______ 195
Figure 5.2 ______ 196
ii) Peripheral NMDA receptor activation ______ 196
Figure 5.3 ______ 197
Figure 5.4 ______ 197
Figure 5.5 ______ 198
Figure 5.6 ______ 199
Figure 5.7 ______ 200
5.3.2 Hypersensitivity following spinal nerve ligation model ______ 200
Figure 5.8 ______ 201
Figure 5.9 ______ 202
5.3.3 Peripheral MK-801 following spinal nerve ligation ______ 202
Figure 5.10 ______ 203
Figure 5.11 ______ 204
Figure 5.12 ______ 205
Figure 5.13 ______ 206
5.4 Discussion ______ 206
5.4.1 Peripheral NMDA receptors when activated by intraplantar NMDA ______ 207

5.4.2 Peripheral NMDA receptors and neuropathic pain 208
5.4.3 Conclusion 211

Chapter 6 A study investigating the roles of each of morphine and ondansetron, and their combination, on the hypersensitivity which occurs following the spinal nerve ligation model 214

6.1 Introduction 214
6.2 Methods 217
6.3 Results 218
6.3.1 Ondansetron in normal animals 218
Figure 6.1 219
Figure 6.2 219
6.3.2 Response to thermal, tactile and cold stimuli in rats with spinal nerve ligation and an intrathecal cannula 219
Figure 6.3 220
Figure 6.4 221
Figure 6.5 221
6.3.3 Saline experiments 221
i) Thermal response 222
Figure 6.6 222
Figure 6.7 223
Figure 6.8 223
ii) Tactile response 224
Figure 6.9 224
Figure 6.10 225
Figure 6.11 226
iii) Cold response 226
Figure 6.12 227
6.3.4 Morphine experiments 227
i) Thermal response 227
Figure 6.13 228
Figure 6.14 229
ii) Tactile response 229
Figure 6.15 230
Figure 6.16 231
Figure 6.17 232
iii) Cold response 232
Figure 6.18 233
6.3.5 Ondansetron experiments 233
i) Thermal response 233
Figure 6.19 234
Figure 6.20 234
ii) Tactile response 234
Figure 6.21 235
Figure 6.22 236
Figure 6.23 237

iii) Cold response 237
Figure 6.24 238
6.3.6 Morphine and ondansetron in combination 238
i) Thermal response 238
Figure 6.25 239
Figure 6.26 240
ii) Tactile response 240
Figure 6.27 241
Figure 6.28 242
Figure 6.29 243
iii) Cold response 243
Figure 6.30 244
6.3.7 A comparison between morphine, ondansetron and the combination of the two 244
i) Thermal response 244
Figure 6.31 245
Figure 6.32 246
ii) Tactile response 246
Figure 6.33 247
Figure 6.34 248
Figure 6.35 249
iii) Cold response 249
Figure 6.36 250
6.4 Discussion 250
6.4.1 Ondansetron in normal and neuropathic rats 251
6.4.2 Morphine in neuropathic rats 253
6.4.3 Morphine and ondansetron in neuropathic pain 257
6.4.4 Conclusion 259

Chapter 7 Discussion 262

7.1 Summary 262
7.2 General discussion 262
7.3 Future studies 273

References 275

Chapter 1:

Introduction

1. Introduction

1.1 Pain transmission, neuropathic pain and aim

1.1.1 Pain transmission

Pain has been defined by the International Association for the Study of Pain as "An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Merskey and Bogduk 1994). Pain is normally transmitted from the periphery, through the spinal cord, to supraspinal sites. However, the spinal cord is more than just a relay for pain transmission, as this is the level where afferent input from the periphery and descending controls from supraspinal sites converge and 'gate' pain (Melzack and Wall 1965).

Additionally, the neurotransmission of pain in the spinal cord dorsal horn involves complex interactions between many excitatory and inhibitory neurotransmitters/neuromodulators, receptors and channels (Dickenson 1995). These transmitters, receptors and channels come from either the afferent fibres, intrinsic neurones or descending fibres (Wilcox and Seybold 1997). The balance between excitatory and inhibitory events is what leads to whether or not pain transmission occurs, and with which intensity. Therefore, pain transmission is not straightforward and demonstrates plasticity. Pain control is normally achieved either by selective blockade of excitatory transmission, or enhancement of inhibitory transmission (Dickenson 1995; Dickenson 1997).

1.1.2 Neuropathic pain

Neuropathic pain has been 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". Neuropathic pain may be subdivided according to the location of the lesion/dysfunction (peripheral versus central). Part of this study will revolve around peripheral neuropathic pain. Peripheral neuropathic pain can

arise from diseases such as diabetes mellitus, herpes zoster, HIV, from nerve compression such as that resulting from tumours, or from neuroma formation following amputation or trauma (Suzuki and Dickenson 2000).

Symptoms of neuropathic pain are both negative, such as sensory deficits and positive. The positive symptoms are subclassified as evoked pain to different mechanical and thermal stimuli such as tactile allodynia, hyperalgesia, continuous pain, such as burning or stabbing pain, or paroxysmal pain, such as shooting or lancinating pain (Attal and Bouhassira 1999). Positive symptoms seem to occur when the nervous system 'compensates' for the sensory loss and becomes hyperexcitable (Suzuki and Dickenson 2000).

1.1.3 Hyperalgesia and allodynia

i) Definitions

As mentioned above, the overall painful sensation experienced, following neurotransmission, from the periphery to the spinal cord, is not directly proportional to the intensity of the painful stimulus at the periphery. Pain transmission is 'plastic', and not a linear relationship between stimulus and response. Hyperalgesia is the term used to describe an amplified pain response to a mildly noxious stimulus, whereas allodynia is the term used to describe pain due to a non-painful stimulus (Petrenko, Yamakura et al. 2003).

Earlier, there were two theories that attempted to explain the mechanisms underlying neuropathic pain. One of them postulated peripheral mechanisms were involved, whilst the other postulated central mechanisms were involved (Treede, Meyer et al. 1992). Nowadays, it is recognised that the mechanisms underlying neuropathic pain include a combination of both peripheral and central mechanisms of sensitisation (Treede, Meyer et al. 1992).

Following tissue damage in the periphery, there is a primary area of pain around the injury site, this is termed primary hyperalgesia and is thought to involve

sensitisation of nociceptors (C- and Aδ- diameter primary afferent fibres which convey the sensation of pain from the periphery to the spinal cord) at the periphery (peripheral sensitisation) (Caterina, Gold et al. 2005; Meyer, Ringkamp et al. 2006). Whereas a secondary area of pain not related to the injury site is termed secondary hyperalgesia/allodynia, the mechanisms of which are thought to be sensitisation of spinal cord dorsal horn neurons (central sensitisation) (Treede, Meyer et al. 1992; Urban, Zahn et al. 1999). Sensitisation is the neurophysiological correlate of hyperalgesia and is defined as a leftward shift of the stimulus-response curve, relating the size of the neural response to the intensity of the stimulus (Treede, Meyer et al. 1992).

ii) Thermal hyperalgesia

Hyperalgesia due to heat occurs only at the primary injury site, and is thought to involve the sensitisation of C- and Aδ-fibres. At the secondary injury site, heat responses are either unchanged or decreased (Meyer and Campbell 1981; Raja, Campbell et al. 1984; Treede, Meyer et al. 1992; Magerl, Fuchs et al. 2001; Meyer, Ringkamp et al. 2006).

iii) Mechanical hyperalgesia/allodynia

Hyperalgesia/allodynia due to mechanical stimuli occurs both at the primary and secondary injury sites, due to both peripheral and central sensitisation mechanisms, respectively. Mechanical allodynia is demonstrated by a lowering of the activation threshold to mechanical stimuli, and the increase of both the responses to suprathreshold stimuli and the receptive fields of both Aδ- and C-fibres following injury (Meyer, Ringkamp et al. 2006). Finally, there are two different types of mechanical allodynia: dynamic allodynia, which is thought to be mostly transmitted by Aβ-fibres and static allodynia, which is thought to be mostly transmitted by Aδ-fibres (Field, Bramwell et al. 1999).

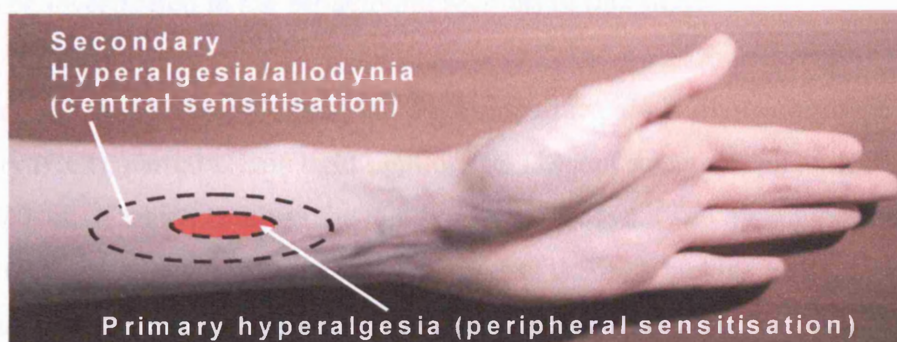


Figure 1.1- The red circle represents the primary area around the injury site where heat and mechanical hyperalgesia occur due to peripheral sensitisation and the larger circle represents the secondary uninjured site where mechanical hyperalgesia and allodynia occur due to central sensitisation.

1.1.4 Animal models of neuropathic pain and what they measure

Experimentally, at the dorsal horn neuronal level, nerve injury causes increases in spontaneous activity of dorsal horn neurons and receptive field sizes of neurons in response to low-intensity mechanical stimuli (Chapman, Suzuki et al. 1998; Suzuki, Kontinen et al. 2000; Suzuki and Dickenson 2002). However, asides from the previously discussed, it has been shown that behavioural and neuronal studies show opposite sides to neuropathic pain, where behavioural studies seem to reflect mostly positive symptoms, whereas studies undertaken at the neuronal level reflect mostly negative symptoms, such as the decrease in the size of C-fibre-evoked responses (Suzuki and Dickenson 2002).

In behavioural studies investigating hyperalgesia and allodynia, researchers usually measure threshold responses, since the animals are free to move once a stimulus is noxious. Hyperalgesia and allodynia to any stimuli investigated in my study will be termed 'hypersensitivity', since it is important to differentiate between measurements made on humans to those made on animals (Per Hansson,

personal communication). Hypersensitivity to mechanical, heat and cold stimuli will be investigated in the behavioural sections of this study.

There are three widely used models of neuropathic pain: chronic constriction injury (CCI), partial sciatic tight ligation (PSTL) and selective spinal ligation (SNL), all of which result in the rapid onset of allodynia and hyperalgesia (Kim and Chung 1992). Evoked hypersensitivities to thermal, tactile and cold stimuli are usually the responses measured in most models of neuropathic pain. These stimulus-dependent responses vary in their onset, intensity and existence between the different models, and are measured because they are readily quantifiable and are also experienced to some degree in humans clinically. Stimulus-independent responses such as spontaneous pain remain harder to quantify in behavioural studies of animal models (Koltzenburg and Scadding 2001; Mogil and Crager 2004).

The model which will be used in this study will be Kim and Chung's selective spinal nerve ligation, which involves the complete ligation of L5 and L6 spinal nerves branching off the sciatic nerve. The sciatic nerve is the major nerve providing the sensory and motor innervation of the lower hindlimb and foot. The advantages of this model over the other two are that the number and types of injured fibres are well controlled, and that the levels of injured and intact spinal segments are completely separated. This model gives rise to thermal, tactile and cold hypersensitivity, as well as spontaneous, stimulus independent, dorsal horn neuronal activity (Kim and Chung 1992).

1.1.5 Pharmacological treatments in neuropathic pain

So far, only "1 in 3 patients get adequate pain relief" (Sindrup and Jensen 1999) and "At best, no more than 50% of patients benefit from available drugs" (Hansson and Dickenson 2005). Neuropathic pain management in patients has included the use of morphine, tramadol, gabapentin, different sodium channel blockers and the use of combination therapy. There is clearly not one drug which may decrease all of the symptoms of neuropathic pain, simply because so many

mechanisms are underlying the pathology of this disease, from the periphery, to the spinal cord to supraspinal sites. Additionally, new directions of research have included investigating the use of peripheral NMDA receptor antagonists in neuropathic pain (Jang, Kim et al. 2004), as well the use of the 5HT3 antagonist ondansetron, the latter of which has caused some pain relief in neuropathic patients (McCleane, Suzuki et al. 2003).

1.1.6 Morphine

Morphine is an opioid that is derived from opium, which is released by scraping the seed pod of the opium poppy, or '*papaver somniferum*'. The analgesic properties of opioids have been discovered and used by mankind for thousands of years, in many different cultures. Morphine exerts its analgesic action by acting on μ opioid receptors, which are located on nociceptive primary afferent fibres, the spinal cord and supraspinal sites as well, and is thought of as the 'gold standard in pain control' (Dickenson and Kieffer 2006; Schug and Gandham 2006). Morphine is available in many different forms, and may be delivered *via* different routes, such as tablets, capsules, suspension and through epidural and intrathecal routes. However, the side-effects associated with morphine, such as respiratory depression, sedation, euphoria, constipation, as well as tolerance, lead to limits in dose-escalation of this drug (Dickenson and Kieffer 2006; Schug and Gandham 2006).

Furthermore, plasticity is also seen with morphine, since there have been some reports of 'morphine-insensitive' pain, where morphine was shown to have a reduced efficacy, such as some types of neuropathic pain (Woolf and Wall 1986; Arner and Meyerson 1988), whereas morphine has been shown to be even more efficient in inflammatory pain states (Kayser, Chen et al. 1991; Dickenson 1997).

Now it is recognised that neuropathic pain is not 'resistant' to morphine (Rowbotham, Reisner-Keller et al. 1991; Yamamoto and Yaksh 1992; Sindrup and Jensen 1999; Suzuki, Chapman et al. 1999; Attal, Guirimand et al. 2002; Hansson and Dickenson 2005), but variably responsive to morphine, according to

the dose, route of administration and type of neuropathic pain treated (Suzuki, Chapman et al. 1999). Therefore, dose-escalation is needed for morphine to inhibit some types of neuropathic pain. However, in order to not resort to higher doses which inevitably lead to unacceptable side-effects, the combination of opioids with other agents, such as gabapentin, have been used to treat neuropathic pain (Gilron, Bailey et al. 2005; Raja and Haythornthwaite 2005).

1.1.7 General aim

The general aim of this study was to investigate the modulation of excitatory transmission in the rat spinal cord by morphine. To do this, morphine efficacy in two different models of nociception where the 'nociceptive balance' was tipped towards spinal excitation, an acute chemical and a chronic surgical neuropathic model of nociception, was investigated. Additionally, in the neuropathic pain model, the combination of morphine with ondansetron, an anti-emetic drug which has been newly found to have analgesic properties (McCleane, Suzuki et al. 2003), was also studied. Furthermore, peripheral NMDA receptors in neuropathic pain were also investigated, since this area has not been extensively studied. In vivo electrophysiology and behavioural techniques were used as investigative tools.

1.2 Primary afferent fibres

There are highly specialised sensory fibres that convey to the central nervous system information about the state and the environment of the organism, and the most investigated and of relevance in this study are cutaneous sensory fibres. Primary afferent fibres have a peripheral terminal that comprises of sensory nerve endings, a central terminal that comprises of dendrites, and their cell bodies lie in the dorsal root ganglion (DRG). Three types of primary afferent fibres have been described (Meyer, Ringkamp et al. 2006):

Small-diameter (0.4-1.2μm) primary afferent fibres are called C-fibres, they are unmyelinated, and conduct slowly (0.5-2.0m/sec). Medium-diameter (2-6μm) primary afferent fibres are called Aδ-fibres and are myelinated and of intermediate speed (12-30m/sec). Large-diameter (>10μm) primary afferent fibres are called Aβ-fibres, these fibres transmit non-noxius stimuli, are myelinated and fast-conducting (30-100m/sec) (Millan 1999).

A subpopulation of C-fibres respond exclusively to gentle warming, whereas a subpopulation of the Aδ-fibres, as well as some C-fibres, respond exclusively to gentle cooling (Caterina, Gold et al. 2005; Meyer, Ringkamp et al. 2006).

Texture and shape of touch sensation are encoded by activity in different classes of low-threshold myelinated afferents (Aβ-fibres), which end in specialised cells. The slowly adapting type 1 afferents end in Merkel cells, whereas type 2 end in Ruffini corpuscles. Rapidly adapting afferents end in Meissner corpuscles or surrounding hair follicles, whereas Pacinian afferents end in corpuscles of the same name (Caterina, Gold et al. 2005).

1.3 Nociceptors

In normal acute pain, noxious stimuli are transmitted from the skin in the periphery to the dorsal horn *via* nociceptors. Nociceptors is the term given for the C- and Aδ-fibres which respond to high-threshold, noxious stimuli, encoding its location and intensity. Some of these nociceptors are polymodal in that they encode thermal, mechanical and chemical stimulus modalities, which all cause injury, whereas others have more specialised response properties (Caterina, Gold et al. 2005; Meyer, Ringkamp et al. 2006). As opposed to the previously mentioned low-threshold myelinated afferents, the axons of nociceptors end as free nerve endings in dermal and epidermal layers of the skin (Patapoutian, Peier et al. 2003).

Furthermore, nociceptors do not exhibit spontaneous activity, and even though they have a higher threshold of activation than low-threshold myelinated afferents, their threshold of activation is still lower than what can be perceived as a painful sensation. It is thought that a painful sensation is only evoked following temporal and/or spatial summation of nociceptor input, as shown when the firing of a single action potential by a C-fibre in human microneurography studies did not cause pain (Treede, Meyer et al. 1992).

1.4 Stimulus modality conduction in unmyelinated and myelinated fibres

Unmyelinated nociceptors that respond to mechanical and heat stimuli are termed CMH (C-fibre mechano-heat-sensitive nociceptors), whereas Aδ-fibres that respond to the aforementioned stimuli are called AMH (Aδ-fibre mechano-heat-sensitive nociceptors), and most of these two fibre types also respond to chemical stimuli and are thus considered polymodal (Caterina, Gold et al. 2005; Meyer, Ringkamp et al. 2006). Furthermore, although in both groups, many nociceptors are Mechanically Sensitive Afferents (MSA), Mechanically Insensitive Afferents (MIA) have also been described. The latter either have high mechanical thresholds or are insensitive to mechanical stimuli, are sometimes termed 'silent nociceptors' (Meyer, Ringkamp et al. 2006), and become sensitised following injury (Caterina, Gold et al. 2005).

CMH fibres exhibit both fatigue, and sensitisation to repeated stimuli. Fatigue is when the response to the second of two identical stimuli is less than the response to the first, which is why in some cases, time for recovery of the nociceptors must elapse between each painful stimuli. Sensitisation is when the response to the second of two (or more) identical stimuli is greater than the response to the first stimulus (Slugg, Meyer et al. 2000; Meyer, Ringkamp et al. 2006).

Furthermore, there are two types of Aδ-fibre nociceptors. 'Type 1' A-fibre nociceptors are activated by heat, mechanical and chemical stimuli, but their heat threshold to short stimuli is so high that they have previously been called 'High

Threshold Mechanoceptors'. They have a low threshold in response to long-duration stimuli and become sensitised after repetitive heat stimulation and tissue injury. Their conduction velocity is between that of Aδ- and Aβ-fibres, and they are located in both hairy and glabrous skin. As opposed to 'Type 1', most 'Type 2' A-fibre nociceptors are MIAs, have a low activation threshold to heat stimuli of both short and long-duration and have a conduction velocity similar to that of normal Aδ-fibres and are found in hairy skin only (Millan 1999; Meyer, Ringkamp et al. 2006).

These aforementioned primary afferent fibres will relay the noxious and innocuous thermal and mechanical stimuli, in the parts of the study using electrophysiological and behavioural techniques, and these primary afferents will also be electrically evoked and separated according to their conduction speed in the part of the study using in vivo electrophysiology.

1.5 Primary afferent fibres may be grouped using different molecular markers

Finally, nociceptors may be grouped according to molecular markers, which are molecules that are either found on the cell surface, or stored and released from inside primary afferents or enzymes. Nociceptors are therefore divided into two groups, which mildly overlap. Group 1 are peptidergic neurons, which contain peptides such as substance P, calcitonin gene related peptide, somatostatin and are TrkA-positive (TrkA- nerve growth factor receptor). These peptidergic neurons comprise almost 40% of DRG cells, and centrally project to lamina (layer) I and lamina II outer, of the spinal cord dorsal horn (mostly C-fibres). Group 2 are non-peptidergic, contain fluoride-resistant acid phosphatase (FRAP), bind the plant lectin IB4 (from *Griffonia simplicifolia*) and comprise almost 30% of DRG neurons. These non-peptidergic neurons project mostly to lamina II inner (mostly Aδ-fibres), of the spinal cord dorsal horn (Urban and Randic 1984; Snider and McMahon 1998; Meyer, Ringkamp et al. 2006).

1.6 Peripheral terminal

At their peripheral level, nociceptors have receptors/channels that are either directly or indirectly (through secondary mechanisms) activated by damage to tissues/nerves via different kinds of stimuli (thermal, mechanical, chemical). The functional receptors that detect noxious stimuli then transduce the different forms of stimuli into action potential generation in the primary afferent fibres, the result of which is neurotransmitter release onto dorsal horn neurons, at the central terminal of these fibres. Furthermore, primary afferent fibres also have the capacity to release substances peripherally as well, by a process called the axon reflex, all of which will be described in the following section (Caterina, Gold et al. 2005).

1.6.1 Channels and receptors at the periphery

Sequentially, a noxious stimuli of thermal, chemical and/or mechanical nature needs to be transduced into electrical signals in order for spike initiation, followed by action potential generation, to result in transmitter release. Furthermore, action potential generation only occurs if the depolarising currents generated following signal transduction are large enough. Although there is still a lot to be known about how different stimulus modalities can cause the initiation and the generation of an action potential, it is thought that this mechanism is spun into action once there is an exchange of ions across the cell membrane, which changes the electrical properties of the cell. There are many receptor and channel families throughout the nervous system, whose activation leads to membrane depolarisation, either directly *via* the opening of cation channels or indirectly *via* the production of intracellular signalling cascades which sensitise depolarising cation channels to the aforementioned noxious stimuli (Woolf and Salter 2000; Caterina, Gold et al. 2005).

1.6.2 Signal transduction receptors/channels

i) TRP channels

TRP (Transient Receptor Potential) family of proteins is named after its role in Drosophila phototransduction. TRP receptors are involved in coding for light, cold, mechanical distension, acidity and heat, and they are expressed in sensory neurons, as well as other locations. TRP channels have four subunits (thought to be homo- or heteromeric), with each subunit having six transmembrane domains, a pore formed between the 5th and 6th transmembrane domains and a voltage sensor. Most TRP channels have variable permeability to Ca^{2+}, and they are non-selective cation channels (Lee, Lee et al. 2005; Meyer, Ringkamp et al. 2006).

TRPV1 (TRP-vanilloid receptor) is a channel which is activated by noxious heat (≥42°C), capsaicin (chemical found in red peppers) and activated as well as potentiated by protons. TRPV1 is expressed highly in small to medium diameter neurons within dorsal root ganglia, especially capsaicin sensitive peptidergic neurons (Caterina, Schumacher et al. 1997). Therefore, most unmyelinated fibres, and some of the type 2 Aδ-fibres, are capsaicin-sensitive (Magerl, Fuchs et al. 2001). In mice lacking this channel, noxious heat-evoked responses were decreased, and this channel was found to contribute significantly to some types of tissue injury- induced thermal hypersensitivity (Complete Freund's Adjuvant and mustard oil) (Caterina, Leffler et al. 2000). But many other TRP as well as other types of channels, are also involved in thermosensation, and thus the fact that in these TRPV1-deficient mice, heat response was not abolished completely (Patapoutian, Peier et al. 2003; Meyer, Ringkamp et al. 2006).

TRPV2 is another channel that transduces heat responses higher than, or equal to, 52°C, and is expressed predominantly on medium-diameter DRG neurons. This channel is thought to underlie the heat response of high-threshold type 1 Aδ-fibres, and is not sensitive to either capsaicin or protons (Patapoutian, Peier et al. 2003; Meyer, Ringkamp et al. 2006).

TRPV3 and TRPV4 are two more TRP channels that are thought to contribute to warm sensation, with TRPV4 also thought to be activated by stretch, making it

have a role in mechanotransduction as well (Wood 2004; Meyer, Ringkamp et al. 2006).

ii) ASIC channels

The Acid Sensing Ion Channel belongs to the epithelial amiloride sensitive Na^+ channel/degenerin (ENaC/DEG) family, and is gated by a reduction in pH. The ASIC subunits may assemble in homo- or heteromultimeric channels, with two transmembrane domains each, and distinct acid sensitivities, ion selectivities and activation/desensitisation kinetics (Caterina, Gold et al. 2005; Lee, Lee et al. 2005). This channel is also found in sensory neurons, and to date, at least three subtypes have been identified (ASIC1-ASIC3) (Wood, Abrahamsen et al. 2004).

iii) P2X channels

Purinergic receptor subtype X channels (P2X) are ATP-gated (adenosine trisphosphate- released from damaged tissues and elicits pain sensation in humans) extracellular ion channels, that allow the entry of Ca^{2+} and Na^+ once opened and have seven different subunits (P2X1-P2X7). Some of these receptor subunits can be found both at the peripheral and central terminal of primary afferent fibres, as well as in dorsal horn neurons and microglia (Nakatsuka and Gu 2006). Of these, P2X3, expressed highly in non-peptidergic, IB4-positive neurons, and P2X4, expressed in microglia (Lee, Lee et al. 2005; Meyer, Ringkamp et al. 2006), have been shown to play an important role in both acute and pathological pain states, of inflammatory and neuropathic nature, respectively (McCleskey 2003; Tsuda, Shigemoto-Mogami et al. 2003; Tsuda, Inoue et al. 2005).

iv) P2X channels and microglia in neuropathic pain

5-10% of the glia in the CNS are microglia, which are thought of as the macrophages of the CNS. Normally, microglia are thought to be in a resting state, however, following peripheral nerve injury, spinal microglia become

activated, change their shape and increase their expression of the P2X4 receptor (McCleskey 2003; Salter 2005). This increased expression of the P2X4 receptor by microglia is thought to be one of the mechanisms underlying tactile allodynia following neuropathic pain (Tsuda, Inoue et al. 2005), since the pharmacological blockade of this receptor, or the administration of its antisense in neuropathic animals has been shown to alleviate tactile allodynia. Furthermore, in normal rats, the administration of microglia with induced and stimulated P2X4 receptors was sufficient to cause tactile allodynia (Tsuda, Shigemoto-Mogami et al. 2003).

1.6.3 Signal transduction due to thermal versus mechanical stimuli

As opposed to thermal and chemical transducing receptors and channels, it is still not well known which receptors/channels are involved in transducing mechanosensation. Some of the channels thought to be involved include the bacterial osmosensitive ion channels MscL and MscS, TRPV4 and P2X3 (Wood, Abrahamsen et al. 2004).

Therefore, in my study, these aforementioned receptors and channels are some of the receptors/channels that are postulated to be activated when the neurons that are being recorded from are responding to thermal and mechanical stimuli. This is also true for the thermal, mechanical and cold response elicited during behavioural tests in the free-moving animal.

1.6.4 Mechanisms involved in peripheral tissue damage and axonal reflex

i) Inflammatory mediators

Following tissue damage in the periphery, many substances are released, which either directly, by acting on their own receptors, or indirectly, by enhancing the nociceptive response of other substances, cause hyperalgesia. Some of the many inflammatory substances include bradykinin, prostaglandins, serotonin, histamine, protons, adenosine, ATP, nerve growth factor (NGF), cytokines,

substance P and possibly glutamate (Kidd and Urban 2001; Caterina, Gold et al. 2005; Meyer, Ringkamp et al. 2006).

ii) Substance P, calcitonin gene-related peptide and the axon reflex

Substance P (SP) and calcitonin gene-related peptide (CGRP) are neuropeptides that are released from both the peripheral and the central terminals of small-diameter peptidergic nociceptors. When one branch of the nociceptor terminal is activated by injury in the periphery, this leads to orthodromic action potential generation into the axon, as well as antidromic action potential generation into other branches. SP and CGRP are thus released by this 'axon reflex' from the peripheral terminal of nociceptors (Burnstock 1977), where they act as vasoactive substances and cause plasma extravasation from post-capillary venules (SP) and vasodilatation from arterioles (CGRP) (Hagermark, Hokfelt et al. 1978). These events lead to the wheal and flare response seen post-cutaneous injury. SP can also cause the release of histamine from mast cells, whereby histamine, in turn, can lead to more vasodilation and oedema (Caterina, Gold et al. 2005; Meyer, Ringkamp et al. 2006). The axon reflex will be further discussed later, when investigating the role of peripheral NMDA receptors in neuropathic pain.

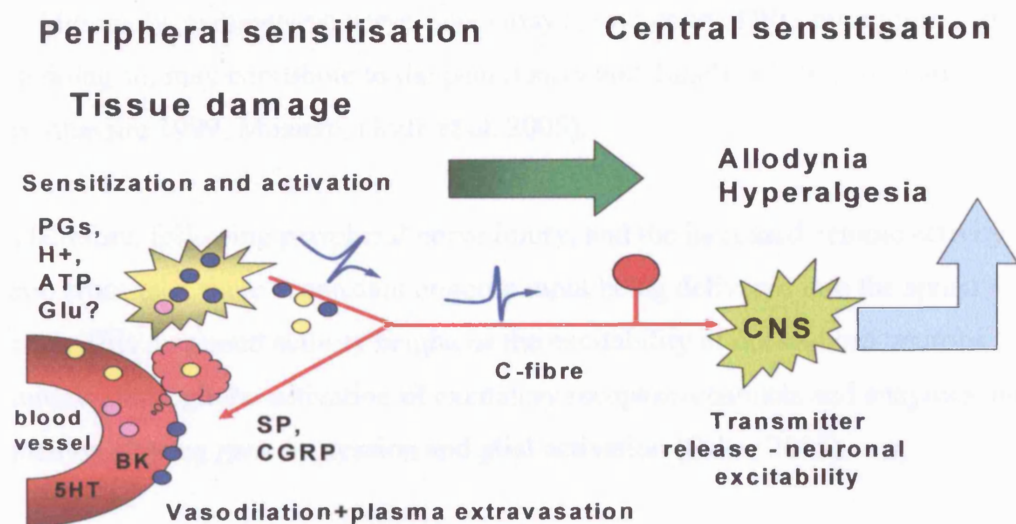


Figure 1.2- Following tissue damage, numerous inflammatory mediators are released at the periphery, such as prostaglandins (PGs), protons (H^+), adenosine trisphosphate (ATP), bradykinin (BK), serotonin (5HT), glutamate (Glu), substance P (SP) and calcitonin gene related peptide (CGRP), which lead to the activation and sensitisation of peripheral receptors and channels (peripheral sensitisation) and thus action potential generation. Transmitter release and dorsal horn neuronal excitability follow the action potential generation, and lead to central sensitisation. Adapted with permission from Prof. A. H. Dickenson.

1.6.5 Peripheral mechanisms in neuropathic pain

In the periphery, after nerve injury, the electrical properties of primary afferent fibres changes, and a newfound hyperexcitability, shown by ongoing spontaneous activity, arises. This activity is called 'ectopic' activity, in that it arises independent of any stimulus, and its points of origin include the DRG and the injured peripheral nerve (neuroma and regions of demyelination) (Song, Hu et al. 1999) (Kajander, Wakisaka et al. 1992). Even though ectopic activity arises from all types of primary afferent fibres, it seems to mostly occur in A-fibres (Attal and Bouhassira 1999).

Furthermore, when the insulation/myelination of the nerves is disrupted following nerve injury, impulses carried in one nerve fibre may be transmitted to

a neighbouring fibre. This is called ephatic transmission, or cross-talk. Additionally, sympathetic nerve fibres may sprout on the DRG after injury, and in doing so, may contribute to the pain (Garry and Tanelian 1997; Attal and Bouhassira 1999; Moalem, Grafe et al. 2005).

Therefore, following peripheral nerve injury, and the increased ectopic activity and cross-talk, there is constant ongoing input being delivered into the spinal cord. This increased activity heightens the excitability of dorsal horn neurons, initially through the activation of excitatory receptors/channels and enzymes, and then by altering gene expression and glial activation (Salter 2005).

1.6.6 Channels involved in signal propagation

Action potential generation is due to two channels, a tetrodotoxin-sensitive voltage-gated sodium (Na^+) channel and a delayed rectifier type of potassium (K^+) channel. The voltage-gated Na^+ channel opens rapidly and transiently, when the membrane is depolarised beyond –60 to –40mV, and causes Na^+ to enter the neuron and further depolarise it. The membrane potential goes back to normal when these Na^+ channels are inactivated, and when the delayed opening on K^+ channels allows K^+ out of the cell (repolarisation) (Caterina, Gold et al. 2005).

i) Na channels

Voltage-gated Na^+ channels in mammals consist of a single family of nine related functional α-subunits ($Na_V1.1$-$Na_V1.9$), associated with two accessory β-subunits. The α-subunit is large and has four homologous domains, which form the voltage sensor and channel pore, whereas the β subunit plays a role in the biophysical properties of the channel (density and plasma membrane distribution). The best way to distinguish between Na^+ channels is based on their sensitivity to tetrodotoxin (TTX): $Na_V1.1$, 1.2, 1.3, 1.4, 1.6 and 1.7 are TTX-sensitive, whereas $Na_V1.5$, 1.8 and 1.9 are TTX-resistant. $Na_V1.6$ is the TTX-sensitive channel found on myelinated axons, whereas $Na_V1.7$ is expressed

preferentially in the periphery, on unmyelinated sensory neurons (Caterina, Gold et al. 2005; Lee, Lee et al. 2005; Julius and McCleskey 2006; Meyer, Ringkamp et al. 2006). $Na_V1.8$ and $Na_V1.9$ are preferentially expressed on sensory neurons, both peptidergic and IB4-positive, but $Na_V1.9$ is found mostly on IB4-positive neurons. Since there are no subtype-specific antagonists which exist, experiments are usually carried out with mutant mice lacking the specific channels (Wood and Baker 2001). Work with mutant mice, which lack either the $Na_V1.7$ or $Na_V1.8$ channel or both, showed that these subtypes may be involved in setting pain thresholds and inflammatory pain, but not neuropathic pain (Akopian, Souslova et al. 1999; Nassar, Levato et al. 2005; Amir, Argoff et al. 2006).

ii) Na channels in neuropathic pain

Following nerve injury, changes in Na ion channel expression and redistribution seems to be involved in ectopic activity generation. There seems to be an up-regulation of $Na_V1.3$ in injured neurones, which was a previously silent, embryonic type of Na channels. This Na channel subtype is of the TTX-S variety, and after nerve injury, allows the Na current in DRG neurones to reprime and recover from inactivation rapidly, which seems to be a feature well needed for the generation of ectopic activity (Woolf and Mannion 1999; Suzuki and Dickenson 2000; Zimmermann 2001; Ji and Strichartz 2004). Furthermore, $Na_V1.1$, $Na_V1.8$ and $Na_V1.9$ are downregulated, after nerve injury. However, following nerve injury, $Na_V1.8$ seems to accumulate in uninjured axons found in the hyperexcitable sciatic nerve (Wang, Sun et al. 2002; Ji and Strichartz 2004). Nevertheless, the role of $Na_V1.8$ in neuropathic pain is questionable, since it was previously shown that treatment with antisense to this channel decreased neuropathic pain in the SNL model in rats, whereas a newer study using $Na_V1.8$-null mice showed that abnormal neuropathic pain developed even in these animals (Nassar, Levato et al. 2005; Amir, Argoff et al. 2006).

Additionally, the importance of Na channels in the pathophysiology of neuropathic pain was further proved when the administration of Na channel blockers such as local anaesthetics (eg: lignocaine) (Rowbotham, Reisner-Keller

et al. 1991; Ossipov, Lopez et al. 1995) and some anticonvulsants (eg: carbamazepine and lamotrigine) (Sindrup and Jensen 1999) was shown to reduce pain associated with nerve injury. These drugs are all use-dependent, and block Na channels at therapeutic doses (Dickenson, Matthews et al. 2002). Furthermore, the ability of topical lignocaine to also reduce neuropathic pain in patients shows that these drugs exert their actions partly at the periphery (Campbell, Raja et al. 1988). Indeed, the local administration of bupivacaine at the sciatic nerve 15 minutes before injuring it in rats delayed the onset of thermal hyperesthesia, and thus injury discharge induced facilitation of dorsal horn neurons in the CCI model (Yamamoto, Shimoyama et al. 1993).

iii) K channels

K^+ channels are extremely diverse in structure and function, and thus have been classified and reclassified numerous times, with two types of nomenclature still used for these channels (Gutman, Chandy et al. 2003). The delayed rectifier K^+ channels mentioned above are voltage-gated, with six transmembrane (TM) domains where the pore lies between the fifth and sixth TM domain. The $K_V1.4$ subtype is thought to be the nociceptive delayed rectifier, since it is found in most sensory neurons where $Na_V1.8$ is expressed, whereas $K_V1.1$ and $K_V1.2$ are found on large-diameter sensory neurons (Lee, Lee et al. 2005; Julius and McCleskey 2006).

Another class of K^+ channels that is not voltage-gated is coded by the gene KCNQ, two of which, the M channel and the H channel, return the potential to resting after the activation of many G protein-coupled receptors (Gutman, Chandy et al. 2003). The M channel has been found to be expressed in both capsaicin-sensitive and insensitive small-diameter primary afferents, as well as in large-diameter non-nociceptive primary afferents (Lee, Lee et al. 2005; Julius and McCleskey 2006). The importance of the currents of the M channel in the latter neurons was not great, since the subthreshold currents were dominated by other types of K^+ currents, and retigabine, an M channel opener, was not found to

have a great effect of the Aβ fibre-mediated response during in vivo electrophysiology (Passmore, Selyanko et al. 2003).

iv) K channels in neuropathic pain

Less is known about the role of K channels in neuropathic pain, however, many voltage-gated K channels seem to be downregulated in the DRGs ipsilateral to nerve injury (Wang, Sun et al. 2002; Lee, Lee et al. 2005). Furthermore, the M channel has been shown to play a role in controlling neuronal excitability, since retigabine inhibited nociceptive fibre-mediated responses in normal and neuropathic rats (SNL model), as well as showed an analgesic effect in an inflammatory model of pain (carrageenan) (Passmore, Selyanko et al. 2003).

1.6.7 Channels involved in neurotransmitter release

i) Ca channels

Following the generation of the action potential generation and the depolarisation of the cell, neurotransmitters are released at the central terminal of primary afferent fibres, onto spinal cord dorsal horn neurons. If the neurotransmitters were excitatory, then secondary excitatory events in spinal cord dorsal horn neurons also occur, and these excitatory events depend on the entry of extracellular Ca^{2+} into the cell, one way of which is *via* voltage-dependent Ca^{2+} channels (VDCC) (Caterina, Gold et al. 2005).

VDCCs include the L (dihydropyridine, phenylalkylamine and benzothiazepine-sensitive), N (ω-Conotoxin-GVIA and SNX-sensitive), P (ω-Agatoxin-IVA-sensitive), R and T subtypes. The L subtype belongs to gene family 1 (Ca_V1), the N, P and R subtypes belong to gene family 2 (Ca_V2), whereas the T subtype belongs to gene family 3 (Ca_V3). The L, N, P and R subtypes are VDCCs that are activated by large depolarisation (high threshold) (Vanegas and Schaible 2000; Caterina, Gold et al. 2005). The L subtype is found mostly at cell bodies and

dendrites, whereas the N, P and R subtypes are located at synaptic sites and the N and P are involved with transmitter release. The T subtype is activated by low voltage, is found in neuronal and non-neuronal cells and is thought to play a role in regulation of neuronal excitability (Vanegas and Schaible 2000; Julius and McCleskey 2006). Each of these channels consists of a large $\alpha1$-subunit, which forms the pore and voltage sensitivity, as well as an intracellular β-subunit, a trans-membrane $\alpha2\delta$-subunit and a γ-subunit (in skeletal muscle), the last three subunits being involved in modulating expression and gating (Caterina, Gold et al. 2005).

The N and P channels are found throughout the brain and spinal cord, but the N channel is predominantly found in laminae I and II of the spinal cord dorsal horn (Vanegas and Schaible 2000), further showing its role in neurotransmitter release, as this is the primary site for neurotransmitters released from the central terminal of primary afferents to exert their effects on dorsal horn neurons. The N channel is thought to mediate the spinal release of the peptides substance P (Holz, Dunlap et al. 1988) and calcitonin gene-related peptide, whereas both the N and P channels are thought to mediate the release of the excitatory amino acid glutamate (Dickie and Davies 1992; Turner 1998).

ii) Ca channels in neuropathic pain

The increase of intracellular calcium is linked to both neurotransmitter release, and thus the ensuing synaptic transmission, as well as phosphorylation and activation of many excitatory channels, receptors and enzymes (see section **1.7.4**). The N channel has been shown to be involved in both acute and chronic pain transmission. Following neuropathic pain, the efficacy of ω-Conotoxin-GVIA, the N channel antagonist, was increased, as well as the expression of its pore-forming $\alpha1\beta$ subunit in the superficial dorsal horn (Matthews and Dickenson 2001; Cizkova, Marsala et al. 2002; Suzuki and Dickenson 2006). Furthermore, this antagonist also inhibited both phases of the formalin response (Diaz and Dickenson 1997). The P channel antagonist, ω-Agatoxin-IVA, has

been shown to inhibit both inhibitory and excitatory transmission (Vanegas and Schaible 2000). As opposed to the N channel, the P channel is thought to play a smaller role in pain transmission, since the administration of its antagonist did inhibit the second phase of formalin inflammation (Diaz and Dickenson 1997), however, the same antagonist did not inhibit neuronal responses in normal and neuropathic rats as much as the N channel blocker did (Matthews and Dickenson 2001).

Additionally, gabapentin, one of the most effective drugs licensed for the treatment of neuropathic pain, has been found to bind to a unique site on the $\alpha 2\delta$ subunit which is found in all VDCCs, and which is upregulated in injured DRGs following nerve injury (Wang, Sun et al. 2002; Ji and Strichartz 2004), leading to increased neurotransmission and hyperexcitability of dorsal horn neurons. Consequently, gabapentin has been shown to inhibit both stimulus-independent and evoked responses of dorsal horn neurons (Dickenson, Matthews et al. 2002; Suzuki, Rahman et al. 2005; Suzuki and Dickenson 2006), as well as abnormal pain behaviour following nerve injury (Field, McCleary et al. 1999).

1.7 Spinal cord dorsal horn

1.7.1 Dorsal horn anatomy

The spinal cord is made up of segments, each of which corresponds to relaying information to and from the periphery and the central nervous system. Each segment of the spinal cord is made up of the dorsal horn, which is involved with receiving, transmitting and altering sensory information from primary afferent fibres to supraspinal sites, and the ventral horn which is involved with the generation of motor effects from spinal and supraspinal sites to the periphery (Willis 1985; Todd and Koerber 2006). The spinal cord dorsal horn is therefore the 'first synapse' where the central terminals of the primary afferent fibres impinge, mostly through dorsal roots, on dorsal horn neurons. Information from the periphery is either transmitted to supraspinal sites unaltered, or altered by

spinal events before it reaches higher sites. Furthermore, local reflexes are produced by neurons in the spinal cord (Willis 1985; Todd and Koerber 2006).

This study investigates cutaneous sensory information from the hindlimbs of rats, which means the area of focus where neurons are recorded from, where some of the drugs used will be applied in both the electrophysiological and behavioural parts of the study, and where neuropathic surgery is performed to affect, is the L4-L6 (lumbar) segments of the spinal cord dorsal horn.

Besides the central terminals of primary afferent fibres which arborise in the different layers of the dorsal horn, there are different types of neurons which exist in the dorsal horn too. Neurons intrinsic to the dorsal horn are called interneurons. The majority of dorsal horn neurons are interneurons, which can be either excitatory (glutamatergic) or inhibitory (enkephalinergic/GABA-ergic), and thus may modify pain transmission at the spinal level. Furthermore, the dorsal horn has neurons which project to supraspinal sites, called projection neurons, and descending neurons, which descend from supraspinal sites onto the dorsal horn and also modify pain transmission (Todd and Koerber 2006). Moreover, spinal cord dorsal horn neurons are divided into two main classes: nociceptive-specific neurons, which respond to stimuli of a noxious nature, and wide dynamic range neurons, which respond to noxious, as well as innocuous stimuli (Menetrey, Giesler et al. 1977; Fields and Basbaum 1978; Coghill, Mayer et al. 1993).

The spinal cord was organised into ten laminae by Rexed (1952), according to local cytoarchitecture (pattern of arrangement- size and packing density of neurons). Laminae I-VI make-up the dorsal horn, laminae VII-IX the ventral horn and lamina X is the substantia grisea centralis (or gray matter surrounding the central canal). Laminae I-II compose the superficial dorsal horn, whereas laminae IV-VI compose the deep dorsal horn (Rexed 1952; Sorkin and Carlton 1997). Nociceptive-specific cells (NS) are predominant in the superficial dorsal horn, whereas wide-dynamic range cells (WDR) are predominant in the deep dorsal horn, although both types can be found in the superficial and deep dorsal

horn (Menetrey, Giesler et al. 1977; Fields and Basbaum 1978; Treede, Meyer et al. 1992; Coghill, Mayer et al. 1993). In vivo electrophysiology recordings in this study are made from deep, wide dynamic range dorsal horn neurons.

i) Lamina I

This 'marginal layer' is a thin layer which covers the most dorsal and lateral side of the dorsal horn. It contains interneurons (mostly), projection neurons (larger than interneurons), as well as the central terminations of some Aδ- and C-fibres (Light and Perl 1979; Light and Perl 1979; Millan 1999; Todd and Koerber 2006). Neurons in this layer have different sizes and shapes, which include fusiform, pyramidal and multipolar shapes (Rexed 1952; Lima and Coimbra 1986). It is thought that each cell morphology mainly subserves different stimulus modalities, so that nociceptive-specific cells (responding to heat/pinch) are mostly fusiform, thermoceptive-specific cells (responding to innocuous cooling) are mostly pyramidal, and polymodal cells (responding to heat/pinch/cold) are mostly multipolar in shape (Han, Zhang et al. 1998). Furthermore, 80% of the lamina I projection neurons have been shown to drive pathological pain states (Suzuki, Morcuende et al. 2002; Todd 2002), and some projection neurons have been shown to be modality specific (Dostrovsky and Craig 1996). Therefore, the neurons in this area of the dorsal horn are thought to relay to supraspinal sites sensory information of a mainly nociceptive and thermoceptive nature (Han, Zhang et al. 1998).

ii) Lamina II

This lamina is also called the 'Substantia Gelatinosa', due to its gelatinous appearance in unstained sections owing to the lack of myelinated fibres (Todd and Koerber 2006). This layer is divided into lamina IIo (outer) and lamina IIi (inner). Cells in this lamina are very tightly packed in general, smaller in size and more densely packed in lamina IIo than lamina IIi. The majority of neurons in this lamina are interneurons, as well as some central terminals of primary afferent fibres (Rexed 1952; Light and Perl 1979; Todd and Koerber 2006).

Lamina IIo receives the central terminations of C-fibres, whereas lamina IIi receives central terminations from both Aδ- and C-fibres (Light and Perl 1979; Light and Perl 1979; Todd and Koerber 2006).

iii) Laminae III and IV

These laminae are also called the 'Nucleus Proprius' (Rexed 1952; Millan 1999). Lamina III is parallel to laminae I and II, but also broader, with cells larger and less densely packed than those in lamina II (Rexed 1952). Most cells in lamina III are interneurons, and there are also some projection neurons in the nucleus proprius (Todd and Koerber 2006). Compared with lamina III, lamina IV has more cells with different shapes and sizes, and overall larger cells (Rexed 1952). The central terminals of Aβ- and Aδ-fibres terminate in the nucleus propius. Some nucleus proprius cells also receive polysynaptic input from C-fibres (Light and Perl 1979; Light and Perl 1979; Millan 1999; Dostrovsky and Craig 2006).

iv) Laminae V and VI

With overall less cells than lamina IV, lamina V has a lateral zone, with many large, darkly stained cells, and a medial zone with medium-sized, lighter cells. Lamina VI is also made up of a medial zone, with compactly grouped small- or medium-sized cells, and a lateral zone with looser grouped larger cells (Rexed 1952). In these deeper laminae, Aδ- and Aβ-fibres terminate in lamina V, whose cells also receive polysynaptic input from C-fibres (Light and Perl 1979; Millan 1999; Dostrovsky and Craig 2006; Todd and Koerber 2006).

1.7.2 Dorsal horn pharmacology

The pharmacology of the dorsal horn is rich, with neurotransmitters and neuromodulators coming from the central terminals of primary afferent fibres, the dorsal horn neurons themselves, as well as from descending fibres. Neurotransmitters and modulators are predominantly either excitatory or

inhibitory, according to the type and the location of the receptor they act on. In the spinal cord dorsal horn, the major excitatory transmitters are glutamate and substance P, and their activation of the NMDA and the NK-1 receptors respectively, is thought to play a role in central sensitisation. The major inhibitory systems in the spinal cord are GABA (gamma-amino butyric acid) and the opioids (Dickenson 1995).

1.7.3 Excitatory transmitter systems

1.7.3.1 Glutamate

One of the most important excitatory amino acids in the transmission of pain is glutamate. It has been previously shown that microiontophoretic administration of glutamate causes neuronal excitation in the spinal cord (Curtis, Phillis et al. 1960). Furthermore, glutamate has been shown to be released after electrical and noxious stimulation, and its uptake and release are decreased after dorsal rhizotomy (De Biasi and Rustioni 1988). Glutamate is found in many primary afferent terminals, both large and small, which end in laminaes I, III and IV and in small dorsal root ganglion cells (Battaglia and Rustioni 1988; De Biasi and Rustioni 1988). Additionally, glutamate has also been shown to be co-localised with peptides such as SP and/or CGRP (Battaglia and Rustioni 1988; De Biasi and Rustioni 1988), and the release of all of these after a noxious stimulus seems to show that they play a role in nociception. This release however, is differential between peptides and amino acids (Aanonsen and Wilcox 1987; Mjellem-Joly, Lund et al. 1992; Dickenson 1997).

Glutamate has been shown to be involved in the transmission of both acute and long-lasting pain, and its actions are exerted through its ionotropic receptors kainate, the alpha-amino3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA) receptor and N-methyl-D-aspartate (NMDA) receptor and its metabotropic receptors (mGlu) (Watkins and Evans 1981; Woodruff, Foster et al. 1987; Dickenson 1995).

i) Actions of glutamate at the NMDA receptor

The NMDA receptor-channel is important in memory, plasticity, motor function and neurodegenerative disorders in the central nervous system (Petrenko, Yamakura et al. 2003). This receptor-channel is found at peripheral, spinal, as well as supraspinal sites (Jacquet 1988; Carlton, Hargett et al. 1995; Coggeshall and Carlton 1997). Furthermore, even though the activation of the NMDA receptor in the rat periacqueductal gray (PAG) has been shown to cause analgesia (Jacquet 1988), the activation of the NMDA receptor in the spinal cord dorsal horn is known to play a major role in the development of plastic changes which lead to spinal hyperexcitability and persistent pain (Dickenson 1997).

a) NMDA receptor location

Most spinal NMDA receptors have been found, using autoradiographic receptor binding studies, to be within lamina II of the dorsal horn, and although most receptors were thought to be only postsynaptic, the presence of presynaptic NMDA receptors on the central terminals of primary afferent C-fibres has recently been shown (Liu, Wang et al. 1994; Coggeshall and Carlton 1997; Petrenko, Yamakura et al. 2003). These 'autoreceptors' are thought to provide a positive feedback for postsynaptic NMDA receptor activation, by increasing the release of SP from these primary afferent terminals on which substance P (SP) and glutamate are co-localised (Liu, Mantyh et al. 1997; Malcangio, Fernandes et al. 1998).

b) NMDA receptor activation and wind-up

The physiological activation of the NMDA receptor-channel requires many processes, as it is both ligand and voltage gated (Dickenson 1990). One of the processes needed to take place is the binding of glutamate and the co-agonist glycine to their respective sites. The second is the summation of non-NMDA depolarisations, caused by excitatory amino acids and peptides acting on their respective receptors, which removes the resting magnesium block of the channel

(Salt 1986; Xu, Dalsgaard et al. 1992; Dickenson 1997). Due to these processes which need to occur before NMDA receptor-channel activation, this receptor-channel is thought to be involved in polysynaptic, and not monosynaptic, pain transmission, since it is not involved in baseline pain transmission but becomes activated following repetitive noxious stimuli (Cahusac, Evans et al. 1984; Dickenson and Sullivan 1990; Yamamoto and Yaksh 1992; Yaksh, Hua et al. 1999).

When the magnesium block in the NMDA receptor channel is removed, the channel becomes permeable to calcium and monovalent ions (Petrenko, Yamakura et al. 2003). This influx of calcium ions, if it lasts for a long period, could lead to cell death, and could be one of the way genes are induced (eg: c-Fos, which has been taken to be a marker for neuronal activity), thus affecting both the cell and its surroundings in the long term (Dickenson 1994; Carpenter and Dickenson 1999; Carpenter and Dickenson 2001). Following the activation of the NMDA receptor channel, there is an increased state of responsiveness in spinal neurones, which is termed wind-up, and which is sensitive to blockade by antagonists at the NMDA receptor (Dickenson and Sullivan 1987). Wind-up was first described by (Mendell 1966) as the increase in responses of the deep dorsal horn neurones to repetitive C-fibre stimulation, despite the input into the spinal cord staying constant. Wind-up lasts for a couple of minutes, and shows that the NMDA receptor-channel could play a role in central hypersensitivity (Dickenson 1990).

c) NMDA receptor subunits

NMDA receptors comprise of NR1, NR2 (A, B, C, and D) and NR3 (A and B) subunits. The formation of functional NMDA receptor channels needs a combination of NR1, and at least one of the NR2 subunits, with the glutamate binding site located on the NR2 subunit, whereas the glycine binding site is located on the NR1 subunit (Petrenko, Yamakura et al. 2003). All inotropic glutamate receptor families are thought to be tetrameric assemblies, with each subunit consisting of an extracellular N-terminal, four transmembrane domains

(TM1-4) and an intracellular C-terminal (Palmer, Cotton et al. 2005). The ligand-binding domain is thought to reside in a pocket made between the extracellular N-terminal region and the extracellular loop between TM3 and TM4, whereas the pore is formed from a membrane-residing domain which follows TM1 (Kew and Kemp 2005; Palmer, Cotton et al. 2005).

The most obvious feature of this receptor channel, which is subunit dependent, is its sensitivity to magnesium block (eg: NR2A and NR2B subunits have a high sensitivity for magnesium block whereas NR2C and NR2D containing receptors have a low sensitivity for magnesium block). The NR2B and NR2D subunits have been identified in the central terminal of some primary afferents (Marvizon, McRoberts et al. 2002; Petrenko, Yamakura et al. 2003).

Additionally, the two most common subunits which are found in the spinal cord are NR2A and NR2B, found mostly in laminae III/IV and laminae I/II respectively, however, some studies have detected low levels of mRNA for both the NR2C and the NR2D subunits in the spinal cord as well. Whilst the NR2C subunit has been shown to be mostly found in the cerebellum by some studies, the NR2D subunit has been shown to be found 'extrasynaptic' in the spinal cord (Petrenko, Yamakura et al. 2003; Nagy, Watanabe et al. 2004).

d) The role of the NMDA receptor in pain states

The NMDA receptor mediated events have physiological roles, as was first shown using the formalin test in an in vivo electrophysiological study. This test showed that after formalin injection in the hindpaw, there is a first immediate phase of activity in the dorsal horn neuron corresponding to where the formalin was injected (both superficial, 0-250μm, and deep, 500-1000μm, neurones were tested), which lasts for 10 minutes, and a second phase which occurs 25 minutes after that and lasts for 60 minutes. Only the second phase involved NMDA receptor activation, since it was reduced by NMDA antagonists. Furthermore, this second phase was independent of the first phase, but both phases required afferent input, since blockade of peripheral inputs with local anaesthetics before

each phase blocked the two phases for 15 minutes (Haley, Sullivan et al. 1990). This experiment has also been done behaviourally (Coderre and Van Empel 1994).

Therefore, whereas the AMPA receptor has been shown to be involved in the mediation of acute spinal pain transmission, the NMDA receptor is involved in prolonged pain (Chapman and Dickenson 1995; Dickenson 1997), in both the induction and initiation of hyperalgesia and allodynia underlying central hypersensitivity, as well as the maintenance of neuronal responses in pain models such as neuropathic and inflammatory ones, since the 'abnormal' pain of these, and not the acute nociception, is reduced by NMDA receptor antagonists (Coderre and Van Empel 1994; Chapman and Dickenson 1995; Dickenson 1997; Yaksh, Hua et al. 1999; Yoshimura and Yonehara 2001).

e) The behaviour following NMDA receptor activation in animal studies

The intrathecal application of NMDA receptor agonists causes nociceptive behaviour and leads to thermal hyperalgesia and mechanical allodynia. Hyperalgesia due to intrathecal NMDA has also been shown by the tail-flick and pressure tests in both rats and mice (Mjellem-Joly, Lund et al. 1992; Yaksh, Hua et al. 1999). The behaviour which arises following intrathecal administration of NMDA in rat and mouse includes biting, scratching and licking behaviour (BSL), as well as vocalisation at higher doses, which has been taken to indicate that sensory pathways have been activated and could be thus concluded to be nociceptive. However, the intrathecal administration of quisqualate and kainic acid, which are excitatory amino acids that do not act on the NMDA receptor, also cause caudally directed biting, which is not followed by hyperalgesia. This means that care must be taken when drawing conclusions from BSL behaviour (Aanonsen and Wilcox 1987). This behaviour also arises from the administration of intrathecal tachykinins (Aanonsen and Wilcox 1987; Raigorodsky and Urca 1987; Sakurada, Manome et al. 1990; Davis and Inturrisi 1999).

ii) Actions of glutamate at other receptors

a) The AMPA receptor

This receptor is involved in synaptic transmission and thus responsible in setting baseline nociceptive transmission (Chapman and Dickenson 1995; Dickenson 1997; Yaksh 2006). Furthermore, the AMPA receptor is found primarily in lamina II of the dorsal horn, as well as other laminae within the spinal cord, with some reports showing that they also exist on primary afferent fibres (Coggeshall and Carlton 1997; Millan 1999). Additionally, even though each inotropic glutamate receptor family was named after its selective agonist, the AMPA receptor also responds to kainate (Kew and Kemp 2005). Finally, the AMPA receptor, once activated, is more selective to Na^+ than Ca^{2+}, in dorsal horn neurons (Millan 1999).

b) Peripheral receptors

Glutamate receptors have been identified in sensory neuron cell bodies in the DRG and on the peripheral terminals of primary afferent axons in both rat hairy and glabrous skin (Carlton, Hargett et al. 1995; Coggeshall and Carlton 1998), and in normal hairy skin (Kinkelin, Brocker et al. 2000).

In addition, numerous studies have shown that glutamate and its ionotropic receptors (NMDA, AMPA and kainate) may play a role in peripheral nociceptive transmission. Mechanical hyperalgesia (Zhou, Bonasera et al. 1996), allodynia (Carlton, Hargett et al. 1995) and thermal hyperalgesia (Jackson, Graff et al. 1995) have been demonstrated following intraplantar glutamate and glutamate agonists, which can blocked by the corresponding intraplantar antagonists.

Additionally, some studies show that peripheral glutamate receptors may play a role in inflammatory pain. The study by Wang *et al*, in 1997, has shown that the unilateral intraplantar administration of NMDA causes a dose-dependent increase in c-Fos (marker for neuronal activity) expression on the ipsilateral spinal cord dorsal horn (Wang, Liu et al. 1997). Furthermore, the latter study also showed

that the intraplantar co-administration of the NMDA antagonist MK-801 with formalin decreased c-Fos expression usually seen when formalin is administered on its own. In another study, the intraplantar administration of the NMDA antagonist MK-801 or the AMPA/kainate antagonist CNQX decreased the thermal hyperalgesia which occurs following carrageenan inflammation (Jackson, Graff et al. 1995). Furthermore, the intraplantar administration of glutamate antagonists decreased the lifting and licking behaviour which occur due to formalin behaviour, but not the flinching behaviour (Davidson, Coggeshall et al. 1997; Davidson and Carlton 1998).

In yet another study, NMDA, AMPA and kainate receptors in cutaneous nerves seem to be upregulated in unmyelinated and myelinated axons, 48 hours after CFA inflammation in the inflamed paw as opposed to the non-inflamed one. This could be part of the mechanism underlying peripheral sensitisation (Carlton and Coggeshall 1999). Moreover, the intraplantar administration of the NMDA antagonist MK-801, but not that of the non-NMDA antagonist CNQX, reduced the mechanical hyperalgesia seen following CFA inflammation (Leem, Hwang et al. 2001). Furthermore, in a study using an in vitro skin-nerve preparation, the levels of peripheral NMDA receptors were increased and decreased in conjunction with the level of the CFA inflammation. Additionally in this study, a lower dose of NMDA was required to sensitise nociceptors to thermal stimuli in inflamed skin, when compared to normal skin and the sensitisation was reversed by the intraplantar application of the NMDA antagonist MK-801 (Du, Zhou et al. 2003).

There is no shortage of endogenous glutamate to act on these receptors, since it has been postulated to originate from keratinocytes, macrophages, Schwann cells, blood serum as well as possibly from the primary afferents themselves (Kinkelin, Brocker et al. 2000; Ji and Strichartz 2004; Meyer, Ringkamp et al. 2006). Indeed, the study by deGroot *et al*, found that glutamate content increased significantly in the dialysate collected from rat hindpaw following electrical stimulation of the sciatic nerve, using parameters which would activate low threshold mechanoceptors (Aβ fibres), which convey non-noxious input, and

high threshold afferents (Aδ+C fibres), which convey noxious input. Furthermore, selective activation of C-fibres, by capsaicin treatment to the sciatic nerve, also resulted in an increase in peripheral glutamate in this study (deGroot, Zhou et al. 2000).

iii) Peripheral NMDA receptors and neuropathic pain

Not many investigations have been done to establish whether peripheral glutamate receptors may play a role in neuropathic pain. One study by Jang *et al*, has shown that by performing an L5 spinal nerve ligation (SNL) which was preceded by L5 DR (dorsal rhizotomy, to stop the signal from getting to the spinal cord and thus allow only peripheral access of SNL-induced signals), long-term mechanical hyperalgesia in the affected hindpaw of the rat occurred, whose induction and maintenance was significantly reduced by the intraplantar administration of the NMDA antagonist MK-801 (Jang, Kim et al. 2004).

Furthermore, one human study showed that the administration of topical ketamine cream (an NMDA antagonist) reduced the hyperalgesia and allodynia seen in CPRS I (complex regional pain syndrome type I) (Ushida, Tani et al. 2002), whereas another study showed that the topical administration of a cream containing a combination of amitriptyline (tricyclic antidepressant, prevents the reuptake of noradrenaline and serotonin) and ketamine decreased neuropathic pain (Lynch, Clark et al. 2005).

To summarise, glutamate receptors have been localised in the periphery, have been shown to be exogenously activated and the local administration of antagonists at the glutamate receptors has been shown to decrease pain states of inflammatory as well as neuropathic nature. Furthermore, glutamate has been shown to be released peripherally following stimulation of the sciatic nerve. Therefore, it could be postulated that endogenous glutamate could be released from the peripheral terminals of primary afferent fibres, possibly in a mechanism akin to the axon reflex (described in section **1.6.4 ii)**, following neuropathic pain states. Consequently, the possibility that glutamate, acting on the peripheral

NMDA receptor, has a role in the mechanism underlying the pain behaviour due to neuropathic pain, will be investigated in this study.

1.7.3.2 Substance P

Substance P (SP) is a neuropeptide from the neurokinin (tachykinin) family, found in many small diameter primary afferents, which terminate mainly in the superficial laminae, in the dorsal horn; although some penetrate deeper (Lamotte, Pert et al. 1976; Randic and Miletic 1977; Yaksh, Jessell et al. 1980; Laneuville, Dorais et al. 1988; Todd, McGill et al. 2000; Todd 2002). SP is implicated in anxiety, mood, stress, smooth muscle contraction, regulation of immune response, as well as secretion from both exocrine and endocrine glands (Garret, Carruette et al. 1991; Yip and Chahl 1999; Suzuki, Hunt et al. 2003). However, SP also plays a role as a neurotransmitter in the central nervous system, which is released after high intensity noxious stimulation (Yaksh, Jessell et al. 1980; Dickenson 1997; Suzuki, Hunt et al. 2003).

i) SP is co-released with other peptides from primary afferent fibres

After C-fibre activation, there is also co-release of neurokinin A (NKA) (Laneuville, Dorais et al. 1988) and calcitonin-gene related peptide (CGRP), which are also primary afferent peptides released by noxious stimulus and excite dorsal horn neurones, as well as co-release of the excitatory amino acid glutamate (Battaglia and Rustioni 1988; De Biasi and Rustioni 1988; Doyle and Hunt 1999; Yaksh, Hua et al. 1999). Without CGRP, SP released by C-fibres will only activate the NK-1 receptor in the vicinity of the site of release. However, because CGRP binds to the peptidase which degrades SP, CGRP extends the release zone for SP to cover much of the dorsal horn (Hokfelt, Zhang et al. 1994; Dickenson 1995; Dickenson 1997; Dray 1997). Therefore, transmission following the release of SP is through volume transmission (Coggeshall and Carlton 1997).

Furthermore, SP has been shown to cause further release of glutamate from primary afferent fibres (Hua, Chen et al. 1999). In turn, as mentioned previously, glutamate, *via* presynaptic NMDA receptor activation, has been shown to increase the evoked release of SP (Malcangio, Fernandes et al. 1998). Finally, the release of SP from the activation of TRPV1 receptors in primary afferent axons has also been shown to be dependent on the activation of presynaptic NMDA receptors, in vitro (Lao, Song et al. 2003).

As mentioned previously, the peripheral release of SP and CGRP from the peripheral endings of sensory neurons leads to neurogenic inflammation and thus peripheral sensitisation, whereas their central release in the spinal cord leads to dorsal horn excitability and possibly central sensitisation, which will be discussed next (Dray 1997).

ii) The NK-1 receptor composition and internalisation

SP acts preferentially on the neurokinin 1 (NK-1) receptor. The NK-1 receptor is a G-protein-coupled receptor, whose activation leads to an increase in inositol trisphosphate levels, which in turn causes the release of calcium from intracellular stores (Rusin, Ryu et al. 1992; Alvarez-Vega, Baamonde et al. 1998). Furthermore, like other G-protein-coupled receptors, the NK-1 receptor is composed of an extracellular N (amino) terminal domain, seven transmembrane domains and an intracellular C (carboxy) domain (Khawaja and Rogers 1996).

Once the NK-1 receptor has been activated by SP or any NK-1 agonist, both agonist and receptor are rapidly internalised (within 5 minutes) into the cytoplasm (Trafton, Abbadie et al. 1999). The receptor is phosphorylated prior to internalisation, which uncouples it from the G-protein and which means the signalling ends before internalisation. This internalisation does not occur when an antagonist binds the NK-1 receptor, and lasts for 60 minutes after agonist binding, with the 'recycled' receptors returning and becoming evenly distributed on the surface around 30 minutes after that (Trafton and Basbaum 2000; Wang and Marvizon 2002).

iii) The NK-1 receptor location and projections

NK-1 receptors are found on intrinsic dorsal horn neurons, in the superficial layers predominantly, with lower levels found in the deep layers (Todd 2002; Suzuki, Hunt et al. 2003). Some studies have also postulated the existence of presynaptic NK-1 'autoreceptors', on the central terminals of primary afferent fibres (Malcangio and Bowery 1999). NK-1-expressing lamina I neurons make some collateral projections to deeper layers, but are mostly nociceptive-specific projection neurons (nearly 80% of lamina I projection neurons express the NK-1 receptor), which terminate mainly within the parabrachial area of the brainstem. These cells greatly outnumber the laminae III-IV cells with this same receptor, which also project to regions of the brainstem involved in pain mechanisms (Todd, McGill et al. 2000; Todd, Puskar et al. 2002).

iv) The role of the NK-1 receptor in pain states

Numerous studies over the years have shown that the NK1 receptor only has roles in central states of hypersensitivity and not acute pain (Xu, Dalsgaard et al. 1992; Chapman and Dickenson 1993; Dickenson 1995; Ma and Woolf 1997; Yaksh, Hua et al. 1999; Yoshimura and Yonehara 2001; Suzuki, Hunt et al. 2003). The application of NK-1 agonists has been shown to produce slow excitatory transmission of dorsal horn neurons (Urban and Randic 1984) and decrease pain thresholds, whilst antagonist administration blocked excitation of dorsal horn neurons and prevented the development of inflammation-induced thermal and mechanical hyperalgesia in behavioural studies (Randic and Miletic 1977; Henry 1980; Kellstein, Price et al. 1990; Dickenson 1997; Ma and Woolf 1997; Field, McCleary et al. 1998; Li and Zhao 1998; Doyle and Hunt 1999). Furthermore, RP-67,580, an NK-1 antagonist, was capable of inhibiting the second phase of the formalin response (Chapman and Dickenson 1993; Seguin, Le Marouille-Girardon et al. 1995; Honore, Menning et al. 1999).

Additionally, the activation of the NK-1 receptor in the dorsal horn is thought to play an important role in enhancing NMDA transmission, by aiding in the removal of the magnesium block. The final result of which is wind-up and central hypersensitivity (Urban and Randic 1984; Dougherty and Willis 1991; O'malley, Calligaro et al. 1991; Xu, Dalsgaard et al. 1992; Dickenson 1997; Doyle and Hunt 1999). The NK-1-mediated enhancement of NMDA-mediated events was shown when NK-1 antagonists were shown to reduce half of the pain behaviour which occurs following intrathecal NMDA administration (Trafton and Basbaum 2000) and to reduce the wind-up response (Xu, Dalsgaard et al. 1992).

Furthermore, following chronic inflammation using complete Freund's adjuvant (CFA), there has been a reported increase in the expression of both SP and the NK-1 receptor in the dorsal horn, and the normally SP-free large diameter sensory neurons also begin to express tachykinins. The latter was thought to contribute to the development of mechanical allodynia following injury, since these large diameter neurons respond to non-noxious stimuli that can now release SP. However, electrical stimulation of these large diameter sensory neurons following inflammation did not cause any NK-1 receptor internalisation, so it is still uncertain how relevant the expression of SP on large diameter primary afferents is (Trafton and Basbaum 2000).

v) The behaviour which follows the activation of the NK-1 receptor in animal studies

Moreover, intrathecal SP and NK-1 agonist administration has been shown to cause thermal and mechanical hyperalgesia, allodynia, and compulsive biting, scratching and licking of the hindlimbs by rats in behavioural studies (Seybold, Hylden et al. 1982; Holland, Goldstein et al. 1993; Picard, Boucher et al. 1993; Dickenson 1997). The aversive behaviour elicited by intrathecal SP is thought to involve the spinal NK-1 as well as the NMDA and non-NMDA receptors, since not only is SP thought to act directly on the NK-1 receptor, but it is also thought

to induce the release of glutamate from the spinal cord (Hylden and Wilcox 1982; Okano, Kuraishi et al. 1993).

vi) Ablating the NK-1-expressing neurons

Finally, the fact that the NK-1 receptor, like other G-protein-coupled receptors, internalises when activated, made it possible to selectively ablate the neurons this receptor is expressed on, by administering a conjugate of its agonist 'SP' with the toxin 'saporin'. This meant that the neuron would take up the toxin, as the NK-1 receptor was activated and internalised by SP, which would lead to cell death (Mantyh, Rogers et al. 1997). Selectively ablating lamina I neurons expressing the NK-1 receptor led to a great loss of capsaicin-induced hyperalgesia (Mantyh, Rogers et al. 1997). This ablation also removed wind-up and decreased central sensitisation, as seen in an electrophysiological experiment testing the formalin response (Suzuki, Morcuende et al. 2002). The receptive field (peripheral area of the cell which responds to the stimuli) size of spinal neurons, and their ability to code accurately for both mechanical and thermal stimuli were also reduced in this experiment (Suzuki, Morcuende et al. 2002). Furthermore, it was shown that these NK-1 expressing lamina I neurons are needed for the generation of long-term potentiation (LTP, "use-dependent, long-lasting modification of synaptic strength") in deep dorsal horn neurons (Rygh, Suzuki et al. 2006).

1.7.4 The postulated role of the NMDA and NK-1 receptors in central sensitisation

The mechanism underlying secondary hyperalgesia following injury, where a painful sensation to mechanical stimuli is felt outside the area of injury in the periphery, has been postulated by most to be due to the central sensitisation of dorsal horn neurons (Treede, Meyer et al. 1992).

A cascade of events is thought to underlie the increase in dorsal horn excitability following injury. The activation of both the NMDA and the NK-1 receptors by glutamate and SP from primary afferent fibres, and by intrathecal NMDA and

NK-1 receptor agonists, leads to increases in intracellular calcium, either through a direct increase in calcium influx through an open channel or through the generation of inositol trisphosphate, which leads to the release of calcium from intracellular stores, respectively. This rise in intracellular calcium causes the activation of many enzymes, including cyclo-oxygenase 2 (COX-2) and nitric oxide synthase (NOS), which are required for the generation of prostanoids and NO respectively, and the activation of many kinases (eg: protein kinase C 'PKC'). These prostanoids and NO retrogradely diffuse after their synthesis and release in the spinal cord, and facilitate further transmitter release from the primary afferents, and thus more glutamate and peptides to be released, whereas the kinases lead to phosphorylation and further activation of membrane receptors and channels, which subsequently causes a further increase in intracellular calcium (Chen and Huang 1992; Rusin, Ryu et al. 1992; Dougherty, Palecek et al. 1993; Rusin, Bleakman et al. 1993; Rusin, Jiang et al. 1993; Yaksh, Hua et al. 1999).

In my study, in order to investigate excitatory transmission in the rat spinal cord, each of the NMDA receptor agonist NMDA, and the NK-1 receptor agonist [Sar^9 Met $(O_2)^{11}$]-Substance P (Sar-SP), will be administered intrathecally, and their effects on the deep wide dynamic range neurons will be investigated using in vivo electrophysiology. Furthermore, each of intrathecal NMDA, and intrathecal Sar-SP will also be used to induce the acute chemical model of pain, in the behavioural part of the study.

1.7.5 The postulated role of the NK-1 and the NMDA receptors in neuropathic pain

Changes in the excitatory transmitter systems occur following peripheral nerve injury, as both SP and CGRP immunoreactivity in both the DRG and the dorsal horn is decreased, whereas spinal NK-1 receptor levels are increased (Sommer and Myers 1995). Furthermore, even though NMDA receptor immunoreactivity in the dorsal horn remains unchanged following peripheral nerve injury, glutamate uptake in the dorsal horn has been shown to be decreased, which

means that more glutamate is available to act at its respective receptors (Binns, Huang et al. 2005).

In addition, the role of the NK-1 and the NMDA receptors in central sensitisation (see section **1.7.4**) means that these two receptors, *via* the mechanisms discussed above, also play a role following nerve injury. Indeed, the administration of NK-1 and NMDA antagonists, as well as antiserum against SP (Wu, Schwasinger et al. 2005), in different models of neuropathic pain, has been shown to decrease the behavioural pain hypersensitivities (Davar, Hama et al. 1991; Yamamoto, Shimoyama et al. 1993; Hao and Xu 1996; Chaplan, Malmberg et al. 1997; Wegert, Ossipov et al. 1997; Coudore-Civiale, Courteix et al. 1998; Field, McCleary et al. 1998; Coudore-Civiale, Courteix et al. 2000; Gonzalez, Field et al. 2000; Cahill and Coderre 2002) and dorsal horn neuron changes (Cumberbatch, Carlson et al. 1998) associated with the disease. However, the clinical use of many of the NK-1 and NMDA antagonists at effective doses is usually hampered by unacceptable side-effects, such as motor disturbances (Coderre and Van Empel 1994; Suzuki, Matthews et al. 2001).

1.7.6 Inhibitory transmitter systems

1.7.6.1 GABA

Even though no GABA receptor agonist or antagonist is used in this study, I will briefly detail GABA, since it is a very important inhibitory transmitter system. GABA (gamma-amino butyric acid) is the major inhibitory transmitter in the central nervous system (Dickenson, Chapman et al. 1997). GABA in the spinal cord is used in 20-50% of all neuronal synapses and acts *via* two receptors, GABA-A (predominantly) and GABA-B (Coggeshall, Zhou et al. 1997; Sieghart, Fuchs et al. 1999).

Both GABA-A and GABA-B receptors are found on the central terminals of primary afferents and spinal cord interneurons, predominantly in lamina II as

well as the rest of the spinal cord (Coggeshall and Carlton 1997; Slonimski, Abram et al. 2004).

It has been shown that GABA acts as a tonic control for excitatory transmission, more specifically, that GABA interneurons control the input of low-threshold afferent fibres (Dickenson, Chapman et al. 1997; Dickenson, Matthews et al. 2002). This tonic GABA control seems to be enhanced in inflammation and decreased in neuropathic pain (Dickenson, Chapman et al. 1997). Indeed, it has been shown that the spinal administration of bicuculline, a GABA-A antagonist, caused facilitation of superficial and deep dorsal horn neuron responses in normal animals (Reeve, Dickenson et al. 1998; Seagrove, Suzuki et al. 2004), whilst the administration of midazolam, a benzodiazepine which enhances GABA-A function by binding to a modulatory site, decreased dorsal horn responses in neuropathic animals (Kontinen and Dickenson 2000).

1.7.6.2 Opioids

The opioidergic system is also a very important inhibitory system in the transmission of pain. Opioid is a term given to any substance with 'morphine-like' effects, which is blocked by antagonists like naloxone. Endogenous opioids include Met- and Leu-Enkephalins, β-Endorphin and Dynorphin. The three main types of opioid receptors, which are G-protein-coupled receptors, are μ, δ and κ. Each of these receptors is associated with different functional effects. Naloxone has a high affinity for the μ receptor, but will also act on δ and κ, in moderately higher concentrations (Kosterlitz 1985; Dickenson and Kieffer 2006).

A fourth opioid receptor, 'opioid receptor-like (ORL1) orphan receptor' was isolated by severeal laboratories between 1994 and 1995. This latest receptor has no affinity for opioid ligands, and stayed an 'orphan' until late 1995, when two groups separately isolated its endogenous ligand, called nociceptin or orphanin FQ (Meunier 1997). Most of the analgesic opioids, as mentioned above, are μ-receptor agonists. It is this receptor which is responsible for most of the analgesic

effects and major side-effects (respiratory depression, sedation, euphoria, dependence) of opioids (Dickenson 1994).

i) The location of opioid receptors

μ opioid receptors are found most abundantly (approximately 70%) on the central terminals of small- and medium-diameter (C and Aδ) primary afferent terminals (Besse, Lombard et al. 1990; deGroot, Coggeshall et al. 1997; Zhang, Bao et al. 1998). In the spinal cord, autoradiographic and immunohistochemical studies have shown that the highest levels of opioid receptors are present around the C-fibre terminal zones in lamina I and II, whereas lower levels are found in deeper laminae. It is suggested that μ receptors form 70% of the total opioid sites in the rat spinal cord, whilst δ receptors form 24% and κ receptors form 6% of these (Lamotte, Pert et al. 1976; Duggan, Hall et al. 1977; Besse, Lombard et al. 1992; Dickenson 1994; Mansour, Fox et al. 1994; Dickenson and Kieffer 2006). Furthermore, there are opioid receptors located on supraspinal sites, such as the rostroventral medulla (Yaksh 1997; Dickenson and Kieffer 2006), which will be discussed in section **1.8.2 iv**.

ii) The mechanisms of action of opioid receptors

The opioid receptors are coupled to Go/Gi inhibitory proteins. Like other receptors which belong to the G-protein family, these receptors have an extracellular N terminal domain, seven transmembrane domains and an intracellular C terminal tail. At the membrane level, morphine binds to the μ-opioid G-protein-coupled receptor, causing a conformational change which leads to the opening of inwardly rectifying potassium channels, which causes a neuronal hyperpolarisation (Dickenson 1994; Matthews and Dickenson 2002; Dickenson and Kieffer 2006).

The hyperpolarisation of the neurons due to the membrane actions of opioids ultimately reduces calcium entry into all of the fibres and neurons which contain

these receptors, such as the central terminals of the primary afferent fibres and spinal cord neurons. The three different locations of opioid receptors, which include the primary afferent terminals, the spinal cord and the midbrain/brainstem, leads to opioids exerting their effects *via* three mechanisms of action. Opioids primarily have an inhibitory presynaptic action on the central terminals of primary afferent fibres, which leads to the inhibition of transmitter release (Lombard and Besson 1989; Kangrga and Randic 1991; Besse, Lombard et al. 1992; Mansour, Fox et al. 1994; Dickenson and Kieffer 2006). Furthermore, opioids acting on their postsynaptic receptors cause a decrease in synaptic transmission. Alternatively, this postsynaptic action may produce disinhibition by inhibiting the first neuron in a circuit of two neurons, where the second cell is controlled by the first inhibitory neuron. This leads to the second cell becoming active and thus the term disinhibition. The overall actions of opioids cause decreased transmission and neuronal excitability and are thus mostly inhibitory (Lamotte, Pert et al. 1976; Duggan, Hall et al. 1977; Jessell and Iversen 1977; Yaksh, Jessell et al. 1980; Duggan and North 1983; Hylden and Wilcox 1983; Lombard and Besson 1989; Kayser, Chen et al. 1991; Dickenson 1997).

iii) The efficacy of opioids in different pain models

Plasticity is manifested in opioid systems, since morphine has been reported to have reduced efficacy in neuropathic pain (Arner and Meyerson 1988), detailed in section **1.7.7**, whilst being more effective in some inflammatory models (Kayser, Chen et al. 1991) than in normal animals (Dickenson and Kieffer 2006).

As mentioned previously, opioids exert most of their actions on their presynaptic receptors, which are higher in number than their postsynaptic receptors found on intrinsic spinal cord dorsal horn neurons (Lamotte, Pert et al. 1976; Duggan, Hall et al. 1977; Besse, Lombard et al. 1992; Dickenson 1994; Mansour, Fox et al. 1994). However, the theory that opioid effectiveness is decreased when used in a situation where opioids are limited to act on their postsynaptic receptors, has yielded conflicting results (Henry 1976; Dickenson 1997; Cheunsuang, Maxwell

et al. 2002). Most studies wishing to investigate this in acute pain models usually use the activation of the NMDA or the NK-1 receptors, both known to be located postsynaptic on the spinal cord dorsal horn, and whose activation shifts the balance in the spinal cord towards excitation.

One study has investigated the efficacy of different intrathecal opioid receptor agonists on inhibiting the behaviour and thermal hyperalgesia (using the tail flick test) associated with intrathecal SP in mice. It was found that all agonists inhibited both the behaviour and the hyperalgesia, but that δ agonists were more potent than morphine in doing so (Hylden and Wilcox 1982; Hylden and Wilcox 1983), but this was contradicted when Trafton *et al* showed that it was μ receptor activation which was most potent in reducing NK-1 internalisation (Trafton, Abbadie et al. 1999).

However, the study by Alvarez-Vega *et al* showed that morphine was not very efficient in reducing the BSL behaviour which arises following intrathecal septide, and was even less effective in reducing the BSL behaviour following intrathecal NMDA administration in mice (Alvarez-Vega, Baamonde et al. 1998). This was also shown in the study by (Bossut, Frenk et al. 1988), where it was demonstrated that different doses of intrathecal morphine were ineffective in inhibiting the behaviour which arises following intrathecal SP. The study by Aanonsen *et al*, showed that intrathecal DAGO (an agonist more μ selective than morphine) was more effective than intrathecal morphine in reducing the behaviour and hyperalgesia associated with intrathecal NMDA administration in mice, and that the doses of both agonists required to inhibit the hyperalgesia were less than those needed to inhibit the behaviour (Aanonsen and Wilcox 1987).

Another study investigated the effect of each of Sar-SP (NK-1 agonist) and DAMGO (μ opioid agonist) in vitro on postsynaptic superficial dorsal horn neurons which express the NK-1 receptor (Cheunsuang, Maxwell et al. 2002). They showed that Sar-SP depolarised these cells initially, whereas DAMGO produced small direct postsynaptic hyperpolarisations of these cells, which were

not as profound as the presynaptic block caused by DAMGO on the C-fibre evoked response, thus showing the smaller effect of μ opioid agonists when they are not acting presynaptically.

Wind-up of dorsal horn neurons, as mentioned in section **1.7.3**, is a response mediated by the activation of the NMDA receptor channel (Dickenson and Sullivan 1987), found mostly postsynaptic on spinal cord neurons (Coggeshall and Carlton 1997). This response has been shown to be resistant to low doses of intrathecal morphine, and needs higher doses of morphine to inhibit it (Chapman and Dickenson 1992; Chapman, Haley et al. 1994), since the intrathecal morphine needs to act on both its presynaptic and less numerous postsynaptic μ-opioid receptors (Lombard and Besson 1989; Besse, Lombard et al. 1990), to abolish it.

Finally, the study by Yamamoto *et al* showed that the administration of intrathecal morphine was effective in reducing the behaviour elicited by treatment with formalin, when morphine was given both pre- and post-formalin treatment in rats, whereas the NMDA receptor antagonist MK-801 was more effective only when administered pre-formalin treatment (Yamamoto and Yaksh 1992). Therefore, this study showed morphine to be effective as an analgesic in a situation where both the NMDA and the NK-1 receptors are thought to be activated.

In the first part of my study, the inhibitory effect of morphine on spinal cord dorsal horn neurons will be investigated. Following that, the modulation, by morphine, of the excitation of the spinal cord dorsal horn neurons and the behavioural and nociceptive response, which occur when each of the NMDA and the NK-1 receptors is activated by its respective agonist, will be studied.

1.7.7 Morphine and neuropathic pain

As discussed previously, opioid receptors are located on the central terminals of the primary afferent fibres, mostly the peptidergic, small- and medium-diameter sensory neurons (deGroot, Coggeshall et al. 1997; Zhang, Bao et al. 1998), on descending controls from the RVM, such as ON cells (Porreca, Burgess et al. 2001), and to a lesser degree on neurons intrinsic to the spinal cord dorsal horn (Zhang, Bao et al. 1998). Therefore, theoretically, opioid receptors seem to be well placed to control the excess activity which occurs in all three aforementioned locations, and which contribute to the plasticity seen in neuropathic pain.

Following nerve injury, the changes in inhibitory transmitter systems vary, with reports showing that GABA-ergic tone is decreased, increased or unchanged following peripheral nerve injury (Sommer and Myers 1995; Hwang and Yaksh 1997; Kontinen, Stanfa et al. 2001), whereas Met-enkephalin immunoreactivity is increased in the dorsal horn. Additionally, μ opioid receptor immunoreactivity increased following the C.C.I model and decreased after the tight ligation model of neuropathy. Therefore, the type of injury sometimes dictates the reorganisation which occurs in the central nervous system following peripheral nerve injury. Additionally, the two 'morphine-resistant' substances, cholecystokinin (CCK) and dynorphin, are both increased in primary afferent fibres and the spinal cord dorsal horn respectively, and are detailed further below (Hokfelt, Zhang et al. 1994; Nahin, Ren et al. 1994; Sommer and Myers 1995; Cameron, Cliffer et al. 1997; Goff, Burkey et al. 1998; Malmberg and Basbaum 1998; Honore, Rogers et al. 2000; Lee, Sohn et al. 2001; Wang, Sun et al. 2002).

Peripheral nerve injury also results in the activation and up-regulation of the NK-1 receptor expressed on projection neurons, which are the origin of the descending serotonergic facilitations (see section **1.9.4**), which are enhanced following neuropathy and which cause further central sensitisation in the dorsal horn spinal cord (Nichols, Lopez et al. 1997). Therefore, the peripheral and spinal events occurring following peripheral nerve injury shift the balance of the spinal cord towards excitation (Dickenson and Kieffer 2006).

Furthermore, μ-receptor-expressing neurons in the RVM seem to be implicated in the increase of descending facilitations following peripheral nerve injury, since the ablation of these cells with the μ-receptor agonist 'dermorphin' conjugated with the toxin 'saporin' decreased abnormal pain following neuropathy and not normal pain. These μ-receptor expressing cells are postulated to include ON cells. Therefore, supraspinal sites also serve to enhance spinal cord excitation, following peripheral nerve injury (Porreca, Burgess et al. 2001; Porreca, Ossipov et al. 2002; Vera-Portocarrero, Zhang et al. 2006).

i) Clinical studies

There have been conflicting results as to the responsiveness of neuropathic pain syndromes to opioids. Some studies have shown that neuropathic pain states are less responsive to opioids (Arner and Meyerson 1988), whereas others have shown that opioid responsiveness depended on dose titration, using more potent opioid agonists than those previously used, as well as on the type of neuropathic pain syndrome (Portenoy, Foley et al. 1990; Rowbotham, Reisner-Keller et al. 1991; Jadad, Carroll et al. 1992; Sindrup and Jensen 1999; Attal, Guirimand et al. 2002; Hansson and Dickenson 2005).

ii) Dynorphin and cholecystokinin

The decrease in opioid responsiveness following neuropathic pain injury has been originally attributed to the loss of μ-opioid receptors on the central terminals of primary afferent fibres. However, even though following some types of peripheral nerve injury, such as peripheral axotomy, dorsal root section and dorsal rhizotomy, μ-opioid receptors have been shown to be down-regulated in DRG and dorsal horn neurons (Lamotte, Pert et al. 1976; Besse, Lombard et al. 1992; deGroot, Coggeshall et al. 1997; Zhang, Bao et al. 1998; Dickenson and Suzuki 2005), in other types of nerve injury, such as the SNL and other sciatic nerve ligation models, the loss of these receptors is too small to be the main cause of decreased opioid responsiveness (Besse, Lombard et al. 1992; Nichols,

Lopez et al. 1997; Porreca, Tang et al. 1998). Furthermore, in the CCI model of nerve injury, there is an increase in the number of μ-opioid receptors (deGroot, Coggeshall et al. 1997; Porreca, Tang et al. 1998).

Therefore, it has been shown that one of the reasons opioids have been less effective in some types of neuropathic pain syndromes is due to increases in spinal dynorphin and cholecystokinin levels. Since this study uses morphine administration in neuropathic rats, both dynorphin and cholecystokinin will be detailed.

a) Dynorphin

Dynorphin is a neuropeptide which is a kappa opioid agonist and thought to have both antinociceptive opioid properties, as well as non-opioid pro-nociceptive properties (thought to be mediated *via* the NMDA receptor). Spinal dynorphin increases in segments which belong to the entry of the injured nerve as well as other segments adjacent, once descending facilitations are activated following peripheral nerve injury (Bian, Ossipov et al. 1999; Dickenson and Kieffer 2006). This means that dynorphin, like descending facilitations, is involved with the maintenance of neuropathic pain and not the initiation (Ossipov, Lai et al. 2001; Burgess, Gardell et al. 2002). The increase in spinal dynorphin correlates well with neuropathic pain symptoms such as thermal hyperalgesia, as well as morphine resistance (Ossipov, Lai et al. 2000). Furthermore, it has been shown that when an antiserum against dynorphin was administered intrathecally following peripheral nerve injury, this aided intrathecal morphine in alleviating tactile allodynia, which was previously resistant to spinal morphine (Bian, Ossipov et al. 1999; Wu, Schwasinger et al. 2005).

b) CCK

Cholecystokinin (CCK) is a neuropeptide, which has been shown to be a physiological antagonist to the effects of opioids (Sullivan, Hewett et al. 1994). This neuropeptide acts on both CCK_A and CCK_B receptors, and both

neuropeptide and receptors are located in primary afferent fibres and dorsal horn neurons (Xu, Puke et al. 1993; Zhang, Bao et al. 1998). Following nerve injury, both CCK and CCK_B receptors are upregulated in the DRG and in the spinal cord (Xu, Puke et al. 1993; Nichols, Bian et al. 1995; Zhang, Bao et al. 1998). The efficacy of spinal morphine following peripheral nerve injury has been shown to be decreased in some neuropathic pain models. The administration of intrathecal morphine in combination with a CCK_B receptor antagonist has been shown to restore the efficacy of spinal morphine following peripheral nerve injury, and enhance the antinociceptive effects of spinal morphine in normal animals (Xu, Puke et al. 1993; Sullivan, Hewett et al. 1994; Nichols, Bian et al. 1995).

iii) Dynamic/static allodynia, thermal hyperalgesia and morphine

As mentioned previously, different primary afferent fibres relay the sensations of dynamic allodynia (Aβ-fibres), tactile (static) allodynia (Aδ-fibres) and thermal hyperalgesia (C-fibres) (Field, Bramwell et al. 1999). There has been conflicting results following numerous studies as to which one of the previously discussed hypersensitivities is reduced by morphine, following peripheral nerve injury. Some studies have shown that morphine was able to alleviate thermal hyperalgesia (Yamamoto and Yaksh 1991; Yamamoto and Yaksh 1992; Lee, Kayser et al. 1994; Backonja, Miletic et al. 1995; Wegert, Ossipov et al. 1997; Catheline, Le Guen et al. 2001; Wu, Schwasinger et al. 2005), other studies have shown that morphine was effective in reducing tactile allodynia (Bian, Nichols et al. 1995; Lee, Chaplan et al. 1995; Field, McCleary et al. 1999; Zhao, Tall et al. 2004) or pain due to mechanical pressure (Attal, Chen et al. 1991).

The majority of the studies which showed morphine having an effect on thermal hyperalgesia were using spinal morphine, whereas the majority of the studies which showed morphine having an effect on tactile allodynia were using the systemic or supraspinal route of administration. This led to the conclusion that following nerve injury, thermal hyperalgesia seems to be mediated mostly spinally, whereas tactile allodynia seems to be mediated supraspinally. Furthermore, this further proves that thermal hyperalgesia is mainly relayed *via*

C-fibres, since these fibres are where most of the presynaptic μ-opioid receptors are expressed and this stimulus modality seems to be the most responsive to opioid therapy, following peripheral nerve injury (Wegert, Ossipov et al. 1997; Ossipov, Bian et al. 1999).

To date only the clinical study by (Attal, Guirimand et al. 2002), using systemic morphine, has shown an effect of this drug on dynamic allodynia following central pain states such as pain following a stroke or spinal cord injury. Since dynamic allodynia is mediated *via* primary afferent fibres which do not express μ-opioid receptors (Aβ fibres), it is not surprising that another study showed that morphine was not effective against this type of pain following neuropathy (Field, McCleary et al. 1999). It is known that there are less postsynaptic μ opioid receptors than there are presynaptic ones and therefore, in order for morphine to be able to alleviate dynamic allodynia, morphine must be acting predominantly *via* its postsynaptic receptors, and thus higher doses of morphine are needed (Dickenson and Suzuki 2005).

iv) Route of administration of morphine

Even though some studies have shown that intrathecal morphine was still effective in decreasing abnormal pain/ hyperexcitability of dorsal horn neurons due to peripheral nerve injury (Yamamoto and Yaksh 1991; Suzuki, Chapman et al. 1999; Suzuki and Dickenson 2002; Zhao, Tall et al. 2004; Suzuki and Dickenson 2006), most studies have shown that the efficacy of spinal morphine is lost following nerve injury, and that morphine is more effective when administered systemically or supraspinally (Bian, Nichols et al. 1995; Lee, Chaplan et al. 1995; Nichols, Bian et al. 1995; Nichols, Lopez et al. 1997; Wegert, Ossipov et al. 1997; Bian, Ossipov et al. 1999; Pertovaara and Wei 2003). Additionally, many studies have shown that the efficacy of spinal morphine is restored following the administration of topical bupivacaine (Na channel blocker) or spinal MK-801 (NMDA receptor antagonist) (Yamamoto

and Yaksh 1992; Ossipov, Lopez et al. 1995; Nichols, Lopez et al. 1997; Bian, Ossipov et al. 1999).

In the part of my study investigating neuropathic pain, systemic morphine will be administered on its own in SNL rats, to study its ability to inhibit the thermal, cold and tactile hypersensitivities seen in this model.

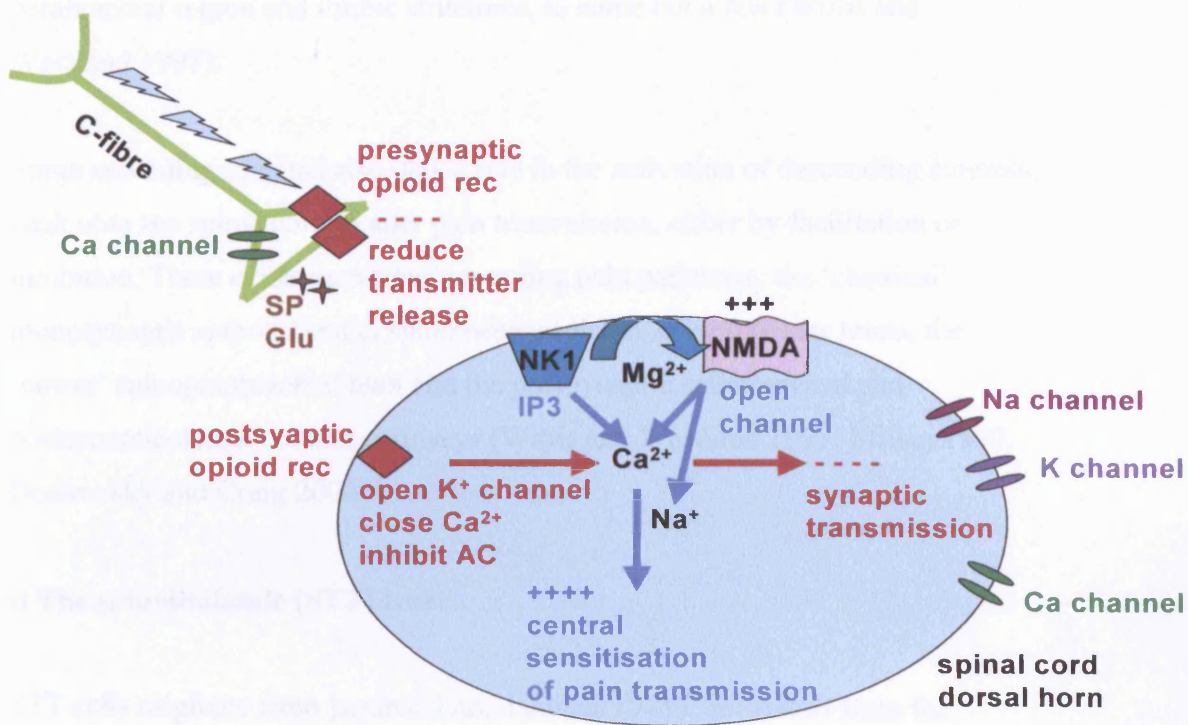


Figure 1.3- A summary of some of the excitatory and inhibitory transmitters and the receptors and channels they affect, which have been investigated in this study, in the spinal cord dorsal horn. The presynaptic and postsynaptic opioid receptors, and the inhibitory effects of their activation are shown in red. The excitatory effects of the activation of the NK-1 and the NMDA receptors are shown in blue. AC- adenylate cyclase, IP3- inositol trisphosphate, SP- substance P, Glu- glutamate, NK1- neurokinin 1 receptor, NMDA- n-methyl-D-aspartic acid receptor, Mg^{2+}- magnesium, Ca^{2+}- calcium, Na^{+}- sodium and K^{+}- potassium.

1.8 Ascending and descending systems

1.8.1 Ascending systems

The axons of projection neurons in the spinal cord traverse to numerous supraspinal sites *via* tracts located in the white matter of the spinal cord (Willis and Westlund 1997; Dostrovsky and Craig 2006). Because pain is made up of an affective as well as a discriminative sensory component, these supraspinal sites are numerous and varied and include the thalamus, the periacqueductal gray, the parabrachial region and limbic structures, to name but a few (Willis and Westlund 1997).

Some ascending systems also play a role in the activation of descending controls, back onto the spinal cord to alter pain transmission, either by facilitation or inhibition. There exists numerous ascending pain pathways: the 'classical' monosynaptic spinothalamic, spinomesencephalic, spinoreticular tracts, the 'newer' spinoparabrachial tract and the polysynaptic spinocervical and postsynaptic dorsal column pathways (Willis and Westlund 1997; Millan 1999; Dostrovsky and Craig 2006).

i) The spinothalamic (STT) tract

STT cells originate from laminae I and laminae IV-VI, as well as from the ventral horn, and project to the contralateral (most cells) thalamus (Fields and Basbaum 1978; Willis 1985; Willis and Westlund 1997; Dostrovsky and Craig 2006). It has been shown that many STT cells are nociceptive, and this pathway has now been shown to be linked with the sensations of pain, temperature and itch. Lesions in this pathway lead to the loss of sensation on the contralateral side (Willis 1985; Craig, Zhang et al. 2002; Dostrovsky and Craig 2006). As well as the sensory component of pain, cells in this tract are also thought to play a role in the motivational-affective aspect of pain, in addition to activating descending controls (Millan 1999).

ii) The spinomesencephalic tract

Many of the cells in the spinomesencephalic tract are nociceptive, and the origins of these cells are distributed in the spinal cord in the same way as those of the STT (Willis 1985; Willis and Westlund 1997). The cells, through this tract, project mainly contralaterally to the periacqueductal gray (PAG), which could contribute to aversive behaviour (Willis and Westlund 1997; Millan 1999).

iii) The spinoreticular tract

Many of the cells of this tract originate from the deep dorsal horn, the ventral horn and as has been shown lately, some cells also originate from the superficial dorsal horn (Fields and Basbaum 1978; Willis 1985; Willis and Westlund 1997). These cells project, mainly contralaterally but also ipsilaterally, to the reticular formation of the brainstem (lateral reticular nucleus LRN, nucleus parogigantocellularis NPGC/NGC), are mostly nociceptive and could activate descending inhibition (Millan 1999).

iv) The spinoparabrachial tract

A group of cells that surround the brachium conjunctivum from the dorsolateral pons (pPB) to the mesencephalon (mPB) division is called the parabrachial area (Bester, Menendez et al. 1995). Cells which project to the parabrachial area are mostly from lamina I neurons, the majority of which express the NK-1 receptor, with a few projections from lamina IV-VI neurons (Todd 2002; Suzuki, Rygh et al. 2004). These cells are predominantly nociceptive specific (Bester, Matsumoto et al. 1997; Bester, Chapman et al. 2000), and project to the parabrachial nucleus (PBN). The parabrachial nucleus in turn, has numerous links to the brainstem reticular formation cells, and projects to the hypothalamus, amygdala and the midline, intralaminar and ventrobasal thalamus, the periacqueductal gray (PAG) and the ventrolateral medulla (Gauriau and Bernard 2002; Rahman, Suzuki et al. 2003; Hunt and Bester 2005; Dostrovsky and Craig 2006). This means that cells in this tract play a role in the motivational-affective component of pain, as well as autonomic and endocrine roles (Millan 1999; Suzuki, Rygh et al. 2004; Dostrovsky and Craig 2006).

v) The spinocervical tract

This pathway starts from the spinal cord dorsal horn and relays in the lateral cervical nucleus, at the C1-C3 level. The neurons of the lateral cervical nucleus then ascend to many thalamic nuclei, through the cervicothalamic tract; and to the midbrain (including the PAG), through a cervicomesencephalic pathway. Spinocervical tract cells respond mostly to tactile stimuli, however, some of them do respond to stimuli of a noxious intensity (Willis and Westlund 1997; Millan 1999).

vi) The postsynaptic dorsal column (PSDC-lemniscal) tract

The PSDC pathway which starts from lumbosacral segments projects to the gracile dorsal column nuclei (DCN) of the caudal medulla. The origins of the PSDC are cells that are innervated by 'serotonin-only' fibres, and are located in laminae III-V and X. Direct projection neurons to the DCN from the dorsal horn also exist, and these are thought to modulate tactile information. DCN neurons then project to thalamic nuclei, to the superior colliculus, with some neurons descending back to the spinal cord. Finally, due to the effect of descending serotonergic pathways, through PSDC cells, on the rostral transmission of tactile information, this tract is postulated to play a role in the mechanical allodynia seen in neuropathic pain states (Fields and Basbaum 1978; Willis and Westlund 1997; Millan 1999).

vii) Labelling and convergence

The question of labelled lines, where pain transmission was thought to occur *via* distinct pathways from the periphery to supraspinal sites, versus convergence, where pain transmission was thought to occur *via* numerous factors which interacted at many levels from the periphery to supraspinal sites, is one that has been asked for a very long time (Melzack and Wall 1965). The new way of looking at pain processing is that it is through both labelled lines and convergence (Millan 1999; Craig 2002; Craig 2003). Therefore, even though the

description of the aforementioned tracts is accurate, the general consensus now is that projections from superficial laminae modulate mostly the motivational-affective component of pain, whereas projections from deeper laminae modulate mostly the discriminative-sensory component of pain, and that these two components of pain do not work independently of each other (Millan 1999; Hunt and Mantyh 2001; Gauriau and Bernard 2002; Hunt and Bester 2005; Dickenson and Kieffer 2006).

1.8.2 Descending controls

As mentioned previously, ascending systems may modulate descending controls, either inhibitory or facilitatory, onto the spinal cord. These descending controls, in turn, may also modulate the activity in ascending systems. Thus by a neuronal loop, pain transmission is either amplified or decreased, at the spinal level. Since most descending pathways originate from the brainstem, this loop is called 'the spino-bulbo-spinal loop'. Furthermore, the pharmacology of descending controls involves many transmitter systems including the opioidergic, noradrenergic and serotonergic networks. In my study, the descending serotonergic system will be investigated (Besson and Chaouch 1987; Millan 1999; Millan 2002).

i) Origins of descending controls

Reynolds, in 1969, achieved analgesia during abdominal surgery on rats, by electrically stimulating a region of the midbrain periacqueductal gray (Reynolds 1969). This and many other studies have consequently showed that there are supraspinal sites that exert control of pain transmission at the spinal level *via* descending fibres. The interaction of these descending controls with spinal transmission can occur at many levels, including the central terminals of primary afferent fibres, intrinsic excitatory/inhibitory interneurons and projection neurons. Furthermore, each of the many transmitters involved may exert different effects on pain transmission, according to the receptor subtype it acts on (Basbaum, Fields et al. 1984; Wei, Dubner et al. 1999; Millan 2002).

Descending controls to the spinal cord dorsal horn arise from numerous brainstem nuclei, including the rostroventral medulla and the parabrachial nucleus. The most investigated of the descending pathways is the one from the PAG *via* the Rostroventral Medulla (RVM), the latter being where most of the dorsolateral funiculus (DLF) and ventrolateral funiculis (VLF) axons descend to the spinal cord from the brainstem. The spinal cord terminations of these projections occur at laminae I, II, and V, which contain neurons that respond to stimuli of a noxious intensity (Fields and Heinricher 1985; Besson and Chaouch 1987; Fields, Heinricher et al. 1991; Heinricher 1997; Millan 2002).

ii) The PAG

The PAG has many neurons using numerous transmitters and receives and projects to several areas. Rostrally, the PAG projections include the diencephalons, hypothalamus and limbic forebrain, whereas caudally, the PAG projects to many nuclei in the parabrachial area (including the locus coeruleus), the RVM (including the Nucleus Raphe Magnus- NRM), with very few direct spinal projections (Fields and Heinricher 1985; Besson and Chaouch 1987; Heinricher 1997; Willis and Westlund 1997). Opioid peptides and receptors are prominent in the PAG, and a microinjection of morphine or other opioid peptides in this area causes analgesia which can be reversed by naloxone. Opioids are thought to activate descending inhibitory neurons from the PAG to the spinal cord *via* the RVM by inhibiting GABA interneurons, which otherwise inhibit this output neuron to the RVM (disinhibition) (Heinricher 1997).

iii) The RVM

The ventral aspect of the rostral medulla (RVM), as mentioned above, is a very important connection between the PAG and the spinal cord dorsal horn. The RVM contains the nucleus raphe magnus (NRM), as well as the nearby reticular formation, which includes the nucleus reticularis magnocellularis (NRMC). Within the RVM, the areas where the descending cells originate include the NRM, the nucleus reticularis gigantocellularis pars α (Rgcα) and the nucleus

reticularis paragigantocellularis lateralis (Rpgl) (Basbaum and Fields 1984; Besson and Chaouch 1987; Heinricher 1997; Willis and Westlund 1997; Suzuki, Rygh et al. 2004).

Inactivation of the RVM (using electrolytic lesions or a local anaesthetic) decreases PAG-induced analgesia. However, the fact that the activation of the RVM (electrically or with glutamate/morphine microinjections) causes analgesia, as well as the fact that there are reciprocal connections back to the PAG, as well as other supraspinal sites from the RVM, show that this area is not just a relay for PAG-induced analgesia. Indeed, the RVM is not just involved in controlling sensory information, but also plays a role in homeostatic functions. Furthermore, even though there are intrinsic spinal cord neurons that contain 5-HT, the RVM is an important source of this transmitter in the spinal cord (Basbaum, Fields et al. 1984; Fields and Heinricher 1985; Besson and Chaouch 1987; Heinricher 1997; Willis and Westlund 1997; Suzuki, Rygh et al. 2004).

iv) ON, OFF and neutral cells

Three different classes of cells have been shown to exist in the RVM: ON cells, OFF cells and neutral cells. They have been classified according to the abrupt change in firing that they show, just before a lightly anaesthetised rat flicks its tail in the tail flick test. ON cells suddenly increase their firing before the tail flick occurs, OFF cells suddenly stop firing before the tail flick occurs and neutral cells show no change in their activity with relation to the tail flick (Fields, Heinricher et al. 1991; Heinricher 1997). Morphine administration in the RVM has been shown to suppress ON cell firing, activate OFF cell firing and have no effect on neutral cell firing. Opioid receptors are expressed on ON cells and therefore, morphine administration into the RVM directly inhibits these cells. These ON cells are also inhibited when morphine is administered into the PAG, causing disinhibition of inhibitory PAG-RVM outputs, as mentioned above. OFF cells on the other hand are activated by opioid-mediated disinhibition, and by output from the PAG. Therefore, ON cells are thought to play a facilitatory role in pain transmission, OFF cells are thought to play an inhibitory role, whereas

neutral cells do not seem to play a role in pain transmission (Fields, Heinricher et al. 1991; Heinricher 1997; Porreca, Burgess et al. 2001; Dickenson and Kieffer 2006).

v) Descending inhibitions or descending facilitations?

Whereas it was previously shown that electrical and glutamate microinjections in the RVM inhibited spinal nociception (Reynolds 1969; Besson and Chaouch 1987), it was later discovered that RVM stimulation both inhibits and facilitates spinal cord nociception. Therefore, low intensity electrical and low concentration of chemical stimulation in the RVM is thought to mostly facilitate spinal cord transmission (both noxious and non-noxious), *via* descending projections in the ventrolateral funiculi and spinal serotonin (5-HT) and cholecystokinin receptors, whereas high intensity/concentration of electrical/chemical stimulation in the RVM is thought to mostly inhibit spinal cord transmission, *via* dorsolateral funiculi projections and spinal monoaminergic receptors (Besson and Chaouch 1987; Urban and Gebhart 1999; Zhuo and Gebhart 2002).

Furthermore, studies using spinalisation, lesions and local anaesthetic injections in the RVM have shown that descending facilitations from the RVM may play an important role in secondary hyperalgesia, and thus central sensitisation arising from different pain states of chemical, inflammatory and neuropathic origin (Urban, Jiang et al. 1996; Mansikka and Pertovaara 1997; Urban and Gebhart 1999; Urban, Zahn et al. 1999). Therefore, it is now known that both inhibitory and facilitatory descending controls arise from the RVM at any one time, that these descending controls are enhanced following persistent pain states, the extent of which depends on the pathophysiological state of the animal and the stimulus modality being tested (Kauppila, Kontinen et al. 1998; Wei, Dubner et al. 1999; Zhuo and Gebhart 2002).

vi) Descending controls in neuropathic pain

Central sensitisation does not just rely on events which occur in the spinal cord, but also relies on descending controls from supraspinal sites onto the spinal cord. Following peripheral nerve injury, spinal sensitisation is initiated by afferent input and maintained by afferent input (to a certain extent) and descending facilitations (Vera-Portocarrero, Zhang et al. 2006). Descending facilitations from the RVM are activated and enhanced 6 days following peripheral nerve injury (Kauppila, Kontinen et al. 1998; Burgess, Gardell et al. 2002), and serve to heighten the excitability of the spinal cord and thus also aid in non-noxious stimuli being perceived as noxious. Therefore, the role of these descending facilitations seems to be the maintenance of neuropathic pain, and not the initiation, which also means that the mechanisms underlying the initiation of nerve injury are different from those needed to maintain it (Ossipov, Lai et al. 2001; Burgess, Gardell et al. 2002).

vii) Thermal versus mechanical hypersensitivity in neuropathic pain- from the periphery to supraspinal sites

As discussed previously, thermal hyperalgesia and allodynia to mechanical stimuli are mechanistically distinct, following neuropathic pain. Whereas thermal hyperalgesia is thought to be conducted mostly *via* C-fibres and to be mostly spinally mediated, mechanical allodynia has different subtypes, is thought to be conducted *via* myelinated primary afferents, and is thought to be mediated supraspinally (Kauppila 1997; Kauppila, Kontinen et al. 1998; Ossipov, Lai et al. 2000; Chen, King et al. 2004).

a) Thermal hyperalgesia

Thermal hyperalgesia is thought to be conducted mostly *via* C-fibres, since it is blocked by capsaicin and resiniferatoxin administration, each of which is known to desensitise C-fibres. Furthermore, this symptom of neuropathic pain is responsive to morphine administration, and it is also known that μ-opioid receptors are located on C-fibres. Finally, thermal hyperalgesia is also thought to be mostly spinally mediated, since it was reduced by both local Na channels

blockers which do not penetrate the CNS, and amplified by increased spinal dynorphin levels. However, intra-RVM Na channel blockers and spinal transection also decreased thermal hypersensitivity following neuropathic pain, therefore descending facilitations are also partly involved in the maintenance of thermal hypersensitivity (Bian, Ossipov et al. 1998; Field, Bramwell et al. 1999; Ossipov, Lai et al. 2000; Chen, King et al. 2004).

b) Mechanical allodynia

It has long been known that brush-evoked pain is distinct from pain due to punctuate stimuli (Price, Bennett et al. 1989; Koltzenburg and Handwerker 1994). To explain how touch-conducting Aβ-fibres could signal pain, it was initially suggested that a structural reorganisation in the spinal cord dorsal horn had occurred, where Aβ-fibres started sprouting into lamina II, which is where most pain-signalling fibres terminate (Woolf, Shortland et al. 1992). However, it was later shown that the tracer used 'CB-HRP' (B unit of cholera toxin-horseradish perodixase), which normally only labels the central terminals of myelinated afferents, was also taken up by small DRG ganglions following peripheral axotomy. Therefore, central sensitisation, due to the initial afferent barrage, and maintained by the enhanced descending facilitations, is now thought to be the reason behind large-diameter Aβ-fibres conveying painful sensation instead of innocuous touch (Campbell, Raja et al. 1988; Koltzenburg and Handwerker 1994; Suzuki and Dickenson 2000).

The increase in descending facilitations, and thus spinal sensitisation, following peripheral nerve injury is part of the mechanism underlying tactile and cold evoked responses more than it is for thermal hyperalgesia (Pertovaara, Wei et al. 1996; Kauppila 1997; Kauppila, Kontinen et al. 1998). This was shown when lidocaine, administered intra-RVM was capable of reversing both tactile allodynia and thermal hypersensitivity, whereas lidocaine in the periphery, which does not have central effects, was only capable of reducing thermal hypersensitivity (Chen, King et al. 2004). Furthermore, in the SNL model,

lidocaine in each of the RVM and PAG caused an antiallodynic effect, which was not opioid-mediated since it was not reversed by naloxone (Pertovaara, Wei et al. 1996). Additionally, spinalisation completely abolished tactile allodynia in SNL rats (Bian, Ossipov et al. 1998).

1.9 Serotonin

Whereas SP and glutamate play an exclusively excitatory role in the spinal cord dorsal horn, serotonin (5-HT), due to its complex action on many serotonergic receptors, has been shown to play a dual, pronociceptive (Jordan, Kenshalo et al. 1978; Ali, Wu et al. 1996; Oyama, Ueda et al. 1996; Calejesan, Ch'ang et al. 1998; Green, Scarth et al. 2000; Zeitz, Guy et al. 2002; Suzuki, Rahman et al. 2004) and antinociceptive role (Jordan, Kenshalo et al. 1978; Yaksh and Wilson 1979; Alhaider, Lei et al. 1991; Crisp, Stafinsky et al. 1991; Oyama, Ueda et al. 1996; Jones, Peters et al. 2005).

Most of the serotonin in the spinal cord comes from descending controls, which, like serotonin, have been previously shown to play an inhibitory, rather than a facilitatory role in pain transmission (Reynolds 1969; Fasmer, Berge et al. 1983; Maxwell, Leranth et al. 1983; Besson and Chaouch 1987; Zhuo and Gebhart 2002). However, newer studies have shown that the dual action of serotonin is probably due to the type of serotonergic receptor being activated (with the 5HT3 receptor being responsible for the excitatory effects), the dose of the agonist used (most studies showing the inhibitory actions of serotonin used high doses of agonists), the stimulus modality used and the pathophysiological state of the animal (Fasmer, Berge et al. 1985; Bardin, Bardin et al. 1997; Suzuki, Rahman et al. 2004; Conte, Legg et al. 2005; Donovan-Rodriguez, Urch et al. 2006).

1.9.1 5-HT receptor subtypes

To date, seven families of serotonergic receptors have been indentified (5HT1-5HT7), each with many subgroups, all of which belong to the G-protein family of receptors, except the one investigated in this study, the 5HT3 receptor. The

5HT3 receptor is a ligand-gated ion channel receptor which, when activated, is permeable mostly to monovalent cations, and to Ca^{2+}. Two receptor subunits have been identified so far, the 5HT3A and the 5HT3B. It has been shown that functional receptors are either made from 5HT3A homopentamers, and 5HT3A and 5HT3B heteromers. The homomeric receptors are thought to be preferentially located in the central nervous system, whereas the heteromeric receptors are thought to be found in the periphery (Maricq, Peterson et al. 1991; Dubin, Huvar et al. 1999; Morales and Wang 2002; Conte, Legg et al. 2005).

1.9.2 5HT3 receptor location

5HT3A immunoreactivity was most densely found in the superficial dorsal horn, most of which is thought to be due to 5HT3 receptors on the terminals of primary afferent fibres. Rhizotomy did not abolish binding sites and immunoreactivity for 5HT3 receptors, which means that some of the receptors are found on intrinsic dorsal horn neurons. Furthermore, 5HT3 receptors are thought to be predominantly (80%) located on a subset of primary afferent fibres which are myelinated (as shown by N52 immunoreactivity- a marker for neurofilaments linked with myelinated primary afferents), and thus of the Aδ-fibre variety, since these fibres terminate in the superficial layers of the dorsal horn. Less (13%) 5HT3A immunoreactivity was found in capsaicin-sensitive neurons, and even less of these receptors were found to be located in peptidergic and IB4-positive primary afferent fibres (Maxwell, Leranth et al. 1983; Kidd, Laporte et al. 1993; Tecott, Maricq et al. 1993; Kia, Miquel et al. 1995; Zeitz, Guy et al. 2002; Maxwell, Kerr et al. 2003; Conte, Legg et al. 2005).

1.9.3 5HT3 receptor- nociceptive or antinociceptive?

Initially, the studies which attributed an antinociceptive role to the 5HT3 receptor claimed that this was done *via* this receptor's activation of inhibitory GABA-ergic (Alhaider, Lei et al. 1991; Peng, Lin et al. 1996) and enkephalinergic interneurons (Tsuchiya, Yamazaki et al. 1999). However, even though 5-HT was found to be co-localised with glutamate decarboxylase (GAD), the enzyme

which synthesises GABA, 5HT3A immunoreactivity was not found to be co-localised with this enzyme, which means that this receptor is not located on inhibitory cells (Maxwell, Maxwell et al. 1996; Zeitz, Guy et al. 2002; Conte, Legg et al. 2005).

Furthermore, even though 5-HT caused the depression of most spinothalamic tract neurons in a study using primates, some of these STT neurons were also excited by 5-HT (Jordan, Kenshalo et al. 1978). Indeed, the intrathecal injection of 5-HT in mice has been shown to cause biting, scratching and licking behaviour reminiscent of that of intrathecal SP, which was reduced by the same compound which reduced the behaviour arising from SP. SP and 5-HT have thus been shown to have an interaction at the spinal level, further proved by the finding that a subpopulation of NK-1 receptors in laminae III-IV receive numerous contacts from 5-HT axons (Fasmer, Berge et al. 1983; Fasmer and Post 1983; Eide and Hole 1989; Stewart and Maxwell 2000).

Moreover, the blockade of the 5HT3 receptor by its antagonist ondansetron has been shown to inhibit both phases of the formalin response (Green, Scarth et al. 2000). It has also been recently shown that the blockade of the 5HT3 receptor with ondansetron reduces tactile allodynia following spinal cord injury (Oatway, Chen et al. 2004), and natural-evoked responses of dorsal horn neurons, which arise from cancer-induced bone pain (Donovan-Rodriguez, Urch et al. 2006) and nerve injury (Suzuki, Rahman et al. 2004), and may cause analgesia in humans with chronic neuropathic pain (McCleane, Suzuki et al. 2003).

However, serotonin does not facilitate acute or carrageenan-induced pain (Green, Scarth et al. 2000; Rahman, Suzuki et al. 2004), and thus the conclusion is that descending serotonergic facilitations, and their actions on the excitatory 5HT3 receptor, are enhanced following chronic pain states. Furthermore, even though the RVM is thought to be the primary source of descending serotonergic control, and some RVM neurons that respond to noxious stimuli have been labelled as serotonergic, neither ON nor OFF cells seem to contain 5-HT (Gao and Mason 2000).

Nearly 80% of the spinothalamic and spinoparabrachial projection neurons from lamina I express the NK-1 receptor. Additionally, it has been shown that the parabrachial area connects with both the amygdala and hypothalamus, among other areas in the brainstem, which in turn may modulate descending monoaminergic controls onto the spinal cord. The axons of descending serotonergic neurons have been shown to terminate in lamina I, as well as have an association with projection neurons in this lamina (Todd, McGill et al. 2000; Todd 2002; Todd, Puskar et al. 2002). The ablation of lamina I projection neurons which express the NK-1 receptor, using the intrathecal administration of SP-saporin (Mantyh, Rogers et al. 1997), has led to a decrease in receptive field size of neurons, an altered coding of mechanical and thermal stimuli and decreased wind-up of deep dorsal horn neurons (thus decreased central sensitisation) (Suzuki, Morcuende et al. 2002). Most of the aforementioned effects were also achieved when ondansetron was intrathecally administered to block descending serotonergic facilitations (Suzuki, Rahman et al. 2004). Therefore, the NK-1-positive projection neurons are thought to be the origin of the spino-bulbo-spinal loop which affects pain transmission at the spinal cord level, *via* the activation of descending serotonergic facilitations (Conte, Legg et al. 2005; Suzuki, Rahman et al. 2005).

1.9.4 The 5HT3 receptor in neuropathic pain

The enhanced descending facilitations following neuropathic pain belong to the spinal-bulbo-spinal loop which has NK-1 expressing projection neurons at its origin, and descending serotonergic facilitations at its 'end'. The ablation of these neurons with the use of SP conjugated to 'saporin' result in a decrease in both hypersensitivity responses and excitability of deep dorsal horn neurons. These effects, as mentioned above, also occur when the 5HT3 receptor is blocked with spinal ondansetron (Suzuki, Rahman et al. 2004; Suzuki, Rahman et al. 2005; Rahman, Suzuki et al. 2006).

The 5HT3 receptor antagonist ondansetron has been clinically used for many years as an anti-emetic drug. However, this drug has been shown to be able to

decrease mechanical-evoked response in deep dorsal horn neurons, which occurs following experimental peripheral nerve injury, to a greater extent in nerve injured than normal animals (Suzuki, Rahman et al. 2004). Furthermore, in a model of spinal cord injury, ondansetron also reduced mechanical allodynia which occurred in this model (Oatway, Chen et al. 2004). Finally, in a clinical study, ondansetron has been shown to be effective in patients with neuropathic pain, two hours following a single intravenous administration (McCleane, Suzuki et al. 2003).

In my study, the 5HT3 antagonist ondansetron will be intrathecally administered in rats with a model of neuropathic pain, to see whether this antagonist can reduce some of the hypersensitivities seen in neuropathic animals, and thus further investigate whether the descending serotonergic facilitations are enhanced following peripheral nerve injury, as has been previously shown (Suzuki, Rahman et al. 2004). In addition, ondansetron, in combination with morphine, will be used to compare the efficacy of the former on its own, and to investigate whether its effects increase when combined with morphine, to inhibit the thermal, tactile and cold hypersensitivity seen in neuropathic rats.

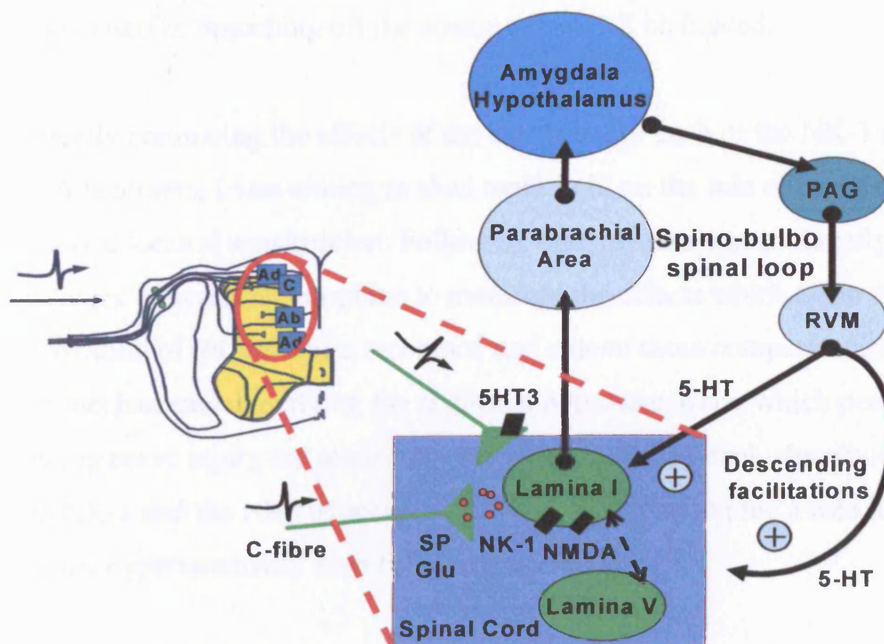


Figure 1.4- A summary of the spino-bulbo-spinal loop theory. Noxious input from the periphery arrives *via* nociceptive primary afferent fibres, such as C-fibres, which release substance P (SP) and glutamate (Glu) and impinge upon superficial (laminae I-II) spinal cord dorsal horn neurons, which express the neurokinin 1 (NK-1) receptor and the n-methyl-D-aspartic acid (NMDA) receptor. NK-1 expressing lamina I projection neurons also project supraspinally *via* the parabrachial area to the amygdala and hypothalamus, which in tun project to the periacqueductal gray area (PAG). The PAG has neurons projecting to the rostral ventral medulla (RVM), which has descending fibres projecting back to the spinal cord. Following nerve injury, descending facilitations from the RVM are enhanced and facilitate pain transmission in the spinal cord by acting on the serotonergic ion channel receptor 5HT3, which is expressed on a subset of primary afferent fibres. Adapted with permission from Dr. R. Suzuki.

1.10 Aim

The general aim of my study is to investigate the interaction between the excitatory systems in the spinal cord with the inhibitory actions of morphine, in rats. In order to achieve this, I have used two pain models which reflect central sensitisation: an acute, chemical model of nociception, whereby each of the spinal NK-1 and NMDA receptors is activated using Sar-SP and NMDA,

respectively; and a chronic, surgical model of nerve injury, whereby the L5 and L6 spinal nerves branching off the sciatic nerve will be ligated.

By directly comparing the effects of the activation of each of the NK-1 and NMDA receptors, I was aiming to shed more light on the role of each of these receptors in central sensitisation. Following that, my aim was to directly compare the efficacy of systemic morphine to modulate the effects which occur following the activation of each of these receptors, and extend these comparisons as to which mechanisms underlying the abnormal hypersensitivity which occur following nerve injury are more sensitive to morphine control, since both the spinal NK-1 and the NMDA receptors have been shown to play a role in the abnormal hypersensitivity seen following nerve injury.

Furthermore, I will investigate whether peripheral NMDA receptors are involved in the abnormal pain behaviour seen in this model of nerve injury, using the intraplantar administration of MK-801, the NMDA receptor antagonist, since it would be clinically beneficial for topical NMDA antagonists to be used in treating pain following nerve injury, devoid of central side effects. And finally, I will further investigate whether descending serotonergic facilitations are enhanced following the SNL model, by using the 5HT3 antagonist ondansetron *via* the intrathecal route, and also study whether the effectiveness of either ondansetron or systemic morphine is increased when these two drugs are used in combination, and thus their interaction in the central processing of chronic pain transmission.

Chapter 2:

Methods

2. Methods

2.1 Animals

The animals used were male, adult (190-250g) Sprague-Dawley rats, obtained from University College London. All rats were housed in a 12 hour light/dark cycle with free access to food and water. All experimental procedures were approved by the UK Home Office and follow the guidelines of the International Association for the Study of Pain (Zimmermann, 1983).

2.2 In vivo electrophysiology

Electrophysiological studies were conducted in the same way as previously described (Urch *et al.*, 2003a). Anaesthesia was induced with 3.5% Halothane (Concord Pharmaceuticals Ltd) in N_2O ($300cm^3/min$) and O_2 ($150cm^3/min$) in the anaesthesia box. As soon as the rat lost its righting reflex, it was placed on a heating blanket (regulated to stay at 37°C with a rectal probe- Harvard Homeothermic Blanket Control Unit), the anaesthetic was reduced to 2.8% and was given through a nose cone. Before proceeding, deep anesthesia was checked for by pinching the hindpaw, to test for complete areflexia (loss of reflexes). A tracheotomy was made, and the shaped end of a polyethylene tube (80-100mm) was inserted into the trachea. At this point, the cone was removed and the anaesthetic was delivered straight into the cannula, with the dose lowered to 1.5%.

The rat was then secured into the ear bars of the stereotaxic frame, after which the anesthetic delivery and rectal probe/temperature were checked. A laminectomy was made in a small area above and below the base of the ribs, to expose the spinal cord at L1-L3 vertebral level. A parylene-coated tungsten electrode (A-M Systems, USA) was inserted, medial to the central vessel, into the cord. This was used to extracellularly record dorsal horn neurons receiving afferent input from the glabrous tissue of the hindpaw. Gentle tapping and

noxious pinch were used as search stimulus to find the neuron, the depth of which was made and identified by the number of revolutions done by the fine control manipulator on the headset (Narishige, Japan).

The responses of each of input, Aβ fibres, Aδ fibres, post-discharge, wind-up (after electrical stimuli), brush, von Frey filaments (Scientific Marketing Associates, UK) 1, 5, 9, 15, 30, 75g and heat 35, 40 45 48 and 50°C were investigated in this experiment. The system used to amplify, filter, discriminate the cells and apply isolated electrical stimuli was Neurolog (Digitimer, UK) and Tektronix TDS 1002 two digital storage oscilloscope was used to visualise neurons. This system was coupled to a Dell computer with Spike 2 software through a CED interface (Cambridge Electronic Design, Cambridge, UK).

First, the signal from the recording electrode is transferred to the Neurolog data capture system, which amplifies, filters and feeds the neuronal signal to the audio speakers and the oscilloscope. This is followed by the action potentials being discriminated and in turn fed to the computer system through the CED 1401 interface. The action potentials evoked were recorded on the latch counter and visualised and counted on the oscilloscope, with the post-stimulus time histogram (PSTH) shown on the computer display.

Transcutaneous electrical stimuli were given via needles inserted in the area most responsive in the receptive field (the area on the hindpaw which responds to electrical and natural stimuli) of the cell. These needles are connected to a Neurolog stimulus isolator which is linked to the Neurolog system. This system pre-sets the frequency of stimulation at 0.5Hz, duration for 2 milliseconds and amplitude of the current at 3x the C-fibre threshold, as well as the number of pulses (16) via a period generator, digital width, pulse buffer and counter. Therefore, a train of 16 stimuli was given at 3x the C-fibre threshold current, which was taken as the lowest amplitude required to evoke a C-fibre response. The natural stimuli was applied on the same area for 10 seconds, with care taken not to touch the needles, as this would lead to the cell being stimulated further.

Heat responses were done with a constant water jet over the same area, at the specific temperature needed, for the same time.

Input is a measure of neuronal activity after the first electrical stimulus, before any hyperexcitability has taken place. Aβ, Aδ and C-fibre evoked responses (after the train of 16 electrical stimuli) were separated according to latency, and these were 0-20, 20-90 and 90-300ms respectively. Any response after the C-fibre band (300-800ms) was considered as the cell's post-discharge, due to its hyperexcitability. Wind-up is calculated by subtracting (16 x input) from the total number of evoked action potentials.

For the characterisation experiments, each neuron isolated was tested electrically and naturally as above, but without drug addition. One extra temperature was performed, and this was 42 °C.

2.3 Acute chemical model of nociception

2.3.1 Lumbar catheterisation

Rats weighing 190-205g were housed separately in cages before and after the surgical procedure, to avoid damage to the catheters. The lumbar catheterisation was undertaken using a modified method by Storkson *et al* (Storkson *et al.*, 1996).

Before the surgery, the catheters were prepared. Each catheter was made by cutting PE-10 tubing (outer diameter 0.6mm, Portex) into 28cm, and stretching 10cm at one end of the tube in 60°C water and then dipping it in room temperature water to retain the stretch. The stretched end was then threaded through a sterile, 4cm length, 19-gauge needle, which served as a guide cannula during surgery. The catheter was then shortened to 28cm again, and flushed through with saline. Furthermore, two marks were made on the catheter, one was 3cm from the stretched tip and was covered under the guide cannula, whereas the second was made 3cm from the hub of the needle, and was visible at all time.

Each rat was induced with 5% halothane and 2% oxygen. The latter was maintained throughout surgery. When the rat lost its righting reflex, it was put on a nose cone at 3% halothane, and its back was shaved. Once the rat had lost its flexor reflex, anaesthesia was lowered to 1.5%, and an incision was made with a scalpel blade in the midline which extended from its caudal ribs to below its hip bones. The guide cannula was inserted between the two vertebrae which lie on the same horizontal level as the ventral iliac spine, at a 45° angle. After that, the catheter was pushed through rostrally until the visible mark reached the hub of the needle, which was 3cm into the spinal subarachnoid space and ended by the caudal ribs, or L1-L3 vertebrae. This area corresponds to the L4-L6 lumbar spinal segment area, which also corresponds to where the input from the hindpaws comes in.

The insertion was sometimes met with a bit of resistance and so to make it easier at times, the spinal cord was raised manually and made kyphotic. The right position of insertion was confirmed by three things: the first was a flick of the tail or twitch of the hindpaws, the second was the easy insertion of the catheter after the initial resistance was overcome, and the third was the spinal fluid backflowing through the catheter. After that, the guide cannula was removed carefully, and the catheter was fixed with cyanoacrylate gel (RS) at the puncture site. A hole was made using another needle, 3cm from the occipital region, and the free end of the catheter was tunnelled under the skin and out using this hole. The catheter was fixed at 2 other places on the inside of the skin, as well as on the hole it was coming out of the skin from, and it was cut down to 20cm and cauterised. The wound was sealed using wound clips, and the rat was left to recover in a heated chamber at 32°C.

After the surgery, rats were checked for motor dysfunction. If the rats showed any sign of motor deficit, they were not used in this study and were humanely culled. The location of the catheters was verified at the end of every experiment by three ways: the first was hindpaw paralysis after the intrathecal administration of Lidocaine (Dobos *et al.*, 2003). The second was by the intrathecal injection of

Pontamine Blue dye (Raymond A. Lamb), after humanely culling the rat, and finding the dye localised on the dorsal area by the caudal ribs following dissection. Finally, the third method was by dissecting the rat and finding the catheter in the middle of the spinal cord, at the dorsal area, by the caudal ribs. Any animal whose catheter was not found in the aforementioned location had its results discarded.

2.3.2 Biting, scratching and licking behaviour (BSL)

The biting, scratching and licking and grooming behaviour which followed every intrathecal administration of each of NMDA and Sar-SP was observed and timed using a stopwatch.

2.3.3 Thermal hypersensitivity

Thermal hypersensitivity was assessed using the Hargreaves test (Hargreaves *et al.*, 1988). Each rat was placed in a clear plastic chamber with a glass bottom, which was covered with a grill on top, and allowed to acclimatise for at least 15 minutes. Testing only began when the rats had stopped their exploratory behaviour and their grooming. A radiant heat source was placed under the glass floor, exactly in the middle of the plantar side of the hindpaw. The intensity of the light beam was set so that the baselines of the rats fell between 10-15 seconds, and cut-off was set at 30 seconds to prevent tissue injury (Davis *et al.*, 2001; Davis *et al.*, 1999).

The radiant heat source was made up of a high intensity projector lamp bulb which projected through the aperture in the top of a movable case. Any movement of the rat which involved its hindpaw was detected by a photoelectric cell which was aimed at the aperture and which turned off both the lamp and the electric clock. Finally, withdrawal latency to the nearest 0.1 second was determined using the electronic clock circuit and microcomputer (Ugo Basile, Italy). Unless the animal was already moving and exploring, withdrawal of the hindpaw and/or licking were considered as positive responses. Furthermore, if the animal urinated or defecated in the cage, it was removed, the bottom of the cage was wiped and it was placed back inside it and given time to re-acclimatise

before being tested. This re-acclimatisation did not take more than 1 minute once the animal had already acclimatised to the cage before.

Three baselines which lay between 10-15 seconds were taken each time, before any drug was added. The rats were tested every 5 minutes for the baselines and for the duration of the experiment (which was 60 minutes), after drug administration. However, in the experiments where two drugs were being administered, some timepoints were missed in order to prepare the second injection.

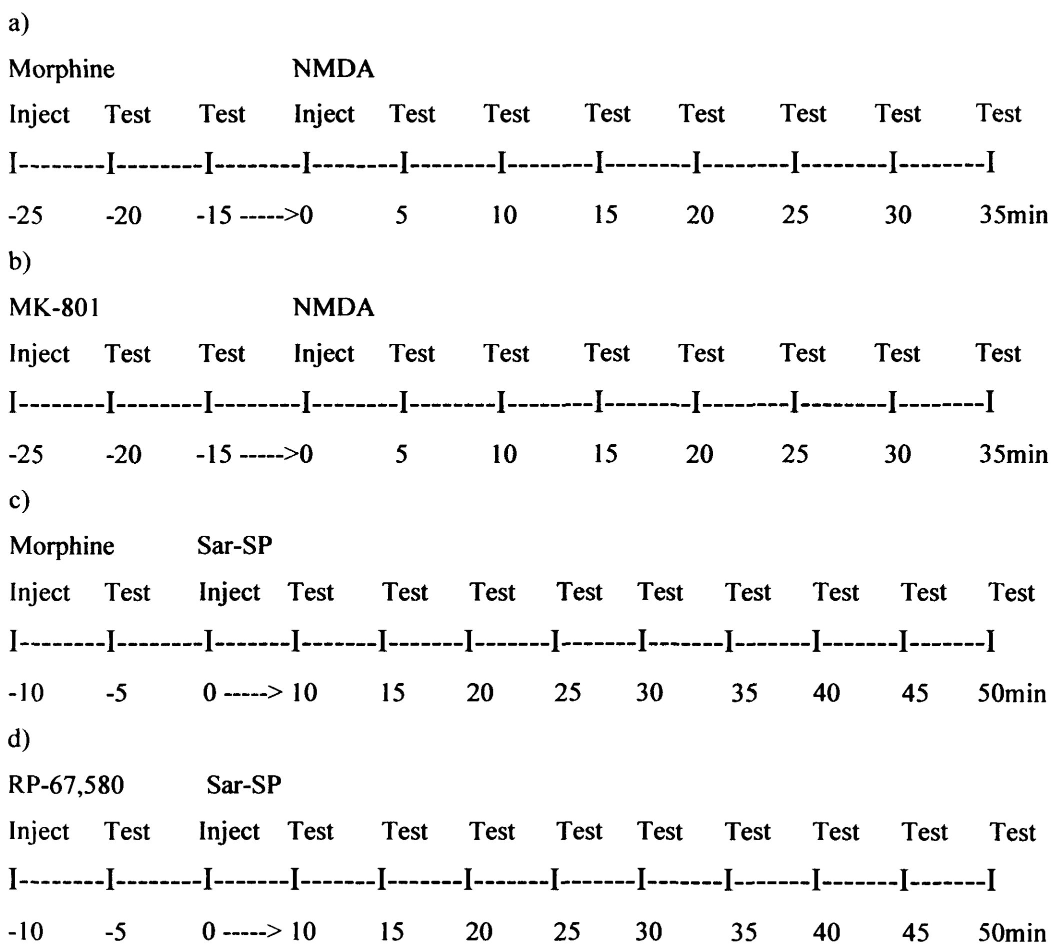


Figure 2.1. Timecourse of each set of thermal hypersensitivity experiments which involved two drugs: a) morphine+NMDA b) MK-801+NMDA c) morphine+ Sar-SP d) RP-67,580+Sar-SP

2.3.4 Paw withdrawal threshold assessment

Tactile hypersensitivity was measured using the Chaplan method, which is based on the Dixon up-down method (Chaplan *et al.*, 1994). Each rat was placed in a plastic cage with a wire mesh bottom (Ugo Basile, Italy), and they were left to acclimatise until all exploratory/grooming behaviour ceased. A series of eight von Frey filaments (North Coast Medical Inc, Morgan Hill, California) were used in this method, and these were 0.4, 0.6, 1, 2, 4, 6, 8 and 15g. The von Frey hair was placed perpendicular to the middle of the plantar surface of the rat hindpaw, and force was applied until the von Frey hair bent against the paw and was kept there for 6 seconds.

Testing began with von Frey hair 2g, consecutively, in an ascending manner, until a positive response was reached. A positive response was taken as licking, withdrawal of the paw or flinching. In the absence of a positive response, the next von Frey up was used, and when there was a positive response, the previous von Frey was used. Once the response threshold has been crossed, which is when the first positive response occurs, this is considered as the first response and is followed by a total of six responses (positive or negative). Positive responses were assigned the letter X and negative responses were assigned the letter O. When there were continuous positive responses starting from 2g down to 0.4g, the paw withdrawal threshold was taken to be 0.25g and when there were continuous negative responses from 2g up to 15g, the paw withdrawal threshold was taken to be 15g.

Paw withdrawal threshold in grams (g) was calculated using 'FlashDixon' software, a kind gift from Dr.Micheal Ossipov, from the University of Arizona. The XO pattern derived from each rat was typed into the formula, along with the number of the last von Frey hair used, and the paw withdrawal threshold was calculated. The calculation used in the software was based on Chaplan et al, 1994. The formula was:

50%g threshold= $(10^{[Xf+K\delta]})/10{,}000$

Xf here was the value (in log units) of the final von Frey hair used, K was the tabular value which was based on the response pattern and modified from Dixon, and δ was the mean difference (in log units) between stimuli.

The rats were tested at three timepoints for each experiment.

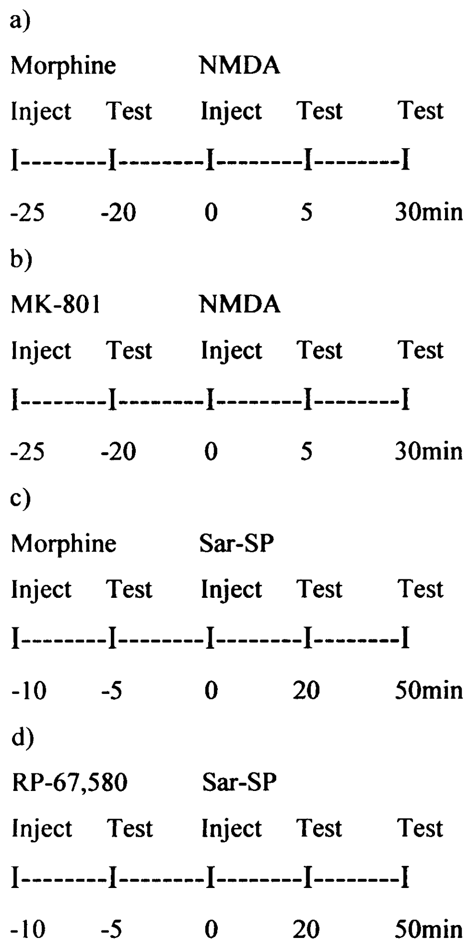


Figure 2.2. Timecourse of each set of tactile hypersensitivity experiments which involved two drugs: a) morphine+NMDA b) MK-801+NMDA c) morphine+Sar-SP d) RP-67,580+Sar-SP

2.3.5 Assessment of ambulation

Rotarod (model 7750, Ugo Basile, Italy) experiments were undertaken when a specific drug was known to cause sedation, or known to cause motor effects. Rats were left in their cages to acclimatise to the room where the rotarod instrument is placed until all grooming and exploratory activity ceased. The

rotarod used was set to accelerate from 0-20 revolutions per minute (rpm) over 60 seconds, and the time taken for the rat to fall from the beam was timed using a stopwatch. Cut-off was taken as 180 seconds, and only the rats which stayed on the beam, for a minimum of 60 seconds in two previous training session were chosen for this experiment (Donovan-Rodriguez *et al.*, 2005).

2.4 Peripheral NMDA receptor study- Spinal Nerve Ligation model

In this part of the study, rats weighing 140-170g were used at the time of surgery. Anaesthesia was induced with 4% halothane in 66% nitrous oxide and 33% oxygen. When the rat lost its righting reflex, it was removed out of the induction box and its nose placed inside a nose cone, lying on its front, and the halothane was reduced to 3%. The back was shaved for 2cm above and below the hipbone, and once areflexia was achieved, a longitudinal incision was made to part the skin around the hipbone area. Anaesthesia was reduced to 1.5%, and the left paraspinal muscles were separated, with pointed tweezers, from the spinous processes at the L4-S2 level (Kim *et al.*, 1992).

Once the sacrum, a shiny, crescent-shaped bone, was identified, the L6 transverse process was gently removed with a small rongeur. The L5 and L6 spinal nerves were identified and ligated, using 6-0 silk thread, distal to the dorsal root ganglion and proximal to the sciatic nerve formation. Once haemostasis was achieved, the wound was sutured with 3-0 thread and the skin was closed with wound clips. For the sham operated animals, all the aforementioned steps were done, except the actual tying of the nerves (Kim *et al.*, 1992).

Following the surgery, the rats were observed for normal weight gain and behaviour. The lesioned paw was mildly deformed, resembling a claw, and the rats exhibited some guarding behaviour at times, but that was acceptable as long as the rats were gaining weight, grooming and were normally active.

Placed in the same mesh-bottom Plexiglas cages that were described above and following the same behavioural guidelines, rats were tested for tactile hypersensitivity using von Frey filaments 1, 5 and 9g. These filaments were placed 10 times for 2-3 seconds each time, at the 'hotspots' (areas on the plantar surface that are responsive to stimuli). A positive response was recorded when the rat either withdrew, licked or held its paw in the air following withdrawal for a few seconds (Kim *et al.*, 1992). The number of times, out of ten, that the rat withdrew its contralateral paw was subtracted from the number of times the rat withdrew its ipsilateral paw. This was called the 'difference score'.

Rats were also tested for cold hypersensitivity, by placing a single drop of acetone on the plantar surface of their paws, five times. The 'difference score' was also calculated here (Chapman *et al.*, 1998).

If sham rats demonstrated tactile and cold hypersensitivity, they were not used in the study and were humanely culled.

2.5 Morphine and ondansetron in neuropathic pain study- Spinal nerve ligation and lumbar catheterisation

For this part of the study, the spinal nerve ligation was performed on rats weighing 190-205g, since the rats were also to undergo lumbar catheterisation at the same time of their spinal nerve ligation surgery. The lumbar catheterisation was performed before the spinal nerve ligation, and care was taken not to tug at the cannula when performing the spinal nerve ligation surgery.

If the rat was dragging its paw, the animal was not used and was humanely culled.

2.6 Drugs

2.6.1 In vivo electrophysiology drugs

Morphine Sulphate (morphine, 0.1, 1 and 10μg, Thornton and Ross), naloxone (50μg, Sigma) [Sar9, Met $(O_2)_{11}$]-Substance P (Sar-SP, 1 and 10μg, Tocris) and N-methyl-d-aspartic acid (NMDA, 5, 50 and 500ng, Sigma) were administered intrathecally in a 50μl volume with a Hamilton syringe.

For the set of pharmacological experiments using morphine alone, three consecutive controls, where the readings for all of the above were not more than 10% different, were followed by each of three morphine doses. Each dose was left for 20 minutes, then followed for one hour, with testing being done every 20 minutes. This was followed by a reversal with naloxone (same route and test time).

For the set of pharmacological experiments using the different doses of the NK-1 agonist Sar-SP alone and the NMDA agonist NMDA alone, the three consecutive stable controls were followed by the intrathecal administration of each agonist. Each agonist was left for 10 minutes, then followed for one hour, with testing being done every 20 minutes.

For the set of pharmacological experiments using morphine and Sar-SP, the three consecutive stable controls were followed by the intrathecal administration of 1μg Sar-SP. This was left for 10 minutes before a set of electrical and natural stimuli was performed. This was followed by adding 1μg morphine, which was left for 20 minutes, then tested 3 times every 20 minutes for one hour. Finally, 50μg naloxone was added, left for 20 minutes and tested as mentioned above. All drugs were dissolved in saline.

2.6.2 Acute chemical model of nociception

Morphine (3mg/kg) and dizocilpine maleate (MK-801, 0.1mg/kg, Tocris) were administered subcutaneously. NMDA (0.3μg), [Sar9, Met $(O_2)_{11}$]-Substance P (Sar-SP, 3μg), a racemic form of 7,7-diphenyl-2-[1-imino-2(2-methoxyphenyl)-ethyl] perhydroisoindol-4-one (RP-67580, 3μg, Tocris), Dimethylsulphoxide

(DMSO, 20% in saline, Sigma) and Lidocaine (200μg, Hameln Pharmaceuticals Ltd) were all administered intrathecally. All drugs were dissolved in 0.9% saline, except for RP-67,580, which was dissolved in DMSO, but diluted in saline after that, and Lidocaine which was ready for use from the ampoule. The drugs which were diluted in saline had saline administered *via* the same route as the drug as a vehicle control, whereas RP-67,580 had intrathecal 20% DMSO as a vehicle control. NMDA was neutralised to pH 7 with 1N sodium hydroxide (NaOH, sigma). All intrathecal injections were flushed with 0.9% sterile saline (Braun), and the total volume in the catheter was 24μl for a 20cm catheter.

Each drug was initially tested alone to check the exact timepoint it reached its peak effect, and all drugs were compared to saline controls. When two drugs were tested together, they were administered so that they reached their peak action at the same time: Morphine and MK-801 reached their peak each at 30 minutes post subcutaneous injection, NMDA reached its peak at 5 minutes post intrathecal administration, Sar-SP reached its peak at 20 minutes post intrathecal administration, and RP-67,580 reached its peak at 30 minutes after intrathecal administration. Furthermore, DMSO was tested in the thermal and mechanical experiments to check if it had any effects of its own.

In the NMDA part of the study, 'NMDA treated rats' refers to the group of rats which received NMDA intrathecally only, 'morphine+NMDA treated rats' refers to the group of rats which received morphine subcutaneously, followed by intrathecal NMDA and 'MK-801+NMDA treated rats' refers to the group of rats which received MK-801 subcutaneously, followed by intrathecal NMDA. In the NK-1 part of the study, 'Sar-SP treated rats' refers to the group of rats which only received intrathecal Sar-SP, 'morphine+Sar-SP treated rats' refers to the group of rats which received subcutaneous morphine followed by intrathecal Sar-SP and 'RP-67,580+Sar-SP treated rats' refers to the group of rats which received intrathecal RP-67,580 followed by intrathecal Sar-SP. In the parts of the study where two drugs have been administered to the rats, 'timepoints' will be used to refer to the minutes post NMDA administration in the NMDA study and

post Sar-SP in the NK-1 study. When only one drug or saline alone has been administered, 'timepoint' will refer to the minutes post administration of the drug/saline.

2.6.3 Peripheral NMDA receptor study

NMDA (0.7, 3.7 and 7.3μg) and MK-801 (0.4μg) were administered intraplantar, in a volume of 50μl when each drug was administered alone. Testing for thermal hypersensitivity was done every 5 minutes, for one hour, with the three different doses of NMDA, and at 5, 30 and 60 minutes post-administration for each of NMDA and MK-801 during tactile hypersensitivity testing.

NMDA (7.3μg) and MK-801 (0.4μg) were also co-administered in a total volume of 50μl (25μl each), and testing was at 5, 30 and 60 minutes after administration, for tactile hypersensitivity testing.

Morphine (3mg/kg) was administered subcutaneously and tested at 5, 30 and 60 minutes alone and when administered 25 minutes previous to NMDA intraplantar, for tactile hypersensitivity testing.

NMDA and saline were neutralised to pH 7 with 1N sodium hydroxide. Saline vehicle controls were administered *via* the same route as each drug tested.

2.6.4 Morphine and ondansetron in neuropathic pain

Morphine was administered via the same route and using the same dose as the previous experiment. Ondansetron (10 and 20μg, Zofran, Glaxo-Smithkline) was administered intrathecally in a 10μl volume. Both drugs were also administered as a combination at the same time, but *via* the different routes, and saline was administered *via* the same routes as the drugs as a vehicle control.

When testing for thermal hypersensitivity, testing was done at 5, 15, 30, 45 and 60 minutes following the administration of each drug alone, as well as when both drugs were administered in combination.

When testing for tactile and cold hypersensitivity, testing was done at 15, 30, 45 and 60 minutes following the administration of each drug alone and in combination.

In this part of the study, an Area Under the Curve (AUC) was constructed and calculated using the GraphPad Prism 4.02 software, to compare whether an overall, global effect had occurred with each drug/drug combination used.

2.7 Statistical analysis

All data was graphed and analysed using GraphPad Prism 4.02 and SPSS v12. Unless otherwise stated, all data is presented as mean±SEM.

Continuous data was analysed using a student's *t*-test, to compare two groups. One way ANOVA, followed by a Dunnett's post-hoc test was used to compare more than two groups to a single control. One way ANOVA, followed by a Tukey post-hoc test was used to compare more than two groups of treatment over a time course in the behavioural experiments, or over numerous temperatures or von Frey strengths in the in vivo electrophysiology experiments. Non-continuous data was analysed using a Mann-Whitney test when comparing two groups.

Chapter 3:

A study investigating the effects of morphine, [Sar^9 Met $(O_2)^{11}$]-Substance P and NMDA on rat deep wide dynamic range dorsal horn neurons, using in vivo electrophysiology

3. A study investigating the effects of morphine, [Sar^9 Met $(O_2)^{11}$]-Substance P and NMDA on rat deep wide dynamic range dorsal horn neurons, using in vivo electrophysiology

3.1 Introduction

The opioid system is one of the most important inhibitory ones in the central nervous system. Morphine, acting on the μ-opioid receptor, exerts its inhibitory action along three important sites: the central terminals of the primary afferent fibres, the spinal cord dorsal horn and on supraspinal sites such as the PAG and the RVM (Dickenson, 1994) (see section **1.7.6.2**).

At the spinal cord level, it has been shown that the number of presynaptic μ-opioid receptors exceeds that of the postsynaptic ones (Besse *et al.*, 1992; Besse *et al.*, 1990; Lombard *et al.*, 1989; Mansour *et al.*, 1994). Additionally, these μ-opioid receptors have been found to be located on mostly small- (C) and medium-diameter (Aδ) peptidergic primary afferent fibres (deGroot *et al.*, 1997; Zhang *et al.*, 1998). During in vivo electrophysiology, the electrical response evoked by large-diameter Aβ-fibres, as well as brush, the natural response evoked conveyed by these fibres, remains unchanged following morphine administration. This has been concluded to be due to the lack of μ-opioid receptors on Aβ-fibres (deGroot *et al.*, 1997; Zhang *et al.*, 1998), which means that morphine has to act mostly on its postsynaptic receptors, which are less than the presynaptic ones, and therefore, larger doses of morphine may be needed to inhibit this response (Besse *et al.*, 1992; Besse *et al.*, 1990; Dickenson, 1997b; Lamotte *et al.*, 1976; Mansour *et al.*, 1994).

Therefore, morphine was postulated to be less effective in inhibiting responses once its action is mostly limited to its postsynaptic receptors (Dickenson, 1997b). This can be also seen with the decreased efficacy of low doses of morphine on the electrically evoked wind-up response of dorsal horn neurons (Chapman *et al.*, 1992; Chapman *et al.*, 1994b; Dickenson *et al.*, 1986). Wind-up, as mentioned

previously, is dependent on the activation of the NMDA receptor, which is located mostly postsynaptic on the spinal cord dorsal horn neurons (Coggeshall *et al.*, 1997a; Liu *et al.*, 1994) and whose activation depends on non-NMDA depolarisations following the activation of both the AMPA and the NK-1 receptors (Salt, 1986; Urban *et al.*, 1984; Xu *et al.*, 1992). The NK-1 receptor is also located mostly on the postsynaptic dorsal horn neurons, although along with the NMDA receptor, both receptors have been shown by some studies to be located presynaptically as well (Coggeshall *et al.*, 1997a; Liu *et al.*, 1994; Malcangio *et al.*, 1999; Todd, 2002).

The intrathecal administration of each of SP (or other NK-1 agonists) and NMDA has been shown to cause the excitation of the responses of dorsal horn neurons (Bentley *et al.*, 1995; Chapman *et al.*, 1994a; Henry, 1976; Randic *et al.*, 1977), as well as behaviour indicative of sensation, such as biting, scratching and licking (BSL) (Aanonsen *et al.*, 1987; Seybold *et al.*, 1982).

Therefore, the aim of this part of my study was to establish how effective intrathecal morphine administration would be on the dorsal horn neuronal responses elicited, once the balance of the spinal cord pharmacology was shifted towards postsynaptic excitation, using each of the potent and selective NK-1 and NMDA receptor agonists Sar-SP and NMDA, to activate their respective receptors, and thus once morphine has to act predominantly *via* its postsynaptic receptors.

3.2 Methods

In this part of my study, the animals and the method which will be used are the same as those described in sections **2.1** and **2.2**.

Briefly, a tracheotomy was performed on an anaesthetised male, Sprague-Dawley rat, weighing between 200-250g, and this was followed by a laminectomy which exposed the L1-L3 vertebral levels. A tungsten electrode

was then used to record extracellularly from deep wide dynamic range dorsal horn neurons (Urch *et al.*, 2003a).

After three consecutive stable controls, where the evoked responses to electrical and natural stimuli were not different by more than 10%, each drug was applied intrathecally in a volume of 50μl, as the protocol described in section **2.6.1**. The three doses of morphine, 0.1, 1 and 10μg, and the three doses of NMDA, 5, 50 and 500ng, were administered cumulatively, on the same day and animal. However, the two doses of Sar-SP, 1 and 10μg, were administered separately, on different experimental days.

In the part of my study investigating morphine on its own, morphine was left for twenty minutes following its administration, after which, the testing began every twenty minutes for one hour. This protocol was used since it has been shown in previous studies that peak morphine action occurred between 20 to 40 minutes after administration (Suzuki *et al.*, 1999).

In the part of my study investigating Sar-SP and NMDA each on its own, each agent was left for 10 minutes before testing began, after which testing was done every 20 minutes for one hour. This dosing protocol was loosely based on the study by Chapman *et al*, however, whereas their study tested only electrical parameters every 10 minutes, I had to extend the testing to every 20 minutes, since I was testing both electrical and natural evoked responses (Chapman *et al.*, 1994a).

In the part of my study investigating the effects of morphine (1μg) on the responses of deep WDR neurons following the activation of the NK-1 receptor, Sar-SP (1μg) was left for 10 minutes, following which, the aforementioned electrical and natural evoked parameters were tested, before the addition of morphine. Morphine (1μg) was left on the spinal cord for 20 minutes, and then the electrical and natural evoked responses were tested every 20 minutes for one hour. Since the largest changes from baseline following each of Sar-SP and

morphine administration occurred between 20 and 40 minutes (Rygh *et al.*, 2006), I felt the two timepoints of administering Sar-SP and morphine were justified.

3.3 Results

3.3.1 Characterisations of deep, wide dynamic range and nociceptive-specific neurons

A total of 75 cells were used to characterise deep wide dynamic range (WDR) dorsal horn neurons. The way these 75 cells coded to the mechanical von Frey and thermal heat stimuli categorises them as wide dynamic range cells (figs. 3.1 and 3.2) (Menetrey *et al.*, 1977; Urch *et al.*, 2003b). All cells used in my study were deep, wide dynamic range (WDR) dorsal horn neurons.

	mean±SEM
cell depth (μm)	830±29
Aβ-fibre threshold (mA)	0.8±0.02
C-fibre threshold (mA)	2±0.1
input evoked response	26±2
Aβ-fibre evoked response	90±5
Aδ-fibre evoked response	80±5
C-fibre evoked response	309±18
post-discharge response	168±21
wind-up evoked response	143±29
brush evoked response	234±26

Table 3.1. Electrophysiological characteristics of the electrical and brush evoked responses in rat deep wide dynamic range dorsal horn neurons. Response= number of action potentials evoked, n=75.

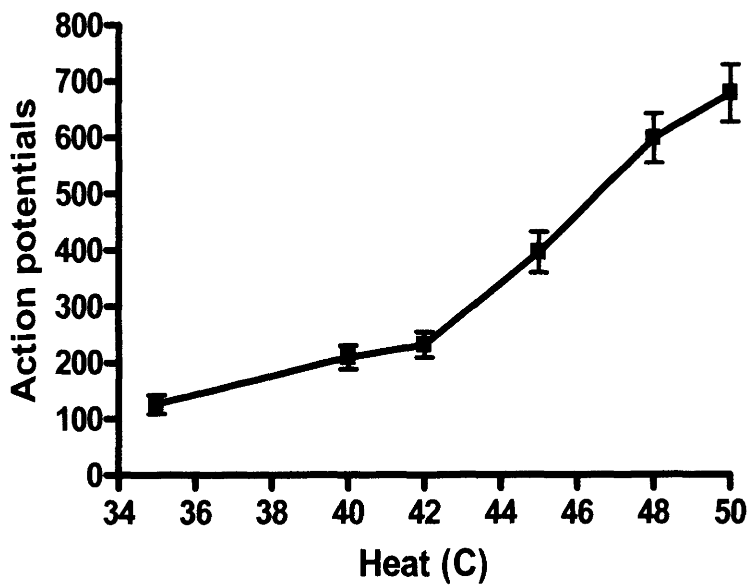


Fig. 3.1- Heat evoked responses of wide dynamic range neurons in the deep dorsal horn (n=75).

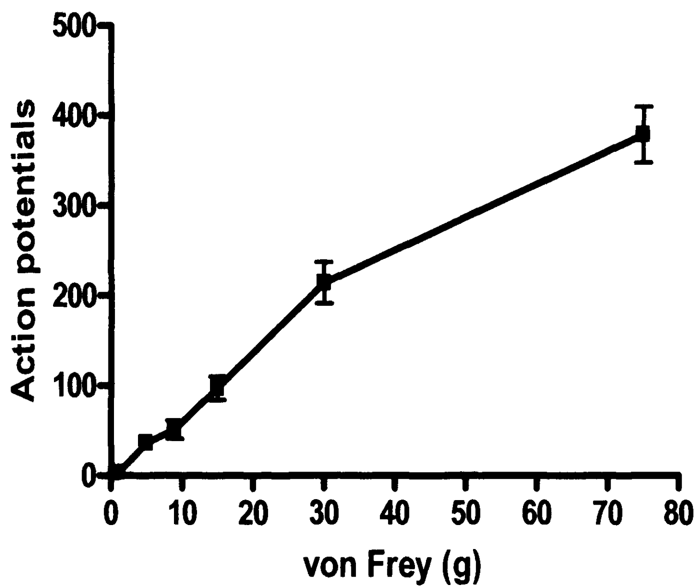
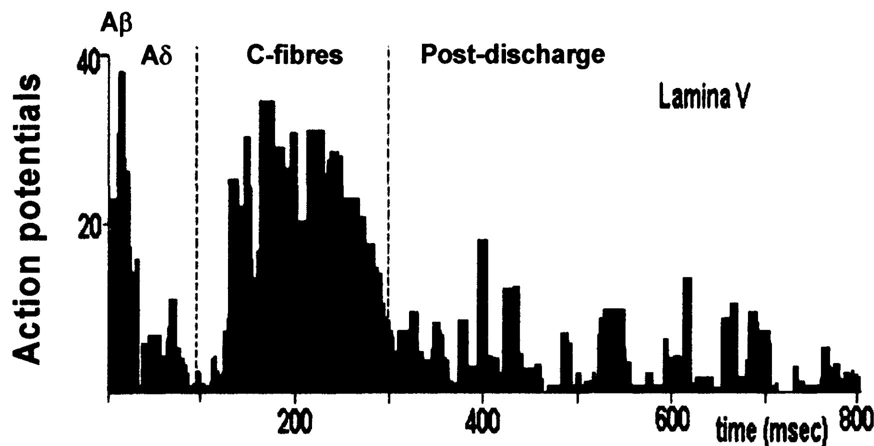


Fig. 3.2- von Frey evoked responses of wide dynamic range neurons in the deep dorsal horn (n=75).



Trace 1- A post-stimulus time histogram (PSTH) showing the evoked Aβ- (0-20msec), Aδ- (20-90msec), C-fibres (90-300msec) and post-discharge (300-800msec) responses of a deep WDR dorsal horn neurons following a train of 16 electrical stimuli. msec- latency in milliseconds. Adapted with permission from Dr. R. Suzuki.

A total of 33 'atypical' deep cells were also characterised. The electrical evoked responses of the deep WDR and 'atypical' neurons were comparable, however, the natural evoked responses of the deep WDR neurons were larger than those of deep 'atypical' neurons (tables 3.1, 3.2 and figs. 3.1-3.4).

	mean±SEM
cell depth (μm)	790±92
Aβ-fibre threshold (mA)	0.9±0.04
C-fibre threshold (mA)	2.2±0.1
input evoked response	19±3
Aβ-fibre evoked response	95±10
Aδ-fibre evoked response	60±9
C-fibre evoked response	326±29
post-discharge response	205±29
wind-up evoked response	264±40
Brush evoked response	59±10

Table 3.2- Electrophysiological characteristics of the electrical and brush evoked responses in rat deep 'atypical' dorsal horn neurons. Response= number of action potentials evoked, n=33.

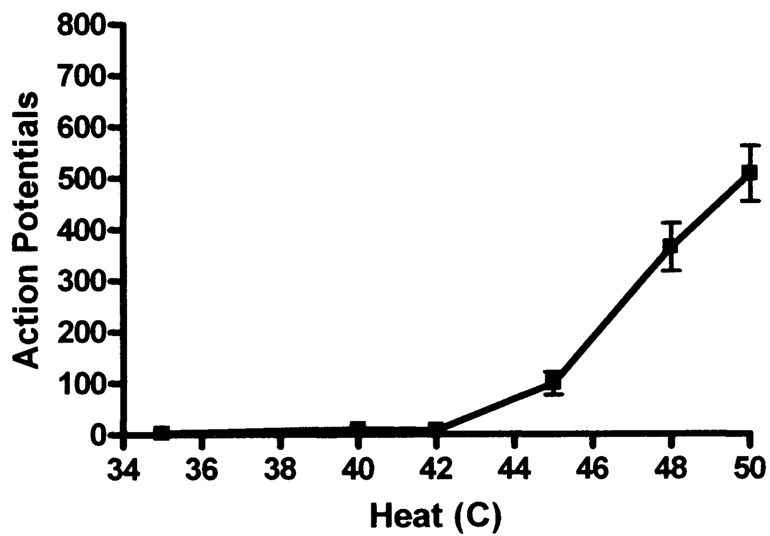


Fig. 3.3- Heat evoked responses of 'atypical' neurons in the deep dorsal horn (n=33).

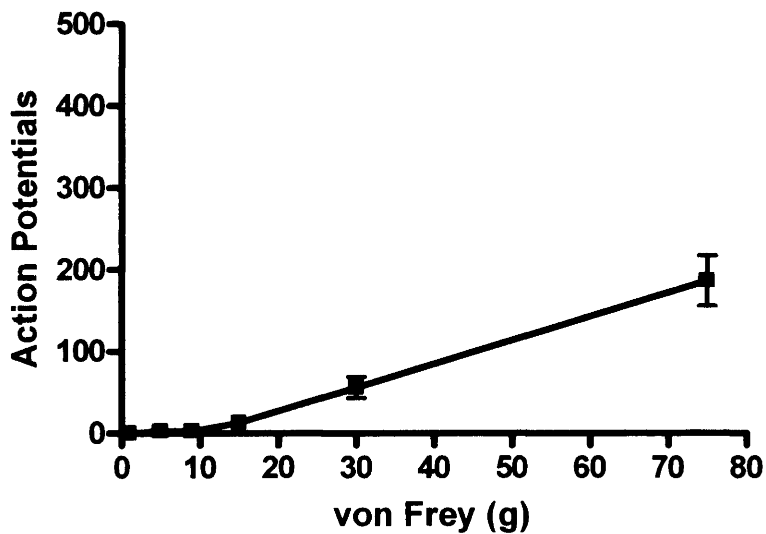


Fig. 3.4- von Frey evoked responses of 'atypical' neurons in the deep dorsal horn (n=33).

3.3.2 The effect of intrathecal morphine on the evoked responses of deep WDR neurons

	mean±SEM
cell depth (μm)	831±40
Aβ-fibre threshold (mA)	0.8±0.03
C-fibre threshold (mA)	1.4±0.2
No. of cells with wind-up	4 out of 8

Table 3.3- Electrophysiological characteristics of the rat deep wide dynamic range dorsal horn neurons.

The intrathecal administration of morphine dose 0.1μg did not have an effect on any of the electrically or naturally evoked responses of deep WDR neurons (figs. 3.5-3.14). Furthermore, any significant inhibitory effect of the other doses of morphine used was reversed by intrathecal 50μg naloxone (n=7).

i) Electrical parameters

Electrically evoked input response was significantly inhibited by both 1 and 10 μg of intrathecal morphine ($p<0.05$, n=8) (fig. 3.5).

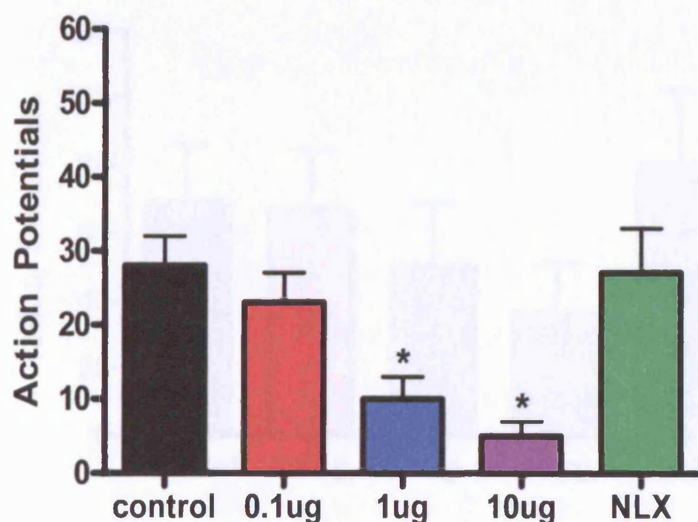


Fig. 3.5- The effect of intrathecal morphine doses 0.1 (red), 1 (blue) and 10μg (purple) on the electrical evoked input response in deep WDR dorsal horn neurons. Reversal by naloxone dose 50 μg is shown in green. *$P<0.05$, where a significant inhibition from the control (black) is seen. n=8 for control and morphine, n=7 for naloxone reversal, one way ANOVA followed by Dunnett's post-hoc test.

Neither the electrically evoked Aβ- nor the Aδ responses were significantly inhibited by any of the doses of intrathecal morphine (n=8) (figs. 3.6 and 3.7).

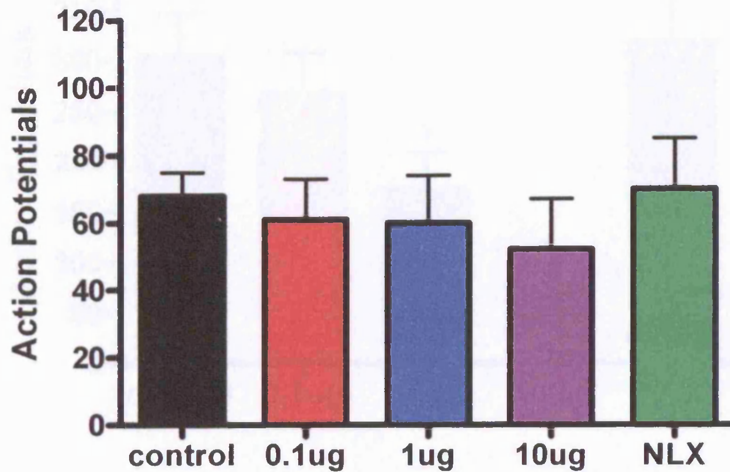


Fig. 3.6- The lack of effect of intrathecal morphine doses 0.1 (red), 1 (blue) and 10μg (purple) on the electrical evoked Aβ-fibre response in deep WDR dorsal horn neurons. Reversal by naloxone dose 50 μg is shown in green. n=8 for control and morphine, n=7 for naloxone.

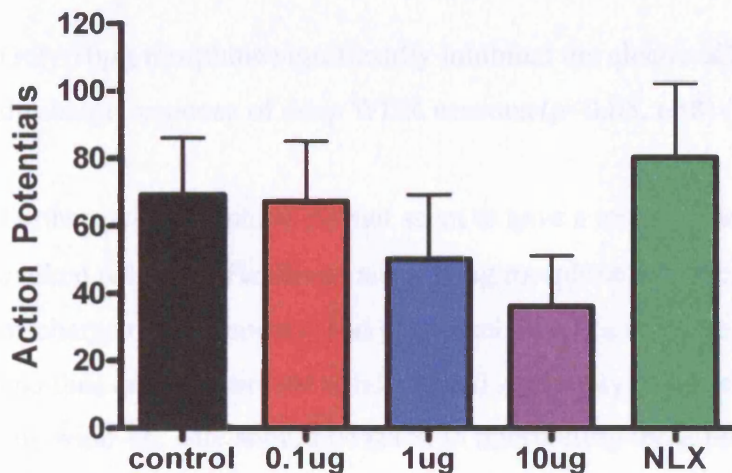


Fig. 3.7- The lack of effect of intrathecal morphine doses 0.1 (red), 1 (blue) and 10μg (purple) on the electrical evoked Aδ-fibre response in deep WDR dorsal horn neurons. Reversal by naloxone dose 50 μg is shown in green. n=8 for control and morphine, n=7 for naloxone reversal.

The C-fibre evoked response was significantly reduced by morphine doses 1 and 10μg ($p<0.05$, n=8) (fig. 3.8).

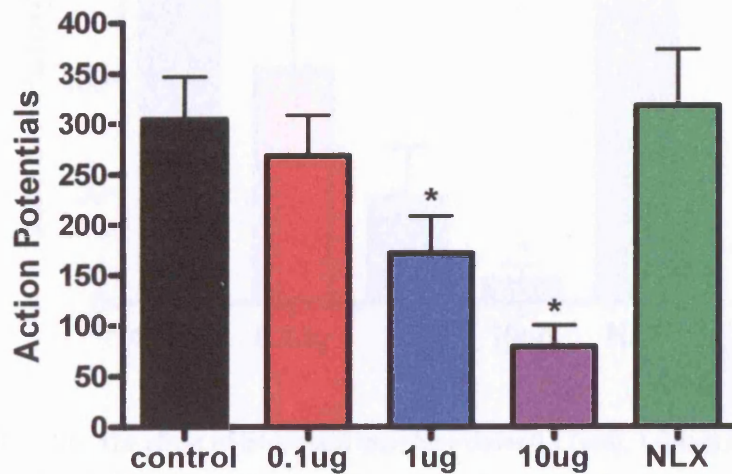


Fig. 3.8- The effect of intrathecal morphine doses 0.1 (red), 1 (blue) and 10μg (purple) on the electrical evoked C-fibre response in deep WDR dorsal horn neurons. Reversal by naloxone dose 50 μg is shown in green. *$P<0.05$, where a significant inhibition from the control (black) is seen. n=8 for control and morphine, n=7 for naloxone reversal, one way ANOVA followed by Dunnett's post-hoc test.

Only 10μg morphine significantly inhibited the electrically evoked post-discharge response of deep WDR neurons ($p<0.05$, n=8) (fig. 3.9).

Furthermore, morphine did not seem to have a tendency to inhibit the wind-up evoked response. However, since 10μg morphine significantly inhibited the post-discharge of the neurons, and post-discharge is a measure of neuronal excitability and thus an indication of wind-up, and since only 4 out of the 8 cells exhibited any wind-up, care should be taken in interpreting these results, as an 'n' number of at least 6 is needed before valid analysis is made (fig. 3.10). However, even when the wind-up of a single cell was plotted (fig. 3.11), it was seen that morphine had no tendency to change the slope of the wind-up response (n=1).

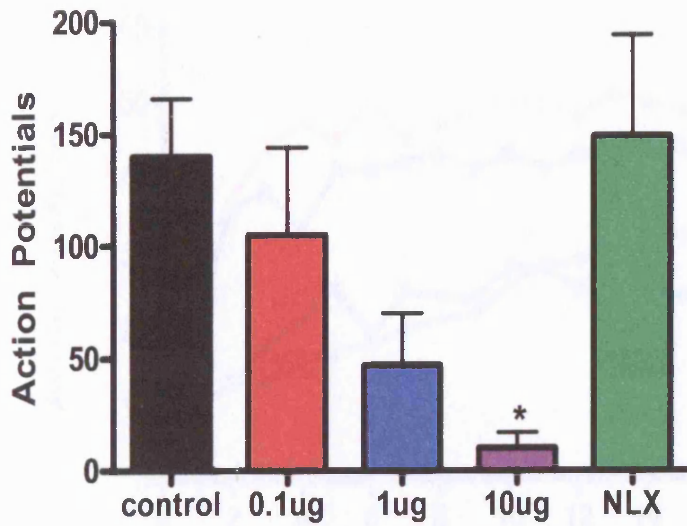


Fig. 3.9- The effect of intrathecal morphine doses 0.1 (red), 1 (blue) and 10μg (purple) on the electrical evoked post-discharge response in deep WDR dorsal horn neurons. Reversal by naloxone dose 50 μg is shown in green. *P<0.05, where a significant inhibition from the control (black) is seen. n=8 for control and morphine, n=7 for naloxone reversal, one way ANOVA followed by Dunnett's post-hoc test.

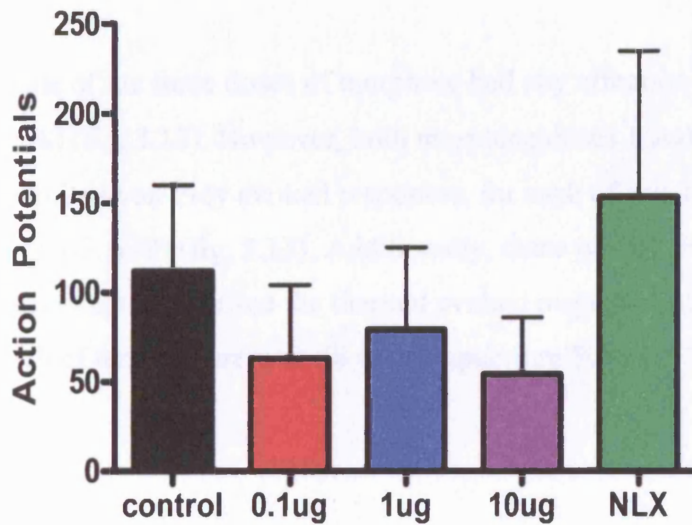


Fig. 3.10- The lack of effect of intrathecal morphine doses 0.1 (red), 1 (blue) and 10μg (purple) on the electrical evoked wind-up response in deep WDR dorsal horn neurons. Reversal by naloxone dose 50 μg is shown in green. n=4 for all.

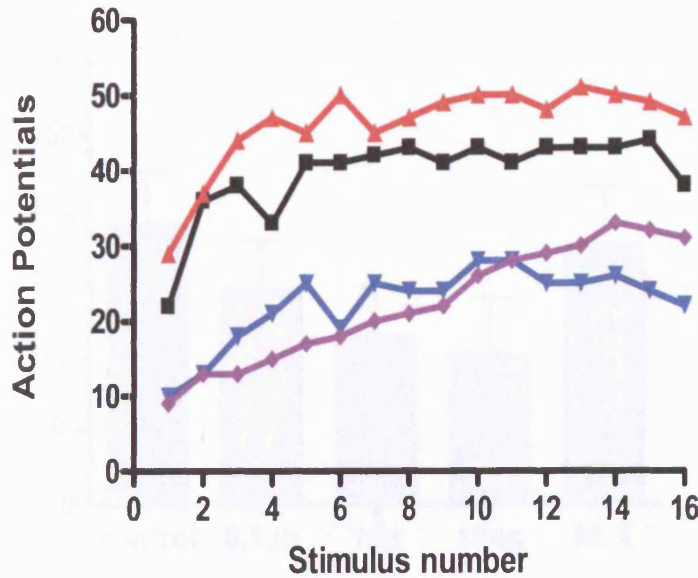


Fig. 3.11- The lack of effect of intrathecal morphine doses 0.1 (red), 1 (blue) and 10μg (purple) on the slope of electrical evoked wind-up response of a single, deep, WDR dorsal horn neuron (black). n=1.

ii) Natural parameters

None of the three doses of morphine had any effect on the brush evoked response (n=8) (fig. 3.12). However, both morphine doses 1 and 10μg significantly inhibited von Frey evoked responses, for each of von Frey 5, 15, 30 and 75g ($p<0.05$, n=8) (fig. 3.13). Additionally, these two doses of morphine also significantly inhibited the thermal evoked responses of deep WDR neurons, at each of temperatures 45, 48 and temperature 50°C ($p<0.05$, n=8) (fig. 3.14).

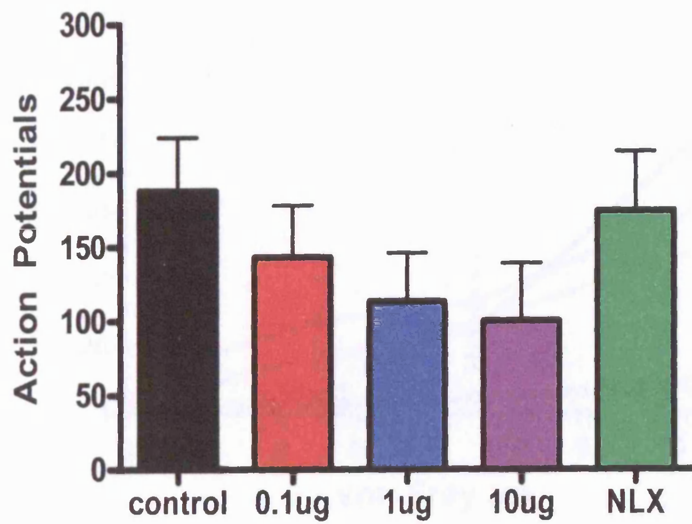


Fig. 3.12- The lack of effect of intrathecal morphine doses 0.1 (red), 1 (blue) and 10μg (purple) on the brush evoked response in deep WDR dorsal horn neurons. Reversal by naloxone dose 50 μg is shown in green. n=8 for control and morphine, n=7 for naloxone reversal.

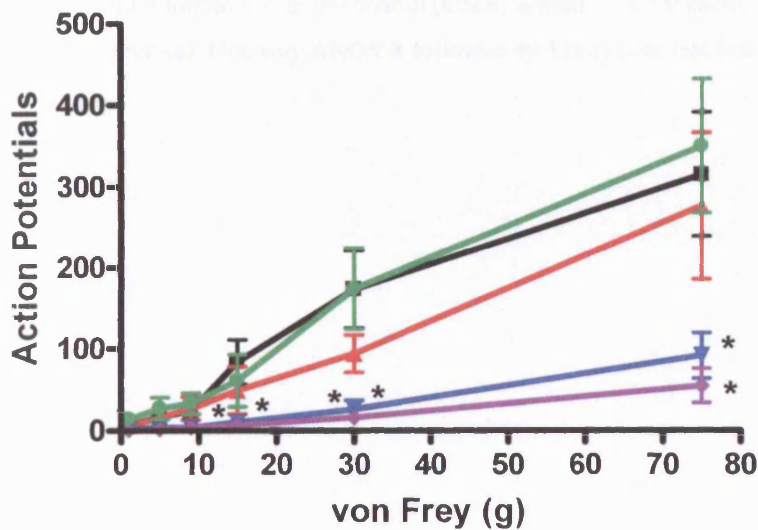


Fig. 3.13a- The effect of intrathecal morphine doses 0.1 (red), 1 (blue) and 10μg (purple) on the von Frey evoked response in deep WDR dorsal horn neurons. Reversal by naloxone dose 50 μg is shown in green. *P<0.05, where a significant inhibition from the control (black) is seen. n=8 for control and morphine, n=7 for naloxone reversal. One way ANOVA followed by Tukey post-hoc test.

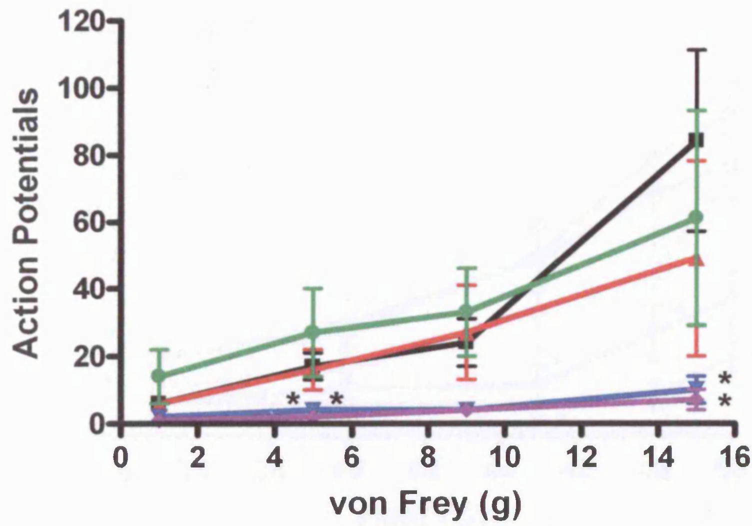


Fig. 3.13b- The effect of the three doses of intrathecal morphine (0.1μg- red, 1μg- blue, 10μg- purple) on the 1, 5, 9 and 15g von Frey evoked responses in deep wide dynamic range dorsal horn neurons in closer detail. Reversal by naloxone dose 50μg is shown in green. *P<0.05, where a significant inhibition from the control (black) is seen. n=8 for control and morphine, n=7 for naloxone reversal. One way ANOVA followed by Tukey post-hoc test.

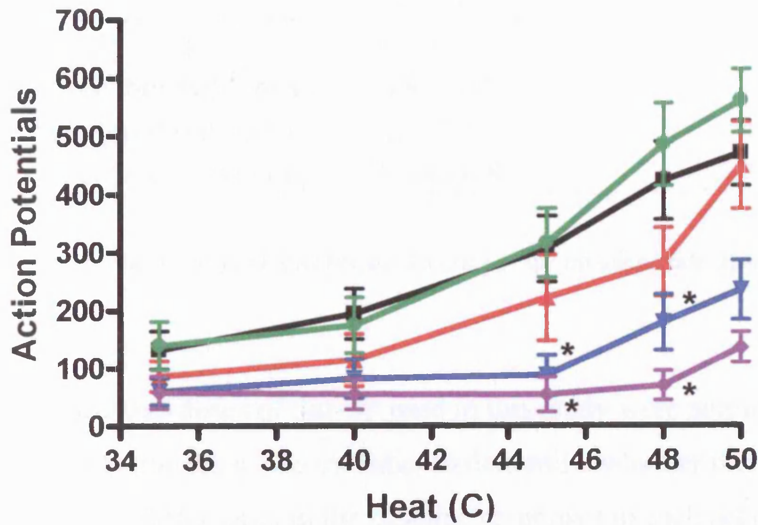


Fig. 3.14- The effect of intrathecal morphine doses 0.1 (red), 1 (blue) and 10μg (purple) on the heat evoked response in deep WDR dorsal horn neurons. Reversal by naloxone dose 50 μg is shown in green. *$P<0.05$, where a significant inhibition from the control (black) is seen. n=8 for control and morphine, n=7 for naloxone reversal. One way ANOVA followed by Tukey post-hoc test.

3.3.3 The effect of intrathecal Sar-SP on the evoked responses of deep WDR neurons

	Mean±SEM
cell depth (μm)	680±58
Aβ-fibre threshold (mA)	0.7±0.01
C-fibre threshold (mA)	1.7±0.1
no. of cells with wind-up	6 out of 6

Table 3.4- Electrophysiological characteristics of the rat deep wide dynamic range dorsal horn neurons.

	Mean±SEM
cell depth (μm)	856±56
Aβ-fibre threshold (mA)	0.9±0.07
C-fibre threshold (mA)	2.5±0.3
no. of cells with wind-up	8 out of 8

Table 3.5- Electrophysiological characteristics of the rat deep wide dynamic range dorsal horn neurons.

Since the two doses of Sar-SP used in this study were administered on different days and animals, it was essential to determine whether there were significant differences in the baseline responses in each set of animals used. As can be seen from figures 3.15-3.18, there were no significant differences in the baseline responses between both sets of animals.

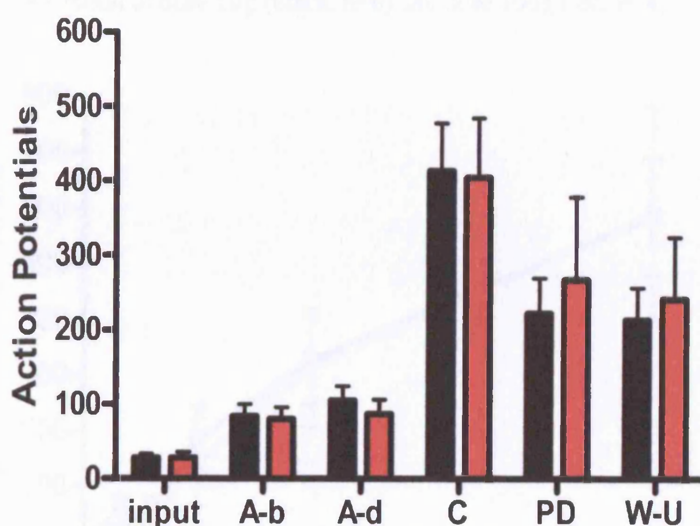


Fig. 3.15- A comparison of the baseline electrical evoked responses of the deep WDR dorsal horn neurons used in the study investigating the different doses of Sar-SP, prior to the administration of dose 1μg (black, n=6) and dose 10μg (red, n=8).

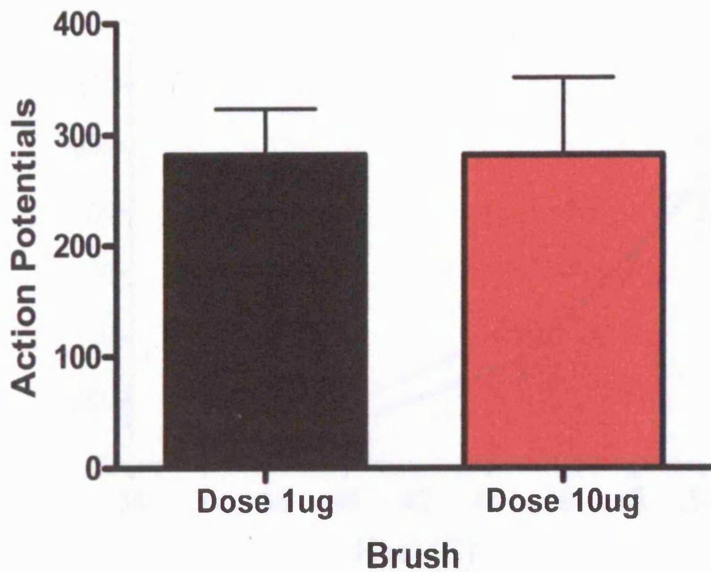


Fig. 3.16- A comparison of the baseline brush evoked response of the deep WDR dorsal horn neurons used in the study investigating the different doses of Sar-SP, prior to the administration of dose 1μg (black, n=6) and dose 10μg (red, n=8).

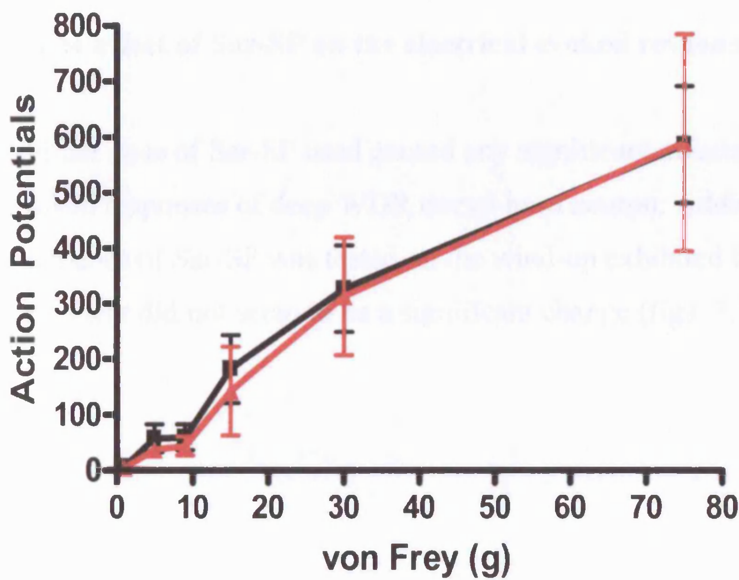


Fig. 3.17- A comparison of the baseline von Frey evoked responses of the deep WDR dorsal horn neurons used in the study investigating the different doses of Sar-SP, prior to the administration of dose 1μg (black, n=6) and dose 10μg (red, n=8).

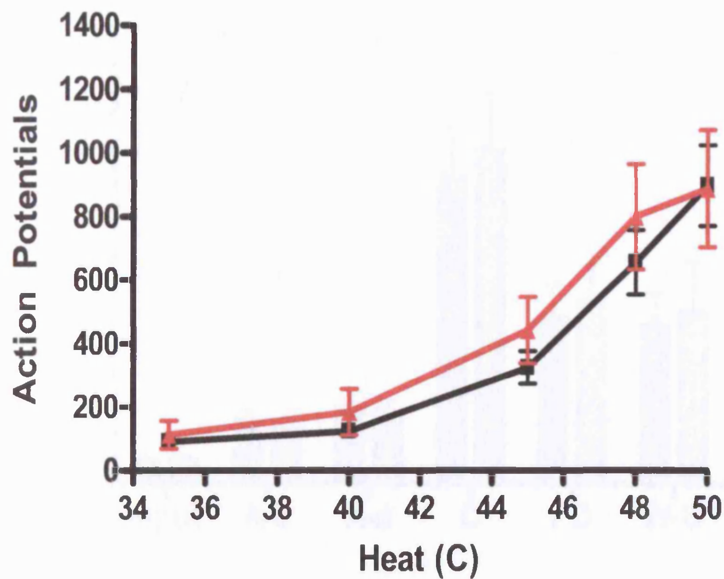


Fig. 3.18- A comparison of the baseline thermal evoked responses of the deep WDR dorsal horn neurons used in the study investigating different doses of Sar-SP, prior to the administration of dose 1μg (black, n=6) and dose 10μg (red, n=8).

i) The effect of Sar-SP on the electrical evoked responses

Neither dose of Sar-SP used caused any significant effects on the electrical evoked responses of deep WDR dorsal horn neuron. Additionally, even when each dose of Sar-SP was tested on the wind-up exhibited by its respective single cell, there did not seem to be a significant change (figs. 3.19-3.22).

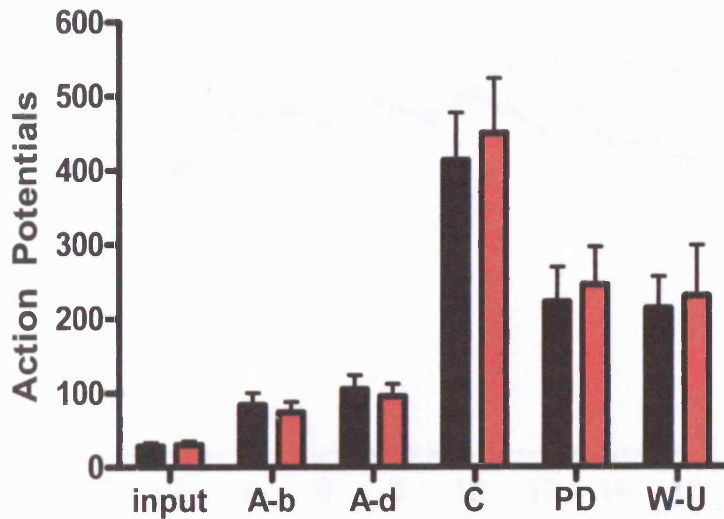


Fig. 3.19- The lack of effect of intrathecal 1μg Sar-SP (red) on electrical evoked responses of deep WDR dorsal horn neurons. Control responses are shown in black. n=6.

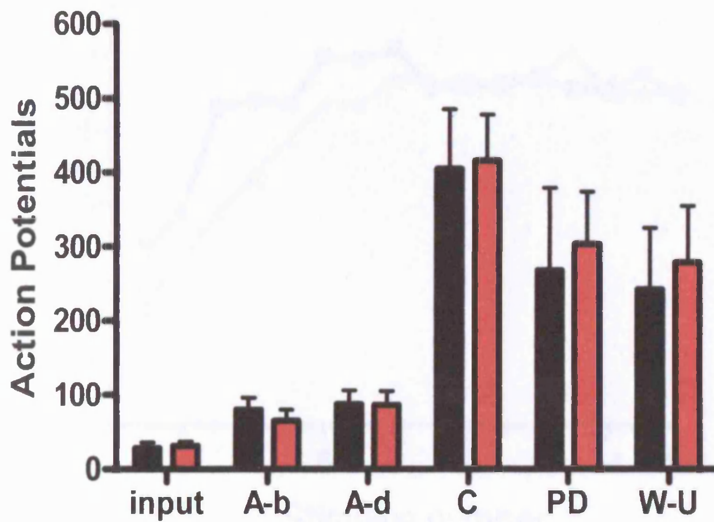


Fig. 3.20- The lack of effect of intrathecal 10μg Sar-SP (red) on electrical evoked responses of deep WDR dorsal horn neurons. Control responses are shown in black. n=8.

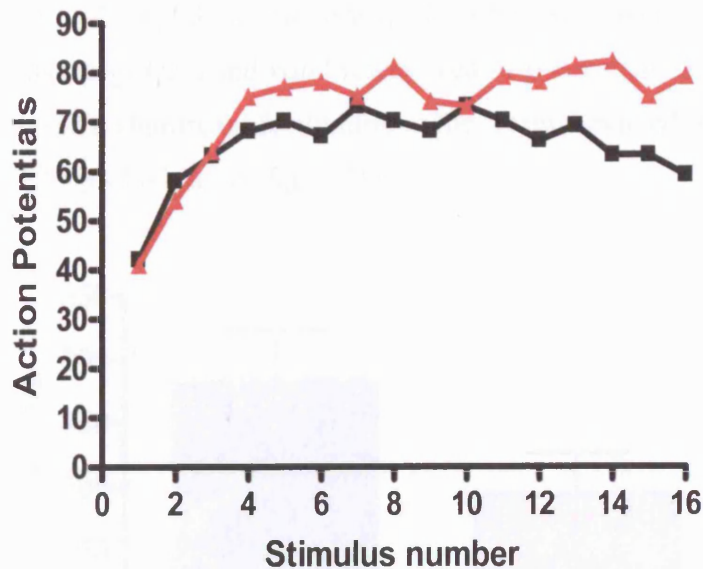


Fig. 3.21- The lack of effect of intrathecal 1μg Sar-SP (red) on the slope of the electrical evoked wind-up response of a single, deep, WDR dorsal horn neuron (black). n=1.

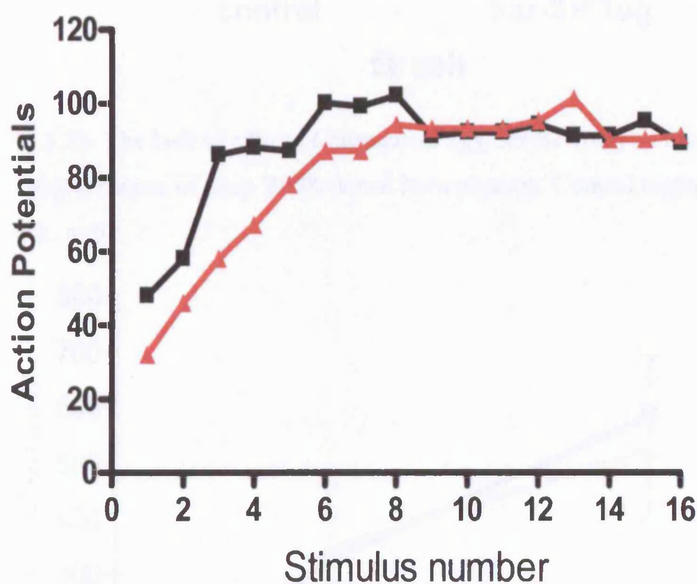


Fig. 3.22- The lack of effect of intrathecal 10μg Sar-SP (red) on the slope pf the electrical evoked wind-up response of a single, deep, WDR dorsal horn neuron (black). n=1.

ii) The effect of Sar-SP on the natural evoked responses

The intrathecal administration of Sar-SP dose 10μg did not cause a significant difference in any of the brush, von Frey and thermal evoked responses (see figs.

3.26, 3.27 and 3.28). However, whilst Sar-SP dose 1μg had no effect on the brush (fig. 3.23) and von Frey evoked responses (fig. 3.24), this dose of Sar-SP caused a significant facilitation in the thermal evoked response at temperature 45°C ($p<0.05$, n=6) (fig. 3.25).

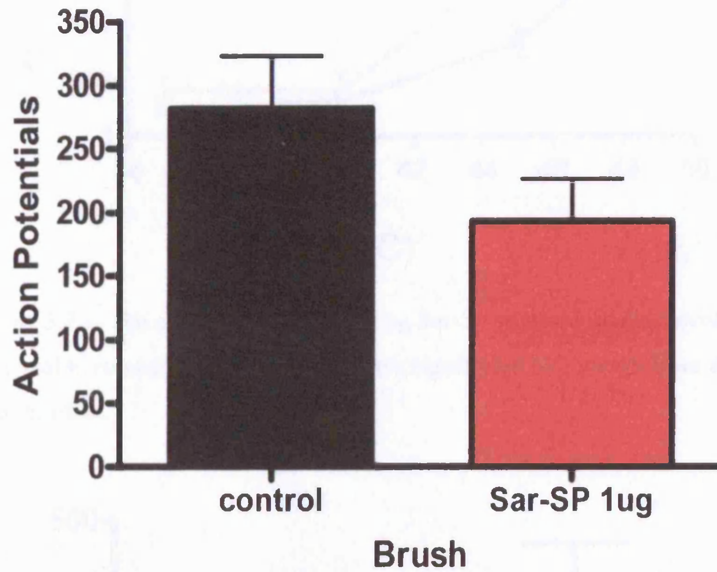


Fig. 3.23- The lack of effect of intrathecal 1μg Sar-SP (red) on brush evoked response of deep WDR dorsal horn neurons. Control responses are shown in black. n=6.

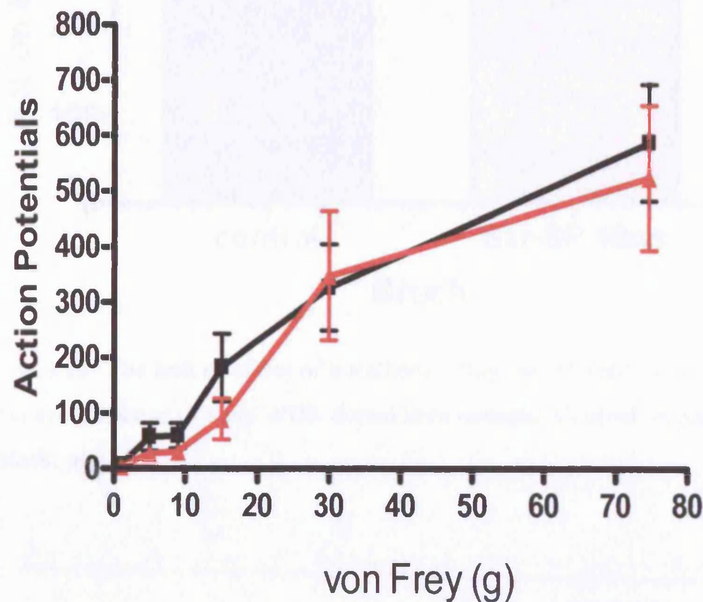


Fig. 3.24- The lack of effect of intrathecal 1μg Sar-SP (red) on von Frey evoked responses of deep WDR dorsal horn neurons. Control responses are shown in black. n=6.

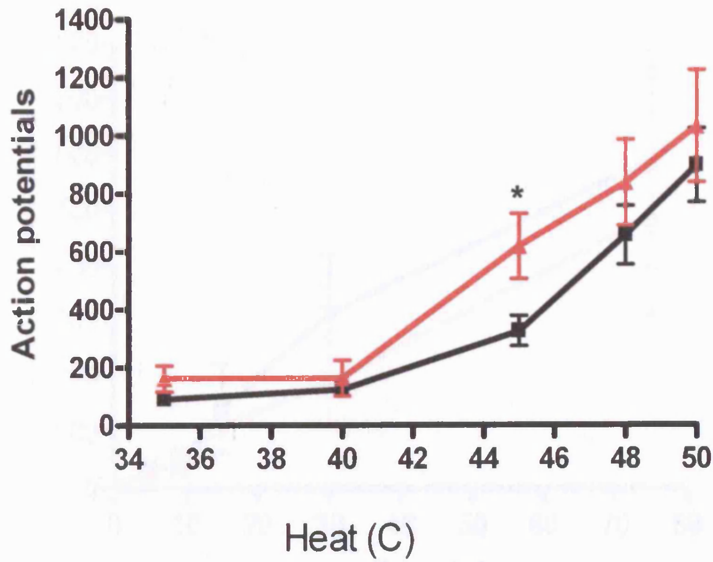


Fig. 3.25- The effect of intrathecal 1μg Sar-SP (red) on thermal evoked responses of deep WDR dorsal horn neurons. *P<0.05, where a significant facilitation from the control (black) is seen. n=6, *t*-test.

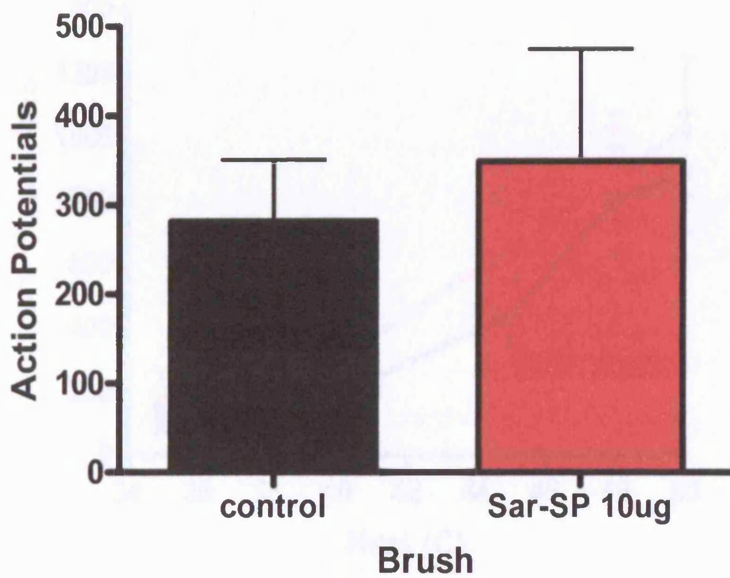


Fig. 3.26- The lack of effect of intrathecal 10μg Sar-SP (red) on brush evoked response of deep WDR dorsal horn neurons. Control responses are shown in black. n=8.

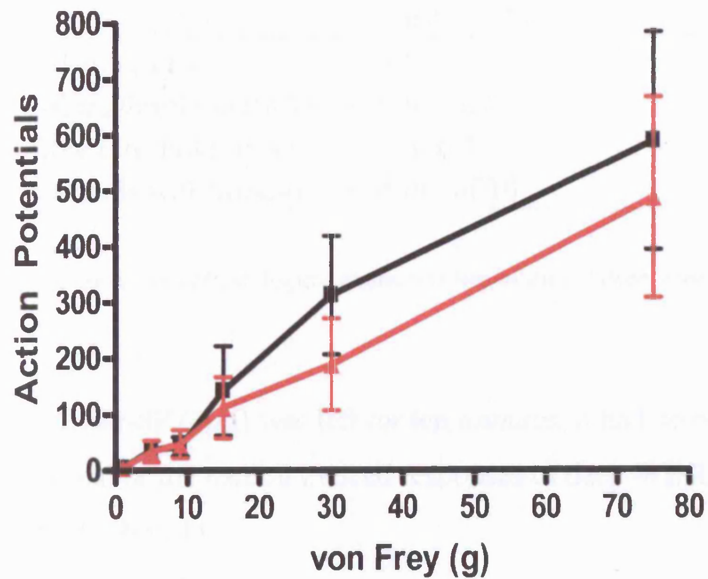


Fig. 3.27- The lack of effect of intrathecal 10μg Sar-SP (red) on von Frey evoked responses of deep WDR dorsal horn neurons. Control responses are shown in black. n=8.

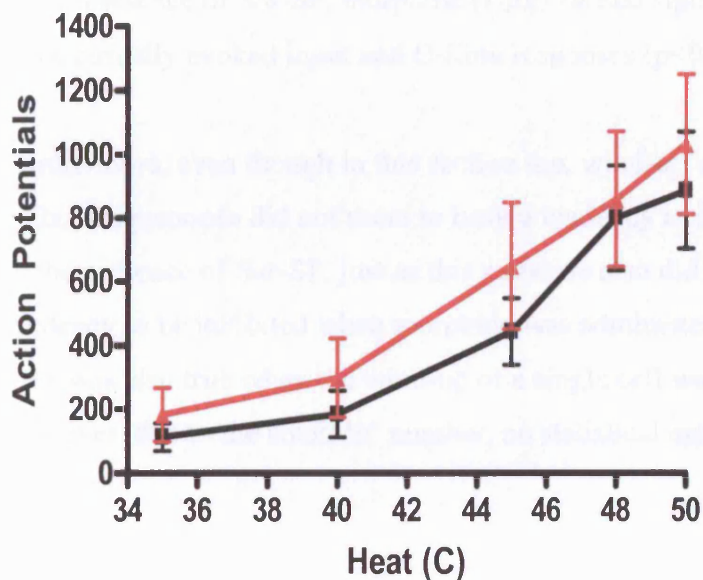


Fig. 3.28- The lack of effect of intrathecal 10μg Sar-SP (red) on thermal evoked responses of deep WDR dorsal horn neurons. Control responses are shown in black. n=8.

3.3.4 The effects of Sar-SP on morphine's inhibitory action of the evoked responses of deep WDR neurons

	mean±SEM
cell depth (μm)	822±50
Aβ-fibre threshold (mA)	0.9±0.07
C-fibre threshold (mA)	1.3±0.2
no. of cells with wind-up	5 out of 10

Table 3.6- Electrophysiological characteristics of the rat deep wide dynamic range dorsal horn neurons.

When Sar-SP (1μg) was left for ten minutes, it had no effect on any of the electrical or the natural evoked responses of deep WDR dorsal horn neurons (figs. 3.29-3.31).

i) Effects on the electrical evoked response of deep WDR neurons

In the presence of Sar-SP, morphine (1 μg) caused significant inhibition on each of electrically evoked input and C-fibre responses ($p<0.05$, $n=10$) (fig. 3.29).

Furthermore, even though in this section too, wind-up was exhibited by only 5 cells, this response did not seem to have a tendency to be inhibited by morphine, in the presence of Sar-SP, just as this response also did not seem to have a tendency to be inhibited when morphine was administered on its own (fig. 3.10). This was also true when the wind-up of a single cell was plotted (fig. 3.30). However, due to the small 'n' number, no statistical analysis could be made.

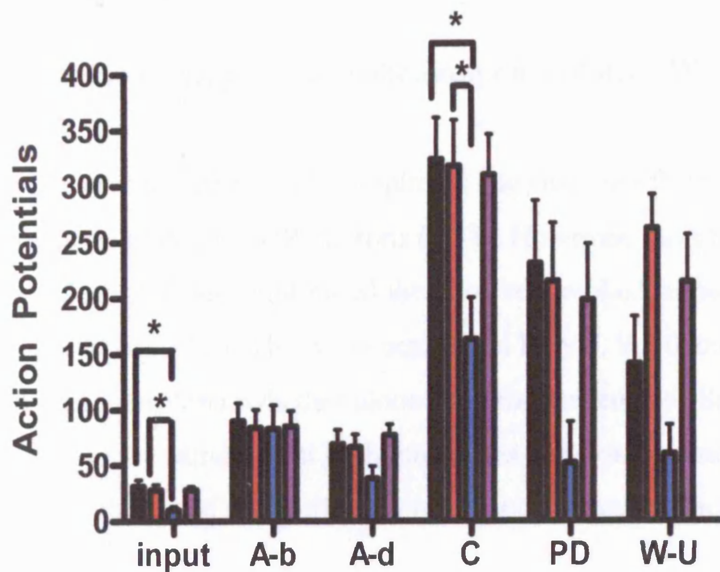


Fig. 3.29- The effect of intrathecal morphine (1 µg, blue), when preceded by intrathecal Sar-SP (1 µg, red), on electrical evoked responses of deep WDR dorsal horn neurons. Reversal by naloxone dose 50 µg is shown in purple. *P<0.05, where morphine induces a significant inhibition from the control (black) and following intrathecal Sar-SP administration (red). n=10 for all except for wind-up evoked response, where n=5, and naloxone reversal, where n=9, one way ANOVA followed by Dunnett's post-hoc test.

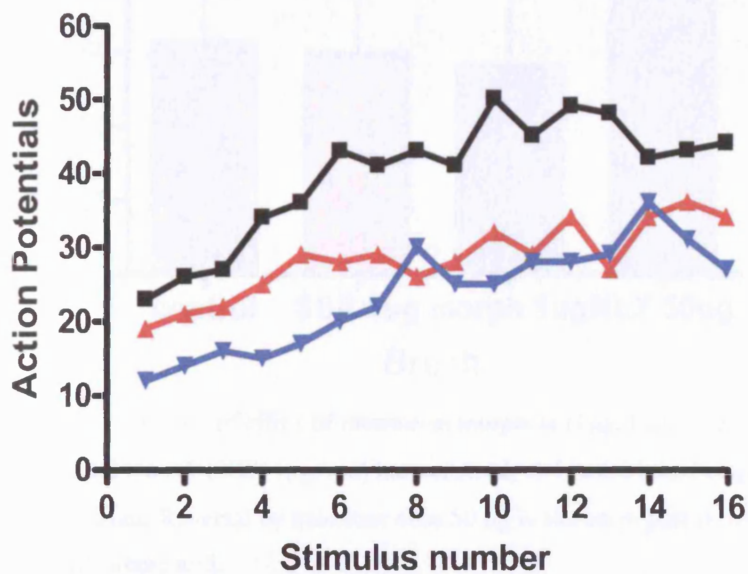


Fig. 3.30- The lack of effect of intrathecal 1 µg morphine (blue), when preceded by intrathecal 1 µg Sar-SP (red), on the slope of the electrical evoked wind-up response of a single, deep, WDR dorsal horn neuron (control=black). n=1.

ii) Effects on the natural evoked response of deep WDR neurons

In the presence of Sar-SP, morphine (1 μg) had no effect on brush evoked responses of deep WDR neurons (3.31). However, morphine, in the presence of Sar-SP, significantly inhibited the von Frey evoked response of deep WDR neurons (fig. 3.32). This was seen at von Frey 5, 9, 30 and 75g ($p<0.05$, n=9). Overall, morphine, whether alone or in the presence of Sar-SP, was shown to inhibit to the same extent both innocuous and noxious mechanical stimuli, as seen by the broad range of von Frey monofilaments which were inhibited.

Furthermore, morphine, in the presence of Sar-SP, significantly inhibited the thermal evoked response of deep WDR dorsal horn neurons (fig. 3.33). This significant inhibition was seen at temperatures 45, 48 and 50°C ($p<0.05$, n=10).

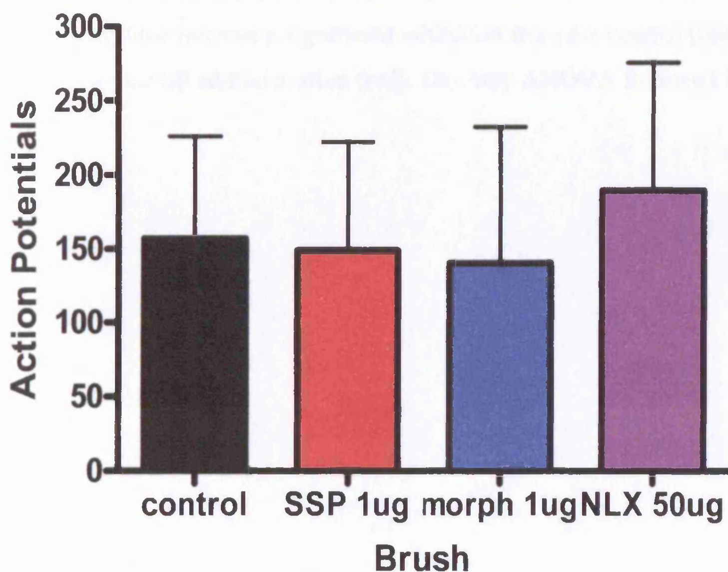


Fig. 3.31- The lack of effect of intrathecal morphine (1 μg, blue), when pre-treatment with intrathecal Sar-SP (SSP, 1 μg, red) has occurred, on brush evoked response of deep WDR dorsal horn neurons. Reversal by naloxone dose 50 μg is shown in purple. n=6 for all except naloxone reversal, where n=5.

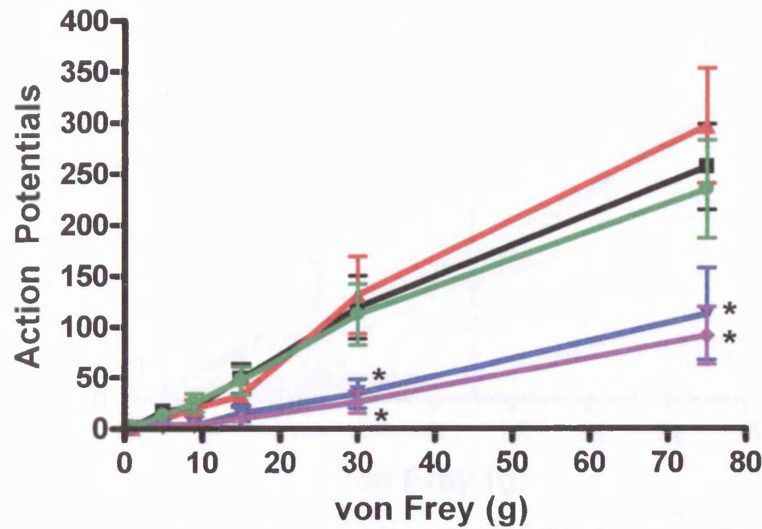


Fig. 3.32a- The effect of intrathecal 1μg morphine alone (purple, n=8) and the effect of morphine when pre-treatment with intrathecal Sar-SP has occurred (blue, n=10) on von Frey evoked responses of deep WDR dorsal horn neurons. The effect of intrathecal 1μg Sar-SP alone (red, n=10) on these responses is also shown. Naloxone reversal is shown in green (n=8). *P<0.05, where morphine induces a significant inhibition from the control (black, n=10) and following intrathecal Sar-SP administration (red). One way ANOVA followed by Tukey post-hoc test.

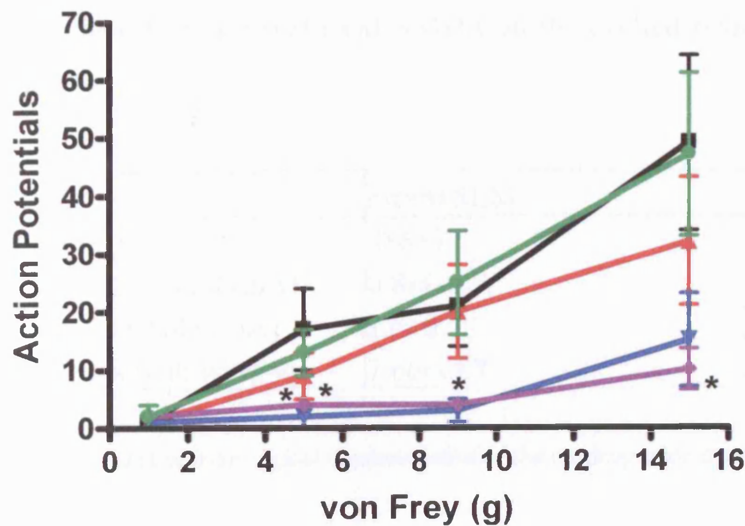


Fig.3.32b- The effect of intrathecal 1μg morphine alone (purple, n=8) and the effect of morphine when pre-treatment with intrathecal Sar-SP has occurred (blue, n=10) on the 1, 5, 9 and 15g von Frey evoked responses in deep wide dynamic range dorsal horn neurons in closer detail. Reversal by naloxone dose 50μg is shown in green (n=8). *P<0.05, where a significant inhibition from the control (black, n=10) is seen, on von Frey 5 and 15g evoked response by morphine alone and on von Frey 5 and 9g evoked response by morphine when pre-treatment with Sar-SP has occurred. One way ANOVA followed by Tukey post-hoc test.

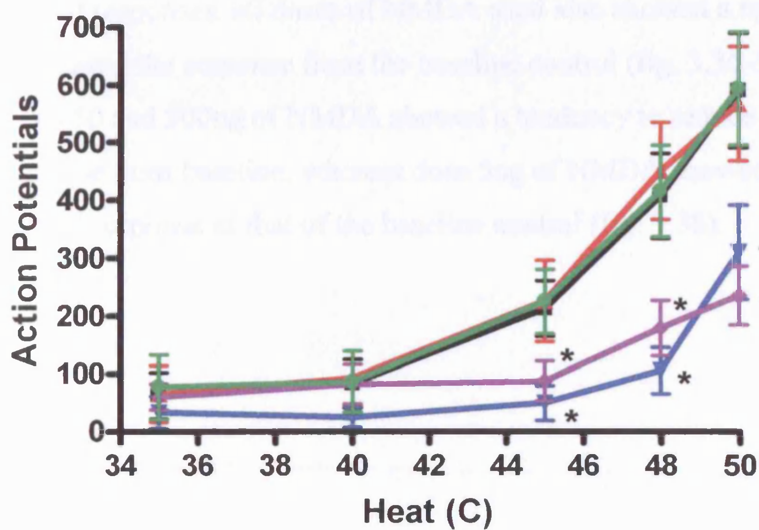


Fig. 3.33- The effect of intrathecal 1μg morphine alone (purple, n=8) and the effect of morphine when pre-treatment with intrathecal Sar-SP has occurred (blue, n=10) on thermal evoked responses of deep WDR dorsal horn neurons. The effect of intrathecal 1μg Sar-SP alone (red, n=10) on these responses is also shown. Naloxone reversal is shown in green (n=8). *P<0.05, where morphine induces a significant inhibition from the control (black, n=10) and following intrathecal Sar-SP administration (red). One way ANOVA followed by Tukey post-hoc test.

3.3.5 The effect of intrathecal NMDA on the evoked responses of deep WDR neurons

	mean±SEM
cell depth (μm)	886±77
Aβ-fibre threshold (mA)	0.8±0.02
C-fibre threshold (mA)	1.6±0.2
no. of cells with wind-up	7 out of 7

Table 3.7- Electrophysiological characteristics of the rat deep wide dynamic range dorsal horn neurons.

None of the doses of NMDA used exerted any significant effects on any of the electrical and natural evoked responses of deep WDR dorsal horn neurons (figs. 3.34-3.38). In the case of the electrical evoked responses, all the doses of NMDA used showed a tendency to decrease the response from the control, except for the Aδ (all doses) and wind-up evoked responses (doses 50 and 500ng), which showed a tendency to increase (fig. 3.34). In the case of brush and von Frey evoked responses, all doses of NMDA used also showed a tendency towards decreasing the response from the baseline control (fig. 3.36-37). Finally, both doses 50 and 500ng of NMDA showed a tendency to reduce the thermal evoked response from baseline, whereas dose 5ng of NMDA showed the same thermal evoked response as that of the baseline control (fig. 3.38).

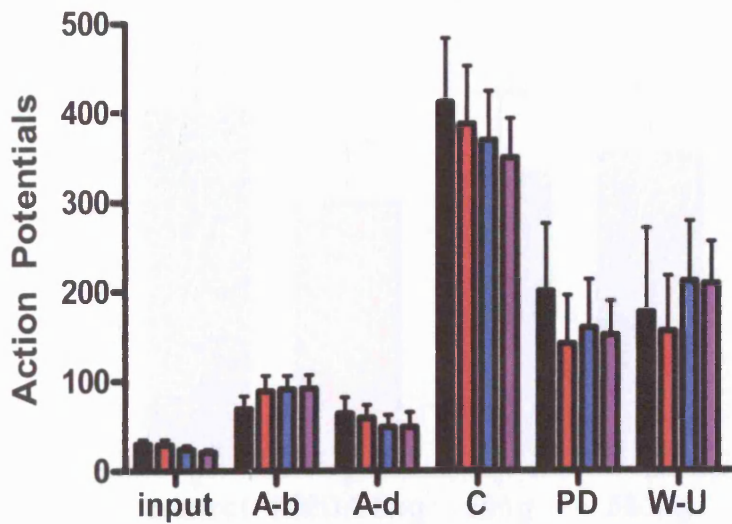


Fig. 3.34- The lack of effect of intrathecal NMDA doses 5 (red), 50 (blue) and 500ng (purple) on electrical evoked responses of deep WDR dorsal horn neurons. Control responses are shown in black. n=7.

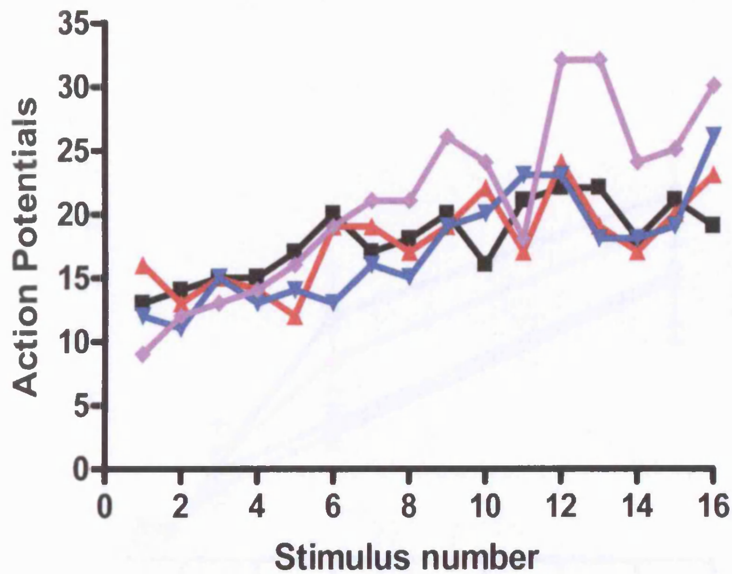


Fig. 3.35- The lack of effect of intrathecal NMDA doses 5 (red), 50 (blue) and 500ng (purple) on the slope of the electrical evoked wind-up response of a single, deep, WDR dorsal horn neuron (black). n=1.

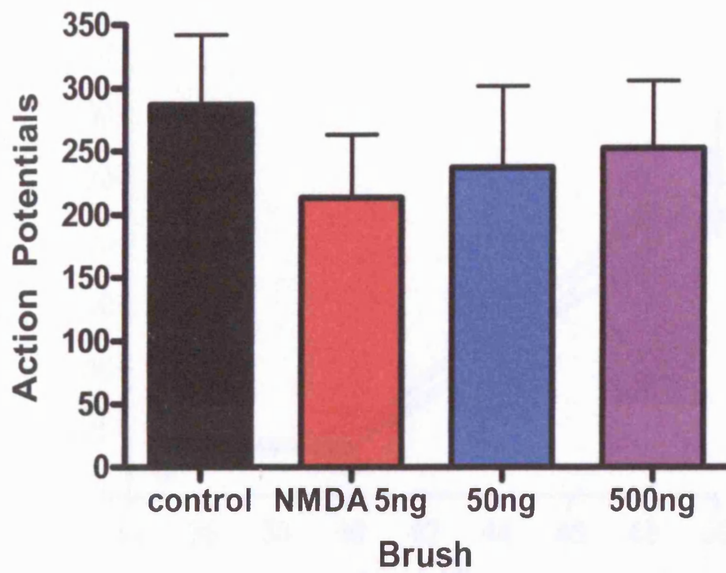


Fig. 3.36- The lack of effect of intrathecal NMDA doses 5 (red), 50 (blue) and 500ng (purple) on brush evoked responses of deep WDR dorsal horn neurons. Control responses are shown in black. n=6.

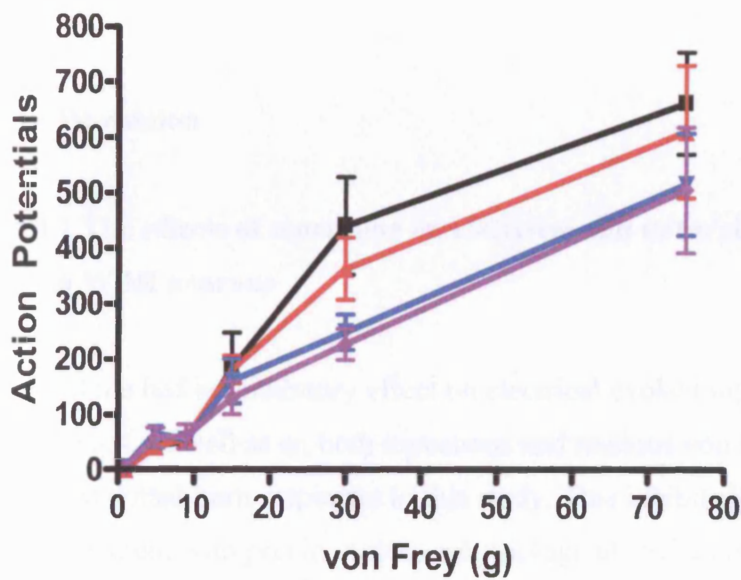


Fig. 3.37- The lack of effect of intrathecal NMDA doses 5 (red), 50 (blue) and 500ng (purple) on von Frey evoked responses of deep WDR dorsal horn neurons. Control responses are shown in black. n=6.

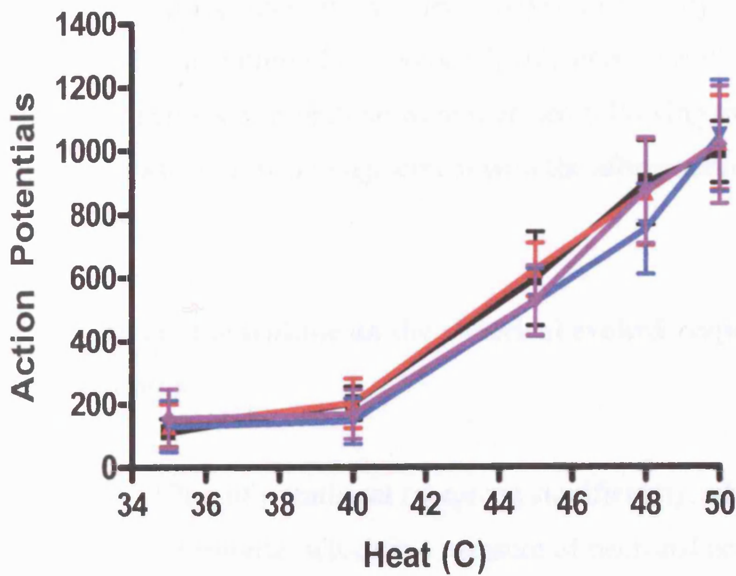


Fig. 3.38- The lack of effect of intrathecal NMDA doses 5 (red), 50 (blue) and 500ng (purple) on thermal evoked responses of deep WDR dorsal horn neurons. Control responses are shown in black. n=6.

3.4 Discussion

3.4.1 The effects of morphine on electrical and natural evoked responses of deep WDR neurons

Morphine had an inhibitory effect on electrical evoked input and C-fibre responses, as well as on both innocuous and noxious von Frey forces and thermal evoked dorsal horn responses in this study. This inhibitory effect of morphine is in agreement with previous electrophysiological studies in the cat (Duggan *et al.*, 1983) and rat spinal cord (Dickenson, 1997b; Dickenson *et al.*, 1986; Duggan *et al.*, 1983).

Whereas a previous study (Dickenson *et al.*, 1986) showed a facilitatory effect of low doses of morphine (0.5 and 0.8μg) on the C-fibre response of spinal cord dorsal horn neurons, the lowest dose of morphine used in this study (0.1 μg) did not cause any effects on the responses of deep WDR neurons, which is in

agreement with another in vivo electrophysiology study using the same dose and route of administration of morphine (Suzuki *et al.*, 1999). Furthermore, all of the inhibitory actions of morphine were reversed following the administration of naloxone, which is also in agreement with the aforementioned study (Suzuki *et al.*, 1999).

i) The effect of morphine on the electrical evoked responses of deep dorsal horn neurons

Both 1 and 10μg of intrathecal morphine significantly inhibited the electrically evoked input response, which is a measure of neuronal activity after the first electrical stimulus, before any hyperexcitability occurs (Urch *et al.*, 2003b). Furthermore, these two doses of intrathecal morphine also inhibited the C-fibre response, which is in agreement with a previous electrophysiological study which used the same doses of morphine as those used here (Suzuki *et al.*, 1999).

The hyperexcitability of the cell occurs following numerous electrical stimuli and is an indication of postsynaptic factors at play. Furthermore, it has been previously shown that most (approximately 70%) μ-opioid receptors are located on the presynaptic central terminals of C-fibres, since levels of these receptors decrease greatly following peripheral axotomy and dorsal rhizotomy (Besse *et al.*, 1992; deGroot *et al.*, 1997; Zhang *et al.*, 1998). Therefore, since postsynaptic μ-opioid receptors are less in number than their presynaptic counterparts, it is not surprising that morphine acting on μ-opioid receptors would be more effective in inhibiting neuronal responses which occur prior to any neuronal hyperexcitability.

Furthermore, even though the C-fibre evoked response is measured after 16 electrical stimuli at 3x the C-fibre threshold, and thus when neuronal hyperexcitability has occurred, morphine is able to inhibit the C-fibre response, since the majority of presynaptic μ-opioid receptors reside on the central terminals of small-diameter primary afferent fibres (Besse *et al.*, 1990; deGroot

et al., 1997; Zhang *et al.*, 1998). Moreover, the activation of the presynaptic μ-opioid receptors by morphine leads to decreased transmitter release, by opening K^+ channels and causing neuronal hyperpolarisation, which leads to a reduction in the opening of voltage-dependent Ca^{2+} channels (Besse *et al.*, 1990; Dickenson, 1994; Kangrga *et al.*, 1991).

Neither the Aβ-fibre nor the Aδ-fibre responses were affected by any of the morphine doses. However, there was a non-significant tendency with the highest morphine dose used in reducing the Aδ-fibre response, whereas no such tendency was seen with the Aβ-fibre response. μ-opioid receptors, as mentioned previously, are mostly found on the central terminals of small diameter (C-fibres), and to a certain extent medium-diameter (Aδ-fibres), primary afferent fibres, but not on large-diameter primary afferent fibres (Aβ-fibres) (Besse *et al.*, 1992; Besse *et al.*, 1990; deGroot *et al.*, 1997; Lamotte *et al.*, 1976; Mansour *et al.*, 1994; Zhang *et al.*, 1998). Since there are no μ-opioid receptors located on large-diameter primary afferent fibres, this means that morphine needs to act on its postsynaptic μ-opioid receptors, which are less in number than its presynaptic receptors, to inhibit the Aβ-fibre response, which means really large doses of morphine would be needed to inhibit the Aβ-fibre response. However, since some of the medium-diameter primary afferent fibres do have μ-opioid receptors, it was expected that the Aδ-fibre response would be significantly inhibited by morphine, which did not happen in my study. Perhaps higher doses of morphine were also required to decrease the Aδ-fibre response, since the highest dose of morphine used in my study was starting to show a tendency to reduce this response.

Post-discharge was significantly inhibited only by morphine dose 10μg. The post-discharge of the neuron is a measure of the number of action potentials after the C-fibre response, and is thus a function of the hyperexcitability of the cell. As mentioned above, neuronal hyperexcitability is a measure of postsynaptic events. μ-opioid receptors located postsynaptically have been shown to be less numerous than their presynaptic counterparts (Besse *et al.*, 1992), therefore, only the larger

dose of morphine was able to inhibit the postsdischarge response (Lombard *et al.*, 1989).

Furthermore, morphine had no effect on the electrically evoked wind-up response. Wind-up occurs due to the activation of the NMDA receptor channel, and is manifested as the summation of the cell's response, whereby the same repeated stimulus leads to a larger response (Dickenson, 1990; Mendell, 1966). Therefore, even though lower doses of morphine which act on the presynaptic μ-opioid receptors inhibit transmitter release from C-fibres and thereby decrease input, which in turn decreases postsynaptic excitation, low doses of morphine which do inhibit input and C-fibre evoked responses do not significantly inhibit wind-up response (Chapman *et al.*, 1994b; Dickenson *et al.*, 1986). That is because lower doses of morphine only decrease and do not abolish input, which means that there will still be some input causing some postsynaptic excitation, and thus wind-up breaks through these inhibitions as stimulation continues (Chapman, 1994; Chapman *et al.*, 1994b).

Furthermore, since wind-up also has a postsynaptic component, as it is mediated by the NMDA receptor which is located postsynaptically, low doses of morphine are not enough to cause inhibition since morphine's action, as has been established before, is predominantly presynaptic (Besse *et al.*, 1992; Dickenson, 1997a; Zhang *et al.*, 1998). Indeed, a previous study has shown that a higher dose of morphine (50μg) can inhibit wind-up by acting on both pre- and postsynaptic receptors (Chapman *et al.*, 1992; Chapman *et al.*, 1994b). However, since 10μg of morphine significantly inhibited the electrically evoked post-discharge response, and sine the cell's post-discharge is an indication of the cell 'winding-up', and since there were only 4 neurons which exhibited wind-up in my study, I cannot make a conclusion on the efficacy of morphine in inhibiting the electrically evoked wind-up response in my study.

ii) The effect of morphine on the natural evoked responses of deep dorsal horn neurons

None of the doses of morphine had any effect on the brush evoked response, which is thought to be mediated via Aβ-fibres (Koltzenburg *et al.*, 1994a), which was expected, since there are no opioid receptors on the central terminals of large-diameter primary afferent fibres (Besse *et al.*, 1992; Besse *et al.*, 1990; deGroot *et al.*, 1997; Lamotte *et al.*, 1976; Mansour *et al.*, 1994; Zhang *et al.*, 1998).

Furthermore, both doses 1 and 10μg morphine significantly inhibited the von Frey, innocuous and noxious, and the noxious thermal evoked responses. Noxious mechanical and thermal stimuli are encoded by numerous channels on the peripheral terminals of small- and medium-diameter primary afferent fibres (see sections **1.6.1**, **1.6.2** and **1.6.3**), and innocuous mechanical stimuli is encoded by large-diameter primary afferent fibres (Caterina *et al.*, 2005; Meyer *et al.*, 2006; Wood *et al.*, 2004). Therefore, since μ-opioid receptors exist on the central terminals of both small- and medium-diameter primary afferent fibres, morphine inhibited both noxious mechanical and thermal evoked responses.

Moreover, doses 1 and 10μg of morphine have been shown to inhibit innocuous as well as noxious mechanical evoked neuronal responses, of wide dynamic range neurons (Dickenson *et al.*, 2006; Suzuki *et al.*, 1999), which is what happened in this part of my study when even innocuous von Frey 5g evoked responses were significantly inhibited by both doses. μ-opioid receptors, as mentioned previously, are not found on the large-diameter primary afferent fibres which mediate innocuous mechanical evoked responses. Therefore, the fact that these low doses of morphine inhibited this innocuous mechanical response (von Frey 5g) and not the electrical evoked Aβ response or brush evoked response could be due to the fact low doses of morphine are not enough to act on the less numerous postsynaptic μ-opioid receptors (Besse *et al.*, 1990; Lombard *et al.*, 1989) to inhibit electrically evoked Aβ response or the brush evoked response.

However, it could be postulated that the innocuous von Frey 5g evoked response was a weak response, mediated by a mixture of Aβ- and Aδ-fibres, which could be inhibited by the low doses of morphine acting on their postsynaptic receptors (Suzuki *et al.*, 1999; Suzuki *et al.*, 2002a).

iii) Conclusion

The majority (70%) of μ-opioid receptors are located on the central terminals of small- and medium-diameter primary afferent fibres (Besse *et al.*, 1992; Besse *et al.*, 1990; deGroot *et al.*, 1997; Zhang *et al.*, 1998). Therefore, the effects of intrathecal low doses of morphine would be mostly the consequence of presynaptic receptor activation. Indeed, intrathecal morphine was effective in inhibiting the presynaptically-mediated responses, such as electrically evoked input response, and the small- and medium-diameter primary afferent fibre responses to mechanical and thermal stimuli.

3.4.2 The effects of each of Sar-SP and NMDA on electrical and natural evoked responses of deep WDR neurons

i) The interaction between the NK-1 and the NMDA receptor

The NK-1 receptor is activated by the peptide SP, which is found co-localised in primary afferent fibres with glutamate, one of the agonists needed to activate the NMDA receptor (Battaglia *et al.*, 1988; De Biasi *et al.*, 1988). It has been shown by previous studies that the release of each of SP and glutamate positively modulates the release of the other, probably *via* presynaptic NK-1 and NMDA receptors (Lao *et al.*, 2003; Liu *et al.*, 1997; Liu *et al.*, 1994; Malcangio *et al.*, 1998a), as well as *via* the diffusion of prostanoids and nitric oxide, which are generated by the activation of each of the NK-1 and NMDA receptors through second messengers and enzymes, and which cause retrograde transmitter release from primary afferent fibres following injury (Rusin *et al.*, 1993a; Yaksh *et al.*, 1999).

The activation of the NMDA receptor causes an increased state of spinal neuronal responsiveness, termed wind-up, which is blocked by NMDA receptor antagonists (Dickenson *et al.*, 1987), and partly blocked by NK-1 receptor antagonists (Xu *et al.*, 1992). Wind-up was initially described by Mendell, in 1966, as the increase in response of deep dorsal horn neurons following repetitive C-fibre stimulation, even though the input to the spinal cord stays constant. Wind-up can be initiated by electrical stimulation at rates of nearly 0.5Hz, but not at 0.1Hz (Dickenson *et al.*, 2002b). The physiological activation of the NMDA receptor complex requires glutamate and glycine binding (ligand-gating), as well as the summation of slow non-NMDA depolarisations (voltage-gating), which remove the Mg^{2+} block *via* the action of excitatory amino acids and neuropeptides (including SP) acting on their respective receptors (Kellstein *et al.*, 1990; O'malley *et al.*, 1991; Salt, 1986; Urban *et al.*, 1984; Xu *et al.*, 1992).

Indeed, the activation of the NK-1 receptor has been shown to enhance the activation and the effects of the NMDA receptor channel, *via* both the aforementioned summation of the slow depolarisations which occur following the NK-1 receptor activation (and non-NMDA receptor activation) and the activation of second messenger systems. The former was seen when an NK-1 antagonist was effective in partly reducing the wind-up response of the flexor reflex elicited by the electrical conditioning stimulus, and following states of increased release of peptides and excitatory amino acids, such as inflammation (Thompson *et al.*, 1990; Xu *et al.*, 1992). The latter was seen following the activation of the second messenger cascade, which occurs after NK-1 receptor activation, which leads to increases in intracellular calcium and protein kinase levels, that activate more enzymes, receptors and channels, including the NMDA receptor, and leads to facilitation of dorsal horn nociceptive transmission and central sensitisation (Chen *et al.*, 1992; Dougherty *et al.*, 1993; Ikeda *et al.*, 2003; Rusin *et al.*, 1993a; Rusin *et al.*, 1993b; Rusin *et al.*, 1992; Yaksh *et al.*, 1999).

There are many studies charting the spinal interaction between the NK-1 and NMDA receptor activation, and their role in central sensitisation. A behavioural study showed that the co-administration of SP and NMDA potentiated the biting and scratching behaviour seen following the application of each agonist alone in mice (Mjellem-Joly *et al.*, 1991). Additionally, the studies by Cumberbatch *et al* (1995), and Chizh *et al* (1995) showed, using in vivo electrophysiology of spinalised rats, that NK-1 agonists increased dorsal horn neuronal responses (former) and NK-1 antagonists reduced dorsal horn neuronal responses (latter) following NMDA receptor activation (Chizh *et al.*, 1995; Cumberbatch *et al.*, 1995). Moreover, the studies by Dougherty *et al* (1991, 1993 and 1995), showed the potentiation of primate spinothalamic tract neuronal responses following the co-administration of NMDA and SP, as well as increased responses to mechanical stimuli (noxious and innocuous), in cells which were already excited by the administration of excitatory amino acids alone (Dougherty *et al.*, 1995; Dougherty *et al.*, 1993; Dougherty *et al.*, 1991).

In addition, in vitro studies showed that SP potentiated the effects of NMDA induced depolarisations, using superficial spinal dorsal horn neurons isolated from young rats (Rusin *et al.*, 1993a; Rusin *et al.*, 1993b; Rusin *et al.*, 1992) and rat primary sensory neurons (Wu *et al.*, 2004). Furthermore, the study by Chapman *et al* (1994) showed that the intrathecal co-administration of SP and NMDA facilitated all of the Aδ-fibre evoked responses, post-discharge and wind-up of rat deep dorsal horn neurons (Chapman *et al.*, 1994a). Finally, it was shown that the activation of the spinal NK-1 and the NMDA receptors is needed in order to induce the long-term potentiation (LTP , "use-dependent, long-lasting modification of synaptic strength") of superficial neurons in spinalised rats (Liu *et al.*, 1998).

Further linking the NK-1 and NMDA receptors with each other and the generation of central sensitisation are studies using the formalin response, an inflammatory model whereby there are two phases of dorsal horn neuronal activity following the intraplantar injection of formalin. Using in vivo electrophysiology, it was shown that the second phase of the formalin response,

which starts after the acute phase in 25 minutes and lasts for 60 minutes, is reduced by each of NMDA (Haley *et al.*, 1990) and NK-1 receptor antagonists (Chapman *et al.*, 1993). An NK-1 antagonist also reduced the second phase of the formalin response in a behavioural study, which measured mouse hindpaw licking and biting as an indication of pain behaviour (Gonzalez *et al.*, 2000), whereas in another behavioural study, the intrathecal co-administration of both SP and NMDA decreased the biting and licking response in both phases of the formalin test in mice (Mjellem-Joly *et al.*, 1992). In addition, c-Fos immunoreactivity expression measured in the superficial and deep dorsal horn three hours after formalin injection in freely moving rats was also significantly reduced by a high dose of an NK-1 antagonist, and by the combination of both the NK-1 and NMDA receptor antagonists at doses where each of these two antagonists was not very effective (Chapman *et al.*, 1996).

ii) Sar-SP

In my study, the intrathecal administration of the NK-1 agonist Sar-SP, which is longer acting and more potent than SP (Yip *et al.*, 1999), did not cause a significant facilitation in any of the electrical and natural evoked responses of deep WDR neurons, except for the 45°C thermal evoked response. The thermal facilitation seen in my study is in agreement with two earlier studies which showed the facilitation of dorsal horn neuronal responses to thermal stimuli in the cat by SP (Henry, 1976), and in the rat by Sar-SP (Rygh *et al.*, 2006). The study by Rygh *et al*, showed, in rat superficial NK-1 expressing projection neurons, that Sar-SP facilitated the heat-evoked response to temperatures 45 and 48°C, and that also following LTP induction, that noxious heat evoked responses are facilitated in deep WDR neurons. Thus further proving the role of NK-1 receptors in central sensitisation states, as well as in facilitating heat-evoked responses of dorsal horn neurons.

The role of the NK-1 receptor in pain transmission is still not clear, with some stating that SP is more of a neuromodulator, than a 'traditional' neurotransmitter (Chapman *et al.*, 1996; Dougherty *et al.*, 1995; Kellstein *et al.*, 1990). Previous

studies investigating the effects of the intrathecal administration of SP, or other NK-1 agonists, have resulted in these agents causing mostly excitatory responses, whether on a neuronal level (Cheunsuang *et al.*, 2002; Henry, 1976; Kellstein *et al.*, 1990; Nagy *et al.*, 1993; Randic *et al.*, 1977; Xu *et al.*, 1992), or on a behavioural level (Seybold *et al.*, 1982). The study by Cheunsuang *et al* showed, using in vitro electrophysiology, that the application of Sar-SP, at a dose higher than the ones used in my study, caused great depolarisations of superficial dorsal horn neurons, whereas that of Nagy *et al* showed similar prolonged postsynaptic depolarisations of neurons in laminae II-IV, using SP-methyl ester.

The studies by Henry (1976) and Randic *et al* (1977), both used in vivo electrophysiology of the cat dorsal horn, whereas Kellstein *et al* (1990) investigated the rat dorsal horn. Henry, investigating deep dorsal horn neurons showed excitation of half of the units tested by SP, whereas Randic *et al* showed the excitation of all of the superficial dorsal horn neurons tested. Kellstein *et al* showed, in laminae III-VII neurons, an increased C-fibre evoked response with SP, a decrease in C-fibre evoked response with an NK-1 antagonist, and no effect by either NK-1 agonist or antagonist on A-fibre evoked response and spontaneous activity of the neurons.

In addition, also in agreement with our study, Chapman *et al*, in 1994, showed that different doses of intrathecal SP on its own, had no effect on the electrically evoked deep WDR neuronal responses (Chapman *et al.*, 1994a). Furthermore, some studies have shown that NK-1 receptor activation only serves to enhance the effects of NMDA receptor activation, and that NK-1 agonists on their own had no effects on dorsal horn neuronal responses of spinalised rats (Cumberbatch *et al.*, 1995), or on all of the background activity, mechanical and thermal cutaneous stimulation in primate spinothalamic tract neurons (Dougherty *et al.*, 1995). Moreover, the study by Fleetwood-Walker *et al* (1990) showed that SP and other NK-1 agonists, only caused a decrease in the brush-evoked responses, but caused no changes in responses to noxious heat or noxious mechanical stimuli and the spontaneous activity in cat spinocervical tract neurons (Fleetwood-Walker *et al.*, 1990).

Upon closer scrutiny, most of the spinal cord dorsal horn neuronal studies showing a facilitatory effect of NK-1 receptor agonists were using superficial dorsal horn neurons, where nociceptive-specific neurons outnumber WDR neurons (Cheunsuang *et al.*, 2002; Coghill *et al.*, 1993; Randic *et al.*, 1977; Rygh *et al.*, 2006), whereas my study investigated deep WDR neurons. The study by Henry, in 1976, which was performed on laminae IV-VI of the cat spinal cord, showed that only half the units investigated were excited by intrathecal synthetic SP (Henry, 1976). Therefore, one reason for why only one heat evoked response was facilitated in my study and not other heat or mechanical evoked responses, could be due to the fact that deep WDR neurons exhibit larger dorsal horn neuronal evoked responses than the superficial dorsal horn neurons, as shown in my study as well as in a previous study (Seagrove *et al.*, 2004). Therefore, it could be easier to significantly facilitate heat and mechanical evoked responses in superficial neurons than in deep neurons.

Furthermore, thermal stimuli is conducted mainly *via* C-fibres and some type 2 Aδ-fibres, with the TRPV1 channel at their peripheral termination (Caterina *et al.*, 1997; Magerl *et al.*, 2001), and most of the C-fibres are peptidergic (Meyer *et al.*, 2006; Snider *et al.*, 1998; Urban *et al.*, 1984), releasing SP at their central terminals and thought to also express the NK-1 receptor at their central terminals (Malcangio *et al.*, 1999). Therefore, even though NK-1 receptors have been shown to be located mostly postsynaptic, in superficial laminae (Todd, 2002), one could conclude that another reason why Sar-SP facilitated thermal and not mechanical evoked responses could be due to Sar-SP activating some presynaptic NK-1 receptors on peptidergic C-fibres which convey thermal sensation. However, previous studies on presynaptic NK-1 receptors have postulated that these were inhibitory autoreceptors (Malcangio *et al.*, 1999) and furthermore, none of the other heat-evoked responses were facilitated in my study. Therefore, there must be other factors involved in why Sar-SP only facilitated the heat-evoked response of one temperature and not the others, such as the neuronal population (discussed above) and/or the dose of Sar-SP used.

iii) NMDA

In my study, the intrathecal administration of NMDA (5, 50 and 500ng) did not cause any significant excitation of the electrical evoked responses, which could be attributed to the fact that electrical stimulation was made at 3x the C-fibre threshold, whereas it should have been done at 1.5x the C-fibre threshold (Chapman *et al.*, 1994a), and that different doses of intrathecal NMDA should have been used to cause increases in some of the natural evoked responses of deep dorsal horn neurons (Sher *et al.*, 1990). Additionally, shorter testing times might have been needed to show the effects of NMDA, since I showed in my behavioural study (**chapter 4**) that the excitatory effects of intrathecal NMDA disappear after 5 minutes, and in the study by Chapman *et al*, only ten minutes were given between each set of testing. However, due to the fact that in my study, all of electrical, brush, von Frey and heat evoked responses were measured, a 20 minute gap between testing was needed to fit in all these stimuli, as well as give the neuron time to recuperate.

In previous studies, the antagonism of the NMDA receptor in the dorsal horn has been shown to decrease the hyperexcitability of dorsal horn neurons, including wind-up of dorsal horn neurons in vivo (Chapman *et al.*, 1995; Dickenson, 1997a; Haley *et al.*, 1990), reduce c-Fos expression in deep dorsal horn neurons following the spinal nerve ligation model (Lee *et al.*, 2002), as well as pain behaviour in different pain models, such as inflammation (Coderre *et al.*, 1994; Ma *et al.*, 1998), and nerve injury (Wegert *et al.*, 1997). However, the activation of the NMDA receptor channel in previous electrophysiological experiments, as in my study, using the intrathecal administration of the agonist NMDA, has not always yielded clear-cut excitations of rat dorsal horn neurons (Chapman *et al.*, 1994a), and primate spinothalamic tract neurons (some which were actually inhibited) (Dougherty *et al.*, 1995), which was also the case with the NK-1 receptor.
Indeed, the study by Chapman *et al*, in 1994, showed that when the neuron was being electrically stimulated at 3x the C-fibre threshold, the intrathecal administration of the same doses of NMDA as the ones used in my study was

ineffective in exciting the neuron (Chapman *et al.*, 1994a). This electrical stimulation had to be reduced to 1.5x the C-fibre threshold for a significant excitation of the deep WDR neurons to be achieved: dose 5ng significantly facilitated wind-up, whereas dose 50ng significantly facilitated the C-fibre, Aδ-fibre and post-discharge response, and higher doses showed a tendency to inhibit the responses, showing a biphasic effect of NMDA, as well as a fine line between the doses that were excitatory and those that were ineffective (Chapman *et al.*, 1994a). Furthermore, a previous study showed an NMDA-induced increase in spontaneous activity, noxious pinch and brush evoked responses of rat deep dorsal horn neurons, but this study used spontaneously firing neurons, did not use any electrical stimulation of the neurons, and the doses of intrathecal NMDA used were higher than those used in my study (Sher *et al.*, 1990).

iv) Conclusion

Even though the effects of NK-1 and NMDA receptor activation have been shown by the majority of studies to be excitatory on the spinal cord dorsal horn neurons (Chapman *et al.*, 1994a; Henry, 1976; Randic *et al.*, 1977; Sher *et al.*, 1990), some studies have shown no effects (Dougherty *et al.*, 1995; Fleetwood-Walker *et al.*, 1990), others have shown both excitation and inhibition (Chapman *et al.*, 1994a; Dougherty *et al.*, 1995) and others still have shown the excitatory effects of only the combined activation of both receptors (Chapman *et al.*, 1994a; Dougherty *et al.*, 1992; Dougherty *et al.*, 1995; Dougherty *et al.*, 1993).

In my study, there was no overall excitation of dorsal horn neuronal responses by either of intrathecal Sar-SP or NMDA (except for the facilitation of the 45°C thermal evoked response by 1μg Sar-SP), which could be due to the doses used, the depth and type of the neurons studies and the electrical stimulation being at 3x the C-fibre threshold, rather than a lack of effect of the activation of each of these two receptor systems on dorsal horn neuronal responses.

3.4.3 The effects of morphine, in the presence of Sar-SP, on electrical and natural evoked responses of deep WDR neurons

Some studies show a reduced efficacy of morphine inhibition in pain states, such as neuropathic pain (Arner *et al.*, 1988), and the wind-up model, which is known to occur following spinal NMDA receptor activation (Chapman *et al.*, 1992; Chapman *et al.*, 1994b), which in turn requires the NK-1 receptor activation to enhance its (NMDA receptor) effects (Dougherty *et al.*, 1995; Rusin *et al.*, 1993a). As it turns out, it is more specifically the dynamic allodynia, resulting from nerve injury, and thought to be conveyed mostly by Aβ-fibres, which is less responsive to morphine administration (Field *et al.*, 1999a; Field *et al.*, 1999b), than other types of hypersensitivities such as thermal and static allodynia (Field *et al.*, 1999b; Wegert *et al.*, 1997).

Both the NK-1 and the NMDA receptors are found located mostly postsynaptic in the spinal cord dorsal horn (Coggeshall *et al.*, 1997a; Liu *et al.*, 1994; Todd, 2002), an area where less postsynaptic μ-opioid receptors exist, in relation to their presynaptic counterparts (Besse *et al.*, 1992; Besse *et al.*, 1990; Zhang *et al.*, 1998). Additionally, μ-opioid receptors are mostly found on small- and medium- primary afferent fibre terminals, and not on large-diameter Aβ-fibres (Besse *et al.*, 1992; Besse *et al.*, 1990; deGroot *et al.*, 1997; Lamotte *et al.*, 1976; Mansour *et al.*, 1994; Zhang *et al.*, 1998), which means that any effect of morphine on Aβ-fibres or responses conveyed by Aβ-fibres such as brush/dynamic allodynia would require morphine to act *via* its less numerous postsynaptic μ-opioid receptors. Therefore, higher doses of morphine would be required to inhibit wind-up and even higher doses would be needed to inhibit Aβ-fibre responses than those required to inhibit responses where morphine can act predominantly on its presynaptic receptors (Chapman *et al.*, 1992; Chapman *et al.*, 1994b), such as electrically evoked input and C-fibre responses, as well as noxious thermal and mechanical evoked responses.

Since both the NK-1 and the NMDA receptors are found mostly postsynaptic (Coggeshall *et al.*, 1997b; Todd, 2002), and the activation of each is known to facilitate dorsal horn neuronal responses *via* the postsynaptic receptors, it was hoped that the administration of each of Sar-SP and NMDA would induce the facilitation of the responses of dorsal horn neurons, which would be followed by investigating the efficacy of morphine in inhibiting these responses.

Unfortunately, the only response which was facilitated was the 45°C thermal evoked response by Sar-SP, and it was seen that morphine was as effective following Sar-SP administration, in inhibiting the dorsal horn neuronal responses, as it was when administered alone.

3.4.4 Conclusion

Even though morphine in my study was as effective in inhibiting dorsal horn neuronal responses on its own, as following the administration of Sar-SP, and even though at higher doses morphine can inhibit postsynaptic responses of dorsal horn neurons, such as wind-up, the administration of either Sar-SP and NMDA in my study did not yield a clear-cut response. This could be due to the depth and the type of the neurons used, the doses used, or the gaps left in between testing time, rather than a conclusive lack of effect of the activation of either the NK-1 or the NMDA receptor on dorsal horn neurones. Therefore, the overall conclusion from this study will be that morphine is effective in inhibiting dorsal horn neuronal responses following the activation of the NK-1 receptor with Sar-SP, but a different technique will be used in the next chapter to further investigate the modulation of excitatory transmission in the rat spinal cord by morphine.

In this chapter, I regrettably could not fully investigate the effects of morphine on excitatory transmission in the rat spinal cord, due to the fact that no overall dorsal horn neuronal excitation could be induced following either of intrathecal Sar-SP or NMDA administration. Therefore, in the next chapter, I will

investigate the effects of morphine on the BSL behaviour and thermal/mechanical hypersensitivities that arise following the intrathecal administration of each of the NMDA and the NK-1 receptors, using as agonists NMDA and Sar-SP, respectively. Furthermore, it would be interesting to directly compare the effects of the activation of each of these receptors, as well as morphine's effect on them, and try to relate them to what is actually seen in some chronic pain states.

Chapter 4:

A study investigating the effectiveness of morphine in inhibiting the behaviour and nociceptive response which arise following the activation of each of the NMDA and NK-1 receptors

4. A study investigating the effectiveness of morphine in inhibiting the behaviour and nociceptive response which arise following the activation of each of the NMDA and NK-1 receptors

4.1 Introduction

The NMDA and NK-1 receptors are located mostly postsynaptic in the spinal cord dorsal horn (Coggeshall and Carlton 1997), and their activation and interaction in the spinal cord is thought to partly underlie the hyperexcitability of spinal cord dorsal horn neurons, such as wind-up and central sensitisation (Dickenson and Sullivan 1987; Xu, Dalsgaard et al. 1992; Liu and Sandkuhler 1998), as well as thermal and tactile hypersensitivities which occur following different models of nerve injury (Yamamoto and Yaksh 1992; Wegert, Ossipov et al. 1997; Field, McCleary et al. 1998; Wu, Schwasinger et al. 2005) and inflammation (Ma, Allchorne et al. 1998).

Previous studies have shown the reduced efficacy of morphine in decreasing wind-up (Dickenson and Sullivan 1986) and abnormal pain following nerve injury (Arner and Meyerson 1988), whereas other studies have shown that it is a question of increased doses, route of administration and timing of administration which determines the efficacy of morphine in these models (Woolf and Wall 1986; Portenoy, Foley et al. 1990; Rowbotham, Reisner-Keller et al. 1991; Chapman and Dickenson 1992; Jadad, Carroll et al. 1992; Chapman, Haley et al. 1994; Suzuki, Chapman et al. 1999).

Many previous studies have investigated the effects of morphine on the biting, scratching and licking behaviour that arises following the direct activation of each of the spinal NMDA and the NK-1 receptors by their respective receptors, and these either concluded significant inhibition (Hylden and Wilcox 1982; Hylden and Wilcox 1983) or decreased effects of morphine in these pain states (Aanonsen and Wilcox 1987; Bossut, Frenk et al. 1988; Alvarez-Vega, Baamonde et al. 1998) (see sections **1.7.3.1**, **1.7.3.2**, **1.7.4** and **1.7.6.2**).

Furthermore, some studies have investigated the effects of morphine on either thermal or tactile hypersensitivity, which arises following either the direct activation of each of the these two receptors with their respective agonists (Hylden and Wilcox 1982), or the secondary activation of these two receptors following inflammatory (Yamamoto and Yaksh 1992; Ma, Allchorne et al. 1998) and neuropathic pain states (Yamamoto and Yaksh 1991). However, not many studies have investigated and directly compared the effects of morphine on both thermal and tactile hypersensitivity, which arise following the direct activation of each of these receptors, with their respective agonists.

Since the activation of the spinal NMDA and NK-1 receptors partly underlie states of central sensitisation, such as the wind-up model (Dickenson 1997) and the pain hypersensitivity seen following nerve injury (Field, McCleary et al. 1998; Suzuki, Matthews et al. 2001), then the modulation of each of these two receptors by morphine would help shed more light on the efficacy of morphine in these pain models.

Therefore, the effects of morphine on the BSL behaviour and thermal/mechanical hypersensitivities that arise following the activation of each of the NMDA and the NK-1 receptors, using as agonists NMDA and Sar-SP, respectively, will be investigated in this chapter.

4.2 Methods

The methods used in this section have been described in detail in the methodology chapter, in sections **2.1** and **2.3**.

Briefly, male, Sprague-Dawley rats weighing 190g will be intrathecally catheterised in the lumbar area by using a modified version of the Storkson *et al* method (Storkson, Kjorsvik et al. 1996). This method of catheterisation *via* the sacral route was thought to cause less motor damage than the one involving catheterisation through the atlanto-occipital membrane (Yaksh and Rudy 1976), since the cannula length would be only 3cm, as opposed to 6cm, in an adult rat.

However, both methods were comparable in terms of success rate (personal communication with R. Suzuki and Y. Wong).

The rats were then habituated, then tested for thermal hypersensitivity, using the Hargreaves test (Hargreaves, Dubner et al. 1988), and for tactile hypersensitivity using the Chaplan method of determining the paw withdrawal threshold (Chaplan, Bach et al. 1994), following the intrathecal administration of each of the NMDA receptor agonist NMDA, and the NK-1 receptor agonist Sar-SP.

The subcutaneous administration of morphine (3mg/kg), and the intrathecal administration of each of NMDA (0.3μg) and Sar-SP (3μg), will be done, and responses to thermal and tactile stimuli will be measured, in order to determine the efficacy of morphine on such responses following the activation of each receptor. Furthermore, the reversal of the tactile and the thermal hypersensitivity induced by intrathecal NMDA and Sar-SP will be attempted with the intrathecal administration of the non-competitive NMDA receptor antagonist MK-801 (0.1mg/kg) (Kemp, Foster et al. 1987), and that of the potent, non-peptide NK-1 receptor antagonist RP-67,580 (3μg), respectively (Rupniak, Carlson et al. 2003). The dosing protocol is explained in detail in section **2.6.2**.

In the section of this chapter investigating the effects of intrathecal Sar-SP, the grooming behaviour following the BSL behaviour which occurred after Sar-SP administration lasted for 21±7 minutes. The grooming was too excessive for me to be able to quantify whether any paw withdrawal before timepoint 20 minutes was due to thermal hypersensitivity or grooming. Therefore, in this part of the study, it is only starting from 20 minutes post-intrathecal Sar-SP that paw withdrawal will be considered to be due to thermal hypersensitivity only and not grooming movements which cut off the Hargreaves beam prematurely. This timing of the actions of Sar-SP has been confirmed by an earlier study (Rygh, Suzuki et al. 2006).

Finally, the tactile hypersensitivity which occurred following either of intrathecal NMDA or Sar-SP administration could not be followed as extensively as thermal hypersensitivity, because the series of stimuli needed to determine tactile hypersensitivity took longer than just placing a radiant heat source under the hindpaw in the Hargreaves test. Therefore, for tactile hypersensitivity, there were only three timepoints to draw conclusions from, whereas with thermal hypersensitivity, testing was done every 5 minutes for 60 minutes.

4.3 Results

4.3.1 Biting, scratching and licking behaviour

i) Intrathecal Saline

None of the rats that were intrathecally injected with 0.9% sterile saline exhibited any biting, scratching and licking behaviour (BSL, n=6).

ii) Intrathecal NMDA

a) NMDA alone- The intrathecal administration of NMDA (0.3μg, n=8) immediately caused 5±1 minutes of caudally directed biting, scratching and licking, as opposed to the intrathecal saline controls which did not cause any of this behaviour.

b) Morphine+NMDA- The subcutaneous administration of Morphine (3mg/kg), 25 minutes before that of NMDA (0.3μg) in the morphine+NMDA treated rats caused a significant decrease in the BSL behaviour, when compared to the NMDA treated rats, from 5±1 to 2±1 minutes ($p<0.05$, n=8, *t*-test).

c) MK-801+NMDA- The subcutaneous injection of MK-801 (0.1mg/kg), 25 minutes prior to that of NMDA (0.3μg) in the MK-801+NMDA treated rats

(n=8) caused no significant difference in the BSL behaviour, when compared to the NMDA treated rats.

iii) Intrathecal Sar-SP

a) Sar-SP alone- The intrathecal administration of Sar-SP (3μg, n=6) immediately led to 21±7 minutes of caudally directed biting, scratching and licking and grooming, as opposed to the intrathecal administration of saline alone, which did not cause this behaviour.

b) Morphine+Sar-SP- The subcutaneous injection of Morphine (3mg/kg), 10 minutes before administering Sar-SP (3μg) in the morphine+Sar-SP treated rats (n=8) did not cause any significant difference in the BSL and grooming behaviour, when compared to the Sar-SP treated rats.

c) RP-67,580 alone- The intrathecal administration of RP-67,580 (3μg, n=6) on its own caused 2±1 min of caudally directed BSL and grooming behaviour.

d) DMSO alone- The intrathecal administration of 20% DMSO (n=10) caused no BSL or grooming behaviour.

e) RP-67,580+Sar-SP- The intrathecal administration of RP-67,580 (3μg), 10 minutes before that of Sar-SP (3μg) in the RP-67580+Sar-SP treated rats (n=8) caused no significant difference in the BSL and grooming behaviour when compared to the Sar-SP treated rats.

4.3.2 Behavioural response to thermal stimuli using radiant heat

i) Saline control experiments

Neither the subcutaneous (n=11), nor the intrathecal (sterile, n=6) administration of saline resulted in a significant change in paw withdrawal latency following

thermal stimuli, from the baseline response. Additionally, there was no significant difference in paw withdrawal latency to thermal stimuli between subcutaneous and intrathecal saline, when the two different routes of administration were compared (fig. 4.1).

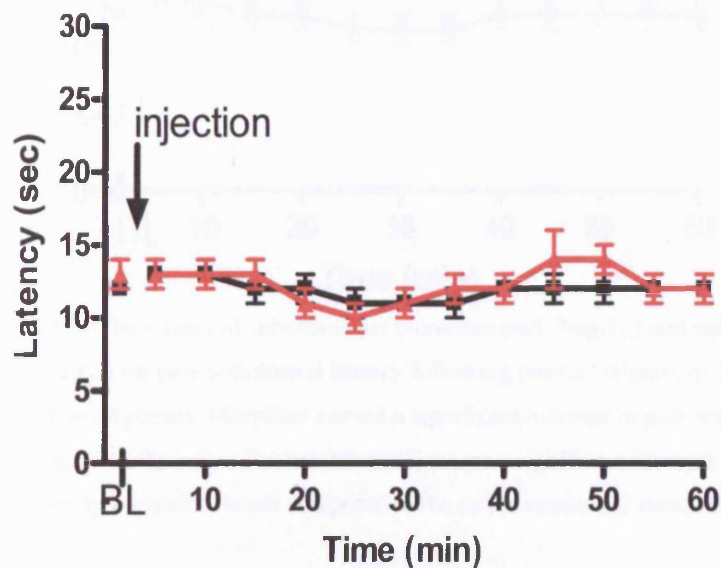


Fig. 4.1- The lack of effect of subcutaneous (black, 0.25ml, n=11) and intrathecal (red, 24µl, n=6) saline on the rat paw withdrawal latency following thermal stimuli. BL depicts pre-drug baseline responses.

ii) Morphine experiments

The subcutaneous administration of morphine (3mg/kg) caused a significant increase in paw withdrawal latency, and thus analgesia, when compared to the saline control in all of the timepoints tested ($p<0.05$, n=11) (fig.4.2).

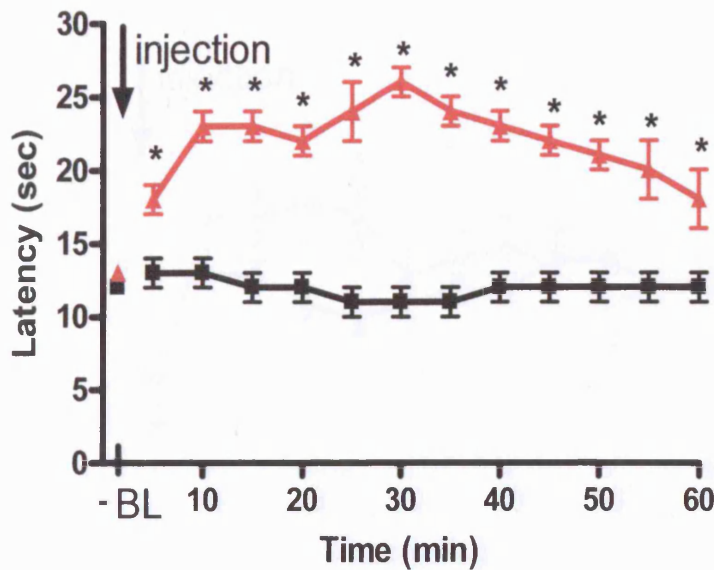


Fig. 4.2- The effects of subcutaneous morphine (red, 3mg/kg) and subcutaneous saline (black, 0.25ml) on rat paw withdrawal latency following thermal stimuli, n=11. BL depicts pre-drug baseline responses. Morphine caused a significant increase in paw withdrawal latency when compared to the saline control. *P<0.05, where a significant increase in paw withdrawal latency, caused by morphine, when compared to the saline control, is seen, *t*-test.

iii) NMDA experiments

a) NMDA alone- When compared to the intrathecal saline control, NMDA (0.3μg) significantly decreased paw withdrawal latency to thermal stimuli and caused thermal hypersensitivity, 5 minutes after its intrathecal administration ($p<0.05$, n=8). After 5 minutes, the response to thermal stimuli resumed baseline latencies, until timepoints 20, and 25 minutes, when the paw withdrawal latency was significantly increased ($p<0.05$, n=8), and timepoint 50 minutes, when the paw withdrawal latency was significantly decreased ($p<0.05$, n=8). The latter will be noted as a return to baseline level since it still falls within 10-15 seconds, which were taken as baseline during the control experiment (fig.4.3).

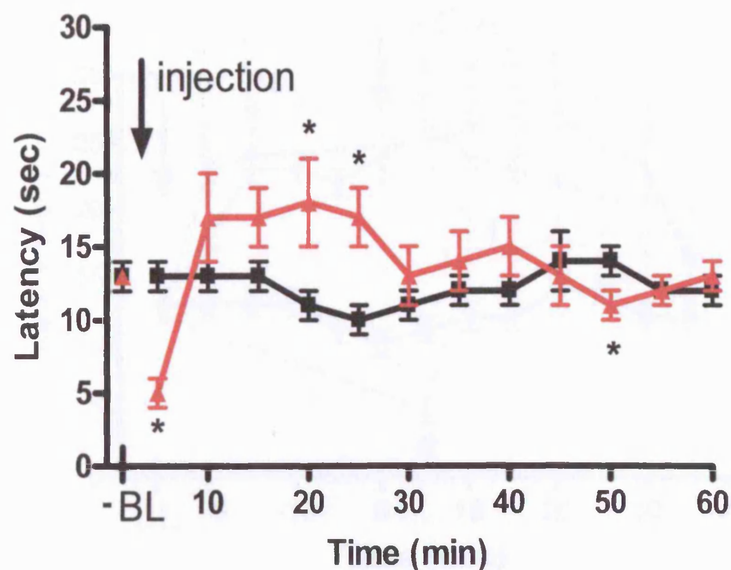


Fig. 4.3- The effects of intrathecal NMDA (red, 0.3μg, n=8) and intrathecal saline (black, 24μl, n=6) on rat paw withdrawal latency following thermal stimuli. BL depicts pre-drug baseline responses. NMDA caused thermal hypersensitivity when compared to the saline control. *P<0.05, where a significant change in paw withdrawal latency caused by NMDA, when compared to the saline control, is seen, *t*-test.

b) Morphine+NMDA- The subcutaneous administration of morphine (3mg/kg) twenty-five minutes before intrathecal NMDA (0.3μg) in the morphine+NMDA treated rats prevented NMDA-induced thermal hypersensitivity following NMDA administration. Furthermore, morphine, in the morphine+NMDA treated rats, seemed to synergise with the previously-seen NMDA-induced increase in paw withdrawal latency to thermal stimuli, since paw withdrawal latency in these animals was increased, albeit non-significantly, to a higher level than in the rats which were only treated with morphine. Moreover, there was a significant increase in the baseline latency following morphine administration, in the morphine+NMDA treated rats, when compared to the NMDA treated rats ($p<0.05$, n=8). This was taken into consideration and was the baseline used to statistically analyse the data. Finally, the administration of morphine pre-NMDA in the morphine+NMDA treated rats seemed to increase paw withdrawal to higher latencies than those seen with morphine administration on its own (fig. 4.4).

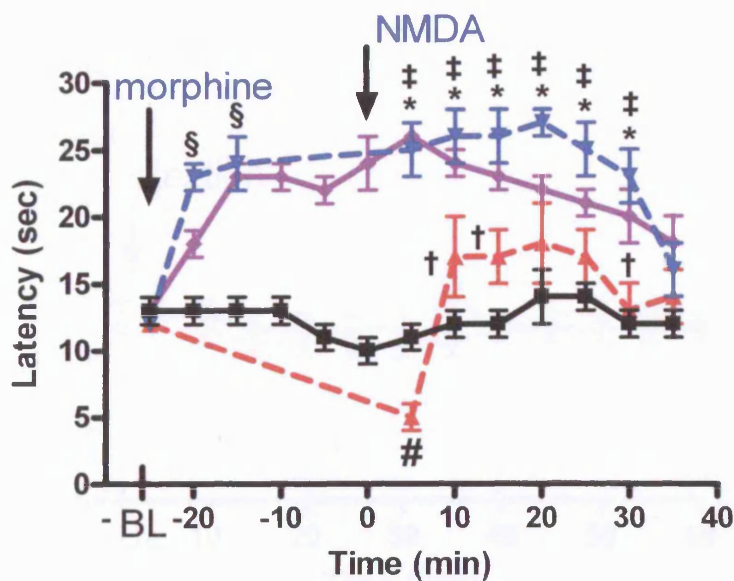


Fig. 4.4- The effects of the combination of morphine+NMDA (blue, n=8) and NMDA alone (red, 0.3μg, n=8), morphine alone (purple, 3mg/kg, n=11) and saline control (black, 24μl, n=6) on the rat paw withdrawal latency following thermal stimuli. BL depicts pre-drug baseline responses. NMDA caused a significant thermal hypersensitivity, when compared to saline control. Morphine, when administered prior to NMDA, was seen to significantly inhibit the NMDA-induced thermal hypersensitivity and also significantly increased paw withdrawal latency following thermal stimuli when compared to saline control. Furthermore, morphine on its own also caused an increase in paw withdrawal latency to thermal stimuli when compared to NMDA on its own and saline control. §$P<0.05$, where a significant increase from the baseline is seen following morphine administration (blue), *t*-test. *$P<0.05$, where a significant increase in paw withdrawal latency caused by morphine+NMDA, when compared to NMDA alone, is seen. #$P<0.05$, where a significant decrease in paw withdrawal latency was caused by NMDA alone, when compared to saline control. ‡$P<0.05$, where a significant increase in paw withdrawal latency was caused by each of morphine alone and morphine+NMDA, when compared to saline control. †$P<0.05$, where a significant increase in paw withdrawal latency was caused by morphine alone, when compared to NMDA alone. One way ANOVA followed by Tukey post-hoc test.

c) MK-801 alone- The subcutaneous administration of the NMDA receptor antagonist MK-801 (0.1mg/kg) caused no significant change from the subcutaneous saline control in all of the timepoints tested (n=7) (fig. 4.5).

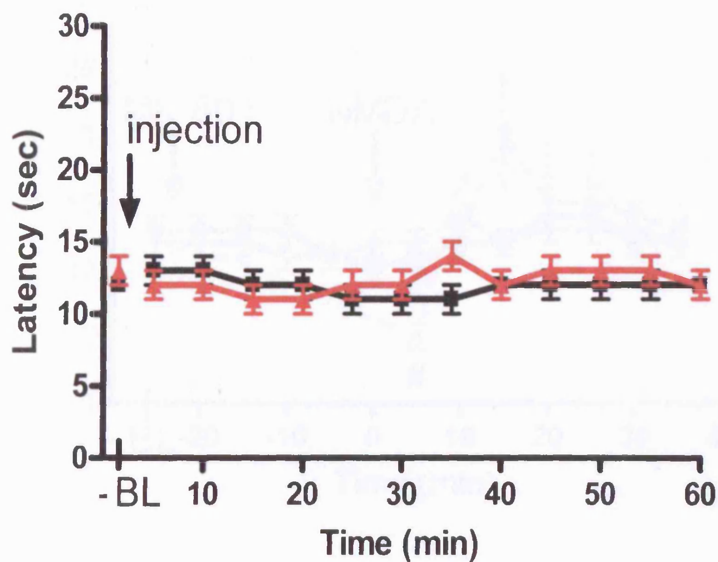


Fig. 4.5- The effects of subcutaneous MK-801 (red, 0.1mg/kg, n=7) and subcutaneous saline (black, 0.25ml, n=11) on rat paw withdrawal latency following thermal stimuli. BL depicts pre-drug baseline responses.

d) MK-801+NMDA- The subcutaneous administration of MK-801 (0.1mg/kg) twenty-five minutes before intrathecal NMDA (0.3μg) in the MK-801+NMDA treated rats prevented the occurrence of NMDA-induced thermal hypersensitivity at timepoint 5 minutes ($p<0.05$, n=8) (fig. 4.6).

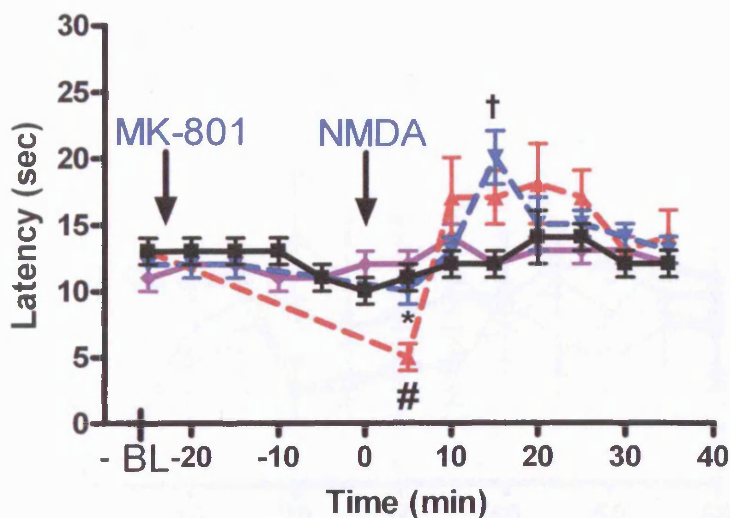


Fig. 4.6- The effects of the combination of MK-801+NMDA (blue, n=8) and NMDA alone (red, 0.3μg, n=8), MK-801 alone (purple, 0.1mg/kg, n=7) and saline control (black, 24μl, n=6) on the rat paw withdrawal latency following thermal stimuli. BL depicts pre-drug baseline responses. NMDA caused a significant thermal hypersensitivity, when compared to saline control and MK-801 on its own. MK-801, when administered prior to NMDA, significantly inhibited the NMDA-induced thermal hypersensitivity, returning the response to baseline level, and significantly increased paw withdrawal latency to thermal stimuli at timepoint 15min, when compared to saline control and MK-801 on its own. *$P<0.05$, where a significant increase in paw withdrawal latency was caused by MK-801+NMDA, when compared to NMDA alone. #$P<0.05$, where a significant decrease in paw withdrawal latency was caused by NMDA, when compared to saline control and MK-801 on its own. †$P<0.05$, where a significant increase in paw withdrawal latency was caused by MK-801+NMDA, when compared to saline. One way ANOVA followed by Tukey post-hoc test.

iv) NK-1 experiments

a) Sar-SP alone- Three doses of Sar-SP, 9, 3 and 1μg, were tested on the thermal response, in order to choose which one caused a significant thermal hypersensitivity with a short-lived BSL behaviour which would not interfere greatly with the testing. Doses 9μg and 1μg both caused 55±5 minutes of BSL behaviour, whereas dose 3μg, as well as causing significant thermal hypersensitivity, only led to 21±7 minutes of BSL behaviour (fig. 4.7). Therefore, Sar-SP dose 3μg was used for the subsequent experiments.

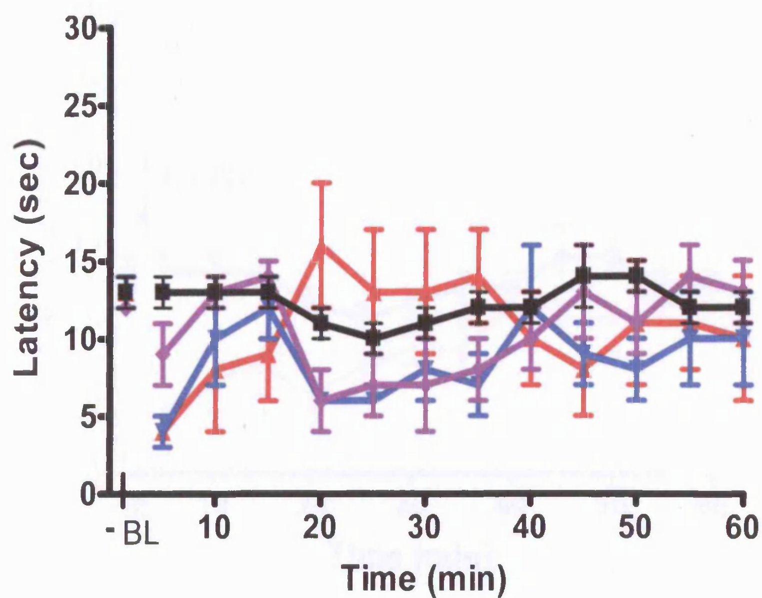


Fig. 4.7- The effects of doses 9 (red), 3 (blue) and 1μg (purple) Sar-SP and intrathecal saline (black, 24μl, n=6) on the rat paw withdrawal latency following thermal stimuli. BL depicts pre-drug baseline responses.

When compared to the intrathecal saline control, intrathecal Sar-SP (3μg) caused a significant decrease in paw withdrawal latency to heat stimuli, and thus thermal hypersensitivity ($p<0.05$, n=7) (fig. 4.8).

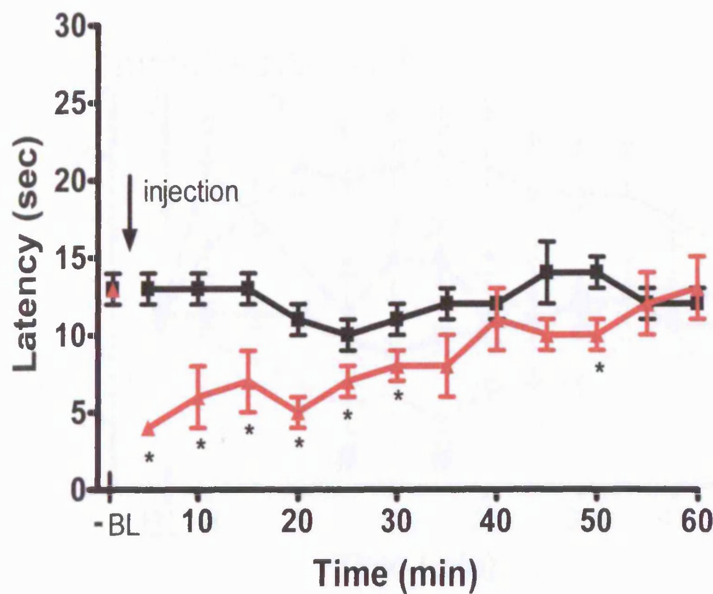


Fig. 4.8- The effects of intrathecal Sar-SP (red, 3µg, n=7) and intrathecal saline (black, 24µl, n=6) on rat paw withdrawal latency following thermal stimuli. BL depicts pre-drug baseline responses. Sar-SP caused thermal hypersensitivity when compared to the saline control. *P<0.05, where a significant decrease in paw withdrawal latency caused by Sar-SP, when compared to the saline control, is seen, *t*-test.

b) Morphine+Sar-SP- The subcutaneous administration of morphine (3mg/kg) ten minutes prior to intrathecal Sar-SP (3µg) in the morphine+Sar-SP treated rats resulted in a significant increase in paw withdrawal latency to thermal stimuli (p<0.05, n=7) (fig.4.9), when compared to the Sar-SP treated rats. This increase in paw withdrawal latency in the morphine+Sar-SP treated rats shifted the whole response back to baseline level, but not to the same level as when morphine was administered alone, except at timepoints 45 and 50 minutes, when morphine administered on its own was resuming baseline levels. Finally, there was an increase in the baseline in the morphine+Sar-SP treated rats, following morphine administration, which was significantly different to the old baseline before any drug addition, and this new baseline was the one used in the statistical analysis.

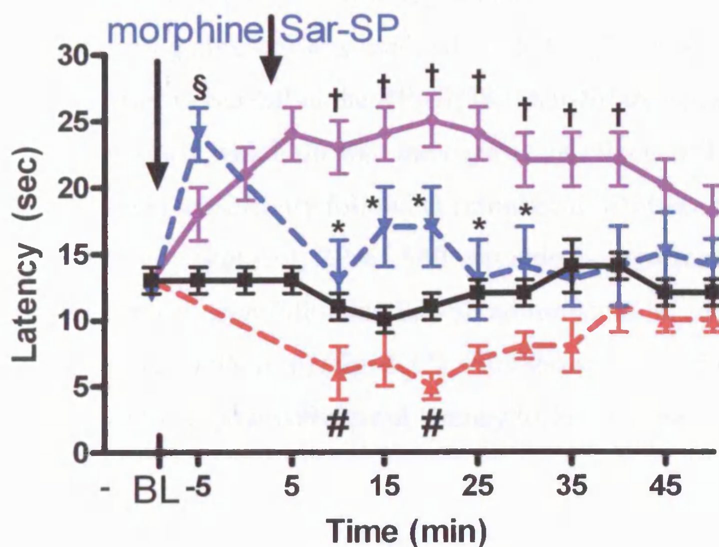


Fig. 4.9- The effects of the combination of morphine+Sar-SP (blue, n=7) and Sar-SP alone (red, 3μg, n=7), morphine alone (purple, 3mg/kg, n=11) and saline control (black, 24μl, n=6) on the rat paw withdrawal latency following thermal stimuli. BL depicts pre-drug baseline responses. Sar-SP significantly decreased paw withdrawal latency to thermal simuli, when compared to saline control. Morphine, when administered prior to Sar-SP, significantly inhibited the Sar-SP-induced thermal hypersensitivity, returning the response to baseline level. §P<0.05, where a significant increase from the baseline is seen following morphine administration (blue), *t*-test. *P<0.05, where a significant increase in paw withdrawal latency caused by morphine+Sar-SP, when compared to Sar-SP alone, is seen. #P<0.05, where a significant increase in paw withdrawal latency caused by morphine on its own, when compared to Sar-SP, saline control and the combined administration of morphine+Sar-SP is seen. †P<0.05, where a significant increase in paw withdrawal latency is seen following morphine on its own, when compared to saline control. One way ANOVA followed by Tukey post-hoc test.

c) RP-67,580 experiments- Initially, it seemed that the intrathecal administration of RP-67,580 (3μg) alone caused a significant increase in paw withdrawal latencies to thermal stimuli at timepoints 50 and 60 minutes (p<0.05, n=6) (fig. 4.10), when compared to the intrathecal saline control. Therefore, the timing of the administration of each of RP-67,580 (3μg) and Sar-SP (3μg) in the RP-67,580+Sar-SP treated rats, so that both agents peaked together, was based on the aforementioned, in that RP-67,580 was given 30 minutes prior to Sar-SP. However, this protocol turned out to be inaccurate, as when RP-67,580 was given 30 minutes before Sar-SP, in the RP-67,580+Sar-SP treated rats, it did not

reverse the thermal hypersensitivity seen due to Sar-SP (fig. 4.11). Therefore, another dosing protocol was used, where RP-67,580 was administered 10 minutes prior to Sar-SP in the RP-67,580+Sar-SP treated rats. This was based on a previous study which showed the significant effects of RP-67,580 in reversing thermal hypersensitivity following intrathecal SP (Hua, Chen et al. 1999). With the new dosing protocol, RP-67,580 was able to significantly reverse the thermal hypersensitivity seen following Sar-SP administration, in the RP-67,580+Sar-SP treated rats ($p<0.05$, n=6) (fig. 4.12), thus showing that there was no true effect of RP-67,580 on paw withdrawal latency following thermal stimuli.

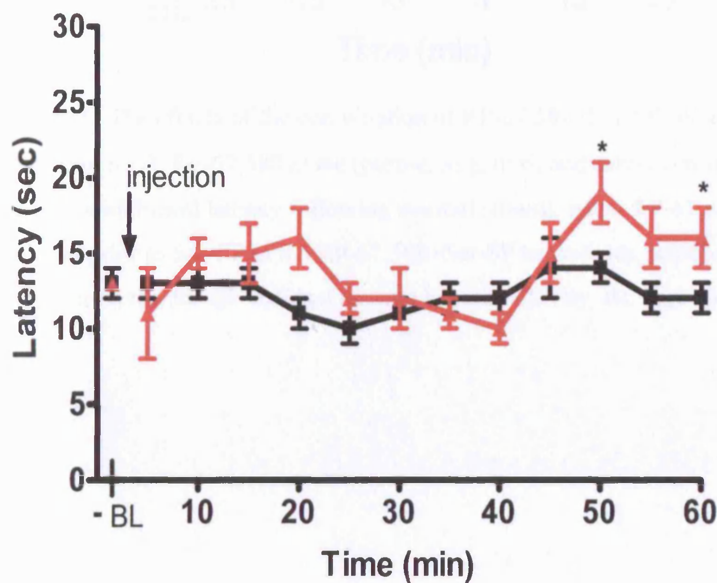


Fig. 4.10- The effects of intrathecal RP-67,580 (red, 3µg) and intrathecal saline (black, 24µl) on rat paw withdrawal latency following thermal stimuli. n= 6. BL depicts pre-drug baseline responses. RP-67,580 caused a significant increase in paw withdrawal latency to thermal stimuli when compared to the saline control. $^*P<0.05$, where a significant increase in paw withdrawal latency caused by RP-67,580, when compared to the saline control, is seen, *t*-test.

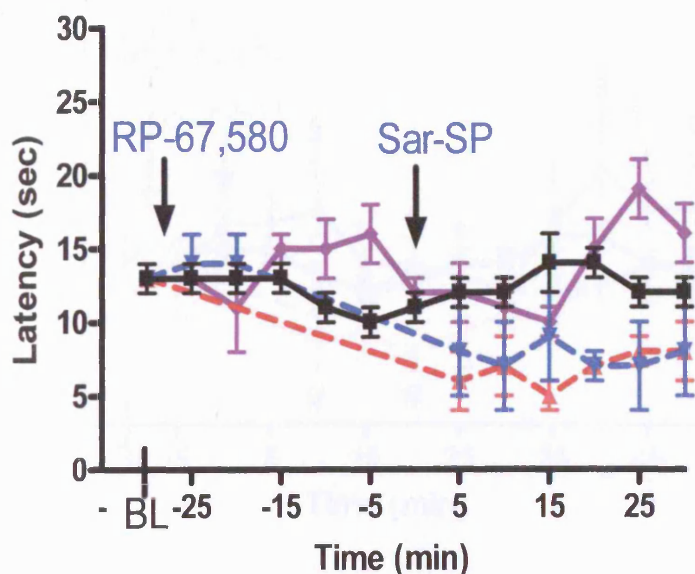


Fig. 4.11- The effects of the combination of RP-67,580+Sar-SP (blue, n=12) and Sar-SP alone (red, 3μg, n=7), RP-67,580 alone (purple, 3μg, n=6) and saline control (black, 24μl, n=6) on the rat paw withdrawal latency following thermal stimuli, when RP-67,580 is administered 30 minutes prior to Sar-SP in the RP-67,580+Sar-SP treated rats, which showed that RP67,580 had no effect on the Sar-SP-induced thermal hypersensitivity. BL depicts pre-drug baseline responses.

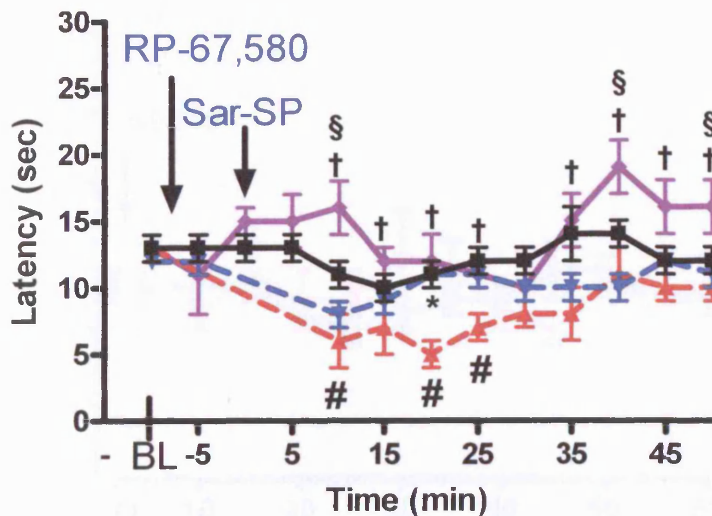


Fig. 4.12- The effects of the combination of RP-67,580+Sar-SP (blue, n=6) and Sar-SP alone (red, 3μg, n=7), RP-67,580 alone (purple, 3μg, n=6) and saline control (black, 24μl, n=6) on the rat paw withdrawal latency following thermal stimuli, when RP-67,580 is administered 10 minutes prior to Sar-SP in the RP-67,580+Sar-SP treated rats. BL depicts pre-drug baseline responses. Sar-SP caused a significant decrease in paw withdrawal latency to thermal stimuli, when compared to saline control. RP-67,580, when administered prior to Sar-SP, was seen to significantly inhibit the Sar-SP-induced thermal hypersensitivity, returning the response to baseline level. *P<0.05, where a significant increase in paw withdrawal latency caused by RP-67,580+Sar-SP, when compared to Sar-SP alone, is seen. #P<0.05, where a significant decrease in paw withdrawal latency was caused by Sar-SP, when compared to saline control. †P<0.05, where a significant increase in paw withdrawal latency was seen, when comparing RP-67,580 to Sar-SP. §P<0.05, where a significant increase in paw withdrawal latency was seen, when comparing RP-67,580 to the combined effect of RP-67,580+Sar-SP. One way ANOVA followed by Tukey post-hoc test.

d) DMSO alone- Since RP-67,580 was dissolved in 20% DMSO, it was essential to investigate whether this agent caused any effects when administered on its own. I found that 20% DMSO resulted in no significant change in paw withdrawal latency to thermal stimuli, when compared to the intrathecal saline control (n=6) (fig. 4.13).

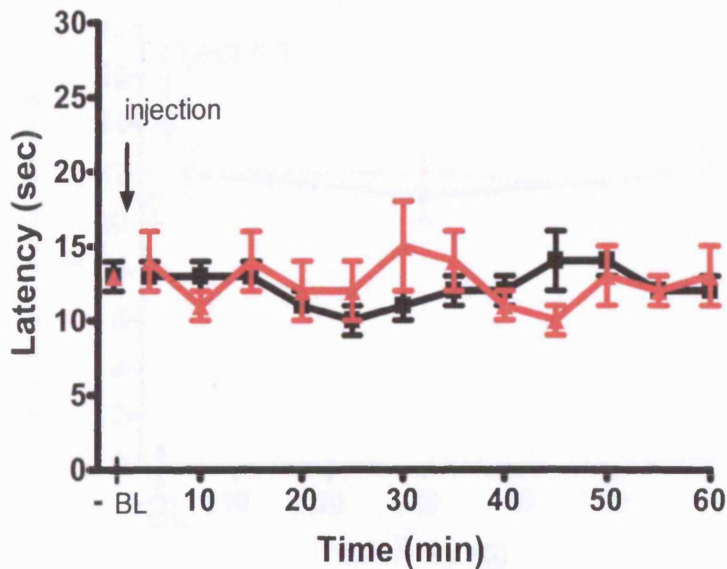


Fig. 4.13- The lack of effect of intrathecal DMSO (red, 24µl) and intrathecal saline (black, 24µl) on rat paw withdrawal latency following thermal stimuli. n= 6. BL depicts pre-drug baseline responses.

4.3.3 Behavioural response to tactile stimuli using von Frey monofilaments

i) Saline control experiments

Neither the subcutaneous (n=11), nor the intrathecal (sterile, n=10) administration of saline resulted in a significant change from the baseline in the paw withdrawal threshold following tactile stimuli. Furthermore, no significant change in the paw withdrawal threshold was seen when the two routes of saline administration were compared (figs 4.14).

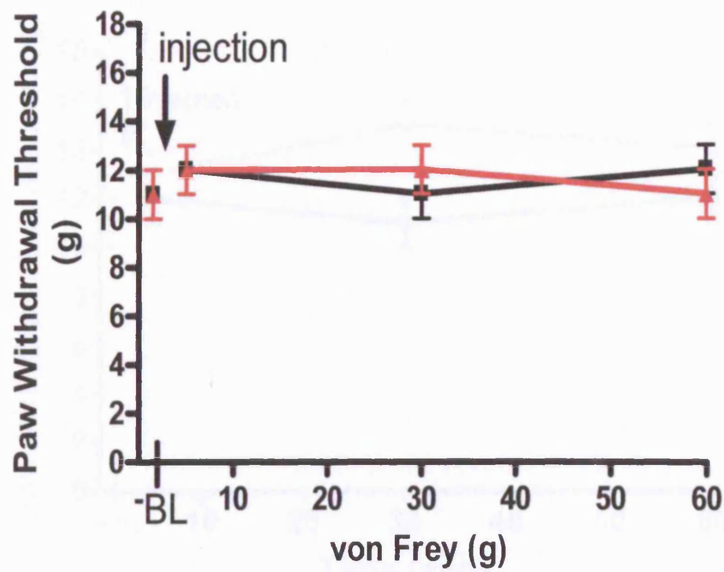


Fig. 4.14- The lack of effect of subcutaneous (black, 0.25ml, n=11) and intrathecal (red, 24μl, n=10) saline on the rat paw withdrawal threshold following tactile stimuli. BL depicts pre-drug baseline responses.

ii) Morphine experiments

When compared to the subcutaneous saline control, the subcutaneous administration of morphine (3mg/kg) caused a significant increase in paw withdrawal threshold to mechanical stimuli, and thus analgesia, at timepoint 30 minutes ($p<0.05$, n=11) (fig. 4.15).

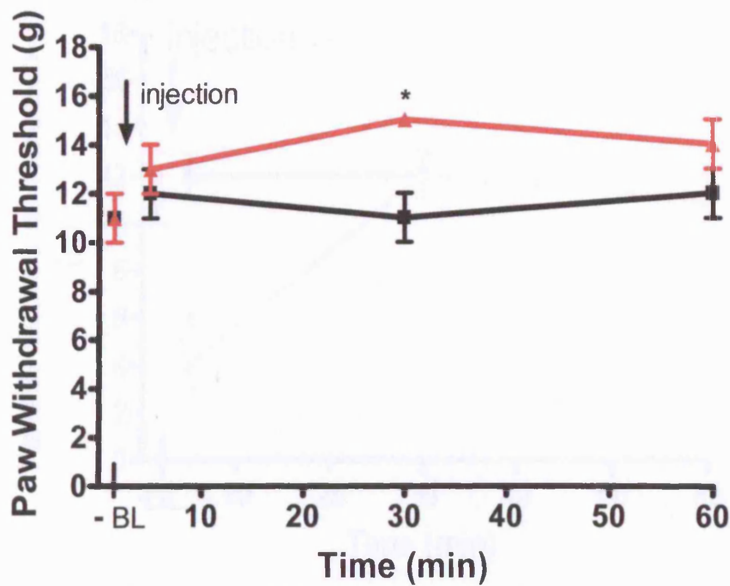


Fig. 4.15- The effects of subcutaneous morphine (red, 3mg/kg) and subcutaneous saline (black, 0.25ml) on rat paw withdrawal threshold following tactile stimuli, n=11. BL depicts pre-drug baseline responses. Morphine caused a significant increase in paw withdrawal threshold when compared to the saline control. *P<0.05, where a significant increase in paw withdrawal threshold caused by morphine, when compared to the saline control, is seen, *t*-test.

iii) NMDA experiments

a) NMDA alone- The intrathecal administration of NMDA (0.3μg) on its own caused a significant decrease in paw withdrawal threshold, and thus caused tactile hypersensitivity, at timepoint 5 minutes (p<0.05, n=8), when compared to the intrathecal saline control (4.16).

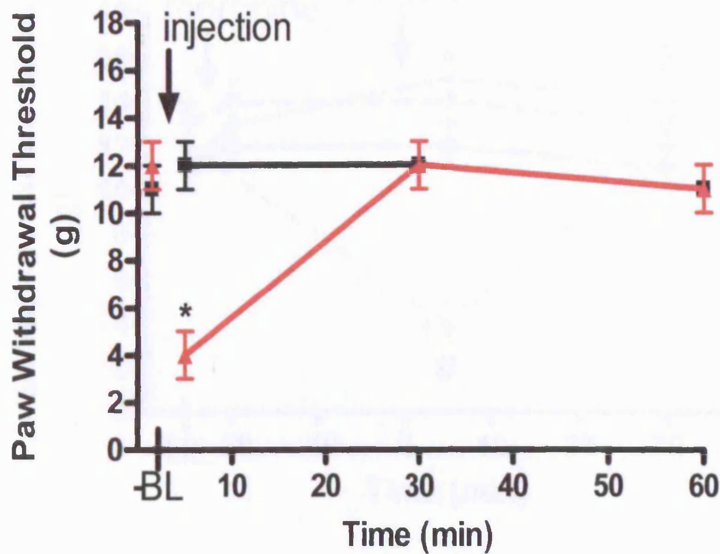


Fig. 4.16- The effects of intrathecal NMDA (red, 0.3μg, n=8) and intrathecal saline (black, 24μl, n=10) on rat paw withdrawal threshold following tactile stimuli. BL depicts pre-drug baseline responses. NMDA caused tactile hypersensitivity when compared to the saline control. *P<0.05, where a significant decrease in paw withdrawal threshold caused by NMDA, when compared to the saline control, is seen, *t*-test.

b) Morphine+NMDA- The subcutaneous administration of morphine (3mg/kg) twenty-five minutes prior to the intrathecal administration of NMDA (0.3μg) in the morphine+NMDA treated rats caused analgesia, increasing the paw withdrawal threshold, when compared to the NMDA treated rats (fig. 4.17).

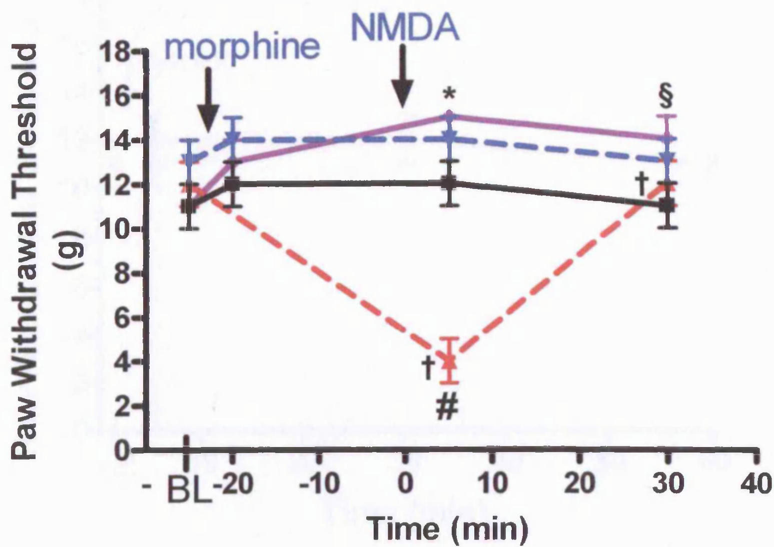


Fig. 4.17- The effects of the combination of morphine+NMDA (blue, n=8) and NMDA alone (red, 0.3μg, n=8), morphine alone (purple, 3mg/kg, n=11) and saline control (black, 24μl, n=10) on the rat paw withdrawal threshold following tactile stimuli. BL depicts pre-drug baseline responses. NMDA caused a significant decrease in paw withdrawal threshold, when compared to saline control, and to morphine on its own. Morphine, when administered prior to NMDA, prevented the NMDA-induced tactile hypersensitivity, returning the response to higher than saline control level. *P<0.05, where a significant increase in paw withdrawal threshold is caused by morphine+NMDA, when compared to NMDA alone, is seen. #P<0.05, where a significant decrease in paw withdrawal threshold was caused by NMDA, when compared to saline control. §P<0.05, where a significant increase in paw withdrawal threshold was seen when comparing morphine on its own to saline control. †P<0.05, where a significant increase in paw withdrawal threshold was seen when comparing morphine on its own to NMDA on its own. One way ANOVA followed by Tukey post-hoc test.

c) MK-801 alone- The subcutaneous administration of MK-801 (0.1mg/kg) did not cause the paw withdrawal threshold to significantly change, when compared with the subcutaneous saline control (n=8) (fig. 4.18).

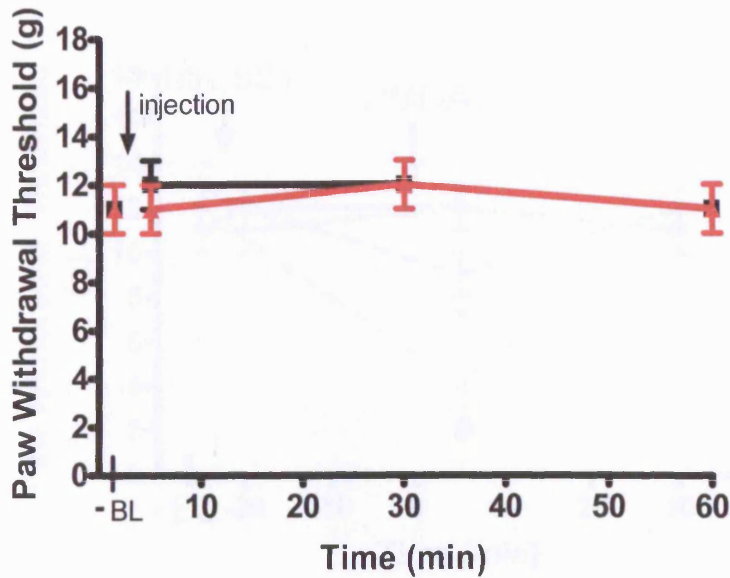


Fig. 4.18- The lack of effect of subcutaneous MK-801 (red, 0.1mg/kg, n=8) and subcutaneous saline (black, 0.25ml, n=11) on rat paw withdrawal threshold following tactile stimuli. BL depicts pre-drug baseline responses.

d) MK-801+NMDA- The subcutaneous administration of MK-801 (0.1mg/kg) twenty-five minutes before the intrathecal administration of NMDA (0.3μg) in the MK-801+NMDA treated rats caused a significant increase in paw withdrawal threshold at timepoint 5 minutes ($p<0.05$, n=8), when compared to the NMDA treated rats (fig. 4.19).

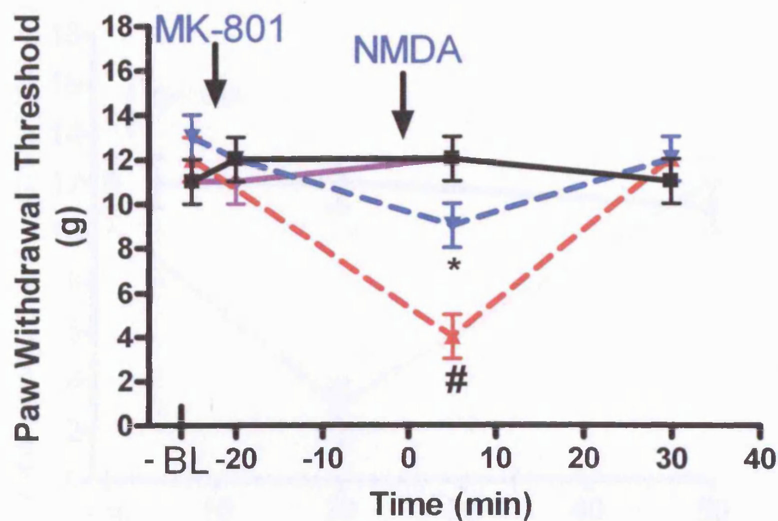


Fig. 4.19- The effects of the combination of MK-801+NMDA (blue, n=8) and NMDA alone (red, 0.3μg, n=8), MK-801 alone (purple, 0.1mg/kg, n=8) and saline control (black, 24μl, n=10) on the rat paw withdrawal threshold following tactile stimuli. BL depicts pre-drug baseline responses. NMDA caused a significant decrease in paw withdrawal threshold, when compared to saline control and to MK-801 on its own. MK-801, when administered prior to NMDA, prevented the NMDA-induced tactile hypersensitivity, but did not return the response to baseline level. *P<0.05, where a significant increase in paw withdrawal threshold caused by MK-801+NMDA, when compared to NMDA alone, is seen. #P<0.05, where a significant decrease in paw withdrawal threshold was caused by NMDA, when compared to saline control and MK-801 on its own. One way ANOVA followed by Tukey post-hoc test.

iv) NK-1 experiments

a) Sar-SP alone- When compared to the subcutaneous saline control, the intrathecal administration of Sar-SP (3μg) caused a significant decrease in paw withdrawal threshold at timepoints 5 and 20 minutes ($p<0.05$, n=6) (fig. 4.20). The greater tactile hypersensitivity seen at timepoint 20 minutes could be seen as further proof that the activity of Sar-SP peaks at this time.

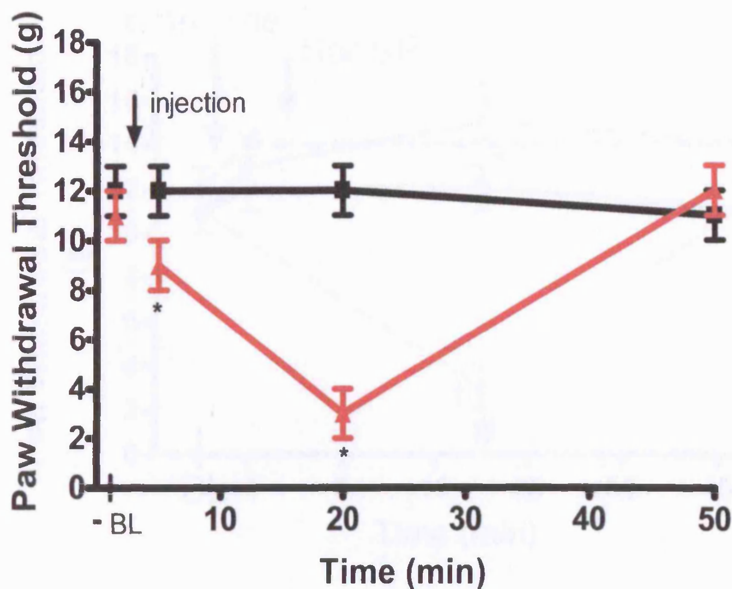


Fig. 4.20- The effects of intrathecal Sar-SP (red, 3µg, n=8) and intrathecal saline (black, 24µl, n=10) on rat paw withdrawal threshold following tactile stimuli. BL depicts pre-drug baseline responses. Sar-SP caused tactile hypersensitivity when compared to the saline control. *P<0.05, where a significant decrease in paw withdrawal threshold caused by Sar-SP, when compared to the saline control, is seen, *t*-test.

b) Morphine+Sar-SP- The subcutaneous administration of morphine (3mg/kg) twenty-five minutes before the intrathecal administration of Sar-SP (3µg) in the morphine+Sar-SP treated rats caused analgesia at timepoints 20 minutes ($p<0.05$, n=8), when compared to the Sar-SP treated rats (fig. 4.21). This increase was enough to make the paw withdrawal threshold at this timepoint at the same level as when the rats were treated only with morphine.

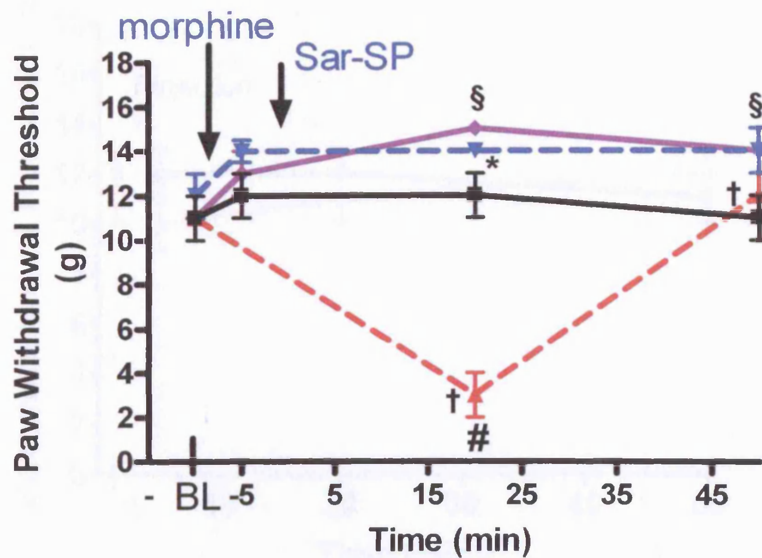


Fig. 4.21- The effects of the combination of morphine+Sar-SP (blue, n=8) and Sar-SP alone (red, 3μg, n=8), morphine alone (purple, 3mg/kg, n=11) and saline control (black, 24μl, n=10) on the rat paw withdrawal threshold following tactile stimuli. BL depicts pre-drug baseline responses. Sar-SP caused a significant decrease in paw withdrawal threshold, when compared to saline control and to morphine on its own. Morphine, when administered prior to Sar-SP, prevented the Sar-SP-induced tactile hypersensitivity, returning the response to higher than saline control level. *$P<0.05$, where a significant increase in paw withdrawal threshold caused by morphine+Sar-SP, when compared to Sar-SP alone, is seen. #$P<0.05$, where Sar-SP caused a significant decrease in paw withdrawal threshold, when compared to saline control. †$P<0.05$, where Sar-SP caused a significant decrease in paw withdrawal threshold, when compared to morphine on its own. §$P<0.05$, where morphine on its own, and when combined with Sar-SP, caused a significant increase in paw withdrawal threshold, when compared to saline control. One way ANOVA followed by Tukey post-hoc test.

c) RP-67,580 experiments- The intrathecal administration of RP-67,580 (3μg) on its own did not cause any significant difference in the paw withdrawal threshold following mechanical stimuli, when compared with the intrathecal saline control (n=7) (fig. 4.22).

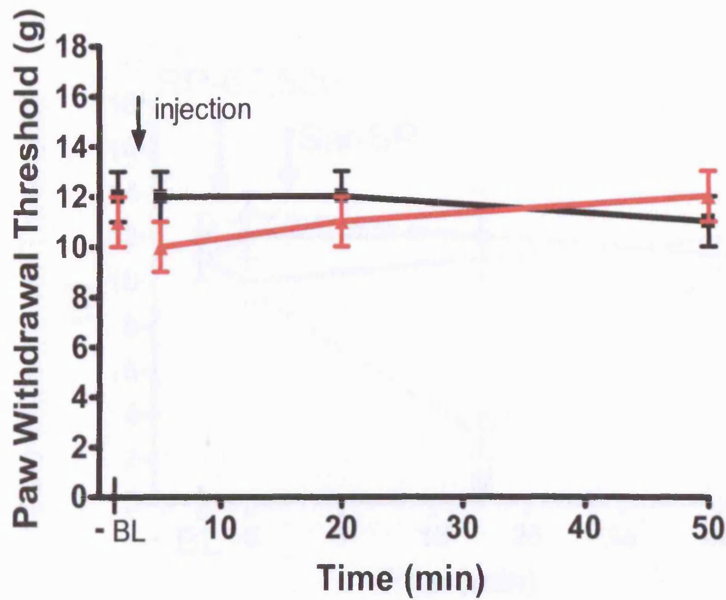


Fig. 4.22- The lack of effect of intrathecal RP-67,580 (red, 3μg, n=7) and intrathecal saline (black, 24μl, n=10) on rat paw withdrawal threshold following tactile stimuli. BL depicts pre-drug baseline responses.

The intrathecal administration of RP-67,580 (3μg), thirty minutes prior to that of Sar-SP(3μg), in the RP-67,580+Sar-SP treated rats caused a significant increase in the paw withdrawal threshold at timepoint 20 minutes, when compared to the Sar-SP treated rats ($p<0.05$, n=8) (fig. 4.23).

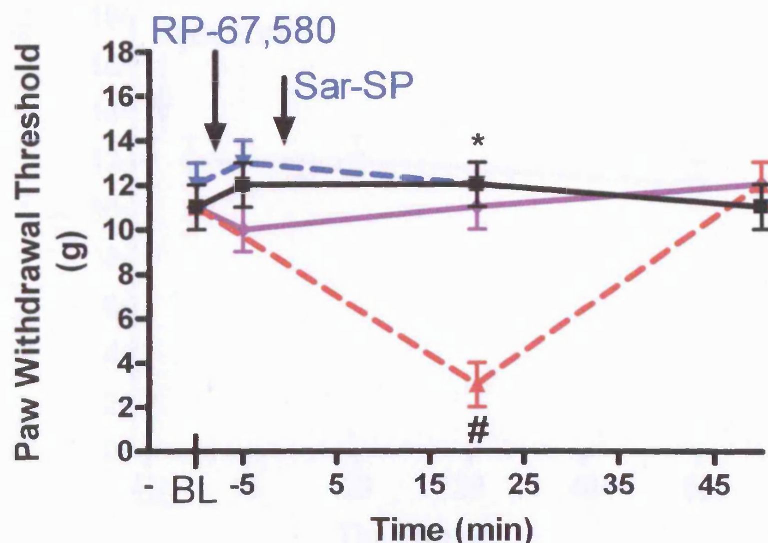


Fig. 4.23- The effects of the combination of RP-67,580+Sar-SP (blue, n=6) and Sar-SP alone (red, 3μg, n=8), RP-67,580 alone (purple, 3μg, n=7) and saline control (black, 24μl, n=10) on the rat paw withdrawal threshold following tactile stimuli, when RP-67,580 is administered 10 minutes prior to Sar-SP in the RP-67,580+Sar-SP treated rats. BL depicts pre-drug baseline responses. Sar-SP caused a significant increase in paw withdrawal threshold, when compared to saline control. RP-67,580, when administered prior to Sar-SP, prevented the Sar-SP-induced tactile hypersensitivity, returning the response to baseline level. *P<0.05, where a significant increase in paw withdrawal threshold caused by RP-67,580+Sar-SP, when compared to Sar-SP alone, is seen. #P<0.05, where Sar-SP caused a significant decrease in paw withdrawal threshold, when compared to saline control and to RP-67,580 on its own. One way ANOVA followed by Tukey post-hoc test.

d) DMSO alone- The intrathecal administration of 20% DMSO did not cause any significant change in the paw withdrawal threshold when compared to the intrathecal saline control (n=10) (fig. 4.24).

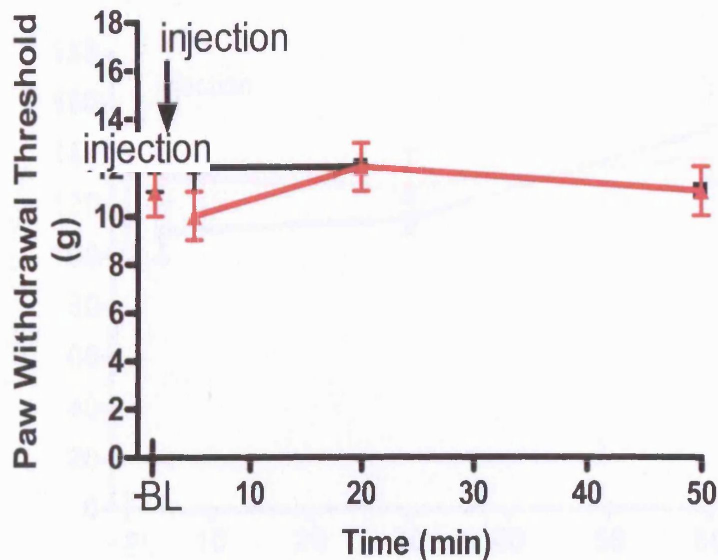


Fig. 4.24- The lack of effect of intrathecal DMSO (red, 24μl) and intrathecal saline (black, 24μl) on rat paw withdrawal threshold following tactile stimuli. n= 10 for both. BL depicts pre-drug baseline responses.

4.3.4 Rotarod experiments

i) Morphine experiment

Morphine (3mg/kg) was the only drug used in this study which significantly increased both the paw withdrawal latency to thermal stimuli and the paw withdrawal threshold following mechanical stimuli, before the chemical induction of hyperalgesia with NMDA and Sar-SP. Therefore, rotarod experiments were performed on rats injected with morphine (3mg/kg), and compared to subcutaneous saline controls, to make sure that morphine at this dose and *via* this route was not causing sedation. In agreement with an earlier study (Urch, Donovan-Rodriguez et al. 2005), these experiments showed that there was no significant difference in the time spent on the beam between the rats that were injected with subcutaneous saline (n=6) and those that were injected with subcutaneous morphine (n=7) (fig. 4.25).

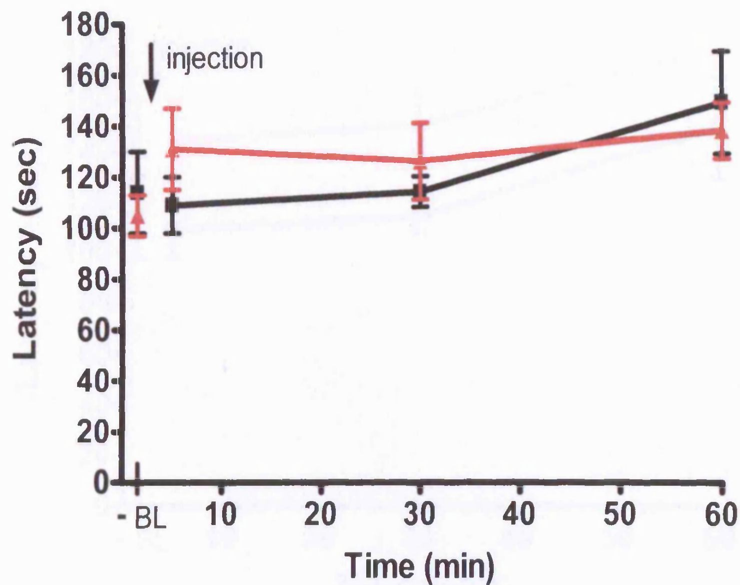


Fig. 4.25- The time in seconds taken to fall off the rotarod beam following subcutaneous saline (black, 0.25ml, n=6) and subcutaneous morphine (red, 3mg/kg, n=7) administration in normal rats. BL depicts pre-drug baseline responses.

ii) MK-801 experiment

MK-801 (0.1mg/kg) has been shown to cause motor disturbances by a previous study (Coderre and Van Empel 1994), which is why I have tested if the dose used in this study had any effects on ambulation, using the rotarod. These results show that there was no significant difference in the time spent on the beam between the rats that were injected with subcutaneous saline (n=6) and those that were injected with 0.1mg/kg subcutaneous MK-801 (n=8) (fig. 4.26).

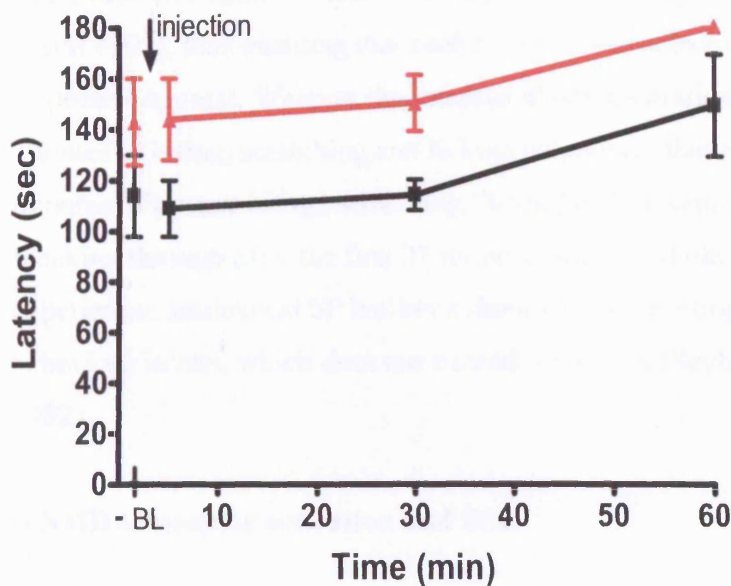


Fig. 4.26- The time taken to fall off the rotarod beam following subcutaneous saline (black, 0.25ml, n=6) and subcutaneous MK-801 (red, 3mg/kg, n=8) administration in normal rats. BL depicts pre-drug baseline responses.

4.4 Discussion

I have shown in my study that the activation of both NMDA and NK-1 receptors caused biting, scratching and licking behaviour. However, grooming was only observed after NK-1 receptor activation, which also induced a longer lasting nociceptive hypersensitivity. Each respective antagonist used was more effective in inhibiting the hypersensitivities than the biting, scratching and licking behavioural response. Furthermore, even though morphine significantly inhibited the thermal and tactile hypersensitivity which arose following the activation of these two receptors, morphine was more effective in inhibiting the NMDA receptor activation induced events, than the ones due to NK-1 receptor activation.

4.4.1 Biting, scratching and licking behaviour

NMDA is a very potent and selective NMDA receptor agonist, and Sar-SP is an NK-1 selective agonist which is more potent and longer lasting than SP (Yip and Chahl 1999), thus ensuring that each receptor was selectively activated by its respective agonist. Whereas the intrathecal administration of NMDA caused 5 minutes of biting, scratching and licking behaviour, that of Sar-SP caused 21 minutes of intense biting, scratching, licking and grooming behaviour which kept breaking through after the first 21 minutes, for the whole duration of the experiment. Intrathecal SP has been shown to cause biting, scratching and licking behaviour in rats, which does not exceed 3 minutes (Seybold, Hylden et al. 1982).

i) NMDA receptor activation and BSL

An important point which needs to be made is that even though glycine, and glutamate are available in the spinal cord to act on the NMDA receptor, this receptor still needs the summation of the depolarisations caused by glutamate and SP, acting on non-NMDA receptors and the NK-1 receptor respectively, in order for the magnesium block to be removed and the receptor's activation to occur (Thompson, King et al. 1990; Xu, Dalsgaard et al. 1992). However, the intrathecal administration of NMDA in this and previous studies (Aanonsen, Lei et al. 1990; Davis and Inturrisi 2001) did cause a short-lasting hyperalgesia, following the BSL behaviour, which means that the receptor was activated and did move the dorsal horn spinal cord into increased excitability.

Several subunits of the NMDA receptor exist, with different sensitivities to the Mg^{2+} block. The NR2D subunit has a low sensitivity to Mg^{2+} block, but it is mostly found extrasynaptic in the spinal cord, with lower levels found in the central terminal of primary afferent fibres. However, the two subunits found mostly in the spinal cord are the NR2A and NR2B subunits, and they have a high sensitivity to the Mg^{2+} block (Petrenko, Yamakura et al. 2003). Therefore, I will postulate, like the study of Chapman *et al* suggested, in 1994, that the NMDA receptor activation could be submaximal and still lead to facilitation in dorsal horn neuronal responses (Chapman, Dickenson et al. 1994). Additionally, it

could be that the BSL behaviour which arises immediately after the administration of intrathecal NMDA, is responsible for aiding in the activation of the NMDA receptor and the resulting hypersensitivities (discussed in more detail below in paragraph **v**).

ii) The effect of morphine on BSL behaviour

In my study, morphine only significantly decreased the behaviour which arised following the activation of the NMDA receptor, but did not abolish it. This was also true in the study by (Aanonsen and Wilcox 1987), which used intrathecal morphine and NMDA in mice, and showed that a higher dose of morphine was needed to block the behavioural response following intrathecal NMDA, than that needed to reduce the hyperalgesic response. Furthermore, the finding in my study that intrathecal morphine did not inhibit the behaviour due to the activation of the NK-1 receptor was also shown in the study by Bossut et al, using intrathecal SP to activate the NK-1 receptor (Bossut, Frenk et al. 1988).

iii) The effect of the antagonists on BSL behaviour

Previous studies have shown that the spinal NK-1 and NMDA receptors interact, since the co-administration of SP and NMDA potentiate each other's effects. The latter was shown in previous behavioural studies, where the intrathecal co-administration of SP and NMDA, each in a lower dose that did not lead to effects in mice, caused the potentiation of the biting and scratching response, as well as the potentiation of both phases of the formalin response (Mjellem-Joly, Lund et al. 1991; Mjellem-Joly, Lund et al. 1992), which could also mean that the effects seen after the activation of one of these receptors is partly due to the activation of the other. Indeed, one behavioural study has shown that the intrathecal administration of an NMDA antagonist decreases the biting, scratching and licking behaviour which occurs following both intrathecal SP and NMDA in rats (Okano, Kuraishi et al. 1993). In addition, the intrathecal administration of each of SP and NMDA facilitated the flexor reflex in decrebrated rats, and the administration of an NMDA antagonist was effective in reducing both the SP-

and NMDA-induced facilitation of the flexor reflex (Xu, Dalsgaard et al. 1992). Furthermore, the nociceptive behaviour which occurred following intrathecal NMDA was partly reduced by the NK-1 receptor antagonist RP-67,580 (Tan-No, Esashi et al. 2004).

The concentration of the NK-1 and NMDA antagonists used in this study, RP-67,580 and MK-801, respectively, must have been too low to inhibit the behaviour seen due to the activation of any of the NK-1 and NMDA receptors. The lack of effect of low-dose MK-801 on the BSL behaviour was shown in previous studies, the first using intrathecal D-APV in mice (Aanonsen and Wilcox 1987), and the second using intrathecal CPP in rats (Alvarez-Vega, Baamonde et al. 2000), where it was shown that higher doses of NMDA receptor antagonists are needed to inhibit the behavioural response than those needed to inhibit the hyperalgesic and analgesic responses following intrathecal NMDA, respectively. The low dose of MK-801 was chosen in order to cause significant block of the NMDA receptor, whilst avoiding the motor effects associated with higher doses of MK-801 (Coderre and Van Empel 1994). Additionally, the dose and route of administration of RP-67,580 were also based on a previous study, which reported a weak CNS penetration of RP-67,580 after systemic administration (Parsons, Honda et al. 1996; Pawlak, Schmidt et al. 2001; Rupniak, Carlson et al. 2003). Therefore, RP-67,580 was administered intrathecally.

Earlier studies have shown that each of SP and Glutamate enhance each other's release (Malcangio, Fernandes et al. 1998; Hua, Chen et al. 1999), and that the NK-1 antagonist RP-67,580 was able to significantly reduce part of the BSL behaviour arising from intrathecal NMDA (Liu, Mantyh et al. 1997). However, in my study, the intrathecal administration of saline (0.9%) did not cause any of the BSL behaviour, and the behaviour which occurred following the activation of each of the NK-1 and NMDA receptors was different in quality, with NMDA receptor activation causing biting, scratching and licking, whereas NK-1 receptor activation causing BSL as well as excessive grooming. Therefore, I conclude that the BSL following the activation of each of the NK-1 and NMDA receptor is due

partly to the activation of each receptor by its agonist, and partly due to the effects of the other receptor.

iv) What does BSL behaviour show?

A previous study has shown that excitatory amino acids which do not cause nociception and which do not act on the NMDA receptor also cause the BSL behaviour seen in this study (Aanonsen and Wilcox 1987), and that only if morphine or any other known analgesic can reduce that behaviour, should the behaviour be linked to pain sensation. Furthermore, other studies postulated that the behaviour elicited by intrathecal SP, as opposed to that elicited by intrathecal glutamate, is not indicative of pain, but rather, that this behaviour indicates a motoneuron response (Nagy, Maggi et al. 1993), and a spinal convulsive state (Bossut, Frenk et al. 1988). The latter study also showed that spinalisation did not affect the SP-induced behaviour, whereas spinalisation potentiated the behaviour due to intrathecal glutamate. Additionally, the same behaviour seen following intrathecal SP is also seen following acetic acid (0.1N) or hypertonic saline (6%) administration (Wilcox 1988). Therefore, care must be taken when extrapolating from any BSL behaviour.

Higher doses of intrathecal NMDA have led to vocalisation (Aanonsen and Wilcox 1987; Wilcox 1988), but not excessive grooming, whereas even the highest dose of intrathecal SP does not cause vocalisation (Wilcox 1988), and in my study, low to high doses of Sar-SP all cause grooming and no vocalisation, whereas the dose of NMDA used here was not enough to cause vocalisation. If vocalisation is a marker for the sensation of pain, and since morphine only inhibited the BSL behaviour due to the activation of the NMDA receptor, then one could postulate that only the BSL behaviour which occurs following the NMDA receptor activation depicts pain sensation (Aanonsen and Wilcox 1987; Wilcox 1988; Alvarez-Vega, Baamonde et al. 2000).

v) Conclusion

Even though the activation of each the NMDA and the NK-1 receptors caused thermal and tactile hypersensitivities, BSL behaviour due to the activation of each of these receptors still constitutes a grey area as to which sensation is being depicted.

However, since it has been shown by my study, as well as previous ones, that lower doses of antagonist or morphine were required to reduce the hyperalgesic responses seen following the activation of each of the NK-1 and the NMDA receptor, than the dose needed to block the behaviour seen following the activation of each of these receptors (Aanonsen and Wilcox 1987; Wilcox 1988; Alvarez-Vega, Baamonde et al. 2000), then one could postulate that the secondary mechanisms underlying BSL behaviour and hypersensitive responses could be distinct, even though both responses partly begin with the activation of the receptor in question.

In addition, since the BSL behaviour following the activation of one receptor can be partly inhibited by antagonists at the other receptor (Okano, Kuraishi et al. 1993; Liu, Mantyh et al. 1997; Tan-No, Esashi et al. 2004), and since other compounds besides those acting at the NK-1 and the NMDA receptors may also lead to BSL behaviour (Aanonsen and Wilcox 1987; Wilcox 1988), this behaviour which arises immediately following the intrathecal administration of each agonist can serve to 'prime' the spinal cord dorsal horn neurons into excitability, by aiding to remove the Mg^{2+} block of the NMDA receptor for example, and thus causing the manifestation of the hypersensitivities seen in previous studies, as well as mine.

4.4.2 NMDA and NK-1 receptor activation and hypersensitivity response

Each of NMDA and Sar-SP caused thermal hypersensitivity in the rats, which manifested itself as a decrease in the latency of paw withdrawal to radiant heat. However, NMDA only caused 5 minutes of such a hypersensitive response, and then caused analgesia for some minutes, until the response returned to baseline,

whereas Sar-SP caused a more prolonged hypersensitivity to thermal stimuli, which lasted for half of the duration of the experiment.

Furthermore, each of NMDA and Sar-SP also caused tactile hypersensitivity in the rats. Again, whereas NMDA only caused 5 minutes of tactile hypersensitivity in the rats, with the response normalising 30 minutes after its administration, Sar-SP caused significant tactile hypersensitivity at both 5 and 20 minutes following its administration.

i) NMDA receptor activation

The dose of NMDA used in this study was based on a previous study showing that this dose caused both BSL and thermal hyperalgesia (Davis and Inturrisi 2001). The biphasic effect of NMDA receptor activation has been shown by previous studies, both electrophysiological (Chapman, Dickenson et al. 1994) and behavioural (Raigorodsky and Urca 1987; Kolhekar, Meller et al. 1993). The analgesia seen following the administration of NMDA could be due to the activation of descending inhibitions. Descending inhibitions, as discussed in section **1.8.2**, originate from the RVM and are thought to inhibit spinal cord dorsal horn excitability (Millan 2002). NMDA administration in the rat PAG has been previously shown to cause analgesia (Jacquet 1988), and in agreement with my study, intrathecal NMDA has been previously shown to have a bidirectional effect causing both nociception and antinociception (Raigorodsky and Urca 1987; Chapman, Dickenson et al. 1994). Indeed, in one study, spinalisation blocked the NMDA-induced inhibition in a previous behavioural study (Kolhekar, Meller et al. 1993). However, in another study, spinalisation seemed to enhance NMDA-mediated analgesia, which was taken to mean that the mechanisms underlying NMDA-induced analgesia could be spinal (Raigorodsky and Urca 1987). The differences between these last two studies are the area and method of spinalisation, whereas Kolkehar *et al* spinalised the rats reversibly by cold block at the segments T8-T10, Raigorodsky *et al* transected the spinal cord at the T5-T7 segments.

ii) NK-1 receptor activation

The hypersensitive effects of Sar-SP seem to be distinct from its behavioural BSL action: the latter starts immediately after administration and lasts for 21 minutes. In fact, it seems as though the hypersensitive action of Sar-SP starts when its behavioural action subsides, because even though it might seem that the BSL behaviour could be masking the hypersensitivity in the hargreaves test, the mechanical test involving the von Frey monofilaments showed that the peak action of Sar-SP was at 20 minutes following its intrathecal administration. Therefore, as was confirmed in a previous study (Rygh, Suzuki et al. 2006), intrathecal Sar-SP exerted its maximal hypersensitive effect 20 minutes after administration.

4.4.3 Antagonist studies

In my study, the administration of each of MK-801 and RP-67,580 on its own did not alter the baseline level of pain transmission, but did reduce the thermal and tactile hypersensitivity which occurred following the administration of each of NMDA and Sar-SP, respectively. This is in agreement with many studies which showed that neither the NMDA (Dickenson and Sullivan 1987; Haley, Sullivan et al. 1990; Davar, Hama et al. 1991; Yamamoto and Yaksh 1992; Ma, Allchorne et al. 1998) nor the NK-1 receptor (Seguin, Le Marouille-Girardon et al. 1995; Parsons, Honda et al. 1996; Coudore-Civiale, Courteix et al. 1998; Ma, Allchorne et al. 1998) is thought to be involved in the baseline transmission of noxious stimuli.

These previous studies showed that antagonists at each of the NMDA and the NK-1 receptor are effective in decreasing hyperalgesia due to: surgical (Davar, Hama et al. 1991; Yamamoto and Yaksh 1992; Coudore-Civiale, Courteix et al. 1998) or diabetes-induced neuropathy (Coudore-Civiale, Courteix et al. 1998), the second but not the first phase of the formalin response (Seguin, Le Marouille-Girardon et al. 1995) and in the CFA model of inflammation (Parsons, Honda et al. 1996; Ma, Allchorne et al. 1998), as well as decreasing the second but not

first phase of dorsal horn neuronal excitability following formalin (Haley, Sullivan et al. 1990), and the hyperexcitability of spinal cord dorsal horn neurons (Dickenson and Sullivan 1987), but not baseline responses. The fact that each of MK-801 and RP-67,580 reversed the thermal and tactile hypersensitivity due to the administration of each of NMDA and Sar-SP, respectively, shows that each of the nociceptive responses seen in this study was due to the activation of each specific receptor.

4.4.4 Morphine modulation of the thermal and tactile hypersensitivity

The administration of morphine on its own led to analgesia, causing significant increases in paw withdrawal latency and threshold to thermal and tactile stimuli, respectively, to levels higher than baseline responses. With the morphine dose used in my study, which was based on previous studies (Field, McCleary et al. 1999; Urch, Donovan-Rodriguez et al. 2005), the analgesia due to morphine occurred without sedation. The lack of sedation was demonstrated when I showed that the rats injected with this dose of morphine stayed on the rotarod beam for the same period of time as those injected with saline.

i) Thermal hypersensitivity

When morphine was administered with each of NMDA and Sar-SP, it was effective in increasing the latency of paw withdrawal to radiant heat. However, the effect of morphine, when NMDA activation had occurred, was even greater than when morphine was acting on its own, thus showing some kind of synergism between the analgesic effects of the μ-opioid receptor and those of the NMDA receptor, as the increase in morphine analgesia when the NMDA receptor was activated, correlated well in time with the analgesia due to when only the NMDA receptor was activated. As was previously mentioned above, supraspinal (Jacquet 1988) and spinal (Raigorodsky and Urca 1987; Chapman, Dickenson et al. 1994) NMDA receptor activation has been shown to cause antinociception, and morphine exerts part of its analgesic action *via* supraspinal sites (Heinricher 1997). Contrary to the synergism with NMDA, the effect of

morphine, when the NK-1 receptor was activated, only extended to normalising the response to baseline, and was less than when morphine was administered on its own.

ii) Tactile hypersensitivity

When morphine and each of NMDA and Sar-SP were administered together, morphine significantly increased the responses to tactile stimuli to levels higher than baseline in each case.

4.4.5 NMDA versus NK-1

The activation of each of the spinal NMDA and NK-1 receptors has been shown to be involved in wind-up and central sensitisation (see sections **1.7.4** and **3.4.2**) (Xu, Dalsgaard et al. 1992; Dougherty, Palecek et al. 1993; Rusin, Bleakman et al. 1993; Rusin, Jiang et al. 1993; Yaksh, Hua et al. 1999). Central sensitisation is thought to underlie abnormal pain sensations, such as hyperalgesia and allodynia, seen in models of chronic pain states, such as neuropathic pain.

However, although the behaviour arising after intrathecal NMDA and Sar-SP is quite distinct in quality and time, numerous studies have shown that the activation of one of the NMDA or NK-1 receptors ultimately leads to the activation of the other, since presynaptic NMDA receptors cause further release of SP from primary afferent terminals (Liu, Wang et al. 1994; Liu, Mantyh et al. 1997; Malcangio, Fernandes et al. 1998), and since SP and glutamate co-exist on some primary afferent terminals (Battaglia and Rustioni 1988; De Biasi and Rustioni 1988). In addition, following a strong stimulus/injury, the activation of each of NMDA and NK-1 will cause the phosphorylation and activation of the other, as well as the synthesis of nitric oxide which will retrogradely release more transmitter from the primary afferent terminals (Rusin, Bleakman et al. 1993; Hua, Chen et al. 1999; Yaksh, Hua et al. 1999). Therefore, this means that part of the response involved with the spinal activation of one receptor is due to the enhanced spinal activation of, and interaction with, the other.

Since the activation of one of the NMDA and NK-1 receptors ultimately leads to the activation of the other, one could postulate that an agent which reduces the effects of the activation of one receptor should, at least partly, reduce the effects of the activation of the other. However, even though morphine significantly reduced both thermal and tactile hypersensitivity following the activation of each of the NMDA and the NK-1 receptors, morphine was more effective in reducing the behavioural and thermal hypersensitivity response due to the activation of the NMDA receptor, than those same responses following the NK-1 activation. This could show that even though the spinal NMDA and NK-1 receptors interact, this interaction is probably only at the spinal level, at the initiation of the central sensitisation cascade.

Subsequently, the activation of each receptor is then responsible for the activation of a distinct secondary pathway/cascade of events that lead to the full response seen with each receptor activation. One proof for this is that the activation of the NMDA receptor causes analgesia after the hyperalgesia, and it has been shown that spinalisation reduced this analgesic response (Kolhekar, Meller et al. 1993), which could mean that the response following the NMDA receptor activation is greatly dependent on a pathway/secondary mechanisms which activates descending inhibitions. This activation of descending inhibitions is in contrast with what happens when the NK-1 receptor is activated, since it has been shown that 80% of the lamina I neurons which express the NK-1 receptor project to the parabrachial area and are the origin of the spino-bulbo-spinal loop, which predominantly drives descending serotonergic facilitations (Todd, McGill et al. 2000; Suzuki, Morcuende et al. 2002; Todd 2002; Todd, Puskar et al. 2002), although it has been postulated that the NK-1-expressing lamina I projection neurons also play a small role in driving descending adrenergic inhibitions (Rahman *et al*, unpublished observations). Therefore, although descending inhibitions and facilitations are both physiologically active at any one time, some chronic pathological pain states (Kauppila, Kontinen et al. 1998), or perhaps even acute activation of different spinal receptors may shift the

activation of one descending pathway more than the other (Rahman *et al*, unpublished observations).

Even though my study was not done on spinalised rats, the activation of the NK-1 receptor in my study led to a longer behavioural response and thermal hypersensitivity than the NMDA-mediated response, and only the NMDA receptor activation led to analgesia after the initial hypersensitivity. Although morphine also acts in the RVM to activate OFF cells, inhibit ON cells, causing analgesia *via* its supraspinal μ-opioid receptors (see section **1.8.2 iv**) (Fields, Heinricher et al. 1991; Heinricher 1997; Porreca, Burgess et al. 2001), the predominant activation of descending facilitations driven by the activation of the NK-1 receptor, could be the reason behind the reduced efficacy of systemic morphine, on the Sar-SP-induced hypersensitivity: morphine was still effective, yet less so than following the activation of the NMDA receptor.

Finally, I could extrapolate from my results that since morphine was effective in reducing abnormal pain due to the activation of each of the NMDA and NK-1 receptors, therefore morphine could be effective in treating the types of pain which involve the activation of these two receptors. Neuropathic pain is an example of such pain states, and was initially thought of as refractory to morphine (Arner and Meyerson 1988). Therefore, provided morphine is given *via* the right dose, route and at the right time of injury (Suzuki, Chapman et al. 1999), this drug can be effective in decreasing abnormal pain which occurs following nerve injury.

4.4.6 Conclusion

I have found that morphine, *via* the route and non-sedating dose used in this study, to be effective in inhibiting the thermal and tactile hypersensitivity which arise following the activation of each of the NMDA and the NK-1 receptor, but more effective following the NMDA receptor activation.

Therefore, the NMDA receptor activation might require secondary pathways which are more sensitive to morphine inhibition, than those following the NK-1 receptor activation. This could mean that even though these two receptors are involved with the activation of the postsynaptic drive, and the central sensitisation associated with it, there are differences in how each receptor is modulated, and the neural pathways each receptor is involved in and thus how each receptor is involved with central sensitisation in chronic pain states.

In the next chapter, the involvement of peripheral NMDA receptors in the hypersensitivity arising following the spinal nerve ligation model will be investigated.

Chapter 5:

A study investigating whether peripheral NMDA receptors play a role in the hypersensitivity following peripheral nerve injury

5. A study investigating whether peripheral NMDA receptors play a role in the hypersensitivity following peripheral nerve injury

5.1 Introduction

The composition of the functional NMDA receptor includes the NR1 subunit, with one of the NR2 subunits (A-D) (Petrenko, Yamakura et al. 2003). Centrally, NMDA receptors have been shown to play a role in plasticity due to chronic pain, as well as central hypersensitivity. Peripherally, recent studies have suggested that NMDA receptors may be functional, since they were acutely activated by the intraplantar injection of NMDA, causing mechanical hyperalgesia, which was blocked by the intraplantar administration of the NMDA receptor antagonist MK-801 (Zhou, Bonasera et al. 1996), and may be implicated in peripheral sensitisation due to inflammation (Wang, Liu et al. 1997; Carlton and Coggeshall 1999) (see sections **1.7.3.1 ii b** and **1.7.3.1 iii**). Indeed, peripheral NMDA receptors have been identified on the peripheral terminals of primary afferent axons in both rat hairy and glabrous skin, using immunohistochemical techniques which stained only for the NR1 subunit (Carlton, Hargett et al. 1995; Coggeshall and Carlton 1998).

Furthermore, the intraplantar injection of NMDA administration has been shown to cause c-Fos expression in the dorsal horn side ipsilateral to the injection, whereas intraplantar MK-801, an NMDA receptor antagonist, has been shown to decrease c-Fos expression following intraplantar formalin (Wang, Liu et al. 1997). Other studies that used the formalin model also showed that glutamate receptor antagonists, including the NMDA receptor antagonists ketamine and MK-801, decreased the lifting and licking behaviour seen in this model (Davidson, Coggeshall et al. 1997; Davidson and Carlton 1998). Another study showed that following carrageenan inflammation, the intraplantar administration of the NMDA receptor antagonist MK-801, or the AMPA/kainate antagonist CNQX, decreased the thermal hyperalgesia seen in this model (Jackson, Graff et al. 1995). Additionally to these inflammatory models, other studies have used the Freund's complete adjuvant (CFA) model of inflammation and have shown that

the NMDA, AMPA and kainate receptors significantly increase 48 hours following the induction of the CFA model (Carlton and Coggeshall 1999), that NMDA receptors increase and decrease according to the level of CFA inflammation (Du, Zhou et al. 2003), and that intraplantar MK-801 reduced the mechanical hyperalgesia seen in this model (Leem, Hwang et al. 2001).

Only one study on peripheral NMDA receptors and neuropathic pain in animals has been undertaken to date, and it showed that the intraplantar administration of MK-801 reduced the mechanical hyperalgesia which occurred following L5 spinal nerve ligation, which was preceded by L5 dorsal rhizotomy (Jang, Kim et al. 2004). The L5 dorsal rhizotomy was performed in order to prevent the ectopic activity which arises following a nerve injury from reaching the spinal cord, and thus permitting only peripheral access of the spinal nerve ligation signals.

Normally, endogenous glutamate has been shown to originate from sources which include macrophages and Schwann cells (Kinkelin, Brocker et al. 2000). However, electrical stimulation of the sciatic nerve, with an intensity to activate both low- (Aβ fibres) and high-threshold (Aδ and C fibres), has been shown to increase the content of glutamate in the dialysate collected from the rat hindpaw (deGroot, Zhou et al. 2000), showing that glutamate may be released peripherally following the electrical stimulation of the sciatic nerve (deGroot, Zhou et al. 2000; Jin, Nishioka et al. 2006). Furthermore, glutamate has been shown to be released peripherally following heat stimulation and capsaicin administration to the rat hindstep (Jin, Nishioka et al. 2006).

These peripheral NMDA receptors could be located on either injured or uninjured fibres in the periphery. Therefore, if by a process akin to the axon reflex, whereby SP is released from the peripheral terminals of primary afferent fibres, primary afferent fibres could release glutamate peripherally, following peripheral nerve injury, then the local administration of NMDA receptor antagonists could block the activation, and thus the ensuing hyperalgesia and allodynia. Clinically, this could be of great advantage, since the peripheral administration of NMDA receptor antagonists can reduce the incidence of

unacceptable central side-effects, which include hallucinations and motor-effects, and which previously limited the use of these agents (Coderre and Van Empel 1994).

Therefore, the aim of this part of my study was to determine whether peripheral NMDA receptors could be activated and blocked pharmacologically, in normal animals, and then if peripheral NMDA receptors have a role in the tactile and cold hypersensitivity seen following peripheral nerve injury, using the spinal nerve ligation model.

5.2 Methods

The methods used in this chapter are detailed in sections **2.1**, **2.4** and the drugs used in section **2.6.3**.

Briefly, peripheral NMDA receptors were activated and blocked by the intraplantar administration of NMDA, and the NMDA receptor antagonist MK-801 respectively, and the responses to thermal and tactile stimuli were investigated. Subsequently, the spinal nerve ligation model described by Kim and Chung in 1992 was used, intraplantar MK-801 was administered and the responses to tactile and cold stimuli were investigated.

In the first part of the study investigating the pharmacological activation of the peripheral NMDA receptor with exogenous NMDA, I used 0.7, 3.7 and 7.3µg, which was based on doses reported in a previous study (Zhou, Bonasera et al. 1996). The dose of MK-801 used was 0.4µg, and was also based on a previous study (Du, Zhou et al. 2003) and the morphine dose used, 3mg/kg, was the same non-sedating dose used in the previous chapter of my study. Morphine was administered systemically, and was injected 25 minutes before intraplantar NMDA, in order for the peak effects of both agents to coincide, as the effects of morphine have been shown to be maximal at 30 minutes, whereas those of NMDA have been shown to be maximal at 5 minutes post administration, by my

study (see **chapter 4**) and a previous study (Davis and Inturrisi 2001). In conjunction with a previous study which showed that intraplantar MK-801 may block peripheral NMDA receptor activation when administered at the same time as NMDA (Zhou, Bonasera et al. 1996), MK-801 (0.4μg) was co-administered into the same paw with NMDA (7.3μg). Furthermore, in order to rule out systemic effects, MK-801 was also administered into the paw contralateral to the one which received NMDA. Paw withdrawal to tactile and thermal stimuli was done in the same manner as in chapter four (detailed in sections **2.3.3** and **2.3.4**), with testing being done every 5 minutes for 60 minutes in the Hargreaves test, and at 5, 30 and 60 minutes in the mechanical test using von Frey filaments, post drug administration.

In the second part of this study, peripheral nerve injury was achieved by ligating spinal nerves L5 and L6, as described by the Kim and Chung study, in 1992 (Kim and Chung 1992). Following spinal nerve ligation or sham surgery, the rats were monitored for normal weight gain and behaviour. Rats with nerve ligation showed abnormal ipsilateral hindpaw posture, but no dragging.

Paw withdrawal following tactile stimuli, using von Frey 1, 5 and 9g, and following cold stimuli, using an acetone drop, was tested in both ipsilateral or contralateral to the nerve injury paw. A positive response was noted when the rat lifted its paw, licked it or withdrew it. Data was plotted as the average difference in paw withdrawal between ipsilateral and contralateral paws, with the number of contralateral paw lifts being subtracted from the ipsilateral paw lifts, thus providing a 'difference score'. In the graphs, the difference score was a positive value, showing that rats with spinal nerve ligation lifted the paw ipsilateral to nerve injury more than they did the paw contralateral to nerve injury, demonstrating that hypersensitivity to thermal, tactile and cold stimuli was present. The antinociceptive effects of morphine and ondansetron were shown when the difference score was reduced, and thus, when the rats were not lifting their ipsilateral paw as much as they did prior to drug(s) administration.

The same dose of MK-801 was used in the rats with nerve injury, and paw withdrawal to tactile stimuli was also tested at 5, 30, 45 and 60 minutes post drug administration. Throughout this chapter, all intraplantar saline and drug administration was given in a 50μl volume. Additionally, the pH of intraplantar saline and NMDA was neutralised to pH 7 with 1N sodium hydroxide.

5.3 Results

5.3.1 Intraplantar injections in normal rats

i) Intraplantar saline

The intraplantar administration of saline in normal rats did not cause any significant change from the baseline response, whether using thermal (fig. 5.1) or tactile (fig. 5.2) stimuli.

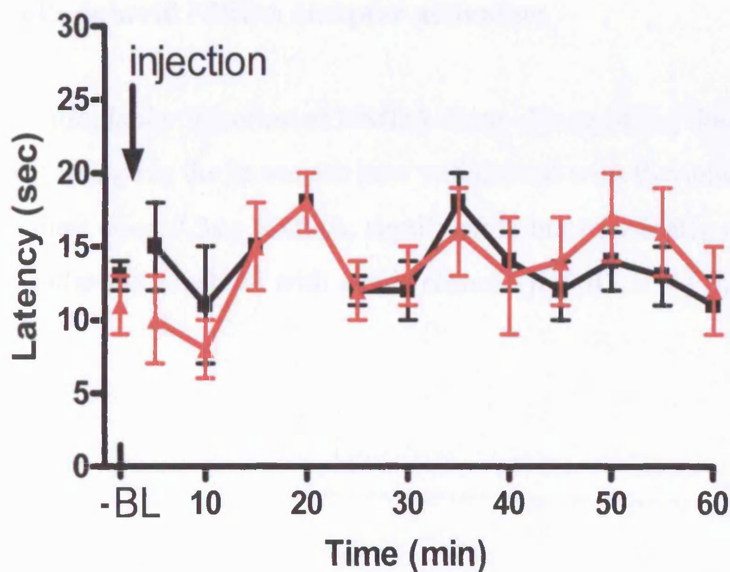


Fig. 5.1- The lack of effect of saline administered in the left hindpaw (black) and in the right paw (red, 50μl) on paw withdrawal latency following thermal stimuli. n=6. BL depicts pre-drug baseline responses.

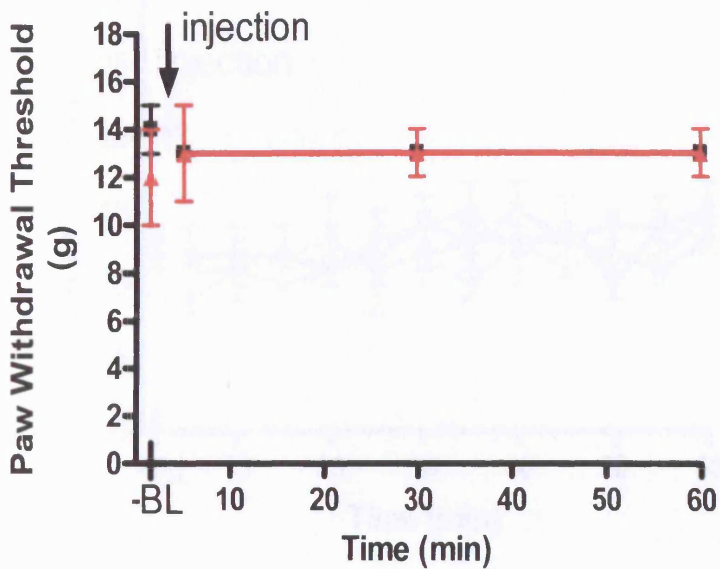


Fig. 5.2- The lack of effect of saline administered in the left hindpaw (black) and in the right paw (red, 50μl) on paw withdrawal threshold following tactile stimuli. n=6. BL depicts pre-drug baseline responses.

ii) Peripheral NMDA receptor activation

a) Intraplantar injection of NMDA alone- None of the doses of NMDA caused any change in the latency to paw withdrawal with thermal stimuli (fig. 5.3). The highest dose, 7.3μg NMDA, significantly but transiently reduced the paw withdrawal threshold with tactile stimuli ($p<0.05$, $n=6$) (fig. 5.4).

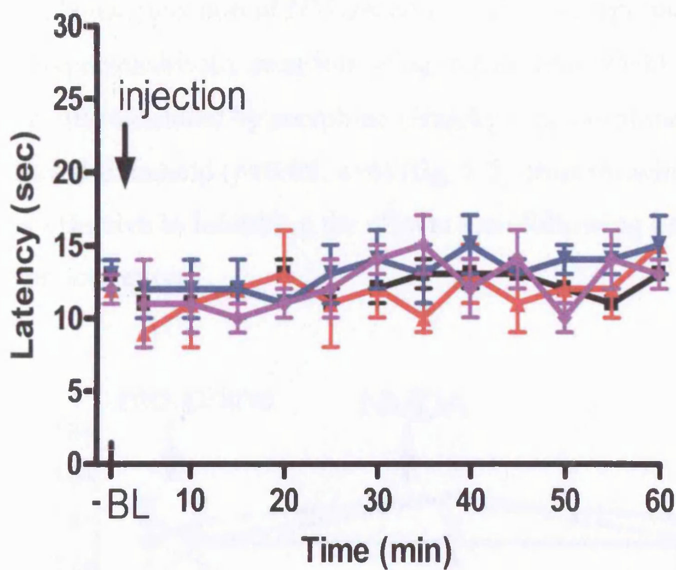


Fig. 5.3- The lack of effect of intraplantar NMDA (n=6 for all) doses 0.7 (red), 3.7 (blue) and 7.3μg (purple) on paw withdrawal latency following thermal stimuli, when compared to the saline control (black, 50μl, n=12). BL depicts pre-drug baseline responses.

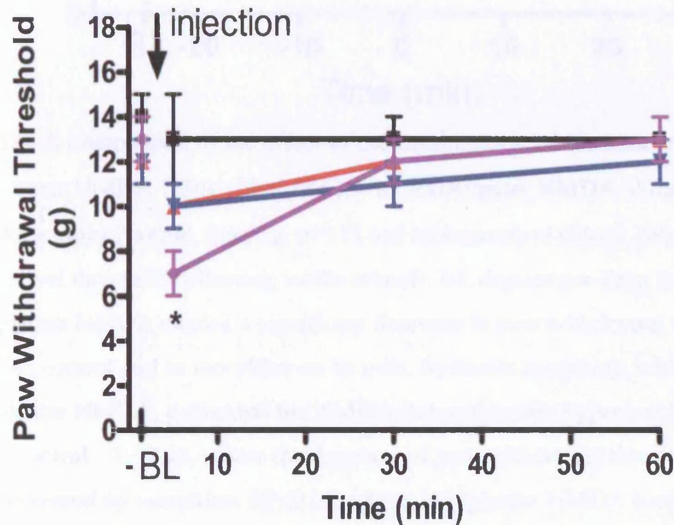


Fig. 5.4- The effect of intraplantar NMDA doses 0.7 (red, n=8), 3.7 (blue, n=12) and 7.3μg (purple, n=6) on paw withdrawal latency following tactile stimuli when compared to the saline control (black, 50μl n=6). BL depicts pre-drug baseline responses. The largest dose of intraplantar NMDA caused significant tactile hypersensitivity, when compared to the saline control. *P<0.05, where a significant decrease by NMDA dose 7.3μg from the saline control is seen, one way ANOVA followed by Dunnett's post-hoc test.

b) Intraplantar injection of NMDA and systemic morphine administration- The tactile hypersensitivitiy seen following intraplantar NMDA (dose 7.3μg) was significantly inhibited by morphine (3mg/kg), as morphine increased the paw withdrawal threshold ($p<0.05$, n=6) (fig. 5.5), thus showing that morphine at this dose is effective in inhibiting the effects seen following peripheral NMDA receptor activation.

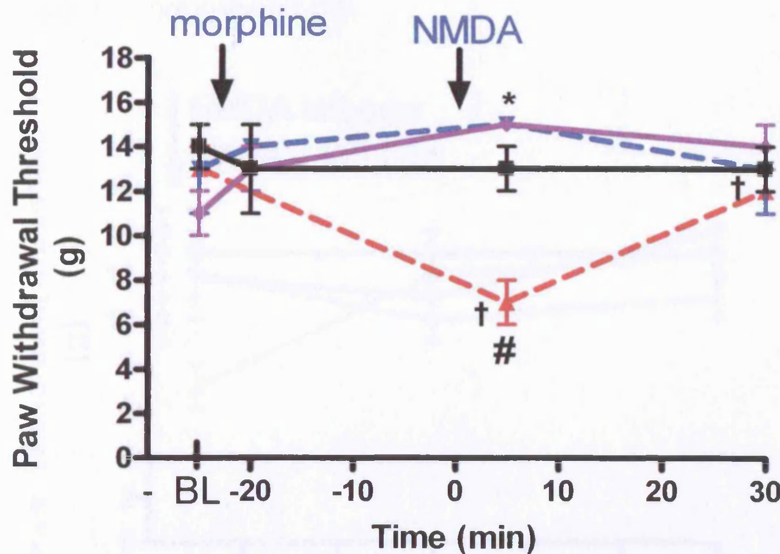


Fig. 5.5- A comparison of the effect of the combination of systemic morphine 3mg/kg with intraplantar NMDA 7.3μg (blue, n=6), with intraplantar NMDA alone 7.3μg (red, n=6), morphine alone (purple, 3mg/kg, n=11) and saline control (black, 50μl, n=6) on the rat paw withdrawal threshold following tactile stimuli. BL depicts pre-drug baseline responses. Intraplantar NMDA caused a significant decrease in paw withdrawal threshold, when compared to saline control and to morphine on its own. Systemic morphine, when administered prior to intraplantar NMDA, prevented the NMDA-induced tactile hypersensitivity when compared to the saline control. *$P<0.05$, where the decrease of paw withdrawal threshold produced by NMDA was prevented by morphine. #$P<0.05$, where intraplantar NMDA caused a significant decrease in paw withdrawal threshold when compared to saline control. †$P<0.05$, where intraplantar NMDA caused a significant decrease in paw withdrawal threshold when compared to morphine on its own. One way ANOVA followed by Tukey post-hoc test.

c) Intraplantar NMDA and MK-801 administration- When the NMDA receptor antagonist MK-801 (0.4μg) was administered alone by the intraplantar route, no significant change from the saline control values was seen (fig. 5.6). However,

when MK-801 was co-administered with NMDA (7.3μg) in the same hindpaw, it significantly blocked the NMDA-induced tactile hypersensitivity (fig. 5.6), by increasing the paw withdrawal threshold. Additionally, when MK-801 was administered alone, contralateral to the paw that received NMDA, no effect on the NMDA-induced tactile hypersensitivity was seen (fig. 5.7). This shows that the tactile hypersensitivity seen following intraplantar NMDA administration is due to the activation of peripheral NMDA receptors, since MK-801 was acting locally and not systemically.

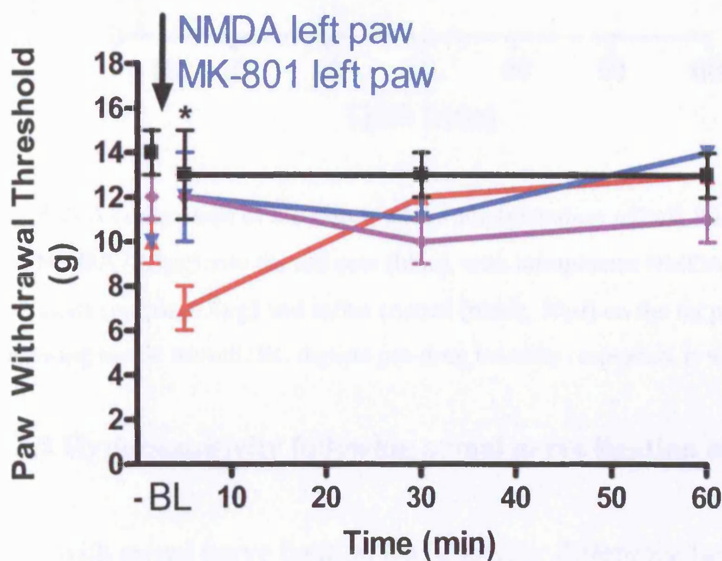


Fig. 5.6- A comparison of the effects of the co-administration of MK-801 (0.4μg) and NMDA (7.3μg) into the same paw (blue), with intraplantar NMDA alone (red, 7.3μg), intraplantar MK-801 alone (purple, 0.4μg) and intraplantar saline control (black, 50μl) on the rat paw withdrawal threshold following tactile stimuli. BL depicts pre-drug baseline responses. n=6. Intraplantar injection of MK-801 in the same paw that received NMDA prevented the NMDA-induced tactile hypersensitivity. *P<0.05, where the significant decrease in paw withdrawal threshold by NMDA, was prevented by MK-801. <P<0.05, where intraplantar NMDA caused a significant decrease in paw withdrawal threshold, when compared to intraplantar saline control and intraplantar MK-801 on its own. *t*-Test.

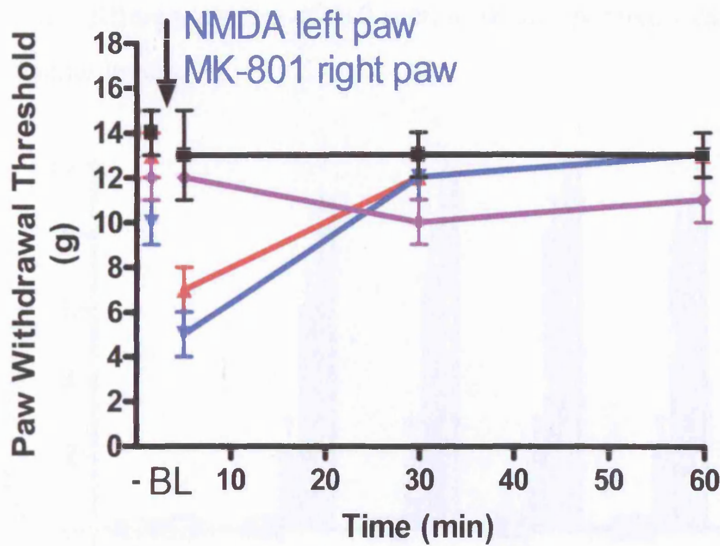


Fig. 5.7- A comparison of the effects of the administration of MK-801 (0.4μg) into the right paw and NMDA (7.3μg) into the left paw (blue), with intraplantar NMDA alone (red, 7.3μg), MK-801 alone (purple, 0.4μg) and saline control (black, 50μl) on the rat paw withdrawal threshold following tactile stimuli. BL depicts pre-drug baseline responses. n=6.

5.3.2 Hypersensitivity following spinal nerve ligation model

Rats with spinal nerve ligation had a greater difference between ipsilateral and contralateral paw withdrawal to tactile (fig. 5.8) and cold stimuli (fig. 5.9), exhibiting greater ipsilateral paw withdrawal, when compared to sham-operated rats and their own baseline, which was tested before the surgery. In order to calculate the difference score, the number of times the rat withdrew its contralateral paw following tactile (out of 10 times) and cold stimuli (out of 5 times) was subtracted from the number of times the rat withdrew its ipsilateral paw following the same stimuli.

Indeed, in rats with spinal nerve ligation, the difference score between ipsilateral and contralateral paw withdrawal to von Frey 1g was increased to a maximum of 2±1, to von Frey 5g was increased to a maximum of 8±1 and to von Frey 9g was increased to a maximum of 10±0, from a difference score of 0±0 seen in sham-operated rats, and pre-surgery baseline levels. The difference score to cold

stimuli in rats with spinal nerve ligation was increased to a maximum of 4±1, from a difference score of 0±0 seen in sham-operated rats and pre-surgery baseline levels.

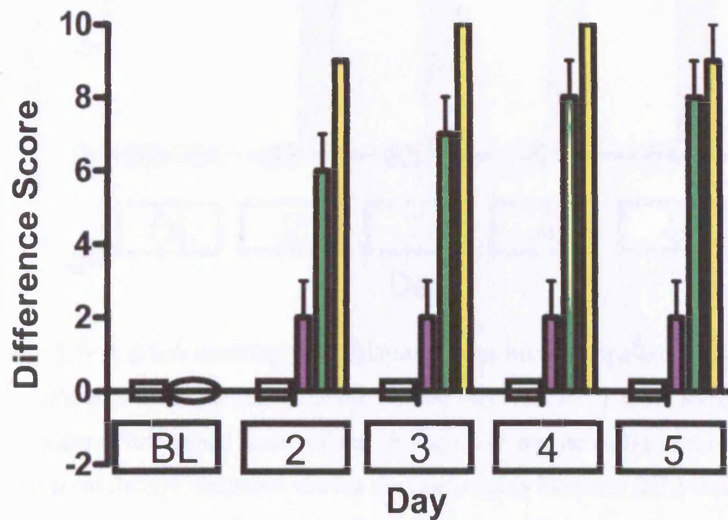


Fig. 5.8- The difference score between ipsilateral and contralateral rat hindpaw to von Frey 1 (purple), 5 (green) and 9g (yellow), in peripheral nerve injured rats (n=7). Tactile hypersensitivity is shown as an increase in difference score values. Since there was no difference in the score between ipsilateral and contralateral hindpaws during the pre-surgery baseline (BL) testing of the spinal nerve ligated rats (open circle) and for the sham-operated rats (small open rectangles), these appear as zero difference score (n=6).

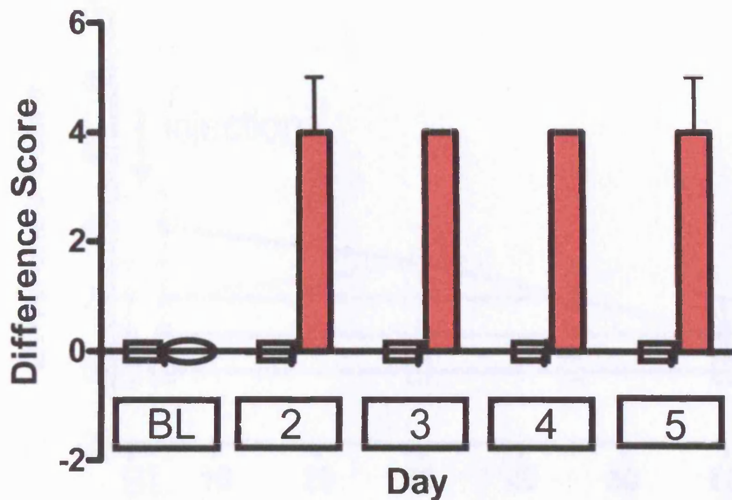


Fig. 5.9- A graph showing the difference score between ipsilateral and contralateral rat hindpaw to cold stimuli, in peripheral nerve injured rats (red, n=7). Cold hypersensitivity is shown as an increase in difference score values. Since there was no difference in the score between ipsilateral and contralateral hindpaws during the pre-surgery baseline (BL) testing of the spinal nerve ligated rats (open circle) and for the sham-operated rats (small open rectangles), these appear as zero difference score (n=6).

5.3.3 Peripheral MK-801 following spinal nerve ligation

In order to determine whether peripheral NMDA receptors play a role in the tactile and cold hypersensitivity that occurs following peripheral nerve injury, the NMDA receptor antagonist MK-801 (0.4μg) was administered intraplantar in the paw ipsilateral to the injury.

The intraplantar administration of MK-801 did not cause any significant inhibition of the hypersensitivity to von Frey 1 (fig. 5.10), 5 (fig. 5.11), 9g (fig. 5.12) and to cold stimuli (fig. 5.13).

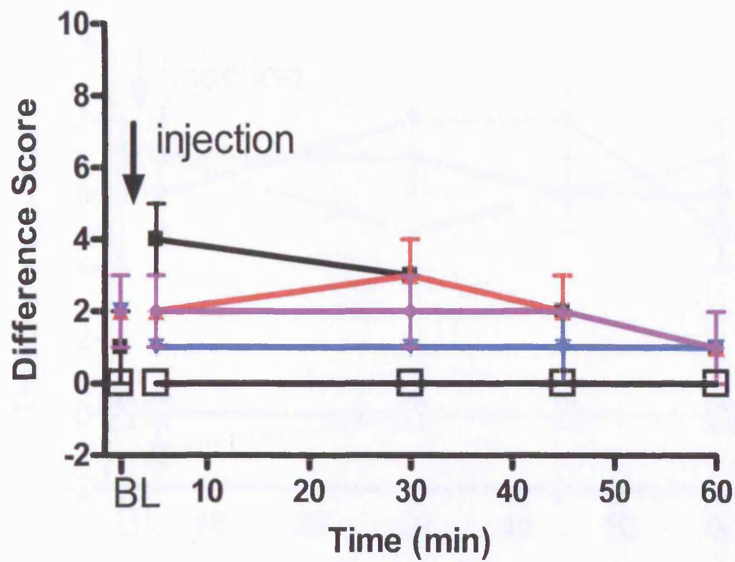


Fig. 5.10- The lack of effect of MK-801 (0.4μg) administered in the ipsilateral paw (blue), and the contralateral paw (purple) on different withdrawal score to von Frey 1g, when compared to saline administered in the ipsilateral paw (black) and the contralateral paw (red, 50μl), in spinal nerve ligated rats (n=7). Tactile hypersensitivity is shown as an increase in difference score values. The responses of the sham-operated rats which received saline and MK-801 in the ipsilateral and contralateral paws are represented by the open squares linked with a black line (n=6). BL depicts pre-drug baseline responses.

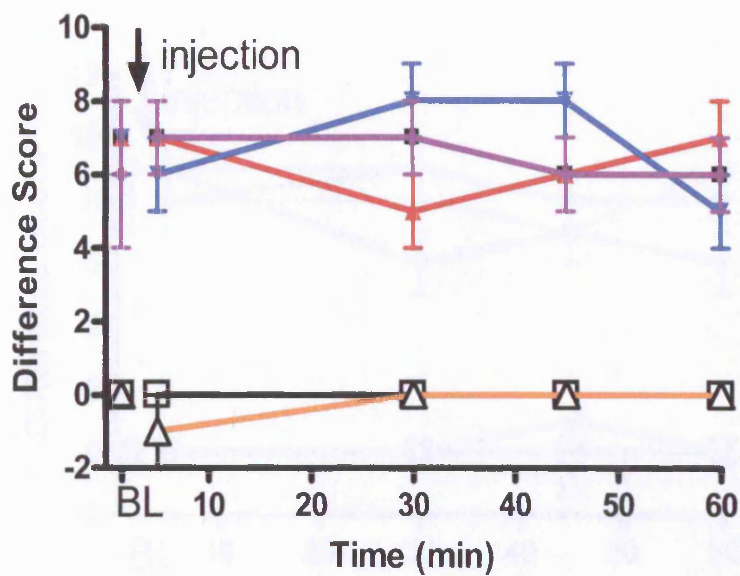


Fig. 5.11- A graph showing the lack of effect of MK-801 (0.4μg) administered in the ipsilateral paw (blue), and the contralateral paw (purple) on different withdrawal score to von Frey 5g, when compared to saline administered in the ipsilateral paw (black) and the contralateral paw (red, 50μl), in spinal nerve ligated rats (n=7). Tactile hypersensitivity is shown as an increase in difference score values. The responses of the sham-operated rats which received either saline or MK-801 in the ipsilateral paw, and the responses of the sham-operated rats which received saline in the contralateral paw are represented by the open squares linked with a black line. The responses of the sham-operated rats which received MK-801 in the contralateral paw are represented by the open triangle linked with an orange line (n=6). BL depicts pre-drug baseline responses.

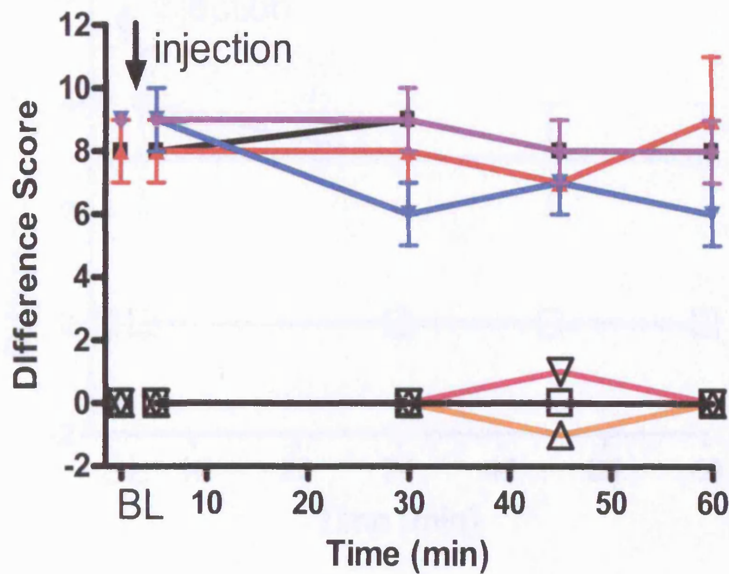


Fig. 5.12- A graph showing the lack of effect of MK-801 (0.4μg) administered in the ipsilateral paw (blue), and the contralateral paw (purple) on different withdrawal score to von Frey 9g, when compared to saline administered in the ipsilateral paw (black) and the contralateral paw (red, 50μl), in spinal nerve ligated rats (n=7). Tactile hypersensitivity is shown as an increase in difference score values. The responses of the sham-operated rats which received MK-801 in the ipsilateral and contralateral paw are represented by the open squares linked with a black line. The responses of the sham-operated rats which received saline in the ipsilateral paw are represented by the open triangle linked with an orange line, and the responses of the sham-operated rats which received saline in the contralateral paw are represented by the inverse open triangle linked with a pink line (n=6). BL depicts pre-drug baseline responses.

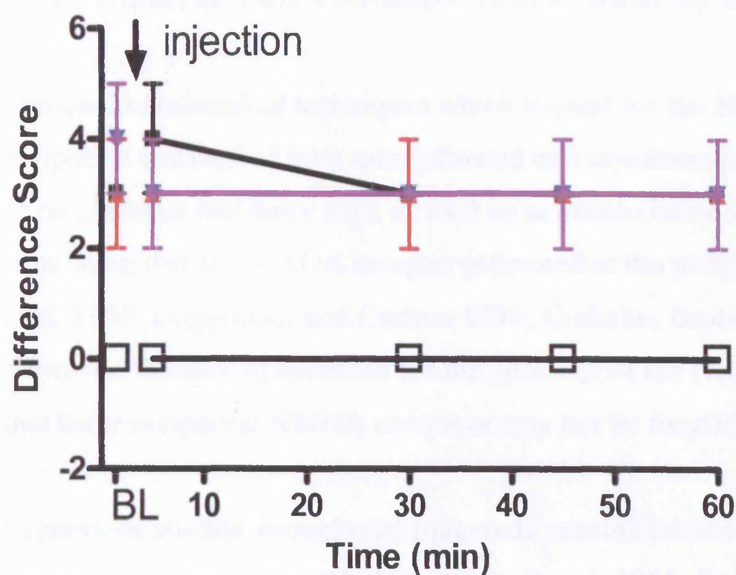


Fig. 5.13- A graph showing the lack of effect of MK-801 (0.4μg) administered in the ipsilateral paw (blue), and the contralateral paw (purple) on different withdrawal score to cold stimuli, when compared to saline administered in the ipsilateral paw (black) and the contralateral paw (red, 50μl), n=7. Cold hypersensitivity is shown as an increase in difference score values. The responses of the sham-operated rats which received saline and MK-801 in the ipsilateral and contralateral paws are represented by the open squares linked with a black line (n=6). BL depicts pre-drug baseline responses.

5.4 Discussion

In my study, peripheral NMDA receptors were activated by a large dose of NMDA, causing tactile hypersensitivity, and this activation was blocked by MK-801 acting locally. However, although it has been postulated that peripheral NMDA receptors may be involved in the mechanical hypersensitivity seen following peripheral nerve injury (Jang, Kim et al. 2004), in my study, the same dose of MK-801 that could normalise the hypersensitive response to tactile stimuli following intraplantar NMDA administration in normal rats was unable to reduce tactile and cold hypersensitivity seen following the spinal nerve ligation model. Therefore, even though they may be pharmacologically activated, peripheral NMDA receptors do not seem to be pathophysiologically active in the spinal nerve ligation model.

5.4.1 Peripheral NMDA receptors when activated by intraplantar NMDA

Immunohistochemical techniques which stained for the NR1 subunit at the peripheral terminal of both unmyelinated and myelinated primary afferent fibres of rat glabrous and hairy skin, as well as in human hairy skin, has led to the stipulation that the NMDA receptor is located at the periphery (Carlton, Hargett et al. 1995; Coggeshall and Carlton 1998; Kinkelin, Brocker et al. 2000). However, the lack of evidence for the presence of the NR2 subunit may suggest that these peripheral NMDA receptors may not be functional.

In previous studies, intraplantar glutamate administration caused both tactile and thermal hypersensitivity (Carlton, Hargett et al. 1995; Jackson, Graff et al. 1995; Zhou, Bonasera et al. 1996; Leem, Hwang et al. 2001; Jang, Kim et al. 2004). However, whereas intraplantar NMDA has been shown to cause tactile hypersensitivity in a previous study using von Frey filaments in awake rats (Zhou, Bonasera et al. 1996), intraplantar NMDA has only been shown to sensitise nociceptors to thermal stimuli in an in vitro skin-nerve preparation (Du, Koltzenburg et al. 2001). In my study, the intraplantar administration of NMDA caused hypersensitivity to tactile, but not thermal, stimuli. Therefore, intraplantar glutamate causing thermal hypersensitivity (Jackson, Graff et al. 1995), and sensitisation of nociceptors to thermal stimuli (Du, Koltzenburg et al. 2001), could be an effect of glutamate on one of its other peripheral receptors.

Indeed, this is the first study that investigated the effect of intraplantar NMDA on paw withdrawal to tactile and thermal stimuli in normal awake animals. An earlier study investigated the effect of NMDA on peripheral NMDA receptors, using an in vitro skin preparation, and found that the same dose of NMDA as the one used in this study sensitised nociceptors in normal and inflamed skin to heat (Du, Zhou et al. 2003). The discrepancy between my study and the study by Du *et al*, could be due to the difference in techniques. Whereas they used the in vitro skin preparation and recorded the response of isolated C-fibre units in dissected skin, using a broad range of temperatures including supra-threshold

temperatures, my study used behavioural techniques in whole, awake, free-moving animals and used threshold temperature.

However, the activation of the peripheral NMDA receptor causing tactile hypersensitivity in this study is in agreement with many previous studies that used intraplantar glutamate (Carlton, Hargett et al. 1995), intraplantar NMDA (Zhou, Bonasera et al. 1996), and intraplantar MK-801 to block the tactile hypersensitivity following inflammation (Jackson, Graff et al. 1995; Leem, Hwang et al. 2001), and nerve injury (Jang, Kim et al. 2004). Furthermore, the reduction in NMDA-induced tactile hypersensitivity, in my study, by intraplantar MK-801, is also in agreement with a previous study which activated the peripheral NMDA receptor with intraplantar NMDA administration (Zhou, Bonasera et al. 1996). Moreover, the effects of the NMDA receptor antagonist seem to be acting solely locally, since the contralateral administration of MK-801 had no effect.

To conclude, the fact that in my study, intraplantar NMDA administration caused tactile and not thermal hypersensitivity could be hypothesised to be due to the NMDA receptor channel in the periphery being expressed on nociceptive primary afferent fibres which do not convey a response due to thermal stimuli, perhaps a subset of the non-peptidergic, IB4-positive Aδ-fibres (Snider and McMahon 1998; Caterina, Gold et al. 2005; Meyer, Ringkamp et al. 2006). However, since immunohistochemical data is not available yet, the conclusion is purely speculative at this point.

5.4.2 Peripheral NMDA receptors and neuropathic pain

In my study, as in previous studies (Kim and Chung 1992; Chapman, Suzuki et al. 1998), the spinal nerve ligation model caused the long-lasting behavioural manifestation of tactile and cold hypersensitivity.

Previous studies have implicated the peripheral NMDA receptor to have a role in inflammation. One study showed the reduction in c-Fos expression, following

formalin, when intraplantar NMDA and MK-801 are administered together (Wang, Liu et al. 1997). Another study showed the reduction of the lifting and licking behaviour seen following formalin administration, by intraplantar NMDA antagonists, such as MK-801 and ketamine (Davidson, Coggeshall et al. 1997; Davidson and Carlton 1998). Additionally, other studies showed that intraplantar MK-801 reduced both the thermal hypersensitivity seen following carrageenan inflammation (Jackson, Graff et al. 1995), and mechanical hypersensitivity seen following Freund's complete adjuvant (CFA) inflammation (Leem, Hwang et al. 2001). Finally, peripheral NMDA receptor subunits have been shown to be upregulated in both myelinated and unmyelinated primary afferent fibres, 48 hours following CFA inflammation in the ipsilateral, and not the contralateral paw (Carlton and Coggeshall 1999).

Despite the mechanisms underlying inflammation being different from those underlying neuropathic pain, the spinal NMDA receptor plays a role in the central sensitisation which occurs following both inflammation and neuropathy (Dickenson 1997). Therefore, one could postulate that since the peripheral NMDA receptor plays a role in inflammation, this receptor could also play a role in the hypersensitivity seen following neuropathic pain. A study by deGroot *et al* has shown that following electrical stimulation of the sciatic nerve, the glutamate content in the dialysate collected from the rat hindpaw increased significantly. Jin *et al* further showed that glutamate is released peripherally from primary afferent fibres following each of electrical stimulation of the sciatic nerve, as well as heat stimulation and capsaicin administration to the rat hindpaw instep (Jin, Nishioka et al. 2006), by measuring the level of glutamate in the perfusate collected from the rat hindpaw instep using in vivo microdyalisis.

Since SP, *via* the axon reflex, has been shown to be released by primary afferent fibres at the periphery (Burnstock 1977; Hagermark, Hokfelt et al. 1978), glutamate may be released *via* a similar mechanism, from injured primary afferent fibres, and could then activate the NMDA receptors located on uninjured fibres, causing some of the hyperalgesia and allodynia seen following neuropathic pain. However, although following inflammation, peripheral

sensitisation and the release of SP by the axon reflex have been proven to occur, the basis for this possible action in neuropathy is unclear. One could postulate that there could be ectopic activity firing antidromically back to the periphery following nerve injury, which could lead to peripheral glutamate release from the injured nerve. This glutamate could then act on the NMDA receptors at the periphery, activate them, and the signal could be transmitted centrally through the uninjured fibres.

In my study, intraplantar NMDA only caused tactile and not thermal (heat) hypersensitivity, therefore, once peripheral nerve injury was established, the effect of MK-801 was tested on tactile hypersensitivity. However, in keeping with previous studies which investigated the spinal nerve ligation model of nerve injury (Chapman, Suzuki et al. 1998; Matthews and Dickenson 2002), cold hypersensitivity was also investigated.

I found that the intraplantar administration of MK-801, which was previously shown to decrease tactile hypersensitivity due to intraplantar NMDA, was ineffective in reducing the tactile and cold hypersensitivity following peripheral nerve injury. This finding differs from the only other study which investigated peripheral NMDA receptors in neuropathic pain (Jang, Kim et al. 2004), where a lower dose than the one used in my study of intraplantar MK-801, was shown to reduce tactile hypersensitivity following L5 spinal nerve ligation. The discrepancy between my study and that of Jang *et al*, could be due to the fact that the nerve injury model was different, as they only ligated the L5 spinal nerve, and preceded that ligation with an L5 dorsal rhizotomy, whereas both the L5 and L6 spinal nerves were ligated in my study and no dorsal rhizotomy was performed. Therefore, in my study, ectopic signals from the neuromas on the damaged nerves reached the dorsal horn and caused central sensitisation, whereas in the Jang *et al* study, any ectopic activity from the neuroma could not reach the dorsal horn.

Clinically, it would have been very important to find a role for the peripheral NMDA receptors in neuropathic pain, since the use of NMDA antagonists is

riddled with undesirable side-effects, such as hallucinations, which limit their use (Dickenson 1997). Two studies by Lynch *et al* were performed, using a cream containing ketamine, amitriptyline and a combination of both, in patients with neuropathic pain. The first study showed that there was no effect of ketamine or amitriptyline cream on the pain rating in patients with neuropathic pain in the placebo-controlled two day trial, but that there was a significant decrease in patients using the combination cream in the seven day trial which did not have a placebo control (Lynch, Clark et al. 2003), and the second study, also lacking a control, showed the effectiveness of the combination cream when used for 6-12 months (Lynch, Clark et al. 2005). Unfortunately, both these studies, as well as not having controls, also allowed patients to keep taking any previous analgesics, including opioids and non-steroidal anti-inflammatory drugs, which means that more rigidly controlled clinical studies are needed before conclusions can be drawn.

5.4.3 Conclusion

Previous studies have shown the existence of the NR1 subunit of the NMDA receptor at the peripheral terminals of primary afferent fibres (Carlton, Hargett et al. 1995; Coggeshall and Carlton 1998). However, the functional NMDA receptor needs both the NR1 and the NR2 subunit (Petrenko, Yamakura et al. 2003). These peripheral NMDA receptors were pharmacologically activated following the administration of a large dose of agonist, however, they do not seem to play a role in the tactile and cold hypersensitivity seen in the spinal nerve ligation model.

However, one could speculate that since intraplantar NMDA administration caused tactile, and not thermal hypersensitivity, and since this static hypersensitivity has been shown by previous studies to be conveyed mostly by Aδ-fibres (Field, Bramwell et al. 1999; Ossipov, Bian et al. 1999), therefore the peripheral NMDA receptor channel may be expressed in a subset of Aδ-fibres which do not express the channels which transduce thermal stimuli, such as the

TRPV family of channels (Caterina, Schumacher et al. 1997; Snider and McMahon 1998; Meyer, Ringkamp et al. 2006). This hypothesis could be tested by repeating my experiments using capsaicin-treated rats.

In the next chapter, I will investigate the role of descending serotonergic facilitations in the hypersensitivities which arise following the SNL model, as well as whether the blockade of these descending facilitations causes synergism with the effects of morphine in this model.

Chapter 6:

A study investigating the roles of each of morphine and ondansetron, and their combination, on the hypersensitivity which occurs following the spinal nerve ligation model

6. A study investigating the roles of each of morphine and ondansetron, and their combination, on the hypersensitivity which occurs following the spinal nerve ligation model

6.1 Introduction

Previous clinical studies have debated the efficacy of morphine in neuropathic pain states. Initially, it was stated that opioids were ineffective in some types of neuropathic pain, as only 1 in 8 patients responded positively to opioids (Arner and Meyerson 1988). That initial study led to debate from other studies, which is still ongoing in clinical (Portenoy, Foley et al. 1990; Rowbotham, Reisner-Keller et al. 1991; Jadad, Carroll et al. 1992) as well as in animal studies (Backonja, Miletic et al. 1995; Suzuki, Chapman et al. 1999; Joshi, Hernandez et al. 2006), as per the efficacy of opioids in neuropathic pain, and even the authors of the original study had to state that although the title of their paper had a 'provocative phrasing', they did not mean that all types of neuropathic pain were resistant to opioids and that opioids should never be withheld from patients with chronic neuropathic pain (Arner and Meyerson 1991).

Indeed, the study by Rowbotham *et al* showed that intravenous morphine was effective in reducing pain intensity in patients with postherpetic neuralgia, whereas the study by Jadad *et al* showed that half the patients with neuropathic pain responded well to intravenous morphine. In addition, the study by Attal *et al* showed that intravenous morphine was effective in reducing dynamic allodynia in patients with spinal cord injury, but that not many of the patients kept taking morphine in the long-term (Attal, Guirimand et al. 2002). Furthermore, the study by Gilron *et al* showed that systemic morphine was effective in patients with neuropathic pain, but that the combination of gabapentin with morphine was more effective in these patients, at doses lower than those that were effective singly (Gilron, Bailey et al. 2005). Therefore, type of neuropathic pain and dose titration play a role in neuropathic pain 'responding' to morphine and opioids (Portenoy, Foley et al. 1990; Dellemijn 1999; Sindrup and Jensen 1999; Hansson

and Dickenson 2005), as well as route of administration (Bian, Nichols et al. 1995; Suzuki, Chapman et al. 1999).

However, the study by Arner *et al* did point to a significant finding: that neuropathic pain is less responsive to morphine than acute or inflammatory pain, (Lemberg, Kontinen et al. 2006). This was shown by the finding that dynamic allodynia, an important nociceptive manifestation of neuropathic pain, remains mostly unaffected by morphine control (Field, McCleary et al. 1999). Indeed, even though systemic morphine acts on μ-opioid receptors located on primary afferent fibres, spinal and supraspinal sites, neuropathic pain has so many underlying mechanisms, such as increased spinal afferent drive (Ossipov, Lopez et al. 1995), changes in levels of spinal transmitters and receptors (Honore, Rogers et al. 2000; Wang, Sun et al. 2002) and alterations in descending controls (Porreca, Burgess et al. 2001; Suzuki, Rahman et al. 2004), that it is quite difficult for one drug to decrease all of the symptoms which arise with this disease (Hansson and Dickenson 2005; Dickenson and Kieffer 2006) (see sections **1.6.2 iv**, **1.6.5**, **1.6.6 ii** and **iv**, **1.6.7 ii**, **1.7.5**, **1.7.7**, **1.8.2 vi** and **vii** and **1.9.4**).

Furthermore, the 'numbers needed to treat', a method used to assess the clinical efficacy of analgesics and which shows how many patients need to be treated before one patient shows more than 50% pain relief, of morphine and other drugs used to treat neuropathic pain, is approximately 3, and the use of these drugs at effective doses is still hampered by unacceptable side effects, such as respiratory depression and sedation for morphine (Sindrup and Jensen 1999). Therefore, drug combinations are required, in order to decrease the side effects of each drug on its own whilst maintaining its efficacy, to treat neuropathic pain.

Ondansetron, a 5HT3 antagonist, has long been licensed as an anti-emetic. The analgesic properties of this drug have only been recently investigated, which is not surprising since serotonin (5-HT) has the confusing dual property of being both anti- (Jordan, Kenshalo et al. 1978; Yaksh and Wilson 1979; Alhaider, Lei et al. 1991; Crisp, Stafinsky et al. 1991; Oyama, Ueda et al. 1996; Jones, Peters

et al. 2005) and pro-nociceptive (Jordan, Kenshalo et al. 1978; Ali, Wu et al. 1996; Oyama, Ueda et al. 1996; Calejesan, Ch'ang et al. 1998; Green, Scarth et al. 2000; Zeitz, Guy et al. 2002; Suzuki, Rahman et al. 2004), depending on which one of its many receptors is being activated. The terminals of the descending fibres from RVM neurons are an important source of 5-HT in the spinal cord (Fasmer, Berge et al. 1983; Besson and Chaouch 1987). Furthermore, the descending controls which were previously investigated were predominantly of the inhibitory type (Reynolds 1969; Besson and Chaouch 1987) and it has only been recently shown, that in some chronic pain states such as neuropathic pain, descending serotonergic facilitations predominate and serve to maintain the abnormal pain seen following nerve injury (Urban and Gebhart 1999; Porreca, Burgess et al. 2001; Suzuki, Rahman et al. 2004; Suzuki, Rahman et al. 2005). Indeed, it has been now shown that descending serotonergic facilitations are part of a spino-bulbo-spinal loop that has, at its origins, NK-1-expressing lamina I neurons which project to the parabrachial area, which relays to the RVM, and then back down from the RVM onto the spinal cord, thus affecting pain transmission at the level of the spinal cord (Mantyh, Rogers et al. 1997; Todd, McGill et al. 2000; Suzuki, Morcuende et al. 2002; Todd 2002; Todd, Puskar et al. 2002; Rahman, Suzuki et al. 2003; Conte, Legg et al. 2005; Dickenson and Kieffer 2006).

In animal models, ondansetron has been shown to reduce the at-level mechanical allodynia which arises in spinal cord injury (Oatway, Chen et al. 2004), as well as the mechanical and thermal evoked responses of spinal cord dorsal horn neurons in rats with cancer-induced bone pain (Donovan-Rodriguez, Urch et al. 2006) and spinal nerve ligation (Suzuki, Rahman et al. 2004). Additionally, in a clinical study, a single dose of intravenous ondansetron also caused analgesia in patients with chronic neuropathic pain (McCleane, Suzuki et al. 2003).

One cannot predict the onset of a nerve injury, but can focus on treating the mechanisms which underlie the maintenance of the pain seen following nerve injury (Porreca, Burgess et al. 2001). Furthermore, ondansetron has never been tested in rats with spinal nerve ligation in a behavioural setting. Therefore, it is of

clinical relevance to investigate what effects blocking descending serotonergic facilitations with the spinal administration of the 5-HT3 antagonist ondansetron could have on the maintenance of the hypersensitivities seen following nerve injury. Therefore, in the next series of experiments, the effects of spinal ondansetron, alone and in combination with systemic morphine, will be investigated in animals with spinal nerve ligation.

6.2 Methods

The methods used in this chapter are detailed in sections **2.1**, **2.3.1**, **2.4**, **2.5**, and the drugs used in section **2.6.4**.

Briefly, normal rats were intrathecally cannulated using a method based on the work of Storkson *et al*, and the effects of different doses of ondansetron (10 and 20μg/10μl) on paw withdrawal latency to thermal stimuli and paw withdrawal threshold to tactile stimuli, were investigated.

Following that, rats underwent a combination of either spinal nerve ligation (Kim and Chung 1992) and intrathecal cannulation, or sham-operation and intrathecal cannulation. The effects of morphine, ondansetron and their combination on paw hypersensitivity to thermal, tactile and cold stimuli were investigated in these animals. In the case of tactile and cold stimuli, the response was plotted as 'difference score'. The 'difference score' is calculated as the subtraction of the number of times the rat withdrew its contralateral paw from the number of times the rat withdrew its ipsilateral paw, out of a total of 10 times, following mechanical stimuli and 5 times, following cold stimuli. The reason for choosing two different methods to determine tactile hypersensitivity, such as the paw withdrawal threshold for normal animals and the 'difference score' of withdrawal to von Frey 1, 5 and 9g, is because the latter cannot be used in normal animals, since normal animals do not respond to innocuous tactile stimuli, whereas it is also an efficient way of showing tactile hypersensitivity in rats with the SNL model of nerve injury.

In rats with spinal nerve ligation and sham-operation, the concentration of systemic morphine used was 3mg/kg and the dose of spinal ondansetron used was 20μg. The total volume used for intrathecal injections was 24μl. Saline was administered subcutaneously, intrathecally and both subcutaneously and intrathecally as a control for each of morphine, ondansetron and their combination, respectively. Both morphine and ondansetron showed a peak action at 30 minutes following their administration, therefore, they were administered at the same time. Since hypersensitivity to tactile and cold stimuli is measured as the 'difference score', then this hypersensitivity is represented on the graph as an increase in difference score, and the antihyperalgesic effects of morphine and ondansetron will be shown as a reduction in the 'difference score'.

6.3 Results

6.3.1 Ondansetron in normal animals

Intrathecal ondansetron at doses 10 and 20μg, did not cause any significant change in paw withdrawal latency to thermal stimuli (fig. 6.1), and paw withdrawal threshold to tactile stimuli (fig. 6.2), in normal animals, when compared to the responses following intrathecal saline.

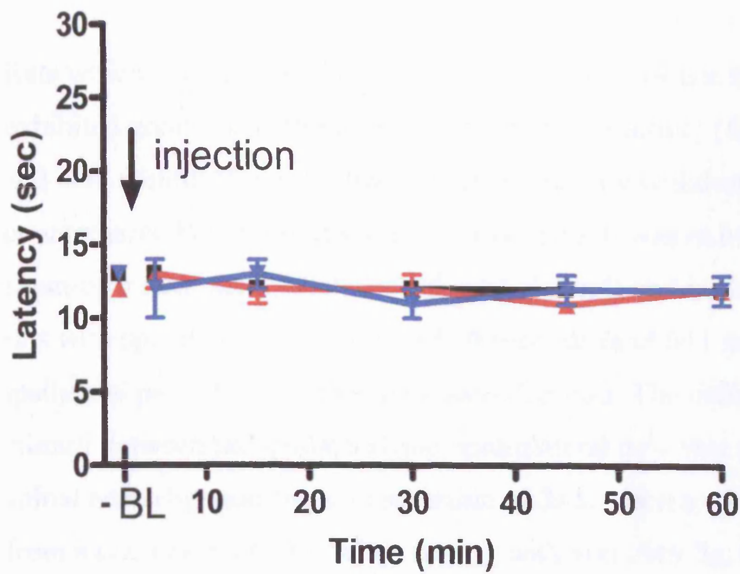


Fig. 6.1- The lack of effect of ondansetron doses 10 (red) and 20μg (blue), on paw withdrawal latency following thermal stimuli, when compared to saline control (black, 24μl). BL depicts pre-drug baseline responses.

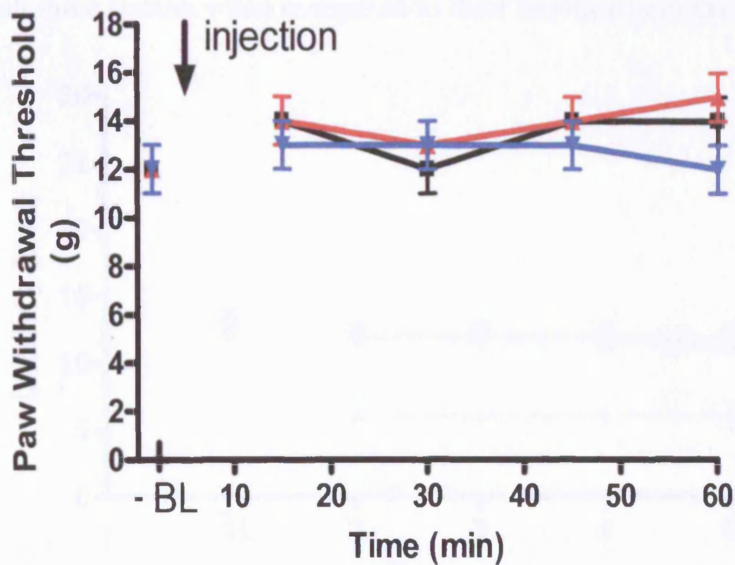


Fig. 6.2- The lack of effect of ondansetron doses 10 (red) and 20μg (blue), on paw withdrawal threshold following tactile stimuli, when compared to saline control (black, 24μl). BL depicts pre-drug baseline responses.

6.3.2 Response to thermal, tactile and cold stimuli in rats with spinal nerve ligation and an intrathecal cannula

Rats which had the spinal nerve ligation, whether or not they were cannulated, exhibited greater ipsilateral paw withdrawal to thermal (fig. 6.3), tactile (fig 6.4) and cold stimuli (fig. 6.5), than contralateral paw withdrawal and than their sham counterparts. Paw withdrawal to thermal stimuli was reduced from 12±1 in sham-operated rats (ipsilateral and contralateral) and in the contralateral paw of rats with spinal nerve ligation, to 6±0 seconds (and 6±1 seconds on day 5), in the ipsilateral paw of rats with spinal nerve ligation. The difference score to tactile stimuli between the ipsilateral and contralateral paw was reduced in rats with spinal nerve ligation from a maximum of 2±1, when testing with von Frey 1g, from a maximum of 7±1, when testing with von Frey 5g, and from a maximum of 9±0, when testing with von Frey 9g, to 0±0 when testing all the aforementioned von Frey forces in sham-operated rats. The difference score to cold stimuli was reduced from a maximum of 2±1 in rats with spinal nerve ligation, to 0±0 in sham-operated rats. Additionally, whether cannulated or not, rats with spinal nerve ligation also exhibited greater ipsilateral paw withdrawal to all three stimuli when compared to their baseline response.

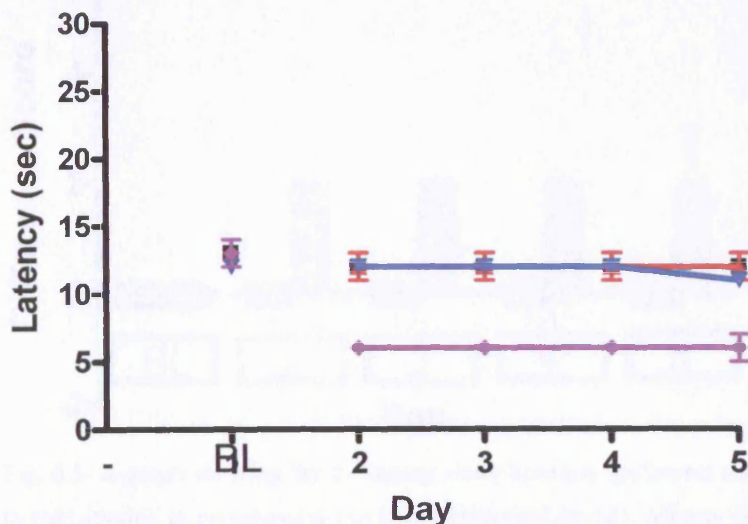


Fig. 6.3- The decrease in paw withdrawal latency to thermal stimuli in the ipsilateral paw (purple, n=16) of rats with spinal nerve ligation, when compared to the contralateral paw (contralateral=blue, n=16) and sham-operated rats (ipsilateral=black, contralateral=red, n=6). All rats also underwent intrathecal cannulation. BL depicts pre-surgery baseline responses.

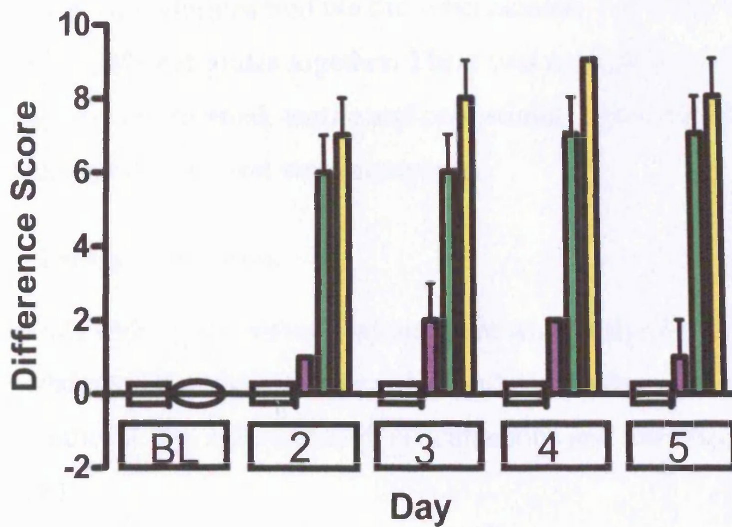


Fig. 6.4- The difference score between ipsilateral and contralateral rat hindpaw to von Frey 1 (purple), 5 (green) and 9g (yellow, n=16 for all), in peripheral nerve injured rats. All rats also underwent intrathecal cannulation. Tactile hypersensitivity is shown as an increase in the value of the difference score. Since there was no difference in the score between ipsilateral and contralateral hindpaws during the pre-surgery baseline (BL) testing of spinal nerve ligated rats (open circle) and for the sham-operated rats (small open rectangles), these appear as zero difference score (n=6).

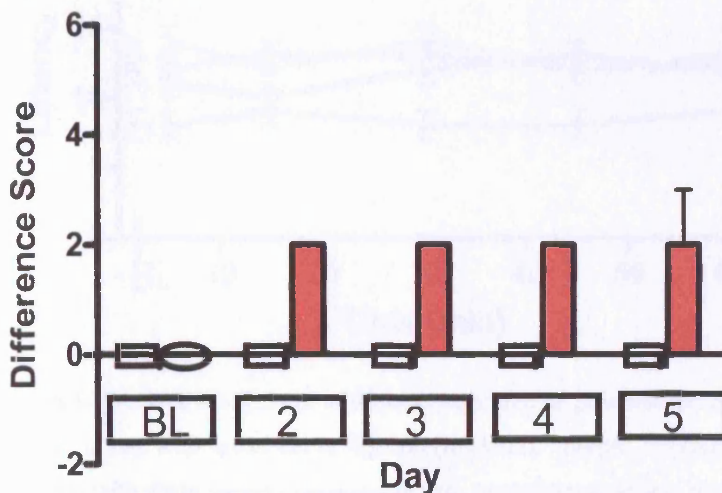


Fig. 6.5- A graph showing the difference score between ipsilateral and contralateral rat hindpaw to cold stimuli, in peripheral nerve injured rats (red, n=16). All rats also underwent intrathecal cannulation. Cold hypersensitivity is shown as an increase in the value of the difference score. Since there was no difference in the score between ipsilateral and contralateral hindpaws during the pre-surgery baseline (BL) testing of the spinal nerve ligated rats (open circle) and for the sham-operated rats (small open rectangles), these appear as zero difference score (n=6).

6.3.3 Saline experiments

Saline was administered *via* the subcutaneous, intrathecal and both subcutaneous and intrathecal routes together. There was no significant difference in the responses to thermal, tactile and cold stimuli, when the three different routes of saline administration were compared.

i) Thermal response

In rats with spinal nerve ligation, there was no significant difference in paw withdrawal threshold to thermal stimuli, following subcutaneous (fig. 6.6), intrathecal (fig. 6.7) and both subcutaneous and intrathecal saline administration (6.8).

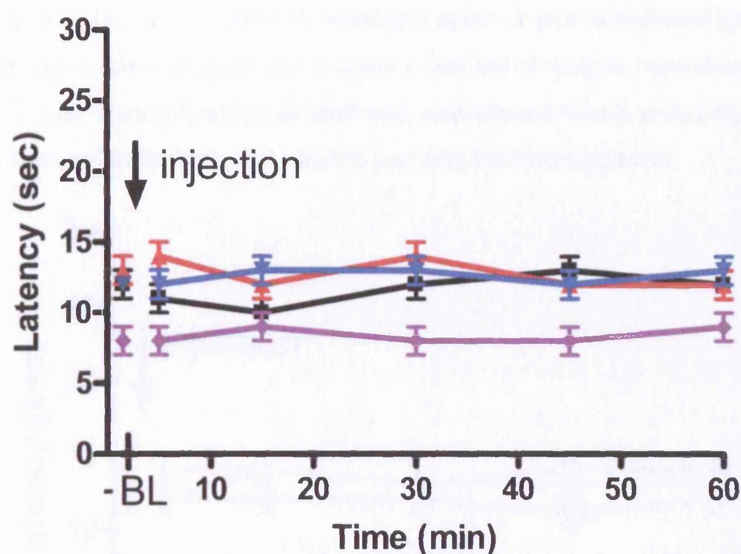


Fig. 6.6- The lack of effect of subcutaneous saline on paw withdrawal latency following thermal stimuli in rats with spinal nerve ligation (ipsilateral=purple, contralateral=blue, n=10, 0.25ml), and rats with sham surgery (ipsilateral=red, contralateral=black, n=6, 0.25ml). All rats also underwent intrathecal cannulation. BL depicts pre-drug baseline responses.

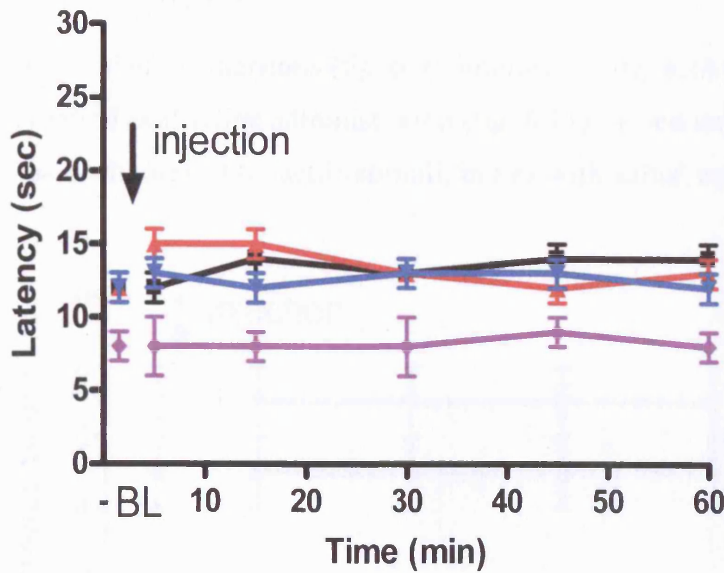


Fig. 6.7- The lack of effect of intrathecal saline on paw withdrawal latency following thermal stimuli in rats with spinal nerve ligation (ipsilateral=purple, contralateral=blue, n=9, 24μl), and rats with sham surgery (ipsilateral=red, contralateral=black, n=6, 24μl). All rats also underwent intrathecal cannulation. BL depicts pre-drug baseline responses.

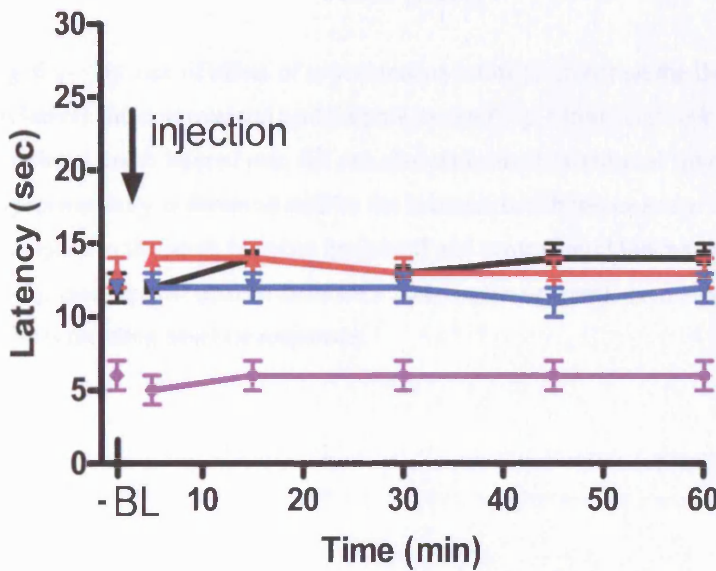


Fig. 6.8- The lack of effect of the combined administration of intrathecal (24μl) and subcutaneous (0.25ml) saline on paw withdrawal latency following thermal stimuli in rats with spinal nerve ligation (ipsilateral=purple, contralateral=blue, n=7), and rats with sham surgery (ipsilateral=red, contralateral=black, n=6). All rats also underwent intrathecal cannulation. BL depicts pre-drug baseline responses.

ii) Tactile response

None of the subcutaneous (fig. 6.9), intrathecal (fig. 6.10) and both subcutaneous and intrathecal saline administration (fig. 6.11) caused any significant difference in paw withdrawal to tactile stimuli, in rats with spinal nerve ligation.

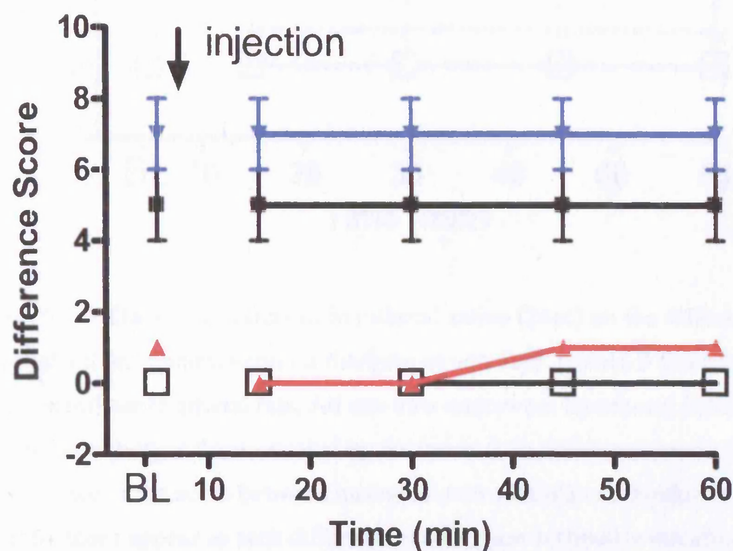


Fig. 6.9- The lack of effect of subcutaneous saline (0.25ml) on the difference score between ipsilateral and contralateral rat hindpaw to von Frey 1 (red), 5 (black) and 9g (blue, n=10), in peripheral nerve injured rats. All rats also underwent intrathecal cannulation. Tactile hypersensitivity is demonstrated by the increase in difference score. Since there was no difference in the score between ipsilateral and contralateral hindpaws for the sham-operated rats (n=6), these appear as zero difference score (open squares), even after saline administration. BL depicts pre-drug baseline responses.

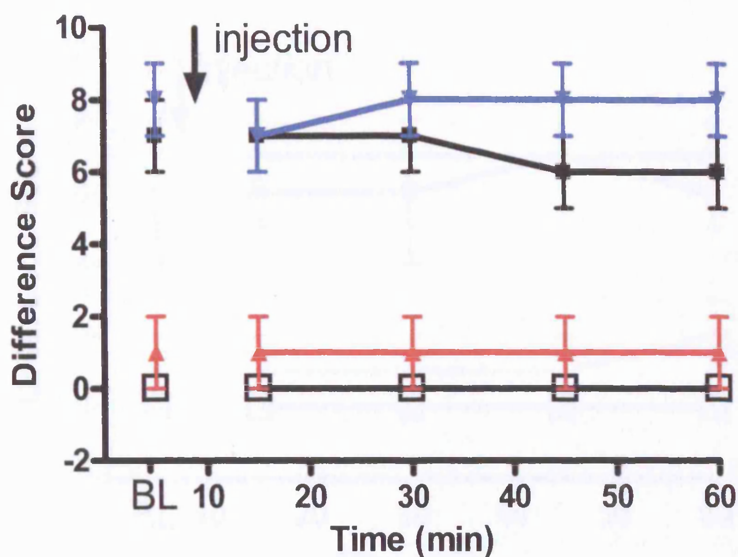


Fig. 6.10- The lack of effect of intrathecal saline (24μl) on the difference score between ipsilateral and contralateral rat hindpaw to von Frey 1 (red), 5 (black) and 9g (blue, n=9), in peripheral nerve injured rats. All rats also underwent intrathecal cannulation. Tactile hypersensitivity is demonstrated by the increase in difference score. Since there was no difference in the score between ipsilateral and contralateral hindpaws for the sham-operated rats (n=6), these appear as zero difference score (open squares), even after saline administration. BL depicts pre-drug baseline responses.

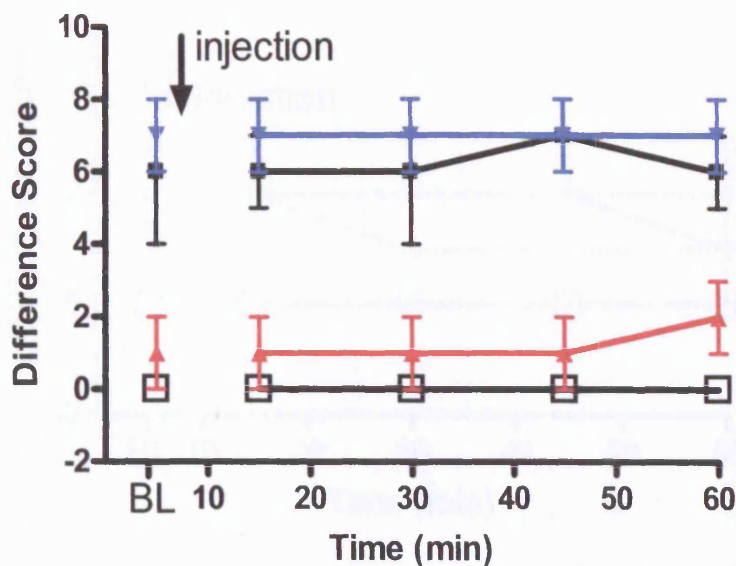


Fig. 6.11- The lack of effect of the combined administration of intrathecal (24μl) and subcutaneous saline (0.25ml) on the difference score between ipsilateral and contralateral rat hindpaw to von Frey 1 (red), 5 (black) and 9g (blue, n=7), in peripheral nerve injured rats. All rats also underwent intrathecal cannulation. Tactile hypersensitivity is demonstrated by the increase in difference score. Since there was no difference in the score between ipsilateral and contralateral hindpaws for the sham-operated rats (n=6), these appear as zero difference score (open squares), even after saline administration. BL depicts pre-drug baseline responses.

iii) Cold response

Saline administration in neuropathic animals did not cause any significant difference in paw withdrawal to cold stimuli, whether *via* the subcutaneous route, the intrathecal route or both the intrathecal and subcutaneous routes together (fig. 6.12).

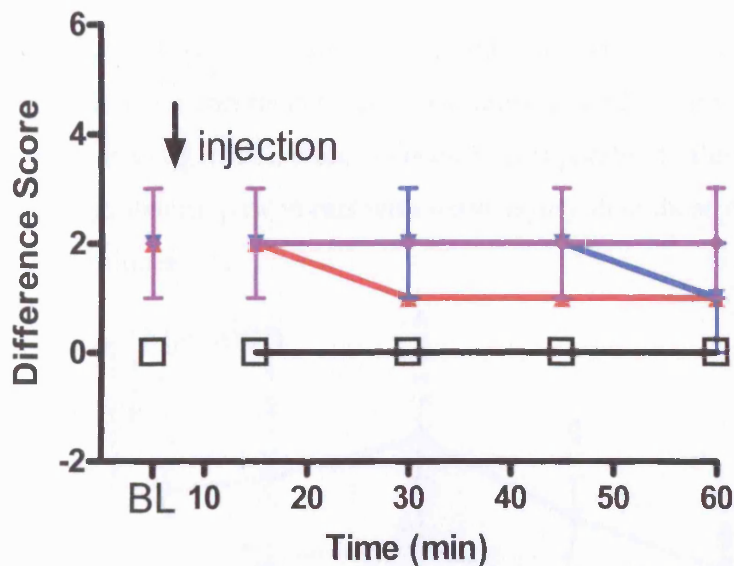


Fig. 6.12- The lack of effect of subcutaneous (red, n=8, 0.25ml), intrathecal (blue, n=7, 24µl) and the combined administration of intrathecal (24µl) and subcutaneous (0.25ml) saline (purple, n=7) on the difference score between ipsilateral and contralateral rat hindpaw to cold stimuli, in peripheral nerve injured rats. All rats also underwent intrathecal cannulation. Cold hypersensitivity is demonstrated by the increase in difference score. Since there was no difference in the score between ipsilateral and contralateral hindpaws for the sham-operated rats (n=6), these appear as zero difference score (open squares), even after saline administration. BL depicts pre-drug baseline responses.

6.3.4 Morphine experiments

i) Thermal response

The subcutaneous administration of morphine, in neuropathic and sham-operated rats, caused a significant increase in the paw withdrawal latency to thermal stimuli. In neuropathic rats, ipsilateral paw withdrawal latency to thermal stimuli significantly increased at 15 and 30 minutes following morphine administration, when compared to the ipsilateral saline control. Contralateral paw withdrawal latency to thermal stimuli was significantly increased by morphine at 5, 15, 30 and 45 minutes post-administration, when compared to the contralateral saline control (fig. 6.13). Therefore, in rats with nerve injury, morphine had a delayed onset and shorter-lasting effects in the ipsilateral paw when compared to the

contralateral paw. In sham-operated rats, morphine significantly increased both ipsilateral and contralateral paw withdrawal latency with thermal stimuli at all timepoints (fig. 6.14), which was more comparable to the effect of morphine on the contralateral paw in rats with nerve injury than those on the ipsilateral paw of nerve-injured rats.

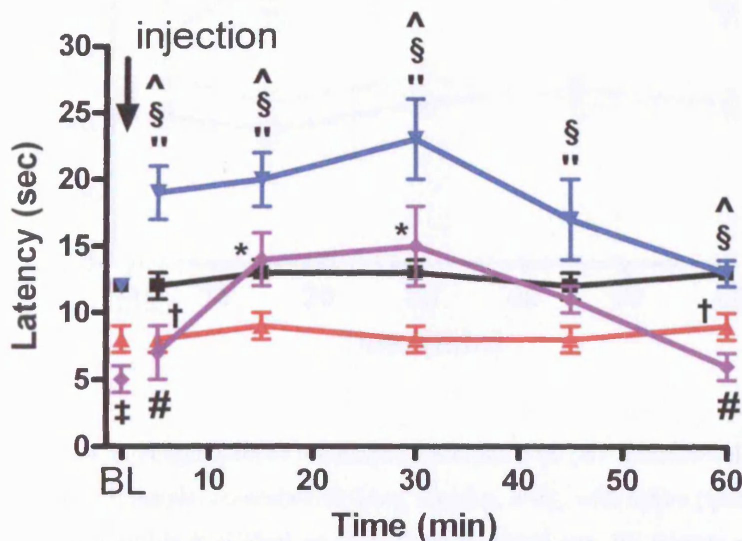


Fig. 6.13- A comparison of the effect of morphine on paw withdrawal latency to thermal stimuli (ipsilateral=purple, contralateral=blue, 3mg/kg, n=6), with saline (ipsilateral=red, contralateral=black, 0.25ml, n=10), in rats with spinal nerve ligation. BL depicts pre-drug baseline responses. Spinal nerve ligation caused thermal hypersensitivity in the paw ipsilateral to nerve injury. Morphine caused a significant increase in both ipsilateral and contralateral paw withdrawal latency following thermal stimuli, when compared to saline control, however, this significant increase was at more timepoints in the contralateral than the ipsilateral paw. *P<0.05, where a significant increase in ipsilateral paw withdrawal latency is seen following morphine administration, when compared to saline control. "P<0.05, where a significant increase in contralateral paw withdrawal latency is seen following morphine administration, when compared to saline control. ‡P<0.05, where spinal nerve ligation caused a significant decrease in ipsilateral paw withdrawal latency to thermal stimuli, when compared to contralateral paw withdrawal latency, during baseline testing. §P<0.05, where morphine administration caused a significant increase in contralateral paw withdrawal latency, when compared to ipsilateral paw withdrawal latency following saline. #P<0.05, where there was a significant decrease in ipsilateral paw withdrawal latency in the group which received morphine, when compared to the contralateral paw withdrawal latency in the group which received saline. ^P<0.05 contralateral paw withdrawal versus ipsilateral paw withdrawal latency, in the group that received morphine. †P<0.05 contralateral paw withdrawal versus ipsilateral paw withdrawal latency, in the group that received saline. One way ANOVA followed by Tukey post-hoc test.

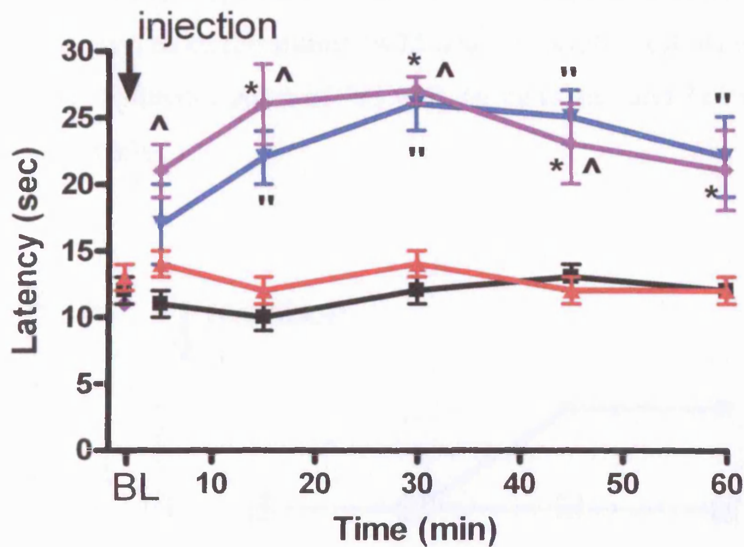


Fig. 6.14- A comparison of the effect of morphine on paw withdrawal latency to thermal stimuli (ipsilateral=purple, contralateral=blue, 3mg/kg, n=6), with saline (ipsilateral=red, contralateral=black, 0.25ml, n=6), in sham-operated rats. BL depicts pre-drug baseline responses. Morphine significantly increased paw withdrawal latency to thermal stimuli at all timepoints tested in both ipsilateral and contralateral paws in rats with sham surgery, when compared to saline control. *P<0.05, where a significant increase in ipsilateral paw withdrawal latency is seen following morphine administration, when compared to saline control. †P<0.05, where a significant increase in contralateral paw withdrawal latency is seen following morphine administration, when compared to ipsilateral and contralateral paw withdrawal latency in the group that received saline. #P<0.05, where morphine caused a significant increase in ipsilateral paw withdrawal latency, when compared to contralateral paw withdrawal latency in the group that received saline. One way ANOVA followed by Tukey post-hoc test.

ii) Tactile response

The subcutaneous administration of morphine, in neuropathic rats, caused a significant decrease in the difference score following von Frey 5 (fig. 6.16) and 9g (fig. 6.17), but not following von Frey 1g (fig. 6.15), and thus a significant reduction in tactile hypersensitivity. In the case of the response following von Frey 5g, morphine significantly decreased ipsilateral paw withdrawal to tactile stimuli at 30 minutes following its administration, which was shown as a

decrease in the difference score from 5±1 with saline to 2±1 with morphine, whereas following von Frey 9g, morphine significantly decreased ipsilateral paw withdrawal to tactile stimuli at 15 and 30 minutes following its administration, from a difference score of 7±1 with saline to 4±1 and 3±1 with morphine, respectively.

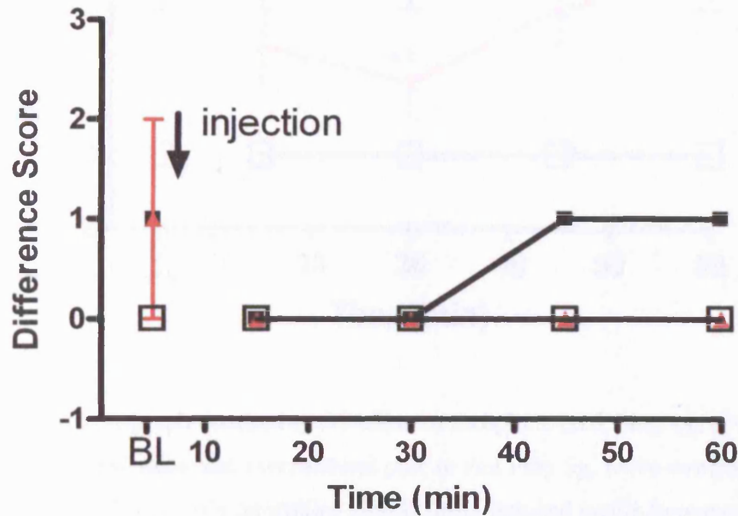


Fig. 6.15- The lack of significant effect of morphine (red, 3mg/kg, n=6) on the difference score between ipsilateral and contralateral paw to von Frey 1g, when compared to the saline control (black, 0.25ml, n=10), in neuropathic rats. Tactile hypersensitivity to von Frey 1g was not very pronounced to begin with, in rats with nerve injury, which was demonstrated as a low difference score baseline value. Since there was no difference in the score between ipsilateral and contralateral hindpaws for the sham-operated rats (n=6), these appear as zero difference score (open squares), even after saline and morphine administration. BL depicts pre-drug baseline responses.

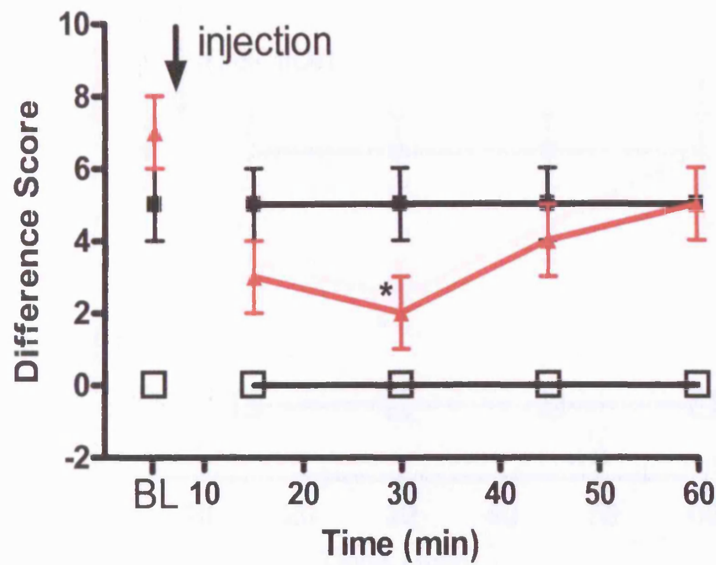


Fig. 6.16- A graph comparing the effect of morphine (red, 3mg/kg, n=6) on the difference score between ipsilateral and contralateral paw to von Frey 5g, when compared to the saline control (black, 0.25ml, n=10). Morphine significantly reduced tactile hypersensitivity in the ipsilateral paw of rats with nerve injury, as demonstrated by a decrease in the difference score. Since there was no difference in the score between ipsilateral and contralateral hindpaws for the sham-operated rats (n=6), these appear as zero difference score (open squares), even after saline and morphine administration. BL depicts pre-drug baseline responses. *$P<0.05$, where the increase in difference score after spinal nerve ligation was prevented by morphine administration (red), when compared to saline control (black). Mann-Whitney test.

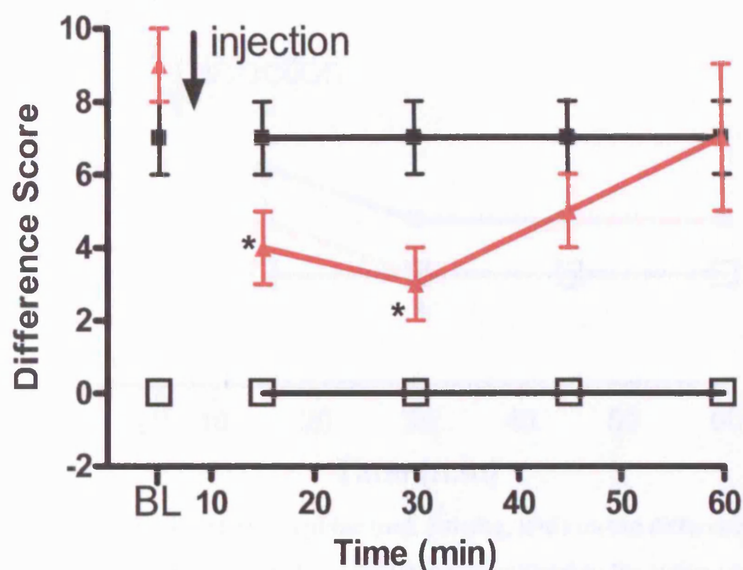


Fig. 6.17- A graph comparing the effect of morphine (red, 3mg/kg, n=6) on the difference score between ipsilateral and contralateral paw to von Frey 10g, when compared to the saline control (black, 0.25ml, n=10). Morphine significantly reduced tactile hypersensitivity in the ipsilateral paw of rats with nerve injury, as demonstrated by a decrease in the difference score. Since there was no difference in the score between ipsilateral and contralateral hindpaws for the sham-operated rats (n=6), these appear as zero difference score (open squares), even after saline and morphine administration. BL depicts pre-drug baseline responses. *$P<0.05$, where the increase in difference score after spinal nerve ligation was prevented by morphine administration (red), when compared to saline control (black). Mann-Whitney test.

iii) Cold response

The subcutaneous administration of morphine, in neuropathic rats, significantly decreased the difference score between ipsilateral and contralateral paw following cold stimuli, 30 minutes after its administration (fig. 6.18), when compared to the saline control, from 1±1 to 0±0. Morphine showed maximal activity 30 minutes following its administration, when each of thermal, tactile and cold was tested.

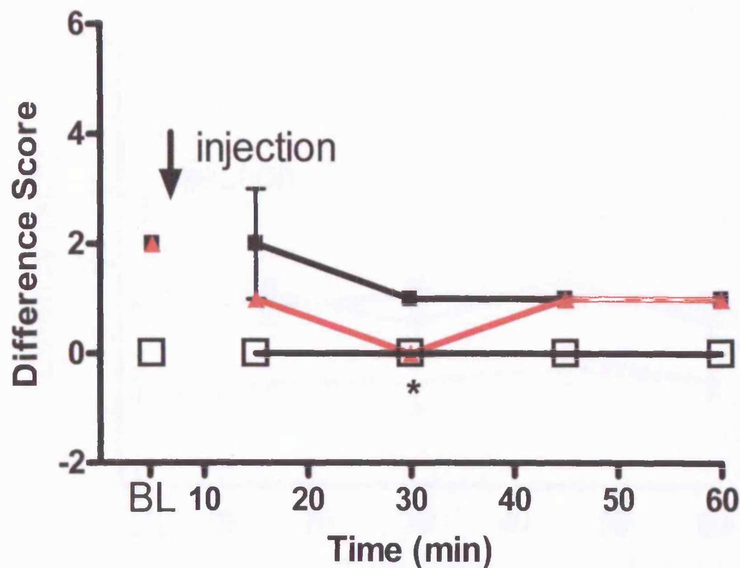


Fig. 6.18- The effect of morphine (red, 3mg/kg, n=6) on the difference score between ipsilateral and contralateral paw to cold stimuli, when compared to the saline control (black, 0.25ml, n=10), in neuropathic rats. Morphine significantly reduced cold hypersensitivity in the ipsilateral paw of rats with nerve injury, as demonstrated by a decrease in the difference score. Since there was no difference in the score between ipsilateral and contralateral hindpaws for the sham-operated rats (n=6), these appear as zero difference score (open squares), even after saline and morphine administration. BL depicts pre-drug baseline responses. *$P<0.05$, where the increase in difference score after spinal nerve ligation was prevented by morphine administration (red), when compared to saline control (black). Mann-Whitney test.

6.3.5 Ondansetron experiments

i) Thermal response

In spinal nerve-ligated rats, the intrathecal administration of ondansetron did not significantly change the thermal hypersensitivity seen following neuropathy, when compared to the saline control (fig. 6.19). In sham-operated rats, ondansetron did not significantly change paw withdrawal latency to thermal stimuli, when compared to the saline control (fig. 6.20)

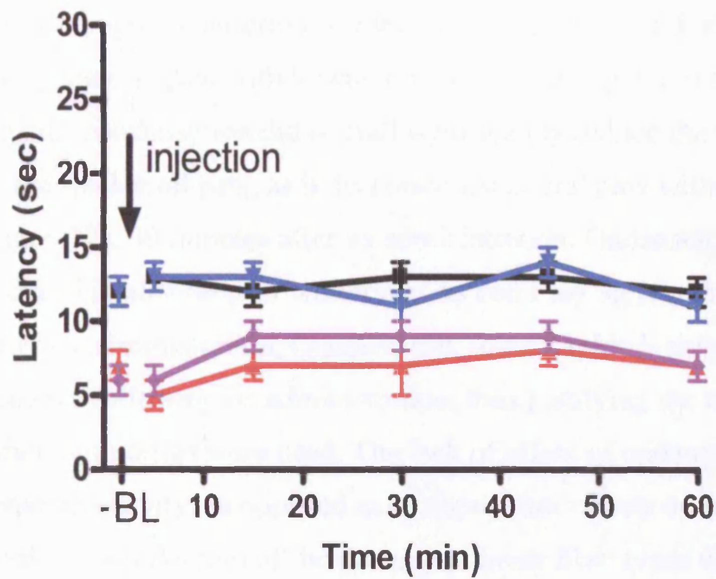


Fig. 6.19- The lack of effect of ondansetron on paw withdrawal latency following thermal stimuli (ipsilateral=purple, contralateral=blue, 20μg, n=9), when compared to the saline control (ipsilateral=red, contralateral=black, 24μl, n=9), in rats with spinal nerve ligation. BL depicts pre-drug baseline responses.

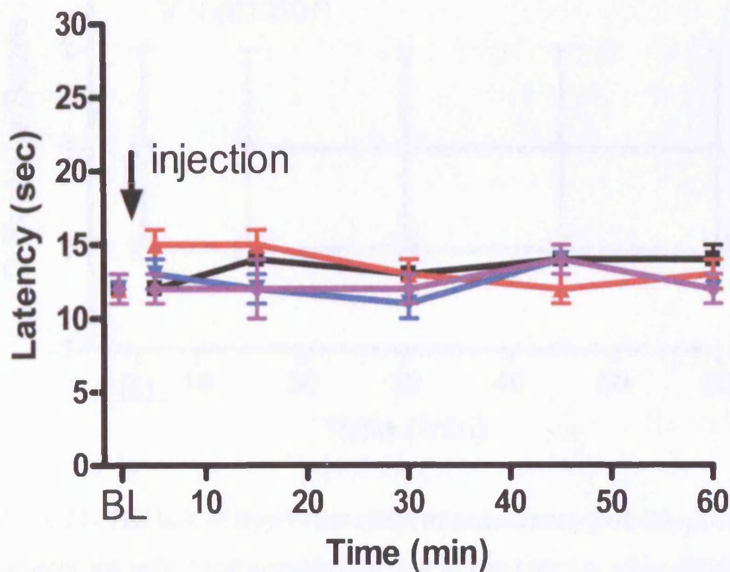


Fig. 6.20- The lack of effect of ondansetron on paw withdrawal latency following thermal stimuli (ipsilateral=purple, contralateral=blue, 20μg, n=6), when compared to the saline control (ipsilateral=red, contralateral=black, 24μl, n=6), in sham-operated rats. BL depicts pre-drug baseline responses.

ii) Tactile response

Even though ondansetron, *via* the intrathecal route, did not significantly change the ipsilateral paw withdrawal to von Frey 1g (fig. 6.21) in rats with spinal nerve ligation, ondansetron did overall significantly reduce the tactile hypersensitivity in the ipsilateral paw, as it decreased ipsilateral paw withdrawal to von Frey 5g (fig. 6.22), 30 minutes after its administration. Ondansetron also significantly reduced ipsilateral paw withdrawal to von Frey 9g (fig. 6.23), 30 and 45 minutes after its administration. Ondansetron, like morphine, showed maximal effect 30 minutes following its administration, thus justifying the timing of administration when both drugs were used. The lack of effect of ondansetron on thermal hypersensitivity, as opposed to its significant effects on tactile hypersensitivity, could be a reflection of the primary afferent fibre types that the 5HT3 receptor is expressed on, which is a subset of the myelinated primary afferent fibres (Maxwell, Kerr et al. 2003).

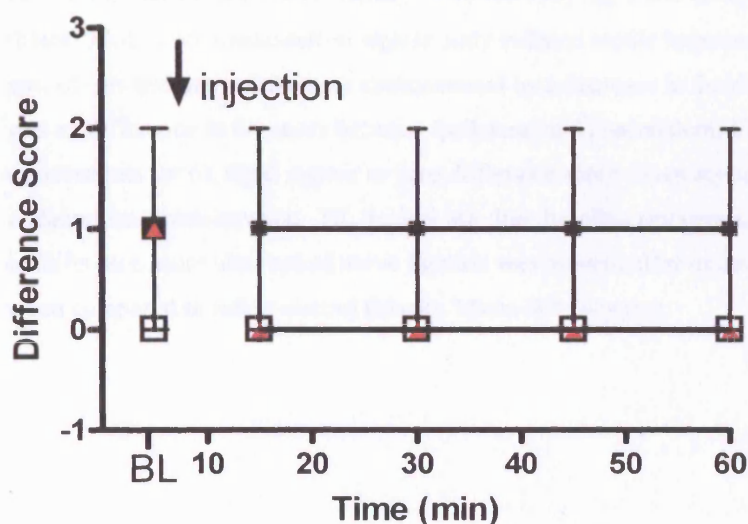


Fig. 6.21- The lack of significant effect of ondansetron (red, 20μg, n=9) on the difference score between ipsilateral and contralateral paw to von Frey 1g, when compared to the saline control (black, 24μl, n=9). Tactile hypersensitivity to von Frey 1g was not very pronounced to begin with, in rats with nerve injury, which was demonstrated as a low difference score baseline value. Since there was no difference in the score between ipsilateral and contralateral hindpaws for the sham-operated rats (n=6), these appear as zero difference score (open squares), even after saline and ondansetron administration. BL depicts pre-drug baseline responses.

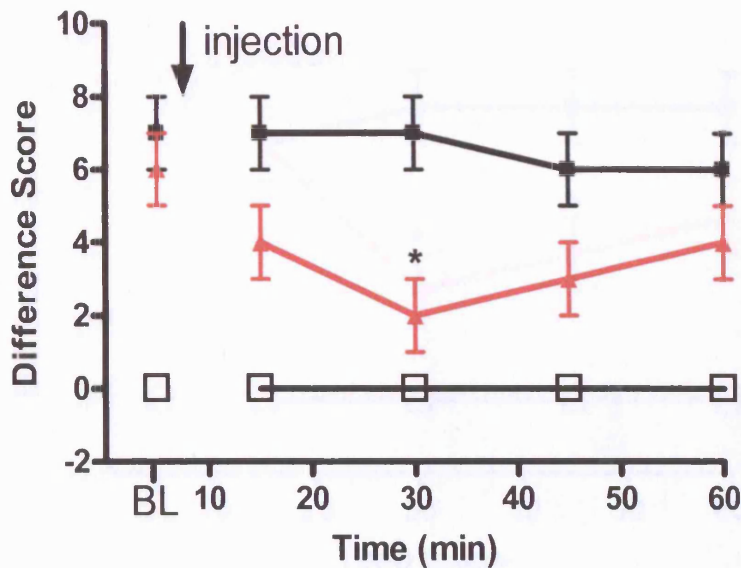


Fig. 6.22- A comparison of the effect of ondansetron (red, 20μg, n=9) on the difference score between ipsilateral and contralateral paw to von Frey 5g, when compared to the saline control (black, 24μl, n=9). Ondansetron significantly reduced tactile hypersensitivity in the ipsilateral paw of rats with nerve injury, as demonstrated by a decrease in the difference score. Since there was no difference in the score between ipsilateral and contralateral hindpaws for the sham-operated rats (n=6), these appear as zero difference score (open squares), even after saline and ondansetron administration. BL depicts pre-drug baseline responses. *$P<0.05$, where the increase in difference score after spinal nerve ligation was prevented by ondansetron administration (red), when compared to saline control (black). Mann-Whitney test.

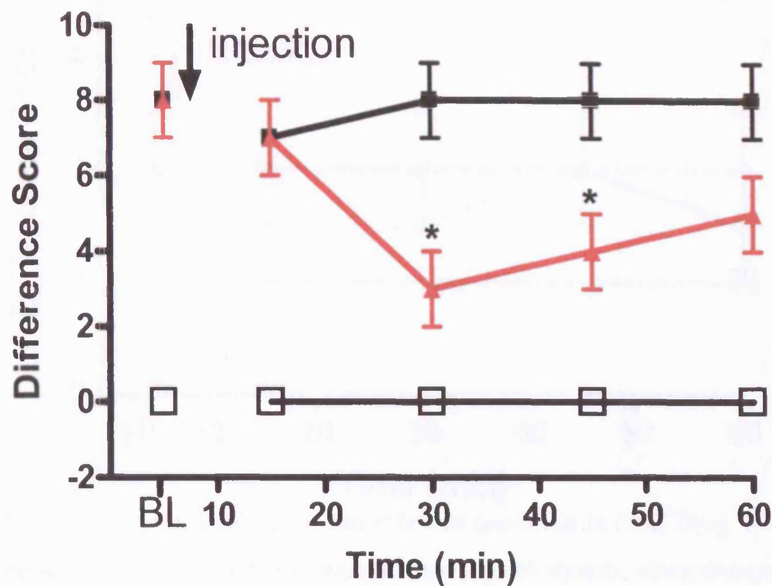


Fig. 6.23- A comparison of the effect of ondansetron (red, 20μg, n=9) on the difference score between ipsilateral and contralateral paw to von Frey 9g, when compared to the saline control (black, 24μl, n=9). Ondansetron significantly reduced tactile hypersensitivity in the ipsilateral paw of rats with nerve injury, as demonstrated by a decrease in the difference score. Since there was no difference in the score between ipsilateral and contralateral hindpaws for the sham-operated rats (n=6), these appear as zero difference score (open squares), even after saline and ondansetron administration. BL depicts pre-drug baseline responses. *P<0.05, where the increase in difference score after spinal nerve ligation was prevented by ondansetron administration (red), when compared to saline control (black). Mann-Whitney test.

iii) Cold response

In rats with spinal nerve ligation, ondansetron, *via* the intrathecal route, did not significantly change the hypersensitivity to cold stimuli in the ipsilateral paw, when compared to the saline control (fig. 6.24).

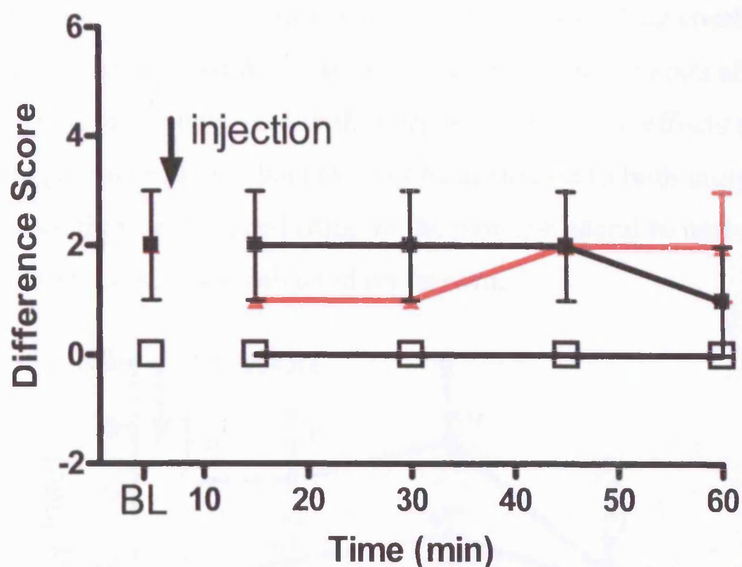


Fig. 6.24- The lack of significant effect of ondansetron (red, 20μg, n=9) on the difference score between ipsilateral and contralateral paw to cold stimuli, when compared to the saline control (black, 24μl, n=9). Since there was no difference in the score between ipsilateral and contralateral hindpaws for the sham-operated rats (n=6), these appear as zero difference score (open squares), even after saline and ondansetron administration. BL depicts pre-drug baseline responses.

6.3.6 Morphine and ondansetron in combination

i) Thermal response

Subcutaneous morphine and intrathecal ondansetron together led to a significant increase in paw withdrawal latency in response to thermal stimuli, in rats with spinal nerve ligation. Ipsilateral paw withdrawal latency to thermal stimuli significantly increased at 15, 30, 45 and 60 minutes following morphine and ondansetron administration, when compared to the ipsilateral saline control, and contralateral paw withdrawal latency to thermal stimuli was also significantly increased by morphine and ondansetron at 5, 15 and 30 minutes post-administration, when compared to the contralateral saline control (fig. 6.25). In sham-operated rats, morphine and ondansetron also led to analgesia in the response to thermal stimuli at timepoints 5, 15, 30 and 45 minutes (fig. 6.26). Therefore, the combination of morphine and ondansetron inhibited thermal hypersensitivity to the same extent in both the ipsilateral and the contralateral paw of rats with nerve injury, and this effect was also comparable to that seen in

sham-operated rats. However, the effect of this drug combination on the ipsilateral paw withdrawal latency in nerve-injured rats also had a delayed onset, as was previously seen with morphine alone. The effects of the drug combination on paw withdrawal latency may be attributed to both morphine and ondansetron, since they are longer-lasting in the paw ipsilateral to nerve injury than when morphine was administered on its own.

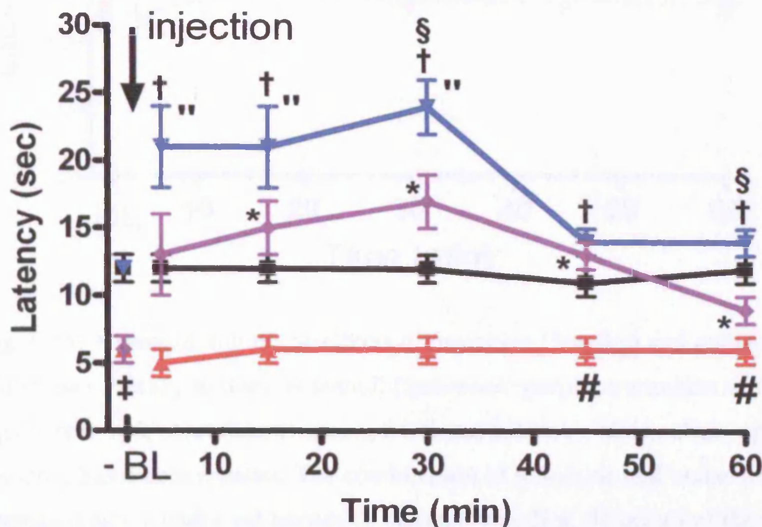


Fig. 6.25- A comparison the effect of morphine (3mg/kg) and ondansetron (20μg) on paw withdrawal latency to thermal stimuli (ipsilateral=purple, contralateral=blue, n=7), with saline (ipsilateral=red, contralateral=black, 0.25ml and 24μl, n=7), in rats with spinal nerve ligation. Spinal nerve ligation caused thermal hypersensitivity in the paw ipsilateral to nerve injury. The combination of morphine and ondansetron caused a significant increase in paw withdrawal latency to thermal stimuli to the same extent in both the ipsilateral and the contralateral paw in rats with nerve injury, when compared to saline control. BL depicts baseline responses. *P<0.05, where a significant increase in ipsilateral paw withdrawal latency is seen following morphine and ondansetron administration, when compared to saline control. "P<0.05, where a significant increase in contralateral paw withdrawal latency is seen following morphine and ondansetron administration, when compared to saline control. †P<0.05, where a significant increase in contralateral paw withdrawal latency was caused by morphine+ondansetron, when compared to ipsilateral paw withdrawal latency in the group that received saline. §P<0.05, contralateral versus ipsilateral paw withdrawal latency in the group that received morphine+ondansetron. #P<0.05, contralateral versus ipsilateral paw withdrawal latency in the group that received saline. ‡P<0.05, where spinal nerve ligation caused a significant decrease in ipsilateral paw withdrawal latency to thermal stimuli, when compared to contralateral paw withdrawal latency, during baseline testing. One way ANOVA followed by Tukey post-hoc test.

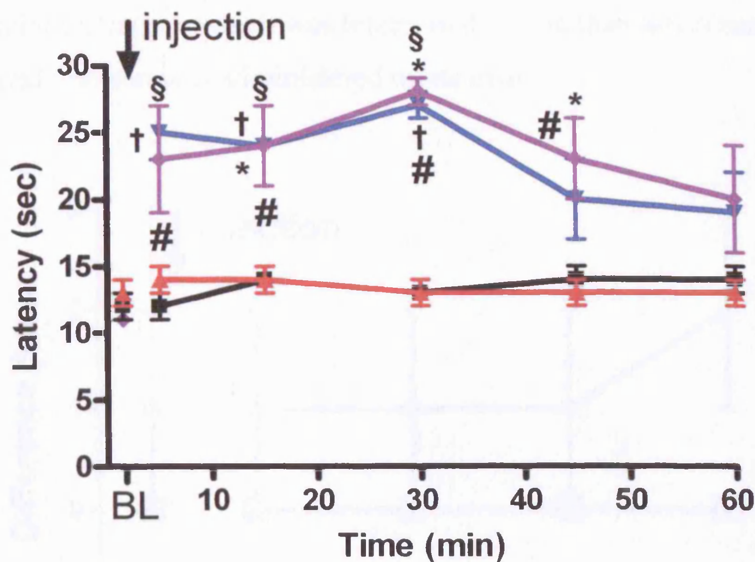


Fig. 6.26- A comparison of the effects of morphine (3mg/kg) and ondansetron (20□g) on paw withdrawal latency to thermal stimuli (ipsilateral=purple, contralateral=blue, n=6), with saline (ipsilateral=red, contralateral=black, 24□l and 0.25ml, n=6), in sham-operated rats. BL depicts pre-drug baseline responses. The combination of morphine and ondansetron significantly increased paw withdrawal latency to thermal stimuli at almost all of the timepoints tested in both ipsilateral and contralateral paws in rats with sham surgery, when compared to saline control. *P<0.05,where a significant increase in ipsilateral paw withdrawal latency is seen following morphine and ondansetron administration, when compared to saline control. †P<0.05, where a significant increase in contralateral paw withdrawal latency is seen following morphine and ondansetron administration, when compared to saline control. §P<0.05, where morphine+ondansetron caused a significant increase in contralateral paw withdrawal latency, when compared to ipsilateral paw withdrawal latency in the group that received saline. #P<0.05, where morphine+ondansetron caused a significant increase in ipsilateral paw withdrawal latency, when compared to contralateral paw withdrawal latency in the group that received saline. One way ANOVA followed by Tukey post-hoc test.

ii) Tactile response

Subcutaneous morphine and intrathecal ondansetron together significantly decreased tactile hypersensitivity in the ipsilateral paw following von Frey 5 (fig. 6.28) and 9g (fig. 6.29), but not following von Frey 1g (fig. 6.27), in rats with spinal nerve ligation. The significant reductions in ipsilateral paw withdrawal

were seen at 15, 30 and 45 minutes following morphine and ondansetron administration, which was longer in duration than when each of morphine and ondansetron was administered on its own.

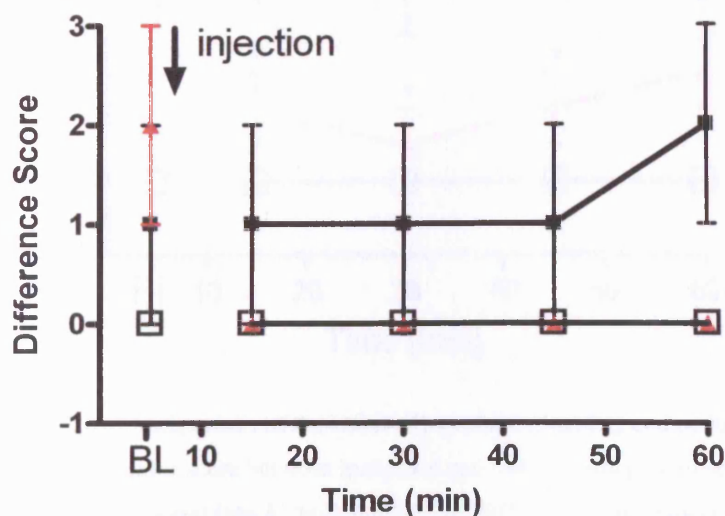


Fig. 6.27- The lack of significant effect of morphine (3mg/kg) and ondansetron (20μg) (red, n=7) on the difference score between ipsilateral and contralateral paw to von Frey 1g, when compared to the saline control (black, 24μl and 0.25ml, n=7). Tactile hypersensitivity to von Frey 1g was not very pronounced to begin with, in rats with nerve injury, which was demonstrated as a low difference score baseline value. Since there was no difference in the score between ipsilateral and contralateral hindpaws for the sham-operated rats (n=6), these appear as zero difference score (open squares), even after saline, and morphine and ondansetron administration. BL depicts pre-drug baseline responses.

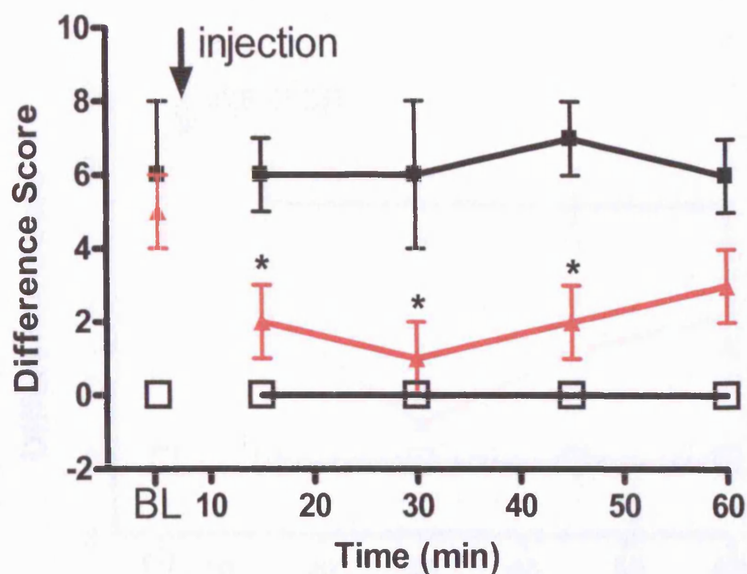


Fig. 6.28- A comparison of the effect of morphine (3mg/kg) and ondansetron (20μg) (red, n=7) on the difference score between ipsilateral and contralateral paw to von Frey 5g, when compared to the saline control (black, 24μl and 0.25ml, n=7). The combination of morphine and ondansetron significantly reduced tactile hypersensitivity in the ipsilateral paw of rats with nerve injury, as demonstrated by a decrease in the difference score. Since there was no difference in the score between ipsilateral and contralateral hindpaws for the sham-operated rats (n=6), these appear as zero difference score (open squares), even after saline and morphine and ondansetron administration. BL depicts pre-drug baseline responses. *$P<0.05$, where the increase in difference score after spinal nerve ligation was prevented by morphine and ondansetron administration (red), when compared to saline control (black). Mann-Whitney test.

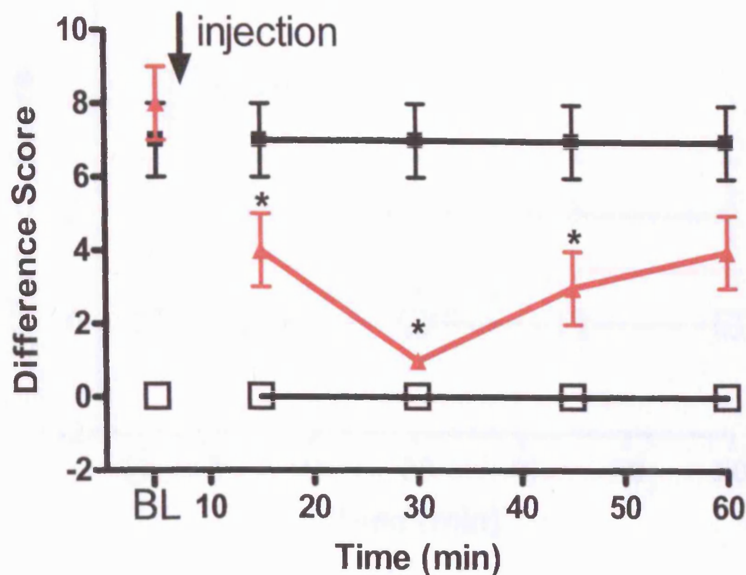


Fig. 6.29- A comparison of the effect of morphine (3mg/kg) and ondansetron (20μg) (red, n=7) on the difference score between ipsilateral and contralateral paw to von Frey 9g, when compared to the saline control (black, 24μl and 0.25ml, n=7). The combination of morphine and ondansetron significantly reduced tactile hypersensitivity in the ipsilateral paw of rats with nerve injury, as demonstrated by a decrease in the difference score. Since there was no difference in the score between ipsilateral and contralateral hindpaws for the sham-operated rats (n=6), these appear as zero difference score (open squares), even after saline and morphine and ondansetron administration. BL depicts pre-drug baseline responses. *P<0.05, where the increase in difference score after spinal nerve ligation was prevented by morphine and ondansetron administration (red), when compared to saline control (black). Mann-Whitney test.

iii) Cold response

The combination of systemic morphine and intrathecal ondansetron also caused a significant decrease in ipsilateral paw withdrawal in rats with spinal nerve ligation (fig. 6.30), as seen by a reduction in the difference score, 30 minutes following the administration of both morphine and ondansetron.

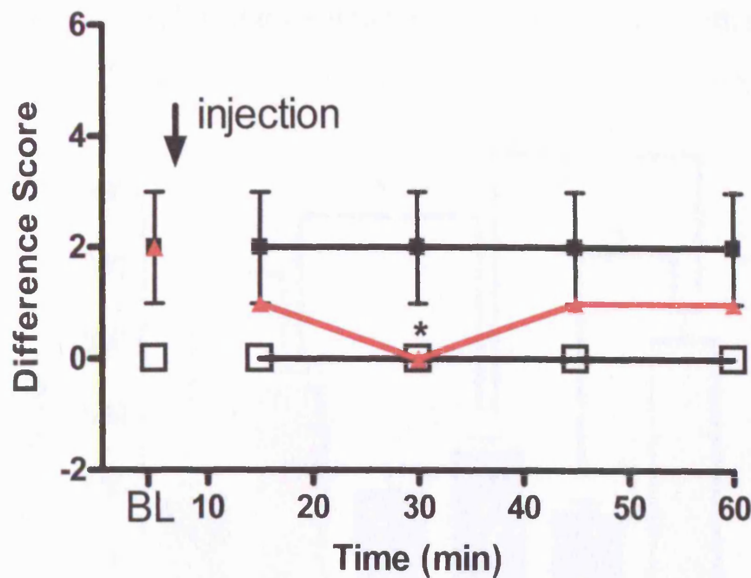


Fig. 6.30- A comparison of the effect of morphine (3mg/kg) and ondansetron (20μg) (red, n=7) on the difference score between ipsilateral and contralateral paw to cold stimuli, when compared to the saline control (black, 24μl and 0.25ml, n=6). The combination of morphine and ondansetron significantly reduced cold hypersensitivity in the ipsilateral paw of rats with nerve injury, as demonstrated by a decrease in the difference score. Since there was no difference in the score between ipsilateral and contralateral hindpaws for the sham-operated rats (n=6), these appear as zero difference score (open squares), even after saline and morphine and ondansetron administration. BL depicts pre-drug baseline responses. *$P<0.05$, where the increase in difference score after spinal nerve ligation was prevented by morphine and ondansetron administration (red), when compared to saline control (black). Mann-Whitney test.

6.3.7 A comparison between morphine, ondansetron and the combination of the two

i) Thermal response

Systemic morphine alone and in combination with intrathecal ondansetron, was significantly more effective than ondansetron alone, and saline control, in reducing ipsilateral (fig. 6.31) and contralateral (fig. 6.32) paw withdrawal to thermal stimuli, in rats with spinal nerve ligation. In addition, when the combination of morphine and ondansetron was compared to morphine alone, the

combination seemed to cause a non-significant increase in the inhibition of ipsilateral paw withdrawal to thermal stimuli in rats with spinal nerve ligation (fig. 6.31).

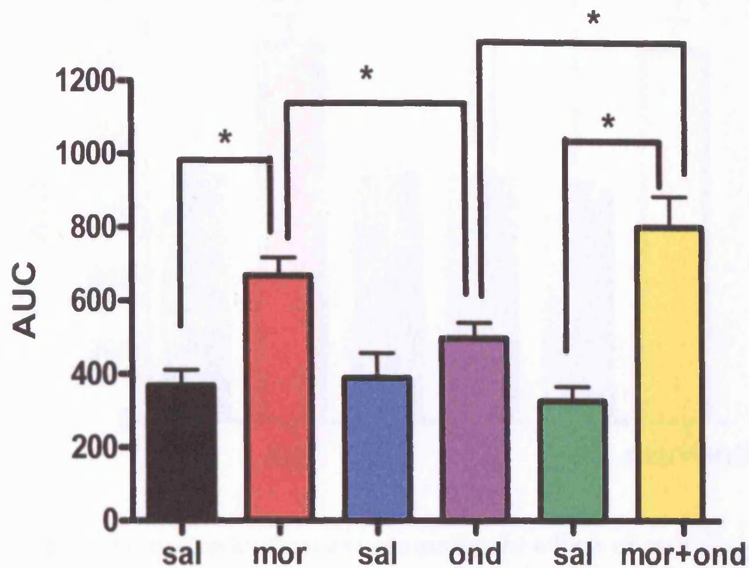


Fig. 6.31- An area under the curve comparing the effects of each of morphine (red, 3mg/kg, n=6, saline control=black, 0.25ml, n=6), ondansetron (purple, 20μg, n=7, saline control=blue, 24μl, n=7) and their combination (yellow, n=7, saline control=green, 24μl and 0.25ml, n=7) on ipsilateral paw withdrawal to thermal stimuli, in rats with spinal nerve ligation. Morphine, and the combination of morphine and ondansetron, were effective in reducing thermal hypersensitivity in the ipsilateral paw of rats with nerve injury, which was demonstrated by a significant increase in the area under the curve, when compared to ondansetron alone and saline controls. *P<0.05, where a significant difference is seen between two groups linked with the lines. *t*-test.

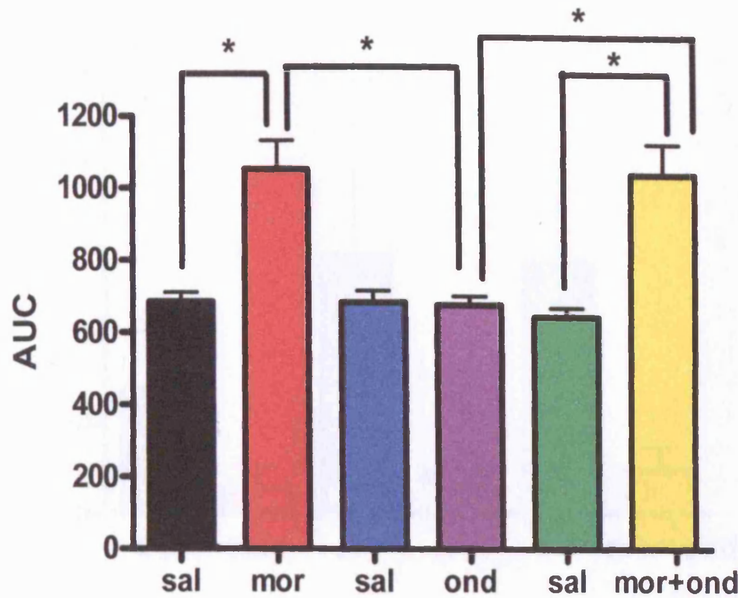


Fig. 6.32- An area under the curve comparing the effects of each of morphine (red, 3mg/kg, n=6, saline control=black, 0.25ml, n=6), ondansetron (purple, 20μg, n=7, saline control=blue, 24μl, n=7) and their combination (yellow, n=7, saline control=green, 24μl and 0.25ml, n=7) on contralateral paw withdrawal to thermal stimuli, in rats with spinal nerve ligation. Morphine, and the combination of morphine and ondansetron, were effective in reducing thermal hypersensitivity in the contralateral paw of rats with nerve injury, which was demonstrated by a significant increase in the area under the curve, when compared to ondansetron alone and saline controls. *P<0.05, where a significant difference is seen between two groups linked with the lines. *t*-test.

ii) Tactile response

Systemic morphine and intrathecal ondansetron, whether alone or in combination, exerted the same effects on ipsilateral paw withdrawal to tactile stimuli, in rats with spinal nerve ligation (fig. 6.33-6.35). However, there was a non-significant tendency for the drug combination to cause more analgesia than each drug alone to von Frey 9g (fig. 6.35) in rats with spinal nerve ligation, as seen by the lower difference score area produced by the combination of morphine and ondansetron.

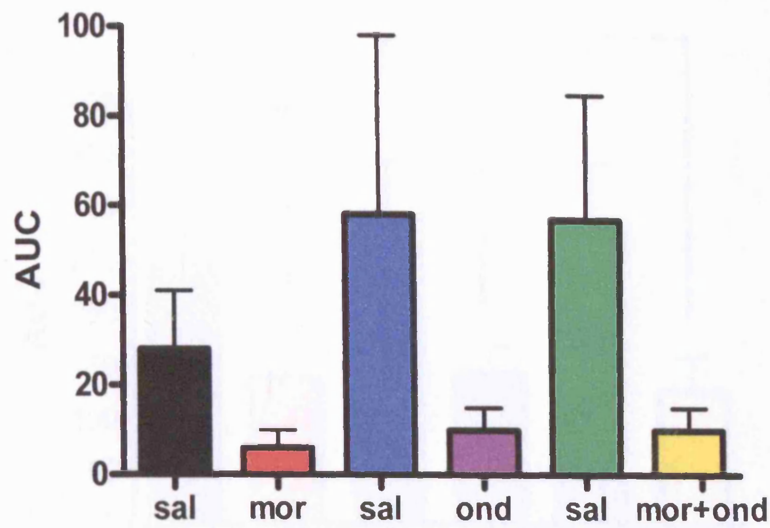


Fig. 6.33- An area under the curve comparing the effects of each of morphine (red, 3mg/kg, n=6, saline control=black, 0.25ml, n=6), ondansetron (purple, 20µg, n=7, saline control=blue, 24µl, n=7) and their combination (yellow, n=7, saline control=green, 24µl and 0.25ml, n=7) on the difference score between ipsilateral and contralateral paw withdrawal to von Frey 1g, in rats with spinal nerve ligation.

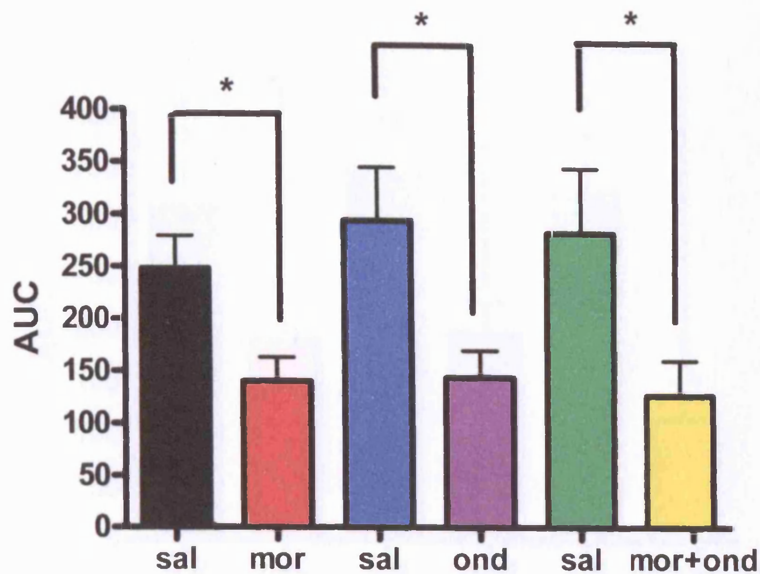


Fig. 6.34- An area under the curve comparing the effects of each of morphine (red, 3mg/kg, n=6, saline control=black, 0.25ml, n=6), ondansetron (purple, 20μg, n=7, saline control=blue, 24μl, n=7) and their combination (yellow, n=7, saline control=green, 24μl and 0.25ml, n=7) on the difference score between ipsilateral and contralateral paw withdrawal to von Frey 5g, in rats with spinal nerve ligation. Morphine and ondansetron, alone or in combination, significantly reduced tactile hypersensitivity to von Frey 5g in the ipsilateral paw in rats with nerve injury, which was demonstrated by a decrease in the area under the curve of the difference score, when compared to saline control. *P<0.05, where a significant difference is seen between two groups linked with the lines. Mann-Whitney test.

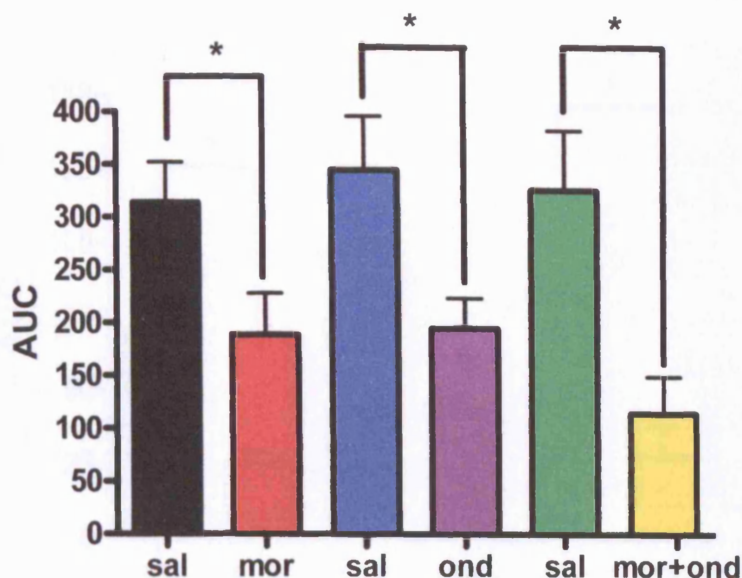


Fig. 6.35- An area under the curve comparing the effects of each of morphine (red, 3mg/kg, n=6, saline control=black, 0.25ml, n=6), ondansetron (purple, 20μg, n=7, saline control=blue, 24μl, n=7) and their combination (yellow, n=7, saline control=green, 24μl and 0.25ml, n=7) on the difference score between ipsilateral and contralateral paw withdrawal to von Frey 9g, in rats with spinal nerve ligation. Morphine and ondansetron, alone or in combination, significantly reduced tactile hypersensitivity to von Frey 9g in the ipsilateral paw in rats with nerve injury, which was demonstrated by a decrease in the area under the curve of the difference score, when compared to saline control. *P<0.05, where a significant difference is seen between two groups linked with the lines. Mann-Whitney test.

iii) Cold response

Morphine alone, and in combination with ondansetron, was significantly more effective than ondansetron alone, in reducing ipsilateral paw withdrawal to cold stimuli, in rats with spinal nerve ligation (fig. 6.36).

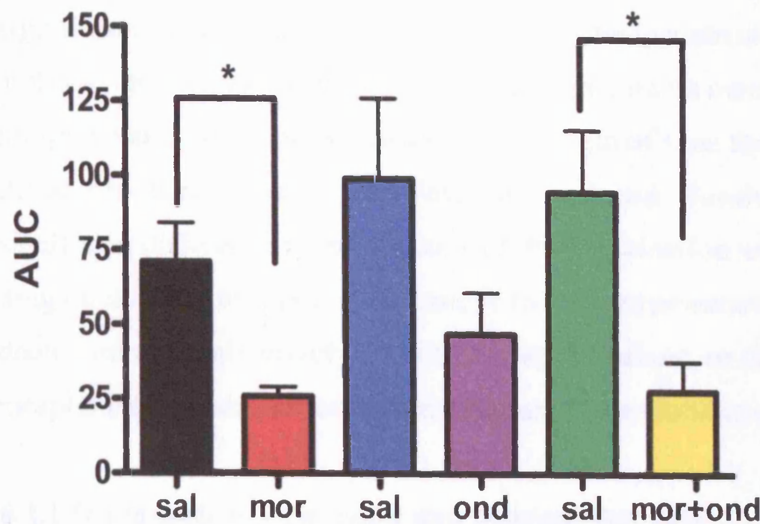


Fig. 6.36- An area under the curve comparing the effects of each of morphine (red, 3mg/kg, n=6, saline control=black, 0.25ml, n=6), ondansetron (purple, 20μg, n=7, saline control=blue, 24μl, n=7) and their combination (yellow, n=7, saline control=green, 24μl and 0.25ml, n=7) on the difference score between ipsilateral and contralateral paw withdrawal to cold stimuli, in rats with spinal nerve ligation. Morphine alone, and in combination with ondansetron, significantly reduced cold hypersensitivity in the ipsilateral paw in rats with nerve injury, which was demonstrated by a decrease in the area under the curve of the difference score, when compared to saline control. *P<0.05, where a significant difference is seen between two groups linked with the lines. Mann-Whitney test.

6.4 Discussion

In my study, the ligation of the L5 and L6 spinal nerves led to the manifestation of all of thermal, tactile and cold hypersensitivities in the injured paw, which also occurred in previous studies which investigated this model of nerve injury (Kim and Chung 1992; Wegert, Ossipov et al. 1997; Chapman, Suzuki et al. 1998; Ossipov, Bian et al. 1999).

Ondansetron, in normal rats, did not cause any analgesia. However, when ondansetron was administered in rats with peripheral nerve injury, it significantly decreased the hypersensitivity to tactile stimuli, in the injured paw. Morphine

alone was analgesic in normal rats (see **chapter 4**) and was also analgesic in neuropathic rats, significantly decreasing ipsilateral paw hypersensitivity to thermal, tactile and cold stimuli. The combination of morphine and ondansetron significantly reduced the hypersensitivity of the ipsilateral paw to thermal, tactile and cold stimuli. In comparison with each drug on its own, the combination of morphine and ondansetron increased the length of time the hypersensitivity of the paw to thermal and tactile stimuli was reduced. However, there was no significant difference in the efficacy of the combination when compared to each drug on its own, except for the case of thermal hypersensitivity, which was decreased more effectively by either morphine alone, or the combination of morphine and ondansetron, when compared to ondansetron alone.

6.4.1 Ondansetron in normal and neuropathic rats

Initial electrical and microinjection studies in the RVM showed that descending inhibitions were activated (Reynolds 1969; Fields and Heinricher 1985). Indeed, it was previously thought that descending inhibitions predominate over descending facilitations. Following that however, it became apparent that different doses and frequencies used in the RVM, also led to descending facilitations becoming activated (Besson and Chaouch 1987; Urban and Gebhart 1999; Zhuo and Gebhart 2002). It has now been shown that both inhibitions and facilitations descend from the RVM at any one time, and the intensity of each is dictated by the pathophysiological state of the animal and the stimulus modality used (Kauppila, Kontinen et al. 1998; Wei, Dubner et al. 1999; Zhuo and Gebhart 2002).

5-HT was found to be both antinociceptive (Alhaider, Lei et al. 1991; Peng, Lin et al. 1996; Tsuchiya, Yamazaki et al. 1999), and pronociceptive (Jordan, Kenshalo et al. 1978; Ali, Wu et al. 1996; Oyama, Ueda et al. 1996; Calejesan, Ch'ang et al. 1998; Green, Scarth et al. 2000; Zeitz, Guy et al. 2002; Suzuki, Rahman et al. 2004), in the spinal cord dorsal horn. The RVM is the primary source of 5-HT in the spinal cord dorsal horn (Fasmer, Berge et al. 1983; Besson and Chaouch 1987), however, neither ON nor OFF cells seem to contain 5-HT

(Gao and Mason 2000). Furthermore, descending facilitations have been shown to be enhanced following chronic pain states, since treatment with the 5HT3 antagonist ondansetron decreased tactile hypersensitivity in rats with spinal cord injury (Oatway, Chen et al. 2004), and decreased thermal and tactile evoked dorsal horn responses in rats with peripheral nerve injury (Suzuki, Rahman et al. 2004), and cancer-induced bone pain (Donovan-Rodriguez, Urch et al. 2006) as well as caused analgesia in patients with neuropathic pain (McCleane, Suzuki et al. 2003).

My study shows that the 5HT3 receptor does not play an important role in thermal and cold responses. The subset of myelinated primary afferent Aδ fibres where the 5HT3 receptor is predominantly located (Maxwell, Leranth et al. 1983; Kidd, Laporte et al. 1993; Tecott, Maricq et al. 1993; Zeitz, Guy et al. 2002; Maxwell, Kerr et al. 2003; Conte, Legg et al. 2005) may be one of the reasons this receptor is not involved in thermal and cold responses, since the receptors which convey these modalities, TRPV1 and TRPA1 respectively, are predominantly located on unmyelinated, peptidergic fibres (Caterina, Schumacher et al. 1997; Magerl, Fuchs et al. 2001; Patapoutian, Peier et al. 2003; Lee, Lee et al. 2005; Meyer, Ringkamp et al. 2006). Indeed, the study by Maxwell *et al* in 2003, using immunohistochemistry, shows that the 5HT3-A receptor subunit, which forms functional homopentameric receptors, is not located on peptidergic primary afferent fibres, thus supporting my findings. Furthermore, the TRPV2 receptor which is located primarily on myelinated fibres, codes a suprathreshold heat (Patapoutian, Peier et al. 2003; Meyer, Ringkamp et al. 2006), which was not tested in this study.

Furthermore, even though a previous study using in vivo electrophysiology showed that ondansetron reduced the thermal evoked response of dorsal horn neurons, the dose of ondansetron used was 100μg, which was higher than the dose used in my study (Suzuki, Rahman et al. 2004). Additionally, the latter study also showed that the effect of ondansetron on mechanical evoked dorsal horn neuronal responses was more pronounced in rats with peripheral nerve

injury than sham-operated rats, and that the effects of ondansetron on the thermal evoked response were less pronounced, since ondansetron reduced the thermal evoked response of dorsal horn neurons in both neuropathic and sham-operated rats to the same extent (Suzuki, Rahman et al. 2004).

Furthermore, in my study, the 5HT3 antagonist ondansetron decreased paw hypersensitivity to tactile stimuli in neuropathic rats, with no effect on normal rats. Although previous electrophysiological studies showed that ondansetron also reduced mechanical evoked dorsal horn neuronal responses in sham-operated rats, which were considered the control 'normal' rats, they used doses 50 and 100μg of ondansetron (Suzuki, Rahman et al. 2004), whereas I used doses 10 and 20μg in normal rats, and only dose 20μg in spinal nerve ligated and sham-operated rats.

Therefore, my study confirms that the status of descending serotonergic facilitations is enhanced following nerve injury (Burgess, Gardell et al. 2002; Suzuki, Rahman et al. 2004; Vera-Portocarrero, Zhang et al. 2006), and that when the increased surge of 5-HT acts on the spinal 5HT3 receptor, it causes nociception (Ali, Wu et al. 1996; Calejesan, Ch'ang et al. 1998; Green, Scarth et al. 2000; Zeitz, Guy et al. 2002).

6.4.2 Morphine in neuropathic rats

Some studies showed that spinal morphine has reduced efficacy in neuropathic pain and that systemic and supraspinal morphine are more effective (Bian, Nichols et al. 1995; Lee, Chaplan et al. 1995; Nichols, Bian et al. 1995; Nichols, Lopez et al. 1997; Wegert, Ossipov et al. 1997; Bian, Ossipov et al. 1999; Pertovaara and Wei 2003), whereas other studies show that spinal morphine is effective in neuropathic pain (Yamamoto and Yaksh 1991; Suzuki, Chapman et al. 1999; Suzuki and Dickenson 2002; Zhao, Tall et al. 2004). Two important events which have been attributed to the 'reduced' efficacy of spinal morphine in neuropathic pain are the increases in levels of spinal dynorphin (Bian, Ossipov et

al. 1999; Ossipov, Lai et al. 2000; Dickenson and Kieffer 2006) and cholecystokinin (Xu, Puke et al. 1993; Nichols, Bian et al. 1995; Zhang, Bao et al. 1998) (see section **1.7.7 ii**).

In my study, systemic morphine was used, since it acts at all three sites where μ-opioid receptors are located: on primary afferent fibres, to decrease neurotransmitter release, in the dorsal horn, to decrease synaptic transmission, and on RVM neurons to activate OFF cells, inhibit ON cells and have an overall inhibitory effect on the spinal cord pain transmission (Fields, Heinricher et al. 1991; Dickenson 1994; Heinricher 1997; Porreca, Burgess et al. 2001; Dickenson and Kieffer 2006).

No study has disputed the effectiveness of morphine in inhibiting thermal hyperalgesia in numerous nerve injury models, however, the effectiveness of morphine on tactile allodynia has been questioned. Morphine (systemic and spinal) effectively inhibited thermal hyperalgesia in the chronic constriction model (Yamamoto and Yaksh 1991; Lee, Kayser et al. 1994; Backonja, Miletic et al. 1995) and the spinal nerve ligation model (Wegert, Ossipov et al. 1997), as well as in my study. In addition, morphine reduced c-Fos expression following noxious thermal stimuli, in the spinal cord dorsal horn side ipsilateral to the chronic constriction of the sciatic nerve (Catheline, Le Guen et al. 2001). The effectiveness of morphine on this stimulus modality is due to the fact that thermal stimuli has been shown to be mostly conveyed by channels of the TRPV family, which reside on the peripheral terminals of C-fibres (Caterina, Schumacher et al. 1997; Ossipov, Bian et al. 1999), the central terminals of which are μ-opioid receptor rich (Besse, Lombard et al. 1992; deGroot, Coggeshall et al. 1997; Zhang, Bao et al. 1998).

There are two types of tactile allodynia, dynamic allodynia, which has been shown to be conveyed mostly by Aβ- and capsaicin-insensitive Aδ-fibres, and static allodynia, which was measured in my study and which has been shown to be conveyed mostly by Aδ-fibres (Field, Bramwell et al. 1999). Static allodynia,

as measured by a decrease in paw withdrawal threshold to von Frey monofilaments, has been shown to be inhibited by systemic and supraspinal, but not spinal, morphine in the spinal nerve ligation model (Bian, Nichols et al. 1995; Lee, Chaplan et al. 1995; Pertovaara and Wei 2003) and in the streptozocin model (Field, McCleary et al. 1999), whereas spinal morphine was shown to be effective in a behavioural study using the spared nerve injury model (Zhao, Tall et al. 2004). In addition, systemic morphine was shown to decrease vocalisation due to mechanical pressure, in the chronic constriction model (Attal, Chen et al. 1991). However, other studies have shown that systemic morphine was only effective in reducing static allodynia, as measured by changes in the paw withdrawal threshold (Joshi, Hernandez et al. 2006), and paw pressure test (Lemberg, Kontinen et al. 2006), when used at sedating doses, in the spinal nerve ligation model, whereas another study has shown the reduced efficacy of systemic morphine, even at sedating doses, to inhibit static allodynia, also measured as a decrease in paw withdrawal threshold, in the spinal nerve ligation model (Kontinen, Paananen et al. 1998).

In my study, systemic morphine, at a non-sedating dose (see **section 4.3.4 i**), was effective in reducing static allodynia, which has been shown previously in studies investigating the chronic constriction model (CCI) (Field, Bramwell et al. 1999) and the streptozocin model of nerve injury (Field, McCleary et al. 1999), as well as in a study investigating cancer-induced bone pain (Urch, Donovan-Rodriguez et al. 2005). The channels which transduce mechanosensation are less known than their thermal and chemical transducing counterparts, but are thought to include the bacterial osmosensitive ion channels MscL and MscS, TRPV4 and P2X3 (Wood, Abrahamsen et al. 2004). Of these, the P2X3 channel has been shown to be expressed in non-peptidergic, IB4 positive primary afferent fibres, which are mostly Aδ-fibres (Tsuda, Shigemoto-Mogami et al. 2003; Tsuda, Inoue et al. 2005; Meyer, Ringkamp et al. 2006), which confirms the other studies that show that static allodynia is mostly conveyed by Aδ-fibres (Field, Bramwell et al. 1999; Ossipov, Bian et al. 1999). However, tactile allodynia following nerve injury has been shown by many previous studies (see section

1.7.7 iii) to be mediated mostly supraspinally, since it was mostly inhibited by systemic and supraspinal, but not spinal morphine (Wegert, Ossipov et al. 1997; Ossipov, Bian et al. 1999), and thus the effectiveness of systemic morphine in inhibiting tactile hypersensitivity in my study.

The effect of morphine on cold allodynia has not been investigated by many studies. Cold stimuli has been found to be coded for by the TRPM8 and the TRPA1 channels, the latter of which is found mostly colocalised with TRPV1, on the peripheral terminals of C-fibres (Patapoutian, Peier et al. 2003; Wood 2004; Lee, Lee et al. 2005), which renders this stimulus modality partly sensitive to morphine control. In my study, morphine was effective at significantly reducing cold allodynia, however, two previous studies also using systemic morphine (Lee, Kayser et al. 1994; Kontinen, Paananen et al. 1998) have found the drug to be ineffective in reducing cold allodynia. Whereas the study by Lee *et al* used the chronic constriction model of nerve injury, as well as paw immersion in a 10°C water bath to test for cold allodynia, the study by Kontinen *et al* used both the same nerve injury model, species and the same cold allodynia test as the ones used in my study. The only difference between the Kontinen *et al* study and my study was the dose of morphine used, I used 3mg/kg, whereas they used an acute systemic dose of 10mg/kg, as well as slow-release pellets resulting in a total dose of 75, 150 and 375mg. However, cold allodynia in the SNL model has been shown to be variable and not strongly demonstrated in some cases (Ossipov, Lai et al. 2006). The study by Kontinen *et al* also could not find a manifestation of thermal hyperalgesia following nerve injury.

Systemic morphine, as was previously discussed, acts on μ-opioid receptors which are located on primary afferent fibres, on dorsal horn neurons, as well as on supraspinal sites, such as the RVM, to exert its effects (Dickenson 1994; Dickenson and Kieffer 2006), whereas intrathecal ondansetron only acts on the 5HT3 receptors which are mostly located in the vicinity of the superficial dorsal horn (Maxwell, Leranth et al. 1983; Maxwell, Kerr et al. 2003). Therefore, the fact that systemic morphine was more effective in my study, than spinal

ondansetron in reducing hypersensitivity to all of thermal, tactile and cold stimuli following peripheral nerve injury, was not surprising.

6.4.3 Morphine and ondansetron in neuropathic pain

Descending serotonergic facilitations are part of a spino-bulbo-spinal loop, which has NK-1-expressing lamina I projection neurons at its origin. These projection neurons ascend to the parabrachial area, which has fibres that activate pronociceptive pathways from the RVM onto the spinal cord (Bester, Matsumoto et al. 1997; Mantyh, Rogers et al. 1997; Bester, Chapman et al. 2000; Gauriau and Bernard 2002; Todd 2002; Rahman, Suzuki et al. 2003; Suzuki, Rahman et al. 2004; Hunt and Bester 2005). The fact that ondansetron, acting on spinal 5HT3 receptors, has been shown to decrease tactile hypersensitivity (Oatway, Chen et al. 2004) and dorsal horn neuronal excitability (Suzuki, Rahman et al. 2004; Donovan-Rodriguez, Urch et al. 2006; Rahman, Suzuki et al. 2006) following chronic pain states, whilst affecting baseline transmission to a lesser extent, shows that descending serotonergic facilitations are enhanced, following some chronic pain states, including those of a neuropathic nature (Suzuki, Rahman et al. 2004).

Indeed, it has been shown that following peripheral nerve injury, continuous afferent input initiates spinal sensitisation, and 6 days following nerve injury, descending facilitations are enhanced and serve to maintain central sensitisation (Kauppila, Kontinen et al. 1998; Burgess, Gardell et al. 2002; Vera-Portocarrero, Zhang et al. 2006). Furthermore, the ablation of μ-expressing cells in the RVM with dermorphin-saporin, which are thought to include ON cells, decreased hypersensitivity due to nerve injury and did not affect normal pain, which means that these μ-expressing cells are also involved in the increased descending facilitations seen after nerve injury (Porreca, Burgess et al. 2001; Porreca, Ossipov et al. 2002; Vera-Portocarrero, Zhang et al. 2006). If these μ-opioid receptor expressing cells are inhibited by morphine, therefore, following nerve injury, systemic morphine should inhibit the increased descending facilitations at

a supraspinal level, and ondansetron should inhibit the enhanced descending serotonergic facilitations at a spinal level and therefore, the combination of morphine with ondansetron was postulated to be more effective than each drug on its own, in reducing the hypersensitivity seen following peripheral nerve injury. In addition, this drug combination could be clinically beneficial in reducing the dose of morphine used and thus the unwanted side-effects seen with high doses of morphine, such as sedation and respiratory depression (Dickenson 1994).

In my study, the combination of morphine and ondansetron only served to increase the efficacy of ondansetron alone in reducing the thermal and cold hypersensitivity seen following nerve injury, since ondansetron alone only reduced the tactile hypersensitivity. Additionally, the combination of morphine and ondansetron in my study increased the duration of analgesia for both thermal and tactile hypersensitivity, when compared to each drug alone. Indeed, thermal hypersensitivity, when the combination was used, was reduced for 45 minutes, as opposed to when only morphine was used, where the reduction was seen for only 15 minutes. Tactile hypersensitivity on the other hand was reduced by the combination of morphine and ondansetron for 30 minutes, whereas when each drug was acting alone, the reduction of tactile hypersensitivity only lasted for half that time.

Even though morphine was administered via the systemic route in my study, previous studies have shown, using spinal morphine, that thermal hypersensitivity following nerve injury is mediated mostly spinally (see section **1.7.7 iii**) (Yamamoto and Yaksh 1991; Yamamoto and Yaksh 1992). Therefore, the fact that morphine, whether alone or in combination with ondansetron, was more effective than ondansetron alone in decreasing the thermal and cold hypersensitivity seen following neuropathic pain could be further proof that thermal and cold hypersensitivity are predominantly conveyed by the unmyelinated, capsaicin-sensitive C-fibres, since most of the μ-opioid receptors are expressed on these fibres (Wegert, Ossipov et al. 1997; Ossipov, Bian et al. 1999), whereas only a small amount of 5HT3 receptors is found on these fibres

(Maxwell, Leranth et al. 1983; Kidd, Laporte et al. 1993; Tecott, Maricq et al. 1993; Zeitz, Guy et al. 2002; Maxwell, Kerr et al. 2003; Conte, Legg et al. 2005).

However, when comparing the area under the curve for each drug alone and the combination, the only significant decrease seen was between morphine with ondansetron alone, and the combination with ondansetron alone, in decreasing the thermal response following peripheral nerve injury, even though there was a non-significant trend for the drug combination to be more effective than each drug in inhibiting thermal hypersensitivity and hypersensitivity to von Frey 9g. The lack of synergism seen in my study could be due to the fact that the doses of morphine and ondansetron used were already effective doses, whereas previous studies investigating the synergism between two drugs usually use the combination of an ineffective dose of each drug (Mjellem-Joly, Lund et al. 1991; Matthews and Dickenson 2002).

Furthermore, synergism studies tend to use more than one dose of each drug and construct an isobologram, whereby the different doses of each drug are plotted against each other and the ED50, the effective dose required to produce a specific effect in 50% of an animal population, of each drug is calculated (van Rijn, Sun et al. 2004). Nevertheless, since only one dose of each drug was used in this study, an area under the curve, normally reserved to determine the total exposure of the body to a given drug over time, was used to compare whether an overall effect had occurred with each drug and drug combination used.

6.4.4 Conclusion

Even though the doses used in my study did not allow for a proper judgement to be made on whether there is synergy between morphine and ondansetron, the combination of the two drugs provided a longer-lasting reduction of the hypersensitivities seen following nerve injury, which could translate into longer-lasting analgesia for patients with neuropathic pain.

Furthermore, morphine alone, the gold standard of pain control, was shown to be more effective than ondansetron alone in my study, which could be due to the fact that morphine acts on the μ-opioid receptors found on primary afferent fibres, spinal cord dorsal horn neurons and supraspinal sites, and thus on all three important sites of the pain transmission pathway (Dickenson 1997; Dickenson and Kieffer 2006). Therefore, morphine is effective in decreasing some of the pain abnormalities which occur in neuropathic pain states (Rowbotham, Reisner-Keller et al. 1991; Yamamoto and Yaksh 1992; Sindrup and Jensen 1999; Suzuki, Chapman et al. 1999; Attal, Guirimand et al. 2002; Hansson and Dickenson 2005). However, clinical testing should be done with different doses of morphine and ondansetron together, before the final ruling is made on this drug combination.

Chapter 7:

Discussion

7. Discussion

7.1 Summary

In my study, morphine was found to inhibit: dorsal horn neuronal responses following the activation of the spinal NK-1 receptor, thermal and mechanical hypersensitivity following the activation of each of the spinal NMDA and NK-1 receptor, tactile hypersensitivity following the activation of the peripheral NMDA receptor as well as thermal, tactile and cold hypersensitivity following the spinal nerve ligation model. Therefore overall, morphine was able to inhibit the excitatory transmission in the spinal cord, when this excitatory transmission was directly activated by specific agonists at excitatory receptors, and when activated secondary to nerve injury.

In addition, peripheral NMDA receptors were found to be pharmacologically activated by a high dose of NMDA, but not pathophysiologically involved in the tactile hypersensitivity seen following the spinal nerve ligation model, whereas descending serotonergic facilitations were found to be enhanced following this model of nerve injury, as blocking the spinal 5HT3 receptor with ondansetron reduced tactile hypersensitivity in neuropathic animals, with no effect in normal animals.

Finally, there was no synergy observed, when morphine and ondansetron were administered together, on thermal, tactile and cold hypersensitivity following the spinal nerve ligation model.

7.2 General discussion

An earlier study has been done to attempt to gauge the importance of pre- versus postsynaptic effects of morphine on inhibiting dorsal horn neuronal responses (Lombard *et al.*, 1989), by investigating the potency of the same dose of systemic morphine on the spontaneous hyperactivity of dorsal horn neurons in the spinalised, decrebrated rats, when primary afferent fibres are left intact or

removed. They concluded that the efficacy of morphine to inhibit responses whereby morphine can act predominantly on its presynaptic μ-opioid receptors, is more than when morphine has to act mostly on its postsynaptic μ-opioid receptors. This finding was postulated to be due to the fact that at the spinal cord level, as has been revealed by dorsal rhizotomy (Besse *et al.*, 1990), peripheral axotomy (deGroot *et al.*, 1997; Zhang *et al.*, 1998) and expression studies (Mansour *et al.*, 1994), the number of presynaptic μ-opioid receptors is larger than postsynaptic μ-opioid receptors.

Furthermore, in previous studies (Chapman *et al.*, 1992; Chapman *et al.*, 1994b; Dickenson *et al.*, 1986; Suzuki *et al.*, 1999), as well as in my study, it was shown that the dorsal horn neuronal responses which reflect the action of morphine on its presynaptic receptors include electrically evoked input, a measure of neuronal activity after the first electrical stimulus and prior to any hyperexcitability (Urch *et al.*, 2003a), and C-fibre responses. Even though the final C-fibre response measured in all these studies occurs following the 16th electrical stimuli, and thus following some hyperexcitability, most of the presynaptic μ-opoid receptors have been shown to reside on the central terminals of these fibres and therefore, this response is highly sensitive to morphine control (Besse *et al.*, 1990; deGroot *et al.*, 1997; Mansour *et al.*, 1994; Zhang *et al.*, 1998).

However, electrically evoked Aβ-fibre response has been shown to represent a response which would require morphine to act predominantly on its postsynaptic μ-opioid receptors to inhibit it (Chapman *et al.*, 1992; Chapman *et al.*, 1994b; Dickenson *et al.*, 1986), since it has been shown that there are hardly any μ-opioid receptors found on Aβ-fibres. The latter was revealed in studies which showed that most μ-opioid receptors are found located in the superficial dorsal horn of the spinal cord, an area where Aβ-fibres do not terminate in (Besse *et al.*, 1990; Zhang *et al.*, 1998).

Another response which would require morphine to act partly *via* its postsynaptic μ-opioid receptors is wind-up. Wind-up is the frequency dependent potentiation

of deep dorsal horn neurons after C-fibre stimulation (Mendell, 1966), and includes both a pre- and a postsynaptic-dependent component. Indeed, wind-up response has been shown to occur due to the activation of the NMDA receptor channel, since this response was inhibited by an NMDA receptor antagonist (Dickenson, 1990; Dickenson *et al.*, 1990; Dickenson *et al.*, 1987). Furthermore, the activation of the NK-1 receptor is also needed for the full expression of the wind-up response, since the administration of an NK-1 antagonist has been shown to partly decrease wind-up response (Xu *et al.*, 1992). Both the NMDA and the NK-1 receptors are mostly found on the spinal cord dorsal horn neurons (Coggeshall *et al.*, 1997a), where postsynaptic μ-opioid receptors are fewer in numbers (Besse *et al.*, 1992; Besse *et al.*, 1990; Zhang *et al.*, 1998).

However, wind-up generation is also due to the electrical stimulation of the neuron at 3x the C-fibre threshold and requires input, a presynaptic component which is sensitive to morphine control, to activate the postsynaptic NMDA receptor. Therefore, previous studies have concluded that morphine is not completely ineffective in inhibiting wind-up response, but that a higher dose of morphine is required than that needed to inhibit presynaptic-mediated dorsal horn neuronal responses. The latter is due to the fact that with a lower dose of morphine, the small amount of electrically evoked input response which is left is able to break through, and is enough to generate wind-up (Chapman, 1994; Chapman *et al.*, 1992; Chapman *et al.*, 1994b; Dickenson *et al.*, 1986).

Since wind-up leads to the sensitisation of dorsal horn neuronal responses, and since both the NMDA and the NK-1 receptors are involved in this response, the hypothesis of the first part of my study was that the activation of each of these two excitatory receptors, the NMDA and the NK-1 receptor, by its respective agonist, would cause the facilitation of some of the dorsal horn neuronal responses, which has been shown in previous studies and discussed in **chapters 3** and **4** (Chapman *et al.*, 1994a; Dougherty *et al.*, 1991; Henry, 1976). In addition, due to the fact that both the NMDA and the NK-1 receptors are located mostly postsynaptic (Coggeshall *et al.*, 1997a) on the spinal cord dorsal horn neurons, and due to the above-mentioned electrophysiological studies showing that

morphine was less able to exert its effect when acting mostly on its postsynaptic μ-opioid receptors (Chapman, 1994; Chapman *et al.*, 1992; Chapman *et al.*, 1994b; Dickenson *et al.*, 1986; Lombard *et al.*, 1989), then it was of relevance to investigate which dorsal horn neuronal responses morphine could effectively inhibit when administered alone, and compare these to when morphine was administered following the administration of each receptor agonist.

Furthermore, the NMDA and NK-1 receptors not only play a role in the wind-up response of dorsal horn neurons, but also play a role in longer-lasting models of central sensitisation. Central sensitisation is thought to underlie abnomal pain sensations such as hyperalgesia and allodynia seen in chronic pain states such as neuropathic pain (Treede *et al.*, 1992). The activation of both the spinal NK-1 and the NMDA receptors are thought to be involved in the abnormal pain sensations following nerve injury, since these hypersensitivities are reduced following the administration of NK-1- (Cahill *et al.*, 2002; Coudore-Civiale *et al.*, 2000; Field *et al.*, 1998) and NMDA receptor antagonists (Davar *et al.*, 1991; Malcangio *et al.*, 1998b; Wang *et al.*, 2001; Wegert *et al.*, 1997; Yamamoto *et al.*, 1992b). Moreover, abnormal dorsal horn neuronal activity following nerve injury is also reduced by NK-1- (Cumberbatch *et al.*, 1998) and NMDA receptor antagonists (Suzuki *et al.*, 2001). Therefore, it was interesting to investigate and compare the effects of the activation of each of these receptors in an acute model of nociception, and their modulation by morphine.

In the part of my study using in vivo electrophysiology however, due to either the frequency of C-fibre stimulation (Chapman *et al.*, 1994a), the neuronal population used, the dosage of each agonist used or a combination of these factors, the only dorsal horn neuronal response which was facilitated was the 45°C thermal evoked response with the NK-1 receptor agonist Sar-SP, and morphine was able to inhibit the same responses it was effective in inhibiting when acting alone, including the thermal-evoked response, and to the same extent. This could lead to the conclusion that morphine is effective in inhibiting the dorsal horn neuronal responses, once the NK-1 receptor is activated, or that

this part of my study should remain inconclusive, and investigated using behavioural techniques.

In the part of my study using behavioural techniques, I show that intrathecal Sar-SP (3μg) causes both thermal and mechanical hypersensitivity, as well as long-lasting biting, scratching and licking (see **chapter 4**). One of the reasons that a facilitation of mechanical evoked neuronal responses tested was not seen in my electrophysiological study could be due to the fact that electrophysiological and behavioural studies measure different parameters, and we cannot always corroborate the findings fully. Behavioural studies measure threshold responses in whole animals, as the response is usually terminated by the free-moving animal withdrawing its paw from the stimulus, whereas in electrophysiological studies, supra-threshold stimuli to a single neuron is measured, since the animals are anaesthetised (Suzuki *et al.*, 1999). However, numerous von Frey forces were used in the electrophysiological part of my study, ranging from innocuous to noxious, and yet there was no significant excitatory effect of Sar-SP on any of them. Therefore, threshold versus suprathreshold mechanical stimulation could not be the reason why Sar-SP did not facilitate the mechanical-evoked response in this part of my study.

Therefore, since 3μg of Sar-SP effectively caused both thermal and mechanical hypersensitivity in my chapter using behavioural techniques, then the most probable reason for the lack of effect of Sar-SP on the mechanical evoked response of dorsal horn neurons is that there is a fine line between the dose of Sar-SP which excites and that which is ineffective in nociceptive transmission, causing 1μg of this agonist to be sufficient to excite only some of the thermal, and not the mechanical evoked responses, whereas 10μg would have been an ineffective dose. To date, this is the first study investigating the effects of Sar-SP (1 and 10μg), using in vivo electrophysiology, on mechanically-evoked spinal cord dorsal horn neuronal responses.

Furthermore, in my chapter using behavioural techniques, (see **chapter 4**), 0.3μg of intrathecal NMDA caused all of BSL behaviour, thermal and mechanical hypersensitivity, 5 minutes after administration, followed by analgesia. The fact that the intrathecal administration of NMDA (5, 50 and 500ng) did not cause any significant excitation of the electrical evoked responses in my study using in vivo electrophysiology could be attributed to the fact that electrical stimulation was made at 3x the C-fibre threshold, whereas it should have been done at 1.5x the C-fibre threshold (Chapman *et al.*, 1994a), and that different doses of intrathecal NMDA should have been used to cause increases in some of the natural evoked responses of deep dorsal horn neurons (Sher *et al.*, 1990). Additionally, shorter testing times might have been needed to show the effects of NMDA, since I showed in my behavioural study that the excitatory effects of intrathecal NMDA disappear after 5 minutes, and in the study by Chapman *et al*, only ten minutes were given between each set of testing. However, due to the fact that in this part of my study, all of electrical, brush, von Frey and heat evoked responses were measured, a 20 minute gap between testing was needed to fit in all these stimuli, as well as give the neuron time to recuperate.

The results that arose following the behavioural part of my study (**chapter 4**) showed that even though the activation of each of the NMDA and the NK-1 receptor caused an initial BSL response, followed by thermal and tactile hypersensitivity, that each receptor led to a different quality of BSL behaviour, as well as different extents of thermal hypersensitivity (which was observed more closely than the tactile response, due to the ease of the thermal stimulus administration). In my study, NMDA receptor activation leads to caudal biting, scratching and licking behaviour, whereas NK-1 receptor activation leads to caudal biting, scratching, licking and also excessive grooming behaviour. Indeed, whereas the spinal NMDA receptor activation has been previously thought to be nociceptive (Aanonsen *et al.*, 1987; Davis *et al.*, 2001; Wilcox, 1988), the spinal NK-1 receptor activation has yielded different conclusions, as per its role in nociceptive transmission, with some studies labelling SP as a neuromodulator, rather than a neurotransmitter (Chapman *et al.*, 1996; Dougherty *et al.*, 1995; Kellstein *et al.*, 1990), and with other studies concluding that the BSL behaviour

following SP is indicative of a spinal convulsive state, rather than pain, since no vocalisation occurs and since the BSL is not inhibited by morphine (Bossut *et al.*, 1988a; Wilcox, 1988).

It could be argued that the fact that morphine, in my study, was more efficient in inhibiting those responses following the NMDA receptor activation rather than the NK-1 receptor activation means that only the NMDA receptor activation led to nociception (Wilcox, 1988). I however, concluded that both these receptors participate in the pain response, but that each receptor leads to the activation of different secondary mechanisms/pathways which are either more, or less, susceptible to morphine control. Indeed, the NMDA receptor activation caused inhibition after the initial hypersensitivity, which could mean that NMDA receptor activation resulted in the activation of descending inhibitions onto the spinal cord (Kolhekar *et al.*, 1993), and which could be one reason why morphine synergised with the intrathecal NMDA administered, to cause an even larger inhibition of the response than when morphine acted on its own. Even though I did not use spinalised rats, the study by Kolkehar *et al* in 1993 showed that spinalisation blocked the NMDA-induced analgesia.

In the case of the NK-1 receptor activation, previous studies have shown that NK-1-expressing lamina I projection neurons are at the origin of the spinal-bulbo-spinal loop (Todd, 2002), which predominantly drive descending serotonergic facilitations needed to maintain neuropathic pain (Burgess *et al.*, 2002; Suzuki *et al.*, 2002b; Suzuki *et al.*, 2004a; Suzuki *et al.*, 2005) and these neurons are needed for the expression of LTP in deep dorsal horn neurons (Rygh *et al.*, 2006). Therefore, even though the NK-1-expressing lamina I projection neurons have been shown to play a role in driving descending noradrenergic inhibitions in normal animals (Rahman *et al*, unpublished observations), I will speculate that in my study, the activation of the NK-1 receptor could have shifted the descending control balance towards facilitations, leading to an acute increase in descending serotonergic facilitations, *via* the activation of the NK-1 receptors on lamina I projection neurons. Consequently, even though morphine was able to inhibit the effects following the activation of the NK-1 receptor with Sar-SP, this

was to a lesser degree than when morphine inhibited the effects following intrathecal NMDA. Could this reduced efficacy of morphine in inhibiting the NK-1-mediated responses be compared to the reduced efficacy of morphine in neuropathic pain reported in some studies? (Field *et al.*, 1999b; Gilron *et al.*, 2005; Joshi *et al.*, 2006; Kontinen *et al.*, 1998; Lemberg *et al.*, 2006).

Neuropathic pain is a chronic pain state that displays numerous symptoms, such as sensory deficits, spontaneous and touch-evoked pain (Suzuki *et al.*, 2000a), which have complex mechanisms that underlie their basis (see **chapter 1**). Indeed, pain following nerve injury involves a peripheral, spinal and supraspinal component (Attal *et al.*, 1999; Porreca *et al.*, 2001). Peripherally, ongoing 'ectopic' activity arises and has been shown to mostly involve changes in the expression and generation of Na^+ channels, an example of which is the upregulation of the $Na_V1.3$ in injured neurons (Amir *et al.*, 2006; Dickenson *et al.*, 2002a), and which is sensitive to block with local anaesthetics and anticonvulsants (Dickenson *et al.*, 2002a; Ossipov *et al.*, 1995; Rowbotham *et al.*, 1991). In the spinal cord, numerous changes occur, such as the increase in dynorphin and cholecystokinin (CCK), two neuropeptides which have been shown to be 'morphine-resistant'. Indeed, the anti-allodynic efficacy of spinal morphine was restored, following nerve injury, when morphine was administered with an antiserum against dynorphin (Wu *et al.*, 2005) or with a CCK_B receptor antagonist (Nichols *et al.*, 1995).

Whereas most previous studies have concentrated on peripheral and spinal mechanisms underlying neuropathic pain, less studies have focussed on investigating the role of descending serotonergic facilitations following nerve injury (McCleane *et al.*, 2003; Suzuki *et al.*, 2004a), as well as investigating whether peripheral NMDA receptors play a role in neuropathic pain (Jang *et al.*, 2004). Therefore, in my study, I investigated the role of descending serotonergic facilitations in the maintenance of hypersensitivities following nerve injury, and whether peripheral NMDA receptors play a role in hypersensitivity following nerve injury.

Clinically, it would be beneficial for pain due to nerve injury to be caused by peripheral factors, as that would make drug administration easy and free of unacceptable side-effects. Initial studies have shown that the intraplantar administration of NMDA receptor antagonists reduced the lifting and licking behaviour which occurs following formalin administration (Davidson *et al.*, 1998; Davidson *et al.*, 1997), the thermal hyperalgesia seen following carrageenan inflammation (Jackson *et al.*, 1995) and the mechanical hyperalgesia seen following CFA inflammation (Leem *et al.*, 2001), indicating a role for the peripheral NMDA receptors in inflammation. To date, only one study has investigated the role of peripheral NMDA receptors in neuropathic pain and showed that blocking peripheral NMDA receptors with an antagonist led to the reduction in tactile hypersensitivity following nerve injury (Jang *et al.*, 2004), and no studies have been undertaken yet to determine whether the levels of peripheral NMDA receptors change following nerve injury.

In my study, I investigated whether peripheral NMDA receptors are involved in the tactile and cold hypersensitivity seen following the spinal nerve ligation model. I showed that, in normal animals, following a high dose of intraplantar NMDA, tactile, but not thermal hypersensitivity arose, which was blocked with the intraplantar administration of the NMDA receptor antagonist MK-801. The fact that tactile and not thermal hypersensitivity resulted following the activation of the peripheral NMDA receptor could show that peripheral NMDA receptors are expressed on nociceptive primary afferent fibres which do not transmit thermal sensation, thus maybe a subset of the non-peptidergic, IB4-positive Aδ-fibres (Caterina *et al.*, 2005; Meyer *et al.*, 2006; Snider *et al.*, 1998).

However, following peripheral nerve injury, MK-801 was ineffective in reducing the tactile and cold hypersensitivity which arose in the spinal nerve ligation model. The discrepancy between my study and that of Jang *et al* could have been due to the fact that different nerve injury models were used, whereas I used the L5 and L6 spinal nerve ligation model, the study by Jang *et al* used an L5 spinal nerve ligation, which was preceded by an L5 dorsal rhizotomy, to avoid the central effects of nerve injury. Since the initiation of neuropathic pain has been

shown to depend on ongoing-input, due to ectopic activity, from the injured nerve onto the spinal cord (Attal *et al.*, 1999; Ossipov *et al.*, 1995), which leads to the sensitisation of spinal cord neurons (Salter, 2005) and subsequently, to increased descending facilitations to maintain the pain (Burgess *et al.*, 2002), the model by Jang *et al* is not a true representation of the mechanisms which underlie neuropathic pain in the clinical setting.

Descending serotonergic facilitations have been shown to exert their excitatory effects *via* their action on the ionotropic 5HT3 receptor, which is predominantly located on a subset of myelinated primary afferent fibres which terminate in the superficial dorsal horn, thus of the Aδ-fibre type, and which are predominantly not peptidergic (Conte *et al.*, 2005; Maxwell *et al.*, 1983; Zeitz *et al.*, 2002). Previous studies investigating the role of descending serotonergic facilitations in neuropathic pain have found that these are enhanced following nerve injury, since the administration of spinal ondansetron, a 5HT3 receptor antagonist, causes a more pronounced reduction of the mechanical-evoked responses of dorsal horn neurons in rats with nerve injury than in the controls (Suzuki *et al.*, 2004a). Indeed, another study showed that depleting endogenous spinal 5-HT reduces both tactile and cold hypersensitivity following spinal nerve ligation, and this effect was seen on day 5 following nerve injury, further proving the role of descending serotonergic facilitations in maintaining pain following nerve injury (Rahman *et al.*, 2006a).

Furthermore, as mentioned previously, descending serotonergic facilitations are driven by NK-1-expressing lamina I projections neurons *via* a spino-bulbo-spinal loop, which relays in supraspinal sites that include the parabrachial area and the thalamus (Suzuki *et al.*, 2005). Each of the ablation of the NK-1-expressing projection neurons, with SP-SAP, and the blockade of the spinal 5HT3 receptors with ondansetron leads to similar electrophysiological changes following nerve injury, further proving the role of this loop in neuropathic pain (Suzuki *et al.*, 2004a; Suzuki *et al.*, 2005). In addition, gabapentin, a drug licensed for the treatment of neuropathic pain, needs the NK-1-expressing lamina I projection

neurons to be intact, or the 5HT3 receptors on primary afferent fibres to be active, in order to be fully effective (Suzuki *et al.*, 2005).

In the case of descending serotonergic facilitations and their role in the spinal nerve ligation model, I found, as has been shown before, that descending serotonergic facilitations are enhanced following peripheral nerve injury (Suzuki *et al.*, 2004a), and that by blocking their effects with spinal ondansetron, the static hypersensitivity seen following nerve injury was reduced. However, neither thermal, nor cold hypersensitivity which arose following nerve injury were affected by ondansetron, which could be could be due to the low dose of ondansetron used in my study, or a reflection of the type of primary afferent fibres on which the 5HT3 receptor is found. Indeed, the 5HT3 receptor is predominantly found on a subset of myelinated primary afferent fibres (Aδ-fibres), whereas thermal and cold responses are transduced mainly by the TRPV1 and the TRPM8 receptors, which are predominantly located on unmyelinated primary afferent fibres (Conte *et al.*, 2005; Maxwell *et al.*, 1983; Zeitz *et al.*, 2002).

In my study, morphine was more effective than ondansetron. This was not surprising, since morphine acts at the three pain transmission levels altered by nerve injury: the first synapse at the primary afferent fibres, where it inhibits transmitter release, the spinal cord dorsal horn, where it inhibits synaptic transmission and at supraspinal sites, where it inhibits ON cells and disinhibits OFF cells in the RVM, thus causing an overall inhibitory effect (Dickenson, 1994; Fields *et al.*, 1991; Heinricher, 1997; Porreca *et al.*, 2001). Therefore, even though some studies have shown that neuropathic pain is less responsive to morphine (Arner *et al.*, 1988), I have shown, using systemic morphine in the spinal nerve ligation model, that this is not the case, in agreement with numerous studies which have shown that the responsiveness of neuropathic pain to morphine depends on route of administration, dose titration and type of nerve injury (Bian *et al.*, 1995; Hansson *et al.*, 2005; Jadad *et al.*, 1992; Portenoy *et al.*, 1990; Rowbotham *et al.*, 1991; Suzuki *et al.*, 1999). Furthermore, it seems that there is only one neuropathic pain symptom which remains resistant to morphine

control, at the dose used in a previous study, and that is dynamic allodynia (Field *et al.*, 1999b).

What was surprising in my study however, was the fact that the combination of morphine and ondansetron did not yield a greater inhibition of the hypersensitivities seen following nerve injury, than each drug alone. One reason for the latter could be that the dose of each drug used was already effective, and in order to assess drug synergism, lower doses of each drug should have been administered (Matthews *et al.*, 2002; Mjellem-Joly *et al.*, 1991).

7.3 Future studies

In chapter 3, I showed that morphine was effective in inhibiting the mechanical evoked response to von Frey 5g, an innocuous mechanical response. Innocuous mechanical responses, such as touch and brush, are conveyed by Aβ-fibres, which do not express μ-opioid receptors (Besse *et al.*, 1990; Zhang *et al.*, 1998) and are less sensitive to morphine control (Dickenson *et al.*, 1986). One future study could involve investigating which primary afferent fibre(s) is involved in conveying the von Frey 5g evoked response, to determine whether this response is conveyed purely *via* Aβ-fibres, or *via* a combination of Aβ- and Aδ-fibres, by using a teased-fibre technique to record from single primary afferent fibres in the rat hindpaw (Leem *et al.*, 1993; Slugg *et al.*, 2000).

Moreover, it would be interesting to investigate the effects of ondansetron on the 5HT3 receptors located on primary afferent fibres, using patch-clamping techniques, and to measure how much ondansetron affects calcium flow into the cell, *via* both patch-clamping and fluorescent techniques, in normal and neuropathic rats.

Furthermore, dynamic allodynia is clinically the most debilitating response in patients with nerve injury, because normal daily routines, such as wearing clothes, becomes painful (Rasmussen *et al.*, 2004), and remains the most

resistant nociceptive response to morphine control (Field *et al.*, 1999b). In animal studies, dynamic allodynia is determined by lightly stroking the plantar surface of the rat hindpaw with a wisp of cotton from a cotton bud: if flinching occurs before 8 seconds, then dynamic allodynia is present, and the cut-off time is 15 seconds (Field *et al.*, 1999b).

Therefore, another future study could involve investigating dynamic allodynia in the spinal nerve ligation model and assessing whether morphine or ondansetron, each on its own and in combination (in lower doses) can inhibit this response. Following that, more clinical studies using different doses of ondansetron alone and in combination with morphine, could be carried out.

In addition, studies using functional magnetic resonance imaging (fMRI) could also be carried out in humans to assess and compare the effects of each of ondansetron and morphine and their combination, on the brain processing following different stimuli in normal individuals as well as in patients with neuropathic pain. Indeed, in a former study which investigated the effects of gabapentin (GBP) in normal and capsaicin-sensitised skin, using fMRI, it was found that GBP was more effective in decreasing neural transmission in central sensitisation states than in normal pain transmission states (Dickenson *et al.*, 2005; Iannetti *et al.*, 2005)

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